Genetics

G001. **Rare *KIAA1109* Compound Heterozygous Truncating Variants Resulting in Alkuraya-Kučinskas Syndrome**

Y. Akgun, E. Solem, L. Le
Vanderbilt University Medical Center, Nashville, TN.

Introduction: Alkuraya-Kučinskas syndrome is a rare autosomal recessive disorder characterized by multiple anomalies, including polyhydramnios, arthrogryposis, clenched hands, clubbed feet, brain abnormalities associated with cerebral parenchymal underdevelopment, dysmorphic facial features, and renal involvement. This disease was first characterized and named in 2018, and biallelic pathogenic variants in the *KIAA1109* gene were determined to be the cause of this severe disease. At our institution, trio testing was ordered on a male newborn with overlapping features. **Methods:** Genomic DNA extracted from submitted specimens from the proband and both parents was enriched for coding and adjacent non-coding regions by hybridization-capture using IDT xGen Exome Research Panel v2 (Integrated DNA Technologies, Coralville, IA). Library products were sequenced on the Illumina NovaSeq. After alignment to the reference genome (UCSC hg19), variants were detected using several different variant calling algorithms. Sequence alterations were described according to Human Genome Variation Society (HGVS) nomenclature guidelines and were classified according to variant interpretation guidelines of the American College of Medical Genetics and Genomics (ACMG). **Results:** Trio whole-exome sequencing identified compound heterozygous pathogenic variants in the *KIAA1109* gene. The first variant (c.6398_6402delinsCAAAGTTACC [p.Leu2133Serfs*6]) is a paternally inherited deletion-insertion variant. This alteration is predicted to shift the reading frame with substitution of leucine by serine at amino acid position 2133 followed by introduction of a premature stop codon. The second variant (c.12272_12273dup [p.Pro4092Ilefs*32]) is a maternally inherited 2-bp duplication. This alteration is predicted to shift the reading frame with substitution of proline by isoleucine at amino acid position 4092 followed by introduction of a premature stop codon. Both variants are predicted to result in loss of *KIAA1109* function due to premature protein truncation and/or nonsense-mediated mRNA decay. Both variants were classified as pathogenic per ACMG guidelines and were confirmed by Sanger sequencing. **Conclusions:** Here we report compound heterozygous frameshifting variants in the *KIAA1109* gene, which were revealed by trio testing to be inherited from reportedly unrelated parents, that resulted in early death of the proband due to Alkuraya-Kučinskas syndrome. In the literature, similar truncating *KIAA1109* variants in both the homozygous and compound heterozygous state have been reported in cases of early death, whereas missense variants have been reported in milder disease cases with occasional survival. To our knowledge, this represents the second reported case of Alkuraya-Kučinskas in the United States.

G002. **Two *SLC25A15* Variants, Including a *de novo* Variant in a Patient with Suspected Diagnosis of Hyperornithinemia-Hyperammonemia-Homocitrullinuria Syndrome**

B. Azab, J. Liao
Columbia University-New York Presbyterian Hospital, New York, NY.

Introduction: An 18-month-old male with concerns for liver failure was referred to the genetics department. The patient was born at full term with macrocephaly, mild axial hypotonia, and distal brisk reflexes. He has been suffering from recurrent infections, febrile seizures, and mild developmental delay restricted mostly to receptive language. He has been admitted for elevated transaminase (AST/ALT) levels and elevated INR, and he has been evaluated by early intervention. Biochemical testing revealed elevated orotic acid in the urine, ammonia of 67 µmol/L, and plasma amino acids with elevated glutamine, alanine, ornithine, and lysine. Elevated orotic acid can be seen in patients with primary or secondary impairment of the urea cycle. **Methods:** To identify the underlying molecular etiology, a battery of molecular investigations was performed for the proband and family members including: SNP microarray, mtDNA sequencing, Southern blot for mtDNA deletions/rearrangements, and whole-exome sequencing (WES). **Results:** Mitochondrial and microarray results were negative, although trio-based WES identified two candidate variants in the *SLC25A15*. The first was a rare, maternally inherited missense variant (c.706A >G, p.Arg236Gly). It is predicted to be damaging to the protein structure and/or function by multiple *in silico* prediction tools and has been interpreted as likely pathogenic by a carrier screening study without any clinical details. The second was also a rare missense variant (c.68G >A, p.Cys23Tyr). *In silico* analyses are inconsistent in their prediction about the effect of this variant. It has been reported with another missense *SLC25A15* variant in an individual with hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (HHHS). Interestingly, it was not detected in either parent and therefore is *de novo* by origin. *SLC25A15* causes HHHS, which is characterized by hyperammonemia accompanied by vomiting, ataxia, lethargy, confusion, and coma in the acute phase. Therefore, the two variants detected in *SLC25A15* are most likely to be causative for the patient's phenotype, though their phase is unknown due to the *de novo* origin of the second variant. **Conclusions:** The differential diagnosis of pediatric liver failure with abnormal biochemical profile is broad and could be guided by genetic testing. We identified two *SLC25A15* variants by WES, which confirmed the suspected clinical diagnosis of HHHS and consequently its clinical management. Noteworthy, the patient harbored a rare scenario of a combination of one *de novo* and one inherited variant that led to an autosomal recessive disorder.

G003. **Genetic Perturbation of Phosphatidylcholine and Phosphatidylethanolamine in Fat Is Associated with Cardiac Remodeling and NLRP3 Inflammasome in Cardiovascular Patients with Insulin Resistance Risk**

M. Corsi Romanelli[1], M. Kalousová[3], F. Ambrogi[4], E. Dozio[1], L. Tacchini[1], T. Zima[3], E. Vianello[1]
[1]Università degli Studi di Milano "LA STATALE," Milan, Italy; [2]Department of Biomedical Sciences for Health Università degli Studi di Milano "LA STATALE," UOC-SMEL1 IRCCS Policlinico San Donato, Milan, Italy; [3]Institute of Medical Biochemistry and Laboratory Diagnostic, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic; [4]Department of Clinical Sciences and Community Health, Università degli Studi di Milano "LA STATALE," IRCCS Policlinico San Donato, Milan, Italy.

Introduction: Lipolysis occurs in adipose tissue during the development of cardiovascular disorders due to sympathetic stimulation and insulin resistance. The bioactive lipid species are a new class of cardiovascular biomarkers involved in cell integrity and inflammation. Indeed, the perturbation in cardiac lipidome promotes left ventricle (LV) remodeling through the activation of NLRP3 inflammasome, a key regulator of chronic inflammation in obesity-related disorders, including cardiac remodeling and type 2 diabetes. In this field, we focused our attention on phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipid class because a reduction of their ratio in plasma and tissues is associated with cardiac disorders. Our study aimed to understand if a reduction of PC/PE ratio in epicardial adipose tissue (EAT) is associated with NLRP3 inflammasome activation and maladaptive LV remodeling in cardiovascular diseases (CVDs) patients with insulin resistance risk. **Methods:** Forty patients from IRCCS Policlinico San Donato were enrolled, and their EAT and plasma were drawn during surgery. LV geometry was evaluated by echocardiography, and the anthropometric and clinical data were collected. Insulin resistance risk was classified according to HOMA-IR index. Microarray technology was used to evaluate all the genes associated to PC and PE expression and NLRP3 inflammasome in EAT. PC and PE in EAT were quantified by ESI-MS/MS with methods using a Quattro Ultima triple-quadrupole mass spectrometer. NLRP3 plasma level was measured by ELISA assay. **Results:** Microarray results show that CVDs patients with HOMA >2.5 had a downregulation of genes involved in neosynthesis of PC and PE species in EAT. Indeed, *PCYT1A* gene involved in phosphocholine cytidylyltransferase expression and SREBFs family

genes involved in *de novo* synthesis of lipids are down-expressed in CVDs patients with HOMA >2.5, underlying the effect of insulin resistance in lipid metabolism. In CVDs patients with HOMA >2.5, NLRP3 inflammasome genes were upregulated and their expression was inversely associated with PC/PE tissue production. Circulating levels of NLRP3 increased in patients with concentric remodeling and were inversely associated with PC/PE ratio in plasma, denoting the pro-remodeling impact of this mediator. **Conclusions:** In summary, these findings suggest that insulin is a key trigger of maladaptive cardiac response in patients with HOMA >2.5 through NLRP3. The lipid changes in EAT lipidome induced by insulin resistance promote a metabolic switch that reduces the PC/PE ratio in tissue and plasma, losing its cardiac protection and promoting cardiac remodeling through NLRP3 inflammasome.

G004. **Cytogenomics Analysis Identifies Three Interstitial Deletions Located within the Chromosome 18q Deletion Syndrome in a Patient Presenting with Developmental Delays**

J. Olayiwola[1], E. Hamelberg[2], S. Leeper[1], C. Myers[1], B. Stone[1], K. Manickam[1], M. Mathew[2], M. Leung[2]
[1]Nationwide Children's Hospital, Columbus, OH; [2]The Steve and Cindy Rasmussen Institute for Genomic Medicine, Nationwide Children's Hospital, Columbus, OH.

Introduction: Chromosome 18q deletion is a contiguous gene deletion syndrome due to the interstitial or terminal deletion of the long arm of chromosome 18. Chromosomal microarray (CMA) is the recommended first-tier clinical diagnostic test for individuals with unexplained developmental delays, congenital anomalies, and autism spectrum disorder due to its higher sensitivity for identifying microdeletions and microduplications compared to conventional karyotyping analysis. Here, we report the cytogenomic findings of a 14-month-old female, who presents with dysmorphic features, speech and motor delays, eczema, bronchiolitis, hypotonia, and a negative family history of developmental delays. **Methods:** Genomic DNA from peripheral blood was extracted for CMA and Fragile X testing. CMA and Fragile X syndrome testing were performed concurrently due to overlapping clinical phenotypes. CMA was performed using a custom-designed Agilent 180k CGH +SNP array to detect regions of homozygosity and copy number variants, and CMA analysis was performed using Genoglyphix software. Fragile X testing was performed using the AmplideX *FMR1* CGG Repeat PCR technique. High-resolution karyotyping analysis of peripheral blood specimen was performed using Leica Biosystems CytoVision. **Results:** Fragile X testing was non-diagnostic, whereas CMA analysis showed three distinct, interstitial deletions in the long arm of chromosome 18, arr(GRCh37) 18q11.2q12.2(21220372_33581798)x1,18q12.3(37279784_39000986)x1,18q21.1(44401023_45650840)x1, resulting in partial monosomy of chromosome 18. Genetic counseling and follow-up chromosomal analysis were recommended. Subsequent high-resolution chromosomal analysis showed that the interstitial deletions occurred in a single chromosome 18 homolog, producing a derivative chromosome 18 and a 46,XX,der(18)del(18)(q11.2q12.2)(q12.3q12.3)(q21.1q21.1) karyotype. **Conclusions:** These interstitial 18q deletions are located within the Chromosome 18q deletion syndrome, but previously reported 18q deletions are described as single events. Therefore, this patient's presentation may vary from the known clinical features of this syndrome, which can include: hypotonia, short stature, developmental delays, obesity, and behavioral problems. The complexity of this karyotype increases the possibility that the derivative chromosome is the result of a balanced translocation inherited from an unaffected parent, or other rearrangements. This complex karyotype highlights the importance of follow-up chromosomal analysis for genomic imbalances identified by microarray, to further characterize the rearrangement. Follow-up parental karyotyping testing was also recommended to determine the origin of the rearrangement and to provide appropriate genetic counseling for recurrence risk estimates.

G005. **Prevalence and Spectrum of Cancer Predisposition Gene Germline Mutations in Young Patients across Six Late-Onset Cancer Types**

X. Zhao, T. Ma, B. Guo, X. Zhang
Genetron Health (Beijing) Co. Ltd., Beijing, People's Republic of China.

Introduction: Our knowledge of pathogenic germline variations (PGVs) in cancer predisposition genes (CPGs) is constantly widening; however, large-cohort and comprehensive screening study of cancer-related PGVs is still lacking. Previously, researchers reported an 8% overall carrier rate of CPG germline mutations in adult cancer and 4%-8.5% in pediatric cancer, which showed no obvious difference. But it's also reported that the prevalence rates and/or spectrum of PGVs varied between young and aged patients in some cancer types, e.g., colorectal cancer and lung adenocarcinoma. Hence, we conducted a novel retrospective study aiming to evaluate the prevalence rates and variation spectrums of PGVs carried by young patients under 30 years old, but who were affected by one of six usually late-onset cancer types with an average onset age of more than 60 years, which were lung cancer (LC), liver cancer (LiC), colorectal cancer (CC), gastric cancer (GC), renal cancer (RC) and head-neck cancer (HNC). **Methods:** Patients affected by one of the six cancer types under the age of 30 years were included, all of whom provided informed consent. Onco PanScan, which is a next-generation sequencing-based multi-gene panel, was applied to detect variants in solid tumor and blood samples. All variants with a minor allele frequency of >0.01 were considered benign. Pathogenic (P) and likely pathogenic (LP) variants were further defined by 1), classified as P/LP in ClinVar; or 2), truncating variants upstream of any reported ClinVar P/LP truncating variant. **Results:** A total of 11,156 patients were enrolled in this study, of which 134 (1.2%) were young patients, with an average age of diagnosis of 23.0 (range, 2-29) years. For each cancer type, the numbers of young patients and overall patients were as follows: LC (56/7,849, 0.7%); LiC (18/645, 2.8%); CC (28/1,393, 2.0%); GC (13/805, 1.6%); RC (15/428, 3.5%), and HNC (4/265, 2.4%). Remarkably, PGV carriers accounted for as many as 17.9% (24/134) of all young patients, and were seen throughout all six cancer types with high penetrance rates: 12.5% (7/56) in LC; 11.1% (2/18) in LiC; 21.4% (6/28) in CC; 15.4% (2/13) in GC; 33.3% (5/15) in RC, and 50.0% (2/4) in HNC. The PGVs were identified in 17 genes: *APC*, *BLM*, *BRCA2*, *FANCA*, *FH*, *MLH1*, *PALB2*, *PDE11A*, *PRSS1*, *RAD50*, *RAD51B*, *RAD51D*, *SBDS*, *SDHB*, *SLX4*, *TP53,* and *VHL*. **Conclusions:** Our results showed a high prevalence (17.9%) of cancer-related PGV carriers in young cancer patients affected by usually late-onset cancers, and clarified the PGV prevalence rates in each cancer type as well as their variation spectrums. These results suggested that comprehensive screening for cancer PGVs in the above-mentioned cohorts would be of great importance for diagnosis of cancer predisposition syndrome.

G006. **Seventy Clinical Genomes: Initial Experience from Texas Children's Hospital**

R. Kumar, L. Saba, H. Streff, D. Lopez-Terrada, J. Scull
Baylor College of Medicine, Houston, TX.

Introduction: Clinical genome sequencing (GS) promises to incorporate features of multiple prior testing modalities, including identifying copy number variants, single nucleotide variants, mitochondrial DNA variants, and repeat expansions. However, accepted indications for GS remain poorly defined. Moreover, it remains unknown what types of misorders (e.g., redundant testing, clerical errors, better alternative test available, etc.) may occur as this clinical assay becomes more widespread. **Methods:** Texas Children's Hospital (TCH) initiated its Genetic Testing Stewardship committee in 2021, with representatives from clinical genetics, genetic counseling, clinical molecular genetics, and molecular pathology. With the goal of improving GS utilization at TCH, the committee reviewed the charts of all admitted patients who received GS between March 2020 and May 2022 and extracted data necessary to address the study questions. **Results:** Seventy admitted patients received GS during the study period. The average age was 4.7 years (two days to 21 years). The

majority of patients were in intensive care units (40/70, 57%), especially the general pediatric ICU (23/70, 33%). The most common classes of phenotypes were neurologic or metabolic (43/70, 61%), hematologic or immunologic (9/70, 13%), and cardiac (7/70, 10%). The clinical genetics consult service ordered most GS (66/70, 94%). Nearly half were ordered "rapid" with results on an expedited basis (33/70, average turn-around time 13.3 days versus 84.6 for non-rapid tests), and all had at least one parent participating. More than half of the tests were characterized as misordered (37/70, 53%), most often due to redundant testing (29/70, 41%). Chromosomal microarray (CMA) was the most frequent prior test (25/70, 36%), though exome sequencing (ES) was also common (19/70, 27%), and patients often had both (13/70, 19%). Patients with prior negative ES had markedly lower diagnostic rates with GS (2/17, 12%) than patients without negative ES (23/48, 48%, OR=0.15, 95% CI 0.015-0.75). The two patients with positive GS but negative ES had final diagnoses likely identifiable by ES reanalysis. Among patients with completed testing, GS had a high diagnostic yield overall (25/65, 38%). However, there were only two cases (3%) in which GS yielded a diagnosis that CMA or ES is unlikely to identify. One patient had a heterozygous 4-Kb single-exon deletion, and the other a mitochondrial DNA variant. **Conclusions:** Clinical GS is growing in availability, but experience with this testing modality is limited in clinical settings. GS has a high diagnostic yield when used as a first-line test and has the potential to shorten the diagnostic odyssey, but the incremental benefit over other available testing methods may be limited.

G007. **A Familial 4p Interstitial Deletion Case Report and New Insights into Molecular Interpretations**

J. Di, S. Wei, S. Zhang, H. Rieger, L. Yenwongfai
University of Kentucky, Lexington, KY.

Introduction: Two distinct phenotypes have been associated with deletions on chromosome 4p: Wolf-Hirschhorn syndrome (a terminal deletion) and more proximal interstitial deletions (4p11-p16), which have been less commonly reported, with distinct mild to moderate mental retardation, multiple dysmorphic features including long face, up-slanted palpebral fissures with epicanthal folds, tall thin body habitus, and hyperextensible joints. The phenotypic manifestation of proximal 4p deletion is less well known among healthcare workers, and it may go undiagnosed until the affected person is older. In the literature, 35 cases of 4p interstitial deletions have been described from prenatal to 77 years. To date, only two familial cases have been reported, and most of the reported deletions were detected by conventional chromosome analyses alone. The critical region for proximal 4p deletion syndrome has been localized to 4p15.2-15.33, but the causative genes remain elusive. In this study, we report a familial 4p interstitial deletion case with insightful molecular interpretations. **Methods:** Routine G-banded karyotyping was performed on cultured peripheral blood lymphocytes from the patients, at the 550-band level according to the International System for Human Cytogenomic Nomenclature (ISCN). Subsequently, genomic DNA was isolated from peripheral blood lymphocytes using the QIAamp DNA Blood Mini kit. DNA sample (250 ng) of the proband was hybridized to CytoScan HD arrays on an Affymetrix SNP array which contains more than 2.6 million markers for the copy number analysis. Using ChAS 4.2.0.80 software based on the reference genome sequence of the University of California, Santa Cruz database, the aberrations were filtered up to a minimum size of 25 kbp for deletions and 50 kbp for duplications. **Results:** We report a three-year-old female (proband) with failure to thrive, global developmental and gross motor delays, and morphological features. The mother (23 years) has intellectual disability, large knuckles, tall and slender habitus, a heart murmur, and facial abnormalities. The mother's karyotype had a 4p15.2p13 deletion, the same as our proband. Chromosome microarray analysis on the proband showed a 13.4-Mb 4p15.2-p14 deletion encompassing 16 genes, five of which are more likely to have phenotypic effects (pLI of more than 0.98): *PPARGC1A, DHX15, RBPJ, STIM2, and PCDH7*. **Conclusions:** Facial/habitus deformations and intellectual disability are the most common features of familial 4p interstitial deletion syndrome and should be considered along with other possible genetic abnormalities. In this 4p deletion, 16 genes were found to be in this region, and presumably contribute to the phenotypic features, due to haploinsufficiency.

G008. **Retrospective Optical Genome Mapping Analysis of FSHD1 and 2 Negative Patients with Diminished Methylation Revealed Exon Deletions of *SMCHD1***

A. Stence[1], A. Bossler[2], S. Moore[1], D. Ma[1]
[1]University of Iowa Hospitals and Clinics, Iowa City, IA; [2]Moffitt Cancer Center, Tampa, FL.

Introduction: Facioscapulohumeral muscular dystrophy (FSHD) is a genetic disorder that leads to the weakening of skeletal muscles. More than 90% of individuals affected with FSHD have a chromosome (chr) 4q35 deletion (FSHD1) which comprises a copy number loss (CNV) of 3.3 kb tandem repeats (D4Z4 repeats) at the subtelomeric chromosomal region 4q35, of which the pathogenic allele contains 1-10 repeats. Furthermore, pathogenic alleles have an A haplotype. Another ~5% of patients have FSHD2 due to heterozygous mutations in the *Structural Maintenance of Chromosomes Flexible Hinge Domain Containing 1* (*SMCHD1*) gene on chr18p. These mutations cause chromatin relaxation and are independent of the size of the D4Z4 array. Here, we performed retrospective *de novo* analysis of patients who were negative for FSHD1, chr18p CNV loss, and *SMCHD1* next-generation sequencing (NGS), but who had diminished D4Z4 methylation. We hypothesized that some of these individuals may have *SMCHD1* structural variants. **Methods:** Blood was processed using the SP Blood & Cell Culture DNA Isolation Kit, labeled using the DLS DNA Labeling Kit and run on the Saphyr system using ICS v5.2, Solve v3.7, Access v1.7, and the Bionano EnFocus FSHD Analysis v1.0 pipeline (Bionano, San Diego). FSHD1-negative specimens that had at least one permissive 4qA haplotype were reflexed to Southern blot analysis to quantify the percentage of CpG methylation using the methylation-sensitive FseI endonuclease and radioactively labeled p13e-11 probe. Specimens that had ≤28% methylated D4Z4 fragments were reflexed to *SMCHD1* NGS using Ion AmpliSeq 2.0 and Ion S5 XL technology (Thermo Fisher Scientific). Those who had diminished methylation (≤28%) but no FSHD1, chr18p CNV loss, or NGS *SMCHD1* mutation underwent *de novo* analysis. Guidelines for reviewing variants in Optical Genome Mapping specifically looking for structural variants in *SMCHD1* larger than 500 bp were used. **Results:** Among 320 cases tested for FSHD, five FSHD1/2-negative patients with diminished D4Z4 methylation (ranging from 12%-17%) were identified. *De novo* analyses found three of them harbored structural variants of deleted exons (ex) in *SMCHD1* (53,54 bp deletion of ex2-3, 235,668 bp deletion of ex26-48, and a 2,662 bp deletion of ex37) The presence of these structural variants was confirmed by Invitae (San Francisco). **Conclusions:** The ability to detect structural *SMCHD1* variants in patients who have undergone Bionano Enfocus FSHD analysis can enhance the diagnosis of FSHD2. Given the high percentage of FSHD1/2-negative patients who were retrospectively found to harbor a structural variant in *SMCHD1,* incorporation of this analysis into the diagnostic workflow for FSHD patients should be strongly considered.

G009. **Interpretation of *SLC3A1* and *SLC7A9* Variants in Cystinuria Patients: A Single Center Experience Implying the Significance of the PM3 Criterion and Protein Stability**

B. Lee, H. Park, S. Lee, D. Han
Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea.

Introduction: Cystinuria is a genetic disorder caused by defects in the dibasic amino acid transporters. The $b^{0,+}$ transporter system is composed of rBAT and $b^{0,+}$AT coded by *SLC3A1* and *SLC7A9*, respectively, which form a heterodimer with disulfide bond. Cystinuria results from variants in *SLC3A1* and *SLC7A9*, which follow autosomal recessive inheritance and autosomal dominant inheritance with reduced penetrance, respectively. Owing to these inheritance patterns,

the interpretation of cystinuria-related variants could be challenging. **Methods:** We collected cystinuria patients with *SLC3A1* and *SLC7A9* sequencing orders. The variants were interpreted based on the ACMG/AMP 2015 guidelines. For *in silico* analysis, REVEL and SpliceAI were utilized. To estimate the change in protein stability, DyanMut2 was used. The capability of REVEL and DynaMut2 in predicting the pathogenicity was evaluated using a previous functional study of *SLC7A9* variants. **Results:** There were a total of 10 cystinuria cases (seven males and three females) with a median onset age of 20 years. Urinary stones were composed of 100% cysteine in all patients. A total of 13 different variants were identified, which consisted of seven different *SLC3A1* variants and six different *SLC7A9* variants. Among these variants there were two novel variants previously not reported: *SLC3A1* c.223C >T and *SLC7A9* c.404A >G. Among the *SLC3A1* variants, there were three pathogenic variants (PVs), three likely pathogenic variants (LPVs), and one variants of uncertain significance (VUS). Among the *SLC7A9* variants, there were one PV, two LPVs, and three VUSs. *In silico* analysis using REVEL correlated well with the functional loss upon *SLC7A9* variants with scores of 0.8560-0.9200 and 0.4970-0.5239 for severe and mild decrease in transport activity, respectively. In addition, protein stability change obtained with DynaMut2 was able to predict decreased protein expression level resulting from the *SLC7A9* variant c.209C >T with $\Delta\Delta G^{Stability}$ -1.65 kcal/mol. **Conclusions:** We report interpretations of *SLC3A1* and *SLC7A9* variants identified from cystinuria patients, which include two novel variants. We believe our study adds to the literature as the current PM3 criterion could be applied with a stronger level with additional cases of the corresponding variant. Moreover, we suggest the clinical utility of REVEL and DynaMut2 in interpreting *SLC3A1* and *SLC7A9* variants. Although decreased protein expression level is not embraced in the current variant interpretation guidelines, we believe *in silico* protein stability predicting tools could serve as evidence of protein function loss.

G010. **A Novel Missense Variant in *COL2A1* Strengthens the Pathogenic Role of the C-propeptide Domain in the Torrance Type of Platyspondylic Lethal Skeletal Dysplasia**

M. Hsiao, S. Wontakal, K. Bogyo
Columbia University Medical Center, New York, NY.

Introduction: The Torrance type of platyspondylic lethal skeletal dysplasia (PLSDT) (OMIM# 151210) is a rare autosomal dominant disorder that is characterized by varying platyspondyly, rib abnormalities, hypoplastic pelvis, and shortening of tubular bones with cupped metaphyses leading to perinatal lethality. All known pathogenic variants, including missense, nonsense, and frameshift variants, associated with PLSDT cluster in exons 53 and 54 of *COL2A1*. These exons code for part of the C-propeptide domain, which encompasses exons 51-54. Therefore, no deleterious variants have been reported in exons 51 or 52. **Methods:** A male born at 34 weeks gestation passed away within two hours of life from a severe skeletal dysplasia. Neonatal X-rays were suggestive of PLSDT based on severe platyspondyly, hypoplasia of the iliac bones, bowing of the radii, and diffuse metaphyseal cupping. Trio-based whole-exome sequencing on peripheral blood samples was performed to identify a possible genetic etiology. **Results:** A *de novo* missense variant in *COL2A1* (NM_001844.5) that results in p.Cys1283Trp (c.3849C >G) was identified in the proband. This variant is absent from gnomAD, indicating it is a rare variant in the populations represented in this database. This highly conserved residue is located in exon 51 and is found within the C-propeptide domain. Indeed, 20/20 *in silico* algorithms predict this variant would be detrimental to the protein function and/or structure, suggesting this variant may interfere with the assembly of type II collagen molecules, leading to skeletal abnormalities characteristic of PLSDT. **Conclusions:** Unlike previously reported PLSDT disease-causing variants clustered in the last two exons, we identified a novel *COL2A1* missense variant in exon 51 associated with PLSDT. Our findings further strengthen the association of an altered *COL2A1* C-propeptide domain with PLSDT.

G011. **Eighth Case of Neurodevelopmental Disorder with Central Hypotonia and Dysmorphic Faces (NEDCHF) Caused by Missense Variant Outside *HDAC4* Binding Motif**

J. Lopes[1], C. Nagaraj[2], B. Simpson[2]
[1]Cincinnati Children's Hospital, Villa Hills, KY; [2]Cincinnati Children's Hospital, Cincinnati, OH.

Introduction: A 14-year-old male presented to clinic for unprovoked frequent falls in context of presumed cerebral palsy (negative brain MRI), distal muscle weakness, fatigue, learning concerns, dysphagia, bicuspid aortic valve, and excessive weight gain. The patient indicated he had no warning signs for the falls except for occasional dizziness. Electroencephalogram was negative for seizures without spell capture during monitoring. Before a trio whole-exome sequencing (WES) was considered, neuromuscular and neuropathy panel testing were non-diagnostic. **Methods:** WES was performed on genomic DNA with the Human Comprehensive Exome kit from Twist Bioscience using an Illumina sequencing system with paired end reads at a minimum coverage of 20X of 95% of the target regions. Sequences were aligned to the human reference genome (build UCSC hg19) with BWA-mem. Variants were called using GATK. Variant call files were uploaded to the Fabric Genomics Analysis platform for annotation, analysis, and classification. **Results:** A *de novo* heterozygous missense variant, c.1979C >T p.(Thr660Met) was identified in *HDAC4*, a gene associated with recently described neurodevelopmental disorder with central hypotonia and dysmorphic faces (NEDCHF, OMIM #605314). This variant is absent from publicly available population databases (1000 Genomes, ESP, or gnomAD) and has not been reported in HGMD, ClinVar, or peer-reviewed published literature. *In silico* analyses predict this missense alteration as damaging, and the *HDAC4* gene is considered constrained for missense variation. This variant is likely pathogenic according to ACMG criteria. His five-year-old sister with a history of acid reflux, constipation, possible ADHD, reactive airway disease, insomnia, falls, and leg pain was negative for this variant. **Conclusions:** NEDCHF was described in a 2021 case series of seven affected individuals (Wakeling, *et al*., 2021). Clinical features include intellectual disability, hypotonia, speech delay, and difficulty or inability to walk. One patient reported “drop attacks” beginning in adolescence in the setting of epilepsy. Additional overlapping findings with our proband include subtle cerebral atrophy, scoliosis, dysphagia, sleep disturbance, visual problems, full lower lip, and long palpebral fissures. Like our patient, all previously reported variants were found to occur *de novo.* However, the reported variants each reside in a key *HDAC4* binding motif, whereas c.1979C >T lies outside of this domain. To our knowledge, this case represents the first to indicate pathogenicity of *HDAC4* variants outside of its 14-3-3 binding in the context of this novel syndrome. It is possible that variants outside of this domain may lead to a less severe phenotype, as observed in our case.

G012. **C282Y, H63D, and S65C *HFE* Gene Mutation Testing at Geisinger: A Five-Year Retrospective Study and Workflow Improvement**

H. Kavus, Y. Ding, A. Campbell, J. Kershner, B. Knock, L. Scicchitano, M. Scicchitano
Geisinger Medical Center, Danville, PA.

Introduction: Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism and is characterized by an accelerated rate of intestinal iron absorption. *HFE* gene mutations are the most common cause and account for up to 85% of HH patients. *HFE* C282Y and H63D mutations are widely studied, whereas data for S65C are inconclusive. We assessed the clinical utility of all three mutations in *HFE* at Geisinger Health System over the past five years and lessons learned in workflow improvement. **Methods:** The cohort comprised 1,656 individuals, which represent all *HFE* mutation testing done at Geisinger Molecular Diagnostic Laboratory since 2018. The study used either multiplex PCR followed by multiplexed primer extension for mass-modified base labeling (MassARRAY) or real-time qPCR (7500FAST) methods. Patients tested as part of research or clinical trials were excluded. The clinical indication of *HFE* mutation

testing includes chronically elevated serum ferritin and transferrin saturation levels with the exclusion of inflammatory conditions and alcohol abuse. A workflow analysis identified the improvement opportunity, which resulted in test revalidation and platform change from MassARRAY to 7500FAST in January 2021. For test validation, a total of 75 samples including 68 previously known patient specimens and seven CAP proficiency testing were included. Sanger sequencing was used for genotype confirmation. **Results:** A total of 52.0% (861/1,656) of patients were positive for *HFE* mutation(s). The most common mutation is heterozygous H63D with 22.8% (378/1,656) followed by heterozygous C282Y 12.6% (208/1,656), compound C282Y/H63D heterozygous 6.6% (109/1,656), homozygous C282Y 5.0% (82/1,656), homozygous H63D 2.6% (43/1,656), heterozygous S65C 1.7% (28/1,656), compound H63D/S65C heterozygous 0.4% (7/1,656), and compound C282Y/S65C heterozygous 0.4% (6/1,656), which was the least common genotype in this cohort. Besides workflow time, *HFE* allelic dropout in patients with compound H63D/S65C heterozygous was identified in four cases that were previously genotyped by MassARRAY as compound homozygous H63D and heterozygous S65C. Risk management and clinical providers of these four patients were contacted. Based on no significant management impact, an additional *HFE* mutation test was obtained for these patients. **Conclusions:** Although *HFE* C282Y is generally associated with more severe clinical presentation, detection of H63D and S65C mutations in *HFE* is clinically valuable by adding additional information to the diagnosis of HH and treatment. The analytical assessments between MassARRAY and real-time qPCR were comparable for inter-run reproducibility, limit of detection, sensitivity, and specificity, whereas real-time qPCR has improved accuracy, improved workflow as well as decreased hands-on time.

G013. **Clinical Laboratory Experience of Hereditary Cancer Panel Test in a Single Center**

E. You[1], H. Kim[1], J. Lee[1], J. Shin[1], H. Kim[1], K. Park[2]
[1]Inje University College of Medicine, Busan Paik Hospital, Busan, Republic of Korea; [2]Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, Republic of Korea.

Introduction: The use of multigene testing panels to provide comprehensive analysis of cancer susceptible genes has proven to be a viable option. Through hereditary cancer panel testing, they can provide an opportunity to apply targeted therapies to patients and contribute to the control of cancer occurrence in families. The purpose of this study is to retrospectively analyze the prescription status and results after the implementation of the hereditary cancer panel test in a single center. **Methods:** A total of 215 hereditary cancer panel test results requested between July 2020 and June 2022 at Inje University Busan Paik Hospital were included. We used a custom target next-generation sequencing 29-gene panel. Analysis was performed on an Ion Torrent S5 XL and Ion Reporter Software (Thermo Fisher Scientific). **Results:** The hereditary cancer panel test was performed in 215 cases for about two years, and the requested departments were obstetrics and gynecology (155 cases), breast surgery (51 cases), colorectal and anal surgery (five cases), and gastroenterology (five cases). The patient group consisted of 211 females and four males, with an average age of 53 years (19 to 79 years). The diseases of the patients were 126 of gynecological malignancies (58.6%) such as ovarian, endometrial, and cervical cancer, 73 of breast cancer (23.9%), 10 of colorectal cancer (4.7%), and six of multiple cancers (2.8%). Among the 215 patients, 7.9% were found to carry at least one pathogenic (PV) or likely pathogenic (LPV) variant. A total of 247 variants were detected, 11 PVs (4.5%), six LPVs (2.4%), and 230 VUSs (93.1%). Disease-related mutations were identified in 15 patients with breast cancer or gynecologic malignancies (7.3%). Four mutations were identified in the *BRCA2* gene, three in *BRCA1*, two in *ATM*, and one each in *PTEN, NF1, CHEK2, RAD51C*, *RAD51D*, and *MLH1*. PV and LPV were identified in two out of 10 colon cancer patients (20%), and *PTEN* and *MLH1* mutations were identified. Among the 29 genes included, the most frequent gene with VUS was identified in the order of *MUTYH, PALB2, ATM*, and *BRCA1*. **Conclusions:** This study was conducted at a single center and has a limitation in that the number of tests is small. However, compared with previous studies, it was confirmed that the detection frequency of pathogenic mutations was similar. This analysis revealed very limited clinical departments using this panel test. This suggests the need to provide test information to hereditary cancer-related clinical departments that do not prescribe this test. Also, since most of the mutations were VUS, periodic reclassification is considered necessary. Hereditary cancer panel testing has been confirmed to play an important role in identifying disease-associated mutations in real-world clinical settings.

G014. **Significance of Exon 327 in Titin Truncating Variants in Dilated Cardiomyopathy: Comparison with Population Database and Relevance to Reduced Nonsense-Mediated mRNA Decay Efficiency**

C. Ha, C. Ha, Y. Kim, S. Shin, J. Park, J. Jang, J. Kim
Samsung Medical Center, Seoul, Republic of Korea.

Introduction: Titin truncating variants (TTNtvs) are the most frequent genetic cause of dilated cardiomyopathy (DCM). Within the *TTN* gene, A-band has been repeatedly reported as a hotspot of DCM-causing TTNtvs, whereas only a few studies reported that exon 327, within A-band, is the most frequently mutated *TTN* exon. We aimed to reassess the TTNtv enrichment degree of the four domains of *TTN* and exon 327 regardless of the effect of confounding variables, including length of the region and level of expression, to find out whether exon 327 is the real hotspot of TTNtv. In addition, since exon 327 is by far the longest exon in *TTN* and the dominant negative mechanism recently evidenced, we tried to determine whether the exon 327 clustering of TTNtv in DCM patients is the consequence of reduced nonsense-mediated mRNA decay (NMD) efficiency caused by the long premature termination codon-to-intron (PTC-to-intron) distance. **Methods:** Research papers regarding TTNtv and DCM were searched and TTNtvs found in DCM patients were collected. Required information such as proportion-splice-in value and location of PTC were annotated using public data. In the four domains of *TTN* and exon 327, the density of TTNtvs was calculated in a way that the effect of the distribution of highly expressed exons (PSI >0.9) was normalized. Calculated TTNtv density of each region was compared between the DCM and control groups (gnomAD ver. 2.1.1). Utilizing cases whose RNA-seq results were available, the correlation between PTC-to-intron distance and observed level of NMD was calculated. **Results:** A total of 672 TTNtvs from DCM patients were obtained from 10 studies. Based on the density calculated, A-band TTNtv was significantly more frequent in the DCM group than in the control group ($P = 0.0026$). However, the difference was even greater in exon 327 ($P = 4.953*10^{-21}$), and the frequency of non-exon 327 A-band TTNtvs was comparable in the DCM group and the control group ($P = 0.2836$). Review of published RNA-seq data revealed that PTC-to-intron distance was positively correlated with the level of expression of altered allele, which is a surrogate for the level of NMD escape ($P = 0.0440$). Different PTC-to-intron distance was observed along with population database allele frequency ($P = 0.0212$). Among exon 327 TTNtvs, expression level of altered allele was negatively correlated with heart transplantation age ($P = 0.0257$). Mean altered allele frequency of exon 327 TTNtvs was 0.46, which was close to 0.5, the value indicating no NMD. **Conclusions:** Our results confirmed the significant enrichment of DCM-related TTNtvs in exon 327. Widely accepted A-band clustering is mostly attributable to exon 327. Reduced NMD efficiency may play an important role in the pathogenesis of TTNtvs in DCM, especially in exon 327.

G015. **Comprehensive Genomic Characterization of Congenital and Infantile Cancers Reveals High Yield of Medically Meaningful Findings**

M. Mathew, B. Bucknor, K. Schieffer, E. Varga, S. Choi, K. Miller, B. Kelly, P. White, R. Wilson, E. Mardis, C. Cottrell
Nationwide Children's Hospital, Columbus, OH.

Introduction: In the setting of a pediatric tertiary care hospital, we have established a patient-focused translational protocol supporting genomic profiling of children, adolescents, and young adults with rare or treatment refractory cancer. Infantile and congenital cancers represent a unique cohort among our study group on the basis of disease spectrum and complexity, and associated comorbidities in this medically fragile population. Comprehensive genomic profiling has been initiated to aid in diagnosis, prognostication, treatment, and detection of germline disease predisposition in patients with rare and difficult-to-treat tumors. **Methods:** Enhanced exome sequencing of disease and comparator tissue was coupled with RNA sequencing of the disease-involved specimen to assess for single nucleotide variation (SNV), insertions/deletions (indels), copy number alteration (CNA), structural variation (SV), fusions, and gene expression. Additional methodologies were applied where applicable, including tumor classification by DNA-based methylation, long-read sequencing, targeted Sanger and immune profiling. **Results:** Between 2018-2022, 398 patients were consented onto the protocol with genomic profiling and analysis completed in 373. Among analyzed patients, 40 (11%) were infantile cancers diagnosed at ≤1 year of age, and of those, 15 were congenital, diagnosed at ≤3 months of age. In total, the infantile/congenital cohort consisted of 16 females and 24 males, who among them harbored 21 central nervous system tumors, 17 solid tumors (six sarcoma, three embryonal, eight other), and two hematologic diseases. Germline genetic alteration was frequent among the infantile/congenital cohort with 12/40 patients (30%) harboring a pathogenic change in a tumor suppressor (n=11) or oncogene (n=1). RNA analysis yielded gene fusion events in 25% (10/40) of the cohort. A medically relevant somatic SNV/indel or CNA event was detected among 75% of the cohort (n=30) using exome sequencing. In total, a medically meaningful finding impacting diagnosis, prognosis, therapy, or surveillance was identified in 36/40 patients (90%) ≤1 year of age within our study. **Conclusions:** Genomic alterations represent a fundamental basis of cancer. Inclusion of germline analysis and RNA sequencing data was of particular utility for disease characterization in this infantile/congenital cancer cohort. These data support that a pediatric cohort gains greater potential benefit from a comprehensive profiling approach than by analysis of a single analyte alone, with a high yield of medically meaningful findings in an infantile/congenital cancer population.

G016. **Enhancer-Promoter Interactome Drives Neuronal Cell Differentiation and Contextualizes Alzheimer's-Associated Polymorphisms**

C. Padilla, M. Bhakta, M. Blanchette, L. Munding
Cantata Bio, Scotts Valley, CA.

Introduction: Chromatin immunoprecipitation, coupled with sequencing, has shaped our understanding of how transcriptional machinery interacts with genomes to facilitate gene regulation. These protein-DNA interactions are fundamental components of primary-feature epigenetic signatures that are informative across development, gene regulation, and disease research. A remaining challenge is that these data depict protein anchors in linear space and do not consider the complex and hierarchical DNA folding packaged in chromatin and further compacted into a nucleus. **Methods:** Here we combine chromatin immunoprecipitation with Hi-C in a single assay, known as HiChIP, to describe the interactome associated with protein anchors. We use the protein-directed interactome to assess the key enhancer-promoter dynamics at key pluripotency maintenance factors, *NANOG* and *OCT4*. Furthermore, the interactome is used to annotate Alzheimer's-associated SNPs at distal regulatory elements. **Results:** By comparing the interactome of H3K27ac (enhancers), promoters (H3K4me3), and topological boundaries (CTCF) in iPSC and NSC, we can describe the different transcriptional landscape of the essential genes involved with pluripotency maintenance, *NANOG* and *OCT4*. We then investigate the enhancer interactome of NSC with regard to Alzheimer's polymorphisms identified through genome-wide association studies, enabling the annotation of distal SNPs at enhancer sites greater than 50 kbp away to the APOE promoter. **Conclusions:** These results posit that SNPs occurring at enhancer kilobases away from a gene promoter could impact gene regulation. These data introduce an additional dimension to ChIP-seq experiments to more completely describe the mechanism of gene activation and provide a more robust annotation of both protein anchors to their target gene as well as disease-associated polymorphisms.

G017. **Characterization of Reference Materials for *TPMT* and *NUDT15*: A GeT-RM Collaborative Project**

V. Pratt[1], W. Wang[3], E. Boone[3], U. Broeckel[4], N. Cody[5], L. Edelmann[5], A. Gaedigk[3], T. Lynnes[6], E. Medeiros[6], M. Mitchell[7], A. Moyer[8], S. Scott[9], P. Starostik[10], A. Turner[4], L. Kalman[2]
[1]Optum, Indianapolis, IN; [2]Centers for Disease Control and Prevention, Atlanta, GA; [3]Children's Mercy Kansas City, Kansas City, MO; [4]RPRD Diagnostics, Milwaukee, WI; [5]Sema4, Stamford, CT; [6]Indiana University School of Medicine, Indianapolis, IN; [7]Coriell Institute for Medical Research, Camden, NJ; [8]Mayo Clinic, Rochester, MN; [9]Stanford University, Palo Alto, CA; [10]University of Florida College of Medicine, Gainesville, FL.

Introduction: Pharmacogenetic testing for *TPMT* and *NUDT15* alleles can be used to predict a patient's risk for myelosuppression when prescribed thiopurines. Test results can guide drug choice and dosing, which may help to prevent myelosuppression or other adverse events. Pharmacogenetic testing is increasingly provided by clinical and research laboratories; however, only a limited number of quality control and reference materials are currently available for many of the *TPMT* and *NUDT15* variants included in clinical tests. To address this need, the Division of Laboratory Systems, Centers for Disease Control and Prevention-based Genetic Testing Reference Material Coordination Program (GeT-RM), in collaboration with members of the pharmacogenetic testing and research communities and the Coriell Institute for Medical Research, has characterized 19 reference material samples that are available through Coriell. **Methods:** DNA samples from 19 cell lines were distributed to four volunteer testing laboratories for genotyping. Laboratories used a variety of commercially available and laboratory-developed tests including exome and whole-genome sequencing, as well as Sanger sequencing and long read phasing. Pre-existing data from two additional laboratories were also used as part of this study. **Results:** Of the 12 samples characterized for *TPMT*, newly identified variants include *TPMT*2, *6, *12, *16, *21, *24, *32, *33,* and **40*. Newly identified variants for the seven *NUDT15* reference material samples are *NUDT15*2, *3, *4, *5, *6,* and **9*. In addition, a novel haplotype, *TPMT*46*, was identified in this study. Pre-existing data for an additional 11 Coriell samples allowed us to create comprehensive reference material panels for *TPMT* and *NUDT15*. Taken together, GeT-RM provides information on the 30 characterized, publicly available genomic DNA reference materials for the following alleles: *TPMT*2, *3A, *3C, *6, *8, *12, *16, *21, *24, *32, *33, *40,* and **46* (18 samples) and *NUDT15*2, *3, *4, *5, *6,* and **9* (12 samples). **Conclusions:** The reference materials for *TPMT* and *NUDT15* generated by this study support the development of consensus recommendations for these genes by the Association for Molecular Pathology Pharmacogenetics Work Group, which has developed recommendations for alleles that should be included in clinical testing using a tiered-based system. These publicly available and well-characterized genomic DNA reference materials can be used to support the quality assurance and quality control programs of clinical laboratories performing pharmacogenetic testing.

G018. **When Pharmacogenetics TaqMan Genotyping Is Led Astray: Presence of rs58973490 in *CYP2C19* Causes a False-Positive *CYP2C9*8* Genotyping Result on TaqMan OpenArray**

R. Ly[1], C. Granfield[1], T. Lynnes[1], E. Medeiros[1], T. Shugg[1], T. Skaar[1], V. Pratt[2], J. Hodge[1]
[1]Indiana University School of Medicine, Indianapolis, IN; [2]Optum Genomics, Indianapolis, IN.

Introduction: *CYP2C9* is important in the metabolism of drugs, such as warfarin and phenytoin. Genetic variation in *CYP2C9* can cause inter-individual differences in drug response and adverse events. *CYP2C9* is located on chromosome 10 within the *CYP2C* locus near the highly homologous *CYP2C8*, *CYP2C18*, and *CYP2C19* genes. *CYP2C9* genotyping using TaqMan assays requires specific primers to avoid off-target amplification, especially of the nearby *CYP2C19* gene. The computational tool Aldy was evaluated for pharmacogenetic genotyping from clinical exome sequencing (ES) data. A discrepancy in *CYP2C9*8* (c.449G >A, p.R150H, rs7900194) genotyping results between ES and OpenArray targeted genotyping platform was identified in two individuals. For both individuals, Aldy results indicated *CYP2C9*1/*1*, whereas the OpenArray results indicated *CYP2C9*1/*8*. Sanger sequencing confirmed that both individuals were *CYP2C9*1/*1*. Given the high homology between *CYP2C9* and *CYP2C19*, we hypothesized that a variant in *CYP2C19* is responsible for the false-positive TaqMan OpenArray *CYP2C9*8* genotyping result. **Methods:** BAM files were used to analyze the *CYP2C9* and *CYP2C19* ES data. Germline DNA isolated from whole blood was genotyped using individual TaqMan assays for *CYP2C9*8* (C__25625804_10) and *CYP2C19*11* (C__30634129_20). **Results:** Review of *CYP2C19* genotyping from ES revealed these individuals to be heterozygous for *CYP2C19*11* (c.449G >A, p.R150H, rs58973490), which has the same change as *CYP2C9*8* (c.449G >A, p.R150H, rs7900194). Amplification curves from the TaqMan OpenArray revealed altered *CYP2C9*8* amplification in individuals who were heterozygous for rs58973490 compared to those who were not. We also identified additional individuals who had false-positive TaqMan OpenArray *CYP2C9*8* calls; they had similar amplification curves and were found to be heterozygous for *CYP2C19*11*. When tested in a 96-well format TaqMan assay, the same individuals were found to be wild-type for *CYP2C9*8* with no altered amplification. **Conclusions:** False-positive TaqMan *CYP2C9*8* genotyping on OpenArray can occur in individuals who are carriers for *CYP2C19*11*, resulting in incorrect prediction of *CYP2C9* metabolizer status; this could affect drug treatment decisions. rs58973490, which defines the *CYP2C19*11* haplotype, can also be found in the *CYP2C19*2* haplotype as the *CYP2C19*2.010* sub-allele. rs58973490 interference should be considered when testing for *CYP2C9*8* on OpenArray by including appropriate controls to ensure accurate *CYP2C9* calling. This false-positive result can occur in an assay-specific manner and should be considered during assay development and validation.

G019. **Determination of DNA-Incorporated 6-Thioguanine and Erythrocyte Thioguanine Nucleotide in Relation to *TPMT* and *NUDT15* Genotypes in Acute Lymphoblastic Leukemia**

J. Kim[1], R. Choi[2], M. Chun[1], H. Ju[1], H. Koo[1], J. Lee[1], S. Lee[1]
[1]Samsung Medical Center, Seoul, Republic of Korea; [2]Green Cross Laboratories, Yongin, Republic of Korea.

Introduction: Large inter-individual variations in drug metabolism pose a challenge in monitoring therapeutic effect and life-threatening cytotoxicity of thiopurine drugs during treatment of acute lymphoblastic leukemia (ALL). DNA-incorporated 6-thioguanine (DNA-TG), the last product of 6-mercaptopurine (6MP) metabolism, has been suggested to reflect the efficacy of 6MP, in particular with patients harboring variants in the 6MP metabolism pathway. In this study, we explored the DNA-TG levels during 6MP therapy in Korean pediatric patients with ALL in relation to erythrocyte levels of the metabolite (RBC-TGN), and investigated the clinical applicability of DNA-TG monitoring according to *TPMT* and *NUDT15* genotypes. **Methods:** We investigated DNA-TG and RBC-TGN levels in a total of 911 blood samples from 112 pediatric ALL patients under 6MP maintenance therapy. DNA-TG (extracted from leukocytes) and RBC-TGN levels were measured simultaneously in patient samples using liquid chromatography-tandem mass spectrometry. Genotyping of *TPMT* and *NUDT15* was performed before the start of the 6MP therapy. PCR and direct sequencing of all exons of *TPMT* and *NUDT15* genes were performed. **Results:** The median DNA-TG level was 125.5 (interquartile range [IQR], 84.1–184.4) fmol TG/μg DNA, and the median RBC-TGN level was 233.9 (IQR, 163.1–333.1) pmol/8x10^8 RBCs. Thiopurine metabolite was measured at a median of 7.0 (range, 1–26) times per patient. Among the 112 pediatric patients, 87 had wild-types for both *TMPT* and *NUDT15,* three patients had *TPMT*3* (intermediate metabolizer), and 22 had at least one variant *NUDT15* allele (15 intermediate metabolizer, one poor metabolizer, and six indeterminate phenotype). DNA-TG and RBC-TGN levels showed strong correlation in all 911 measurements ($\rho = 0.7053$, $P < 0.0001$). The DNA-TG/RBC-TGN ratio was significantly higher in patients with *NUDT15* variant than in those with wild-type alleles (0.74 vs. 0.47, $P < 0.0001$). **Conclusions:** The DNA-TG/RBC-TGN ratios reflect the genotypic status of *TPMT* and *NUDT15*, particularly in patients with *NUDT15* variants showing significantly higher DNA-TG/RBC-TGN ratios than those with wild-type alleles. Further studies would be needed to clarify the relationships between DNA-TG and *TPMT* and *NUDT15* genotypes, as DNA-TG could be a useful marker in therapeutic drug monitoring for thiopurine treatment.

G020. **Validation and Performance of Pharmacogenomics Panel Using MassARRAY System**

S. Harada, J. Holliday, B. Davis, D. Morlote, N. Singh, G. DeFrank, A. Mackinnon
University of Alabama at Birmingham, Birmingham, AL.

Introduction: Variation in genes involved in drug-related processes, including drug target receptors, metabolizing enzymes, and drug transporters, influences individual response to medications across drug classes (e.g., antidepressants, antipsychotics, antithrombotic agents, opioids, etc.). The strength of the evidence supporting these associations has led to the development of guidelines for prescribers based on genetic variants. However, genotype-guided therapy is not widely accepted in clinical practice. There are several different methodologies to test these genotypes such as next-generation sequencing (NGS) or real-time PCR. NGS is expensive, laborious, and requires complicated bioinformatics analysis. The MassARRAY system provides accurate, low-cost, facile, multiplexed analysis of hundreds of clinically relevant mutations with relatively simple analytics. In this study, we evaluated the utility of this system for germline variants (single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels) found in drug-related genes as part of a pharmacogenomics (PGx) assay. **Methods:** The PGx custom panel targets 95 SNPs/small indels and several copy number variants in 29 genes implicated in drug-related processes. Genomic DNA is extracted from peripheral blood samples using Ion Torrent Genexus Purification System when needed. Genetic targets are PCR amplified followed by primer extension using allele-specific, specialized primers. Allotypes are detected by the MassARRAY system and results are generated using the MassArray software (the Typer). **Results:** Forty-eight Coriell samples with known various genotypes, four CAP survey samples and blood samples from 12 volunteers were analyzed using the PGx assay. Each assay has a total of 95 alleles to evaluate. Of 48 Coriell samples with a total of 4,560 alleles, 3,689 alleles have both reference information and MassARRAY data, of which 3,673 are concordant, making 99.57% concordance. Allele type calls for key genes (*CYP2C19, CYP2D6, SLCO1B1, VKORC1,* and *CYP2C9*) are 100% concordant when allele type was called. Reproducibility was demonstrated using seven Coriell samples, two intra-run and two inter-run, with all in agreement. Different DNA inputs (1, 2, 5, and 10 ng) were tested to determine the limit of detection. Both 5 and 10 ng input resulted in 100% concordance without no call, whereas 1 and 2 ng input had some drop out. Overall, conservative estimate of the limit of detection is 5 ng. **Conclusions:** Overall, the MassARRAY system is

reliable, cost effective, and easy to operate. Performance is consistent and analysis is streamlined. The PGx assay could facilitate utilization of genotype-guided therapy in a clinical setting. Furthermore, the MassARRAY platform has broad application across multiple indications for molecular diagnostic testing.

G021. **Comprehensive Genomic Profiling to Examine Predisposition to Fluoropyrimidine Toxicity Caused by Germline *DPYD* Variants in Cancer Patients**

S. Szelinger[1], J. Aldrich[2], G. Basu[1], M. Evans[2], M. Halbert[2], D. Hall[2], J. Hoag[2], S. Thakkar[1], J. Lobello[1]
[1]Exact Sciences, Phoenix, AZ; [2]Exact Sciences, Redwood City, CA.

Introduction: Fluoropyrimidines such as 5-fluorouracil and capecitabine are used routinely in the management of colorectal cancer (CRC), breast, pancreatic, and head and neck carcinomas. Some patients suffer severe adverse events due to reduced activity of their dihydropyrimidine dehydrogenase (DPD) enzyme, which is encoded by the *DPYD* gene and acts to detoxify these cytotoxic agents. Several *DPYD* variants reduce DPD function and *DPYD* genotyping can thus be used to alert clinicians to guideline-based chemotherapy dosage adjustment. Here we describe the potential utility of a tumor-normal comprehensive genomic profiling assay, Oncomap ExTra, to detect clinically and functionally relevant *DPYD* alleles in the germline of cancer patients who received tumor profiling results. **Methods:** In this retrospective study, 1,563 cancer patients who received Oncomap ExTra testing were randomly selected and germline *DPYD* variants covered by the assay were identified. Covered variants were cross-referenced with 83 variants currently annotated in the Clinical Pharmacogenetics Implementation Consortium (CPIC) database. Variant status and associated enzyme activities were assigned to each patient, who was then classified as normal, intermediate, or poor metabolizers. Intermediate metabolizers are likely susceptible to fluoropyrimidine toxicity. **Results:** The Oncomap ExTra assay can detect 82 of the 83 CPIC-listed *DPYD* variants with an average coverage of >100X. Among the 1,563 patients, 28 different CPIC alleles were identified: 23 normal function, two decreased function, and three non-functional. There were 31 genotypes in 174 (11.2%) patients that were not in the CPIC database, which prevented assignment of metabolic activity. Metabolic activity could be assigned to the other 1,389 (88.8%) patients: 1,355 (86.6%) were normal, and 34 (2.2%) were intermediate metabolizers. No poor metabolizers were identified in the cohort. There were two (6.3%) head-and-neck, two (3.6%) pancreatic, five (2.4%) CRC, and 12 (2.3%) breast cancer patients who were intermediate metabolizers. **Conclusions:** The Oncomap ExTra assay can identify 82 of 83 CPIC-listed *DPYD* variants and was able to classify 2.2% of cancer patients as intermediate metabolizers and thus susceptible, or likely susceptible, to fluoropyrimidine toxicity. Comprehensive tumor-normal profiling assays could potentially be utilized to identify both tumor-specific mutations and germline variants that indicate possible treatment toxicity, further helping to guide personalized treatment choices when fluoropyrimidines are considered in the advanced cancer setting.

G022. **Landscape of Clinically Significant *DPYD* Mutations in Indian Patients**

R. Iyer, O. Shetty, N. Karnik, G. Wagh, T. Pai, R. Rumde, S. Hathkar, S. Rane, R. Kumar, S. Epari, S. Desai
Tata Memorial Hospital, Parel Mumbai, Maharashtra, India.

Introduction: Mutations and polymorphisms in the Dihydropyrimidine Dehydrogenase (*DPYD*) gene lead to intolerance to 5-fluorouracil (5-FU) causing toxicity build up. Partial or complete deficiency of the *DPYD* gene is found to be variable in Caucasians, White population, Africans, and South Asians due to ethnic differences. The Clinical Pharmacogenetics Implementation Consortium (CPIC) 2017 update has recommended dosing guidelines based on the allele status of the *DPYD* gene. Four mutations, *DPYD*2A* (c.1905+1G >A), *DPYD*13* (c.1679T >G), *DPYD*949* (c.2846A >T), and *HapB3* (c.1129-5923C >G), have been identified as clinically significant to predict toxicities. The prevalence of these four mutations has been very sparsely documented among the Indian population. The present study explores the frequency of these four mutations in Indian cancer patients. **Methods:** A total of 1,418 samples tested for *DPYD* allele status at Molecular Pathology Laboratory, Tata Memorial Hospital, Mumbai, India, during the period from May 2020 to May 2022. Peripheral blood samples of patients were used for extraction of DNA and real-time PCR assay was set up using DIATECH EASY DPYD kit having TaqMan probe labelled specific primers for *DPYD*2A, DPYD*13, DPYD*949*, and *HapB3* domains. The assay was performed on ABI Quant Studio 12K Flex with appropriate controls, followed by data analysis using expression suite software. **Results:** A majority of samples were from head and neck cancer patients (n=814) followed by gastric cancer (n=450), lung cancer (n=124), breast cancer (n=13), gynaecology and urology cancer groups (n=4 each), and one each from neurology and bone and soft tissue cancer groups. Of the 1,418 patients tested, no significant mutations were identified in 1,368 (96%) patients. Forty-one patients showed heterozygous *HapB3* mutation, and two patients showed homozygous mutation. *DPYD*2A* mutation was positive in six patients, and only one patient was positive for *DPYD*949* allele. Two patients having *DPYD*2A* mutations had mucositis and hand and foot syndrome, whereas no 5-FU was provided to the other four patients after assessing the *DPYD* status. Dosage was reduced to 50% for the patient with *DPYD*949* allele status. Twelve patients harbouring *HapB3* mutations were given a reduced dose of 5-FU, as they showed toxicities of grade 2/3 diarrhoea, grade 1 mucositis, and grade 3 neutropenia. **Conclusions:** Genotyping of *DPYD* prior to 5-FU treatment is beneficial to ascertain the toxicity tolerance associated with the drug. Occurrence of clinically significant variants for *DPYD* is found to be low in Indian patients.

G023. **Next-Generation Sequencing (NGS) Testing Trends among Adult Cancer Patients with Select Advanced Tumor Types: A Real-World Evidence Evaluation**

A. Ferreria-Gonzalez[1], B. Hocum[2], G. Ko[2], S. Shuvo[2], S. Appukkuttan[2], S. Grabich[3], T. Kozlek[4]
[1]Virginia Commonwealth University, Richmond, VA; [2]Bayer, Whippany, NJ; [3]Xcenda, Chapel Hill, NC; [4]Bayer, Brookline, MA.

Introduction: Wider adoption of next-generation sequencing (NGS) testing increases targeted care and understanding of underlying mechanisms improving survival, potentially reducing cost. Evaluation of NGS biomarker testing across cancerous tumor types known to harbor actionable gene mutations is key to understanding utilization in the changing policy and treatment landscape. **Methods:** A retrospective cohort study was performed using administrative health claims data from Optum Clinformatics Data Mart. Inclusion for patients aged ≥18 years included ≥2 diagnoses for non-small cell lung cancer (NSCLC), soft tissue sarcoma (STS), melanoma, head and neck cancer, thyroid carcinoma, colorectal cancers (CRC), or non-secretory breast cancer (BC) indexed on first metastatic claim between 1/1/2015-3/31/2021. In the same timeframe, patients with primary central nervous system (CNS) tumors were indexed on the first of ≥2 claims. NGS panel tests were identified using Current Procedural Terminology codes. Outcomes by tumor type included NGS testing rate, time to first NGS test, and annual prevalence of NGS testing each year of study period and across key guideline/therapy timeframes. Data were summarized descriptively and Cox proportional hazards calculated for time to NGS. **Results:** A total of 26,086 BC, 14,620 NSCLC, 9,538 CRC, 5,835 CNS, 2,751 thyroid, 2,360 head and neck, 1,740 melanoma, and 279 STS patients met the inclusion criteria. All tumor types experienced an increase in NGS testing prevalence between 2015 and 2021. For example, NGS testing among patients with BC was 0.1% for those indexed in 2015 and increased to 0.8% in 2021. Among patients with NSCLC, NGS testing increased from 1.5% in 2015 to 8.8% in 2020. In the earliest key period (1/1/1015-8/31/2016), 3.3% of NSCLC patients and 0.2% of BC patients had an NGS test. When UnitedHealthcare expanded NGS testing (9/1/2016-3/31/2018), NGS use increased in NSCLC patients (6.4%) but remained low among BC patients (0.1%). After Centers for

Medicare & Medicaid Services recommended NGS use in diagnostics (4/1/2018-11/25/2018), NGS rates increased to 7.0% among NSCLC patients and 0.4% in BC patients. After the FDA approved larotrectinib (11/26/2018-6/30/2021), 9.1% of NSCLC patients and 0.6% of BC patients received an NGS test during the period. The median time from index to NGS test was 7.9 months in patients with BC and 1.4 months in patients with NSCLC. In adjusted models, age, region, and Charlson Comorbidity Index were significant predictors ($p < 0.05$) of time to NGS test in patients with NSCLC, but not BC. **Conclusions:** NGS test use increased across all tumor types assessed during the study period and in relation to key guideline changes but remained quite low given the potential benefits of biomarker-based targeted therapy.

G024. **Somatic Synonymous Variants (Silent Mutations) Are Highly Enriched in Hypermethylated or Hypomethylated CpG Sites Compared to Missense Variants**

J. Dermawan, L. Villafania, J. Benhamida, M. Ladanyi, C. Vanderbilt
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: Synonymous variants (silent mutations) are single nucleotide variations in the coding sequence that do not alter amino acid sequence. Despite not resulting in changes in the encoded protein, synonymous variants are known to be positively selected in cancer. Observed in both germline and somatic settings, the biological functions and consequences of synonymous variants are poorly understood and primarily studied in splice sites. To date, the association between synonymous variants and methylation in human cancers has not been studied. **Methods:** We interrogated somatic silent variants from variant call format files of all solid tumors profiled by MSK-IMPACT, a targeted next-generation DNA sequencing panel. Inclusion criteria included a minimum tumor variant allele frequency (VAF) of 5%, a tumor-to-normal VAF ratio >2, and a minimum tumor alternate read count of 8. Of these, 195 cases over different tumor types (gliomas, colorectal carcinomas, endometrial carcinomas, esophagogastric carcinomas) also underwent methylation profiling by Illumina EPIC 850k platform. After filtering out single nucleotide polymorphism- and sex chromosome-related probes and keeping only CpG sites annotated as "islands", 123,038 CpG sites remained. Methylation levels of CpG sites were measured by β values (ratio of methylated signal relative to total signal). **Results:** Across 25,881 unique solid tumors profiled by MSK-IMPACT, we identified 166,861 somatic silent variants. We found an inverse relationship between the frequencies of these silent variants and distance from coding start sites and exon boundaries. Since CpG islands are known to be enriched in transcription start sites and exon boundaries, we interrogated the association between silent variants and CpG islands. We found that silent variants that overlap with CpG islands were highly enriched in G>A or C>T transversion mutations. Further, compared to 257,009 somatic missense variants, silent variants were significantly more likely to be located near CpG islands (chi-square $P < 0.0001$). We then matched silent variants to CpG sites by genomic positions. Within the same case, the methylation levels of these CpG sites showed a bimodal distribution: majority being hypermethylated and minority hypomethylated. This bimodal distribution was not observed with random CpG sites. However, on cross-sample comparison, these CpG sites were hyper- or hypomethylated across all cases regardless of the presence of silent variants and tumor types. **Conclusions:** Somatic synonymous variants in solid tumors occur preferentially in CpG islands compared to missense mutations. Compared to other CpG sites, these CpG sites are hyper- or hypomethylated, but the presence of the silent mutation does not affect their methylation status.

G025. **Standardized Assessment of Oncogenicity and Clinical Significance of *NTRK* Fusions**

J. Saliba[1], A. Church[2], A. Danos[1], L. Furtado[3], T. Laetsch[4], V. Nardi[5], L. Zhang[6], S. Golem[7], S. Garin[8], A. Venigalla[1], W. Lin[9], D. Ritter[10], M. Li[4], O. Griffith[1], M. Griffith[1], G. Raca[11], A. Roy[10]
[1]Washington University School of Medicine, St. Louis, MO; [2]Boston Children's Hospital and Harvard Medical School, Boston, MA; [3]St. Jude Children's Research Hospital, Memphis, TN; [4]Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [5]Massachusetts General Hospital and Harvard Medical School, Boston, MA; [6]David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA; [7]University of Kansas Medical Center, Kansas City, KS; [8]University of Maryland School of Medicine, Baltimore, MD; [9]Mayo Clinic Florida, Jacksonville, FL; [10]Baylor College of Medicine and Texas Children's Hospital, Houston, TX; [11]Children's Hospital of Los Angeles, Los Angeles, CA.

Introduction: Gene fusions involving neurotrophic receptor tyrosine kinase genes (*NTRK1*, *NTRK2*, and *NTRK3*) are well-established oncogenic drivers and important diagnostic and therapeutic markers in cancers. Accurate interpretation of their clinical significance is a high priority given FDA approval of TRK inhibitors, but remains challenging due to the rapid pace of new fusion discovery, diversity of fusion partners and tumor types, inconsistent and incomplete reporting of data elements, and lack of standardized classification rules specific for gene fusions. The ClinGen NTRK Fusions Somatic Cancer Variant Curation Expert Panel (SC-VCEP) was formed to address these challenges, including development of *NTRK* fusion-specific oncogenicity rules, and to create a resource of high-quality clinically significant variant assertions in the Clinical Interpretation of Variants in Cancer (CIViC; civicdb.org) knowledgebase. **Methods:** During monthly calls, experts discussed important structural and oncogenic features of *NTRK* fusions and outlined standardized criteria to assess their oncogenicity. To aid development, a list of *NTRK* fusions was aggregated from public fusion databases and member diagnostic laboratories. More than 40 CIViC Evidence Items from more than 30 publications were submitted and revised. *NTRK* fusion classification guidelines were reviewed by the ClinGen Somatic Cancer Clinical Domain Working Group and ClinGen Cancer Variant Interpretation Committee. **Results:** Through the systematic review and compilation of fundamental fusion element annotations, *NTRK* fusions are classified as Oncogenic, Likely Oncogenic, Unknown Significance, or Benign with regards to oncogenicity. Fusion Structure (FS1-3) is evaluated based on partner gene orientation, breakpoint/transcript junction, and reading frame retention. *NTRK* must be the 3' partner and retain the intact tyrosine kinase domain for a fusion to be classified as Oncogenic or Likely Oncogenic. Fusions are further assessed based on: Cancer Association (CA1-3) defined by the number of unique positive cases found in the literature; Clinical Validity (CV1-2) based on reports of objective clinical response to targeted inhibitors; and Functional Status (FS1-2) defined as *in vivo* or *in vitro* evidence of pathway activation and evidence of TRK protein expression. SC-VCEP fusion oncogenicity classification is integrated within the overall AMP/ASCO/CAP guidelines and will be publicly recorded as structured CIViC Oncogenic Assertions. **Conclusions:** The ClinGen NTRK SC-VCEP created a standardized evidence-based scoring and classification system for assessing the oncogenicity of *NTRK* fusions. Our guidelines are being piloted on common, intermediate, and rare *NTRK* fusions to guide refinements and ensure creation of high-quality publicly available clinical interpretations.

G026. **Comparison of *PIK3CA* Genetic Variation Specific to Disorders of Somatic Mosaicism (DoSM) and Variants Associated with Neoplasms**

P. Hernandez, B. Mojarad, M. Evenson, M. Corliss, M. Schroeder, K. Bowling, K. Krysiak, J. Heusel, J. Neidich, Y. Cao
Washington University School of Medicine, St. Louis, MO.

Introduction: Activating variants in the *PIK3CA* gene upregulate the PI3K/AKT/MTOR signaling pathway, contributing to uncontrolled cell proliferation and resistance to cell death. Patients with segmental overgrowth phenotypes that harbor somatic variants in *PIK3CA* have been given the name PROS (*PIK3CA*-related overgrowth spectrum). Many of the same *PIK3CA* variants identified in PROS are also associated with various types of malignancies. Here we review a cohort of cases that underwent clinical testing for disorders of somatic mosaicism (DoSM) to compare with known variants associated with neoplasms. **Methods:** DNA extraction and sequence analysis were performed on affected tissues including fresh tissue; formalin-fixed, paraffin-embedded tissue; fibroblasts; and buccal swabs. Target enrichment was performed using oligonucleotide-based targeted capture of all coding exons and flanking regions for gene variants that cause DoSM, including the gene *PIK3CA*. Sequencing of enriched libraries was performed in multiplex, using the paired-end configuration with an average unique on-target coverage depth of ~2,000x. The detailed descriptions of the DNA isolation, library preparation, sequencing, bioinformatic analyses, and variant interpretation were described in our previous publication. Finally, we compared DoSM *PIK3CA* variants to those observed in COSMIC (cancer.sanger.ac.uk/cosmic) in tumor samples. **Results:** In a cohort of 943 individuals with DoSM, 371 pathogenic or likely pathogenic *PIK3CA* variants were identified across 370 clinical cases with one case harboring two *PIK3CA* variants. We identified 70 unique variants, being 12 small insertions or deletions (indels) and 58 single nucleotide substitutions (SNVs). Although, in general, the helical domain and the kinase domain are known to be most frequently mutated in cancer and DoSM, all *PIK3CA* indel variants identified in this DoSM cohort resided in the P85 adaptor-binding domain, C2 membrane domain, and the extra-domain regions, whereas in tumor samples, they were mostly found in the P85 adaptor-binding domain. Furthermore, we identified seven SNVs and 12 indel *PIK3CA* variants that are frequently mutated in DoSM cases, but are rarely observed in tumors, including Glu453 (23/370) and Cys378 (11/370) in the C2 membrane domain, Gly914 (17/370) in the kinase domain, and Glu726 (16/370) in the extra-domain region. **Conclusions:** This study compared *PIK3CA* variants in neoplasms and cases of DoSM using a large cohort of clinically significant *PIK3CA* variants observed at a single clinical testing center. We identified 19 DoSM-specific *PIK3CA* variants that are rarely observed in cancer, including seven SNVs and 12 indels. This study sheds light on the genotype-phenotype correlation of *PIK3CA*, expanding the knowledge of *PIK3CA* variant contribution to DoSM.

G027. **The Challenges in Classifying Fusion Transcripts**

S. Deans[1], P. Rehal[2], H. Mugalaasi[3], J. Fairley[1]
[1]GenQA, Edinburgh, United Kingdom; [2]Central and South Genomic Laboratory Hub, Birmingham, United Kingdom; [3]The Royal Marsden, London, United Kingdom.

Introduction: The detection of variants through somatic testing requires the classification of the pathogenicity of the variant and interpretation of clinical significance. This is challenging for fusion genes as the prediction of a functional protein must be determined, and with the availability of pan-cancer treatments based on the presence of *NTRK* gene fusions this has become more of a requirement. Education and standardisation are required. External quality assessments (EQAs) for variant interpretation have been provided by GenQA since 2013 and demonstrate variable approaches. The somatic variant EQA 2021 required the classification of an *NTRK2* fusion. **Methods:** The clinical case supplied was a female with an osteosarcoma and lung metastases. Gene fusion testing detected a fusion transcript, *UFD1::NTRK2*, encompassing the whole of *UFD1* exon 6 and the whole of *NTRK2* exon 6. Participants were required to classify the pathogenicity and indicate if the finding would be reported by the laboratory. The results were assessed against peer-ratified criteria. **Results:** The *UFD1::NTRK2* fusion resulted in a transcript with a STOP codon present at the start of the *NTRK2* part of the fusion; a productive *NTRK* fusion is therefore unlikely to be produced. This fusion is described in osteosarcoma and suggested to be non-functional and would be classed as a variant of unknown significance, i.e., AMP/ASCO/CAP Tier III. Thirty-six laboratories participated and six (13%) received critical classification errors. Five of these did not correctly determine the production of a non-functional protein. The remaining classed the fusion as Tier I. Most laboratories (81%) applied the AMP/ASCO/CAP guidelines to classify the variants, either standalone or in combination with other guidance. There were differences in the reporting of variants which are not classification specific. **Conclusions:** The EQA has demonstrated variability in the interpretation of fusions and the need to understand the biological impact of the creation of the fusion with regards to protein function and subsequent predicted effect of the variant. Guidelines are used in different ways and standardisation is required to ensure optimal clinical management is available.

G028. **Loss of *BRD7* Promotes Breast Cancer Lung Metastasis by Reprogramming the Tumor Immune Microenvironment**

J. Mondal, J. Zhang, F. Giancotti, J. Huse
University of Texas MD Anderson Cancer Center, Houston, TX.

Introduction: Thwarting metastasis is regarded as the holy grail in the treatment of cancer. Intriguingly, even though epigenetic alterations are a universal feature of all cancer types, little is known about key epigenetic events that lead to metastasis. Additionally, the mechanism(s) by which the loss of SWI/SNF complex subunits-like Bromodomain-7 (BRD7) induce dormant breast cancer cells to metastasize remain to be elucidated. **Methods:** We established a novel high-throughput *in vivo* screening platform which enables the identification of specific epigenetic entities that regulate metastatic reactivation. RNA-seq enabled the identification of the core signaling pathways that govern metastatic dormancy, too. Furthermore, tumor sphere and invasion assays were performed. To decipher the chromatin accessibility regions regulating metastatic reactivation, ChIP-seq and ATAC-seq were conducted. A magnetic bead-based multiplex cytokine assay informed us about the regulation of cytokines. *In vivo* experiments were performed in the 4TO7-TGL and D2A1-d dormancy models and the NSG immunodeficient mice model. Further, FACS immune-phenotyping coupled with scRNA-seq was implemented to comprehend the changes in the tumor immune microenvironment upon loss of BRD7. **Results:** The loss-of-function screen revealed that BRD7 is essential for the maintenance of the dormant state of breast cancer cells *in vivo*. Interestingly, RNA-seq revealed that BRD7 knockout promotes the expression of genes involved in inflammation, hypoxia, and EMT. Intriguingly, the top signatures enriched in BRD7-silenced cells were IL6-JAK-STAT3 signaling, TNF-α signaling, and interferon-gamma responses. Further, ATAC-seq and ChIP-seq experiments indicated that inactivation of BRD7 causes an increased accessibility of enhancer sites that were enriched for interferon-regulated response element sites. Additionally, we found that BRD7-inactivation induces expression of *IL6, IL33, CXCL10*, and *CXCL12*, all of which have been implicated in metastasis. Finally, the loss of BRD7 led to upregulation of the tumor-promoting N2 neutrophil population and downregulation of the tumor-suppressive M1 macrophages and dendritic cells *in vivo*. **Conclusions:** Our novel functional genomic platform shall enable the identification of single genes that enforce dormancy or mediate metastatic reactivation of breast cancer. Furthermore, our findings that BRD7 is a suppressor of breast cancer lung metastasis and a predictive cancer biomarker could have major implications in the formulation of myriad chemotherapeutic strategies for metastatic cancers. Taken together, we anticipate that our study could potentially bring about a paradigm shift in our understanding of how epigenetic

regulators, like BRD7, mechanistically regulate breast cancer metastasis and reactivation.

G029. **Clonal Hematopoiesis and Therapy-Related Myeloid Neoplasms in Patients with Breast Cancer**

H. Kim, J. Lee, H. Jin, J. Kim, A. Oh, Y. Hong
Korea Cancer Center Hospital, Seoul, South Korea.

Introduction: Clonal hematopoiesis (CH) is a precursor clonal state of hematologic stem cells that increases the risk of subsequent hematologic malignancies. Therapy-related myeloid neoplasms (t-MNs) are fatal secondary malignancies that occur in patients who have undergone cytotoxic treatment. Herein, we retrospectively investigated the prevalence and features of CH mutations and risk of t-MN in patients with breast cancer (BC) after chemotherapy and/or radiotherapy. **Methods:** Genomic DNA was isolated from blood and/or bone marrow aspirates of 10 patients with BC and 31 genes associated with CH were sequenced. **Results:** The overall incidence of CH mutations in our cohort was 29% in eight of the 31 genes. Co-mutations and the variant allele frequency were significantly increased upon cytotoxic treatment (p = 0.000 and 0.008, respectively). Nine of the 107 patients developed t-MN, five of whom carried CH mutations. *TP53* was the most frequently mutated gene in patients who developed t-MN; therefore, *TP53* mutation in CH is a risk factor for t-MN development (relative risk 7.1). **Conclusions:** In conclusion, cytotoxic treatments can induce mutational events in critical genes related to hematopoiesis, leading to t-MN through clonal gene expansion. Screening and monitoring of CH mutations may help identify a higher risk of t-MN in patients with BC.

G030. **A Unique Case with Immunophenotypic Features of Acute Promyelocytic Leukemia by Flow Cytometry, Non-Descript Myeloid Blast Morphology, der(10) inv ins(10;11), and Mutations in *KRAS*, *NRAS*, and *SUZ12***

J. Xu[1], C. Julius[1], M. Leffew[2], M. Sampson[1], M. Stalling[1]
[1]Akron Children's Hospital, Akron, OH; [2]Summa Health System, Akron, OH.

Introduction: This study aims to characterize cytogenomic abnormalities (CA) in acute myeloid leukemia (AML). **Methods:** A 19-year-old female presented with increased blasts in the peripheral blood (PB). The blasts by flow cytometry were CD33+, CD117+, MPO+, CD34-, and HLA-PB-, the typical phenotype in acute promyelocytic leukemia (APL). Her white blood cell and platelet counts, hemoglobin, and coagulation tests were normal. The myeloid blasts had polygonal nuclei with smooth chromatin, discernible nucleoli and moderate cytoplasm with identifiable Auer rods. No marked cytoplasmic granulation or Faggot cells were seen, but some blasts showed >1 Auer rods. At diagnosis, PB and bone marrow (BM) were studied by fluorescence *in situ* hybridization (FISH), G-banding, and myeloid neoplasm next-generation sequencing (NGS) panel with mutation hotspots of 50 genes interrogated. **Results:** Interphase FISH of the BM was 59.5% positive for *KMT2A* rearrangement and negative for *PML/RARA* fusion. Sixteen of the 20 cells analyzed by G-banding had a complex karyotype with two related clones. A stemline (14 cells) had der(10) with insertion of 11q23q2?5 with *KMT2A* at 10p12 near MLLT10. This was likely followed by inversion of the inserted 11q segment leading to break apart of *KMT2A* and *KMT2A/MLLT10* fusion. A sideline (two cells) had additional del(X)(p11.2). This interpretation was supported by sequential FISH/G-banding finding of der(10) with break apart *KMT2A* signals and the deleted 11q with no *KMT2A* signal. The ISCN was 46,XX,der(10)inv ins(10;11)(p12;q23q2?5),del(11)(q23q2?5)[14]/idem,del(X)(p11.2)[2]/46,XX[4].ish der(10)(5`*KMT2A*+,3`*KMT2A*+),del(11)(*KMT2A*-). *KMT2A/MLLT10* fusion is recurrent in AML, acute lymphoblastic leukemia, and therapy-related cases. The NGS testing was positive for three mutations. 1) A missense variant in *KRAS* (NM_004985.5): c.183A >C, p.Gln61His, variant allele frequency (VAF): 12% with strong clinical significance. This is a recurrent gain-of-function mutation seen in multiple cancers including AML. 2) A missense variant in *NRAS* (NM_002524.5): c.35G >A, p.Gly12Asp, VAF: 27.8% with strong clinical significance. This is a recurrent somatic mutation seen in various neoplasms including AML. 3) A nonsense variant in *SUZ12* (NM_015355.4): c.361C >T, (p.Arg121Ter), VAF: 5.6%. It is reported as a somatic variant (COSMIC, cBioPortal) or germline variant in population databases (ExAC, gnomAD) and once as a pathogenic germline variant (ClinVar). This truncating variant is expected to have loss-of-function effect and thus interpreted as being potentially clinically significant. **Conclusions:** The complex karyotype with clonal evolution and mutations in *KMT2A, KRAS, NRAS*, and *SUZ12* indicates a poor prognosis. We hypothesize that the CA detected may be a cause of the unique pathological features of this case. More reports can help test this hypothesis and confirm if the CA may be markers for diagnosis, prognosis, and treatment of this type of tumor.

G031. **Genetic Mutation Profile and Prognostic Significance in Acute Leukemia Based on Next-Generation Sequencing Technique: A Single-Institute Study**

M. Kwak, J. Han, J. Shim, S. Park, S. Kim, J. Lee, H. Chueh
Dong-A University Hospital, Busan, Republic of Korea.

Introduction: Cytogenetic analysis is known to be crucial for the diagnosis and prognostic stratification of acute leukemia. Recently, the development of molecular biology technology, especially next-generation sequencing (NGS), has enabled the simultaneous screening of multiple genes and the construction of a molecular prognostic evaluation system. A combination of cytogenetic analysis and molecular diagnostics has been integrated into the classification and risk assessment of patients with acute leukemia. The aim of this study was to investigate somatic mutational spectrum and risk stratification in patients with acute leukemia using cytogenetics and targeted gene panel sequencing. **Methods:** Between November 2018 and January 2021, a total of 67 patients who were newly diagnosed with acute leukemia (57 for acute myeloid leukemia [AML], 10 for acute lymphoblastic leukemia (ALL]) at Dong-A University Hospital were screened for AML or ALL associated gene mutations (49 genes for AML and 49 genes for ALL) using NGS. Risk stratification for AML patients was determined according to the 2017 ELN guidelines. We retrospectively analyzed the collected data from chromosomal analysis and AML/ALL-targeted gene panel sequencing. **Results:** A total of 163 genetic variants were detected from 57 patients (30 males and 27 females) diagnosed with AML. Among these patients, 91.2% (n=52) had at least one genetic mutation classified in tier 1, 2, or 3 (strong, potential, and unknown clinical significance). In 10 patients with ALL (six males and four females), 17 genetic variants were detected. Seventy percent (n=7) of the patients had at least one genetic mutation. The most frequently detected variants in AML were *DNMT3A* and *CEBPA*, each in 9.0% (n=15) of patients, followed by *TET2* 8.4% (n=14), *TP53* 6.6% (n=11), and *FLT3*-ITD 6.0% (n=10). The most frequently detected variants in ALL were *KMT2D* (18.2%), followed by *WT1* (9.1%), *ETV6* (9.1%), *SETD2* (9.1%), and *FLT3* (9.1%). In 28.1% (n=16) of AML patients, there was a discrepancy in prognostic stratification according to the results of chromosomal analysis and the targeted gene panel sequencing test. **Conclusions:** There are limitations in diagnosing and determining AML/ALL prognosis using the conventional cytogenetics. Targeted gene panel sequencing analyses can be clinically useful for patients with acute leukemia. It is expected that NGS technology will be an essential diagnostic tool for acute leukemia as a complementary test with conventional cytogenetics.

G032. **Comprehensive Tumor Profiling Reveals High Concordance of Gene Amplification with RNA Expression**

A. Alontaga, T. Boyle, K. Furlan, L. Saeed, J. Puskas, S. Reddy, M. O'Leary, A. Bossler, P. Cano, J. Koomen, E. Haura
Moffitt Cancer Center, Tampa, FL.

Introduction: Next-generation sequencing (NGS) is routinely performed to match patients with targeted therapies. Gene amplification is commonly reported, but the relationship between gene amplification and abnormal gene expression remains poorly understood. We report

concordance between gene amplification by NGS and gene expression by an RNA expression panel, RNA Salah Targeted Expression Panel (STEP). **Methods:** The RNA STEP (204-gene panel, NanoString platform) was designed based on input from oncologists to identify genes with abnormal expression for clinical trial prescreening and selection of therapy. Gene expression profiling with RNA STEP was performed with 102 remnant RNA samples with amplified genes detected using a clinically validated NGS panel referred to as Moffitt STAR (TST170 panel, Illumina Inc.), including 10 samples with *MET* exon 14 skipping. STAR NGS detects copy number variants (CNV) for 59 genes, and 38 of these genes are mutually covered by RNA STEP. We evaluated accuracy, positive percent agreement (PPA), negative percent agreement (NPA), and Pearson correlation for detection of CNV by STAR NGS versus increased RNA expression by RNA STEP. **Results:** Accuracy was 93%, PPA was 69.4%, and NPA was 93.8% for all comparable results of DNA-detected amplification (NGS, copies ≥5) versus increased RNA expression (RNA STEP, log2 ratio ≥2) of the 38 mutually covered genes (N=102 samples). Specific genes had higher PPA, such as *CDK4* (100%), *ERBB2* (HER2, 92.3%), *MDM2* (85.7%), and *MYC* (88.9%). Excellent concordance was observed for *MET* exon 14 skipping between assays (accuracy = 98%, PPA = 100%, NPA = 97.8%). Moderate correlation was observed between NGS and RNA STEP for all genes ($r = 0.53$, $p < 0.0001$). The RNA STEP assay demonstrated high reproducibility with good correlation ($r > 0.97$, $p < 0.0001$) of results within runs, between runs, with different operators, days, instruments, and reagent lot numbers. **Conclusions:** RNA STEP provides robust results for gene expression using clinical formalin-fixed, paraffin-embedded tissue samples. Accuracy between gene amplification (DNA-based NGS) versus increased gene expression (RNA-based expression profiling) was higher than expected (93%). The relationship between gene amplification and increased gene expression varied by specific gene. The utility of the RNA STEP assay will be evaluated by prescreening patients for clinical trials at the Moffitt Cancer Center.

G033. **Post-Therapy Samples Collected through Rapid Tissue Donation Confirms the Necessity of Tissue-Based Next-Generation Sequencing in Identifying Driver Mutations**

L. Saeed, H. Ozakinci, A. Majumder, G. Nazario, R. Macaulay, A. Bossler, B. Pellini, A. Beg, E. Haura, T. Boyle
Moffitt Cancer Center, Tampa, FL.

Introduction: Lung tumor biopsy specimens are intended to diagnose patients and guide therapy. They are often small and become depleted during clinical care to support later research. Post-treatment samples are not routinely collected even though they can be informative in identifying resistance mechanisms to therapy. The thoracic Rapid Tissue Donation (RTD) program procures invaluable, abundant, high-quality, post-therapy tissue within 48 hours after death from primary and metastatic sites. RTD samples can be evaluated with multiple different assays, including DNA and RNA sequencing, mass spectrometry, immunohistochemistry, and multiplex immunofluorescence to support studies of therapy resistance and tumor heterogeneity. **Methods:** Between 11/2015 and 06/2022, a total of 74 patients enrolled into Moffitt's thoracic RTD program. Demographic, clinical, genetic, and radiology information was collated in a RedCap database. Of 33 RTD donations from consented patients, 19 had a diagnosis of adenocarcinoma, one squamous cell carcinoma, and 13 small cell lung cancer. Of the 19 adenocarcinoma cases, prior molecular testing of clinical samples identified nine with *KRAS* mutations, three with *EGFR* mutations, three with *EML4-ALK* fusions, one with *MET* exon 14 skipping, and three with no driver identified. RTD samples from 10 donors with adenocarcinoma were sequenced with a 170-gene Moffitt STAR Next-Generation Sequencing (NGS) panel. These patients received various therapies including pembrolizumab, nivolumab, capmatinib, bevacizumab, osimertinib, brigatinib, erlotinib, and alectinib, as well as sotorasib. **Results:** *MET* exon 14 skipping and *NRAS* Q61L were detected by STAR NGS in two cases without drivers identified by prior targeted genomic testing. In a patient with *KRAS* G12C treated with sotorasib, STAR NGS revealed *KRAS* G12V, but not G12C, in the RTD-collected tissue, possibly a form of resistance. Two cases showed potential resistance alterations in addition to primary driver mutations detected during clinical care. One had a resistance *AGK-BRAF* fusion in addition to the primary *EML4-ALK* fusion; the other had a *CTNNB1* T41A mutation in addition to an *EGFR* exon 20 insertion, both previously reported. Three cases harbored *PIK3CA* mutations that were not identified earlier. **Conclusions:** Collection and sequencing of RTD samples provides an opportunity to detect resistance mutations and to understand more about post-treatment disease progression and tumor heterogeneity. The identification of drivers by comprehensive NGS tissue testing in two cases serves as a reminder about the value of such testing when no drivers are identified by small panels or blood-based NGS testing.

G034. **Molecular and Clinical Characteristics of Glioma Patients with Germline Pathogenic Mutations of Lynch Syndrome Genes**

X. Zhang[1], A. Yang[2], B. Guo[1], T. Ma[3], X. Zhao[1]
[1]Genetron Health Co., Ltd., Beijing, People's Republic of China; [2]Beijing Tiantan Hospital, Capital Medical University, Beijing, People's Republic of China; [3]Genetron Health Co., Ltd., Hangzhou, People's Republic of China.

Introduction: Lynch syndrome (LS) is an inherited disease caused by germline mutations of DNA mismatch repair genes. LS patients are mainly predisposed to gastrointestinal and endometrial cancers. However, little was known about glioma patients with LS. **Methods:** We retrospectively analyzed 5,594 glioma patients and enrolled 54 patients with germline pathogenic mutations of LS genes ("Lynch glioma patients") and 119 patients without germline pathogenic mutations of LS genes ("none-Lynch glioma patients"). Meanwhile, we enrolled 38 colorectal cancer (CRC) patients with germline pathogenic mutations of LS genes ("Lynch CRC patients") and 143 CRC patients without germline LS gene mutations. Next-generation sequencing was performed to analyze somatic and germline variants of those patients. Microsatellite instability and PD-L1 expression were examined, and tumor mutation burden was calculated. **Results:** We found that only 0.97% (54/5,594) glioma patients were Lynch glioma patients. The median age of Lynch glioma patients was 37.5, 68.5% (37/54) of them were high-grade (grade III or IV) glioma, and 16.7% (9/54) of them were low-grade (grade I or II) glioma, which were comparable to "none-Lynch glioma patients." Moreover, we found that among the 12 most frequently mutated genes in glioma patients, five genes were significantly hypermutated in Lynch glioma patients, including *TP53* (39/54 vs. 63/119, $p = 0.0197$), *CDKN2A* (14/54 vs. 9/119, $p = 0.0028$), *NF1* (27/54 vs. 36/119, $p = 0.0168$), *PIK3CA* (21/54 vs. 26/119, $p = 0.0265$), and *PDGFRA* (22/54 vs. 23/119, $p = 0.0047$). Also, we found that Lynch glioma patients primarily carried germline mutations of *MSH2* and *MSH6* genes, which was different from Lynch CRC patients. In addition, through comparison of LS and none-LS patients, we found that the percentage of TMB-H, MSI-H, or PD-L1 positive patients was significantly lower in glioma than those in CRC patients, indicating the unique molecular characteristics of Lynch glioma patients. Finally, we found that Lynch glioma patients were not initially resistant to TMZ treatment and may benefit from immunotherapy. **Conclusions:** In summary, we characterized molecular and clinical features of these Lynch glioma patients, which extended our knowledge about Lynch glioma patients and may shed a light on their personalized management.

G035. **Comparing the Differences of *ALK* Fusions between DNA NGS and RNA NGS**

S. Hao[1], Y. Fang[3], X. Zhang[3], J. Kong[3], J. Zhang[3], W. Guo[3], G. Wang[4], X. Sun[5], T. Ma[3]

[1]Cancer Hospital affiliated to Shandong First Medical University, Jinan, People's Republic of China; [2]Genetron Health (Beijing) Co. Ltd., Beijing, People's Republic of China; [3]Cancer Hospital Chinese Academy of Medical Sciences, Beijing, People's Republic of China; [4]Shandong Provincial Hospital, Jinan, People's Republic of China; [5]Cancer Hospital affiliated to Xinjiang Medical University, Urumchi, People's Republic of China.

Introduction: *ALK* fusion accounts for about 3%-7% of non-small cell lung cancer, and the most common form is *EML4-ALK*. With the development of next-generation sequencing (NGS) technology, DNA NGS had been widely used in clinic, and not only can identify *EML4-ALK* variants, but also identify many new *ALK* fusions that can't be classified into variants. Previous studies have found some *ALK* fusions, especially uncommon ones, were inconsistent at the DNA and RNA levels. Here, we retrospectively analyzed the *ALK* fusions in lung cancer which were detected with DNA NGS, and verified them with RNA NGS, and compared the variance between DNA NGS and RNA NGS. **Methods:** Samples with *ALK* fusions were extracted from a Chinese lung cancer cohort, which were detected by OncoPanscan (Genetron Health)-based DNA sequencing of tissue. These samples were validated by Fusioncapture (Genetron Health) in RNA level. **Results:** A total of 125 DNA NGS tested samples with *ALK* fusions were used for analysis. These samples could be classified into two cohorts: cohort A included all *EML4-ALK* variants (n=67), and cohort B contained all other *ALK* fusions (n=58). In cohort A, there were seven kinds of variants, including V1 (n=29), V2 (n=5), V3 (n=23), V5 (n=1), V5' (n=2), V7 (n=5), and V8 (n=2). Except for the 13 samples (including all samples of V7) that failed due to poor quality, the other 54 samples verified by RNA NGS were consistent with DNA level. The consistency between DNA NGS and RNA NGS of the six kinds of variants was 100%, whereas the consistency of V7 was unclear. In cohort B, the samples were classified into 5'*ALK* (n=9) and 3'*ALK* (n=49) according to the position of *ALK*. Then 41 samples were successfully sequenced by RNA NGS, and the samples of 5'*ALK* and 3'*ALK* were five and 36, respectively. Among the five samples of 5'*ALK*, three of them transcribed to *EML4-ALK* variant (V1[n=1] and V2 [n=2]), one transcribed to a non-variant *EML4-ALK*, and one was negative. Among the 36 samples of 3'*ALK*, 10 samples were consistent with DNA, 14 samples transcribed to *EML4-ALK* variant (V1 [n=3], V2 [n=6] and V3 [n=5]), three samples transcribed to other non-variant *EML4-ALK*, two samples transcribed to rare partner *ALK*, and seven were negative. On the whole, no consistent samples were identified in 5'*ALK*, whereas 27.78% (10/36) samples of 3'*ALK* were consistent. There were 60% (3/5) and 38.89% (14/36) of 5'*ALK* and 3'*ALK*, respectively, transcribed to *EML4-ALK* variants, and the variants focused on V1-V3. **Conclusions:** The *EML4-ALK* variants detected in DNA NGS had no changes at RNA level. About 41.46% (17/41) of non-variant *ALK* fusions were transcribed into *EML4-ALK* variants. The ratio of 5'*ALK* transcribed to variant is higher than that of 3'*ALK*. Therefore, for patients with non-variant *ALK* fusions, further RNA verification is needed to identify their functional *ALK* fusions.

G036. **Characteristics of Patients with Multiorgan Primary Cancers Based on NGS Detection**

G. Xiong[1], X. Zhang[3], x. Wang[1], H. Yuan[2], W. Li[2], T. Ma[3]

[1]The First Affiliated Hospital of Kunming Medical University, Kunming, People's Republic of China; [2]Genetron Health (Beijing) Co. Ltd., Beijing, People's Republic of China; [3]Genetron Health (Beijing) Co. Ltd, Hangzhou, People's Republic of China.

Introduction: Multiorgan primary malignant neoplasms (MOPMNs) refer to the presence of multiple primary cancers of separate organ sites in one patient. According to the different time of the occurrence of each tumor, it can be divided into synchronous MOPMNs (sMOPMNs) and metachronous MOPMNs (mMOPMNs). So far, there have been few studies of them. Next-generation sequencing NGS detection of tumor tissue and ctDNA has been widely used, whereas the genomic characteristics of these patients have not been well studied. **Methods:** Patients who clinically confirmed as MOPMNs were screened and their basic and treatment information were analyzed. NGS detection (Oncopanscan Panel [Genetron Health Co. LTD]) was performed on the tumor tissues or ctDNAs of these patients to analyze their genomic characteristics. **Results:** A total of 80 MOPMN patients were screened. The ratio of male/female was 1.13, and sMOPMNs/mMOPMNs was 2.11 (54:26). A total of 36 types of cancer pairs were found. The top three were lung-digestive tumors (n=16, 20%), lung-breast cancer (n=11, 13.75%), and digestive-digestive tumors (n=9, 11.25%). In all, 38.89% (21/54) of sMOPMNs and 42.31% (11/26) of mMOPMNs underwent only surgical resection. mMOPMN patients had a wider age distribution and were relatively younger than sMOPMN patients (8-61 years vs. 52-88 years, P=0.0021), with a higher proportion of patients with family history (45.45% vs. 28.57%). In 50 out of 80 patients, NGS was performed on ≥2 primary tumor tissues of each patient. The results confirmed that somatic mutations were completely different among primary tumor tissues. Eight patients (four sMOPMNs and four mMOPMNs) had both NGS testing of tissue and ctDNA. In sMOPMNs, ctDNA could only reflect the mutation information of one tumor (n=2); or could detect the mutations of two primary tumors simultaneously, but the mutations of one tumor were dominant (n=1); or could not detect mutations of any tumor (n=1). ctDNA testing after the identification of the first primary cancer tissue of mMOPMNs could (n=1) or could not (n=1) detect the mutations of the first primary cancer. When ctDNA was tested after all of the patient's mMOPMNs occurred, ctDNA could detect the mutations of resected cancer tissue close to the detection time (n=1) or could not feedback the mutation of any primary tumor tissue (n=1). On the whole, ctDNA was not good at detecting mutations in each primary cancer. **Conclusions:** Compared to previous reporting, patients with MOPMNs in this study were more diverse in cancer pairs, although the surgery ratio was similar. NGS was helpful in determining MOPMNs, but ctDNA could not detect the mutations of each primary tumor well. Personalized ctDNA panel based on the mutations of each primary tumor may be an effective measure to dynamically monitor the tumor development of MOPMN patients.

G037. **The Frequency of *NTRK1-3* Alterations across Solid Tumors Using the Oncomap ExTra Assay**

M. Wang[1], F. Alyaqoub[1], M. Barbi[1], T. Chandrasekaran[1], T. Dogruluk[1], S. Dombrowski[1], D. Driscoll[1], L. Hong[1], A. Jalkanen[1], P. Noel[1], S. Szelinger[1], S. Udhane[1], D. Hall[2], j. Hoag[2], F. Baehner[2], J. Routh[1], S. Thakkar[1], J. Lobello[1], G. Basu[1]

[1]Exact Sciences, Phoenix, AZ; [2]Exact Sciences, Redwood City, CA.

Introduction: Genomic profiling of tumors to identify actionable alterations is rapidly becoming the standard of care and is critical in selection of targeted therapies. Many alterations can be identified using DNA-based panels; however, some are difficult to detect without the use of RNA sequencing. Gene fusions, especially those with novel partners, are particularly challenging using DNA-based sequencing. Given that some fusions are associated with highly effective targeted therapies, the use of RNA-based assays in addition to DNA-based sequencing seems warranted. Here we examine the frequency of *NTRK1-3* fusions, identified using DNA- versus RNA-based analysis, which can be targeted across multiple cancer types using the FDA-approved tropomyosin kinase receptor inhibitors larotrectinib or entrectinib, as well other investigational agents. **Methods:** Tumor samples submitted from April 2018 to April 2022 were sequenced with the Oncomap ExTra assay, which uses tumor-normal paired whole-exome DNA sequencing and whole-transcriptome RNA sequencing to detect alterations that are clinically actionable, defined as associated with FDA-approved therapies or clinical trial eligibility. Here we focus on *NTRK1-3* alterations, specifically fusions, focal amplification and acquired missense mutations. **Results:** Tumors from 6,456 patients were assayed and 19 (0.29%) carried an *NTRK1-3* alteration (two *NTRK1*, five *NTRK2*, 12 *NTRK3*). Among these patients, 16 (0.26%)

had an *NTRK1-3* fusion, two had copy number gains, and one patient with metastatic mammary analogue secretory carcinoma had both an *ETV6/NTRK3* fusion and an acquired secondary resistance mutation indicative of resistance to TRK inhibitors. *NTRK1-3* alterations were found in thyroid (five of 98, 5.1%), breast (four of 1,599, 0.25%), sarcoma (three of 286, 1.0%), melanoma (two of 134, 1.5%), small bowel (one of 40, 2.5%), urothelial (one of 167, 0.60%), head/neck (one of 164, 0.61%), brain (one of 296, 0.34%), and prostate (one of 432, 0.23%) cancers. *NTRK* alterations were not detected in other cancers tested. Of the 17 fusions detected using RNA sequencing data, only six (35%) were also detected by the whole-exome DNA sequencing data. All of the five thyroid cancers with *NTRK* alterations had fusions, four were of predominantly papillary histology, and two were in pediatric patients. **Conclusions:** Though rare, *NTRK1-3* fusions are critically important for therapy selection in advanced cancers. Assays such as Oncomap ExTra that utilize RNA sequencing allow detection of actionable fusions that might otherwise be missed. The patient with an *ETV6/NTRK3* fusion along with an acquired resistance mutation in *NTRK3* may be eligible for clinical trials with second-generation *TRK* inhibitors, such as repotrectinib.

G038. ***FGFR1-3* Alterations in Cholangiocarcinoma, Urothelial, and Other Cancers**

S. Udhane[1], F. Alyaqoub[1], M. Barbi[1], T. Chandrasekaran[1], T. Dogruluk[1], S. Dombrowski[1], D. Driscoll[1], L. Hong[1], A. Jalkanen[1], P. Noel[1], S. Szelinger[1], M. Wang[1], D. Hall[2], J. Hoag[2], F. Baehner[2], J. Routh[1], S. Thakkar[1], J. Lobello[1], G. Basu[1]

[1]Exact Sciences, Phoenix, AZ; [2]Exact Sciences, Redwood City, CA.

Introduction: Although rare in most cancers, *FGFR1-3* alterations are more frequent in metastatic cholangiocarcinoma and urothelial cancers. Both of these cancers have poor prognoses but if they have an actionable driver alteration in *FGFR2* or *FGFR3*, they are candidates for FDA-approved therapies. Pemigatinib and infigratinib are FDA approved for use in cholangiocarcinoma with *FGFR2* fusions, and erdafitinib is FDA approved in urothelial cancers harboring one of four missense mutations, or two gene fusions, in *FGFR3*. Here we examine the frequency of these and other *FGFR1-3* alterations in cholangiocarcinoma and urothelial cancer as well as across other tumor types. **Methods:** Tumor samples submitted between April 2018 and April 2022 were sequenced with the Oncomap ExTra assay, which uses tumor-normal paired whole-exome DNA sequencing and whole-transcriptome RNA sequencing to detect alterations that are clinically actionable, defined as associated with FDA-approved drugs or clinical trial enrollment. The assay detects single-nucleotide variants, indels, copy number variants, and fusions. In addition to determining the frequency of *FGFR* alterations across cancers, we also calculated the frequency of patients with alterations that made them candidates for FDA-approved, *FGFR*-targeted drugs. **Results:** Across multiple cancers, 6,456 patients were assayed and 268 (4.2%) carried an *FGFR1-3* alteration (120 *FGFR1*, 93 *FGFR2*, 59 *FGFR3*). Focal gains were most prevalent (133, 2.1%), missense mutations were somewhat less frequent (103, 1.6%), and fusions were least common (48, 0.7%). *FGFR* alterations occurred in >5% of the following cancers: urothelial (22.2%), cholangiocarcinoma (14.6%), endometrial (12.5%), small cell lung (9.5%), gynecologic (6.7%), breast (6.5%), skin (5.9%), and cervical (5.5%). In the 167 urothelial cancer patients, 37 had *FGFR1-3* alterations, and 32 had an *FGFR3* alteration specifically associated with FDA-approved erdafitinib therapy: 23 S249C missense, three R248C missense, three Y373C missense mutations, and three *FGFR3/TACC3* fusions. In the 82 cholangiocarcinoma patients, 12 had *FGFR1-3* alterations, which included six (7.3%) with *FGFR2* fusions (three with *BICC1*, and one each with *GAB2*, *HSPB6*, and *PAWR*). Such patients are eligible for FDA-approved pemigatinib or infigratinib therapy. **Conclusions:** The Oncomap ExTra assay, which includes whole-transcription RNA sequencing, identified actionable *FGFR1-3* alterations, including fusions, across multiple tumor types. A total of 19.2% of urothelial cancers and 7.3% of cholangiocarcinomas had alterations associated with FDA-approved FGFR therapies. The prevalence of *FGFR* alterations across tumor types suggests *FGFR*-targeted therapy may become important in several other cancers.

G039. **Establishing Criteria for Classifying Variants of Potential Clinical Significance in the Context of Myeloid Malignancies**

T. Roman[1], I. Shyu[2], L. Schultz-Rogers[2], T. Sneddon[2], M. Gulley[2], J. Merker[2], J. Booker[2], N. Montgomery[2], L. Ramkissoon[2], J. Galeotti[3], K. Weck[2]

[1]University of North Carolina Medical Center, Chapel Hill, NC; [2]University of North Carolina at Chapel Hill, Chapel Hill, NC.

Introduction: Identification of oncogenic variants provides critical diagnostic, prognostic, and therapeutic information for myeloid malignancies. A tiered system for interpreting clinical actionability of somatic variants in cancer was recommended by AMP/ASCO/CAP in 2017 (Li, *et al.,* 2017). More recently, a standard operating procedure (SOP) for classifying somatic variant oncogenicity was published, to be used in combination with the 2017 guidelines (Horak, *et al.* 2022). In light of these new recommendations, our laboratory aimed to build on this SOP to refine variant classification, focusing on Tier II (variants of potential clinical significance) and Tier III (variants of unknown clinical significance) in the context of myeloid malignancies. **Methods:** We are evaluating 34 genes associated with hematologic myeloid malignancies using a custom hybridization capture-based assay from SOPHiA Genetics. To establish gene-specific criteria for Tier II variant classification, we assessed: 1) variant allele frequency in population and cancer databases, 2) molecular mechanisms for each gene in the context of myeloid malignancies, 3) gene regions encoding functional domains, 4) published literature, 5) consensus expertise from genetics and pathology professionals (i.e., molecular and hematopathologists) in our group. We also used the National Comprehensive Cancer Network (NCCN) Guidelines for evaluating variants observed in the context of specific myeloid neoplasms (myelodysplastic syndromes, myeloproliferative neoplasms and acute myeloid leukemia). **Results:** To date, we defined Tier II criteria for variants in *CEBPA*, *ETV6*, *EZH2*, *PPM1D*, and *WT1* in the context of myeloid neoplasia. These include variants with 20+ entries in the COSMIC database for *CEBPA*, *PPM1D,* and *EZH2*. Truncating variants in *WT1* codons 458, 462 or exons 6-9; exons 1-5 of *PPM1D;* prior to the penultimate exon of *ETV6;* or outside the N- and C-terminal regions of *CEBPA* were also classified as Tier II. In these five genes, we have currently classified 36 variants as Tier II and 52 variants as Tier III, in addition to 70 variants classified as Tier I (variants of strong clinical significance). **Conclusions:** Creating gene-specific criteria for variants of potential clinical significance in the context of hematological malignancies provides consistency and reproducibility for variant classification and determining variant oncogenicity. These criteria build off the framework proposed by the AMP/ASCO/CAP guidelines and standardize clinical reporting of somatic variants.

G040. **Differences of Molecular Profiles of Indeterminate Thyroid Nodules between Chinese and American Cohorts**

Y. Song[1], D. Zhao[2], J. Liu[2], W. Wang[2], J. Zhu[2], B. Wang[2], X. Wang[2], X. Liu[2], C. Wang[2], T. Ma[2], B. Zhang[1]

[1]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Head and Neck Surgery, Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; [2]Department of Translational Medicine, Genetron Health (Beijing) Technology, Co. Ltd., Beijing, People's Republic of China.

Introduction: Thyroid nodules are the main manifestation of thyroid cancer, of which a definite diagnosis of thyroid nodules is key to the management. Given the differences in guidelines and molecular profiles of pre-operative nodules of thyroid fine-needle aspiration (FNA) among different ethnicities, there are differences in FNA cytology and post-operative pathology in diagnosis. The variation of genomics among different ethnicities, especially in Bethesda III/IV indeterminate nodules that need an auxiliary means to help in decision-making on whether the thyroid nodule is benign or malignant, which impacts

clinical treatment decisions. Therefore, there is great need to investigate the difference in the molecular profile of thyroid nodules to guide clinical practice. **Methods:** A prospective study was performed on 383 FNA samples of thyroid nodules from 352 Chinese thyroid patients, evaluated using the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) and subjected to the Onco-Thyroid v1 panel (Genetron Health) for next-generation sequencing. The molecular profiles of our pre-operative indeterminate thyroid nodules or post-operative malignant nodules (Bethesda III/IV) were then compared with the cohorts selectively ordered in the University of Pittsburgh Medical Center (UPMC) study using ThyroSeq v2, respectively. **Results:** When compared to the pre-operative thyroid nodules with AUS/FLUS (Bethesda III) in the University of Pittsburgh Medical Center (UPMC) study, *BRAF* V600E mutation was significantly higher in Bethesda III nodules (19.7% vs. 0.2%, P <0.0001) in our cohort. Meanwhile, the *TERT* promoter mutation (4.2% vs. 0%, P <0.01) and *RET* fusion (2.8% vs. 0%, P <0.05) were also significantly higher in Bethesda III nodules in our study. The frequency of *RAS* mutation was relatively higher (5.6% vs. 3.7%, P >0.05), but there was no significant difference. Further, the comparative result of the molecular profiles of post-operative Bethesda III malignant thyroid tumors between Chinese and American cohorts also showed remarkably different genomic characteristics, among which presented significantly high frequency of *BRAF* V600E mutation (55% vs. 4.6%, P <0.001) and profoundly low frequency of *RAS* mutation (5% vs. 50%, P <0.01) in the Chinese cohort. However, in terms of *TERT* promoter mutation (5% vs. 0%, P >0.05), *RET* fusion (10% vs. 0%, P >0.05), and other variations, no significant differences were observed. Due to the limited sample size of Bethesda IV thyroid patients in our cohort, all the above mutations/fusions showed similar incidence in Chinese and Caucasian cohorts. **Conclusions:** In this study, the genomic characteristics of indeterminate thyroid nodules in Chinese and Caucasian populations were compared, providing reference for accurate clinical diagnosis of thyroid cancer.

G041. **Targeted RNA-Based NGS Significantly Enhanced the Proportion of Druggable Patients in Melanoma**

H. Feng[1], K. Jiang[2], T. Ma[3], B. Wang[3], C. Wang[3]
[1]Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan, People's Republic of China; [2]The Second Affiliated Hospital of Dalian Medical University, Liaoning, People's Republic of China; [3]Genetron Health (Hangzhou) Co. Ltd., Hangzhou, People's Republic of China.

Introduction: Previous results still showed that 40%-42% of melanoma patients could not get benefits from DNA-based next-generation sequencing (NGS) detection. This suggests additional techniques should be utilized to enhance the proportion of druggable patients. **Methods:** Formalin-fixed, paraffin-embedded tissues from 469 Chinese patients with melanoma between March 2018 and September 2021 were used to perform DNA-based NGS (Onco Panscan) and RNA-based NGS (Fusioncapture) at Genetron Health, a laboratory accredited by the College of American Pathologists and Clinical Laboratory Improvement Amendments. **Results:** At DNA level, only 75% (352/469) patients were druggable. To explore the therapy opportunity, the undruggable group identified by DNA NGS was further sequenced by RNA NGS (Fusioncapture). In total, 117 patients were identified as undruggable based on the DNA NGS result. Sixty patients with enough samples were sequenced by RNA NGS and positive fusions were found in 15 samples (25%). Those fusions involved in multiple driver genes: *BRAF* (5%, n=3), *RAF1* (3%, n=2), *ALK* (2%, n=1), *FGFR1* (2%, n=1), *ETV6* (2%, n=1), *AKT3* (2%, n=1), *TCF12* (2%, n=1), *ATF* (2%, n=1), *ZCCHC7* (2%, n=1), *ARHGEF2* (2%, n=1), *KDM6A* (2%, n=1), and *WHSC1L1* (2%, n=1). Based on the RNA NGS results, seven patients present druggable potential, which accounted for 47% in the fusion-positive group, including *PUM2-ALK*, *FGFR1-TACC1*, *AGAP3-BRAF*, *AGK-BRAF*, *ZKSCAN1-BRAF*, *SOX6-RAF1,* and *CTDSPL-RAF1*. Most of the rearrangements occurred in the same chromosome, except *SOX6-RAF1* (chr11-chr3). All the fusions with druggable potential could be targeted by the drugs with FDA approval. In total, the RNA NGS utility increased the proportion of druggable patients from 75% to 78%. **Conclusions:** To fully reveal targetable gene fusions or oncogenic isoforms in melanoma, targeted RNA-based NGS can be used as a supplement to DNA-based NGS testing.

G042. **Analysis of Genetic Alterations Using Next-Generation Sequencing in Primary and Metastatic Colorectal Adenocarcinomas**

J. Kaz, S. Deharvengt, D. Green, B. Houde, P. Shah, S. Sukhadia, G. Tsongalis, K. Winnick
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Colorectal adenocarcinoma still remains the third leading cause of cancer-related deaths in the United States today, with an incidence of 150,000 new cases estimated per year. Among the various mechanisms of pathogenesis, molecular studies can provide clinically relevant markers for determining prognosis and treatments, with clinical importance often placed upon genes related to microsatellite instability, *KRAS*, and *BRAF*. Using the TruSight Tumor 170 (Illumina) next-generation sequencing assay, we recorded the number of single nucleotide variants, insertions, and deletions of 151 genes from 139 histologically diagnosed colorectal adenocarcinoma cases from primary and metastatic sites over the course of two years to identify the prevalence of these genetic alterations. **Methods:** Genomic material was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues, processed at an academic, tertiary care center using the Qiagen AllPrep DNA/RNA FFPE Kit on the QIAcube instrument. DNA and RNA Libraries were prepared using the TruSight Tumor 170 Kit (Illumina) and sequenced on the NextSeq 500 (Illumina). Alignment and variant calling was performed using the TruSight Tumor 170 Local App v1 housed in the Clinical Genomics Workspace (CGW) (PierianDx). The CGW Platform was utilized for variant filtering. Variants retained after filtering were classified as IA through III per AMP/ASCO/CAP guidelines. **Results:** A total of 109 cases from March 2020 to May 2022 were analyzed and showed 1,425 genetic alterations with 478 classified as IA through IID in 151 genes tested. Of these, the following most common alterations were found: *APC* (demonstrating 161 total insertion, deletion, or substitution alterations out of 478, constituting 33.6% of those total alterations observed), *TP53* (105/478, 21.9%), and *KRAS* (61/478, 12.8%). Other notable genes include *BRAF* (16/478, 3.3%), *FBXW7* (16/478, 3.3%), *PIK3CA* (35/478, 7.3%), *MLH1* (2/478, 0.4%), *MSH2* (2/478, 0.4%), and *MSH6* (1/478, 0.2%). **Conclusions:** This study shows that a majority of the genetic alterations within the colorectal adenocarcinomas sequenced involved the *APC* gene. With this expanded genetic analysis, we are able to provide more potential avenues of treatments for those patients who initially fail currently recommended therapies.

G043. **Value of NGS Analysis for Benign Liver Disease Diagnosis**

R. Khalife, S. Park, A. Magliocco
Protean BioDiagnostics, Orlando, FL.

Introduction: Accurate diagnosis of cancer type is crucial to guide patients to a correct drug for therapy, prevent unnecessary procedures, and increase life expectancy. However, patients are frequently misdiagnosed, as evidenced by our case. Some cancers are especially prone to misdiagnosis. The diagnosis of hepatocellular carcinoma can be particularly challenging for even experienced pathologists. **Methods:** A male in his 50s was originally diagnosed with hepatocellular carcinoma and underwent immunohistochemistry (IHC) and genetic testing. This case was then referred for Protean MAPS (Protean BioDiagnostics) analysis, a diagnostic testing service including pathology review, comprehensive in-house molecular testing, and virtual molecular tumor boards. Patient results were further analyzed using SOPHiA DDM, which identifies rare large-scale insertion or deletions (indels). Results were then compared to findings from the COSMIC and cBioPortal databases. **Results:** Initial blood test and genetic testing results were unremarkable. SOPHiA DDM, however, uncovered a somatic catenin (*CTNNB1* c.52_414del)

deletion. This rare indel features deletion of exons 3 and 4 of CTNNB1. The exon 3 region contains proteasomes, important for β-catenin degradation, and the deletion causes a gain-of-function resulting in degradation-resistant β-catenin protein. Data compared to findings in the COSMIC database confirmed liver adenoma. Review of cBioPortal also confirmed liver adenoma, as *CTNNB1* mutations without *TP53, RYR2*, and/or *MUC16* alterations were more likely to be classified as adenomas. Out of three possible distinct adenomas, patient's results aligned with the benign β-catenin–mutated hepatocellular adenoma. This revised diagnosis was confirmed by the IHC result, where the stains depicted upregulated glutamine synthetase – a feature consistent with hepatic adenoma. The patient underwent partial liver resection to remove the adenoma, which does have a potential risk of malignant transformation. **Conclusions:** In this case, the patient was initially misdiagnosed with a hepatocellular carcinoma, which could have led to aggressive treatments and severe morbidities including liver transplantation. A comprehensive review of next-generation sequencing results using SOPHiA DDM's use of rare indel detection enabled the correct diagnosis to be rendered. We conclude that NGS may also have roles for correct diagnosis and management of benign lesions.

G044. **Analysis of Tumor Mutation Signature in a Colorectal Cancer Patient with Two Germline *MUTYH* Mutations**

M. Hawley[1], J. Zhao[2], M. Barbosa[3], M. Emery[1], L. Thompson[4], D. Kingma[4]
[1]Invitae, Iselin, NJ; [2]Invitae, Dresher, PA; [3]Invitae, Keyport, NJ; [4]TN Oncology, Franklin, TN.

Introduction: *MUTYH* encodes adenine DNA glycosylase, which is involved in base excision repair. Adenine DNA glycosylase recognizes and excises misincorporated adenine bases that result from the formation 8-oxo7,8-dihydro-2'-deoxyguanosine under oxidative stress. MUTYH deficiency in tumors has been shown to lead to an increase in G:C >T:A transversions. Somatic tumor profiling was performed on a cecal adenocarcinoma sample from a 48-year-old male who underwent a right hemicolectomy that included excision of an 8.2 cm tumor, and two *MUTYH* mutations (p.Gly396Asp and p.Tyr179Cys) were detected along with other mutations and high tumor mutation burden (TMB). After somatic testing was performed, the patient underwent germline genetic testing and the *MUTYH* mutations were confirmed to be germline in origin. **Methods:** The OneTumor Molecular Profile assay was used for somatic variant analysis. It includes DNA analysis for sequence variants (523 genes) and copy number variants (59 genes) plus RNA analysis for gene fusions (55 genes) and splice variants (three genes), and reports microsatellite instability and TMB. The mutational profile of single nucleotide variants (SNVs) detected in the tumor sample was analyzed to determine the rate of G:C >T:A transversions versus other types of SNVs. Variants were filtered to detect non-synonymous SNVs that were rare in gnomAD (maximum alternate allele frequency of 0.5%) and had not been previously classified as benign variations. Two additional colorectal cancer (CRC) tumors without associated germline *MUTYH* mutations but with a similarly high TMB tested by the same assay were used for comparison. **Results:** The MUTYH deficient tumor had a TMB of 78.6 mutations per Mb (muts/Mb) and showed a rate of 30.2% (19/63 SNVs) for G:C >T:A transversions compared to rates of 16.7% (17/72 SNVs) and 13.5% (10/74 SNVs) in the two other colorectal cancer samples, which had TMB of 84 muts/Mb and 79.4 muts/Mb, respectively. **Conclusions:** Approximately double the amount of G:C >T:A transversions were observed in the *MUTYH* mutated CRC when compared to CRCs with similarly high TMB, but without *MUTYH* mutations. This result suggests the two mutations are biallelic and resulted in MUTYH deficiency. This mutational signature defines a distinct subset of colorectal cancers associated with MUTYH deficiency and has been observed previously using whole-exome sequencing. These data demonstrate that the increased amount of G >T and C >A transversion mutations in individuals with two *MUTYH* mutations can also be detected using a somatic tumor panel. The presence of this mutational signature may provide insights into tumor development and have clinical implications in the future.

G045. **Rare Expression of t(11;16)/*KMT2A-CREBBP* in Therapy-Related Myelodysplastic Syndrome**

A. Alswied, D. Fuchs, M. Proytcheva, I. Lai
The University of Arizona, Tucson, AZ.

Introduction: Exposure to chemotherapeutic agents has been linked to developing secondary hematologic neoplasms, specifically acute myelocytic leukemia (AML) and myelodysplastic syndromes (MDS). Among the described cytogenetic abnormalities is t(11;16)(q23;p13)/*KMT2A-CREBBP*, which carries a dismal prognosis and has been observed mainly in the pediatric age group. **Methods:** Giemsa banding karyotype and fluorescence *in situ* hybridization (FISH) molecular panels. **Results:** The patient is an 18-year-old Caucasian male with no significant past medical history who presented with chief complaints of left hip and low back pain. Imaging studies revealed the presence of left sacral mass. A mass biopsy was performed, and a diagnosis of Ewing sarcoma was established based on the morphology, staining profile, and the presence of *EWSR1* gene rearrangement at 22q12. Five months after completion of therapy, laboratory studies showed marked leukopenia with absolute neutropenia, lymphopenia, monocytopenia, and 1% blasts. Bone marrow showed a hypocellular marrow with multilineage hematopoiesis and dysplastic features. MDS and AML interphase FISH molecular panels and next-generation hematology molecular profile were negative. Within six months, the patient's laboratory values returned to normal except for persistent mild macrocytic anemia. Bone marrow was performed and showed a hypocellular marrow with trilineage hematopoiesis with a normal myeloid-to-erythroid ratio. Rare erythroid elements show dysplastic features, including nuclear/cytoplasmic asynchrony and rare binucleate cells. Chromosome analysis studies showed t(11;16)(q23;p13.3) rearrangement, and FISH was positive for *KMT2A* rearrangement. At diagnosis, the FISH study revealed *KMT2A/CREBBP* in 20.0% of nuclei. The karyotype in 20 metaphases showed 46,XY,t(11;16)(q23;p13.3 [15]). Next-generation hematology molecular profile was negative at the time of diagnosis, however. The patient was diagnosed with therapy-related neoplasm based on the morphologic and cytogenetic findings. The clinical team proceeded with a haploidentical bone marrow transplant and the patient was given fludarabine, busulfan, and low-dose total body irradiation. Furthermore, he was given fludarabine on days +3/4. After approximately five months, the patient achieved complete engraftment with no evidence of residual disease. **Conclusions:** The active Children's Oncology Group phase 3 trial has classified t(11;16) in patients over 90 days as high risk. Accordingly, t(11;16) should be used for risk stratification in patients with a history of exposure to chemotherapeutic agents than presumptive evidence of MDS or AML with myelodysplasia-related changes. Moreover, a hematopoietic stem cell transplant should be an early therapeutic consideration in such cases.

G046. **Genetic Ancestry Associations with Actionable Somatic Mutations from Tumor Profiling Data of 100,000 Cancer Patients**

F. De La Vega, B. Rhead, Y. Pouliot, J. Guinney
Tempus Labs, Inc., Chicago, IL.

Introduction: The incidence and mortality of cancer vary widely across race and ethnicity. This is attributed to an interplay of socioeconomic factors, environmental exposures, and genetic background. Cancer genomic studies have underrepresented minorities and individuals of non-European descent, thus limiting a comprehensive understanding of disparities in the diagnosis, prognosis, and treatment of cancer among these populations. Furthermore, the social constructs of race and ethnicity are far from precise categories to understand the biological underpinnings of such differences. In this study, we use a large real-world data (RWD) patient cohort to examine associations of genetic ancestry with somatic alterations in cancer driver genes. **Methods:** We used 654 ancestry informative markers selected to overlap the capture regions of the assay to infer global ancestry proportions at the

continental level: Africa (AFR), America (AMR), Europe (EUR), East Asia (EAS), and South Asia (SAS). Whereas most patients are of European descent (72%), our cohort includes 4.7- and 3.8-fold more patients with substantial (>50%) AFR and AMR ancestry, correspondingly, than The Cancer Genome Atlas. Logistic regression was used to directly test for associations between continental ancestry proportions and presence of actionable somatic mutations (OncoKB, Levels 1 &2, R1/2) in cancer genes, controlling for assay version, gender, and age. P-values were adjusted for multiple testing by the Benjamini-Hochberg method to control the false discovery rate at 5%, and all associations reported were significant with p <0.0001. **Results:** We identify an association between actionable somatic mutations in *EGFR* with EAS ancestry in lung cancer (OR=1.48, per 20% increase in ancestry proportion), which has been previously reported using race categories. Furthermore, we also identify five novel significant associations with AFR ancestry including: *PIK3CA* in breast (OR=0.91) and colorectal (OR=1.11) cancers; *BRAF* in colorectal cancer (OR=0.73); *EGFR* in lung cancer (OR=0.77); and *KIT* in sarcomas (OR=1.23). **Conclusions:** Our results support the use of genetic ancestry inference on RWD to improve upon the use of race and ethnicity to understand the impact of ancestry cancer incidence, progression, and outcomes.

G047. **Supporting Evolving Clinical Guidelines Requires Updated Cancer Variant Interpretation Resources**

K. Krysiak[1], A. Coffman[1], S. Kiwala[1], J. McMichael[1], A. Danos[1], J. Saliba[1], C. Grisdale[2], A. Suda[1], A. Venigalla[1], M. Khanfar[1], K. Singhal[1], A. Wagner[3], M. Griffith[1], O. Griffith[1]
[1]Washington University School of Medicine, Saint Louis, MO; [2]Canada's Michael Smith Genome Sciences Centre, Vancouver, Canada; [3]Nationwide Children's Hospital and The Ohio State University College of Medicine, Columbus, OH.

Introduction: The Clinical Interpretation of Variants in Cancer (CIViC, civicdb.org) knowledgebase provides a community curation platform with expert moderation. This platform is designed to capture evidence from the published literature that supports or refutes the relevance of specific variants in various cancer types. As clinical guidelines and variant detection methods increase in their complexity, variant interpretation tools need to evolve with them. In multiple cancer types, combinations of variants, including the co-occurrence of variants (e.g., double hit lymphoma) and complex variant types (e.g., microsatellite instability) detected by variable molecular methods have become critical to clinical decision making. Unfortunately, these have not been well represented in variant knowledgebases. **Methods:** CIViC has been embraced by the community, engaging >320 individual contributors entering evidence from >3,100 sources representing >3,100 variants from >470 genes in >330 cancer types. CIViC has always supported a wide variety of variant types (e.g., fusions, copy number variants (CNVs), overexpression). Nevertheless, with the complete redesign of the data model and user interface, CIViC 2.0 is able to further expand relationships to capture more complexity in how these variants are represented and related to one another. The new API utilizes GraphQL, supporting complex queries across relationships not possible with our previous RESTful design. The recently launched CIViC 2.0 provides enhanced support for evolving standards and guidelines in the field of cancer variant interpretation. **Results:** The redesign of CIViC provides the flexibility to create Molecular Profiles and Regions to represent these more complex concepts in variant interpretation. Molecular Profiles utilize boolean operators (AND, OR) to connect individual variants and are assigned individual evidence pertaining to that combination while maintaining simple connections to evidence underlying individual constituent variants. Assigning Regions (currently in development) to each variant will allow for support of multiple coordinate systems, each designed for the specific type of variant being addressed, whether a CNV or more genome-wide phenomena. By supporting Regions and Molecular Profiles, the combination of variants detected by multiple assay types that are part of routine clinical care (e.g., next-generation sequencing and fluorescence *in situ* hybridization) can be curated together. **Conclusions:** CIViC 2.0 will support the combination of complex variants detected by multiple methods, enhancing support for existing and evolving clinical guidelines. This approach will provide the community with first-of-its-kind access to structured and highly curated data which are assay agnostic while representing complex variant relationships.

G048. **Validation of a Germline Genetic Testing Panel for Hematologic Malignancies Using Nail Specimens**

O. Ceyhan-Birsoy, S. Rana, V. Rai, Y. Li, C. Yang, Z. Yelskaya, I. Rijo, J. Casanova, J. Somar, P. Salazar, E. Fiala, M. Sheehan, A. Syed, R. Ptashkin, Z. Stadler, M. Walsh, K. Nafa, M. Arcila, D. Mandelker
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: Identifying hereditary cancer predisposition in patients with hematologic malignancies is critical for their clinical management. Broad genetic testing strategies revealed that a substantial proportion of patients with hematologic malignancies have hereditary cancer predisposition, including those with sporadic and non-syndromic disease. One major limitation in genetic testing of patients with hematologic malignancies is the difficulty to obtain suitable DNA for germline analyses, as peripheral blood, saliva, and buccal swab specimens are often contaminated with malignant cells. Therefore, current approaches rely on DNA isolated from fibroblasts, which are obtained by invasive skin biopsies, require long culturing time, and are not appropriate for large-scale testing. Nail clippings are easily accessible DNA sources for germline analyses, although isolating high yield and quality of intact DNA from nails has been challenging. **Methods:** We developed and clinically validated a next-generation sequencing panel for germline testing of patients with hematologic malignancies, which can be performed using DNA isolated from nail and saliva specimens. The panel targets 82 cancer predisposition genes on the MSK-IMPACT Heme (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets for hematologic malignancies) assay and uses a protocol to extract high quality and quantity of genomic DNA from nails. To validate the Germline MSK-IMPACT Heme panel performance, equivalency of coverage and variant calling was tested against the previously validated MSK-IMPACT assay using blood specimens from 21 individuals run on MSK-IMPACT and specimens (nail clippings [N=14], saliva [N=11], or blood [N=19]) from the same individuals that were run on Germline MSK-IMPACT-Heme. **Results:** All 1,482 variants called by MSK-IMPACT, including 1,355 single nucleotide variants, 109 small insertion/deletions, and 18 copy number variants (deletion/duplication of single or multiple exons), were correctly detected by Germline MSK-IMPACT Heme, showing 100% concordance between the two assays. All target exons had higher than 20X coverage, with an average coverage of 416X for nail, 229X for saliva, and 255X for blood specimens. Early clinical testing detected previously undiagnosed hereditary cancer syndromes in patients, including Fanconi anemia and ataxia telangiectasia. **Conclusions:** We developed a clinical gene panel for hereditary cancer predisposition testing of patients with hematologic malignancies that can be performed using nail or saliva specimens. Our assay provides a rapid and efficient alternative for germline testing of patients with hematologic malignancies that is suitable for large-scale broad genetic testing approaches and minimizes the need for skin biopsies.

G049. **Labor Cost Comparison between the Thermo Fisher Scientific Genexus Integrated Sequencer and the Illumina MiSeq**

J. Chang, A. Hutchison, D. Joun
Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Lab economics play a significant role in determining the purchase of a next-generation sequencing (NGS) platform. Ease of use, reproducibility of results, and overall turnaround time are important attributes of an NGS platform and have a direct impact on the overall operating costs of a lab. In this new study we conduct an Activity Based Costing (ABC) comparison to determine the full-time equivalent (FTE) cost of running the Thermo Fisher Scientific Genexus Integrated

Sequencer workflow and the Illumina MiSeq workflow. **Methods:** Five operators were instructed to follow steps to prepare a two-pool Ion Ampliseq library and initiate sequencing of eight purified nucleic acid samples using the Thermo Fisher Scientific Genexus Integrated Sequencer. Each operator was then asked to prepare libraries and initiate sequencing of the same eight samples a second time but this time using the Ampliseq for Illumina MiSeq workflow. An auditor measured and recorded operator hands-on time for every step of each respective workflow. Publicly available lab technician cost was then applied to model and compare FTE cost for both the Genexus Integrated Sequencer and Illumina MiSeq workflows. **Results:** Despite varying levels of experience amongst the five operators, the Genexus Integrated Sequencer required an average of 13 minutes of hands-on time and a standard deviation of two minutes to complete all necessary steps to prepare library and initiate sequencing of eight nucleic acid samples. Comparatively, the same five operators required an average of almost three hours to complete the Ampliseq for Illumina MiSeq workflow with a standard deviation of 43 minutes. Modeled lab FTE expense suggests that preparing library and setting up a sequencing run for eight samples on the Genexus Integrated Sequencer will cost a lab $7.15 in operator expense per run, whereas the equivalent workflow on the Illumina MiSeq will cost a lab $95.70 in operator expense per run. As sample volume increases the modeled operator cost savings from using the Genexus Integrated Sequencer over the Illumina MiSeq also increases from $4,605 at 416 samples to $23,023 at 2,080 samples. **Conclusions:** This study suggests that in an eight-hour workday the Genexus Integrated Sequencer's ease of use and automation yields a 33% saving of operator time which can be used to perform other tasks in the lab. Both the empirically determined operator time to perform a run and modeled FTE cost per run are significantly lower for an equivalent workflow on the Illumina MiSeq platform, suggesting that the Genexus Integrated Sequencer's automation and ease of use are better suited for labs that desire lower operating expenses. Currently, the Genexus Integrated Sequencer is the only rapid NGS workflow that enables automated nucleic acid sample-to-variant report in a single day.

G050. **Non-Invasive Prenatal Testing (NIPT): A Reliable Accurate Prenatal Non-Invasive Diagnosis Setting in Nepal**

S. Thapa[1], S. Khanal[2], A. Kunwar[2], N. Thakur[2]
[1]Kathmandu Center for Genomics and Research Laboratory (KCGRL), Gwarko, Bagmati, Nepal; [2]Kathmandu Center for Genomics and Research Laboratory (KCGRL), Lalitpur, Bagmati, Nepal.

Introduction: Non-invasive prenatal testing (NIPT) for fetal aneuploidies utilizing cell-free DNA (cfDNA) has become widely used in clinical practice for its enhanced accuracy. The core goal of cell-free-based prenatal testing is to provide minimally invasive, clinically accurate screening for fetal chromosomal aneuploidies in the early stages of pregnancy. The purpose of this study was to establish a validated NIPT workflow for cell-free fetal DNA sequencing from maternal plasma for the detection of particularly autosomal trisomies (involving chromosomes 13, 18, and 21) and sex chromosomes on an ion semiconductor sequencing technology. **Methods:** A total of 91 standard samples from healthy pregnant women were provided by Yourgene Health and their cfDNA library was prepared. We performed emulsion PCR on One Touch 2 machine, enriched ISPs on One Touch ES machine, then loaded ISPs on an Ion 540 chip for sequencing. The sequencing output data were analyzed by using the bioinformatics pipeline of Yourgene Health. Eighty-one samples were successfully validated out of a total of 91 samples. However, 3% of samples did not meet quality, and 3% of sample libraries had low reads and failed validation. The method included a fetal DNA fraction calculation for all samples as well as additional quality indicators. After the establishment of validated workflow, following an assessment of the continuing pregnancy's data, NIPT was offered to 174 pregnant women after the informed consent form. Maternal BMI, maternal age, gestational age along with the latest copy of ultrasound sonography was received and noted. Pre-test counseling was provided to all participants and addressed the main testing procedures, their benefits, limitations, turnaround time, and interpretation of the results. **Results:** From the total clinical samples, 18 samples showed an abnormal result: 13 trisomy 21 samples, three trisomy 18, and two samples with sex chromosome abnormalities were reported. One case had to discontinue pregnancy due to complications in later pregnancy but unrelated to the NIPT results. Twelve samples were needed to repeat due to the low fetal fraction of cfDNA, and later reported normal after the resampling and repetition of the test. During a post-test counseling session, the findings were explained. **Conclusions:** This research aims to introduce and set up Nepal's first next-generation sequencing laboratory for NIPT, utilizing the Ion Torrent technology to provide a complete in-house solution for pregnant women in Nepal. The established laboratory standards for testing and reporting, as well as educational resources and counseling approaches, will contribute to the acceptance of NIPT in society and clinical practice.

G051. **Evaluation of *SDC2* Methylation-Based Stool DNA Test for Early Detection of Colorectal Neoplasia**

S. Kim[1], E. Hong[1], C. Im[1], J. Lee[1], T. Oh[2], H. Kang[2], S. An[1], S. Beqaj[1], B. Bang[1]
[1]Promis Diagnostics, Irvine, CA; [2]Genomictree, Daejeon, Republic of Korea.

Introduction: A sensitive and accurate non-invasive early detection tool for colorectal cancer (CRC) is urgently needed to reduce disease-related mortality and incident rate. We previously identified aberrant methylation of the *SDC2* gene (meSDC2) as a specific diagnostic biomarker for colorectal cancer and validated its diagnostic potential in a large-scale prospective clinical study for detecting early-stage CRC in South Korea. In addition, the same marker-based stool DNA test was repeatedly validated for early detection of CRC in several other studies including in China. However, its performance in individuals in the United States remains unclear. Here, we develop a sensitive and accurate stool DNA-based *SDC2* methylation detection assay, meSDC2 LTE/qMSP (ColonoChek), and aim to demonstrate its performance to detect CRC in a clinical study in the U.S. **Methods:** Stool samples collected from 166 participants who were scheduled to undergo colonoscopy examination in multiple sites were transported using a long-term preservation medium to the central CLIA lab in PromisDx. The presence of methylated *SDC2* and control DNA in stool was measured by two consecutive steps of PCR reactions: a linear target enrichment step, and a quantitative methylation-specific PCR by real-time PCR (ColonoChek). The presence of any signal (Ct value) of meSDC2 from at least one reaction was considered as test positive. The result of this study was compared to that of a prospective clinical study in South Korea. In the previous clinical study conducted in Korea, a total of 1,210 patients scheduled for colonoscopy were enrolled in a number of institutes, and a total of 1,124 patients' data were evaluable. The results of the test were independently analyzed by comparison with colonoscopic findings and pathology outcomes as reference standards. **Results:** ColonoChek in this study detected all CRC patients (n=5). The sensitivity and specificity were 100% and 80.6%, respectively. The sensitivity for advanced adenoma (AA, ≥1.0 cm) was 36.4%. As comparative data, in the previous clinical study in South Korea, ColonoChek had a sensitivity of 95% (n=20) and a specificity of 81.6% for detecting CRC. The sensitivity for early-stage CRC (stage 0-1) was 100%. The sensitivity for detecting AA was 47.9%. **Conclusions:** The performance of ColonoChek in the U.S. is comparable to that of the clinical study in Korea. It showed a high sensitivity for CRC detection at an early stage. This study suggests that *SDC2* methylation-based stool DNA test (ColonoChek) is highly sensitive and specific for the detection of early-stage CRC. It is warranted to conduct large-scale prospective clinical trials for translating to clinical practice the use of ColonoChek for CRC screening.

G052. **Precision Genotyping for *EGFR* Mutation-Positive Non-Small Cell Lung Cancer (NSCLC) Using Multiplexed Digital PCR on Cell-Free DNA**

A. Horning[1], R. Feigelman[1], L. Jiang[1], D. Joun[1], K. Kao[2], M. Kim[1], W. Wei[1]
[1]Thermo Fisher, South San Francisco, CA; [2]Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan (Republic of China).

Introduction: *Epidermal Growth Factor Receptor* (*EGFR*) mutations can be drivers of cancer and cause resistance to treatments in patients with non-small cell lung cancer, the leading cause of cancer-related deaths in the U.S. There are multiple tyrosine kinase inhibitors (TKIs) that can be used to specifically target *EGFR*-mutated NSCLC for potential treatment. However, cancer cells frequently mutate and acquire new TKI-resistant *EGFR* mutations that may reduce efficacy. Identifying *EGFR* mutations in patient cell-free DNA (cfDNA) may enable researchers to detect cancer early, measure therapeutic response, quantify residual tumor burden, and monitor emerging resistance to potential therapies. Here, we present three multiplex *EGFR* mutation panels compatible with cfDNA for use on the Applied Biosystems QuantStudio Absolute Q digital PCR (dPCR) system. This platform consists of a single instrument with five optical channels and microfluidic array plate which can compartmentalize ≤16 samples each into 20,480 micro-chambers and acquire multiplexed results in <2 hours. We demonstrate the application of Absolute Q with the three *EGFR* mutation panels to detect multiple mutations with high sensitivity (0.1 % allele frequency, AF) simultaneously, thus enabling liquid biopsy cancer research for early detection and monitoring, which is critical to understand how a patient's tumor may respond to potential treatment. **Methods:** We developed three separate Absolute Q *EGFR* multiplex panels targeting various alleles on *EGFR*:

A. Major Driver – Mutant targets L858R and p.E746-A750del. Wild-type target L858L.
B. Drug Resistance – Mutant targets T790M, C797S (T >A and G >C), L792F and L718Q. Wild-type target T790T.
C. Low Frequency – Mutant targets L861Q, G719C/S, and S768I. Wild-type target G719G.

Each multiplex panel has a single VIC wild-type probe and the rest of the mutant probes in the panel had either FAM, ABY, or JUN. We tested the panels on mutant *EGFR* cfDNA reference standards with 5%, 1%, 0.1%, and 0% AF. Candidate assays were screened in duplex with one mutant FAM, ABY or JUN probe, and one wild-type VIC probe. Selected mutant assays were pooled together and tested against different wild-type assays. All experiments were performed on the Absolute Q dPCR system. **Results:** We optimized the multiplex dPCR assay to detect 5%-0.1% AF sensitively and specifically for each mutation with 10-40 ng of *EGFR* mutation-positive reference DNA. **Conclusions:** We developed a comprehensive multiplex genotyping method with high sensitivity and specificity for detection of *EGFR* driver, drug resistance, and low frequency mutations using a fast and simple workflow. This study demonstrates a powerful multiplex dPCR application for *EGFR* mutation detection.

G053. **Validation of the Agilent Magnis and Alissa Clinical Informatics Platform for Germline Whole-Exome Sequencing in a Rural Academic Center**

E. Hughes, D. Toledo, E. LaRochelle, D. Green, G. Tsongalis, W. Khan
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Whole-exome sequencing (WES) has become a common application used in the diagnosis of inherited disease. We present the validation of a benchtop Magnis DNA library preparation system and a cloud-based clinical informatics platform (Alissa Align & Call and Interpret; Agilent) for WES. This approach provides an end-to-end solution for downstream clinical-grade next-generation sequencing (NGS) that includes automation, reproducibility, and bioinformatics support for clinical laboratories implementing germline testing. **Methods:** Pooled Agilent SureSelect Human All Exon V8 DNA libraries, derived from blood or buccal swabs, were prepared on the Magnis, quality assessed on the Agilent TapeStation 4150, and sequenced on the Illumina NextSeq 500. FASTQ analysis and sequence read alignment were performed using Align and Call (A&C). Classification tree pipelines on Alissa Interpret performed single sample and cohort analysis for precision and accuracy using de-identified clinical samples with known variants. Analytical limit of detection (LOD) and sensitivity/specificity testing were performed with Genome in a Bottle (GIAB) standards for all exonic and splice-region variants. Mosaic minor allele assessment LOD was simulated by blending two patient samples with known variants. **Results:** Automated library preparation took 9.5 hours with an extra two hours of hands-on time. Quality assessment of prepared libraries averaged concentrations of 18.5 nmol/l and fragment size of 379.8 bp with 95.3% between 200-700 bp. A&C classification compared GIAB samples to reference variant calls, with an average sensitivity of 99.98% for SNPs and 93.80% for indels, and overall specificity of 99.98%. Reproducibility and repeatability of exonic variants averaged 97% and 96%, respectively. Accuracy was measured on residual clinical DNA samples; 47 de-identified blood extracted DNA with specific variant and human phenotype ontology findings resulted in a 99.3% positive percent agreement. LOD studies with a minimum of 50 ng input as the lowest concentration provided no notable loss in the number of variants detected. This was assessed at 95% confidence interval and when compared to the baseline 200 ng input, maintained a duplication rate under 10%. For interrogating blended mosaic patient samples, an LOD variant allele frequency of 10% was selected in regions with adequate read depth (>20x). Interference studies showed no obvious deficit in performance. **Conclusions:** The Magnis system provided consistent automated NGS library preparation for WES resulting in high QC sequencing performance with minimal technician oversight. Alissa Interpret and Align & Call provides an end-to-end NGS informatics solution for clinical laboratories to perform a successful validation of exome-scale sequencing.

G054. **A Probabilistic Graphical Modeling Approach to Variant Interpretation That Improves VUS Resolution and Increases Actionability of Genetic Testing by Providing Continuous Estimates of Pathogenicity**

R. Nussbaum, T. Manders, J. Nicoludis, A. Nampally, K. Nykamp, Y. Kobayashi, A. Colvin
Invitae, San Francisco, CA.

Introduction: Current variant interpretation frameworks cast clinical, allelic frequency, functional and computational data into discrete evidence categories that are combined following heuristic rules to provide a five-tier classification of pathogenicity. These frameworks provide consistency and scalability to variant interpretation across laboratories as genetic testing expands to more patients, larger panels, and exome analysis. However, more than 50% of variants in disease-associated genes are classified as variants of uncertain significance (VUS) using these frameworks, which limits the utility and actionability of genetic testing. **Methods:** We developed a variant interpretation approach that uses probabilistic graphical models to determine a continuous estimate of a variant's probability of pathogenicity. This approach addresses some of the challenges facing rules-based five-tier classification frameworks, namely information loss when converting quantitative data (e.g., allele frequencies) into discrete categories, how different evidence types are combined, and the inability to distinguish VUS that warrant attention and monitoring from VUS leaning benign. We built a model using a limited set of distinct evidence types that represents a causal understanding of the pathogenesis of disease. We trained the model with more than 125,000 confidently classified variants from more than 1,000 genes. This allowed us to determine the complete joint probability distribution of evidence used for classification. **Results:** The trained model provides a continuous estimate of pathogenicity probability. The model performs as well as rules-based frameworks while substantially decreasing the number of missense VUS observed at our lab by up to 25%, demonstrating that joint

probabilities improve evidence weighting compared to current frameworks. A VUS just above the likely benign threshold, for example, is more likely to be benign than is a VUS just below the pathogenic threshold, suggesting that probabilities can be used to prioritize VUS for resolution. Furthermore, with the probabilistic graph model, we can simulate the effect of hypothetical additional data on the pathogenicity estimate for a specific variant, thereby targeting the types of evidence that will most effectively resolve VUS and increase certainty in classifying specific variants. **Conclusions:** We developed a novel variant interpretation approach that resolves a significant proportion of VUS observed by our lab, and provides probabilities of pathogenicity for all variants. We propose that estimating quantitative probabilities of pathogenicity will better inform clinical actionability and enable a more nuanced use of genetic information for healthcare decision making.

G055. **An Amplification-Based, Long-Read Sequencing Assay Accurately Identifies Clinically Relevant SNVs, Duplications, and Deletions in Hemoglobinopathy Relevant Genes**

J. Turner, S. Statt, R. Routsong, C. Fraher, G. Latham, B. Hall
Asuragen, a Bio-Techne Brand, Austin, TX.

Introduction: Hemoglobinopathies, including thalassemias and abnormal structural variants such as HbS, HbE, and HbC, are among the most common inherited genetic diseases; at least 5% of the global population carries a pathogenic variant. Deletions affecting the four hemoglobin α subunit gene copies (*HBA1* and *HBA2*) cause 90% of α thalassemia, whereas *HBB* mutations account for 99% of β thalassemia. Homologous gene sequences and complex recombination events cause deletions and duplications that are challenging to resolve and often require distinct workflows. Here, we combine scalable PCR enrichment with long-read nanopore sequencing in a prototype assay that accurately identifies common pathogenic single nucleotide variants (SNVs), deletions, and duplication events across *HBA1/2* and *HBB*. **Methods:** Genomic DNA from whole blood samples and cell lines (Coriell Cell Repository) was PCR amplified in a single tube, barcoded, pooled, and prepared using a ligation sequencing kit (Oxford Nanopore, ONT). Sequencing was performed using R9.4.1 flow cells and an Mk1B or Mk1C (ONT). Custom software was developed to manage projects, start sequencing, review run-time information, automate data analysis, and report genotypes. A set of 47 samples was used to train the algorithm to identify deletion and duplication events. Performance was tested using a second set of 49 unique challenge samples bearing additional variants. Two hundred previously untested whole blood samples were then screened using the prototype assay. Comparator methods (e.g., melt curve analysis, Sanger sequencing) were developed to determine concordance for *HBA1/2* copy number and SNVs. **Results:** The prototype PCR/Nanopore assay correctly identified at least 15 cases of -α3.7, -α4.2, --SEA, and --FIL deletions, *HBA1/2* SNVs (e.g., Hb Constant Spring, Hb G-Philadelphia) and *HBB* SNVs (e.g., HbS, HbC) in the challenge sample set. Comparator methods confirmed these variants, some of which were expected based on available sample information, whereas others were identified for the first time using the prototype assay. Finally, carrier status calls showed ≥95% agreement to comparator methods in 200 previously untested whole blood samples. **Conclusions:** The data demonstrate feasibility for integrated PCR enrichment and nanopore sequencing to identify the most common deletions and SNVs relevant to hemoglobinopathies and carriers. This assay leverages a simple workflow to resolve structural variants across homologous *HBA1/2* genes and mutations in *HBB* with custom automated analysis software to genotype at least 96 samples in a single run. The approach is responsive to recent American College of Medical Genetics and Genomics (ACMG) guidelines for carrier screening and may be combined with other genes to improve laboratory operations and economies and deliver more equitable screening and diagnostic solutions.

G056. **Circulating Cell-Free DNA Mutation and Methylation NGS Analysis for Early-Stage Colorectal Cancer Diagnosis**

P. Kaur[1], M. Kulak[1], Z. Cui[1], A. Babu[1], S. Tran[1], H. Mansour[2], A. Zhang[1], M. Sha[1]
[1]Diacarta Inc, Pleasanton, CA; [2]Mohamed 1st University, Oujda, Morocco.

Introduction: Circulating cell-free DNA (cfDNA) profiling has emerged as a promising biomarker for cancer diagnosis and prognosis. Here, we assessed the usefulness of integrating genomic and epigenomic signatures in cfDNA to detect colorectal cancer (CRC). **Methods:** We developed targeted next-generation sequencing (NGS)-based panels for loci-specific mutations (43 genes, 437 targets) and methylation (40 genes, 56 targets) for early-stage CRC detection using low-input clinical material. The analytical and clinical performance was validated using plasma cfDNA of 25 CRC patient samples for the mutation panel and six for the methylation panel plus 42 healthy donor samples as controls. **Results:** The limit of detection for CRC mutation panel was 0.25% variant allele frequency for single nucleotide variants and indels with 10 ng DNA input in 2,000x coverage. The mutation assay is highly reproducible with inter-assay CV <4%, intra-assay and lot-to-lot variation CV <5%, operator and technical variability CV <2%. Clinical sample testing showed that the mutation panel yielded sensitivity of 88% (95% CI: 67.7-96.8) and specificity of 100% (95% CI: 89.5-100), whereas the methylation panel had sensitivity of 100% (95% CI: 30.9-100.0) and specificity 75% (95% CI: 42.8-93.3). Combining these two analyses, their positive predictive value and negative predictive value are 100% (95% CI: 51.6-100.0) and 100.0% (95% CI: 59.7-100.0), respectively. **Conclusions:** We have developed and validated the targeted NGS-based panels to detect clinically relevant mutations and methylation patterns and demonstrate their potential as a highly sensitive tool for CRC diagnosis.

G057. **A Streamlined PCR/Nanopore Sequencing Carrier Screening Panel for Cystic Fibrosis, Spinal Muscular Atrophy, and Fragile X Syndrome**

J. Kemppainen, P. Rao, R. Routsong, B. Killinger, C. Fraher, G. Latham, B. Hall
Asuragen, Inc., Austin, TX.

Introduction: Cystic fibrosis (CF), spinal muscular atrophy (SMA), and Fragile X syndrome (FXS) are three of the most common inherited genetic disorders, each with high carrier rates (~1/30 for CF, ~1/50 for SMA and ~1/250 for FXS). Historically, screening has required separate assays for each gene, often with inefficient workflows and uneven detection rates across ethnicities. Additionally, genes for SMA and FXS (*SMN1* and *FMR1*, respectively) are technically challenging to characterize due to homologous or GC-rich sequences. Repetitive polymorphisms, exon deletions, and other CF variants can also be problematic to resolve. Here, we demonstrate a prototype long-read sequencing (LRS) trio assay on Oxford Nanopore Technologies' (ONT) MinION platform. The assay identifies pathogenic variants across all exons of *CFTR* and *SMN1/2*, determines *SMN1* and *SMN2* copies, and accurately genotypes *FMR1* repeats with AGG status in a single workflow. **Methods:** Cell-line genomic DNA (gDNA) samples (n=100) were obtained from Coriell Cell Repository including HapMap controls, 0 to ≥4 copies of *SMN1/2*, 47 pathogenic single nucleotide variants (SNVs)/insertion-deletions (indels) in *CFTR*, and all major repeat expansion categories for *FMR1*. gDNA was also isolated from whole blood of 200 presumed healthy donors. DNA was amplified, barcoded, pooled, prepped by ligation sequencing kit (ONT), and run on R9.4.1 flow cells using the Mk1B or Mk1C (ONT). Data were analyzed using custom-developed software. The AmplideX PCR/CE *CFTR* Kit, PCR/CE *SMN*1/2 Plus Kit, *FMR*1 PCR/CE Kit, Xpansion Interpreter, and Sanger sequencing were used as comparator methods. **Results:** The PCR/Nanopore Trio assay detected variants with >95% accuracy versus comparator methods across the 300 samples tested. More than 40 *CFTR* variants were correctly identified in carrier and affected samples representing 87% prevalence in the U.S. *SMN1* and *SMN2* copy numbers, SNVs and indels were quantified from 0 to ≥3 with

>95% accuracy. The assay reported deletions of exon 7 and/or 8 in *SMN1* in all samples confirmed by comparator method. *FMR*1 CGG repeats were correctly categorized as normal, intermediate, premutation, or full mutation, and also fell within the reference ACMG precision ranges. AGG interruptions associated with risk of *FMR1* expansion from mother to child agreed with the reference Xpansion Interpreter results. **Conclusions:** The prototype LRS Trio assay accurately resolves multiple classes of challenging variants using a streamlined workflow. This method may help labs shift to more comprehensive, affordable, and equitable carrier screening practices using panels of conventional and challenging genes in combination with diagnostic-grade PCR enrichment and more accessible and capable LRS methods.

G058. **Artificial Intelligence Delivers Deeper Insights of Fabry Disease Using Large Scale Longitudinal Electronic Health Records**

K. Zhang, C. Wei, M. Ma, A. Valencia, D. Corrigan, L. Havens, J. Wang, T. O'Connell, X. Zhou, C. Yu, E. Schadt, W. Oh, R. Chen, P. Kruszka
Sema4, Stamford, CT.

Introduction: Fabry disease (FD) is a rare X-linked lysosomal storage disease due to alpha galactosidase A deficiency. The classic form of FD in males has disease onset in childhood or adolescence with acroparesthesia, angiokeratomas, hypohidrosis, and proteinuria. Female heterozygotes have milder symptoms and later onset than males. Therefore, patients may be asymptomatic at birth, but develop cardiac, neurological, and renal manifestations in their lifetime. It is recommended that enzyme replacement therapy (ERT) be initiated as early as possible in all males with Fabry disease. However, there is no consistent clinical recommendation for female patients whose clinical disease course is highly variable and not well understood. **Methods:** We developed a hybrid rule and deep learning-based clinical natural language processing (NLP) system to extract various clinical data elements from the electronic health records. We executed the analyses of de-identified clinical data from one large academic medical center, which also includes gold standard assessment of NLP-extracted clinical data for accuracy and completeness. Similar analyses were also performed on the whole exome sequence data from a clinical reference laboratory. **Results:** From the academic medical center data set, we identified 105 Fabry patients with *GLA* mutations and clinical symptoms by reviewing the electronic medical records. We found a higher number of females (n=54) than males (n=51). The average age of diagnosis is 30.2 years for males, 36.9 years for females. We found 58 unique variants in the *GLA* gene from this cohort, including five new variants that have not been previously reported in peer-reviewed literature. ERTs were effective, and could delay and/or prevent the occurrences of life-threatening complications. However, none of the females (n=8) and only 20% of males (n=20) in the 0–17-year-old group received ERT. In many cases, diagnoses were made when non-reversible organ damages have already developed. There are 14 *GLA* pathogenic variants identified from the clinical reference lab group, of which 10 are previously unreported variants. Four patients were confirmed with clinical FD, and the rest are at risk of developing FD in their lifetime. Interestingly, from this group, 10 are females and four are males. **Conclusions:** We observed that more females were affected or at risk of developing Fabry-associated diseases than males in their lifetime of this X-linked condition. The NLP powered health insight system is a useful tool for large cohort longitudinal study. Our findings call for a revised professional management guideline for female FD patients, and a nationwide medical record system for better patient care and clinical studies.

G059. **High-Fidelity CRISPR-Cas12a-Based *APOL1* Genotyping**

R. Haun, C. Larsen, J. Wilson, D. Kenan
Arkana Laboratories, Little Rock, AR.

Introduction: Approximately 13% of African Americans have a combination of risk variants of the *APOL1* gene, often termed the *APOL1* "high-risk genotype," which predisposes individuals to serious kidney diseases such as focal segmental glomerulosclerosis. Despite the potential severity of *APOL1*-linked kidney disease, genetic testing for *APOL1* variants is uncommonly performed. To address this unmet need, we developed a CRISPR/Cas12a-mediated genotyping assay for the detection of pathological *APOL1* variants from patient samples for potential use in a point-of-care setting. **Methods:** A series of crRNA guides were screened to identify crRNAs that could discriminate either between a single base change at the *APOL1 G0* locus (*G1* variant, rs73885319) or the six-base indel at the *G2* variant (rs71785313) utilizing the *trans*-cleavage properties of Cas12a. Briefly, DNA gene blocks were synthesized representing each of the four *APOL1* variants and amplified with *Taq* polymerase. For each variant, a series of six crRNA guides were prepared that were offset by one base relative to each other and the target variant sequence. The efficiency of the crRNAs to discriminate between the wild-type and variant sequence was then examined using a fluorescence-based CRISPR-Cas12a assay in the presence of a FAM-labeled ssDNA reporter probe and relevant DNA target. The selected crRNA guides were used in genotyping assays of DNA prepared from patient blood samples comprising all six potential *APOL1* variants: G0/G0, G1/G0, G1/G1, G2/G0, G2/G2, and G1/G2. The crRNA guides were then examined in a lateral flow assay with a FAM- and biotin-labeled reporter. **Results:** For each variant, crRNA guides were identified that could readily discriminate between either the wild-type or variant sequence in a Cas12a-*trans*-cleavage assay. Interestingly, no protospacer-adjacent-motif (PAM) sequence was required for the CRISPR/Cas12a-based detection of any of the variants. CRISPR/Cas12a fluorescence-based genotyping assays performed on patient samples achieved 100% sensitivity and specificity compared to genotypes obtained using a clinically validated TaqMan assay. Similar results were also obtained in lateral flow assay format. **Conclusions:** In the present study, we have developed a CRISPR/Cas12a-based *APOL1* genotyping assay with very high specificity and sensitivity. Based on current population estimates, more than 5 million Americans would be at increased risk for serious kidney diseases based on their *APOL1* genotype alone. Thus, it would be immensely helpful if we can translate this genotyping assay for rapid point-of-care testing to determine if an individual has an increased risk of serious kidney disease based on *APOL1* genotype.

G060. **Spinal Muscular Atrophy/SCID Control Panel: A Control Panel of Biologically Relevant, Allele-Based, and Copy Number Controls to Accurately Monitor SMA/SCID Assay Performance**

T. c, A. Karaczyn, M. Escott, J. Gordon, N. Sivaji, M. Steffen, N. Wilkinson
Maine Molecular Quality Controls Inc, Saco, ME.

Introduction: Spinal muscular atrophy (SMA) and severe combined immunodeficiency (SCID) are two diseases recommended as part of routine newborn screening due to the severity and recently available therapies. SMA, an autosomal recessive disease affecting motor neurons caused by a homozygous mutation or loss of the *SMN1* gene, results in progressive muscular atrophy. Testing is primarily associated with determination of *SMN1* and *SMN2* copy numbers. Early diagnosis allows for critical intervention with new treatments to prevent further damage to nerve cells. SCID comprises a group of disorders caused by mutations in genes involved in the development and function of immune cells. SCID is recognized as a pediatric emergency since it leads to severe and recurrent infections and death in the first two years when untreated. SCID is primarily detected by quantifying T-cell receptor excision circles (TREC) and kappa-deleting element recombination circles (KREC) on newborn dried blood spots. As more assays and technologies are developed for newborn screening diagnostics, the need for a comprehensive control to accurately monitor

both SMA and SCID assay performance is critical. **Methods:** A panel of synthetic plasmids were created to include important SMA genetic markers within all exons plus intronic borders of *SMN1* and *SMN2* genes, TREC and KREC sequences, and gene segments of numerous reference genes. The plasmids were quantified by UV spectrophotometry and suspended in a proprietary blood-like mimic and spotted on Whatman 903 Protein Saver cards to create an SMA/SCID dried blood spot (DBS) control panel. The control panel consists of four cards with different SMA genotypes and SCID levels. Samples were extracted using QuantaBio DBS Extracta and tested by qPCR, digital droplet PCR (ddPCR), and multiplex ligation-dependent probe amplification (MLPA). To represent a "patient" sample, whole blood was tested alongside the controls for comparison. Ten DBS extraction methods were evaluated using multiplex SMA/SCID quantitative PCR (qPCR) and ddPCR *SMN1/SMN2* copy number determination kits. **Results:** All methods resulted in 100% accurate genotype calls across multiple lots. Extraction methods resulted in varying recovery based on extraction efficiencies, however, were reproducible with %CV <10% tested by qPCR. Eight punches across a single DBS show homogeneous distribution with no significant variability (SD = <1 and %CV <5%) tested by qPCR. **Conclusions:** The SMA/SCID control panel provides the ability to assess assay determination of copy number of *SMN1* and *SMN2*, properly detects clinically relevant variants for the accurate detection of SMA, and monitors the performance of quantification of TREC/KREC levels for detection of SCID. The SMA/SCID control panel demonstrated reproducible compatibility across multiple platforms, including qPCR, ddPCR, and MLPA test methods.

G061. **Cytogenomic Evaluation of Plasma Cell Neoplasms: A Novel Integrated Approach**

M. Aarabi[1], S. Yatsenko[1], J. Yoest[2], R. Farah[1], A. Rajkovic[3], S. Swerdlow[1]
[1]University of Pittsburgh Medical Center, Pittsburgh, PA; [2]Case Western Reserve University School of Medicine, Cleveland, OH; [3]University of California, San Francisco, CA.

Introduction: Plasma cell neoplasms (PCN) are associated with characteristic chromosomal aberrations of diagnostic and prognostic significance. The presence of a small proportion of neoplastic cells is a drawback in karyotyping and fluorescence *in situ* hybridization (FISH) for evaluation of bone marrow aspirates. Although enrichment for CD138+ cells has improved the FISH detection rate, FISH requires several probes and a sufficient number of isolated cells. **Methods:** We developed a novel integrated protocol for microarray analysis of amplified DNA obtained from isolated CD138+ cells, and one FISH assay for balanced *IGH* rearrangements. Bone marrow aspirates from 56 patients with clinical suspicion for plasma cell neoplasms were used to evaluate the proposed approach. **Results:** Among patients affected by PCN, whole genome profiling results were fully concordant with the positive findings of karyotype and conventional FISH in 87.5% of patients, and detected abnormalities characteristic of PCN in four patients (12.5%) with normal karyotype and uninformative FISH. This method uncovered abnormalities of prognostic value including *MYC* alterations, 16q loss, and hypodiploidy that were otherwise undetectable by conventional methods. **Conclusions:** We propose a cost-efficient approach that, regardless of the initial plasma cell percentage, provides a superior detection rate required for proper risk stratification and differential diagnosis of PCN.

G062. **A Novel Approach for Genetic Testing of Autosomal Dominant Polycystic Kidney Disease That Includes Analysis of the Highly Homologous *PKD1* Gene**

L. Train, S. Peterson, S. Lagerstedt, M. Kluge, S. Kester, D. Kronemann, B. Moore, E. Blake, C. Sosa, R. Setlem, R. Gnanaolivu, N. Mistry, E. Thoreson, K. Kotzer, L. Coon, S. Senum, P. Harris, C. Marcou, A. Moyer, L. Baudhuin
Mayo Clinic, Rochester, MN.

Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is primarily characterized by bilateral kidney cysts, liver cysts, and an increased risk for intracranial aneurysm. Multiple genes are associated with ADPKD, with *PKD1* accounting for the majority of cases. *PKD1* is a highly homologous gene and is challenging to resolve by standard short-read next-generation sequencing (NGS). Here, we describe our novel approach to develop testing for a panel of genes involved in ADPKD, including the highly homologous *PKD1* gene. **Methods:** The IDT xGEN Exome Research Panel v1 capture and amplicon-based NGS were utilized to interrogate eight genes (*ALG8, ALG9, DNAJB11, GANAB, HNF1B, PKD1, PKD2, UMOD*) associated with ADPKD. Due to high homology, sequence variants (SV) in the *PKD1* gene were identified by combining the results of the exome capture with four PCR amplicons created from specific primers to uniquely amplify the region of interest (highly homologous exons 1-33). The amplicons were pooled together and run by Illumina TruSeq Nano NGS chemistry and aligned to a pseudogene masked reference genome file. The results for the two methods were then combined to definitively determine the variants located within. **Results:** Fifteen well-characterized DNA samples from a research laboratory with deep expertise in the *PKD1* gene were run, resulting in an 80% concordance for SV calls using the IDT xGEN Exome Research Panel v1 capture. Discordances seen with the IDT cGEN Exome capture included variants at very low variant allele frequencies that did not match criteria to be called heterozygous SVs. Inclusion of data for the amplicon-based TruSeq Nano NGS method increased the concordance of the SV calls to 96%. The two variants seen as discordant in the TruSeq Nano NGS data may be somatic changes that are not targeted by this test. **Conclusions:** We developed an approach to offer genetic testing for ADPKD, including the highly homologous *PKD1* gene. This approach provides greater accuracy than can be seen with an NGS capture chemistry alone and will provide a cost-effective testing option for ADPKD.

G063. **A Highly Multiplexed Non-Invasive Prenatal Test Utilizing Digital PCR**

M. Smith, A. Aguiar, P. Belitz, P. Berroteran, J. Enciso, L. Herdt, K. Jeevaprakash, G. Ornelas, O. Pererra, S. Rosenberg, M. Wang, J. Gole
ChromaCode, Carlsbad, CA.

Introduction: Non-invasive prenatal testing (NIPT) via next-generation sequencing (NGS) has become increasingly adopted in the United States as a screening test to determine the risk of fetal aneuploidy. The high cost and slow turnaround time associated with NGS have hampered wider adoption. Digital PCR (dPCR) is a sensitive technique that can be utilized for cell-free DNA testing at a lower price point compared to many NGS applications. Here we describe a dPCR NIPT assay to estimate the risk of aneuploidy of chromosomes 13, 18, 21, X, and Y leveraging ChromaCode's High Definition PCR (HDPCR). HDPCR enables high multiplexing within fluorescence channels on dPCR instrumentation yielding a 10X target density over traditional dPCR. The assay was optimized using contrived samples, then tested against a cohort of clinical samples, yielding a high concordance. **Methods:** The prototype HDPCR assay utilized the Bio-Rad QXONE dPCR instrument. Monte Carlo simulations determined the number of targets per chromosome required to detect aneuploidy at fetal fraction as low as 4%. A three-well assay was developed: a core trisomy assay to detect trisomy of 21, 13, and 18; a sex chromosome assay to detect the presence of Y, Turner's syndrome (X0), and Klinefelter's syndrome (XXY); and an assay to quantify fetal fraction. The assay was first optimized using contrived samples of cell line DNA with known

karyotypes sheared to ~150 bp to simulate cell free DNA. Next, euploid and aneuploid cell lines (Coriell) from various ethnicities were spiked into either euploid cell lines or cell free DNA from donor blood at different simulated fetal fractions. Then, 5-15 ng of DNA per reaction were used per assay and data were analyzed using ChromaCode's proprietary algorithm. Z-scores were calculated to call the aneuploidy status. Finally, the assay was verified on 66 euploid and 30 trisomy 21 clinical samples. **Results:** The assay showed promising results on the 520-570 contrived samples. Sensitivities at 4% fetal fraction were 89.5%, 91.1%, 45.6%, 91.1% 99.2%, and 55.8% for T21, T18, T13, XXX, XXY, and X0, respectively. Sensitivities at 8% fetal fraction were 98.1%, 97.8%, 93.3%, 100%, 100%, and 94.2% for T21, T18, T13, XXX, XXY, and X0, respectively. Overall specificities were 90.7%, 93.2%, 83.3%, 99.2%, 98.2%, and 83.9% for T21, T18, T13, XXX, XXY, and X0, respectively. The assay was tested using 96 clinical samples with fetal fractions ranging from 3%-17% and DNA inputs ranging from 3-12.5 ng. The overall positive percent agreement and negative percent agreement across targets were 91.1% and 98.5%, respectively. **Conclusions:** HDPCR was demonstrated to be an advantageous approach to NIPT on a dPCR platform utilizing this prototype three-well assay. We have demonstrated proof of concept for a viable alternative to current NGS-based NIPT assays with lower cost and faster turnaround time, enabling wider adoption of NIPT.

G064. **Highly Multiplexed SNP Discrimination Utilizing Digital PCR**

A. Richards, J. Enciso, O. Pererra, M. Smith, L. Herdt, K. Jeevaprakash, M. Wang, S. Rosenberg, J. Gole
ChromaCode, Carlsbad, CA.

Introduction: Digital PCR (dPCR) enables highly sensitive detection of single-nucleotide polymorphism (SNPs) by partitioning a single reaction into thousands of partitions. Here, we demonstrate the ability to detect SNPs and quantify minor allele fraction down to 4% from liquid biopsy in a highly multiplexed reaction using ChromaCode's High Definition (HDPCR) technology. Whereas conventional multiplexing limits the number of detectible targets by the number of available fluorescence detection channels on the instrument, HDPCR enables 10X the number of detectible targets per color channel in a single dPCR reaction. This prototype assay serves as a demonstrator assay for HDPCR to highly multiplex SNPs on a dPCR instrument for a wide variety of applications. **Methods:** Digital PCR (dPCR) enables highly sensitive detection of single-nucleotide polymorphism (SNPs) by partitioning a single reaction into thousands of partitions. Here, we demonstrate the ability to detect SNPs and quantify minor allele fraction down to 4% from liquid biopsy in a highly multiplexed reaction using ChromaCode's High Definition (HDPCR) technology. Whereas conventional multiplexing limits the number of detectible targets by the number of available fluorescence detection channels on the instrument, HDPCR enables 10X the number of detectible targets per color channel in a single dPCR reaction. This prototype assay serves as a demonstrator assay for HDPCR to highly multiplex SNPs on a dPCR instrument for a wide variety of applications. **Results:** A total of 20 assays were multiplexed across a total of four fluorescence channels. Nine assays were found to be highly sensitive and specific to the allele of interest. Correlation to spike in percentage for each of these nine assays was high, with the majority greater than 95%. For each assay, accurate quantification of the minor allele down to 4% allele fraction was achieved. **Conclusions:** The ability to accurately detect SNPs in a highly multiplexed manner utilizing dPCR is demonstrated herein. The applications of this technology include fetal fraction determination for NIPT, single nucleotide, and other variants for oncology, and forensics. Highly multiplexed dPCR assays for these applications will enable reduced cost and increased throughput over next-generation sequencing-based methods.

G065. **Supplemental NGS Method for Homologous Genes in Inborn Errors of Immunity (Primary Immunodeficiency) Comprehensive Genetic Panel**

S. Lagerstedt, A. Kumanovics, C. Sosa, L. Baudhuin, L. Train, S. Peterson, B. Johnson, J. Hardcastle, A. Moyer
Mayo Clinic, Rochester, MN.

Introduction: Inborn errors of immunity are genetic defects of the immune system that result in immune dysregulation, greater susceptibility to infection, autoimmunity, and autoinflammation. Appropriate diagnosis and treatment require identification of the underlying cause. Next-generation sequencing (NGS) is routinely used as a clinical diagnostic tool, yet numerous homologous regions in relevant genes (*C4A, C4B, CD46, CFH, CFTR, CORO1A, DUOX2, EFL1, IKBKG, NCF1, SBDS, STAT5B,* and *UPS18*) can't be effectively analyzed by standard short-read NGS technology. **Methods:** We have developed a strategy for supplementing our IDT xGEN Exome Research Panel v1 capture NGS method. The supplemental method ensures specificity in two ways. Gene-specific long-range amplification separates the gene from its homologous counterparts, and a custom reference file, in which regions of homology have been removed, force alignment to the specific target. This allows reads to be uniquely mapped despite significant homology, a requirement for variant calling. Multiple amplicons may be pooled together and prepared for sequencing on an Illumina MiSeq instrument using TruSeq Nano DNA Library Prep, making the method cost effective. **Results:** We prepared specific amplicons for several homologous gene targets. Amplicons were sequenced and were compared with the standard capture method. Variants, not called in the standard-capture method due to poor mapping scores (non-uniquely mapped reads), are called in the amplicon method. In the capture method, the variants are visualized in the BAM as a mixture of gene and pseudogene, whereas gene and pseudogene variants are clearly separated and identified in the amplicon method. In some situations, we are able to creatively leverage copy number variant (CNV) analysis from the capture NGS method to identify alleles that misalign to the pseudogene. The pathogenic *NCF1* GT deletion in Exon 2 appears to resemble a copy number deletion event when present as reads from one allele misalign to the *NCF1B* and *NCF1C* pseudogenes. Due to high variability in alignment, many homologous regions do not provide reliable CNV results preventing standard CNV analysis. Information from both the capture and amplification methods may be used in tandem to assess copy number state. From the capture method, a combined gene/pseudogene copy number analysis is investigated where "normal" copy number represents four alleles rather than two alleles. From the amplification method, the variant allele frequency may indicate which gene is affected. **Conclusions:** We plan to include these strategies in our new Inborn Errors of Immunity Panel which will enable us to provide a more comprehensive and cost-effective analysis than is currently available.

G066. **Genetic Testing in Renal Disease: A Combined NGS Approach Using Capture-Based Exome Plus Amplicon-Based Chemistry for Comprehensive Testing Including Analysis of the Homologous *CFH* Gene Family**

S. Peterson, L. Train, S. Lagerstedt, M. Kluge, S. Kester, D. Kronemann, B. Moore, E. Blake, C. Sosa, R. Setlem, R. Gnanaolivu, N. Mistry, E. Thoreson, K. Kotzer, L. Coon, C. Marcou, L. Baudhuin, A. Moyer
Mayo Clinic, Rochester, MN.

Introduction: Chronic kidney disease (CKD) is a common condition worldwide that can have a genetic etiology with hundreds of genes implicated. Although next-generation sequencing (NGS) is an ideal platform for testing large numbers of genes, standard short-read NGS is often unable to resolve the sequence of homologous genes, including the *CFH* gene family, which is associated with complement-mediated thrombotic microangiopathy and C3 glomerulopathy. Thus, regions of homology are often unavailable in standard exome testing and may be excluded from targeted panels. Here, we describe our approach to develop testing for the *CFH* gene family for inclusion in a

comprehensive genetic test for hereditary renal disease. **Methods:** The IDT xGEN Exome Research Panel v1 capture and amplicon-based NGS were utilized to interrogate 302 genes associated with hereditary renal diseases. Genes were assessed for homology and an accuracy comparison of previously run samples was performed. Fourteen genes (97 exons) had homology requiring a supplemental method, with the *CFH* gene family requiring the most complex approach. For the *CFH* gene family, sequence variants (SV) were identified by combining the results of the exome capture with six PCR amplicons (three short-range, three long-range) created using specific primers that uniquely amplify the region of interest. The amplicons were pooled together and run by Illumina TruSeq Nano NGS chemistry and aligned to a modified reference genome file with select regions of homology masked. The results for the two methods were then combined to definitively determine which gene the variant was located within. Copy number variation (CNV) was bioinformatically detected for non-homologous regions by the pipeline. Due to the polymorphic *CFHR1* and *CFHR3* deletions, as well as complex haplotype structure, CNV calls for homologous regions were made by comparison to a training set created with samples known to have two copies of each gene. **Results:** Across all 302 renal genes, SV in 40 samples were 100% concordant with expected results. A set of 18 samples demonstrated 100% concordance for *CFH* family SV using this approach as compared to a long-range Sanger sequencing plus NGS approach. CNV were 97.5% concordant with multiplex ligation-dependent probe amplification (MRC Holland P236-B1 CFH Region probemix). The 2.5% discordance was due to a *CFH*-Ex20 probe false positive deletion in the presence of a *CFHR1* deletion. **Conclusions:** We developed an approach to offer genetic testing for hereditary renal diseases, including highly homologous genes resulting in complement-mediated disorders. The approach provides an accurate, comprehensive, and cost-effective testing method and can be applied to other genetic testing panels that include highly homologous genes.

G067. **Data Curation for NGS Gene Panels: Guideline-Driven Assessment of Clinical Significance of Genes Contributing to Cardiovascular Disease**

A. Levitas[1], J. Neidich[2], M. Schroeder[2], K. Bowling[2], Y. Cao[2], M. Corliss[2], J. Heusel[2]
[1]Washington University School of Medicine, Department of Pathology & Immunology, St. Louis, MO; [2]Washington University School of Medicine, St. Louis, MO.

Introduction: Heterogeneity in the manifestation of human diseases requires not only robust detection but also interpretation of potentially causal or contributory genetic variants leading toward accurate diagnoses. Our laboratory had clinically validated an exome capture next-generation sequencing (NGS) assay and applied bioinformatic filters to construct disease-specific diagnostic panels of genes, including one for cardiovascular diseases. Ongoing quality assurance for NGS testing requires proactive reevaluation of gene-disease and variant-disease associations. This curation process is facilitated to an increasing degree by the guidelines arising from dedicated ClinGen working groups. We undertook a reanalysis of our cardiovascular disease panels to assure the clinical relevance of the included genes. **Methods:** To restructure our existing cardiovascular disease panels, we curated 35 genes following version 8 of ClinGen's standard operating procedure for the gene clinical validity curation process. Published literature associated with the cardiovascular conditions and associated genes was reviewed in depth, and data from various databases including gnomAD and Online Mendelian Inheritance in Man were interrogated. These data were used to guide the classification of gene-disease associations categorizing genes as being associated with disease or as genes of uncertain clinical significance (GUS). These classifications informed decision-making regarding which genes would be included in the updated panels. **Results:** The curation process led to restructuring the full cardiovascular disease gene panel, including 150 genes, and nine disease-specific subpanels. The panels were additionally curated according to the strength of the evidence of clinical impact on disease. The genes with sufficient evidence for clinical impact for each disorder formed core panels that would constitute a molecular diagnosis when a clinically significant variant was identified. The genes with less evidence for clinical impact, or where emerging data may indicate an impact on disease, formed expanded panels that could be ordered as a reflex clinical assay (GUS). **Conclusions:** We used the ClinGen standard operating procedure version 8 to guide the curation of genes in a set of NGS panels for cardiovascular diseases. These panels were divided into core and expanded genes subpanels, with GUS included in the expanded panels. The identified GUS have an undetermined clinical impact, yet may allow for the diagnosis of cardiovascular disorders as more evidence is accrued, or may guide additional testing. We will discuss the clinical systematic use of the ClinGen process for gene inclusion in the cardiovascular panels and other disease-associated panels.

G068. **Microsatellite Instability Analysis of Circulating Tumor DNA Using Capillary Electrophoresis**

S. Lewis, M. Ensenberger, K. Oostdik
Promega Corporation, Madison, WI.

Introduction: Microsatellite instability (MSI) analysis is often required in situations where a large tissue sample or normal reference sample cannot be obtained. Access to adequate samples can be a challenge for assessment of this critical biomarker. Similarly, tumor-only analysis, though possible, is less sensitive to subtle shifts present in certain tumor and tissue types. In this work, we present a way to obtain sensitive results with circulating cell-free DNA (ccfDNA) and matched buffy coat or whole blood from the same sample using two MSI panels, one containing the gold standard markers, and the other containing novel markers optimized for alternative sample type assessment. **Methods:** A cohort of 26 samples from individuals with MSI-high endometrial adenocarcinomas with associated staging at time of collection were used. High quality DNA was obtained from matched formalin-fixed, paraffin-embedded (FFPE) normal, FFPE tumor, circulating tumor DNA (ctDNA) from plasma samples for all individuals. Sixteen samples also had associated matched buffy coat for comparison. **Results:** High concordance was observed between profiles obtained in ctDNA and DNA isolated from the solid tumor of origin. Limit of detection for selected representative samples was calculated for differing shift sizes to demonstrate the sensitivity of this technique with each tissue type. **Conclusions:** PCR followed by capillary electrophoresis has several advantages for MSI testing in circulating DNA and other limited or precious sample types. Extremely low DNA input requirement, a single amplification step to minimize error introduction, and low cost all highlight the benefits of this technique for targeted MSI analysis. In this study we show that PCR followed by capillary electrophoresis is a valuable and effective tool for MSI analysis with challenging samples and across tissue types.

G069. **Design and Validation of a Whole-Genome Sequencing-Based Assay for Population Health**

J. Welle[1], B. Piening[1], J. Wagner[1], I. Lucas-Beckett[2], T. Ward[1], B. Cosgrove[1], E. Shull[1], R. Rattray[1], M. Pukay[1], M. Ricks[1], K. Childers[3], J. Linehan[4], M. Campbell[1], O. Gordon[4], C. Bifulco[1]
[1]Providence Health System, Portland, OR; [2]Institute for Systems Biology, Seattle, WA; [3]Providence Health, Burbank, CA; [4]Providence Health, Santa Monica, CA.

Introduction: Next-generation sequencing (NGS) is rapidly emerging as a key methodology in the clinical laboratory for a wide array of clinical uses, including heritable disease testing, somatic tumor profiling, and microbiology. Whereas current clinical methods are largely focused on targeted gene panels, the low cost and high throughput of modern sequencing platforms are making it possible to use whole-genome sequencing (WGS) for routine clinical applications. Here we developed a clinical WGS-based lab developed test (LDT) for heritable disease gene testing and pharmacogenomics (PGx) and performed extensive validation across a large cohort of blood and saliva specimens. **Methods:** DNA was isolated from both whole blood

(EDTA) and saliva (Oragene collection) using the Qiagen QIAsymphony DSP Midi Kit. Whole genome libraries were prepared from 300 to 500 ng gDNA with the Illumina DNA PCR-Free Tagmentation kit. Sequencing (30X) was performed on the Illumina NovaSeq 6000. Pharmacogenetic genotyping and variant detection analysis (82 genes) were performed using standard analysis pipelines on the Illumina Dynamic Read Analysis for GENomics (DRAGEN) platform. Interpretation of heritable disease gene variants as well as PGx star allele assignment were performed by experts utilizing the Fabric Genomics interpretation platform as well as a novel in-house developed platform for clinical reporting. **Results:** WGS was performed on a combined retrospective and prospective validation cohort of 119 whole blood and 69 saliva specimens that were orthogonally tested via single gene or small panel tests at commercial laboratories. Validation was performed across a set of 77 actionable disease genes and resulted in 100% agreement in called pathogenic and likely pathogenic single nucleotide variants, indels, and copy number variants when compared to the reference method. Genotyping from WGS across a panel of PGx genes (*CYP2C19, CYP2C9, VKORC1*, and *CYP4F2*) in the 119 whole blood and 69 saliva gDNA samples resulted in 100% concordance with the reference method (MALDI-TOF mass spectrometry). **Conclusions:** WGS exhibited equivalent performance with targeted gene panel testing across a large cohort of clinical specimens. The comprehensive nature of a WGS backbone for clinical testing has the added benefit of facilitating expanded reanalysis of new actionable genes, rapid redeployment for use in other clinical contexts, as well as for use by researchers in supporting novel genomic discoveries. Given the anticipated upcoming reductions in cost for WGS, a singular clinical genome-based platform will likely represent a viable streamlined option for supporting clinical genetic/genomic testing across the clinical spectrum.

G070. **Evaluation of the CystoChek, a Single Methylation Biomarker-Based Urine Test for the Selection of Hematuria Patients for Cystoscopy**

C. Im[1], S. Kim[1], E. Hong[1], J. Lee[1], S. Beqaj[1], J. Lim[2], T. Oh[3], S. An[1], B. Bang[1]

[1]Promis Diagnostics, Irvine, CA; [2]Chungnam National University, Daejeon, Republic of Korea; [3]Genomictree, Daejeon, Republic of Korea.

Introduction: Generally, patients with gross or increased risk of microscopic hematuria are recommended to examine with a cystoscopy by current clinical guidelines to rule out bladder cancer (BC). However, only 5% to 20% of hematuria patients are diagnosed with BC. In terms of diagnostic procedures, cystoscopy is associated with high costs and patient burdens. Therefore, there is a great need for a urine-based molecular marker test that can focus specifically on the hematuria workup. We previously identified aberrant methylation of the *PENK* gene (mePENK) as a specific diagnostic biomarker for BC and validated its diagnostic potential in clinical studies for detecting BC. Here, we aim to develop a sensitive and accurate urine-based *PENK* methylation detection assay, mePENK LTE-qMSP (CystoChek), and validate its performance to detect BC at an earlier stage in the prospective clinical study. **Methods:** The CystoChek test is designed to perform two-step reactions, linear target enrichment and real-time PCR (qMSP), in a single tube, which is applied to measure a single *PENK* methylation biomarker and control DNA. In the clinical validation, CystoChek measured *PENK* methylation level in the DNA extracted from voided urine samples collected from 183 hematuria patients who were scheduled for cystoscopy examination. In the analytic validation, the limit of detection (LoD) test and sample stability test were conducted using the contrived samples containing a range of 0% to 10% of RT4 (*PENK*-methylated cancer cell) in urine from healthy donors. DNA was extracted from either the contrived samples or the urine samples from hematuria patients using our proprietary extraction method. **Results:** CystoChek showed a sensitivity of 93% in the detection of Ta high-grade and greater stages of BCa at a specificity of 90%. *PENK* methylation was not significantly correlated with sex, age, or stage, but was associated with grade. The positive and negative predictive values were 51.9% and 99.2%, respectively. Analytical validation showed that CystoChek has an LoD as low as 0.1% (one cancer cell in 1,000 normal cells) which was determined by Probit analysis. The stability test demonstrated that the urine samples were well preserved in a transport medium for at least seven days at either 4°C or room temperature. **Conclusions:** This study suggests that urine-based *PENK* methylation assay (CystoChek) is highly sensitive and specific for the detection of BC. It is warranted for CystoChek to conduct large-scale prospective clinical trials for translating its use to clinical practice for selecting hematuria patients for cystoscopy.

G071. **Development of a Cell-Based Pan-Cancer Fusion FFPE RNA Reference Standard for Use in Targeted Oncology NGS and RT-PCR Applications**

C. McGee[1], M. Fernandez-Munoz[1], A. Van Brabant Smith[2], B. Freeman[1], J. Keynton[1], E. Chin[3]

[1]Horizon Discovery, Cambridge, United Kingdom; [2]Horizon Discovery, Lafayette, CO; [3]PerkinElmer, Sugar Land, TX.

Introduction: Targeted next-generation sequencing (NGS), quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and RT-droplet-digital PCR (RT-ddPCR) platforms offer researchers tremendous potential for profiling biomarkers in tumor samples. However, data interpretation can be challenging due to inherent variabilities in samples, RNA extraction procedures, RNA quantification, and platform biases. This can result in low confidence results and a potential failure to detect biomarkers. To aid in the development, validation, and routine monitoring of these assays, Horizon has developed the Pan-Cancer 6-Fusion Panel which is a multiplexed cell-line blend verified to contain *TPM3-NTRK1*, *QKI-NTRK2*, *ETV6-NTRK3*, *EML4-ALK*, *CCDC6-RET,* and *SLC34A2-ROS1* mRNAs in a formalin-fixed, paraffin-embedded (FFPE) format. **Methods:** Cell-lines, which were either engineered by Horizon to express the fusions of interest or derived from cells expressing fusions endogenously, were single-cell diluted to produce clonal cell populations for each fusion variant. Fusions were then confirmed by Sanger sequencing. After fusion expression profiling by RT-ddPCR, the cell-lines were blended at defined ratios to ensure consistent expression levels of each fusion. The multiplexed cell blend was then fixed in 10% formalin, before being embedded in FFPE cores and finally embedded in paraffin blocks. Three FFPE blocks were sectioned into 15 µm sections. RNA was extracted from sections using the Maxwell RSC RNA FFPE Platform (Promega) and assessed for:

- Average RNA yield by Qubit RNA HS Assay (Thermo Fisher Scientific)
- RNA integrity (DV_{200}) using the High-Sensitivity ScreenTape Assay (Agilent)
- Fusion biomarker expression by RT-ddPCR using fusion specific probes on the QX200 (Bio-Rad)

Results: All RNA yields (n=90) were above the >100 ng minimum QC acceptance criteria, with average yields of 413 ng, 679 ng, and 585 ng, respectively, from the three blocks tested. DV_{200} values for all the sections tested (n=90) were greater than the minimum acceptance criteria of 65%, with averages of 84%, 90%, and 87%, respectively, for three blocks. Fusion expression for the six fusion biomarkers was consistent across the three blocks (n=90) with average copies/ng RNA well above the minimum acceptance criteria for each fusion.

- *NTRK1*: 411 copies/ng (≥100)
- *NTRK2*: 605 copies/ng (≥100)
- *ALK*: 155 copies/ng (≥40)
- *ROS1*: 175 copies/ng (≥40)
- *NTRK3*: 40 copies/ng (≥4)
- *RET*: 42 copies/ng (≥4)

Conclusions: The Pan-Cancer 6-Fusion Panel FFPE RNA is a highly characterised, cell-line derived reference material that is commutable with biopsy samples. It serves as an appropriate control for end-to-end validation and optimization of RNA-seq, RT-qPCR or RT-ddPCR assays aimed at detecting *TPM3-NTRK1, QKI-NTRK2, ETV6-NTRK3, EML4-ALK, CCDC6-RET,* and *SLC34A2-ROS1* fusions.

G072. **Quantifying Fetal DNA in Maternal Blood Plasma by ddPCR Using DNA Methylation**

E. Hall, T. Riel, M. Ramesh, M. Gencoglu, O. Mikhaylichenko, R. Dannebaum, S. Margeridon, M. Herrera
Bio-Rad Laboratories, Pleasanton, CA.

Introduction: The proportion of cell-free DNA (cfDNA) circulating in maternal blood that originates from the fetus, the fetal fraction, is an important quality control metric when performing tests on fetal-derived cfDNA. Epigenetic differences produce dissimilar DNA methylation patterns, allowing for leveraging regions of high methylation contrast using methylation-sensitive restriction enzyme (MSRE) digestion to quantify fetal and maternal DNA via droplet digital PCR (ddPCR). This advancement positions ddPCR as a faster and less expensive alternative to next-generation sequencing (NGS) for fetal fraction estimation. **Methods:** Assays were designed to target MSRE-compatible regions with high methylation contrast between maternal and fetal cfDNA. Fetal assays targeted sites hypermethylated in fetal cfDNA and maternal assays targeted sites hypermethylated in maternal cfDNA. The assay multiplex was tested against contrived and clinical samples using an in-droplet MSRE-ddPCR workflow. The reaction mix was dropletized to create about 20,000 droplets per 24-µL reaction, thermocycled, and analyzed in a QX ONE instrument. The thermocycling profile included a 45-minute MSRE incubation step prior to PCR amplification. Contrived samples were constructed by spiking DNA-free plasma with micrococcal nuclease-digested DNA from an amniotic fluid cell line ("fetal" component) and a B-lymphocyte cell line ("maternal" component). Clinical samples were remnant diagnostic samples with existing NGS non-invasive prenatal testing results attached. DNA was extracted from all samples with the Apostle MiniMax kit on the KingFisher Flex. **Results:** From an initial set of 15 assays, a final five-assay multiplex was produced following amplicon sequencing with NGS and ddPCR screening. Although amplicon sequencing did not completely predict ddPCR performance and non-specific interactions, it was valuable for guiding the final ddPCR screen. The five-assay multiplex, consisting of three fetal assays and two maternal assays, produced an excellent linear response against contrived samples from 0% to 25% fetal fraction (R^2 >0.99). Similarly, a high correlation was observed between ddPCR-estimated fetal fraction and NGS fetal fraction for a set of clinical samples (n = 6, plus two non-pregnant controls, R^2 >0.94). **Conclusions:** As an epigenetic trait, DNA methylation is a useful way to discriminate between otherwise highly similar DNA sequences in an efficient and effective manner. Leveraging DNA methylation may be done with minimal impact to the standard ddPCR workflow. The high sensitivity, speed, and direct quantification of ddPCR make it an attractive alternative to NGS for fetal fraction estimation.

G073. **Higher Resolution and Accurate Breakpoint Determination of a Balanced Translocation by Optical Genome Mapping**

M. Serrano[1], B. Clifford[2], N. Al-Sweel[1]
[1]Bionano Genomics, Salt Lake City, UT; [2]Bionano Genomics, San Diego, CA.

Introduction: Optical genome mapping (OGM) can detect and characterize structural variants present in the human genome. Whereas fluorescence *in situ* hybridization (FISH) and karyotype are considered standard of care for identifying translocations, OGM can be used to identify otherwise cryptic abnormalities and to better characterize breakpoints and gene content. **Methods:** We present a 12-year-old male with severe sensorineural deafness, reduced DNA repair, and atypical pigmentations. The subject had no history of basal cell or squamous cell carcinomas. The subject was identified to have an apparently balanced translocation between 9 and 22 (46, XY, t[9;22][p22;q11.2]) via routine FISH and karyotype but it was unclear whether it was causative of the clinical features observed. **Results:** OGM was performed to further delineate the translocation and revealed that the breakpoint regions mapped to the *CDKN2A* gene on chromosome 9 and a segmental duplication region (LCR-B) on chromosome 22. A similar translocation was reported previously in the literature. The authors of this case characterized a novel balanced t(9;22)(p21;q11.2) translocation in a patient with melanoma, DNA repair deficiency, and features of DiGeorge syndrome. They identified a unique fusion transcript encoding portions of p14ARF CDKN2A isoform) and a novel gene, *FAM230A*(LCR-B) that was suspected to be causative of the patient's clinical features. **Conclusions:** Importantly, the authors required multiple techniques including karyotype, FISH, bivariate flow sorting, and sequencing to characterize the translocation, whereas in our case, OGM achieved a close level of breakpoint resolution in a single step. This case highlights the benefit of OGM in characterizing complex rearrangements compared to standard of care.

G074. **Invitae's High-Throughput AMP-Based RUO VariantPlex NGS Workflow Offers Scalable and Customizable Targeted-Detection of Germline SNVs, Indels, and CNVs**

C. Walker, L. Hartje, K. Hedges, C. Picard
Invitae, Boulder, CO.

Introduction: Accessible and reliable detection of germline genetic variations has become a key diagnostic tool in personalized healthcare. However, high costs and scalability challenges have limited laboratory adoption of germline testing for both preventative and cascade screening. Next-generation sequencing (NGS) assays designed for platform agnostic automation can improve overall implementation of germline testing by increasing throughput. Invitae's NGS-based anchored multiplex PCR (AMP) enrichment chemistry allows for scalable and customizable target selection. AMP panels enrich target regions using gene-specific primers opposing molecular barcoded adapters containing a universal primer binding site, enabling each primer to function independently. Primers can be added or removed from panels without negatively affecting assay performance, meaning that regions of interest can be effectively targeted. **Methods:** Here, we demonstrate an automation-friendly, platform-agnostic, liquid VariantPlex workflow that enables scalable germline testing with small, medium, or large AMP panels. We tested the equivalence of this liquid-based library prep kit to a commensurate lyophilized workflow, evaluating coverage depth and on-target percent across our small CFTR panel (one gene), and medium cardiomyopathy panel (20 genes), to our large expanded carrier panel (127 genes). Copy number variation (CNV) detection performance was tested using our Expanded Carrier panel and input sample material containing known *SMN1* and *SMN2* CNVs associated with spinal muscular atrophy. **Results:** Our liquid VariantPlex workflow resulted in equivalent target and base coverage compared to the lyophilized workflow across all panel sizes. For the small CFTR and large expanded carrier panel, >99.5% of targeted bases were covered at a depth of >50X for both the liquid and lyophilized assays. The medium cardiomyopathy panel achieved 96.1% and 94.7% coverage of targeted bases at >50X for the liquid and lyophilized assays, respectively. For all panels, >96.3% of all unique fragments were on-target. Finally, we saw high concordance when testing 16 samples containing known *SMN1* and *SMN2* CNVs using the liquid-based workflow. **Conclusions:** Invitae's lyophilized and fully liquid VariantPlex assays reliably detect germline variations across small (<20 gene), medium (20-100 gene), or large (>100 gene) panels without requiring assay re-optimization, primer redesign, or balancing. We have shown strong concordance between the liquid and lyophilized workflows for target coverage and on-target percent. Moreover, the liquid-based workflow is automation compatible and platform agnostic, making it ideal for scalable and customizable germline testing.

G075. **High-Precision Copy Number Variation (CNV) Measurement Achieved on Droplet Digital PCR with a High Multiplexing Approach**

R. Dannebaum, M. Herrera, R. Abayan, N. Dzvova, M. Gencoglu, A. Henriquez, S. Margeridon, O. Mikhaylichenko
Bio-Rad, Pleasanton, CA.

Introduction: The ability to test circulating cell-free DNA in the bloodstream has been a revolution for the development of liquid biopsies for clinical applications. Likewise, digital PCR (dPCR) offers a fast and cost-effective means to target and accurately count DNA molecules of interest. However, due to the limited amount of material available in a single tube of blood, high levels of precision are difficult to achieve without sufficiently large numbers of multiplexed PCR assays. In this study, we demonstrate the ability to achieve high levels of precision and accuracy for copy number variation (CNV) detection in dPCR by combining highly multiplex assays with a universal probe approach. **Methods:** This study focused on the use of universal dPCR probes, which enable the combination of several target molecules to be combined in the same digital channel. In contrast to hydrolysis probes (e.g., TaqMan), which require a probe to be defined for each PCR target, universal probes are hybridization probes that have a reduced cost and decreased manufacturing complexity while also maintaining high specificity in the assay. Highly multiplexed dPCR assays were tested on the QX series of droplet digital PCR (ddPCR) instruments. Contrived and clinical samples were tested to evaluate the performance of the multiplexed primers. Furthermore, an *in silico* model was constructed to evaluate the performance of multiplexed assays for CNV detection applications. **Results:** Seventy-eight universal dPCR probe combinations were analyzed, (13 universal probes combined with six dyes). Out of these probes, six were selected as best performers based on probe-probe interactions, ddPCR fluorescence amplitude, lack of ddPCR positivity in no template controls, and ddPCR concentration accuracy. Using these universal probes, more than 100 PCR assays were combined in a single reaction achieving precise measurements of wild-type copies and low positivity in no template controls. Using an *in silico* model, we demonstrate the potential for dPCR applications to detect CNV in sample mixtures as low as 4% with greater than 95% sensitivity. **Conclusions:** ddPCR using high multiplex assays and universal probes enable high-precision CNV detection. This innovative approach can be quickly adapted to other applications, such as liquid biopsies, without the need to redesign the universal ddPCR probes.

G076. **Clinical Validation of Whole-Genome Sequencing for the Detection of Copy Number Variation**

V. Thayanithy[1], B. Thyagarajan[2], M. Bower[1], S. Munro[2], H. Lam[2], S. Bray[1], S. Vivek[2], M. Schomaker[1], J. Daniel[2], C. Henzler[2], A. Nelson[2], S. Yohe[2], K. McIntyre[3]
[1]University of Minnesota Health Fairview, University of Minnesota Medical Center, Minneapolis, MN; [2]University of Minnesota, Minneapolis, MN; [3]Invitae, San Francisco, CA.

Introduction: Structural variants (SVs), traditionally defined as genomic variants >1 kb in length, are an important contributor to Mendelian disease. Several methods have been developed to identify structural variants from whole-genome sequencing (WGS) data. We adopted SpeedSeq software, which integrates both a read-depth-based algorithm (CNVnator) and a breakpoint detection algorithm (Lumpy) for SV detection. SpeedSeq detects an average of 13,684 SV calls per sample, the majority of which are irrelevant to the reported clinical phenotype. Here, we present the development and clinical validation of WGS SV analysis workflow that we have implemented in our lab which produces a minimal number of relevant variants for efficient review. We focused on copy number variations (CNVs), i.e., deletions and duplications – the subset of SVs that are most relevant to germline mutation detection. **Methods:** We used an in-house standard (MDL-GS1) to determine optimal coverage depth for WGS-based SV detection. We have developed a pipeline that filters and integrates the outputs into a manageable list of candidate variants. For the initial development of the workflow (Phase I), seven samples with a broad range of known causative SVs were analyzed. By validating a select list of 122 calls by qPCR, we developed criteria and custom scripts to filter, stitch, intersect, and combine Lumpy and CNVnator outputs into a single annotated manifest. In addition, we automated the identification of common and clinically irrelevant CNVs using DGV, gnomAD, and an in-house developed database of control samples without known genetic disease (n= 34). Finally, 27 unique samples containing 30 known diagnostic structural variants were analyzed and validated (Phase II). **Results:** We have developed an automated workflow that filtered 96.5% of the SV calls from the raw SpeedSeq output and still identified 100% of the pathogenic CNVs identified in the seven samples. Using this automated workflow, our analysis of 27 unique samples (Phase II) resulted in 481 CNV calls (average of 20 CNV calls per sample). Our workflow classified 84 (99.5%) CNVs correctly as not clinically significant, with an average specificity of 97%. All of 30 known tested causative CNVs were detected across 27 samples, with 100% analytical sensitivity. One sample that was prepared in duplicate and analyzed separately revealed identical calls with 100% precision. **Conclusions:** Overall, our WGS analysis workflow demonstrated high sensitivity and specificity, providing a streamlined scalable solution for robust clinical detection of CNVs at genomic scale.

G077. **Application of Long Read Sequencing with Cas9 Target Enrichment for Myotonic Dystrophy Type 1**

J. Jang, J. Cha, J. Park
Samsung Medical Center, Seoul, Republic of Korea.

Introduction: Repeat expansion is a relatively common mechanism in human genetic disorders. Repeat-primed PCR (RP-PCR) is widely used for the diagnosis of repeat expansion disorders as an alternative method to the Southern blot. However, RP-PCR could not provide information about the exact number of repeats and the presence of interrupted sequences, which may be associated with disease severity. In the present study, we applied the Nanopore sequencing technology with cas9 target enrichment to investigate the feasibility of long-read sequencing for the diagnosis of repeat expansion disorders. **Methods:** We used positive reference materials which have information on the number of repeats of expanded alleles of *DMPK*. Eleven patient samples from myotonic dystrophy type 1, who were diagnosed by a typical saw-tooth pattern of RP-PCR, were also used. For enrichment of *DMPK* and/or *CNBP* which is the causative gene of myotonic dystrophy type 2, CRISPR RNAs were designed by using CHOPCHOP software. Library preparation was SQK-LSK110 and long-read sequencing was performed with either Flongle or Spoton flow cells. Base calling and read alignment were done by MinKNOW and using Minimap2-2.17, respectively. **Results:** For two positive reference materials with 1,000 (NA04034) and 2,000 repeats (NA03759), 80~160X sequence reads generated from Spoton flow cells were aligned to the region of interest of *DMPK* and repeat expansions were detected. When *DMPK* and *CNBP* genes were targeted for eight patient samples, and 10~60X and 30~70X coverage was obtained, respectively. When using Flongle flow cell for three patient samples for a single target of *DMPK*, 50~20X coverage was obtained. Repeat expansion was detected in all patient samples. **Conclusions:** We confirmed the feasibility of the Nanopore sequencing technology with cas9 target enrichment for the diagnosis of myotonic dystrophy type 1. The platform could be also be applied for other repeat expansion disorders.

G078. **Clinical Validation of Optical Genome Mapping for Postnatal Application**

N. Sahajpal[1], R. Kolhe[1], A. Mondal[1], A. Hastie[2], A. Chaubey[2]
[1]Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA.

Introduction: Constitutional disorders – including birth defects and neurodevelopmental disorders – warrant genetic/genomic testing for accurate disease diagnosis and clinical management. The current standard-of-care (SOC) workflow includes tiered testing using several cytogenetic and molecular methods to reach a diagnosis, but several

cases remain undiagnosed. The cytogenetic methods employed for the detection of sequence variants (SVs) and copy number variants (CNVs) are low resolution (karyotype), targeted, and require prior knowledge (fluorescence *in situ* hybridization [FISH]), or cannot detect balanced SVs and orientation of duplicated segments of genome (chromosomal microarrays). Repeat array analysis methods are time consuming and have low accuracy (Southern blotting) or low dynamic range (PCR, next-generation sequencing [NGS]). Optical genome mapping (OGM) provides a unique opportunity to consolidate these multiple technologies by detecting all classes of SVs in a single assay. **Methods:** This retrospective validation study analyzed 70 unique and well-characterized samples that received traditional cytogenetic testing with karyotyping, FISH, chromosomal microarray analysis, Southern blot, or PCR assay. Additionally, 10 phenotypically "normal" and cytogenetically "negative" controls were analyzed to access true/false positive rate. Six samples were run in triplicate to evaluate inter-run, intra-run, and inter-instrument reproducibility. Clinically relevant SVs and CNVs were reported using the Bionano Access software with standardized and built-in filtration criteria and phenotype-specific analysis. **Results:** OGM was 100% concordant in identifying the aberrations reported with clinical testing, which included samples with *FMR1* repeat expansion, *DUX4* repeat contraction, aneuploidies, interstitial deletions, interstitial duplications, triploidy, absence of heterozygosity (AOH), and translocations. **Conclusions:** This study demonstrates that OGM has the potential to identify unique genomic abnormalities such as CNVs, AOHs, and several classes of SVs including complex structural rearrangements. OGM has a standardized laboratory workflow and reporting solution that can be adopted in routine clinical laboratories and demonstrates the potential to replace the current SOC methods for postnatal diagnostic testing. We highly recommend its use as a first-tier genetic diagnostic test for constitutional tests.

G079. **Optical Genome Mapping: A Potential Tier I Test for Prenatal Diagnostic Testing**

N. Sahajpal[1], R. Kolhe[1], A. Mondal[1], T. Fee[2], A. Hastie[3], A. Chaubey[3], B. DuPont[2]

[1]Augusta University, Augusta, GA; [2]Greenwood Genetic Center, Greenwood, SC; [3]Bionano Genomics, San Diego, CA.

Introduction: The standard-of-care (SOC) diagnostic prenatal testing includes a combination of cytogenetic methods such as karyotyping, fluorescence *in situ* hybridization (FISH), and chromosomal microarray (CMA) using either direct or cultured amniocytes or chorionic villi sampling. However, each technology has its limitations: karyotyping has a low resolution (>5 Mb), FISH is targeted, and CMA does not detect balanced structural variants (SVs) or decipher complex rearrangements in the genome. These limitations necessitate the use of multiple tests, either simultaneously or sequentially, to reach a genetic diagnosis. This long-standing prenatal testing workflow demonstrates the need for an alternative technology that can provide high-resolution results in a cost- and time-effective manner. Optical genome mapping (OGM) is an emerging technology that has demonstrated its ability to detect all classes of SVs, including copy number variations (CNVs) and balanced abnormalities in a single assay, but has not been evaluated in the prenatal setting. **Methods:** This retrospective validation study analyzed 114 samples (including replicates), representing 94 unique and well-characterized samples that were received in our laboratory for traditional cytogenetic analysis with karyotyping, FISH, and/or CMA. These comprised 84 cultured amniocytes, and 10 phenotypically "normal" and cytogenetically "negative" controls. Six samples were run in triplicate to evaluate inter-run, intra-run, and inter-instrument reproducibility. Clinically relevant SVs and CNVs were reported using the Bionano Access software using a phenotype-specific analysis. **Results:** OGM was 100% concordant in identifying the 101 aberrations that included 29 interstitial/terminal deletions, 28 duplications, 26 aneuploidies, six absence of heterozygosity (AOH), three triploid genomes, four isochromosomes, one translocation, and revealed the identity of the three marker chromosomes, and one chromosome with additional material not determined with karyotyping. Additionally, OGM detected 64 additional clinically reportable SVs in 43 samples. **Conclusions:** This study demonstrates that OGM has the potential to identify genomic abnormalities such as CNVs, AOHs, and several classes of SVs including complex structural rearrangements. OGM has a standardized laboratory workflow and reporting solution that can be adopted in routine clinical laboratories. This study demonstrates the potential to replace the current SOC methods for prenatal diagnostic testing with a single assay. We highly recommend its use as a first-tier genetic diagnostic test in a prenatal setting.

Hematopathology

H001. **Chimerism Monitoring for the Multiple Allogeneic Stem Cell Transplantations Using PCR of Short Tandem Repeats: 10-Year Experience**

J. Han[1], M. Kwak[2], J. Shim[2], J. Lee[2], S. Kim[2]

[1]Dong-A University Hospital, Busan, South Korea; [2]Dong-A University College of Medicine, Busan, South Korea.

Introduction: PCR of short tandem repeats (STR) is regarded as the gold standard method for chimerism analysis, although more sensitive techniques such as next-generation sequencing-based single nucleotide polymorphisms or digital PCR have been recently introduced. Multiple hematopoietic stem cell transplantations (HSCT) can present technical problems for chimerism monitoring. In this study, we investigated the effects of multiple different or shared alleles provided by multi-donors for the laboratory monitoring of chimerism. **Methods:** For the years between January 1, 2012, and May 31, 2022, we enrolled 585 chimerism analyses for the study. STR analysis was performed with DNA from peripheral blood using AmpFISTR Identifier kit (Applied Biosystems, UK) and 16 STR markers. PCR-STR products were electrophoresed using ABI 3100 Genetic Analyzer, and GeneMapper ID software (Applied Biosystems) was used for the genotyping and quantification of allele peaks. **Results:** During this period, a total of 12 patients received more than two allogeneic HSCTs. Two patients received three HSCTs, with the rest having two. Seven acute lymphoblastic leukemia (ALL), two acute myeloid leukemia, two myelodysplastic syndrome (MDS), and one case of primary myelofibrosis were included. The first donors were related in seven and related in five patients. Before the second HSCT, recipient DNA originated from the first donor in 10 double-donor transplants. One MDS patient had mixed DNA of his (58.1%) and the first donor (41.9%) before the second HSCT. One ALL patient who received triple-donor transplant showed DNA from the second donor, substituting his totally original DNA before HSCT. **Conclusions:** In summary, the present study shows that even with the multi-donor transplants, STR chimerism measurements can be done accurately, examining the allele peaks carefully shared or not shared between donor-recipient pairs. Emerging new technologies may provide alternative advantages, whereas PCR-STR method still remains the most powerful in differentiating between different individuals.

H002. **Going beyond Karyotyping and FISH: Impact of Optical Genome Mapping (OGM) with Additional Clinically Relevant Information in 75 Hematological Malignancy Cases**

N. Sahajpal[1], R. Kolhe[1], A. Mondal[1], T. Tvrdik[2], J. Hauenstein[3], K. Deeb[2], H. Shi[1], D. Saxe[2], A. Hastie[3], N. Savage[1], V. Kota[1], A. Chaubey[3]

[1]Augusta University, Augusta, GA; [2]Emory University, Atlanta, GA; [3]Bionano Genomics, San Diego, CA.

Introduction: The current standard of care (SOC), cytogenetic techniques for the analysis of hematological malignancies include karyotyping, fluorescence *in situ* hybridization FISH, and chromosomal microarray (CMA), which are labor-intensive, time and cost-prohibitive, and often do not reveal the genetic complexity of the tumor, demonstrating the need for alternative technology for better characterization. **Methods:** In this retrospective study, we evaluated

75 hematological malignancy samples that were received in our laboratories for clinical testing by karyotyping, FISH, and/or CMA. The samples included diagnoses of adult acute myeloid leukemia (n=26), chronic lymphocytic leukemia (n=16), myelodysplastic syndromes (n=14), plasma cell myeloma (n=6), lymphoma (n=6), myeloproliferative disorders/myeloproliferative neoplasms (n=5), and chronic myeloid leukemia (n=2). The structural variations (SVs) detected by optical genome mapping (OGM) were filtered against >300 healthy controls to enrich for rare variants, and against genes/loci included in the NCCN and NHS guidelines for hematological malignancies. After concordance assessment, additional clinically relevant information was evaluated for 1) detected SV included in NCCN/NHS guidelines; 2) changed interpretation of an event previously impacting a gene/loci in NCCN/NHS guidelines; 3) changed karyotype classification (simple/complex); 4) detected SV of potential clinical significance that overlapped a gene included in NCCN/NHS guidelines. **Results:** In addition to achieving a 99% concordance with SOC methods, OGM detected additional SVs including translocations and copy number variations. Importantly, OGM identified additional clinically relevant information in 24% of cases, which include aberrations listed in NCCN/NHS guidelines in 22% of cases, and OGM changed the interpretation of SVs impacting NCCN/NHS genes in 2.6% of cases. Also, OGM found potential clinically relevant information in 25% of cases. Further, OGM detected cytogenetic aberrations in 40% of cases that were previously negative by karyotyping and FISH, and 21% of these cases were reclassified as complex (>4 aberrations). **Conclusions:** This study demonstrates the clinical utility of OGM compared to current SOC methodologies and shows that the clinical use of OGM will not only increase the yield of actionable targets, leading to improved clinical outcomes, but also help resolve our ongoing conundrum of apparently genomically normal myeloid cancers by providing more answers.

H003. **WITHDRAWN**

H004. **Routine Use of cfDNA Isolated from Nail Clippings as a Source of Matched Non-Tumor DNA for Genetic Studies in Hematologic Malignancies**

M. Krystel-Whittemore, M. Arcila, C. Vanderbilt, R. Ptashkin, K. Nafa
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: Genetic characterization is a critical analysis for hematologic malignancy, with implications for diagnosis, prognosis, therapeutics, and disease monitoring. Large-panel next-generation sequencing (NGS) has become a practical approach for upfront comprehensive genetic assessment. However, a distinct challenge for efficient analysis is to determine that the variants are somatic in origin, requiring a paired non-tumor sample. Peripheral blood samples, buccal swabs, and saliva have traditionally been used as the main source of control samples for solid tumors, yet these control samples are not suitable in many hematologic tumors due to the presence of neoplastic cell contamination. The utilization of nail tissue as a source for a paired sample is advantageous due to the lack of contaminating myeloid and lymphoid cells. Traditional extraction methods for obtaining DNA from nails presented a problem, as extraction was a multi-day process, which could delay sequencing. In this work we describe the use of nail tissue as a control for hematologic tumor genotyping using a rapid method for DNA extraction. **Methods:** A new bead blaster-based nail extraction protocol which allows for single-day DNA nail extraction was validated. The extracted DNA from nail clippings was used for matched analysis of clinical samples using MSK-IMPACT Heme, a hybrid capture-based NGS assay that queries genetic alterations in all coding regions of 400-468 genes. Performance from using nails as a control for a wide range of both myeloid and lymphoid malignancies was assessed, including frequency of presumed somatic mutations from tumor DNA contamination in nails. **Results:** The new extraction method resulted in a 110% increase in mean DNA yield compared to the traditional method. We analyzed results from nails obtained from 2,636 samples from 2,610 unique patients, and a total of 10,925 somatic mutations were identified. The somatic mutation was observed in the paired nail 201 (1.8%) and 470 (4.3%) times with thresholds of 5% and 2%, respectively. The variant allele frequency of mutations in myeloid neoplasm was higher in myeloid neoplasms compared to lymphoid neoplasms (0.0095 vs. 0.018, p <0.002). The somatic mutations associated with the highest variant allele frequencies in nails were mutations associated with chronic myeloid neoplasms (e.g., *JAK2* and *SRSF2*) and clonal hematopoiesis (e.g., *TET2* and *DNMT3A).* **Conclusions:** DNA isolated from nail tissue can be extracted efficiently and expeditiously, and is a suitable non-tumor control for NGS assessment of hematologic malignancies. Contaminating tumor DNA is seen in a subset of patients, resulting in somatic mutations being present in the nail. However, these variants are present at lower levels and allow for the distinction between somatic and germline variants.

H005. **A Rare Autosomal Dominant β-Thalassemia Due to a Novel *de novo* Null *HBB* Gene Variant: Hb-Jordan**

W. Naser[1], A. Tbakhi[1], M. Taha[2], N. Kamal[1], R. Abu Aboud[1], R. Abu Khashabah[1], I. Sultan[1], M. Abu Shanap[1]
[1]King Hussein Cancer Center, Amman, Jordan; [2]University of Jordan, Amman, Jordan.

Introduction: Unstable hemoglobinopathies are rare, mostly dominantly inherited hemoglobin disorders frequently misdiagnosed. Mutations in one of the globin-coding genes can destabilize the hemoglobin tertiary or quaternary structure, leading to intracellular precipitation of the hemoglobin tetramer. The affected red blood cells (RBCs) usually have a shortened life span, producing hemolytic anemia of heterogeneous severity. In cases of unexplained hemolytic anemia, testing for unstable hemoglobinopathies is opted. **Methods:** A 10-year-old boy was presented with non-immune hemolytic anemia and splenomegaly at five months of age (7 to 8 g/dL baseline hemoglobin, reticulocyte of 10%-14%). The peripheral blood smear showed normochromic normocytic anemia with target cells, polychromasia, and nucleated RBCs with double nucleoli basophilic stippling. Hb electrophoresis was typical. The patient had a presumed diagnosis of congenital dyserythropoietic anemia (CDA) based on dyserythropoiesis seen in a bone marrow study. At three years old, the patient underwent splenectomy due to severe hemolysis and massive splenomegaly. Two years later, he developed priapism and was referred for allogenic hematopoietic stem cell transplantation. We opted to do whole *HBB* gene Sanger sequencing since the patient lacked the most common CDA variants. All probands' first-degree relatives were subjected to *HBB* gene mutation analysis. We assessed the functional impact of the newly discovered mutation using homology modeling and *in silico* tools. **Results:** We discovered a novel likely pathogenic variant in exon 3 of the *HBB* gene. The proband has *HBB*:c.391_394delTATC (p.Y131Rfs*27) in heterozygosity. All family members tested had normal genotypes indicating that the new variant is *de novo*. The newly discovered null (frame-shift) deletion variant created an early stop codon and was predicted to cause loss of function (LOF) due to nonsense-mediated decay. The *HBB* gene harbors 270 pathogenic LOF variants linked to Heinz body anemias and other hemoglobinopathies. In particular, the truncated region contains 150 pathogenic disease-causing LOF variants mimicking the effect of the newly discovered variant. Moreover, homology modeling revealed loss of the alpha-helix domain harboring L141 residue required for heme hydrophobic stabilization within the binding pocket of wild-type hemoglobin. **Conclusions:** We report a novel *de novo* β-globin mutation in a patient with features of non-spherocytic hemolytic anemia of Middle Eastern/Arab descent. We named this variant as Hb-Jordan. *HBB* gene sequencing is crucial in the diagnostic work-up of rare unstable hemoglobinopathies. Lastly, the reported discovery adds to the human globin database and sheds light on the future diagnosis of hemoglobinopathies and genetic counseling.

H006. **Performance Comparison of a Capture-Based Oncology NGS Panel with SNP Array and FISH for the Detection of Genome-Wide Copy Number Variants and Copy Number Neutral Loss of Heterozygosity**

P. Szankasi[1], A. Kellogg[1], A. Bolia[1], C. Paxton[1], B. O'Fallon[1], P. Rindler[1], D. Close[1], P. Li[2], J. Patel[2]
[1]ARUP Laboratories, Salt Lake City, UT; [2]University of Utah, Salt Lake City, UT.

Introduction: Massively parallel sequencing (MPS) enables the detection of nearly all genetic alterations ranging from single nucleotide variants and exon-level insertions/deletions to inter- and intra-chromosomal rearrangements and copy number alterations (CMAs). Although whole genome-based approaches offer the least bias in sequencing data, routine testing in the field of oncology requires more sensitive and cost-effective methods, such as capture-based target enrichment. Here we describe a test utilizing a combination of gene-based and genome-wide single-nucleotide polymorphism (SNP) backbone capture allowing sensitive concurrent detection of both gene variants and genome-wide copy number variants (CMVs), including copy number neutral loss of heterozygosity (CN-LOH). We compare the performance characteristics of this method to those of SNP microarray and interphase fluorescence *in situ* hybridization (FISH). **Methods:** DNA was sheared and converted to adapter-ligated sequencing libraries using Kapa Hyper Prep (Roche) and capture enriched for 700+ cancer-related genes and 12,000 genome-wide SNPs (spaced by 200 kb, heterozygous in at least 40% of the population) using oligonucleotide probes (IDT). Enriched libraries were sequenced on the NovaSeq 6000 instrument (Illumina). Sequencing reads were demultiplexed and aligned to a reference genome. Copy number gains and losses among all captured sequences were detected using GATK4, comparing to a pool of normal male and female reference samples. CN-LOH was detected using a custom script, essentially intersecting segments with aberrant B-allele frequencies with areas of normal copy number state. **Results:** Ninety-six hematologic malignancy cases with CNVs confirmed by SNP array or FISH were tested by MPS. Among copy number gains and losses of ≥2 Mb (on average 10 backbone probes) present in ≥30% of the sample, 156/164 (95%) were detected. CMAs <2 Mb, such as *KMT2A* partial tandem duplications, could still be detected if a captured gene was present in the interval. Complex patterns, such as chromothripsis, were readily identified. Except for hyperdiploid cases, copy number determined by MPS correlated well with SNP array (R^2=0.991) and was highly reproducible, with a CV <10% for 95% of variants. Filtering CNV calls by length (≥10 datapoints) and exclusion of areas of inconsistent performance eliminated most false positives (PPV=0.975). Fifty backbone probes (on average 10 Mb) were necessary for consistent detection of CN_LOH (29/29) at ≥30% of the sample. **Conclusions:** We describe a robust method for detecting CNVs by MPS with a limit of detection of 30% and resolution of 2 Mb (gains and losses) and 10 Mb (CN-LOH). The method can be used as an adjunct to any gene panel with a genome-wide SNP backbone and does not require additional laboratory steps.

H007. **Molecular Profiling of Pediatric and Young Adult Acute Leukemia with *PICALM::MLLT10* Fusion**

A. Palagani, L. Wang, S. Foy, M. Umeda, J. Ma, M. Wilkinson, J. Rubnitz, J. Klco
St. Jude Children's Research Hospital, Memphis, TN.

Introduction: The gene fusion *PICALM::MLLT10*/t(10;11)(p12;q14) has been described in patients with undifferentiated acute leukemia, T-lymphoblastic leukemia/lymphoma (TLL) and acute myeloid leukemia (AML). However, data on recurrent molecular events associated with the *PICALM::MLLT10* fusion are scarce. To better understand the genetics of *PICALM-MLLT10*-positive leukemias, we performed comprehensive molecular profiling in a series of pediatric and young adult acute leukemia patients with the fusion. **Methods:** *PICALM::MLLT10* fusion was detected by RNA-seq at St. Jude Children's Research Hospital in 15 patients (7-20 yrs) from 05/2017 to 12/2021, including eight AMLs, six TLLs and one mixed phenotype acute leukemia (T/B-MPAL). Fresh samples from bone marrow (n=10), peripheral blood (n=3) and pleural fluid (n=1) with ≥30% blasts were used. For one TLL case, a formalin-fixed, paraffin-embedded (FFPE) lymph node tissue was tested. Whole-genome sequencing (WGS) was performed on seven AMLs, five TLLs and the one T/B-MPAL to achieve an unbiased comprehensive analysis of single nucleotide variants (SNVs)/indels, structural alterations, and copy number variations (CNVs) across the genome. The FFPE tissue was evaluated by whole-exome sequencing for SNVs/indels only, and the remaining AML case was tested by targeted next-generation sequencing assay. **Results:** The comprehensive molecular profiling demonstrated distinct recurrent molecular alterations in *PICALM-MLLT10*-positive acute leukemia subtypes, respectively. In AMLs, the most common finding was *TP53* alterations, which were detected in 6/8 (75%) cases, and inactivation of both alleles of *TP53* by "two hits" (one mutation coupled with 17p loss/loss of heterozygosity or two *in-trans* mutations) was observed in five cases. Another common finding was loss-of-function (LOF) alterations in *PHF6* (6/8). *PHF6* is on the X chromosome and 5/6 cases with *PHF6* alterations are male. The only female showed bi-allelic LOF of *PHF6*. Other recurrent alterations in the AML group included *NF1* LOF variants (5/8, including four cases with bi-allelic LOF) and *SUZ12* (3/8, including two with bi-allelic LOF). In contrast to the AMLs, *TP53* alterations (SNVs/indels/focal CNVs) were not detected in any TLLs or T/B-MPAL; instead, in the non-AML group (n=7), the most frequently affected single genes were *PHF6* (4/7, three males and one female with a heterozygous nonsense mutation) and *EZH2* (4/7). *JAK-STAT* pathway-activating mutations, affecting *JAK1*, *JAK3*, *IL7R*, or *STAT5B*, respectively, were noted in 5/7 cases. Notch signaling-activating mutations were observed in 3/7 cases (*NOTCH1* variants in two cases and *FBXW7* in one). **Conclusions:** Our study demonstrated that *PHF6* LOF is the most common genetic alteration associated with *PICALM-MLLT10*-positive acute leukemias. Intriguingly, *TP53* bi-allelic LOF seems to be specifically associated with *PICALM-MLLT10*-positive AMLs. In contrast, *EZH2* alterations is enriched in *PICALM-MLLT10*-positive TLL cases.

H008. **Bringing a New Technology in CLIA Laboratory: Our Experience of Clinical Validation, Getting AMA PLA-Code, and the Moldx Z-Code for Optical Genome Mapping for Evaluation of Hematological Neoplasms**

N. Sahajpal, R. Kolhe
Augusta University, Augusta, GA.

Introduction: Any new promising and cutting-edge technology needs thorough clinical evaluation, validation, and assessment of incremental clinical utility. Coding, coverage, and reimbursement are the cornerstone of any breakthrough platform. Upon robust and reproducible performance and established additional clinical utility, laboratories shift from traditional or current testing methodologies and implement these novel technologies of that time for better diagnosis, prognostication, and ultimately better patient management. However, laboratories are faced with challenges from desiring to use the technology for patient care to implementing it in their laboratories. In this study, we discuss our experience with optical genome mapping (OGM), from clinical validation to implementation for clinical use. **Methods:** The OGM technology was validated as per CLIA standards, assessing the sensitivity, specificity, accuracy, reproducibility, and limit of detection using clinical specimens. After successful validation, the documentation was submitted to AMA for PLA code, and the PLA code was then submitted to the Moldx for Z-Code. **Results:** The clinical validation of the instrument might take from six months to two years, depending upon the lab's experience in validating technologies and the complexity of the test and the platform. The PLA code application opens twice a year, and the codes are issued within two to three months. The Moldx submission opens a few times a year and the codes are issued within a few months. **Conclusions:** Herein, we describe our experience, which is essential for each laboratory to validate new technologies to implement clinically. The entire process

can take from one to two years in the United States, and a streamlined and clear plan helps in a smooth flow from clinical validation to implementation of the technology for clinical use.

H009. **Rare *KMT2A-USP2* Fusion Using RNA Next-Generation Sequencing in a Pediatric Mixed Phenotype Acute Leukemia**

P. Gerasimou[1], P. Costeas[1], L. Koumas[1], J. Chi[1], K. Nicolaou[1], A. Mitsidou[1], A. Miltiadous[1], I. Kyprianou[1], R. Gavrielides[1], E. Socratous[1], L. Loizou[2]
[1]Karaiskakio Foundation, Nicosia, Cyprus; [2]Pediatric Oncology-Hematology, Archbishop Makarios III Hospital, Nicosia, Cyprus.

Introduction: According to WHO, updated classification of acute myeloid leukemia (AML) has segregated novel subtypes according to the molecular characteristics they possess. AML with *KMT2A* rearrangements includes such an entity, where prognosis relies on the *KMT2A* translocation partners. We herein describe a case of a pediatric patient with Down syndrome with mixed phenotype acute leukemia (MPAL). **Methods:** The patient is a 3.5-year-old girl with trisomy 21, who presented with pancytopenia and frequent febrile episodes with signs of upper respiratory tract infection for three weeks. Flow cytometric assessment was performed using multi-parametric immunophenotypic analysis on a bone marrow aspiration sample. Molecular investigation was performed using RNA next-generation sequencing (NGS) and high-resolution 400K oligonucleotide array comparative genomic hybridization analysis. **Results:** Flow cytometric assessment showed a predominant progenitor population reaching 72.1% of total cellularity with ambiguous lineage immunophenotypic characteristics. Differential diagnosis included mixed phenotype acute leukemia (MPAL), acute leukemia with ambiguous lineage or B-cell acute lymphoblastic leukemia (ALL) with aberrant myeloid markers. Confirmation of MPO positivity with cytochemistry suggested a final diagnosis of MPAL. Molecular investigation using RNA NGS revealed the presence of an extremely rare *KMT2A::USP2* fusion. Array CGH revealed arr[GRCh37]11p13p11.2(32045836_46548468)x 1~2,19p13.3(3437994_4920784)x1,21q11.2q22.3(14571110_4809585 6)x3. **Conclusions:** The *KMT2A::USP2* fusion has only been reported in selected cases in the literature, initially in an infant with AML and subsequently in isolated cases of ALL and AML. Since disease prognosis has been reported to be related to the localization of breakpoints, the importance of developing methodologies to detect additional *KMT2A* fusion partners is highlighted within this case. Intriguingly, the finding of this rare *KMT2A* fusion across different lineage acute leukemias and our recent report of its presence in MPAL may provide links of a common pathway in myeloid/lymphoid lineage derivation. Interestingly, *KMT2A* rearranged acute leukemia has also increasingly appeared in patients following treatment with CD19-directed therapy, thereby reinforcing the concept of lineage conversion from lymphoid to myeloid lineage following selective CD19 pressure. Lineage conversion as an evasion system further sheds insight into the myeloid-based model of hematopoiesis, and the finding of the *KMT2A::USP2* fusion at this crossroad may suggest a possible involvement of key players in the equivocal hematopoietic lineage decision.

H010. ***KIT* (D816V) Digital PCR Testing**

L. Cai, D. Overman, S. Hood, E. Kallam, T. Jensen, D. Wang, L. Kam-Morgan, A. Chenn
Labcorp, RTP, NC.

Introduction: Systemic mastocytosis (SM) is a hematopoietic neoplasm characterized by an abnormal growth of clonal mast cells in bone marrow and other extracutaneous organs. *KIT* is a receptor tyrosine kinase involved in proliferation of mast cells, melanocytes, germ cells, and hematopoietic stem cells. The vast majority (>90%) of SM cases have a somatic missense mutation c.2447A >T (p.D816V) of the *KIT* gene in exon 17. This change results in ligand-independent constitutive activation of *KIT* and leads to increased cell proliferation and accumulation in various organs, and a reduction in cell death. The detection of *KIT* D816V is one of the minor diagnostic criteria for SM per the WHO system. Quantitative detection using digital PCR (dPCR) may aid physicians in diagnosis and therapeutic monitoring of patients with SM. In this study, we have evaluated the clinical and analytical performance features of the assay. **Methods:** Total genomic DNA is extracted and amplified using a multiplex dPCR with wild-type and mutant-specific probes on QIAcuity One 5plex Digital PCR system. DNA from SM specimens was used to evaluate accuracy, repeatability, reproducibility, analytical detection sensitivity, and stability of the assay. **Results:** Of the 21 specimens (blood, bone marrow, and cell pellet) tested during validation, results from 11 specimens with p.D816V mutation and 10 specimens without the mutation were 100% concordant with results obtained by next-generation sequencing. Repeatability was 100% and reproducibility was 95% concordant using 10 specimens with p.D816V mutation and 10 specimens without the mutation. This assay has a sensitivity to detect approximately 0.03% *KIT* p.D816V somatic mutations. The DNA stored at 2°-8°C was stable for at least one year. The EDTA, heparin blood, and bone marrow specimens were stable for at least 30 days stored at 15°-30°C. The *KIT* (D816V) Test has been offered as a clinical test at LabCorp. Of the 258 specimens tested, 80.23% (207) were negative, 17.44% (45) were positive. The age distribution for patients with a positive result is 30-79 in this set of data. Results could not be obtained in 2.33% (six) specimens due to low wild-type copy number (specimen degradation and limited amount of DNA). **Conclusions:** The *KIT* (D816V) Test is a robust, reproducible, and sensitive assay using blood, bone marrow, and cell pellet specimens for systemic mastocytosis assessment and therapeutic monitoring.

H011. **Evaluation of Molecular Methodologies for DNA-Based Measurable Residual Disease Detection in Acute Myeloid Leukemia**

L. Dillon, N. Ravindra, G. Andrew, G. Gui, C. Hourigan
National Heart, Lung, and Blood Institute, Bethesda, MD.

Introduction: Acute myeloid leukemia (AML) is a genetically heterogeneous disease with a five-year survival rate of only 30%. High sensitivity measurements of anti-leukemic treatment effect would speed novel drug development. Absence of measurable residual disease (MRD) is associated with superior outcomes, but a lack of standardization has limited the adoption of such testing. To date, AML MRD guidelines have focused on the use of flow cytometry or molecular assays for somatic structural variants, but next-generation sequencing (NGS) detection of well-defined DNA mutations could expand applicability to nearly all patients. Here we perform a technical comparison of DNA-based techniques for MRD detection, focusing on well-characterized AML hotspot mutations. **Methods:** AML patients with DNA available from diagnostic peripheral blood or bone marrow were chosen based on the presence of known AML hotspot mutations, including *IDH1* R132, *IDH2* R140/R172, *NPM1* type A/B/D, *KIT* D816, *FLT3* D835, and *FLT3* internal tandem duplication (ITD). Patient DNA was serially diluted into healthy donor DNA at expected variant allele fractions (VAF) ranging from 0.005% to 5%. Samples were evaluated using four methodologies: 1) digital PCR (dPCR), 2) single amplicon NGS with single-strand unique molecular identifier (UMI) error-correction (saNGS), 3) anchored multiplex PCR panel NGS with single-strand UMI error-correction (ampNGS), and 4) capture panel NGS with duplex UMI error correction (dupNGS). **Results:** For hotspot single nucleotide variants (SNVs), saNGS and dupNGS exhibited the lowest limit of detection (LOD), ranging from 0.005%-0.01%, followed by dPCR (0.01%-0.05%) and ampNGS (0.05%-0.1%). For small insertions within *NPM1* exon 11, dPCR and ampNGS showed improved sensitivity (0.001%-0.005%), now equal to or better than dupNGS. Additionally, specific bioinformatic and experimental modifications improved *NPM1* insertion discovery at the lowest levels by NGS. Similarly, *FLT3*-ITD detection was equivalent across all NGS methods (0.01%-0.05%), with ITD length serving as a contributing factor. **Conclusions:** With prior knowledge of the mutation of interest, dPCR is the most economical method for MRD detection at hotspots. saNGS performs similarly while evaluating any variant within the single

amplicon region. NGS panels are most useful for MRD detection without prior mutation knowledge, with dupNGS affording the greatest sensitivity for SNVs. Overall, the LOD of indels was lower than that of SNVs, did not require error correction, and could be improved with region-specific techniques. This pilot study lays the groundwork for the FNIH AML MRD Biomarkers Consortium, a public-private partnership aimed at validating new methods of MRD detection as a measure of response and trial endpoints in AML.

H012. **High-Resolution Melting Assay for Rapid Simultaneous Detection of JAK2, *MPL*, and *CALR* Variants in Patients with Clinical Suspicion for Myeloproliferative Neoplasms**

C. Sande[1], G. Yang[1], A. Mohamed[2], B. Legendre[2], D. Pion[2], S. Ferro[2], K. Grimm[2], K. Elenitoba-Johnson[1]
[1]Hospital of the University of Pennsylvania, Philadelphia, PA; [2]Precipio, Inc., New Haven, CT.

Introduction: Identification of recurrent genetic alterations in *JAK2*, *MPL*, and *CALR* remains crucial in the diagnosis of Philadelphia-negative myeloproliferative neoplasms (MPN). Current laboratory testing algorithms often depend on batching and/or sequential testing in order of mutation prevalence, involving multiple testing modalities and send-out testing that increase lab technical and economic demands while delaying patient diagnoses. To address this gap in testing, we employed a novel protocol based on high-resolution melting (HRM) analysis, embodied in the HemeScreen MPN Assay, for multiplex testing of *JAK2* exons 12-14, *MPL* exon 10, and *CALR* exon 9, validated with a cohort of ~1,000 patient samples. **Methods:** Blood and bone marrow samples from 982 patients with clinical suspicion for MPN were tested with the HemeScreen MPN Assay and Sanger sequencing in independent, CLIA-certified laboratories. The samples carried disease variants ranging from <5%-95% variant allele frequency. The HRM assay was performed on a Quantstudio 3 Real Time PCR System and analyzed with HRM v3.1 software (Thermo Fisher). Sanger sequencing was performed on an ABI 3730XL DNA Analyzer (Life Technologies) and analyzed with Sequencher 5.4.6 software (Gene Codes Corp.). Analytical and clinical performance was calculated with Sanger sequencing as the gold standard, considering disease-associated (DA) variants and variants of uncertain significance (VUS) to be clinically relevant findings. **Results:** HRM and Sanger sequencing had an overall concordance of 99.4% with HRM detecting 133/139 (96%) variants confirmed by sequencing (9/10 *MPL*, 25/25 *CALR*, 99/104 *JAK2*). Variants included 114 single nucleotide variants, three insertions (5 bp), 21 deletions (3-52 bp), and one deletion-insertion. Although no HRM runs failed, Sanger sequencing was unsuccessful in 26 cases (three with positive results), resulting in the inability to completely corroborate results across platforms. The HRM-based assay identified known DA variants (89%), VUS (2%), and non-DA variants (9%) with a positive predictive value of 92.3% and negative predictive value of 99.5%. Non-DA variants were predominantly benign 3 or 9 bp *CALR* deletions. Among *JAK2* V617F cases, 94/96 (98%) were detected by the HRM assay. **Conclusions:** Our studies demonstrate the exquisite accuracy, sensitivity, and specificity of the HemeScreen MPN Assay in detecting genetic variants observed in MPN. The multiplex design with HRM technology offers an opportunity for a low-cost approach with a simplified workflow for laboratories while providing a quick turnaround time and accurate results. Overall, these data demonstrate the capability of HRM-based assays to serve as powerful, clinically applicable platforms for rapid, simultaneous molecular detection of clinically relevant, somatic disease variants.

H013. ***STAT5B-RARa* Fusion Positive Variant Acute Promyelocytic Leukemia: Role of Next-Generation Sequencing in Detection of a Rare Malignancy**

I. Dey, S. Vinarkar, M. Parihar, A. Mandloi, K. Saha, A. Nag, D. Mishra
Tata Medical Center, Kolkata, Kolkata, West Bengal, India.

Introduction: Variant acute promyelocytic leukemia (APL) due to *STAT5B-RARa* fusion t(17;17) is a rare Retinoic acid-unresponsive APL. The *RARa* fusion partner plays a critical role in the therapy and prognosis of APL and needs to be distinguished from the classic Retinoic acid responsive *PML-RARa* APL and other *RARa* partners (i.e., *PLZF, NuMA,* etc.). We present a rare case of *STAT5B-RARa* translocation positive APL and highlight the role of next-generation sequencing (NGS) as an important diagnostic modality in its definitive diagnosis. **Methods:** A 42-year-old male presented with complaints of severe weakness, decreased appetite, loss of weight, fever, visual disturbances, and slurred speech for three months. During the assessment of this patient in our hospital, peripheral blood smear, bone marrow examination, cytogenetic studies, and molecular genetic studies including NGS were performed. **Results:** Clinical examination of the patient revealed only mild splenomegaly. Comprehensive blood count showed leukocytosis with white blood cell of 42,200/µl along with thrombocytopenia and anemia. PBS examination revealed 76% blast/abnormal promyelocyte/atypical monocytoid cells with many of them showing salmon-pink cytoplasmic granules and strong cytochemical MPO staining. No Auer rods were seen. Flow cytometric immunophenotyping on peripheral blood revealed 60% blasts/abnormal promyelocytes with high side scatter, dim CD45, showing expression of CD117, CD13, CD33, CD56, cMPO, and negativity of CD34, HLA DR. The PBS morphology and immunophenotyping were suggestive of possibility of APL. Fluorescence *in situ* hybridization (FISH) for *PML-RARa* using dual-color dual-fusion probe (MetaSystem, Germany) was negative. RT-PCR for *PML-RARA* (*BCR1, BCR2*, and *BCR3* fusion transcripts) was negative. FISH utilizing *RARa* break-apart probe (Kreatek, Leica, Amsterdam) revealed an atypical *RARa* rearrangement (1F1G signal pattern). Bone marrow aspirate revealed a cellular marrow with 30% MPO positive blasts/abnormal promyelocytes with many of them showing prominent Auer rods and occasional Faggot cells. NGS-based Oncomine Myeloid Research Assay (Thermo Fisher Scientific) was performed. RNA sequencing revealed *STAT5B-RARa* fusion positivity (6,094 read-counts). A missense variant in *GATA2* gene NM_032638.5:c.1084C >G;p.(Arg362Gly) was detected on DNA sequencing. The patient received ATO, steroid and azacytidine; however, within seven days he developed respiratory distress followed by cardiac arrest which proved fatal. **Conclusions:** *STAT5B-RARa* t(17;17) is a rare variant of APML. It exhibits diagnostic, therapeutic, and prognostic difference with the common *PML-RARa* positive APL and hence the need for its prompt identification. Our case demonstrates the valuable role of NGS in reaching a definite diagnosis of such challenging and rare malignancy.

H014. **A Pitfall of Fragment Analysis in *NPM1* Mutation Analysis**

R. Wang, R. He, M. Mai, P. Ollila, D. Viswanatha
Mayo Clinic, Rochester, MN.

Introduction: *NPM1* mutations are seen in approximately 30% of acute myeloid leukemia (AML). *NPM1*-mutated AML in the absence of *FLT3*-ITD portends a more favorable outcome. *NPM1* mutations are commonly 4-bp insertions involving the last exon (exon 11). They have been shown to be a reliable AML measurable/minimal residual disease (MRD) marker. *NPM1* MRD testing is usually composed of a quantitative reverse transcription-PCR (RT-PCR) targeting the three most common mutation types (A/B/D, 90% of cases). It offers a high analytical sensitivity given the nature of the platform and overexpression of mutant *NPM1* on the RNA transcript level. RT-PCR may be paired with a DNA-based fragment sizing analysis (FA) to cover all insertion events (with a lower analytic sensitivity on the DNA level). Here we report an AML case with apparently discordant RT-PCR and fragment sizing results, highlighting the limitation of FA in

NPM1 mutation detection. **Methods:** A bone marrow sample was received for *NPM1* testing. DNA and RNA were extracted using the Qiagen EZ1 DNA extraction kit (Germantown, US) and Promega SimplyRNA kit (Madison, US), respectively. For the quantitative RT-PCR, RNA was reverse transcribed into cDNA (Thermo Fisher High-capacity cDNA Reverse Transcription Kit, Waltham, US) followed by quantitative PCR on Roche LightCycler 480 (Indianapolis, US) using reverse primers specific for types A/B/D. For the paired qualitative FA, PCR was performed on DNA using primers flanking exon 11. The amplicons were then analyzed by capillary electrophoresis on an ABI 3700 (Waltham, US). Next-generation sequencing (NGS) was performed using a targeted panel with capture-based chemistry on an Illumina HiSeq sequencer (San Diego, US). **Results:** RT-PCR detected a mutant *NPM1* transcript at 8,064/10,000 *ABL1* copies (80.64%). The FA, however, showed no apparent insertional event and revealed a 3-bp deletion. To further investigate the discrepancy seen between the two assays, which was not adequately explained by the overexpression of a mutant *NPM1*, NGS testing was performed on the DNA sample. NGS showed a 3-bp deletion (c.847-7_847-5del; p.?) in 40.3% of sequencing reads, along with an *in-cis* type A *NPM1* mutation (c.860_863dup; p.Trp288Cysfs*12) in a subset (11.2%) of the reads harboring the 3-bp deletion. **Conclusions:** Although FA offers quicker turnaround time and lower cost compared to RT-PCR and NGS, it may miss pathogenic *NPM1* mutations (insertions) co-occurring *in cis* with a deletion, as the latter alters the variant amplicon size interrogated by the FA platform. NGS should be performed at the time of AML diagnosis to identify the *NPM1* mutation type and delineate rare events of complex insertion/deletion and their *in cis/in trans* status to guide future MRD monitoring.

H015. **Evaluation of the Oncomine Myeloid Assay GX v2 NGS Panel on the Ion Torrent Genexus System with Previously Benchmarked Clinical Nucleic Acid Samples**

M. Dina, J. Blommel, J. Balan, D. Viswanatha, S. Murphy
Mayo Clinic, Rochester, MN.

Introduction: As the number of actionable somatic nucleic acid mutations expands, the clinical oncology genomic testing space is increasingly pressured to deliver rapid, accurate, and sensitive variant reporting. Innovative approaches to decrease the overall turnaround time (TAT) have been developed and continuously improved upon to accomplish this goal. The Thermo Fisher Genexus Integrated Sequencing platform can deliver rapid 24-hour variant reporting. In this study, we evaluate the Oncomine Myeloid Research Assay GX v2 (OMAv2) panel on the Genexus System. **Methods:** DNA and RNA samples previously sequenced on a clinically verified 42-gene hybrid-capture myeloid neoplasm panel or fusion detection platforms, along with well-characterized cell lines and reference materials, were used for this evaluation. Initial testing compared the Genexus OMAv2 allele frequency (AF) calling on 11 well-characterized patient DNA samples. Lower limits of AF detection were further evaluated in one of the samples in a dilution series targeting 10%, 7%, 6%, 5%, 4%, and 3% variant levels. For the RNA panel, a dilution series was made to evaluate the lowest number of fusion reads detectable for *BCR*::*ABL1* translocation. Beginning with a 2:1 by volume mix of *BCR*::*ABL1* p210 and p190 cell lines, the mix was subjected to a 1:4 serial dilution with a fusion negative sample. **Results:** The average DNA base coverage depth was 2,127x and the average target base coverage at 350x was 99.51%. The average run time was 23 hours. Of 36 variants present in the 11 DNA samples, 33 (92%) were detected by OMAv2, with 32 (89%) reported by the software. AFs were consistent with previous assays. Two *CEBPA* (c.436del and c.827_828ins25) and one *STAG2* (c.1400dup) variants in complex regions were present in the raw sequencing reads but not annotated. Upon limited dilution, *FLT3* c.1809_1810ins33 and *TET2* c.2704_2705insT variants were consistently detected down to 1.6% and 2.3% AF. In the RNA study, the 2:1 by volume mix resulted in 7,967 fusion reads. The subsequent 1:4 serial dilution read counts for the p210 fusion were reported by the OMAv2 software down to 27 reads. **Conclusions:** OMAv2 panel variant detection was consistent with current benchmarked assays down to 5% AF. Serial dilutions provided AF calls down to 1.6% for *FLT3* and *TET2* variants. Variant calls below 5% AF were more inconsistent. The RNA fusion detection was sensitive, with as few as 27 reads passing reporting thresholds. The Genexus OMAv2 assay effectively integrates library assembly, sequencing, and clinical bioinformatics reporting with minimal hands-on time and rapid TAT of 23 hours, but more in-depth studies are needed to fully evaluate the performance characteristics of the assay and bioinformatics reporting.

H016. **Comprehensive Analysis of Combined Variant Callers Manta, Pindel, and TNhaploytyper2 for Detection of SNVs and Indels in a Clinical Myeloid Neoplasm NGS Panel**

M. Dina, S. Basu, J. Balan, P. Tandale, T. Koganti, C. Artymiuk, K. Bessonen, R. He, D. Viswanatha
Mayo Clinic, Rochester, MN.

Introduction: Oncology specimens may contain DNA alterations in the form of single nucleotide variants (SNV), and insertion/deletion (indel) events ranging in size from one base to several megabases. Although capture-based next-generation sequencing (NGS) panels can capably detect SNVs and smaller indels, the bioinformatics algorithms utilized can vary in ability to accurately profile larger indel events, particularly with short-read methods. Ideally, the use of multiple variant callers may overcome deficiencies in individual programs and enhance overall calling accuracy and sensitivity. Here we benchmark performance of variant callers to better understand the strengths and limitations of each. **Methods:** DNA extracted from 151 unique bone marrow and peripheral blood specimens was used in this study, constituting a total of 262 previously characterized samples including replicates and sample mixes. NGS library preparation was performed with the KAPA HyperPrep protocol (Roche) using IDT xGen Dual Index UMI adapters (IDT), captured with a 50-gene custom-designed IDT xGen Lockdown Probe Pool, and sequenced on a NovaSeq 6000 (Illumina). Following primary analysis, FASTQ files from the sequencer were passed through an internal bioinformatics pipeline using TNhaplotyper2 (Sentieon), Manta (Chen, *et al.*, 2016), and Pindel (Ye, *et al.,* 2009) for variant calling with various filtering thresholds applied. **Results:** TNhaplotyper2 called 9,248 true variants of which 8,289 were SNVs and 959 were indels ranging in size from 1 bp to 104 bp. The accuracy for detected SNVs was 100%. Collectively, Manta and Pindel detected 141 true indels ranging in size from 25 bp to 26.8 Mb, including 108 (77%) that overlapped with TNhaplotyper2. Of these 141 indels, Manta exclusively detected seven calls (5%), Pindel detected 83 (59%), and 51 variants (36%) were found by both callers. Manta had a higher detection rate for large events including six calls ranging from 848 bp to 26.8 Mb. Pindel detected only two of these six calls (33%). Conversely, Pindel demonstrated higher sensitivity for small indels by detecting 83 events (25 bp to 243 bp) not identified by Manta, including 49 with allele frequencies (AF) from 2.1% to 5.3%. Pindel also detected 15 calls between 1.0% to 1.9% AF that were below the filter threshold of Manta given its poor specificity within this AF range. Together, all three callers detected ≥99% of expected variants. **Conclusions:** Three variant callers were evaluated in a clinical myeloid NGS panel. Each caller varied in analytic sensitivity and size limitations for detecting indels across a broad range of variants. We demonstrate that an optimized multi-algorithmic caller approach can ensure high accuracy for calling complex oncogenic events in the clinical laboratory.

H017. **SNPrint: Customizable Genotyper for Clinical Sample Tracking and Disease Progression Monitoring for NGS-Based Targeted Gene Panels**

J. Wemmer[1], P. Sanchez[2], J. D'Angelo[1]
[1]Invivoscribe, San Diego, CA; [2]LabPMM, San Diego, CA.

Introduction: Clinical labs performing regular genetic testing to monitor and evaluate for refractory and relapsed disease rely on accurate sample labeling for quality assurance; however, sample swapping may occur at any step between sample collection and

sequencing. For next-generation sequencing (NGS) data, one strategy to ensure that data correspond to the expected individual is to genotype based on single nucleotide polymorphisms (SNPs). For NGS-based targeted gene panels (TGPs), specific SNPs used for genotyping are constrained by the genes targeted in the assay. To robustly and accurately track sample integrity during hematological testing in our clinical lab, LabPMM, we present SNPrint, a customizable genotyper developed for our NGS-based TGPs, MyAML and MyMRD. **Methods:** For internal quality control (QC) purposes, the SNPrint program was designed to genotype 47 SNPs targeted in two of Invivoscribe's heme-oncology TGPs, MyAML and MyMRD. SNPrint takes in binary alignment map (BAM) files as input, calculates the variant read frequency (VRF) and determines the genotype for each SNP based on predetermined VRF thresholds. SNPrint outputs a barcode or an "SNPrint" for each sample which contains the genotypes of all 47 SNPs. An additional quality check is performed by ensuring each SNP meets a read depth threshold. To ensure that an SNPrint belongs to an expected patient, we then pair-wise contrast groups of SNPrints to match those that are identical. **Results:** SNPrint has helped resolve issues around sample swapping, but also has the added benefit of identifying whether a patient has recently undergone a bone marrow transplant or change in disease progression. When a patient has not undergone a bone marrow transplant or had significant disease progression, we expect two samples with the same unique patient ID to have identical barcodes. However, these aforementioned cases can result in discordant barcodes containing multiple ambiguous SNPs (no sample swap occurred). In these ambiguous cases it is possible to see SNPs with no distinct genotype called, due to VRFs falling between canonical thresholds (i.e., homo-/heterozygous frequencies). Further analysis of each VRF is then performed to determine the true genotypes for ambiguous SNPs. **Conclusions:** SNPrint has allowed for robust and streamlined QC of clinical samples, and has been proven to mitigate sample swap issues. SNPrint also has the added benefit of being able to track disease progression and engraftment analysis of bone marrow transplants, which has helped resolve past sample-to-patient discrepancies. Lastly, the customizability of SNPrint lends itself to ubiquitous implementation for any NGS-based TGP.

H018. **Detection of *UBA1* Mutations Using a Droplet Digital Polymerase Chain Reaction Assay for the Diagnosis of VEXAS Syndrome**

A. Boerger, D. Milosevic, R. He, D. Viswanatha
Mayo Clinic, Rochester, MN.

Introduction: VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome is associated with somatic mutations in the *UBA1* gene, which encodes ubiquitin-activating enzyme E1. This syndrome is characterized by adult-onset rheumatologic and hematologic manifestations, the symptoms of which overlap with both myelodysplastic syndrome and autoimmune disorders. The total number of cases has rapidly increased since its discovery in 2020, suggesting its prevalence is not yet sufficiently understood. To date, all documented cases have been caused by seven mutations that occur at three different sites within exon 3 of the *UBA1* gene: p.Met41, p.Ser56, and the splicing region. **Methods:** A droplet digital polymerase chain reaction (ddPCR) assay was designed to detect each of the seven mutations. Two sets of primers were designed to amplify 1) the splicing and p.Met41 mutation regions, and 2) the p.Ser56 mutation region. TaqMan hydrolysis probes were designed to detect each of the mutant and corresponding wild-type (WT) targets and included locked nucleic acids to increase specificity. In each assay, the competing WT sequence was targeted using a dark probe, ensuring only mutant targets were detected. A reference sequence was labeled with a fluorophore and included in one assay to act as an internal control. In total, eight detectable targets (seven mutant and one reference) were assessed across four duplex assays using probes labeled with FAM and HEX fluorophores. Samples were combined with master mix in cartridges and loaded onto the QX200 Automated Droplet Generator (Bio-Rad). Partitioned samples were amplified on the Veriti Thermal Cycler (Applied Biosystems) and analyzed using the QX200 Droplet Reader (Bio-Rad). **Results:** The assay successfully detected and differentiated all seven mutant targets and the internal control. No cross-reactivity was observed between the mutant or WT targets across all four reactions. Three VEXAS patient samples with three different known *UBA1* mutations (previously confirmed by Sanger sequencing) were correctly identified by the assay. The analytical sensitivity for all mutant targets was determined to be ≤1%. **Conclusions:** Given the localized distribution of currently known mutations in *UBA1*, ddPCR offers a sensitive method for detecting and discriminating common somatic alterations associated with VEXAS syndrome. We demonstrate that this four-duplex assay allows for the efficient, simultaneous detection of all seven mutations and a WT internal control in a single run with better sensitivity than Sanger sequencing.

H019. **Validation of a 54-Gene Myeloid NGS Panel Using an Independent Tertiary Analysis Software Program**

L. Bonomi, A. Tyropolis, C. Labaj, J. Deslauriers, K. Lindsey, F. Moore
Baystate Health, Springfield, MA.

Introduction: Sequencing DNA of tumor cells from patients with myeloid neoplasms has therapeutic, diagnostic, and prognostic implications. Presented here are validation data combining one commercial sequencing method with an independent commercial tertiary analysis software program. **Methods:** DNA extracted using either a manual or automated method from 13 peripheral blood and 38 bone marrow specimens from patients with a myeloid malignancy was sequenced. Additionally, 15 CAP PT specimens and five commercial standards (Horizon Tru-Q 1-4, Horizon OncoSpan) were analyzed. Sequencing was performed on two Illumina MiSeq Dx instruments using the amplicon-based Illumina TruSight Myeloid 54-gene Sequencing Panel. Genome.vcf files were generated in the Illumina DNA Amplicon app with alignment to the hg19 reference genome. These files were then uploaded into the Qiagen Clinical Insight Interpret software for tertiary analysis using custom filters. All results were compared to those obtained from reference labs (patient specimens) or CAP PT results/known controls. **Results:** The 71 samples resulted in 186 detected variants consisting of 147 single nucleotide variants, 37 indels, and two *multi-nucleotide variants*. For the indels, detected size range was 1 bp to 66 bp for insertions and 1-52 bp for deletions. Detection of the 52-bp type-1 *CALR* deletion required secondary analysis with the Illumina BaseSpace Pindel app. Analysis of *FLT3* and *CEBPA* variants were not evaluated in the present study as they may not be amenable to the present next-generation sequencing methodology secondary to large duplications or being GC-rich, respectively. In total, 185 positive variant calls and 1,173 negative variant calls matched expected results, with one false negative and two false positives, yielding a sensitivity of 99.5%, a specificity of 99.8%, a positive predictive value of 98.9%, and a negative predictive value of 99.9%. The one false negative was due to the variant being in a region with strand bias and thus was not called. Orthogonal analysis was not performed on the two potential false positive variants and thus their true presence or absence could not be confirmed. The mean difference in variant allele frequency (VAF) between the present study and the reference labs was 2.5% $\pm$ 3.3% (S.D.) with 98% of variants within 10% VAF. Lastly, the overall clinical interpretation (e.g., tier classification, predicted response to therapy, etc.) of all variants was concordant with those reported by the reference labs in patient specimens. **Conclusions:** The combination of the Illumina Trusight Myeloid Sequencing Panel and the Qiagen Clinical Insight Interpreter tertiary analysis software is a sensitive and specific approach for the detection and interpretation of clinically important variants in myeloid neoplasms.

H020. **Evaluation of Genexus System for Rapid Comprehensive Genomic Profiling of Myeloid Malignancies in Clinical Samples**

N. Maharjan[2], K. Champion[1], K. Blakely[2], L. Blann[3], X. Dai[2], S. Dhungana[2], T. Freitas[2], S. Glidewell[2], J. Graham[2], S. Khadgi[2], G. Kilaru[2], S. Powers[2], G. Sun[2], A. Tigh[2], J. Zhang[3]
[1]Quest Diagnostics, Irving, TX; [2]Quest Diagnostics, Lewisville, TX; [3]Thermo Fisher Scientific, Waltham, MA.

Introduction: The World Health Organization (WHO) and the European Leukemia Net (ELN) urged the use of molecular testing for disease subclassification, prognosis, risk stratification, and therapeutic treatment for myeloid malignancies such as acute myeloid leukemia (AML). In addition, results of molecular analysis for key biomarkers such as nucleophosmin 1 (*NPM1)* and fms-related tyrosine kinase 3 (*FLT3)* should be available within 48-72 hours, and the remaining molecular results within the first treatment cycle. The standard next-generation sequencing (NGS) approach to provide these results can take anywhere from a few weeks to months. In contrast, the Oncomine Myeloid Assay GX v2 kit using Ion Torrent Genexus System detects and quantifies critical biomarkers in myeloid malignancies in one day. Because expedited stratification and initiation of appropriate therapy is important to improve patient outcomes, we evaluated the platform's performance against an in-house NGS panel. **Methods:** The study was conducted using Oncomine Myeloid Assay GX v2 (DNA only) on 123 samples including commercial controls and previously extracted DNA from clinical bone marrow, whole blood, paraffin-embedded tissue, and fresh tissue specimens that were previously tested positive by our in-house NGS panel for hematological malignancies (HemeSEQ). The Genexus Sequencer dilutes extracted and qubit-quantified DNA to 1.1 ng/µL, which is then processed through automated library preparation, templating, sequencing, and fully annotated reporting in a single day. **Results:** The overall sequencing metrics showed good uniformity (>94.4%), read depth (>4,000X) with on-target reads >96.3%, and 100% concordance was observed between the two platforms for variant calls in all clinical samples. Variants were detected down to 3.0% variant allele frequency (VAF). *FLT3*-internal tandem duplication (ITD) variants that were previously undetectable by our in-house NGS method, long calreticulin (*CALR)* deletions, and mutations within GC-rich region of CCAAT enhancer binding protein alpha (*CEBPA)* gene were all sequenced and detected successfully. Ion Torrent Genexus System completed 16 DNA samples from nucleic acid to reporting in 23 hours with a total hands-on time of 20-30 minutes, as opposed to HemeSEQ which takes 72 hours and 10-12 hours of hands-on time. **Conclusions:** Based on our evaluation, the Ion Torrent Genexus system ultra-rapid NGS assay produces highly reproducible data, which is highly concordant with our in-house NGS assay but obtained in a single day with minimal specimen manipulation. In addition, difficult-to-sequence targets, such as *NPM1*, GC-rich *CEBPA*, and *FLT3*-ITDs, were identified without orthogonal specimen triaging and at lower expense because of the elimination of multiple workflows.

H021. **Rapid Detection of Pathogenic *UBA1* Variants by MassARRAY in Patients with VEXAS Syndrome**

S. Chen[1], L. Lawrence[2], c. Ho[3], J. Le[3], P. Libiran[3], J. Zehnder[4], C. Suarez[4]
[1]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [2]Guardant Health, Redwood City, CA; [3]Stanford Health Care, Palo Alto, CA; [4]Stanford University School of Medicine, Palo Alto, CA.

Introduction: Somatic pathogenic variants in the *UBA1* gene on the X chromosome have been causally implicated in the VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome affecting men. Given the severe clinical sequelae common to the disease, rapid testing for pathogenic *UBA1* variants that permits expedited diagnosis and clinical management is critical. Our study aims to provide a cost-effective and rapid method that could be easily adapted for routine clinical testing. **Methods:** We developed, validated, and clinically deployed a rapid mass spectrometry-based single-base extension product detection (MassARRAY System, Agena Bioscience), for detection of *UBA1* variants at the commonly mutated positions c.121, c.122, and c.118-1. The *TGIF2LY* gene is also assessed as a sex marker to support specimen identification. The assay consists of polymerase chain reaction amplification of targeted region with custom amplification primers, followed by single base-pair extension at assayed positions with custom extension primers. Extension products are differentiated by matrix-assisted laser desorption/ionization mass spectrometry. Base identity at each assayed position is determined from molecular mass of extension product. In validation, 44 control samples were tested, including eight samples positive for mutation (all extracted DNA), and 36 samples expected to be negative for mutation (31 peripheral blood and five bone marrow aspirates). Limit of detection (LOD), analytical accuracy, sensitivity, specificity, and precision, and clinical sensitivity and specificity were determined. **Results:** Serial dilution study showed a lower LOD of 10% variant allele fraction (VAF) for assayed variants in positive control samples (c.121A >C, c.121A >G, c.122T >C, c.118-1G >C). At LOD, analytical accuracy, sensitivity, and specificity were 100% among the control samples (8 true positives and 36 true negatives). For intra-run reproducibility, VAF coefficients of variation (CV) ranged from 1% to 5% undiluted, and from 2% to 18% at LOD. For inter-run reproducibility, VAF CV ranged from 1% to 6% undiluted, and from 3% to 14% at LOD. Clinical sensitivity and specificity, based on known *UBA1* pathogenic variants, are 98% and 100%, respectively, for VEXAS syndrome. In clinical deployment, testing of 10 samples yielded two positive results (both c.122T >C) and eight negative results. Turnaround time from sample receipt to result reporting of four days minimum, with instrumentation test run time of nine hours. Cost analysis indicates a per-sample reagent cost of as little as USD $12. **Conclusions:** Rapid testing for *UBA1* pathogenic variants with MassARRAY detection for diagnosis of VEXAS syndrome has high analytical performance and is a cost-effective approach for testing.

H022. **Targeted Genetic Profiling of Myeloid Malignancies Using Next-Generation Sequencing: Prevalence and Clinical Impact in a Cohort of Lebanese Patients**

L. Hamadeh, R. Abdul-Khalik, R. Mahfouz
American University of Beirut Medical Center, Beirut, Lebanon.

Introduction: Myeloid neoplasms comprise a group of biologically and clinically heterogeneous clonal disorders characterized by ineffective hematopoiesis, due to hematopoietic stem cell excessive proliferation and defective myeloid lineage differentiation. Molecular profiling plays an important role in diagnosis, prognosis, and treatment of these malignancies. Current methods for studying blood disorders can be effective but are time consuming and expensive when looking at multiple variants, and may not determine the underlying genetic cause of the disease. The introduction of next-generation sequencing (NGS) into the routine clinical practice allowed for a highly sensitive simultaneous screening of clinically relevant genomic mutations, thus transforming the disease management landscape. Herein, we evaluate the clinical utility of a 54-gene panel in a cohort of Lebanese patients. This panel targets mutations in tumor suppressor, and oncogenic hotspots with potential involvement in acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), chronic myelogenous leukemia, chronic myelomonocytic leukemia, and juvenile myelomonocytic leukemia. **Methods:** Genomic DNA from 258 patients was extracted from bone marrow or peripheral blood. Library preparation was performed using the Trusight Myeloid Sequencing Panel according to the manufacturer's instructions. Pooled libraries were loaded on the MiSeq system for automated sequencing and data analysis. Variants were annotated using variant interpreter. **Results:** Clinically relevant mutations were detected in 91.08% of samples with a total of 720 variants. Only 23 cases were negative for any variants in the targeted regions. *TET2* showed the highest mutation rate among our cohort with a total of 111 variants mainly in AML, MDS, and MPN cases. *ASXL1* and *DNMT3A* variants were detected in 50 and 55 samples, respectively. Other commonly

mutated genes were *EZH2* with 39 variants, *RUNX1* (30 variants), *SRSF2* (27 variants), *TP53* (27 variants), *IDH1* (26 variants), *BCOR* (24 variants), *NRAS* (20 variants), *BCORL1* (19 variants), and *IDH2* (18 variants). Isolated, fewer than 10 mutations were also observed in other genes such as *ABL1*, *ATRX*, *CDKN2A*, *GNAS*, *JAK2*, *KMT2A*, *PDGFRA*, *SF3B1*, *WT1*, and *ZRSR2*. Of note, point mutations, insertions, and deletions were detected in challenging regions such as *CALR*, *CEBPA*, *FLT3*, and *NPM1*. **Conclusions:** The implementation of this NGS assay allows for a comprehensive evaluation of the mutational spectrum of myeloid malignancies in Lebanese patients. The detection of these actionable gene mutations contributes to an early diagnosis, appropriate individualized treatment, enhanced prediction of target therapy response, and improved clinical outcome.

H023. **Development of Synthetic External RNA Controls for Quantitative Detection of the *BCR-ABL1* p190 (e1a2) Translocation**

J. Salem[1], T. Spenlinhauer[2], M. Escott[1], E. Farrell[1], J. Gordon[1], M. Held[1], L. Krott[1], G. MacLeod[1], D. Magoon[1], A. Olsen[1], B. Parker[1], S. Pelsue[1], A. Rietdyk[1], J. Ross[1], N. Wilkinson[1]

[1]MMQCI, Saco, ME; [2]Maine Molecular Quality Controls Inc., Saco, ME.

Introduction: Chronic myeloid leukemia (CML) is a disease caused by a translocation between chromosomes 9 and 22, forming the truncated Philadelphia chromosome. In this translocation event the *ABL1* gene on chromosome 9 is translocated to the break point cluster region (*BCR* gene) on chromosome 22. Expression of the *BCR/ABL1* fusion genes in myeloid cells promotes aberrant proliferation, increased cell survival, and enhanced migration and invasion through the activation of key regulatory pathways. One such translocation, called p190, occurs at exon a2 of *ABL1* and exon e1 of *BCR*, forming a 190-kDa protein. One method for monitoring CML treatment is measuring levels of *BCR/ABL1* transcripts in peripheral blood by RT-PCR. The molecular response (MR) is the log of ratio of *BCR*/*ABL1* to *ABL1* transcripts and guides treatment. A reduction in the *BCR*/*ABL1* to *ABL1* ratio results in a higher MR demonstrating a reduction in CML; a 3-log reduction of *BCR*/*ABL1* transcript is defined as the major molecular response, indicating the patient is responding well to therapy. Here, we describe the development of a control panel that can be used to monitor performance of assays that detect e1a2 (p190) and report a range of p190 *BCRABL1*/*ABL1* levels. **Methods:** Gene segments of *ABL1* and *BCR* were designed *in silico* to create DNA constructs and cloned to create stable frozen clone stocks. *In vitro* RNA transcripts were generated, quantified by UV spectrophotometry and formulated in a proprietary matrix that carries the RNA through extraction processes and provides stability. *BCR*/*ABL1* and *ABL1* transcripts were combined to generate a panel of five *BCR*/*ABL1* e1a2 levels (0%, 0.02%, 0.1%, 1%, 10%). Three lots of the panel were tested across three lots of the Xpert *BCR-ABL* Ultra p190 assay over multiple days and operators to determine the linearity and reproducibility. **Results:** Out of a total 195 Xpert *BCR-ABL* Ultra p190 runs, there were three invalid runs. Of 192 valid runs, 100% had correct results with reported percent ratio values within expected limits of assays performance. Performance data demonstrated that the control panel was linear with R^2 values of 0.9914, 0.9888, and 0.9958, reported Ct values for each level were within expected targeted range with standard deviations of less than two and percent CV <10% across the three lots tested, and the percent ratios were within twofold of targeted value, i.e., 0.02%, 0.1%, 1%, 10%. Stability studies and historical data for similarly formulated control material demonstrate ≥12 months stability when stored at -20°C. **Conclusions:** The synthetic *BCR*/*ABL1* p190 external control demonstrated reproducible and robust performance when tested on Xpert *BCR*-ABL Ultra p190 assay. These controls are beneficial for routine monitoring of p190 *BCRABL1*/*ABL1* assay performance.

H024. **Performance Evaluation of an Automated Genomic Diagnostic Assay for Myeloproliferative Neoplasms**

A. Campbell[1], B. Knock[1], M. Scicchitano[1], C. Hall[1], G. Chen[2], H. Li[1], Y. Ding[1]

[1]Geisinger Health, Danville, PA; [2]Penn State Health, Hershey, PA.

Introduction: Diagnosis of myeloproliferative neoplasms (MPNs) depends on a close correlation among clinical presentation, molecular diagnostics, and morphological evaluation of bone marrow biopsy. *BCR::ABL1* fusion and mutations in *JAK2*, *CALR*, *MPL,* and *CSF3R* are considered driver events. Conventional diagnostic pathway usually includes a multi-step reflex single gene mutation testing algorithm, whereas *BCR::ABL1* fusion is detected by a different method. The Genexus platform was previously validated as a multi-gene panel for automated, rapid, comprehensive profiling of suspected MPNs. Despite reducing the need for multi-step reflex testing, the *CALR* type 1 and type 2 insertion/deletion mutations were not correctly identified in nearly half of the validation samples. Presumptive *CALR* mutant samples thus required confirmation testing. This study compares the validation of the customized Myeloproliferative Neoplasm Diagnostic Assay on the Genexus system using the first- and second-generation assay with their associated versions of bioinformatics software. **Methods:** For each run, seven DNA, seven RNA, and one no template control were loaded on the Genexus for *BCR::ABL1*, *CALR*, *CSF3R*, *JAK2*, and *MPL* testing. A total of 124 clinical, control, and CAP specimens were tested using assay version 1, and data analysis was completed using version 6.2.1 software. A total of 54 clinical, control, and CAP specimens previously tested by reference methods were tested with the second-generation assay, and data were analyzed by version 6.6.0.3 software. Fusions and single nucleotide variants/insertion/deletions (indels) were reviewed using no filter, Oncomine Variants 5.14 filter (assay version 1) or Oncomine Variants 5.16 Extended filter (assay version 2), and Integrative Genomics Viewer (IGV). **Results:** The Myeloproliferative Neoplasm Diagnostic Assay achieved >95% sensitivity and specificity for *CALR, CSF3R*, *JAK2*, and *MPL* mutations at a lower limit of detection (LOD) of 5% when using either the Oncomine Variants 5.16 Extended filter or "No Filter" setting. In contrast to the first-generation assay and bioinformatics version 6.2.1, *CALR* indel mutations were accurately called in 100% (17/17) of the samples. *BCR::ABL1* p190, p210, and p230 transcripts were detected, and the sensitivity and specificity of *BCR::ABL1* p190 and p210 fusion RNA at an LOD of 0.1% was >95% using both versions of the assay. **Conclusions:** The Myeloproliferative Neoplasm Diagnostic Assay on the Genexus system provides a comprehensive solution for the molecular diagnosis of MPNs. Assay version 2 and 6.6.0.3 software demonstrated significant improvement in *CALR* variant detection, thus eliminating the need for automatic reflexive secondary confirmation. The assay remains sufficient to measure *BCR::ABL1* at ≥0.1%.

H025. **Development of a Synthetic RNA Control Panel for Monitoring Detection of "Type A" *NPM1* Mutant Transcripts**

M. Held, T. Spenlinhauer, M. Escott, E. Farrell, J. Gordon, L. Krott, G. MacLeod, D. Magoon, A. Olsen, B. Parker, S. Pelsue, A. Rietdyk, J. Ross

Maine Molecular Quality Controls, Inc., Saco, ME.

Introduction: Nucleophosmin-1 (NPM1) is a versatile chaperone protein with chief roles in nucleocytoplasmic protein shuttling, ribosome biogenesis, and cell survival. Tetranucleotide insertions in *NPM1*'s endmost exon 12 occur in one-third of adult acute myeloid leukemia cases. The "Type A" *NPM1* (*NPM1mutA*) insertion is a TCTG tandem duplication and represents approximately 80% of all *NPM1* mutations. The result is a frameshift leading to aberrant accumulation of NPM1 in the cytoplasm and instability of tumor-suppressors p53 and $p14^{ARF}$. Notably, NPM1 mutant leukemias are recognized as a distinct class by the World Health Organization. Diagnostic assays for *NPM1mutA* quantitation are valuable tools for defining treatment responses in patients. To monitor performance of NPM1 assays, a novel synthetic control panel was developed comprising a set of

relevant concentrations of wild-type *ABL1* and *NPM1* transcripts mixed with varying levels of *NPM1mutA* within a stabilizing matrix formulation. **Methods:** Partial gene sequences of *ABL1*, *NPM1,* and *NPM1mutA* were synthesized, ligated into engineered vectors, and transformed to generate stable frozen clones. All sequences were confirmed via bidirectional Sanger sequencing. *In vitro* transcripts were generated, quantified by UV-spectrophotometry, and combined with proprietary stabilizing matrix to create a panel of *NPM1mutA* to *ABL1* levels; 0%, 0.1%, 5%, and 20% *NPM1mutA*. Three lots of the NPM1 control panel were tested across five lots of Xpert NPM1 Mutation cartridges (Cepheid) with four different operators. **Results:** NPM1 control panel was tested on-site with a total of 152 Xpert NPM1 Mutation cartridges. Linear regression analysis across the ~3-log range of *NPM1mutA*:*ABL1* levels demonstrated R^2 values >0.98 for each Xpert cartridge lot, and >0.97 across all five cartridge lots. Combining data from all three NPM1 control panel lots showed high accuracy and precision at all positive *NPM1mutA* levels: 20% level, 95% CI (18.22, 21.11); 5% level, 95% CI (4.69, 5.72); 1% level, 95% CI (0.89, 1.08); 0.1% level, 95% CI (0.113, 0.139). **Conclusions:** The synthetic NPM1 *"Type A"* external control panel demonstrated high accuracy, precision, and linearity when utilized on Xpert NPM1 Mutation cartridges. The external control panel is vital for monitoring instrument and assay performance consistency in clinical labs worldwide. The proprietary matrix formulation enhances stability and longevity of synthetic transcripts for routine usage, and facilitates extraction of the RNA via Xpert NPM1 cartridges. The controls are processed in the same manner as patient blood samples, which provides operational familiarity for the end-user and monitors performance deviations of the assay.

H026. **Cytogenetic Analysis of Mixed Phenotype Acute Leukemia Reveals Frequent MDS-Related Abnormalities**

M. Hennawi, M. Hussaini, S. Kapadia, S. Osman, f. Qaudeer, H. Tashkandi
Moffitt Cancer Center, Tampa, FL.

Introduction: Mixed phenotype leukemia (MPAL) is a rare form of acute leukemia (2%-5% of acute leukemia) with poor prognosis. The diagnosis of MPAL requires either single blast population expression of multiple lineage markers (myeloid, B-cell, and/or T-cell) or two concurrent blast populations with distinct lineages. Given the rarity of MPAL, cytogenetic studies investigating the genomic aberrations are limited. **Methods:** IRB approval was obtained. Central MCC database was searched for any patient with a diagnosis of acute undifferentiated leukemia, acute leukemia of ambiguous lineage, and MPAL from a database of >600,000 entries. All patient diagnoses were manually reviewed by a hematopathologist to confirm the diagnosis. Cytogenetic and fluorescence *in situ* hybridization (FISH) results were manually curated from the EMR. **Results:** Twenty-nine patients with MPAL were identified. Fourteen were female and 15 were male. Average age was 54 years old (range=28-81). Diagnoses were as follows: B/myeloid (n= 18), T/myeloid (n=10), and T/B (n=1). Overall survival was 18.8 months (range=0-70). Cytogenetic analysis (karyotype +/- FISH) was available for 28 patients. The most frequent recurrent abnormalities were complex karyotype (n=8), *BCR/ABL1* translocation (n=6), Del 5q/-5 (n=5), polysomy 21 (n=4). Additional myelodysplastic syndrome (MDS)-related cytogenetic abnormalities included: del 7/- 7 (3), Trisomy 8 (1), Del 17p/- 17 (2), del 20q (1), del 9q (1), and del 13q (1). For those who were *BCR/ABL1* positive (n=6), three were p210, two were p190 positive, and one was positive for both p210 and p190. For patients with mutational data, *TP53* mutations were present in four patients, two of which had complex karyotype. Novel rearrangements included t(2;14;5)(q23;q32;q13), der (12;16) (q10;p10); t(2;14)(p13;q32); t(1;16)(q12;q11.2); t(2;17;8)(p23;q25;q22); t(1;3)(q21;p25), t(8;14)(q24;q32); and t (5;14) *TLX3-BCL11B*. **Conclusions:** The largest series of MPAL (n=117) reports frequent *BCR/ABL1* fusion, complex karyotype, polysomy 21, -7, and polysomy 8 as the most frequent alterations. We expand on this data, with one of the largest series of MPAL cases with cytogenetic data by reporting the frequent occurrence of MDS-defining cytogenetic abnormalities (n=13, 46%), excluding cases with complex karyotype. Our findings expand on the molecular underpinnings of MPAL and may carry prognostic implications in a disease subset with already dismal prognosis.

H027. **Detection of Clonotypic Rearrangements by NGS Can Be Used to Track Minimal Residual Disease in Mixed Phenotype Acute Leukemia**

M. Hennawi, M. Hussaini, S. Kapadia[3], S. Osman, F. Quadeer, H. Tashkandi
Moffitt Cancer Center, Tampa, FL.

Introduction: Multiplex PCR and next-generation sequencing (NGS) (clonoSEQ Assay) can be used to identify a neoplastic clonotype by targeting *CDR3* and assessing rearrangements in *IgH, IgK, IgL, TCR-β* and *TCR-g* loci. The clonotypic sequence can be robustly used to track measurable residual disease (MRD). Although calibration rates of >90% are reported in myeloma, chronic lymphocytic leukemia, and acute lymphoblastic leukemia (ALL), in some cases of primitive ALL no dominant sequence can be identified due to lack of VDJ rearrangement in these early precursors. The ability to track MRD by NGS in mixed phenotype acute leukemia (MPAL), a rare leukemia, warrants investigation and is explored in this study. **Methods:** Central MCC database was searched for any patient to locate patients with MPAL from more than 600,000 entries. All patient diagnoses were manually reviewed by a hematopathologist. Patient charts were manually curated to identify those with Clonoseq data, and clinical data were procured from the EMR. **Results:** Twenty-nine patients with MPAL were identified. Only two patients with Clonoseq testing were found. Both demonstrated a B/myeloid phenotype and both were bilineal (two distinct blast populations). NGS (Clonoseq) identified four dominant (*IGH*) (patient A; 8/2019) and two dominant sequences (patient B; 10/2019), respectively. Patient A had 28% blasts by morphology and sample clonality by NGS was 27%. For Patient B, flow cytometry showed the myeloid component was 26% and the CD19+ lymphoid component was 16%. Sample clonality by NGS was 15%. For patient A, subsequent tracking demonstrated 366, 0, 8, 0, 0, and 0 residual clonal cells (between 9/2019 to 9/2020). In 11/2019 MRD flow cytometry was negative but NGS showed eight residual clonal cells. The patient was treated with hyperCVAD, then modified POMP, followed by dose-modified R+mini-CVD+Inotuzumab. The presence of MRD(-) led to the clinical decision to avoid additional (6MP) low-dose therapy. Overall survival was 16 months. For patient B, subsequent tracking showed 24,352 residual clonal cells (3/2020) correlating with relapsed *FLT3*+ MPAL after a negative marrow 12/2019. Flow cytometry showed 44% myeloblasts and <1% B lymphoblasts. NGS showed 45% clonality with dominant sequence 1 comprising all 24,352 reads and dominant sequence two undetected. Notably, a new dominant sequence with partial identity to prior dominant sequence 2 was detected (21,963 clonal cells). Patient B was treated for relapse with azacitidine + gilteritinib and repeat biopsy showed no excess blasts. **Conclusions:** This report confirms the utility of Clonoseq NGS-based MRD tracking in patients with MPAL and increased sensitivity over MRD flow cytometry. Both patients had bilineal disease, and future studies are warranted on larger cohorts to determine the feasibility of NGS-MRD on biphenotypic MPAL.

H028. **Simultaneous Detection of Mutations in Codon 505 and 515 of *MPL* by qPCR and Melting Curve Analysis Using a Single Pair of Hybridization Probes**

C. Courtemanche, E. Winstall, M. Gagnon, O. Larochelle
CHU de Quebec/Université Laval, Quebec City, Quebec.

Introduction: Myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal hematopoietic disorders associated with driver mutations in *JAK2, CALR,* or *MPL* genes. Mutations in the *MPL* gene are found in 3% of essential thrombocythemia and 10% of primary myelofibrosis and are found in exon 10, mostly at codon 515 (W515L, W515K, W515A, W515R) and, to a lesser extent, at codon

505 (S505N). As codon 505 and 515 are 30 pb apart, most assays will use two different PCR reactions, done separately or in multiplex. To optimize the detection of *MPL* mutations, we designed a qPCR assay that, with only one primer pair and a single set of fluorescence resonance energy transfer (FRET) hybridization probes, has the ability to detect *MPL* exon 10 mutations at both codon 515 and 505 by melting curve analysis of the amplified products. **Methods:** A pair of DNA primers was designed to amplify a 147bp fragment in exon 10 of *MPL* encompassing both codon 505 and 515. A pair of hybridization probes was carefully designed to allow the detection of mutations in both codons by FRET and melting curve analysis. The 3' fluorescein-labeled sensor probe covers the sequences of codon 515, whereas the 5' LC640-labeled anchor probe sits on the 505 codon. Starting from 10 ng of genomic DNA (gDNA) isolated from blood or bone marrow, PCR amplification was done on a LightCycler480 instrument followed by melting curve analysis on a temperature range from 40°C to 80°C. **Results:** Serial dilutions of double-stranded DNA fragments (gBlocks) containing the W515L, W515K, or S505N mutations into gDNA from individuals that previously tested negative for *MPL* exon 10 mutations were used to assess the capacity of our single-tube assay to detect the different mutations. Melting analysis of the mutated amplicons had a clearly distinctive denaturation pattern from the wild-type amplicons. Although the wild-type amplicons displayed a Tm of 70°C, the W515K, W515L, and S505N controls yielded amplicons with Tm values of 58°C, 61°C, and 65°C, respectively. Lower limit of detection of the assay for variants tested is estimated at 2% to 5% of variant allele frequency, a range expected for melting analysis assays. The assay was further validated on more than 100 previously analyzed patients and showed a 100% sensitivity/specificity. **Conclusions:** Our results show that a single PCR assay composed of a unique primer pair and a single set of hybridization FRET probes has the ability to detect the most frequent mutations at different locations in exon 10 of the *MPL* gene. This assay circumvents the decrease in sensitivity generally associated with multiplex PCR assay. It represents a rapid and cost-effective solution for sensitive screening of *MPL* mutations in patients with suspected MPN.

H029. **Tracking Measurable Residual Disease in Acute Myeloid Leukemia with Error Corrected Sequencing**

J. D'Angelo, Y. Huang, Y. Kebede, S. Miick, N. salva, C. Troung, S. Turner

Invivoscribe, San Diego, CA.

Introduction: Measurable or minimal residual disease (MRD) detection has proven to be useful in the clinical management of patients with leukemia and can facilitate the development of new therapies. DNA-based focused target enrichment strategies are an attractive solution to detect MRD using next-generation sequencing (NGS), as they can be applied to a broader population of patients as compared to a single gene. However, NGS approaches have a relatively high error rate; approximately one erroneous base call per 100-1,000 sequenced nucleotides. To circumvent this limitation, we present our MyMRD Myeloid Gene Panel Assay, which uses a proprietary probe design targeting 53 select genes with an innovative approach of duplexed sequencing and tag-based error correction method that improves sequencing accuracy. In this RUO assay, unique molecular identifiers (UMIs) are physically incorporated into sequencing libraries. During the error correction process, duplicate reads (determined by UMIs) allow for the assessment of whether a variant is an error or a true mutation, thereby enabling accurate tracking of measurable residual disease. **Methods:** A commercially available myeloid reference standard DNA containing 14 hotspot mutations (11 single nucleotide variants [SNVs] and three insertion or deletions [indels]) was diluted in background DNA to a variant allelic frequency (VAF) of $5x10^{-4}$. The contrived DNA was then enzymatically fragmented using the Twist Library Preparation Enzymatic Fragmentation Kit 2.0. UMIs were then ligated to the fragmented DNA to facilitate error correction. Illumina-style adapters were then incorporated to generate NGS libraries, which were quality checked and hybridized with MyMRD Myeloid probes. Enriched libraries were sequenced on the NovaSeqTM 6000 sequencer, and sequencing data were analyzed using proprietary MyMRD Myeloid analysis pipeline software. **Results:** Twenty-two samples were sequenced with error-corrected duplex sequencing to a mean duplex molecular depth of 41,461x. At a VAF of $5x10^{-4}$, nine of the SNVs interrogated were detected ≥95% of the time. Two other SNVs were detected at 91% and 86% frequency. The three indels we evaluated (*JAK2* F537-K539 >L deletion, a 30 bp *FLT3*-ITD, and the *NPM1* W288fs*12 insertion) were detected at 100%, 100%, and 82% frequency, respectively. Overall, in the 22 samples evaluated with the MyMRD Myeloid Gene Panel Assay, one or more hotspot mutations were detected at $5x10^{-4}$ VAF with 96% frequency. **Conclusions:** The MyMRD Myeloid Gene Panel Assay demonstrates excellent accuracy and high sensitivity in the detection of AML driver mutations at MRD levels. Its ability to reliably detect multiple mutations within one sample enables comprehensive myeloid studies.

H030. **Development of Seraseq ctDNA Myeloid Mutation Mix: A Reference Material to Monitor Sensitivity and Specificity of NGS-based Testing in Myeloid Blood Cancers**

C. Huang[1], D. Ruminski Lowe[1], S. Jamindar[1], Y. Konigshofer[1], M. Butler[1], I. Chivukula[2], R. Garlick[2], B. Anekella[1]

[1]LGC Clinical Diagnostics, Gaithersburg, MD; [2]LGC Clinical Diagnostics, Milford, MA.

Introduction: Myeloid malignancies are characterized by excessive proliferation, abnormal self-renewal, and differentiation defects of hematopoietic stem cells and myeloid progenitor cells. The detection of these types of malignancies using the traditional procedures by obtaining bone marrow sample is invasive and risky. Hence, liquid biopsy-based methods are in high demand to monitor and detect new cases of blood cancer. Here we present the development of four new Seraseq ctDNA reference materials specifically for myeloid malignancies. **Methods:** Genomic DNA (gDNA) from the GM24385 cell line was blended with 22 clinically relevant biosynthetic myeloid DNA variants that were quantified using droplet digital PCR. The formulated gDNA blends were adjusted to contain allelic frequencies of 0.1%, 0.5%, and 1%, and variant allele frequencies (VAFs) of each mix were verified with digital PCR. A 0% (WT) reference material with no added myeloid variants was prepared for comparison and identification of background variants. Fragmentation and size selection were performed to obtain circulating tumor DNA (ctDNA)-like size range DNA molecules. The resulting gDNA blend was assessed with custom next-generation sequencing (NGS) assays from Invitae (LiquidPlex) for all the 22 variants in the mixes to demonstrate compatibility with assays in common use in laboratories. **Results:** All biosynthetic variants in the 0.1%, 0.5%, and 1% mixes were detectable at the anticipated VAFs. The Agilent Bioanalyzer data revealed a size distribution of DNA fragments in the range of 100-300 bp, which is the desired range for ctDNA products. The VAFs observed in the Invitae data were consistent with those that were obtained by digital PCR (dPCR). The 0% WT product served as a negative and did not show elevated background variants over intact gDNA from the GM24385 cells. **Conclusions:** Seraseq ctDNA myeloid mutation mixes were demonstrated to provide reliable reference to 22 clinically relevant myeloid variants at VAFs from 1% down to 0.1%. These reference materials support ctDNA NGS assay development and routine quality control, and hence may be highly beneficial for the development of liquid biopsies for myeloid blood cancers without the need for flow cytometry. These reference materials can be used to monitor both sensitivity and specificity of variant detection using both dPCR and NGS-based assays.

H031. **Expanded International Scale (IS) Value Assignments to our *BCR-ABL1* Panel and Development of Daily Controls Traceable to WHO International Standards**

M. Wagner, H. Chan, T. Sung, N. Umblas, M. Macalik, R. Hu, L. Liu
Thermo Fisher Scientific, Fremont, CA.

Introduction: Chronic myeloid leukemia (CML) is driven by the *BCR-ABL1* fusion gene, formed by the t(9;22) translocation. Regular monitoring of the expression of the resulting *BCR-ABL1* oncogene mRNA in CML patients is critical for optimal disease management. The expression of *BCR-ABL1*, measured by RT-qPCR or digital PCR, is relative to a reference gene (i.e., *ABL1*), and results are expressed on the International Scale (IS). These methods require a full-process IS value assigned control that captures any variability during RNA extraction, complementary DNA (cDNA) synthesis, and qPCR. We (Thermo Fisher Scientific) have developed a full-process *BCR-ABL1* panel (for research use only, not for clinical use.) with IS traceability; however, this control panel is traceable to the *ABL1* gene only, and there is a lack of third-party daily controls in the market. Therefore, we extended our in-market cell-line-based *BCR-ABL1* control panel and assigned additional IS values to *BCR*, and *GUSB* control genes. In addition, 10% and 0.1% daily controls were also developed to allow laboratories to monitor assay performance as well as operator training **Methods:** The *BCR-ABL1* and *ABL1* gene copy numbers of HL60 (*BCR-ABL1* negative) cells and K562 (*BCR-ABL1* positive) cells were calculated before mixing to the approximate target ratios. The 10%^{IS} (high) and 0.1%^{IS} (low) daily controls were also created in full process and in buffer formats. The cell mixtures were lyophilized and stored at -20ºC. After reconstitution, RNA was extracted and cDNA was synthesized with a cDNA reverse transcription kit. The copy numbers of *BCR-ABL1*, *ABL1*, *BCR*, and *GUSB* were then determined using custom assays on the Bio-Rad droplet digital PCR system. Precise IS values were assigned by reference to the WHO international primary panel. Stability of the controls has been monitored in accelerated and real-time studies. **Results:** Two panel members of *BCR-ABL1* control material were created at approximately 10% and 0.1% as per target (*BCR-ABL1/ABL1*) percent values by mixing HL60 and K562 cells at different ratios. The panel was calibrated using the WHO international primary *BCR-ABL1* panel values, and performed well in external testing. In addition, the IS value assignment was determined for *ABL*, *BCR*, and *GUSB* reference genes. **Conclusions:** The *BCR-ABL1* panel was generated and value assigned using *ABL1*, *BCR,* and *GUSB* genes traceable to WHO IS values. The only commercially available panel and daily controls that have been assigned IS values to all three reference genes as the WHO standard were generated. These controls could standardize routine analytical validation of assays, enable monitoring of assay variation over time, and expand adoption by more testing laboratories through the additional value assignment of the *BCR*, and *GUSB* reference genes.

H032. **Compound Heterozygous Events in Myeloid Tumors: Next-Generation Approach with Optical Genome Mapping and a 523-Gene NGS Panel**

N. SAHAJPAL[1], R. Kolhe[1], S. Ananth[1], A. Chaubey[2], A. Hastie[2], V. Kota[1], D. Saul[2], N. Savage[1], S. Shams[2], A. Mondal[1], H. Singh[1], A. Vashisht[1]
[1] Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA.

Introduction: The standard of care (SOC) for genomic testing of myeloid cancers primarily relies on karyotyping/fluorescent *in situ* hybridization (FISH) (cytogenetic analysis) and targeted gene panels (≤54 genes) that harbor hotspot pathogenic variants (molecular genetic analysis). Both cytogenetic and molecular testing work-up are necessary for the identification and detection of large structural variants (SVs), single nucleotide variants (SNVs), and indels. Despite this combinatorial approach, ~50% of myeloid cancer genomes remain cytogenetically normal, and the limited sequencing variant profiles obtained from targeted panels are unable to resolve the genetic etiology of these myeloid tumors. The current testing methodology does not allow for the simultaneous analysis of SVs and SNVs, which might obscure the detection of compound heterozygous pathogenic variants. The current study aimed to detect/estimate the pathogenic compound heterozygous events in myeloid tumor samples. **Methods:** In this retrospective study, we evaluated the performance and clinical utility of combinatorial use of optical genome mapping (OGM) and a 523-gene next-generation sequencing (NGS) panel for comprehensive genomic profiling of 31 myeloid tumors and compared it to SOC cytogenetic methods (karyotyping and FISH) and a 54-gene NGS panel. Importantly, we used the NxClinical v6.1 software for simultaneous analysis of CNVs and small sequencing variants (SNVs and indels) from a single sample to detect pathogenic compound heterozygous events, which remain refractory to the SOC testing. **Results:** In the 31 samples, the complementary analysis identified 405 compound heterozygous events, with 13 (±4.2) events per sample. Of these 405 events, 15 events of potential clinical relevance were detected in 13 cases (12/31, 41.9%), of which five events detected in five cases (5/31, 16.1%) were loss of function (LoF) variants in genes included in the NCCN guidelines, whereas 10 events detected in 10 cases (10/31, 32.2%) were LoF variants in genes implicated in the hematological malignancies (genes not included in NCCN or NHS guidelines). **Conclusions:** This study demonstrates the clinical utility of OGM and a 523-gene NGS panel for comprehensive profiling of myeloid tumors that not only adds to the respective additional advantages to cytogenetic and molecular analysis, respectively, but the combinatorial analysis enables the detection of novel compound heterozygous events that remain intractable with the current diagnostic approach. These novel compound heterozygous LoF variants detected with combinatorial analysis might play a critical role towards patient management.

H033. ***FLT3* ITD Gene Detection by PCR/CE and NGS Method: Performance Comparison**

L. Mazur, M. Patel, M. Cuaresma, L. Ma, S. Spirtovic, P. Sok, M. Mihalov
ACL Labs, Rosemont, IL.

Introduction: The *FLT3* gene internal tandem duplication (ITD) is caused by duplication and insertion of a portion of the *FLT3* gene that includes the region in and around the juxta membrane (JM) region of the *FLT3* gene exons 14 and 15. International consensus AML guidelines incorporated *FLT3*-ITD as a risk stratification marker, and an *FLT3*-ITD/wild-type allelic ratio (AR) of 0.5 is used for high/low AR classification. Unfortunately, next-generation sequencing (NGS) short-read sequencers as well as bioinformatics algorithms are not accurate in detection of *FLT3*-ITD. This study describes the performance of two methods, PCR capillary electrophoresis (PCR/CE) and NGS, in detection and clinical reporting AR. **Methods:** Fifty-seven clinical samples were tested in parallel by the following methods; ACL LDT *FLT3*-ITD PCR/CE assay versus NGS Oncomine Myeloid panel from Thermo Fisher. 1) ACL LDT *FLT3*-ITD assay is using PCR amplification and capillary electrophoresis fragment analysis on Applied Biosystems 3500 Genetic Analyzer from Thermo Fisher. Raw data were collected and analyzed using GeneMapper 5.0 from Thermo Fisher. Limit of detection of ITD variant allelic fraction (VAF) 3% and AR=0.03. 2) Oncomine Myeloid Research Assay on Ion Chef and GenStudio S5' (Thermo Fisher). AR was determined as the VAF of the *FLT3*-ITD mutation divided by the VAF of the wild-type allele. **Results:** Accuracy for NGS method was determined as 54/57 (94.7%). The sensitivity and specificity of NGS in detecting *FLT3*-ITD was 90% and 100%, respectively. Three samples representing medium and large ITD fragments (76, 82, 278 bp) failed detection of ITD by NGS. For PCR/CE AR varied from 0.03 to 28.76, and for NGS AR 0.007 to 3.1. The AR correlation coefficient between methods (r) was 0.64 confirming significant bias present in AR values between the two methods. Samples affected the most by AR discrepancy have ITD fragment size of >60 bp. Three out of 27 cases (11%) with AR-high (>0.5) determined per PCR/CE method would have been misclassified as AR-low (<0.5) by the NGS method. The major limitations of NGS for

FLT3-ITD variant detection are: lack of sensitivity for detection of large ITD (>150 bp) and lack of AR value precision for ITD (>60 bp). **Conclusions:** The analytical correlation between both assays for detection of *FLT3*-ITD was satisfactory; however, based on ACL data NGS Myeloid panel should not be used as an independent method for AR calculation. PCR/CE method has been the gold standard for unbiased AR determination. For the best interest of the patient, ACL Lab retained PCR/CE method for detection of ITD variants and determination of AR values.

H034. **Utility of Molecular Testing as a Screening Test for Detection of Myeloproliferative Neoplasms**

D. Mignogna III, M. Al-Kawaaz, S. Al-Quran
University of Louisville, Louisville, KY.

Introduction: Molecular testing is integral for diagnosis of myeloproliferative neoplasms (MPN) in its ability to identify driver mutations. Due to cost and presence of clonal hematopoiesis of undetermined potential, it is better utilized along with clinical and/or laboratory findings that raise a suspicion of an underlying MPN. This study aims to investigate the value of molecular testing for MPN driver mutations associated with different clinical and laboratory abnormalities. **Methods:** We retrospectively identified send-out requests for *JAK2* (V617F and exons 12-14), *MPL* (W515, S505), and *CALR* (exon 9) mutations. For each, the clinical findings and comprehensive blood counts were reviewed to identify the primary indication for testing and the associated lab values. **Results:** Fifty-two patients were identified, 29 female (56%) and 23 male (44%). The average age was 50.2 years (range: 22-73). Five were positive for driver mutations (5/52, 9.6%). Two were *JAK2* V617F, two were *MPL*, and one was *CALR* mutated. A total of 61 clinical indications were identified. Most were due to thrombosis (23/61, 38%) or thrombocytosis (19/61, 31%), followed by leukocytosis (9/61, 15%) and erythrocytosis (5/61, 8%). Others (5/61, 8.1%) were also cited. Three of 23 cases (13%) with thrombosis involved the splanchnic circulation and one tested positive for a driver mutation (1/3, 33%). Three of 19 cases with thrombocytosis tested positive for driver mutations (3/19, 16%; average platelet count 800k/ml; all were persistent), including one with concurrent leukocytosis. Sixteen of 19 cases (84%) with thrombocytosis had platelet counts above the WHO cut-off of 450k/ml, including 13 (13/16, 81%) with persistent (~3 months) elevation. Two of five cases of erythrocytosis were positive for driver mutation (2/5, 40%), including one case (1/2, 50%) with splanchnic thrombosis. The second patient had a strong family history of venous thromboembolic events. One of nine cases with leukocytosis was positive for mutation (1/9, 11%) but also had concurrent thrombocytosis and thrombosis (cerebral). Of five total positive cases for driver mutations, two (2/5, 40%) also had thrombosis. As stated, one of the two (1/2, 50%) had splanchnic thrombosis; the other case (1/2, 50%) had cerebral thrombosis. **Conclusions:** These data support prior evidence that MPNs are prevalent in cases of unprovoked splanchnic thrombosis. The value of testing in the setting of unprovoked clotting outside the splanchnic circulation remains uncertain and a larger sample size may be needed. Erythrocytosis and thrombocytosis appear to show a stronger association with MPNs versus isolated leukocytosis in this limited cohort. This study emphasizes the value of utilizing WHO cut-offs to guide ordering of molecular testing for MPN driver mutations. Future direction will involve further data collection to investigate these trends.

H035. **AML-Like Gene Mutations Occur in MDS**

P. Hernandez, T. Wang, F. Du, D. Spencer, E. Duncavage
Washington University School of Medicine, St. Louis, MO.

Introduction: The distinction between acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) is based on the presence of >20% myeloblasts in the blood or marrow. Both MDS and AML share a common biology and have overlapping genomic findings; however, the distribution of mutations in AML and MDS is different. Whereas mutations in *FLT3*, *NPM1*, and *IDH1/IDH2* are among the most commonly detected in AML and present in ~50% of patients, these mutations are generally rare in MDS. Here we evaluate the incidence and clinical significance of "AML-like" mutations in MDS. **Methods:** Newly diagnosed MDS and AML patient samples collected between January 2018 and May 2022 were evaluated. All patients had targeted DNA gene sequencing (MyeloSeq) which evaluates 40 genes recurrently mutated in myeloid malignancies with a sensitivity of 2% variant allele frequency (VAF). All patients had blood or bone morphologic evaluation. **Results:** A total of 369 patients with one or more *FLT3*, *IDH1/IDH2* or *NPM1* mutations were identified. Among them, 46 patients were diagnosed with MDS. Thirty-three of the 46 MDS patients (73%) had *IDH1/IDH2* mutations, 10 (22%) had *FLT3*, and four (8.9%) had *NPM1* mutations. The median VAFs were 27%, 31%, 44%, respectively. Only 24% of these 46 patients were classified as high grade MDS (blasts >5%). Twenty-five MDS patients were followed with serial bone marrow biopsies with a median follow-up time of 9.5 months (range 2-26 months). Six of 25 (23%) progressed to AML within a median of 5.5 months (range 2-12 months). No correlation was seen between AML transformation and specific variants (P >0.05) although the sample size was small. Median IPSS-M scores of patients who transformed to AML was 1.88 versus 0.73 for patients who did not progress (P >0.05). **Conclusions:** AML-like gene mutations in MDS patients are reportedly rare. Here we identify 46 MDS patients with AML-like mutations, the most common of which were *IDH1/IDH2* mutations. Although AML-like mutations in MDS have been associated with increased risk of AML transformation by NCCN guidelines, in this cohort only 23% of MDS patients progressed to AML, suggesting a more heterogeneous clinical outcome. No correlation was seen between AML transformation and any specific AML-like gene variant.

H036. **"Rule Out MPN": Evaluation of Molecular Testing Patterns for Suspected Myeloproliferative Neoplasms**

A. Stone, P. Mroz, C. Arries
University of Minnesota, Minneapolis, MN.

Introduction: Testing for *JAK2, MPL*, and *CALR* gene mutations is indicated in the diagnostic work-up of a myeloproliferative neoplasm (MPN) and for monitoring disease status. This may have inadvertently led to the increase in requests for testing to "rule out MPN" including clinical situations with low pretest clinical suspicion representing a potentially significant use of laboratory resources. Here we evaluated the ordering patterns of *JAK2, MPL*, and *CALR* gene testing with the goal of formulating practical guidelines that could improve our test utilization and increase the rate of detection of MPN. **Methods:** Next-generation sequencing (NGS) results for MPN testing between 2015 and March 2022 were retrieved from EHR. This search identified 2,289 individual tests, from which the test ID and patient record numbers were used to retrieve NGS result reports and additional clinical data from the electronic medical record (Epic), including bone marrow biopsies and comprehensive blood counts (CBC) performed within 180 days of NGS testing. Patients who had opted out of research were excluded. **Results:** Results for 1,981 eligible patients were included in the final analysis; 251 (12.6%) were "positive" (defined as a pathogenic/likely pathogenic variant in *JAK2, CALR*, or *MPL*); 80% of tests were performed on peripheral blood specimens, showing a much lower positivity rate (13.4%) than bone marrow samples (28.9%). When analyzed by patient age, the positivity rate ranged from 5.6%-8.9% for patients <60 years old, rising to 12.9%-15.3% for those 60-79 years old, and further increasing to 30.0%-33.3% for those 80 and above. Positivity rates also notably varied by ordering provider specialty, ranging from 4.4% for testing ordered by internal medicine/primary care to 18.8% for hematology-oncology to 38.0% for pathologists. **Conclusions:** We hypothesize that these notable differences in positivity rates/test utilization reflect the variable clinical contexts in which testing is being ordered. For example, 94% of testing ordered by internal medicine is on peripheral blood, compared to 4% of tests ordered by pathologists. This striking disparity suggests pathologists are ordering testing after being able to review the

morphologic results of a bone marrow biopsy, and thus likely have a higher pretest suspicion. Our next step will therefore include statistical analysis of NGS results compared to CBC results and other clinical data to help identify potential guidelines that could improve the positivity rate of our NGS testing for MPNs in other clinical settings. Finally, we will use the results of this study to create ordering guidelines and an education program for clinicians to improve utilization of NGS testing for suspected MPNs.

H037. **Cohesin Complex Mutations in Myeloid Neoplasms Reveal Frequent Co-Mutations in Spliceosome Genes, *NRAS*, and *RUNX1* with a Normal Karyotype**

C. Hirt, P. Michaels
Beth Israel Deaconess Medical Center, Boston, MA.

Introduction: The genetic landscape of cohesin complex (CC) mutations has only recently been described in myeloid neoplasms (MNs) with varying reports of co-mutations and potential for leukemogenesis. As CC mutations have been identified in a variety of MNs, with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) being the most common, the reporting of CC mutations in other MNs has also been lacking. As such, we present the initial results of a systematic analysis of CC mutations in the context of co-mutations, karyotype, and morphologic diagnosis. **Methods:** Patients with a targeted next-generation sequencing (NGS) panel of myeloid associated genes submitted from 2017 to 2021 were reviewed for the presence of mutations in *RAD21*, *SMC1A*, *SMC3*, and *STAG2*. For patients with NGS demonstrating CC mutations, concomitant electronic pathology records were reviewed for final diagnostic interpretation and cytogenetic results. Statistical analysis for Fisher's exact test was performed using GraphPad QuickCalcs. **Results:** From 2017 to 2021, 965 unique patients had a targeted NGS panel of myeloid associated genes submitted. Forty patients were identified as having CC mutations (40/965; 4.15%); 35.0% of CC mutations occurred in *de novo* AML, 35.0% in MDS, 10.0% in MDS/MPN, 7.5% in AML-MRC, 5.0% in MPN, 5.0% of unclassifiable MN, and 2.5% of t-MN. Meanwhile, 55.0% of patients with CC mutations had a *STAG2* mutation, 22.5% with *SMC1A*, 17.5% with *SMC3*, and 7.5% with *RAD21*. A total of 12.5% of patients had a co-occurring mutation within the CC. Spliceosome mutations were the most frequent co-mutations (47.5%; p=0.0001) with *SRSF2* being prevalent (35.0%; p=0.0001). *RUNX1* (32.5%; p=0.0001) and *NRAS* (25.0%; p=0.0001) were also significantly associated with CC mutations. Lastly, 17.5% of patients' CC mutations had a complex karyotype, 10.0% with a monosomal karyotype, and 10.0% with both a complex and monosomal karyotype. **Conclusions:** CC mutations are an infrequent event in MN, but our data indicate that they run the spectrum of MN with enrichment in *de novo* AML and MDS. Next, upon analysis for co-mutations, spliceosome genes, particularly *SRSF2*, were significantly associated with CC mutations, as were *RUNX1* and *NRAS*. Additionally, as it has been hypothesized that *STAG2* mutations lead to chromosomal instability, this was not observed in our cohort, as the relative frequency of a complex or monosomal karyotype was low. Lastly, our cohort demonstrated that 12.5% of patients had co-occurring CC mutations. Overall, we present the initial results of our analysis identifying unique relationships of CC mutations with spliceosome genes, *RUNX1*, and *NRAS* while frequently displaying a normal karyotype.

H038. **Unraveling the Somatic Mutational Landscape in Myelodysplastic Syndrome: A Single Center Experience**

I. Dey, S. Vinarkar, M. Parihar, K. Patel, R. Demde, K. Saha, S. Banerjee, D. Nathi, A. Nag, j. Kumar, R. Nair, M. Chandy, D. Mishra
Tata Medical Center, Kolkata, West Bengal, India.

Introduction: Myelodysplastic syndromes (MDS) are clonal haematological disorders characterized by cytopenias, haematopoietic cell dysplasia, and a predisposition to transform into leukaemia. There is tremendous interest in the complex genetics of MDS which is evident by the emerging diagnostic, prognostic, therapeutic genetic biomarkers, and as highlighted by the recent integration of mutation profile into the prognostic scoring of MDS (IPSS-M). High-throughput next-generation sequencing (NGS) has been instrumental in unraveling this complex genetic landscape of MDS. **Methods:** The Routine workflow in assessment of MDS at our center involved peripheral blood smear, bone marrow examination, cytogenetic study, and comprehensive myeloid panel (NGS). We studied the mutational profile of 42 cases of *de novo* MDS diagnosed during the period of March 2019 to May 2022. DNA/RNA was extracted from bone marrow samples, followed by library preparation using the Oncomine Myeloid Research Assay. The DNA and RNA sequencing was performed on the Ion Torrent PGM platform. Sequencing reads were aligned using Torrent Suite 3.4.2, followed by annotation of .vcf files with Ion Reporter (v 5.6). **Results:** Over the period of three years, 42 cases were diagnosed as MDS based on the clinical, morphological, and cytogenetic findings. Out of these 42 cases of MDS, 57% of patients were ≥60 years of age, M:F ratio was 1.7:1 and 78% had good cytogenetic risk (IPSS-R). Additionally, 60% of the cases had at least one pathogenic variant (n=25/42); 40%(n=10/25) of mutated cases had a single gene variant, whereas the rest had multiple (two to four) mutations. MDS with multilineage dysplasia (41%, n=17/42) was the most common morphological classification and most frequent mutated subgroup, followed by MDSEB (33%, n=14/42)**.** According to functional genetic categories, the majority of the mutated genes belonged to regulators of RNA splicing (48%, n=12/25) followed by DNA methylation genes (44%, n=11/25) and transcription regulating genes (36%, n=9/25). The most frequently mutated gene was *TET2* (40%, n=10/25), followed by *STAG2* (20%, n=5/25). The most commonly co-mutated genes were *TET2* and *SRSF2* (27% n=4/15). Patients were treated as per WHO classification and IPSS-R risk stratification with either hypomethylating agents alone or in combination (with Venetoclax), Lenalidomide and supportive care. Three patients transformed to acute myeloid leukemia, out of which one had a *TP53* gene variant. Five patients (15%) succumbed to disease/therapy-related complications, out of which two harbored mutations in the *TP53* gene. **Conclusions:** We studied the genetic profile of 42 MDS cases using NGS. The mutation frequency of MDS at our center was 60%, which was in concordance with published literature. *TET2* and *STAG2* genes were the most frequently mutated genes and *TP53* gene mutated patients showed progression and poor outcomes

H039. **An Assessment of Somatic Variant Interpretations and Classifications across Multiple Platforms**

E. Miller, S. Sperber, C. Lamberson, R. Akabari, M. Nasr, E. Ruckdeschel, S. Smith
SUNY Upstate Medical University, Syracuse, NY.

Introduction: Identification of genetic alterations and proper classification of these variations, including a comprehensive therapeutic program, is the aim of personalized patient care. Improved diagnostics have increased over the past decade as a result of enhanced next-generation sequencing (NGS) technologies intersecting with robust bioinformatic platforms and expanding knowledgebases. Though the combination of these advancements has been a boon for the clinical community, novel insights and increased knowledge have led to conundrums regarding properly classifying and interpreting observed variations. Despite standardization efforts, classifications can vary, causing discordance and potentially affecting patient care. This study demonstrates the variability observed when classification is performed by different institutions and third-party platforms. **Methods:** Genomic DNA was extracted from peripheral blood or bone marrow and tested using a 73-gene myeloid NGS panel LDT at SUNY Upstate Molecular Diagnostics Laboratory. All patients tested were diagnosed with acute myeloid leukemia, myelodysplastic syndrome, chronic myeloproliferative disorder, chronic myelomonocytic leukemia, or myeloproliferative neoplasms. Three third-party bioinformatic analysis platforms, in addition to the clinical laboratory, were employed to classify somatic variants identified from NGS testing and subsequent 1°, 2°, and 3° analyses. These

classifications were performed under the guidance of the AMP/ASCO/CAP 2017 guidelines. Variant classifications were then compared and analyzed to determine concordance and observe any patterns in discordant classifications. **Results:** An analysis and classification of >200 variants from >50 patients demonstrated a concordance of 46% between two different variant classification services and SUNY Upstate Molecular Laboratory. Whereas the laboratory was in agreement with both variant classification services in >60% of variants analyzed, both services exhibited 55% agreement when compared with each other. Furthermore, discrepant results were often observed from a third variant analysis service which uses a modified version of the 2017 AMP/ASCO/CAP guidelines. **Conclusions:** This analysis and comparison demonstrates the high degree of variability when a standardized process is not used for molecular profile assessment and analysis. Furthermore, the data presented here reveal the need for further standardization in this process. The analysis and classification of somatic variants is not a straightforward task, and many variants can be difficult to classify. This is especially true considering that those analyzing the data are not only trained differently, but also have developed their own methods to classify variants. The data and analysis presented here encourage continued discussion on the standardization of variant analysis and harmonization among the molecular pathology community.

H040. **Next-Generation Sequencing Identifies Frequent *FLT3* N676K Mutations in Myeloid Neoplasms**

M. Magallanes-Hoyos[1], C. Mani[2], W. Xie[1], J. Dunlap[1], H. Wang[3], D. Peker[4], K. Bessonen[5], K. Karner[6], D. Viswanatha[5], T. Kovacsovics[7], P. Shami[7], X. Xu[5], R. He[5], P. Li[6]

[1]Oregon Health and Science University, Portland, OR; [2]ARUP Laboratories, Salt Lake City, UT; [3]University of California San Diego Health System, La Jolla, CA; [4]Emory University, Atlanta, GA; [5]Mayo Clinic, Rochester, MN; [6]University of Utah Health/ARUP Laboratories, Salt Lake City, UT; [7]University of Utah Health/Huntsman Cancer Institute, Salt Lake City, UT.

Introduction: *FLT3*-mutated acute myeloid leukemia (AML) accounts for approximately 30% of all newly diagnosed AML and generally confers a poor prognosis. Next-generation sequencing (NGS) identified more non-canonical *FLT3* tyrosine kinase domain (TKD) mutations, but the prevalence, prognostic significance, and response to FLT3 inhibitors of these mutations in myeloid neoplasms (MN) including AML remain uncertain. **Methods:** Using NGS, we retrospectively identified 123 MN patients with *FLT3* N676K variants (100 AML and 13 other MNs) from five academic institutions, along with a control cohort of 132 MN with D835 and 168 AML patients with *FLT3*-internal tandem duplication (ITD) variants. Clinicopathologic and demographic features, and genomic profiles were analyzed. Fisher's exact and Mann-Whitney tests were performed on categorical and continuous variables, respectively, and overall survival (OS) was assessed using the Kaplan-Meier method. Multivariate Cox proportional hazard analysis (MVA) was performed to determine the impact of variables on OS for all AML patients and those treated with induction therapy. **Results:** The prevalence of the most common non-canonical TKD variant, N676K, was estimated at 3% of MN. The median age of N676K mutant patients was similar to those carrying either *FLT3*-ITD and the typical D835 variants. Like *FLT3*-ITD and D835 variants, N676K mutations were commonly observed in *de novo NPM1* mutant AML and fewer in AML with recurrent cytogenetic abnormalities. Altogether, 36% of N676K, 21% of D835, and 18% of all *FLT3*-ITD variants (P >0.05) were identified in patients with secondary AML. The variant metrics and genetic profiles were similar in MNs with *FLT3* N676K, D835, and ITD variants, demonstrated by the distribution of variant allele frequency, average mutation burden per case, and the number and diversity of concurrent mutations. The MVA of the OS identified bone marrow transplant predicts more favorable outcomes in AML patients with N676K and D835 mutations treated with induction. **Conclusions:** This is the first study to comprehensively evaluate *FLT3* N676K variants in MN patients. Similar to the canonical TKD variants, N676K mutations were frequent in patients with *de novo* and secondary AML. Compared to ITD, N676K and D835 mutant AML conferred significantly better OS after bone marrow transplant. In the context in which the D835 mutation resulted in resistance to most type II FLT3 inhibitors, thus leading to poor disease-free survival, our findings of the similarity in non-canonical TKD variants underscores the importance of more comprehensive detection of TKD mutations to include the new variants. The proposed new approach might benefit AML patients for targeted therapy.

H041. **Mutational Landscape of Mixed Phenotype Acute Leukemia Reveals Targetable Mutations**

M. Hennawi, M. Hussaini, F. Quadeer, S. Kapadia, S. Osman, H. Tashkandi

Moffitt Cancer Center, Tampa, FL.

Introduction: Mixed phenotype leukemia (MPAL) is a type of acute leukemia, in which the blast population shows mixed features of myeloid, T-lymphoid, and/or B-lymphoid differentiation. MPALs are rare and carry a poor prognosis, thus often posing both a diagnostic and therapeutic challenge. Conventionally, the diagnosis of MPAL requires either a single blast population with lineage-defining phenotypic expression of multiple lineages (myeloid, B-cell, and/or T-cell) (biphenotypic) or two distinct blast populations that each independently satisfy criteria for designation as acute myeloid leukemia, B-cell acute lymphoblastic leukemia, and/or T-cell acute lymphoblastic leukemia (bilineage). Given the rarity of MPAL, minimal studies have been performed to describe the genomic landscape of these neoplasms. **Methods:** Central MCC database was searched for any patient with a diagnosis of acute undifferentiated leukemia, acute leukemia of ambiguous lineage, and MPAL. All patient diagnoses were manually reviewed by a hematopathologist to confirm the diagnosis. Genomic and molecular data were collated from the EMR and bioinformatically from MCC genomics repositories. IRB approval was obtained. **Results:** Twenty-nine patients with MPAL were identified. Fourteen were female and 15 were male. Average age was 54 years old (range=28-81). Ten cases were biclonal and 19 were biphenotypic. Diagnoses were as follows: B/myeloid (n= 18), T/myeloid (n=10), and T/B (n=1). Overall survival was 18.8 months (range=0-70 m). Ten patients were treated with an allogeneic stem cell transplant. Mutational analysis was available for 18 patients wherein mutations were detected in 45 unique genes. The most frequently mutated genes were *TP53* (7), *RUNX1* (6), *WT1* (4), *MLL2* (3), *FLT3* (2), *CBL* (2), *ASXL1* (2), *TET2* (2), *MAP3K6* (2), *MLL* (2), and *MAP3K1* (2). Of the two patients with *FLT3*-internal tandem duplication (ITD) mutations, one was treated with adjuvant midastaurin initially and adjuvant gilteritinib at relapse with response. The other patient was diagnosed prior to release of *FLT3*-targeted therapy. One patient with targetable *IDH2* R140Q was detected but he expired in 14 days. We also note recurrent involvement (n=3) of MAPKKK family of serine/threonine-specific kinases involved in proliferation and differentiation, which warrants additional investigation as a target. Lastly, KMT2 (MLL) family was also noted to be involved (n=3), raising the possibility of targeting with anti-KMT2A KO-539, which is in trial. **Conclusions:** In one the largest series of MPAL cases to date, we corroborate previous findings with enriched detection of *RUNX1* and *FLT3*-ITD mutations along with discovery of unreported mutations (*MAP3K*) that may be amenable to therapeutic manipulation. Our findings support the need to genomically profile MPAL cases to exploit opportunities for targeted therapies in this orphan disease with dismal prognosis.

H042. **ClinGen *FLT3* Somatic Cancer Variant Curation Expert Panel: Refining Oncogenicity Classification of Clinically Relevant *FLT3* Aberrations**

H. Williams[1], H. Williams[2], J. Saliba[3], N. Kopp[4], C. Mani[5], Y. Li[6], W. Zhang[7], J. Zhao[8], K. Krysiak[9], A. Danos[3], X. Shao[10], R. He[11], C. Kesserwan[12], H. Kantarjian[13], T. Haferlach[14], P. Blombery[15], M. Ouseph[16], E. Thompson[15], Y. Zhong[17], X. Zhao[18], Y. Jaufeerally-Fakim[19], S. Pullarkat[20], O. Griffith[3], M. Griffith[3], R. Kanagal-Shamana[21], X. Xu on behalf of the ClinGen FLT3 Somatic Cancer Variant Curation Expert Panel[11]

[1]Tempus Labs, Inc., Cleveland Heights, OH; [2]Tempus Labs, Inc, Chicago, IL; [3]Department of Medicine, Washington University School of Medicine, St. Louis, MO; [4]David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA; [5]ARUP Laboratories, University of Utah, Salt Lake City, UT; [6]Hayward Genetics Center, Department of Pediatrics, Tulane University School of Medicine, New Orleans, LA; [7]Cincinnati Children's Hospital Medical Center, Division of Human Genetics, Department of Pediatrics, University of Cincinnati, Cincinnati, OH; [8]Invitae Corp, San Francisco, CA; [9]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO; [10]Department of Pediatrics, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI; [11]Division of Hematopathology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN; [12]National Cancer Institute, Bethesda, MD; [13]Department of Leukemia, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX; [14]Munich Leukemia Laboratory (MLL), Munich, Germany; [15]Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia; [16]Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY; [17]Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia; Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [18]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX; [19]University of Mauritius, Réduit, Moka, Mauritius; [20]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, CA; [21]Department of Hematopathology, Division of Pathology/Lab Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX.

Introduction: The recently formed (2021) FMS-like tyrosine kinase 3 (*FLT3*) Somatic Cancer Variant Curation Expert Panel (SC-VCEP) within the ClinGen Somatic Cancer Clinical Domain Working Group (CDWG) is composed of 56 multidisciplinary experts including oncologists, hematopathologists, molecular pathologists, clinical lab directors, and biocurators with expertise in hematological malignancies. The SC-VCEP aims to drive consensus of *FLT3* variant interpretation aligned to the AMP/ASCO/CAP guidelines and the most recent ClinGen/CGC/VICC oncogenicity classification standards. **Methods:** Given inconsistency in the curation, interpretation, and reporting, specifically of rarer tyrosine kinase domain *FLT3* sequence variants within the clinical diagnostic setting, this SC-VCEP utilized clinical laboratory practice standards for the evaluation of clinically relevant variants. Using the Clinical Interpretations of Variants in Cancer (CIViC; civicdb.org) platform, predictive, prognostic, and diagnostic evidence was collected to support variant interpretations that are finalized as assertions following the AMP/ASCO/CAP guidelines. All assertions underwent expert panel approval prior to final submissions; consensus classifications will be made publicly available through ClinVar. In addition, the SC-VCEP is establishing specifications to apply the recent ClinGen/CGC/VICC oncogenicity standards for *FLT3* variants to assess the oncogenicity classification. These specifications include defining the consensus structure and functional domain of *FLT3*, establishing *FLT3*-specific criteria for variant hotspots, and rules to apply functional evidence. **Results:** The *FLT3* SC-VCEP has published the assertions of the *FLT3* internal tandem duplication (ITD) and D835 variants as Tier 1 - level A. Pilot assessment to apply ClinGen/CGC/VICC oncogenicity standards has classified the following variants as oncogenic: c.2505T >A, p.Asp835Glu (rules applied: OS2, OS3, OP1, OP4); c.2503G >T, p.Asp835Tyr (OS2, OS3, OP1, OP4); c.2503G >C, p.Asp835His (OS2, OS3, OP1, OP4); c.2504A >T, p.Asp835Val (OS2, OS3, OP1, OP4). One variant as likely oncogenic: c.2028C >A, p.Asn676Lys (OS2, OM4, OP4), and a variant of uncertain significance: c.2503_2504delinsAT, p.Asp835Ile (OM1, OP1, OP3, OP4). **Conclusions:** Routine screening for *FLT3* variants is part of diagnostic work-up in acute myeloid leukemia patients as recommended by several international guidelines given the critical diagnostic, prognostic, and therapeutic implications. Therefore, standardized classification of *FLT3* variants is necessary. The *FLT3* SC-VCEP will continue to evaluate and refine *FLT3*-specific rules to classify *FLT3* variants using the AMP/ASCO/CAP guidelines and the ClinGen/CGC/VICC oncogenicity classification standards as a framework.

H043. ***FLT3* Mutations in Myeloid Malignancies: A Single Institution Retrospective Review**

S. Miller[1], V. Wang[2], M. Chen[2], W. Chen[2], F. Fuda[2], M. Juntilla[2], O. Weinburg[2], D. Oliver[2], J. Xu[2]

[1]UT Southwestern Medical Center, Sunnyvale, TX; [2]UT Southwestern Medical Center, Dallas, TX.

Introduction: FMS-related tyrosine kinase 3 (*FLT3*) is one of the most frequently mutated genes in acute myeloid leukemia (AML). Two types of *FLT3* mutation types have been extensively studied: the internal tandem duplications (ITD) in the juxtamembrane domain, and point mutations in the tyrosine kinase domain (TKD). *FLT3* mutations are considered high-risk mutations in AML that impart shorter survival times and increased risk of relapse. Here, we present a review of our institution's positive *FLT3* molecular tests with the patients' associated clinical, laboratory, and other genetic findings. **Methods:** We reviewed all the *FLT3* mutational assays performed in our institution since 2019. Patients with positive results were chart reviewed and pertinent data such as age, gender, survival, associated mutations and cytogenetics, white blood cell (WBC) count, blast percentage, or other relevant history were gathered. Positive patients were further separated into three groups: ITD only mutant, TKD only mutant, and ITD and TKD double mutants. *FLT3* analysis was done using a lab-developed PCR-based fragment analysis assay. **Results:** A total of 612 assays on 308 patients have been performed since 2019. Of these, 35 patients were positive for ITD mutations (11.3%), nine for TKD mutations (2.9%), and four patients (1.3%) carried both ITD and TKD mutations. These patients had myeloid malignancies including AML and myelodysplastic syndrome/myeloproliferative neoplasm. ITD mutation size ranged from 15 bp to 237 bp (median: 57 bp) and mutant to wild-type allelic ratio ranges from 0.02 to 12.3. *FLT3* mutations are associated with high WBC counts (median: 39.6x 10^9/L), and no significant difference was seen among the ITD only, TKD only, and double positive groups. Interestingly, patients carrying both ITD and TKD mutations tended to be older than the patients with only ITD mutations or TKD mutations (average age: 78 vs. 67 vs. 68), although not statistically significant. Patients with only TKD mutations frequently had AML with monocytic differentiation (4/9, 44%). Per EMR, patients with *FLT3* mutations occasionally harbored additional mutations in *NPM1, IDH, DNMT3A*, and *TET2*. Out of the total 48 *FLT3* positive patients, six (12.5%) tested negative for *FLT3* mutations on initial work-up but developed mutations during follow-up, suggesting clonal evolution of the leukemia cells. **Conclusions:** Our study generally agrees with and expands the literature on *FLT3* mutations and their associations in the context of myeloid malignancies. Six patients with wild-type *FLT3* at the time of initial diagnosis gained mutations later, indicating that it is important to monitor the *FLT3* mutation status in patients with persistent/recurrent AML even if the initial *FLT3* assay is negative.

H044. **Features of *RAD21* Mutated AML**

D. Laczko, G. Yang, J. Morrissette, A. Bagg
Hospital of the University of Pennsylvania, Philadelphia, PA.

Introduction: Mutations in genes encoding proteins in critical pathways play a key role in the initiation and propagation of acute myeloid leukemia (AML). Cohesin complex is a ring-shaped protein structure that is involved in DNA repair and chromosome segregation, with loss-of-function mutations associated with diseases including myeloid malignancies. *STAG2* is the most commonly mutated and investigated cohesin member in AML; however, the exact role of other members such as *RAD21* in myeloid transformation remains controversial. In this study, we aimed to evaluate the morphologic and immunophenotypic features and coexistent mutations of our cohort of patients with *RAD21* mutated AML. **Methods:** A total of 1,152 unique patient samples diagnosed with AML were analyzed for *RAD21* variants by next-generation sequencing (NGS) using the Penn hematologic malignancy panel. Only cases with disease associated variants (DAVs) in *RAD21* were included. We performed a detailed evaluation of morphological and immunophenotypic features for the positive patients. **Results:** Review of NGS data identified 11 unique AML patients with *RAD21* DAVs categorized as *de novo* AML (8/11) and AML with myelodysplasia related changes (3/11). The majority of these changes were frameshift indels (72%), and the remaining cases were nonsense mutations (28%). Seventy-two percent of *RAD21* DAVs co-occurred with mutations in epigenetic regulators, such as *DNMT3A* (4/11), *BCOR* (2/11), *TET2* (2/11), and *ASXL1* (1/11). *RAD21* DAVs never co-occurred with other cohesin mutations. Four out of 11 cases showed normal karyotype and 54% of cases had morphologic evidence of monocytic differentiation. By flow cytometry blasts showed a typical myeloid immunophenotype with expression of CD13, HLA-DR, CD33, CD117. Expression of CD34 was observed in eight (72%) patients. Aberrant CD7 expression was seen in 72% of cases. **Conclusions:** Our study provides a detailed evaluation of *RAD21* mutated AML for the first time, showing that it is frequently associated with monocytic differentiation and aberrant CD7 expression. About one-third of cases showed normal karyotypes, suggesting that
RAD21 mutations may not induce chromosomal instability. Our cohort also demonstrated a strong association with mutations in epigenetic regulators, suggesting that *RAD21* may exert a synergistic effect in epigenetic dysregulation, raising the possibility that patients with *RAD21* mutated AML could potentially benefit from epigenetic modifying drugs that could be explored through multicentric prospective studies.

H045. **Examination of Mutant *NPM1*: RNA versus DNA versus Morphology**

P. Ollila, R. He, R. Wang, M. Mai, B. Bailey, M. Ollila, B. Gutierrez, D. Viswanatha
Mayo Clinic, Rochester, MN.

Introduction: *NPM1* mutations occur in approximately 30% of acute myeloid leukemia (AML) and indicate a more favorable prognosis in the absence of an *FLT3* internal tandem duplication (ITD). They were shown to be a reliable and sensitive marker for measurable residual disease (MRD) monitoring and described as an overexpressor on the mRNA level. However, the degree and variability of overexpression has not been well documented. This study aimed to evaluate and further characterize mutant *NPM1* transcript overexpression in AML. **Methods:** DNA and RNA were extracted from bone marrow using the Qiagen EZ1 DNA extraction kit (Germantown, US) and Promega SimplyRNA kit (Madison, US), respectively. For the reverse transcription real-time PCR (RT-PCR), RNA was reverse transcribed into complementary DNA (Thermo Fisher, Waltham, US) followed by quantitative PCR on Roche LightCycler 480 (Indianapolis, US) using reverse primers specific for mutation types A/B/D. For the fragment analysis (FA), PCR was performed on DNA using primers flanking exon 11 with subsequent capillary electrophoresis on an ABI 3700 (Waltham, US). Next-generation sequencing (NGS) was performed on DNA using a targeted, 42-gene panel with capture-based chemistry on an Illumina Hiseq sequencer (San Diego, US). Blast percent (morph) was collected from available pathology reports. Results from RT-PCR ($_{mut}$*NPM1/ABL1*, n=30), FA (n=30) and NGS (n=27) mut/wild-type (wt) *NPM1*, and morph (n=20) were compared. **Results:** Among 30 RT-PCR-positive cases (25 type A, four type D, one type B), $_{mut}$*NPM1/ABL1* showed a mean of 508% (median 423%, range 17%-1,727%), whereas the DNA-based mut/wt *NPM1* by FA and NGS revealed a mean of 64% (54%, 1%-149%) and 52.7% (53.8%, 0.7%-88.7%), respectively. Morph had a mean of 58% (61%, 3%-92%). Comparison of RT-PCR/FA and RT-PCR/NGS, as well as RT-PCR/morph exhibited a mean ratio of 12.4 (7.2, 1.8-85.0), 16.1 (8.5, 2.2-127.5), and 13.5 (7.2, 1-114), respectively. Results of FA and NGS were highly concordant with a mean ratio of 1.3 (1.2, 0.4-1.2). The FA/morph and NGS/morph showed a mean of 2.1 (0.9, 0.06-17.4) and 0.8 (0.9, 0.04-2.5), respectively. The larger ranges seen in FA/morph and NGS/morph were likely attributable to sampling bias between the molecular and morph samples seen in few cases and/or clonal heterogeneity of the blast population. **Conclusions:** Mutant *NPM1* is overexpressed on the mRNA level with varying degrees in different individuals (up to more than 100-fold) in comparison to its DNA mutation level and cell number-based blast percentage. RNA-based RT-PCR, therefore, offers a robust platform for MRD monitoring in *NPM1*-mutated AML. The quantitative results from the RT-PCR are generally higher than those observed from a DNA-based assay and blast percentage by morphology, and confusion among the three results should be avoided clinically.

H046. **Evaluation of Incomplete DJ-Rearrangements by Next-Generation Sequencing of Immunoglobulin Genes: The Canary in the Coal Mine**

K. Miller, P. Mbonu, L. Haley, K. He, C. Gocke, M. Borowitz, M. Lin, R. Xian
Johns Hopkins University School of Medicine, Baltimore, MD.

Introduction: PCR analysis of *IGH* is a standard molecular test used in the clinical work-up of suspicious lymphoproliferations. Targeting multiple complete VDJ- and incomplete DJ-rearrangements improves diagnostic sensitivity. However, the interpretation of isolated DJ-rearrangements is challenging, as its clinical significance is not always clear when considering a mature lymphoid neoplasm. In this study, we sought to characterize the clinical significance of lymphoproliferations with uncommon DJ-rearrangements by immunoglobulin next-generation sequencing (NGS). **Methods:** All formalin-fixed, paraffin-embedded tissues that underwent clinical *IGH* PCR analysis (FR1-, FR2-, FR3-VDJ; DJ) at Johns Hopkins Hospital from 2016-2019 were reviewed to identify cases with isolated DJ-rearrangements (iDJ; DJ without any complete VDJ-rearrangements) and extraneous DJ-rearrangements (eDJ; DJ in addition to ≥2 VDJ rearrangements). Samples with sufficient DNA underwent *IGH* and *IGK* NGS by LymphoTrack. Clonality by NGS was compared to PCR and reviewed with pathologic and clinical findings to determine final diagnoses. **Results:** From 300 cases showing clonal, indeterminate, or oligoclonal patterns, 39 iDJ (13%) and 13 eDJ (4%) cases were identified. Eighteen iDJ and five eDJ cases underwent NGS, including 12 B-cell lymphomas (BCLs), one monoclonal gammopathy of uncertain significance (MGUS), five atypical B-cell proliferations (atypical), three T-cell lymphomas (TCLs), and two reactive B-cell processes (reactive). Half (9/18) of the iDJ cases showed clonal or indeterminate clonal sequences by *IGH* and/or *IGK* NGS consistent with the diagnosis in eight cases (6 BCL/MGUS, one atypical, one TCL with EBV+ B-cells). A positive NGS clone upgraded a single atypical case to BCL. The remaining iDJ cases were negative by NGS with no change in diagnoses (4 BCL, one atypical, two TCLs, and two reactive). In total, 72% (13/18) of iDJ cases represented BCL/MGUS, all of which were germinal center (GC) and post-GC cell of origin, and 28% (5/18) represented other lymphoproliferations. NGS of the five eDJ cases showed either biclonal or oligoclonal patterns in both *bona fide* lymphoma and atypical lymphoproliferations. **Conclusions:** Isolated

DJ-rearrangement occurs in approximately 13% of *IGH*-PCR clonal cases submitted for clinical evaluation of suspicious lymphoproliferations. In conjunction with NGS, iDJ cases represent BCL/MGUS in 72% of cases, suggesting that up to 9% of lymphomas may be missed in routine work-up if *IGH*-VDJ rearrangements are assessed alone without targeting *IGH*-DJ or *IGK*. Extraneous DJ rearrangements always herald the presence of biclonal or oligoclonal proliferations, even in BCL, indicating NGS improves comprehensive clone identification for diagnosis and disease monitoring.

H047. **Plasma Circulating Tumor DNA Mirrors Tumor Tissue in Identification of Somatic Mutations in Diffuse Large B-Cell Lymphoma Using Onco-LymScan Panel**

C. Wang, X. Wang, J. Zhu, Q. Yang, D. Zhao, T. Ma
Department of Translational Medicine, Genetron Health (Beijing) Technology, Co. Ltd., Beijing, China; Beijing, People's Republic of China.

Introduction: Diffuse large B-cell lymphoma (DLBCL) is the most prevalent subtype of non-Hodgkin lymphoma, with a high clinical and biological heterogeneity. Circulating tumor DNA (ctDNA) as a biomarker of liquid biopsy has become a promising noninvasive and feasible tool in many solid tumors. However, there are relatively few clinical uses of ctDNA in patients with DLBCL who have solid mass similar to solid tumors. In this study, we assessed the potential of ctDNA to serve as an alternative biomarker to tumor tissue biopsy for molecular profiling in DLBCL patients. **Methods:** A total of 52 peripheral blood and 43 tumor tissue samples were collected from 73 newly diagnosed DLBCL patients before treatment. Among them, 22 patients were available with matched blood and tumor samples. DNA libraries were prepared with the genomic DNA (gDNA) extracted from tumors/blood lymphocytes and cfDNA extracted from plasma. Libraries were captured by a custom target sequencing panel (Onco-LymScan) including 188 lymphoma-related genes (Genetron Health, Beijing) and sequenced on the NovaSeq6000 platform (Illumina, San Diego, CA) with a mean coverage depth of at least 100× for lymphocytes gDNA controls, 1,000× for tissue gDNAs and 3,000× for cfDNAs after removing duplicates. **Results:** For tissue biopsy samples, our panel identified nonsynonymous somatic mutations in all the patients (100%, 43/43) with a median of 13 mutations per sample (range, one to 41). The most frequently mutated genes were *DTX1* (37%), *CD79B* (35%), *BTG1* (30%), *BTG2* (30%), *TMSB4X* (30%), and *PIM1* (26%). Among the 52 patients with cfDNA samples, 51 (98%) were detected with at least one mutation with a median of 15 mutations per sample (range, 0 to 63). According to the cfDNA mutation profile, the most frequently mutated genes in the cfDNA were *CD79B* (42%), *PIM1* (42%), *PCLO* (40%), *BTG1* (33%), *KMT2D* (33%), and *HIST1H1E* (31%). Mutations identified in 22 patients with matched cfDNA and tumor samples were compared. The concordance of each individual was analyzed using the mutations identified in the tumor tissues as a reference and the median concordance was 79.1%. The overall sensitivity of cfDNA to detect the mutations present in paired tumor samples was 78.2% (223 of 285). In addition, ctDNA allowed for the identification of additional 173 somatic mutations that were undetectable in tumor gDNA, which demonstrated that ctDNA might overcome tumor spatial heterogeneity. **Conclusions:** Onco-LymScan panel showed a robust performance to detect somatic mutations in tumor and plasma samples for DLBCL patients, and plasma ctDNA was an alternative to tumor tissues for noninvasive mutational profiling by NGS.

H048. **Clinicopathologic and Clinical Genomic Profiling of Systemic EBV-Associated T-Cell Lymphoproliferative Disorders of Childhood**

C. Yasa Benkli, N. El-Mallawany, A. Marcogliese, T. Elghetany, J. Punia, A. Gaikwad, K. McClain, T. John, C. Allen, K. Fisher, C. Curry
Baylor College of Medicine and Texas Children's Hospital, Houston, TX.

Introduction: Systemic Epstein-Barr virus (EBV)-associated T-cell lymphoproliferative disorders of childhood (S-EBV-T-LPD) comprise three major forms: EBV-positive hemophagocytic lymphohistiocytosis (EBV-HLH), systemic EBV-positive T-cell lymphoma of childhood (S-EBV-TCL), and systemic T-cell chronic active EBV infection (S-T-CAEBV). These disorders are rare in childhood in Western countries and are associated with poor outcomes. We report the clinicopathologic findings and clinical genomic profiling (CGP) of a cohort of 17 children and young adults diagnosed at our tertiary pediatric academic institution expanding on a previously published cohort (n=8). **Methods:** Seventeen cases of S-EBV-T-LPD were identified from 1990 to 2022. Clinicopathologic and relevant laboratory data were collected and next-generation sequencing using a capture-based 152-gene custom-designed hematologic malignancy panel was performed on five cases including one case of paired diagnostic and disease progression samples. **Results:** Patients (6F, 11M) ranged in age from 1-22 years (median: four yrs). All patients presented with fever, hepatosplenomegaly, cytopenias, abnormal EBV serologies, and markedly elevated peripheral blood EBV-DNA load. Histologic features ranged from EBV+ subtle T-cell infiltrates with mild atypia to clusters or sheets of lymphoma cells with marked cytologic atypia (overt lymphoma). All showed aberrant T-cell populations by immunohistochemistry (n=4) or marrow flow cytometry (n=13, abnormal T-cells 1%-50% [of total nucleated cells], mean 13.9%). Diagnoses included overt lymphoma in the setting of S-EBV-TCL (n=5), overlapping features of S-EBV-TCL and EBV-HLH (S-EBV-TCL/HLH, n=10), and S-T-CAEBV (n=2) with subsequent overt lymphoma in one. Abnormal karyotypes were identified in all patients with overt lymphoma, except one. CGP of paired diagnostic and disease progression samples (lymph node) of S-T-CAEBV with subsequent overt lymphoma revealed activating *STAT3* p.G618R and pathogenic *SMARCA4* p.E882D mutations. An additional *BCOR* p.K742fs loss-of-function mutation was observed in the disease progression sample with features of overt lymphoma. Variants of unknown significance were detected in *APC, ATM, CREBBP, CUX1, KMT2C, SETBP1*, and *TP53* whereas *KMT2D, PRDM2*, and *SMARCA4* VUS were detected in multiple samples. Outcomes were poor after HLH-directed immuno- and chemotherapy protocols with or without bone marrow transplant as 10 (58.82%) died of disease or complications, and only seven (41.18%) were alive at 0.5 months to seven years follow-up (median: three months). **Conclusions:** This series details the clinicopathologic features of rare childhood S-EBV-T-LPD disorder spectrum, and demonstrates the diagnostic and management challenges. CGP might help identify novel clinically relevant alterations in this cohort.

H049. **Transcriptome Sequencing (RNA-Seq) Allows Comprehensive Genomic Characterization of Pediatric B-Lymphoblastic Leukemia (B-ALL) in the Clinical Setting**

G. Raca[1], A. Kovach[1], Z. Hu[2], V. Yelapantulla[1], D. Ostrow[1], A. Doan[1], J. Ji[1], R. Schmidt[1], J. Biegel[1], Z. Gu[2], D. Bhojwani[1]
[1]Children's Hospital Los Angeles, Los Angeles, CA; [2]City of Hope, Monrovia, CA.

Introduction: Application of risk-adapted and targeted therapies for pediatric B-cell lymphoblastic leukemia/lymphoma (B-ALL) calls for rapid and accurate identification of genomic lesions and subtypes. Due to the genetic heterogeneity of B-ALL, this typically requires simultaneous or sequential use of multiple cytogenetic and molecular assays. Studies have shown the power of RNA-Seq in identifying known and novel oncogenic drivers and molecular subtypes of B-ALL. We hypothesized that our clinically validated RNA-Seq fusion assay,

coupled with a custom data analysis pipeline, could be applied for comprehensive B-ALL diagnostic evaluation. **Methods:** RNA-Seq was performed on 78 retrospective B-ALL cases, 28 with known subtype and 50 in which the subtype remained undetermined following clinical karyotype analysis, fluorescence *in situ* hybridization (FISH), chromosomal microarray (CMA), and next-generation sequencing (NGS) panel testing (OncokKids). RNA-Seq was conducted by enrichment with the Twist Comprehensive Exome capture probes, followed by NGS (Illumina Inc., San Diego, CA). Integrated data analysis was performed to define expression profiles and identify genetic lesions for molecular subtyping. Selected RNA-Seq findings (nine cases) were verified by optical genome mapping (OGM) (Bionano Genomics, San Diego, CA). **Results:** Subtypes were accurately identified by blinded RNA-Seq analysis in all the 28 known cases, and successfully determined in 39/50 unknown cases (78%). The subtypes of the unknown cases included: *PAX5*Alt (n=12), *DUX4*-rearranged (n=6), Philadelphia (Ph)-like (n=5), low hyperdiploid (n=5), *ETV6*::*RUNX1*-like (n=3), *MEF2D*-rearranged (n=2), *PAX5* P80R (n=2), *ZEB2*/*IGH*::*CEBPE* (n=1), *NUTM1*-rearranged (n=1), *ZNF384*-rearranged (n=1) and *TCF3*::*PBX1* (n=1). In 19/39 cases, classification based on expression profile was corroborated by detection of a subtype-defining oncogenic driver and/or by OGM, and is in process for the remaining 20 cases. Among the 11 cases unclassified by RNA-Seq, six had suboptimal samples (4: <30% blasts, 2: >70% post-transplant donor chimerism). RNA-Seq analysis also allowed detection of large copy number abnormalities, showing concordance for whole-chromosome gains and losses with karyotype, FISH and CMA. Finally, RNA-Seq detected hot-spot oncogenic variants in *NRAS, KRAS, PTPN11, TP53, JAK3,* and *ZEB2*, with 74% concordance with the NGS results. **Conclusions:** RNA-Seq coupled with a custom analytic pipeline showed full concordance in defining B-ALL subtypes with clinical testing, and allowed molecular classification in an additional 78% of previously undetermined cases. We show the feasibility of implementing an RNA-Seq workflow for clinical diagnosis of molecular subtypes in pediatric B-ALL, and confirm that RNA-Seq represents a promising global genomic assay for this leukemia.

H050. **Validation and Adoption of an *IGVH* Mutational Status Assay in Chronic Lymphocytic Leukemia (CLL) using Next-Generation Sequencing (NGS) in a Clinical Laboratory**

R. Prathapam[1], B. Lemoine[1], x. Dai[1], T. Frietas[1], S. Khadgi[1], j. Graham[1], Z. Tan[1], G. Sun[1], B. Pugh[1], I. Marmolejo[1], B. Shahbahrami[2], D. Cacho[2], D. Ngo[2], C. Ma[2], K. Champion[1]
[1]Quest Diagnostics, Lewisville, TX; [2]Quest Diagnostics, San Juan Capistriano, CA.

Introduction: Somatic hypermutation (SHM) status of the *Immunoglobulin variable heavy chain region* (*IGVH*) is a well-established prognostic and predictive biomarker in chronic lymphocytic leukemia (CLL). SHM analysis determines whether the *IGVH* gene sequence is significantly modified from the closest germline reference sequence. The gold standard method for *IGVH*-SHM analysis has been Sanger sequencing using RNA or DNA isolated from a patient's blood. However, this method is labor intensive, complex, technically challenging, hard to scale, and not standardized across the labs. In addition to these limitations, cases with more than one dominant *IGH* rearrangement could be under-detected. On the other hand, the next-generation sequencing (NGS) methodology offers superior benefits in overcoming the limits of the Sanger sequencing method, making it more attractive for SHM analysis. Hence, we validated an NGS-based assay and in this study, we present the results of analytical validation of our NGS-based assay compared to the Sanger-based assay. We will also share our experience adopting the assay in a clinical laboratory. **Methods:** Total RNA extracted from mononuclear cells isolated from peripheral blood or bone marrow aspirate of patient specimens with >10% monoclonal B-cell population by flow cytometry were evaluated using NGS-based and clinically validated Sanger-based assays. The NGS-based assay is a modified version of the Oncomine BCR IGH-LR assay kit by Thermo Fisher Scientific. The assay utilizes Ion AmpliSeq multiplex PCR technology to amplify framework 1 and the constant gene region of the *IGH* gene. Libraries prepared using RNA as input were templated and sequenced using Ion Chef and Ion GeneStudio S5 Prime system. Following sequencing, the sequencing files were analyzed in Torrent Suite and Ion Reporter. **Results:** SHM analysis using NGS methodology demonstrated excellent performance characteristics with an overall accuracy of (54/55) 98.2%, positive percent agreement of (24/24) 100% and negative percent agreement of (30/31) 96.7% in comparison to the Sanger sequencing method. Intra- and inter-run assays performed showed 100% concordance, indicating high precision. Moreover, the NGS method detected more than one clonal *IGH* rearrangement in ~10% of samples, highlighting the NGS approach's benefits. We processed more than 200 samples in our clinical laboratory using the validated assay, which demonstrated a failure rate of <1% and was able to provide 100% conclusive results. **Conclusions:** We successfully validated and adopted an NGS-based assay to evaluate *IGVH* SHM status in patients with CLL. Our clinical laboratory experience performing this assay has demonstrated superior performance by the NGS method in terms of ease of use, assay robustness, reliability, and clinical usefulness.

H051. **Clinical Implications of Circulating Tumor DNA for Minimal Residual Disease Detection and Relapse Prediction in Diffuse Large B-Cell Lymphoma Patients**

M. Li[1], L. Mi[3], C. Wang[4], X. Wang[4], J. Zhu[4], F. Qi[3], H. Yu[3], Y. Ye[3], D. Wang[3], J. Cao[3], D. Hu[3], Q. Yang[2], D. Zhao[4], T. Ma[2], Y. Song[3], J. Zhu[3]
[1]Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; [2]Genetron Health (Beijing) Technology, Co. Ltd., Beijing, People's Republic of China; [3]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Lymphoma, Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; [4]Department of Translational Medicine, Genetron Health (Beijing) Technology, Co. Ltd., Beijing, People's Republic of China.

Introduction: Precise detection of minimal residual disease (MRD) to predict early relapse of diffuse large B-cell lymphoma (DLBCL) patients is needed to guide individual therapy. Detectable plasma circulating tumor DNA (ctDNA) in the radiographic remission status has been considered as a noninvasive biomarker of MRD in multiple solid tumors, but ctDNA-based MRD detection in DLBCL remains poorly understood. This study sought to assess the clinical performance of ctDNA through targeted next-generation sequencing to detect radiographically occult MRD and predict early relapse for DLBCL patients who underwent standard first-line therapy. **Methods:** A total of 25 DLBCL patients diagnosed and treated at Peking University Cancer Hospital and Institute were enrolled retrospectively in this study. All of them reached a radiographic complete remission (CR) during or after first-line treatment. With a median 38 months follow-up period, 19 patients survived free of disease and six experienced relapse. Tumor biopsy tissues were collected at diagnosis and peripheral blood samples were serially obtained before treatment and at the time of remission, relapse, or follow-up time visits. Tumor genomic DNA (gDNA) and cell-free DNA (cfDNA) were extracted from tumor tissues and plasma samples, respectively. Germline gDNA was extracted from paired white blood cells for normal controls. Onco-LymScan panel sequencing of 188 lymphoma-associated genes (Genetron Health) was performed in all samples. **Results:** All of the 25 patients were identified with at least one trackable variant from their pretreatment tumor and/or plasma samples. CtDNA-MRD positive was defined as any basal variant that could be detected in any of the post-treatment plasma samples at CR status; otherwise, it was termed ctDNA-MRD negative. Only two of the 18 ctDNA-MRD negative (11.1%) patients developed recurrence or disease progression. However, four of the seven (57.1%) ctDNA-MRD positive patients exhibited a relapse. Survival curves demonstrated that relapse-free survival was significantly poor in ctDNA-MRD positive patients than ctDNA-MRD negative ones (HR 6.471; p = 0.014). A similar, albeit

insignificant, trend was also observed with regards to overall survival (OS; HR 6.515; p = 0.079). Especially, patient RR821, who achieved a CR and ctDNA negativity after treatment, was detected with a ctDNA reappearance during follow-up, although the corresponding CT scans did not show any signs of clinical recurrence and eventually relapsed. CtDNA monitoring indicated relapse with lead time of 4.8 months than CT scans. **Conclusions:** These findings indicated that ctDNA could not only track MRD during or after first-line treatment but also is a promising noninvasive biomarker for predicting the risk of recurrence in DLBCL.

H052. **Whole-Genome Sequencing for Copy Number Abnormalities in Multiple Myeloma Supersedes Karyotyping and Fluorescent *in situ* Hybridization**

M. Mao[1], L. Gao[2], Z. Li[3], Y. Chang[4], X. He[4], Y. Cheng[5], Y. Li[6], C. Yao[4], S. Li[7], M. Li[4], D. Zhu[4], M. Mao[7], X. Zhang[2]
[1]SeekIn Inc., Shenzhen, People's Republic of China; [2]The Xinqiao Hospital of Third Military Medical University, Chongqing, Chongqing, People's Republic of China; [3]China-Japan Friendship Hospital, Beijing, Beijing, People's Republic of China; [4]Clinical Laboratories, Shenyou Bio, Zhengzhou, Henan, People's Republic of China; [5]Yancheng No. 1 People's Hospital, The Yancheng Clinical College of Xuzhou Medical University, Yancheng, Jiangsu, People's Republic of China; [6]The Affiliated Cancer Hospital of Zhengzhou University and Henan Cancer Hospital, Zhengzhou, Henan, People's Republic of China; [7]Research and Development, SeekIn Inc., Shenzhen, Guangdong, People's Republic of China.

Introduction: Prognosis and management of multiple myeloma (MM) relies on risk stratification based on copy number abnormality (CNA) detection results. CNA is conventionally evaluated by cytogenetic methods including karyotyping and fluorescence *in situ* hybridization (FISH), both limited by the invasiveness, low proliferative activity of plasma cell, and the low plasma cell count. LeukoPrint is a novel shallow whole-genome sequencing (sWGS)-based approach to profile CNA in bone marrow cells and/or circulating cell-free DNA (cfDNA). We compared the CNA detection results by LeukoPrint, karyotyping, and FISH using bone marrow or plasma samples from multiple myeloma patients. **Methods:** A total of 63 patients were enrolled in this study. Karyotyping and FISH were performed by conventional approaches. CNA was detected by sWGS in genomic DNA (gDNA) from bone marrow aspiration samples and CD138+ enriched plasma cells as well as in plasma cfDNA. CNA degree was measured by Log R ratio. Significant mutational peak region was identified by GISTIC2. Risk stratification was performed following the Mayo Stratification of Myeloma and Risk-adapted Therapy (mSMART) criteria. **Results:** In this study, 458 CNA events were identified by sWGS in 47 (74.6%, 47/63) patients, whereas the positive rate of CNA detected by karyotyping and FISH was 17.5% and 55.6%, respectively. A total of 182 whole chromosome aberration events were identified by sWGS, 65.4% (119/182) of which were trisomy that defined 27.0% (17/63) of patients as hyperdiploidy and the remaining 47.6% (30/63) as non-hyperdiploidy. The most frequently mutational CNA peak regions were amp(19p13.3), amp(6p21.2), del(1p22.1), del(4q34.1), del(8p21.2), del(14q24.1), and del(16p13.3). The concordance rate between sWGS and FISH in detecting the four conventional loci (1q21 gain, 13q14 del, 13q14.3 del, and 17p13 del) was 76.4%. Compared to the conventional approaches, sWGS provided new CNA information for 74.6% of the patients, and changed the risk stratification for 20.6% of patients according to mSMART criteria. CNA detection results using paired bone marrow aspiration samples and enriched plasma cell samples from eight patients were compared. We found that more CNA events (92 vs. 60) and higher Log R ratio of CNAs were detected using enriched plasma cells than those using bone marrow aspiration samples. The concordance rate of the CNA detection results by sWGS between paired bone marrow genomic DNA and plasma cfDNA from another nine patients was 86.3%. **Conclusions:** LeukoPrint is an automated, convenient, and cost-effective approach to depict CNA profile in gDNA or cfDNA. This method is superior to conventional approaches when used for CNA testing, and the practice of this method could improve prognostic stratification of MM patients.

H053. **Validate an NGS-Based Measurable Residual Disease (MRD) Assay for B-Cell Lymphoproliferative Diseases**

P. Shah[1], Y. Huang[1], S. Glenn[2], A. Herrera[3], C. Morrison[2], A. Wong[5], L. Hebert[1], J. Li[1], E. Rudenko[1], A. Zlotnicki[1], A. Street[1], O. Diaz[1]
[1]Invivoscribe, San Diego, CA; [2]Roswell Park Comprehensive Cancer Center, NY; [3]Salamanca University Hospital, Salamanca, Spain; [5]Lahey Hospital and Medical Center, Burlington, MA.

Introduction: Measurable residual disease (MRD) assessment can provide useful information when assessing response to therapy, refining treatment, and predicting clinical outcome for patients with B-cell lymphoproliferative disease. One method of MRD detection is to identify and track specific clonal *Immunoglobulin heavy chain* (*IGH*) gene rearrangement sequences on a next-generation sequencing (NGS) platform. Here, we present the validations of a CAP/CLIA-accredited NGS-based B-cell MRD Assay with 1.0 x 10^{-6} sensitivity for malignancies such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM), and mantle cell lymphoma (MCL). **Methods:** The B-cell MRD Assay workflow consists of DNA extraction, DNA concentration, and PCR-based library preparation with proprietary multiplex master mixes (MMx) targeting either *IGH* Framework 1 (FR1) or Framework 3 (FR3). Purified libraries are then equimolar pooled and sequenced on Illumina's NextSeq Dx platform. The FASTQ output files from sequencing are analyzed using Invivoscribe's B-Cell MRD Software-NextSeq Dx. Contrived DNA panels were prepared by diluting DNA from either cell lines or clinical bone marrow (BM) samples (ALL, CLL, MM, and MCL) into DNA from healthy BM samples. The B-cell MRD Assay was validated for limit of detection (LoD), limit of quantitation (LoQ), limit of blank (LoB), linearity, precision, and accuracy. **Results:** Using FR1 PCR MMx, the B-Cell MRD Assay exhibited an LoB at 0, LoD at 2.3x10^{-6}, LoQ at 2.4x10^{-6}, linearity with R^2 >0.88 across 4.5 logs, and precision with %CVs (coefficient variation) ranging from 27.6% to 63.5% at the LoD. Using FR3 PCR MMx, the B-Cell MRD Assay exhibited an LoB at 0, LoD at 1.8x10^{-6}, LoQ at 1.7x10^{-5}, linearity with R^2 >0.78 across 4.5 logs, and precision with %CVs ranging from 46.6% to 89.4% at the LoD. In addition, the assay demonstrated 100% agreement in an accuracy study that included 20 clinical specimens and 10 healthy donors. **Conclusions:** The results demonstrate that the B-Cell MRD Assay is highly sensitive (FR1 LoD is 2.3x10^{-6} and FR3 LoD is 1.8x10^{-6}) and accurate (100% agreement) to meet the clinical MRD threshold requirement of 10^{-5} for B-cell malignancies.

H054. **Single Multiplex Master Mix versus Dual Master Mix Method Comparison for T-cell Receptor Clonality Assessment**

B. Drumheller, B. Broadfoot, R. Saade, Y. Ma, C. Lyons, H. Jones, C. Hill, L. Zhang
Emory University Hospital, Atlanta, GA.

Introduction: T-cells rearrange the T-cell receptor gamma (*TRG*) locus early in development, which is retained regardless of the α/β or γ/δ protein expression. Each *TRG* locus can produce a single V-J combination with a unique length and sequence; detecting a product with the same length or sequence in a population of T-cells indicates a clonal T-cell proliferation. PCR amplification followed by fragment length analysis generates distinct peaks when a clonal population is present. This clonal population can be tracked for disease recurrence. Our old assay (TRG 1.0) utilized a dual master mix protocol to generate eight size distribution ranges. It is labor intensive and difficult to interpret with potential false positive peaks. Invivoscribe's *TRG* gene rearrangement assay 2.0 (TRG 2.0) uses a single multiplex master mix with amplified products falling within a single size range. Here we describe our experience validating TRG 2.0. **Methods:** Archived nucleic acid samples from 2021-2022 (n=51) tested by TRG 1.0 were selected to include a variety of clonal (n=25; 1-2 peaks), oligoclonal (n=9; ≥3 peaks), and polyclonal (n=17) cases. TRG 2.0 was performed per the manufacturer's instructions, with minor modifications. Controls

were performed on each run. By definition, a clonal TRG 2.0 peak is at least 3x the amplitude height of either adjacent peak. Peaks with a fluorescent intensity <1,000 were below our detection threshold. Comparison, dilutional, and replicate studies were performed. **Results:** All cases were reinterpreted for consensus agreement. Two clonal TRG 1.0 cases were retrospectively reclassified as negative. Of the 23 samples considered clonal by TRG 1.0, there were three false negative samples by TRG 2.0. Chart review demonstrated T-cell lymphoproliferative disorders for two cases, with prior peaks located within primer regions not covered by TRG 2.0. The third discrepant case showed a peak outside the polyclonal distribution but a fluorescence intensity below our cut-off for TRG 2.0. All nine oligoclonal patterns were concordant. Clinical sensitivity and specificity were 87% (20/23) and 100% (19/19), respectively. The limit of detection was 2.5% by serial dilutions of clonal control samples. The replicate samples gave consistent results across three runs (reproducibility 100%). **Conclusions:** Previously reported peak sizes by TRG 1.0 were not directly comparable with TRG 2.0 due to the differences in assay design and analysis. TRG 2.0 primers lose Vγ1, Vγ6, and Vγ7, and gain JγP detection. The two assays showed high concordance. TRG 2.0 had a lower false positive rate. Overall, the simplified TRG 2.0 was quicker to perform and easier to interpret; however, our experience shows some possible loss of sensitivity in previously covered primer regions.

H055. **Ring-Seq: A Novel Technology for Multiplex PCR Based Detection of Gene Fusions with Novel Partners and Breakpoints from Highly Degraded Material**

T. Looney[1], R. Que[2], K. Fichter[2], S. Owens[2], S. Shore[2]
[1]Singular Genomics, Austin, TX; [2]Singular Genomics, San Diego, CA.

Introduction: Gene fusions are an important type of genetic aberration in cancer with relevance to therapy selection and as a marker for measurable residual disease (MRD) monitoring. Traditional multiplex PCR (mPCR) is unable to detect gene fusions with novel partners or breakpoints. Here we introduce Ring-Seq, a novel mPCR technology for the targeted detection of gene fusions, including those with unknown partners or breakpoints. Using the Singular Genomics G4 sequencing platform, we apply the Ring-Seq protocol to simultaneously identify clinically relevant translocations and V(D)J rearrangements of the *IGH* locus from highly degraded material. **Methods:** The Ring-Seq method begins with a highly efficient intramolecular ligation of DNA fragments followed by a multiplex inverse PCR that preferentially amplifies breakpoint junction containing fragments. Amplicon libraries are sequenced on the G4 platform via 2x150bp reads and analyzed to detect translocations. We applied the method to simultaneously detect *IGH* V(D)J rearrangements, and *BCL1*-JH and *BCL2*-JH translocations from fragmented IVS-0010 and IVS-0030 reference control genomic DNA (Invivoscribe Cat #40880550 and 40881750) and healthy donor PBL gDNA. **Results:** *BCL1*-JH and *BCL2*-JH translocations were detected from 50 ng of fragmented gDNA (200 bp avg template length) from IVS-0010 and IVS-0030 reference controls, respectively. Translocations were also detected from 50ng samples consisting of fragmented reference control material spiked at 1% frequency into a background of fragmented healthy donor PBL gDNA. We observe preferential amplification of translocation-containing templates, enabling detection from <1M reads/sample in all conditions tested. V(D)J rearrangements were successfully detected from PBL gDNA using the same multiplex inverse PCR reaction. **Conclusions:** Ring-Seq enables mPCR-based detection of novel gene fusions from highly degraded material with a sequencing efficiency similar to that of traditional mPCR. As a first application, we have applied Ring-Seq to simultaneously detect B-cell V(D)J rearrangements and clinically relevant JH translocations from a limited amount of degraded gDNA. In this respect, the Ring-Seq method represents a significant advance over current mPCR-based approaches for antigen receptor sequencing. We expect the method to have broad utility for molecular diagnostics and MRD monitoring of cancer.

H056. **Using Next-Generation Sequencing and Genetic Profiling to Distinguish *STAT3/STAT5B* Mutated Large Granular Lymphocyte Leukemia from Myeloid Neoplasms**

M. Riveros Angel[1], M. Kavesh[2], M. Mohebnasab[1], W. Xie[1], P. Raess[1], W. Cui[3], R. Press[1], P. Li[4]
[1]Oregon Health and Science University, Portland, OR; [2]University of Utah, Salt Lake City, UT; [3]University of Kansas Medical Center, KS; [4]Genomic Laboratory, ARUP, Salt Lake City, UT.

Introduction: Activating mutations in *STAT3* and *STAT5B* are frequently found in large granular lymphocyte leukemias (LGLLs) and myeloid neoplasms (MNs), whereas large granular lymphocyte (LGL) clonal expansion is common in MNs including myelodysplastic syndrome (MDS). Distinguishing LGLLs from MNs in patients with cytopenia becomes necessary for appropriate management. **Methods:** Retrospectively we identified 117 patients with pathogenic/likely pathogenic *STAT3* or *STAT5B* variants as well as 18 MDS patients with clonal LGL expansions in the absence of *STAT3/5B* mutations, amongst an unselected cohort of 6,690 patients with hematologic neoplasms using a targeted panel of myeloid mutations using next-generation sequencing. We analyzed the demographic and genomic profiles, the clonal metrics of the *STAT3/5B* variants, T-cell clonality, and flow cytometric studies, and these data were organized by diagnosis. **Results:** *STAT3* variants were predominant in LGLLs (96% in 54 patients), whereas the *STAT5B* variants were more represented amongst MNs (34% in 50 patients, P <0.0001). Other genetic features distinguishing LGLL from MN cases included a lower median variant allele frequency (VAF) of *STAT3/5B* variants (8.9 ± 1.5 versus 12.0 ± 2.7, P=0.01) and often absence of and less diversity of concomitant variants. Furthermore, in LGLLs, the VAFs of *STAT3/5B* variants correlated with neoplastic T or NK lymphocytes using flow cytometry (R^2=0.92), and *STAT3/5B* variants indicated the presence of the founder clones (R^2=0.91) in contrast to MNs. **Conclusions:** Our study demonstrates that the characteristic molecular features and *STAT3/5B* clonal metrics in LGLLs and MNs can facilitate accurate diagnosis to inform patient care.

H057. **Non-Invasive Detection of Lymphoma with Circulating Tumor DNA Features and Plasma Protein Marker**

M. Mao[1], Y. Chang[2], S. Li[3], Z. Li[4], X. Wang[2], F. Chang[5], S. Geng[5], D. Zhu[5], G. Zhong[3], W. Wu[3], Y. Chang[5], S. Tu[5], M. Mao[3]
[1]SeekIn Inc., Shenzhen, People's Republic of China; [2]The First Affiliated Hospital of Zhengzhou University, Zhengzhou, People's Republic of China; [3]Research and Development, SeekIn Inc., Shenzhen, People's Republic of China; [4]Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, People's Republic of China; [5]Clinical Laboratories, Shenyou Bio, Zhengzhou, People's Republic of China.

Introduction: Lymphoma is a diverse group of malignancies originating from B, T, and NK cells, which can be divided into more than 30 unique subtypes. Lymphoma is traditionally diagnosed by excisional lymph node biopsy which shows significant limitations including invasiveness and difficulty in sample obtaining, and most lymphoma patients are diagnosed at an advanced stage at which treatment is not curative. We developed a novel blood-based liquid biopsy assay (SeekInCare) for pan-cancer detection which integrated multi-omics and multidimensional cancer-associated hallmarks. We evaluated the SeekInCare performance for lymphoma detection in this study. **Methods:** A total of 144 untreated lymphoma patients and 396 healthy individuals were enrolled in this study. One tube of blood (10 ml) was collected from each participant after enrollment. Expression of seven tumor protein markers (*AFP, CA125, CA153, CA199, CA724, CEA*, and *CYFRA21-1*) were quantified by electrochemiluminescence (ECL) assay, and cell-free DNA (cfDNA) was extracted and analyzed by shallow whole-genome sequencing (sWGS) for copy number aberrations (CNA), fragment size (FS), end-motif, and Epstein-Barr virus (EBV) profiling. The cancer risk score (CRS) of each sample was calculated based on the genomic DNA features and protein expression by a machine learning approach developed for the early diagnosis of

lymphoma. **Results:** After comparing each protein marker between lymphoma and healthy groups, ROC curve analysis showed only CA125 achieved a moderate sensitivity (28.5%) and a high specificity (97.7%), with an area under the curve (AUC) of 0.702. The sWGS analysis results showed that CNA, FS, end-motif, and EBV profile each distinguished lymphoma plasma samples from healthy counterparts (p value <0.001 by Mann-Whitney U test), which showed improved sensitivity (63.9%, 61.8%, 45.1%, and 38.9%, respectively) compared to CA125. A CRS model incorporating the ECL assay results and sWGS data achieved the best performance, allowing 108 lymphoma cases to be identified with an overall sensitivity of 75.0% at 97.7% specificity, resulting in 91.7% accuracy. In early-stage lymphoma, sensitivity was 36.8% and 55.0% for stage I and II, respectively, and among all lymphoma subtypes, Hodgkin lymphoma had the highest sensitivity (92.9%). **Conclusions:** Our study found CA125 and EBV profiles as potential biomarkers for lymphoma screening, and confirmed that the non-invasive SeekInCare assay could be used to detect lymphoma with accuracy. Positive predictive value and negative predictive value were more than 90.0%, especially for which biopsies that were inconclusive.

H058. **Prompt Assessment of Tumor Load and Treatment Response in Patients with Lymphoma by a Blood-Based Multi-omics Approach**

M. Mao[1], X. Wang[2], Z. Li[3], Y. Chang[2], S. Li[4], X. Hu[4], W. Wu[4], F. Chang[5], Y. Chang[5], D. Zhu[5], M. Zhang[2], M. Mao[4]
[1]SeekIn Inc., Shenzhen, People's Republic of China; [2]The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, People's Republic of China; [3]Sun Yat-sen University Cancer Center, Guangzhou, Guangdong People's Republic of China; [4]Research and Development, SeekIn Inc., Shenzhen, Guangdong People's Republic of China; [5]Clinical Laboratories, Shenyou Bio, Zhengzhou, Henan People's Republic of China.

Introduction: Lymphoma represents a diverse group of cancer that arises from clonal proliferation of lymphocytes. Periodic imaging is the current standard of care for clinical evaluation of treatment response. Liquid biopsy provides an alternative non-invasive method for quantitative assessment of therapeutic response. In this lymphoma prospective study, we applied non-invasive multi-omics assay (SeekInClarity) to evaluate lymphoma treatment response and predict patient prognosis. **Methods:** A total of 149 patients meeting the WHO diagnosis criteria of lymphoid neoplasm were prospectively recruited from two clinical sites. In each participant's plasma sample, seven plasma tumor protein markers (PTMs, including *AFP, CEA, CA153, CA125, CA199, CYFRA21-1*, and *CA724*), were evaluated by electrochemiluminescence assay, and the copy number aberration (CNA) index and the fragment size (FS) index of the cell-free DNA were analyzed by shallow whole-genome sequencing. Molecular tumor burden (MTB) score of each patient was determined before and after two, four, and six cycles of treatment based on the number of abnormalities detected in PTM, CNA index, and FS index. We defined an MTB score of 0 as MTB negative (MTB-) and the score greater than 0 (1, 2) as positive (MTB+). **Results:** The values of each dimension (PTMs, CNA and FS) in the lymphoma baseline sample were significantly higher than those in healthy control samples (Mann-Whitney test: P <0.001). The sensitivity of CNA/FS/CA125 was 60.4%/40.9%/34.2%, respectively, at 95% specificity. After combining these dimensions, 108 out of 149 patients (72.5%) were MTB+. The proportion of patients with MTB+ was higher in patients with higher tumor stage. After two cycles of treatment, the proportion of final progression patients with each MTB scores 0/1/2 was 1.5%/18.8%/50%, respectively, which indicates that higher MTB score has a significantly higher proportion of progression patients. After treatment, patients with MTB- at the monitoring test point had a better progression-free survival than those with MTB+ [HR: 17.9, 95% CI:5.2-61.4; P=0.00011]. After two cycles of treatment, 62.5% of patients with MTB+ would change their treatment regimen, and the median event time was 152 days after enrollment. However, in the MTB- patients, only 16.7% of patients changed their treatment regimen and had not reached median event time. **Conclusions:** This prospective study demonstrated SeekInClarity, a non-invasive, multidimensional blood-based assay, can promptly evaluate the treatment response of lymphoma patients, and showed an excellent correlation between MTB status and the clinical outcome. It challenged the status quo of employing imaging as a sole standard for evaluating early treatment response.

H059. **Identification of *IKZF1* Exon Skipping by a Custom Designed Targeted NGS Assay**

N. Guseva[1], A. Stence[1], R. Sompallae[1], S. Hornberg[1], S. Bhagavathi[1], A. Snow[1], A. Bossler[2], D. Ma[1]
[1]University of Iowa Hospitals and Clinics, Iowa City, IA; [2]Moffitt Cancer Center, Tampa, FL.

Introduction: *IKZF1* (IKAROS) encodes a transcription factor that is essential for lymphocyte development. IKAROS has four N-terminal zinc fingers for DNA binding and two C-terminal zinc fingers for mediating dimerization of IKAROS with itself and other family members. Isoforms lacking the N-terminal domain have attenuated DNA binding capacity but retain their ability to undergo homo- and heterodimerization, and act as dominant-negative inhibitors of IKAROS. Substitutions, deletions, and alternative splicing can lead to exon skipping aberrant expression and dysfunction of *IKZF1*. In B-cell acute lymphocytic leukemia (B-ALL), *IKZF1* deletion is associated with a higher relapse risk and worse prognosis. We evaluated an RNA-based custom-designed next-generation sequencing (NGS) assay for detection of oncogenic isoforms of *IKZF1* to assist in diagnosis, prognosis, and therapy selection in ALL patients. **Methods:** A total of 175 cases of hematological malignancies were tested using a custom-designed, laboratory-validated targeted RNA-based NGS assay using the anchored multiplex PCR (Invitae) technology. This assay multiplexes gene fusion and WHO-defined variants important for lymphoid and myeloid malignancies, and is capable of detecting novel fusions and exon skipping. A total of 250 ng RNA extracted from blood or bone marrow was used for library preparation. Sequencing was performed on the MiSeq (Illumina). Data were analyzed using the Archer Analysis software (v.6.0.4). *IKZF1* exon skipping was divided into high/true positive (TP), low positive (LP), and suspected false positive (FP), based on criteria established during test validation. RT-PCR and gel electrophoresis were used for confirmation of *IKZF1* exon skipping. **Results:** *IKZF1* exon skipping was reported by the Archer software in 101 cases (57%) including 79 suspected FP, 10 LP, and 12 TP cases. Among these 101 cases, the oncogenic isoforms ex1-8 and ex3-8 skipping (NM_006060.6) were reported in 74 and nine cases, respectively. Both transcripts coexisted in 18 cases. Truncated transcripts were confirmed by RT-PCR in LP and TP cases using specific primers designed for each isoform. FP cases revealed wild-type transcript. In the *IKZF1* exon skipping positive cases, coexisting *BCR::ABL* were detected in two LP (20%) and five TP (45%) cases. Other coexisting pathogenic variants included *KRAS* p.Q61H and p.G12D, and *ABL1* p.T315I. One TP case had three additional mutations in *JAK1* and *NRAS* genes. **Conclusions:** *IKZF1* exon skipping isoforms were reliably identified by our RNA-based NGS assay. Further studies are warranted on the morphologic and clinical correlation, the clinical relevance of *IKZF1* exon skipping with coexisting aberrations, and the prognostic effect of low positive *IKZF1* exon skipping.

H060. **Comparative Utility of *IGHV* Leader Targeted NGS versus Framework 1 Targeted NGS for Somatic Hypermutation Status Evaluation in CLL/SLL**

B. Tandon[1], M. You[2], W. Swat[2]
[1]Pathline Laboratory, Ramsey, NJ; [2]BioReference Laboratories/GenPath Oncology, Elmwood Park, NJ.

Introduction: Somatic hypermutation (SHM) status of rearranged *IGHV* is a key requirement for prognostic risk stratification in chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), the most common leukemias in adults. Due to costs and other practical factors, next-generation sequencing (NGS) workflows targeting only VH Framework 1 (FR1) remain widely utilized in routine clinical practice; however, use of FR1 primers may limit accuracy of *IGHV* SHM mutation rates, due to amplification of only a portion of the rearranged *IGHV* gene. To date, clinical data comparing FR1 NGS to Leader NGS for *IGHV* SHM analysis in CLL/SLL remains limited. **Methods:** Fifty cases of CLL/SLL, diagnostically confirmed by flow cytometry, comprehensive blood count, and fluorescence *in situ* hybridization, were studied. Previously extracted DNA was analyzed by Illumina sequencing targeting VH FR1, and Leader NGS was also performed. NGS results were compared to original Leader-based Sanger sequencing results. *IGHV* SHM mutation rates were designated as follows: 2%-3%=Borderline, <2%=Unmutated, and >3%=Mutated. **Results:** NGS demonstrated categorically discordant mutation rates in 5/50 cases (10%). In 4/50 cases (8%), FR1 NGS results were Borderline, whereas Leader NGS demonstrated >3% mutation in these cases. In the remaining fifth discordant case, FR1 NGS mutation rate=1.33% (unmutated CLL), whereas NGS Leader mutation=4.73% (mutated CLL). VJ family calls were also different among two of the five NGS FR1/NGS Leader discordant cases. Leader SS and Leader NGS demonstrated complete categorical concordance among 100% of cases. **Conclusions:** Clinically significant discordances in *IGHV* SHM mutation rates were identified among 10% of cases studied. In 8% of cases, FR1 NGS yielded clinically indeterminate, borderline results, whereas Leader NGS yielded clinically informative results in all cases, corroborated by the original Leader SS results. Further, in 2% of cases, FR1 NGS generated an inaccurate call of unmutated disease status, predictive of poor prognosis that may have necessitated exposure to toxic therapy, whereas Leader NGS, corroborated by Leader SS, demonstrated findings indicative of prognostically favorable, mutated status. These results support European Research in CLL (ERIC) recommendations that suggest using Leader primers to ensure accurate assessment of *IGHV* SHM status in CLL.

H061. **Cancer Personalized Profiling by NGS-Based Deep Sequencing (CAPP-SEQ) Panel in B-Cell Lymphoid Malignancies (Tissue Genotyping)**

S. Cheng[1], K. Nie[2], B. Liu[2], E. Fernandez[2], W. Tam[2]
[1]Icahn School of Medicine at Mount Sinai, New York, NY; [2]Weill Cornell Medicine, New York, NY.

Introduction: Genomic alterations found in B-cell lymphoid malignancies are becoming increasingly important for diagnosis, classification, prognostication, and initiation of targeted therapy. For example, a characteristic mutation identified in *MYD88* (p.L265P) was found in about 90% of lymphoplasmacytic lymphoma (LPL) cases and can be used for LPL differential diagnosis with other small mature B-cell neoplasms. Therapeutically, oncogenic mutations in *EZH2* are an FDA-approved biomarker for tazemetostat treatment for *EZH2*-mutated relapsed or refractory follicular lymphoma. In hairy cell leukemia (HCL), mutations in *BRAF* (p.V600E) and *MAP2K1* appear to sensitize HCL to *BRAF* and *MEK* inhibitors, respectively. Emerging certain mutations in *BTK* are predictive of resistance to Ibrutinib when treated for chronic lymphocytic leukemia patients. A practical targeted next-generation sequencing (NGS) sequencing assay is needed to meet this clinical demand. **Methods:** CAPP-Seq assay interrogates 1,487 exons and 29 introns of 144 genes mutated in B-cell malignancies. It utilizes hybridization capture-based target enrichment and massive parallel sequencing by NextSeq. Single nucleotide variants (SNVs) and insertions/deletions (indels) of six genes (*ATM*, *CREBBP*, *KMT2D*, *TP53*, *MYD88,* and *NOTCH1*) were evaluated in a total of 58 clinical samples (44 frozen/fresh and 14 formalin-fixed, paraffin-embedded specimens) chosen from patients with B-cell tumors. We assessed the analytical sensitivity, precision, reproducibility, and accuracy of the assay. **Results:** Analysis shows that the mean coverage for ~97%-97.4% of the samples tested (n=58) was greater than >500X, with median and mean coverages of 2,236X and 2,163X, respectively. Ninety-seven percent of the samples also have good coverage uniformity with a median value of 90.6%. A total of 104 variants were identified, including SNVs and indels. The limits of detection for the variants of *ATM*, *CREBBP*, *KMT2D*, *TP53*, *MYD88,* and *NOTCH1* ranged from 0.5% to 1.56%, with observed variant allele frequency (VAF) of 0.86%, 0.68%, 0.7%-2.7%, 1.29%-1.83%, 1.18%-1.52%, and 0.5%, respectively. Analysis of precision showed the observed average VAFs had low variability (SE: 0.15%-1.40%) in the 10 SNVs and one indel with both low (<4%) and higher mutation burdens (30%-45%). In the inter-run study with four independent runs, the average observed VAFs range from 0.6%-40.1% with SE of 0.19%-0.78% across all evaluated 10 SNVs and four indels, demonstrating excellent inter-run reproducibility. The accuracy study showed 100% concordance between CAPP-Seq and orthogonal assays. **Conclusions:** We conclude that this panel demonstrates excellent performance characteristics with ability to detect SNVs and indels of as low as 0.5%-1% on average, and it has recently been conditionally approved for clinical use by New York State. For hematologists and hematopathologists, it should serve as an excellent tool to improve clinical management of patients with B-cell lymphoid malignancies.

H062. **Utility of Molecular Testing for Subtyping of New Precursor B-Cell Neoplasm Entities (WHO-HAEM5 Classification, 2022): A Single Center Experience**

K. Reddy
Northwell Health System, Mineola, NY.

Introduction: Though the majority of precursor B-cell neoplasms are classified in WHO-HAEM5 (2022) according to ploidy changes, chromosomal rearrangements, or the presence of other genetic drivers, molecular genetic subtyping is required for some entities based on the current requirements. We highlight our experience at a single center identifying newly included entities of B-cell lymphoblastic leukemias/lymphomas over a period of 4.5 years in adult and pediatric patients. **Methods:** Next-generation sequencing was performed on fresh blood/marrow aspirates at an external CLIA-certified, CAP-accredited laboratory. The hematology panel sequenced DNA of 406 genes, introns of 31 gene rearrangements, and RNA of 265 genes. Successful comprehensive sequencing was completed in 116 out of 121 B-lymphoblastic leukaemia/lymphoma (B-ALL) patients (ages 8 - 57 years). **Results:** Focusing only on newly included subtypes of B-ALL, we noted the following categories in our cohort: 1) B-ALL with *ETV6::RUNX1*-like features (n=1) was identified in an 18-year-old male; 2) B-ALL with *TCF3::HLF* fusion (n=3) with concurrent *BLM/CHEK2/LRPIB* mutations in 16-19-year-old males and very poor prognosis; 3) B-ALL with *BCR::ABL1*-like features (now an entity, previously provisional). Under the category of B-ALL with other defined genetic abnormalities the following are included: B-ALL with 1) *DUX4*-rearranged (n=3) in two females and one male (aged 19-27 years) with *KRAS/NRAS/PTPN11* mutations. This subtype is associated with excellent prognosis even with poor prognostic co-occurring mutations like *IKZF1*; 2) *MEF2D*-rearranged (n=0); 3) *ZNF384* (n=4) with *EP300* and *TCF3* in 14-17-year-old males showing *FLT3* D835V and *RAS/PTPN11* mutations, considered as intermediate prognosis; 4) *NUTM1* rearranged (n=0); 5) *IG::MYC* fusion; 6) with PAX5alt or *PAX5* P80R mutation (n=2) of intermediate prognosis, seen with co-occurring *NRAS* Q61L mutation in an eight-year-old female. **Conclusions:** Clinical molecular testing in our cohort revealed these gene alterations mainly in pediatric patients and enabled diagnosis, prognosis, and risk

stratification allowing the use of clinically actionable therapeutic targets in some cases. It also contributed toward a useful data set for further analysis and potential for novel drug development. Longer-term follow-up incorporating therapy and outcomes information would be valuable.

Infectious Diseases

ID001. **Clinical Evaluation of the Accelerate Arc Module and BC Kit for Isolation of Microorganisms from Positive Blood Culture Broths and Suitability for MALDI-ToF Analysis**

B. Buchan[1], A. Cruz[2], M. faron[2], D. Gerstbrein[2], b. Mesich[2], B. Sullivan[3]
[1]The Medical College of Wisconsin, Children's Wisconsin, Brookfield, WI; [2]The Medical College of Wisconsin, Milwaukee, WI; [3]Accelerate Diagnostics, Tucson, AZ.

Introduction: Accurate identification of bacterial and fungal organisms in positive blood culture broths (PBCBs) enables appropriate antibiotic selection and is central to reducing morbidity and mortality associated with bloodstream infections. MALDI-ToF provides rapid identification of cultured isolates; however, it requires purification of microorganisms from PBCBs prior to analysis. We conducted a clinical performance evaluation of the Accelerate Arc module and BC kit (Accelerate Diagnostics, US) for automated sample preparation from PBCBs for subsequent MALDI-ToF analysis. **Methods:** We enrolled 50 prospective clinical PBCBs (VersaTREK, Thermo Fisher, US) and augmented these with 25 seeded PBCBs to evaluate less common organisms. Following positivity, a 1.5-2.0 mL portion of each PBCB was loaded to the Arc capsule and inserted with the reagent cartridge into the Arc module for automated processing (~70 min.). Following processing, 1 µL of processed sample was spotted in triplicate for MALDI-ToF (Bruker, RUO library) analysis. In parallel, each broth was plated to solid culture medium and incubated overnight. Resulting colonies were spotted in triplicate for MALDI-ToF analysis. The final identification, mean scores, and standard deviation were compared between methods. **Results:** Among prospective and seeded PBCBs, 94.6% of plate grown isolates generated high confidence scores (≥2.0), 4.0% generated low confidence scores (1.7-1.99), and one culture failed to grow when plated to solid medium. In comparison, microorganisms isolated directly from PBCBs using Arc yielded 68.0% with high confidence scores, 20.0% with low confidence scores, and just 12.0% with failed identifications (score <1.7). Importantly, 98.5% species level ID agreement was observed between plate grown organisms and Arc processed samples regardless of score. This suggests the use of a high confidence score threshold of 2.0 for species-level reporting may be overly conservative. The single species level discordance was reported as *Aeromonas jandaei* (1.96) following Arc processing and *Aeromonas veronii* (2.16) by direct colony analysis. Among nine PBCBs with failed ID following Arc processing, the processed sample was noted to be pink in 33.3% cases, suggesting the presence of residual red blood cells. Of note, use of Arc enabled identification of *Prevotella* spp. in the PBCB that failed to grow on solid medium. The average MALDI-ToF score for organisms identified by Arc was 0.17 lower than the direct colony scores, which is only slightly outside two standard deviations of the precision of replicate tests (0.10). **Conclusions:** The Arc enables efficient automated isolation of microorganisms from PBCBs which can in turn be subjected to MALDI-ToF analysis without the need for subculturing.

ID002. **Confirmation of Aptima-Positive *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* Samples Using Extracted Nucleic Acid in the Xpert CT/NG Assay**

A. Rahman[1], D. Cuevas[1], M. Ha[1], T. Luong[1], A. Rector[1], A. Garcia[2], T. Ton[1], J. Dunn[3]
[1]Texas Children's Hospital, Houston, TX;, [2]Baylor College of Medicine, Houston, TX; [3]TCH/Baylor College of Medicine, Houston, TX.

Introduction: Because of the overall low prevalence of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) among children, the CDC recommends that all specimens that are initially positive be confirmed either by retesting the original specimen or obtaining another. Our lab performs initial testing using the Aptima Combo 2 (AC2) Assay on the Panther System (Hologic, San Diego, CA) and previously used the individual CT and NG Aptima assays on the DTS System (Hologic) for confirmation in children less than 14 years of age or in cases of suspected sexual assault. Since the DTS platform is no longer supported, we evaluated the performance of the Xpert CT/NG assay as a confirmatory nucleic acid amplification test for specimens initially positive using the AC2 Assay from patients younger than 14 years of age or in cases of suspected sexual assault. The Xpert CT/NG assay (Cepheid, Sunnyvale, CA) is a qualitative *in vitro* real-time PCR test for the automated detection of genomic DNA targets in CT and NG that are distinct from those in the AC2 Assay. **Methods:** A total of 89 samples (55 urine and 34 swabs) in Aptima collection tubes were evaluated. These consisted of 59 retrospectively collected patient specimens and 30 samples contrived using NATtrol CT and NG control material (Zeptometrix, Buffalo, NY). Specimens in Aptima collection tubes are incompatible for testing with the Xpert CT/NG assay. Therefore, 1 mL of Aptima tube sample material was extracted on the NucliSENS easyMAG (bioMerieux, Durham, NC) and eluted in 100 µL of buffer. Nine-hundred microliters of nuclease-free water was added to the eluate, mixed, and added to the Xpert CT/NG cartridge per the manufacturer's instructions. **Results:** The overall sensitivities of detection using the material extracted from the Aptima collection tubes using the easyMAG and tested by the Xpert CT/NG assay were 86.1% for CT and 88.6% for NG. Of 13 patient specimens with co-infection from the AC2 assay, CT was detected in 12 and NG in 10 using the easyMAG/Xpert CT/NG procedure. In patient samples with only a single target detected by the AC2 assay, all CT and NG false-negatives from the easyMAG/Xpert CT/NG procedure had RLU values <1,000. Clinical specificities and reproducibility were 100% for both CT and NG using the easyMAG/Xpert CT/NG procedure. **Conclusions:** Nucleic acid extraction of CT- and/or NG-positive samples from Aptima collection tubes with application of the eluate into the Xpert CT/NG test system is an effective process for the confirmatory testing required for young children or suspected sexual assault victims. Confirmation test false-negatives can occur in some samples with AC2 RLU values <1,000.

ID003. **Prevalence of Polymicrobial Urinary Tract Infections: Molecular Diagnostic Approach**

M. Shanmugam, R. Elliott, S. Khwaja, K. Mayilvahanan, M. Habib, L. Pham, A. Johnathan lee, M. Akhtar, C. Podszus, T. Fernandes, S. liao, F. Lach, Y. Rashed, A. Hughes, L. Christ, C. Tobia, S. Qaiser, M. Leger, D. Goldberg, T. Schutzbank, M. Bon Homme
Acutis Diagnostics Inc., Hicksville, NY.

Introduction: Urinary tract infections (UTIs) are the most common bacterial infections, impacting approximately 150 million individuals globally. UTI-related spending is estimated to cost the US healthcare system ~$3.5 billion annually. Recent clinical studies point toward the polymicrobial nature of UTIs. Identification of polymicrobial infection among different populations may be an important factor for balancing the risk and benefits of prescribing antibiotics. To assist clinicians, a rapid, robust assay for the accurate identification of key UTI-associated pathogens was developed and validated. **Methods:** From May 1, 2022, to June 15, 2022, 3,325 urine samples were collected consecutively. Pathogen identification and antimicrobial sensitivity testing were ordered by a licensed clinician during the course of

routine patient care. All specimens were tested in a CLIA licensed laboratory. The samples were interrogated using a nucleic acid amplification assay designed to detect 31 pathogens (14 gram negative, 14 gram positive, and three yeast). The culture and sensitivity were performed on an automated system. Data sets including demographic information (age and gender) were collected from the laboratory information system and de-identified. **Results:** The overall PCR assay positivity rate was 30% (1,013/3,325). The female positivity rate was 82% (830/1,013). The male positivity rate was 18% (183/1,013). UTIs were observed in the elderly population (>66 years): males 67% (123/183) and females 33% (278/830). The prevalence of the fastidious organism *Aerococcus urinae* among the elderly population was 27% (33/123) for males and 32% (89/278) for females. The antimicrobial sensitivity data showed that *A. urinae* was resistant to levofloxacin 29% (12/41). The total number of polymicrobial infections detected by PCR was 37% (375/1,013), whereas culture identified only 12% (122/1,013). *Escherichia coli* was associated with 54% of polymicrobial infections (202/375). *E. coli* was associated with *A. urinae* 28% (56/202), *Enterococcus species* 45% (90/202), *Klebsiella species* 4% (8/202), *Corynebacterium* 7% (15/202) and *Candida species* 3% (6/202). *E. coli* was resistant to ampicillin 47% (166/352), ciprofloxacin 24% (86/352), and TMP-SMX 21% (74/352). **Conclusions:** We have developed a robust assay that detects 25% more polymicrobial infections compared to routine culture. The identification of polymicrobial infections along with antimicrobial sensitivity data will assist clinicians providing personalized treatment resulting in better patient outcomes. Widespread adoption of this approach should improve antibiotic stewardship.

ID004. **Limit of Detection Comparison of Two Molecular Extended Enteric Bacterial Panel Assays to Culture Methods for the Detection of *Y. enterocolitica, P. shigelloides*, and *V. parahaemolyticus* in Stool**

M. Faron[1], C. Clark[2], A. Cruz[1], A. Jiang[2], B. Mesich[1]
[1]The Medical College of Wisconsin, Milwaukee, WI; [2]Hologic Inc, San Diego, CA.

Introduction: Enteric pathogens cause a significant burden to US hospital systems, with >50,000 hospitalizations a year. Traditional methods of bacterial culture are labor intensive, take days to obtain actionable results, and can be difficult to isolate pathogens due to the abundance of commensals. Molecular panels have greatly increased turnaround time and improved sensitivity with previous reports identifying common pathogens as low as 10^3CFU/mL, whereas detection rates for culture are typically below 90% at 10^5CFU/mL. The current study evaluated the performance of two molecular GI assays and culture for detection of *Yersinia, Plesiomonas*, and *Vibrio*. **Methods:** To evaluate sensitivity, three isolates for each pathogen were tested. Isolates were serially diluted in saline and spiked into nine Cary-Blair stools from unique donors ranging from 10^7-10^3CFU/mL and confirmed by plate counts (saline dilutions). Stool matrices were de-identified patient specimens sent for clinical GI testing and were molecular negative for all three pathogens. Each isolate and dilution were tested in triplicate using bacterial culture and two molecular assays, Hologic's Panther Fusion GI Expanded Bacterial Panel in development and BD Max Extended Enteric Bacterial Panel. Bacterial culture included plating to Blood, MacConkey, CIN, and TCBS agar. Cultures were reviewed (blinded) by a medical technologist and resulted as positive for targets at the lowest concentration of each replicate, which was confirmed by MALDI-TOF MS. Molecular testing (blinded) followed manufacturer's recommendations and results were reported as a percentage of positive detections at each concentration. **Results:** The Hologic assay was the most sensitive among the three methods and was 100% sensitive for all three targets down to 10^4CFU/mL. At 10^3CFU/mL, detection on the Hologic assay ranged from 44%-93% of samples tested. BD Max was less sensitive, with 100% detection down to 10^5CFU/mL for all samples tested, with the exception of one strain of *P. shigelloides* that was 100% at 10^7CFU/mL. Bacterial culture was the least sensitive of the methods used. Selective agar was essential for detection of pathogens with *Y. entercolitica* detected at 100% down to 10^5CFU/mL. *V. parahaemolyticus* was detected at 85% at 10^7CFU/mL using TCBS agar, and *P. shigelloides* was only detected in 11% of the specimens from MAC agar. **Conclusions:** Similar to previous reports, molecular stool panels were more sensitive than selective agar culture by 1-2 logs. The ability of culture to Identify these pathogens from commensals on MAC or Blood agar was nearly impossible, which was demonstrated in the lack of *P. shigelloides* detection. The Hologic Panther Fusion GI Expanded Bacterial Panel in development provides an additional tool for rapid and sensitive detection of enteric pathogens.

ID005. **A Cellular Host Response Test May Provide a Window to Immune Dysregulation in Infected Patients with Elevated SOFA Scores Enabling Rapid Sepsis Risk Stratification**

H. O'Neal[2], R. Sheybani[1], H. Tse[1], A. Shah[1], C. Thomas[2]
[1]Cytovale, Inc., San Francisco, CA; [2]LSU Health Sciences Center, Baton Rouge, LA.

Introduction: Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer, 2016), is a common and costly condition encountered in US emergency departments (ED). Advancements have been made in diagnostic tools to determine the presence of infection, along with scoring systems (e.g., the sequential organ failure assessment (SOFA) score) to assess organ dysfunction. However, currently available solutions for sepsis detection cannot distinguish between chronic and acute organ dysfunction, assess whether the organ dysfunction has an explanation other than infection, or attribute dysfunction specifically to a dysregulated host response (Seymour, 2016). The objective of this study was to evaluate an investigational rapid cellular host response test as a tool for appropriate sepsis risk stratification in those with confirmed infection and organ dysfunction per the SOFA criteria. **Methods:** The IntelliSep test is an investigational *in-vitro* diagnostic that quantifies the state of immune activation by measuring the biophysical properties of leukocytes from a routine whole blood sample in less than 10 minutes. The result is the IntelliSep Index (ISI), ranging between 0.1-10.0, stratified into three discrete interpretation bands of increasing disease severity risk: Green, Yellow, and Red. Adult patients presenting to the ED with signs or suspicion of infection were prospectively enrolled in four discrete but similar cohorts at multiple sites in the US (Feb. 2016-Apr. 2020). All patients had ISI performed and were followed by retrospective chart review for outcome information and sepsis adjudication. Treating clinicians did not have access to assay results. Only those determined to be both infected and have a SOFA ≥2 on day of enrollment were included in the final analysis. **Results:** A total of 176 study patients (sepsis prevalence 76.7%) were stratified by the ISI as 36 (20%) Green, 41 (23%) Yellow, and 99 (56%) Red. The test achieved a positive percent agreement (sensitivity) of 92.6 (86.8-96.4, 95% CI), Negative Percent Agreement (specificity) of 82.9 (74.7-88.3, 95% CI), and Red to Green Diagnostic Odds Ratio of 34.2 (24.3-43.4, 95% CI). Compared to those in the Red band, Green band subjects had significantly lower mortality ($p < .05$), blood culture positivity ($p < .001$), and hospital length of stay among survivors ($p < .05$). Additionally, severity of illness, as measured by the need for ICU level of care, trended positively with the ISI bands. **Conclusions:** The ISI, a rapid, quantitative measure of immune activation, may offer ED clinicians insight into the presence of dysregulated host response in patients presenting with infection and increased SOFA scores to aid in rapid sepsis risk stratification and guide appropriate treatment.

ID006. **Molecular Detection of Emerging Carbapenemases from Rectal Swab Colonization Screenings in the Northeast**

K. Cummings, S. Morris, L. Rose, L. Leach, J. Bodnar, C. Jacobsen, K. Wahl, C. Prussing, K. Musser, M. Rowlinson, E. Nazarian
Wadsworth Center NYSDOH, Albany, NY.

Introduction: The detection of carbapenemase-producing organisms (CPOs) can facilitate the mitigation of rapid containment and reduced transmission in healthcare facilities. The Wadsworth Center (WC) performs testing for CPO colonization screenings (CS) throughout the Northeast (NE) as part of the Antimicrobial Resistance (AR) Lab Network to detect the "Big Five" carbapenemases (*KPC, NDM, OXA-48, VIM*, and *IMP*) from rectal swabs using the Cepheid Xpert Carba-R (Carba-R) assay. Carba-R cannot detect all IMP variants, or other emerging carbapenemases (*OXA-23, OXA-24/40, OXA-58, SIM, SPM*, and *IMI*). CS to identify emerging carbapenemases currently requires culture which is labor intensive and increases time to identification to one to two weeks. WC validated three multiplex real-time PCR assays to be performed on CPOs as a rapid method for identification of novel carbapenemases from rectal swabs: "OXA" (OXA-23, OXA-24/40, OXA-58), "IMP" (IMP), and "Novel" (SIM, SPM, and IMI). PCR-positive swabs are cultured to recover the CPO for further characterization. **Methods:** Dual rectal swabs are collected for CS. One swab is tested with Carba-R and if positive, the remaining swab is used for culture. Remaining Carba-R lysis reagent is used for DNA extraction with the addition of an internal control. Extracted DNA is tested in the appropriate PCR (OXA, IMP, or Novel). PCR-positive swabs are inoculated to CHROMagar, mSuperCARBA, and MacConkey broth. Suspect colonies are isolated to blood agar and tested by PCR for gene confirmation. Once confirmed, organism identification, phenotypic carbapenemase-production, and antimicrobial susceptibility testing are performed. Additionally, whole-genome sequencing is performed which provides the gene variant, and multi-locus sequence type. **Results:** A total of 1,370 swabs were tested by either OXA, IMP, or the Novel PCR from January 2019 to June 2022. Carbapenemase genes were detected in 138/1,370 (10%) followed by culture isolation of 90/138 (69%). The majority (1,320) of CS requests were for *OXA*, where 130 (*OXA-23*) and four (*OXA-24/40*) were detected. Eighty-two of the 130 (63%) OXA-23 and four (100%) of the OXA 24/40 were isolated from culture. Only 48 rectal swabs were tested by the IMP PCR with three positives and isolated from culture. Two swabs were tested by the Novel PCR with one positive for IMI and isolated. Time to results were 24 to 48 hours. **Conclusions:** The use of the OXA, IMP, and Novel PCR assays for detection of emerging carbapenemase genes from rectal swabs has enhanced our CPO CS capacity. This workflow decreases turnaround-time to result and streamlines culture isolation and additional testing to provide data to prioritize infection prevention measures.

ID007. **Multi-Site Validation of EDTA Blood as a Sample Type for SeptiCyte RAPID CE-IVD, a Near-Patient Diagnostic Test for Sepsis on the Biocartis Idylla System**

J. Kirk[1], M. Parcina[2], M. Vasse[3], I. Hannet[1]
[1]Immunexpress, Inc, Seattle, Washington; [2]Universitatsklinikum Bonn, Bonn, Germany; [3]Hospital Foch and UMRS1176, Suresnes, France.

Introduction: The SeptiCyte RAPID test is an FDA 510(k) cleared, CE marked gene expression assay using RT-qPCR to quantify the relative expression levels of host response genes isolated from whole blood in patients suspected of sepsis. The test generates a continuous score between 0-15 (SeptiScore) that falls within one of four discrete interpretation bands based on the increasing likelihood of infection-positive systemic inflammation (sepsis) in a single-use cartridge format processed in about one hour. The test was initially validated for use with PAXgene Blood RNA tubes; however, these tubes are not commonly used in clinical practice. The purpose of this study was to validate the use of undiluted EDTA blood as an additional sample type for the SeptiCyte RAPID CE-IVD test. **Methods:** EDTA blood and PAXgene Blood RNA tubes were simultaneously collected from a total of 46 suspected sepsis patients at two European hospitals (n=26, Hospital Foch, France – "Foch"; and n=20, Universitatsklinikum Bonn, Germany – "Bonn"). Bonn additionally performed reproducibility testing using three replicate EDTA samples. Due to phlebotomy limitations at Foch, PAXgene samples were not drawn directly into a PAXgene tube but were prepared upon arrival at the laboratory by diluting EDTA blood in PAXgene stabilizing reagent (1:2.75). All samples were tested with SeptiCyte RAPID cartridges capable of processing either 900 µL diluted PAXgene or 240 µL EDTA whole blood. **Results:** Both clinically confirmed sepsis and non-sepsis patients were studied. A high degree of correlation (R^2 = 0.95) was observed when comparing the average SeptiScore for EDTA blood samples to results from PAXgene samples. SeptiScore values were consistent between EDTA blood and PAXgene samples with an average absolute difference of 0.39 score units, when calculated over all 46 clinical patients. Replicates for EDTA blood samples tested were also highly consistent, with the SeptiScore standard deviation less than 0.5 score units for all samples (n=60). Results from EDTA blood samples diluted in PAXgene stabilizing reagent (Foch, n = 26) were strongly correlated (R^2 >0.9) with undiluted EDTA blood samples. EDTA blood samples maintained consistent SeptiScore values within 0.5 unit for at least six hours prior to testing. **Conclusions:** The results of this study confirm the equivalence of PAXgene and EDTA blood sample types in the clinical setting when processed with the SeptiCyte RAPID CE-IVD cartridge. Combined with the small 240 µL blood volume requirement and the convenient and long storage conditions, the addition of the widely used EDTA blood tube as a validated sample type has the potential to greatly enhance the clinical utility of this new near patient sepsis diagnostic.

ID008. **Validation of the DiaSorin LIAISON MDX Platform for Qualitative Real-Time PCR Detection of *Candida auris* in Hospital Inpatients**

M. Ahmed[1], M. Sperling[1], K. Van Benten[2], R. Relich[2], K. Gavina[2]
[1]Indiana University Health, Indianapolis, IN; [2]Indiana University School of Medicine, Indianapolis, IN.

Introduction: *Candida auris* is an emerging, often multidrug resistant, opportunistic fungal pathogen that causes nosocomial outbreaks. For infection control purposes, pre- or post-admission patient screening is becoming commonplace and is often accomplished by cultivation-based testing of swabs obtained from various anatomic sites (e.g., axial and inguinal skin). This approach is labor- and time-intensive, and is relatively insensitive. These drawbacks demonstrate a need for a rapid, highly sensitive molecular diagnostic solution. The purpose of this study was to investigate the merit and implementation of quantitative PCR (qPCR) as an alternative to culture-based *C. auris* surveillance. **Methods:** We evaluated the DiaSorin *C. auris* analyte-specific reagent using the LIAISON MDX platform and compared results to fungal culture. Analytical sensitivity was determined using serial dilutions of culture-quantified *C. auris* (107–10-1 colony forming units [CFU]/mL); the limit of detection (LOD) was determined by probit analysis with a 95% confidence interval. Analytical specificity was determined by screening for *C. auris* in no-template controls, non-*C. auris* specimens, and common skin microorganisms. Precision was assessed by measuring the coefficient of variation (%CV) with multiple replicates of a quantitated *C. auris* control. Clinical sensitivity and specificity are currently being assessed through a prospective study screening for the prevalence of *C. auris* among hospital inpatients within the Indiana University (IU) Health System, in which full clinical results will be made available at the completion of the study. **Results:** The LOD of the *C. auris* qPCR assay was 879.5 CFU/mL with an analytical specificity of 100.0%. Coefficients of variation for the *C. auris* target and internal control were 2.3% and 1.1%, respectively. As of abstract submission, a total of 74 patients have been screened for *C. auris* as part of the prospective study at IU; clinical sensitivity and specificity of *C. auris* qPCR were determined to be 100.0% compared to fungal culture. From receiving the specimen to generating a result, the average turnaround-time (TAT) for qPCR was approximately 2.5 hours, whereas specimens assessed by fungal culture required

incubation for a minimum of three days before reporting a negative result. **Conclusions:** Our findings suggest that qPCR provides a superior alternative to culture for screening inpatients for *C. auris* colonization. qPCR offers a more sensitive and labor-saving approach based on LOD, analytical specificity, precision, and process TAT. Improved screening methods will directly, and positively, impact infection prevention and control, patient care, and laboratory throughput.

ID009. **Validation of Real-Time PCR Assays for the Rapid Detection of Vancomycin Resistant *Staphylococcus aureus* and Vancomycin Resistant *Enterococcus* in Nares/Axilla/Groin Surveillance Samples**

L. Leach, E. Nazarian, G. Nattanmai, K. Musser, M. Rowlinson, K. Mitchell
Wadsworth Center NYSDOH, Albany, NY.

Introduction: Vancomycin-resistant *Staphylococcus aureus* (VRSA) is an emerging public health concern in the United States. In the last two decades 16 VRSA cases have been reported, and typically these patients are also colonized with vancomycin-resistant *Enterococcus* (VRE). VRSA can spread through close contact with infected patients or contaminated material, so detection is important for infection control. Patients may be colonized and asymptomatic; therefore, screenings should be performed to detect and prevent VRSA exposure to staff and other patients in healthcare facilities. Implementation of a screening protocol to allow for the rapid identification of VRSA is valuable for healthcare facilities where there is suspected or a high risk of transmission. Since individuals with VRSA infection may be colonized with VRE, it is critical that screening methods can link the resistance genes to the organism. In this study, two real-time PCR assays were developed and evaluated for the rapid detection of VRSA and VRE in nares/axilla/groin surveillance samples in conjunction with conventional culture methods. **Methods:** Two multiplex real time PCR assays targeting the resistance genes *vanA*, *vanB,* and *mecA*, and targeting the organisms *S. aureus* (*nuc* gene) and *E. faecalis* and *E. faecium* (*ddl* gene) were evaluated for use with nares/axilla/groin ESwabs. DNA was extracted on an eMAG instrument following off-board lysozyme treatment and lysis in easyMAG Lysis Buffer. A spiked matrix was used to determine the PCR assays' performance, after which nares/axilla/groin samples were evaluated from both healthy and high-risk populations. Samples were also cultured on CHROMagar *Staph aureus*, 5% sheep blood agar, and Columbia CNA Agar. Subsequent isolates were tested using both assays to confirm which resistance genes were present in the organisms. **Results:** The limits of detection for all targets in the two real-time PCR assays were lower than 100 CFU/PCR reaction. Initial testing of the high-risk population group yielded a 20% positivity rate for *vanA, nuc*, and *E. faecalis* suggesting the possible presence of VRSA. Culture of those samples combined with real-time PCR showed that none of the samples contained VRSA, but rather VRE. None of the samples from the healthy population were positive for *vanA.* **Conclusions:** The validation of these two real-time assays and screening results from their application to selected patient populations indicate that these are powerful tools for the direct detection of the resistance genes and bacteria when used in conjunction with culture methods. The accurate and rapid screening for VRSA allows for the implementation of infection control mitigation measures for these resistant bacteria in healthcare facilities.

ID010. **Towards a High-Throughput Workflow Using the LGC sbeadex Pathogen Nucleic Acid Purification Kit for Studying Common Human Pathogens in Multiple Sample Matrices**

E. von der Heide[1], M. Siewert[2], A. Brinckmann[1], C. Dowman[3], M. Hoffmann[1], M. Kohlenberg[1], J. Weiser[1], H. Hauser[1]
[1]LGC Biosearch Technologies, Berlin, Germany, [2]LGC Biosearch Technologies, Middleton, WI; [3]LGC Biosearch Technologies, Hoddesdon, United Kingdom.

Introduction: Being able to streamline efficient nucleic acid purification from different pathogens in various sample matrices into a purification workflow is crucial for high-throughput methods. LGC, Biosearch Technologies has verified the sbeadex Pathogen Nucleic Acid Purification Kit for the purification of nucleic acids from yeast, various bacterial and viral pathogens, including SARS-CoV-2 in the background of multiple sample matrices. Here, we show that all tested pathogens and sample matrices could be streamlined into a single protocol with an optional and adjustable lysis step making the kit suitable for high-throughput pathogen detection workflows. **Methods:** We conducted a verification study with commercially available viral and bacterial panel reference materials spiked into various human matrices and purified nucleic acids using the automated purification platform KingFisher Flex (Thermo Fisher Inc.). We particularly investigated suitable lysis time durations (0, 3, 10, 20 min) for each sample matrix spiked with SARS-CoV-2 and cytomegalovirus extraction standards, respectively, as a detection model with subsequent detection of each pathogen using (RT-)qPCR. Moreover, we compared our extraction kit with market-leading competitor kits. Automated extraction was also carried out on the LGC oKtopure purification platform for several viral and bacterial targets in serial dilutions to evaluate extraction efficiency on this high-throughput platform. **Results:** We show that, depending on sample matrix and pathogen, the lysis step could be either omitted (UTM, urine) or performed for 3 min (plasma, serum) or for ≥10min (stool) at elevated temperature. Interestingly, pathogenic DNA in whole blood could be detected reliably for all analyzed time points but pathogenic RNA was most efficiently detected when limiting the lysis time to 3 min in the background of whole blood. By showing linear correlation reflecting serial dilutions, we demonstrate efficient purification of multiple bacterial and viral pathogens involved in, e.g., respiratory diseases and pneumonia (SARS-CoV-2, influenza, adenovirus, RSV, mycobacterium tuberculosis, etc.) using both LGC oKtopure and KingFisher Flex. Equivalent performance of our products was shown compared to competitor extraction kits and 1-Step RT-qPCR systems. **Conclusions:** The sbeadex Pathogen Nucleic Acid Purification Kit is compatible with multiple sample matrices delivering high-quality pathogen RNA and DNA suitable for multiple downstream analyses. The lysis step can be individually adjusted to the respective matrix and pathogen of interest while using a single purification protocol starting from nucleic acid binding step in a high-throughput purification manner.

ID011. **Development and Performance Evaluation of a Multiplex Real-Time PCR Panel for the Qualitative Detection and Differentiation of Enteric Bacterial Pathogens**

C. Wei, G. Wu, S. Gregory, M. Tanner, I. Pagani, E. Swartzman, R. Cao, N. Pabbati, G. Govoni, S. Leow, P. Hoener, M. Gandhi, K. Li
Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Bacterial enteritis represents the most common cause of infective gastroenteritis, worldwide, especially in adults. The TaqPath Enteric Bacterial Select Panel (CE-IVD) is a multiplex real-time PCR test designed to detect and differentiate the most common bacterial causes: *Campylobacter (jejuni, coli, and upsaliensis)*; *Salmonella* spp.; *Shigella* spp./*enteroinvasive E. coli (EIEC),* in a single reaction. In this study, we evaluated the analytical and the clinical performance of the TaqPath Enteric Bacterial Select Panel (Enteric Panel). **Methods:** Sample extraction was performed using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit with the Microbiome Bead Plate on the KingFisher Flex and bead beater

instrument. PCR was performed on the QuantStudio 5 Dx system and data were analyzed using the Pathogen Interpretive Software. Analytical validation (AV), including limit of detection (LoD), linearity, and precision, was performed using contrived stool samples from pooled negative stool specimen and cultured bacteria. Clinical validation (CV) was performed using banked and prospectively collected remnant stool samples from patients with suspected gastrointestinal disease from three sites: Germany, France, and Ivory Coast. The RIDA GENE Bacterial Stool Panel I was used as a comparator. Clinical sensitivity and specificity were determined after arbitration testing of discordant samples using a different set of primers and probe that bind to unique genetic sequences of each target. **Results:** The LoD of the Enteric Panel was between 20 to 500 CFU/mL for all seven bacteria pathogens, which equates to 0.7 to 10.8 genomic copies/well. Linearity studies showed dynamic range of the Enteric Panel from 10 to 10^7 GCE/reaction. Precision studies showed 98.7%-100% PPA at 1.5x LoD and 99.3%-100% at 3x LoD with 100% NPA for the negative panel. For CV, 395 samples were included in the study. After arbitration testing of discordant samples, the clinical sensitivity and specificity of TaqPath for each of the targets were as follows: *Campylobacter* spp. (97.9% and 100%); *Salmonella* spp. (100.0% and 97.8%); *Shigella* spp./*EIEC (*100.0% and 100.0%). The Enteric Paneltest also detected 19 coinfections (10 *Campylobacter*/*salmonella*; six *Campylobacter*/*Shigella*, and three S*almonella/Shigella*) and two triple infections in the CV study. **Conclusions:** The TaqPath Enteric Bacterial Select Panel is a highly accurate method for the detection and differentiation of the most common enteric bacterial pathogens including identification of coinfections. With a quick turnaround time of less than two hours from sample extraction to report, molecular detection of enteric bacterial pathogens significantly reduces time-to-result as compared to traditional culture-based methods.

ID012. **The Gut Microbiome in Autism Spectrum Disorder**

M. Dadlani, K. Moffat
CosmosID, Germantown, MD.

Introduction: In the United States, autism spectrum disorder (ASD) affects one in 44 children, an increase in prevalence of >150% from 2000 to 2021. Unfortunately, there are no approved therapies that address the core ASD symptoms, and in the United States this disease drives $268 billion in costs per year. Since more than 30% of those with ASD report gastrointestinal symptoms, the role of the gut microbiome in this disease is of increasing interest. Examination of the gut microbiome in ASD may lead to identification of microbial differences that may drive symptoms characteristic of ASD. Most studies of the ASD gut microbiome have employed 16S rRNA sequencing and analysis. This technology is limited in its ability to fully characterize the microbiome since only one gene is evaluated. Whole genome shotgun sequencing utilizes the entire genomic material of microorganisms present in a sample, allowing a broader range of species detection as well as increased specificity and accuracy. Further, whole-genome sequencing results are not complicated by PCR amplification bias. **Methods:** To address the need for a more complete and comprehensive analysis of the gut microbiome in ASD, we employed whole genome shotgun sequencing-based metagenomics of stool samples from children affected by ASD. This enables evaluation of bacterial strain level differences between children affected by ASD and neurotypical children. We have observed differences in occurrence of pathogenic bacteria and commensal bacteria between these two groups. Additionally, we have compared incidence of antimicrobial resistance genes, virulence factors, fungi, protists, and functional profiling of genes and pathways between children with ASD and neurotypical children. **Results:** Differential analysis at the genus level showed increases in abundance of *Akkermansia, Adlercreutzia, Collinsella, Coprococcus, Dorea, Prevotella, Clostridia*, and *Blautia* in children with ASD and decreased *Bacteroides, Megasphaera*, and *Lactobacillus*. Additionally, functional analysis revealed children with ASD have lower levels of the genes and pathways involved in short chain fatty acid production compared to non-affected children. **Conclusions:** By further studying these microbial signatures in ASD, we will improve understanding of how the microbiome can be employed in symptom management and early diagnosis and treatment of this condition.

ID013. **Performance Evaluation of COVID-19 Ag Home Test for the Diagnosis of COVID-19**

Y. Kim[1], S. Kim[2]
[1]Samsung Changwon Hospital, Changwon, Republic of Korea, [2]Gyeongsang National University Changwon Hospital, Changwon, Republic of Korea

Introduction: Control of SARS-CoV-2 outbreak through a gold standard detection, i.e., real-time polymerase chain reaction (RT-PCR), becomes a great obstacle, especially in the Omicron pandemic situation, due to lack of reagents and experienced medical personnel. Rapid antigen home test (RAHT) may be useful to supplement RT-PCR when prevalence of COVID-19 is surged. We evaluated the performance of RAHT compared to RT-PCR to observe the eligibility of RAHT in the community. **Methods:** Seventy-nine COVID-19-positive and 217 COVID-19-negative patients confirmed by RT-PCR were enrolled for this study. Duration from symptom onset to COVID-19 confirmation of less than five days was considered a recruiting criterion for COVID-19-positive cases. A nasal cavity specimen was collected for the RAHT carried out by each participant, and a nasopharyngeal swab specimen was collected for RT-PCR by attending technicians. **Results:** Sensitivity of the STANDARD Q COVID-19 Ag Home Test (SD Biosensor, Korea), compared to RT-PCR, was 94.94% (75/79) (95% [confidence interval] CI, 87.54-98.60%), and specificity was 100%. Sensitivity was significantly higher in symptomatic patients (98.00%) than in asymptomatic (89.66%) patients (p-value = 0.03). There was no difference according to the duration of symptom onset to confirmation (100% for 0-2 days: 96.97% for 3-5 days, respectively) (p-value=1.00). The RAHT detected all 51 COVID-19 patients whose Ct values were ≤25 (100%), whereas sensitivity was 85.71% (24/28) among patients with Ct values >25 (*p*-value=0.01). **Conclusions:** The RAHT revealed an excellent sensitivity for COVID-19-confirmed cases, especially for those with symptoms. There was a decrease in sensitivity when the Ct value was high, indicating that RAHT screening may be useful during the early phase of symptom onset, when the viral numbers are high and it is more transmissible.

ID014. **Balancing Act in the Face of Variants: Testing Strategies to Minimize Infections and Maximize In-Person Learning in Schools**

T. Proctor[1], O. Sorel[1], M. Gandhi[1], J. Walson[2], K. Tickell[2]
[1]Thermo Fisher Scientific, South San Francisco, CA; [2]Walson Consulting LLC, Seattle, WA.

Introduction: As COVID-19 transitions into an endemic state, school administrators are still concerned about how to best minimize infections to maintain staff and student safety, while, at the same time, maximize school days and reduce days lost to infection and quarantine. Using a mathematical model, we estimated the impact of different testing strategies on COVID-19 transmission and school days lost across different variants. **Methods:** We adapted an existing deterministic compartmental model assuming a school district of 1 million students; 53,000 teachers and staff, with 75 school days per term and a 1% disease prevalence on day 0. A positive test resulted in a 10-day quarantine, during which students were unable to return to class. We used data provided in recent Cochrane and FIND reviews to quantify sensitivity (99% for PCR and 58% for antigen) and specificity (99.5% for PCR and 98% for antigen). We evaluated different testing strategies (symptomatic vs. asymptomatic screening); different screening frequency (every other day, weekly, and every 10 days); different screening methodologies (PCR, antigen, or both) across different transmissibility settings. **Results:** When limited to symptomatic testing, a COVID variant similar to Delta (R_o=1.60) would result in in 600,000 infections over the entire term, whereas with a R_o similar to Omicron (R_o=3.6) >85% of students and staff would become

infected by the midpoint of the term. Expanding any sort of testing (different frequencies of PCR or antigen) to include asymptomatic screening significantly reduced the number of infections as compared to symptomatic-only testing. For a variant similar to Delta, weekly PCR testing reduced the number infected to 100,000 infections, whereas weekly antigen testing resulted in 2X the number of infections over the entire term (200,000). For higher R_o variants such as Omicron, weekly testing was not sufficient to control spread. With such a variant, every-other-day testing with PCR resulted in 200,000 infections and antigen testing resulted in twice as many infections. Antigen testing resulted in significantly more false positive results and school days lost to infection over the term in both scenarios. With a variant like Delta, there were 356,000 lost days of school when PCR testing was used vs. 1 million with antigen testing, and with an Omicron-like variant, there were 318,000 lost days of school when PCR testing was used vs. 894,000 with antigen testing. **Conclusions:** A testing strategy must balance disease transmission and time lost due to false positives in a way that is acceptable to parents, schools, and the community. Implementing a testing program with low diagnostic accuracy may not only not change the epidemic curve but instead result in unnecessary absences from school and lack of confidence in the testing program.

ID015. **Comparison of the Diagnostic Accuracy of Nasal Cavity and Nasopharyngeal Swab Samples for SARS-CoV-2 Detection with Rapid Antigen Test**

Y. Kim[1], S. Kim[2]
[1]Samsung Changwon Hospital, Changwon, Republic of Korea; [2]Gyeongsang National University Changwon Hospital, Changwon, Republic of Korea.

Introduction: Rapid diagnosis of SARS-CoV-2 is critical to detect and prevent spread of COVID-19 efficiently. The rapid antigen test (RAT) has been widely used due to short turnaround time and lower cost compared to the RT-PCR. Nasal cavity swab is not so painful and uncomfortable compared to nasopharyngeal swab. Therefore, we evaluated the accuracy of RAT from nasal cavity and nasopharynx analyzed by sample collection timing and viral load. **Methods:** A total of 71 COVID-19-positive and 104 COVID-19-negative subjects were enrolled. Nasal cavity and nasopharyngeal swab samples taken from each participant were tested using STANDARD Q COVID-19 Ag test (Q Ag, SD Biosensor, Korea). Sensitivities from nasal cavity and nasopharynx were compared using Fisher's exact test. Sensitivity analyses were performed according to days from symptom onset to sample collection (DSO) as well as Ct values of RT-PCR. **Results:** The sensitivity of the Q Ag test was 77.5% (95% confidence interval [CI], 67.8%–87.2%) for nasal cavity specimens and 81.7% (95% CI, 72.7%–90.7%) for nasopharyngeal specimens. The RAT results showed a substantial agreement between two sampling sites. Cohen's kappa index was 0.78. The sensitivity of the RAT for nasal cavity specimens exceeded 89% for <5 DSO and 86% for Ct of *E* and *RdRp* gene <25. **Conclusions:** The Q Ag test performance revealed excellent especially in the early DSO with high viral load, and the nasal cavity swab could be considered as a primary site for RAT of COVID-19.

ID016. **SARS-CoV-2 Variant Identification in Long-Term Viral Shedding and Reinfection Cases at a Tertiary Cancer Center**

K. Jani[1], E. Babady[2], T. McMillen[2]
[1]Memorial Sloan Kettering Cancer Center, Jersey City, NJ; [2]Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: Oncology patients are prone to prolonged SARS-CoV-2 infections. Surveillance for SARS-CoV-2 infection is a common practice at many institutions to identify asymptomatic patients. Additionally, re-infection cases have also been documented. Whole-genome sequencing has been heavily used in this pandemic to identify SARS-CoV-2 variants. The purpose of this study was to determine if patients with prolonged SARS-CoV-2 infections were infected with the same variant (long-term viral shedding [LVS]) or if they were being infected with a different SARS-CoV-2 variant (re-infection). **Methods:** This was a retrospective study that included all patients tested at Memorial Sloan Kettering Cancer Center between March 2020 and June 2022. The samples were tested using multiple for SARS-CoV-2 RT-PCR including a laboratory-developed test and several commercial platforms. All remnant positive samples with Ct value less than 30 were sequenced according to the Scripps PrimalSeq-Nextera XT protocol with modifications and PANGO lineage assigned using Illumina's DRAGEN COVID Lineage app. The data were analyzed for LVS and re-infections. An LVS was described as consecutive positive SARS-CoV-2 test for at least 35 days (five weeks) since the first positive test result. A re-infection was described as repeat detection of SARS-CoV-2 following a negative result for at least 30 days after the last positive sample. **Results:** A total of 13,058 (2.82%) samples resulted as positive out of 462,831 total samples tested for SARS-CoV-2 between March 2020 and June 2022. Fifteen (0.11%) patients were LVS and 75 (0.57%) patients had re-infections. The range of LVS was 36 days to 343 days, with mean of 113 days. Different variants including Alpha, Delta, Iota, Omicron, and some lesser-known subtypes of B.1 were associated with LVS. The range of days to re-infection was 45 days to 798 days, with mean of 380 days. Most re-infection cases (98.66%) were identified as initial infection with Delta and re-infection with Omicron variants. Two out of 75 patients were re-infected more than once with three different variants including B.1 subtypes, Delta, and Omicron. **Conclusions:** As previously reported, LVS was observed in cancer patients with some patients positive for more than 300 days. In all cases, patients were shedding the variant responsible for the initial infection. On the other hand, the majority of re-infection cases were caused by a different variant, primarily Delta to Omicron.

ID017. **Rapid Detection of SARS-CoV-2 Variants by Molecular Clamping Technology Based RT-qPCR**

S. Shen[1], A. Fu[1], M. Jamba[1], J. Li[1], z. Cui[1], L. Pastor[1], d. Cataldi[1], Q. Sun[1], J. Pathakamuri[2], D. Kuebler[2], M. Rohall[2], M. Krohn[2], D. Kissinger[2], J. Neves[2], I. Archibeque[3], M. Powell[4], A. Zhang[1], C. Lu[5], M. Sha[1]
[1]Diacarta Inc, Pleasanton, CA; [2]Franciscan University of Steubenville, Steubenville, OH; [3]Diacarta Inc., Richmond, VA; [4]UCSF, San Francisco, CA.

Introduction: Two years after its initial emergence, the SARS-CoV-2-fueled COVID-19 pandemic continues to spread globally with 6.2 million deaths to date. Despite increasing vaccination levels and a rising number of COVID-19 recovered people who have acquired some degree of natural immunity, the pandemic has continued. Given the challenges that fast-changing SARS-CoV-2 variants have caused in terms of rapid spread and reduced vaccine efficacy, a rapid and cost-effective assay that can detect new and emerging variants is greatly needed worldwide. Toward this end, target-specific RT-PCR screens for specific mutations within the spike protein have been developed based on molecular-clamping technology and use xenonucleic acids (XNA) as molecular clamping probes. **Methods:** Five groups of XNAs, each specific to one of five SARS-CoV-2 mutations (D614G, N501Y, T478K, L452R, K417T/K417N), were designed to be exact matches with the wild-type (WT) sequence to facilitate selective blocking of qPCR amplification of WT targets. The QuantiVirus SARS-CoV-2 Variants Detection Kit assay consisted of three multiplex PCR tubes: tube A tested for the presence of D614G and L452R, tube B tested for the presence of K417 T, N501Y, and the human *Rp* gene as internal control, and tube C tested for the presence of T478K, K417N, and ORF1ab as wild-type target. SARS-CoV-2 Delta or Omicron variant is identified when different combinations of D614G, T478K, L452R, N501Y, K417T, and K417N are detected from sample. **Results:** We have successfully applied the XNA-based molecular-clamping technology to develop a multiplex RT-qPCR assay for SARS-CoV-2 multivariant detection. The assay was tested on 649 nasopharyngeal swab samples including 447 SARS-CoV-2-positive samples and 202 negative samples that were collected from California and Ohio. The assay was able to correctly identify all 36 Delta variant

samples as it accurately detected D614G, T478K, and L452R mutations. The assay's positive predictive value (PPV) for Delta variant detection was 100% (95% CI: 0.89-1.0) and its negative predictive value (NPV) was 100% (95% CI: 0.91-1.0). In addition, the assay was able to correctly identify all 34 Omicron samples by detecting K417N, T478K, N501Y, and D614G mutations. The assay's PPV for Omicron detection was 100% (95% CI: 0.87-1.00) and its NPV was 100% (95% CI: 0.92-1.00). This technique reliably detects a variety of variants and has an analytical sensitivity of 100 copies/mL. **Conclusions:** We demonstrated for the first time the feasibility, efficiency, reliability, and applicability of using an XNA-based RT-qPCR method to detect various SARS-CoV-2 variants. This assay provides a rapid, reliable, and cost-effective testing platform for rapid detection and monitoring of known and emerging SARS-CoV-2 variants.

ID018. **Highly Sensitive Multiplex RT-qPCR for SARS-CoV-2 and Influenza A and B Detection**

J. Li, Q. Sun, L. Pastor, S. Shen, R. Ni, A. Zhang, M. Sha
Diacarta Inc., Pleasanton, CA.

Introduction: Two years after its initial emergence, the SARS-CoV-2-fueled COVID-19 pandemic continues to spread globally with 6.2 million deaths to date. By increasing vaccination levels and a rising number of COVID-19 recovered people who have acquired some degree of natural immunity, the pandemic has been slowed down recently. However, flu season will occur in the coming fall and continually up to the peak in Dec. and Feb. of the year. Given the challenges that fast-changing SARS-CoV-2 variants and influenza A and B have caused in terms of rapid spread and shown similar symptoms, a rapid and cost-effective assay that can detect SARS-CoV-2 and flu A and B viruses simultaneously is greatly needed worldwide. Toward this end, target-specific RT-PCR screens for these viruses have been developed. **Methods:** Primers and TaqMan probes designed for conserved regions of the SARS-CoV-2 and influenza virus genomes allow specific amplification and detection of the viral RNA from all strains of SARS-CoV-2 and Influenza from respiratory specimens. The Human RNase P gene is used as an internal control to monitor viral RNA extraction efficiency and assess amplifiable RNA in the samples to be tested. The assay is a multiplex RT-qPCR assay consisting of one reaction with primers and probes for all the viral targets (SARS-CoV-2's Orf 1ab, influenza A's *NP* and *M2* gene [H1N1 and H3N2] and flu B's *NA* and *HA* genes [Yamagata and Victoria]) together with internal control in one tube, thus with increased assay throughput and ease of use and other advantages as a multiplex assay. **Results:** To evaluate the clinical performance of the QuantiVirus SARS-CoV-2 and Flu AB Detection Test Kit in detection of SARS-CoV-2 virus, a total of 363 SARS-CoV-2-positive samples and 997 negative samples were tested on the Bio-Rad CFX 384 instrument. The clinical samples were all NP samples with their COVID-19 infection determined by FDA-authorized RT-qPCR assay QuantiVirus SARS-CoV-2 Test (FDA EUA 200176). The data show that the positive predictive agreement (PPA) is about 99.7% (95%CI: 0.98-0.99) and negative predictive agreement (NPA) is about 99.9% (95%CI: 0.99-0.99) for SARS-CoV-2 specimen; for influenza A and B detection, 48 positive samples and 44 negative samples were collected and tested. The status of these samples was confirmed by FDA-authorized RT-qPCR assay CDC Flu SC2 Multiplex Assay. The result indicated that its PPA is about 100% (95%CI: 0.91-1.00) and NPA is about 100% (95%CI: 0.90-1.00). **Conclusions:** We have successfully developed a multiplex RT-qPCR assay for SARS-CoV-2 and influenzas A and B detection. This assay provides a rapid, reliable, and cost-effective multiplex testing platform for detection and monitoring of COVID-19 and influenza during the coming flu season.

ID019. **Implementation of a CRISPR-Based SARS-CoV-2 Screening Assay**

R. Barney, J. Barbuto, P. Carnaroli, J. Ellis, P. Shannon, S. Allen, H. Steinmetz, S. Goodwin, P. Boyle, P. Adamo, G. Tsongalis, W. Khan
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: With the continuation of COVID-19, the ability to screen unvaccinated individuals can help slow transmission of the virus through asymptomatic carriers. Screening tests are intended to identify carriers with SARS-CoV-2 viral infections who are asymptomatic and do not have known or suspected exposure to the virus. We have previously validated a CRISPR-based SARS-CoV-2 screening assay. Here we report the results from individuals screened in a clinical setting. **Methods:** Our sample cohort comprises asymptomatic individuals with exemptions on vaccination, who were serially tested on a weekly schedule. This assay was partially automated on the Hamilton Microlab STAR Liquid Handling System using saliva. Saliva samples did not require RNA extraction and were instead lysed with proteinase K and subjected to a brief reverse-transcriptase loop-mediated isothermal amplification. The amplified products were incubated with a Cas-13 family of enzymes complexed with CRISPR guide RNAs specific to SARS-CoV-2 targets (Sherlock BioSciences). Detection of fluorescent readouts was performed in 10 minutes on a microplate reader (BioTek). SARS-CoV-2 was considered detected when the Nucleocapsid (N) and Open Reading Frame 1ab (ORF1ab, O) fluorescent ratio was >5. SARS-CoV-2 was considered not detected when the N/O ratio was <5 and the human RNaseP gene (*RP*) extraction control ratio was >5. A sample was considered invalid when both ratios were <5. **Results:** Between November 2021 and June 2022, a total of 3,430 saliva samples were screened over 100 runs for COVID-19. The average run size was approximately 34 samples per batch. SARS-CoV-2 was detected in 74 samples (2.16%) from the total cohort using CRISPR-based screening. The N/O SARS-CoV-2 target ratios ranged from 5.97 to 53.01 ($\bar{x}$ = 29.09, 95% CI: 26.292-31.888) for "detected" samples. The average percent invalid rate on the total number of saliva samples submitted was 6%, with 199 invalid samples from 93 unique individuals. Sample processing to result time was approximately three hours so that appropriate quarantine measures could be rapidly deployed. **Conclusions:** Implementing weekly screening assays for SARS-CoV-2 can help minimize the spread of the virus through vulnerable populations. We learned that when implementing a screening assay for COVID-19, it is important to focus on ease of use, sample batching, automation, and a test that offers reasonable sensitivity/specificity. In addition to the non-invasive saliva self-collect that was implemented for this screening program, the cost of implementing a CRISPR-based assay was nearly 50% lower than conventional RT-PCR-based approaches.

ID020. **Colorimetric RT-LAMP as an Effective Tool for Rapid Molecular Diagnostics**

N. Tanner, Z. Li, E. Tamanaha, Y. Zhang
New England Biolabs, Ipswich, MA.

Introduction: Loop-mediated isothermal amplification (LAMP) has become a standard molecular diagnostic technique, and its robustness and flexibility have enabled rapid, simple tests outside traditional laboratory environments. The need for additional diagnostic methods became urgent during the SARS-CoV-2 pandemic, and LAMP was extensively evaluated and validated for numerous testing applications. We have extended the utility of LAMP to enable colorimetric detection, multiplex assays, variant identification, and robust direct tests for point-of-care and workplace surveillance testing. **Methods:** We utilized fluorescent and colorimetric reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for assay development, validation, and our workplace screening program. A simple lysis buffer was applied to whole saliva samples and added directly to our colorimetric LAMP SARS-CoV-2 tests, with more than 60,000 samples tested to date. For variant calling we developed novel hybridization probes with single-base sensitivity to regions of sequence changes, and we characterized more than 5,000 LAMP assays intentionally positioning

mismatches in the LAMP primer regions to ensure robustness to and tolerance of mutations. **Results:** RT-LAMP is remarkably tolerant of sequence variation, with no individual positional mutation significantly affecting assay performance, and by combining target primer sets, any minor effect is additionally suppressed. Our CLIA-certified workplace screening has resulted in a positivity rate of 0.6% and a 0.1% inconclusive rate over the course of the program, with >200 unique positive individual patients identified and quickly removed from the workplace environment to minimize onsite transmission events. By designing primers around sites of mutation interest and using simple hybridization probes, we can accurately call SARS-CoV-2 variants with a universal primer set, distinguishing Omicron subtypes with multiple unique probes. **Conclusions:** RT-LAMP is a robust and simple molecular diagnostic method, and over the course of the SARS-CoV-2 pandemic it has been utilized for point-of-care and decentralized testing around the world. By extensively profiling its tolerance to mutations and extending its ability to identify variants to be used for multiplex testing, we aim to firmly establish RT-LAMP as a validated tool for increased access to molecular diagnostic testing.

ID021. **Clinical Severity and Cyclic Threshold Values of Real-Time Polymerase Chain Reaction at Hospital Admission of COVID-19 Patients**

E. Yoo, C. Jeon, S. Kim, A. Lee
Daegu Catholic University Medical Center, Taegu, Taegu-jikhalsi, Republic of Korea.

Introduction: Reverse transcription polymerase chain reaction (RT-PCR) testing is considered the gold standard for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The patient's viral load can be deduced from the cyclic threshold (Ct) value. In this study, the correlation between the initial SARS-CoV2 viral load and clinical features of hospitalized patients in Daegu, South Korea, was explored. **Methods:** The clinical and RT-PCR data of 750 patients with COVID-19 on admission to eight hospitals in Daegu, Korea, were investigated. The correlation between Ct values and COVID-19 severity was assessed. The Wilcoxon signed-rank and Kruskal-Wallis tests were used to compare Ct values between sample types and severity groups. **Results:** Patients' median age was 59.0 ± 20.7 years and 62.1% (N = 466) were female. There was a difference in Ct values between the specimens of upper and lower respiratory tract for the *E* and *RdRP* genes. There was no difference in Ct values between the *E* and the *RdRP* genes ($p = 1.000$); however, there was a statistical difference in Ct values among the four genes ($p < 0.001$) for the same specimen type. Of the 716 patients classified according to clinical severity, the Ct value was found to be significant for predicting disease severity at the time of admission. Ct values for the E, RdRP, and N genes showed significant differences among the eight severity groups ($p < 0.001$). Compared to the clinical severity in groups 1–4, groups 5–8 had a relatively lower Ct value distribution of E ($p < 0.001$), RdRP ($p = 0.029$), ORF1a ($p = 0.009$), and N genes ($p = 0.003$). **Conclusions:** The Ct value estimate of viral load can be a supportive indicator to predict the severity of disease at the time of hospitalization.

ID022. **Whole-Genome Sequencing Reveals Major Shifts in SARS-CoV-2 Genotypes in the Greater NYC Region**

V. Streva[1], S. Duong[2], S. Juretschko[2], J. Lemon[2], V. Streva[2], F. Zhang[3]
[1]Northwell Health, Queens, NY; [2]Northwell Health Laboratories and Donald and Barbara Zucker School of Medicine, New York, NY; [3]Northwell Health Laboratories, New York, NY.

Introduction: The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019 initiated a global pandemic which continues to this day nearly three years later. The initial SARS-CoV-2 viral strain was first isolated from Wuhan, China, and quickly spread globally, including to the United States. Subsequently, viral genetic mutations resulted in the evolution of SARS-CoV-2 variants, several of which were classified as variants of interest (VOI) or variants of concern (VOC) by the World Health Organization (WHO). Several of these variants conferred replication benefits to the SARS-CoV-2 virus, resulting in the establishment of concurrent waves of novel SARS-CoV-2 variants globally. **Methods:** Information about the timing and prevalence of SARS-CoV-2 variants is significant not only for epidemiological purposes, but also because some SARS-CoV-2 variants have been shown to have increased infectivity, to escape immune protection from infection with previous variants, and/or to render some SARS-CoV-2 clinical therapeutics ineffective. Additionally, early recognition of a new variant and its virulence can aid in targeted response to adequate laboratory staffing. **Results:** One way to identify and track SARS-CoV-2 variants is through whole-genome sequencing (WGS) of SARS-CoV-2-positive patient specimens. In this study, we analyzed one year of SARS-CoV-2 variant sequencing data from our healthcare system. In this time, we sequenced more than 10,000 SARS-CoV-2 isolates from the Greater New York City Metropolitan Area within our 23+ hospital catchment region. Our sequencing data show patterns of SARS-CoV-2 variant shifts mirroring those seen nationwide, including establishment of the Alpha, Delta, and Omicron SARS-CoV-2 variants, as well as emergence of the SARS-CoV-2 Omicron BA.1, BA.2, and BA.2.12 subtypes. Interestingly, we also describe the recent emergence of the SARS-CoV-2 Omicron BA.4 and BA.5 subtypes in the Greater New York City Metropolitan Area. Finally, we correlate the emergence of new SARS-CoV-2 variants with the SARS-CoV-2 positivity rate to show the impact new viral variants have on driving continued SARS-CoV-2 transmission. **Conclusions:** WGS of SARS-CoV-2 is an important tool for monitoring emerging and ongoing pandemics.

ID023. **Clinical Validation of an Extraction-Free Multiplex PCR-Based Diagnostic Assay for the Detection of SARS-CoV-2, Influenza A and Influenza B**

D. Koirala[1], C. Sesler[1], D. Buller[1], R. Boos[1], A. Adams[2], A. Roth[3], S. Doerfert[4]
[1]Eurofins ARCA Technology, Inc., Madison, WI; [2]Eurofins ARCA Technology, Inc., Huntsville, AL; [3]UNT Health Science Center, Fort Worth, TX; [4]Tec-Masters, Inc., Huntsville, AL.

Introduction: The onset of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has created challenges for clinical diagnostics. The massive influx in demand for molecular testing to identify SARS-CoV-2 infection led to shortages of diagnostic resources including enzymes, extraction, and PCR reagents; plastic consumables; and lab personnel. The ability of an assay to identify multiple targets with fewer resources will be very beneficial to clinical diagnostics. Here, we describe an extraction-free multiplex assay for the detection of SARS-CoV-2, influenza A, and influenza B in a single reaction that significantly reduces the burden for clinical diagnostics while maintaining sensitivity and improving result turnaround time (TAT). **Methods:** We developed and validated an RT-PCR-based multiplex assay for the detection of SARS-CoV-2, influenza A, and influenza B in a single reaction. In this retrospective, blinded study, we evaluated 50 influenza A, 30 influenza B, 30 SARS-CoV-2, and 111 negative clinical samples. The nasopharyngeal samples were collected in universal transport medium and initially tested through the standard of care with Cepheid Xpert Xpress Flu/RSV or Xpert Xpress SARS-CoV-2 assays. Eleven of the influenza A and influenza B negative specimens were tested using the Luminex Verigene Respiratory Pathogen Flex Test. Specimens were frozen by the clinical site post-processing and transferred for clinical validation testing with the new assay. **Results:** Among the 221 nasopharyngeal swab specimens used in this study, 92% (46/50) of influenza A, 93% (28/30) of influenza B, and 97% (29/30) of SARS-CoV-2 samples were detected with the extraction-free multiplex assay when compared to the test performed at clinical site. The negative percent agreement (NPA) for both influenza A and B was 100%. The NPA for SARS-CoV-2 was 99%. **Conclusions:** A multiplex, extraction-free assay that provides sensitive and specific clinical detection of common respiratory viruses was developed. It provides faster TAT and decreases the cost

burden of health care. Moreover, the assay performance is similar in comparison to an FDA-approved assay used for clinical testing.

ID024. **PCR-Based Genotyping: Better, Faster and Cheaper Than Whole-Genome Sequencing for Known Viral Strain Identification**

N. Pinkhover[1], E. Sanchez[1], K. Fletcher[1], K. Pontbriand[1], T. Proctor[2], R. Schorr[2], J. Feenstra[2], O. Sorel[2], M. Gandhi[2], J. Auclair[1]
[1]Northeastern University, Burlington, MA; [2]Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Whole-genome sequencing (WGS) has been the preferential technology used in viral tracking and surveillance. However, variable viral loads have been a technical limiting factor for use of WGS in generating consistent variant data. In addition, WGS is more labor intensive and takes days to yield a result. PCR-based genotyping is an alternative approach for viral strain monitoring, especially for known variants. The aim of this study was to compare the performance of the two approaches across a range of viral loads. In addition, an assessment of turnaround time, and total cost analysis was conducted to compare these two approaches for rapid variant evaluation. **Methods:** Thirty known SARS-CoV-2-positive remnant nasal swab samples were selected as part of the study cohort. Samples were selected to reflect a range of viral loads. Variant determination was performed using the following methods: 1) PCR-based genotyping using a panel of eight TaqMan SARS-CoV-2 single nucleotide polymorphism assays (d.H69V70; T95I; G339D; K417T; L452R; Q493R; P681R; and Q27x) on the QuantStudio6 PCR instrument (Thermo Fisher Scientific); 2) WGS using two different platforms: a) Ion Torrent GeneStudio S5 Plus system (Thermo Fisher Scientific); b) MinION Mk1B sequencer (Oxford Nanopore Technologies). **Results:** Based on overall study, the following SARS-CoV-2 variants were identified: Delta (N=2), Omicron BA.1 (N=22), and Omicron BA.2 (N=5). PCR-based genotyping panel was able to accurately assign lineages in 96.66% (29/30) of all samples. Across the two different WGS platforms, variant identification could be performed in approximately 50.0%-62.5% of all tested samples (50.0% on Ion Torrent, and 62.5% on MinION). In samples with low viral loads (Ct >25), PCR-based genotyping was able to accurately identify SARS-CoV-2 variants in 93.33% (14/15) of the low viral load samples. In contrast, WGS using either of the two methods was able to identify variants in no more than 12.5% of samples (0% for Ion Torrent, and 12.5% for MinION). The average time to result from sample extraction to variant reporting was ≤12 hours using PCR-based genotyping as compared to ≥3 days for WGS. The total cost for running the 8-assay genotyping panel was >10X cheaper when compared to both Ion Torrent, and MinION WGS. **Conclusions:** SARS-CoV-2 variant monitoring is critical not only from a public health standpoint but may also have implications for managing patients with COVID-19. PCR-based genotyping outperforms WGS for variant identification across a range of viral loads, especially in samples with low viral loads. PCR-based genotyping provides an alternate approach that is more sensitive, faster, and cheaper than WGS for identification of known SARS-CoV-2 variants.

ID025. **Validation of SARS-CoV-2 Whole-Genome Sequencing and Epidemiologic Surveillance in a Private Hospital in Sao Paulo, Brazil**

R. Santana, D. Amgarten, E. Dorlass, F. Malta, M. Basqueira, R. Petroni, A. Salles, A. Takara, A. Santos, B. Oliveira, N. Cagini, R. Sitnik, M. Cervato, N. Muto, A. Doi, J. Pinho
Albert Einstein Hospital, Sao Paulo, Brazil.

Introduction: Human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread worldwide from December 2019. RNA viruses constantly change their genome during the replication process, and thus new variants emerge over time. Those variants could improve the virus' ability to spread more quickly, evade natural or vaccine-induced immunity, cause more severe disease, and impair the detection by specific diagnostic test. The SARS-CoV-2 genomic surveillance is essential to the characterization of emerging variants and monitoring their potential impact on SARS-CoV-2 control measures. **Methods:** We validated a commercially available amplicon-based next-generation sequencing library preparation kit for SARS-CoV-2 genome sequencing according to CAP guideline. For accuracy, SARS-CoV-2-positive samples previously sequenced and genotyped were used. For inter-assay reproducibility we tested three samples in three different routines, and for intra-assay reproducibility three samples were tested in triplicate in a unique routine. And finally, for sensitivity assay we tested a set of samples with cycle threshold (Ct) of qPCR ranging from 28 to 36.5 to stablish the Ct limit for SARS-CoV-2 genome sequencing. The validated test was available in our molecular menu since March 2021. **Results:** In our test validation, we obtained 100% correlation with previous virus genotype and 100% intra- and inter-assay reproducibility. The sensitivity was established as qPCR Ct 30 to ensure at least 90% at 10x genome coverage. During the period from March 2021 to May 2022 we sequenced about 3,300 samples, with a mean coverage of 5,780x and median 99.83% at 10x genome coverage. In the last month Omicron BA.2 represented 35.9% of cases, whereas in the total dataset, we found prevalence of Omicron BA.1 (25.6%), Gamma P.1 (25.6%), Omicron BA.2 (15.5%), Delta B.1.617.2 (11.1%), and minor frequencies of other variants. **Conclusions:** The validated test was shown to precisely cover the SARS-CoV-2 genome with high performance. The sequenced cases allowed us to determine the prevalence of variants, and the viral dissemination patterns along time in our service. All sequences are available on the GISAID platform to contribute to the rapid global flow of SARS-CoV-2 sequence data.

ID026. **Molecular Controls for Influenza A/B and SARS-CoV-2 Testing in Microchip-Based RT-PCR Test System**

S. Gill[1], R. Gill[1], C. Dancey[1], N. Varankovich[1], I. Gelimson[1], K. Jackson[3], S. Bailey[3], A. Brown[3], F. Hui[3], R. Elliot[3], M. Slyadnev[1], D. Eveleigh[3]
[1]Lumex Instruments Canada, Mission, British Columbia, Canada; [2]RAS Medical Solution, Riverdale, GA; [3]Asuragen, a Bio-Techne Brand, Austin, TX.

Introduction: Seasonal respiratory viral pathogens can often be challenging to distinguish from one another symptomatically. For accurate and early detection of these viruses, extremely sensitive molecular detection methods like real-time PCR demand availability and inclusion of standard RNA positive controls in the test system. Here we describe a microchip-based influenza A, influenza B, and SARS-CoV-2 rapid detection system that utilized Armored RNA Quant controls to achieve these objectives. **Methods:** The primers and probe sets of *Inf*A/*Inf*B, N1/HsRP30 targets were pre-loaded as a 2-plex and lyophilized in the microwell array (5x6 or 6x8) formats of AriaDNA microchips. Armored RNA Quant (ARQ) Influenza (P/N52013), SARS-CoV-2 (P/N 52030), RNAse P (P/N52031), and SARS-CoV-2 Panel (P/N 52036) obtained from Asuragen were used as positive test controls (PTC) at the time of performing PCR on the AriaDNA Microchip Analyzer. Technical limit of detection (LOD) of ARQ SARS-CoV-2 and reproducibility as PTC were tested at the microchip manufacturing site. Quality control (QC) checks of microchip production was also performed using the ARQ. **Results:** Technical LOD of the ARQ SARS-CoV-2 and ARQ RNase P was determined to be one copy/PCR for N1 and HsRPP30. These data encouraged the use of Armored RNA as a QC tool for microchips and PTC in the test. For QC, the ARQ influenza, SARS-CoV-2, and RNase P were applied as PTC onto the entire microchip generating Ct values within acceptable range of Ct 29±2 for *Inf*A/*Inf*B, and Ct 28±2 for N1/HsRPP30. The resulting intra-chip CV% of Ct values among replicates (n=15) for each target was highly reproducible with CV% of 0.4/0.4 and 0.1/0.2, respectively. The inter-chip variability of Ct values and the qualitative scores reported as +ve or -ve by the analyzer indicated that ARQ controls worked well to reflect the quality of lyophilized reagents in the microchips. At the clinical sample testing facility of the end-user lab, the ARQ influenza and SARS-CoV-2 panel were used as PTC along with samples loaded onto the microchip.

Seventeen randomly picked RT-PCR runs resulted in CV% of 5.4/6.59, and 2.34/2.63 for *InfA*/*InfB*, and N1/HsRPP30 targets, respectively, indicating acceptable stability and reproducibility of ARQ controls. As a solution to supply bottleneck due to the pandemic, ARQ Respiratory Triplex that included all four targets (*InfA*/*InfB*, and SARS-CoV-2-N1/HsRPP30) was also studied providing CV% of 0.3/0.7 and 0.1/0.2 across five replicates of the respective targets. **Conclusions:** The characteristics of the ARQ including resistance to nuclease degradation, safety being non-infectious in nature, and availability at high titer contributed to the success of the launch of AriaDNA test system.

ID027. **Developing a Loop-Mediated Isothermal Amplification Assay for the Rapid Detection of Multiple Respiratory Viruses including SARS-CoV-2**

M. Lee[1], W. Lee[2], S. Kang[2], M. Kim[2]
[1]Kyung Hee University Hospital at Gangdong, Seoul, Republic of Korea; [2]Kyung Hee University School of Medicine, Seoul, Republic of Korea.

Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still a pandemic disease. Since the symptoms of SARS-CoV-2 are not significantly different from those of other respiratory viruses, it is important to differentially diagnose them from infections of other respiratory viruses. Currently, the most widely used method for the multiplex respiratory virus detection is the real-time PCR (RT-qPCR) assay. However, the RT-qPCR assay relies on sophisticated facilities and well-trained personnel. It is urgent to develop a platform that quickly determines whether a patient has respiratory infection without going to a large medical institution. Isothermal amplification assays, such as loop-mediated isothermal amplification (LAMP) is a promising point-of-care testing (POCT) method with rapidity and simplicity. In this study, we aimed to evaluate the analytic performance of LAMP assays for the detection of common respiratory viruses and SARS-CoV-2 and assessed the correlation between LAMP assay and RT-qPCR. **Methods:** Nasopharyngeal (NP) swabs were obtained from symptomatic patients. All specimens were confirmed by RT-qPCR. The primers for RT-qLAMP were designed based on the sequence information stored in the NCBI. RNA/DNA from standard strains for respiratory viruses and heat-inactivated preparations of standard strain for SARS-CoV-2 were used to evaluate the accuracy and specificity of the RT-qLAMP assay. **Results:** We developed a multiplex RT-qLAMP assay of six respiratory viruses: RSV A, RSV B, ADV, influenza A (H1N1 and H3N2), influenza B, and SARS-CoV2. LAMP was carried out in a final reaction volume of 9.6 μL. When the 10 standard strains' RNA of respiratory viruses was added, no cross-reactivity was observed. Among the 316 specimens included in the study, 186 were detected as positive by the RT-PCR assay, and 176 (94.6%) were detected as positive by the RT-qLAMP assays. All 130 NP specimens that were negative by RT-PCR for 16 respiratory viruses were negative in the developed RT-qLAMP assays. Compared to the RT-PCR, the results of the developed RT-qLAMP assay showed good sensitivity (94.6%) and specificity (100%). The agreement between these two methods was 96.8%. The median amplification time to positivity for RT-qLAMP was 22:34 min (range, 6:80-47:98 min). **Conclusions:** The developed RT-qLAMP assay requires only a very small amount of reagents and samples and performed with an isothermal reaction. This study established a fast, simple, and sensitive RT-qLAMP that can apply to POCT to facilitate the detection of respiratory viruses including SARS-CoV-2. It also performed high diagnostic sensitivity and specificity among clinical samples. The developed RT-qLAMP assay is an attractive and efficient tool for infection control.

ID028. **Evaluation of SARS-CoV-2 Commercial RT-PCR Test S-Gene Target Failure (SGTF) Signature for Identification of the BA.1 Subvariants during the 2021 Omicron Wave**

T. McMillen, K. Jani, T. Kalghatgi, D. Saintine, A. Quianio, N. Babady
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: The SARS-CoV-2 variant of concern (VOC) designated as Omicron appeared in the United States in late November of 2021. This VOC (subvariants BA.1), like the VOC Alpha, is characterized by mutations that result in the lack of detection of the *Spike* gene target for nucleic acid amplification tests that target the *S* gene for detection of SARS-CoV-2 RNA in clinical samples. This genomic signature is referred to as S-gene Target Failure (SGTF). The SGTF signature has been proposed as a preliminary indicator of Omicron VOC. Although Whole-genome sequencing (WGS) is established as the gold standard for lineage assignments, the cost and turnaround time limits its wide use for most clinical laboratories. In this study we aimed to evaluate the accuracy of the SGTF signature from the Thermo Fisher TaqPath RT-PCR to correctly identify the Omicron BA.1 variant during the initial Omicron-associated SARS-CoV-2 surge in New York City in December 2021. **Methods:** Samples collected between December 1, 2021, and January 31, 2022, testing positive on the cobas 6800 SARS-CoV-2 assay were selected for repeat testing on the TaqPath SARS-CoV-2 assay to identify samples with the SGTF genomic signature. All samples were tested according to manufacturer's instructions. A subset of samples with Ct values <30 for all targets underwent SARS-CoV-2 WGS using Nextera-XT reagents and ARTIC primers according to the Scripps Primal Seq protocol with modifications. **Results:** A total of 30,991 samples were tested for SARS-CoV-2 during the study period with 13,886/30,991 (44.8%) tested on the cobas 6800 and 1,798 positive for SARS-CoV-2 RNA. A total of 929/1,798 (51.7%) samples met criteria for testing on the TaqPath assay. Additionally, 917/929 (98.7%) were available for testing and 893/917 (97.4%) were positive for the SGTF signal. A total of 515 samples with Ct values <30 and SGTF signature were sequenced and 512/515 (99.4%) were classified as Omicron (BA.1, BA.1.1, BA.1.1.529). **Conclusions:** In this study, the SGTF signature showed high accuracy for the identification of the Omicron VOC, particularly BA.1. For institutions that may not have the availability to perform large scale WGS testing, the use of this signature to confirm the presence of this variant is an accurate and less labor-intensive option than WGS methods.

ID029. **Diagnostic Testing Sample-Type Preferences for the Detection of SARS-CoV-2: Results from an International Survey**

S. Akbarzada, L. Salzano, E. Tobik, S. Megiel, C. Duni, B. Choate, Y. Wu, S. Sunder, A. Wyllie
Yale School of Public Health, New Haven, CT.

Introduction: Strategies for diagnostic testing rapidly evolved during the COVID-19 pandemic in response to the unprecedented worldwide demand. Testing remains the most reliable tool for understanding the current state of the pandemic and directing efforts and resources. The purpose of this study was to better understand the public view regarding different sample types to inform future surveillance planning and diagnostics development. **Methods:** To assess testing preferences for the detection of SARS-CoV-2, we employed a non-experimental research design: an online survey created in Qualtrics that was distributed via social media and connections in >100 countries. In addition to testing preferences, the questionnaire covered demographic information such as sex, age, education, occupation, and country of residence. Python was used to analyze the raw data. **Results:** From 03/30/2022 to 06/15/2022, a total of 1,533 responses were collected from six regions: Africa (12.8%), Asia (9.2%), Europe (5.8%), North America (51.4%), South America (9.5%), and Oceania (11.2%). Participants were 58.9% female, 39.1% male, and 1.3% non-binary, and ranged in ages from 18-24 (6%), 25-34 (29%), 35-44 (25%), 45-54 (17.6%), 55-64 (14.4%), and 65+ (7.7%). Respondent education level was skewed, with 60.7% holding a graduate degree and 29% holding a bachelor's degree. Additionally, 34.3% were

scientists/researchers and 25.4% were healthcare workers. By rank sum analysis, the most preferred testing method globally was the oral swab, followed by saliva testing; the least preferred method was deep coughing, followed by nasopharyngeal swab. By region, the most preferred type in Africa was the oral swab, in Asia saliva testing, in Europe the oral swab, in North America the oral swab, in South America saliva testing, and in Oceania saliva testing. When respondents were asked about their preferred testing method for their children (if applicable), respondents favored drooling saliva into a small plastic tube (saliva testing) in Africa, Asia, North America, South America, and Oceania; and using a swab to collect a sample from about halfway up the nose (anterior-nares swab) in Europe. **Conclusions:** This study identified a preference for oral sample types for the detection of SARS-CoV-2 in the international population surveyed, with sampling saliva as the most preferred testing method for children in five out of the six regions. Large-scale, easily accessible diagnostic testing is vital for detecting and containing SARS-CoV-2; utilization of oral sample types makes this endeavor feasible if individuals are then more likely to test. Results from this study should be considered when new testing practices are designed to encourage maximum participation from individuals and improve community health.

ID030. **The Evolution of a Novel Nationwide Network of Clinical Diagnostic Laboratories to Enhance the COVID-19 Pandemic Response**

S. Sunder, B. Choate, L. Kitfield-Vernon, A. Wyllie
Yale School of Public Health, New Haven, CT.

Introduction: The COVID-19 pandemic presented an unprecedented demand for diagnostic testing. Testing is essential for isolating infected individuals, contact tracing, and epidemiological surveillance for public health countermeasures, but at times has been strained by inadequate infrastructure and supply chain disruptions. To overcome these challenges, we developed an open-source, low-cost, sensitive *in vitro* diagnostic assay in an effort to deliver equitable testing across the US. **Methods:** SalivaDirect was developed as a simple diagnostic test by 1) eliminating collection tubes with preservatives, 2) developing clear self-collection instructions, 3) replacing nucleic acid extraction with a simple enzymatic step, 4) testing specimens in dualplex RT-qPCR, and 5) establishing a sustainable umbrella emergency use authorization (EUA) regulatory model. Around our mission to provide accessible and affordable testing, any qualified CLIA lab is welcome to test under the SalivaDirect EUA, negating the need for an independent EUA or lab developed test. This approach led to the formation of a national network of labs designated to test with SalivaDirect. **Results:** Since receiving EUA on August 15, 2020, 182 labs in 41 states have been designated to test using SalivaDirect. With more than 6.3 million+ tests performed, designated labs administer 36,000+ SalivaDirect tests/day with a projected capacity of 228,000+ tests/day. Reports of very few false-positive (eight) and false-negative (five) results with low rates of sample rejection (0.62%) and invalid tests (0.38%) demonstrate reliable, effective implementation of the SalivaDirect assay across a diverse lab network. The collective expertise of the network guided the expansion of SalivaDirect protocol, with more than 20 amendments in the last two years in response to lab needs. The SalivaDirect EUA now includes dozens of validated reagents and instruments for increased testing flexibility and supply-chain resilience. In collaboration with lab network partners, we verified test accuracy in asymptomatic individuals and developed unsupervised and at-home saliva collection kits for individuals aged ≥2 years. Remarkably, the lab network became a platform for sharing knowledge, expertise, samples, and resources, which proved particularly important when responding to new variants. **Conclusions:** The open-source SalivaDirect PCR test evolved into a nationwide lab network. Leveraging upon the upfront validation work performed by our team, labs could easily implement SalivaDirect into their workflow process. Together with our network we formulated an effective testing solution for local communities. The SalivaDirect assay has strengthened the public health response for a future pandemic and laid the groundwork for others to build upon its research.

ID031. **Optimization and Utilization of COVID-19 Next-Generation Sequencing Assay with Custom Bioinformatics Pipeline for Genomic Surveillance and Public Health Monitoring**

J. Starbuck[1], J. Spildener[1], K. McDonnell[1], D. Plunkett[1], J. Brock[2], J. Nakitandwe[1], Z. Tu[1], D. Bosler[1], D. Rhoads[1], E. Azzato[1]
[1]Cleveland Clinic, Cleveland, OH; [2]Akron Children's Hospital, Akron, OH.

Introduction: Since first published in January 2020, the viral genomic sequence of SARS-CoV-2 (SC2) has developed multiple variants, which circulated during the COVID-19 global pandemic. Public health departments monitor variants to determine resource allocations but often do not have infrastructure for rapid development of assays suitable for genomic surveillance. Here we present our clinical laboratory's experience optimizing and utilizing a next-generation sequencing (NGS)-based assay for whole-genome sequencing of the SC2 viral genome, in partnership with Ohio Department of Health. **Methods:** NGS libraries were generated from viral RNA extracted from a subset of COVID-19 diagnostic specimens, using Illumina COVIDSeq kit (Illumina, San Diego, CA, US), and sequenced on the Illumina NextSeq platform with mid-output v2 reagents at 2 × 149 bp and dual-indexing. Laboratory optimizations included sequencing specimens with diagnostic Ct values ≤28, staggering bead washes, adjusting positive control dilution and final library loading concentration. Bioinformatics analysis was originally performed using DRAGEN COVID Lineage Application in the BaseSpace Sequencing Hub (BSSH, Illumina) with in-house scripts. Due to limitations with BSSH, we transitioned to a combination of in-house scripts and publicly available databases. Our pipeline uses BWA alignment for read mapping, Freebayes and LoFreq for mutation detection, snpEff for mutation annotation, and Pangolin COVID-19 Lineage Assigner (https://pangolin.cog-uk.io/) and Nextclade (https://clades.nextstrain.org/) for variant classification. Coverage information, CDC and WHO variants of concern, and mutation spectrums were reported. **Results:** Since March 2021, ~11,000 SARS-CoV-2 genomic sequences were generated (188 specimens per week), with >100 variants and subvariants detected. Mutation status (e.g., L452R, N501Y, and E484K) was tracked, regardless of lineage. Monitoring genome-wide coverage and mutational spectrum, especially in the *S* gene, allowed bioinformatics pipeline adjustments to detect emerging variants before they were widely recognized, such as R346K mutation in BA.1 (BA1.1) and L452Q in BA.2 (BA.2.12.1). Sequencing data allowed us to troubleshoot diagnostic PCR assay quality concerns. We assisted in surveillance of variant surges, including Alpha, Delta, and Omicron in northeast Ohio and contributed ~10,000 sequences (~0.1% of global SARS-CoV-2 sequences) to the GISAID database. **Conclusions:** Clinical molecular pathology laboratories with NGS capabilities can leverage their resources and infrastructure to assist in public health epidemiological surveillance of emerging pathogens. Robust monitoring of quality metrics is necessary to make dynamic bioinformatics analysis pipeline adjustments as new variants emerge.

ID032. **An Epidemiological and Clinical Characteristics Study of SARS-CoV-2 Delta and Omicron Lineages in Northern New England**

S. Sathyanarayana[1], P. Shannon[1], S. Deharvengt[1], K. Winnick[1], D. Green[1], S. Soucy[2], W. Thomas[3], G. Tsongalis[1], M. Whitfield[2], A. Hoen[2], J. Lefferts[1]
[1]Dartmouth Health, Lebanon, NH; [2]Dartmouth College, Hanover, NH; [3]University of New Hampshire, Durham, NH.

Introduction: The SARS-CoV-2 Delta variant and its subtypes arose in the US in June 2021 and developed as a dominant strain internationally. Later in November 2021, WHO recognized another variant of concern, Omicron, with increased transmission and decreased disease severity compared to the Delta and other lineages.

Regardless of vaccination, symptomatic or asymptomatic, Omicron can be contagious to others. The NIH has sponsored several locations in the US to conduct SARS-CoV-2 surveillance studies in rural and underrepresented populations by genome sequencing. This study evaluates the incidence, severity, and expansion of Delta and Omicron variants and their clinical features by sequencing the SARS-CoV-2 genome. **Methods:** Positive residual nasopharyngeal collections from SARS-CoV-2-positive patients are stored at -80ºC. The SARS-CoV-2 samples are processed and analyzed at the University of New Hampshire (UNH) and the CGAT laboratory. At the UNH, RNA extraction is carried out using Thermo Fisher Scientific's MagMax Viral/Pathogen nucleic acid isolation kit, followed by ARTIC v3 amplicon library generation. The sequencing was performed using NovaSeq 6000 (Illumina). In addition, the CGAT laboratory extracted viral RNA from the automated EZ1 Virus Mini Kit v2.0 (Qiagen). Library preparation was done using the Ion AmpliSeq SARS-CoV-2 Insight Research Assay – GS Chef Ready, followed by sequencing on the Ion GeneStudio S5 (Thermo Fisher). The Pangolin COVID-19 lineageID v5 was employed to derive different phylogenetic lineages. **Results:** Out of 2,672 SARS-CoV-2-positive samples, 1,212 are males and 1,460 females. Among Delta infections, 53.8% were female, 46.1% were male; 55.3% of Omicron infections were female, and 44.6% were male. Among individuals with Omicron, the age group 25-49 years were 40.2% and 5-24 years 31.1%. Similarly, for the Delta infection, the age group 25-49 years were 35.8% and 5-24 years 29.3% which were relatively higher compared to other age groups. In this study, healthcare workers comprised 12% of Omicron infections and 7.7% of the Delta infections. Further, 91.9% of Omicron samples were from symptomatic patients, and 90.8% of the Delta infections were symptomatic. Considering the ability of the acute infection and its spread, the transition from Delta to Omicron from late December 2021 was very rapid. **Conclusions:** Our study emphasizes the significance of prolonged surveillance specific to variant types. Further, this approach helps monitor new variants and their epidemiological and biological dynamics, enabling time-specific interference and adequate healthcare planning.

ID033. **Evaluation of Alternate Collection Media for Molecular Detection of SARS-CoV-2 on cobas 6800 System**

S. McCune[1], E. Gick[1], J. Baron[1], A. McMullen[1], P. Uprety[1]
[1]Roche Diagnostics, Indianapolis, IN; [2]Roche Diagnostics, Augusta, GA.

Introduction: cobas SARS-CoV-2 assay received Emergency Use Authorization (EUA) from the FDA for qualitative detection of SARS-CoV-2 nucleic acid on the cobas 6800/8800 systems. Four types of collection media (Copan Universal Transport Medium System [UTM-RT], BD Universal Viral Transport System [UVT], cobas PCR Media, and 0.9% physiological saline) are considered on-label for use per the package insert. There have been shortages of most of these collection media throughout the pandemic; hence, labs have been using off-label media types for SARS-CoV-2 testing. There are limited publications on the performance of the cobas SARS-CoV-2 assay with alternate off-label collection media. **Methods:** Eight different collection medias (UTM-RT, UVT, Copan eNat, Copan ESwab, Hardy Diagnostics Viral Transport Medium, Thermo Fisher/Remel M4RT, Thermo Fisher/Remel M6, Puritan UniTranz-RT) were spiked with AccuPlex SARS-CoV-2 full genome positive control material (SeraCare, Cat# 0505-0226). Tenfold serial dilutions were made from each collection media, from 10,000 copies/mL to 1 copy/mL. Each dilution level was then split into three secondary tubes and tested with the cobas SARS-CoV-2 assay. **Results:** At higher viral concentrations (100, 1,000 and 10,000 copies/mL), both viral targets were detected across all replicates for all media. Likewise, at these higher concentrations, the mean Ct values across replicates for each off-label media were equivalent (with 95% statistical confidence) to corresponding mean Ct values obtained using each on-label media (BD UVT and Copan UTM) using an equivalence threshold of +/-1.5 Ct units. At lower viral concentrations (1 and 10 copies/mL), Target 1 was detected in 27 out of 48 tests (3 replicates X 8 media X 2 concentrations=48 tests) and Target 2 was detected in 25 out of 48 tests. There were no statistically discernible differences between media in detection rates above the assay LOD. Below the LOD, media appear to be similarly equivalent; however, more data are needed for statistical analysis. **Conclusions:** This study demonstrates the potential compatibility of these alternative collection media for use with the cobas SARS-CoV-2 assay on the cobas 6800/8800. Laboratories will need to complete additional studies for validating these devices before utilizing them for clinical testing. We are planning to test additional specimens at the lower viral concentrations to assess statistical equivalence in detection rates, as well as evaluate additional media.

ID034. **Performance of the Seegene Technologies Novaplex SARS-CoV-2 Variants VII Assay Compared to Whole-Genome Sequencing**

R. Yee[1], M. Codoy[2], M. Hailu[2], J. Flores-Vasquez[3], Z. Sands[2], O. Kochar[2], J. Dien Bard[3]
[1]George Washington University School of Medicine and Health Sciences, Washington, District of Columbia; [2]George Washington University Hospital, Washington, District of Columbia; [3]Children's Hospital Los Angeles, Los Angeles, CA.

Introduction: Genotyping plays a key role in detecting SARS-CoV-2 variants and may provide information on therapeutic effectiveness to monoclonal antibodies. Although next-generation sequencing (NGS) methods are necessary in identifying novel mutations, allele-specific real-time polymerase chain reaction (RT-PCR) tests allow for variant detection in laboratories that lack NGS capabilities, in samples with low viral loads, and with a faster turnaround time. The Seegene Technologies Novaplex SARS-CoV-2 Variants VII assay (VarVII PCR) is a RT-PCR test that can detect SARS-CoV-2 variants (Alpha, Beta/Gamma, Delta, or Omicron) by targeting key mutations, E484A, RdRP, N501Y, and 69/70del, in the genome in <2 hours. We determined the performance of the VarVII PCR on clinical specimens using whole-genome sequencing (WGS) as the gold standard. **Methods:** RT-PCR was performed on 121 specimens (78 nasopharyngeal [NP] swabs and 43 saliva specimens) using the TaqPath COVID-19 Combo kit (Thermo Fisher), CDC SARS-CoV-2 RT-PCR, or Cepheid Xpert Xpress *CoV-2*/Flu/RSV plus. Nucleic acid was extracted using the Thermo Fisher KingFisher processing system with Applied Biosystems MagMAX Extraction kits. WGS was performed on SARS-CoV-2-positive samples (Ct <30) using the AmpliSeq SARS-CoV-2 Insight Research Assay on Ion Torrent Genexus system. All samples were tested using VarVII PCR per the manufacturer's protocol. A clinical validation was performed. **Results:** Among 121 specimens, 30 NP swabs and 15 saliva specimens were positive for SARS-CoV-2. WGS characterized the isolates as B.1.1.7 (Alpha, n=2), P.1 (Gamma, n=5), B.1.617.2 (Delta, n=14), and BA.1/BA.2 (Omicron, n=25). The VarVII PCR achieved 100% sensitivity and specificity for both specimen types and accurately differentiated Omicron BA.1 (n=20) versus BA.2 (n=5). The coefficients of variation from precision studies were 1.2%, 0.35%, 0.42%, and 1.3% for targets E484A, RdRP, N501Y, and 69/70del, respectively. Specimens were stable at 25°C for 48 hrs and -70°C for 14 days. No cross-reactivity with circulating strains of influenza, human rhinovirus/enterovirus, respiratory syncytial virus (RSV), coronavirus NL63, human metapneumovirus, and parainfluenza virus was detected. Replicate testing of dilutions from positive specimen showed that samples with Ct ≥36 resulted in some mutations not detected. **Conclusions:** The VarVII PCR is an accurate test with high correlation to WGS in identifying known SARS-CoV-2 variants, including BA.1 and BA.2 variants of the Omicron lineage. The VarVII PCR may allow for increased accessibility in clinical labs to detect variants for surveillance and treatment purposes. However, allele-specific RT-PCR assays require continuous development and validations to ensure detection of novel variants.

ID035. **Anti-SARS-CoV-2 Antigen Home Test Rapidly Detects SARS-CoV-2 Omicron and Delta Variants**

M. Tahsin[1], R. Chuang[1], S. Shen[1], L. Pastor[1], J. Li[1], H. Yang[1], J. Pathakamuri[2], A. Zhang[1], M. Sha[1]
[1]Diacarta Inc., Pleasanton, CA; [2]Franciscan University of Steubenville, Steubenville, OH.

Introduction: COVID-19 is an infectious disease caused by the SARS-CoV-2 virus affecting the respiratory system and other major organ systems. Multiple new variants of concern have emerged, such as the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529), which are associated with increased virulence and transmissibility. In this study, we are evaluating the clinical performance of the QuantiVirus SARS-CoV-2 Antigen Rapid Test kit and its ability for detecting Omicron and Delta variants. **Methods:** A prospective clinical study was conducted to evaluate the clinical validity of QuantiVirus SARS-CoV-2 Antigen Rapid Test kit for direct nasopharyngeal swab specimens compared to RT-qPCR test where 69 patients were enrolled. To validate whether this kit can detect SARS-CoV-2 variants Delta and Omicron, we collected 14 Delta, one Delta plus, and 18 Omicron variants, a total of 33 variant samples which were confirmed by QuantiVirus SARS-CoV-2 variant Detection Test Kit (CE Mark) and Sanger Sequencing. We have also assessed 151 samples for self-test to determine the positive percentage agreement (PPA) and negative percentage agreement (NPA). **Results:** The clinical validity study showed the QuantiVirus SARS-CoV-2 Antigen Rapid Home Test has clinical sensitivity of 88.2% (95% CI: 0.75.4-0.951) and specificity of 100% (95% CI: 0.934-1.00). Its Positive Predictive Value (PPV) is 100% (95% CI: 0.90.2-1.00) and its Negative Predictive Value (NPV) is 92% (95% CI: 0.83-0.97). On the other hand, the QuantiVirus SARS-CoV-2 Antigen Test Kit can 100% detect SARS-CoV-2 Delta, Delta plus, and Omicron variants. Its PPV is about 100% (95% CI:0.87-1.00) and NPV about 90.9% (95% CI: 0.75-0.98). In summary, the overall PPA and NPA of QuantiVirus SARS-CoV-2 Antigen Rapid Home Test for coronavirus (COVID-19) are 91.1 % (95% CI: 0.80-0.96) and 100% (95% CI: 0.95-1.00), respectively, for all subjects including symptomatic and asymptomatic subjects. **Conclusions:** QuantiVirus SARS-CoV-2 Antigen Rapid Home Test can detect variants of interest including Omicron and Delta variants with sensitivity of 88.2% and specificity of 100%. which make it a precise, efficient, and effective self-used test in detecting COVID-19 and its variants of concern.

ID036. **Wastewater Monitoring Provides a Rapid, Cost-Effective Approach to Assess Community-Wide SARS-CoV-2 Variant Emergence and Longitudinal Monitoring**

M. Klomp, M. Stischer, E. Gordon, R. Skerjanec, J. Peat, C. McKee, A. Robison, S. Kane
GT Molecular, Fort Collins, CO.

Introduction: Several academic, industry, and public health laboratories implemented wastewater-based epidemiology (WBE) to track community-wide COVID-19 trends. Within, we employed a mutational signature WBE approach to track SARS-CoV-2 variant emergence and spread. Namely, we developed assays and longitudinally monitored the signatures of the α, β, γ, δ, λ, μ, and o variants beginning January 2021 in various communities across the United States. **Methods:** To identify variant mutational signatures, we first performed *in silico* analyses. To consider a target for assay development (n≥2 per variant), mutations must represent ≥95% inclusivity of a given variant and show <5% exclusivity of non-variant sequences. We then developed multiplexed digital PCR (dPCR) assays to detect wild-type and mutated signatures at a given locus. Wastewater sample processing included spiking an internal process control to assess viral recovery, filtration to remove solids, dead-end ultrafiltration to concentrate viruses, and RNA extraction. Droplet digital PCR was performed targeting: 1) total SARS-CoV-2 (N1), and 2) variant-specific *S*-gene mutations. Viral load, mutational prevalence, and rolling clinical cases were tracked over time, and statistical analyses were performed in GraphPad Prism. **Results:** Early in 2021, we detected and reported the first indication of the α (del69-70 and N501Y) variant emergence. The transition from α variant dominance to δ variant (L452R and T478K) dominance showed a gradual slope with a transition period in mid-June 2021. We sporadically detected β (K417N, E484K, N501Y), γ (K417T, E484K, N501Y), λ (L452Q and F490S), and μ (R346K and ins145) mutational signatures and did not observe evidence of increasing viral load and presumptive transmissibility. The transition from δ variant dominance to o (N679K and Q954H) variant dominance rapidly occurred and showed a shorted transition period compared to the α-δ transition. In spring-summer 2021, o sublineages BA.2, BA.2.12.1, BA.4, and BA.5 emerged and overshadowed the BA.1 variant. Collectively, these datasets mirror clinical epidemiology studies. **Conclusions:** WBE offers a complementary approach to clinical-based epidemiology. Specifically, WBE not only represents an average sample of an entire community, but also circumvents various hesitations to clinical visitations. Within, we employed WBE approaches to track the emergence and spread of SARS-CoV-2 variants spanning α through o sublineages. To our knowledge, we are the only group that gathered national variant trends with dPCR. These datasets complement clinical-based epidemiology and offer near real-time information to public health agencies within an actionable time frame.

ID037. **Investigating the Long-Term Kinetics of SARS-CoV-2 Anti-nAB Antibodies Based on the Cohort of COVID-19 Vaccinated Health Workers**

A. Vashisht[1], R. Kolhe[1], S. Ananth[1], R. Bloomquist[1], D. Bradshaw[1], A. Chaubey[2], J. Farmaha[1], D. Fransoso[1], J. James[1], K. Jones[1], V. Kota[1], A. Kothandaraman[3], C. Marie Carlock[1], A. Mondal[1], P. Ray[1], N. Sahajpal[1], H. Singh[1]
[1]Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA; [3]PerkinElmer, Waltham, MA.

Introduction: Vaccination has been the most impressive and significant measure to halt the progression of COVID-19. In addition to this, several studies have shown that infection with SARS-CoV-2 provides some degree of protection against reinfection, and vaccination substantially enhances the immune response and confers strong resistance against variants of concern. Specifically, the high-spike antibody levels such as neutralizing antibody (nAB) have been reported to be important in building a proactive immune response against SARS-CoV-2 infection. As the data related to the effectiveness of these antibodies are only beginning to surface, it is important to document the longitudinal effects of the vaccines to determine the appropriate cycle for the administration of booster doses. **Methods:** This study analyzed healthcare workers (employees, students, and volunteers) enrolled in the SeroPrevalence And Respiratory Tract Assessment (SPARTA) study at Augusta University, GA. Only those participants who had a minimum of six months of follow-up were included for analysis. The study participants were divided into vaccinated participants with either BNT162b2 (Pfizer–BioNTech) or mRNA-1273 (Moderna). The participants were further divided based on their vaccination status and gender. The detection of SARS-CoV-2 was performed by TaqMan-based real-time PCR assay. The anti-SARS-CoV-2 neutralizing nAB levels were determined by the SuperFlex Anti-SARS-CoV-2 Neutralizing Antibody Kit and SuperFlex Chemiluminescent Immunoassay System (PerkinElmer Inc., Waltham, US). Statistical analysis of the data was performed using GraphPad Prism 8 (version 8.0.2, San Diego, CA, US). **Results:** The nAB levels were monitored monthly for 16 months from Nov. 2020 to Feb. 2022. Significant differences were observed in the nAB levels between different study groups and within the groups at different time points. The vaccinated participants who were vaccinated with mRNA-1273 showed initial higher antibody levels after vaccination as compared to BNT162b2. But later on, the nAB levels started declining in participants with mRNA-1273 vaccine as compared to BNT162b2. No disparity was observed after the administration of booster doses. Further, the nAB levels remained comparable in both females and males at all the time points. **Conclusions:** This study showcased the

importance of vaccination and its subsequent booster doses in establishing the required immune strength against SARS-CoV-2 infection. The data showed that both the vaccines require timely administration of booster doses. No difference in antibody levels was observed concerning gender showing comparable kinetics after vaccination and booster doses, reflecting the equivalent immunization achieved with the same dose in these groups.

ID038. **Heat Shock-Viral RNA Release for COVID-19 Testing**

C. Ledesma
VA Medical Center, Oklahoma City, OK

Introduction: The public health emergency of the COVID-19 pandemic emphasized the crucial role of medical laboratory professionals and scientists in molecular diagnostics laboratories to ensure success in infection control strategies. The demand for laboratory testing using nucleic acid amplification tests to detect SARS-CoV-2 RNA imposed strains in laboratory supplies. Here, we explored an alternative cost-effective solution that will simplify the pre-PCR steps by using a simple heating method to release viral RNA. **Methods:** Samples tested using the reference automated extraction method were used: 100 samples identified as positive for SARS-CoV-2 RNA and 500 samples tested as negative for SARS-CoV-2 RNA were used for the study and sorted with equal distribution according to Ct values of 1) <20, 2) 20–30, and 3) >30.100 µl from swab preserved in Universal Transport Medium treated with 30 µg of proteinase K, and another set was tested without proteinase K pretreatment. All samples with or without proteinase K were diluted to minimize PCR inhibitors. The thermal shock protocol was set at (98°C, 5 minutes; 4°C, 2 minutes) and screened for purity. Performance and method verification studies were performed. Internal extraction, positive template, and no template controls were markers used for testing quality. **Results:** The Ct values from the thermal shock RNA release were compared to the automated extraction method and statistically analyzed. The criteria for acceptability for validation of this new RNA extraction proceeding were set to 100% concordance compared to the commercial kit using an automated extraction. PCR efficiency was at 98% and a slope of -3.3. Within run precision of 2% and limits of detection from 200 to 20,000 copies/µL the method showed 100% (50/50) concordance on samples previously identified as negative by automated methods and identified 86% (86/100) with a mean difference of 3 Ct. **Conclusions:** Our findings suggest that the thermal shock treatment of nasopharyngeal swabs in viral transport media can successfully extract viral nucleic acid for nucleic acid amplification and is a reasonable alternative for chemical extraction methods when molecular diagnostic laboratories persistently encounter supply chain issues.

ID039. **Longitudinal Analysis of IgG Antibody Levels in SARS-CoV-2 Vaccinated Healthcare Workers**

H. Singh[1], R. Kolhe[1], S. Ananth[1], R. Bloomquist[1], D. Bradshaw[1], A. Chaubey[2], J. Farmaha[1], D. Fransoso[1], J. James[1], K. Jones[1], v. Kota[1], A. Kothandaraman[3], C. Marie Carlock[1], A. Mondal[1], P. Ray[1], N. Sahajpal[1], A. Vashisht[1]
[1]Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA; [3]PerkinElmer, Waltham, MA.

Introduction: The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has infected more than 545 million people, causing more than 6.3 million deaths worldwide due to coronavirus disease 2019 (COVID-19). Studies have found that natural infection with SARS-CoV-2 provides some degree of protection against reinfection, but vaccination substantially enhances the immune response and confers strong resistance against variants of concern. Thus, we aimed to investigate the long-term immunological response to SARS-CoV-2 after the first, second, and booster vaccination dose, and to understand the impact of two vaccines (Moderna and Pfizer) on the levels of antibodies and for the future deployment of vaccination/booster strategies. **Methods:** This study included healthcare workers (employees, students, and volunteers) in the age group 18 to 90 years, enrolled in the SeroPrevalence And Respiratory Tract Assessment (SPARTA) study at Augusta University, GA. The study participants were separated into two groups, which were further divided based on their infection/vaccination status and age (below 50 and above 50). We collected saliva and peripheral blood to measure antigen (SARS-CoV-2 viral RNA by RT-PCR assay, PerkinElmer Inc.) and levels of IgG antibodies in serum (Anti-SARS-CoV-2 QuantiVac ELISA (IgG); EUROIMMUN). **Results:** From November 2020 to February 2022, 164 participants who met the inclusion criteria were included in the study. A total of 60.6% had received BNT162b2 and 39.3% received the mRNA-1273 vaccine as either their first, second, or booster dose; 66% of participants were female and 34% were male. The IgG levels were significantly higher in the participants that received the Moderna vaccine after the first (time point 2) and second (time point 3) dose as compared to the Pfizer. Notably, the IgG levels at nine months (before booster dose, time point 11) were higher in participants that received Pfizer compared to Moderna. But the participants receiving BNT162b2 and mRNA-1273 showed comparable IgG levels after booster doses until five months of follow-up. Further, no significant differences were found in the levels of IgG antibody levels between below-50 and above-50 age groups. **Conclusions:** The immunological response to SARS-CoV-2 after vaccination provides important insights into the longitudinal kinetics and the timeline in which the booster dose strategies should be made. Both vaccines induced significant immune response after second dose, but IgG antibodies decreased over time in all subjects assessed, with the most significant drop after six months since the last immunization dose, which further encourages the deployment of booster dosing.

ID040. **A Single Drop of Fingerstick Blood for Quantitative Antibody Response Evaluation after SARS-CoV-2 Vaccination**

M. Sha[1], J. Pathakamuri[2], D. Kuebler[2], Y. Loginova[1], R. Chuang[1], C. Lu[3], A. Zhang[1]
[1]Diacarta Inc, Pleasanton, CA; [2]Franciscan University of Steubenville, Steubenville, OH; [3]University of California, San Francisco, CA.

Introduction: COVID-19, caused by infection with the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is associated with a spectrum of clinical manifestations ranging from asymptomatic infection to minor flu-like illness to acute respiratory distress syndrome to severe pneumonia and death. Testing and vaccination have been major components of strategies for combating the ongoing COVID-19 pandemic. In this study, we developed and evaluated the feasibility of using a sample consisting of a single drop of fingerstick blood collected with nylon flocked swabs for the quantitative detection of anti-SARS-CoV-2 S1 IgG antibody on a microsphere platform. Our results demonstrated that fingerstick blood is sufficient for quantitative measurement of antibody responses after receiving a mRNA COVID-19 vaccine. **Methods:** We have developed a quantitative anti-SARS-CoV-2 spike (S1) IgG antibody assay using a single drop of fingerstick blood by microsphere immunoassay. Recombinant SARS-CoV-2 spike protein 1 (S1) RBD was covalently coupled to the surface of MagPlex Microspheres. S1 RBD protein-coated magnetic beads and human specimens were mixed and incubated at room temperature for one hour. After washing, phycoerythrin-conjugated anti-human IgG antibody was added to the reaction mixture. The IgG concentration (mg/mL) was calculated according to the standard curve generated by the Luminex xPONENT software based on the Five Parameter Logistic (5PL) curve fit of the five standards. **Results:** We have demonstrated that the assay clinical performance showed a positive percent agreement of 100% (95% CI: 0.89-1.00) and a negative percent agreement of 100% (95% CI: 0.93-1.00). More importantly, we evaluated the feasibility of this high-throughput and quantitative anti-SARS-CoV-2 spike IgG antibody testing after vaccination. Fingerstick blood samples collected from 137 volunteers (313 data points) before and after receiving the Moderna or Pfizer mRNA vaccine were used. Anti-SARS-CoV-2 S1 IgG antibody could not be detected prior to or on day 7 after receiving the first vaccine dose, but antibodies could be measured from day 14 onwards.

No anti-SARS-CoV-2 nucleocapsid protein IgG antibody was detected in any of the vaccinated or healthy participants, indicating that the anti-SARS-CoV-2 S1 IgG assay is specific for the mRNA vaccine-induced antibodies. **Conclusions:** This method can be used for quantitative detection and monitoring of post-vaccination anti-SARS-CoV-2 spike IgG responses.

ID041. **Validation of Whole-Genome Sequencing of Bacterial Isolate**

N. Muto, M. Basqueira, R. Franco, F. Malta, A. Soares, P. Oliveira, P. Koga, R. Petroni, R. Sitnik, M. Cervato, M. Martino, J. Pinho, C. Mangueira, A. Doi
Albert Einstein Hospital, Sao Paulo, Brazil.

Introduction: The incidence of hard-to-treat bacterial infections is increasing in hospitals worldwide and enhancing the central role of rapid and accurate identification of the agent, for timely and effective treatment for the patients. The bacterial whole-genome sequencing (WGS) technique is becoming widely used in clinical diagnostic, public health laboratories, and in research. This technique can help in accurate identification of the causative bacterial pathogen, and can potentially evaluate virulence and antibiotic resistance, as well as providing data for molecular epidemiology in outbreaks, by clonality analysis. **Methods:** We selected 34 bacteria strains of different species previously identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Seven of the 34 samples were also submitted to antimicrobial susceptibility test (AST) in the VITEK2 AST cards (Cards for Antimicrobial Susceptibility Testing). For reproducibility, we selected three samples for triplicate intra-assay and three samples for triplicate inter-assays. DNA from isolated cultures was extracted and used on library preparation for next-generation sequencing (NGS) using 300pb paired end sequencing run. For data analysis, the genome taxonomy was assigned using GTDB-Tk v2.1.0. The mean nucleotide identity (ANI) between the genome and the closest reference was determined using FastANI. Resistance-encoding mutations were predicted using the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) v5.1.1. **Results:** The sequencing quality metrics were high satisfactory. All the strain presented a genomic coverage of at least 30x and mean of 98.8% and 0.93 for the ANI and alignment fractions parameters, respectively. We found correlation of 91% (31/34 samples) comparing the species identification by conventional MALDI-TOF and NGS, and 03 samples presented different species due to database update. Regarding resistance in the AST method, six strains presented resistance to imipenem and meropenem (carbapenems antibiotics class) and one strain only to imipenem. The WGS method was able to identify in the seven samples the antimicrobial resistance gene *NDM*-New Delhi metallo-β-lactamase (five samples NDM-1, one sample NDM-5 and one sample NDM-7) that is responsible in hydrolyzing a wide range of β-lactam antibiotics, including carbapenem, agreeing with the AST results. Intra- and inter-assay reproducibility showed 100% correlation between replicates. **Conclusions:** The WGS for bacterial isolate characterization demonstrated a high performance in the validation. The use of this technique in addition to conventional methods can help to improve the diagnostic of hard-to-treat infections worldwide.

ID042. **Mutation Analysis in Bedaquiline, Delamanid and Linezolid of Rifampicin Resistant Mycobacterium Tuberculosis in Korea Using WGS**

H. Lee[1], H. Kim[2], R. Heo[2], Y. Jun[2], K. Kim[2], K. Seok[2], S. Kim[2], H. Kim[2], J. Song[2], D. Han[2], S. Nam[2], S. Chae[2], C. Jimin[2], S. Shin[2], G. Lee[2], S. Lee[2], H. Kim[2], J. Yang[2], J. Whang[2]
[1]Korean National Tuberculosis Association, Seoul, Republic of Korea; [2]Korean National Tuberculosis Association, The Korean Institute of Tuberculosis, Cheongju, Ch'ungch'ong-bukto, Republic of Korea.

Introduction: We analyzed 8,943 isolates on the Lowenstein-Jensen (L-J) phenotypic DST from January 2018 to June 2018 and 16,454 isolates on the L-J phenotypic DST from April 2020 to August 2021, a total of total 25,397 isolates in Korea. Rifampicin resistant (R-R) isolates were categorized according to MICs using 7H9 broth microdilution method (7H9 BMD). Bedaquiline (BDQ) showed 82.16% of ≤0.03125 ~0.125 μg/mL area, delamanid (DLM) showed 88.78% of ≤0.00625 ~0.0125 μg/mL area, together with a relatively large distribution (8.03%) of 0.4 ~0.8 μg/mL area despite its high concentration. The aim of this study is to analyze mutations associated with high MIC cases using whole-genome sequencing (WGS). **Methods:** We selected 101 R-R isolates with high BDQ, DLM, or linezolid (LZD) MICs among 25,397 isolates. We tested 101 isolates using Miseq benchtop sequencer (Illumina, Inc., San Diego, CA) for WGS. We analyzed the *atpE, mmpR* and *pepQ* regions for 32 isolates that were >0.25 μg/mL in BDQ. For DLM, we analyzed the *ddn, fgd1, fbiA, fbiB,* and *fbiC* regions for 48 isolates which were >0.2 μg/mL. For LZD, we analyzed *rrl* and *rplC* regions for 11 isolates which were >1.0 μg/mL. Additionally, we analyzed 15 isolates of ≤0.25 μg/mL in BDQ, ≤0.2 μg/mL in DLM, and ≤1.0 μg/mL in LZD. **Results:** Among 33 isolates, 27 (84.38%) had mutations in *mmpR (Rv0678)* for BDQ. We found correlation between phenotypic DST and WGS. We were unable to find the mutant types (MT) in *atpE* and *pepQ*. For DLM, 41/48 (85.41%) isolates were of MT in *ddn*. For LZD, 10/11 (90%) isolates were found to be of MT in *rplC* region. Among low MIC isolates, 2/15 (13.33%) isolates were of silent MT in *rplC* and *fbiA*. In this study, we were able to find mutations for BDQ, DLM, and LZD in 96 isolates from 2020 to 2021, but we were unable to find the mutation for BDQ and DLM, and only found *rplC* mutation for LZD in five isolates in 2018. **Conclusions:** By matching MIC results and WGS, we found that *mmpR*, *ddn,* and *rplC* mutations are correlated with high MICs of new drugs. However, in this study, only five isolates in 2018 were used to analyze WGS, Further investigation is necessary to analyze large numbers of isolates and confirm the mutations associated with resistance.

ID043. **Deciphering the Microbiology of Respiratory Infection Using Precision Metagenomics Analysis**

R. Carpenter[1], R. Sharma[2], S. Almas[1], E. Brown[1], T. Vine[1]
[1]Advanta Genetics, Tyler, TX; [2]Advanta Genetics, Coppell, TX.

Introduction: Metagenomic sequencing using next-generation sequencing (NGS) technology has unparalleled power of comprehensive microbial profiling. NGS can detect unknown target organism(s) often undetected by routine microbiological and polymerase chain reaction (PCR) methods. We deployed a comprehensive NGS panel for simultaneous detection of 187 bacteria, 42 viruses, 53 fungi, and 1,218 antimicrobial resistance markers in 20 respiratory infection samples. NGS results were compared to a 36-target PCR panel commonly used for molecular diagnosis of respiratory infection. **Methods:** DNA and RNA were extracted separately and we prepared libraries using the Illumina RNA Prep with Enrichment kit. Indexed libraries were enriched for microbial content by hybridization capture with the Respiratory Pathogen ID-AMR panel (Illumina). Final libraries were sequenced using the Illumina MiniSeq instrument to yield paired-end reads of 75 bp length. Sequencing data were analyzed by the IDbyDNA-Explify portal. A final explify report was generated containing the quantitative identification of viruses, bacteria, and fungi in each sample, including the antibiotic resistance marker. Same samples were also analyzed with a 36-target semi-quantitative PCR panel, and results obtained from both technologies were compared. **Results:** All the organisms identified by the PCR panel were likewise detected in microbiome analysis. Three bacteria and 10 viruses were commonly detected by both PCR and NGS with *Streptococcus pneumoniae, Moraxella catarrhalis,* influenza A virus, human rhinovirus, and human metapneumoviruses most common concurrently detected organisms. However, the PCR panel only identified the co-infection in 2/20 samples, whereas the NGS panel detected a minimum of three and up to 20 organisms in a single sample. Additionally, 25 organisms (three viruses, 20 bacteria, and two fungi) were exclusively identified by the microbiome analysis; the most

prevalent – not targeted on PCR – were *Elizabethkingia species* (12/20) and *Dolosigranulum pigrum* (9/20). Furthermore, *Moraxella catarrhalis* was detected in only 3/20 samples by PCR compared to 9/20 samples by NGS. **Conclusions:** Although PCR testing for respiratory pathogen detection remains a valuable tool, concurrent NGS analysis suggests that the etiological restriction of PCR testing may have clinical limitations. The NGS-based microbiome analysis revealed a much more comprehensive microbiology profile. Moreover, NGS-based analysis has the potential to improve clinical outcomes in complicated co-infection cases. NGS results can also be used for improving PCR panels by adjusting the target organism(s) according to clinical significance. Further investigations are required to establish the clinical utility of the precision metagenomic analysis using NGS assays.

ID044. **WITHDRAWN**

ID045. **The Vaginal Microbiome: Its Composition and Function in Three Countries**

M. Dadlani, B. Ozdinc, D. Walsh
CosmosID, Germantown, MD.

Introduction: The healthy vaginal microbiome is a unique community that tends to be dominated by a single species of *lactobacilli*; *L. crispatus, L. jensenii, L. iners,* or *L. gasseri.* A fifth healthy community exists that is non-*lactobacilli* dominated and has much higher diversity. These are termed community state types (CSTs) and they may be associated with risk of vaginal infection. CST I, the *L. crispatus*-dominated community, seems to be the most protective, followed by CSTs II (*L. gasseri*) and V (*L. jensenii*). CST III is dominated by *L. iners*, which displays fewer protective characteristics. Finally, CST IV, the diverse, polymicrobial community, may be least protective and most prone to infections such as bacterial vaginosis (BV). BV-affected women often display a dominance of *Gardnerella vaginalis* in their vaginal microbiota but the presence of this species does not always indicate that the disease is present. Regardless, detection of these microbiota and classification into CSTs could help physicians predict a woman's chance of vaginal infection and lead to closer monitoring and earlier treatment. **Methods:** In this work we compare the vaginal microbiota of women from three different countries: groups from Sweden (N = 45), China (N = 35), and two groups from the US (N = 51 and N = 30). We imported this whole-genome sequencing data from publicly available studies and performed a comparative microbiome taxonomic and functional analysis using the CosmosID-HUB. We performed alpha diversity, beta diversity, and differential abundance analyses for the taxonomic and functional data on the HUB. **Results:** We found every CST within each group and the samples clustered according to CST rather than country, implying a broad applicability of the CST classification system. However, when we predicted functionality of the microbial communities we found that they clustered by country rather than CST. This suggests that vaginal community functionality may be influenced by location, regardless of CST. Community functionality may be influenced by ethnicity, lifestyle practices, diet, or some other environmental factor. One functional group consisted of samples with enriched abundance of genes coding for mucin degradation, which has been previously associated with pathogenic potential. The women in this group were from a mix of the three countries. **Conclusions:** These results suggest that, in a healthy state, CSTs are globally present but their functionality may be influenced by location-specific factors. However, there may be a subset of functions with greater pathogenic potential that is not location-associated. These women may be more prone to vaginal infections, and their identification may aid physicians in catching and treating vaginal disease early on.

ID046. **Evaluation of Aptima HIV-1 Quant Dx Assay on the Panther System in a Pediatric Hospital**

A. Rector[1], D. Cuevas[1], A. Crowell[1], Y. Wongworawat[2], T. Ton[1], J. Dunn[3]
[1]Texas Children's Hospital, Houston, TX; [2]Baylor College of Medicine, Houston, TX; [3]Baylor College of Medicine, Texas Children's Hospital, Houston, TX.

Introduction: Rapid and accurate diagnosis of human immunodeficiency virus type 1 (HIV-1) infection is critical for patient management, reduction of mortality, and prevention of forward transmission. The Aptima HIV-1 Quant Dx Assay (Aptima) (Hologic, Inc., San Diego, CA) is an *in vitro* nucleic acid amplification test for both the diagnosis of HIV-1 infection and quantitation of viral RNA in plasma samples on the fully automated Panther System (Hologic) in <4 hours. The assay quantitates HIV-1 RNA between 30 to 10 million copies/mL. Target capture, target amplification by transcription-mediated amplification, and detection of the amplicon by fluorescent labeled probes (torches) occurs in a single tube within the fully automated system. **Methods:** A total of 199 plasma samples were included in the study. Thirty-four were previously quantitated samples obtained from BioCollections Worldwide, Inc. (BWI), (Miami, FL). One hundred and thirty-one prospective and retrospective patient plasma samples, along with 34 contrived samples, previously tested at Texas Children's Hospital (TCH) by either the cobas AmpliPrep/cobas TaqMan HIV-1 Test (cobas) (Roche Diagnostics, Indianapolis, IN) and/or the APTIMA HIV-1 RNA Qualitative Assay (Hologic, Inc., San Diego, CA). To assess the accuracy of testing low volume plasma samples, a total of 43 samples (33 patient plasma samples and 10 contrived samples) originally tested by BWI and/or TCH using the cobas were diluted in HIV-1-negative normal human plasma and tested at TCH using the Aptima assay on each Panther system. All retrospective samples remained frozen until retested on the Panther using the Aptima assay. **Results:** The overall sensitivity of the Aptima assay for HIV-1 RNA detection was 92% (113/123). Ten samples with original results ranging from <20 copies/mL to 608 copies/mL were not detected by the Aptima assay. These discrepancies are likely due to sample degradation after multiple freeze-thaws or target loss from low level of viral content. Linearity of diluted and undiluted samples were analyzed by simple linear regression and showed correlation with R^2 values of 0.8867 and 0.8871 respectively. Limit of detection studies included dilutions of the SeraCare AccuSpan HIV-1 RNA Linearity Panel member 6 (viral load 2,014 copies/mL) tested in 10 replicates at 30 copies/mL, which demonstrated detectable HIV-1 RNA at 100% (10/10). Analytical specificity and precision/reproducibility were both 100%. **Conclusions:** The Aptima assay on the Panther system is a relatively easy-to-perform, sample-to-answer test with minimal hands-on time. The assay delivers sensitive and reliable results which enable early diagnosis of HIV-1 infection and quantitative information for patient staging and monitoring.

ID047. **Development of a Novel Triplex Reverse Transcription Loop Mediated Amplification (RT-LAMP) Assay**

Y. Chander, D. Mead, P. Brumm, A. Hassinger, K. Throckmorton
Varigen Biosciences, Middleton, WI.

Introduction: Most loop-mediated isothermal amplification (LAMP)-based diagnostic methods are generally used for the detection of a single target per reaction, as current methods of detection in a LAMP assay are not sequence-specific and lack in differentiation among multiple targets amplified in a single reaction. To overcome this challenge, we have developed a novel method for detection of three targets in a single LAMP reaction using labeled primers. **Methods:** Triplex RT-LAMP assay was developed for detection of foot-and-mouth disease virus (FMDv) in cattle and pigs. LAMP primers targeting two different regions of FMDv were designed. For internal control (IC), LAMP primers were designed targeting a conserved region of the 18S rRNA gene. Primer designs were evaluated for specificity and sensitivity in an RT-LAMP assay. Each target (FMDv-1, FMDv-2, and 18S rRNA) was detected in a separate RT-LAMP assay, and reactions

were performed using Isothermal Master Mix. This master mix contains all the reagents required for RT-LAMP, including proprietary enzymes (Bst polymerase and RT enzyme) and intercalating dye (SYTO-82) for real-time monitoring of amplification. Master Mix was combined with 10-fold serial dilutions of template (synthetic RNA for each target) and target-specific primer sets. The reactions were performed in a real-time thermocycler at 72°C for 30 minutes, with data collected every 30 seconds. Best performing primer sets for each target were selected for developing Triplex RT-LAMP. For multiplexing, best performing primer sets for each target were labeled with a 5'-fluorophore and a 3'-quencher (BHQ-1). FMDv-1 primers were labeled with Cy5 fluorophore, FMDv-2 primers with Texas Red (TxR) fluorophore, and 18S rRNA with FAM fluorophore. Triplex RT-LAMP assay was performed using a specially formulated Multiplex Isothermal Master Mix but without any intercalating dye added to the mix. All three primer mixes were added to the Master Mix and reactions were carried out in a real-time thermocycler at 72°C for 30 minutes, with data collected every 30 seconds. Assay sensitivity was determined by testing 10-fold serial dilution of respective template, individually as well as mixed together. **Results:** Results obtained showed feasibility of triplex RT-LAMP assay for simultaneous detection of three targets in a single reaction. Sensitivity and specificity of triplex RT-LAMP were comparable to control single target reactions with intercalating dye. No cross-reactivity or non-specific amplification was observed with any of the primer designs. **Conclusions:** This newly developed method allows detection of up to three targets in a single reaction without affecting the assay performance. This method is much easier to develop and optimize than previously reported methods, as this approach uses standard LAMP primers without need of any additional primers/probes.

ID048. **Detection and Quantification of EBV Transcript in Whole Blood, Plasma and Paraffin-Embedded Tissue Samples from Organ Transplant Recipients with Post-Transplant Lymphoproliferative Disorder (PTLD)**

R. Martinez, P. Mroz, A. Farah, A. Keeler
University of Minnesota, Minneapolis, MN.

Introduction: Epstein-Barr virus (EBV) infection occurring in organ transplant recipients is associated with development of post-transplant lymphoproliferative disorder (PTLD), a serious post-transplant complication and comorbidity. The early and accurate detection and quantification of EBV DNA copy numbers in the whole blood and/or plasma specimens are an excellent way to identify patients who have acute infection or reactivation of their EBV infection and are at increased risk of developing PTLD. To date, no EBV DNA quantitative assay has been FDA approved for this purpose. Furthermore, there is no WHO EBV DNA standard available, making the comparisons of EBV DNA quantitative results among different laboratories particularly challenging. Here, we developed and validated a droplet digital PCR (ddPCR) assay for the quantification of EBV viral load in whole blood, plasma, and formalin-fixed, paraffin-embedded (FFPE) tissue for both the monitoring and adjunct diagnosis of EBV+ PTLD. **Methods:** Validation experiments involved optimization of DNA extraction methods as well as ddPCR parameters including primer efficiency evaluation, annealing temperature, cycle number, primer concentration, and droplet generation. Droplets were collected by a QX200 Droplet Reader and analyzed with QuantaSoft software (Bio-Rad) or a novel method using flow cytometry software (FlowJo, BD). **Results:** Assay validation experiments demonstrated a limit of detection of ~111 copies/ml (positive results in ≥95% repeated measurements) and linearity over 4.5 $\log_{10}$. Interestingly, the ddPCR assay was consistently measuring 1 $\log_{10}$ lower EBV DNA concentration than the commercial assay. Measurement of paired whole blood and plasma samples demonstrated reduced EBV viral loads in plasma. When normalized to a housekeeping gene Abl1, plasma samples demonstrated higher normalized viral loads than whole blood. FFPE results from lymph nodes from healthy controls and diagnostic EBV+ PTLD cases showed healthy controls with a 2 $\log_{10}$ range of normalized EBV DNA, and PTLD cases demonstrating a 7 $\log_{10}$ range. Interestingly, some PTLD samples had similar normalized EBV loads to healthy controls. **Conclusions:** We developed, optimized, and validated a single-step, multiplexed, and automated ddPCR assay for the detection of EBV DNA from whole blood, plasma, and FFPE samples. Ongoing studies include correlating morphologic variants of EBV+ PTLD with normalized EBV DNA quantity and analyzing EBV RNA from FFPE to determine if active viral replication helps to further distinguish healthy controls or PTLD morphologic variants. We strongly believe that the improved quantification and detection of EBV by ddPCR may lead to earlier detection of PTLD in organ transplant recipients and translate to better clinical outcomes.

ID049. **Use of Encapsulated RNA as a Stable and Quantitative Positive Control for Infectious Disease Detection, Wastewater Surveillance and More**

M. Maharjan, Y. Bao, R. Kadam, C. Sontag, J. Wang, A. Farmer
TakaraBio, San Jose, CA.

Introduction: External and internal positive controls are an integral part of molecular assays, as they not only eliminate false negatives and positives, but also can verify extraction and quantify samples. Workflows involving RNA demand a highly stable control, due to ubiquitous ribonucleases (RNases). Unreliable RNA controls make data interpretation challenging and cost a great deal of time, effort, and expense during both validation and execution. **Methods:** Here we describe a new technology of a single-plasmid double-expression system to encapsulate target-specific RNA sequences in stable, non-infectious phage-like particles. To validate our technology, we produced encapsulated RNA (En-RNA) containing sequences specific to the CDC assays for SARS-CoV-2, and evaluated production, purification, extraction, and detection via RT-qPCR using a commercially available SARS-CoV-2 detection kit. **Results:** We demonstrate that large-scale production of En-RNA followed by affinity purification, absolute quantification with digital droplet PCR, and rigorous quality-control tests ensure precise copy number and very low variability from lot to lot. Next, we show that our En-RNA particles have very similar extraction efficiencies using simple extraction methods like heat lysis at 85°C, to harsher methods like TRIzol/Chloroform extraction or commercial RNA extraction kits, making them an ideal positive control even when sample volume is a limiting factor. Finally, using a commercially available SARS-CoV-2 detection kit, we demonstrate that our En-RNA performed better in comparison to conventional controls when working with either saliva or wastewater samples. **Conclusions:** These data demonstrate that our controls offer the unique ability for precise and stable performance throughout all stages of the RNA processing workflow. Performance remains, regardless of the sample type (such as saliva, nasal swabs, blood, plasma, wastewater, etc.), sample volume, extraction method, and the desired En-RNA sequence. Moreover, En-RNA outperforms standard controls in RT-qPCR, enabling more precise sensitivity for pathogen detection. Thus, En-RNA is a unique and ideal control for infectious disease detection, gene expression experiments, biomolecular studies, wastewater surveillance, and more.

ID050. **Prevalence of High-Risk HPV Genotypes in Cervical Specimens from Kosovo**

R. Barney[1], N. Georgantzoglou[1], K. Hershberger[2], W. Keegan[1], E. LaRochelle[1], L. Kennedy[1], S. Manxhuka-Kerliu[3], S. Bejarano[4], P. Palumbo[4], G. Tsongalis[1], J. Bentz[1]
[1]Dartmouth-Hitchcock Medical Center, Lebanon, NH; [2]Virginia Commonwealth University, Richmond, VA; [3]University of Pristina, Pristina, Albania; [4]Hospital of la Liga Contra el Cáncer, San Pedro Sula, Honduras.

Introduction: Nearly all cervical cancers are caused by persistent high-risk human papillomavirus (HPV) infection. There are 14 recognized high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and HPV genotypes 16 and 18

comprise ~66% of all cases worldwide. Another 15% of cervical cancers are caused by HPV genotypes 31, 33, 45, 52, and 58. Screening patients for high-risk HPV is a proven strategy for decreasing the incidence of cervical carcinogenesis caused by HPV. Here, we present population data from an HPV screening initiative in Kosovo to better understand the prevalence of HPV in this region. **Methods:** A total of 863 cervical swabs were collected in Kosovo and sent to Dartmouth Health in 50mL conical tubes. Viral particles were resuspended in lysis buffer and then isothermally amplified using the Atila AmpFire HPV Screening 16/18/HR. This kit qualitatively detects all high-risk HPV genotypes (listed above) and separately subtypes HPV 16 and 18. Samples that were positive on the screening assay were further genotyped using the MeltPro High Risk HPV Genotyping Assay (QuanDx, San Jose, CA) and the ZSLAN real-time PCR instrument. **Results:** Eight hundred sixty-three cervical swabs were screened, with 116 swabs (13%) identified as positive and 16 swabs (2%) as invalid. Of the 116 positive swabs, the screening assay identified 28 swabs (24%) with HPV 16, and six of these cases also harbored a coinfection with other high-risk genotypes. Four samples (3%) harbored both HPV 16 and 18, with three coinfected by additional high-risk genotypes. One sample was positive for HPV 18 and other high-risk HPV types. The remaining 83 samples (72%) contained only non-16/18 high-risk HPV genotypes. Twenty-nine of the 116 positive swabs (25%) were invalid on the MeltPro genotyping assay, and five swabs (4%) were negative by the assay. Of the 81 remaining positive swabs, 59 (73%) contained HPV 51; 32 (40%) were positive for HPV 68; 22 (27%) harbored solely HPV 16; 20 (25%) were positive for HPV 35; and 16 (20%) had an isolated infection with HPV 18. HPV 58 was found in 11 samples (14%) and HPV 52 was identified in eight swabs (10%). Of the 81 genotyped samples, 31 samples (37%) were simultaneously coinfected with two HPV genotypes; 23 (28%) with three types; six (7%) with four types; two (2%) with five types; and one (1%) with six types. **Conclusions:** Three of the top five HPV genotypes in this Kosovo cohort only comprise ~19% of cervical cancers worldwide, which supports a geographic distribution of the most common high-risk HPV genotypes. These findings also suggest consideration for targeted vaccine coverage and treatment guidelines based on the most prevalent genotypes in different geographic regions.

ID051. **Expanded PCR Panel Testing for Identification of Respiratory Pathogens and Coinfections in Influenza-Like Illness**

P. Upadhyay[1], F. Surur[1], G. Kim[1], V. Singh[1], J. Reddy[1], H. Veereshlingam[2], T. Proctor[2], O. Sorel[2], M. Gandhi[2]
[1]HealthTrackRx, Denton, TX; [2]Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Although COVID-19 has dominated influenza-like illness (ILI) over the past two years, there are many other pathogens responsible for ILI. It is not uncommon to have coinfections with multiple pathogens in patients with ILI. The goal of this study was to identify the different organisms in symptomatic patients presenting with ILI. **Methods:** Specimens were collected from 381 subjects presenting with ILI symptoms. All samples (nasal and nasopharyngeal swabs) were simultaneously tested on two expanded panel PCR platforms: Applied Biosystems TrueMark Respiratory Panel 2.0, OpenArray plate (OA) (32 viral and bacterial targets); and Applied Biosystems TrueMark Respiratory Panel 2.0, TaqMan Array card (TAC) (41 viral, fungal, and bacterial targets). Results were analyzed for concordance between the platforms as well for identification of organisms responsible for the clinical presentation including possible coinfections. **Results:** Very good agreement was observed between the OA and TAC. The organisms that showed 100% agreement were SARS-CoV-2, coronaviruses (NL63, OC43, 229E), *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, parainfluenza virus (PIV) 2, PIV 3, pan-enterovirus, enterovirus D68, RSVB, HHV4-EBV, HHV5-CMV, and HHV6. A 99% positive percent agreement (PPA), 100% negative percent agreement (NPA) was observed for *Staphylococcus aureus,* and 94% PPA, 99% NPA for rhinovirus. One sample was detected positive for metapneumovirus on TAC, but not on OA. *Moraxella catarrhalis* and *Pneumocystis jirovecii*, which were detected positive on TAC (18 and two samples, respectively), were not included on OA; hence, an agreement could not be established. Results from TAC platform showed 35% positivity rate for one organism, 20% for two organisms, and 3% for three organisms, whereas 42% of the samples were negative for all organisms. *S. aureus* was the most prevalent (29%) followed by SARS-CoV-2 (21%), rhinovirus (12%), HHV6 (8%), and *M. catarrhalis* (5%). Of all coinfections, bacterial-viral was the most common (68%), followed by viral-viral (22%), bacterial-bacterial (8%), bacterial-fungal (1%). SARS-CoV-2/*S. aureus* coinfections were the most common (29%), followed by rhinovirus/*S. aureus* (12%), HHV6/SARS-CoV-2 (8%), HHV6/*S. aureus* (7%), and *M. catarrhalis*/*S. aureus* (5%). **Conclusions:** Although targeted RTI panels are useful in most outpatient settings**,** expanded panels are recommended for in-patient settings for ILI, where diagnosis turnaround time and accuracy are key to a precise and personalized treatment plan and reduced length of hospitalization. Coinfections are common in RTIs, and syndromic panel-based multiplex PCR tests can enable the identification of pathogens contributing to coinfections and help guide patient management, thereby improving clinical outcomes and supporting antimicrobial stewardship.

ID052. **Validation of a Laboratory Developed BKV qPCR Assay on the NeuMoDx 288 Molecular System**

E. Bixler, J. Davidson, S. Cowden, J. Grantham, J. Nutt, B. Neil, J. Huang
Eurofins-Viracor, Lenexa, KS.

Introduction: The NeuMoDx 288 Molecular System is a fully automated sample-to-result liquid handling system capable of performing automated extraction and isolation of nucleic acids, as well as amplification and detection of target nucleic acid sequences by fluorescence-based qPCR. Here we describe the performance and validation of an in-house laboratory-developed BKV qPCR assay using the NeuMoDx 288 platform. **Methods:** The NeuMoDx platform utilizes proprietary NeuDry extraction and amplification reagents that are lyophilized in the bottom of disposable strips. Native sample nucleic acids are extracted and bound to magnetic silica, then transferred to a microfluidics cartridge to separate beads from lysate and wash/elute the nucleic acid. Eluate is then added to NeuDry master mix reagents and analyte-specific primers/probes before being returned to the microfluidics cartridge for amplification and data acquisition. For this study, BKV virus was spiked into BKV-negative plasma and urine to assess the analytical sensitivity, analytical accuracy, intra-assay/inter-assay precision (reproducibility), linearity, and dynamic range of the BKV qPCR assay performed using the NeuMoDx 288 platform. Analysis of BKV qPCR reactions was performed using an auto-cycle threshold (C_t) function that calls the C_t using a NeuMoDx algorithm based on the second derivative of the amplification curve. Prior to validation, the BKV qPCR assay was calibrated to the first World Health Organization (WHO) International Standard (IS) for BK virus. **Results:** Full-process standards generated linear plots with confident predictive power (R^2 >0.99). Slope and y-intercept for plasma were -3.3199 and 38.7133, respectively, with urine exhibiting a slope and y-intercept of -3.2886 and 38.7933, respectively. Standardizing the quantitative results to the first WHO BKV IS allowed for reporting standardized IU/mL quantitative results. The LOD was determined to be 7 IU/mL in plasma and 19 IU/mL in urine. Linearity and dynamic range of whole virus spiked in plasma and urine was assessed. Linear regression analysis yielded a slope of 0.9452 (R^2 = 0.9919) for plasma and 0.0404 (R^2 = 0.9877) for urine. Intra- and inter-assay precision IU/mL %CV ranges were 1.3% to 22% and 17% to 19%, respectively, in plasma and 5.8% to 40% and 41% to 50%, respectively, in urine. Accuracy was demonstrated to be 100% in plasma and 97% in urine. **Conclusions:** Validation of the BKV laboratory-developed test on NeuMoDx 288 Molecular System has demonstrated that this platform can deliver a rapid sample-to-result turnaround and a sensitive means

of detecting BKV in plasma and urine. The NeuMoDx platform is an option for high-complexity molecular labs looking to increase testing throughput using automation.

ID053. **A Lyo-Ready 1-Step RT-qPCR Master Mix: Utilising a Warm-Start Thermostable Reverse Transcriptase to Enable Sensitive Pathogen Detection with Difficult RNA Targets**

A. Burgess[1], M. Siewert[2], M. Auldridge[2], J. Koelbl[2], T. Rockweiler[2]
[1]LGC Biosearch Technologies, Hoddesdon, United Kingdom, [2]LGC Biosearch Technologies, Middleton, WI.

Introduction: Reverse transcriptases are typically inactive at high reaction temperatures, which reduces the ability to detect certain targets. RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has been specially developed with a heat-activated thermostable reverse transcriptase, capable of maintaining activity in higher temperatures, therefore enabling the sensitive pathogen detection of difficult targets such as those with high %GC content or with double-stranded RNA genomes requiring pre-denaturation. **Methods:** Rotavirus A genomic doubled-stranded RNA (ATCC VR-2018DQ) was serially diluted to achieve a final reaction concentration of 5-50,000 genomic copies/reaction. The samples were either directly added to the RT-qPCR reaction or were incubated at 95°C for five minutes to pre-denature the dsRNA. The samples were tested in 20µL reactions with either RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix or a competitor master mix and a Dual-labeled BHQ Probe Assay targeting the *Nsp3 Rotavirus A* gene. The reactions were set up and run with thermocycling parameters according to the manufacturer's recommendations. **Results:** With pre-denatured samples, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix achieved 100% detection down to five copies/reaction versus the competitor master mix only detecting half of samples at 50 copies/reaction. Without pre-denaturation, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix achieved 100% detection down to 50 copies/reaction versus the competitor master mix detecting less than half at 50 copies/reaction. **Conclusions:** RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix can maintain a higher sensitivity for the detection of double-stranded RNA Rotavirus without the need for pre-denaturation steps compared to a competitor master mix.

ID054. **Detection of High-Risk Human Papillomavirus (hr-HPV) in Head and Neck Carcinoma Samples from Honduras**

N. Georgantzoglou[1], C. Alas-Pineda[2], K. Hershberger[1], T. MacKenzie[1], A. Suresh[1], L. Kennedy[1], S. Bejarano[3], G. Tsongalis[1], D. Kerr[1]
[1]Dartmouth-Hitchcock Medical Center, Lebanon, NH; [2]Facultad de Medicina y Cirugía, Universidad Católica de Honduras-Campus San Pedro y San Pablo, San Pedro Sula, Honduras; [3]La Liga Contra el Cáncer, San Pedro Sula, Honduras.

Introduction: Head and neck (HandN) carcinoma is the sixth most common solid malignancy worldwide. High-risk human papillomavirus (hr-HPV) has emerged as the etiologic driver for an increasing proportion of these cancers. In oropharyngeal squamous cell carcinoma (SCC), hr-HPV carries prognostic, staging, and treatment implications. Most new cancer diagnoses occur in low- and middle-income countries (LMIC), and Honduras is an LMIC with a high prevalence of hr-HPV-related cervical carcinoma. In Honduras, the most commonly seen hr-HPV serotypes in cervical cytology are different from those in the US. Given the high burden of hr-HPV-related cervical cancer and the clinical implications of hr-HPV status in a subset of HandN cancers, we aimed to identify, for the first time in the English literature, the prevalence of hr-HPV in HandN cancer specimens from Honduras and describe the relevant histopathologic findings. **Methods:** We retrospectively searched the pathology archives of a Honduran cancer center, identifying nine cases of HandN carcinoma with available material. Hematoxylin and eosin (HandE) slides were reviewed by a subspecialized HandN pathologist. DNA isolation from formalin-fixed, paraffin-embedded tissue blocks was performed using Purigen Ionic Purification. MeltPro high-risk genotyping reagents with extraction eluate were mixed and loaded directly onto the SLAN-96 real-time PCR instrument (QuanDx/Zeesan Biotech) and run per the manufacturer's protocol. This assay detects and distinguishes 14 hr-HPV types. **Results:** The mean patient age was 70 (range: 44-84) and the female-to-male ratio was 5:4. Three tumors were in the tongue, two in the hypopharynx, two in oral mucosa, and one each in the uvula and soft palate. Five had no detectable hr-HPV, two were positive for hr-HPV, and two yielded invalid PCR results. The hr-HPV-positive tumors were oropharyngeal: a soft palate tumor with HPV16 and a uvula tumor with coinfection of HPV16 and 45. Histologically, the HPV16-positive tumor was a non-keratinizing SCC, whereas the HPV16/45-positive tumor was an invasive moderately differentiated, keratinizing SCC. Among the hr-HPV-negative tumors, three were invasive keratinizing SCCs (two poorly differentiated, one well differentiated), one was a low-grade adenocarcinoma, and one was *in situ* neoplasia, mild to moderate keratinizing dysplasia. **Conclusions:** In this Honduran cohort, hr-HPV-related SCC appears to have a predilection for the oropharynx, whereas hr-HPV-negative tumors commonly involve the oral cavity. Keratinizing morphology was typical of hr-HPV-negative SCC, whereas hr-HPV-positive SCC showed either keratinizing or non-keratinizing morphology. Testing a larger cohort is essential for a fuller picture of hr-HPV serotypes and the overall burden of hr-HPV in HandN carcinoma in Honduras.

ID055. **Surveillance of Infectious Agents with a Highly Multiplexed, Quantitative Wastewater Surveillance Using Digital PCR**

B. Brown[1], I. Regoli[1], J. Schmidt[2], H. Steeves[2]
[1]ChromaCode, Carlsbad, CA; [2]LuminUltra, New Brunswick, Canada.

Introduction: A large, global increase in wastewater surveillance infrastructure was due to COVID-19. Wastewater has the capability to detect community risks such as influenza A/B and multi-drug resistance. The WHO, in May 2022, urged countries worldwide to increase their wastewater surveillance for monkeypox following outbreaks in several non-endemic countries. The number of detectible targets in a dPCR is unfortunately limited by the number of available fluorescence channels. Thus, as the desire to surveil more targets in wastewater increases, there arises a need for higher-level multiplex on dPCR instrumentation. Here we demonstrate a single well assay capable of detecting and quantitating influenza A, H3, H12009pdm, influenza B, SARS-CoV-2, bla_{NDM}, *mcr*-1, and monkeypox from wastewater samples. **Methods:** Wastewater samples collected in early 2021 from sites across North America were extracted using the GeneCount SARS-CoV-2 Wastewater Test Kit. In addition, 124 contrived samples were evaluated. The extracted samples and contrived samples were evaluated on the Qiagen QIAcuity using a highly multiplexed assay developed by ChromaCode and leveraging their HDPCR technology, which contained primers and probes for eight analytes in a single well. Positive partitions were identified using a proprietary algorithm and temporally trended across location. **Results:** Three samples showed high levels of SARS-CoV-2 (>~10 copies/µL of eluate) as measured by dPCR. The remaining samples showed low-level SARS-CoV-2 detection ranging from not detected to two copies/µL. One sample showed a high level of detection for influenza A and H3 (~10 copies/µL of eluate) using the flu A universal primer/probe set and H3 primer/probe set, respectively. No H1 2009pdm, flu B, monkeypox, bla_{NDM} or mcr-1 was detected in the samples evaluated, but all were detected as expected in contrived samples with concentrations ranging from two copies/µL to 2,000 copies/µL. **Conclusions:** We successfully detected viral targets in wastewater samples and quantified their rise and fall over time. Furthermore, the ability to heavily multiplex in a single well on dPCR while maintaining sensitivity and quantitation allows for a cost-effective, high-throughput, and reliable tool for monitoring infectious agents in wastewater. These results, coupled with ChromaCode Cloud, ChromaCode's analysis and data aggregation platform, demonstrate that highly multiplex dPCR, enabled by HDPCR, can

provide comprehensive wastewater monitoring beyond only SARS-CoV-2 that could provide quick and actionable results for public health.

ID056. **A Molecular Platform That Interfaces Directly with Blood Culture Bottles Enables Earlier Pathogen Identification**

L. Huning, D. Varma, A. Jackson, P. Bohlen, R. Ng, V. Pamula
Baebies Inc., Durham, NC.

Introduction: Prolonged antibiotic exposure in infants with negative blood cultures is associated with adverse outcomes, disruption of the developing microbiome, and increased carriage of antibiotic resistance genes. Platforms capable of accelerating bloodstream infection diagnosis can improve patient outcomes and promote antibiotic stewardship. Here, we present a digital microfluidic (DMF) platform that combines blood culture (BC) and PCR-based pathogen identification (ID) to monitor pathogen growth and reflex positive samples directly to ID without operator intervention. Our device enables bacteria to be detected at quantities 1,000-fold lower, and therefore earlier than CO_2 detection chemistries. **Methods:** To maximize the detection sensitivity of pathogens in the BC bottle, we optimized sample preparation methods and reagent formulations to enable PCR to be performed directly on the contents of the BC bottle without dilution. Custom primer/probe sets were designed to report Gram status as well as species-specific ID of the five most commonly implicated bacteria in neonatal sepsis (*K. pneumoniae, E. coli, S. aureus, S. agalactiae*, or *S. epidermidis*). Commercial BC bottles were inoculated with whole blood and a single bacteria species at 2e5 CFU/mL, then placed onto the BC bottle receptacle on our platform. A sample from the BC bottle (75 µl) was transferred immediately into a DMF cartridge using an integrated peristaltic pump. The cartridge was preloaded with all reagents necessary for sample prep and multiplex PCR, and all liquid handling steps, including sample preparation, were performed by the cartridge automatically. PCR positivity was determined using cycle threshold and fluorescence intensity cutoffs. **Results:** Each of the five bacteria species, including Gram status, was correctly identified directly from BC bottles with no cross-reactivity. Gram status was reported in ~60 minutes, followed by species ID results. In controls that were inoculated with blood only, no amplification was observed except for housekeeping gene controls. **Conclusions:** Our integrated molecular platform provides Gram status and pathogen ID directly from BC bottles containing 2e5 CFU/mL of bacteria, which is 1,000-fold fewer bacteria than the limit for a positive BC result using traditional continuous monitoring systems. By interfacing sensitive molecular detection methods directly to BC bottles and automating liquid handling and sample preparation steps to eliminate hands-on time, we expect to reduce the time to Gram status and species ID results by eight to nine hours compared to traditional diagnostics. Future work will include reduction of assay run-time, expansion of pathogen panels, and development of the cartridge and hardware to perform repeated PCRs from the same BC bottle.

ID057. **Development of a Multiplex RT-qPCR Assay for the Simultaneous Detection and Differentiation of Human Adenovirus, Metapneumovirus, Parainfluenza Virus and Rhinovirus/Enterovirus**

Q. Sun[1], L. Wong[2], F. Hu[2], J. Du[2], E. Kyger[2], A. Hurley[2], F. Rathi[2], J. Yu[2], X. Wang[2], K. Li[2], S. Williams[2], M. Gandhi[2], K. Norman[2]
[1]Thermo Fisher Scientific, Fairfield, CA; [2]Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Viruses other than SARS-CoV-2, influenza, and respiratory syncytial virus (RSV) represent a significant proportion of respiratory tract infections in outpatient settings as well as hospitalized patients. The TaqPath Respiratory Viral Select Panel is a multiplex RT-qPCR test designed to detect and differentiate essential respiratory pathogens including human adenovirus, metapneumovirus (hMPV), parainfluenza virus (hPIV) and rhinovirus/enterovirus (hRV/hEV). In this study, we evaluated the analytical and the clinical performance of the TaqPath Respiratory Viral Select Panel (TaqPath). **Methods:** Target-specific TaqMan probes were used in the five-plex RT-qPCR assay to allow simultaneous detection and differentiation of hPIVs (probes labeled with FAM), hRV/hEV (VIC), hMPV (ABY), adenovirus (Alexa647) and human RNase P as internal controls (JUN). Using synthetic DNA templates and *in vitro* transcribed RNA (IVT-RNA) serial dilutions, the PCR performance of the multiplex panel was evaluated. Analytical performance including Limit of detection (LoD), precision, cross-reactivity, and competitive interference was evaluated using contrived samples with inactivated virus strains. Clinical performance was evaluated in a retrospective study with 350 nasopharyngeal (NP) swab samples using a FDA 510(k)-cleared assay (Luminex NxTAG Respiratory Pathogen Panel) as a comparator. Clinical sensitivity and specificity were determined after arbitration testing of discordant samples using BioFire Respiratory Panel 2, which is also an FDA 510(k)-cleared test. **Results:** For analytical performance, LoD of TaqPath was between 0.060-37.500 $TCID_{50}$/mL for the targeted pathogens. TaqPath showed good dynamic range with linearity over five orders of magnitude from 10^5 to 10 copies/reaction. Precision studies showed high repeatability (SD ≤0.68, CV% ≤1.85) and reproducibility (SD ≤1.09, CV% ≤2.95). No cross-reactivity with 52 common respiratory microorganisms tested was observed, and there was no competitive interference observed in the tested target combinations. For clinical performance, after arbitration testing of discordant samples, the clinical sensitivity and specificity of TaqPath for each of the targets were as follows: adenovirus (100%, 99.4**%**); *hMPV* (92.7%, 100%); *hPIV* (100%, 100%); *hRV/hEV* (80.6%, 98.8%). **Conclusions:** TaqPath Respiratory Viral Select Panel is a highly sensitive and specific molecular assay for simultaneous detection and differentiation of common respiratory viruses in a single test format. The assay allows for accurate detection of the targeted pathogens in a clinical setting including presence of co-infections.

ID058. **Integration of Viral Probes into a Clinical NGS Cancer Panel**

J. Adler, J. Segal
University of Chicago, Chicago, IL.

Introduction: The identification of tumor-associated viruses is an important component of the assessment of solid tumors. Myriad tumor types are defined diagnostically and prognostically by the presence of viral DNA, and the diagnostic inference gained in otherwise difficult cases can be significant. Despite these considerations, typical workflows for identification of viruses in tumor samples are one-at-a-time-type operations. Incorporating pan-tumor virus discovery into large-scale cancer panels would allow for universal detection, even when a tumor-associated virus is not predicted. **Methods:** The University of Chicago Oncoplus is a 1,005-gene hybrid capture NGS panel with a 168-gene subset reported into the medical record. Covaris-sheared DNA was assembled into libraries using the HTP library preparation kit (KAPA) and captured with a panel of oligonucleotide xGen probes (IDT) purchased via the GOAL consortium. Final libraries are sequenced on a NovaSeq 6000 system (2x101 bp, ~25M read pairs per sample). Probes for 29 viral genomes were included in the capture pools during the design phase. Viral genomes were added to the hg19 reference genome as additional "chromosomes" to permit optimal read partitioning during BWA-MEM alignment. Quantification of each virus was accomplished by extracting read counts associated with each viral "chromosome," expressed as viral reads per million total reads. Approximately 3,000 patient samples submitted for our clinical NGS cancer panel were analyzed alongside control samples. **Results:** Analysis of tumor-associated viral sequences in large-scale next-generation sequencing (NGS) panel data was able to correctly identify known cases of virus-associated tumors such as HPV-related squamous cell carcinoma, Merkel cell carcinoma, and Kaposi sarcoma with 100% sensitivity. High viral counts were able to provide significant clinical inference into a number of challenging cases, such as a case of metastatic cervical squamous cell carcinoma (pancreatic mass initially diagnosed as pancreatic ductal adenocarcinoma), as well as a likely case of Epstein-Barr virus-associated smooth muscle tumor initially diagnosed as

myofibromatosis. Using dual-index libraries compared with single-index libraries, cross-contamination of viral reads into negative samples within sequencing batches was substantially minimized. **Conclusions:** This study demonstrates the utility and practicality of integrating viral probes into a clinical NGS cancer panel as an additional layer of scrutiny for challenging or confounding cases, additionally providing a level of quality control prior to downstream analysis. Including pan-virus discovery across all specimens tested in the lab, such as those evidenced by the two cases highlighted above, has the potential to produce substantial improvement to patient management.

ID059. **New HIV-1 Drug Resistance Genotyping Assay of the Protease, Reverse Transcriptase and Integrase Gene Regions in Major Group-M Subtypes**

A. Garcia[1], A. Wong[2], D. Jensen[2], J. Fedynyshyn[2], C. Kronfel[1], J. Young[2], P. Kambham[3], H. Luu[2], S. Gorday[1], C. Garcia[1], A. Gupta[2], A. Liu[2], R. Cao[1], P. Chua[2], E. Bolchakova[3], S. Williams[2]
[1]Thermo Fisher Scientific, Austin, TX; [2]Thermo Fisher Scientific, South San Francisco, CA; [3]Thermo Fisher Scientific, Pleasanton, CA.

Introduction: To meet the UNAIDS 95-95-95 Fast-Track Targets by year 2030, robust drug-resistance (DR) genotyping solutions for HIV-1 are urgently needed. Presented here is the development and performance of a novel HIV-1 genotyping assay to address this market need. The assay is designed to aid in detecting genomic mutations (in the protease (PR), reverse-transcriptase (RT), and integrase (IN) regions of the *pol* gene) in HIV-1 viral RNA extracted from EDTA plasma and dried blood spots (DBS). The assay is part of a workflow that combines both targeted PCR amplification and Sanger sequencing technology to provide comprehensive drug-resistance profiles in HIV-1 subtypes A, B, C, D, F, G, CRF01_AE, CRF02_AG, and CRF06_cpx. **Methods:** A combination of HIV-1-positive EDTA plasma clinical specimens and viral isolates were procured ranging across nine different HIV-1 subtypes with varying viral loads. Clinical specimens and viral isolates were also spiked into healthy donor EDTA whole blood to create DBS. RNA was extracted from plasma and DBS samples using the Applied Biosystems MagMAX Viral/Pathogen Nucleic Acid Isolation Kit, for HIV-1 DBS (Cat# A53770) and the Thermo Fisher Scientific KingFisher Flex Purification System. The extracted RNA samples proceeded through the HIV-1 genotyping assay workflow, using the Applied Biosystems Veriti Dx 96-well Thermal Cycler and the Applied Biosystems 3500xL Dx Genetic Analyzer. Resulting data files were analyzed using the Exatype Platform by Hyrax Biosciences. **Results:** This novel prototype HIV-1 genotyping assay successfully amplified HIV-1 RNA recovered from EDTA plasma (A, B, C) with 100% positivity in samples with viral loads ≥1,000 cps/mL for PR/RT and ≥5,000 cps/mL for IN. Amplifiable HIV-1 RNA was recovered from DBS (subtypes A, B, C) with 100% positivity in samples with viral loads ≥5,000 cps/mL for PR/RT and IN. Inclusivity of the HIV-1 genotyping assay with RNA extracted from plasma and DBS was also established across nine different HIV-1 subtypes with viral loads ≥1,000 cps/mL and ≤300,000 cps/mL using three different lots of assay. Analyses of reproducibility and precision indicated a mean of 99.7% nucleotide alignment for the PR/RT gene region, and 99.8% nucleotide alignment for the IN gene region. **Conclusions:** Overall, the new HIV-1 genotyping assay presented here provides a robust Sanger sequencing-based assay alternative to existing on-market solutions for HIV-1 DR surveillance.

ID060. **The Value of RT-PCR for Pediatric Patients with Influenza-Like Illness in a Point of Care Setting**

A. Tice, A. Styer, D. Wolk
Geisinger, Danville, PA.

Introduction: Influenza infections represent an opportunity for rapid testing because of illness severity and the requirement for prompt treatment. For pediatric outpatients with influenza-like illness (ILI) visiting community medicine clinics for care, the lack of timely and accurate respiratory virus testing can result in preventable overutilization of antiviral therapy and delays in treatment. **Methods:** A retrospective cohort study, IRB# 2016-0497, was performed to examine the impact of point-of-care testing (POCT) on clinical and operational outcomes. ILI cases were defined as pediatric patients with a reverse-transcriptase polymerase chain reaction (RT-PCR) ordered for influenza and respiratory syncytial virus (fluA/B_RSV) and collected at Rapid Response Laboratories between 8 a.m.-5 p.m. (on-site study arm). Pediatric patients with samples collected at 87 smaller clinic sites and transported by courier to the Geisinger Core Laboratory for testing served as the comparator (off-site study arm). The Xpert Flu/RSV XC test (Cepheid, Sunnyvale, CA) was used for the comparison during influenza virus season 2017-2018. A data set with more than 350 data variables was pre-designed and mapped to Geisinger's electronic health record for subsequent analysis. The off-site study arm contained n=1,160 (44.4%) and 1,459 (55.7%) for the on-site arm. **Results:** No significant differences were observed for patient sex or virus prevalence. The mean age was 5.2 yrs and 5.6 yrs for off-site and on-site, respectively, and did not differ by 95% CI. The median collect-to-result (CTR) time decreased from 7.0 hrs to 0.8 hrs with on-site testing ($p < 0.0001$). The mean CTR decreased from 7.7 hrs (±5.1) to 1.5 hrs ± 2.4 ($p < 0.0001$). The sample type varied significantly; more nasopharyngeal (NP) samples were collected off-site (41%) versus 33% from on-site ($p < 0.001$). The sample differences did not impact the distribution of viruses detected. Overall, negative RT-PCR was 51.2%, with flu A 20.5%; flu B 14.6%; RSV 13.4%; and mixed 0.3%. Multiple clinical visits were statistically reduced with on-site testing (33% vs. 41% for off-site) ($p = 0.0002$). When flu RT-PCR was positive, 7.5% of off-site patients received antivirals before the testing without provider knowledge of results (a stewardship opportunity) versus only 0.5% from on-site testing sites ($p < 0.001$). Likewise, 6.7% versus only 1.9% for on-site received their antivirals late, the day after testing ($p < 0.001$). Antiviral prescriptions were better matched with RT-PCR results more often, with on-site testing 83.8% versus 79.1%, respectively ($p=0.002$). **Conclusions:** POCT for influenza and RSV improved precision medicine (targeted therapy), reduced clinic visits, and better optimized the administration of antiviral medication.

ID061. **Validation of Applied Biosystems HIV-1 Genotyping Kit with Integrase (Thermo Fisher Scientific) Performed on the ABI 3500xL Genetic Analyzer Platform**

J. Schroeder[1], J. Sen[1], L. Cheng[1], K. Kirsits[1], R. Relich[1], R. Sperling[2]
[1]Indiana University Health, Indianapolis, IN; [2]IU Health Pathology Laboratory, Indianapolis, IN.

Introduction: HIV-1 is the etiologic agent responsible for the AIDS pandemic. Treatment of HIV-1 infections with potent antiretroviral therapy can result in a profound and durable suppression of HIV-1 replication. However, emergence of drug-resistant viral strains frequently results in treatment failure. These HIV-1 mutant strains can be resistant to one or more drugs in each of the four antiretroviral drugs classes; nucleoside reverse-transcriptase inhibitors, non-nucleoside reverse-transcriptase inhibitors, protease inhibitors, and integrase strand transfer inhibitors. The Applied Biosystems HIV-1 Genotyping Kit with Integrase (Thermo Fisher Scientific) is designed to detect HIV-1 mutations in the protease, reverse transcriptase and integrase regions of the *pol* gene in RNA isolated from HIV-1. **Methods:** Twenty-one in-house, archived plasma samples, previously tested for HIV-1 genotype using the Abbott ViroSeq HIV-1 Genotyping Kit and the Abbott ViroSeq HIV-1 Integrase Genotyping Kit, were included in this study. HIV-1 RNA was extracted using the QIAamp Viral RNA Mini kit. Samples were analyzed using the Applied Biosystems HIV-1 Genotyping Kit with Integrase. The following steps were performed: reverse transcription-PCR, nested PCR, gel analysis, clean-up of nested-PCR products, cycle sequencing, and purification of sequencing products. The purified sequencing products were analyzed using an Applied Biosystems 3500xL Genetic Analyzer. Sequence data were analyzed for presence of mutations conferring drug resistance and level of resistance to the four classes of

antiretroviral drugs using Exatype Sanger Software (Hyrax Biosciences). **Results:** Of the 21 samples studied, 15 cases showed the presence of mutations and drug resistance. Six cases did not have any mutations leading to drug resistance. All 21 cases correlated with the expected results, for overall drug resistance. Three samples had additional mutations detected; however, none of the additional mutations translated into changes in drug-resistance patterns. Two samples exhibiting additional mutations showed resistance to the same set of drugs; however, the level of resistance showed changes. **Conclusions:** Our testing, using the Applied Biosystems HIV-1 Genotyping Kit with Integrase, matched the expected results with previously tested samples for HIV-1 genotype. The five samples where mutations did not exactly match were determined not to be true discrepancies since there was no significant alteration in drug-resistance patterns. The HIV-1 genotype analysis protocol, using the Applied Biosystems HIV-1 Genotyping Kit with Integrase, was deemed acceptable based on our validation studies and will be used for clinical testing.

ID062. **Exploration of Whole Blood Testing on the cobas CMV Test**

R. Hein, N. Robinson, E. Gick, S. McCune
Roche Diagnostics, Indianapolis, IN.

Introduction: Cytomegalovirus (CMV) is an important opportunistic pathogen causing increased morbidity and mortality after solid organ and hematopoietic stem cell transplantation. Quantitative CMV DNA testing with real-time PCR assays is widely used for clinical management of CMV disease and to detect active disease, determine when to initiate pre-emptive therapy, and to monitor response to therapy. The Roche cobas CMV test is approved for use with plasma specimens but the performance of whole-blood samples has not been widely established. **Methods:** In preliminary feasibility studies, whole blood (WB) was diluted at three concentrations (1:9, 1:14, 1:19) in either cobas omni Lysis Reagent (LYS) or cobas PCR Media. Dilutions were split and half were incubated for 15 minutes at room temp (RT), and the remaining half were frozen at -20°C for 30 minutes and then incubated for 15 minutes at RT. The samples were then tested on the cobas 6800 system (Roche Diagnostics, Indianapolis, IN). Based on these studies, a 15-minute incubation in cobas omni Lysis Reagent (LYS) was used for further studies. CMV standard material from Exact Diagnostics (Fort Worth, TX) was spiked into plasma at two concentrations and WB at four concentrations. The spiked WB samples were diluted, pretreated, and tested in triplicate over three runs. **Results:** The WB protocol providing the best sensitivity while avoiding invalid results was a dilution of 1:14 with LYS reagent followed by a 15-minute RT incubation. Based on these results, this pre-analytic treatment method was chosen for further investigation utilizing CMV spiked specimens. WB was spiked with CMV standard at 10,000, 5,000, 1,000, and 500 IU/ml, and each concentration was diluted in triplicate for pre-analytic treatment prior to loading onto the cobas 6800 for testing. Plasma was also spiked at 1,000 and 100 IU/ml to compare WB performance with the approved sample type. There were no invalid results in all three CMV runs, demonstrating that the pre-analytic treatment was able to overcome PCR inhibition caused by WB. **Conclusions:** The cobas 6800/8800 systems were not designed to process undiluted whole blood, and previous approaches have required dilution prior to processing. In addition to viscosity challenges, whole blood contains inhibitors that can result in invalid assay results. Pre-analytic dilution of contrived WB samples at 1:14 with cobas omni Lysis Reagent followed by incubation for 15 minutes at RT reduced the invalid rate to 0% in this study. Further testing is required to establish a WB limit of detection with the cobas CMV test.

ID063. **Exploration of Whole Blood Testing on the cobas EBV Test**

R. Hein, N. Robinson, E. Gick, S. McCune
Roche Diagnostics, Indianapolis, IN.

Introduction: Epstein Barr virus (EBV) is an important opportunistic pathogen causing increased morbidity and mortality after transplantation. Quantitative EBV DNA testing with real-time PCR assays is widely used for clinical management of EBV post-transplant to help identify those at risk of EBV disease and post-transplant lymphoproliferative disorder (PTLD). The Roche cobas EBV test is approved for use with plasma specimens but the performance of whole-blood samples has not been widely established. **Methods:** In preliminary feasibility studies, whole blood (WB) was diluted at three concentrations (1:9, 1:14, 1:19) in either cobas omni Lysis Reagent (LYS) or cobas PCR Media. Dilutions were split and half were incubated for 15 minutes at room temp (RT) and the remaining half were frozen at -20°C for 30 minutes and then incubated for 15 minutes at RT. The samples were then tested on the cobas 6800 system (Roche Diagnostics, Indianapolis, IN). Based on these studies, a 15-minute incubation in cobas omni Lysis Reagent (LYS) was used for further studies. EBV standard material from Exact Diagnostics (Fort Worth, TX) was spiked into plasma at two concentrations and WB at five concentrations. The spiked WB samples were diluted at either 1:9 or 1:14, pre-treated and tested in triplicate. **Results:** The WB protocol providing the best sensitivity while avoiding invalid results was a dilution of 1:9 with LYS reagent followed by a 15-minute RT incubation. Based on these results, this pre-analytic treatment method was chosen for further investigation utilizing EBV spiked specimens. WB was spiked with EBV standard at 10,000, 5,000, 1,000, 500, and 100 IU/ml, and each concentration was diluted in triplicate for pre-analytic treatment prior to loading onto the cobas 6800 for testing. Plasma was also spiked at 1,000 and 100 IU/ml to compare WB performance with the approved sample type. There were no invalid results in all four EBV runs, demonstrating that the pre-analytic treatment was able to overcome PCR inhibition caused by WB. **Conclusions:** The cobas 6800/8800 systems were not designed to process undiluted whole blood, and previous approaches have required dilution prior to processing. In addition to viscosity challenges, whole blood contains inhibitors that can result in invalid assay results. Pre-analytic dilution of contrived WB samples at 1:9 with cobas omni Lysis Reagent followed by incubation for 15 minutes at RT resulted in a 0% invalid rate in this study. Further testing is required to establish a WB limit of detection with the cobas EBV test.

ID064. **MassArray Human Papillomavirus (HPV) Genotyping in Head and Neck Cancers: Resolving Inconsistencies across Conventional Detection Platforms**

S. Bhardwaj, A. Paniz-Mondolfi, J. Ramirez, M. Hernandez, W. Westra
Icahn School of Medicine at Mount Sinai, New York, NY.

Introduction: Human papillomavirus (HPV) status is a powerful prognostic marker for patients with head and neck squamous cell carcinomas (HNSCC). Given HPV's expanding importance in treatment and surveillance, accurate detection is critical without margin for erroneous results. Widely used HPV testing includes p16 immunohistochemistry (IHC), PCR-based DNA assays, and RNA *in situ* hybridization (RISH), but each carries inherent limitations that may give rise to discordant results across testing platforms. The purpose of this study was to validate the MassArray platform for HPV testing of HNSCC, and then to use this novel assay to resolve HPV status of equivocal cases. **Methods:** Targeted single nucleotide polymorphism genotyping was performed using a mass-array-based system (MassARRAY Agena Bioscience) and the Early Access HPV Genotyping Panel v2.0 Protocol for the detection of 24 HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 34, 53, 66, 67, 70, 73, 82, 6, 11, 42, 81). MassArray testing was first validated using HNSCCs with known HPV genotypes. At our institution, HPV testing by p16 IHC and PCR-based HPV DNA genotyping is routinely performed for all patients with oropharyngeal SCCs (OPSCC) and sinonasal SCCs (SNSCC) as part of standard clinical care. The

pathology databases were then searched for OPSCCs and SNSCCs with discrepant HPV results (i.e., p16+/HPV DNA- and p16-/HPV DNA+). Discrepant cases were re-evaluated using wide spectrum HPV E6/E7 RISH followed by MassARRAY. **Results:** HNSCCs from 562 patients underwent HPV testing by p16 IHC and HPV DNA PCR. There were 23 (4.1%) discrepancies (p16+/HPV DNA-, n=22; and p16-/HPV DNA+, n=1). MassARRAY genotyping was performed on 15 cases. There was strong correlation between the presence of HPV E6/E7 mRNA as detected by RISH, and the presence of HPV as detected by MassARRAY. HPV was not detected in the eight RISH-negative cases, but it was detected in six of seven RISH-positive cases. All six of the positive cases were non-16 HPV variants (genotypes 33, n=2; 58, n=2; 59, n=1; and 82, n=1). The validation cohort consisted of one negative control and 10 positive controls, including HPV genotypes 6, 11, 16, 18, 33, 35, 45, 56, 59, and 67. HPV was detected and typed correctly in all positive controls, and it was not detected in the negative control. **Conclusions:** HPV MassARRAY testing is a highly effective method for determining HPV status in HNSCC. Its ability to detect extraordinarily rare HPV variants such as 58, 59, and 82 appears to enhance the sensitivity of HPV detection. These rare variant forms that elude detection by conventional PCR-based assays appear to be a major contributor to detection inconsistencies that can confound clinical management.

ID065. **Validation of the AltoStar AM16 System for Cytomegalovirus (CMV) Quantitative Viral Load Assay Using Altona Diagnostics RealStar CMV ASR**

D. Banerjee[1], N. Kanwar[1], S. Ghanshani[2], R. Selvarangan[1]
[1]Children's Mercy Hospital, Kansas City, MO; [2]Altona Diagnostics USA, Inc., Plain City, OH.

Introduction: Human cytomegalovirus (CMV) virus, a member of the beta herpes family, is a common infectious cause of birth defects in the United States. It can remain latent and reactivate in immunocompromised patients causing opportunistic infections in hematopoietic stem cell transplant or solid organ transplant patients. Rapid and accurate diagnosis of CMV is critical for administering appropriate patient management to reduce morbidity and mortality in such patients. We evaluated the Altona Diagnostics RealStar CMV analyte-specific reagents (ASR) on the AltoStar AM16 System and compared its performance to CMV ELITe MGB Kit on the InGenius for quantitative detection of CMV in plasma specimens. **Methods:** To validate the RealStar ASR CMV assay, a dilution series ranging from 10^7 to 38 IU/ml of CMV was contrived in pooled human plasma using a reference standard (Exact Diagnostics, Texas) and tested on the AltoStar AM16 to determine the linear range, preliminary limit of detection (LOD), and precision. Evaluation of additional replicates at and near the initial LOD confirmed assay LOD and limit of quantitation (LOQ). Control material for CMV, Epstein-Barr virus, human herpesvirus 6, herpes simplex virus (HSV)-1, HSV-2, varicella-zoster virus, and adenovirus was tested for assay cross-reactivity. For method comparison, 20 frozen plasma specimens (10 positive and 10 negative) and five contrived samples (125 IU/mL, 250 IU/mL, and 500 IU/mL) were tested concurrently by both AltoStar AM16 and ELITe InGenius and results compared. **Results:** The RealStar ASR CMV assay exhibits a linear range between 100 IU/mL-10^7 IU/mL in human plasma with an observed LOD and LOQ of 100 IU/mL. Intra- and inter-assay precision of 250, 10^4, and 10^6 IU/ml tested in triplicate over three days by two operators demonstrated total within-laboratory %CV of 0.7%-10.3%. The CMV assay did not cross-react with other viral targets tested. Of the 25 samples tested on the two platforms for method comparison, two exhibited discrepant results with viral loads below the LOD and were excluded from analysis. Fifteen of the remaining 23 plasma samples tested positive, and eight samples tested negative by both methods with a 100% positive percent agreement and negative percent agreement between the two assay methods. The correlation coefficient between both assays was 97.3% with average error index of 0.49. **Conclusions:** The Altona Diagnostics RealStar ASR CMV assay, deployed on the AltoStar AM16 system, is sensitive and reproducible for determination of CMV viral loads. The AltoStar AM16 offers high-throughput quantitative viral load testing, including the capacity to test different specimen types on the same run.

ID066. **An Institutional Review of Molecular Testing Patterns in Bronchoalveolar Lavage Specimens to Determine Clinical Utility of In-House Next-Generation Sequencing for Pathogen Detection**

A. Dillard[1], P. Velu[2], S. Dzedzik[1], L. Cong[1], J. Sipley[1], L. Serrano[1], H. Rennert[2]
[1]New York-Presbyterian Hospital, New York, NY; [2]Weill Cornell Medicine, New York, NY.

Introduction: Next-generation sequencing (NGS) can be performed directly on clinical samples for simultaneous detection of multiple pathogens and antimicrobial resistance alleles as well as assembly of whole genome sequences. An analysis of three years of institutional send-out testing of plasma samples for The Karius Test, a microbial cell-free DNA sequencing test, revealed the most detections and changes in clinical management for immunocompromised patients with fever of unknown origin and/or pulmonary infiltrates. This observation, along with a large send-out test volume for lower respiratory samples, prompted us to review institutional bronchoalveolar lavage (BAL) fluid specimens to determine how molecular testing was utilized in standard of care. **Methods:** A total of 262 BALs from 194 patients over a four-month period were reviewed for clinical indication, test orders, and test results. Data were extracted from the laboratory information system used by our clinical pathology laboratories (Cerner Millenium) and the electronic medical record (EPIC). Data were analyzed using Excel v.16.45 and R v.4.1.2. **Results:** Ordered tests included culture, antigen testing, ova and parasite, and molecular testing. Respiratory culture was first-line and performed on all specimens, with only a subset also receiving acid-fast bacteria (AFB) (n=143, 55% of total specimens), fungal (n=135, 52%), or *Legionella* and/or *Nocardia* (n=85, 32%) cultures. Aspergillus Galactomannan Antigen by EIA (n=45, 17%), Fungitell (n=4, 2%), PJ-DFA (n=51, 19%), and ova and parasite (n=27, 10%) were also performed on a subset of BALs. Molecular testing was performed on 85 (32%) specimens and consisted of quantitative (adenovirus, cytomegalovirus, human herpesvirus-6, herpes simplex virus 1/2, influenza, varicella-zoster virus, *P. jirovecii*) or qualitative (*Aspergillus spp.*, *C. pneumoniae*, *M. pneumoniae*, *Mucorales spp.*, *Nocardia spp.*, SARS-CoV-2) pathogen detection by PCR methods (range n=1-37, 1%-44% of specimens with molecular testing), the BioFire Respiratory Pathogen Panel (RPP) (n=74, 87%), or ITS sequencing of fungal culture isolate (n=1, 1%). Thirty-six BALs from 30 patients had >3 molecular tests ordered on the same specimen; these patients tended to be immunocompromised. RPP and SARS-CoV-2 PCR were the only in-house molecular tests, and send-out tests were often ordered after initial in-house testing. **Conclusions:** Immunocompromised patients may benefit from broad pathogen detection tests like shotgun metagenomics or targeted next-generation sequencing pathogen panels that are ordered in the early phases of a diagnostic work-up in parallel to standard-of-care methods. This may reduce the costs and turnaround-times associated with multiple asynchronously ordered send-out tests and the longer wait times associated with AFB and fungal cultures.

ID067. **Development of Monkeypox Virus Molecular Control as Reference Material for Molecular Diagnostic Tests**

K. Banjara, A. Tong, Q. Gan, R. Hu, L. Liu, T. Sung, N. Umblas
Thermo Fisher Scientific, Fremont, CA.

Introduction: In 2022, new cases of humans contracting monkeypox virus have been detected in multiple countries that historically do not report monkeypox virus infection cases, according to the Centers for Disease Control and Prevention (CDC). Monkeypox infection is a disease caused by the monkeypox virus that is part of the *Orthopoxvirus* genus. Many molecular diagnostic tests are in development for detecting this virus and improving sensitivity for early detection. However, the lack of proper monkeypox-positive control

materials for assay development and validation greatly impacted the establishment of the molecular testing in clinical laboratories. In response to the urgent need of such control materials, we developed a positive control for monkeypox assays; this control also shows positive reactions to assays that target human RNase P gene. **Methods:** Based on CDC monkeypox virus testing guidance, 5 µL of the positive control material was used in 20 µL reactions, and the results should have Ct cut-off value between 22-28 in 40 cycle PCR reaction. Therefore, the positive control material was serially diluted and tested using CDC monkeypox virus assay, CDC RNase P assay, and the Thermo Scientific monkeypox virus assay on the QuantStudio 5 Real-Time PCR system as well as the Bio-Rad Droplet Digital PCR (ddPCR) instrument. Three replicates were tested at each dilution level. **Results:** This molecular control was tested with the CDC monkeypox virus assay, CDC RNase P assay, and the Thermo Scientific monkeypox virus assay and showed positive reactions, and linearity studies showed R^2 value above 0.99 on both qPCR and ddPCR platforms. When using 5 µL of $2x10^2$ cp/µL -$2x10^3$ cp/µl dilution level, the results fall within the 22-28 Ct cut-off values. **Conclusions:** Surveillance and detection of the monkeypox virus is crucial to combat its spread, and this molecular control would play an integral role for validating and monitoring molecular diagnostic tests' performance. A positive control contains partial sequence of the monkeypox virus, and the human RNase P genes was created in response to the emergence of the monkeypox virus infection. Similar technology can be applied to fulfill any future demand for molecular testing on potential emerging infectious disease. (This positive control is for Research Use Only and Not for Clinical Use.)

ID068. **Using the Digital Phenotype of Disease in Human Plasma for Simplified and Accurate Prediction of Tuberculosis and HIV Infection**

C. Gupta[1], K. Murugesan[2], Z. Jia[1], S. Fischer[1], J. Hui[1], E. Quevy[1], G. Theron[3], N. Banaei[2]
[1]Probius, Fremont, CA; [2]Stanford University, Palo Alto, CA; [3]NRF/DST Centre of Excellence for Biomedical Tuberculosis Research, Cape Town, South Africa.

Introduction: Approximately 1.5 million died from tuberculosis (TB) disease in 2020. Nucleic acid amplification tests (NAAT) have greatly improved the detection of the *Mycobacterium tuberculosis* and HIV comorbidity. However, the frequently challenging sputum collection for NAAT creates a risk of infection, restricting testing to facilities that are far removed from the patients. NAAT workflows also require expensive instrumentation and consumables suited to centralized and better-resourced labs. To address these limitations, we developed a new, label- and probe-free electrochemical sensing modality, Quantum Electrochemical Spectroscopy (QES), for the rapid detection of TB and HIV infection from 2 µl of patient plasma in 40 minutes. **Methods:** A total of 2 µl aliquots of human plasma were pipetted into a consumable sensor coupled to a Probius QES instrument that performs a voltage scan of the sample at a nanoscale electrochemical interface and measures the resulting charge transfer current, which contains discrete, vibration-correlated signatures of species in the sample. We collected QES signatures from a cohort of 30 training and 10 blinded samples from South African symptomatic patients. The training samples were equally portioned between rule-out and TB-positive samples. Blood and sputum samples collected from pulmonary TB patients and rule-out individuals were assigned TB phenotypes after analysis of their sputum by the culture and or GeneXpert System. A separated subset of the training samples (~10) was used for validation of a trained k-nearest neighbor's classifier. Once validated, the classifier was used to predict the TB disease phenotype on the 10 blind samples. In addition, without re-assaying the samples with new reagents, we developed an HIV classifier using the same training and validation protocols, by adding available information on patient HIV burden to the training data and re-training *in silico*. **Results:** Our method demonstrated 90% overall accuracy on the 10 validation and 10 blinded samples for the TB phenotype. When repurposing the data for HIV, we also found that the classifier was 70% and 80% accurate on 10 validation and 10 blinded samples, respectively. The extension of these results to a larger cohort and different disease models is ongoing. **Conclusions:** We demonstrate that the use of vibrational molecular signatures obtained with QES can be successfully used as a scalable platform for the detection of TB and HIV coinfection in 2 ul of plasma samples. Classifiers built using vibrational signatures can predict disease phenotypes for TB and HIV infection with 90% and 80% accuracy, respectively. The ability to repurpose the same sample set with different clinical phenotype associations also facilitates "over the air" updates to perform multiple assessments on one specimen.

ID069. **cobas 6800 HPV Assay Verification Performed at CGAT DHMC Lab**

P. Shannon, J. Barbuto, P. Carnaroli, S. Sathyanarayana, L. Thompson, G. Tsongalis
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: The CGAT Lab performed/finalized a human papillomavirus (HPV) assay verification process of the Roche cobas 6800 to improve testing capabilities and turnaround time. The cobas HPV test is an FDA-approved IVD assay for the detection of HPV DNA in cervical specimens collected in ThinPrep Pap Test PreservCyt Solution. This qualitative real-time PCR test detects 14 high-risk (HR) HPV genotypes using a pool of primers and probes targeting approximately 200 nucleotides within the L1 region of the HPV genome. The 14 HR HPV genotypes can be distinguished and were reported as genotype calls 16 and 18. The other 12 HR HPV genotypes were grouped together and reported as "Other HR HPV," when one or more were detected. The human beta-globin gene was used as an internal control to confirm proper collection, processing, and amplification. During the testing process, an aliquot of the PreservCyt specimen was transferred from the primary specimen vial to a secondary tube using the cobas p480 instrument. Next, the specimen was loaded onto the cobas 6800 instrument for analysis and resulting. Here we describe our verification of the HPV assay. **Methods:** The verification process followed three steps before the assay was implemented in the lab for clinical use. Precision testing was accomplished by testing QC material over 10 days using material from Roche. The accuracy of test results was assessed by testing a panel provided of known HR, positive 16, positive 18, and negative samples previously performed on the cobas 4800 and calculating positive and negative percent agreement. Lastly, a method correlation was performed between the cobas 4800 and the cobas 6800 using clinical patients. **Results:** All positive and negative controls were completed with 100% expected results for the precision testing. The comparison run from the cobas 4800 consisting of 29 positive patients and 71 negative patients was performed on the cobas 6800 with 100% expected results as well. Finally, there was 100% positive percent agreement and 100% negative percent agreement from the results of accuracy testing of known positive and negative samples. **Conclusions:** Implementation of the HPV assay from the prior instrument, cobas 4800, to the newly acquired cobas 6800 worked as expected following the verification procedure determined by CGAT personnel. To support our clients with HPV testing, a turnaround time of more than four hours on the cobas 4800 was decreased to about 3.5 hours on the cobas 6800. The cobas 6800 also allows a better workflow by performing HPV concurrently with other assays.

ID070. **Clinical Performance and Relative Sensitivity of BK Virus RT-PCR Using Viral Loads Derived from Parallel Urine and Plasma Collections**

Z. Sands[1], M. Codoy[2], R. Yee[2]
[1]The George Washington University Hospital, Alexandria, VA; [2]The George Washington University Hospital, Washington, District of Columbia.

Introduction: BK virus (BKV) reactivation is a major concern for transplant patients as reactivation can cause nephritis, nephropathy, hemorrhagic cystitis, viremia, and graft rejection. Quantitative PCR for

BKV can be useful for monitoring and diagnosing BKV-associated complications. Here, we report the validation of the Roche cobas 8800 BKV RT-PCR test on both plasma and urine specimens. **Methods:** Validation was performed by using sourced clinical specimens (plasma and urine) previously tested on a validated platform for BKV or contrived specimens using Exact Diagnostics Verification Panels to evaluate for precision, accuracy, limit of detection (LoD), lower limit of quantitation (LLoQ), and linearity. A total of 28 urine (28 pos, 0 neg) and 37 plasma (23 pos, 14 neg) were tested for accuracy. A total of 84 blinded, prospective specimen pairs (urine and plasma) collected from February to May 2022 submitted at our institution for BKV viral loads were tested and results were compared to evaluate clinical performance. **Results:** The Roche cobas 8800 BKV RT-PCR test detected 94.1% of previously tested samples, with an overall concordance of 95.4%. The sensitivity was 91.3% and 96.4% for plasma and urine, respectively, and the specificity was 100% for plasma. Although the LoD was the same for both specimen types at 21.5 IU/mL, the LLoQ for urine was higher than plasma (200 IU/mL vs. 21.5 IU/mL). The coefficients of variation at mid-range and high-range viral load measurements were 2.31%/0.38% and 3.61%/0.53% for inter-assay and intra-assay precision, respectively. Linearity reported had R^2 of 0.9988, which corresponded to a PCR efficiency of 104.1%. Among the 84 prospective patients, 16.7% (14/84) were positive. Positivity was higher in urine (13/84, 15.5%) compared to plasma (8/84, 9.5%). Concordance was seen in 91.7% (77/84) of the paired-samples. Of the seven discordant pairs, 85.7% (6/7) were positive by urine only; 66.7% (4/6) had viral loads <2,000 IU/mL observed. In patients (n=7) with BKV detected in both sample types, the mean viral load presented as 2.40Log10 IU/mL and 5.59Log10 IU/mL for plasma and urine, respectively. **Conclusions:** The Roche cobas 8800 BKV RT-PCR is an easy-to-use commercial option for sensitive and specific detection of BKV in plasma and urine. Although low-grade BKV viruria (<2,000 IU/mL) may not be considered clinically significant, our comparison study suggested that certain patients may benefit from prompt serial monitoring of BKV due to detectable BKV in urine but not plasma. Testing urine specimens may increase diagnostic yield, while offering the test on both specimen types can allow for a more comprehensive monitoring of systemic viremia. Further clinical chart review may be insightful to determine the clinical utility of testing plasma versus urine in different patient populations.

ID071. **Quantitation of Replication Competent Viruses Using ddPCR**

N. Tusneem, P. Halvorsen, a. Wali, L. Frady
Q2 Solutions, Inc., Durham, NH.

Introduction: Viral vectors used in cell and gene therapy products are typically engineered to be devoid of genes required for replication to prevent the formation of replication-competent virus during therapy. This addresses potential safety concerns such as off-target effects including disruption of gene function via unintended insertion into protein coding sequences. Accordingly, regulatory agencies require testing for replication-competent viruses in cell and gene therapy products prior to treatment, during treatment, and as part of long-term follow-up. Here we describe the development and validation of assays for quantitation of replication-competent retrovirus (RCR) and replication-competent lentivirus (RCL) in whole blood samples. **Methods:** The RCR and RCL assays have been developed to target the gibbon ape leukemia virus (GALV) and vesicular stomatitis virus G (VSV-G) envelope sequences, respectively, along with a human reference gene to enable reporting of results in copies per microgram of human genomic DNA (gDNA). **Results:** The RCR and RCL assays utilize droplet digital PCR (ddPCR) technology to achieve high sensitivity, with a limit of detection of ≤10 copies/µg gDNA and a lower limit of quantitation of ≤50 copies/µg gDNA. **Conclusions:** Analytical validation of RCR (GALV) and RCL (VSV-G) assays using ddPCR meets the FDA recommendation for lower limit of quantitation for monitoring of replication-competent virus during treatment with cell and gene therapy products in clinical studies.

Informatics

I001. **Impact of Concurrent Conditions on Methylation Detection Profiled by ctDNA NGS Assay**

M. Cai[1], S. Zhang[3], J. Kurata[3], S. Chen[3], T. Jiang[3], J. Yen[3], T. Rich[3], K. Price[3], Y. Hong[3], K. Banks[3], H. Chuang[3]
[1]Guardant Health, Livermore, CA; [2]Guardant Health, San Diego, CA; [3]Guardant Health, Palo Alto, CA.

Introduction: DNA methylation has emerged as a promising signal for tracking the occurrence, progression, and recurrence of cancer. Guardant Reveal is a blood-only liquid biopsy test that incorporates both genomic and epigenomic signals to detect circulating tumor DNA (ctDNA) for minimal residual disease assessment in cancer patients. Tumor-derived methylation is determined by a bioinformatics classifier that provides a quantitative assessment across all epigenomic regions; positive calls are made in samples that score above a predefined threshold. Although unique methylation signatures have been established in the early-stage cancer setting, the specificity of methylation signals in relation to age, clonal hematopoiesis (CH), and comorbidities are not well understood. In this study, we investigate the impact of age and CH upon the detection of methylation signatures. In addition, exploratory analysis of the effect of common comorbidities including asthma, chronic obstructive pulmonary disease (COPD), and rheumatoid arthritis (RA) was also performed. **Methods:** Samples from 734 healthy and 195 lung cancer samples were processed on Guardant Reveal Multi-Cancer assay. Methylation scores were calculated to predict ctDNA positive or negative status and estimate tumor fraction. To approximate prevalence of CH, we used the count, mean mutant allele frequency and maximum MAF of annotated CH variants for each sample. The relationship among age, CH, and methylation score was therefore interrogated. For comorbidity analysis, we leveraged a subset of cancer-free samples with known comorbidities including asthma (n=64), COPD (n=13), and RA (n=38). **Results:** The presence of CH was significantly correlated with increasing age ($p < 0.005$) as has been previously reported. However, neither CH nor age was found to be significantly associated with the methylation score in cancer-free samples, indicating that the Reveal mutation signature is independent of these confounding factors. To demonstrate that the signature is unique to the cancer, we showed a positive correlation between genomic and methylation tumor fractions holds regardless of the presence of CHIP mutations in lung cancer samples. Moreover, the exploratory analysis of cancer-free samples with asthma, COPD, or RA showed no statistical difference in methylation scores compared to cancer-free samples without those comorbidities ($p > 0.05$). **Conclusions:** We demonstrate that the methylation signature used in Guardant Reveal Multi-Cancer panel is specific to the tumor fraction and independent of CH and age. In addition, the exploratory analysis based on a subset of samples shows comorbidities including asthma, COPD, and RA don't affect the methylation scores in the Reveal Multi-Cancer panel.

I002. **Monitoring Specimen Molecular Identity Signatures to Capture Pre-Analytic and Analytic Errors**

J. Nakitandwe, E. Azzato, Y. Cheng, D. Bosler, Z. Tu
Cleveland Clinic, Cleveland, OH.

Introduction: Next-generation sequencing (NGS) is widely used for molecular diagnosis. Due to the complex workflow, various errors can be introduced, with serious consequences to patient care. Fidelity of sample identity is critical, especially as multiple samples are pooled together for sequencing. However, despite molecular barcoding, human error remains possible and utilization of additional molecular identity (ID) confirmation assays may not be feasible for all samples. In this study, we explored the feasibility of using panel content of a hematologic neoplasm NGS assay to help assess specimen molecular ID as part of routine quality control. **Methods:** Our custom hematologic neoplasm NGS test panel evaluates 63 genes with 122 kb of target region. We define informative single nucleotide variants (SNVs) that meet coverage ≥100x with allele frequency (AF) at least 0.1 and

strand bias ratio <0.1. For each sample, SNV pattern was created and compared to any other sample in the same sequencing batch as an N x N crossmatch table, as well as against the last reported 1,000 samples. Sample potential gender was generated using SNV AF in target genes on chromosome X. **Results:** The average number of informative SNVs was 33.2 (SD = 6.3). The average of sharing ratio of informative SNVs between any sample other than self was 0.4; maximum sharing ratio is 0.86 from 1,000 samples analyzed. There are nine genes on chromosome X in our test panel, with four to five informative SNVs per patient, which allowed for comparison of calculated gender identity versus the documented medical record. Considering this to be informative, we implemented the crossmatch table and presumed gender identity as part of our routine workflow. The overall match per sequencing batch (20-23 samples) helped pinpoint abnormal sample patterns. Through this approach, we were able to detect potential mismatches and instances of contamination, which were further evaluated using SNV AFs, data viewing via Integrative Genomic Browser, or STR analysis. Most commonly, we detected repeat testing of the same individual, often due to clinician order error or multiple subpanels requested. Gender mismatches were most often due to bone marrow transplantation or transgender patients. **Conclusions:** Through monitoring of informative SNVs, we reliably matched samples with gender. Same gender sample swaps are difficult to detect except when in concert with other errors, or in repeat samples with the integration of historical data. Despite not being foolproof, this approach uses existing data, is easy to interpret, and provides additional sample ID review, making it worth the simple implementation to include in a clinical workflow.

I003. **Detecting Germline Structure Variants in Cancer Predisposition Genes Using Whole Genome Sequencing**

L. Choate, H. Sellers, M. Stein, P. Tandale, J. Tan, R. Setlem, Y. Sakai, C. Sosa, M. Hoenig, M. Holdren, R. Urban, S. Gupta, E. Thorland, N. Boczek, H. Kearney, W. Shen
Mayo Clinic, Rochester, MN.

Introduction: Although technological advancements have enabled structural variant (SV) detection using next-generation sequencing (NGS), limitations remain. For example, the inability to fine-map and sequence breakpoints by targeted gene panels and exome sequencing hinders accurate interpretation of the effects of duplications on genes. With uniform coverage across the entire genome, whole-genome sequencing (WGS) maximizes the ability to capture aberrant reads across breakpoint junctions for SV detection and interpretation. Detecting small or complex SVs such as those in cancer can be challenging. Although SVs are identified in at least 30% of tumor genomes, they also account for 7.2% of overall pathogenic germline variants in cancer predisposition genes and are more prevalent in *APC, BRCA1, MSH2, PMS2, STK11, TP53,* among others (van Belzen, *et al.,* 2021; Pocza, *et al.,* 2021; Mancini-DiNardo, *et al.*, 2019). Therefore, accurate detection of SVs is pivotal for cancer diagnosis and treatment selection. **Methods:** Library preparation was performed using Illumina DNA PCR-Free Prep kit and sequenced on a NovaSeq 6000 instrument. Mapping and variant calling (including SV calling) of sequencing data was performed using the DRAGEN v3.8.4 pipeline (Illumina) and GRCh38 genome build. To evaluate the general performance of copy number variant (CNV) detection using WGS, we sequenced 45 patient samples that had 142 high-quality CNVs (70 duplications, 72 deletions) previously characterized by chromosomal microarray. To investigate the utility of WGS in profiling SVs in cancer predisposition genes, 13 samples with challenging intragenic SVs, including *Alu* insertions and single exon deletions/duplications, were sequenced. **Results:** All 142 CNVs were detected by WGS. Supporting SV calls were made by the DRAGEN SV caller (Manta) for 56.3% (80/142) of the CNVs, which helped refine the breakpoints or further characterize them. Among these calls, breakpoint analysis fine-mapped all 45 deletions to the base pair level, and confirmed that all 35 duplications were in direct tandem orientation. WGS also detected all expected intragenic SVs in the cancer predisposition genes: one *Alu* insertion, two single exon deletions, two single exon duplications, four multi-exon deletions, two multi-exon duplications, and two complex intragenic CNVs. Breakpoint analysis confirmed that all five intragenic duplications disrupted the respective genes, thus improving their interpretation. **Conclusions:** Our results demonstrate the promising performance of WGS for sequencing breakpoints for the accurate interpretation of the genetic consequences of SVs. In addition to tandem duplications and simple deletions, we were able to detect breakpoints and interpret more challenging SVs in cancer predisposition genes.

I004. **Application of Whole Transcriptome Sequencing to Pediatric Cancer Diagnosis and Patient Management**

A. Gout, L. Furtado, M. Cardenas, S. Foy, D. Rahbarinia, J. Neary, A. Thrasher, Z. Shi, V. Pastor Loyola, J. Zhang, L. Wang, D. Wheeler
St Jude Children's Research Hospital, Memphis, TN.

Introduction: Whole-transcriptome sequencing (RNA-Seq) is a powerful tool for interpreting tumor pathology. Diagnostic applications of RNA-Seq include detecting gene fusions and evaluating multi-gene expression signatures for risk stratification. Methods for visualizing high-dimensional data, such as t-distributed stochastic neighbor embedding (tSNE), have proven useful for classifying tumors based on whole-transcriptome expression profile in the research setting. We have analyzed more than 2,500 tumor samples by RNA-Seq. By combining our clinical samples from patients consented for research with samples from our research cohorts, we have generated a pan-cancer transcriptome reference set of 2,000 samples enabling rapid classification of cancer entities and subtypes. Here, we report integration of unsupervised analysis of whole-transcriptome gene expression into our pediatric tumor clinical genomic testing service, as an adjunct to germline and tumor DNA testing, and demonstrate the diagnostic potential of gene expression profiling. **Methods:** Reference samples were annotated using a common ontology and augmented with subtype-specific genetic biomarkers from matching DNA sequence data (available at https://stjude.cloud). These were in turn divided into separate hematologic, central nervous system, and other solid tumor cancer type reference sets. For each new patient's clinical RNA-Seq data, gene read counts were normalized, batch corrected, and analyzed alongside reference samples using tSNE to predict their diagnostic category and subcategory. Resulting predictions are compared to the tumor's histological diagnosis and corroborated by relevant genetic biomarkers (e.g., gene fusions, and/or other pathognomonic mutations). **Results:** For more than 80 cancer subtypes with five cases or more in the reference set, the resultant tSNE maps cluster more than 90% of all samples at the cancer type or subtype level. Further, tSNE clustering alone is ~95% concordant with the integrated diagnosis from molecular pathology for many cancers. The classification is particularly valuable for predicting tumors characterized by enhancer hijacking fusions (e.g., IGH@ DUX4) and other structural alterations that are otherwise difficult to detect using conventional approaches due to minimal read evidence. In addition, this approach led to the identification of novel putative driver events. **Conclusions:** The integration of RNA expression profiling into the routine work-up of pediatric tumors demonstrated improved diagnostic precision and may lead to a revised classification system for multiple tumors. Our results demonstrate the potential for using gene expression profiling in a validated clinical diagnosis setting across most pediatric tumors.

I005. **A Comparative Analysis of Somatic Cancer Variant Interpretation Using the ClinGen/CGC/VICC Framework and QIAGEN Clinical Insight Decision Support Software**

A. Goverdhan[1], L. Mullineaux[2], A. Pryzbylski[2], C. Teigen[2], S. Elkin[1], B. Pitel[2]
[1]QIAGEN, Redwood City, CA; [2]Mayo Clinic, Rochester, MN.

Introduction: With adoption of comprehensive next-generation sequencing panels for tumor genomic profiling, high-volume somatic variant interpretation poses a challenge for molecular pathologists. QIAGEN Clinical Insight Interpret One (QCI) automates the somatic cancer variant interpretation process by applying a framework based on ACMG/AMP sequence variant interpretation guidelines and AMP/ASCO/CAP cancer variant actionability guidelines. The ClinGen/CGC/VICC (ClinGen) somatic standards are the first collaborative guidelines tailored for the assessment of somatic variant oncogenicity. We performed a comparison using sets of variants classified by QCI and separately by experts utilizing the ClinGen framework. The 94 variants evaluated by ClinGen in their publication were used for the initial analysis. The study was extended to a set of variants from real-world oncology cases. **Methods:** The ClinGen variant set was analyzed in QCI in a tumor type-agnostic context. The QCI computed classification was compared with the ClinGen classification. For the second analysis, genes interrogated in the ClinGen publication were omitted. The most frequently reported variant in each remaining gene from the MayoComplete Solid Tumor panel was included if reported more than once; variants with n=2 were retained if listed in COSMIC. The final set of variants was analyzed in QCI using the method described above. Variants were also assessed according to the ClinGen somatic standards by two independent groups of scientists. Finally, the QCI and ClinGen classification frameworks were compared. **Results:** For the ClinGen variant set, the QCI computed classification was 80.9% concordant with the ClinGen classification: pathogenic or likely pathogenic (P-LP; 43 of 43), variants of uncertain significance (VUS; 24 of 39), benign or likely benign (B-LB; 9 of 12). Fifteen variants classified as VUS by ClinGen were P-LP in QCI. Following expert scientific review, six variants were reclassified as VUS; for the other nine variants, P-LP classification was accepted by consensus. Analysis of the Mayo variant set revealed a similar trend in classification. Overall, the ClinGen guidelines led to more conservative classifications than QCI. Differences were attributed to nuances in ClinGen guidelines distinct from the ACMG/AMP guidelines adapted by QCI for somatic variant interpretation. **Conclusions:** Along with the AMP/ASCO/CAP guidelines, the ClinGen guidelines provide a foundation for standardizing the somatic variant interpretation process. QCI automates this process using a framework that largely aligns with the ClinGen standards. The overall difference in ClinGen and QCI classification falls within the range previously reported by studies evaluating variability in interpretation by experts.

I006. **Rapid Targeted Germline and Somatic Variant Detection Using the G4 Sequencing System**

T. Looney[1], K. Gouin[2], A. Tong[2], S. Shore[2], R. Shultzaberger[2], A. Sethia[3], B. Zamirai[3], M. Samadi[3], M. Fabani[2], E. Glezer[2]
[1]Singular Genomics, Austin, TX; [2]Singular Genomics, San Diego, CA; [3]NVIDIA, Santa Clara, CA.

Introduction: Next-generation sequencing has become an indispensable tool for the diagnosis of genetic disease, though there remains a need to reduce turnaround for time-sensitive applications such as newborn genetic screening and somatic variant detection. Reducing turnaround requires faster sequencing and accelerated data analysis. We recently introduced the Singular Genomics G4 platform for rapid sequencing-by-synthesis (SBS), which can deliver four human whole genomes at ~30x coverage in less than 19 hours. Here we present accelerated pipelines for whole-exome germline and somatic variant detection on the G4 that leverage the NVIDIA Clara Parabricks platform. **Methods:** The G4 sequencer employs a novel fluorescently labeled reversible terminated nucleotide chemistry, optimized fluidics and enzymes, and a rapid acquisition camera to deliver a cycle time of ~2.5 minutes. To maximize speed and performance for germline variant detection, we trained a custom DeepVariant model for exome analysis using a warm start from the Illumina WES model and data from Genome in a Bottle samples HG002-6 (2x150 bp reads; multiple library preparation kits used in generation of exome data). We iteratively explored the DeepVariant parameters alt_align_pileup, opt_channels (insert_size and gc_content), vsc_min_fraction_snps, and vsc_min_fraction_indels before validating performance on HG001. Separately, we applied the Parabricks umi_fgbio workflow to perform single family UMI error correction following targeted enrichment and sequencing of control genomic DNA consisting of an equimolar pool of 24 reference cell lines derived from the 1000 Genomes Project. **Results:** The baseline DeepVariant Illumina whole-exome model delivered a precision and recall of 99.23% and 98.36% for single nucleotide polymorphisms (SNPs), and 97.73% and 92.02% for indels, respectively, for HG001 at 100x coverage. The trained model showed improved performance for both SNPs and indels, with a precision and recall of 99.53% and 98.53% for SNPs, and 97.76% and 93.09% for indels, respectively, from the same library. The model was adapted to Parabricks to deliver a fastq-to-vcf turnaround of four minutes for 100x whole-exome analysis. Single family UMI-based error correction and variant calling from 50M input reads were completed in 15 minutes on the Parabricks platform using the same instance type. **Conclusions:** We have successfully implemented a GPU-accelerated DeepVariant whole-exome model for the G4. We further demonstrated accelerated single family UMI error correction and somatic variant detection via the Parabricks umi_fgbio workflow. We anticipate that the combination of rapid-SBS and GPU-based acceleration will significantly reduce turnaround for the most time-sensitive variant detection applications.

I007. **Creation of a Workflow for Germline Whole Exome Sequencing Gene Prioritization, Variant Curation, and Paternity Confirmation**

E. Hughes, D. Toledo, E. LaRochelle, D. Green, G. Tsongalis, W. Khan
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: The analysis of whole-exome sequencing (WES) data can present a bottleneck for small academic clinical laboratories expanding to genomic-scale sequencing. Here we describe a workflow in which a clinical informatics platform (Alissa "Interpret" and "Align and Call," Agilent) is operationalized to provide a solution for singleton or trio data analysis, variant classification, and ascertaining whether *de novo* variants from trio WES germline studies are confirmed *de novo* events versus assumed. **Methods:** Align and Call was used for FASTQ analysis and alignment. An in-house designed classification tree pipeline set up within Interpret performed singleton or trio analysis. Exome data triage occurred with a user-defined protocol for gene prioritization that utilized classification tags. This workflow grouped gene-variant pairs by "tags." It then filtered them to meet requirements of HPO relevance, population/disease databases, splice site variants, damaging and/or missense variants, as well as *in silico* prediction values. Curated managed variant lists (MVLs) were generated from validated clinical samples as well as curated literature that recognize ACMG/AMP guidelines. WES trios were assessed for Mendelian violations (e.g., *de novo* events) using inheritance mode filtering. **Results:** The generation of a variant call format file resulted in approximately ~70,000 variants as starting input. Each classification "tag" filter combination resulted in 5-20 gene-variant pairs shortlisted from a full exome cross-referenced to OMIM. The germline MVL was populated with more than 300 pre-curated variants. A second MVL was constructed containing next-generation sequencing and Sanger dead zones in hard-to-sequence regions as well as recurrent variants from previous cases (>5 times observed). To this "noise" MVL, ~ 5,000 variant flags in problematic regions were populated. The clinical informatics tool was further leveraged to support WES Genome in a Bottle (GIAB) trios to generate a reference standard range for biological *de novo* rates in real and false GIAB trios. For our two "true"

trio sets, the percentage of Mendelian violations was <1%. When a single false parent was deliberately introduced, the percentage of Mendelian violations increased to 8%-9%. When both parents had misattributed paternity, the percentage of Mendelian violations increased to 13%-16%. This approach also helped baseline true *de novo* versus presumed *de novo* events. **Conclusions:** We present a customizable laboratory accessible workflow for successful exome variant review and parental status confirmation in trios. We also find a well-maintained MVL knowledgebase represents a useful strategy for clinical laboratories starting germline testing in which sample availability and previous clinical grade benchmark data are sparse.

I008. **Assessing Completeness of Exome Sequencing to Identify Low Performing Regions in OMIM Morbid Genes**

D. Green, W. Khan, E. Hughes, M. Dinulos, N. Shah, S. Sukhadia, P. Shah
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Utilization of whole-exome sequencing (WES) for diagnosis of hereditary disease provides an opportunity to discover causative variants in a wide breadth of human illnesses. However, the current practice of exon hybrid capture subsequent to next-generation sequencing using short read chemistry has well-known limitations. Long stretches of homology within the genome confound alignment and accurate variant calling in regions having insufficient depth or poor mappability. Here we describe the computational evaluation of sequence depth outputs from WES to flag recurrent regions with low coverage in OMIM morbid genes. **Methods:** Automated library preparation was preformed using SureSelect chemistry (v8) on the Magnis robot (Agilent). Sequencing was performed on the NextSeq 500 system (Illumina). After local demultiplexing using BCL2FASTQ, FASTQ files were uploaded to Alissa Align and Call for alignment and variant calling. Automatically generated coverage QC files across 40 exomes were summarized using a custom Python script. Specifically, exon portions with less than 20x read depth and the total percentage of the low coverage region relative to the size of the exon were calculated. Files were subset to determine frequency and percentage of these problematic regions in OMIM morbid genes from the full exome, a neurodevelopmental (NDD) panel and a connective tissue disorder (CTD) panel. **Results:** Of the total 15,144 OMIM morbid genes evaluated, 3,984 had at least one low coverage region in at least one exome sample. Across this subset, approximately 3 million base pairs of exon coverage were below 20x, this being equivalent to roughly 225 exons, of which 94 were entirely <20x in morbid genes. In terms of exon size, 70% of exons within the above subset were below 20x for less than 10% of their length. To implement this analysis, as part of a QC review, exons with low coverage in all samples (n = 40) where at least 50% of an exon was poorly covered (<20x) were used to identify problematic genes. Such an analysis produced 247 genes, 61 genes, and a single gene in the germline exome, NDD, and CTD panels, respectively. Recurrent variants coincident in the exons of these genes were automatically flagged in a managed variant list. **Conclusions:** We performed a detailed review for completeness of WES coverage which permitted identification of underperforming exonic regions in clinically relevant genes. This analysis can be potentially leveraged to other WES library preparation methods. Prospectively evaluating the depth and breadth of exome sequencing, as performed here, will add a QC checkpoint for accurate variant calling and avoid unnecessary curation of variants that are recurrent noise.

I009. **MPath Archer: Clinical Software for Targeted RNA-Based Panel Utilizing Anchored Multiplex Polymerase Chain Reaction for Next-Generation Sequencing**

S. Lachhander[1], J. Ziegler[3], J. Du[2], A. Agarunov[2], K. Mullaney[2], S. DiNapoli[2], P. Sukhadia[2], M. Zaidinski[2], R. Benayed[4], A. Zehir[4], A. Syed[2]
[1]Memorial Sloan Kettering Cancer Center, Clifton Park, NY; [2]Memorial Sloan Kettering Cancer Center, New York, NY; [3]Flatiron Health, New York, NY; [4]AstraZeneca, New York, NY.

Introduction: The MSK-Solid Fusion and MSK-Heme Fusion assays are utilized to detect gene fusions in solid tumor and sarcoma samples using a 123 specific gene panel, and samples with hematological malignancies using a 199 specific gene panel, respectively. The detection of fusion structural variants associated with these genes may provide critical diagnostic or prognostic information regarding patient disease and can identify a target for therapies which are approved or in clinical trials. Here, we present MPath Archer, a reporting and sign-out software which alleviates the manual burden of paper record analysis by providing end-users with auto-generation of structural variant metadata with OncoKB information, integrated text reporting capabilities, retrieval, and curated views of sample results on a per-run basis and robust application programming interfaces (APIs) for comprehensive data mining use-cases. **Methods:** MPath Archer is designed using a multi-tier client-server architecture to generate, store, and report Archer Solid and Archer Pan-Heme next-generation sequencing (NGS) assay results. This software consists of dynamically generated and responsive front-end portals, robust backend APIs, and a relational and non-relational hybrid database layer. The front-end layer is built using an in-house-developed ReactJS component library, whereas the backend APIs are engineered in Python 3.8. The run and sample results are stored in a MySQL database, whereas gene and structural variant annotation data are stored in MongoDB. MPath Archer integrates with the laboratory information management system (LIMS) to capture NGS-specific sample and run metadata. During the loading process, a job-scheduler also initializes an MPath Lab-QC instance to report run metrics and sequencer details. MPath Archer also provides an on-demand generation of clinical text reports on a sample-to-sample basis after analysis is complete and cases are ready to be signed out and finalized. **Results:** Since MPath Archer was implemented in 2017, our clinical laboratory staff has digitally registered more than 1,200 Archer Solid runs comprising more than 8,700 samples, and more than 1,100 Archer Heme runs comprising more than 3,700 samples. MPath Archer has eliminated the need for paper analysis and provides end-users with robust web services and rich graphical user interfaces during run and sample sign-outs. **Conclusions:** MPath Archer provides a centralized system to facilitate MSK-Solid Fusion and MSK-Heme Fusion analysis and transparent review. This software system digitizes, enhances, and streamlines the curation process for pathologists by giving them a standard user-interface and reporting format. MPath Archer also assists end-users by auto-loading the Archer sample and run metadata along with quality-control metadata.

I010. **Developing a Robust Bioinformatics Pipeline for NCI Clinical Whole Exome Sequencing Assay**

L. Chen[1], S. Narava[1], N. Nair[1], A. Hayes[1], L. Romero[1], A. Chapman[1], R. Thorton[1], S. Jiwani[1], C. Camalier[1], G. Rivera[1], E. Cantu[1], M. Williams[1], L. Harris[2], J. Doroshow[2], C. Karlovich[1], B. Das[1]
[1]The Frederick National Laboratory for Cancer Research, Frederick, MD; [2]National Cancer Institute, Bethesda, MD.

Introduction: NCI Clinical Whole Exome Sequencing Assay (NcWES) is an analytically validated assay that detects substitutions (single nucleotide variants [SNVs]), insertions and deletions (indels), and copy number variants (CNVs). It also reports tumor mutational burden (TMB). The assay is intended to be used for prospective patient selection and stratification, as well as retrospective correlative analyses in NCI sponsored Precision Medicine clinical trials. A fast and robust bioinformatics pipeline has been developed to generate variant

and TMB data with high sensitivity and specificity. **Methods:** The NcWES bioinformatics pipeline starts demultiplexing raw Illumina sequencing data and generates somatic and germline SNV/indel, CNV, TMB, and associated quality control (QC) data. The pipeline is based on Illumina's DRAGEN Bio-IT Platform (v3.10.8). Additional modifications were implemented to improve the analytical performance of the assay: 1) To eliminate formalin-fixed, paraffin-embedded (FFPE) tissue-related and other assay-specific artifacts, we built a systematic noise bed file based on 217 libraries from normal FFPE specimens with varying qualities. 2) We identified and blacklisted low coverage regions, which were defined as <0.5x normalized coverage in >80% of the normal FFPE libraries, based on normalized coverage analysis. 3) We applied more stringent criteria (total reads ≥150 and altered reads ≥10) to increase specificity of variant calls. 4) We generated a reference file from a panel of 177 libraries from normal FFPE and fresh frozen specimens for robust CNV calling. 5) We built a high-confidence region of coding exons (32.6 Mb) to call TMB. Additional QC metrics were identified which are critical for variant and TMB calling. **Results:** The pipeline was first tested on 39 adjacent normal FFPE libraries (eight individual specimens with replicates) for specificity. The number of false positives for SNV/indel on 671 cancer-related genes had reduced from 30 to 0 after we implemented all the modifications in the pipeline, which resulted in >99.99% specificity. Based on the performance assessment on six individual clinical specimens with seven replicates, the sensitivity for variants was >95% at variant allele frequency ≥5%, and it was >90% for CNV at copy number ≥5, by comparing to the orthogonal assay. TMB data (range: 2-20 mut/Mb) showed high correlation (Pearson's r = 0.99) between NcWES and the orthogonal assay. Finally, high-precision data were observed for all variants and TMB with %CV <5%. **Conclusions:** We have developed and validated a fast and robust bioinformatics pipeline for NcWES assay that can generate highly accurate SNV/indel, CNV, and TMB data. The pipeline is scalable and can be easily adopted and harmonized among multiple sites for use in clinical genomics workflows.

I011. ***In silico*** **Replacement for Molecular Barcoding with High Sensitivity and Specificity Variant Calling in Liquid Biopsy NGS Assays with Low Input and Tumor Content**

A. Conley, T. Modak, S. Polvino, L. Rishishwar, A. Kotlar
Pillar Biosciences, Natick, MA.

Introduction: Early detection of recurrence is important for improving patient survival outcomes. Ultra-deep sequencing of cfDNA (cell-free DNA) is an attractive and affordable approach for periodic MRD (minimal residual disease) monitoring post-treatment. However, reaching sufficient sensitivity and specificity with low DNA inputs and low tumor concentrations remains a challenge. Stochastic noise arises throughout the isolation and sequencing process and can appear at a low frequency similar to true variants. Molecular barcoding can reduce this noise but is complex. Here we describe the performance of a novel denoising algorithm HBDNA (Hierarchical Bayes DeNoising Algorithm) implemented in the PiVAT (Pillar Biosciences variant analysis toolkit) variant caller. HBDNA achieves high specificity variant calling with low DNA input and tumor concentrations, without the use of molecular barcoding. **Methods:** We assessed the performance of PiVAT's HBDNA in a dilution series of contrived cfDNA samples. Seraseq ctDNA Mutation Mix v2 positive samples were generated with dilutions at 0.025%, 0.05%, 0.1%, and 0.2%, and 5, 10, 20, and 30 ng of DNA input, 1,500-9,000 genome equivalents. For each concentration and input pair we generated three replicates, for a total of 36 positive samples. For each DNA input, three wild-type samples were also generated, for a total of 12 wild-type samples. Libraries were prepared using Pillar's SLIMamp (Stem-Loop Inhibition-Mediated amplification) technology, and sequenced on Illumina NextSeq. Variants were called using PiVAT with HBDNA. **Results:** The amplicon panel targeted 663 sites, including 12 expected variants (10 SNVs and two indels) in the Seraseq ctDNA Mutation Mix v2 material. Samples were sequenced to a mean coverage of 52,000X. After denoising, we saw a per-variant sensitivity of 90% down to 10 ng at 0.1% concentration. Per-sample sensitivity (≥2 positive calls within sample) was 100% across all concentration-input pairs, except at the lowest concentration and input pair (0.025%, 5 ng), where we identified 2/3 samples. In wild-type samples, we achieved panel-wide per-variant specificity of 99.65%, 99.93%, and 99.97% with 5, 10, and 20 ng DNA input, improving on the 97% per-variant specificity with PiVAT alone. **Conclusions:** HBDNA is a novel denoising algorithm that achieves high sensitivity and specificity in variant calling from cfDNA. In ≥20ng input samples, we would expect a per-sample false-positive rate of 0.3% based on performance in wild-type samples. At very low DNA input (≤10 ng) and low tumor concentrations (0.025%), per-variant sensitivity is reduced, but per-sample sensitivity remains high. This high accuracy makes PiVAT's HBDNA ideal for MRD monitoring and a viable alternative to the complexity of molecular barcoding.

I012. **Evaluation of *RET* Fusion Identification by the Idylla GeneFusion Assay**

M. Lacy[1], N. Sireci[2], N. Marella[2], T. Marquart[3]
[1]Eli Lilly and Company, Indianapolis, IN; [2]Loxo@Lilly, Stamford, CT; [3]Loxo@Lilly, Des Peres, MO.

Introduction: The Biocartis Idylla GeneFusion assay uses RT-qPCR for the detection of gene fusions and expression imbalance in seven genes, including *RET*. The assay requires small amounts of tissue and hands-on time, and offers quick turnaround times, allowing for quick identification of *RET* fusion-positive patients who may benefit from *RET* targeted therapy, in addition to other actionable fusions and targeted therapies. The recent approval of targeted *RET* therapies makes it essential to evaluate the Idylla GeneFusion Assay in a clinical laboratory setting. **Methods:** Fifty formalin-fixed, paraffin-embedded (FFPE) tumor samples with known *RET* fusion status from previous PCR, next-generation sequencing (NGS), or fluorescence *in situ* hybridization (FISH) testing were assessed using the GeneFusion Assay and analyzed for concordance. One to three 5µ FFPE sections with at least 20 mm² of tissue area were placed between wet filter paper, loaded into a GeneFusion cartridge, and run on the Idylla platform. Both *RET* fusion status and *RET* expression imbalance detection results were reviewed. *RET* fusions with *KIF5B* and *CCDC6* fusion partners are reported as *RET* Fusion Detected. *RET* fusions with other partners are reported through *RET* Expression Imbalance (EI) Detection and were interpreted as *RET* fusion positive. **Results:** Of 50 total samples tested, 37 were known *RET* fusion-positive and 13 were known *RET* fusion-negative. All *RET* fusion-negative samples were correctly identified as no *RET* fusion or *RET* EI detected, therefore having 100% concordance with previous results. Of the 37 known *RET* fusion-positive samples, three samples had invalid tests due to RNA degradation. No retest was performed, as tissue had been exhausted. Three of the 37 known *RET* fusion-positive samples had no *RET* fusion or *RET* EI detected. These discrepant samples were previously tested by NGS and found to have fusion partners other than *KIF5B* and *CCDC6*. Two samples were previously *RET* fusion-positive by FISH, but negative by the GeneFusion assay. These samples were also determined to be *RET* fusion-negative by NGS. Overall, these results provide 85% sensitivity, and 100% specificity. Sensitivity is increased to 91% by removing the FISH discrepancies from analysis. **Conclusions:** The Biocartis Idylla GeneFusion assay was able to detect *RET* fusions in our sample set with a sensitivity of 91% and specificity of 100%. Its concordance with NGS suggests it is a suitable method for rapid screening of *RET* fusions.

I013. **Automated Mutation Impact Analysis Tool for Real-Time Tracking of SARS-CoV-2 Variant Impact on COVID Diagnostic Assays**

X. Wu, M. Manivannan, R. Martinez, I. Mujawar, I. Pagani, D. Wang, X. You, P. Brzoska, S. Gummidipundi, H. Woo
Thermo Fisher Scientific, South San Francisco, CA.

Introduction: Due to its RNA genome, SARS-CoV-2 virus mutates more quickly than DNA viruses, acquiring an average of two mutations per month. Several global lineages have been observed since the beginning of the pandemic in 2019. PCR is one of the most widely used techniques for SARS-CoV-2 detection. PCR primer hybridization can be impacted by arising mutations if a mutation is located at the primer or probe target sequences, thus affecting the overall performance of the assay. A well-known example is the *S* gene target failure (SGTF) due to the delH69V70 mutation in Alpha and some Omicron lineages which eliminate the *S* gene signal of the TaqPath COVID-19 assay. As SARS-CoV-2 mutations continue to arise, it remains critical to monitor PCR assays against the emerging mutations and assess the impact. Health agencies such as FDA (US) and PHE (UK) require assay providers to regularly monitor and report any mutations that may affect assay performance. Here we describe a comprehensive mutation monitoring pipeline for the TaqPath COVID-19 Combo Kit 2.0 , TaqPath COVID-19 Combo Kit, and the TaqPath COVID-19, FluA, FluB Combo Kit. **Methods:** To track new mutations and the impact on PCR assays in real-time, a fully automated pipeline was developed and hosted on AWS. COVID-19 genetic sequences are regularly downloaded from the GISAID Initiative. In-house developed bioinformatics analysis pipeline and open-source third-party tools were wrapped in a Docker container. The AWS hosted pipeline used S3 for data storage, lambda function for task scheduling, and elastic computation for running analysis. **Results:** The tool can be run as scheduled tasks on AWS or on-demand in Docker container. Using the state-of-the-art Cloud computation and data storage technologies, the analysis time was shortened from multi-days to hours and from multiple manual steps to one-line command run. For each major variant, the tool analyzed the type and position of mutations relative to the primer or probe, and the mutation frequency. Validating against the open-source data confirmed our tool output variant mutation frequency with good concordance. Primers and probes of Thermo Fisher's diagnostic products listed above are included in the analysis. Predicted melting temperatures for mismatched primers and probes are calculated and evaluated against the annealing temperature. **Conclusions:** Overall, this fully automated tool allowed us to monitor the robustness of our products in the context of existing and emerging viral variants. Mutations that are predicted to impact assay performance are closely monitored, further evaluated, and reported to regulatory agencies and customers.

I014. **Development, Validation and Clinical Implementation of Reporting Pipeline for PGx Results from Targeted Genotyping Using Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline Database**

P. Mroz[1], J. Allen[2], S. Bradley[1], T. Butler[3], S. Johnson[1]
[1]University of Minnesota, Minneapolis, MN; [2]College of Pharmacy, Minneapolis, MN; [3]M Health Fairview, Minneapolis, MN.

Introduction: Pharmacogenomics (PGx) has been consistently cited as a major opportunity for improving patient care. The key requirement for effective clinical use of PGx results is the development of an appropriate clinical decision support that will allow for automated and simultaneous incorporation of complex genotyping information and translate it into actionable recommendations. Here we describe development and validation of a custom-designed genotype-phenotype caller called Go4PGx that provides reporting and interpretation of pharmacogenomic results from targeted genotyping analysis and automatically generates an individual report for each sample containing Clinical Pharmacogenetics Implementation Consortium (CPIC) phenotype and drug dosing recommendations. **Methods:** The Go4PGx software was written in Ruby on Rails and used a PostgreSQL database. The system supports uploading of variant SNP call data from a .csv file. The variant data are evaluated to produce diplotype matches based on information in the CPIC database. Go4PGx produces a web page and static portable document format (PDF) report that details genotype data and phenotype translation for all designated genes on the PGx testing panel, incorporation of copy number variation results for *CYP2D6*, as well as prescribing recommendations based on published CPIC guidelines. Forty samples with established diplotypes were uploaded in duplicates including 21 samples from the GeT-RM Pharmacogenomics project. Results were validated against genotype calls provided by two external laboratories (n=27) as well as by a complete *in silico* regression test. **Results:** There were no discrepancies in haplotype/diplotype/phenotype calls among the sample duplicates, and all results were concordant and called correctly by the portal algorithm. In one case the portal did not generate the result for the *CYP2D6* gene (which was the expected behavior) and the result was manually reviewed and reported as indeterminate. Diplotype call validation against external genotyping results showed 100% accuracy with the exception of when the number of SNPs tested differed among laboratories. **Conclusions:** The Go4PGx reporting pipeline was developed to analyze targeted genotyping data and produce a concise, human-readable PDF-based PGx report for nine CPIC genes. The annotation regarding phenotype significance and drug interactions and other PGx content in the report comes from the CPIC guideline database. The pipeline was implemented in the clinical PGx testing workflow and integrated with electronic health records. The Go4PGx PDF-based report was developed according to AMP recommendations for laboratories providing pharmacogenomic tests. Ongoing work includes adoption pipeline to accept .vcf (next-generation sequencing) type data and cross validation against other publicly available PGx pipelines.

I015. **Comprehensive *SMN1* and *SMN2* Profiling for Spinal Muscular Atrophy Analysis Using Long-Read PacBio HiFi Sequencing**

X. Chen[1], J. Harting[2], M. Eberle[2]
[1]PacBio, Richland, WA; [2]PacBio, Menlo Park, CA.

Introduction: Spinal muscular atrophy (SMA), a leading cause of early infant death, is a genetic neuromuscular disease caused by biallelic mutations of the *SMN1* gene. *SMN1* and its paralog *SMN2* are near identical in sequence with just one functionally different single nucleotide polymorphism (SNP), c.840C >T. Current SMA screening tests determine the *SMN1* copy number by distinguishing *SMN1* from *SMN2* based on c.840C >T and a few nearby differentiating bases. However, detailed sequence analysis of the complete genes is impossible due to the high sequence similarity. Furthermore, current tests are unable to accurately identify silent carriers with two copies of *SMN1* on one chromosome and zero copy on the other. The g.27134T >G SNP commonly tested for silent carriers does not satisfactorily distinguish two-copy *SMN1* alleles from singleton *SMN1* alleles. **Methods:** We developed an informatics method that accurately identifies complete *SMN1* and *SMN2* haplotypes throughout a 45kb long region (gene body plus upstream/downstream regions) using long-read PacBio HiFi sequencing data (whole-genome sequencing or hybrid capture-based enrichment). Our method determines *SMN1* and *SMN2* copy numbers based on the number of haplotypes and depth, and makes phased variant calls throughout the genes. We validated this method and applied it to 350 samples across five ethnic populations to conduct, to our knowledge, the first population-wide full-gene haplotype analysis of this complex region. **Results:** The *SMN1* and *SMN2* copy number calls by our method are highly concordant with orthogonal methods (>99% for both genes). In addition, a population-wide haplotype analysis identified 10 major *SMN1* haplotype clusters, four of which are specific to Africans. To study the two-copy *SMN1* alleles that contribute to silent carriers, we identified such alleles in samples with three or four copies of *SMN1* and examined the haplotype pairing on these alleles through trio-based

analyses. We identified a common two-copy *SMN1* allele (comprising ~65% of all two-copy *SMN1* alleles) that contains two *SMN1* haplotypes which are rarely present as singletons (<0.1% probability of being present on different chromosomes in the same individual). **Conclusions:** Here we provide the most comprehensive solution to one of the darkest regions of the human genome. Extending beyond copy number testing based on c.840C >T, our method can detect other pathogenic variants that cause SMA and enable potential haplotype-based screening of silent carriers. Testing the common two-copy *SMN1* allele that we identified for silent carriers will have >50 times higher positive predictive value than current tests. Future analysis of large population data with our caller will allow identification of more diverse haplotypes and more genetic markers for silent carrier detection.

I016. **Variant Annotation Tool Comparability: A Tie between Alamut and Nirvana**

M. Barbosa[1], J. Zhao[2], J. Reuther[3], R. Daber[3], N. Malani[3]
[1]Invitae, Keyport, NJ; [2]Invitae, Dresher, PA; [3]Invitae, Iselin, NJ.

Introduction: Accurate variant annotation is a critical component of clinical bioinformatics pipelines that ultimately impacts clinical reporting of genetic results. Therefore, selection and validation of variant annotation tools are vital for genetic result reporting. A comparison of two variant annotation tools, Alamut and Nirvana, available by license or as open-source, is summarized here along with methods for transcript and variant effect prioritization. **Methods:** To evaluate concordance and accuracy between Alamut and Nirvana, 2,435 variants from the Catalogue Of Somatic Mutations in Cancer (COSMIC) cancer mutation census with tier 1 or 2 mutation significance or reported in at least 50 samples within the census were used to generate an *in silico* variant caller file that served as the input to Alamut and Nirvana. Transcript-based annotations between the two annotators and COSMIC annotations were compared including HGVS nomenclature and variant effect concordance. Nirvana variant annotations were retained if they occurred in a preferred transcript or if the variant effect, defined by biological features based on sequence ontology terms, in a non-preferred transcript was predicted to have a major protein impact. **Results:** With respect to each other, Alamut and Nirvana provided a comparable number of annotations, 2,589 and 2,510, respectively, with near equivalent processing time after accounting for post-processing. The slightly higher numbers for both tools as compared to COSMIC is a result of the provision of additional variant effects and annotations in multiple transcripts. Of the total number, 0.27% (7/2,589) of variants annotated by Alamut had duplicate calls versus 2.7% (68/2,510) by Nirvana. Variants impacting splicing had the highest duplicate annotation rate among different variant effects by Nirvana (97.1%, 66/68). Alamut had slightly higher concordance to COSMIC regarding the predicted variant effect (97.2%, 2,417/2,589) than Nirvana (96.7%, 2,427/2,510). Between Alamut and Nirvana 93.4% of variants had concordant variant effects. Of the variant effect annotations incongruent with COSMIC, discordant Nirvana annotations typically involved COSMIC annotated in-frame delins, whereas discordant Alamut annotations typically involved COSMIC annotated nonsense variants. Five variants had incongruent annotations across all three sources. **Conclusions:** This study compared the variant annotation tools Alamut and Nirvana for use in clinical variant reporting. The two tools exhibited a high level of concordance with COSMIC; however, the discordant calls could impact clinical reporting. Both tools may require additional processing, including the transcript and variant effect prioritization described here, to ensure accurate variant effect representation.

I017. **Efficient Biorepository Development Using Oncomine Precision Assay and the Genexus Integrated NGS Platform**

S. Wunsch[1], V. Mittal[2], J. Schageman[3], A. Marcovitz[4], D. Garcia[3], A. Bigley[3], N. Ezzedine[3], T. Cooper[5], M. Taylor[6], C. Taylor[6], D. Tsavachidou[6], C. Smith[7], D. Payne[6], T. Zachary[7], L. Blann[3], D. Hassell[3], A. Hatch[8], S. Sadis[2], K. Bramlett[3]
[1]Thermo Fisher Scientific, San Diego, CA; [2]Thermo Fisher Scientific, Ann Arbor, MI; [3]Thermo Fisher Scientific, Austin, TX; [4]Thermo Fisher Scientific, South San Francisco, CA; [5]Thermo Fisher Scientific, Woodbridge, VA; [6]Precision For Medicine, Houston, TX; [7]BioIVT, Detroit, MI; [8]Thermo Fisher Scientific, Carlsbad, CA.

Introduction: The development of tumor sample biorepositories is critically important to enable the development and analytical validation of next-generation sequencing (NGS) and other molecular assays. However, screening formalin-fixed, paraffin-embedded (FFPE) and plasma samples for the presence of relevant variants of interest has traditionally been a slow, labor-intensive, and expensive endeavor. To this end, we paired the targeted Oncomine Precision Assay (OPA) with the Genexus integrated NGS platform and successfully screened more than 20,000 FFPE and plasma specimens, allowing low input of DNA and RNA, rapid turnaround time (TAT), and limited hands-on time. **Methods:** OPA is a pan-solid tumor research NGS panel covering small variants, copy number variants (CNV), and gene fusions across 50 genes including *ALK, BRAF, EGFR, ERBB2, FGFR1-4, KRAS, NTRK1-3, PIK3CA, RET, ROS1*, and others. The OPA panel utilizes AmpliSeq-HD technology to afford low analytical sensitivity and accurate detection of somatic variants in FFPE and plasma. The Genexus integrated platform provides automated sequencing within a 24-hour period, analyzing up to 16 FFPE tissue samples, or 32 plasma samples, simultaneously per run. Samples were chosen from common cancer types including lung, breast, colon, prostate, melanoma, thyroid, bladder, endometrium, and others. Data were analyzed using Ion Reporter informatics workflows generating complete variant reports. **Results:** Genexus sequencing of more than 18,000 FFPE samples generated a pass rate of 93%. Average (avg) DNA mapped reads were >900,000 with avg. read length of 84 base pairs (bp); avg RNA mapped reads were >100,000 with avg. read length of 61 bp. More than 77% of FFPE samples had at least one small variant detected with an avg. of 2.3 small variants per sample. The prevalence of individual small variants closely corresponded to estimated frequencies derived from public data repositories such as the "cBio Portal." Sixteen percent of FFPE samples contained at least one CNV event, and 4.5% of samples contained an identified gene fusion. Comparison of sequencing results with orthogonal variant detection methods yielded concordant results. **Conclusions:** The combination of OPA with the Genexus integrated sequencer enabled high-volume, fast, and accurate sample screening with low input and limited hands-on-time. Sequencing results provided novel insights into the distribution of specific variants across cancer types and the co-occurrence and mutual exclusivity of certain variants. The technology now enables laboratories to efficiently generate their own sample biorepositories to support their assay development and validation requirements.

I018. **Apollo-CNV: A Validated Exon Level CNV Calling Algorithm for Next-gen Sequencing (NGS) Data**

G. Liu, J. McCarthy, R. Littin, A. Bhattacharjee, M. Reese, E. Frise
Fabric Genomics, Oakland, CA.

Introduction: Detecting germline copy number variants (CNVs) is essential for inherited disease screening or diagnosis. Based on a reported survey of 50 genes associated with hereditary cancer and cardiovascular diseases, about 60% of deletions and 45% of duplications involve only one to three exons. Although several software tools are available to detect large, gene-level CNVs, detection of CNVs involving one or a few exons remains challenging. **Methods:** Here, we report performance of a method (Apollo-CNV) to confidently identify deletions or duplications at a single exon level. Apollo-CNV is implemented to work with hybrid capture-based panel or

exome sequencing data. The approach normalizes read depth of capturing-probes, utilizes a batch of unrelated individual samples (sequenced in the same laboratory with the same experiment procedures) as a reference, and calculates the probability for a region to harbor a deletion or duplication. For whole-exome sequencing (WES) data, where the read depth is low and therefore noisier, the algorithm further calculates a p-value and uses that to control false positives. We found false-positive calls tend to concentrate in samples with noisier read depth distribution and, accordingly, developed a mechanism to label those noisy samples. **Results:** To validate our approach, we obtained a next-generation sequencing dataset (ICR96, Illumina TruSight Cancer Panel v2) from Professor Nazneen Rahman's team at The Institute of Cancer Research in London. In this dataset, exon-level CNVs and CNV-neutral regions were verified with Multiplex Ligation-dependent Probe Amplification. There are 68 true CNV events in the dataset and we detected 66 of them. At the probe level, the sensitivity is 97.9% (412 out of 421 detected) and false discovery rate is 8.6% (39 false-positive calls). When the noisy samples were removed, 57 of the 58 true CNV events were detected, and probe-level sensitivity became 98.5% (260 out of 264) and FDR dropped to 1.9% (five false calls). We further validated the algorithm by running CNV calling with eight WES samples. All known pathogenic exon-level deletions and duplications were detected. As expected, some unknown CNVs were also reported. Interestingly, most of those CNVs were filtered out after applying GEM, a clinical variant prioritization algorithm from Fabric Genomics. **Conclusions:** The Apollo-CNV algorithm offers a reliable solution for detecting exon-level CNVs in both panel and whole-exome data. Combined with a downstream variant prioritization system, the method can contribute significantly to identifying pathogenic CNV variants in the clinical sequencing field.

I019. **Quantifying and Accounting for Reproducibility Issues of Copy Number Variation Detection with High-Density Methylation Arrays in a Clinical Setting**

T. Michael, P. Blackburn, A. Breuer, M. Eldomery, L. Furtado, Y. Liu, P. Stow, L. Wang

St. Jude Children's Research Hospital, Memphis, TN.

Introduction: DNA methylation profiling with Illumina Infinium Methylation Array platform has emerged as a powerful diagnostic tool to assist in the characterization and classification of different tumor types. These arrays can detect copy number variations (CNVs) via signal intensity, which we utilize for clinical reporting. However, we found that inter-instrument variability could influence the reliability of CNV calling. Despite improvements by changing from SWAN to FUNNORM preprocessing and segmentation parameter tuning, we were unable to obtain satisfactory reproducibility for clinical service. Furthermore, there was no standardized definition for borderline calls, which could lead to inter-clinician variability. In response, we developed a CNV pipeline built on conumee that allows for more reliable calling of CNVs using known reproducibility bounds. **Methods:** We developed an inter-run model that quantifies the expected variability between repeat runs of the same sample across different iSCAN machines. The model is based on a multiplicative scalar for describing reproducibility. In other words, we found the absolute magnitude of the CNV was correlated to its variability on repeat runs. To establish these bounds, we: 1) obtained seven paired runs of the same tissue sample with independent lab processing and scanning devices; 2) compared summed overall intensity of the copy number profile; 3) constructed a Bayesian predictive model based on an assumption of an unknown variance of mean intensity and a Gaussian distribution of intensities; 4) used leave-one-out cross-validation to validate the model from step 3 on the data from step 1; and 5) constructed the final model on all seven pairs. **Results:** In cross-validation, we found that 17 out of 17 clinically reportable chromosomal alterations fell in the 98% confidence intervals on repeated runs. For the final model, we find 0.17 to be the multiplicative scalar which represents the 95% confidence interval. We therefore adopted this interval into our clinical service for defining borderline calls to identify findings that may need further verification. **Conclusions:** The reproducibility issues that are intrinsic to using methylation array data for CNV detection cause difficulty in interpreting cases with copy number alterations close to cutoff thresholds. Although stricter segmentation parameter tuning and FUNNORM preprocessing improves reproducibility, we find that implementing a multiplicative confidence interval has enabled our clinical pipeline to better control reproducibility, allows for objective identification of borderline CNV calls, and prompts further verification when needed. Overall, the algorithm and approach we presented helps to improve the analytical validity of methylation array CNV calling.

I020. **Impact of Tumor Fraction and Batch Variation on Differential Gene Expression Analysis from Whole Transcriptome Sequencing of Metastatic Breast Carcinoma Biopsies**

C. Suciu, A. Creason, J. Letaw, C. Beadling, T. Neff, k. Onwuzu, Z. Mitri, E. Hobbs, B. Johnson, A. McGraw, G. Mills, S. McWeeney, C. Corless

Oregon Science & Health University, Portland, OR.

Introduction: Whole-transcriptome sequencing is commonly performed in the setting of clinical trials to assess changes in gene expression in solid tumors undergoing therapeutic challenge. There is interest in moving this approach into clinical practice, but CLIA validation is complicated by the lack of standard quality metrics and the wide variation in tumor fraction seen in core biopsies. **Methods:** Ninety-eight formalin-fixed, paraffin-embedded core biopsies were collected from 54 patients with metastatic breast cancer in an ongoing precision oncology trial at our cancer institute. RNA prepared from tumor-enriched tissue was sequenced using Illumina's TruSeq RNA Exome Library Prep on an Illumina NextSeq instrument (2 x 75 bp). FASTQ files were processed by Kallisto to yield per-gene transcripts per million (TPM). About 15,000 genes were ranked by TPM abundance, and differential expression of hallmark gene sets was quantified with Singscore to produce normalized scores between 0 and 1. A universal human reference (UHR) RNA sample (Agilent) was included in each run. Data from UHRs served to define batch variation, and this informed statistical evaluation of score differences among serial biopsies from individual patients. A normalization strategy was developed and used to assess the impact of tumor purity on scores. This was applied in an organ-specific manner to breast (n=10), liver (n=28), and lymph node (n=32) biopsies ranging from 30%-90% tumor cellularity. Estrogen receptor and Ki-67 immunohistochemistry (IHC) results were correlated with estrogen and proliferative pathway gene sets. **Results:** High reproducibility of single gene ranks for hallmark gene set members was seen across technical replicates of UHR and clinical samples. In both groups, >82% of pairwise comparisons of rank percentiles were within 5% of one another, and >96% were within 10% of one another. Effect size between IHC data and early estrogen response (r^2: 0.35-0.61) and four proliferative (r^2: 0.36-0.89) gene set scores varied by biopsied organ. Linear regression analysis identified a significant relationship ($p < 0.05$) between TPM abundance and tumor cellularity for 4.6%, 13.4%, and 6.9% of genes in the context of breast, liver, and lymph node specimens, respectively. Organ-specific cellularity adjustments of these 3,131 total genes, which included 26.6% of hallmark gene set members, improved the accuracy of gene set expression scores. **Conclusions:** Whole-transcriptome gene set expression analysis of tumors can be a valuable tool for predicting treatment response. Incorporating UHR batch controls and adjusting for tumor fraction are novel approaches that can be used to support CLIA validation and the routine deployment of transcriptome data in the analyses of solid tumors.

I021. **Profile Building of Patients with Hematological Cancers Using Machine Learning**

V. Williamson, B. Bullard, T. Olson, F. Sábato, E. Barrie, S. Turner, A. Ferreira-Gonzalez
Virginia Commonwealth University Health System, Richmond, VA.

Introduction: Increasing evidence suggests that next-generation sequencing (NGS) can be used to model and track the genomic profiles of hematological patients over time. There is little consensus in the proper approach for tracking these patients, however, and its ramifications for minimal residual disease. Clear definitions of "driver" and "passenger" mutations are lacking, and variation in sequencing quality across multiple runs can lead to the apparent loss of critical variants. Further, patients with these disorders are variable in terms of their responses to treatment, increasing uncertainty if new variants are found. Factors such as disease type, and the timing of the test within the context of the patients' treatment cycle, are therefore important. **Methods:** Here, we use a naïve-Bayes classifier and natural language processing (NLP) to build profiles for 336 patients sequenced with the Oncoheme NGS assay (Invitae FusionPlex/Myeloid, Illumina NextSeq500). A total of 56 diagnoses, ranging from acute myeloid leukemia to myelodysplastic syndrome, were included in this study. Each patient was sequenced between two and 12 times in a three-year period. All variants were clustered to identify the genomic profile of each sample. NLP was used to screen the clinical histories for these patients, creating a common vocabulary documenting patient prognosis and treatment course. Long-range linkage disequilibrium (LRLD) was used to address the absence of expected variants potentially resulting from sequencing error. Ten features including age, gender, and %blasts were used along with sequencing results and clinical histories to model the likelihood of specific profiles occurring within the course of a patient's treatment history. Profiles were divided into a training/test dataset (20/80) to predict the likelihood of a subsequent sequencing event. A brier score and an ROC-AUC curve were used to model accuracy and precision of the probabilities over time. **Results:** Overall, we found this approach to be highly effective in predicting the likelihood of the multiple sequencing events as well as the patients' state when that event occurs. Brier scores ranged from 0.98 to 1.406 ([max = 2] depending on disease type/treatment) and AUC scores were centered at 0.81. LRLD explained a small subset of the missing variants (11.6%), likely due to inconsistencies in self-reported ethnicity. **Conclusions:** By using ML-based profiles derived from NGS we can better understand disease course and identify variant artifacts arising due to technical variation.

I022. **Using a Machine Learning (ML) Algorithm to Predict Quantity/Quality Not Sufficient (QNS) Samples in a Clinical Massively Parallel Sequencing (MPS) Laboratory**

W. Mahmud[1], P. Velu[2], S. Priore[3]
[1]Stony Brook University Hospital, Stony Brook, NY; [2]NewYork-Presbyterian Hospital, New York, NY; [3]Hospital of the University of Pennsylvania, Philadelphia, PA.

Introduction: Massively parallel sequencing (MPS) is frequently used to identify genomic variants that have clinical significance. Quantity/quality not sufficient (QNS) rates for samples submitted for MPS vary depending on the assay's requirements; however, failure rates ranging from 10%-15% have been reported. QNS samples increase the cost of testing, as failed testing often requires retesting and increased wait times for the patient. Using ML models to accurately predict QNS samples before sequencing can help labs avoid suboptimal samples or identify alternate specimens for high-risk specimens. In this study we trained and evaluated a random forest classifier using clinico-pathologic and pre-analytic features to predict QNS specimens. **Methods:** Consecutive samples sent to the MPS lab at the University of Pennsylvania were included in the data set (Aug. 2018-Sep. 2020). The dependent variable was specimen outcome (binary; successfully tested or QNS). Independent variables (features) selected from the dataset included patient sex, sample type, assay type, tumor percentage, tissue type, sample received as tissue or DNA, sample age (days), 260nm/280nm ratio, 260nm/230nm ratio, and sample volume. Features that directly determine sample failure in our existing clinical algorithm such as DNA concentration and degradation were excluded. Samples with missing data were excluded. The final data set had 1,398 samples. Python 3 SciKit-learn and NumPy libraries were used for the analysis. Feature encoding and scaling were performed using OneHotEncoder and SimpleScaler. Random forest classifier was used for training the model and predicting the outcome. Eighty percent of the data (n=1,118) was used for training the model, cross-validation, and hyperparameter tuning (GridSearchCV). The remaining data (n=280) were used for testing. Performance of the model was evaluated using accuracy, true negative (TN) rate, and negative predictive value (NPV). Gini index was used to determine features' importance. **Results:** The model accurately classified 237 of 245 successfully sequenced samples and 16 of 35 QNS samples. Accuracy of the model was 90.3% with a TN rate of 45.7% (16/35). Sample failure rate based on the model's prediction was 7.4% as compared to the true QNS rate of 12.5%. NPV of model was 66% (16/24). The top five features were 260nm/230nm ratio, 260nm/280nm ratio, sample volume, sample age, and cytology sample type. **Conclusions:** This study provides a proof of principle that an ML approach using a limited feature set can identify QNS samples with a 66% NPV. Additional features may help refine this model. Thus, an ML algorithm may provide a tool to identify suboptimal specimens prior to testing, facilitating timely arrangement of alternate samples for retesting.

I023. **Single Source Product: An Authoring Suite to Generate the College of American Pathologists (CAP) Cancer Protocols and Electronic Cancer Protocols (eCP) from a Single Database**

E. Daley, K. Hulkower, J. Bodner, T. Carithers, K. Durham, C. Hebert, F. Marsh, S. Krejci, R. Moldwin, A. Patel, S. Spencer
College of American Pathologists, Northfield, IL.

Introduction: The CAP produces cancer and biomarker reporting protocols in both paper and electronic formats. The content for these formats had been archived separately, resulting in disparities in modeling structure and duplication of effort. In response, the Cancer Protocol and Data Standards team harmonized content modeling within a single database to improve workflow and the user experience. **Methods:** A relational database structure was employed to construct the CAP Single Source Product (SSP) tool for modeling both protocol formats. The architectural design of the database previously used for the eCP was modified and expanded to include the paper format with input from pathologists and CAP staff. The SSP tool architecture was designed as a web-developed software written in C# with the database hosted on the Azure Cloud. A dashboard aligns the user's workflow to navigate the process for protocol selection and editing. Editing and modeling are performed within the SSP Protocol Editor module utilizing a three-panel view to split the workflow into a template Telerik tree, a central metadata entry panel, and an output preview pane. **Results:** The SSP dashboard was designed to provide an activity synopsis for the status of any given protocol. A landing page provides a graphical representation of the state of the protocol and highlights any changes that have occurred since a user last accessed the tool. An import function allows users to easily incorporate elements from other protocols. A comparator function allows visualization of marked-up edits between versions. The SSP tool has a specific module for the creation and editing of the explanatory notes and references along with dynamic links to the case summary. A user-centric workflow was designed and integrated within the SSP tool allowing authors, modelers, and administrators to advance the protocols and templates through distinct stages within the publication process. This complex workflow aligns the release state to a corresponding version of the protocol in the SSP database. **Conclusions:** Since the launch of the SSP tool in October of 2020, the CAP has continued to optimize the tool and has published nine releases providing consistency in content in Word, XML, and HTML outputs. The CAP is exploring opportunities to extend the utilization of the SSP tool to capture biomarker and

micromolecular reporting results, and embed resource links transmitted from reference laboratories. The CAP continues to optimize and enhance the tool and is currently developing an XML-centric model to remove relational database dependencies.

I024. **MPath Reporter: A Scalable Solution to Integrate Ancillary NGS-Based Clinical Systems with Medical Informatics Infrastructure**

O. Adhali, A. Syed, A. Balakrishnan, J. Du, N. Fuks, J. Gurman, L. Plate, J. Schachter, V. Schuessler, J. Wang, A. Zehir
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: It is common for legacy pathology laboratory information systems to interface with ancillary clinical systems to pull reporting data elements, specifically around next-generation sequencing (NGS)-based assays. Such interfacing systems are usually proprietary and are not easy to customize, which could involve integration with other systems via web application programming interfaces (APIs), easy deployment in Linux server environments, and make use of open stack technology. Here, we describe MPath Reporter, a molecular interfacing server capable to receive and transmit HL7 messages, and interface with ancillary NGS API servers to generate PDF and text-based diagnostic reports. Also, MPath reporter interfaces with electronic medical records (EMRs) to transmit the diagnostic reports. **Methods:** MPath Reporter is a TCP/IP socket-based, n-tiered horizontally and vertically scalable software system. A non-blocking implementation allows MPath reporter to scale for high-volume cases. For each HL7 message received for an NGS case, an API request is posted to the report generation server to generate the case clinical report, and subsequently send the report to EMR. Unstructured HL7 transactions are stored in the MongoDB database, and structured workflow data are stored in the PostgreSQL database. A web-based report administrator connects with databases and API-based ancillary servers to help monitor system health and troubleshoot problems. The front-end of the application is architected using ReactJS, and Redux. The backend systems are built using Python (Twisted, and Flask), and hosted through Gunicorn. **Results:** Since April 2016, MPath Reporter has generated and transmitted roughly 81,800 NGS-based clinical reports. Decoupling the reporting web API server(s) enabled horizontal scalability to rapidly add new ancillary reporting systems. Initially created to support NGS solid tumor reports, the system has grown to include other NGS-based assays for heme malignancies, RNA fusions, and germline testing. In addition to molecular cases, the system has been expanded to intercept HL7 results for laboratory medicine cases to integrate into heme molecular workflows. On average, the system handles approximately 5,500 HL7 laboratory medicine transactions per day, accounting for 7 million transactions since June 2018. The n-tier approach has demonstrated the flexibility of the system to expand on the number of clinical tests and handle a high volume of cases. **Conclusions:** MPath Reporter is necessary to seamlessly integrate NGS-based reporting into existing and customizable clinical workflows. Such a system fosters integration of cutting-edge ancillary systems with other clinical, medical, and patient care informatics systems.

I025. **Development of a Clinical Cancer Variant Database for the Molecular Diagnostic Laboratory**

M. Parida, R. Sompallae, D. Ma
University of Iowa Hospitals and Clinics, Iowa City, IA.

Introduction: Advances in next-generation sequencing (NGS) have led to breakthroughs in diagnosis and targeted therapies for cancer patients. However, the large number of data generated by the NGS assays and the variability in the interpretation of variants in different disease settings have added to the challenges of comprehensive data management. We developed a clinical Cancer Variant Database (CVDB) to support our growing clinical and research needs. This CVDB contains Tier I and Tier II variants identified from our diverse cohort of cancer patients. It is searchable and has links to the variant interpretations and disease classification. **Methods:** The CVDB was implemented using MySQL and paired with an easy-to-use web interface using PHP and HTML. Data elements of CVDB contain genes, variants and their effects, therapies, diagnosis, patient age, and gender that were extracted from molecular pathology reports using a custom Python script. Extracted tables were loaded into the database using the MYSQL LOAD DATA command and hosted by our local web server. We used MySQL queries and PHP to handle user inputs and display search results. We included data from a cohort of 2,285 patients tested at our laboratory in the past two years into our CVDB. **Results:** The CVDB contains 2,064 Tier I and Tier II variants detected across 170 genes from 37 different cancer types. Our web interface provides two simple ways to inquire the contents of CVDB. Users can search by the official gene symbol, which will display variants identified in this gene, the effects of variants, and total number of patients with each variant effect. We assigned an effect ID to every unique variant interpretation associated with the disease. The effect IDs were used to group patient case accession numbers, diseases, and sequencing dates sharing a common variant effect. An alternate search option allows the users to query from the disease perspective by clicking on the “Disease gene network” button. Selection of the disease from the drop-down menu populates a table with a list of altered genes, the frequencies of co-mutated gene pairs observed in this disease, the number of patients harboring the alterations, and the age distribution of the patients. Furthermore, the CVDB contains therapy information that is associated with the Tier I variants. **Conclusions:** Our CVDB provides a solution for storing and searching variants of data generated by multiple platforms of our NGS assays. It also serves as a knowledgebase for research and education. In the future, this CVDB can be expanded to connect via application programming interfaces with the existing databases such as OncoKB and CIViC as a useful resource for integrative analysis.

I026. **Automatic Patient Matching for Genome-Guided Adoptive Cell Immunotherapy Trials at a Large Health System**

A. Dowdell, T. Bower, B. Cosgrove, R. Weerasinghe, A. Vita, B. Piening, C. Bifulco, E. Tran, R. Leidner
Earle A Chiles Research Institute, Providence Health, Portland, OR.

Introduction: As precision cancer therapies and genome-guided immunotherapies are trending to increasing biomolecular specificity, rapid and routine patient screening is essential to quickly identify the subset of patients that meet highly specific enrollment criteria. Here we developed a protocol for rapid clinical genomic data reanalysis, and patient identification for supporting precision medicine trials across a large healthcare system. We applied these approaches to extract human-leukocyte antigen class I (HLA-I) and tumor mutation information from next-generation sequencing data to support a novel investigator-initiated trial that uses the adoptive transfer of T-cells engineered to express T-cell receptors (*TCRs*) that target hotspot mutations. **Methods:** Electronic health record and genomic data from patients (n=8,115) spanning 33 cancer subtypes and with at least one reported pathogenic mutation were mined to identify existing patients that might qualify for an ongoing adoptive cell therapy matching clinical trial. Patients received comprehensive genomic profiling (CGP) using the Illumina TruSight Oncology 500 High Throughput assay (TSO500 HT) within the Providence Health system between September 2019 and May 2022. Previously reported pathogenic mutations were compiled and BAM files from patients with a pathogenic *KRAS* G12V/D mutation and reportedly not deceased were HLA-typed using DRAGEN HLA Caller. Key criteria to qualify for the clinical trial includes the presence of a pathogenic *KRAS* G12D mutation with either HLA-A*11:01 or HLA-C*08:02 match, or a pathogenic *KRAS* G12V mutation with HLA-A*11:01 match, while also assessing feasibility via last known contact date with the patient and other variable factors. **Results:** From the 8,115-patient cohort who received CGP testing, 960 patients (11.8%) harbored a pathogenic *KRAS* G12V/D mutation. The most enriched tumor types include pancreas (28.5%), bowel (25.4%), lung (18.8%), cancer of unknown primary (9.7%), and bladder/urinary tract (4.2%). As of early May 2022, 58.1% (n=558) of patients with a pathogenic *KRAS* G12V/D were not reported

as deceased, and reanalysis for HLA typing was performed on CGP datasets. We identified 17.4% (n=97) of alive *KRAS* G12V/D patients had an HLA-A*11:01 or HLA-C*08:02 genotype matching the trial eligibility. **Conclusions:** Although *KRAS* G12V/D is a relatively common solid tumor driver mutation, adding an HLA matching criterion significantly restricts the number of eligible patients. As more and more neoantigen-targeting TCRs are identified, automated matching algorithms represent a necessary step in rapidly identifying patients who might benefit from highly targeted immunotherapeutics.

I027. **The Development of a Patient Tracking Database with Standardized Capture of Pathology and Genetic Data in a Pediatric Oncology Setting**

L. Lansdon, M. Farooqi
Children's Mercy Kansas City, Kansas City, MO.

Introduction: Cancer registries, such as the National Cancer Institute's (NCI) Surveillance, Epidemiology and End Results (SEER) program, capture valuable information with regards to patient demographics, diagnoses, and outcomes. These types of data are also fairly well captured by electronic health records (EHRs) commonly used by hospitals. In contrast, the lack of ability by many registries and EHRs to capture complex genetic data in a discrete, granular manner presents a barrier to more informed patient monitoring locally and, on a wider level, hampers the field of clinical oncology as a whole. To this end, we developed a translational patient tracking database, presented here, which integrates the NCI's data elements with detailed genetic testing results for each of our pediatric oncology patients. **Methods:** The database was developed using Microsoft Access to capture NCI-recommended fields such as patient demographics (e.g., vital status, race), cancer diagnosis (e.g., SEER ICD-O code), and disease monitoring (e.g., last known disease status). Additional fields were then added to capture sample-specific pathology information and to summarize the genetic testing performed by disease timepoint (e.g., diagnosis versus relapse). The database was password protected and stored on a secured server behind our institution's firewall. Data entry was performed manually by two separate curators with second review by the other for each case. **Results:** We found that a multidimensional database was necessary to capture patient data with the level of detail necessary for clinical monitoring. This included integrating filters to facilitate easy data visualization by disease timepoint and by cancer type. We also developed additional data fields which were not well captured by SEER including a "literature diagnosis" field for diagnostic genetic findings not yet assigned an ICD-O code, a table of all pathology samples collected, a summary page of all clinical genetic testing performed at a particular disease timepoint, and test-specific data entry templates. For each template, we utilized dropdowns to capture data in a fixed manner and drafted rules to ensure uniformity of data entry and visualization. This allowed complex genetic data to be distilled into discrete data elements to facilitate easy informatic comparisons and data mining for future clinical or research purposes. **Conclusions:** Here, we present a novel patient database for the enhanced documentation and tracking of genetic results throughout each patient's oncologic disease course. We anticipate that the utilization of our database will result in improved management of our pediatric oncology patients and help to more uniformly capture data within our healthcare organization.

I028. **Zeta File System (ZFS) Is a Performant and Cost Effective Storage Solution for High Throughput Genomic Data Analysis**

W. Broderick, S. Roy
Cincinnati Children's Hospital Medical Center, Cincinnati, OH.

Introduction: Efficient next-generation sequencing (NGS) data analysis is dependent on many factors including the type of data storage. Zeta File System (ZFS) is a copy-on-write storage protocol that is highly resilient and easy to use. ZFS combines features of traditional volume managers and file systems allowing pooling of multiple drives while allowing users to easily add additional storage. The read-and-write cache algorithms of ZFS, using system memory or solid-state drives (SSD), allow highly performant use of large pools of slow spinning hard disks (HDD). In this pilot project, we assess the utility of ZFS for optimizing the cost and performance of NGS data analysis using very fast storage. **Methods:** Sequencing read sorting, a high disk input-output process, was used to compare the performance of different storage types. Samtools sort was used to sort aligned (hg19) NGS reads from exome sequencing of cancer reference material sample (Oncospan). Storage types included 7200 RPM serial ATA (SATA) HDD (4TB), SATA SSD (1TB), NVME SSD (1TB), a RAID0 ZFS disk pool consisting of a 72TB SATA HDD with a NVME write cache SSD (30GB), and a second RAID0 ZFS configuration using NVME SSD (3TB) with an NVME write cache SSD (30GB). The ZFS storage pools were provisioned as NFS shares (40G bps). The analysis server consisted of a 20 core (40 threads) Intel CPU with 315GB RAM. Available sorting threads were limited by Samtools ranging from five to 35 threads, in five-thread increments. Sorting algorithm was completed for each of the storage configurations across the seven processing thread sets. The analysis time was recorded for each run. **Results:** Sorting was completed on the HDD ZFS pool in 573 seconds (five CPU threads) and decreased to 402 seconds when using 35 CPU threads. The all-NVME ZFS pool finished with similar times, averaging two seconds' difference across all thread counts. In contrast, sorting on the HDD configuration completed in 840 seconds (five CPU threads) and decreased to 629 seconds using 35 CPU threads. On average, the HDD ZFS configurations completed sorting 247 seconds faster than the local HDD configuration, reducing the time required by 38%, while demonstrating almost identical performance to an all-NVME ZFS and local NVME storages. The HDD ZFS storage pool was significantly less expensive than the all-NVME ZFS storage configuration ($33/TB vs. $160/TB). **Conclusions:** Although the cost for storage continues to plummet, high-capacity pure flash storage can be cost prohibitive for NGS data analysis, both on-premise as well as in the Cloud. ZFS storage configurations using HDD disk pool and NVME caching can provide a fast, economical, reliable, and an easy-to-manage storage solution for NGS data analysis.

I029. **Building the Healthcare Metaverse: Leveraging Mixed Reality Guides to Augment Standard Operating Procedures (SOP) in a Precision Medicine Laboratory**

N. Perez, O. Elemento, A. Sboner, A. Sigaras
Weill Cornell Medicine, New York, NY.

Introduction: Recent groundbreaking advances in virtual, augmented, and mixed reality (VR/AR/MR) have paved the way for real-world applications in the Metaverse, an embodied version of the internet that merges the digital and physical worlds into one. The biomedical field already started reaping the benefits of this technology in a variety of situations from FDA-cleared MR applications used for preoperative surgery planning, to VR applications used for physician training, and MR applications used in biomedical labs to increase the safety and productivity of healthcare workers during the COVID-19 pandemic. We report the creation of standard operating procedures in mixed reality (MR-SOPs) in the context of a biology research laboratory to reduce the learning curve when training lab technicians, and increasing knowledge retention and productivity when executing lab tasks and workflows. **Methods:** We developed MR-SOPs: Leveraging Mixed Reality Guides, an MR application for Microsoft HoloLens 2 connected to the Englander Institute for Precision Medicine (EIPM) Laboratory Information Management System (LIMS) at Weill Cornell Medicine, which enables biology lab techs to overlay text, images, videos, and 3D objects to the real world. Information is displayed in the person's field of view and is spatially aware, moving away automatically when blocking the way. Navigation from one step to another can be performed via eye-tracking gazes, voice commands, or hand gestures without the need to touch any surface or remove gloves, thus eliminating the risk of contamination or need for disinfection. 3D objects are placed next to real-world objects, i.e., consumables and lab instruments, allowing for a more hands-on training. **Results:** We identified a set of paper-based SOPs commonly found in a biomedical

lab, i.e., blood fractionation, DNA extraction, as well as more complex ones involving robotic equipment. We then created MR equivalent SOPs, enriching them with video tutorials and 3D objects including arrows pointing at consumables and instruments in the real world. We recruited lab tech volunteers that had never performed these SOPs and split them into two groups: paper-based and MR-SOPs. We recorded execution times for each SOP and then asked lab technicians to perform the task without SOPs measuring their performance. **Conclusions:** The prototyping phase showed promising preliminary results in terms of user satisfaction, productivity increase, and reducing the learning curve for new technicians. The next phase of this study is to conduct a formal clinical trial of larger participant enrollment size and duration, measuring which method (MR-SOPs versus paper-based SOPs) leads to better productivity and shorter learning times compared to a control group of already experienced technicians.

I030. **Clinical Implementation of a Robust Cloud-Based Architecture for the Analysis of Somatic Whole Exome Sequencing Data**

D. Green, S. Sukhadia, E. Hughes, L. Tafe, J. Lefferts, E. Loo, W. Khan, G. Tsongalis, P. Shah
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Over the past decade, gene panels based off of next-generation sequencing (NGS) technology have enabled detection of the most common and clinically significant somatic variants covered in the gene content of such panels. However, such panels are gradually becoming obsolete because of the rapid advent of new targeted therapies and demand for expanded sets of genes to enable more comprehensive profiling of the cancer patient. Whole-exome sequencing (WES) utilizing a hybridization-based DNA capture technique covers the entire coding repertoire of the human genome, allowing laboratories to simultaneously screen all tumor relevant genes using a single library preparation. Concurrently, robust data processing and analysis software is necessary to manage such massive datasets. Here we describe our approach to the analysis of somatic WES data. **Methods:** Libraries were prepared from DNA extracted from formalin-fixed, paraffin-embedded tumor tissue using the Agilent v8 WES kit automated on the Magnis NGS Prep System (Agilent). Sequencing was performed on the NovaSeq 5000 with S4 flowcells and the Xp workflow. This allowed a 16-sample pool to be loaded on each of the four lanes, so a total of 64 somatic WES samples could be sequenced at once. We employed AUGMET, a Cloud-based software package, to validate and benchmark a somatic WES panel (covering >600 reportable tumor genes) against the TruSight Tumor 170 Panel (Illumina). All analysis was performed on the Amazon Web Services Cloud platform using several autoscaling components. **Results:** On average a total yield of 3,931 Gb was obtained per S4 flow cell, with an average of 359 million reads per sample. The average data retained per sample after deduplication and adapter trimming was 38.1 Gb. This resulted in an average of 316x coverage across the full exome target regions and an average coverage of 509.6x in the tumor genes. Sensitivity, positive predictive agreement, and specificity were established at 97%, 98%, and 100%, respectively. All clinically reported variants were detected in the precision replicates. Analysis times per batch of 64 samples were less than 24 hours including data uploads including variant annotation and classification. Average cost per sample was assessed at less than 20 USD per sample in compute costs with further optimizations expected to reduce this. **Conclusions:** Developing somatic testing on a whole-exome backbone provides the ability to analyze a comprehensive list of clinically significant variants without compromising sensitivity. Cloud-based infrastructures are essential for deployment of such software and could be optimized further to improve the turnaround time of patient reports accompanied with the reduced cost of processing, analyzing, and storing such data.

Other (e.g., Education)

O001. **Design and Implementation of a Decentralized Virtual Molecular Tumor Board across a Large, Diverse Community Health System**

B. Piening[1], P. Foley[1], K. Matlock[2], H. Wright[2], L. Harold[1], R. Weerasinghe[1], A. Vita[1], C. Moung-Wen[1], J. Abel[1], V. Kaimaktchiev[1], A. Dowdell[1], R. Meng[1], W. Urba[1], G. Srinivasa[2], P. Gold[3], G. Vaccaro[1], C. Bifulco[1]
[1]Providence Health System, Portland, OR; [2]Omics Data Automation, Inc., Portland, OR; [3]Swedish Cancer Institute, Seattle, WA.

Introduction: Rapid advances in precision cancer therapy and immunotherapy are transforming oncology with a wealth of genomic biomarker-associated drugs recently approved or available through clinical trials. Concomitantly, clinical tumor genomic sequencing has expanded in scope and utilization. As such, oncologists are now faced with treatment decisions of increasing complexity that may span conventional, precision therapies and immunotherapies for a single patient. However, typically supporting tumor board infrastructures are often suboptimal, with isolated boards carried out at individual sites and not utilizing large-scale analytics. Here we describe our efforts in designing and implementing a decentralized virtual molecular tumor board (VMTB) across a large health system spanning 50+ hospitals across seven states. **Methods:** A software platform was developed that integrates clinical genomics datasets, digital pathology imaging, and radiology imaging, as well as restructures key elements from the electronic health records. The platform was deployed in a secure internal cloud environment at Providence and made available system-wide to all clinicians. VMTBs were carried out twice a month via videoconference. Over the initial six-month launch window of the VTMB study, data were manually abstracted for aggregate analysis. **Results:** Key novel features integrated in the platform include real-time clinical trial matching, deep integration of medical imaging, timeline visualizations of a patient's treatment trajectory, and treatment comparisons via patient matching across the health system. Over the pilot phase of the VMTB, the most common tumor types discussed at the conference were of brain origin, followed by colon, lung, and bile duct. For patients discussed at the VMTB, 62% had a recommendation for treatment with a precision therapy or immunotherapy, either standard of care or through a clinical trial enrollment. This is in comparison to historical utilization of 18% for precision therapies, 30% for immunotherapies, and 4% for clinical trial enrollment for genomics-tested patients that were not presented at the VMTB. **Conclusions:** The VMTB developed into a forum for multidisciplinary experts across a broad geographic region to assist with critical treatment decisions for cancer patients. Patients discussed at the VMTB were more likely to receive a precision therapy or immunotherapy. Although part of this is likely due to selection bias (i.e., cases without actionable findings would be less likely to be presented at a molecular tumor board), we also expect the knowledge gained by participating physicians to lead to appropriate increases in precision therapy or clinical trial utilization over the larger patient population.

O002. **COVID-19 Minority Community-Based Health Care Initiative "Meeting People Where They Are": A Pathology Laboratory Perspective**

N. SAHAJPAL[1], S. Ananth[1], R. Bloomquist[1], D. Bradshaw[1], A. Chaubey[2], J. Farmaha[1], D. Fransoso[1], J. James[1], K. Jones[1], V. Kota[1], A. Kothandaraman[3], C. Marie Carlock[1], A. Mondal[1], P. Ray[1], H. SINGH[1], a. Vashisht[1], R. Kolhe[1]
[1]Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA; [3]PerkinElmer, Waltham, MA.

Introduction: There remain major gaps in our understanding of COVID-19, including the roles played by host genetics. A key to addressing these issues will be included in clinical studies of key constituencies, such as rural, African American (AA), and other underserved populations. A substantial percentage of the Georgia population is from rural communities, often with a high percentage of

minorities. These communities are often poorly represented in NIH clinical studies and are historically difficult to engage in such work. Unfortunately, these same populations are at high risk for severe COVID-19. **Methods:** To mitigate the burden of COVID-19 within the underserved AA communities, we decided to engage and "meet people where they are" by implementing participation at local AA churches and AA local barbershops. We are meeting people "where they are" by implementing testing and health information at local AA churches and AA local barbershops. We have partnered with the 100 Black Men of Augusta and have recruited 38% of our study participants from both AA churches and barbershops in local Central Savannah River Area communities identified as high-risk areas based on current COVID-19 incidence rates and neighborhood poverty. This is in contrast to lower AA recruitment in other study sites across the country. **Results:** Prior studies have demonstrated that recruitment and participant involvement in these non-traditional research environments have great benefits including accessing and reaching a demographic that would not otherwise be captured in a clinical setting, increasing diversity of the study sample, and promoting community-based health advocacy. This engagement has a profound impact on our understanding of the impact of COVID-19 in the medically underserved community leading to the identification of vulnerable counties and populations, targeted resource allocation, addressing underlying structural inequities, intervention and education, community-based participatory research, and clinical and translational research (inclusive of minorities). **Conclusions:** Recruitment and participant involvement in these non-traditional research environments have great benefits including accessing and reaching a demographic that would not otherwise be captured in a clinical setting.

O003. **Spatial Localization of Specific Nucleic Acid Sequences in Cells and Tissues with a Novel LoopRNA Technology**

D. Holzapfel, F. Mazzoni, J. Coleman
Enzo Biochem, Farmingdale, NY.

Introduction: *In situ* hybridization (ISH) is a method used for precise localization and detection of specific nucleic acid sequences in formalin-fixed, paraffin-embedded cells and tissue samples with DNA or RNA probes. As the sensitivity of these probes improve, ISH has been gaining a larger role in diagnostic pathology. Both DNA and RNA ISH probes can detect DNA integrated in cells; however, RNA ISH probes can also detect active mRNA. This study introduces AMPIVIEW RNA ISH probes, which combine the precision of targeted, sequence-specific RNA probes with the superior sensitivity of loopRNA technology for the detection of not only infection of viral DNA and RNA such as human papilloma virus and SARS-CoV-2, but also the expression of endogenous genes and mRNA such as *HER2*/neu. **Methods:** Manual protocols were performed starting from baking and deparaffinization with xylene or dewaxing agent before rehydrating the cells or tissue samples, followed by antigen retrieval and proteinase K treatment. Hybridization was performed with AMPIVIEW probes in a hybridization oven. Since the AMPIVIEW RNA probes are conjugated with biotin or digoxigenin, it makes them compatible with current immunohistochemistry (IHC) nanopolymer-based detection reagents and chromogens for signal amplification and detection. Detection with RNAscope probes was performed according to manufacturer's instructions. Results were visualized and imaged with a conventional light microscope. **Results:** Signal comparison between sense probes (target DNA) and antisense probes (target DNA and RNA) showed that the antisense probe yielded stronger signals in cells and tissue samples. Additionally, we show that AMPIVIEW HPV High-Risk signal correlated with p16 detection with IHC methods in tissue samples, and *HER2* DNA and RNA expression signal from ISH correlated with the expression of HER2/neu protein from IHC in tissues, demonstrating the specificity and sensitivity of the probes. The performances of AMPIVIEW HPV High-Risk RNA probes and SARS-CoV-2 RNA probes were compared to the performance of RNAscope from ACDBio. Our results show that the AMPIVIEW RNA probes performance is comparable to RNAscope, if not better. Tissue samples showed reduced background using AMPIVIEW RNA probes as AMPIVIEW protocols do not require lengthy steps to form structures in branched DNA (bDNA) to amplify signal, which can increase background. **Conclusions:** AMPIVIEW RNA probes can be a powerful tool for histopathological examination by ISH techniques for diagnostic purposes and can aid in further understanding the pathophysiology and distribution of nucleic acids, both DNA and RNA of infectious viruses or endogenous genes in cells and tissues.

O004. **Development of a Pathway to Quality-Focused Testing Tool for Validation, Implementation, and Maintenance of Next-Generation Sequencing in Clinical and Public Health Laboratories**

S. Mason-St. Prix[1], D. Arambula[1], G. Langley[3], H. Stang[1], S. Kocherlakota[3], C. Hanigan[4], M. Korth[3], P. Rosenbohm[3], J. Pettis[3], A. Muehlenbachs[1]
[1]Centers for Disease Control and Prevention, Atlanta, GA; [2]Booz Allen Hamilton, VA; [3]Booz Allen Hamilton, Atlanta, GA; [4]Association of Public Health Laboratories, Bethesda, MD.

Introduction: Clinical and public health laboratories (PHLs) are expanding use of next-generation sequencing (NGS) for various applications. Despite the availability and continuing development of guidance for developing performance specifications and supporting test validations for NGS, challenges remain due to the absence of a standardized quality framework. To address this, the Centers for Disease Control and Prevention (CDC) NGS Quality Initiative (QI) and the Association of Public Health Laboratories (APHL) are developing a Pathway to Quality-Focused Testing tool to support assay validation, test implementation, and system maintenance suitable across laboratory settings and applications. **Methods:** Development of this tool involved NGS expertise from CDC, APHL, and state PHLs to establish a product development life cycle. Preliminary analysis and technical requirements gathering sessions were followed by listening sessions to capture lessons learned from CDC and state PHLs that validated and stood up testing. Internal quality assessments were performed followed by stakeholder engagement to obtain feedback. **Results:** The Pathway is part of an adaptive, agnostic NGS quality management system (QMS) with resources that support all phases of the testing process and can be used across laboratory settings and sequencing applications. This tool contains five modules that address pre-analytic considerations through test maintenance, with a stepwise approach to validation and testing to support standing up a validated NGS workflow. With development and refinement ongoing, the current iteration focuses on establishing performance specifications and validating NGS workflows consistent with Clinical Laboratory Improvement Amendments (CLIA). The tool includes checklists and visuals to guide actions, cross-references relevant standards, identifies resources, monitors progress, tracks task completion, and provides an overview to laboratory leadership. **Conclusions:** The NGS QI provides standardized resources for addressing challenges encountered by laboratories implementing NGS-based assays including but not limited to bioinformatics pipelines, database usage, and internal/external assessments. For clinical laboratories and PHLs, the tool can contribute to the development/adaptation of workflows that include the necessary components of a QMS, documentation of quality control, integration of quality practices for development and maintenance of bioinformatics pipelines, and compliance with CLIA requirements. Future phases will include work with stakeholders to refine tool development, address challenges with method validation, and engage additional laboratory personnel (e.g., pathologists, directors) on usefulness and ease of implementation in laboratories performing NGS.

O005. **Clinical Implementation of a Precision Medicine Consultation Service**

A. Finer, A. Pannone, A. Bard, U. Green, L. Ritterhouse, D. Dias-Santagata, J. Lennerz
Massachusetts General Hospital, Boston, MA.

Introduction: To achieve precision medicine, providers must manage an increasing number of diagnostic tests. Each test result is dependent on specific domain expertise, and integration across several diagnostic test results can present distinct challenges. Recent revisions to the 2022 billing code stress the importance of consultations. Here, we report the design and implementation of a consultation pipeline for clinical pathology. **Methods:** The core design team comprised three board-certified molecular-genetic pathologists, one board-certified genetic counselor, and one high-complexity medical technician. Billing operations, electronic medical record (EMR) support staff, and compliance, departmental, hospital, and network leadership were among the additional team members. The team prioritized a modular, EMR-based design with laboratory information management system integration as well as appropriate documentation and invoicing procedures. **Results:** The seven-month design process comprised weekly 30-90–minute sessions with the core team and different meetings with additional team members (estimated total effort 170 hours or 10% of a full-time position). The workflow comprised seven modules: 1) physician order; 2) triaging module; 3) e-reply (no bill); 4) limited written documentation (no billing); 5) extensive written EMR documentation (effort-based billing); 6) denial/appeal module; 7) direct patient query module. Since launch of the EMR-based consultation service in early 2022, discounting the pilot period, we have managed 50 test orders, six billing-related requests, and 14 consultations further subdivided into the following categories: tumor origin (one), variant interpretation (one), clinical actionability (two), impact on diagnosis (two), and guidance on whether to pursue germline testing for variants detected through somatic testing (nine). Thus far, each consultation submitted to insurance using the 2022 pathology clinical consultation codes has successfully been reimbursed. **Conclusions:** Implementation of a consultation workflow for precision medicine needed substantial effort from a multidisciplinary team. The workflow accounted for various administrative and compliance-related aspects as well as reimbursement. Future work will focus on utilization, provider adoption, and optimization of workflows.

O006. **Going Green Step by Step: A Small Molecular Lab on a Path to Reduce Their Environmental Footprint**

T. Jakowczuk, T. Edmonston, S. Carey, K. Behling
Cooper Medical School at Rowan University, Camden, NJ.

Introduction: Medical laboratories contribute significantly to healthcare-related waste worldwide. Aside from hazardous waste, non-hazardous consumables and electrical power contribute to a laboratory's carbon footprint. The need to avoid contamination causes reliance on single-use plastics that are often platform-specific. We decided to formally assess the environmental impact of the molecular pathology laboratory and take action to reduce waste. **Methods:** A waste audit was conducted in March 2022 to evaluate waste associated with the pre-analytical (test ordering, obtaining, receiving specimens), analytical (performing assays), and post-analytical (quality control, interpretation, reporting) phases of the molecular diagnostics process. Paper used in the 12 months prior was weighed and consumption of plastic and gloves was estimated based on orders in the last 12 months. An action plan was established, guided by the principles of reduction, reuse, and recycling. **Results:** The action plan focused on reduction and recycling. Within the pre- and post-analytical phases, paper was identified as the greatest waste. Paperless test requests and paperless professional billing were launched in January 2022. Starting in April 2022, test results, quality control forms, and other reports were no longer printed and instead reviewed digitally, saving $\geq$40 lbs. of paper annually. Within the analytical phase, plastic waste was most significant. The lab joined the Applied Biosystems SeqStudio Cartridge Recycling Program. By using reloadable pipet tip boxes, our lab can avoid up to 57 pounds of plastic waste (about 550 boxes) per year. Nitrile glove recycling (about 200 boxes/year) can avoid landfill contributions of up to 440 lbs. per year. These changes are estimated to reduce CO2 emissions by one metric ton/year. However, the annual cost associated with implementing these solutions is at least $4,400. Turning off lights, instruments, and computers when not in use and setting ultra-low freezers to -70°C instead of -80°C can partially offset that cost. **Conclusions:** Reducing use of paper and energy is an easy first step. Switching to reusable pipet tip boxes and increasing plastic/nitrile recycling is currently associated with higher costs that a lab may or may not be able to afford. As more laboratories look to "go green," vendors have an incentive to develop more environmentally friendly consumables and offer reusable/recyclable packaging. More competition will hopefully bring down prices. Finally, by educating members of the molecular pathology department on the impact of simple steps on waste reduction and recycling, we hope to inspire enthusiasm for the "green lab" effort, and we plan to expand our initiatives to include the entire pathology department.

O007. **Enabling High-Stringency, Clinically Relevant Applications with Highly Scalable and Accurate DNA Library Preparation Workflows**

M. Ranik[1], B. Kudlow[1], P. Benson[1], K. Giorda[1], J. Haimes[1], T. Harrison[1], Z. Jaafar[1], L. Peterkin[1], K. Scott[2]
[1]Watchmaker Genomics, Boulder, CO; [2]Colorado State University, Fort Collins, CO.

Introduction: Next-generation sequencing is increasingly becoming the method of choice for clinical and translational research, replacing several disparate methods with a consolidated and comprehensive molecular profile. With this transition, there is a need to support a variety of challenging clinically relevant sample types, from damaged formalin-fixed, paraffin-embedded (FFPE) material to low input cell-free DNA (cfDNA) for early cancer detection and monitoring. Methods need to be robust and automation-friendly without compromising sensitivity or accuracy. To meet this demand, we developed streamlined workflows that enable DNA sequencing for challenging samples and demanding applications. **Methods:** We utilized precision enzyme engineering and a high-resolution Design of Experiment approach to develop novel library preparation methods. Our PCR-free whole-genome sequencing was compared against commercially available workflows using the Genome in a Bottle (GIAB) pilot genome, NA12878, as a direct measurement of library preparation efficiency, sensitivity, and accuracy. The method was further optimized for FFPE samples and tested across a range of sample qualities and inputs against conventional sonication-based protocols. Finally, cfDNA-like control samples were utilized to assess library conversion efficiency for low inputs. **Results:** Libraries generated using our optimized enzymatic-based fragmentation workflow exhibited a four- to 10-fold reduction in chimeric reads and terminal hairpin artifacts compared to other enzymatic methods and reached comparable levels to mechanically sheared DNA control libraries. Higher coverage combined with lower sequencing artifacts produced the highest variant calling sensitivity and specificity of the enzymatic library preparation methods, as determined using the GIAB high-confidence regions as ground truth. In addition to the data quality benefits, we provide workflow modifications that allow precise insert size tuning and enable rapid protocol customization for variable quality FFPE samples. Higher yields from low input samples, combined with lower sequencing artifacts, resulted in high-accuracy variant calling using contrived cfDNA samples. **Conclusions:** Watchmaker DNA library preparation methods accommodate a range of clinically relevant sample types, broad input ranges, and enable scalable automation workflows while delivering excellent sequence accuracy to support high-stringency applications.

Solid Tumors

ST001. **First Reported Case of Paraspinal Soft Tissue Tumor with *GLI1* Gene Fusion and Complex Karyotype**

R. Wong, N. Protopsaltis, A. Chung
University of California San Diego, San Diego, CA.

Introduction: Soft tissue tumors with *GLI1* gene fusions or amplifications are an emerging class of tumors which have historically been diagnosed as a broad spectrum of entities. Although some *GLI1* tumors have been described as having a benign clinical course, a subset behave aggressively and metastasize. Though no standard treatment exists for *GLI1* fusion tumors, a recent report described a patient with advanced metastatic disease effectively treated with the tyrosine kinase inhibitor pazopanib after failing immunotherapy and chemotherapy. This finding of a potentially effective targeted therapy highlights the importance of identifying *GLI1*-driven tumors. Here we describe the first case of a primary paraspinal *GLI1* fusion tumor and provide the first karyotypic characterization of this entity. **Methods:** Immunohistochemistry (IHC): IHC studies were performed in CLIA-certified and CAP-accredited laboratories. 1) Conventional karyotype: Standard procedures were performed with 20 metaphase cells counted and analyzed. 2) Fluorescence *in situ* hybridization (FISH): FISH analysis was performed using break-apart FISH probes to detect rearrangements of the *DDIT3* (CHOP)/*GLI1* gene at the 12q13 locus. 3) Next-generation sequencing (NGS): Total nucleic acid extracted from formalin-fixed, paraffin-embedded tissue was used in two hybridization capture-based targeted NGS assays for detection of fusions, single nucleotide variants, insertions and deletions, and copy number variants. **Results:** IHC profile: Tumor cells were S100+, CD56+, vimentin+, CD117+, CD10+, and negative for a broad panel of other markers. Karyotype analysis revealed a complex karyotype: 40~44,XY,der(7;17)(q10;q10),del(9)(q13q22),add(11)(p15),-12,del(16)(q23),-17,add(18)(p11.2),-20,+mar[cp11]/46,XY[9]. FISH analysis revealed a break-apart signal pattern suggesting rearrangement in the locus containing the *GLI1* gene. NGS assays identified a *PTCH1-GLI1* t(9;12)(9q22.31;12q13.3) fusion and ARID1B p.Q1144fs. **Conclusions:** GLI1 and PTCH1 are effectors in the Sonic hedgehog (SHh) signaling pathway. PTCH1 is a transmembrane receptor for SHh ligands and GLI1 is a transcription factor. PTCH1-*GLI1* tumors have shown fusions of exon 1 of the PTCH1 to exons 6/7-12 of *GLI1*. This fusion protein is predicted to have the GLI1 binding site of PTCH1 and the functional zinc finger domains of GLI1. This architecture suggests an auto-activating protein with SHh pathway upregulation. Here we describe the first reported paraspinal *GLI1* fusion tumor. This tumor was locally destructive with regional metastasis. Our case provides the first karyotypic characterization of this entity, which was noted as complex with multiple structural and numerical abnormalities. The degree of karyotypic abnormalities may be an indicator of disease progression in this normally described as indolent neoplasm.

ST002. **Identification of a Novel *MET* Exon 14 Skipping Variant in Non-Small Cell Lung Cancer**

Y. Cheng, R. Das, M. Jakubowski, J. Spildener
Cleveland Clinic, Cleveland, OH.

Introduction: Mutations in *MET* oncogene provides a therapeutic opportunity in non-small cell lung cancer (NSCLC) patients. We have identified a novel non-canonical splicing site variant in the *MET* intron 14. Our goal is to determine if this variant causes *MET* exon 14 skipping and to show the usefulness of common cytology slides for RNA-based testing. **Methods:** This new variant was identified in two advanced-stage NSCLC patients from a next-generation sequencing panel test. The impact on *MET* exon 14 splicing was predicted using *in-silico* tools, followed by confirmation using RNA extracted from stained cytology smears for reverse transcription (RT)-PCR and Sanger sequencing analysis. **Results:** The detected variant was a single nucleotide substitution in *MET* intron 14, NG_008996.1:c.3028+3A >T. *In-silico* prediction analysis exhibited reduced splicing strength compared with the *MET* normal transcript. RT-PCR and subsequent Sanger sequencing analyses showed evidence of *MET* exon 14 skipping in both patients' tumor specimens. **Conclusions:** This study demonstrated a non-canonical *MET* splicing site variant that causes exon 14 skipping while providing a rationale to practice *in-silico* prediction and use routine cytology slides for RNA-based confirmatory testing.

ST003. **Primary Intraosseous Rhabdomyosarcoma: Report of Two Cases**

P. Adelhardt[1], T. Liu[2], A. Wolf[2]
[1]ARUP, Salt Lake City, UT; [2]University of Utah, Salt Lake City, UT.

Introduction: Primary intraosseous rhabdomyosarcoma has been recently described as a rare variant of rhabdomyosarcoma (RMS) with recurrent *EWSR1/FUS-TFCP2* gene fusions. These tumors have a propensity for the pelvic and craniofacial bones and a highly aggressive clinical behavior. The lesions typically show a mixed epithelioid and spindle morphology with co-expression of myogenic markers, epithelial markers, and commonly *ALK*. To our knowledge, the number of cases reported in the literature thus far is limited. Herein, we report two additional cases of this rare entity with detailed clinicopathologic description and molecular confirmation. **Methods:** The molecular abnormalities were analyzed with fluorescence *in-situ* hybridization and/or RNA sequencing. **Results:** Case 1: A 34-year-old female was found to have a pelvic lesion in the superior pubic ramus in 2019. She was initially diagnosed with metastatic carcinoma of unknown origin and received radiation in an outside institution. Within a year she developed multiple pulmonary nodules and was treated with stereotactic radiation. Patient then was found to have new lesions in her pelvis involving superior/inferior pubic ramus and acetabulum. She underwent repeat biopsy showing the above diagnosis and received more radiation. In March of 2022, she presented to our institution with progressive pain in the left hip and pelvis. An excisional biopsy of the left femoral head/acetabulum revealed the diagnosis of intraosseous rhabdomyosarcoma with *FUS-TFCP2* fusion and *ALK* overexpression. Patient is currently receiving alectinib, which is being tolerated well. Case 2: A 73-year-old male was transferred to our institution for evaluation of a proximal femur bone mass and management of a presumed pathologic fracture. Imaging studies demonstrated a destructive lesion in left proximal femur with extraosseous spread and adjacent muscle and soft tissue edema. This lesion was previously biopsied, and although the results were interpreted as leiomyosarcoma, there was concern for a different diagnosis. The patient underwent a proximal femur resection and replacement. The final pathology showed the tumor to be consistent with epithelioid/spindle cell rhabdomyosarcoma with *EWSR1* rearrangement. Confirmatory RNA sequencing is in progress and whereas results are pending, the clinical team has concluded that they will move forward with radiation treatment. **Conclusions:** RMS are malignant mesenchymal tumors with skeletal muscle differentiation, currently classified into embryonal, alveolar, pleomorphic, and spindle/sclerosing types. Recent advances in molecular diagnostics have led to identification of a rare type of RMS defined by a recurrent genomic abnormality and distinct clinicopathologic features.

ST004. **Validation of Illumina Infinium Methylation Array to Determine *MGMT* Promoter Methylation Status**

T. Freeman, A. Wald, A. Christner, M. Grupillo, M. Miller, J. Skaugen, M. Nikiforova
University of Pittsburgh Medical Center, Pittsburgh, PA.

Introduction: Methylation status of the *MGMT* promoter in gliomas has been shown to predict patient survival in the setting of temozolimide treatment. With the advent of genome-wide methylation array technology to classify central nervous system tumors, we investigated the concordance of methylation array prediction of *MGMT* promoter methylation status using probes throughout the entire *MGMT* gene with *MGMT* promoter methylation-specific polymerase chain reaction (MS-PCR) assay. **Methods:** Brain tumor biopsies submitted

for clinical Infinium methylation array analysis utilizing the Heidelberg classifier were also analyzed in parallel by conventional *MGMT* promoter MS-PCR. The overall array *MGMT* promoter methylation prediction score and beta-scores associated with specific methylation array probes located in closest proximity to the established conventional MS-PCR assay (cg12434587 and cg12981137; Bady P, *et al*., 2012) were recorded and probe-specific cutoff values for array methylation positivity were determined. Methylation array results were then compared to *MGMT* MS-PCR results to determine sensitivity and specificity of *MGMT* methylation status via Heidelberg classifier prediction and via specific *MGMT* array probes. **Results:** *MGMT* methylation promoter score reported by Heidelberg classifier demonstrated 100% sensitivity and 88% specificity, and 89% concordance compared with *MGMT* promoter MS-PCR. However, specific methylation probes (cg12434587 and cg12981137) demonstrated 100% sensitivity, specificity, and concordance. Upon expansion of the dataset (n=149), these probes together demonstrated a decrease in sensitivity (88.0%), but were more specific for detection of *MGMP* promoter methylation (96.8%) and had a high rate of concordance (95.3%) with conventional *MGMT* methylation array assay. **Conclusions:** Utilization of the two probes (cg12434587 and cg12981137) for detection of *MGMT* methylation using the Infinium methylation array provided higher specificity and concordance with *MGMT* MS-PCR assay as compared to the Heidelberg classifier score. Continued longitudinal clinical monitoring based on methylation status and treatment should be performed to measure the clinical impact of utilizing the two probes for determination of methylation status versus conventional *MGMT* promoter methylation testing.

ST005. **WITHDRAWN**

ST006. **Clinical Validation of an Automated Gene Fusion Assay for Non-Small Cell Lung Carcinoma**

M. Hessler, P. Caffes, A. Buglioni, Y. Lo
Mayo Clinic, Rochester, MN.

Introduction: Targetable oncologic driver gene fusions are present in ~11% of non-small cell lung carcinoma (NSCLC). Next-generation sequencing (NGS) panels have been the preferred method to detect gene fusions; however, they have a long turnaround time. The Idylla GeneFusion assay is a cartridge-based, fully automated multiplex real-time PCR system that rapidly assesses clinically relevant gene fusion status of tissue directly scraped from formalin-fixed, paraffin-embedded (FFPE) slides. We aim to assess the performance characteristics of this recently available assay by NSCLC samples previously profiled with clinically validated NGS assays. **Methods:** The assay utilizes two detection strategies: 1) specific fusion detection, including RT-qPCR primers designed specifically for 17 *ALK*, 13 *ROS1* and seven *RET* rearrangements as well as *MET* exon 14 (*MET* ex14) skipping, and 2) expression Imbalance (EI) detection, which compares the 3' and 5' expression levels in *ALK*, *ROS1*, and *RET* for detection of uncommon fusions. A detected EI result is indicative that a fusion is present. A total of 63 samples were selected, including fusions involving different genes: *ALK* (n=11), *ROS1* (n=10), *RET* (n=12), *MET* ex14 skipping (n=10) and negative samples (n=20). Break-apart FISH testing was performed in discordant cases and in cases where fusions were detected by EI only. Several specimen types were tested, including FFPE (tissue and cell block), cytology smears (Diff Quik and PAP), and pre-extracted RNA. Limits of detection (LOD) were evaluated. **Results:** The sensitivities of the assay for detecting *ALK*, *ROS1*, *RET*, and *MET* ex14 skipping in NSCLC samples examined were all 100%. Of the positive results, 94% (31/33) were detected by specific fusion detection; 6% (2/33) were detected by the EI method only and were confirmed by FISH. The specificities were 98% (52/53) in *ALK* and 100% in others. One case with *RET* rearrangement showed a false-positive fusion call in *ALK*, and confirmatory FISH revealed *ALK* gene amplification. Intra- and inter-assay reproducibility studies demonstrated 100% concordance between replicates. The assay accepted all specimen types examined. LOD studies indicated that the assay can detect fusions down to 5% tumor content, 36 mm^2 input for FFPE scraping, and a cellularity of at least 300-3,000 cells on smears, or a minimum of 20 ng pre-extracted RNA. **Conclusions:** The performance characteristics of this assay is compatible with NGS. Due to rare false-positives by EI detection, we suggest a secondary confirmation test for fusion calls by EI only. The significant reduction in turnaround time, wide material-type acceptance, low sample amount requirements, and minimal labor make the automated gene fusion assay an attractive alternative option to NGS assays.

ST007. **LungLB Four-Color Fluorescent *in-situ* Hybridization for the Classification of Individuals with Indeterminant Pulmonary Nodules by CLIA Validation**

B. Katchman, S. Tahvilian, D. Leventon, L. Baden, P. Pagano
LungLifeAI, Thousand Oaks, CA.

Introduction: Low-dose computed tomography (LDCT) is the standard method by which pulmonary nodules are detected in a lung cancer screening setting and incidentally from other medical events, and it is estimated greater than 1.5 million are found each year in the United States. LDCT has a high sensitivity but is limited by a low specificity and high false-positive rate. It is estimated >40% of biopsies of suspicious pulmonary nodules are not lung cancer. In addition to the increased number of unnecessary biopsy procedures, the high false-positive rate may lead to additional complications including infection, pneumothorax, hemorrhage, and even death. **Methods:** The LungLB test is a four-color fluorescent *in-situ* hybridization (FISH) assay used to identify circulating genetically abnormal cells (CGAC) isolated from peripheral blood of patients with indeterminate nodules suspicious for lung cancer. Clinical blood samples are drawn into a blood collection tube used to stabilize blood samples for use within 24-96 hours. Stabilized blood samples are initially processed by a highly selective immunomagnetic depletion methodology selectively removing erythrocytes and subpopulations of leukocytes to create an enriched cell suspension. Enriched cells are deposited on a glass microscope slide followed by FISH using four-color fluorescent probes targeting regions of the genome known to be amplified in lung cancers. Images of the entire slide are acquired using a BioView wide-field fluorescent microscope and processed using a custom-designed, artificial intelligent-derived algorithm to categorize CGACs, defined as amplifications of two or more probe regions, which are subsequently verified by a trained and licensed technologist. **Results:** LungLB is a Four-color FISH assay utilizing four unique probes to target chromosome locations 3q29 (Green), 3p22.1 (Red), 10q22.3 (Gold), and 10cen (Aqua). Probe hybridization specificity was assessed by processing metaphase cells using the LungLB assay, demonstrating 100% specificity. The threshold of the assay was established by processing 20 healthy normal donors, resulting in a threshold of 2.24 CGAC/10,000 white blood cells screened. The clinical performance was assessed by processing 30 clinical samples from the target population with indeterminate nodules. Based on the CGAC threshold of 2.24, CGAC/10,000 WBCs results in 87.7% sensitivity and 84.6% specificity resulting in an area under the curve of 0.815. **Conclusions:** The performance of the LungLB has been validated according to the standard operating procedure established at LungLifeAI in accordance with CLIA standards. There is an ongoing FDA clinical validation underway to further evaluate the performance of LungLB.

ST008. **Development and Validation of an Ultra-Rapid Assay for Detection of *NTRK1/2/3* Fusions**

P. Caffes, M. Hessler, A. Buglioni, Y. Lo
Mayo Clinic, Rochester, MN.

Introduction: Entrectinib and larotrectinib received tissue-agnostic FDA approvals for treatment of *NTRK*-rearranged cancers. *NTRK* fusions are rare and can be found in a variety of carcinomas and sarcomas. Accurate detection of *NTRK* fusions has been challenging due to homologous gene family members and various splice variants and fusion partners. We reported the development data and product validation study of an ultra-rapid assay for its detection profile of

NTRK1/2/3 fusions. **Methods:** The Idylla GeneFusion Assay is a cartridge-based, fully automated multiplex real-time PCR system. The assay utilizes 3' and 5' expression imbalance strategy to determine if a fusion involving *NTRK1, NTRK2,* or *NTRK3* is present. Forty-five *NTRK* fusion-positive samples (19 sarcoma/spindle cell neoplasm, 18 glioma, five kidney mass, two papillary thyroid carcinoma, and one parotid carcinoma) and 23 fusion-negative cases (21 lung carcinoma and two glioma) were selected. Formalin-fixed, paraffin-embedded scrapes from 10 cases with *NTRK1*, eight with *NTRK2,* and 12 with *NTRK3* fusion, based on clinically validated next-generation sequencing assay, were analyzed with prototype cartridges. Data were used to facilitate defining algorithms. After the release of manufactured cartridges and updated algorithm, we examined 10 *NTRK1* (five from prior beta testing), 10 *NTRK2* (five prior), 14 *NTRK3* (nine prior)-rearranged cases, and 23 negative samples. **Results:** In beta testing, the assay detected 7/10 (70%) *NTRK1*, 0/8 (0%) *NTRK2,* and 11/12 (91.7%) *NTRK3* fusions. Baseline mRNA expression level and difference in 5' and 3' expression was analyzed. The fusion calling algorithm was adjusted accordingly. For manufactured cartridges and commercially launched algorithm, the assay detected 10/10 (100%) *NTRK1*, 1/10 (10%) *NTRK2*, and 14/14 (100%) *NTRK3* fusions. No *NTRK* fusion was detected in all 23 negative samples. Surprisingly, *NTRK3* fusion was detected in five *NTRK2*-rearranged cases, suspected due to sequence homology. No fusion was detected in four *NTRK2*-rearranged cases. Combined reporting for *NTRK2/3* fusions was proposed, as current treatments are the same for different *NTRK* fusions, reasonable from a clinical perspective. Combined *NTRK2/3* fusion detection was 83.3% (20/24) sensitive and 100% (33/33) specific. **Conclusions:** The beta testing and resulting algorithm revision improved the sensitivity of detecting *NTRK1* and *NTRK3* fusions, and 100% specificity remained for *NTRK1* fusion. Performance of standalone *NTRK2* fusion detection remains poor. Applying combined *NTRK2/3* fusion reporting, the assay yielded favorable performance in detecting *NTRK2/3* fusions. The quick turnaround time and overall high specificity suggest this assay is a valuable screening tool for *NTRK* fusions for treatment consideration. Confirmatory NGS testing is recommended for differentiating *NTRK2/3* fusions.

ST009. **Analytical Performance of a Next-Generation Sequencing (NGS) Assay Kit for Assessing Homologous Recombination Deficiency (HRD) from Solid Tumor Samples**

L. Liu, K. Roessler, S. Bilke, Y. Ding, D. Erlandson, Y. Fu, B. Hariharan, S. Katz, J. Lee, C. Schulman, F. Song, R. Vijayaraghavan, P. Wenz, E. Xia, H. Yan, Y. Zhu, C. Zhao, J. Dockter, T. Pawlowski, J. Day

Illumina, Inc., San Diego, CA.

Introduction: Homologous recombination deficiency (HRD) status is predictive of response to PARP inhibitors (PARPi) in ovarian cancer. Developing reliable methods to assess HRD status is important to enable genomic profiling of tumor types with PARPi indications. Here we describe the analytical performance of the TruSight Oncology 500 HRD (TSO 500 HRD) research assay, a distributable next-generation sequencing kit (available outside of the US and Japan) for assessing HRD using DNA from solid tumor samples. **Methods:** The TSO 500 HRD assay was developed by adding an HRD probe pool targeting genome-wide single nucleotide polymorphisms to the TruSight Oncology 500 (TSO 500) assay to enable Genomic Instability Score (GIS) assessment. For each sample, the DNA library was split into two hybridization reactions, one with TSO 500 probes and the other with HRD probes. Then both libraries were pooled for sequencing with eight samples per NextSeq 550 run. The GIS algorithm was licensed from Myriad, re-implemented as part of the DRAGEN Bio-IT software suite and integrated into the TSO 500 analysis workflow. Forty nanograms of DNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples were used as assay input. Samples were also tested with a reference assay as the orthogonal test. **Results:** Testing of serial dilutions of eight ovarian cancer samples showed the limit of detection for small variant calling in *BRCA1/2* genes is at 5%-10% variant allele frequency (VAF), for large rearrangement variant calling in *BRCA1/2* genes is at 30%-50% VAF, and for GIS is at 30%-40% tumor content. Testing with benign-adjacent ovarian FFPE samples showed that the false-positive rate for GIS status and *BRCA* variants is <0.0001%. Testing of ovarian cancer samples and control samples with multiple operators, reagent lots, and library preparation start days showed >95% concordance in *BRCA* variant calling and GIS status. The GIS scores were highly concordant with the reference method assay (R = 0.98). The results were robust on both NextSeq 550 and NovaSeq 6000 with SP flowcells. To confirm that the addition of HRD testing did not impact TSO 500 variant calling, results from the TSO 500 HRD assay were compared to those generated using TSO 500. High concordance was observed between the two assays in various variant types, including single nucleotide variants, insertions, deletions, copy number variants, fusions, splice variants, tumor mutation burden, and microsatellite instability. **Conclusions:** The TSO 500 HRD assay demonstrated high analytical sensitivity and specificity in detecting *BRCA* variants and calculating a GIS for HRD status assessment in ovarian cancer samples. TSO 500 HRD enables high-resolution comprehensive genomic profiling alongside HRD assessment to maximize biomarker detection from limited amount of sample.

ST010. **Detection of Somatic Variants in Breast Cancer Specimens Using the Novel AGENA ClearSEEK *PIK3CA* Panel on the MassARRAY System**

A. Sartori[1], D. Irwin[3], M. Schwab[4], W. Schroth[2]

[1]Agena Bioscience, Hamburg, Germany; [2]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Baden-Wurttemberg, Germany; [3]Agena Bioscience, Bowen Hills, Queensland, Australia; [4]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology and Clinical Pharmacology, Biochemistry and Pharmacy, University of Tübingen, Tübingen, Baden-Wurttemberg, Germany.

Introduction: *PIK3CA* is the most commonly mutated oncogene in hormone-sensitive (HR+) breast cancer with up to 38% of tumors having hotspot mutations. The PI3K inhibitor alpelisib has been recently approved for the treatment of *HR+/HER2-* metastatic breast cancer with *PIK3CA* mutations (*PIK3CA*mut). The AGENA ClearSEEK *PIK3CA* Panel covers all major hotspot mutations assigned with evidence level 1 for the prediction of alpelisib benefit. The panel detects 20 mutations in exons 7, 9, and 20 with an analytical sensitivity down to 1% variant allele frequency (VAF), thereby providing a screening tool for clinical trial evaluation, endocrine resistance, and response to PI3K inhibitors in breast cancer. **Methods:** *PIK3CA* profiling with the AGENA ClearSEEK PIK3CA Panel (Agena Bioscience) was applied in two cohorts (N=84, N=62) of HR+ early and advanced breast cancer, respectively. A total of 20 ng formalin-fixed, paraffin-embedded-derived tumor DNA was subjected to a single global PCR reaction, and subsequently divided into three multiplexed extension reactions. Purified reactions were analyzed with the MassARRAY System (Agena Bioscience). Sensitivity and specificity were tested using commercial *PIK3CA* mutation controls (SensID GmbH) and by a platform performance comparison to digital droplet PCR (ddPCR, Bio-Rad Laboratories GmbH). **Results:** Using commercially available reference material covering 11 clinically relevant *PIK3CA* mutations, a 100% accurate detection between 0.5% and 4% VAF was observed with the AGENA ClearSEEK PIK3CA Panel. Assay readouts were successfully assigned in 100% (N=84) of cohort 1 samples, irrespective of DNA fragmentation or age of tumor material (range 1-11 years). Nine of 20 variants (45%) were detected at VAF between 0.5% and >10%. In 30 tumors (35.7%), a *PIK3CA* mutation was observed with H1047Y (N=10; 33%) being the most abundant variant, similar to large-scale observations based on FDA-approved *therascreen* PIK3CA RGQ PCR Kit. Of the three *PIK3CA* mutations E542K, E545K, and H1047R determined in cohort 2 (N=62) by ddPCR, the ClearSEEK PIK3CA Panel showed 100% concordant variant assignments in tumor samples with robust ddPCR results (>20

droplets). In the low-performance range, 2.7% discordant results were observed between technologies. The overall detected frequency of PIK3CAmut was similar between platforms (42%-43%). **Conclusions:** The AGENA ClearSEEK PIK3CA Panel combines both low DNA input and hands-on time requirements with accurate data assessment and provides a high-sensitive and reliable tool to perform *PIK3CA* analysis of clinically actionable mutations in breast cancer tumors.

ST011. **Assessment and Implementation of the Agilent Magnis for Automated Somatic Whole-Exome Sequencing of DNA Extracted from FFPE Tumor Tissue**

E. Hughes, D. Green, K. Winnick, B. Houde, S. Deharvengt, S. Sukhadia, P. Shah, L. Tafe, G. Tsongalis
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Somatic whole-exome sequencing (sWES) by next-generation sequencing (NGS) offers interrogation of the pan-cancer exome for providing high-quality ancillary testing in patients with cancer. Gene panel assays, the current standard of care for most somatic NGS testing, though crucial, are sometimes limited in scope as more genes are discovered to have functional roles in cancer biology or are linked to prognostic, predictive, or diagnostic relevance in cancer. Here we describe our evaluation of the Agilent Magnis NGS Prep System, which is a genuine walk-away automated NGS library preparation solution from formalin-fixed, paraffin-embedded (FFPE)-derived DNA for genomic-scale sequencing. **Methods:** Sixteen Agilent SureSelect Human All Exon V8 DNA libraries were prepared on the Magnis from 50ng input DNA extracted from FFPE patient specimens with known clinically reported variants that included single nucleotide variants, indels, and copy number variants (≥5 copies). Libraries were quality assessed on the Agilent TapeStation 4150, and sequenced on the Illumina NovaSeq 6000. Sequence alignment and annotation were performed using an in-house integrated, automated bioinformatics pipeline for variant calling and interpretation for sWES libraries. **Results:** Magnis automated library preparation was completed in 9.5 hours. The workflow between extracted DNA through to sequencer-ready libraries took only 1.5 days with approximately four hours of technologist hands-on time. This was far superior to our current gene panel assay that takes two to three days with almost constant technologist involvement. Tapestation quality-control (QC) analysis of prepared libraries showed an average concentration of 17.4 nmol/l and average fragment size of 335 bp with 96.3% fragments between 200-700 bp. All previously clinically reported variants from the current gene panel assay were identified with sWES, including those reported with low VAF (5%). Post-sequencing QC metrics, based on a 684 cancer-specific gene panel that includes genes commonly found on most commercially available solid tumor gene panels, were all high performing with more than 98% of genes achieving ≥250x depth of coverage. **Conclusions:** The Magnis provided consistent automated NGS library preparation for sWES that resulted in high QC sequencing performance that requires minimal technologist oversight. A minor limitation of this system is the throughput where library constructs are restricted to eight samples at a time. This may be overcome by the quick library preparation time which may allow for multiple runs over a single day. Sequencing the human exome in cancers allows for the possible interrogation of any gene of interest and offers exciting possibilities for cancer assay development.

ST012. **ASPYRE: Validation of a Simple, Fast and Robust Novel Method for Complex Genetic Analysis of Actionable NSCLC Variants in Tissue and Plasma**

A. Silva[1], R. Palmer[1], S. Andreazza[1], E. Christoforou[1], J. Mordaka[1], I. Turner[1], C. Xyrafaki[2], M. Stolarek-Januszkiewicz[1], K. von Bargen[1], C. Ho[1], N. Potts[1], E. Gray[1], W. Levin[1], T. Kelley[1], R. Osborne[1], B. Balmforth[1]
[1]Biofidelity Ltd., Cambridge, United Kingdom; [2]Biofidelity Ltd., North Salt Lake, UT.

Introduction: Allele-specific pyrophosphorolysis reaction (ASPYRE) is a novel method relying on enzymatic degradation of probes hybridized with perfect complementarity to target DNA strands. ASPYRE is fast, simple, and inexpensive and can be performed in a platform-agnostic fashion using standard real-time PCR instruments. Using this technology we created ASPYRE-Lung, a targeted panel of 114 actionable genomic variants across 11 genes with simultaneous DNA and RNA workflows. The assay covers the NCCN guideline recommended biomarkers for patients with non-small-cell lung cancer (NSCLC), informing timely treatment decisions in up to 80% of patients. **Methods:** A set of contrived samples was generated for each of the 114 DNA variants or RNA fusions targeted by the assay. Target variant allele fractions were 0.1%, 0.2%, 0.5%, 1.0%, and 5.0% for DNA variants and six or 30 copies for RNA fusions. To mimic liquid biopsy samples, we used a background of genomic DNA (gDNA) from peripheral blood leukocytes or cell-free RNA (cfRNA) derived from healthy individuals (n=1,223). For tissue specimens, samples had a background of gDNA or RNA derived from non-neoplastic formalin-fixed, paraffin-embedded (FFPE) lung tissue (n=1,106). Nine independent operators generated three independent datasets from all samples. Data were analyzed using a support-vector machine (SVM) classifier. To evaluate assay performance we used a cross-validation approach, a resampling method that uses different portions of the data to test and train a model on different iterations. Variants were interpreted as detected or not-detected by the SVM. **Results:** The SVM classifier made a total of 121,718 distinct variant calls. Across all samples, the positive predictive value was 0.997 and the negative predictive value was 0.999. Assay-wide specificity was 99.5% for the set of FFPE-derived DNA and RNA samples and 99.7% for non-FFPE-derived DNA and RNA samples. The typical turnaround time from extracted nucleic acids to result was approximately six hours. Throughput was highly scalable and we routinely processed up to 16 samples per run. **Conclusions:** We describe validation of a novel method for detecting a panel of clinically actionable NSCLC-associated mutations and RNA fusions from FFPE tissue or plasma. The simple workflow includes four steps that involve only reagent transfer and incubation. The assay is robust and precise with performance characteristics approaching that of next-generation sequencing, but with faster turnaround times, lower complexity workflows, simple bioinformatic analysis, reduced cost, and with no variant of uncertain significance-type variants to interpret.

ST013. **Validation of Idylla *EGFR* Assay for Rapid Detection of *EGFR* Mutations in Patients' FFPE Tissues**

E. Ryzhova[1], K. Avila[1], E. Maldonado[2], S. Shah[1], S. Gandhi[1], T. Boyle[1], D. Qin[1]
[1]Moffitt Cancer Center and Research Institute, Tampa, FL; [2]Biocartis US Inc., Jersey City, NJ.

Introduction: Mutations in the epidermal growth factor receptor (*EGFR*) are common alterations in lung adenocarcinoma. The spectrum of actionable *EGFR* mutations consists of both activating and resistance variants that may either promote or preclude responses to targeted therapy. Current guidelines recommend prompt *EGFR* genotyping of patients with advanced disease to facilitate selection of optimal initial treatment. Here we report the validation of the Idylla *EGFR* Mutation Assay for a rapid testing of the Moffitt Cancer Center patients. **Methods:** Idylla *EGFR* Mutation Assay (Biocartis) interrogates 53 mutations in exon 18-exon 21 of *EGFR* gene. *EGFR* genotyping was performed using Idylla System that provides fully automated testing including formalin-fixed, paraffin-embedded (FFPE) sample preparation, real-time PCR amplification, variant detection and reporting. A single-sample test was completed in 90 min, ensuring turnaround time within one to three days depending on sample volume. Accuracy, positive percentage agreement (PPA), negative percentage agreement (NPA), limit of detection (LOD), and precision were assessed using two multiplexed FFPE reference materials and 35 FFPE patient samples encompassing 38 mutations. **Results:** Analytical accuracy of the assay was established based on the cumulative results of reference and patient samples' testing. Discordant result was observed in a sample with low tumor content

(10%), in which T790M mutation had been identified by next-generation sequencing panel but was not called by Idylla assay. Of note, the reduced sensitivity of the assay for T790M variant in samples with tumor content ≤10% has also been documented in the literature. The *EGFR* mutation status was determined correctly for the rest of the cohort. The resultant accuracy, PPA, and NPA of the assay were 97.2%, 97.4%, and 100%, respectively. As reported in the literature, the LOD of the Idylla *EGFR* assay is 5%. To investigate if our LDT attains the same sensitivity, we used the *EGFR* Gene Multiplex Reference Standard 5%, Horizon Discovery. The standard has been repeatedly run throughout a 3-month period using three reagent lots and two instruments. All variants were invariably detected in all runs, thus confirming that we can accurately identify targeted *EGFR* mutations at 5% variant allele fraction. These data were also used to examine assay precision. We demonstrated that the test returned the same results regardless of performing technologists, instruments, reagent lots or days of testing. **Conclusions:** Our validation study affirms that the Idylla *EGFR* mutation assay enables streamline, rapid, accurate and reproducible *EGFR* genotyping of FFPE samples with tumor cellularity above 10%.

ST014. **Evaluation of OncoReveal Dx Lung and Colon Cancer Assay (oRDx-LCCA) for Tumor Profiling Using Multiple Tumor Types**

N. Lodato, N. Taqiya, Z. Liu, J. Aldersley, J. Lim, Y. Ke, S. Polvino, I. Flockhart, M. Urbin, Z. Wang
Pillar Biosciences, Natick, MA.

Introduction: OncoReveal Dx Lung and Colon Cancer Assay (oRDx-LCCA) is an FDA-approved next-generation sequencing (NGS)-based companion diagnostic test for the detection of *KRAS* G12/G13 variants in colorectal cancer (CRC) patients as well as *EGFR* L858R and exon 19 deletions in non-small cell lung cancer (NSCLC) patients. However, oRDx-LCCA can detect >3,600 DNA variants across 22 total genes, including additional sites within *KRAS* and *EGFR*, that are important for tumor profiling applications and are frequently mutated in CRC, NSCLC, and other tumor types. Here we report the results of evaluation experiments using CRC and NSCLC tissues and eight additional tumor types, including bladder, breast, endometrial, liver, melanoma, pancreatic, renal, and thyroid cancers using oRDx-LCCA. **Methods:** Clinical tumor tissues positive or negative for mutations covered in oRDx-LCCA were used for evaluation purposes. DNA input was tested at 30 ng across eight tumor types and the results were compared to CRC and NSCLC tissues tested at comparable input levels. Likely potential endogenous (melanin, triglycerides, and hemoglobin) and exogenous substances (residual extraction kit components) that may interfere with PCR were tested at two relevant concentrations. Assay accuracy was assessed by comparing 3,663 potential CDx and non-CDx variants detected by oRDx-LCCA to two independent, externally validated, comparator methods (CompC and CompO). **Results:** Non-CRC, non-NSCLC samples tested at 30 ng of input produced valid results in 100% of libraries tested (94/94) and had a positive percent agreement (PPA) >95%, which is comparable to CRC and NSCLC samples. PPA analysis of libraries prepared in the presence of PCR interfering substances was 100% for three endogenous derived substances tested at two levels each and 100% for six exogenous derived substances. In the accuracy analysis, PPA and NPA comparing oRDx-LCCA to either CompC or CompO were both >95%. **Conclusions:** oRDx-LCCA is a highly accurate NGS-based assay for the detection >3,600 mutations across 22 genes in multiple tumor types.

ST015. **Updated Interim Results of the Clinical Performance Evaluation of a Novel Deep Learning Solution for Homologous Recombination Deficiency Detection**

A. Buisson[1], P. Saintigny[2], A. Harle[3], D. Vacirca[4], M. Barberis[4], P. Gilson[3], C. Roma[5], F. Bergantino[5], C. Grimm[6], L. Müllauer[6], P. Harter[7], S. Pignata[8], A. Gonzalez-Martin[9], C. Schauer[10], K. Fujiwara[11], I. Vergote[12], N. Colombo[4], E. Pujade-Lauraine[13], I. Treilleux[14], I. Ray-Coquard[15]
[1]Centre Léon Bérard, Lyon, France; [2]Centre Léon Bérard and University Claude Bernard Lyon I, Lyon, France; [3]Institut de Cancérologie de Lorraine, Nancy, France; [4]European Institute of Oncology, Milan, Italy; [5]Istituto Nazionale Tumori-IRCCS-Fondazione G. Pascale, Naples, Italy; [6]Medical University of Vienna, Austria; [7]Kliniken Essen Mitte, Essen, Essen, Germany; [8]Istituto Nazionale Tumori IRCCS Fondazione G. Pascale, and Multicenter Italian Trials in Ovarian Cancer and Gynecologic Malignancies (MITO), Naples, Italy; [9]Clínica Universidad de Navarra, Madrid and GEICO, Madrid, Spain; [10]Hospital Barmherzige Brüder Graz, Graz, Austria; [11]Saitama Medical University International Medical Center, Saitama, Japan; [12]University Hospital Leuven, Leuven Cancer Institute, Leuven, Belgium; [13]Association de Recherche Cancers Gynécologiques (ARCAGY), Paris, France; [14]Centre de Recherche en Cancérologie de Lyon, Centre Léon Bérard, Université de Lyon, Lyon, France; [15]Centre Léon BERARD, and University Claude Bernard Lyon I, Lyon and Groupe d'Investigateurs Nationaux pour l'Etude des Cancers Ovariens (GINECO), Lyon, France.

Introduction: Comprehensive identification of ovarian cancer (OvCa) patients that can benefit from poly (ADP-ribose) polymerase inhibitor treatment is currently based on genome-wide enumeration of known homologous recombination deficiency (HRD) biomarkers and requires deep sequence profiles (>30x). The cost and challenges of implementing available solutions currently hinders clinical adoption of HRD testing. Here we present updated interim results for the ongoing multicenter evaluation study of SOPHiA DDM Dx HRD Solution (SOPHiA GENETICS, SA), a novel deep learning-based approach that leverages the impact of HRD on the coverage profiles from low-pass whole-genome sequencing (lpWGS, 1x) data. **Methods:** In this multicenter study, we processed and analyzed, according to manufacturer's instructions, DNA from 328 formalin-fixed, paraffin-embedded OvCa samples. We assessed the concordance between SOPHiA DDM Dx HRD Solution and Myriad myChoice CDx results. For the subset of our cohort (206 samples) included in the PAOLA-1 clinical trial (Ray-coquard, *et al.,* 2019), we carried out survival analysis to investigate differences in progression-free survival (PFS) in the olaparib and placebo arms of the study between patients, with HRD-positive or HRD-negative test. **Results:** Similar to the earlier interim report, we observed high overall percentage agreement, 90.43% (95% confidence interval [CI], 86.59-93.25), between SOPHiA DDM Dx HRD Solution and Myriad myChoice CDx status. The median PFS time for patients with HRD-positive tumors was 20.8 months higher in the olaparib arm (hazard ratio [HR], 0.44; 95% CI, 0.26-0.76, p=0.003), confirming the findings of our first interim analysis. No significant difference in PFS was observed between treatment arms in patients without HRD-positive tumors (HR, 0.92; 95% CI, 0.59-1.43; p=0.69). The effect of the interaction between olaparib and HRD status on PFS was similar for the two stratification methods (p=0.20). **Conclusions:** The interim results of SOPHiA DDM Dx HRD Solution evaluation study continue to support the value of lpWGS data for patient stratification, making it a reliable option for HRD testing in the clinical setting.

ST016. **Performance of MSI Testing by the Automated Rapid Idylla Assay in Comparison with the Promega Assay in MMR-Deficient Endometrial Carcinomas**

M. Smithgall[1], L. Cong[2], L. Serrano[2], E. Zhong[2], P. Velu[3], J. Solomon[3], H. Rennert[3], A. Yemelyanova[3]
[1]Columbia University Irving Medical Center, New York, NY; [2]New York Presbyterian, New York, NY; [3]Weill Cornell Medicine, New York, NY.

Introduction: Identification of microsatellite instability-high (MSI-H)/mismatch repair protein deficient (dMMR) endometrial cancer (EC) has become routine practice to screen for Lynch syndrome and for identification of cases that could benefit from immunotherapy. Though immunohistochemistry (IHC) is generally deemed an acceptable screening tool for detection of dMMR cases, MSI testing by polymerase chain reaction (PCR) is used as well. It has been acknowledged that MSI testing in endometrial cancer poses interpretative challenges compared to other tumor sites, and some studies suggest that PCR-based methods are less sensitive than IHC in detecting dMMR/MSI-H EC. The automated rapid Idylla (Biocartis) assay is being increasingly used for detection of MSI-H cancers. The goal of this study was to evaluate the performance of the Idylla MSI assay in dMMR endometrial cancers classified by IHC. **Methods:** Thirteen cases of dMMR endometrial cancer were tested. These included five cases of Lynch syndrome (1-*MSH2*, 1-*MLH1,* and three with *MSH6* mutations) and eight cases with MLH1/PMS2 IHC loss and confirmed *MLH1* promoter methylation (*mMLH1*). The neoplastic cellularity ranged from 30% to 90%. The MSI testing was performed using the Idylla MSI assay (Biocartis) according to the manufacturer's instructions. The tissue input met the 50-600 mm^2 requirement. Additionally, the Promega MSI Analysis System was used for comparison. Two ng of DNA extracted from tumor and paired normal tissue were amplified by PCR using fluorescent multiplex primers and subjected to fragment analysis using the ABI PRISM 3130xl Genetic Analyzer. Cases analyzed using GeneMapper Software (v4.0) were considered MSI-H, if instability was detected in two or more loci. **Results:** Of the 13 dMMR cases, Idylla detected five as MSI-H (38.5%), which included three Lynch syndrome and two *mMLH1* cases. The Promega assay confirmed the findings in the five Idylla-MSI-H cases and yielded MSI-H results in an additional five cases, all *mMLH1*. Three cases resulted MSS (microsatellite stable) by both Idylla and Promega assays. Repeating the Idylla assay in three discordant cases (Idylla-/Promega+) with extracted DNA (50ng input) resulted in MSI-H calls in two of three cases. **Conclusions:** The findings highlight challenges in MSI detection in endometrial cancer using PCR-based methods. The imperfect correlation between IHC for MMR and molecular MSI testing has been acknowledged. The automated rapid Idylla MSI assay yields negative results in more than 60% of dMMR endometrial cancer cases when manufacturer's instructions are followed. Rigorous validation, including, potentially, adjustment of tissue/DNA input should be considered before expanding rapid MSI testing to cancer types other than colorectal.

ST017. **Multifaceted Research Application of Comprehensive Genomic Profiling for Detection of Cancer Variants, Gene Fusions, and Complex Oncology Endpoints**

D. Cyanam[1], G. Lowman[2], E. Norris[1], J. Kilzer[2], M. Toro[2], J. Guo[3], P. Bernardo[3], C. Nemes[4], T. Puranik[3], Y. Zhu[3], A. Phan[2], D. Wong[3], M. Gupta[3], Y. Jin[3], S. El-Difrawy[3], Y. Tseng[3], L. Pickle[2], A. Marcovitz[3], V. Mittal[1], S. Myrand[1], S. Bandla[1], P. Williams[1], E. Ingerman[3], E. Wong-Ho[3], S. Sadis[1]
[1]Thermo Fisher Scientific, Ann Arbor, MI; [2]Thermo Fisher Scientific, Carlsbad, CA; [3]Thermo Fisher Scientific, South San Francisco, CA; [4]Thermo Fisher Scientific, Guilford, CT.

Introduction: The Ion Torrent Genexus System has redefined the genomic profiling paradigm as the first fully integrated next-generation sequencing research platform to provide an automated sample-to-report workflow with results in a single day. Coupled with the Genexus Purification System, 15 minutes of hands-on time and just two touch points, the Genexus System enables a convenient solution for oncology research. Here we highlight the high-throughput oncology research capabilities of the Genexus System with Oncomine Comprehensive Assay Plus (OCA Plus), a 500+ gene targeted AmpliSeq-based oncology research panel that can detect variants, fusions, and evaluate key oncology research endpoints. We demonstrate the ability of OCA Plus on Genexus to evaluate tumor mutational burden (TMB), microsatellite instability (MSI), and homologous recombination repair deficiency (HRD). **Methods:** The high-throughput capabilities of the Genexus System enable it to support large oncology research panels such as OCA Plus, which comprises more than 13,000 amplicons. In addition, the AmpliSeq technology enables low sample input requirement of just 20 ng of DNA and RNA. Hence, the extensive per-sample coverage and low-sample input allow for comprehensive DNA and RNA genomic profiling of relevant cancer biomarkers in more than 500 genes including detection of more than 1,300 fusion isoforms. We utilized high-molecular weight and formalin-fixed, paraffin-embedded (FFPE) samples, reference controls, and orthogonally tested research samples to evaluate DNA variant calling, RNA fusion calling, and key oncology research endpoints, including MSI, TMB, and HRD. **Results:** Commercially sourced reference controls and FFPE research samples were sequenced using OCA Plus on the Genexus System. Sequencing metrics showed ≥24 million reads per sample, with four DNA and RNA samples supported per run. Variant calling performance was assessed using the AcroMetrix Oncology Hotspot Control, which has 377 variants covered by OCA Plus and showed sensitivity and PPV >95%. MSI status was assessed using known MSI-High and microsatellite stable FFPE samples of interest and results showed high concordance with orthogonal truth. The TMB endpoint was tested using Seraseq TMB Mix controls and showed a correlation of r^2 >0.90. For the RNA fusions, the Seraseq RNA v4 reference showed 100% positive correlation. **Conclusions:** The increased throughput of the Genexus System combined with minimal touchpoints and a rapid turnaround time enables comprehensive genomic profiling for research assays such as OCA Plus where an increased number of sequencing reads leads to greater sensitivity for detecting rare variants and low-level fusion transcripts. Further, accurate characterization of key oncology research endpoints, such as TMB, MSI, and HRD, allow the Genexus System to accelerate research in the field of oncology.

ST018. **Evaluation of the Stilla *EGFR* 6-Color Crystal Digital PCR Kit for Use with FFPE DNA on the naica System**

E. Hughes, R. Barney, G. Tsongalis
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Digital PCR (dPCR) is an established tool used for the precise and highly sensitive quantification of nucleic acids, detection of copy number variations (CNVs) and mutations, especially at extremely low variant allele frequencies (VAFs). However, most well-established platforms provide limited experimental design with only two-color detection systems. The naica system gives researchers the ability to read up to six fluorescent signatures in one well, providing better sensitivity and increased low-level detection of multiple reactions in parallel. The EGFR 6-color Crystal Digital PCR kit is intended for the detection and quantification of 32 somatic *EGFR* mutations in exons 18, 19, 20, and 21 using the Sapphire chips on the naica system. We explored the potential for using the 6-color naica dPCR system for use with formalin-fixed, paraffin-embedded (FFPE) DNA by evaluating the performance of this *EGFR* kit with previously run clinical samples with known mutations. **Methods:** Nine previously run clinical samples and two FFPE cell line controls with known *EGFR* mutations and VAFs, detected by next-generation sequencing (NGS), were prepared at 40ng and 10ng input according to manufacturer's instructions. *EGFR* mutations included L858R, E746_A750del, T790M, and G719A/C/S. Samples were loaded into Sapphire chips and PCR was performed on-chip inside the Geode instrument. Chips were transferred to the Prism6. Analysis of droplets was performed using the Prism6 and Crystal Reader software. Data analysis was performed with the Crystal Miner software. **Results:** There was 100% concordance between

dPCR and NGS regarding the presence or absence of *EGFR* mutations in all samples. Interestingly, the optimal input was 10 ng, with a Pearson coefficient for NGS and dPCR concordance of 0.997 (p-value <.0001). VAF for dPCR was similar to that ascertained with NGS with an average difference between NGS and dPCR of -0.9% (SD 3.3%; range -4.8%-5.8%). At 40 ng, the advertised maximum input concentration for this assay, some deterioration in the assay performance was detected in comparison to the 10ng counterparts. **Conclusions:** In this study, Stilla dPCR was effective at detecting and quantifying the *EGFR* variants in solid tumor samples compared to NGS. As a rapid, inexpensive technology that has been widely praised for its improved sensitivity compared to traditional molecular technologies, six-color dPCR could be an important tool for interrogating multiple mutations in a single well, a clear advantage over two-color detection systems. Furthermore, the Stilla dPCR technology appears compatible with analysis of FFPE DNA, thus opening exciting possibilities for further studies into assessing other abnormalities such as CNV analysis in samples with extremely low input concentrations.

ST019. **BioCartis Idylla Testing for *EGFR* Mutations in Lung Adenocarcinoma Reveals Low Clinical Sensitivity for Detecting Exon 20 Insertion Alterations**

E. Zhong1, P. Velu[2], L. Cong[2], L. Serrano[2], J. Solomon[2], H. Rennert[2]
[1]New York Presbyterian Weill Cornell Medicine, Little Neck, NY; [2]Weill Cornell New York Presbyterian, New York, NY.

Introduction: The Idylla EGFR Mutation Assay, performed on the Biocartis Idylla System, is a qualitative molecular assay that simultaneously detects 51 single nucleotide variants (SNVs), deletions and insertions in the *EGFR* gene, including mutations in exon 18 (G719A/S/C), exon 21 (L858R, L861Q), exon 20 (T790M, S768I), exon 19 deletions, and five exon 20 insertions. The platform uses allele-specific qPCR for rapid detection of *EGFR* mutations from formalin-fixed, paraffin-embedded tissue samples, from placement in cartridge to result. Our objective was to review the rapid *EGFR* results generated by the Idylla system and compare those to the results obtained by targeted next-generation sequencing (NGS) testing using the Thermo Fisher Oncomine panel to identify any discrepancy. **Methods:** Between 2/2021 and 6/2022, we analyzed 62 specimens from 61 patients with a diagnosis of lung cancer. Tumor percentage ranged from 5% to 90%. Tissue tested was lung in 47 cases (76%), lymph node in six (10%), and a metastatic site (pleura, liver, soft tissue, adrenal, brain) in nine (14%) cases. One to five unstained sections were scraped off the glass slide and run on the Idylla EGFR 2.0 cartridges. Assay results were assessed by the Idylla Explore system and the qPCR curves were manually evaluated as needed. NGS testing was performed using the Oncomine Cancer Gene Mutation Panel v2 assay for detection of genetic mutations from DNA and RNA of 143 cancer-related genes. **Results:** Among the 62 specimens analyzed, 13 (20%) tested positive and 46 (74%) tested negative. Three samples (5%) were inconclusive due to inadequate DNA input. Of the positive samples, 12 (92%) were confirmed by NGS; one was quantity/quality not sufficient for sequencing. Seven had L858R (one of which also had T790M) and six had exon 19 deletion. No exon 20 insertions were detected. Of the negative samples, 38 (83%) were confirmed negative by NGS; four were not sequenced. The remaining four (8.7%) of the 46 Idylla-negative samples all had exon 20 insertion by NGS. The four discrepant mutations were: c.2252_2277delCATCTCCGAAAGCCAACAAGGAAATCinsAT, c.2314_2319dupCCCCAC, c.2317_2319dupCAC, and c.2304_2305insATGGACAGC. Tumor percentage of the discrepant samples ranged from 20% to 40%. Three were primary lung samples and one was a liver metastasis. None of these mutations are included in the current Idylla EGFR mutation assay. **Conclusions:** The Idylla EGFR assay demonstrated high specificity and sensitivity for identifying exon 18 and 21 SNVs, and exon 19 deletions. However, the platform has not detected any of the exon 20 insertions seen in our cohort, leading to erroneous results and potentially compromising patient care if this is the only platform used. These insertion mutations are not included in the Idylla EGFR assay design, which is a significant limitation of this assay.

ST020. **Analytical Validation of Thyroid GuidePx: An RNA-Seq-Based Gene Expression Classifier for Papillary Thyroid Cancer**

A. Hernandez[1], C. Stretch[2], C. Wilson[2], D. Heiney[2], K. Powell[2], E. White[4], G. Tripathi[5], F. Khan[5], M. Khalili[6], O. Bathe[7], A. Magliocco[2]
[1]Protean BioDiagnostics, Saint Cloud, FL; [2]Protean Biodiagnostics, Orlando, FL; [3]Qualisure Diagnostics, Calgary, Alberta; [4]Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL; [5]Department of Pathology and Laboratory Medicine, University of Calgary, Calgary, Canada/OncoHelix, Alberta, Calgary, Canada; [6]Department of Pathology and Laboratory Medicine, University of Calgary, Canada; [7]Department of Surgery and Oncology, University of Calgary, Calgary, Canada.

Introduction: Decisions related to clinical management of papillary thyroid cancer (PTC) require an accurate estimate of recurrence risk. Previously, using a machine learning approach on data from The Cancer Genome Atlas (TCGA), we identified 82 genes most closely associated with recurrence. Three molecular subtypes were apparent based on their patterns of expression, including a high-risk subtype. A targeted assay based on RNA sequencing (Thyroid GuidePx) was designed and tested on formalin-fixed, paraffin-embedded (FFPE) surgical samples. **Methods:** A targeted RNA sequencing assay using the on-bead tagmentation workflow was used to quantify the 82 prognostic genes, as well as 10 internal controls. Positive controls consisted of Ambion ERCC RNA spike-in controls, and water negative controls that were included in every batch. Archival FFPE samples from unifocal PTCs (N=68, from 2010 to 2012) were tested in replicate to determine gene-level correlation and classification concordance. **Results:** In comparison to frozen samples, there was instability in five genes in FFPE samples. A two-step classification algorithm based on random forest that was specific for FFPE samples was constructed, then back-tested on 331 cases from TCGA. Tenfold cross-validation accuracy of the first and second step were 92% and 86%, respectively. There was similar gene representation in samples with DV200 values greater than 36.5 and DV200 values ranging from 20 to 36.5; assay performance declined with lower quality RNA. There was excellent gene-level correlation with intra-batch (N=5), inter-batch (N=27), and inter-lab (N=37) replicates (R >0.94). The classification concordance was 92%. **Conclusions:** Thyroid GuidePx was capable of yielding repeatable gene expression measures. A classification algorithm customized for FFPE enabled reliable and repeatable classification of molecular subtypes.

ST021. **NYSDOH Initial Validation of the Oncomine Precision Assay for Use in Clinical Studies**

M. Aguilar, P. Lao, A. Yuki, J. Chitwood, F. Luo, H. Maydinie, J. Black, K. McCall, S. Salazar, D. Ginzinger, R. Ramsamooj
Thermo Fisher Scientific, West Sacramento, CA.

Introduction: The Oncomine Precision Assay with the Genexus Integrated Sequencer (OPA GX) is a pan-cancer research next-generation sequencing panel designed for detecting cancer driver variants across 50 genes. These variant types include single nucleotide variants (SNV), insertions and/or deletions (indels), copy number variants (CNV), and gene fusions. The Genexus platform was chosen due to its ability to produce results with a quick turnaround time, enabling faster patient enrollment for appropriate targeted therapies in clinical trial settings. This initial validation was performed taking into consideration elements of New York State Department of Health (NYSDOH) guidelines, demonstrating its effectiveness to detect somatic variants in formalin-fixed, paraffin-embedded (FFPE) tumor samples. **Methods:** FFPE samples (n=93) and cell lines (n=5) originating from seven tumor types were tested. Libraries were prepared utilizing 13.4 ng of nucleic acid input, which were templated and sequenced on the Genexus system following manufacturer's instructions. Reads were aligned to genome assembly GRCh37 and

variant calls were made using the Ion Torrent Genexus Software (version 6.2). Limit of detection (LOD) for SNV (*KRAS*) and indel (*EGFR*) was determined by testing two characterized cell lines diluted to five minor allele frequencies (MAF) between 10% and 2.5% with normal genomic DNA. FFPE samples (49 SNV, 10 indel, and 30 wild-type) were sequenced and then orthogonally confirmed via Sanger or Oncomine Focus Assay (OFA) sequencing. Three commercial reference cell lines were sequenced in quadruplicate to calculate analytical accuracy. FFPE samples with known variants were used to measure reproducibility (four replicates by two operators on two different sequencers) and repeatability (three replicates by one operator within 24 hours). **Results:** The LOD for SNV and indel was determined to be 4% MAF with >99% sensitivity. Accuracy was >99% for SNV and indel, meeting the acceptance criteria of >98%. Precision and reproducibility were >99%, exceeding the >95% reproducibility requirement set by NYSDOH. Orthogonally confirmed samples previously characterized by Sanger or OFA (with SNV, indel or wild-type) were 100% concordant when sequenced with OPA GX. **Conclusions:** This initial validation shows targeted sequencing with the Oncomine Precision Assay panel exceeds the analytical accuracy and sensitivity, orthogonal confirmation, precision, and reproducibility performance criteria set by NYSDOH guidelines in a variety of FFPE-derived tumor tissue using only 13.4 ng of input. These results, in conjunction with the Genexus platform's rapid turnaround time, demonstrate the potential suitability for Oncomine Precision Assay to be used in clinical trial settings.

ST022. **A Custom NGS Panel for NSCLC: Making NGS Available and Affordable in India**

D. Kumar, P. Manoj, A. Mehta, S. Nathany, M. Sakshi, D. Surender
Rajiv Gandhi Cancer Institute and Research Center, Delhi, India.

Introduction: Lung cancer, once considered a dismal disease, has witnessed a therapeutic and prognostic paradigm in the era of precision medicine. With newer small molecules gaining approval for first-line treatment, unmet needs, especially in the Indian subcontinent, are availability and affordability of panel-based next-generation sequencing testing. With more than one-third of our patients belonging to lower socioeconomic strata, development of a panel which is both affordable and accurate is warranted. **Methods:** A targeted tumor-specific 16 genes (*AKT1, ALK, BRAF, ERBB2, EGFR, KRAS, MET, NRAS, NTRK1, PIK3CA, PTEN, RB1, ROS1, STK11, TP53, KEAP1*) DNA custom panel using AmpliSeq Designer after data curation from portals like NCBI, ClinVar, COSMIC, cBioportal, UCSC, and TCGA was designed, interrogating for single nucleotide variants and indels. This assay was standardized and optimized, and subsequently validated as per recommended AMP guidelines (Jennings, *et al.,* 2017) before clinical using twenty pre-diagnosed (using Oncomine Focus Assay) samples on Ion Torrent S5 Gene studio platform. For LOD (limit of detection) Seraseq Tri-Level Tumor Mutation DNA Mix v2 HC control DNA was used. Analysis workflow was designed on AmpliSeq Reporter using designed BED files provided by AmpliSeq designer. Analytical accuracy, sensitivity, and specificity, positive predictive agreement (PPA), as well as turnaround time and costs, were assessed. **Results:** The panel was run on 20 previously diagnosed non-small cell lung cancer (NSCLC) cases using formalin-fixed, paraffin-embedded samples. The concordance rate with previously used Oncomine Focus Assay was 97%, with two cases being positive for DNA alterations in *MET* gene (splice site) which were not detected in the Focus Assay, attributed to the assay design. Serial dilution of a known *EGFR* mutant (del19: VAF 50%) was done along with the Seraseq DNA Mix (*EGFR* del19 (known VAF 4%). Serial dilution validated the LOD at 5% VAF for *EGFR* del19 mutation at a median coverage of 500x and a median depth of 3,000x. We could not run 59 samples as recommended owing to limited resources. Other mutations diluted include *ERBBB2, BRAF V600E*, and *KRAS* G12C. The *ERBB2* exon 20 was additionally spiked owing to limited coverage and tiling. The overall sensitivity was 91%, specificity was 90%, and PPA with Oncomine Focus Assay was 89%. The median turnaround time for library preparation and report was ~10 days, and the cost when compared to the already-in-use commercial panel was 48% less. **Conclusions:** This panel designed specifically for NSCLC can be implemented for clinical use based on the performance characteristics. It carries the advantages of not only cost effectiveness, simple design, and workflow, but also has the potential to be spiked with evolving biomarkers of interest.

ST023. **Clinical Validation of an RNA-Based Next-Generation Sequencing Assay for Solid Tumors: Detection of Fusions, Oncogenic Splicing Events and Expressed Hotspot SNVs with RNA for Tissue Conservation**

M. Awobajo[1], M. David[1], H. Fan[1], W. He[1], D. Montes[2], K. Vadlamudi[1]
[1]UT Health San Antonio, San Antonio, TX; [2]Agilent, Austin, TX.

Introduction: Single nucleotide variants (SNVs) are usually identified by high-throughput sequencing of DNA for SNVs, whereas RNA sequencing yields the highest sensitivity for detecting fusions and oncogenic splicing events. However, performing two separate sequencing assays requires additional technologist time, reagents, and tissue, which is not always feasible. Of particular concern are lung core needle biopsies, where repeat biopsy due to insufficient tissue may have significant patient morbidity. The Archer Fusion-Plex Solid Tumor OncoPanel assay, a custom designed next-generation sequencing (NGS) Anchored Multiplex PCR (AMP) assay, can detect gene fusions, oncogenic splicing events, and expressed hotspot SNVs in one assay, maximizing tissue conservation. **Methods:** We validated the OncoPanel NGS assay to detect fusions (101 genes) in a partner-agnostic fashion, oncogenic splicing events (three genes), and expressed SNVs from hotspot regions (39 genes). Total nucleic acid or RNA was extracted from 62 unique samples using the Maxwell RSC RNA FFPE Kit (Promega), ReliaPrep FFPE Total RNA Miniprep Kit (Promega) or ReliaPrep RNA Tissue Miniprep System (Promega). Samples included formalin-fixed, paraffin-embedded (FFPE), cytology FFPE cell blocks, core needle biopsy FFPE, frozen tissue, and samples from cytogenetics. Sequencing was performed on the Illumina MiSeq. Bioinformatics was performed with vendor-supplied Archer Analysis bioinformatics software v6.0. **Results:** A subset of validation samples that were also tested with a highly reliable orthogonal method (NGS, Sanger or Pyrosequencing, fluorescence *in situ* hybridization) demonstrated a fusion and oncogenic splicing event detection sensitivity of 100% (33/33 events), and a hotspot SNV and small indel sensitivity of 96.4% (27/28 events) after identifying one hotspot region of *PIK3CA* with poor coverage which will be excluded from the panel. A subset of validation samples with orthogonal whole-exome sequencing demonstrated a fusion event detection specificity of 99% (400/404 events) and a hotspot SNV specificity of 100% (150/150 events). Different technologists repeating the same sample showed 100% concordance (4/4 samples). The same technologist repeating samples on the same run (2/2 samples) or a different run (3/3) showed 100% concordance. Serial dilutions into wild-type RNA were performed for two samples, demonstrating a lower limit of detection of 5% tumor content. Variants were detected with a minimum of 20ng input nucleic acid in studies with 20ng, 100ng, or 200ng input (4/4 samples). **Conclusions:** We successfully validated the OncoPanel NGS assay to simultaneously detect fusions, oncogenic splicing events, and expressed hotspot SNVs with as little as 20 ng of nucleic acid. This is advantageous for cases with limited input, such as lung core needle biopsies.

ST024. **A Novel Algorithm SvG Facilitates Tumor Mutation Burden (TMB) Evaluation from Tumor-Only Highly Consistent with Paired Tumor-Normal Samples**

C. Liu[1], Z. Zhang[2], Y. Han[2]
[1]Burning Rock Biotech, Shanghai, People's Republic of China, [2]Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, People's Republic of China.

Introduction: Tumor mutation burden (TMB) is an important biomarker associated with immunotherapy, which is calculated by the number of non-synonymous mutations and indels per megabase (Mb). Matched normal samples are often required to filter out germline variants while calculating TMB. However, unreachability of matched normal sample in some cases hampers the clinical application of TMB. Hence, computational algorithms to distinguish somatic from germline mutations are required for TMB evaluation in tumor-only sequencing. **Methods:** We developed a computational algorithm named SvG to predict the origin of mutation based on tumor-only sequencing, and incorporated it into tumor-only workflow for TMB evaluation. The algorithm constructs a comprehensive analysis to filter germline variants from somatic variants based on four modules including 1) population frequency at least 1% using population databases, 2) copy number of the mutation and sample purity, 3) allele frequency of neighboring mutations within 1 Mb around the mutation, and 4) a subclonal model assuming the mutation occurs after copy number variation. A concordance study of tumor-only and tumor-normal workflow for TMB evaluation is performed using a 520-gene panel called OncoScreen Plus and a dataset comprising 4,670 samples from more than 20 tumor types. **Results:** As to all 2,731,778 mutations detected in the dataset, their origin was correctly determined by the SvG algorithm for 99.83% mutations and 95.19% when considering only rare mutations of population frequency less than 0.1%. The accuracy to predict the origin of rare mutations was 58.8% and 84.9% when using only module 3 and module 2, respectively, which showed that the comprehensive analysis of the SvG algorithm improved the prediction performance. TMB values obtained by tumor-only with SvG algorithm and tumor-normal workflow showed very high consistency of adjusted R^2~0.99 using linear regression. More than 62.5% samples obtained exactly the same TMB values, and the differences were less than two for 96.5% samples. **Conclusions:** The computational algorithm SvG can predict variant origin from tumor-only sequencing with high accuracy of 99.83%. This study shows that tumor-only sequencing with the SvG algorithm has comparable performance of TMB evaluation with tumor-normal pipeline (R^2~0.99).

ST025. **Oncomine Precision Assay GX on Genexus Integrated Sequencer to Rescue Quantitative or Quality Insufficient Sample of Non-Small Cell Lung Cancer**

Q. Wei, S. Harada, A. Mackinnon
University of Alabama at Birmingham, Birmingham, Alabama.

Introduction: Molecular testing in non-small cell lung cancer (NSCLC) is part of the standard of care in oncology. However, patients with advanced-stage NSCLC often face limited specimen availability for molecular testing. The goal of this study was to compare the performance of Oncomine Precision Assay GX (OPA) on quantity or quality not sufficient (QNS) samples with our in-house, hybrid-capture next-generation sequencing (NGS) assay. OPA was validated on Ion Torrent Genexus Integrated Sequencer using a "sample-to-result" NGS workflow. OPA detects actionable biomarkers across 50 key genes with rapid turnaround time. Recommended minimum DNA input is 10 ng, which is lower than the minimal input of 100 ng for the hybrid-capture assay. **Methods:** Fourteen QNS cases were selected. DNA was extracted from formalin-fixed, paraffin-embedded NSCLC samples. The quality of DNA was evaluated by Genomic DNA ScreenTape with DNA integrity number (DIN) ranging from 1.9 to 4.5 (our Hybrid Capture NGS assay yields sufficient coverage with DIN ≥4.0). DNA concentrations were too low to be measured by Qubit in five cases. The DNA concentrations ranged from 1.1 to 14 ng/μl in the remaining nine samples and were diluted to the manufacturer's recommended concentration for OPA (0.67 ng/μl). Input DNA volume was 20 μl for all cases. The OPA results were compared to reference methods (i.e., single gene test, send out test, previous patient results, or circulating tumor DNA). **Results:** All OPA samples yielded valid results. OPA detected single-nucleotide variants (SNVs), and copy number variants (CNVs) in 12 of 14 cases, even in the samples with DNA concentrations too low to measure. Six of 11 cases showed a mutation in the *TP53* gene. Twelve of 14 cases with OPA results were compared to other valid assays with an 83% (10 out of 12) concordance. In one discordant case, OPA detected a *KRAS* p.G12A mutation that was not confirmed by Idylla *KRAS* assays, potentially due to sample variation. In the other discordant case, OPA failed to detect an *EGFR* p.E746_A750del mutation, which was detected in resected specimen three years prior. **Conclusions:** In this study, we challenged OPA with QNS samples, and OPA successfully detected SNVs and CNVs with an at least 83% concordance rate, compared to reference methods. We demonstrated that OPA can be a valuable, rapid method for molecular testing of advanced NSCLC cases with scant tissue.

ST026. **Personalized Cancer Monitoring Assay for the Sensitive Detection of MRD in Solid Tumors**

j. Reuther[1], R. Daber[1], C. Cadwell[2], I. Chen[1], A. Chesh[1], M. Emery[1], L. Hartje[2], S. harris[1], M. Hawley[1], L. Johnson[2], V. Johnson[2], M. Barbosa[4], J. Macioszek[2], N. Malani[1], E. Metzger[1], M. Moreau[1], W. O'Callaghan[1], A. O'Connor[2], E. Reckase[4], P. Roberts[4], M. Washburn[2], E. Westheimer[1], Y. Zhang[1], J. Zhao[5]
[1]Invitae, Iselin, NJ; [2]Invitae, Boulder, CO; [3]invitae, Keyport, NJ; [4]Invitae, Golden, CO; [5]Invitae, Dresher, PA.

Introduction: Non-invasive circulating tumor DNA (ctDNA) assays for the detection of minimal residual disease (MRD) are currently not widely used for solid tumors. Emerging evidence indicates ctDNA MRD testing can be clinically important for assessing recurrence risk, therapy response, and early detection of disease relapse. The Personalized Cancer Monitoring (PCM) assay is an MRD assay that utilizes patient-specific tumor mutation signatures to monitor disease progression and treatment efficacy. **Methods:** Paired tumor formalin-fixed, paraffin-embedded tissue, plasma, and whole blood specimens from 1,633 patients with cancer including colorectal, non-small cell lung, melanoma, breast cancer, urothelial cancer, renal cell carcinoma, pancreatic, and head and neck cancers underwent whole-exome sequencing (WES) in a CLIA laboratory to facilitate the identification of a tumor mutation signature featuring 18-50 somatic variants to generate patient-specific panels (PSP). Subsequently, longitudinal ctDNA MRD testing was performed on a total of 4,125 plasma samples using PSPs with next-generation sequencing utilizing anchored multiplex PCR chemistry. Paired tumor-normal WES data were processed using IGT-Capture Pipeline (v2.17.3+), patient-specific panels were designed using PCM-The Gentleman Pipeline (1.7.3-1+), and MRD analysis utilized PCM-Reveal MRD Pipeline (v1.10.0-1+). **Results:** Validation studies demonstrated analytical accuracy, sensitivity, and specificity of 100% for the PCM assay when utilizing 10ng ctDNA input. The limit of detection for the assay ranged from 0.01% to 0.005% allele frequency when utilizing 10 to 60 ng DNA input, respectively. The positive percent agreement for reproducibility and precision was 98.15% and 97.22%. On average, samples passing all quality control (QC) achieved 34.12 million reads (range 10.04-60 million) with an average coverage of 3,055x (range 79-20,752 x). Within the cohort, 94.91% (3,915/4,125) samples passed QC thresholds. Of the failed samples in the cohort, 2.7% (n=112) were due to poor DNA or library quantity/quality, 62 (1.50%) from external sample collection error, 18 (0.44%) from internal processing error, and 18 (0.44%) due to sequencing data quality. Within the samples that passed QC, MRD present was detected in 986/3,915 (25.19%), whereas 2,929/3915 (74.81%) showed MRD absent result. The mean observed allele frequency of targeted variants in the MRD-positive plasma samples was 1.26% (range 0.0097%-35.07%). **Conclusions:** The use of the PCM assay, which featured ctDNA MRD testing

utilizing patient-specific tumor mutation signatures, enabled sensitive detection of MRD in solid tumors with low DNA input. The PCM assay was able to detect the presence of MRD at low allelic fractions, which may enable early detection of MRD and patient relapse.

ST027. **Development of Seraseq FFPE Homologous Recombination Deficiency Reference Materials for NGS Assay Validation**

C. Huang[1], D. Ruminski Lowe[1], R. Whiting[1], Y. Konigshofer[1], M. Butler[1], I. Chivukula[2], R. Garlick[2], B. Anekella[1]
[1]LGC Clinical Diagnostics, Gaithersburg, MD; [2]LGC Clinical Diagnostics, Milford, MA.

Introduction: Homologous recombination deficiency (HRD) arises due to a defect in DNA repair and serves as an important therapeutic biomarker. Next-generation sequencing (NGS) assays that measure HRD status are used to stratify ovarian and breast cancer patients, and determine eligibility for clinical trials and polyADP-ribose polymerase (PARP) inhibitor- and platinum-based therapies. Testing is performed on formalin-fixed, paraffin-embedded (FFPE) biopsied tissue, and despite the challenges of this sample type and complexity of analysis, there are currently no commercially available HRD reference materials that enable standardization among assays. Therefore, such reference materials were developed to aid in developing and validating NGS-based HRD assays and for quality-control monitoring of genomic instability scores (GIS). **Methods:** Tumor cell lines were characterized by sequencing and evaluated in collaboration with several IVD partners. Three breast cancer cell lines (along with their single nucleotide polymorphism (SNP)-matched normal cell lines) were selected based on their GIS. Tumor cells were blended with their SNP-matched normal cells to achieve ~65% tumor content. Biosynthetic DNA containing mutations in homologous recombination repair (HRR) genes (*ATM, BRIP1, RAD51C, RAD51D*) were added to the positive and negative reference materials targeted at >5% variant allele frequency (VAF) and measured by digital PCR (Bio-Rad QX200). FFPE blocks were made and each block was tested for yield per curl using both the Qiagen QIAamp DNA FFPE Tissue Kit and the Maxwell RSC DNA FFPE Kit for extraction, and Qubit dsDNA HS kit for concentration analysis. The DNA quality was assessed using an Agilent gDNA ScreenTape Assay for the TapeStation. **Results:** A breast cancer cell line with a GIS of ~60 was selected for use in the HRD-positive reference material, a second breast cancer cell line with a GIS of ~39 was selected for use in HRD cut-off reference material, and a third breast cancer cell line with a GIS of ~25 was selected as HRD-negative. Representative DNA yields per 10-micron section (determined using the HRD-positive FFPE curls extracted by Qiagen QIAamp method) were 165 ± 28 ng/curl. Digital PCR confirmed the presence of the eight HRR mutations (in four genes) at levels >5% VAF. The HRD status of the samples was evaluated using several IVD and RUO assays using different measurement approaches, confirming the wide applicability of the new reference materials. **Conclusions:** We have developed the Seraseq HRD reference materials to meet the needs of laboratories looking to analyze HRD in cancer patient samples. These reference materials facilitate standardization and quality control in HRD testing by clinical labs for current and new PARP inhibitor treatment stratification in expanded patient populations that may include those with WT *BRCA1/2* genes.

ST028. **Validation of Genomic Profiling by PGDx Plasma Focus to Facilitate Precision Oncology through Cell-Free DNA Testing of Solid Tumors**

E. Verner[1], S. Hastings[3], E. Severson[2], S. Ramkissoon[4], P. Reddy[4], M. Nesline[5], K. Valkenburg[3], A. Greer[3]
[1]Personal Genome Diagnostics, Baltimore, MD; [2]Labcorp, Cary, NC; [3]PGDx, Baltimore, MD; [4]Labcorp, Durham, NC; [5]OmniSeq, Buffalo, NY.

Introduction: Genomic profiling is critical for precision oncology to help guide treatment decisions, and tissue isn't always available for testing. To overcome several challenges of tissue testing, an alternate and complementary method is the assessment of cell-free DNA (cfDNA). The PGDx plasma focus assay is a custom next-generation sequencing cfDNA genomic profiling test intended to identify actionable variants in cancers with FDA-approved targeted therapy indications including non-small cell lung cancer, colorectal cancer, melanoma, breast, esophageal, GEJ, and gastric carcinomas. **Methods:** PGDx plasma focus is a hybrid capture-based assay that uses 25 ng of cfDNA input. It interrogates all coding exons of 33 genes to detect single nucleotide variants (SNVs), insertions and deletions (indels). The assay also reports copy number amplifications (CNAs) in eight genes, translocations in five genes, and microsatellite instability (MSI). Targets were selected for clinical utility and include SNVs/indels in *AKT1, ALK, APC, ARID1A, ATM, BRAF, BRCA1/2, BRIP1, CCND1, CD274, CDH1, CSF1R, EGFR, ERBB2, EZH2, FGFR1/2, HRAS, KIT, KRAS, MET, MYC, NRAS, NTRK1, PDGFRA, PIK3CA, POLD1, POLE, RAF1, RET, ROS1, TP53; CNAs in CCND1, CD274, EGFR, ERBB2, FGFR2, KIT, MET, MYC*; and translocations in *ALK, FGFR2, NTRK1, RET*, and *ROS1*. Validation studies were performed with two reference samples from Genome in a Bottle, 19 cell lines, and 42 clinical samples to assess assay performance against orthogonal cfDNA assays. **Results:** Analytical sensitivity was determined using cell line blends and demonstrated a limit of detection of 0.6%-2.1% variant allele frequency (VAF) for SNVs/indels, 1.3- to 1.4-fold for CNAs, and 0.5%-0.7% VAF for translocations. Analytical specificity was assessed in two reference samples run in duplicate, demonstrating specificity of >99% for SNVs/indels (n=4.9x105), and 100% for amplifications (n=32), translocations (n=20), and MSI (n=4). Reproducibility was determined from sensitivity replicates with an average positive agreement (APA) of 97.5% and an average negative agreement (ANA) of 99.9%. Interim analysis of concordance was performed on data from 42 clinical specimens and 15 cell lines, with further testing underway. APA was 93% for SNVs/indels (135/145), 91% for CNAs (10/11), 100% for translocations (4/4), and 100% for MSI (8/8). ANA was >99% for SNVs/Indels (n=3x106), 99% for CNAs (243/245), 98% (153/156) for translocations, and 96% for MSI (27/28). Finally, across all testing, 97% (122/126) of sample libraries passed quality metrics. **Conclusions:** Taken together, these data demonstrate that PGDx plasma focus is a sensitive, specific, accurate, reproducible, and robust approach for cfDNA genomic profiling to supplement tissue testing and inform precision medicine clinical decision making.

ST029. **Rapid Turnaround Oncomine Precision Assay Enables Global Harmonization for Clinical Trial Testing**

D. Unselt, L. Frady, K. Zhao, F. Torres, J. Sims
Q2 Solutions, Inc., Durham, NC.

Introduction: Genetic variation in neoplasia can take the form of single or multiple nucleotide variants, gene fusions, amplifications, and/or splice variations. This heterogeneity can pose a challenge in comprehensive profiling to guide therapeutic decision making. The Oncomine Precision Assay on the Genexus Integrated Sequencer (OPAGX) is an amplicon-based automated next-generation sequencing (NGS) assay that enables detection of all of these genomic variant classes, spanning 50 genes. The complete workflow, from library prep to final report generation, can be accomplished in less than 30 hours for up to 32 formalin-fixed, paraffin-embedded (FFPE) samples. OPAGX uses AmpliSeq HD technology, utilizing unique molecular tags for sequencing error correction and increased sensitivity of variant detection. The assay, previously characterized at a US-based site, was transferred to and validated by a genomics laboratory in Beijing to enable global harmonization of clinical trial testing. **Methods:** Fully automated processing on Genexus used 10 ng of DNA derived from FFPE tissue or from well-characterized controls. OPA primer pools amplified the targets of interest. AmpliSeq HD chemistry digested the products and added dual molecular barcode adapters via amplification. Purified libraries were normalized, pooled, and sequenced on a GX5 chip, with analysis performed by the Genexus software. A Probit model was utilized to determine the

variant allele frequency (VAF) at which the assay achieves 95% power to call an expected variant, defining the limit of detection (LoD). Other parameters evaluated included analytical sensitivity, analytical specificity, positive predictive value (PPV), accuracy, and precision. Results from the OPAGX assay validation at the Beijing genomic laboratory were compared to those from the US laboratory to guarantee similar performance. **Results:** The estimated LoD for the assay was 4.5% VAF for single nucleotide variants (SNVs) and 3.8% VAF for insertions/deletions, compared to 4.2% VAF and 2.8% VAF, respectively, at the US site. The overall sensitivity for small sequencing variants above the LoD was 97.9%. Below these thresholds, OPAGX maintained sensitivity of 92.6%. At both global sites, above the LoD, the assay demonstrated 99.99% accuracy of base-calling, 99.99% specificity, and >99% PPV. The LoD for fusions was estimated to be 41 fusion-supporting reads, with no false-positive calls at >20 reads. OPAGX demonstrated >90% sensitivity for CNV calling for genes with ≥5 copies. **Conclusions:** OPAGX is a quick-turnaround NGS assay enabling sensitive multivariant genomic profiling to inform therapeutic decision making. Consistent performance at global sites will provide clinical trial organizations with the ability to expand their study populations and obtain results rapidly.

ST030. **Clinical Validation of Anchored Multiplex PCR-Based Next-Generation Sequencing of Gene Fusions in Solid Tumors and Sarcomas**

C. Ho[1], M. Black[2], H. Zhu[1], J. Le[1], C. Kunder[2]
[1]Stanford Health Care, Palo Alto, CA; [2]Stanford University, Palo Alto, CA.

Introduction: Numerous translocations have been established as diagnostic, prognostic, or therapeutic markers in the setting of solid tumors and sarcomas. Recognition of specific gene fusions is now integrated into the diagnostic criteria of many soft tissue sarcomas. The identification of certain translocations is also essential to guide therapeutic decision making. Methods for accurate, sensitive, and rapid detection of a wide variety of gene fusions is increasingly important in routine clinical testing. Limited computational and development resources in clinical laboratories and rapid progression in the diagnostic landscape may delay implementation of custom-designed RNA sequencing panels. Here, we share our validation of a commercially available next-generation sequencing (NGS)-based assay utilizing anchored multiplex PCR (AMP) to target 137 actionable fusion gene partners in solid tumors and sarcomas. Adaptation of commercial products may reduce the time required to develop, optimize, validate, and implement an NGS gene fusion assay to fewer than six months. Furthermore, it empowers molecular laboratories to identify established and novel fusion partners and oncogenic isoforms to improve research efforts and direct patient care. **Methods:** Fifty formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with sarcoma or solid tumors that were previously determined to harbor gene fusions by orthogonal methods and 10 FFPE from non-neoplastic samples were tested using the FusionPlex Pan Solid Tumor v2 Panel (Invitae, Inc.). Total nucleic acid was extracted from FFPE tissue samples using Maxwell RSC RNA FFPE extraction kits (Promega, Inc.). Analytical accuracy, sensitivity, specificity, and precision were determined. A dilution series of FFPE Tumor Fusion RNA v4 Reference material (SeraCare, Inc.) and germline DNA established the limit of detection (LOD). A dilution series of control sample with different library inputs determined minimum required sequencing reads. **Results:** The analytical accuracy, sensitivity, and specificity of FusionPlex was 97.6%, 96.7%, and 100.0%, respectively. Results were highly reproducible with all 18 known fusion calls detected in precision study. The LOD was determined to be 0.1% to 12.5%. The minimum required sequencing reads were determined to be 1 million. **Conclusions:** A commercially available NGS gene fusion assay allowed accurate and reproducible identification of fusions involving 137 target genes. Extraction of total nucleic acid (both DNA and RNA) preserves limited tumor material and allows future genomic testing. The adaption of a commercially available AMP-based NGS gene fusion assay allowed rapid implementation, reduced development cost, and decreased maintenance burden to enhance patient care.

ST031. **A Highly Specific Digital PCR Assay for Detecting *KRAS* Mutations in Liquid Biopsy Samples**

S. Barbari, M. Sticher, C. McKee, S. Kane
GT Molecular, Fort Collins, CO.

Introduction: Liquid biopsies are minimally invasive and can be used by oncologists to make rapid therapeutic decisions, monitor treatment efficacy, and detect therapy resistance. Additionally, circulating tumor cells can provide information about primary and metastatic tumors simultaneously. However, circulating tumor cells are present at very low concentrations. Digital PCR (dPCR) provides a powerful, highly sensitive method for detecting low concentrations of target DNA in a sample. Mutations in *KRAS* are prevalent in several tumor types. Additionally, the FDA recently approved a long-awaited targeted therapy of *KRAS-G12C*, sotorasib, making longitudinal monitoring of treatment efficacy an attractive option for these patients. We developed multiplex *KRAS-G12/G13*-specific dPCR assays for use in detecting the variant allele frequency in a mixed wild-type (WT) and mutant DNA population as seen in blood. **Methods:** Primers and probes were designed to target a ~80bp region surrounding the site of the *G12C/D/V/A* or *G13C* mutation in *KRAS*. A universal set of primers and probes were designed to target a non-mutated region of *KRAS* to determine the variant allele fraction in a duplex dPCR assay. Genomic DNA and synthetic DNA containing *KRAS-WT* or mutant *KRAS-G12/G13* were used in our analytical validation studies. Droplet digital PCR was performed using the Bio-Rad QX200 system. **Results:** We developed highly specific and robust dPCR assays capable of detecting *KRAS-G12C/D/V/A* or *-G13C* in a mixed *KRAS-WT* and mutant *KRAS-G12/G13* DNA sample. Additionally, using our developed patent-pending technology, our assay showed no cross-reactivity with WT genomic DNA, thus minimizing false-positive results. **Conclusions:** Mutations in *KRAS-G12/G13* can be detected with high specificity and sensitivity using our dPCR assays. Future studies will include clinical validation using serum obtained from patients known to harbor tumors with *KRAS-G12C* in collaboration with UCHealth. Our unique technology provides high performance, economy, and fast turnaround for more accessible results, leading to the promise for streamlined development of additional assays targeting other actionable mutations, like those in *EGFR* and *BRAF*, to aid oncologists in their care while also reducing the burden on patients.

ST032. **Development and Analytical Performance of NCI Clinical Whole-Exome Sequencing (NcWES) Assay to Support NCI Precision Medicine Clinical Trials**

N. Nair[1], L. Chen[1], S. Narava[1], A. Hayes[1], L. Romero[1], A. Chapman[1], R. Thorton[1], C. Camalier[1], E. Cantu[1], L. Harris[2], J. Doroshow[2], S. Jiwani[1], C. Karlovich[1], M. Majano[1], L. McShane[2], E. Mosher[1], G. Rivera[1], M. Saeed[1], X. Xu[1], M. Williams[1], B. Das[1]
[1]Frederick National Laboratory for Cancer Research-Leidos Biomedical Research Inc., Frederick, MD; [2]National Cancer Institute, Bethesda, MD

Introduction: We have developed a whole-exome assay, NCI Clinical Whole-Exome Sequencing (NcWES), that can assess complex biomarkers like tumor mutational burden (TMB), loss of heterozygosity, microsatellite instability (MSI), and detect variant types including substitutions (single nucleotide variants), insertions and deletions (indels), and copy number variants with high specificity and sensitivity. We incorporated features in NcWES panel for enhanced variant detection sensitivity in 671 cancer-related genes, seven oncogenic virus families, and translocations in tumor specimens. The analytically validated NcWES assay is intended for use in National Cancer Institute-sponsored Precision Medicine Clinical Trials for both prospective patient selection and retrospective correlative analyses. **Methods:** Formalin-fixed, paraffin-embedded (FFPE) specimens from 95 individual tumors with corresponding germline (i.e., buffy coat

fraction from whole blood) and commercially available reference material were extracted to yield a minimum of 130 ng genomic DNA with DIN (DNA integrity number) score of ≥2.0 (on TapeStation 4200). The NcWES assay is a high-throughput, automated assay that uses Twist Biosciences chemistry for library preparation and hybrid probe capture. Final libraries were sequenced using Illumina NovaSeq 6000 sequencers. TMB was calculated using nonsynonymous variants from the 32.6Mb coding region of the assay. A robust data analysis pipeline has been developed on Illumina's DRAGEN Bio-IT platform for identification of somatic variants and TMB. **Results:** Analytical performance for variant detection and TMB in NcWES assay was assessed in clinical FFPE specimens. Specificity for variants and TMB were >99.99% and 100%, respectively (assessed in 21 adjacent normal FFPE specimens). Sensitivity for variants was assessed in 95 clinical specimens and was >95% (for VAF ≥5%). Copy number amplifications in 10 genes (CN ≥5) were confirmed by orthogonal assay, with high precision (%CV <5%) observed between replicate runs. The Pearson's correlation coefficient of TMB values when compared to an orthogonally validated assay was >0.99 based on 95 individual clinical specimens (slope=1.03 and intercept=-0.48). The precision for TMB values between replicate libraries (n=9) for 10 clinical specimens was high with %CV <5% (TMB range: 2-25 mut/Mb). **Conclusions:** The NcWES assay demonstrated excellent specificity, sensitivity for variant detection, accurate TMB assessment, and reproducibility. Future planned validation studies will incorporate additional capabilities including the determination of MSI status. We envision this versatile assay will be used both prospectively and retrospectively to support a wide range of NCI-sponsored precision medicine initiatives incorporating both immunotherapies and targeted agents.

ST033. **Development of a Modular Set of Hybridization-Capture Panels and Analysis Workflows for Tumor Molecular Characterization**

M. Ruvolo[1], M. Cayuette[2], A. De Peralta[2], K. Felts[2], L. Forsmark[1], S. Happe[3], A. Khare[1], A. Lucas[1], B. Novak[1], H. Shin[1]
[1]Agilent, Santa Clara, CA; [2]Agilent Technologies, Inc., La Jolla, CA; [3] Agilent Technologies, Inc., Cedar Creek, TX.

Introduction: Identification of the molecular changes that drive tumorigenesis is critical for understanding tumor biology. Here we describe a set of hybrid-capture target enrichment next-generation sequencing panels and analysis workflows that provide a modular and flexible approach to tumor characterization. The solution includes a comprehensive genomic profiling (CGP) pan-cancer assay targeting 650+ cancer-specific genes for detection of single nucleotide variants (SNVs), small insertions/deletions (indels), somatic copy number alterations, translocations, tumor mutation burden (TMB), and microsatellite instability (MSI) paired with an 80-gene panel for RNA fusion detection. Along with the pan-cancer panel, nine cancer-specific panels provide actionable molecular information at a reduced sequencing cost. All panels are developed with formalin-fixed, paraffin-embedded (FFPE)-optimized sample prep chemistry and automation as well as analysis, interpretation, and reporting software to maximize workflow efficiency. (For research use only; not for use in diagnostic procedures.) **Methods:** Assay design was performed using optimized probe selection algorithms to give deep and uniform coverage of the target regions while minimizing sequence-specific bias. Libraries were made from 50 ng of DNA/RNA using the Agilent SureSelect XT HS2 kit. Dual unique molecular indexes were added and utilized in the analysis to improve the accuracy of SNV/indel detection at low-allele frequencies. Analysis of TMB and MSI was performed using tumor-normal and tumor-only analysis methods. Gene fusions were detected from both DNA and RNA through identification of chimeric reads, allowing detection of both known and novel fusions. Performance was evaluated using both reference cell lines and FFPE samples using 40M reads per sample for the CGP panel and 10M reads for the tumor-specific and RNA fusion panels. **Results:** Mean sequencing depth for all panels was >600x with >98% of the targeted bases having at least 100x coverage. Performance was uniform across the %GC spectrum, with both high and low GC regions showing good coverage. SNV and indels with allele frequencies as low as 1% were reliably detected. Gene fusion detection from both DNA and RNA inputs showed good agreement with fluorescence *in situ* hybridization (FISH) and dPCR. TMB values and MSI status determined using the CGP panel were concordant with whole-exome sequencing and MSI-PCR, respectively. **Conclusions:** This work demonstrates the utility of a modular and flexible set of tools for tumor molecular characterization. The combination of optimized wet-lab workflows and analysis methods streamlines the workflow while maximizing the information obtained from FFPE samples.

ST034. **Rapid Assessment of Kinase Gene Fusions Using the Idylla GeneFusion Assay in Lung Adenocarcinoma**

D. Nkosi, M. Buldo, H. Liu, Z. Oltvai
University of Rochester Medical Center, Rochester, NY.

Introduction: Receptor kinase gene fusions represent about 8%-10% of the actionable gene mutations in lung adenocarcinoma. Methods currently utilized to detect kinase fusions include fluorescence *in situ* hybridization, immunohistochemistry, quantitative real-time PCR, and next-generation sequencing. However, these different testing modalities may be impacted by long turnaround times and the need for trained personnel to run or interpret assay results. **Methods:** We evaluated the clinical performance of the RNA-based Idylla GeneFusion Assay, which is fully automated multiplex PCR platform with three hours' turnaround time, for the detection of *ALK, ROS1, RET*, and *NTRK1/2/3* fusions and *MET* exon 14 skipping on tissue obtained directly from formalin-fixed, paraffin-embedded slides. We assessed 24 surgical resections/biopsies and 10 cytological blocks paraffin-embedded tumor samples with tumor cellularity between 10%-90%. Among the tumor samples, 13 harbored *ALK* fusion mutation, five harbored *ROS1* fusion mutation, two harbored *RET* fusion mutation, six contained *MET* exon 14 skipping, and eight were negative controls. **Results:** Idylla GeneFusion Assay showed a concordance of 92% (12/13) on *ALK* mutations, 80% (4/5) on *ROS1* mutations, 100% (2/2) on *RET* mutations, and 100% on *MET* exon 14 skipping mutations when compared to results obtained by orthogonal testing methods. Overall the Idylla GeneFusion cartridge demonstrated a clinical sensitivity of 92.3%, and specificity of 100%. Further analyses using cell lines harboring the different targetable kinase mutations demonstrated that the assay had a limit of detection of 10% tumor cells. Varied input limit criteria with sample size ranging from 25 mm to maximum size of 100 mm were used during the evaluation. Additionally, the results from the Idylla molecular testing were highly reproducible with range of specific fusion cycles among triplicate runs of less than 1.5 and the range of ΔCQ values among triplicate runs of less than one cycle. **Conclusions:** Taken together, these results demonstrate the clinical utility of the Idylla GeneFusion Assay for rapid, valid, and reproducible detection of the kinase fusions in lung adenocarcinoma with use of low tissue requirements in comparison to the other established testing modalities.

ST035. **Development and Clinical Validation of an *MLH1* Promoter Methylation Assay by Methylation-Specific Multiplex Real-Time PCR**

M. Morrison, Z. Wang, K. Cardoso de Melo, K. Gwin, G. Rivera-Colón, J. Mitchell, Z. Chi, J. Xu, D. Oliver
University of Texas Southwestern Medical Center, Dallas, TX.

Introduction: *MLH1* promoter methylation is often observed in sporadic microsatellite instability (MSI) tumors such as colorectal and endometrial carcinomas, as compared to inherited Lynch syndrome. Whereas MSI can be seen in both sporadic and inherited tumors, *BRAF* V600E mutations are found almost exclusively in sporadic colorectal cases. Here we discuss the development of an *MLH1* promoter methylation assay to aid in the assessment of sporadic versus Lynch syndrome-associated carcinomas. **Methods:** Samples included ones previously tested at a reference lab for *MLH1*

hypermethylation, stained for MMR-IHC, and/or analyzed for *BRAF* mutations. Tumor types were predominantly colorectal and endometrial carcinomas. Genomic DNA was extracted from microdissected formalin-fixed, paraffin-embedded (FFPE) slides using Qiagen FFPE Tissue Kits, followed by bisulfite treatment using the Zymo EZ DNA Methylation-Lightning Kit. Methylation-specific multiplex real time PCR using lab developed primers and probes targeting eight conserved CpG islands in the "C" region of the *MLH1* promoter was done. The delta C_T values of the methylated versus unmethylated probe for each case were compared to a methylation standard curve (run in triplicate) to determine percentage promoter methylation. **Results:** A total of 33 samples were tested. We established 5% methylation as the assay's limit of detection and used 10% methylation as the cut-off level to call a promoter methylated versus unmethylated. Previous studies have reported low-level (<10%) *MLH1* methylation in patients with Lynch syndrome. Using this 10% parameter we found strong agreement in observed versus expected results. Samples ranged in methylation from 15%-85% across the different tumor types. Of 13 cases tested at a reference lab, there was >92% concordance overall between expected and observed samples. One sample was discordant, likely due to differences in established cut-offs between labs. Specificity for unmethylated cases was 100%. Interestingly, of six colorectal cancer samples with MLH1/PMS2 loss which previously tested negative for *BRAF* mutations, two were shown to be *MLH1* hypermethylated, highlighting the importance of co-testing for both *BRAF* and *MLH1* promoter methylation in these tumors. **Conclusions:** Our assay showed good accuracy, reproducibility, specificity, and sensitivity. The assay shows promising clinical applications in distinguishing between Lynch syndrome and sporadic colorectal and endometrial adenocarcinoma.

ST036. **Global Site Reproducibility of Characterizing Biomarkers in cfTNA from Liquid Biopsy Samples Using the Targeted Oncomine Precision Assay**

D. Unselt, L. Frady, K. Zhao, F. Torres, J. Sims
Q2 Solutions, Inc., Durham, NC.

Introduction: High-complexity next-generation sequencing (NGS) workflows impact the ability to achieve consistent inter-laboratory performance. Often it is highly dependent on operator proficiency and institutional systems designed to minimize variability of testing performance. Here we describe analytical performance and reproducibility of an automated NGS Pan-Cancer DNA and RNA assay at two global laboratory sites located in Research Triangle Park, US, and Beijing, China. The Oncomine Precision Assay on the Genexus Integrated Sequencer (OPAGX) is a targeted, NGS assay that detects biomarkers across 50 genes from both solid tissue and liquid biopsy samples. This assay utilizes unique molecular identifiers (UMIs) for error correction in sequencing and improved sensitivity for detection of single nucleotide variants (SNVs), small insertions or deletions, gene fusions, and amplifications in liquid biopsy samples. **Methods:** The Ion Torrent Genexus Integrated Sequencer is an automated NGS platform requiring minimal hands-on time for library preparation and sequencing. Each reaction requires 20 ng of cell-free total nucleic acid isolated from plasma. Key components of this assay are the use of both DNA and complementary DNA to amplify the gene targets of interest, the use of AmpliSeq HD technology to label amplicons with dual molecular barcodes during library preparation, and sequencing libraries on a GX5 chip to a targeted depth of 12 million reads per sample. Results from the analytical validation performed in Beijing, China, were compared to results achieved in Research Triangle Park, US, to evaluate performance and global site-to-site variability. **Results:** OPAGX variant results of cell-free DNA from well-characterized control samples were evaluated. The estimated allele frequency limit of detection (LoD) for small variants (single nucleotide variants, insertions, and deletions) was 0.64%, which is slightly above the advertised 0.50% provided by the vendor. The following metrics were evaluated for small variants above the estimated LoD. For sequencing runs performed in the US and China, results demonstrated >99.9% accuracy; >95.0% sensitivity, specificity, and positive predictive value; and >88.0% concordance for intra-run, inter-run, and instrument-to-instrument precision. Additionally, when evaluating fusions, although none of our plasma donors had fusions detected, seven out of seven RNA expression controls were present, indicating cfRNA was amplified and should yield fusion supporting reads, if present. **Conclusions:** Liquid biopsy sample performance was characterized for several biomarker classes using the Oncomine Precision Assay. The results from both Research Triangle Park, US, and Beijing, China, genomic laboratories demonstrate interlaboratory accuracy and reproducibility. enabling global implementation and harmonization for clinical testing.

ST037. **A Study to Evaluate Association of Nuclear Grooving in Benign Thyroid Lesions with *RET/PTC1* and *RET/PTC3* Gene Translocation**

A. B R, N. C, D. Biligi
Bangalore Medical College and Research institute, Bengaluru, Karnataka, India.

Introduction: Papillary thyroid carcinoma (PTC) is the commonest malignant lesion of thyroid characterized by unique histological features like nuclear grooving, nuclear clearing, and intra-nuclear inclusions. However, nuclear grooves are observed even in benign thyroid lesions (BTL) like nodular goiter (NG), Hashimoto thyroiditis (HT) and follicular adenoma (FA), resulting in diagnostic dilemma of presence of PTC in such BTLs. *RET/*PCT gene translocation is one of the commonest oncogenic rearrangements seen in PTC, known to be associated with nuclear grooving. Among different types of *RET*/PTC translocations, *RET*/PTC1 and *RET*/PTC3 gene translocations are the commonest. These translocations have also been identified in many BTL-like hyperplastic nodule and HT. Our study was done to determine the frequency of nuclear grooving in BTL and evaluate its association with *RET/*PCT1 and *RET/*PTC3 gene translocation. **Methods:** The formalin fixed, paraffin-embedded tissue blocks of NG, HT, and FA were included in the study. The hematoxylin and eosin stained sections were evaluated for the presence of nuclear grooving/hpf and a scoring of 0 to 3 was used for the number of grooves. Sections of 10 μ thickness were cut and the cells containing the nuclear grooves were picked using Laser-Capture microdissection. About 20 to 50 such cells were microdissected in each of the cases followed by RNA extraction, cDNA conversion, real time-polymerase chain reaction (RT-PCR) for *RET*/PTC1 and *RET*/PTC3 gene translocation, and the findings were checked for statistical significance. **Results:** Out of 87 BTLs included in the study, 67 (77.0%) were NG, 12 (13.7%) were HT, and eight (9.2%) were FA. Thirty-two cases (36.8%) had nuclear grooving with 18 out of 67 NG, six out of 12 HT, and all eight cases of FA showing varying number of nuclear grooves. Significant association between number of nuclear grooves and *RET*/PTC gene translocation (p value of 0.0014) was obtained. Significant association of HT with *RET/*PTC gene translocation (p value of 0.0001) was observed. *RET/*PCT1 and *RET/*PTC3 translocation was seen in five out of 87 cases, with HT showing positivity in two and FA in one case for *RET*/PTC1, and HT in one and FA in two cases for *RET*/PTC3 gene translocation with one case of FA being positive for both *RET/*PCT1 and *RET/*PTC3 gene translocation. **Conclusions:** A statistically significant association was seen between the number of nuclear grooves and *RET/*PTC gene translocation, favoring them as a marker in BTL necessitating further follow-up of the patient. HT had a significant association with *RET/*PTC gene translocation warranting follow-up of patients with HT showing nuclear grooving.

ST038. **Identification of *MET* Fusions in Solid Tumors: A Multi-Center, Large Scale Study in China**

W. Yang[1], Q. Liu[2], W. Li[3], Z. Liu[4], J. Ma[4], T. Ma[3], H. Tao[4], D. Wang[3], X. Zhang[3], Y. Zhang[3], J. Zhu[3], X. Zhao[3], A. Zheng[5]
[1]Shanxi Bethune Hospital Cancer Center, Taiyuan, People's Republic of China; [2]Xiangya Hospital Central South University, Changsha, People's Republic of China; [3]Genetron Health Co. Ltd., Beijing, People's Republic of China; [4]Zhejiang Provincial People's Hospital, Hangzhou, People's Republic of China; [5]Cancer Hospital of the University of Chinese Academy of Sciences, Hangzhou, People's Republic of China.

Introduction: Genomic alterations of *c-Mesenchymal–Epithelial Transition Factor* (*MET*) promote tumor cell proliferation and metastasis in many kinds of human malignancies. *MET* amplifications and *MET* exon 14 skipping mutations are common genomic alterations in solid tumors, and are well studied in clinical samples. However, *MET* gene fusions are poorly implicated due to the rare events and distinct diagnostic challenges in the clinic. To explore the profile and analyze the characteristics of *MET* fusions, and provide molecular evidence on development of *MET* fusion-targeting therapies, we conducted a multi-center, large-cohort study to identify *MET* fusions in common cancer types. **Methods:** In total, 10,882 clinical samples were collected from three hospital centers (Xiangya Hospital Central South University, Shanxi Bethune Hospital Cancer Center, and Zhejiang Provincial People's Hospital) from Dec. 2018 to Sep. 2021, and were sequenced by DNA-based next-generation sequencing (NGS) (Onco PanScan, Genetron Health). To further validate *MET* fusion, total RNA was isolated from fusion-positive samples detected by DNA-based NGS, subsequently analyzed by RNA-based NGS (FusionCapture, Genetron Health). **Results:** In the present study, 37 potentially functional *MET* fusions coding the intact tyrosine kinase domain (TKD) of *MET* were identified by DNA-based NGS. The incidences of *MET* fusion in each cancer type revealed by DNA-based NGS were 1.1% (27/2,440) in brain cancer; 0.07% (4/5,835) in lung cancer; 0.14% (2/1,428) in intestine cancer; 0.25% (2/794) in gastric cancer; and 0.52% (2/385) in bile duct cancer. Remarkably, 15 novel *MET* fusions were identified in five cancer types, and the incidence of novel *MET* fusions accounted for 40.5% (15/37). Moreover, the results displayed that brain cancer had the highest incidence of *MET* fusion, and the most common fusion in which was *PTPRZ1-MET* (37.0%). In addition, we noticed that all *MET* breakpoints in brain cancer (n=27) were in intron 1, whereas all of which in lung cancer (n=4) occurred in intron 1, intron 11, intron 14, and exon 14. Furthermore, the results revealed that the positive consistency of common fusion group was 100% (11/11), whereas the positive consistency of rare fusion group was 53.8% (7/13). Therefore, these results indicated that the consistency between DNA-based NGS and RNA-based NGS was very high in common fusion groups, but relatively low in rare fusion groups. **Conclusions:** We provided a comprehensive genomic landscape of *MET* rearrangement and updated *MET* fusions database for clinical test. In addition, we revealed that DNA-based NGS might meet the clinical test for *MET* common fusions, whereas it was necessary to validate *MET* rare fusions by both DNA-based NGS and RNA-based NGS. Prospective trials are necessary to confirm the efficacy of *MET* inhibitors treatment.

ST039. **Recurrent Patterns of Copy Number Changes in IDH-Wild-Type Glioblastoma Patients**

L. Choate, T. Kollmeyer, C. Praska, R. Jenkins, C. Ida
Mayo Clinic, Rochester, MN.

Introduction: Glioblastoma, IDH-wild-type (GBM-IDHWT) is also defined by molecular features including +7/−10 copy-number changes, *EGFR* amplification, and *TERT* promoter (*TERT*p) mutation. It is unclear if additional copy-number changes can be diagnostically useful in the absence of diagnostic morphological features. We describe copy-number signatures in morphologically defined GBM-IDHWT to investigate potential additional diagnostically useful copy-number patterns. **Methods:** IDH/H3-wild-type diffuse astrocytic gliomas with glioblastoma morphological features (n=425) tested by clinical chromosomal microarray and targeted neuro-oncology next-generation sequencing (187 or 50-gene panel) were collected. Unsupervised hierarchical clustering was performed based on recurrent copy-number changes for *TERT*p-mutant (n=362) and *TERT*p-wild-type (n=63) groups separately. Additional gene mutation status was correlated with clusters defined by copy-number signatures. **Results:** Copy-number clustering resulted in six *TERT*p-mutant clusters and three *TERT*p-wild-type clusters. Partial +7/-10 was more common than whole +7/-10 in both groups (mean difference in percent: *TERT*p-mutant [10: 10.9%; 7: 14.5%] and *TERT*p-wild-type (10: 27.6%, 7: 14.3%). All *TERT*p-mutant clusters were enriched for -10, with enrichment for +7/-10 in clusters 3-6 and *EGFR* amplification in cluster 4. Additional *TERT*p-mutant copy-number features included 2q- with *IDH1* loss (clusters 1, 3, 6), -4 (cluster 3), 11p- (clusters 1, 3, 6), *TP53* loss of heterozygosity (LOH) or deletion (clusters 1, 3, 5, 6) and *CDKN2A/B* LOH or deletion (clusters 1-4). All *TERT*p-wild-type clusters were enriched for *CDKN2A/B* LOH or deletion and 11p-. Only *TERT*p-wild-type cluster 2 was enriched for +7/-10. *TERT*p-wild-type cluster 3 was enriched for *TP53* LOH or deletion. Mutation analysis of copy-number-based clusters showed that *PTEN* mutations were enriched in all clusters. *TERT*p-mutant group was enriched for *EGFR* (clusters 2, 4), *RB1* (clusters 2, 3), and *NF1* (cluster 1) mutations. *TP53* and *CDKN2A/B* mutations were enriched in all *TERT*p-wild-type clusters. Potential alternative telomere maintenance mechanisms were observed equally across *TERT*p-wild-type clusters and included *ATRX* mutations (n=6), 5p+ where *TERT* is located (n=19) and/or 2q- where *SMARCAL1* is located (n=5 total, one case also with 5p+). **Conclusions:** Clustering of *TERT*p-mutant and *TERT*p-wild-type morphologically defined GBM-IDHWT based on copy-number signatures shows additional potential diagnostically useful copy-number patterns. Partial +7/-10 was more common than whole +7/-10 suggesting that partial +7/-10 may be a more sensitive diagnostic criterion than whole +7/-10 for undersampled GBM-IDHWT that lack diagnostic morphological diagnostic features.

ST040. **Prevalence of *PIK3CA* Mutations across Indian Solid Tumor Patients**

M. Gurav, O. Shetty, P. Gogte, T. Pai, G. Deshpande, N. Karnik, S. Epari, S. Desai
Tata Memorial Hospital, Mumbai, India.

Introduction: *PIK3CA* mutations are the most prevalent (10%-20%) oncogenic event observed across all solid tumors viz. breast, bladder, colorectal, lung, and head-neck cancers with varied frequencies. These mutations result in PI3K pathway hyperactivation leading to oncogenesis and contribute to therapy resistance in breast and colorectal cancers. More than 80% of these occur at codons 542, 545, and 1047. These mutations show co-occurence with other hotspot mutations such as *KRAS*, *EGFR*, *BRAF*, etc. The present study evaluated frequency of *PIK3CA* mutations and co-occurring alterations across solid tumors in Indian patients. **Methods:** The study included 1,052 formalin-fixed, paraffin-embedded tissues (January 2021-May 2022) from lung (n=500), gastrointestinal cancers (GI; n=305), head-neck (n=107), breast (n=24), soft tissue sarcomas (n=30), urological (n=36), central nervous system (n=25), gynecological (19), pediatric (n=4), and adult hematological cancers (n=2). Cases were subjected to next-generation sequencing using targeted gene panel. Clinicopathological details were collated from electronic medical records and were correlated with molecular findings. **Results:** In total, 9.3% cases were *PIK3CA* mutant of which 89.9% affected exon 9 and 20 with hotspot codons 542, 545, 546, 1047, 1049, and 1043. They also showed other variants at codon 38, 88, 93, 365, 453, 524, 539, 1004, 1011, and 1023. Highest mutation frequency was detected in breast (20.8%) followed by head-neck (13.1%), gynecological (10.5%), soft tissue sarcomas (10.0%), GI (9.8%), lung (8.6%) and urological tumors (2.8%). *PIK3CA* mutant breast cases (n=4) included three invasive ductal and one invasive lobular carcinoma. Lung and GI tumors showed prominent alterations in exon 9, whereas head-neck tumors showed predominantly exon 20 mutations. *PIK3CA* mutants

showed co-occurring mutations in 81.6% cases with *TP53* (37.8%) followed by *EGFR* (20.4%), *KRAS* (18.4%), *BRAF* (6.1%), *CDKN2A* (6.1%), and 4.1% each of *NRAS*, *HRAS*, *CTNNB1*, *ERBB2* genes. Within lung tumors, *TP53* (45.5%) and *EGFR* (43.2%) were the most common concurrent mutations. *PIK3CA* mutations were commonly associated with *EGFR* exon 19 and 21 alterations and all *EGFR*/*PIK3CA* concurrent mutations were seen in metastatic tumors. Coexistence of *KRAS* mutations was significantly seen in GI tumors (p=0.0001). *PIK3CA* mutants in head-neck tumors showed co-occurrence mainly with *HRAS* (28.6%), *TP53* (28.6%), and *BRAF* (21.4%) mutations. **Conclusions:** *PIK3CA* mutations were detected in 9.3% cases and the majority of these showed concomitant mutations of other hotspot genes. With approved targeted therapies and favorable response to PI3K/AKT/mTOR inhibitors, identification of *PIK3CA* mutations has become important in molecular diagnostics for treatment decision and identifying resistance mechanisms.

ST041. **Idylla *BRAF* and *NRAS***

B. Houde, J. Barbuto, P. Carnaroli, B. Dokus, K. Godwin, D. Green, G. Huang, M. Johnston, K. Rumrill, L. Thompson, T. Wilson, K. Winnick, G. Tsongalis, A. Sriharan
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Common mutations within both the *BRAF* and *NRAS* genes can be used to determine use of target-specific treatment therapies for oncology patients. The Idylla NRAS-BRAF-EGFR S492R Mutation Assay is a cartridge-based system that automates deparaffinization, nucleic acid isolation, and multiplexed real-time PCR in a single use, disposable cartridge. This procedure is used to detect 23 common mutations located in the *BRAF* (five) and *NRAS* (18) genes from formalin-fixed, paraffin-embedded tissues. This is a highly targeted assay where no other mutations are assessed. Here we describe our experience with this assay. **Methods:** The Biocartis NRAS/BRAF assay is performed on the Idylla system using the NRAS-BRAF-EGFR 5492R single-use cartridge. Tumor tissue is enriched by macrodissection after the tumor area and neoplastic cellularity are determined by a trained dermatopathologist to ensure specimens meet assay requirements (>10% neoplastic cellularity and 50-600 mm2 tissue area). Since implementation in January 2021, we tested 147 clinical samples. **Results:** Of the 147 clinical samples analyzed, 44 patients tested positive for variants within the *BRAF* gene (25 p.V600 E/D, 15 p.V600 K/R, and four p.V600 E); 44 patients tested positive for variants within the *NRAS* gene (three *NRAS* p.G13 R/V, six *NRAS* p.Q61L, two *NRAS* p.Q61H, 13 *NRAS* p.Q61K, 15 *NRAS* p.Q61R, and five *NRAS* p.Q61R/K). Fifty-four patient samples were wild-type with no known detectable mutation for those detected by the cartridge. In 5/147 samples, the quality or quantity was not sufficient for analysis. **Conclusions:** The 23 common mutations detected in the *BRAF* and *NRAS* genes using this assay have significant impact on therapeutic selection. Somatic variants in the *BRAF* gene have been found in 37%-50% of all malignant melanomas, whereas somatic variants in the *NRAS* gene are found in 13%-25% of all melanomas. The Biocartis Idylla system offers a simple and rapid method to obtain somatic mutation information in these two genes that are of clinical importance.

ST042. **Mutational Spectrum of *PIK3CA* in Lebanese Breast Cancer Patients: A Pilot Study**

C. Nakad, L. Hamadeh, L. Farhat, R. Mahfouz
AUBMC, Beirut, Lebanon.

Introduction: Breast cancer is the most common malignancy in women worldwide. Although curable in about 70% to 80% of patients with early, non-metastatic disease, it still ranks as the leading cause of cancer-related death in women to date. The phosphatidylinositol-3 kinase (PI3K) is a pathway responsible for the regulation of several cellular processes, such as cell survival, cell growth, and cell cycle progression. The *PIK3CA* pathway is one of the most frequently altered pathways in human cancers, especially in breast cancer with approximately 40% of HR+/HER2- advanced cases exhibiting mutations in the *PIK3CA* gene. *PIK3CA* is a gene that encodes p110α, the catalytic subunit of the PIK3 pathway. There are three mutational hotspots in this gene: E542 and E545 in exon 9, corresponding to the helical domain, and the exon 20 H1047R mutation encompassing the kinase domain. *PIK3CA* mutation assays are intended for use as a companion diagnostic test, to aid clinicians in the identification of breast cancer patients who may be eligible for treatment with alpelisib, an alpha-specific PIK3CA inhibitor, based on a *PIK3CA* mutation detected result. This study constitutes the first attempt at determining the frequency and mutational spectrum in Lebanese breast cancer patients. **Methods:** A total of 280 primary breast cancer tissue samples were collected from across Lebanon. DNA was extracted from 20μm-50μm sections of formalin-fixed, paraffin-embedded tissue. Subsequent mutation detection was accomplished using a real-time qualitative polymerase chain reaction assay for the detection of 11 mutations in the *PIK3CA* gene. **Results:** Our findings indicate that 38.92% of breast cancer patients in Lebanon carry at least one mutation in the *PIK3CA* gene. Among the positive cases, 43 samples have mutations located in exon 20 of the *PIK3CA* gene, 66 samples are mutated in exon 9, and only four samples carry a mutation in exon 7. Seven out of 108 positive samples were double mutants: one case carries two mutations in exon 20 (H1047L and H1047R), one case carries two mutations in exon 9 (E542K and E545K), one case carries two mutations in exon 9 and 7 (E545K and C420R), four cases carry mutations in exons 9 and 20 (E542K and H1047R detected in two cases, and E545D and H1047R, E542K, and H1047Y). Additionally, exon 9 mutations E545A and Q546E were not detected in our cohort of positive samples. **Conclusions:** Screening for *PIK3CA* mutations highlighted the highly diverse mutational status of this gene among breast cancer patients. As the mutational profile of a patient renders them eligible for targeted therapy, an urgent need arises to perform comprehensive next-generation sequencing assays to detect additional hotspot and non-hotspot mutations.

ST043. ***HRAS* Alteration Is Retained in the Heterogeneous Components of the Non-Invasive Upper Tract Urothelial Carcinoma**

B. Ahn, Y. Cho, D. Kim
Asan Medical Center, Seoul, Seoul-t'ukpyolsi, Republic of Korea.

Introduction: Pre-operative risk stratification of upper tract urothelial carcinoma (UTUC) is critical, since gold standard treatment for high-grade or invasive UTUC is radical nephroureterectomy. In particular, non-invasive UTUC (NI-UTUC) also needs prompt and extensive intervention if high-grade component is included; however, only low-grade tumor cells are often obtained by endoscopic biopsy and/or urine cytology due to intratumoral heterogeneity. We herein compared the mutational profile of heterogeneous components within the NI-UTUC to understand the distinct underlying biology of UTUC. **Methods:** Four surgically resected high-grade NI-UTUC cases with intratumoral heterogeneity and its pre-operative biopsy samples discrepantly diagnosed as low grade, were selected as a pilot cohort for whole-exome sequencing and molecular profiles of the high- and low-grade components were compared. Barcode-tagged sequencing (BT-seq) was evaluated in the validation cohort of surgically resected high-grade (n=48) and low-grade (n=76), NI-UTUC. Additionally, public data from cBioportal were analyzed for comparison of the mutational signature of high-grade (n=95) and low-grade (n=23) UTUC. **Results:** Phylogenic tree of the pilot cohort showed clonal evolution starting from the low-grade to high-grade component, and *HRAS* alterations (Q61L, Q61R, Q61H) were shared between the high- and low-grade components of the high-grade NI-UTUC. In the validation cohort, BT-seq results revealed similar *HRAS* alteration frequency in the high-grade and low-grade NI-UTUC (31% vs. 30%, respectively). *HRAS* alteration was correlated to larger size (P = 0.041) in the overall cohort and age less than 60 years (P = 0.016) and cases with intratumoral heterogeneity (P = 0.040) in the high-grade NI-UTUC. In the public data, UTUC showed statistically significant different alteration rates between high-grade and low-grade UTUC in several genes including *HRAS* (16% vs. 0%, respectively) (P <0.05). **Conclusions:** *HRAS*

mutation, a well-known genetic alteration of low-grade tumor in bladder cancer, was detected only in the high-grade UTUC in the public data, suggesting a rather distinct molecular alteration of UTUC. Also, *HRAS* alteration was clonally retained in not only the low-grade but also the high-grade components, and was significantly correlated to intratumoral heterogeneity. Our findings can help understand the underlying biology of NI-UTUC cases with *HRAS* alteration and intratumoral heterogeneity.

ST044. **A Tale of Concurrent Gene Mutations in *EGFR* and *TP53* through a Next-Generation Sequencing Approach**

P. Gogte, M. Gurav, P. Joshi, T. Pai, G. Deshpande, N. Karnik, O. Shetty, R. Kaushal, K. Prabhash, S. Desai
Tata Memorial Hospital, Mumbai, India.

Introduction: Rapid development and applicability of next-generation sequencing (NGS) technology has revealed that concurrent genetic alterations play an important role in the response and resistance of epidermal growth factor receptor (*EGFR*) mutations to *EGFR*-tyrosine kinase inhibitors (TKI). Alterations in *TP53*, a tumor suppressor gene encoding tumor protein p53 have been associated with poor prognosis in a wide variety of cancers. Here, we assess the presence of concomitant *EGFR* and *TP53* mutations by NGS analysis. **Methods:** This is a retrospective study of 1,124 non-consecutive cases (lung adenocarcinomas, small-cell carcinomas, squamous cell carcinomas, sarcomas, thyroid carcinomas, melanomas, colon cancers, astrocytomas, glioblastomas) that were subjected to molecular testing from January 2021 to May 2022. NGS was performed using the Solid Tumor plus Solution (SOPHiA Genetics, Saint-Sulpice, Switzerland) gene panel on Miseq platform (Illumina, San Diego, CA, US) and data were analyzed using SOPHiA DDM software (Version 5.10.20.1). **Results:** NGS analysis revealed the presence of *EGFR* mutations in 197 cases (17.5%). Concomitant *TP53* mutations were detected in 96 cases (48.7%). *EGFR* exon 19 deletions were the most frequently occurring mutations (n=63, 65.6%) followed by *EGFR* exon 21 L858R (n=26, 27.1%) and *EGFR* exon 20 T790M (n=22, 22.9%). *TP53* mutations were detected in exon 5 (n=29, 30.2%), exon 7 (n=21, 21.9%), exon 8 (n=17, 17.7%), exon 6 (n=15, 15.6%), exon 4 (n=11, 11.5%) and exon 10 (n=3, 3.1%). Among the *TP53* alterations, missense mutations (n=68, 70.8%) were the predominant subtype followed by nonsense (n=16, 16.7%) and frameshift mutations (n=12, 12.5%). These alterations were classified as likely pathogenic (Tier II) (n=84; 87.5%) and variants of uncertain significance (VUS, Tier III) (n=12; 12.5%). *TP53* exon 5 mutations were most commonly associated with *EGFR* Exon 19 deletion (n=20, 34.1%) and L858R mutation (n=7, 26.9%) whereas *TP53* exon 8 mutations were associated with T790M mutation (n=7, 31.8%). *PIK3CA* mutations were also detected in *EGFR-TP53* double mutant cases (n=10; 10.4%). **Conclusions:** In the present study, exon 5 was the most common mutation site of *TP53*. *EGFR* resistance mutation T790M was found to be predominantly associated with *TP53* exon 8 mutations. This group of patients who were treated with third-generation TKI, osimertinib, had progressive disease and/or succumbed. This observation is consistent with previous studies that identified *TP53* exon 8 mutations as negative prognostic predictors. This is the first Indian study to analyze the co-occurrence of *EGFR-TP53* mutations in cancer and underscores the importance of molecular profiling to analyze genetic alterations and potential resistance mechanisms.

ST045. **Pan-Solid Tumor Identification of *NTRK* Fusions Utilizing RNA Sequencing Identifies Diverse Fusion Partners**

E. Severson[1], M. Nesline[3], B. Achyut[2], S. Pabla[3], G. Kannan[2], A. Chenn[2], S. Zhang[3], R. Klein[3], J. Conroy[3], M. Sausen[4], P. Sathyan[5], K. Saini[2], T. Jensen[2], P. Reddy[2], S. Ramkissoon[2]
[1]Labcorp, Cary, NC, [2]Labcorp, Durham, NC, [3]OmniSeq, Buffalo, NY, [4]Personal Genome Diagnostics (PGDx), Baltimore, MD; [5]Illumina, San Diego, CA.

Introduction: *NTRK* gene fusions were the first genomic alteration with an FDA-approved pan-solid tumor targeted therapy. Although rare, identification of *NTRK* fusions is critical for optimal patient care but can be challenging to detect due to the variety of fusion partners and large intronic regions. **Methods:** We analyzed data from 6,730 formalin-fixed, paraffin-embedded solid tumor samples that had comprehensive genomic profiling (CGP), including non-small cell lung cancer (NSCLC, n=2,410), breast carcinoma (BC, n=459), skin basal cell carcinoma (BCC, n=2), angiosarcoma (AS, n=10), melanoma (n=135), colorectal carcinoma (CRC, n=815), pancreatic carcinoma (PC, n= 233), and ovarian carcinoma (OC, n=102). CGP included RNA sequencing for gene fusions and DNA sequencing for detection of genomic alterations (GAs). **Results:** We identified *NTRK* fusions (*NTRK1* = 5, *NTRK2* = 3, and *NTRK3*=8) by RNA sequencing in 16 patients (age 26-84 y, mean=60 y, 50% male, 50% female) in NSCLC (n=8, 0.33%), BC (n=1, 0.22%), BCC (n=1, 50%), CRC (n=2, 0.25%), melanoma (n=1, 0.74%), AS (n=1, 10%), PC (n=1, 0.43%) and, OC (n=1, 0.98%). Fusion breakpoints were present in *NTRK1* introns 1 and 11, *NTRK2* introns 2, 3, 9, and 15, and *NTRK3* introns 3, 5, 14, and 19, spanning 0.32 Mb. Ten novel fusions were identified*: HMCN1-NTRK1, ASTN2-NTRK2, MSANTD3-NTRK2, PRKACA-NTRK2, ERBB2-NTRK3, FAM174B-NTRK3, PIAS1-NTRK3, SIN3A-NTRK3*, and *TCF-NTRK3*. Previously described fusions (*LMNA-NTRK1, PEAR-NTRK1, RABGPAP1L-NTRK1, TP53-NTRK1, KANK1-NTRK3, AGBL1-NTRK3*, and *SASH-NTRK3*) were also identified. This clinical cohort did not contain *NTRK*-associated cancers (inflammatory myofibroblastic tumors, secretory breast cancers, or high-grade pediatric gliomas). Despite lacking these tumor types with frequent *NTRK* fusions, the rate of *NTRK* fusions across all solid tumors in this cohort was 0.24%. In NSCLC with *NTRK* fusions, *TP53* was the most common recurrent GA (n=7/8). Half of NSCLC cases had co-occurring driver alterations, with *KRAS* (G12C, G13D), *EGFR* (S752_I759del), *BRAF* (G649A) identified. Outside of NSCLC, the only co-occurring driver GAs were an *ERBB2* amplification (BC) and an *ITSN-ALK* fusion (PC). One CRC sample was MSI-high and 47% of samples had a tumor mutational burden >10 mutations/Mb (TMB-high). Fifty percent of the NSCLCs were TMB-high. **Conclusions:** *NTRK1, NTRK2,* and *NTRK3* fusions are clinically relevant driver alterations across solid tumor types. These fusions are difficult to detect, as the breakpoints occur across large intronic regions and they have many partner genes, with 10 novel fusion partners identified in this study. These data emphasize how important CGP with RNA sequencing is to identify all *NTRK* fusions for optimal patient treatment.

ST046. **Pan-Solid Tumor Identification of *NRG1* Fusions Utilizing RNA Sequencing Identifies Diverse Fusion Partners and Highlights a Lack of Co-occurring Oncogenic Driver Alterations**

E. Severson[1], M. Nesline[3], B. Achyut[2], S. Pabla[3], G. Kannan[2], A. Chenn[2], S. Zhang[3], R. Klein[3], J. Conroy[3], M. Sausen[4], P. Sathyan[5], K. Saini[2], T. Jensen[2], P. Reddy[2], S. Ramkissoon[2]
[1]Labcorp, Cary, NC, [2]Labcorp, Durham, NC; [3]OmniSeq, Buffalo, NY; [4]Personal Genome Diagnostics (PGDx), Baltimore, MD; [5]Illumina, San Diego, CA.

Introduction: Some genomic alterations are oncogenic drivers regardless of tumor type, which has resulted in tumor-agnostic approval for select therapeutic agents. Neuregulin-1 gene (*NRG1*) fusions may result in dysregulated signaling via the *HER3* pathway, resulting in cellular proliferation and growth. Recently, the FDA has granted fast-track approval to a *HER3* inhibitor (seribantumab for the treatment of solid tumors with *NRG1* fusions). Identifying *NRG1*

fusions is critical, as these patients typically lack other driver alterations and targeted treatment options. **Methods:** We analyzed 3,288 formalin-fixed, paraffin-embedded patient samples from non-small cell lung (NSCLC, n=1,696), breast (n=369), colorectal (CRC, n=611), esophageal (EC, n=117), ovarian (OC, n=105), pancreatic (PC, n=157), and unknown primary (CUP, n=233) carcinomas with comprehensive genomic profiling (CGP), including RNA sequencing for gene fusions and gene expression and as well as DNA sequencing for detection of biomarkers and genomic alterations (GAs). **Results:** We identified 19 *NRG1* fusions (involving 3' region of *NRG1*) in 17 unique patients (age 41-86 y, 41% male, 59% female), across multiple tumor types including NSCLC (n=7, 0.4%), BC (n=2, 0.5%), EC (n=2, 1.7%), OC (n=1, 1.0%), PC (n=1, 0.6%), and CUP (n=1, 0.4%). *NRG1* fusions were detected across all histologic types of NSCLC. Fusion breakpoint locations included intron 1 from the type II, IV, and IV *NRG1* promoters, and intron 1, 2, 3, and 9 from the type I promoter, which collectively span 1.4 Mb. Thirteen unique fusion partners were identified: *UBXN8, DDHD2, FUT10, IKBKB, TMEM66, ZCCHC7, TNRFSF10B, BIN3, BRE, CCAR2, CD9, ERO1L*, and *KCTD9,* and three known fusion partners were identified twice each: *CD74, SLC3A2, and PCM1*. Ten of 19 fusions were the result of rearrangements within chromosome 8. In NCLSC, co-occurring driver mutations were not identified, with *TP53* being the only recurrent GA (n=2/7). In other tumor types, *TP53* GAs were most common (n=6/10, three CRC, one EC, one BC, and one OC case). There were two *ERBB2* amplifications (CRC and EC) and one *ERBB2* activating (OC) GA, which fits with the mechanism of action of *NRG1* through *HER2/HER3* heterodimers. In CRC, *APC* GAs were present in two of three samples. **Conclusions:** *NRG1* gene fusions are important driver alterations to identify across all solid tumor types, as these patients typically lack other targetable alterations, and there is a targeted therapy seribantumab with an FDA-fast track designation now available. *NRG1* fusion identification by RNA sequencing is critical, as the genomic region of breakpoints is larger (1.4 Mb) than the size of many DNA CGP panels and there are diverse fusion partners, such that 13 of 19 in this study were unique. Taken together, these data emphasize that it is critical to use RNA sequencing to find *NRG1* fusions for guiding patient treatment.

ST047. ***MET* Genomic Profiling of Lung Cancer: Insights from Comprehensive Genomic Clinical Testing**

Y. Hu[1], X. Pan[2], N. Mohamed[2], M. Hunt[2], H. Muniyappa[2], K. Shilo[2], A. Madrigal[2], D. Chappell[2], K. Kenworthy[2], K. Miller[2], M. Avenarius[2], W. Zhao[2], D. Carbone[2], D. Jones[2]
[1]Ohio State University Wexner Medical Center, Dublin, OH; [2]OSUWMC, Columbus, OH.

Introduction: Targetable dysregulation of c-MET in non-small cell lung cancer (NSCLC) occurs primarily by *MET* gene amplification and mutations in intron (I)13, I14 and boundaries of exon (E)14 that produce E14 skipping (EX14SM). EX14SM are increased in tumors with *MET* amplification. We have tested NSCLC for *MET* amplification by fluorescent *in situ* hybridization (FISH) and a small Hotspot next-generation sequencing (NGS) panel to detect a subset of EX14SM. For full characterization, we introduced and summarize here addition of a comprehensive solid tumor panel (CTP) NGS that determines copy number (CN) and includes *MET* intron sequencing, with reflex to RNA sequencing (RNA-Seq) for uncharacterized EX14SM. **Methods:** From 2013 to 2022, 4,145 cases of NSCLC were assessed by Hotspot NGS and *MET* FISH, with reflex CTP-NGS for cases with *MET* amplification or those without Tier 1/driver mutations with a smoking history of ≤15 pack per year. *MET* FISH was done with probes from Abbot Molecular and/or Empire Genomics with a cutoff of 2.3 *MET/CEP7* ratio for amplification (ratio 1.8 to 2.2: equivocal). Hotspot NGS was performed by AmpliSeq Cancer Hotspot Panel v2 on PGM or Ion S5 sequencers. CTP-NGS was performed by hybrid capture with a custom panel of 542 genes (IDT xGen probes selected by the GOAL consortium) with KAPA HyperPrep/Illumina sequencing. Probes also tiled *MET* introns 1, 11, 13, 14, and 16. *MET* CN and fusions was assessed by CNVkit and Juli software. RNA-Seq was performed with KAPA RNA Hyper+RiboErase HMR/Illumina sequencing and the same xGen probes. EX14SM were detected statistically measuring fold changes in reads from E14 and surrounding sequences. **Results:** Among 4,145 cases, we detected *MET* amplification by FISH in 147 (3.5%) and 18 equivocal cases (0.4%). Among 218 cases meeting reflex criteria for CTP-NGS, 7 EX14SM (3.2%) were detected with three showing co-occurring *MET* amplification. These were all in cases with no other driver mutations by CTP-NGS, with EX14SM missed by Hotspot NGS being deeper in I13 or the E14-I14 boundary outside the primers. The previously uncharacterized c.2942-26_2942del EX14SM showed E14 skipping by RNA-Seq. Among reflexed *MET*-amplified cases, there was 90.9% qualitative match (10/11) using FISH *MET/CEP7* ratio≥3 and/or NGS-CN≥4 cutoff. The amplified region spanning *MET* was typically small (0.9 to 4.7 Mb), with *CAV1/2* always co-amplified but generally not flanking *CDK6* or *POT1* and *SMO*. **Conclusions:** *MET* FISH parallels NGS-CN and with Hotspot NGS provides a cost-effective method for rapidly reporting in limited samples, enriching for cases with possible novel EX14SM needing CTP-NGS. Follow-up with DNA-based coding/intronic sequencing of *MET* will likely identify all possible EX14SM, with RNA-Seq with the same probes providing support for exon skipping for any novel mutations identified.

ST048. **Clinical Implementation of Oncogenic Pathway Analysis to Guide Therapy**

L. Nobre, C. Hawkins, A. Levine, S. Milos, M. Johnson, B. Laxer, M. Ku, S. Ryall, J. Bennett, R. Siddaway, U. Tabori
SickKids, Toronto, Ontario, Canada.

Introduction: Advances in molecular profiling have led to characterization and improved understanding of pediatric cancer. This knowledge propelled the use of cancer precision therapy, particularly in brain tumors. The increasing availability and use of targeted agents have been largely tailored to common driver alterations; however, not all tumors respond the same, and combinatorial alterations may be present. Here we hypothesize that evaluating the transcriptomic and proteomic background of these tumors will lead to improved understanding of the most active oncogenic pathways and help guide therapy. **Methods:** We have developed and validated a custom NanoString-based assay that interrogates four key oncogenic pathways (MAPK, PI3K-AKT-mTOR, JAK-STAT, and NFkB) including RNA, protein, and phosphoprotein expression. The assay was validated using isogenic cell lines and a cohort of 40 gliomas with previous RNA-Seq. We further characterized more than 400 tumor samples, including 15 ependymomas, 11 medulloblastomas, 250 low-grade gliomas (LGG), 145 high-grade gliomas, and 10 control normal brain specimens. **Results:** Pediatric low-grade gliomas, generally driven by Ras-MAPK alterations were found to have higher MAPK activation than control samples and other tumor types. Interestingly, a subset of these tumors present with high activation of parallel pathways including PI3K and JAK-STAT. Particularly high PI3K pathway activation was correlated with worse progression-free survival in a subset of LGG that did not achieve gross-total resection at diagnosis. We further characterized a cohort of low-grade glioma patients that were treated with MEK inhibition; variability found in parallel pathways may explain the diversity in response. This cohort is currently being expanded for further characterization. **Conclusions:** We conclude that assessing key oncogenic pathway activation can add to the molecular characterization of brain tumors and further improve the prediction of prognosis, response, and potentially guide therapeutic strategies. This tool can be further explored in a multitude of tumor types. Through the incorporation in future clinical trials we will have the ability to identify potential responders and combination strategies for non-responders.

ST049. **Spectrum of Exon 21 Insertion Mutations in Non-Small Cell Lung Carcinoma (NSCLC): Indian Tertiary Cancer Centre Experience**

T. Pai, M. Gurav, P. Gogte, G. Deshpande, N. Karnik, P. Joshi, J. Saha, V. Noronha, O. Shetty
Tata Memorial Hospital, Mumbai, India.

Introduction: *EGFR* exon 20 insertions (ex20ins) mutation is the molecular driver and the third most common *EGFR* mutation, accounting for ~1%-10% cases in non-small cell lung carcinoma (NSCLC). It is a highly heterogeneous family of activating mutations comprising in-frame insertions and/or duplications of three to 21 base pairs spanning the regions between AA761 and AA775, often associated with *de novo* resistance to targeted EGFR inhibitors and correlated with a poor patient prognosis in a majority of the subtypes. Although standard chemotherapy is yet preferred for these patients, with the recent development of novel EGFR inhibitors, targeting these mutations might improve the outcome of the patients. To determine the eligibility of the novel EGFR TKI, it is important to know which particular ex20ins subtype is present for a particular case. The present study describes the spectrum of ex20ins mutation seen on targeted gene panel by next-generation sequencing (NGS) among the Indian NSCLC patients at a tertiary cancer centre. **Methods:** The study is the retrospective analysis of NSCLC subjected to NGS performed on formalin-fixed, paraffin-embedded (FFPE) tissue tumour samples (n=800 cases) as part of routine diagnostic work-up between 2019 to 2022 using AmpliSeq Focus and SOPHiA Solid Tumor Plus Solution (STS Plus panel) which could detect both DNA and RNA alterations. The data analysis was performed using LRM and Base space software for AmpliSeq Panel and SOPHiA DDM platform for STS Plus. The analysis and interpretations were done as per the recommended guidelines (AMP and CAP guidelines). **Results:** Out of a total of 800 cases, *EGFR* ex20ins mutation was seen in 17 cases, of which nine (53%) were female patients with an age range of 43-65 years (>90% in the age range of 51-56 years). Samples were from primary lung sites (n=7), and the rest from the metastatic sites (n=10). All cases were histologically adenocarcinoma (100%). The most common ex20ins mutation was A767_V769dup (n=4), followed by p.N771_H773dup (n=3), S768_D770dup (n=3), p.P772_H773dup (n=2), H773dup (n=2), p.Asn771_Pro772insGly (n=1), p.His773_Val774dup (n=1), and p.Val769_Asn771dup (n=1) with a variant allelic frequency ranging from 15.5%-90.1%. None of the cases had co-occurrence of other driver mutations or fusions and all cases were microsatellite stable. Among these patients, the *EGFR* gene was amplified in three cases included EGFR (n=3), followed by gains in *CDK4* gene. **Conclusions:** The present study unravels the diverse spectrum of *EGFR* ex20ins mutations and shows molecular heterogeneity of this rare type of *EGFR* mutation. With the novel EFGR inhibitors it is important to ascertain the exact mutation subtype of ex20ins to determine the sensitivity of the EGFR TKI.

ST050. ***MET* Amplification Prediction in Non-Small Cell Lung Carcinoma Using RNA-Based Next-Generation Sequencing**

P. Desmeules, É. Barrette, N. Bastien, D. Boudreau, P. Joubert
IUCPQ-UL, Québec, Quebec.

Introduction: *MET* gene amplification in non-small cell lung cancer (NSCLC) occurs either *de novo* or as an acquired resistance mechanism to *EGFR* tyrosine kinase inhibitors. It represents an emerging biomarker for targeted therapy along several genes with small variants, fusions, and isoforms, requiring a complex testing strategy. Although *MET* gene amplification status is traditionally assessed using fluorescent *in situ* hybridization (FISH) or DNA-based next-generation sequencing (NGS), we investigated whether it could be predicted using data from an RNA-based anchored multiplex PCR NGS panel. **Methods:** A selection of 30 NSCLC cases assessed with NGS using a 17-gene RNA-based panel (ArcherDx FusionPlex Lung V2) underwent FISH with *MET* and *CEP7* probes. Amplification status was categorized according to the number of *MET* signals per cell and the *MET*/*CEP7* ratio. Tumors were classified as high-amplified *MET* (HAM) if they had >7 *MET* signals/cell on average and/or a *MET*/*CEP7* ratio >2. Overall read metrics and relative expression data were extracted and the numbers of unique reads per gene-specific primers (GSP2) for *MET* were compared to three reference genes on the same or different chromosomes (*KRAS, BRAF, ERBB2*). **Results:** The cohort consisted of 12 non-amplified cases, 12 HAM, and six cases with low/intermediate *MET* amplification. Two non-amplified and two HAM cases were excluded during post-analysis due to a very low number of unique molecular fragments. In non-amplified cases, the average number of reads per GSP2 for *MET* (Chr 7) was 930 compared to 815 for *KRAS* (Chr 12) (P >0,05). In contrast, HAM cases showed a 13-fold increase of the average number of reads per *MET* GSP2 compared to *KRAS* GSP2 (18,340 versus 623, respectively; p<0,05)). The mean value for the number of reads per GSP2 on two other genes (*BRAF*; Chr 7 and *ERBB2*; Chr 17) was combined to determine a baseline reference average value of 1,368 for reference genes, to classify cases according to the *MET* amplification status. Using a cut-off value of 9,138 reads, four out of six low/intermediate (67%) and nine out of 10 HAM (90%) cases were properly classified. **Conclusions:** In this study, simple NGS output data analysis from RNA-based NGS assay allowed reliable prediction of *MET* high-amplification status in NSCLC. Although copy number variation analysis is usually restricted to DNA panels, this study shows that focused analysis can be useful to triage and select samples with candidate *MET* gene amplifications from an RNA-based NGS assay.

ST051. **Looking beyond Genome and Transcriptome in Pancreatic Adenocarcinoma by Darwin OncoTarget and OncoTreat Tests as a New Generation of Diagnosis and Treatment**

I. Manukyan, S. Hsiao, M. Pantrangi, H. Remoti, M. Mansukhani
Columbia University, New York, NY.

Introduction: Pancreatic cancer (PC) remains a dismal disease, with a few non-targetable well-known variants (*KRAS, TP53, CDKN2A*, and *SMAD4*). Clinical trials for PC have failed to improve survival beyond five years. Here we summarize results of transcriptome-based tests using tumor-type-specific interactomes to infer aberrant activation of targetable proteins (Darwin OncoTarget) or to relate inferred protein activity to sublethal drug-induced RNA-expression perturbation data in related tumor cell lines to predict drug susceptibility (Darwin OncoTreat). **Methods:** From January 2020 through March 2022, OncoTarget and OncoTreat analysis were performed on 21 PCs. Gene-specific read information obtained with Illumina whole-transcriptome sequencing was rank-transformed, to a uniform distribution, and the VIPER (Virtual Inference of Protein-activity by Enriched Regulon) algorithm computed relative protein activity levels of 193 actionable onco-proteins. PC regulatory model (interactome) was compared with 11,289 expression profiles from 35 tumor types to determine aberrant activation using a Bonferroni corrected p-value of $\leq$0.00001 (OncoTarget). The VIPER-inferred protein activity of 2,544 transcriptional regulators was also aligned against a proprietary compound perturbation database containing tissue context-specific mechanism of action for 193 FDA-approved drugs and 95 investigational compounds for the ASPC1 and PANC1 PC cell lines cultured with sublethal concentrations of these drugs. Tumor DNA was sequenced using a hybrid-capture library targeting 467 genes, including introns of 72 genes ("CCCP"). Potential fusions were evaluated with the StarFusion tool. **Results:** Activating *KRAS* (14 G12 and two Q61) were seen in all 16 DNA-sequenced cases. DOT1L, SRC, ERBB2, PML, NFKB, MUC1, MAPK3, MSLN, MST1R, HDAC7 and PPARG were the most frequently activated proteins. Eighty-seven percent *KRAS* mutant tumors showed activation of SRC/ERBB2. In two cases without SRC/ERBB2 activation, DOT1L, PML, and HDACs were among the aberrantly activated proteins. OncoTreat test showed significant tumor checkpoint reversion of aberrantly activated DOT1L by Pinometostat in 67% (14/21) in addition to other FDA-approved drugs and experimental compounds. No fusion was identified. **Conclusions:** Our results demonstrate the feasibility of incorporating RNA precision oncology in advanced PC selection of treatment

utilizing master regulator gene targets. Two out of eight patients with recurrent PC in the HIPPOCRATES trial have received treatment based on OncoTarget and OncoTreat results. These ongoing studies will define the role of targeting aberrantly activated proteins in PC.

ST052. **The Clinical Utility of DNA Methylation Profiling in Pediatric CNS Tumors: Comparison of Methylation Classification and Integrated Diagnosis in a Cohort of 184 Pediatric CNS Tumors**

L. Pagliaroli, F. Xu, W. Fu, K. Cao, A. Long, A. Viaene, A. Resnick, P. Storm, M. Santi, M. Li
Children's Hospital of Philadelphia, Philadelphia, PA.

Introduction: DNA methylation profiling has recently emerged as a diagnostic tool for central nervous system (CNS) tumor classification. It is especially helpful when tumor histologic/genomic features are not characteristic. However, the diagnostic implication of methylation profiling in pediatric CNS tumors has not been systematically evaluated. We studied 184 pediatric CNS tumors with Illumina EPIC array to assess the clinical utility of DNA methylation-based classification and discussed its diagnostic role in pediatric neuropathology. **Methods:** One hundred eighty-four pediatric CNS tumors that were previously profiled at CHOP with the Comprehensive Solid Tumor Panel were studied using the Illumina EPIC array (Illumina, Inc., San Diego, CA). DNA samples were bisulfite-treated and fluorescent-labeled before being hybridized to the EPIC array. Raw methylation data (.IDAT files) were uploaded to the DKFZ methylation classifier v12.5, which includes 181 tumor classes and provides scores for different hierarchical levels (superfamily, family, class, subclass) (https://www.molecularneuropathology.org/mnp/). The methylation-based tumor classifications were compared to the integrated diagnoses based on histologic and genomic findings. **Results:** The overall concordant rate between the methylation classification and integrated diagnosis was 89.1% (164/184). When samples were divided into three groups based on the calibrated scores, the concordance rates for group 1 (scores ≥0.8), group 2 (scores <0.8≥0.5), and group 3 (scores <0.5) were 93.1% (134/144), 87.5% (21/24), and 56.3% (9/16), respectively. In five histologically "difficult-to-diagnose" cases, the methylation array was able to help classify them into specific tumor groups, including two CNS sarcomas with a novel *ATXN1::NUTM2A* fusion to CIC-rearranged sarcoma, a high-grade neoplasm to CNS neuroblastoma, FOXR2-activated, and a high-grade brain tumor to diffuse pediatric-type high-grade glioma, H3 wild-type, and IDH wild-type. Follow-up histologic and genomic reviews were performed on eight discordant cases with a calibration score ≥0.8 and reaffirmed integrated diagnoses in six cases with two cases corroborating the methylation classification. **Conclusions:** This large cohort study showed a high concordance between methylation classification and integrated diagnosis in pediatric CNS tumors. Since integrated diagnosis provides detailed histologic features and genomic alterations that are essential to informing patient care, it should remain the primary diagnostic approach for pediatric CNS tumors. However, DNA methylation profiling may be an effective or the only way to identify some of the rare and diagnostically challenging tumors, and should be considered when histologic/genomic findings are not informative.

ST053. **Sentinel-10: A New Multi-Cancer Early Detection Test**

L. Vrba[1], B. Futscher[1], R. Bernert[2], P. Chalasani[3], M. Nelson[1]
[1]Precision Epigenomics, Inc., Tucson, AZ; [2]Precision Epigenomics, Inc., Scottsdale, AZ; [3]University of Arizona Cancer Center, Tucson, AZ.

Introduction: A multi-cancer early detection (MCED) test that can identify cancer from a liquid biopsy, e.g., a blood draw, and can be used across various cancer types is an unmet clinical need. Hypermethylation of DNA is a hallmark of cancer cells and can be exploited for early tumor detection. Since tumors shed DNA into body fluids, DNA methylation analysis of cell-free DNA from blood can be used for minimally invasive cancer tests. Sentinel-10 liquid biopsy is based on a novel set of 10 biomarker loci hypermethylated in carcinomas of bladder, breast, colon, esophagus, head and neck, lung adeno, lung squamous cell, pancreas, prostate, and rectum. We previously demonstrated that Sentinel-10 can detect lung and pancreatic cancers. Here, we present a new bioinformatics analysis revealing the performance of Sentinel-10 in additional cancer types and in a cohort of patients with breast cancer consented to IRB-approved protocol. **Methods:** DNA methylation data from Illumina microarray platforms from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus databases were used to evaluate DNA methylation of the biomarker genomic loci in cancer samples versus normal samples. For the Sentinel-10 liquid biopsy test, methylation-specific qPCR was used to analyze cfDNA extracted from plasma samples obtained from healthy controls (n=44) and women diagnosed with metastatic breast cancer (n=15). **Results:** Our bioinformatics analysis revealed that the Sentinel-10 biomarker loci are hypermethylated in multiple additional cancer types: cervical squamous cell carcinoma, cholangiocarcinoma, diffuse large B-cell lymphoma, glioblastoma, lower-grade glioma, hepatocellular carcinoma, mesothelioma, stomach adenocarcinoma, endometrial carcinoma, and uterine carcinosarcoma (AUC range 0.987-1.0). Therefore, the Sentinel-10 MCED test has the ability to detect 20 cancer types according to TCGA classification with high sensitivity and specificity. Furthermore, the DNA methylation biomarker loci that comprise the Sentinel-10 test are hypermethylated early in cancer progression as illustrated here on prostate and bile duct premalignant lesions. Our clinical study shows that the Sentinel-10 biomarker set can differentiate metastatic breast cancer cases from cancer-free controls with high sensitivity and specificity (AUC=0.982). **Conclusions:** The Sentinel-10 liquid biopsy test can detect breast cancer and has the potential to detect the majority of cancers since the 20 TCGA cancer types presented here account for 73.7% of new cancer cases and 80.5% of cancer deaths worldwide. Sentinel-10 can possibly detect early stages of cancers as soon as tumor DNA becomes present in blood or other biofluids. Sentinel-10 represents an innovative MCED test for cancer patients.

ST054. **Maximizing Molecular Testing Opportunities for Challenging Clinical Samples**

L. Fink[1], B. Jaradi[1], N. Stone[1], L. Anderson[2], P. Leo[2], M. Marshall[2], K. O'Byrne[2]
[1]XING Genomic Services, Sinnamon Park, Australia; [2]Queensland University of Technology, Woolloongabba, Australia.

Introduction: Identification of somatic variants in cancer by next-generation sequencing (NGS) is routine clinical practice to identify predictive biomarkers for targeted therapies. Unfortunately, there can be high-sample QC failure rates for some assays preventing the timely and accurate return of results that may affect patient treatment decisions. SLIMamp is a patented technology that improves PCR efficiency by reducing the generation of unwanted short, overlapping amplicons that compete for PCR reagents. The result is increased sensitivity by preferential amplification of genomic regions of interest and reduction of background sequencing noise. Additionally, SLIMamp allows very high-level multiplexing target enrichment (>1,000 amplicons) in an automatable single tube reaction. Pillar Biosciences has incorporated SLIMamp into NGS cancer test kits with the claim that these kits can successfully interrogate challenging formalin-fixed, paraffin-embedded samples with low tumour content (~10%), poor DNA quality, and/or low input DNA (~2 ng), resulting in a high-sample QC pass rate. The aim of this study was to substantiate that claim using their OncoReveal Solid Tumor Panel (STP) test. **Methods:** We acquired 48 tumour samples that had failed one or more pre-analytical QC sample parameters for comprehensive genomic profiling (CGP) from an independent ISO15189-accredited diagnostic genomics laboratory. We performed an exploratory data analysis using our pre-analytical QC metrics to characterise the samples and then tested the samples in our ISO15189-accredited laboratory using the validated STP test. **Results:** We achieved high sequencing coverage (>3,000X)

for all 48 samples, and we were able to generate clinical reports for 39 samples (81%), of which 36 (75%) contained clinically actionable or significant variants that would not have otherwise been identified. For the nine unreportable samples, we found that the sequencing data were dominated by artefacts and, furthermore, that it was impossible to predict sample unreportability based on pre-analytical QC criteria alone. Instead, we found that applying a novel post-sequencing QC metric, based on the variant allele frequency kernel density, can accurately identify unreportable samples, thereby threading the needle between denying a patient molecular testing and causing patient harm by issuing erroneous test results. **Conclusions:** We demonstrated that the SLIMamp technology is able to successfully test most samples that cannot be tested by CGP or, potentially, conventional amplicon sequencing methods. SLIMamp kits, in combination with our novel post-sequencing QC metric, enable clinical testing for predictive biomarkers on a large proportion of the most challenging clinical samples which would otherwise go untested.

ST055. **Disease Monitoring with a Comprehensive Liquid Biopsy Assay for Patients Receiving Individualized Neoantigen Vaccine**

D. Schenk[1], O. Spiro[2], R. Zhou[2], A. Mantilla[2], A. Ferguson[2], A. Dhanik[2], K. Jooss[2], M. Davis[2]
[1]Gritstone Bio, Cambridge, MA; [2]Gritstone Bio, Emeryville, CA.

Introduction: Circulating-tumor DNA (ctDNA) is an emerging, minimally invasive diagnostic and prognostic biomarker for patients receiving immunotherapy. Tumor-naïve and tumor-informed ctDNA-based assays have been described, yet most assays capture a small fraction of the somatic tumor genome. Comprehensive tumor-informed ctDNA longitudinal monitoring was included in the evaluation of a phase 1/2 clinical trial in patients with late-stage cancers receiving individualized neoantigen-directed immunotherapy in combination with nivolumab and ipilimumab (NCT03639714). We evaluated ctDNA dynamics and tumor evolution over time through a combination approach of a tumor-informed and tumor-naïve ctDNA monitoring assay. **Methods:** Patient-specific hybrid-capture panels were designed for all coding mutations detected in whole-exome sequencing (WES) of archival tissue (median: 123; range: 67-402). A tumor-naïve panel was designed and included in patient panels to monitor recurrently mutated tumor hotspots and genes implicated in immunotherapy resistance. Baseline and on-treatment biopsies were collected when available and subjected to WES. Monthly cell-free DNA (cfDNA) samples were collected on-treatment (mean 7; range: 1-18). Libraries with duplex unique molecular identifiers were prepared from cfDNA, matched normal DNA, and biopsy DNA, and captured using a combination of personalized panels, a universal panel, or WES. Enriched duplex libraries were sequenced to a mean target depth >80,000x prior to consensus deduplication. **Results:** In 24 patients, a median of 92.5% of neoantigens (range: 45%-100%) and a median of 84% (range: 24%-99%) of all targeted variants were found in cfDNA. Indications of heterogeneity were found in both cfDNA and biopsies, and duplex sequencing improved target variant detection in low tumor content biopsies. Combining tumor-informed and tumor-naïve panels, *de novo* variants were found in the cfDNA of 19 patients. The *de novo* variants were discovered in regions targeted by the tumor-naïve panel or where another patient had a targeted variant. For instance, evidence of acquired immune escape was observed in a patient with colorectal cancer via biallelic *TAP1* loss-of-function mutations. Using WES of longitudinal cfDNA in a patient with gastroesophageal adenocarcinoma, copy number changes, including *HLA* loss of heterozygosity, and emerging subclonal variants were corroborated between on-treatment biopsies and cfDNA. **Conclusions:** For patients treated with a neoantigen vaccine, longitudinal monitoring of cfDNA provides early insight into patients responding to treatment. Using a hybrid approach of tumor-informed and tumor-naïve monitoring, the ctDNA dynamics shown by targeting many mutations also track tumor burden, evolution, and emerging resistance.

ST056. **DelPHI: Delivering Precision Health Insights for Timely Treatment of Rhabdomyosarcoma Using Protean MAPS and NAVIFY Digital Tools**

S. Park[1], R. Khalife[1], T. Love[2], A. Gholami[2], A. Markus[2], H. Sorensen[2], A. Magliocco[1]
[1]Protean Biodiagnostics, Orlando, FL; [2]Roche, Santa Clara, CA.

Introduction: Rhabdomyosarcoma (RMS) is the most common of the rare soft-tissue sarcomas that typically affect the head, neck, or genitourinary tract and have a five-year survival rate of <30%. Within the US, the incidence of RMS is six cases per 100,000 per year, and about 87% of cases are individuals under 15 years of age. Here we present a case that underwent pathology review and comprehensive genomic analysis powered by digital decision support tools, which led to the identification of several clinical trials for which the patient was eligible. **Methods:** A 19-year-old male presented with a history of recurrent paratesticular RMS with metastases in the liver. This case was referred for Protean MAPS (Protean Biodiagnostics) analysis, which is a diagnostics service including pathology review, comprehensive in-house molecular testing, and virtual molecular tumor boards. The liver biopsy was available to conduct immunohistochemistry and pathway analyses using OncoSignal (InnoSIGN), a test that determines activity of seven key cancer pathways. Additionally, a liquid biopsy was conducted using Follow It (Canexia Health). Genomic test results were analyzed using NAVIFY Mutation Profiler (RUO – not for diagnostic procedures) and the NAVIFY Clinical Trial Match application (Roche). Subsequently, all Protean MAPS results were aggregated and discussed with the patient's care team using NAVIFY Tumor Board (Roche). **Results:** Molecular analysis of the liver biopsy confirmed a malignant tumor consistent with embryonal RMS and PD-L1 positivity. NAVIFY Mutation Profiler highlighted several potential actionable mutations, most notably, *NRAS* Q61K. *NRAS* is mutated in a significant subset of embryonal RMS cases and is targetable by MEK inhibitors. OncoSignal pathway analysis revealed activation of PI3K and Hedgehog pathways, raising potential for dual blockade by PI3K and MAPK inhibitors. The NAVIFY Clinical Trial Match application provided several relevant, actively recruiting clinical trials targeting PD-L1 and MEK. Two of the trials (NCT04216953; NCT03838042) were further explored by the patient's care team and led to enrollment in a similar trial in their location. This patient's comprehensive testing results were displayed in NAVIFY Tumor Board and discussed with the patient's care team in a virtual molecular Tumor Board meeting, leading to a timely, consensus-driven therapy plan. **Conclusions:** By combining multiple types of molecular analyses, it was uncovered that this patient was eligible for novel clinical trials containing MEK and PD-L1 checkpoint inhibitors. Digital tools for genomic interpretation, clinical trials matching, and molecular tumor board discussions not only provided clinical decision support but also accelerated the path to treatment options.

ST057. **DelPHI: Delivering Precision Health Insights for Timely Diagnosis and Treatment of Epithelioid Sarcoma Using Protean MAPS and NAVIFY Digital Tools**

A. Magliocco[1], S. Park[1], R. Khalife[1], T. Love[2], A. Gholami[2], A. Markus[2], H. Sorensen[2]
[1]Protean Biodiagnostics, Orlando, FL; [2]Roche, Santa Clara, CA.

Introduction: Epithelioid sarcoma (ES) is a rare, commonly misdiagnosed soft-tissue tumor. This rarity poses challenges for diagnosis and understanding disease pathology. We utilized a unique, systematic approach that combines molecular testing with digital decision support tools to diagnose a child's ES and identify viable therapy options. **Methods:** A 10-year-old female presented with a soft-tissue tumor of the mouth and subsequently underwent surgery and traditional chemotherapy. This case was referred for Protean MAPS (Protean Biodiagnostics) analysis, which is a diagnostics service including pathology review, comprehensive in-house molecular testing, and virtual molecular tumor boards. Next-generation sequencing (NGS) was performed using FoundationOne Heme and Tempus xF.

Long-range genome mapping was done using the Saphyr Optical Genome Mapping system (OGM; Bionano Genomics). Genomic test results were analyzed using NAVIFY Mutation Profiler (RUO – not for diagnostic procedures) and the NAVIFY Clinical Trial Match application (Roche). Results were aggregated and discussed with the patient's care team using NAVIFY Tumor Board (Roche). **Results:** NGS and OGM testing revealed findings consistent with an ES diagnosis. Notably, a complete *SMARCB1/INI1* deletion (targetable by tazemetostat) was identified. NAVIFY Mutation Profiler highlighted several key alterations, including a presumed germline splice-altering mutation, *MUTYH* c.934-2A >G. OGM uncovered a partial deletion of chr. 22q and complete deletion of chr. 4, consistent with NGS findings. OGM also revealed a partial deletion of the *FHIT* tumor suppressor, along with structural alterations in 43 other cancer genes. IHC analysis revealed PD-L1 positivity. The NAVIFY Clinical Trial Match application provided several potential clinical trials, including NCT04416568, relevant to *SMARCB1* loss and PD-L1 positivity in children with sarcomas. This patient's comprehensive testing results were displayed in NAVIFY Tumor Board and discussed with the patient's care team in a virtual molecular Tumor Board meeting, leading to a timely, consensus-driven diagnosis and therapy plan. **Conclusions:** NGS in conjunction with OGM demonstrated improved detection of clinically actionable alterations for this patient. Thus, both tests should be routinely considered as a part of the diagnostic regimen to better characterize key mutations in rare tumors and uncover actionable targets for treatment. Combining multiple types of molecular analyses uncovered that this patient was eligible for a novel clinical trial. Digital tools for genomic interpretation, clinical trials matching, and molecular tumor board discussions not only provided clinical decision support but also accelerated the path to treatment options.

ST058. **Mapping Cell Types in Human Breast Ductal Carcinoma Using Xenium *in situ* Platform**

V. Gonzalez Munoz[1], A. Janesick[1], R. Shelansky[1], A. Kim[1], J. Hensel[1], F. Meschi[1], N. Farahani[1], V. Kumar[1], X. Qian[2], S. Taylor[1]
[1]10x Genomics, Pleasanton, CA; [2]10x Genomics, Stockholm, Sweden.

Introduction: Breast cancer accounts for 12% of all new cancer cases worldwide and is the most common cancer globally as of 2021. Although ductal carcinoma *in situ* (DCIS) is this most common type of cancer, it isn't clear which cancers will become metastatic and turn into invasive ductal carcinoma. RNA expression patterns revealed by spatial transcriptomic studies can be used to classify cell types and reveal key gene players in cancer progression to metastasis. **Methods:** Here we use the Xenium platform, a novel, high-throughput *in situ* technology, to map DCIS tumor tissue at the single cell level. Xenium uses a microscopy-based read-out to identify spatial patterns of expression in tissue sections. Using a pre-validated gene panel for human breast, we localized 203 genes with subcellular resolution in a DCIS FFPE sample to identify cell types and clusters. **Results:** Differential expression analysis reveals distinct markers associated with tumor progression. The single cell *in situ* gene expression profiles complement the pathological assessment of the tissue, but provide an additional level of information that is not accessible using traditional methodologies. Here, we were able to identify tumor regions with unique molecular signatures indicative of the progression from DCIS to invasive cancer. **Conclusions:** Using the Xenium workflow in breast DCIS samples, we show that we can accurately identify cell types and discern different tumor regions based on high-resolution spatial gene expression signatures that can complement molecular pathology.

ST059. **Comprehensive Profiling of Copy Number Changes to Assess Genomic Instability**

M. Gupta[1], S. Sadis[2], J. Kilzer[3], V. Mittal[2], D. Cyanam[2], Y. jin[1], G. Lowman[3], E. Wong-ho[1], J. Guo[1], E. Ingerman[1], S. bandla[2], S. Myrand[2], P. Bernardo[1], E. Norris[2], C. Nemes[4], A. Phan[3], L. Pickle[3], T. Puranik[1], M. Toro[3], Y. Tseng[1], P. Williams[2], D. Wong[1], Y. Zhu[1], S. El-Difrawy[1], A. Marcovitz[1]
[1]Thermo Fisher Scientific, South San Francisco, CA; [2]Thermo Fisher Scientific, Ann Arbor, MI; [3]Thermo Fisher Scientific, Carlsbad, CA; [4]Thermo Fisher Scientific, Guilford, CT.

Introduction: Defects in homologous recombination repair (HRR) pathway interfere with the ability to repair DNA double strand breaks, leading to homologous recombination deficiency (HRD). One of the consequences of HRD is accumulation of copy number changes leading to genomic instability. Recently, the sensitivity of certain cancers to DNA damaging therapeutics such as cisplatin and inhibitors of poly (ADP-ribose) polymerase has been associated with HRD. To better understand the incidence and prevalence of HRD in cancer, and its association with HRR gene defects, we developed a novel metric to measure genome instability in formalin-fixed, paraffin-embedded (FFPE) cancer samples. **Methods:** We developed an amplicon-based enrichment next-generation sequencing panel (OCA Plus) using the Ion Torrent Gene Studio S5 system. OCA Plus interrogates 500+ genes relevant to precision oncology including more than 40 genes in the HRR pathway including *BRCA1/2*. To evaluate genomic instability, we measured copy number (CN) log-ratio profiles and determined log odds for thousands of single nucleotide polymorphisms with high minor allele frequencies. Using these measurements, the genome was segmented and the resulting CN profiles for the segments were aggregated into a summary metric to characterize genomic instability. We also used an orthogonal array-based method to determine genomic scarring. **Results:** We profiled hundreds of FFPE cancer specimens of various tumor types including ovarian and prostate cancer. The novel endpoint for genomic instability strongly correlated with genomic scarring as determined by array. As expected, we observed that genomic instability was associated with biallelic mutation of *BRCA1/2*. In addition, we observed high genomic instability scores in samples lacking *BRCA1/2* mutations, suggesting that other components of the HRR pathway may contribute to genomic instability. **Conclusions:** We developed a novel method to determine genomic instability using OCA Plus, which was developed for fast comprehensive genomic profiling of cancer FFPE samples to aid research into precision oncology. By combining genomic instability assessment with DNA repair pathway analysis, OCA Plus will support research into the mechanisms underlying HRD.

ST060. **Combined Low-Pass Whole-Genome and Targeted Sequencing Identifies Causative Mutations and Associated Genomic Scarring Indicative of Homologous Recombination Deficiency**

J. An[1], G. Belbin[2], C. Mazur[2], J. Li[2], J. Pickrell[2], D. Metzger[1], S. Gao[1], E. Van Roey[1], R. Seager[1], S. Pabla[1], D. Dash[1], J. Conroy[1]
[1]OmniSeq (Labcorp), Buffalo, NY; [2]Gencove, New York, NY.

Introduction: Comprehensive genomic profiling (CGP) using targeted next-generation sequencing (tNGS) allows for the simultaneous assessment of multiple therapeutic biomarkers within a single assay. However, most tNGS approaches are not designed to assess genome-wide copy number variation (CNV) or the genomic scars associated with homologous recombination deficiency (HRD), such as loss of heterozygosity (LOH), large-scale state transitions and telomeric allelic imbalance. In this study, a low-pass whole-genome sequencing (LP-WGS)-based assay was developed to run in parallel with tNGS to support simultaneous evaluation of HRD causative mutations and genome-wide scarring. **Methods:** LP-WGS and tNGS were performed on 96 samples from 16 tumor types. LP-WGS libraries were prepared using Nextera (Illumina) using 0.4ng DNA input, and sequenced to 0.5-1x coverage. tNGS libraries were prepared using TSO500 (Illumina) using 40-80ng input, and sequenced to >150x unique read coverage.

Regions of CNV using CNVKit v0.9.6, and regions of LOH were estimated using a novel ancestry-aware method. Small variant detection was performed using the TSO500 v2.2.0.12 analysis pipeline. SNP array analysis of 12 tumor samples using OncoScan (Thermo Fisher) was also performed. CNV and LOH estimates derived from LP-WGS, TSO500 and SNP array data were calculated using Jaccard similarity. **Results:** We observed near-perfect levels of regional concordance between CNV gains across samples (Jaccard index=1.0), as well as for CNV losses between LP-WGS and SNP arrays (Jaccard index=1.0), and additionally noted that LPS-WGS calls captured both CNV loss and gains that were not detectable via the SNP array. We also observed high concordance between regions of the genome called LOH between both platforms (median Jaccard index=0.70, IQR=0.254), but noted an attenuation of sensitivity in samples where estimated tumor heterogeneity was high. We also evaluated LP-WGS CNV calls against the TSO500 assay and noted high sensitivity (79%, 90%) and specificity (97%, 97%) for both CNV gains and losses, respectively. **Conclusions:** CGP workflows incorporating LP-WGS with tNGS can support simultaneous evaluation of *BRCA1/2* mutations, other HRD causative mutations, and genome-wide scarring. Together, this approach can provide a more complete assessment of HRD, which is essential for identifying patients who may obtain clinical benefit from treatment with poly (ADP-ribose) polymerase inhibitors.

ST061. **Application of Whole-Genome Sequencing to Evaluation of Gene Fusions in Solid Tumors**

L. Sun, D. Russler-Germain, M. Schroeder, F. Du, S. Devarakonda, J. Robinson, A. Khanna, D. Spencer, E. Duncavage
Washington University in St. Louis School of Medicine, Saint Louis, MO.

Introduction: The identification of recurrent gene fusions has significant diagnostic, prognostic, and therapeutic implications across many solid tumor types. Current methods of detection such as fluorescence *in situ* hybridization (FISH), PCR, and targeted sequencing are limited by selection of regions of interest. We thus hypothesized that whole-genome sequencing (WGS) would be able to perform comprehensive gene fusion analysis in solid tumors, with simultaneous discovery of additional genetic mutations. **Methods:** This study was conducted with institutional review board approval. Formalin-fixed, paraffin-embedded (FFPE) blocks were retrospectively retrieved from five lung adenocarcinomas, seven diffuse large B-cell lymphomas, and 24 fusion-associated sarcomas. After DNA extraction and WGS library preparation, sequencing was performed on NovaSeq 6000 instruments. A tumor-only custom variant analysis workflow was conducted. Results were reviewed by board-certified molecular pathologists and compared to prior testing results. **Results:** During the clinical work-up, FISH and targeted RNA and DNA sequencing had been previously performed to assess gene fusions in 30 of the 36 samples, with 22 positive fusions/rearrangements identified. Seven cases additionally had pathogenic or targetable single nucleotide variants (SNVs) and small insertions/deletions discovered during prior sequencing. In the current study, all 36 cases passed quality-control metrics after whole-genome library preparation and sequencing. The mean average coverage was 45.8X (standard deviation 6.5) per sample. On average, 99.6% of reads were mapped, with an average of 86.7% unique reads. The mean insert size was 98.2bp. Copy number alterations including large deletions and duplications were detected in 34 of 36 specimens (94%). Thirteen of the 22 previously detected gene rearrangements were identified using WGS (59% positive agreement). New translocations were detected in six of the 14 cases with either no or negative prior testing (43%). Verification of these new translocations is ongoing. The same SNVs were detected in three of the seven cases (43%) with prior pathogenic/targetable variants, including variants in *EGFR* and *KRAS* in lung adenocarcinoma. **Conclusions:** WGS is a potential option for comprehensive genomic profiling in solid tumors, including fusion gene assessment. The methodology and analysis require optimization for FFPE tissue, which presents challenges due to DNA fragmentation. This optimization is part of ongoing work.

ST062. **High-Definition PCR (HDPCR) Detection of Precision Biomarkers in Non-Small Cell Lung Cancer Samples**

B. Leatham, K. McNall, H. Subramanian, J. Alvarado, D. Yurk, L. Jacky, M. Wang, A. Rajagopal, I. Regoli, K. Cabrera, B. Brown, J. Schwartz
ChromaCode, Carlsbad, CA.

Introduction: Based on a 2020 ACS CAN survey of cancer patients and survivors, only 33% had received biomarker testing. Of those patients not tested, more than 25% reported it was because of high cost or insurance issues. Thus, to help ensure that currently underserved cancer patients have access to biomarker testing, a low-cost alternative to next-generation sequencing (NGS) is necessary. Due to high sensitivity, fast turnaround, and familiar workflow, digital PCR (dPCR) is an ideal platform for detecting genomic variants. However, dPCR is limited in practical applications like oncology, where the number of biomarkers exceeds the number of available color channels on the instrument. ChromaCode's High Definition PCR (HDPCR) technology breaks the conventional limitations, by enabling a 10-fold increase in the number of targets detected per color channel. Here we present a prototype, research use only (RUO) HDPCR assay for multiplexed detection of 14 DNA variants and 15 RNA fusion biomarkers associated with non-small cell lung cancer (NSCLC). **Methods:** To characterize assay performance, a mixture of formalin-fixed, paraffin-embedded (FFPE) contrived (negative RNA or DNA FFPE matrix spiked with target RNA or DNA), cell-free contrived (negative plasma spiked with target RNA or DNA), and retrospective NGS characterized samples was evaluated on two different dPCR platforms (QuantStudio Absolute Q and Qiagen QIAcuity). Mutant allele fractions ranging between 0.25%-20% for cell-free contrived samples and between 1%-40% for FFPE contrived samples were tested. Data analysis was performed utilizing proprietary algorithms housed on ChromaCode Cloud software. **Results:** The assay detected as low as 10 variant copies in a 10,000 haploid human genome copy DNA background. The *EGFR* L858R, *KRAS* G12C, *EGFR* exon 20 H773 dup, and *EGFR* E746_A750del mutations all yielded results from the assay that 100% aligned with the specimens tested. All other mutations tested yielded results from the HDPCR assay with >94% negative percent agreement and positive percent agreement. **Conclusions:** This HDPCR prototype assay includes relevant research biomarkers in current National Comprehensive Cancer Network (NCCN) guidelines, including variants in *EGFR, BRAF, KRAS, ERBB2, ALK, ROS1, RET, MET*, and *NTRK1/2/3*, and has shown sensitive and specific performance. In addition, this assay leverages a proprietary bioinformatics suite, the ChromaCode Cloud, to report relevant variants with the click of a button, allowing for an easily adopted solution to testing needs.

ST063. **Exome Capture-Based RNA Sequencing (RNA-Seq) Increases Gene Fusion Detection Clinical Sensitivity in Pediatric Solid Tumors**

R. Schmidt, J. Ji, D. Maglinte, D. Ostrow, G. Raca, M. Sun, J. Buckley, X. Gai, J. Biegel
Children's Hospital Los Angeles, Los Angeles, CA.

Introduction: Gene fusions represent an important class of molecular alterations that drive tumorigenesis in pediatric solid tumors and are frequently associated with diagnostic and/or therapeutic clinical significance. We have developed an RNA-Seq assay for gene fusion detection based on exome hybridization capture that performs well with low-quality and fragmented nucleic acid from formalin-fixed, paraffin-embedded (FFPE) specimens. **Methods:** Sequencing libraries were generated from DNase-treated total RNA using the KAPA RNA HyperPrep Kit (Roche) followed by Comprehensive Exome capture (Twist Biosciences) using the Fast Hybridization Target Enrichment Protocol. Additionally, 2x100 bp paired-end sequencing was performed using either an Illumina NextSeq 500 or HiSeq 4000 sequencer. Gene

fusions were identified using an ensemble of four fusion callers (Arriba, FusionCatcher, STAR-Fusion, Illumina DRAGEN RNA). Candidate gene fusions, identified by at least two fusion callers, were further evaluated for technical validity and potential clinical significance using custom software (Genetrix). Novel gene fusions were confirmed by RT-PCR and Sanger sequencing. **Results:** A pan-cancer validation study was performed with 100 unique specimens which demonstrated greater than 90% agreement with our targeted amplification-based sequencing panel (OncoKids) and 100% concordance for intra-run, inter-run, inter-operator, and inter-instrument comparisons. Fusions were detected in dilutions down to 11% fusion-positive specimen for all specimen types. Ninety-five percent of fusions identified that were not in the OncoKids test design were confirmed. When applied clinically to solid tumors, our assay detected several novel fusions as well as promoter swap gene fusions that included only a short non-coding first exon from the 5' gene partner. Rare or novel fusions identified included an *RCC1*::*LCK* fusion in an unclassifiable malignant spindle cell sarcoma and an *MYB*::*DDX1* fusion in a case of alveolar rhabdomyosarcoma that lacked fusions involving the *PAX3*, *PAX7*, and *FOXO1* genes. Four *USP6*-associated neoplasms that were positive for *USP6* break-apart fluorescence *in situ* hybridization but did not have a *USP6* fusion identified by targeted sequencing were analyzed. *USP6* fusions were identified by RNA-Seq in two of four cases, including a novel fusion with the *ZFX* gene. **Conclusions:** Exome capture-based RNA-Seq is a robust and reproducible method that detects gene fusions with a comparable sensitivity to our targeted amplification-based sequencing panel. Additionally, our test detected rare or novel gene fusion events with high-level agreement with confirmatory testing. Exome capture-based RNA-Seq performs well with low-quality fragmented nucleic acid from FFPE specimens. This method provides an excellent clinical testing platform for the characterization of solid tumors.

ST064. **Clinical Implementation of Pan-Cancer Mutational Signature Analysis and Landscape across 13,000 Solid Tumors**

J. Nowak[1], L. Sholl[1], P. Davineni[1], M. Manam[1], P. Shivdasani[1], F. Dong[1], F. Kuo[1], N. Lindeman[1], L. MacConaill[2]
[1]Brigham and Women's Hospital, Boston, MA; [2]Dana-Farber Cancer Institute, Boston, MA.

Introduction: Mutational signatures represent stereotypic patterns of nucleotide alterations that reflect specific mutagenic events and can harbor diagnostic, prognostic, and predictive potential. Herein, we describe the development and validation of a mutational signature-calling algorithm termed STAMP (Signatures from Targeted-assay Mutational Profiles) for a 447-gene tumor-only next-generation sequencing (NGS) panel and describe the observations derived from application of STAMP in routine reporting of 13,319 tumors sequenced as part of standard clinical care or following consent to an institutional research protocol. **Methods:** Signature rules based on single nucleotide variants, dinucleotide change, and short insertions/deletions were built based on published observations correlating DNA substitution patterns, tumor type, and mutational etiology for six signatures (MMR deficiency, tobacco smoke, UV light exposure, APOBEC activity, *POLE* mutation, alkylating agent exposure). Rule performance was orthogonally validated using MMR protein IHC or MSI status (MMR signature) across 431 cases or known clinical or molecular characteristics associated with each signature (smoking history, sun-exposed anatomic location, HPV-positivity, known *POLE* hotspot mutation, history of temozolomide) across 893 cases. Following validation, STAMP was deployed to our NGS pipeline in 2017, with results available for interpretation and integration with other data at the time of report preparation. **Results:** Across 13,319 cases, 10.2% were positive for any signature, with UV, tobacco, MMR, APOBEC, *POLE*, and alkylating agent signatures identified in decreasing frequency; 0.3% of cases were positive for multiple signatures. Median tumor mutation burden ranged from 15 for tobacco signature cases to 119 for *POLE* signature cases. Although MMR, APOBEC and *POLE* signatures were seen in many tumor types, UV and alkylating agent signatures were largely confined to melanomas and gliomas, respectively. Mutational signatures, most commonly UV, tobacco, and MMR, were detected in 15% of cancers of unknown primary origin. Genomic correlate analyses showed enrichment of MMR gene loss of function alterations in MMR, alkylating agent, and *POLE* signatures cases, whereas *B2M* loss of function alterations were enriched in MMR and *POLE* signatures cases and *CD274* (PD-L1) amplification was enriched in *POLE* signature cases. Tumors with an MMR or *POLE* signature showed less aneuploidy than signature-negative tumors. **Conclusions:** Mutational signatures beyond MMR deficiency can be detected during routine clinical interpretation of NGS results from targeted, tumor-only sequencing. In this setting, signature analysis can contextualize tumor mutational burden, inform diagnosis, and shed light on tumor biology related to common mutagenic processes.

ST065. **Detecting Actionable Structural Variants with 3D Genomics in Solid Tumors with Unknown Drivers from Formalin-Fixed, Paraffin-Embedded Samples**

K. Galbraith[1], Y. Yang[2], M. Hussein[2], K. Sikkink[3], M. Movahed-Ezazi[2], I. Tran[2], B. Zeck[2], L. Chiriboga[2], A. Tsirigos[2], A. Schmitt[3], S. Chiang[4], G. Jour[2], M. Snuderl[2]
[1]NYU Langone Health, Mineola, NY; [2]NYU Langone Health, New York, NY; [3]Arima Genomics, San Diego, CA; [4]Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: Gene rearrangements are drivers and therapeutic targets in various types of solid tumors. However, RNA next-generation sequencing (NGS) panels cover a limited number of genes, are rarely successful in formalin-fixed, paraffin-embedded (FFPE) samples more than five years old, and cannot detect rearrangements between genes and non-coding regulatory regions. We developed a novel whole-genome DNA-based partner-agnostic approach for identifying rearrangements between coding and noncoding DNA regions in solid tumors from archival FFPE samples. **Methods:** DNA for all tumors was extracted from FFPE scrolls of 63 tumors in which clinically validated DNA and RNA NGS panels failed to identify any drivers including primary CNS tumors, gynecological sarcomas, and solid hematological tumors (CNS lymphoma / plasmacytoma) up to 10 years old. Samples were processed using Arima-HiC+ FFPE sample protocol, consisting of chromatin fragmentation, labeling, and re-ligation, followed by DNA purification and library preparation for paired-end Illumina whole genome sequencing with an average of 10X genome coverage (100M PE reads per sample). Data were analyzed using the Arima-SV pipeline using Juicer and HiCUP, SV detection using HiC-Breakfinder, loop calling using Juicer Tools, and integrative data visualization using Juicebox. Overexpression of putative driver genes was confirmed by immunohistochemistry. The clinical significance of detected fusions was assessed using NCCN guidelines, WHO classification criteria, and OncoKB, and classified as a therapeutic target (e.g., *PD-L1, NTRK, MET*), diagnostic/prognostic biomarker, potential significance, or unknown significance. **Results:** HiC profiling identified previously undetected rearrangements in 71% (45/63) of previously molecularly negative tumors. Of those, 39.7% (25/63) of tumors had fusions involving a potential therapeutic target and 12.7% (8/63) of fusions involved a diagnostic or prognostic biomarker, indicating an overall clinical yield of 52.4%. These included actionable fusions such as *NTRK3*, and *PD-L1* fusions and tumor-defining diagnostic fusions such as *MYBL1* rearrangement. In addition, 19% (12/63) had fusions of potential clinical significance, according to OncoKB. We also identified fusions between coding regions and non-coding regulatory domains, such as gene promoters. **Conclusions:** Genome-wide Hi-C NGS is successful in detecting gene fusions and cryptic rearrangements in archival FFPE tissue up to 10 years old, including degraded samples. Whole-genome Hi-C NGS expands our ability to detect actionable and novel drivers, and identifies potentially new therapeutic targets in a single NGS workflow.

ST066. **Pan-Cancer Detection by Cell-Free DNA 5-Hydroxymethylcytosine**

Z. Li[1], J. Shao[2], P. McShane[2], N. Garcia[2], R. Singh[2], R. Olsen[1], C. He[3]
[1]Houston Methodist Hospital, Houston, TX; [2]Houston Methodist Research Institute, Houston, TX; [3]The University of Chicago, Chicago, IL.

Introduction: Early cancer detection enables faster therapeutic intervention, significantly improves clinical outcomes, and greatly reduces financial burden. 5-hydroxymethylcytosine (5hmC) is the first oxidative product in the demethylation of 5-methylcytosine (5mC) by 10 to 11 translocation proteins. 5hmC changes early and dynamically in cancer. Using highly sensitive technologies, genome-wide 5hmC distribution can be quantified using very low DNA input (<10 ng) from plasma. However, the utility of plasma cell-free DNA (cfDNA) 5hmC for the detection of most common malignancies has not been investigated. **Methods:** Using a highly sensitive nano-5hmC-Seal and next-generation sequencing method, we profiled the genome-wide distribution of 5hmC in plasma cfDNA from 412 patients with bladder (n = 41), breast (n = 62), colorectal (n = 45), kidney (n = 54), lung (n = 57), ovarian (n = 11), prostate (n = 125), or uterine (n = 17) cancer and 221 controls. We randomly split the samples into training and validation sets. In the training sets, we analyzed differentially hydroxymethylated regions (DhMRs) to identify plasma cfDNA 5hmC signatures for cancer detection and cancer origin determination and then validated the signatures in the validation sets. **Results:** We discovered a signature of DhMRs that detected all eight cancers with sensitivity of 65.5% and specificity of 95.5%. We also discovered six cancer-specific signatures with a sensitivity of 81.2% for bladder cancer, 84.0% for breast cancer, 88.9% for colorectal cancer, 85.7% for kidney cancer, 86.4% for lung cancer, and 90.0% for prostate cancer, and a specificity of 100% for all except breast (92.0%) and prostate (97.7%) cancer. The area under the curve (AUC) of the signatures ranged from 89.0% [95% confidence interval (CI), 79.2%-98.8%] in breast cancer to 96.9% (95% CI, 92.6%-100.0%) in lung cancer in the validation set. The sensitivity of cancer-specific signatures was high in early-stage cancers (90.0%-92.2%) and post-surgery samples (66.7%-88.2%). Moreover, we discovered a 5hmC signature that could determine cancer origin with a prediction accuracy of 67.9% for all samples, 58.8% for bladder, 83.3% for breast, 100.0% for colorectal, 59.1% for kidney, 83.2% for lung, 67.2% for prostate, and 66.7% for uterine cancer. The AUC of the cancer origin prediction signature was 89.0% (95% CI, 85.7%-92.5%). **Conclusions:** We demonstrated that plasma cfDNA 5hmC is a highly sensitive biomarker in early detection of common cancers. The study also sheds light on the potential of plasma cfDNA 5hmC for minimal residual disease monitoring in cancer. Our results provide a solid foundation for future clinical application of plasma cfDNA 5hmC as an accurate and non-invasive biomarker for pan-cancer.

ST067. **A Novel, Single-Tube, Barcoded Next-Generation Sequencing Library Preparation Approach for Detection of Circulating Tumor DNA Using Personalized Patient Panels**

T. Godfrey[1], E. Kintsurashvili[1], A. Devaiah[1], G. Rasic[1], C. D'Amato[2], J. Kaur[2], R. Meltzer[2]
[1]Boston University, Boston, MA; [2]Fluent Biosciences, Watertown, MA.

Introduction: Circulating tumor DNA (ctDNA) is a promising biomarker for cancer. Applications for ctDNA analysis include screening, diagnosis, treatment selection, minimal residual disease (MRD) detection, and recurrence monitoring. Although some applications are best served by panels covering large regions of the genome, such large panels are not universally appropriate due to limitations in sensitivity, user friendliness, turnaround time, and sequencing cost. One such application where smaller, customized panels are preferable is detection of MRD and cancer recurrence, and studies have shown that ctDNA can detect residual/recurrent disease several months earlier than routine clinical imaging. This application requires high sensitivity and in most cases will involve repeat testing of longitudinal samples collected over several years. In this scenario, the ideal assay characteristics include small, personalized assays (5-20 target mutations), with a simple, rapid workflow that can be performed on-site, and with minimal cost per assay. We have developed our assay to meet these requirements. **Methods:** We have developed a variation on SiMSen-Seq (Anders Ståhlberg A. *et al.*, 2016) that uses digital sample partitioning and modified primer sequences to facilitate uniform amplification of multiple target amplicons in a single-tube library construction protocol that can be completed in less than three hours. Pre-templated Instant Partitions (PIPs) from Fluent BioSciences are used to enable simple, rapid, and uniform sample and reagent partitioning into >100,000 reactions in a PCR-compatible format without complex instrumentation or microfluidic droplet generators. Using this approach, single amplifiable target DNA molecules are stochastically loaded into digital partitions, barcoded, and amplified with adaptors compatible with Illumina sequencers. Single-target loading of partitions facilitates uniform amplification of all targets by eliminating competition and minimizing formation of off-target products. Barcode information is used to compile reads from individual DNA molecules and minimize base calling errors introduced during PCR and sequencing. **Results:** Here we present data demonstrating uniformity and sensitivity of our approach for ctDNA detection using control DNA samples. In addition, we will present results of MRD and recurrence detection studies on longitudinal plasma samples from head and neck squamous cell carcinoma patients using personalized, multiplex assays. **Conclusions:** Our simple and rapid, single-tube next-generation sequencing library preparation protocol enables highly sensitive detection of ctDNA in plasma of cancer patients. This approach improves upon the original two-step SimSen-Seq protocol and could easily be implemented in clinical diagnostics laboratories at the site of care.

ST068. **Overcoming FFPE Hurdles to Enable High-Quality Hybrid Capture Libraries**

M. Heider, P. Liu, J. Sun, B. Sexton, B. Langhorst, A. Gray, L. Higgins, L. Chen, L. Apone, T. Evans, N. Nichols
New England Biolabs, Inc., Ipswich, MA.

Introduction: In cancer genomics, a common source of DNA is formalin-fixed, paraffin-embedded (FFPE) tissue from patient surgical samples, where in most cases high-quality fresh or frozen tissue samples are not available. FFPE DNA poses many notable challenges for preparing next-generation sequencing libraries, including low input amounts and highly variable damage from fixation, storage, and extraction methods. Due to the high cost of sequencing and variability of coverage, regions of interest are often specifically enriched using hybrid capture-based approaches, but these methods require a high input of diverse, uniform DNA libraries to achieve the coverage required for somatic mutation identification in tumor samples. **Methods:** We applied a multifaceted approach to successfully generate high-quality libraries from a broad range of FFPE DNA sample qualities. We developed a new DNA repair enzyme mix, enzymatic fragmentation mix, and PCR master mix, optimizing the activities of these mixes using FFPE samples ranging from DIN 1.8 to 6.8 to maximize yield, pre-capture library quality, and target enrichment library performance. The use of unique molecular identifier-containing adaptors ensured accurate duplicate marking for deep sequencing. Using tumor-normal pairs of FFPE DNA with matched frozen DNA from cancer patient samples as well as formalin-compromised reference standard DNA (Horizon Discovery), we validated the performance of these new enzyme mixes in somatic variant calling with a truth set of known variants. **Results:** Combining DNA damage repair and a novel enzymatic fragmentation mix upstream of library preparation not only reduced the false-positive rate in somatic variant detection by repairing damage-derived mutations, but also improved the library yield, library quality metrics (including mapping, chimeras, and properly paired reads), library complexity, coverage uniformity, and hybrid capture library quality metrics. Finally, a new PCR master mix boosts the library yield without compromising library quality in FFPE samples, allowing flexibility in the PCR cycles

used to accommodate the high-throughput processing of FFPE samples of highly varied quality. **Conclusions:** This new suite of enzyme mixes allows even the most damaged FFPE samples to achieve high-quality libraries with sufficient input for hybrid capture. Increasing the useable reads and coverage enables the robust detection of somatic variants as demonstrated using both reference standard DNA and real patient FFPE samples.

ST069. **Optical Genome Mapping Workflow for Somatic Abnormality Detection in Multiple Solid Tumor Types**

B. Clifford[1], A. Wing Chun Pang[2], M. Oldakowski[1], A. Chaubey[1], A. Hastie[1]
[1]Bionano Genomics, San Diego, CA; [2]Bionano Genomics, Toronto, Ontario, Canada.

Introduction: Solid tumors are often characterized by a high degree of complex somatic structural variants of multiple classes, especially rearrangements and copy number variants. Characterizing this genomic complexity is crucial for understanding the biology behind carcinogenesis – but is challenging because of limitations in current genomic technology classes: cytogenic (low resolution) and molecular (poor sensitivity for structural variation). Accurate assessment of genomic structural variation is important because some tumors acquire growth advantage by amplifying or creating oncogenes by fusing otherwise non-pathogenic genes and by deleting/inactivating tumor suppressor genes. **Methods:** Fusions are generally detected through targeted assays like next-generation sequencing panels, PCR, and fluorescence *in situ* hybridization, or through karyotyping. However, high-resolution genome-wide approaches to detect fusions are needed. Optical genome mapping (OGM) is able to fill this gap, providing high resolution (~3kbp breakpoint precision) and ability to span repetitive and complicated genomic regions. Here we demonstrate a simple OGM workflow for the analysis of tumor biopsies, applying it to varying types (bladder, brain, breast, colon, kidney, liver, lung, ovary, prostate, thyroid, tongue). **Results:** Ultra-high-molecular-weight DNA was isolated from snap-frozen tissue from 6.5-18mg biopsies, then labeled and collected on a Saphyr Instrument. The resulting analyses produced variants annotated against GRCh38 and filtered within Bionano Access software to enrich for somatic variants by filtering against a control database (≤1% presence) and for those that overlap with gene(s). OGM variants are further evaluated in NxClinical variant classification software, which enables automated scoring, additional databases and knowledgebases, and ability to overlay orthogonal data. Based on this analysis, we have identified copy number variants involving oncogenes and tumor suppressors (*ERBB2, CDKN2A, NF1*; many others) as well as numerous novel fusions in our comprehensive characterization of these complex genomes. **Conclusions:** Based on our survey of multiple solid tumor types, OGM data are well suited to identify manifold variants relevant to carcinogenesis of solid tumors. The analytical approach directs focus to the most relevant findings, and newly integrated software tools enable extremely comprehensive variant annotation, providing practical new utilities.

ST070. **Development of AcroMetrix Multi-Analyte cfTNA (Cell-Free Total Nucleic Acid) Human Plasma Controls**

Q. Gan[1], M. Wagner[1], N. Siepert[2], T. Jayaweera[2], R. Hu[1], L. Liu[1]
[1]Thermo Fisher Scientific, Fremont, CA; [2]Thermo Fisher Scientific, Austin, TX.

Introduction: Cell-free total nucleic acid (cfTNA) from liquid biopsy samples is an important biomarker and has enormous potential to be used in clinical diagnostics and treatment monitoring in oncology. Because of this clinical importance, there is a high demand for quality-control products containing specific cell-free DNA (cfDNA) and RNA (cfRNA) targets for molecular diagnostic assays. However, due to the fleeting stability of the naked fragmented nucleic acid, especially RNA, in human plasma or serum, it is challenging to produce a cfTNA plasma control with sufficient sensitivity for specific targets. There are a few cfDNA control products commercially available either in buffer, synthetic plasma matrix, or real human plasma, whereas cfRNA controls are limited to buffer formats only. Therefore, we developed a method to stabilize both fragmented DNA and RNA in real human plasma. We utilized this technology to develop a multi-analyte cfTNA human plasma controls, which contain both DNA and RNA variants that cover many different cancer alterations including single nucleotide variant, insertion and deletion, copy number variation (CNV), and fusion RNA. **Methods:** We created multi-analyte cfTNA controls in human plasma matrix using proprietary method to mimic the patient cfTNA in plasma. (This control is currently in development and Microgenics has not applied for nor received clearance by FDA at this time.) The cfTNA consists of a mixture of synthetic DNA and RNA blended with fragmented genomic DNA and total RNA derived from GM24385, the well-characterized Genome in a Bottle cell line. The endogenous DNA and RNA was stripped from the human plasma matrix and the remnants were characterized. Multi-analyte cfTNA that carries different variant types at the specific targeting frequencies was then spiked into the plasma matrix with a proprietary method. The control was then extracted and tested using Qubit dsDNA assay, RT-qPCR, Bio-Rad ddPCR, and next-generation sequencing assays. **Results:** The AcroMetrix Multi-Analyte cfTNA Plasma Controls have been carefully formulated to mimic naturally occurring cfTNA in human specimens, which allow for effective verification of the cfTNA extraction procedures. DNA and RNA yield may vary depending on the extraction and quantification method, but the DNA variant allele frequency, CNV, and synthetic RNA copy number per nanogram of total RNA are compatible to the controls in the buffer format. Accelerated stability data support 24 months' shelf-life stored at -80°C. **Conclusions:** This full-process cfTNA control can be used for proficiency and training in test procedures designed for extraction and measuring both cfDNA and cfRNA in human plasma. In addition, this technology has the potential to stabilize cfTNA in other human body fluids such as urine and saliva.

ST071. **Evaluation and Reproducibility Study Using NanoString GeoMx Immune Pathway Panels**

M. LaFaivre, J. Sims, E. Parilla Castellar
Q2 Solutions, Inc., Durham, NC.

Introduction: The rapidly advancing field of spatial biology has led to development of new platforms which provide measurements of gene and protein expression and their precise localization to defined tissue regions. The NanoString GeoMx DSP (digital spatial profiling) platform enables spatial profiling using formalin-fixed, paraffin-embedded (FFPE) tumor biopsy samples with a read-out using either the nCounter or next-generation sequencing. In this study, Q^2 Solutions evaluated both healthy tonsil sections and a well-characterized breast carcinoma biopsy in a proof-of-concept study examining variability across days, patient biopsies, and sample stability. **Methods:** FFPE tissue biopsy sections were hybridized to RNA probes or antibodies conjugated with UV photocleavable oligonucleotide tags to a targeted panel of genes or proteins within the Immune Pathways RNA panel or Immune Cell Profiling Core panel. The slide is then scanned by the DSP and the pathologist will then select regions of interest (ROIs). In this study, PanCK (epithelium), CD45 (immune cells), and Syto13 (DNA) were used as morphology markers. The ROIs can then be segmented into discrete biological compartments or areas of illumination (AOI) for precise profiling. Once the ROIs were selected, the oligos were cleaved, deposited into a plate, pooled and processed using the nCounter workflow for quantification. The resulting data were transferred back to the DSP for further analysis as well as evaluated using a Q^2 Solutions developed tool. **Results:** Two healthy donor tonsil biopsies and three breast cancer biopsies were analyzed across two independent runs collecting six AOIs per slide. Variance among samples tested on different days was <30% both within and across tissue samples. Unsupervised clustering of the tonsil biopsies stratified germinal center AOIs from mantle zone AOIs. Similar analysis of the breast cancer biopsies stratified intra-tumoral regions from infiltrating boarder immune microenvironments and *in situ* from invasive

carcinoma regions and normal periductal versus invasive carcinoma immune microenvironments. **Conclusions:** The NanoString GeoMx DSP platform provides a robust method for investigating spatial relationships within an FFPE tissue section. There is low variability across sections and runs, but the platform provides the opportunity to clearly stratify cell populations, tissue areas, and cellular states.

ST072. **NGS Copy Number Signatures in the Assessment of Cancers of Unknown Origin: Targeting Therapy**

B. Hollister[1], C. Lum[2], Z. Halford[3], J. Yip[4], j. Pon[3], A. Woron[3]
[1]John A Burns School of Medicine, Honolulu, HI; [2]Queens Medical Center, Honolulu, HI; [3]Diagnostic Laboratory Services, Inc., Honolulu, HI; [4]Hawaii Residency Program, Honolulu, HI.

Introduction: Thirty to eighty thousand cancers are diagnosed where the site of origin is not known. This can obscure eligibility for targeted therapies and insurance coverage. Standard chemotherapy for cancers of unknown primary is in general more toxic. Copy number variation (CNV) analysis is a component of routine next-generation sequencing (NGS) tumor profiling. CNV can be applied within molecular diagnostics to assess the eligibility for treatment and also generate a signature that can aid in the determination of the primary tumor site. Bionano's NxClinical software compares a patient's tumor to a national database (The Cancer Genome Atlas [TCGA]) to identify the tissue of origin. We tested the performance of the NxClinical software in identifying the tissue of origin compared to standard morphology and immunophenotypic diagnosis. We further compared the ability of this method using a database generated from copy number changes of a trained tumor database using tumor categories from our NGS validation dataset. **Methods:** We challenged this process against 64 solid tumors (21 unique diagnoses across 11 organ systems) using genomic data on CNVs from TCGA tumor signatures. We recorded the five highest percent similarities and their diagnoses for the TCGA dataset. We then tailored our own tumor signature profile based on these 64 tumors and recorded the five highest percent similarities and determined their site of origin as a control series. **Results:** The accuracy of tumor identification for using the TCGA profile was 31.25%. The correct diagnosis was present in the top five options 62.5% of the time. The accuracy of tumor identification using a custom profile based on our own tumor collection was 81.25%. The correct diagnosis was present in the top five options 98.44% of the time. **Conclusions:** A software application compared known tumor copy number signatures to correctly identify known tumor types about one-third of the time. This study suggests that tailoring a custom profile based on similar extraction methods, sequencing methods, and gene coverage to help identify unknown tumor types increased accuracy. An important next step to assess the utility of this process will be to test cases outside of the training dataset. If successful, we hope this workflow will improve the identification of tumors with unknown origins. Adding additional known tumor types to the database may continue to build on this workflow's performance.

ST073. **Development of New Multianalyte NGS Controls with Microsatellite Instability, Tumor Mutation Burden and Loss of Heterozygosity Variants**

L. Liu[1], Q. Gan[1], R. Hu[1], B. Pursley[2], P. Waggoner[2], M. Wagner[1]
[1]Thermo Fisher Scientific, Fremont, CA; [2]Thermo Fisher Scientific, Guildford, CT.

Introduction: As targeted next-generation sequencing (NGS) continues to rapidly grow in the oncology and applied markets, the number and variety of assays, content, and clinically relevant endpoints continue to grow as well. The need for multianalyte NGS controls that cover most of the mutation variants is emerging; however, the existing quality-control materials are limited only to certain variant types. In this study, we developed a more comprehensive multianalyte NGS control that covers most of the cancer hotspot mutations from each variant categories, including single nucleotide variant (SNV), multi-nucleotide variant (MNV), insertion and deletion (indel), copy number variation (CNV) and fusions, as well as for tumor mutation burden (TMB), loss of heterozygosity (LOH) and microsatellite instability (MSI) measurements. (The control is currently in development and Microgenics has not applied for nor received clearance by FDA at this time.) **Methods:** Target variants were selected from cancer hotspot mutations, with representative genes across different cancer types. Genomic DNA from several cell lines was purified and characterized to serve as background. Several somatic variants were also selected and created using synthetic DNA constructs by spiking-in to the genomic background. The controls were tested using Oncomine Comprehensive Assay Plus on Ion Chef and Ion S5 Sequencer and the TMB scores were determined. Additional RNA fusion controls were also created either using synthetic constructs or RNA extracted from specific cell lines. **Results:** Multianalyte NGS controls were created with target somatic hotspot mutations and several additional variants including TMB (high and low), MSI (high and low) and LOH on selected genes. The NGS results showed detection of expected level of variants that were covered in the Oncomine Comprehensive Assay Plus, and assigned the TMB, MSI scores as claimed. **Conclusions:** A multianalyte control that covers many major somatic cancer hotspot mutations in the format of SNV, MNV, indel, as well as additional MSI, TMB, LOH and CMV variants was created. This multianalyte control can be used for NGS assay development, method verification and validation, operator training, and proficiency testing.

ST074. **Implementation of a Liquid Biopsy NGS Assay for Pediatric Brain Tumors**

L. Nobre, C. Hawkins, M. Johnson, R. Siddaway, J. Sheth, M. Ku, R. Lyn, S. Ryall, U. Tabori
SickKids, Toronto, Ontario, Canada.

Introduction: The field of liquid biopsy has seen great improvement over the last 5-10 years with the development of sensitive tools such as ddPCR and ultra-deep next-generation sequencing (NGS). For brain tumors, multiple studies have proven the superiority of cerebrospinal fluid (CSF) over plasma as a source of circulating tumor DNA (ctDNA). For pediatric patients, added challenges include small sample volumes, low shedding, and a large proportion of fusion-driven tumors. The molecular characterization of brain tumors has become imperative not only to classify and prognosticate these lesions but also to guide precision therapeutics. Liquid biopsy is a minimally invasive alternative to surgical biopsies which enables molecular characterization to diagnose specific mutations, monitor response to therapy, and potentially predict progression/recurrence. **Methods:** We have developed a customized capture-based NGS panel (Twist) including 21 commonly altered genes present in pediatric and young adult brain tumors coupled with low pass whole genome sequencing (WGS) as a diagnostic and monitoring liquid biopsy tool. To assess for common fusions, exonic and intronic regions of specific genes were covered to capture different breakpoints. **Results:** To establish the sensitivity and specificity of this assay we used a commercially available control (Seraseq) with 18 known mutated genes of interest and an in-house control sample with two additional mutations. Samples with low ctDNA concentration (10 ng) and a limit of detection of 0.5% variant allele frequency had a sensitivity of 83% and specificity of 100%. Forty CSF samples from 37 pediatric patients (12 high-grade gliomas [HGG]; 25 low-grade gliomas [LGG]) were collected intra-surgically, through ventricular drains or lumbar puncture. Driver alterations, including fusions, were detected in 62% of samples, with a lower detection rate in LGG (14/25) compared to HGG (11/12). Using low-pass WGS, we were able to detect copy number variants in 3/4 samples (concordant with matched tumor). Interestingly, in one HGG patient with mismatch repair deficiency (MMRD) we were also able to identify MMRD signature using ctDNA. **Conclusions:** This work supports further implementation of CSF use as a liquid biopsy source to detect molecular alterations including single nucleotide variants, fusions, copy number alterations, and signatures in pediatric brain tumors. Ongoing work through prospective studies will aid in establishing the sensitivity of different sources of samples such as

lumbar puncture versus ventricular samples and aid in establishing the benefit of monitoring and therapy guidance in this population.

ST075. **Impact of Clinicopathologic Variables on Pre- and Post-Comprehensive Genomic Profiling (CGP) Tumor Content (TC) Limit of Detection (LOD) Discordance**

J. Mowers[1], J. Hipp[2], K. Kwiatkowski[2], S. Drewery[2], T. Reeder[2], D. Rhodes[2], D. Hovelson[2], B. Johnson[2], S. Tomlins[2], H. Vakil[2]
[1]Strata Oncology, Superior Twp, MI; [2]Strata Oncology, Ann Arbor, MI.

Introduction: Clinical genomic profiling (CGP) is increasingly utilized for cancer diagnosis, patient prognosis, and therapy selection. Pre-analytic misestimation of tumor content (TC), from errors during microscopic tissue review and/or macrodissection, may impact performance and/or reporting near the TC-based limit of detection (LOD) for multiple CGP variant classes. Therefore, understanding the frequency and determinants of pre-/post-analytic TC discordance (at LOD) may inform on the reliability of pre-analytic TC estimation. **Methods:** Clinicopathologic variables from consecutive StrataNGS CGP cases reported Jan. 2020 to Jun. 2022 were obtained from the deidentified StrataPOINT database. TC was estimated histologically by one of five experienced pathologists prior to tissue extraction and recorded; the final post-CGP molecularly informed TC was determined by a single pathologist evaluating CGP results at the time of sign-out. As the validated TC LOD for all StrataNGS variant classes is ≥20%, samples were binned based on concordant or discordant pre- and post-CGP TC estimates (<20% to ≥20% and ≥20% to <20% as discordant). Rates of TC discordance across clinicopathologic variables were compared and multivariable linear regression was performed using TC discordance as the dependent variable (MedCalc v20). **Results:** Of 31,660 consecutive formalin-fixed, paraffin-embedded tumor tissue samples tested by StrataNGS CGP, 2,511 (7.3%) had discordant pre- and post-CGP TC estimates (3.0% <20% to ≥20%; 4.3% ≥20% to <20%). Across >35 tumor types, appendiceal (18.9%), pancreatic (18.7%), non-small cell lung (11.9%), esophagogastric (11.3%), hepatobiliary (11.2%), and unknown primary (10.1%) cancers had the highest rates of TC discordance. Compared to the 27.2% of samples with ≥25mm^2 tissue surface area (TSA), the 41.4% of samples with TSA ≤5mm^2 had a significantly higher rate of TC discordance (1.5% vs. 13.6%, Fisher's exact test p <0.0001). Linear regression demonstrated that tumor type, decreasing TSA, increasing number of sections used for tissue isolation, specimen collection type (cytology, excision, and bone biopsy vs. tissue biopsy), received sample type (block vs. unstained slides), and earlier year of test performance were all independently associated with TC discordance, whereas tissue isolation method (e.g., macrodissection) was not. In the linear regression model (AUC 0.786), samples with TSA <2mm^2 and 2-5mm^2 had odds ratios of 59.5 and 25.1, respectively, for TC discordance compared to samples >100m^2. **Conclusions:** Across a large consecutive CGP cohort, the rates of significant TC discordance varied by tumor type and TSA, among other factors. Samples with TSA ≤5mm^2 (41.4% of StrataNGS CGP tested samples) are especially susceptible to TC discordance; therefore, histopathology should not be used solely to determine TC in minute tumor samples.

ST076. **Evaluation of Cell-Free DNA Testing with Guardant360 Cdx for Identifying Clinically Actionable Mutations in Lung Cancer Patients at Weill Cornell Medicine**

C. Ng[1], H. Rennert[1], P. Velu[2], L. Cong[2], A. Ding[2], E. Zhong[2], S. Rand[2], E. Sholle[2], J. Sipley[2]
[1]Weill Cornell New York Presbyterian, New York, NY; [2]Weill Cornell Medical College, New York, NY.

Introduction: Circulating tumor-derived cell-free DNA (cfDNA) can be sequenced for genomic alterations and used in the screening, surveillance, and management of cancer. Advantages of liquid biopsy include its minimally invasive nature, low cost, and ease of longitudinal testing. Given the ongoing implementation of cfDNA testing in oncology, there is a need to compare assays. Here we present an analysis of cfDNA testing with Guardant360 CDx (Guardant Health) at Weill Cornell Medicine (WCM). Guardant360 CDx is an FDA-approved multiplex PCR test for solid tumors. It includes 74 genes covering single nucleotide variants (SNVs), indels, amplifications, and fusions with a 0.2% limit of detection. The Oncomine Lung cfDNA Assay (Thermo Fisher Scientific) was recently implemented at our institution and is an NYS-approved multiplex PCR cfDNA test for non-small cell lung cancer (NSCLC). It covers 11 genes (168 SNVs and indels) with a 0.1% limit of detection. **Methods:** From 2017-2021, 338 patients at WCM received Guardant360 cfDNA testing of peripheral blood (448 tests). Data were extracted from the electronic medical record (EPIC). Technical parameters were obtained from the manufacturer's website (Guardant Health and Thermo Fisher, respectively). **Results:** Liquid biopsies were performed for 19 different tumor types, primarily from prostate (90), lung (88), and pancreas (47). Among patients with NSCLC (84 adeno, one squamous cell carcinoma, three poorly differentiated) the median variable allele frequency was 2.2%. The median mutation number was three, with an average of one test per patient (range: 1-3). Turnaround time from receipt of specimen averaged seven days. Seventeen percent of NSCLC cases were negative. Mutations with FDA-approved targeted therapy for NSCLC (*EGFR* mutations, *ALK* fusions, *ROS1* fusions, *BRAF*-V600E, *KRAS*-G12C, *MET* exon 14 skipping) were identified in 31.8% (28/88) of patients. Of these actionable mutations, 86% (6/7) were identical to those found on tissue biopsy. The one discrepancy was *EGFR* T790M in blood versus *EGFR* exon 19 deletion in tumor in the setting of extensive therapy, likely reflecting clonal outgrowth. In comparison, the Oncomine cfDNA test covers 98.9% (91/92) of pathogenic or likely pathogenic mutations identified by Guardant and all mutations that are targetable by FDA-approved therapies except for *MET* exon 14 skipping. Limitations of this study include a small sample size and the assumption of similar specificity and sensitivity between Oncomine and Guardant. **Conclusions:** Nearly one-third of NSCLC patients were found to have actionable mutations by liquid biopsy, and these were highly concordant with tissue. Oncomine cfDNA testing is predicted to provide similar results with lower cost and faster turnaround time.

ST077. **Assessing Variability across HRD Assays: Findings from the Friends' HRD Harmonization Project**

H. Stires[1], Z. Zhang[2], L. McShane[2], J. Bieler[3], L. Chen[4], M. Gupta[5], A. Lazar[6], B. McKelvey[1], S. Pabla[7], J. Parsons[8], O. Serang[9], S. Shams[10], E. Sokol[11], E. Starks[12], B. Thomas[13], S. Yang[14], J. Yen[15], M. Stewart[1], J. Allen[1]
[1]Friends of Cancer Research, Washington, District of Columbia; [2]National Cancer Institute, Bethesda, MD; [3]SOPHiA GENETICS, Saint-Sulpice, Switzerland; [4]Frederick National Laboratory for Cancer Research, Frederick, MD; [5]Clinical Sequencing Division, Thermo Fisher Scientific, South San Francisco, CA; [6]The University of Texas MD Anderson Cancer Center, Houston, TX; [7]Omniseq, Buffalo, NY; [8]Tempus Labs, Chicago, IL; [9]DNAnexus, Mountain View, CA; [10]Bionano Genomics, San Diego, CA; [11]Foundation Medicine, Cambridge, MA; [12]Invitae, San Francisco, CA; [13]Neogenomics, Fort Myers, FL; [14]Amoy Diagnostics Co., Ltd, Xiamen, Fujian, People's Republic of China; [15]Guardant Health, Palo Alto, CA.

Introduction: Homologous recombination deficiency (HRD) assays determine eligibility for treatment with poly (ADP-ribose) polymerase inhibitors and other DNA repair targeting drugs. The assays measure several factors to define homologous recombination (HR) status including causes (i.e., inactivation in HR pathway genes) and consequences (i.e., genomic instability) of HRD. Methodological variability across HRD assays has not been investigated thoroughly, and an empirical assessment of assay variability may support broader adoption of HRD and strengthen clinical interpretation of test results. **Methods:** Friends of Cancer Research (*Friends*) initiated a unique partnership with HRD assay developers and other key stakeholders to characterize differences in assay factors and assess levels of agreement and variability across HRD assays. First, we surveyed HRD

assay developers (n=20) about factors their assays measure to determine HR status. Subsequently, a subset of assay developers (n=11) measured *in silico* and reported HR status and the contributing factor(s) for 348 TCGA ovarian cancer samples. We performed pairwise comparisons of assay's HR status calls to determine the level of agreement and considered specific factors measured by each assay to identify potential sources of variation. Additionally, we analyzed HR status agreement for *BRCA1/2* mutated versus wild-type *BRCA1/2* samples. **Results:** The 20 surveyed HRD assays are heterogeneous in the factors they measure. Although all assays consider *BRCA1/2* mutations, assays also variously consider genomic loss of heterozygosity (gLOH; 75% of assays), additional HRR genes (55%), telomeric allelic imbalance (TAI; 45%), and large-scale state transitions (LST; 45%). For assays involved in the TCGA analysis, the range of percent positivity (% patients with HRD) was 9%-67% with a median of 49%. Rates of HRD were higher in assays that included gLOH, TAI, and/or LST. Median positive percent agreement (PPA) was 74% and median negative percent agreement was 81%. The presence of *BRCA1/2* mutations was associated with an increase in PPA. The median Spearman correlation for pairwise comparisons of ranked continuous HRD scores was 0.66 and 0.70 for %gLOH. **Conclusions:** Preliminary findings demonstrate variation in the factors measured and the HR status calls made across HRD assays. Some of the variation in HR status calls could be due to the nature of the TCGA dataset, and future studies will aim to understand assay agreement from freshly extracted formalin-fixed, paraffin-embedded human archival ovarian tumor samples. Understanding the agreement among assays will help to inform assay interpretation and improve consistency between HR status calls and alignment of HRD scores across HRD assays to help patients and providers make appropriate treatment decisions.

ST078. **Practice Patterns of *HER2 in situ* Hybridization Testing in the Setting of Equivocal Immunohistochemistry in the United States**

B. Iorgulescu
MD Anderson Cancer Center, Houston, TX.

Introduction: For breast carcinomas with initial equivocal HER2 immunohistochemistry (IHC 2+), the 2013/2018 HER2 testing ASCO/CAP guidelines recommend reflex testing by *in situ* hybridization (ISH) on the same specimen or IHC/ISH on new specimen. Furthermore, for stage IV cancers, novel therapies exist for HER2-low cases (i.e., IHC 1+ or IHC 2+/ISH-negative). Thus, we investigated the US practice patterns of ISH testing/reporting in the setting of equivocal IHC. **Methods:** Patients presenting with a newly diagnosed, pathology-confirmed invasive breast carcinoma from 2010-2019 were identified in the U.S. National Cancer Database (includes 81% of breast cancers diagnosed in the US). Practice patterns of HER2 ISH testing/reporting for IHC 2+ cases were assessed using multivariable logistic regression. **Results:** From 2010-2019, 1,257,425 patients were identified with HER2 data. Among cases with IHC data, 27.3% were IHC 0, 39.1% were IHC 1+, 22.0% were IHC 2+, and 11.7% were IHC 3+. Overall, HER2 ISH testing was reported in 46.3% of cases. ISH testing was reported in 90.6% of IHC 2+ cases, compared to 18.4% of IHC 0, 22.6% of IHC 1+, and 22.2% of IHC 3+ cases. For patients with IHC 2+ tumors that also had ISH testing, ISH was amplified in 14.6% (n=30,781), negative in 80.0% (n=168,709), and equivocal in 5.5% (n=11,513). Dual versus single probe ISH information was only available for ≥2,018, for which 97.2% used dual-probe assays. We explored the ISH testing patterns among IHC 2+ cases. The proportion of cases without coincident ISH fell from 14.2% in 2010 (n=2,317), to 10.5% in 2013 (n=2,153), 6.6% in 2018 (n=1,817), and 5.9% in 2019 (n=1,643). In multivariable regression, lack of coincident ISH was associated with patients' older age at diagnosis, race/ethnicity (Asian/Pacific Islander OR 0.86, 95CI: 0.80-0.93, p <.001 and Hispanic OR 0.90, 95CI: 0.84-0.96, p=.002, vs. non-Hispanic White), and lower socioeconomic status (richest quartile: OR 1.28, 95CI: 1.22-1.34, p <0.001; vs. poorest quartile) – but not patients' insurance status or ER status. Lack of ISH was more likely for stage I and IV disease (p <.001). Comprehensive community programs were most likely to have coincident ISH (OR 1.14, 95CI: 1.10-1.19, p <.001), even compared to academic hospitals (referent). There was substantial variability in coincident ISH geographically, with the highest rates at New England hospitals and lowest rates at Mountain division hospitals (p <.001). **Conclusions:** HER2 ISH testing/reporting for IHC 2+ cases increased from 2010 to 2019; however, even in 2019, coincident ISH testing was not reported for ~6% of IHC 2+ cases. ISH testing underutilization was associated with patients' race/ethnicity and socioeconomic status, hospital's type and location, as well as in stage IV disease – suggesting opportunities for improving HER2 testing.

ST079. **Effects of *EWSR1* Amplification on the Clinical and Histologic Behavior of Non-Ewing Sarcoma Soft Tissue Tumors**

L. Miller[1], C. Bodnar[2], L. Parsons[3], R. Singh[2], K. Bone[2]
[1]University of Michigan, Milwaukee, WI; [2]Medical College of Wisconsin, Milwaukee, WI; [3]Children's Hospital of Wisconsin, Milwaukee, WI.

Introduction: Fusions of *EWSR1* play a significant role in soft tissue sarcomas that are distinct tumor entities from Ewing sarcoma. Due to the morphologic similarities among the small round blue cell tumors, genetic analysis is often required to characterize these tumors. In this series, we present 10 patients with amplification of *EWSR1*, a novel finding in seven cases without concurrent fusion. **Methods:** The 10 cases were identified based on fluorescence *in situ* hybridization (FISH) probe amplification of the 3' end of *EWSR1*. Patient demographics, histologic description, presence of fusions and alterations in other genes, and patient status were extracted from pathology reports from two affiliated institutions from 2019-2022. **Results:** The 10 patients consisted of nine males and one female, with two pediatric patients and eight adults (age range 13-65 years). Nine of the cases were diagnosed as soft tissue sarcoma, and one case was diagnosed as glioblastoma. All 10 cases contained amplification of *EWSR1* with an average of eight copies per cell (range 6-12 copies). The sarcoma cases demonstrated a high proportion of cells affected (50%-97%), and the glioblastoma showed only 12% of cells affected. Histologically, all tumors were cellular and had discohesive cells with high-grade nuclear atypia and pleomorphism. Necrosis was not seen in any of the cases. Clinically, five of 10 patients presented with metastasis of the primary lesion. As of May 2022, the average time since diagnosis was 26 months (range 11-33). Two of the 10 patients were deceased. Three of the eight remaining patients had achieved remission. **Conclusions:** Soft tissue sarcomas with *EWSR1* amplification demonstrated histology that deviated from the expected monomorphic population of small round blue cells arranged in sheets and nests. This suggests that amplification of *EWSR1* contributes to the development of cellular atypia and discohesion. The number of cells containing the amplification appears to be positively correlated with the level of cytologic atypia. In the literature, the expected one-year survival for the Ewing sarcoma family of tumors is 90% for localized tumors, and 40% in metastatic lesions (Valdes, *et al.,* 2015). In comparison, this series demonstrated 100% survival without metastasis and 60% survival with metastasis. These data suggest that *EWSR1* amplification demonstrated clinical behavior similar to *EWSR1* fusions and contributes to the development of histologically atypical soft tissue sarcomas. This study is limited by the low number of cases, and further study and observation of this novel finding is needed.

ST080. **Outcomes of Various Molecular Testing on Thyroid Fine-Needle Aspiration Cytology with Histologic Correlation: A Multicenter Retrospective Study on the Management of Indeterminate Thyroid Nodules**

G. Sura, M. Thrall, P. Hodjat, J. Rogers, P. Christensen, J. Thomas, E. Jacobi
Houston Methodist, Houston, TX.

Introduction: Thyroid malignancy is one of the most common types of cancer in developed nations. Currently, fine-needle aspiration cytology (FNAC) is the most practical screening test for thyroid nodules. However, diagnostically indeterminate samples comprise approximately 15%-30% of cases. Indeterminate cases may undergo molecular testing for more definitive classification to help guide management. This retrospective review evaluated how thyroid FNAC with adjunct molecular testing helped guide patient surgical management and reviewed subsequent histologic diagnoses in resection specimens. **Methods:** We retrospectively reviewed reports from thyroid FNAC specimens, corresponding molecular testing, and subsequent surgical resection specimens over six years. **Results:** A total of 10,253 thyroid FNAC were performed in our hospital system during our study period. Molecular testing was performed in 16% (n=178) of indeterminate cytology cases. The molecular platforms used were as follows: Houston Methodist Thyroid Cancer Test (Agena Biosciences; ArcherDX) (n=40), ThyGeNEXT/ThyraMIR (Interspace Diagnostics) (n=13), ThyroSeq V3 (Sonic Healthcare) (n=13), and Afirma (Veracyte) (n=112). Genetic alterations were identified in 39% (n=69) of the total cases sent for molecular testing. Of the indeterminate cases with identified alterations, 75% (n=52) were treated surgically, whereas only 18% (n=20) of the cases with no genetic alterations were treated surgically. Of the 72 cases receiving surgical treatment, thyroid lobectomy was performed in 75% (n=54) of cases and total thyroidectomy was performed in 25% (n=18) of cases. In indeterminate FNAC cases with a molecular alteration and surgical treatment (n=52), the histologic diagnoses were as follows: malignant (37%, n=19), undetermined malignant potential (6%, n=3), and benign (58%, n=30). In indeterminate FNAC cases with no molecular alteration and surgical treatment (n=20), the histologic diagnoses were as follows: malignant (20%, n=4), uncertain malignant potential (15%, n=3), and benign (65%, n=13). **Conclusions:** Molecular testing on cytologically indeterminate thyroid nodules spared more than half of the patients from surgery. This finding emphasizes the value of adding molecular testing, particularly in patients that are poor surgical candidates. Molecular testing did not affect which surgical treatment was performed. In this study, four fundamentally different molecular testing platforms had an approximately equal ability to identify low-risk patients who may be spared from surgery within the same health care system. The correlation of molecular test results performed on cytology specimens with follow-up surgical pathology specimens can be a valuable quality assurance measure, helping to identify under- or overdiagnosed nodules.

ST081. **Rescuing Low Tumor Cell Content Carcinoma Samples from Formalin-Fixed, Paraffin-Embedded Biopsies for Clinical Next-Generation Sequencing by Dielectrophoretic (DEP) Array Sorting**

M. Snuderl, C. Gao, I. Tran, G. Shen, B. Zeck, K. Galbraith, L. Chiriboga, A. Moreira, G. Jour, X. Feng
NYU Langone Health, New York, NY.

Introduction: Next-generation sequencing (NGS) of carcinomas is performed on formalin-fixed, paraffin-embedded (FFPE) tissue; however, many samples are not suitable for clinical NGS due to their low tumor cell content, leading to either rejection of the sample or a false-negative result. Here we validated dielectrophoretic (DEP) array sorting to capture pure populations of whole tumor cells for NGS. **Methods:** We analyzed 20 carcinoma (16 lung and four GI) samples with tumor cell content between 10%-60%. For each sample, one 60 μM FFPE tissue scroll was dissociated into a single-cell suspension and immunostained with immunofluorescent antibodies for pancytokeratin (CK), vimentin (VIM) and DAPI. The stained single-cell suspension was sorted using DEPArray NxT system based on the immunophenotype and DNA ploidy. Selected cell populations were collected, lysed and NGS was performed using clinically validated Oncomine Focus DNA Assay. Results were compared with the bulk tumor NGS results. **Results:** We collected pools of cells ranging between 7-230 cells. All samples were successfully profiled by NGS with depth of coverage between 100-2,000x. VIM-positive stromal cells served as negative control in each sample. In CK-positive cells, we sorted diploid, hyperdiploid cells, and cells co-expressing CK and VIM. Tumor mutations were not detected in VIM-positive cells, whereas both hotspot single nucleotide variants and indel mutations were detected in CK-positive cells. Overall, increase of variant allele frequency (VAF) ranged between two- to 21-fold in CK-positive DEPArray sorted cells compared to bulk tumor NGS in the entire cohort. For example, *KRAS* G12V VAF increased from 3.2% in bulk tumor to 51% in DEPArray sorted CK-positive cells from the same block, confirming the specificity of sorting. In addition, we identified CK/VIM co-expressing cells that carried the same hotspot mutations as CK-only positive cells suggestive of epithelial mesenchymal transition and tumor heterogeneity. There was 100% concordance between mutations identified in bulk and DEPArray sorted populations, and there were no false-positive NGS results in DEPArray sorted cells. **Conclusions:** DEPArray sorting enables highly specific sorting of whole cells from FFPE tissue. Since each cell contains a complete genome, sorted pools of cells as small as seven cells could be successfully profiled by NGS without amplification to obtain clinically relevant hotspot mutations. Tumor mutation detection rate can increase as high as 21-fold compared to bulk tumor sequencing. DEPArray workflow can be used as enrichment of FFPE samples that would be considered insufficient. Furthermore, DEPArray sorting of FFPE samples enables analysis of tumor heterogeneity based on tumor cell size, ploidy, and immunophenotype.

ST082. **Assessing the Prevalence of Subclonal *BRAF* E/K Alterations in Melanoma**

D. Milosevic, G. Zheng, A. Buglioni, C. Ida, A. Rumilla
Mayo Clinic and Foundation, Rochester, MN.

Introduction: *BRAF* V600E/K are important targetable alterations in melanoma. It was reported that *BRAF* V600E/K could be subclonal, which has important clinical implications. However, the true prevalence of subclonal *BRAF* V600E/K in melanoma is unknown. Droplet digital (ddPCR) is a highly sensitive method for detection of specific gene alterations. With a retrospective analysis of ddPCR-based *BRAF* V600E/K assay, our study aims to determine the prevalence of subclonal *BRAF* V600E/K in melanoma. **Methods:** A retrospective analysis of the ddPCR test (Test ID: BRAFD) data from 10/2020 to 05/2022 was performed. This test was designed to test for *BRAF* V600E/K alterations in FFPE tumor tissues. Tumor cell percentages and allele frequencies by ddPCR were used to determine whether the alterations detected are subclonal. For low-level positive samples, repeating ddPCR with UDG (uracyl deaminase) treatment was used to exclude deamination artifacts. **Results:** During the last two years, we tested a total of 437 FFPE melanoma samples for the presence of the *BRAF* V600E/K alterations using ddPCR method. Altogether, 49.9% (N=218) tested positive for an alteration, among which 15 cases had a ratio between allele frequency by ddPCR to tumor percentage below 0.2, which was used as a conservative cut-off for subclonal alterations. These subclonal *BRAF* V600E/K alterations were confirmed with UDG enzymatic treatment. **Conclusions:** The prevalence of the subclonal *BRAF* V600E/K alterations in melanoma is at least 6.9% (15/218). The subclonal nature of the targetable alteration has important implications for therapy and should be communicated with clinicians.

ST083. ***FGFR* Mutations and Associated Variant Histology in Urothelial Carcinoma**

A. Tharp, J. Sen, M. Sperling, L. Cheng
Indiana University, Indianapolis, IN.

Introduction: In the United States, urothelial carcinoma is one of the costliest cancers to treat. As the oncogenesis of urothelial carcinoma continues to be better understood, *FGFR* mutations have stood out as a valuable target with prognostic and therapeutic utility. **Methods:** The departmental records of a single institution were reviewed for cases of invasive urothelial carcinoma between the years 2019-2022. A total of 190 cases were identified and submitted for molecular analysis. Tumor tissue was sourced from several specimen categories, including: transurethral tumor resection, tumor biopsy, primary surgical resections, and metastasis biopsy. Tumor samples from formalin-fixed, paraffin-embedded (FFPE) blocks were then tested for *FGFR* mutations. Testing was performed on all cases as part of clinical care using Qiagen's therascreen FGFR RGQ RT-PCR companion diagnostic kit. This assay detects four point mutations and two fusions, which result in *FGFR3* amplification. The *FGFR* mutated cases were then reviewed with conventional microscopy in an effort to identify and catalog any morphologic variation. **Results:** Of the submitted cases, 37 were found to harbor *FGFR* mutations. The S249C mutation (n=17 | 45%) was the most common, followed by Y373C mutation (n=12 | 32%). The remaining discovered mutations included *FGFR3:TACC3v1* fusion (n=4 | 11%), R248C (n=3 | 8%) and G370C (n=1 | 3%). Twenty-eight of the 37 cases showed some form of variant histology. Histologic subtypes discovered included: micropapillary (n=13 | 35%), inverted (n=1 | 3%), pushing (n=4 | 11%), clear cell (n=1 | 3%), small cell (n=1 | 3%), large nested (n=2 | 6%), lymphoid rich (n=1 | 3%). Squamous differentiation was also discovered in numerous cases (n=7 | 19%). Lesions with micropapillary variant histology harbored either S249C or Y373C mutations. No other mutations were identified in this variant histology category. Between these two categories, S249C was the most common, representing 69% of all lesions expressing a micropapillary pattern. Y373C mutations made up the remaining 31%. **Conclusions:** Variant histology was appreciated in 76% of *FGFR* mutated cases, with the micropapillary pattern being the most prevalent (n=12 | 32%). The *FGFR* pathway is classically associated with low-grade papillary tumors, which, through additional mutations, can cause invasive, high-grade disease. It is speculated that the initial papillary architecture present in *FGFR* mutated lesions may be preserved, manifesting as the micropapillary pattern appreciated in some invasive lesions. This is supported by the observation that the micropapillary pattern was the most common variant histology appreciated when evaluating invasive *FGFR* mutated tumors.

ST084. **Distinct IDH1/2-associated methylation profile and enrichment of TP53 and TERT mutations distinguish dedifferentiated chondrosarcoma from conventional chondrosarcoma on integrated genomic and epigenomic profiling**

J. Dermawan[2], K. Nafa[2], A. Mohanty[2], Y. Xu[2], I. Rijo[2], L. Villafania[2], J. Benhamida[2], C. Kelly[2], W. Tap[2], P. Boland[2], N. Fabbri[2], J. Healey[2], C. Lu[3], M. Ladanyi[2], M. Hameed[2]
[1]Memorial Sloan Kettering Cancer Center, New York, NY; [2]Memorial Sloan Kettering Cancer Center, New York, NY; [3]Columbia University Medical Center, New York, NY.

Introduction: Dedifferentiated chondrosarcoma (DDCS) is a high-grade subtype of chondrosarcoma characterized histologically by a well-differentiated chondrosarcoma (WDCS) component that abruptly transitions to a high-grade, non-cartilaginous sarcomatous component. Patients with DDCS carry a dismal prognosis. Similar to conventional chondrosarcoma, about 50%-80% of DDCS cases harbor *IDH1*/*IDH2* mutations. It is well documented that *IDH1*/*IDH2* mutations lead to global hypermethylation in conventional chondrosarcoma. To date, the molecular pathogenesis of DDCS and its distinction from conventional chondrosarcoma remain poorly understood. **Methods:** Using MSK-IMPACT, a targeted next-generation DNA sequencing panel, we examined the mutational and copy number profiles of 17 DDCS cases, including microdissected WDCS components when available (eight cases), in comparison to 55 conventional chondrosarcoma cases. Additionally, using the Illumina EPIC array platform, in conjunction with external publicly available methylation and gene expression data, we analyzed the methylation profiles of 33 DDCS cases and 94 conventional chondrosarcoma cases. **Results:** *IDH1*/*IDH2* mutations were present in 36% conventional chondrosarcoma and 71% DDCS cases. Compared to conventional chondrosarcoma, DDCS had significantly higher tumor mutation burden and frequencies of *TP53* and *TERT* promoter mutations and *CDKN2A*/*CDKN2B* copy number losses (P = 0.004). Paired analysis of microdissected WDCS and the high-grade sarcoma components from seven patients revealed *TERT* promoter mutations as common, early events and acquisition of additional copy number gains and losses in the high-grade sarcoma component not seen in the WDCS component. Despite phenotypic similarities, the percentage of genome involved by copy number alterations in DDCS was significantly lower than those in other high-grade sarcomas (osteosarcomas, leiomyosarcomas, undifferentiated pleomorphic sarcomas). The WDCS and high-grade sarcoma components in DDCS showed similar methylation profiles. Differential methylation analysis revealed reversal of *IDH1/IDH2*-dependent hypermethylation of CpG sites in conventional chondrosarcoma to hypomethylation in DDCS. These CpG sites were associated with upregulated expression of gene sets enriched in pathways involved in G2M checkpoints and E2F targets. **Conclusions:** Genomic profiling revealed enrichment of *TP53*, *TERT* promoter, and *CDKN2A*/*CDKN2B* alterations in DDCS. Integrated methylation and gene expression analysis revealed reversal of *IDH1/2*-dependent global hypermethylation as an early event in DDCS, underpinning an important role in the pathogenesis of dedifferentiation in chondrosarcomas.

ST085. **Correlating Splicing Variants with Downstream Effects in RNA-Seq Data**

M. Tjota, P. Wang, J. Segal
University of Chicago Medical Center, Chicago, IL.

Introduction: Thus far, the majority of sequencing studies have been focused on coding regions, but increasing evidence has demonstrated that splice site variants can have significant tumorigenic and prognostic implications. It has been well established that nearly all splice sites conform to consensus sequences: GT at the 5' end of the intron and AG at the 3' end of the intron. Alterations affecting these sites may result in production of an aberrant transcript including loss of an exon, retention of the intron, or creation of an alternative splice site. As we have been increasingly performing concurrent DNA and RNA sequencing in our patient specimens, we wanted to correlate splicing variants at these locations with downstream effects on the RNA. **Methods:** In this study, we retrospectively reviewed the Department of Pathology database from 2018 to 2021 and identified 195 variants involving the consensus splice site that had been categorized as pathogenic (1) or likely pathogenic (2). In addition, these cases had both DNA and RNA sequencing performed. **Results:** The majority of cases were lung adenocarcinomas (n=93). Other tumor types included lung squamous cell carcinomas, colonic adenocarcinoma, melanoma, pancreatic adenocarcinoma, urothelial carcinoma, and others. The most common genes to have splice site variants were *TP53* (n=43), *NF1* (n=17), *RB1* (n=15), and *STK11* (n=10). We separated the variants based on their location (last base, upstream, and downstream) and manually assessed the RNA-Seq results for loss of exon(s), retained intron, alternative splice site, more than one of these alterations, or no effect. Overall, the most common alteration was loss of exon(s), which was seen in 45% of the variants identified. Eighteen percent of the variants had a partial or complete retention of the intron; 13% had an alternative splice site; and 14% had more than one effect. There were 10% of cases where no effect was noted. **Conclusions:** Currently, there are limited data on the downstream effect of variants identified at splice sites. We were able to demonstrate that 90% of

these variants had RNA alterations. Identifying splicing variants and understanding their downstream effect will be helpful in understanding disease pathogenesis, as alternative splicing events have been implicated in various carcinogenic behaviors including metastasis, angiogenesis, apoptosis, and immune evasion. More importantly, identifying splicing variants and understanding their downstream effect may lead to the identification of tumor-specific splicing derived neoantigens, which could be used to identify novel therapeutics or vaccine targets.

ST086. **A Recurrent *ERBB2* Rearrangement in Invasive Mucinous Adenocarcinoma of the Lung**

F. El-Sharkawy, J. Roth, S. Priore
Hospital of the University of Pennsylvania, Philadelphia, PA.

Introduction: Amplification and activating variants of the *ERBB2 (*alias *HER2)* gene are recognized as oncogenic drivers and potential therapeutic targets in non-small cell lung cancer (NSCLC). However, *ERBB2* rearrangements have rarely been reported in NSCLC and their significance is uncertain. A recurrent *ERBB2* rearrangement in NSCLC was identified at our institution during routine clinical practice. This study aimed to evaluate the demographic, clinical, histopathologic, and molecular characteristics of NSCLC cases with *ERBB2* rearrangements. **Methods:** NSCLC specimens are routinely tested at the Hospital of the University of Pennsylvania using an RNA-based, anchored multiplex sequencing assay that detects oncogenic fusions and novel isoforms in 55 genes (Fusion Transcript Panel; FTP). A retrospective review of all FTP data was performed. NSCLC cases with *ERBB2* fusions were selected and respective clinical notes, surgical pathology reports, tissue sections, and molecular results were reviewed. **Results:** Three patients harboring an *ERBB2* rearrangement were identified. Histologically, all tumors were classified as invasive mucinous adenocarcinoma of the lung. Comprehensive genomic profiling of each tumor found no other recurrent oncogenic drivers in *EGFR, ALK, ROS1, BRAF, KRAS, MET, RET, NRG1,* or *FGFR.* The rearrangement consisted of an *ERBB2-SHC1* fusion joining the amino-terminal domain of ERBB2, including almost all of the tyrosine kinase domain, to the full coding sequence and part of the 5' untranslated region of the *SHC1* gene. All patients were female, never-smokers, in the age range of 50 to 60 yrs. Two patients were diagnosed at stage IVA and were treated with multiple chemotherapy regimens with subsequent disease progression (one patient alive with disease, one patient dead from disease). The third patient was diagnosed at stage IB and had no evidence of disease at eight months post-surgery. None of the patients received ERRB2-targeted therapy. **Conclusions:** We present three cases of invasive mucinous adenocarcinoma of the lung with a recurrent *ERBB2-SHC1* fusion. Although *ERBB2* alterations have been described in lung adenocarcinoma, this is the first report of an *ERBB2-SHC1* fusion associated with mucinous histology. This rearrangement, which was shown to have oncogenic activity in NSCLC in one study, likely contributes to increased cell proliferation and survival based on the known functions of the proteins involved. Similar to other NSCLC driver alterations, the demographic, histologic, and mutual exclusivity of other known oncogenic drivers in our cases suggests that the *ERBB2-SHC1* fusion may be a novel driver of a rare molecular subtype of NSCLC.

ST087. **Clinical Performance of Comprehensive Genomic Profiling Using a Wide Range of Input Quantities and Solid Tumor Tissue Types**

J. Wagner[2], S. Reynolds[2], A. Dowdell[2], E. Shull[2], J. Welle[2], M. Pukay[2], R. Rattray[2], C. Moung-Wen[2], T. Ward[2], M. Campbell[2], C. Bifulco[2], B. Piening[2]
[1]Providence Health System, Portland, OR; [2]Providence Health Services, Portland, OR.

Introduction: Identification of solid tumor mutation profiles using comprehensive genomic profiling (CGP) has shown significant utility in predicting response to targeted and immuno-oncology therapies. In clinical practice, tumor samples are primarily preserved as formalin-fixed, paraffin-embedded (FFPE) tissues and can vary highly in nucleic acid quantity and quality. The Illumina TruSight Oncology 500 High-throughput assay (TSO 500 HT) is a CGP next-generation sequencing (NGS) assay designed to detect somatic DNA variants in 523 genes, RNA fusions in 55 genes, tumor mutational burden, and microsatellite instability. Although accumulating evidence supports robust TSO 500 HT performance using a variety of FFPE tissues, few studies have explored how variable initial sample quantity predicts reflex testing success in a high-volume, large healthcare system. **Methods:** We retrospectively assessed TSO 500 HT clinical sequencing results (n = 5,576) from 33 cancer types for nucleic acid quantity, sequencing quality, and the presence of clinically significant DNA or RNA alterations. Adult cancer patients (stages I-IV) in the Providence Healthcare system received CGP testing from January 2021-April 2022 (n = 5,332 patients). DNA and RNA was extracted from FFPE tissues using Qiagen or Promega FFPE Extraction Kit procedures. NGS libraries were prepared using the TSO 500 HT protocol and sequenced on the Illumina NovaSeq 6000 platform. Variant clinical significance was determined using interpretation assistance from the OncoKB precision oncology knowledgebase. **Results:** Overall, 95% of samples had a DNA and RNA result with TSO 500 HT assay metrics in acceptable quality ranges and 91% of samples had at least one clinically significant DNA/RNA biomarker. Initial DNA and RNA stock concentrations were significantly higher in successful assays (DNA mean = 34.1 ng/µl, RNA mean = 135 ng/µl) compared to failed assays (DNA mean = 11.3 ng/µl, RNA mean = 91.1 ng/µl). Notably, acceptable test results and clinically significant biomarkers were obtained in an appreciable number of cases (n = 5,200) that had DNA or RNA stock concentrations within the range of failed tests (failed DNA range 0.018-325 ng/µl, failed RNA range 0.69-1186 ng/µl). Finally, we show several case studies with scant tissue that revealed clinically actionable sequencing results. **Conclusions:** Our results found that initial DNA and RNA concentrations following FFPE extraction are a significant predictor of TSO 500 HT procedure test success. However, testing success occurred even at the lowest DNA and RNA stock concentrations. We therefore propose clinical value in using CGP testing for all non-zero nucleic acid stock concentrations in FFPE solid tumor samples.

ST088. **Sarcoma Targeted Gene Fusion Panel (SARCP): The Mayo Clinic Experience with a Novel Clinical Gene Fusion Detection Panel**

M. Atiq[1], K. Halling[1], P. Blackburn[2], J. Gross[3], J. Voss[1], J. Balan[1], S. Barreto[1], C. Hofich[1], K. Willkomm[1], L. Jin[1], B. Pitel[1], J. Torres-Mora[1], C. Inwards[1], A. Folpe[1], A. Oliveira[1], B. Kipp[1]
[1]Mayo Clinic, Rochester, MN; [2]St. Jude Hospital, Memphis, TN; [3]Johns Hopkins, Baltimore, MD.

Introduction: Accurate classification of sarcomas, of increasing oncologic importance, may be challenging for the general pathologist. Identification of specific gene fusions is increasingly important for sarcoma classification, in particular, for small round cell sarcomas. In addition, sarcomas "defined" by specific molecular genetic signatures (e.g., *CIC* and *BCOR*-rearranged sarcomas) may not be detectable by traditional testing methods. We report our experience with a novel, custom-designed sarcoma fusion detection assay, "SARCP," for the first 652 of >2,800 cases analyzed to date. **Methods:** The SARCP gene fusion panel is a targeted, custom-designed, PCR-based panel that assesses 138 genes for fusions found in 39 sarcoma types. The assay utilizes QIAseq Targeted RNAscan Panel chemistry (Qiagen, Germantown, MD), which uses single primer extension target enrichment and unique molecular identifier technology to identify gene fusions, including novel fusions involving one of the panel genes. The final library is sequenced on an MiSeq (Illumina, San Diego, CA) instrument and next-generation sequencing data are analyzed using a pipeline developed at Mayo Clinic (SeekFusion) that uses a combination of traditional alignment and *de novo* assembly-based approaches. Review by a board-certified pathologist with bone and

soft tumor expertise was performed for all cases exhibiting novel fusions, or where the identified fusion supported a diagnosis significantly different than that suggested by the referring pathologist. **Results:** A total of 584 (90%) of the 652 cases were from referring institutions and 68 (10%) from our internal practice. Mean age was 43 years (SD: 23) with 347 females (53%) and 305 males (47%). Soft tissue tumors were more common (n=379; 58%) than were osseous ones (n=61; 9%). Fusions were identified in 238 samples (37%), with 15 novel fusions and 25 samples with targetable fusions including *NTRK1, NTRK3, ALK,* and *PDGFB*. The most common fusions were *EWSR1::FLI1* (38), *SS18::SSX1/2* (19), *EWSR1::WT1* (14), *PAX3::FOXO1* (10), and *EWSR1::ATF1* (8). *BCOR* internal tandem duplications were identified in eight cases. The assay helped establish a diagnosis in 146 cases (61%), confirmed a suspected diagnosis in 58 (24%), and changed a previously suspected diagnosis in 23 (10%). **Conclusions:** We have designed and clinically implemented a targeted fusion panel assay that allows for detection and characterization of novel and established fusions in sarcomas. Several targetable gene fusions involving *NTRK1, NTRK3, ALK*, and *PDGFB* were identified in a variety of different tumors. Our experience highlights the clinical utility of gene fusion testing for establishing the correct diagnosis and identifying potential targetable alterations.

ST089. **Somatic Reference Sample (SRS) Initiative: A Transformative Model to Improve Molecular Diagnostic Test Validation**

M. de Mars[1], A. Abdulkadir[1], N. Olson[2], J. Zook[2]
[1]Medical Device Innovation Consortium, Arlington, VA; [2]National Institute of Standards and Technology, Gaithersburg, MD.

Introduction: The Somatic Reference Samples (SRS) Initiative is a public-private partnership convened by Medical Device Innovation Consortium (MDIC) guiding the development of reference samples that can be used to develop and validate next-generation sequencing (NGS)-based cancer diagnostics. Ensuring that oncology patients receive accurate results is imperative; however, lack of agreed-upon, well-characterized, and validated reference samples and data benchmarks creates potential challenges. Phase I (completed) was initiated in 2018 with a mission to address the gap in reference material for NGS-based diagnostic tests. The initial output of this working group was MDIC's SRS Landscape Analysis (2019), a comprehensive catalog of existing clinically relevant cancer variants and available reference samples. In Phase II, currently underway, the SRS Initiative has prioritized a subset of 10 variants clinically associated with cancer to be engineered into the GM24385 (PGP/GIAB) cell line. **Methods:** A comprehensive review of existing NGS reference material was undertaken to help identify existing genes/variants and to help avoid duplication of effort. Additionally, NGS users and assay manufacturers were invited to respond to an online survey to help determine unmet needs. 1) Variant prioritization: Somatic variants with established clinical significance were identified and sent out for public comment. The selection process for 10 variants in the pilot project will be discussed. 2) Variant engineering: The 10 selected gene variants will be engineered into the human cell line GM24385 using CRISPR technology. Each individual cell line will be blended with the other engineered cell lines in formalin-fixed, paraffin-embedded blocks. 3) Data analysis/validation: The engineered cell lines will be evaluated to confirm intended mutations are in the correct location and to develop datasets for ongoing research and regulatory applications. **Results:** See MDIC's SRS Landscape Analysis (2019). 4) Variant selection: The 10 variants prioritized: *FGFR3, NTRK1, RET, EGFR, ERBB2, PDGFRA, RET, ERBB2, BRAF, FGFR3.* The 10 backup variants in case of technical difficulty: *FGFR2, NTRK2, ROS1, ALK, EGFR, ERBB2, MET, KRAS, PDGFRA, PIK3CA.* 5) Variant engineering/data validation and integration: CRISPR engineering of the GM24385 cells has been initiated. Results generation is in progress. **Conclusions:** The SRS Initiative is a novel, collaborative approach to improving the validation process and ultimately the accuracy of NGS-based cancer diagnostics by developing highly characterized samples and validated datasets. With key stakeholders' participation, this project has potential to transform the regulatory approval process for NGS-based cancer diagnostics and address critical unmet needs.

ST090. **Evaluation of *TERT* Promoter Mutations and Chromosome 1q Gain as Potential Diagnostic and Prognostic Biomarkers of Hepatocellular Neoplasm-Not Otherwise Specified (HCN-NOS)**

Y. Chen Wongworawat[2], S. Sarabia[2], K. Patel[3], A. Major[3], P. Sumazin[3], M. Urbicain[3], A. Roy[2], K. Fisher[2], D. López-Terrada[2]
[1]Baylor College of Medicine, Loma Linda, CA; [2]Baylor College of Medicine, Houston, TX; [3]Texas Children's Hospital, Houston, TX.

Introduction: Hepatoblastomas (HBs) are the most common primary liver malignancies in children, and the majority carry *CTNNB1* mutations resulting in activation of the WNT-signaling pathway. Most HBs respond to chemotherapy, but some HB patients succumb to this disease. The provisional category hepatocellular neoplasm-not otherwise specified (HCN-NOS) exhibits intermediate or combined HB and hepatocellular carcinoma histology and aggressive behavior was recently proposed. *TERT*-promoter mutations have been observed in clinically aggressive tumors and HBs diagnosed in older patients. MDM4 is an inhibitor of p53 activity and located within the region of 1q. Chromosome 1q gain is a recurrent event in HBs. We compared the molecular features of HBs and HCN-NOS including 1q gain and *TERT*-promoter mutation status and found that both are common in HCN-NOS and HBs in children >8 years old, and may be hallmarks of aggressive disease. **Methods:** HCN-NOS (n=20) and HBs in children >8 years old (n=3) were tested using a 124-gene pediatric solid tumor next-generation sequencing panel and an Affymetrix OncoScan copy number array, and results were compared to HBs control set. mRNA expression levels were determined using a customized NanoString nCounter PanCancer panel. Immunohistochemical (IHC) staining of TERT was performed, using a monoclonal primary antibody (ab32020, Abcam, Cambridge, UK). **Results:** *TERT*-promoter mutations occurred in 13 out of 23 HCN-NOS and HBs in older children. Focal and whole arm chromosome 1q copy gain was observed in 22 of 29 tumors. *MDM4* mRNA expression levels were elevated, correlating with increased 1q copy number (R^2=0.2638), and *p53*-pathway activation inversely correlated with *MDM4* gain. There was significant correlation between *MDM4* copy gain and *TERT* promoter mutation status (p <0.01, Fisher exact). Interestingly, an increased *MDM4* copy gain was observed in HCN-NOSs when compared to HBs (p <0.01, t-test). IHC staining of TERT was performed in HCN-NOS cases with *TERT*-promoter mutations, but results did not correlate with mutation status. **Conclusions:** We confirmed the high frequency of *TERT* promoter mutations and copy number alterations in HCN-NOS and HBs of older children, as well as the high frequency of 1q/MDM4 gains in this high-risk group. A large proportion (17 out of 20) of HCN-NOS cases showed *MDM4* copy gain, correlating with *MDM4* increased expression and reduced *p53*-pathway activity, suggesting a mechanism for their aggressiveness. *TERT*-promoter mutations and chromosome 1q/*MDM4* copy gain may represent potential diagnostic and prognostic biomarkers of HCN-NOS. Molecular testing represents the gold standard to detect *TERT*-promoter mutations in this group of aggressive pediatric liver tumors.

ST091. **A Multi-Country Real-World Analysis to Understand Diagnostics Testing Trends across New and Emerging Actionable Biomarkers**

I. Wang[1], A. Levent[2], L. Zinetti[1], G. Montenegro[1], J. Beer[3], C. Schott[1]
[1]Novartis Pharmaceuticals Corporation, East Hanover, NJ; [2]Ipsos Healthcare, New York, NY; [3]Novartis Pharmaceuticals Corporation, Cambridge, MA.

Introduction: We aim to understand the current biomarker testing pattern in solid tumors in the US, Germany (DE), Canada (CA), China (CH), and Japan (JP), following the approval of biomarker-directed targeted therapies. **Methods:** Retrospective data were collected through online survey of treating physicians (Ipsos Global Biomarker

Testing Rate Monitor). Data included physician practice setting, patient demographics, disease state, biomarker tested, and testing modality. This cohort included patient charts review with a confirmed diagnosis of non-small cell lung cancer (NSCLC), breast cancer, and melanoma. Data analyzed included biopsy sample type, biomarker of interest, test methodology. **Results:** A total of 386 treating physicians completed the survey, and 2,148 patient charts were reviewed in Q1 2022. Of 1,048 NSCLC charts (US: 242; DE: 165; CH: 272; CA: 77; JP: 292), testing rates of any molecular biomarkers (excl. PD-L1) were as follows: 42% US, 48% DE, 83% CH, 50% CA, and 48% JP. Next-generation sequencing (NGS) was the most common utilized technology among US (78%) and CA (85%), with PCR having the highest utilization in CH (42%) and JP (42%). Of 374 melanoma charts (US: 111; DE: 143; CA: 65; CH: 55), *BRAF* testing to guide therapy selection varies across disease stages. Stage IV had higher rates (US 97%, CA 96%, DE 85%, CH 75%) compared to stages 0-III (63% US, 58% CA, 58% CH, and 47% DE). NGS was the most utilized technology for *BRAF* testing in US (81%), CA (62%), and CH (66%); in DE, PCR (31%) and fluorescence *in situ* hybridization (FISH) (29%) were the most utilized. Of 726 breast cancer charts (US:146; DE:139; CA: 77; CH: 172; JP: 192), overall testing rates were high (>93%) in all countries. No difference was observed between academic versus community setting. Among stage IV patients, NGS utilization ranged from 66% (US) to 55% (CH), 48% (CA), 30% (DE), and 29% (JP). Germline *BRCA1/2* testing was higher in triple-negative breast cancer (TNBC) versus other subtypes. In stage IV HR+/HER2- BC, *PIK3CA* was the most common tumor marker (US: 28%; DE: 22%; CA: 42%; CH: 35%; JP: 3%), whereas PD-L1 was commonly tested in TNBC (US: 21%; DE: 21%; CA: 46%; CH: 39%; JP: 58%). Tissue from initial biopsy was the most common sample type. Liquid biopsy was emerging (ranging from 0%-29%). Individual biomarker testing by country/disease/subtype will be described in further details. **Conclusions:** Biomarker testing is recommended by multiple clinical practice guidelines in NSCLC, melanoma, and breast cancer. Real-world data suggested a significant portion of patients were not receiving guideline concordant biomarker testing in advanced/metastatic disease. Further studies to monitor testing rates and strategies to improve clinical adoption are ongoing.

ST092. **Beyond *KIAA1549::BRAF*: Rare and Novel *BRAF* Fusions Identified in Solid Tumors**

N. Guseva[1], A. Stence[1], R. Sompallae[1], S. Hornberg[1], A. Snow[1], S. Bhagavathi[1], A. Bossler[2], D. Ma[1]
[1]University of Iowa Hospitals and Clinics, Iowa City, IA; [2]Moffitt Cancer Center, Tampa, FL.

Introduction: *BRAF* fusions are rare oncogenic drivers in a wide variety of malignancies, but enriched in pilocytic astrocytoma (PA), melanoma, prostate and lung cancer, and papillary thyroid carcinoma. About 650 *BRAF*-fusion-positive cases and 17 different fusion partners were reported in COSMIC. The majority of the *BRAF* fusions were detected in PA and fusions with *KIAA1549* and *FAM131B* account for 95% and 1%, respectively. The *KIAA1549::BRAF* fusion results from a tandem duplication in chromosome 7q34, whereas the *FAM131B::BRAF* fusion likely arises from an interstitial deletion that places *BRAF* under the promoter of *FAM131B*. Other rare *BRAF* fusion partners include *RNF130, CLCN6, MKRN1, GNAI1, FXR1,* and *MACF1*. *BRAF* fusion events are known to activate the mitogen-activated protein kinase pathway important for the development of PA, but little is known about the less common fusions. Here, we report rare and novel *BRAF* fusions identified in the past six years at our institution and correlated the fusions with the pathologic diagnosis and other molecular findings. **Methods:** A total of 2,534 tumors were tested, and 250 ng RNA extracted from formalin-fixed, paraffin-embedded tissue or cytology slides were used for library preparation. Targeted sequencing for fusions was performed using the FusionPlex Thyroid/Lung Panel (Invitae). Data were analyzed using the Archer Analysis software (v.6.0.4). Novel fusions were confirmed by RT-PCR and Sanger sequencing. **Results:** *BRAF* fusions were detected in 48 cases; all retained an intact BRAF kinase domain. The *KIAA1549::BRAF* was identified in 36 cases (33 PA, two ganglioglioma, and one myxopapillary ependymoma) (75%). Rare/novel fusion partners were found in 12 cases (25%), *GNAI1* in a ganglioglioma; *FAM131B*, *GTF2I,* and *PRKAR2B* in three PAs; *CUX1* and *AKAP9* in two young males with desmoplastic infantile gangliogliomas; *SND1* in two prostatic adenocarcinoma, *FAM131B* in a 17-year-old male and *PRKAR2B* in a 25-year-old female with PA. Reciprocal fusions were found in three cases: *BRAF::AGK/AGK::BRAF* in a 70-year-old female with glioblastoma*; BRAF:SND1/SND1::BRAF* in an 81-year-old with metastatic prostate cancer*; BRAF::GTF2I/GTF2I::BRAF* in a 70-year-old male with PA. Three novel fusion partners were detected: an *AFAPIL2* in an 11-year-old female with PA, *TOP2B* in an 18-year-old male with PA, and *SPPL2A* in a 66-year-old male with melanoma. Coexisting pathogenic variants in *PTPN11, ATM, KRAS, TERT* promoter*, TP53,* and *PTEN* were detected in some of the *BRAF* fusion-positive tumors by DNA sequencing. **Conclusions:** Although rare, *BRAF* fusion is an inclusion criterion of several clinical trials for malignant solid tumor. Multiple rare/novel *BRAF* fusion transcripts were detected in our study. Patients with *BRAF* fusion-positive tumors may benefit from therapies targeting this genomic alteration.

ST093. **The Landscape of Somatically Actionable Genes Identified by DNA-NGS in Chinese Melanoma Patients**

C. Bai[1], T. Ma[2], B. Wang[3], C. Wang[3]
[1]Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; [2]Genetron Health (Beijing) Co. Ltd., Beijing, People's Republic of China; [3]Genetron Health (Hangzhou) Co. Ltd., Hangzhou, China, Hangzhou, People's Republic of China.

Introduction: Rarely studies had been done on melanoma in Asian populations due to the lower incidence rate than Caucasian. Given the low prevalence, delayed diagnosis is almost inevitable, resulting in more advanced stages of melanoma. Therefore, effective strategies to mitigate or even cure advanced melanoma in Asians are urgent. **Methods:** A total of 469 patients diagnosed with melanoma between March 2018 and September 2021 were enrolled in this study. Genomic DNA and RNA were extracted from formalin-fixed, paraffin-embedded tissues and targeted by using DNA-based next-generation sequencing (NGS) (Onco Panscan) and RNA-based NGS (FusionCapture) at Genetron Health, a laboratory accredited by the College of American Pathologists and Clinical Laboratory Improvement Amendments. **Results:** The druggable targets were identified in 75% (352/469) patients based on the OncoKB database, including 96 (20.5%) participants presenting high tumor mutation burden (≥10) and one microsatellite instability-high patient. Furthermore, the druggable patients were grouped based on the highest tier level of drug response evidence in OncoKB. Approximately 47% (164/352) of patients could be recommended as tier level 1 of OncoKB, followed by level 2 (29%, 100/352), level 3 (15%, 55/469), and level 4 (9%, 33/469). To explore the specific variations involved in the druggable group, the potential actionable mutations were classified in a mutation type-specific manner: single nucleotide variants (SNVs)/indels, copy number variants (CNVs), and fusions, which were found in 85% (n=299), 13% (n=59), and 2% (n=10) patients, respectively. The number of patients with *BRAF, NRAS,* and *NF1* mutations were the top three in the SNVs/indels group, which accounted for more than half of the total. In the CNV group, the proportion of *CDK4* and *MDM2* amplification was obviously predominant. Only three druggable tyrosine kinase drivers (*ALK, BRAF,* and *NRG1)* were identified at DNA level and *BRAF* fusions owned the dominant position (60%, n=6) in the fusion group. **Conclusions:** In summary, we present the druggable mutation landscape of 469 Chinese melanoma patients, and our work provides a workable translational genome-driven precision oncology strategy in melanoma patients, demonstrating the potential of individualized treatment for this rare but intractable disease.

ST094. **Implementation, Reporting and Clinical Utility of a Large Next-Generation Sequencing Panel at a Safety Net Institution**

B. Baskovich[1], K. Desai[2], M. Jamal[2], T. Rashid[2], I. Badar[2]
[1]Mount Sinai, New York, NY; [2]UF Health Jacksonville, Jacksonville, FL.

Introduction: Though use of next-generation sequencing (NGS) in solid tumors and other neoplasms has grown dramatically and the Center for Medicare and Medicaid Services has created a coverage policy, it may be underutilized in underserved areas, and even if a large send-out lab is used there may be difficulty interpreting the results in smaller institutions. We implemented a large NGS panel at a safety-net hospital, ran it over two years, attended tumor boards, and reviewed the reports and charts to determine the utility. **Methods:** The TruSight Oncology 500 was implemented at our institution, following recommendations of the College of American Pathologists and Association for Molecular Pathology (AMP). The AMP tiering system was used for reporting. Tumor mutational burden (TMB) and microsatellite instability (MSI) were reported. Clinical trials were reported. Interpretations were given for all Tier I and II variants. To evaluate the clinical utility, two molecular pathologists and three residents reviewed 201 cases in total after discussion and consensus on scoring. Metrics included whether Tier IA variants were found, high TMB or MSI, approved therapies in other tumor types, studies suggesting a therapy might have benefit, clinical trials available, prognostic information, and diagnostic information. This information was obtained from our reports. We then reviewed the charts to see if there was specific mention of the NGS results and if it was clearly used to determine the treatment plan. **Results:** We evaluated 201 cases. Of these, 17.9% had a Tier IA actionable mutation; the majority were in lung cancer and prostatic cancer. An additional 11.9% of cases had a contraindicated therapy (predominantly colorectal cancers), and 24.4% of cases had a TMB (above 10). In 22.9% of cases, a therapy was approved in other tumor types; DNA repair defects made up a majority of these. In almost all cases there were at least phase II clinical trials available (89.1%), and in 66% of cases prognostic information could be found (usually poor prognosis). In 52.2% of cases the NGS results were noted in the chart by the clinician. Uses varied, including using an FDA-approved therapy for specific mutations or high TMB, to rare clinical trial enrollment, to noting the results with no clear action taken. **Conclusions:** We demonstrated that implementation of a large NGS panel offered significant value to our patients. Clinical utility could potentially be better evaluated by also recording any altered decisions at tumor boards. Though NGS has grown, it may still be underutilized at certain institutions, and some payers, generally private insurers, lag behind in covering large NGS panels despite critical biomarkers such as TMB that can only be evaluated by such a panel, with therapeutic implications in a large number of cases.

ST095. **Targeted Next-Generation Sequencing Panel Reveals Differences in Mutational Patterns between Endometrial Cancer Molecular Classifier Subgroups**

A. Guimaraes-Young[1], L. Smith[2], D. Aisner[2], R. Wolsky[1], K. Davies[1]
[1]University of Colorado Anschutz Medical Campus, Aurora, CO; [2]University of Colorado Anschutz Medical Campus, Denver, CO.

Introduction: Four molecular subgroups of endometrial carcinomas (ECs) are currently recognized: *POLE* mutated (*POLE*), mismatch repair deficiency (MMRd), *TP53*-abnormal (a*TP53*), and no specific molecular profile (NSMP). These subgroups recapitulate genomic subgroups characterized by The Cancer Genome Atlas (TCGA) which have prognostic implications. NCCN guidelines currently encourage the use of ancillary studies to detect aberrancies in *POLE*, *TP53*, and mismatch repair (MMR)/microsatellite instability as a complement to histopathologic evaluation. In November 2020 we initiated reflex testing of all ECs. Here we summarize our findings from routine molecular analysis of 190 cases. **Methods:** Between Nov. 2020-Dec. 2021, 190 EC specimens across all stages and histotypes underwent molecular subtyping using p53 and MMR immunohistochemistry and next-generation sequencing-based analysis. Each specimen was assigned to a molecular subgroup: *POLE* (n=10), MMRd (n=52), a*TP53* (n=41), or NSMP (n=87) based on combined molecular and IHC data. Mutational load (ML) was determined by absolute mutation count across roughly 45Kb of targeted sequence from 56 genes. PI3K pathway activation was predicted based on the mutation status of four genes: *PTEN*, *PIK3CA*, *PIK3R1*, and *AKT1*. **Results:** ML was examined by molecular subgroup with mean number of mutations as follows: *POLE*, 14.1; MMRd, 6.4; a*TP53*, 3.6; and NSMP, 4.2. The ML was significantly different among the four subgroups. *POLE* tumors had a higher ML than all other subgroups including MMRd. MMRd tumors had a higher ML than a*TP53* and NSMP subgroups. No difference in ML was observed between the a*TP53* and NSMP subgroups. PI3K pathway activating genes were altered in a significant proportion of tumors: *POLE*, 100%; MMRd, 96.2%; a*TP53*, 68.3%; NSMP, 95.4%. Notably, 40% of *POLE* tumors harbored *TP53* mutations, as did 17.3% of MMRd tumors (considered multiple classifier). a*TP53* tumors demonstrated reduced prevalence of mutations in canonical activating kinases including *KRAS* and *FGFR2*. NSMP tumors more frequently contained *CTNNB1* mutations. **Conclusions:** Our findings from real-world implementation of ProMise classification are consistent with prior studies. All subgroups had mutations in genes implicated in PI3K pathway activation, with >95% of tumors within the *POLE,* MMRd, and NSMP subgroups altered. a*TP53* tumors harbored fewer PI3K pathway mutations than the other subgroups. Dual classifier cases may represent acquisition of *TP53* mutations secondary to other pathologic mechanisms such as *POLE* and MMRd.

ST096. **Evaluating the Utility of Thermo Fisher Oncomine NGS Panels in Determining Copy Number Variation among Solid Tumors**

G. Luk[1], W. Huang[1], D. Chan[1], K. Craddock[1], R. Goswami[1], D. Hwang[1], H. Wang[2], B. Wong[1], N. Wong[1]
[1]Sunnybrook Health Science Center, Toronto, Ontario, Canada; [2]North York General Hospital, Toronto, Canada.

Introduction: Targeted next-generation sequencing (NGS) panels are increasingly being used to identify actionable copy number gains (gene amplifications) among solid tumors. However, there remain limited studies comparing the copy number gains detected by NGSs to those detected by orthogonal methods. In this study, we evaluated the analytical performance of two amplicon-based NGS assays, the Oncomine comprehensive panel (OCAv3) and the Oncomine focus assay (OFA), in the detection of copy number gains among formalin-fixed, paraffin-embedded tumors of varying tumor cellularity. **Methods:** Thirty-four tumors with copy numbers greater than four in *ERBB2*, *MET,* and *EGFR* detected via OCAv3 (16 tumors) or OFA (18 tumors) analysis were included in this study. Illumina SNP microarray assay (Infinium CytoSNP-850K v.1.2 BeadChip) was carried out on 28 of 34 tumors. Tumors with a discordance in *ERBB2* copy number changes were further analyzed by fluorescence *in situ* hybridization (FISH) assay. Six of the tumors with *ERBB2* copy number gains were analyzed by FISH only. Two reference samples from Horizon with known copy number gains in *ERBB2*, *MET*, *EGFR*, *FGFR3*, *MYCN,* and *MYC* were serially diluted and tested in duplicate to determine the limit of detection using OCAv3 and OFA. **Results:** The mean MAPD scores of OCAv3 and OFA of the 34 NGS-tested tumors were 0.28 and 0.38, respectively, passing vendor-recommended QC metrics. Although the copy number read-outs from NGS were consistently higher than those obtained via SNP microarray, 26 of 28 samples showed concordance on the calling of copy number gains between the two. SNP microarray failed to detect copy number gains within two tumors with lower *ERBB2* copy number gains called by NGS. Both samples were subsequently confirmed to show *ERBB2* copy number gains by FISH. Five of six tumors analyzed by *ERBB2* FISH showed concordant results with those obtained via NGS. Testing reference materials with 14 copies across six driver genes showed that both OCAv3 and OFA assays failed to detect copy number changes when

tumor cellularity was below 20%. **Conclusions:** Thirty-three of 34 tumor samples with copy number gains called by NGS were confirmed by an orthogonal method. Investigation of the only false-positive calling by NGS showed that the sample exhibited a much higher MAPD score, approaching the manufacturer's established cut-off. Overall, the analytical specificity of OCAv3 and OFA in detecting copy number gain is greater than 95%. Setting the MAPD score at a cut-off of 0.4 or less for OCAv3 could reduce the likelihood of false-positive callings by NGS. Amplicon-based NGS assays, however, are not sufficiently sensitive enough in detecting copy number gains in the presence of low tumor cellularity, which is a significant limitation that should be clearly stated in NGS reporting.

ST097. **Reflex-Ordered Molecular Biomarkers in Early-Stage Lung Adenocarcinoma**

P. Hodjat, J. Kinskey, J. Thomas, F. Zazueta Leon-Quintero
Houston Methodist Hospital, Houston, TX.

Introduction: Reflex-ordered molecular testing in lung adenocarcinomas (LAd) standardizes and significantly decreases the turnaround time for molecular results. Current guidelines recommend molecular testing only in advanced stage/metastatic LAd. However, the potential benefits of molecular testing in early-stage (stage I/II) tumors remain unclear. In our hospital system, a reflex-ordered molecular panel for all newly diagnosed LAds of any stage was approved in 2017. For this study, we retrospectively examined the results of our biomarker testing in early-stage LAds. **Methods:** The reflex-ordered panel includes gene mutations in *EGFR*, *BRAF*, *KRAS*, and *ERBB2* (iPLEX HS Lung v2 and MassARRAY System, Agena) and gene rearrangements of *ALK*, *ROS1*, *RET*, *NTRK*, and *MET* (Archer FusionPlex CTL and NextSeq 550, Illumina). With IRB approval, molecular results for 721 patients diagnosed with LAd from 2017 to 2021 were reviewed. These data were extracted and analyzed using custom Python and SQL scripts and Excel PowerQuery. Staging was obtained by chart review. **Results:** The mean age of this cohort was 68.8 years. Almost half (46%) of the patients were lost to follow-up after the initial diagnosis. Of the remaining 393 patients, 156 (40%) were early-stage at diagnosis. Molecular biomarkers were reflex-ordered at diagnosis for approximately 91% of the cohort. The inconclusive rate was 0.4%-1% for specific gene mutations and 2%-5% for specific gene rearrangements. Only five patients received no molecular testing. Twenty-nine percent of early-stage LAds had no alteration identified, compared to 6% of advanced-stage cases. The most common gene alterations detected in both early- and advanced-stage LAds were mutations in *KRAS* (40% vs. 44%) and *EGFR* (26% vs. 29%). The median turnaround time for gene mutation analysis was 10 days, with an additional five days required for the gene rearrangement analysis. **Conclusions:** Our findings show that a significant number of patients were diagnosed with LAd at an early stage. Early-stage LAds were more frequently negative for all tested gene alterations compared to advanced-stage tumors. For *KRAS* and *EGFR* genes, the mutation frequency was similar for early- and advanced-stage cases. However, alterations of *BRAF*, *ALK*, *ROS1*, and *RET* genes, as well as co-alterations, were more frequent in advanced-stage tumors. The inconclusive result rate was quite low, <5% for each tested gene alteration. The median turnaround time for reflex-ordered molecular results was approximately 15 days, allowing for timely guidance of therapeutic management for these patients. A reflex molecular testing strategy allows the possibility of targeted therapy in early-stage LAd patients, should such therapies prove beneficial. Future work will examine outcomes of molecular biomarker testing in early-stage LAd.

ST098. **Molecular Profiling of *ALK* Fusion Variants Observed among Indian Lung Cancer Patients: Tertiary Cancer Center Perspective**

O. Shetty, M. Gurav, P. Bapat, t. Pai, N. Karnik, G. Wagh, V. Noronha
Tata Memorial Hospital, Mumbai, India.

Introduction: The Anaplastic lymphoma kinase (*ALK*) gene rearrangements are the oncogenic driver fusion oncoproteins. With *ALK* inhibitors of *ALK*-positive lung cancer, patients have benefited tremendously; however, clinical outcomes vary widely in patients. This could be due to different *ALK* fusion variants. The most common *ALK* fusion partner is the *EML4* gene which has >15 *EML4-ALK* variants. The predominant are variant 1 (v1; *EML4*: exon 13 and *ALK*: exon 20) and variant 3 (*EML4*: exon 6a/b and *ALK*: exon 20). Variant 2 (v2; *EML4*: exon 20 and *ALK*: exon 20) is uncommon. The present study describes the frequency and distribution of the *ALK* fusion variants detected among Indian cancer patients treated at a tertiary cancer hospital. **Methods:** This is a retrospective study of formalin-fixed, paraffin-embedded tissue of histologically confirmed lung cancer cases (n=800) between the years 2019-2022. These were subjected to next-generation sequencing (NGS) as part of routine diagnostic work-up using targeted panels viz. AmpliSeq Focus panel and SOPHiA Solid Tumor Plus Solution (STS Plus) which could detect both DNA and RNA alterations. The data analysis was performed using Local Run Manager and Base space software for AmpliSeq Panel and SOPHiA DDM platform for STS Plus. The analysis and interpretations were done as per standard recommended guidelines. **Results:** Of the 800 lung cancer cases, *EML4-ALK* fusion was detected in 47 cases (5.0%). The fusion variants 1, 2, and 3 were stratified. Among the fusions detected, variant 1 was observed in ~51% of the cases (24/47) followed by variant 3, which was observed in 40% of the cases (19/47). Variant 2 was detected in 8% of the cases (4/47). There was 100% concordance observed between *ALK* IHC and the NGS data. Concomitant mutations were detected in 38% of the cases. *EGFR* was the most common Tier I co-mutation detected among all three variants (12%, 6/47) followed by *TP53* which was the most common Tier II mutation observed only with variant 1 (8%; 4/47). *ALK* resistance mutations were detected in variant 1 and variant 3 (6%, 3/47). The other mutations included *PIK3CA, RAS, IDH, CDKN2A,* and *SMAD4.* First-generation TKI was given to 48% of the patients, second-generation TKI was given to 36% of the patients, and 6% of them received third-generation TKI. Among the variant 1 positive groups four patients were deceased due to progressive disease. Among variants 1 and 3, ~50% of the patients had progressive disease. **Conclusions:** The present study illustrates the profile of the *ALK* fusion variants observed in this Indian cohort. The frequency of different fusion variants as well as the occurrence of co-mutations will help unravel the diverse mechanism of response to the *ALK*-directed therapy.

ST099. **Clinical Impact of Targeted Genomic Profiling**

T. Phung, C. Manganti, M. Kasukurthi, K. Hebert, J. Huang
University of South Alabama, Mobile, AL.

Introduction: Next-generation sequencing (NGS) technology is key to identifying gene mutations important in cancer diagnostics and therapeutics. NGS assays for solid tumors range from those that assess dozens of genes with actionable clinical utility (targeted genomic profiling, TGP) to assays that evaluate hundreds to thousands of genes (comprehensive genomic profiling, CGP). Genes included in TGP are limited in number and carefully chosen based on their clinical actionability with mutations that have prognostic and/or therapeutic impact. Although CGP would yield more genomic findings than TGP, our goal is to determine the degree of difference in clinical impact of TGP as compared with CGP. **Methods:** We analyzed 775 unique CGP cases (189 non-small cell lung, 153 colorectal, 108 breast, 78 endometrial, 69 pancreatic, 64 ovarian, 40 hepatobiliary, 33 prostate and 26 cervical carcinoma, and 15 melanoma). CGP covered ~500 genes with variant types including single nucleotide variants, copy number variants, indels, and gene fusions. Pathogenic variants extracted from CGP cases included actionable variants with FDA-approved therapy or standard of care, and variants included in clinical trials specific for cancer type as identified in "Clinicaltrials.gov." Pathogenic variants identified in CGP cases were matched with those covered by TGP of 60 key cancer-related genes. **Results:** Actionable pathogenic variants were identified in 112 out of 775 (14%) CGP cases across multiple tumor types (37 breast, 36 lung, 18 colorectal, 13 endometrial, seven melanoma, and one cervical carcinoma). Among 112 CGP cases with actionable variants, 108 cases (96%) had variants that were covered in TGP across multiple tumor types (33 breast, 36 lung, 18 colorectal, 13 endometrial, seven melanoma, and one cervical carcinoma). There were 348 of 775 (45%) CGP cases with pathogenic variants indicative for cancer-specific and mutation-specific interventional clinical trials as listed in "Clinicaltrials.gov" across multiple tumor types (117 colorectal, 73 lung, 48 endometrial, 39 pancreatic, 29 breast, 18 ovarian, nine cervical, seven hepatobiliary, four melanoma, and four prostate carcinoma). Among these 348 CGP cases, 235 cases (68%) had variants that were covered in TGP across multiple tumor types (81 colorectal, 42 lung, 28 endometrial, 39 pancreatic, 20 breast, 16 ovarian, three cervical, five hepatobiliary carcinoma, and one melanoma). **Conclusions:** TGP covers 96% of actionable mutations, and provides significant coverage (68%) of clinical-trial eligible pathogenic variants identified by CGP. These findings show significant clinical impact of TGP with respect to identifying biomarkers for targeted therapy and clinical trials. Targeted gene profiling has important clinical utility in the setting of cost effectiveness, manageable gene content, and limited tissue resources.

ST100. **A 2-Log Reduction in ctDNA Level Similar to *BCR-ABL1* May Prove Predictive of Response in *EGFR* Mutant NSCLC: An Initial Report**

U. Batra, S. Nathany, M. Sharma, A. Bansal, S. Dhanda, H. Singh, J. Jose
RGCIRC, New delhi, India.

Introduction: With the IASLC recommending use of liquid biopsy in first line as well as FLAURA trial results for use of osimertinib in first line, there is a need for serial monitoring of *EGFR* mutations as well as resistance mechanisms in those patients on TKIs. The present study explored the association between the dynamics of *EGFR* mutations in cell-free DNA and disease evolution in 47 *EGFR* mutant patients receiving first-line EGFR TKI therapy. **Methods:** Forty-seven patients diagnosed with advanced non-small cell lung cancer, with sensitizing *EGFR* mutations (del19 and L858R) on tissue genotyping were enrolled in this study. Serial plasma samples were taken at three- to four-month intervals from these patients, before objective response evaluation. ddPCR-based *EGFR* genotyping was performed, with an assay sensitivity to detect one mutant copy of circulating tumor DNA (ctDNA) per microlitre. Log reduction in *EGFR* mutant copies was computed. Statistical analysis was done using SPSS v23, IBM (Tucson, AZ, US) **Results:** Forty-seven patients were enrolled in this study. The median age was 57 years (range: 31-72 years). Thirty cases had del19 mutation, and 17 patients had L858R mutation. Beyond first response evaluation, 35 (74.4%) patients have progressed, (21 del19 and 14 L858R) with a median progression-free survival (PFS) of nine months. Together with 47 baseline samples, 82 serial samples were taken. In the baseline samples, the corresponding sensitising mutation was detected in 46 (97.8%) cases of which 29 (63%) had del19 and 17 (37%) had L858R mutations. At first serial evaluation, *EGFR* mutations were detected in 15 patients with an average concentration of 5.97 copies/microlitre for del19 and 5.19 copies/microlitre for L858R. Median PFS of ctDNA-positive patients was nine months. Patients who lost their primary mutation (n=21) had a median PFS of 8.8 months (95% CI: 6.95-7.83), whereas the 15 patients who retained their primary mutation showed a median PFS of 7.15 months (95% CI: 4.7-6.27 months). The ddPCR ctDNA concentration preceded objective response evaluation in these patients by a median of 2.7 months (range: 1-8.2 months). A $\geq$2 log reduction in *EGFR* ct DNA concentration was associated with a better PFS (median: 8.8 months) when compared to those with <2 log reduction (7.15 months) ($p < 0.05$). **Conclusions:** This is real-world data from India, depicting the same, with a better PFS in those who lose their primary mutation compared to those who retain it. A 2-log reduction in ctDNA concentration may serve as a predictor of response

ST101. **Hybridization Capture-Based RNA Sequencing Shows Greater Clinical Sensitivity for Oncogenic Fusion Detection Than Amplicon-Based RNA Sequencing**

J. Solomon[1], E. Zhong[2], G. Cheang[2], D. Xu[2], H. Kim[2], E. Fernandez[2], M. Yip[2], S. Parmar[2], R. Purakait[2], E. Hissong[2], P. Velu[2], A. Yemelyanova[2]
[1]Weill Cornell Medicine/New York Presbyterian, New York, NY; [2]Weill Cornell Medicine, New York, NY.

Introduction: The accurate identification of oncogenic gene fusions in solid tumors is important not only because they are drivers of tumorigenesis but also because they are often amenable to targeted therapy. RNA sequencing in the form of targeted next-generation sequencing (NGS) is quickly becoming the standard of care for comprehensive fusion detection. However, there are a few different sequencing chemistry platforms available with important clinical and analytical implications to be considered. Amplicon-based library preparation requires that both fusion partners be covered by the assay to allow for detection. In contrast, hybridization capture-based library preparation enables detection of oncogenic fusions agnostic of the fusion partner. Here, we demonstrate that this theoretical difference in sequencing approach translates to a marked difference in clinical sensitivity for fusion detection in a variety of tumor types. **Methods:** DNA and RNA were separately extracted from formalin-fixed, paraffin-embedded tissue. DNA and RNA underwent amplicon-based library preparation using the Oncomine Comprehensive Assay v2 (Thermo Fisher), and libraries were sequenced on the IonTorrent S5 Sequencer (Thermo Fisher). At the time of clinical signout, if the case was found to be driver-negative in a tumor type where MAP kinase pathway alterations are known to drive tumorigenesis, the RNA was reflexed to the hybridization capture-based TruSight Oncology 500 Assay (Illumina) and sequenced on the NovaSeq 6000 (Illumina). **Results:** From April 2021 through June 2022, 1,093 clinical specimens underwent comprehensive molecular profiling using the amplicon-based NGS panel. Of these, 41 cases negative for MAP kinase pathway driver alterations were reflexed to hybridization capture-based RNA sequencing. Oncogenic fusions were detected in 10 of these cases. Fusions were detected in non-small cell lung cancer (four fusions detected in 26 cases reflexed), histiocytic neoplasms (two of four), cholangiocarcinoma (one of three), thyroid carcinoma (two of four), and other tumor types (one of four). Fusions were in-frame and included the tyrosine kinase domains for the functional partners (*ALK* [n=3], *RET* [n=2], *BRAF* [n=2], *KIT* [n=1], *CSF1R* [n=1], *FGFR2* [n=1]). The other partner was rare or novel in each case, and it was not

covered by the amplicon-based assay. **Conclusions:** Advantages of amplicon-based tumor profiling include ease of implementation, rapid turnaround time, and lower cost. However, hybridization capture methods can identify oncogenic fusions in a partner-agnostic manner and demonstrate improved clinical sensitivity. Here, we present a reflex testing algorithm implemented in a clinical laboratory to leverage the advantages of both systems and improve detection of targetable alterations in solid tumors.

ST102. **Assessment of Germline Splice Variants in Tumor Sequencing**

E. Bogdanova[1], Z. Soens[2], A. Silkov[2], R. Chen[2], L. Edelman[2], B. Funke[2], H. Al-Kateb[2], F. Hantash[2], M. Rossi[2]
[1]Icahn School of Medicine at Mount Sinai, Northbridge, MA; [2]Sema4, Branford, CT.

Introduction: Detection and interpretation of splice variants (SVs) that lie beyond the canonical splice acceptor or donor sites can be challenging. We previously evaluated the utility of SpliceAI (SAI) in reporting cryptic and/or canonical splice variants in the context of tumor-only sequencing in 2,901 formalin-fixed, paraffin-embedded samples. The aim of this study was to investigate the prevalence of germline SVs in the subset of cancer-relevant genes using tumor-normal whole-exome sequencing (WES) and tumor transcriptome sequencing (WTS). **Methods:** We chose 641 matched tumor-normal samples across 46 different tumor types which underwent clinical WES/WTS. Rare germline SVs, restricted to 107 genes associated with known and possible increased risk for cancers, were surveyed for potential splicing impact by SAI and RNA analysis. **Results:** In total, 93 rare germline SAI-annotated SVs were identified across 641 unique samples and were subjected to RNA splicing analysis. A normal splicing pattern was observed for 49.6% (46/93) of annotated SVs. An abnormal splicing pattern was observed for 23.7% (22/93) SVs with a high proportion altering the extended canonical splice-site region. These included: (eight) canonical SVs, (11) non-canonical intronic or cryptic variants, and (three) deep-exonic cryptic SVs. We were unable to determine the splicing pattern for 26.9% (25/93) SVs due to the poor quality of RNA (two), lack of coverage in tumor RNA (15), or absence of the variant in the tumor RNA (eight). SVs with evidence of mis-splicing were identified in known cancer-predisposition genes (*ATM, BRCA1/2, MAX, NF2, PTCH1, RAD51D*) and candidate cancer-susceptibility genes (*ERCC2, ERCC4, NTHL1, FANCA, FANCI, FANCL*). ClinVar entries were available for 81.8% (18/22) of RNA-supported SVs with (eight) pathogenic/LP, (seven) variants of uncertain significance, and (three) Benign/LB. Somatic loss of heterozygosity (LOH) accompanied 31.8% (7/22) SVs. **Conclusions:** Germline SVs in HC susceptibility genes, leading to mis-splicing, were detected in 3.4% (22/641) of WES/WTS specimens in our study. A higher rate of abnormal splicing was noted for non-canonical intronic or cryptic variants (63.6%) versus canonical donor or acceptor sites (36.4%). Using tumor-normal WES and tumor WTS as a standard clinical somatic sequencing approach may assist in reclassification of uncharacterized splicing variants in the known cancer-predisposition genes and extend the spectrum of causative variants in candidate cancer-susceptibility genes. In the somatic context, aberrant germline SVs along with somatic LOH likely contribute to tumorigenesis. However, the significance of some SVs in candidate cancer-susceptibility genes in the germline context remains uncertain and requires further investigation.

ST103. **Value of Comprehensive Genomic Profiling on Clinical Actionability and Treatment in Advanced Cancer Patients**

C. Bifulco[1], B. Bapat[2], R. Weerasinghe[1], R. Meng[1], A. Dowdell[1], A. Vita[1], S. Chang[1], M. Campbell[1], B. Schroeder[2], B. Piening[1]
[1]Providence Health, Portland, OR; [2]Illumina, Inc., San Diego, CA.

Introduction: Precision therapies and immunotherapies have revolutionized cancer care, with novel genomic biomarker-associated therapies being introduced into clinical practice rapidly. We assessed the utility of comprehensive genomic profiling (CGP)-based testing for identifying biomarkers associated with approved therapies, and therapies in precision medicine basket clinical trials (CT) and the therapies received in the line subsequent to CGP testing across a large cohort of advanced cancer patients in the Providence Health System. **Methods:** Advanced cancer patients were tested utilizing the Providence CGP workflow between 2019-2021. Clinical actionability was assessed for patients based on OncoKB Tier 1 AMP/ASCO/CAP categorization: FDA recognized (Level 1), standard of care (Level 2), and standard of care predictive of resistance to an FDA-approved drug (Level R1). CT matching was assessed based on enrollment criteria for ASCO-TAPUR, NCI-MATCH and My Pathway CTs at time of testing. Systemic therapy post CGP-testing was assessed among patients whose care was managed at Providence post-CGP testing. Pooled electronic medical record and genomic data were curated and standardized. **Results:** A total of 2,028 advanced cancer patients were tested with CGP and followed up for cancer care management at Providence; 52% were female, 83% were white, and median age was 66 years. Across 31 tumor types, the most commonly tested were lung (27%), bowel/colon (16%), and breast (10%). Forty-seven percent (964) of patients had at least one actionable biomarker for an approved and/or guideline recommended targeted therapy (based on OncoKB levels 1,2, R1), and 53% were eligible for at least one of three basket trials at the time of testing. Of the CGP tested patients, 14% did not receive any cancer-directed therapy and/or received only supportive care. Patients were treated using the following systemic therapies in line following CGP testing: chemotherapy only (663 [33%]), chemotherapy + immunotherapy (IO) (347 [17%]), IO only (269 [13%]), targeted therapy in community setting (361 [18%]), targeted therapy in CT setting (79 [4%]), and off-label use of targeted therapy (7 [0.3%]). Of 361 patients who received a targeted therapy, 227 (63%) had an associated oncogene whereas 134 (37%) were treated based on approved disease-specific indications. **Conclusions:** The rate of clinical actionability for approved and guideline recommended therapies and for basket CTs is considerable after testing with CGP, resulting in precision therapies (targeted therapy and immunotherapy) rapidly eclipsing chemotherapy as major treatment modalities. We expect the value of CGP to increase as biomarker actionability transforms clinical practice.

ST104. **Co-occurrence of *KRAS* Q61K with Synonymous Mutations and Their Effect on RNA Expression**

T. Gogakos[1], J. Lennerz[1], A. Bagaev[2]
[1]Massachusetts General Hospital, Boston, MA; [2]BostonGene, Waltham, MA.

Introduction: The clinical significance of base substitutions that do not result in amino acid changes (synonymous mutations) remains understudied. It has recently been shown that pathogenic mutations in codon 61 of *KRAS* (p.Gln61Lys or p.Q61K) co-occur exclusively in cis with synonymous mutations in the preceding codons (Gly60 or Ala59), which are required for *KRAS* activation (Kobayashi Y. *et al.*, 2022). Mechanistically, when present in isolation, mutations in codon 61 provide a novel splice donor site that results in a truncated non-oncogenic protein isoform. However, the presence of a synonymous variant in cis abolishes the splice site and results in full-length, mutant, and oncogenic *KRAS*. **Methods:** We analyzed all 14,698 solid tumor specimens sequenced by our institution's targeted next-generation sequencing panel and identified n=8 cases with *KRAS* p.Q61K (0.05%). We supplemented our analysis with RNA sequencing (RNA-Seq) to investigate the effect of these in cis events on *KRAS* isoform composition and level of expression. **Results:** By prevalence and tumor type, our cohort of positive cases was of similar size to that of the Cancer Genome Atlas (TCGA, n=7 vs. MGH, n=8) and their proportion with respect to the total cases tested was comparable to that of the Dana-Farber Cancer Institute (DFCI, fraction=0.05% vs. MGH, fraction=0.09%), suggesting an accurate and unbiased representation of tumors in our patient cohort. In all cases *KRAS* p.Q61K occurred exclusively in cis with a synonymous Gly60Gly or Ala59Ala variant. The synonymous variants represented n=4/6

possible coding single nucleotide changes, with their variant allele fractions ranging from 1% to 39%. To accurately reflect the presence of such variants in clinical samples, we established clinical reporting paradigms via appropriate variant tiering and commenting in our reports. **Conclusions:** To our knowledge, this is the fourth large cohort that shows exclusive co-occurrence of *KRAS* synonymous mutations (p.G60G or p.A59A) with p.Q61K, and the first that provides supporting RNA-Seq evidence. These data showcase the importance of examining and reporting synonymous mutations in cancer and highlight the value of RNA-Seq for the concurrent analysis of their effects on isoform composition and RNA expression. Given the potential for splicing-directed intervention in *KRAS* p.Q61K cancers, our data expand the number of possible patients that can benefit from such potential therapies and raise awareness for more careful annotation of synonymous variants in clinical sequencing panels. Towards that end, our established reporting and variant tiering paradigms could serve as a blueprint for other co-occurring synonymous variants.

ST105. **Reference Materials for Analysis of *BRCA1* and *BRCA2* Gene Variants with Expansion to Large Genomic Re-arrangements (LGRs) to Support Therapy Selection in Breast and Ovarian Cancer Patients**

C. Huang[1], B. Forson[1], D. Ruminski Lowe[1], Y. Konigshofer[1], I. Chivukula[2], E. Kolossovski[2], B. Anekella[1]
[1]LGC Clinical Diagnostics, Gaithersburg, MD; [2]LGC Clinical Diagnostics, Milford, MA.

Introduction: Genetic testing of the tumor suppressor genes *BRCA1* and *BRCA2* allows for the identification of DNA variations, such as those that are associated with a significantly elevated lifetime risk of breast and ovarian cancer. Accurate detection of a *BRCA1* or *BRCA2* pathogenic variant has immense impact on clinical management of disease including the patient's eligibility for new poly (ADP-ribose) polymerase inhibitors. Large genomic rearrangements (LGRs) in *BRCA1* are responsible for up to 27% of all *BRCA1* disease-causing mutations identified in numerous populations, accounting for about one-third of all disease diagnosis. However, these LGRs are frequently missed by PCR-based methods and targeted next-generation sequencing (NGS) assays that do not detect partial or complete exon losses or gains. Given the difficulty in detecting LGRs, there is a need for a comprehensive *BRCA1*/2 testing algorithms including reference materials that incorporate pathogenic *BRCA1*/2 LGRs to support NGS assays that analyze for these mutations at both germline and somatic levels. **Methods:** GM24385 human reference cell line was engineered to contain biosynthetic constructs bearing the *BRCA1*/2 variants of interest. Cell bulks were produced targeting 5%-20% variant allele frequencies (VAFs) as determined by digital PCR (dPCR) using allele-specific assays. Formalin-fixed, paraffin-embedded (FFPE) blocks were prepared using the engineered cells. The QIAamp DNA FFPE Tissue Kit and the Maxwell RSC DNA FFPE Kit were used for DNA isolation from FFPE blocks. DNA was quantified by the Qubit dsDNA HS Assay and the quality was assessed using the Genomic DNA ScreenTape for the Agilent TapeStation. Extracted DNA was analyzed using a targeted NGS assay to verify that all variants were present at appropriate VAFs and that the product was compatible with clinical testing workflows. **Results:** DNA constructs bearing *BRCA1* and *BRCA2* gene variants were designed and synthesized. These included a total of 20 variants, 10 in *BRCA1* and 10 in *BRCA2,* which ranged in size from simple single nucleotide variants to deletions of more than 500 bp. Prototype FFPE blocks were tested for DNA yield per curl and resulted in 193±44 ng (QIAamp) and 124±11 ng (Maxwell). Digital PCR testing confirmed the presence of all variants at >5% VAF. **Conclusions:** We have developed reference materials to support *BRCA1* and *BRCA2* gene testing, providing labs with a tool to optimize and challenge their assay's detection and quantitation of LGRs. The FFPE format is the preferred sample format for breast and ovarian cancer sample testing in clinical labs, and so the reference material may serve as a whole-process control. It can be used in assay development, validation, and application of amplicon and hybrid capture-based targeted NGS assays to analyze *BRCA1* and *BRCA2* mutations and LGRs as well as for developing new analysis pipelines.

ST106. **The Landscape of *BRAF* Alteration in Pediatric and Young Adult Solid Tumors**

J. Schubert, J. Wu, F. Xu, M. Patel, N. Golenberg, J. Chen, E. Denenberg, E. Fanning, W. Fu, K. Cao, R. Bagatell, T. Laetsch, A. Resnick, M. Santi, P. Storm, M. Luo, G. Wertheim, Y. Zhong, L. Surrey, M. Li
Children's Hospital of Philadelphia, Philadelphia, PA.

Introduction: Gain-of-function mutations in the proto-oncogene *BRAF* promote tumorigenesis through activation of MAPK/ERK pathways, and are seen recurrently in various solid tumors, which can be targeted using BRAF or MEK inhibitors. We sought to determine the spectrum of *BRAF* alterations in a cohort of pediatric and young adult patients. **Methods:** We reviewed the data of 2,237 solid tumors sequenced using the CHOP Comprehensive Solid Tumor Panel to detect clinically significant (Tier 1 or 2 variants as defined by AMP/ASCO/CAP guidelines) single nucleotide variants (SNVs), indels, and gene fusions affecting *BRAF* (NM_004333.5) in a pediatric and young adult cohort. **Results:** We identified 318 (14.2%) tumors with a clinically significant *BRAF* SNV or fusion from 302 patients. CNS tumors were predominant (245/318, 77.0%), the most common of which were low-grade gliomas (LGG, 192) and glioneuronal tumors (GN, 43). LGGs were mainly affected by fusions versus SNVs (146 vs. 45, respectively), whereas the opposite was true for GN tumors (37 SNVs and six fusions). Other tumors enriched with *BRAF* SNVs included papillary thyroid carcinoma (PTC, 42 cases, 37 with SNVs) and Langerhans cell histiocytosis (LCH) and non-LCH histiocytosis (16 cases, 14 with SNVs). We found 21 unique fusion genes in 164 tumors. The most common, *KIAA1549::BRAF* (140), was found only in LGG or GN tumors; pilocytic astrocytoma (128) was the most frequent histologic diagnosis. The top recurrent breakpoints between *KIAA1549* (NM_020910.2) and *BRAF* were exons 16::9 (78), 15::9 (36), and 16::11 (12). Other recurring fusions included *GNAI1::BRAF* found in four LGG or GN tumors, and five PTC cases all with different 5' gene partners. Notable novel/rare findings included fusions to *MCC* (infantile fibrosarcoma), *SND1* (pancreatic tumor), and *MTAP* or *MS4A6A* (LCH/non-LCH). *BRAF* SNVs or indels were observed in 154 tumors. We identified 17 unique coding variants resulting in 14 protein-level changes; V600E (126/154) was the most common. Of the V600E tumors, PTCs (35/126) were most frequent, followed by LGG (33), GN (33), LCH/non-LCH (11), and high-grade gliomas (seven). Rarer findings included G469A in 3/3 cases of neuroblastoma and several non-conventional tumor types with diverse SNVs. Notably, 11 unique indels were seen in 20 tumors, emphasizing the utility of next-generation sequencing to detect a range of variation. **Conclusions:** This pediatric and young adult *BRAF*-positive cohort (14% of tested samples) included primarily low-grade CNS tumors, followed by PTC and histiocytosis, indicating that a significant fraction of pediatric tumors may benefit from BRAF inhibitor therapy. The rate of fusion and SNV positivity was nearly equal, though varied by tumor type, demonstrating the utility of a comprehensive panel targeting both DNA and RNA.

ST107. **A Single-Institution Cohort Study on Her2-Low Expression in Hormone-Receptor Positive Metastatic Breast Cancer Treated with Cyclin-Dependent Kinase Inhibition by Palbociclib**

J. Illarramendi, Y. Ruiz de Azua, R. Beloqui, I. Amat, C. Cerezo, D. Guerrero, E. Salgado, S. De La Cruz, A. Cordoba
University Hospital of Navarra, Pamplona, Spain.

Introduction: Recent breakthrough results of trials with antibody-drug conjugates have increased the interest on metastatic breast cancer (MBC) tumors with low expression of the Her2 receptor (Her2-low). Most of these tumors express hormone receptors (HR) and are initially treated with the combination of hormone therapy and inhibitors of cyclin-dependent kinases, like palbociclib (PALB). Further knowledge

on the features and response to PALB therapy of Her2-low tumors is needed to improve the strategies for this disease. **Methods:** Retrospective study. Setting: 1,200-bed university hospital. Study period: March 2018 to March 2022. Inclusion criteria: All the patients with HR+ MBC tumors who had Her2-low expression and were treated with PALB in this center. Protocol for Her2 evaluation was performed by pathologists with experience on BC diagnosis using the Ventana Her2 4B5 antibody on a Ventana BenchMark system. Her2-low (1+, 2+ without gene amplification) expression was defined using the guideline update of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). Further molecular tests were performed in selected patients for *PIK3CA* mutations using the QIAamp DNA FFPE Tissue Kit and for multiple genes using the Oncomine Focus Assay on Ion530 chip. BRCA tests were performed by multiplex ligation-dependent probe amplification. Clinical benefit (CB) was defined as a period of more than six months without disease progression after the start of PALB. **Results:** A total of 66/158 (41.7%) patients (p.) had Her2-low disease. Histology: 52 ductal, 13 lobular, two other. Median age: 62 years (38-85). Tissues positive for Her2-low: breast or chest wall (26), breast before relapse (15), axilla (three), bone (seven), liver (seven), skin (three), ovary (two), pleura (one), lung (one). 3 Tp. had pathogenic *BRCA* mutations. Her2 expression: 1+ (50), 2+ (17). *PIK3CA* mutated in 3/12 (25%) of studied tumors. Line of therapy for MBC: first line (1L) (43), second line (2L) (17), >2 lines (7). Concurrent hormone therapy: letrozole (47), fulvestrant (20). CB was found for 35/39 (89.7%) evaluable p. in 1L and 9/13 (69.2%) in 2L. Of note, *BRCA* mutations were present in 2/4 p. without CB to 1L therapy. Although the number of such p. is small, this is concordant with results from the PADA-1 trial and other studies. With a median follow-up of 31 months for 1L p., median time to progression has not been reached yet, and is estimated in 14.5 months for p. in 2L. **Conclusions:** Her2-low disease was present in more than one-third of patients treated with palbociclib for metastatic breast cancer, and was found in various tumor tissue sites. Clinical benefit of palbociclib was high in our cohort, and lower in the small number of patients who also presented with *BRCA* mutations.

ST108. **The Prevalence and Prognosis of Microsatellite Instability-High/Mismatch Repair-Deficient Colorectal Adenocarcinomas in the United States**

B. Iorgulescu[1], C. Gutierrez[2], S. Ogino[3], J. Meyerhardt[4]
[1]MD Anderson Cancer Center, Houston, TX; [2]Massachusetts General Hospital, Boston, MA; [3]Brigham and Women's Hospital, Boston, MA; [4]Dana-Farber Cancer Institute, Boston, MA.

Introduction: Microsatellite instability (MSI) and DNA mismatch repair (MMR) status is an indispensable biomarker in the management of colorectal cancers. We examine the epidemiology of MSI-high/MMR-deficient colorectal cancers in the US. **Methods:** Adults presenting with colorectal adenocarcinoma in 2018-2019 were identified from the U.S. National Cancer Database. Attributes associated with MSI-high/MMR-deficiency were identified using multivariable logistic regression (2-sided alpha of 0.001) and reported with average adjusted probabilities (%$_{AAP}$) and 99.9% confidence intervals (CI). As a secondary aim, the survival associated with MSI/MMR status was assessed with multivariable Cox regression (p <0.006 for corrected significance). **Results:** Among 101,259 colorectal adenocarcinomas in 2018-2019, 82% were microsatellite stable/MMR-proficient, 3.8% MSI-low (n=3,884), and 14.2% MSI-high/MMR-deficient – including 16.6%, 19.9%, 12.4%, and 8.0% of stage I, II, III, and IV cancers, respectively. In locoregional cancers, MSI-high/MMR-deficiency was independently associated with a bimodal age distribution, female sex, right-sided colonic origin, wild-type *KRAS*, and a prior diagnosis of cancer (all p <0.001). By race/ethnicity, colorectal adenocarcinomas were MSI-high/MMR-deficient in 16.9%$_{AAP}$ of non-Hispanic White (CI=16.5-17.4, referent) patients, compared to 11.3%$_{AAP}$ of non-Hispanic Black (CI=10.3-12.4; p <0.001), 14.7%$_{AAP}$ of Asian/Pacific Islander (CI=11.6-17.8; p <0.001), and 15.1%$_{AAP}$ of Hispanic (CI=13.5-16.7; p <0.001) patients. Histologically, MSI-high/MMR-deficiency was associated with increasing grade, from 11.3%$_{AAP}$ of well-differentiated tumors (CI=10.2-12.4) to 28.4%$_{AAP}$ of poorly differentiated cases (CI=27.1-29.8; p <0.001). Compared to conventional histology (15.2%$_{AAP}$, CI=14.8-15.6), medullary (41.2%$_{AAP}$, CI=33.0-49.3; p <0.001) and mucinous (24.6%$_{AAP}$, CI=22.8-26.3; p <0.001) subtypes – but not signet-ring cell histology (15.5%$_{AAP}$, CI=11.6-19.3, p=0.79) – were more frequently MSI-high/MMR-deficient when adjusting for clinicopathologic features including grade. Tumors with *KRAS* codon 12/13/16 mutations (OR 0.48, CI=0.35-0.64, p <0.001), but not codon 146 mutations (OR 0.76, CI=0.27-2.14, p=0.38), were more likely MSI-high/MMR-deficient compared to wild-type *KRAS*. Even after accounting for patient characteristics and stage-appropriate treatments, MSI-high/MMR-deficient cancers were associated with improved overall survival in stage IV cancers (p <0.001), though not reaching corrected significance for stage I tumors (p=0.02). The OS differences between MSS/MMR-proficient and MSI-low cases did not reach corrected significance at any stage. **Conclusions:** Our findings establish the national epidemiology, correlates, and prognostic implications of MSI-high/MMR-deficiency in colorectal adenocarcinoma patients.

ST109. **Factors Affecting the Performance of Next-Generation Sequencing (NGS) Assays: Experience with the NVIGEN 180 Gene Targeted NGS Panel Cancer Precision Profiling Assay**

A. Fu[1], M. Ton[1], K. Wang[1], W. Gu[1], T. Li[2], M. Liu[3], H. Parsons[4], G. Sledge[5]
[1]NVIGEN Inc., Campbell, CA, [2]UC Davis Medical Center, Davis, CA, [3]Mayo Clinic, Rochester, MN, [4]Dana-Farber Cancer Institute, Boston, MA; [5]Stanford University Medical Center, Stanford, CA.

Introduction: Next-generation-sequencing (NGS) molecular profiling assays are gaining widespread clinical applications. However, existing assays present a limit in sensitivity and accuracy. As reported in the FDA-led-SEQ2 project and other recent publications, only mutations above 0.5% variant allele frequency (VAF) could be detected with high sensitivity and accuracy. Mutations below this limit could not be reliably detected. In addition, massive sequencing reads were needed to achieve the necessary coverage depth for sensitive variant calling; hence, the cost of NGS assays remain high. To address these challenges, we developed a 180-gene cancer precision profiling assay with our novel NGS sample preparation workflow based on magnetic nanobeads capture and enhancement. **Methods:** NVIGEN's 180-gene cancer precision profiling assay uses a hybrid capture approach targeting 225 kb gene regions. These gene targets are customizable and include FDA-approved and emerging therapeutic targets, genes to monitor treatment responses, and genes to investigate integrative biomarker information. DNA reference standards applied in the FDA SEQC2 project and from other commercial vendors were used. Sequencing experiments were performed on Illumina HiSeq and NovaSeq instruments. Data were analyzed with NVIGEN's internal bioinformatics pipeline with unique molecular identifiers. Experiment parameters such as input DNA quantity, NGS library preparation PCR cycles, one- or two-step magnetic nanobeads cleanup, library enhancer buffers, and NGS data quantity were investigated. **Results:** The NGS assay performance is highly correlated with the sample complexity loaded onto the sequencer, which reflects the quantity of original DNA material and the yield of the complete sample preparation workflow. PCR cycles represent a significant factor in the assay performance. By reducing one cycle of PCR in the library preparation, 5x fewer random calls were observed in the sequencing data. Our assay reduces the PCR cycle by 5-10 cycles compared to other commercial assays and has about 10x better NGS data efficiency. With 10M reads, 1,500x coverage depth, and 18 ng DNA, we achieved 100% detection sensitivity for the 0.5% VAF samples and 83% sensitivity for the 0.375% VAF samples. The effect of different experiment factors on improving the assay performance will be reported. **Conclusions:** By understanding and optimizing the factors affecting NGS assay performance, it can be expected for reliable high sensitivity mutation detection of 0.1 to 0.5% VAF. Our continuing assay development and validation experiments will help meet the

increasing need of NGS clinical assays for high sensitivity, high accuracy, quick turnaround time, low DNA input quantity, and affordability.

ST110. **WITHDRAWN**

ST111. **Massive Parallel Sequencing of Archived Formalin-Fixed, Paraffin-Embedded Diffuse Glioma Tissue at 1, 5, and 10 Years from Fixation**

E. Goold[1], C. Davidson[2], R. Jattani[3], J. Kim[2], C. Palmer[2], P. Rindler[3], J. Klonoski[2]
[1]University of Utah, Bountiful, UT; [2]University of Utah, Salt Lake City, UT; [3]ARUP, Salt Lake City, UT.

Introduction: Given the updates and new editions to the WHO Central Nervous System tumors classification, there is greater need for evidence-based anticipatory guidance on retrospective massive parallel sequencing. Herein, we retrospectively sequenced 45 diffuse gliomas up to 14 years after collection and, when available, compared results with prior outside sequencing, fluorescence *in situ* hybridization (FISH), and immunohistochemistry (IHC) results. **Methods:** The Illumina TruSight Oncology 500 Assay (TSO500) and formalin-fixed, paraffin-embedded tissue were used to analyze 15 diffuse astrocytomas, oligodendrogliomas, and glioblastomas. Five clinical cases at one, five, and 10 years after tissue procurement were selected for each of the three groups. DNA and RNA extractions were performed using whole tissue scrolls and the TSO UMI kit. Variant calling and interpretation were aided by the TSO500 Local App and PierianDx Clinical Genomics Workspace. **Results:** Selected cases from 2008-2020 included astrocytomas (11/15), oligodendrogliomas (3/15), and glioblastomas (10/15); cases with prior outside sequencing over total cases in parentheses. One 10-year oligodendroglioma failed to be sequenced. Mean percent tumor and tumor necrosis ranged from 84.5%-98.1% and 0%-13%, respectively. Mean DNA yield ranged from 39.04-80 ng/uL and mean RNA yield ranged from 94.14-253.22 ng/uL. Transition-to-transversion ratio (Ti/Tv) averages at one, five, and 10 years for the astrocytoma group were 2.78, 2.84, and 2.90, respectively; for the oligodendroglioma group the ratios at the same timepoints were 2.92, 2.82, and 2.82, respectively; and for the glioblastoma group at the same timepoints the average ratios were 2.81, 2.95, and 2.89, respectively. The concordance rate of TIER I/II mutations between retrospective TSO500 and prior outside sequencing was 91.7%, 77.8% and 71.4% at one, five, and 10 years, respectively, for astrocytomas, 100% and 75% at one and five years, respectively, for oligodendrogliomas, and 88.9%, 66.7%, and 75% at one, five, and 10 years, respectively, for glioblastomas. Concordance with FISH and IHC at one, five, and 10 years was 85%, 92.9%, and 100% for astrocytomas, 100% and 88.9% at one and five years for oligodendrogliomas, and 95% and 87.5% for glioblastomas at one and five years. One 10-year-old astrocytoma WHO Grade 2 was reclassified under the 2021 WHO guidelines as an IDH-wild-type glioblastoma and three glioblastomas were reclassified as IDH-mutant astrocytoma WHO grade 4. **Conclusions:** Sequencing of archived clinical cases up to 14 years after tissue procurement yields DNA and RNA of adequate quantity to yield sequencing data of high enough quality to be used in research but not for clinical retroactive reclassification. Thus, retrospective genetic analysis may inform clinical case reviews and aid epidemiologic studies. Additional purported uses may include the refinement of the CNS tumor classification system and additional markers of clinical utility.

ST112. **Tissue Quality and False-Negative Homologous Repair Deficiency (HRD) Results Might Deprive Patients of Targeted Therapy Benefits**

P. Constantoulakis[1], S. Samara[1], A. Oikonomaki[1], K. Roumelioti[1], E. Patsea[2], A. Kotsakis[3], V. Georgoulias[2], G. Christopoulou[1]
[1]GENOTYPOS MSA, Athens, Greece; [2]Metropolitan General Hospital, Athens, Greece; [3]University of Thessaly, School of Health Sciences, Larissa, Greece.

Introduction: Homologous recombination deficiency (HRD) may predict sensitivity to poly-ADP ribose polymerase inhibitors (PARPi) and platin-based regimens. Currently, guidelines suggest testing to select ovarian (OC) and prostate cancer (PC) patients who may benefit from such targeted therapy. Still, PARPi demonstrate efficacy in a proportion of HRD-negative patients. Although HRD+ detection rate is significant with comprehensive next-generation sequencing (NGS) approaches, apparently negative results may include false classifications due to low-quality DNA extracted from compromised formalin-fixed, paraffin-embedded (FFPE) samples, as shown from our experience. **Methods:** A total of 543 OC and 180 PC FFPE samples were referred for HRD/homologous recombination repair testing. DNA was extracted (QIAamp◊ DNA Mini kit, Qiagen) followed by library preparation according to the manufacturer (AmoyDx◊ Focus Panel and AmoyDx◊ HANDLE HRR NGS Panel, Amoy Diagnostics Co., Ltd) sequenced on NextSeq550◊ (Illumina). Bioinformatics were performed by proprietary algorithms (ANDAS v1.0.9-CE-B1.0, Amoy Diagnostics Co., Ltd) to identify *BRCA1/2* and other HR genes' pathogenic/likely pathogenic (P/PL) mutations and the Genomic Scar Score (GSS) utilizing >24,000 genome-wide SNPs. **Results:** For OC, 535/543 samples reached a "verdict" leading to 44.3% HRD detection rate: 5% had *BRCA* mutations, 25.6% GSS+ and 13.7% both. Tumor cell content (TCC) was unknown for 87% of samples, partially explaining the deviation from the "expected" 50% HRD+ results. At least one quality control (QC) was compromised in >70% of samples, reflecting low-quality FFPE tissue, most likely contributing to false-negative results. Eight samples (1.5%) with all QCs unreached were rejected. If selecting samples with all QCs valid, HRD+ rises to 54.2%, and when including only samples with all QCs passed and TCC >30%, HRD+ reaches 72.7%, mostly due to optimal GSS calculation. For PC, HR P/LP mutations leading to HRD were detected in 33.2% samples. TCC was unknown in 71% cases and in 76% of samples at least one QC was unmet. Reduction in OC cases led to 46.8% positivity, with TCC still unknown. **Conclusions:** Clinical trial outcomes undoubtedly urge companion diagnostics tests' and newly developed HRD assays' optimization. Nonetheless, sample quality is critical for all methods, especially when complex traits, such as HRD, are measured, as shown by our analyses. Sample quality is adversely affected in numerous steps, from surgery to DNA extraction. To minimize the negative impact, all specialties from anesthesiologists to lab scientists should be well-educated, predominantly pathologists. Having control over the whole specimen "odyssey" and establishing multidisciplinary oncology boards may contribute to detecting larger patient subgroups that may benefit from targeted therapy in the precision medicine era.

ST113. **Evaluating the Performance of Plasma Circulating Tumor DNA Testing for Homologous Recombination Repair Gene Variants in Prostate Cancer: A Multicenter Ring Study**

N. Wolstenholme, S. Patton
EMQN CIC, Manchester, United Kingdom.

Introduction: Tumors in patients with metastatic castration-resistant prostate cancer (mCRPC) often harbor mutations in genes involved in homologous recombination repair (HRR). Plasma circulating tumor DNA (ctDNA) testing is a less invasive alternative to tissue biopsy for the detection of HRR gene mutations, the presence of which can identify patients eligible for targeted therapy. In prostate cancer, ctDNA testing is challenging, as the genes of interest are large, and mutations can be difficult to identify. A ring study was conducted to explore the robustness and technical limitations of ctDNA HRR mutation testing strategies in mCRPC. **Methods:** In total, 2,285 European Molecular

Genetics Quality Network (EMQN) member laboratories were surveyed. Eligible laboratories met these criteria: 1) ISO 15189-accredited; 2) previously participated in a European Quality Assessment scheme for testing cell-free DNA (cfDNA) (in plasma); 3) perform clinical diagnostic testing for prostate cancer using cfDNA (in plasma); 4) use a next-generation sequencing (NGS) strategy for testing cfDNA (in plasma); and 5) use an NGS strategy that screens the study genes of interest. Synthetic samples allow controlled testing on a larger scale than real patient samples. Eight synthetic plasma ctDNA samples were manufactured. Seven contained clinically relevant variants in four HRR genes of interest (*BRCA1*, *BRCA2*, *ATM*, and *CDK12*), and one (a "wild-type") had no variants in these genes. Each sample was replicated at three variant allele frequencies (VAFs) – high, medium, and low. Laboratories tested the 24 samples for variants in the genes of interest using their standard methods. **Results:** Overall, 81 laboratories responded to the survey, of which 34 were invited to participate, and 23 submitted results. There was extensive variation in genotyping performance among the laboratories; the highest number of samples correctly genotyped (true positives/negatives) by a laboratory was 20 (83.3%) and the lowest number was two (8.3%). No laboratories correctly determined the genotype and VAF of all low VAF samples; the low VAF was outside the scope, or below the limit of detection, of many assays. VAFs reported for true positives were consistently lower than the expected value; however, VAFs for endogenous variants were, generally, as expected. The quantity of DNA extracted also varied among the laboratories and tended to be lower than expected; for each sample, the mean yield was ~50% of the total amount available. **Conclusions:** Genotyping accuracy and DNA yield were highly variable among the laboratories, which may be due to the technically challenging nature of this testing and issues with testing strategies and/or with the synthetic samples. Developing technical guidelines is essential to optimize assay performance.

ST114. **Molecular Pathology Quality Assurance Program (MPQAP): Experience of First Solid Tumour EQAS Service by Premier Tertiary Cancer Centre in India**

S. Desai, O. Shetty, R. Iyer, T. Shet, P. Joshi, P. gogte, M. Gurav, N. Karnik, T. Pai, S. Epari, C. Hingnekar, P. C S
Tata Memorial Hospital, Mumbai, India.

Introduction: Participation in external quality assurance schemes (EQAS) ensures calibre, competence among testing laboratories, and achieves confidence in patients' test results. Recent advances in solid tumor diagnostics tests have paved the way for prompt results by array of commercial kits and testing offered by several diagnostic laboratories across India. This trend necessitates the need to monitor quality of testing offered by clinical laboratories. Lack of awareness and exorbitant costs linked to international programs restrict testing laboratories to engage in EQAS program. Molecular Pathology Quality Assurance Program (MPQAP) for solid tumours was introduced by Tata Memorial Hospital, Mumbai, in the year 2019 to address these concerns and ensure uniformity, consistency, and quality in molecular testing services. **Methods:** The program offers a method-based proficiency testing approach and was supported by National Cancer Grid, a cancer care consortium of India. The testing schemes in fluorescence *in situ* hybridization (FISH), PCR, and sequencing techniques were launched by surveying the needs and requirements of testing centres across the country. Initially, 18 centres registered for the program, which gradually increased to 34 centres during a span of three years, including one each from Sri Lanka and Malaysia. Test materials were provided in the form of formalin-fixed, paraffin-embedded tissue sections for PCR and sequencing tests and unstained slides for FISH tests. In the COVID-19 pandemic, digital EQAS was introduced for data analysis and interpretation. Precautionary guidelines while working with diagnostic samples were also released. **Results:** A total of 21 test cycles were released during the span of three years. Evaluation of participants' performance was based on a scoring system comprising pre-analytical, analytical, and post-analytical aspects of testing. Participants scoring >90 were graded "Very Good Performance," scores 70-80 were graded as "Good Performance," and <70 were graded as "Needs Improvement." Overall, 71% of centres scored >90% in all test modules exhibiting quality standards in testing. About 25% scored between 80%-90% presenting baseline acceptable performance and 3.5% centres were outliers to which assistance was provided in terms of recommendations and interpretation of results. Five centres showed gradual improvement in overall performance trend in two subsequent years, underscoring the importance of EQAS in implementing and maintaining quality standards. **Conclusions:** MPQAP has helped monitor performance of testing laboratories and assisted in continuous quality improvement. It has provided an accessible, efficient, and cost-effective EQAS for India and has extended service to neighbouring countries in Asia as well. Future goals entail further expansion of scope of proficiency testing and inclusion of more laboratories within the ambit of MPQAP.

ST115. **Implementation of a Clinical Immuno-Oncology Panel for Pediatric Brain Tumors**

A. Levine, A. Das, C. Hawkins, M. Johnson, B. Laxer, S. Milos, L. Nobre, S. Ryall, R. Siddaway, U. Tabori
Hospital for Sick Children, Toronto, Canada.

Introduction: The tumor immune microenvironment (TIME) is a growing area of interest in neuro-oncology; however, the extent of immune activation in childhood CNS tumors is unknown. Although immunotherapy has not been successful in most adult CNS cancers, our group previously described gliomas with mismatch repair deficiency (MMRD) which exhibit hypermutation and favorable responses to immune checkpoint inhibition (ICI). Therefore, detailed characterization of the CNS TIME is key for the development of novel immunotherapeutic strategies and application of existing ones in childhood brain tumors. **Methods:** We developed a clinical NanoString immuno-oncology panel that includes markers reflecting cell types, therapeutic targets, and cellular pathways, as well as the 18-gene tumor inflammation signature (TIS), a biomarker for ICI response. We tested more than 500 brain tumors, including 266 low-grade gliomas (LGG), 170 high-grade gliomas (HGG), 91 MMRD tumors, 16 ependymomas, 46 medulloblastomas, and 36 non-tumor brain samples. The panel was validated using immunohistochemistry (both conventional and multiplexed). **Results:** Overall, ependymomas and medulloblastomas had low levels of inflammation, although Sonic hedgehog (SHH) medulloblastomas had higher inflammation than other subtypes. IDH-mutant LGG were immunologically cold, whereas many gliomas with pediatric-LGG mutations had high levels of inflammation, including upregulation of immune checkpoints – indicating that ICI may be an effective strategy. Interestingly, in pediatric-LGG inflammation impacted outcome in tumors with the same genetic alterations. *BRAF* V600E-mutant LGG exhibiting high TIS had inferior prognosis (p=0.02), whereas no such relationship was observed in *BRAF*-fused tumors. *BRAF* V600E mutant LGG also formed distinct groups in unsupervised clustering of panel genes, corresponding to different patterns of immune activation. Diffuse midline gliomas had higher inflammation than hemispheric HGG, indicating that these tumors are not immunologically cold, as has been previously reported. Many pediatric HGGs had high expression of immune checkpoint genes, including *PDL1, PD1, LAG3*, and *TIGIT*. In MMRD tumors treated with ICI, high TIS correlated with improved survival (p=0.028) and was uncorrelated with hypermutation and mutational burden. Furthermore, MMRD gliomas had high expression of several other immune checkpoints including LAG3, suggesting its value as an additional therapeutic target. **Conclusions:** In summary, we present a clinically validated immuno-oncology gene expression panel. Our results in a large cohort of pediatric brain tumors demonstrates that characterization of the TIME provides potential prognostic clues and suggests treatment strategies for further investigation.

ST116. **Tumor Mutational Burden Quantification Using a Small (669 kb) Targeted Anchored Multiplex PCR Panel and Next-Generation Sequencing**

G. Bjerke, E. Johnson, R. Rogge
Invitae, Boulder, CO.

Introduction: Tumor mutational burden (TMB) is rapidly gaining acceptance as a biomarker of disease prognosis and response to immune checkpoint inhibitors (ICIs). Whole-exome sequencing (WES) is used as a reference standard for calculation of TMB; however, given the cost and other clinical implications of WES, TMB is frequently derived from next-generation sequencing panels. Herein we describe measurement of TMB using the VariantPlex Pan Solid Tumor panel covering a genomic area of 669 kb. Utilization of Anchored Multiplex PCR (AMP) chemistry with molecular barcoding allows deduplication of PCR and sequencing duplicates, enabling powerful error correction. In addition to TMB measurement, usage of the VariantPlex Pan Solid Tumor panel allows simultaneous detection of single nucleotide variations (SNVs), insertions/deletions (indels), copy number variations (CNVs), and microsatellite instability (MSI) from formalin-fixed, paraffin-embedded (FFPE) tissue samples. **Methods:** Sequencing libraries were produced using the VariantPlex Pan Solid Tumor panel using purified DNA from either cell lines or FFPE samples of differing qualities and sequenced on Illumina NextSeq or NovaSeq instruments. Bioinformatic analyses were performed using Archer Analysis 7.0 with bespoke algorithms to optimize for use of a small targeted panel. Somatic variants were filtered to separate them from germline variants, allowing TMB calculations without a paired normal sample. TMB scores are calculated from somatic variant counts (both nonsynonymous and synonymous) fed into a linear model trained on TCGA variant data to correct for panel size. **Results:** TMB was assayed using 50 ng of input DNA extracted from SeraCare TMB standards from the FOCR TMB Harmonization group or commercial samples characterized by orthogonal methods. Due to the inherent limitations in measuring TMB using a small panel we implemented a user controlled indeterminate range around the standard cut-off of 10 to determine TMB low/high. Using FFPE samples without a matched normal, we show concordance of panel-based TMB as compared to WES or other panel-based TMB assays. **Conclusions:** The addition of TMB measurement to VariantPlex panels allows additional biomarker testing in addition to MSI, SNV, and CNV assessment using the same experimental workflow. Our TMB assay functions on FFPE samples over a range of qualities and without a paired normal sample. Additionally, there is also an ongoing development applying these TMB calling algorithms to a larger VariantPlex panel (>1.4 Mb genomic coverage and 430 genes) to increase gene coverage and improve TMB calling.

Technical Topics

TT001. ***BRAF* V600E Droplet Digital PCR (ddPCR) cfDNA Troubleshooting**

D. Qin, J. Puskas, A. Bossler
Moffitt Cancer Center, Tampa, FL.

Introduction: Quantitative detection of *BRAF* mutations in plasma of melanoma patients may be useful for monitoring therapy response and recurrence. The T-to-A transition is the most common alteration but others occur. Results from ddPCR PCR assays “specific” for the c.1799T >A may be affected by these alterations. We evaluated the impact of alternative substitutions using Bio-Rad’s BRAF V600E ddPCR assay. ddPCR using cell-free DNA quantitates mutant and wild-type allele copy number depicted in two-dimensional (2D) dot plots in four quadrants similar to flow cytometry. The mutant channel threshold (MCT) is set with water and *BRAF* wild-type DNA negative controls. Signal dots above MCT are considered positive and results are reported as copies/ml plasma with the variant allele frequency. Among 225 tests, three cases showed dots just above or intersecting with the MCT, significantly lower than normal positives. Do these data reflect a true positive, a negative result, or something else? **Methods:** Plasma cfDNA extraction used the QIAmp Circulating Nucleic Acid kit (Qiagen, Inc. cat. #55114) according to the suggested protocol. ddPCR was performed using 30 ng of cfDNA, the *BRAF* V600E ddPCR (FAM + HEX) probe assay (Bio-Rad Inc.) using the manufacturer’s protocol, the Bio-Rad QX200 Automated Droplet Generator and Reader, C1000 thermocycler, and QuantaSoft analysis software. **Results:** 1) In normal positive cases dots are located above the MCT. 2) Among 225 tests, data from three cases show an unusual dot plot pattern. The cases show dots in the upper left quadrant of the 2D dot plot. However, these dots were just above the MCT, significantly lower than normal dots. We designate this as "low positive" here. A patient chart review showed case 1 had a subcutaneous nodule of malignant melanoma testing positive for *BRAF* V600K. Case 2 had a dermal nodule of malignant melanoma positive for *BRAF* V600R. Interestingly, no *BRAF* V600E mutation was reported in these two patients. Case 3 had no prior *BRAF* testing. The circulating tumor DNA of this case was tested using next-generation sequencing showing a V600K mutation. **Conclusions:** 1) Although this ddPCR assay is designed specifically for *BRAF* V600E, it is possible the low positive dots reflect V600K or V600R detection. 2) Such low-positive results may be due to the partial annealing of V600E primers to *BRAF* V600K or V600R sequences. 3) We believe that under perfect conditions, the assay has good specificity for V600E. However, if test conditions are suboptimal, like a slight change in the annealing temperature or [Mg^{++}], partial non-specific annealing may occur.

TT002. **Gene-Specific Validation of Copy Number Gains from a Massively Parallel Sequencing Assay**

J. Corines, A. Bigdeli, S. Herlihy, S. Priore, J. Roth
Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Introduction: Validation of clinical laboratory developed tests, including copy number calling from massively parallel sequencing (MPS) assays, establishes various parameters for reporting test results. Here we describe our institutional experience with evaluating gene-specific criteria for the reporting of DNA copy number gains (CNGs) from an oncology-based MPS assay. **Methods:** CNGs were validated from a custom capture-based MPS assay for 19 clinically relevant genes using the open source CNVkit program. CNG calculations were performed at different sample purities (15%, 30%, 55%, and 100%) based on tandem histomorphology assessment. A reference baseline for CNG calling was established from 59 disease-free samples originating from both fresh and paraffin-embedded specimens. Validation of assay cut-offs for positive/negative determination was performed using gene-specific limit of the blank (LOB) studies in 36 unique disease-free samples not included in the reference pool. In addition to performing the LOB studies at 100% sample purity, the 36 samples were processed at incorrect purities (15%, 30%, and 55%) to measure the effect of sample purity on CNG calculations. Assay cut-offs were established on a gene-specific basis by selecting the widest bounds of variation in CNG observed at any sample purity. Additional assay performance characteristics required for a complete assay validation were also assessed. **Results:** Based on the validation, gene-specific copy number cut-offs ranged from three to 11 copies in which calls at or above these numbers are clinically reported. The mean and median copy number cut-offs implemented were 5.4 and 5, respectively. Processing samples at incorrect sample purities had an adverse effect (i.e., noise) with the greatest adverse effect observed when disease-free specimens were processed at 15% purity. Positive concordance between our newly validated assay and the previous comparator assay was similarly lowest at 15% sample purity when compared to the other percentages. Accuracy, precision, analytic specificity, reportable range, and reference range validations all yielded acceptable performance characteristics. **Conclusions:** Patient management decisions rely on accurate copy number calls. Our data demonstrate a wide range of copy number calls in LOB samples, necessitating gene-specific cut-offs for CNG calling on MPS assays to minimize false-positive or false-

negative calls. In addition, our validation of the effect of sample purity on CNG calls reinforces the need for accurate sample purity estimates for CNG calculations.

TT003. **Improved RNA-Seq Results on Recalcitrant FFPE Diseased Liver Resections Using Ultrasonic Isolation**

L. Reising[1], E. Wray[2], J. McElwee[2], E. Rasmussen[2], P. Ebert[2], K. Credille[2], T. Holzer[2]
[1]Eli Lilly, Indianapolis, IN; [2]Eli Lilly, New York, NY.

Introduction: Gene expression profiling on formalin-fixed, paraffin-embedded (FFPE) resections of human liver poses challenges stemming from pre-analytical variables such as surgical duration, ischemia time, fixation and tissue processing, and effects of glycogen on RNA isolation. The impact of these variables is especially apparent during RNA integrity analyses of liver resections from patients undergoing transplants, however; optimization of methods for RNA isolation may allow for robust transcriptomic analysis results for translational molecular characterization. **Methods:** FFPE liver specimens were obtained from biorepositories that included tissue from three autoimmune-related primary biliary cholangitis patients undergoing transplants and non-matched, non-diseased tissue. Routine H&E and pathology reviews were performed. RNA was extracted using AllPrep FFPE kit (Qiagen) or truXTRAC FFPE kit (Covaris) which included ultrasonication. RNA fragment analyses were completed using a high-sensitivity D1000 ScreenTape assay (Agilent). Bulk RNA-Seq was performed using the Transcriptome Human Gene Expression Panel (Thermo Fisher). RNA was profiled by the ligated-probe-based Visium Spatial Gene Expression FFPE transcriptome assay (10X Genomics). **Results:** H&E showed late-stage disease with severe bridging fibrosis, nodular regeneration, and multifocal areas of mononuclear inflammation in fibrotic bands. For diseased livers, mean DV200 values increased from 26.7% (AllPrep) to 55.8% (truXTRAC). Likewise, mean percentage of panel targets detected increased from 48.2% to 69.1%, and mean mapped reads using amplicon sequencing increased from 4.8M to 11.6M reads per sample, comparable to 11.7M reads from non-diseased livers using AllPrep only. Ligated-probe-based detection of mRNA *in situ* was consistent with RNA-Seq with Spearman's r(12816) = 0.27, 0.56, and 0.63 across the diseased specimens ($p < 0.001$). Spatial profiling confirmed hepatocyte-enriched expression of known liver genes (e.g., *AHSG, APOA2, ASGR1*, and *F2*). **Conclusions:** RNA isolation using ultrasonication improved the mean DV200 values above recommended thresholds of 30%-50%, and it enabled deeper sequencing resulting in more diseased liver genes detected. This was presumably due to the method's effects on removal of fragments of degraded RNA, and/or improved homogenization of fibrotic diseased tissue sections. Improved bulk RNA-Seq results and the independent probe-based transcriptome analysis showed weak/moderate to strong rank order of expression level, indicating that the relative abundances of RNAs were consistent between profiling methods and suggesting suitability for further characterization. This work highlights the potential impact of RNA isolation and assessment of next-generation sequencing quality metrics on the ability to profile recalcitrant FFPE liver specimens.

TT004. **Treatment with DNA Repair Enzyme Removes NGS Variant Call Artifacts in Archival FFPE Tissues**

L. Haley[1], P. Mbonu[2], A. Pallavajjala[2], R. Xian[2]
[1]Johns Hopkins, Forest Hill, MD; [2]Johns Hopkins, Baltimore, MD.

Introduction: Archived formalin-fixed, paraffin-embedded (FFPE) tissues are used for clinical and research sequencing. Long-term storage reduces the recovery of high-quality DNA needed for next-generation sequencing (NGS) in as little as two years, which confounds the interpretation of tumor mutations. This study evaluates the effect of DNA repair on the sequencing data generated from older FFPE tissues. **Methods:** DNA was isolated from 10 FFPE tumor samples at time zero (T0). Paired two-year DNA (T2) was isolated from four samples and paired four-year DNA (T4) was isolated from six samples. T2 and T4 DNA was split with an untreated aliquot and an aliquot that was pretreated with a DNA repair enzyme cocktail. All DNA underwent hybrid-capture NGS using a 432-gene panel. Data were processed with the same bioinformatics pipeline. Sequencing quality-control (QC) metrics, variant calls, and tumor mutation burden (TMB) calculations were compared. **Results:** Among five QC metrics, median insert size decreased from 249 bases (T0) to 145 bases (T2, $P=0.0001$) and 157 bases (T4, $P<0.0001$), which was not improved with the repair enzyme. At two years, the remaining QC metrics showed worsened and improved trends when comparing the aged sample with and without repair enzyme, respectively, but did not reach statistical significance. At four years, PCR duplicates worsened from a median of 39% (T0) to 58% (T4, $P=0.0006$) and back to 42% with enzyme repair (T4E, $P=0.0037$). The number of variants called per sample increased from a median of 20 (T0) to 27 (T4, $P=0.0158$) and back to 20 (T4E, $P=0.0235$). Percentage targets with ≥150x coverage showed similar trends: 98% (T0), 60% (T4, $P=0.0029$) and 78% (T4E, $P=0.0865$). All four of the two-year comparators were TMB-high (≥ 10 mutations/Mb) at T0 and remained TMB-high in the aged specimen with and without enzyme repair. All six of the four-year comparators were TMB-low/intermediate at T0 and T4E, but three were TMB-high in the aged untreated DNA. A total of 153 variants were consistently detected across the 10 samples at all time-points (with and without enzyme repair). An additional 191 variants were only called in the aged untreated DNA, and 15 variants and 17 variants, respectively, were only called at baseline or in the treated aged DNA. All concordant variants had high variant allele frequencies (VAFs) (37% mean, 95CI 35%-38%). All variants only detected in the aged untreated DNA had low VAFs (8% mean, 95CI 7%-10%, $P<0.0001$) that were still above the assay limit of detection (LOD) of 5%. **Conclusions:** Sequencing of aged specimens generated twice the number of variant calls (artifacts) above LOD that were only detected in the untreated aged DNA, which may inflate TMB. Enzyme treatment of the aged DNA improved sequencing QC metrics, particularly at four years, and removed these variant call artifacts.

TT005. ***MGMT* Promoter Methylation and *IDH1* Mutation Testing to Prognosticate Glioblastoma Multiforme and Determine Alkylating Agent Treatment Eligibility: KHCC Experience**

W. Naser, B. Muhsen, M. Al-Hussaini, R. Abu Aboud, M. Al Masri, R. Amarin, D. Abu Laban, A. Tbakhi
King Hussein Cancer Center, Amman, Jordan.

Introduction: The existence of *MGMT* promoter methylation and mutated *IDH1* gene in newly diagnosed patients with glioblastoma multiforme (GBM) powerfully forecasts sensitivity to alkylating chemotherapy (e.g., temozolomide) and indicates a favorable prognosis. Thus, at King Hussein Cancer Center (KHCC), we included *MGMT* promoter methylation detection and *IDH1* mutation testing to guide alkylating chemotherapy and predict survival for GBM patients. **Methods:** Methylation Sensitive Restriction Enzyme-Quantitative PCR (MSRE-qPCR) and targeted *IDH1* gene bi-directional Sanger sequencing were implemented to assess the *MGMT* promoter methylation and *IDH1* exon 4 mutation status in KHCC GBM patients. We designed primers to amplify a 155 base pair (bp) product specific to a clinically relevant CpG-rich site upstream from the transcription start site of the *MGMT* gene. An accuracy and precision validation study along with a comparison to an orthogonal method was conducted to assess method characteristics. We assessed method performance using College of American Pathologists (CAP) proficiency testing material and commercial controls (methylation range, 0%-100%). We adopted the validated MSRE-qPCR assay to analyze genomic DNA extracted from 85 formalin-fixed, paraffin-embedded brain tumor tissues (≥40% tumor content) procured during 15 months. The *IDH1* (R132) mutation was tested using an in-house validated Sanger sequencing and/or immunohistochemistry protocols. **Results:** Our MSRE-qPCR assay had 100% analytical precision and analytical specificity. Below 35% methylation, we observed 64.4% and 61.5% analytical accuracy and analytical sensitivity, respectively. However, both parameters improved to 100% at methylation levels above 35%.

At 0% methylation, we detected background noise as high as 15% methylation. In addition, methylation levels measured between 16%-35% were not consistent. Thus, we categorized methylation results into "Negative," "Equivocal," or "Positive" if methylation was 0%-15%, 16%-35%, or ≥36%, respectively. Our validated MSRE-qPCR assay revealed that 60% of the cohort was positive (methylation median 54.21%; range, 37.23%-100%). Finally, we categorized epigenetic and *IDH1* gene mutation results into *MGMT*neg-eq/*IDH1*wt (37.6%), *MGMT*pos/*IDH1*mt (11.8%), and *MGMT*neg-eq/*IDH1*mt or *MGMT*pos/*IDH1*wt (49.4%). **Conclusions:** We successfully validated a semi-quantitative bisulfite conversion-independent *MGMT* promoter methylation assay with minimal failure and false-negative rates. Survival predictions were not feasible because of the study's short duration. Nevertheless, our GBM cohort had methylated *MGMT* promoter prevalence similar to previous reports. Moreover, we expect that 11.8% (i.e., *MGMT*pos/*IDH1*mt) will have the best outcome, whereas 37.6% (i.e., *MGMT*neg-eq/*IDH1*wt) will have the poorest prognosis.

TT006. **Neurodegenerative Disease Differences in Luminex and Lumipulse ATN Categories**

L. Jain[1], M. Khrestian[2], S. Formica[2], E. Tuason[2], J. Pillai[3], S. Rao[3], J. Leverenz[3], L. Bekris[2]
[1]Cleveland Clinic Foundation, Cleveland, OH; [2]Cleveland Clinic Genomic Medicine Institute, Cleveland, OH; [3]Cleveland Clinic Lou Ruvo Center for Brain Health, Las Vegas, NV.

Introduction: In 2011, the National Institute on Aging and Alzheimer's Association created separate diagnostic recommendations for the different stages of Alzheimer disease (AD), which were updated in 2018 to be labeled as the AT(N) research framework. This framework proposes to use biomarkers (namely amyloid [A], tau [T], and neurodegeneration [N]) to categorize individuals with an AD diagnosis. However, it has been used to study trends specific to other conditions, such as Parkinson disease (PD), myopic maculopathy, and subjective cognitive decline. The potential differences across cerebrospinal fluid AD-related biomarker measurement methodologies make it more complicated to identify their patterns in different patient groups. Our objective was to determine if the ATN distribution is the same when utilizing two different assays, Fujirebio Lumipulse G1200 and the Luminex 200, within a cohort of 291 individuals. **Methods:** Participants were recruited from the Cleveland Alzheimer's Disease Research Center (CADRC), Cleveland Clinic Lou Ruvo Center for Brain Health (CBH), and Dementia with Lewy Bodies Consortium (DLBC) and included cognitively normal (CN) (N=55), AD (N=132), dementia with Lewy Bodies (DLB) (N=32), mild cognitive impairment (MCI) (N=49), and PD (N=23) patient groups. $A\beta_{42/40}$ (A), $pTau_{181}$ (T) and tTau (N) were quantitated using both Lumipulse and Luminex platforms. Receiver Operating Characteristic curve was utilized to identify optimal cutpoints for the three biomarkers (A, T, and N) based on the CN and AD cohorts. Youden index was calculated to identify the strength of these biomarkers. **Results:** Out of the overall cohort of 291 individuals, only 59% of the population had identical ATN status for both Lumipulse and Luminex. Correlation analyses between the two platforms indicated that there was no correlation in PD, AD, and MCI for A, all groups had significant correlation for T, and all groups except PD showed significant correlation for N. **Conclusions:** In light of different techniques that are available, it is important to understand the difference among them to inform standardization and data harmonization efforts. Here we used $A\beta_{42/40}$ for A and tTau for N, but it is possible that different results will be observed when utilizing $A\beta_{42}$ for A or Neurofilament Light chain (NfL) for N characterization. Given that the overlap between the two platforms for this study was only found to be approximately 59% and no strong correlation was found between the two platforms, especially for $A\beta_{42/40}$, it is imperative to work towards standardization protocols. A definitive and effective method to quantitate AD-related biomarkers holds the promise of better differential diagnosis in individuals suffering from different neurodegenerative diseases.

TT007. **Multi-omic Analysis of Solid Tumor Biopsies: Incorporating Multiplex Protein and Phospho-Protein Signals Using the Nanostring GeoMx Platform**

C. Corless, J. Lee, J. Letaw, B. Johnson, J. Keck, A. Creason, D. Bottomly, G. Mills, S. McWeeney
Oregon Health and Science University, Portland, OR.

Introduction: Tumor evolution under therapeutic challenge leads to treatment resistance. Multi-modal analysis of serial biopsies can elucidate underlying mechanisms. To date, 205 biopsies (CT and US-guided cores) from 152 patients have been obtained under a clinical research protocol at our cancer institute. In addition to targeted next-generation sequencing (NGS) of tumor DNA and whole-transcriptome sequencing, biopsies are studied with a validated (CLIA LDT) multiplexed antibody assay on the Nanostring GeoMx Digital Spatial Profiler (DSP) system. **Methods:** DNA and RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor sections are sequenced using standard methodologies on Illumina platforms. Two multiplexes of oligonucleotide-tagged antibodies are used to interrogate proteins and phospho-proteins (one FFPE section for each plex). One panel consists of 79 markers related to tumor cell state (ER, PR, HER2) and intracellular signaling (MAPK, PI3K/AKT, cell death, cell cycle regulation), and the other is 25 immune cell markers. Oligo tags released via focused UV light on the DSP are collected separately from CK-positive tumor cells and peritumoral stromal cells across three or more regions of interest in each biopsy. Collected oligos are quantitated on the nCounter MAX system. A tissue microarray consisting of FFPE cancer cell lines is analyzed in every run, signals from which serve for data normalization and batch correction using validated workflows. DSP signals are reported relative to the range observed in a cohort of ~20 previously analyzed biopsies of the same tumor type. **Results:** Biopsies from patients with advanced carcinoma of the breast, ovary, or pancreas, or pleomorphic sarcoma have been analyzed using the multi-omic approach, yielding a wealth of data: mutations, copy number variants, and tumor mutation burden from DNA-Seq; mutation expression, fusion transcripts, and hallmark pathway levels from RNA-Seq; and relative levels of intracellular signaling and local immune cell activity from DSP. The multi-omic approach is particularly powerful when examining the impact of drug treatments. Examples to be shared include changes in phospho-Mek1, p-Erk1/2 and p-P90Rsk signals on MEK inhibitor therapy, as well as tumor and immune contexture changes on drugs targeted to HER2, PARP, MEK, or CDK4/6. Although agreement between the analytic modalities is generally good, differences between protein/p-protein signals and inferences made from NGS are often clinically informative. **Conclusions:** The addition of quantitative high-plex protein analyses to DNA-Seq and RNA-Seq data in the analysis of FFPE core biopsies from patients with advanced solid tumors provides a "third dimension" of information that better informs therapeutic options for individual patients.

TT008. **Are We Using the Right Tools to Calculate Homologous Recombination Deficiency (HRD) Scores?**

N. SAHAJPAL[1], R. Kolhe[1], A. Mondal[1], a. Vashisht[1], H. SINGH[1], S. Ananth[1], D. Saul[2], S. Shams[2], A. Hastie[2], A. Chaubey[2]
[1] Augusta University, Augusta, GA; [2]Bionano Genomics, San Diego, CA.

Introduction: Homologous recombination deficiency (HRD) is characterized by the inability of a cell to repair the double-stranded breaks using the homologous recombination repair (HRR) pathway. The deficiency of the HRR pathway results in defective DNA repair, leading to genomic instability and tumorigenesis. The presence of HRD has been found to make tumors sensitive to ICL-inducing platinum-based therapies and poly(adenosine diphosphate [ADP]-ribose) polymerase inhibitors. However, there are no standardized methods to define, measure, and report HRD in diagnostic laboratories. Herein, we compare optical genome mapping (OGM), chromosomal microarray (CMA), and 523-gene next-generation sequencing (NGS) panel for

HRD scar calculations. **Methods:** This retrospective study included the analysis of 32 samples, of which 10 were gliomas and 22 were myeloid neoplasms. The 10 gliomas were evaluated with CMA and OGM. The 22 myeloid neoplasms were evaluated with a 523-gene NGS panel and OGM. The HRD scores were calculated using a combination of three HRD signatures that included loss of heterozygosity, telomeric allelic imbalance, and large-scale transitions. **Results:** In the 10 glioma cases analyzed with OGM and CMA using the same DNA (to remove any tumor percentage bias), the HRD scores were 13 (±13.7) with OGM compared to 3.7 (±4.5) with CMA. In the 22 myeloid neoplasm cases analyzed with OGM and 523-gene NGS panel, the HRD scores were 6 (±10.5) with OGM compared to 2.1 (±4.3) with 523-gene NGS panel. OGM detected 70.8% and 65% additional structural variants that resulted from HRD in gliomas and myeloid neoplasms, respectively. **Conclusions:** This study highlights the HRD scars that are missed by current technologies used for accessing HRD phenotype, and presents OGM as an alternative tool with high resolution and sensitivity to accurately assess the HRD phenotype.

TT009. **The ColoScape Test: A Novel QClamp XNA Technology-Based Assay to Detect Colorectal Cancer Associated Mutations and Methylations in Plasma Cell-Free DNA**

H. Tanaka, M. Sha, S. Shen, M. Scimia, L. Pastor, J. Li, A. Fu, M. Jamba, Q. Sun, A. Zhang
DiaCarta Inc, Pleasanton, CA.

Introduction: Colorectal cancer (CRC) is the second most common cause of cancer deaths when men and women are combined in the US. Thus, early detection in the precancerous stage is critical to reduce CRC morbidity and mortality. To meet this need, we developed a unique multiplex qPCR assay (ColoScape) to detect CRC-associated genetic and epigenetic changes from liquid biopsies using our proprietary QClamp XNA technology. The QClamp XNA technology enables ColoScape to selectively amplify the mutant and methylated DNA target sequences by using a synthetic DNA analog xenonucleic acid (XNA). This study introduces our new ColoScape test for detecting CRC-associated mutations and methylation markers in plasma cell-free DNA. **Methods:** The ColoScape test is designed to detect mutations in eight genes, 61 mutations, and seven methylated markers. The test consists of two parts: 1) the detection of mutations in eight genes (*APC*, *KRAS*, *BRAF*, *TP53*, *CTNNB1*, *NRAS*, *SMAD4*, and *PIK3CA*), and 2) the detection of seven methylation targeted genes. We designed PCR primers, TaqMan probes, and XNA in each mutation and methylation gene target for multiplexing. The assay analytical performance was verified and validated using various control samples. Furthermore, we have conducted a case-control pilot study in comparison with the clinical status to evaluate the clinical sensitivity and specificity of the ColoScape test. **Results:** We revealed that XNA effectively suppressed the wild-type background amplifications and led to amplification of either mutation or methylation target sequences, providing a high level of clinical sensitivity. The ColoScape test is highly reproducible with intra- and inter-assay coefficient of variation of <10%, and the cross-reactivity within the assay was limited and negligible. The results for the assay analytical sensitivity indicated that each target could be detected between 0.1% and 0.5% variant allele frequency in 10 ng cell-free DNA. The preliminary assay clinical specificity and sensitivity were 100% (95% CI: 91.3-100%) and 86% (95% CI: 66%-95%), respectively, for CRC, and 91% specificity (95% CI: 75%-98%) and 60% sensitivity (95% CI: 17%-93%) for advanced adenomas. **Conclusions:** The ColoScape test utilizing the XNA-based technology provides high sensitivity and high specificity to CRC and advanced adenomas with a great potential to be used as an early screening test.

TT010. **A Comparison of SARS-CoV-2 Genome Sequencing Methods for Surveillance in Rural New England**

S. Deharvengt[1], D. Green[1], K. Winnick[1], S. Sathyanarayana[1], P. Shannon[1], M. Kelly[2], J. Sevigny[2], K. Thomas[2], G. Tsongalis[1], M. Whitfield[3], J. Lefferts[1]
[1]Dartmouth Health, Lebanon, NH; [2]University of New Hampshire, Durham, NH; [3]Dartmouth Geisel School of Medicine, Hanover, NH.

Introduction: Genomic SARS-CoV-2 sequencing is essential to address the global pandemic. The NIH Institutional Development Award (IDeA) program has funded 21 sites in the US to perform SARS-CoV-2 surveillance studies in rural and underrepresented communities. The CGAT laboratory at Dartmouth Health and the University of New Hampshire (UNH) have participated in this project by sequencing SARS-CoV-2-positive cases from their New Hampshire and Vermont populations. The two institutions used different amplicon-based sequencing assays. The aim was to compare the workflow and data obtained using the Ion AmpliSeq Insight Panel to the Illumina CovidSeq Test. **Methods:** The same 19 positive patient samples were sequenced by both AmpliSeq (CGAT) and Illumina (UNH) panels. Viral transport media specimens from positive nasal or nasopharyngeal collections were stored at -80ºC until retrieved for RNA extraction using the automated EZ1 Virus Mini Kit v2.0 (QIAGEN) for CGAT and the MagMax Viral/Pathogen nucleic acid isolation kit on the KingFisher Flex (Thermo Fisher Scientific) for UNH. AmpliSeq sequencing: Libraries were made using the Ion AmpliSeq SARS-CoV-2 Insight Research Assay-GS Chef Ready followed by sequencing on the Ion GeneStudio S5 (Thermo Fisher). Base-calling and alignment were performed on Torrent Suite v5.12.0. Variant calling was performed on the SARS_CoV_2_variantCaller plugin v5.16.0.5. Variant calls were annotated by the SARS_CoV_2_annotateSnpEff v5.16.0.5 plugin, and the generateConsensus plugin v5.16.0.10 was used to assemble consensus FASTA for each sample. Sequence variants were called based on the reference sequence NC_045512.2. Illumina sequencing: Complementary DNA was amplified using the ARTIC v3 primers. Libraries were prepared with the Illumina COVIDSeq Test and sequenced on the NovaSeq 6000. Data were analyzed using The DRAGEN COVIDSeq Test App in BaseSpace. Pangolin lineages for both datasets were assigned using the web-based Pangolin Covid-19 Lineage Assigner v4.0.6. **Results:** The AmpliSeq panel is highly automated but with lower throughput than the Illumina panel. The lineage calls were 100% concordant between methods for all 19 samples. The AmpliSeq panel analysis produced more undetermined nucleotide (N) calls than Illumina (an average of 234.42 and 33.78 N calls, respectively). This resulted in slightly lower lineage designation Scorpio confidence scores in the AmpliSeq samples despite having an average of four times greater coverage. Further investigation revealed an Omicron-specific amplicon dropout with AmpliSeq panel not observed with Illumina. **Conclusions:** The data obtained by both platforms were comparable, but sequencing quality varied with new variants or lineages. The choice of the platform is highly dependent on desired throughput.

TT011. **Comparison of *STK11* and *KEAP1* Mutation Detection in Circulating Tumor DNA (ctDNA) and Tissue from Patients with Non-Squamous Metastatic Non-Small-Cell Lung Cancer (mNSCLC)**

Z. Lai[1], J. Barrett[1], R. Stewart[2]
[1]AstraZeneca, Waltham, MA; [2]AstraZeneca, Cambridge, United Kingdom.

Introduction: First-line treatment with immune checkpoint inhibitors, either alone or combined with chemotherapy, has been shown to improve outcomes for patients with metastatic non-small cell lung cancer (mNSCLC). However, patients with *STK11* and/or *KEAP1* mutations appear to derive reduced benefit from such therapies. Thus, there is a need to identify these patients to test new therapies. Here, we compared the concordance between detection of these mutations in plasma circulating tumor DNA (ctDNA) samples versus formalin-fixed, paraffin-embedded (FFPE) tissue samples from three phase III

studies (NCT02453282, NCT02542293, NCT03164616) in first-line non-squamous mNSCLC. **Methods:** Baseline FFPE tissue samples were profiled using an external vendor's next-generation sequencing (NGS) panel and baseline plasma ctDNA samples using another external vendor's NGS panel. Only loss-of-function *STK11* and *KEAP1* mutations were included in the comparison. **Results:** Sequencing was successful for 919/1,425 (64.5%) tissue samples and 1,589/1,687 (94.2%) ctDNA samples. Roughly two-thirds of tissue sequencing failures were due to minimum pathology requirements for tissue volume or tumor nuclei content, reflecting the challenge of tissue quality in the large real-world global studies. *STK11* and *KEAP1* mutations were detected in 17.1% and 7.3% of patients with successfully sequenced tissue samples and 11.8% and 4.9% of patients with successfully sequenced ctDNA samples, respectively. In 775 patients, both tissue and ctDNA samples were sequenced. Compared with the tissue-based method, detection of *STK11* mutations in ctDNA had a positive percent agreement (PPA) of 62% and negative percent agreement (NPA) of 99.2%, but a false-negative rate (FNR) of 38%. For *KEAP1*, it had a PPA of 62%, NPA of 99.6%, and FNR of 38% versus the tissue-based method. The false-positive rate (FPR) was low, at 0.8% and 0.4% for *STK11* and *KEAP1*, respectively. The high FNR was mainly due to low/non-shedding and limited detection of homozygous deletions and rearrangements in ctDNA. *STK11* and *KEAP1* mutations were also significantly co-occurring in both tissue and ctDNA (odds ratio 5.1 [3.3 to 7.9]; p <0.0001). **Conclusions:** In this *post-hoc* analysis of specimens from phase III studies in non-squamous mNSCLC, ctDNA NGS had a higher success rate than FFPE tissue NGS, but with a high FNR in detecting *STK11* and *KEAP1* mutations due to non-shedders exceeding assay sensitivity and limited detection of complex mutations. Ensuring that tissue samples are of sufficient volume and quality will increase the success rate for sequencing. The low FPR but high success rate for ctDNA suggests it is a feasible tool to aid recruitment of patients with *STK11* and *KEAP1* mutations in prospective clinical trials, whereas the high NPA makes it an attractive alternative in cases where tissue testing fails.

TT012. **Development of Quality-Control Methods for Reliable NGS Measurement of Methylome in Circulating DNA**

J. Willey[1], E. Crawford[2], D. Craig[1], H. He[3], T. Morrison[4], J. Herman[5]
[1]University of Toledo College of Medicine and Life Sciences, Toledo, OH; [2]College of Medicine and Life Sciences University of Toledo, Toledo, OH; [3]NIST, Gaithersburg, MD; [4]Accugenomics, Inc., Wilmington, NC; [5]University of Pittsburgh, Pittsburgh, PA.

Introduction: Recent development of next-generation sequencing methods that measure the methylome in circulating tumor DNA has potential to enable earlier detection of cancer and more sensitive measurement of minimal residual disease. Advances include methods that measure cancer-type-specific methylated regions to enable determination of site of origin and methods that measure hundreds to thousands of methylated regions and thereby enable earlier detection by overcoming stochastic sampling associated with measurement of a small number of targets. Widespread adoption of these methods for clinical diagnostic applications will be facilitated through improved quality controls that reduce false-positive and false-negative results and increase reliability. The purpose of this study was to use recently developed DNA methylation test materials developed by the National Institute for Standards and Technology (NIST) to assess performance of the SNAQ-SEQ method as a quality control to establish limit of detection (LOD) for measurement of methylation at each targeted CpG site. **Methods:** NIST test materials were provided through an inter-laboratory pilot testing program. These materials were formulated by mixing DNA from NA24385 (Coriell Institute for Medical Research) in the "native" state of DNA with *in vitro* methylated genomic DNA (methylated NA24385) at fractions of 0%, 5%, 20%, 50%, and 100% (Samples A-E). The genome-wide DNA methylation pattern was analyzed by reduced representation bisulfite sequencing (RRBS). The Accugenomics, Inc. SNAQ-SEQ Mix4 kit was used in these studies. Synthetic internal standards (IS) are included in this kit, and PCR primers designed for analysis of post-bisulfite treated DNA were used to assess LOD for measurement of methylation at each of 14 CpG sites in the targeted region of the *SOX2* gene. Following mixture of Mix4 IS with each of the NIST test materials, each mixture was subjected to PCR-amplicon library preparation, then sequenced on Illumina MiSeq. **Results:** Based on RRBS conducted at NIST, average methylation across CpG in the *SOX2* gene was 11.6% in native NA24385 DNA (Sample A), and increased as expected to a maximum of 98% (Sample E). In our analysis of the particular *SOX2* region targeted by the SNAQ-SEQ Mix4 kit, average methylation (preserved C nucleotide) was 16.4% (0.5%-50%) in native NA24385 DNA. In contrast, in IS corresponding to the targeted *SOX2* region the average preserved C was 0.32% (0.03%-0.5%). **Conclusions:** Use of synthetic IS enabled determination of LOD for each individual methylated site, controlling for all known sources of interference and technical error, including inefficient bisulfite conversion, nucleotide substitution error, and alignment error.

TT013. **Validation of an Automated Next-Generation Sequencing Workflow Using the Hamilton VANTAGE Platform**

L. Schultz-Rogers, J. Booker, J. Galeotti, R. Kshatriya, N. Montgomery, L. Ramkissoon, K. Weck
UNC Chapel Hill Hospital, Chapel Hill, NC.

Introduction: The UNC Chapel Hill clinical molecular laboratory currently runs a targeted next-generation sequencing assay (customized Myeloid Solution from SOPHiA Genetics) which is used for characterization of clinically actionable variants in hematologic malignancies. To date, wet bench library preparation has been performed manually by technologists and sequencing is performed on an Illumina MiSeq instrument. To improve wet bench consistency, increase throughput, decrease turnaround time, and optimize patient care, our laboratory procured a Hamilton Microlab VANTAGE automated liquid handling platform. Here we present the first validation results of the customized Myeloid Solution using the Hamilton Microlab VANTAGE. **Methods:** To validate the Hamilton VANTAGE platform for library preparation, nine manually processed runs were repeated using a protocol generated in collaboration with Hamilton and SOPHiA. We analyzed on-instrument metrics (cluster density, clusters passing filter, Q30 score) and post-analysis metrics (percent mapped reads, on target percentage, coverage, coverage heterogeneity). We compared variants in all samples, both pre- and post-filtering, using our laboratory standard protocols. **Results:** Results and QC metrics from a total of 87 patient samples were compared between manual and Hamilton VANTAGE processing. All Hamilton runs passed our run-level and sample-level QC criteria and showed a trend toward higher overall QC precision. Of the retained variants passing minimum filtering criteria variant allele frequency (VAF) >1%, indel in homopolymer <10bp, on target region, and coverage above 1,000x), 98.3% (3,245/3,299) were called using both methods. There were 21 and 33 variants called only in samples from the manual or Hamilton runs, respectively; however, none of these met criteria for reporting as they were low-level variants (VAF <3%), known artifacts, or deep intronic. We were able to confirm eight *FLT3* internal tandem duplications ranging from 20 to 188 base pairs, although as expected VAF was more variable than for single nucleotide variants. There was an overall improvement in quality and consistency, with fewer samples with low coverage regions or that had to be repeated compared to manual runs. Finally, the manual method required 635 minutes of hands-on time, whereas the Hamilton method required only 285 minutes of hands-on time. **Conclusions:** Use of the Hamilton Microlab VANTAGE automated liquid handling platform for library preparation with the custom SOPHiA capture assay is superior to manual library preparation in terms of decreased hands-on time and increased precision and run-to-run consistency. There was 100% concordance for calling clinically reportable variants. Overall, this platform increases the quality and output of our next-generation sequencing panel.

TT014. **Reference Standards for the Determination of Limit of Blank (LoB) and Limit of Detection (LoD) in Tumor-Informed, ctDNA Minimal Residual Disease (MRD) Assays**

C. Raymond[1], S. Fransen[2]
[1]Salish Bioscience, Seattle, WA/Ripple Biosolutions, Seattle, WA; [2]Salish Bioscience, Seattle, WA.

Introduction: Monitoring of circulating tumor DNA (ctDNA) in patients undergoing treatment for cancer has the potential to improve oncology care. Limit of blank (LoB) and limit of detection (LoD) are critical assay metrics. Using phased, single-nucleotide variants (SNVs) with a spacing of 3 nt, we created reference samples that can be used to simultaneously measure specificity and sensitivity within the next-generation sequencing (NGS) data of a single sample. Here we demonstrate the performance of these standards using a unique target hybrid capture technology. **Methods:** To approximate the random nature of tumor-specific somatic variants, we chose to place phased variants and assays in 112 genomic loci distributed across chr1 through chr12. The selection criteria were that loci should occur in unique regions of the genome and that the target region should avoid common SNVs; we did not impose restrictions on base composition. Target regions were synthesized as gBlocks, fragmented to the size of cell-free DNA (cfDNA) monomers, and spiked into cfDNA purified in bulk from an anonymous human donor. Reference libraries were tagged with unique molecular identifiers, and target regions were captured using a total of 224 probes designed to independently address both chromosomal strands of the 112 target loci. This was followed by NGS of pooled libraries. Bioinformatic analysis included standard error correction and quantitative analysis of variant allele frequencies. **Results:** Robust platforms for tumor-informed measurable residual disease (MRD) must be able to address large numbers of different tumor markers that are unique to each individual patient. This requires nimble probe performance. We observed one probe failure among 224 possible probes. We scored true positive (TP) sequences as those that had both phased variants. False-positives (FP) were sequences with only one of the two possible variants detected. We evaluated duplicate spike-in samples with spike-in allele frequencies of 0.1%, 0.01%, 0.003%, and 0.001%. The FP detection rate was uniform across all eight samples (6.6 ± 2.4 reads per one million unique reads considered). Detection of TP expressed as a function of phased variants detected per million unique reads scaled as expected (0.1%-1,142; 0.01%-72; 0.003%-19; and 0.001%-11). **Conclusions:** Analytical validation of MRD assays will require determination of the LoB. This in turn will dictate the LoD. In this proof-of-concept (POC) study presented, LoB was 0.0007%. If we argue that LoB and LoD must be separated by four standard deviations of the LoB measurements, then LoD will be approximately 0.0017%. In real patient samples, this observed allele fraction is necessarily the sum of FP + TP. Taken together, this places the LoD for ctDNA detection in this POC pilot at an allele fraction of 1/100,000.

TT015. **Reference Standards for the Development and Analytical Validation of a Next-Generation Lung Cancer Liquid Biopsy Assay**

S. Fransen[1], F. Raymond[2], S. Grindstaff[1], C. Zakarian[1], T. Persse[1], P. Batzel[1], K. Misura[1], C. Raymond[2]
[1]Salish Bioscience, Seattle, WA; [2]Salish Bioscience/Ripple Biosolutions, Seattle, WA.

Introduction: We are developing a next-generation sequencing liquid biopsy for patients with non-small cell lung cancer (NSCLC) that has improved accuracy for the detection of low-frequency variants. Assay validation requires training and test reference standards for optimization/familiarization (O&F) and analytical validation (AV). Here, we describe the results from our assay prototype and a collaboration to develop reference standards that enable O&F and AV. **Methods:** Our technology improves conversion of cell-free DNA (cfDNA) molecules into sequencing fragments, uses unique molecular identifiers for error correction and quantitative data analysis, and improves performance through changes to hybridization chemistries. A research prototype assay was tested using blended cfDNA from two human donors that differed at 352 common single nucleotide polymorphisms. cfDNA was mixed at 0.1% variant allele frequency (VAF) for 279 heterozygous (HET) markers and 0.2% VAF for 73 homozygous (HOM) markers. Next we created an NSCLC assay and corresponding reference standards. These NSCLC reference standards were formulated in a background of human cfDNA purified from whole units of plasma. Spike-in mutations were synthesized as gene segments that were then fragmented and size-selected to the size of cfDNA monomers. They include a large number of target mutations (34 single nucleotide (SN) variants, nine SN indels, 11 indels >1 base, and 12 fusions), all at a consistent VAF. Phased base change "tags" were introduced 3 nt away from target mutations to unambiguously mark reference standard DNA and differentiate this from mutations anticipated in patient samples. **Results:** The research prototype assay was conducted with an input of 50 ng. Greater than 90% of HET markers and 100% of HOM markers were detected on both Watson and Crick strands of the target loci. No false-positive calls were observed at the polymorphic base positions. Next, the lung cancer reference standards, comprising a training set with cancer-like mutations and a test set with mutations commonly found in NSCLC were assessed using our NSCLC assay. Initial analysis of both sets demonstrated that all 66 mutations were present at consistent and uniform VAFs. **Conclusions:** Promising results from the prototype assay prompted us to develop a cfDNA assay for NSCLC. We have collaborated to create NSCLC reference standards that consist of training and test samples for validation. These contain similar but independent mutations at consistent VAF and are suitable for measuring sensitivity and specificity at low VAFs. We believe this large number of mutations in different contexts lends statistical power to AV and anticipate these types of reference standards will be useful for liquid biopsy method validation.

TT016. **SalivaDirect Bridging Study: Validation of the Liberty16 Pro for the Detection of SARS-CoV-2**

Y. Tan[1], A. Wyllie[2], T. Verdun[1], D. Hirst[1], P. Pickering[1]
[1]Ubiquitome Limited, Auckland, New Zealand; [2]Yale School of Public Health, New Haven, CT.

Introduction: The emergence of SARS-CoV-2 highlighted the need for rapid and accurate screening tests to successfully respond to a worldwide pandemic and identify infected individuals to limit pathogen spread. We therefore developed an advanced version of a mobile real-time PCR device, the Liberty16 Pro, to expand testing capacity, permitting pathogen detection with a multiplexed assay, (*e.g.,* FAM and ROX/TexasRed/Cy5) and increased speed (average ramp rate = 4.5°C/s) whilst retaining the simplicity of use. **Methods:** To validate the clinical performance of the SalivaDirect SARS-CoV-2 assay on the Liberty16 Pro, we collaborated with the SalivaDirect team to perform a protocol bridging study at Massey University, in New Zealand. An established set of heat-inactivated clinical samples were provided by the Yale School of Public Health, which were shipped frozen on dry ice and immediately stored at −80°C upon receipt. Both SARS-CoV-2 RNA spiked-in and clinical samples were processed according to the SalivaDirect protocol (for details, see dx.doi.org/10.17504/protocols.io.btdnni5e). **Results:** The limit of detection (LoD) of the SalivaDirect duplex assay on the Liberty16 Pro using the standardized bridging reagents was 3.0 copies/µL. A lower LoD of 1.5 copies/µL was achieved when using the NEB Luna Probe One-Step RT-qPCR 4X Mix with UDG (4X) and IDT primers and probes. Additionally, we showed 100% concordance among six low positive (Mean = 34.08; SD = 1.94) and two negative clinical samples when tested on the Liberty16 Pro as compared to the Bio-Rad CFX96 Touch. **Conclusions:** The Liberty16 mobile PCR instrument has been clinically validated as a flexible and accessible device system for running the affordable SalivaDirect real-time PCR assay for the detection of SARS-CoV-2, especially in low-resource settings. Here, we showed that our new advanced device, Liberty16 Pro, has more than 4× higher sensitivity in comparison to the FDA-EUA single-plex Liberty16 SalivaDirect assay (LoD = 12 copies/µL). Most importantly, it expands testing capacity greatly from six to 14 samples per run with a

shorter run time, providing an opportunity to increase sample daily throughput and turnaround time, ranging from 230% to 700% (depending on the selected thermal cycling protocol), while retaining the unique features which are best fit to a low-resource setting.

TT017. **A Review of Applying Single-plex Liberty16 SalivaDirect Assay as On-Site Surveillance Testing in New Zealand Workplaces**

Y. Tan, T. Verdun, L. Hua, C. Chen, K. Weeks, P. Pickering
Ubiquitome Limited, Auckland, New Zealand.

Introduction: The newly emerged Omicron variant and its subvariants have gained extended functions in adapting to the host-cell environment and therefore are more transmissible than other variants of concern. The outbreak in New Zealand created an enormous wave of COVID-19 cases and paralyzed the testing capacity of IANZ-accredited diagnostic laboratories, leading to delayed results. In response to the crisis, we provide the option of applying single-plex Liberty16 SalivaDirect assay as an on-site COVID-19 surveillance system to ensure workplace health and safety, and to maintain uninterrupted day-to-day business operations. **Methods:** To verify the performance of the CDC 2019-nCoV-N1 primer-probe set in detecting the Omicron variant of SARS-CoV-2, plasmids containing Wuhan/WH01/2019 (wild-type) and B.1.1.529 (Omicron) sequences were serially diluted and subsequently directed for SalivaDirect RT-qPCR detection on Liberty16 using commercially procured reagents. Additionally, to assess the clinical performance of the Liberty16 SalivaDirect assay in particular ethnic groups within the New Zealand population, we retrospectively analyzed the reported cases from local organizations that have been using the Liberty16 SalivaDirect assay as a surveillance system. **Results:** Here, we show that the CDC 2019-nCoV-N1 primer-probe set can detect the Omicron N1 target despite the C28311T mutation using synthetic plasmid DNA. Indeed, the limit of detection of Omicron detection is comparable to SARS-CoV-2 first isolate Wuhan/WH01/2019. Our data further confirmed that the SARS-CoV-2 N1 can be efficiently detected by using either SalivaDirect standard or fast cycling protocols on Liberty16. In addition to analytical performance, we found that the Liberty16 SalivaDirect screening results were 100% concordant with diagnostic testing results provided by IANZ diagnostic laboratories. **Conclusions:** Our findings provide analytical and clinical support for reports that the mutations in the Omicron variant have little or no impact on SalivaDirect assay in terms of amplification efficiency and detection sensitivity using either standard or the recently reported fast Liberty16 SalivaDirect thermal cycling protocols. Most importantly, we demonstrated that the Liberty16 SalivaDirect screening results were 100% concordant with the diagnostic testing results provided by IANZ diagnostic laboratories, indicating the feasibility of applying the Liberty16 SalivaDirect assay for workplace on-site testing. The suggested on-site workplace sentinel surveillance system for COVID-19 not only helps by taking the pressure off centralized labs during major outbreaks but also serves as an early warning system for public healthcare providers.

TT018. **Colli-Pee UAS Combined with nRichDX Revolution System, a Promising Urinary Cell-Free DNA Collection, Preservation and Extraction Workflow**

S. Jordaens[1], A. Arora[2], K. Zwaenepoel[3], A. Vorsters[3], J. Tijsen[1], K. Beyers[1], C. Deben[3], W. Tjalma[3], S. De Wachter[4], P. Van Dam[4], P. Pauwels[4], V. Vankerckhoven[1], N. Jafari[5]
[1]Novosanis, Wijnegem, Antwerpen, Belgium; [2]DNA Genotek Inc., Ottawa, Canada; [3]University of Antwerp, Wilrijk, Belgium; [4]Antwerp University Hospital (UZA), Edegem, Belgium; [5]nRichDX, Irvine, CA.

Introduction: Urine as liquid biopsy for non-invasive cancer diagnostics is gaining interest. Standardization of sample collection, storage, and processing is a critical enabler driving the clinical utility of urine for cancer research, detection (screening), or treatment monitoring. Here, we investigated a first-void urine collection Colli-Pee device and a urine preservative (UAS) from Novosanis, combined with a cell-free DNA (cfDNA) extraction platform, Revolution System (nRichDX), as a seamless workflow for urine sample collection, handling, and processing. **Methods:** First, three commercial cfDNA extraction kits, QIAamp Circulating Nucleic Acid kit (Qiagen), Urine Cell-Free Circulating DNA Purification Maxi kit (Norgen), and Custom Maxwell RSC ccfDNA kit (Promega), input volumes ranging from 4 mL to 12 mL, were compared using samples collected with the Colli-Pee UAS FV-5040 from healthy volunteers (n=27) and cancer patients (n=10). Secondly, the Qiagen kit and the Revolution System were compared by using unpreserved and UAS preserved urine samples held at room temperature (RT) for seven days. Both unpreserved and preserved urine samples were investigated under freeze-thaw cycling conditions to evaluate batch processing compatibility with the Revolution System. The performance was measured using Qubit dsDNA HS assay (Thermo Fisher), cfDNA ScreenTape (Agilent), HS D5000 ScreenTape (Agilent), and β-globin qPCR assay. **Results:** All three extraction kits performed comparably; however, the Qiagen kit showed a higher DNA concentration than the Norgen and Promega kits. Additionally, the Revolution System showed higher cfDNA yield and detection sensitivity. Furthermore, unpreserved samples showed degradation and loss of cfDNA profiles, whereas no significant difference was observed in UAS preserved urine samples under both RT storage and freeze-thaw cycling conditions. Additionally, all used cfDNA extraction kits and platforms utilize different technologies/principles and were compatible with UAS preserved urine samples. Each extraction workflow requires different additional steps, where Qiagen incurs the most steps and transfers, followed by Norgen and Promega kits. The Revolution System showed the lowest number of steps and was least prone to manual error. **Conclusions:** The nRichDX Revolution extraction platform enables processing of large urine volumes leading to increased cfDNA yield and sensitivity. Additionally, UAS preserved samples showed compatibility with all available tested cfDNA extraction kits or platforms, demonstrating the agnostic nature of the UAS chemistry. Overall, the innovative Colli-Pee UAS FV-5040 (RUO), urine collection and analyte preservation, combined with the nRichDX Revolution System, offers an improved workflow for investigating urinary biomarkers.

TT019. **digitalMLPA: Multiplex Tool for Simple and Accurate Detection of Genetic Alterations in Central Nervous System (CNS) Tumors**

N. Kakava-Georgiadou, A. Benard-Slagter, L. Atanesyan, B. Boerrigter, C. Enright, M. Loden-van Straaten, J. Schouten, S. Savola
MRC Holland, Amsterdam, Netherlands.

Introduction: Central nervous system (CNS) tumors have distinct genetic and epigenetic features that play an important role in their diagnosis and clinical management. With an increasing number of genetic markers used in CNS tumor characterization, there is a growing need for fast and affordable multiplex assays for their detection. MLPA has been successfully used in a plethora of studies on CNS tumor-derived DNA samples for copy number (CN), *IDH1/2* mutation, and *MGMT* methylation status detection, thus contributing to diagnosis and treatment selection. For broad genetic profiling of CNS tumors, we utilized digitalMLPA, which allows simultaneous detection of CN, selected mutations and fusions, as well as methylation for up to 1,000 targets in one reaction requiring only 20 ng of input DNA. **Methods:** A digitalMLPA assay of 966 probes was used to detect 1) CN of more than 60 genes (e.g., *BCOR, BRAF, CCND1, CDKN2A/B, CDK4/6, EGFR, FGFR1, MDM2/4, MET, MYB, MYBL1, MYC, MYCN, NF1/2, PDGRFA/B, PTEN, RB1, SMARCB1, TERT, TP53, YAP1)*; 2) chr. 1, 7, 10, 16, and 17p, 19q arms and various other regions (e.g., 9q34, C19MC); 3) methylation status of *MGMT* and *TERT*; 4) selected point mutations in *IDH1/2*, *TERT*, *PIK3CA*, *FGFR1*, *H3-3A* and *BRAF*; 5) various gene fusions (e.g., *BRAF-KIAA1549*, *RELA-c11orf95* and *ROS1-GOPC)* in 24 cancer cell lines, of which eight are of CNS origin. Each digitalMLPA reaction was performed with 20-40 ng of genomic DNA and data were analysed using Coffalyser digitalMLPA software. The results were compared to those reported in public databases. **Results:** Five out of eight CNS tumor cell lines showed a gain of chr. 7

(including *EGFR* amplification) combined with complete or partial loss of chr. 10 and a homozygous *CDKN2A/B* deletion. Interestingly, DK-MG glioma cell line showed a high-level *EGFR* amplification accompanied by *EGFR*vIII deletion and *TERT* c.250C >T mutation, whereas in other CNS tumor cell lines *TERT* c.228C >T was detected. The *ROS1-GOPC* gene fusion was detected in U-118 MG cell line. Furthermore, *BRAF* V600E was successfully detected in three cell lines as well as in Horizon reference standards with allelic frequency as low as 7.7%. Reported point mutations in cell lines and reference standards for *IDH1* R132C/H, *H3-3A* K27M, *PIK3CA* (R88Q, E542K, E545K, H1047R/L) were correctly identified by digitalMLPA. Finally, detected *MGMT* and *TERT* methylation status strongly correlated with publicly available data. **Conclusions:** This novel digitalMLPA Neuro-Oncology panel is suitable for the simultaneous detection of CN, (point) mutation, fusion, and methylation status in samples of somatic origin, thus serving as an affordable and simple tool for CNS tumor research and characterization. Further validation with cancer patient DNA samples in independent laboratories and on a larger scale is required to assess its clinical and diagnostic utility.

TT020. **Using NGS to Detect the *MYD88* p.L265P Mutation Often Requires Distinguishing Low-Level Mutation from Background Using Bioinformatic Analysis and Visual Review in IGV**

S. Hafeez, A. Cushman-Vokoun, S. Rapp, W. Zhang
University of Nebraska Medical Center, Omaha, NE.

Introduction: The MYD88 protein mediates activation of NF-KB. The *MYD88* p.L265P mutation is identified in 95% of lymphoplasmacytic lymphomas (LPLs), 30% of ABC-subtype diffuse large B-cell lymphomas, and 5%-20% of splenic marginal zone lymphomas (splenic MZLs). It is rare in multiple myeloma. The presence of an *MYD88* mutation can be useful, but not definitive, in distinguishing LPL from other B-cell lymphomas. Next-generation sequencing (NGS) can be used to detect the p.L265P mutation, but considerations are required to avoid false-negative results. **Methods:** To assess the analytical validity of NGS detection of the *MYD88* p.L265P (c.794T >C) mutation, we retrospectively identified cases resulted as indeterminate or positive for the p.L265P mutation since 2018 (either by single gene *MYD88* analysis using bioinformatic filtering or by full myeloid panel). Single gene *MYD88* orders require specific review of the mutation location in the Integrative Genomics Viewer (IGV). These specimens and concurrently analyzed specimens on the NGS runs plus two normal (210 specimens) were analyzed bioinformatically to determine background noise of the T >C change at the mutation site. NGS was performed on DNA extracted from fresh bone marrow or blood using the Oncomine Myeloid Assay (5% VAF LOD). Library preparation, clonal amplification and NGS were performed on the Ion Chef System and Ion Gene Studio S5. All specimens (single gene and full panels) were bioinformatically interrogated at chr3:38182641, the site of the p.L265P mutation. Using R programming and Rsamtools, the number of reads supporting all the nucleotides at chr3:38182641 in the aligned bam files were retrieved. The variant allele frequency (VAF) of all alternative alleles was calculated for the background noise evaluation. **Results:** Review of the 210 cases determined a background rate of a T >C change of 0.0081 mean VAF ±2SD of 0.0792 (using the bam file). Forty-two cases were clinically resulted as p.L265P-positive or indeterminate after retrospective review. The corresponding diagnoses included 25 LPLs, seven MZLs, four either LPL or MZL, and six categorized as other. The VAFs ranged from 0.26% to 36.4%, with 20 cases demonstrating a VAF of <5%. Only 28% of the LPL case category had VAFs less than 5%, whereas 71%-83% of the other subgroups had VAFs of <5%. **Conclusions:** MYD88 is being included on many NGS-based panels. However, our results show that the *MYD88* p.L265P mutation can occur at a very low level, especially in cases that cannot be definitively classified as LPL. It is worthwhile for laboratories using NGS to interrogate the specific chr3:38182641 site bioinformatically and/or use IGV visualization to identify a low-level p.L265P mutation. It is also important to determine background noise at the chr3:38182641 site to minimize false-positive results.

TT021. **Reference Materials for the Analysis of Methylation in Circulating Cell-Free DNA**

J. Ramprakash[1], M. Butler[1], D. Xia[2], Y. Konigshofer[1], R. Garlick[2]
[1]LGC Clinical Diagnostics, Inc., Gaithersburg, MD; [2]LGC Clinical Diagnostics, Inc., Milford, MA.

Introduction: Epigenetic modifications such as methylation influence cellular differentiation and gene expression. Liquid biopsies are starting to screen for cancer-derived DNA by looking for unexpected epigenetic modifications in circulating cell-free DNA (ccfDNA) that could also be used to assign a tissue of origin to the cancer to direct confirmatory diagnostic procedures. However, because of the low abundance of ccfDNA, obtaining sufficient amounts for assay development, validation, and proficiency testing is difficult. Furthermore, methods of analyzing the epigenetic modifications, such as bisulfite conversion, can damage a significant fraction of the input material, but failing to carry them out to completion can result in an overestimation of methylation. This makes method optimization very important. To address the challenges associated with assessing the methylation of ccfDNA, we created ccfDNA-sized reference materials (RMs) with defined amounts of methylation. **Methods:** ccfDNA-sized genomic DNA from the GM24385 cell line as well as donor-derived ccfDNA were used as starting points for amplification to create unmethylated and methylated RMs. Methylation was assessed by digital PCR by targeting heterozygous SNPs, where one allele was an MspI site (unknown) and the other was not (reference), and where methylation protects the MspI site from digestion with HpaII. Methylation was also assessed by next-generation sequencing after bisulfite and enzymatic conversion as well as by external testing. **Results:** Digital PCR-based methylation assessment of the RMs was generally in agreement with other methods. Results from bisulfite versus enzymatic conversion were similar. Interestingly, the choice of dU-tolerant proofreading polymerase was found to be important in the amplification of dU-containing bisulfite-converted DNA, where one led to improved yields and more uniform genomic coverage than another, which may explain some of the perceived yield- and coverage-related differences between bisulfite and enzymatic conversion. Results from external testing were usually in agreement with expected levels of methylation in the RMs. **Conclusions:** We have created RMs for the assessment of methylation in ccfDNA. The manufacture of these RMs is scalable so that sufficient quantities can be prepared to support assay design, optimization, validation, and beyond. By having defined degrees of methylation, the RMs should also be useful in establishing the analytical validity of the measurement of methylation.

TT022. **Streamlining Solid Tumor Comprehensive Genomic Profiling Workflows Using Learned Variant and Sequencing Noise Autoclassification**

T. Ward, S. Reynolds, J. Wagner, D. Naidoo, E. Shull, J. Welle, R. Rattray, C. Moung-Wen, M. Campbell, B. Piening, C. Bifulco
Providence St. Joseph Healthcare, Portland, OR.

Introduction: Use of next-generation sequencing (NGS) has seen a dramatic expansion in the clinical setting, with tests for inherited conditions and somatic alterations in cancer. Variant review is a vital part of the NGS pipeline, ensuring that true genetic alterations are being reported for precision matching to the best treatment options and clinical trial eligibility. However, due to an ever-increasing list of cancer-related genes and increased sensitivity of NGS pipelines, this process can be arduous and time consuming. Here, we present an auto-classification system for routinely observed variants and sequencing artifacts developed and refined across a cohort of nearly 10,000 samples. **Methods:** Cancer patients (stages I-IV) in the Providence St. Joseph Healthcare system received comprehensive genomic profiling (CGP) from October 2019 to June 2022 (n=9,237 patients), 9,839 total sequencing samples. DNA and RNA were extracted from formalin-fixed, paraffin-embedded (FFPE) tissues using Qiagen or Promega FFPE Extraction Kit procedures. NGS libraries were prepared using the Illumina TSO 500 HT protocol and sequenced on the Illumina NovaSeq 6000 platform. Average variant allele frequency (VAF) is

calculated from each variant's historical calls. ClinVar and OncoKB were used to determine clinical significance. Variants added to the auto-classification list were decided on by multiple factors such as their known oncogenicity, number of occurrences seen, VAF average for all occurrences, and sequencing read quality. **Results:** We have 800 distinct DNA variants auto-classified: 160 as a reportable variant, 545 as a relatively rare benign polymorphism, and 95 as sequencing noise. These classifications span 248 different genes (of 523 analyzed in the TSO500 panel) with *TP53* having the most (n=83). Across the 9,839 samples, after initial filtering of common polymorphisms, 192,064 variants were (or retroactively could have been) auto-classified, an average of 19.5 variants per sample (0.85 reportable, 11.06 benign polymorphisms, and 7.61 sequencing noise). Conversely, we see a total count of 229,244 variants that were not auto-classified (23.3 variants per sample). This results in an average of 45.56% of the called variants being auto-classified. We also identified several assay chemistry-specific sequencing noise variants that occur routinely that prior to auto-classification required careful investigation to distinguish from real reportable genomic variants. **Conclusions:** With ever-increasing panel size and complexity for CGP testing, auto-classification of variants is a key step in reducing the burden and time commitment for manual variant review, as well as potentially increasing variant designation consistency and identification of assay-specific sequencing noise that would otherwise be reported.

TT023. **Next-Generation Reference Materials for Somatic Mutation Detection in Circulating Cell-Free DNA**

Y. Konigshofer[1], J. Ramprakash[1], M. Butler[1], R. Garlick[2]
[1]LGC Clinical Diagnostics, Inc., Gaithersburg, MD; [2]LGC Clinical Diagnostics, Inc., Milford, MA.

Introduction: Advances in next-generation sequencing (NGS) are enabling larger (e.g., 500 gene) panel sizes and increased sensitivities for liquid biopsies. These advances lead to a need for patient-like reference materials (RMs) with more clinically relevant alterations at lower levels than before. To address this need, we generated novel circulating tumor DNA (ctDNA) RMs that combine much of the content of our ctDNA v2 and ctDNA Complete reference materials and add additional variants and copy number alterations. Because limits of detection of individual mutations are now at or below 0.2% variant allele frequency (VAF) in some ctDNA assays, we also reduced the lowest VAF from 0.1% to 0.01%. Here we provide data on the initial testing of these RMs. **Methods:** As part of manufacture, digital PCR (dPCR) was used to assess the VAFs of variants. Additionally, a custom hybrid/capture-based NGS panel was used, which targeted only the alterations that were expected in the RMs to maximize coverage. The use of molecular barcodes allowed for error correction and for determining the number of unique molecules to assess library incorporation efficiency. An Agilent Bioanalyzer was used to evaluate the size distribution of the RMs. External testing with the Illumina TSO 500 ctDNA assay was used as a representative ~500-gene panel. **Results:** Because dPCR relies on an error-prone polymerase, the data were uninformative at the 0.01% level for many SNVs – likely because these can arise during PCR. However, NGS with molecular barcodes made it possible to observe some variants in the 0.01% RMs above the background noise and showed a library incorporation efficiency of more than 30% for 50 ng of input DNA. Bioanalyzer analyses of the RMs and NGS libraries from the RMs showed a patient-like size distribution. External NGS testing resulted in comparable performance to patient samples with regard to metrics, although variants in the 0.01% VAF RM were essentially undetectable. **Conclusions:** Our next-generation RMs should provide additional content and features to enable the design, optimization, validation, and evaluation of next-generation ctDNA assays while retaining patient-like characteristics that are essential for this.

TT024. **Performance Assessment of the G4 Sequencer: A Novel Platform for Rapid and Flexible Next-Generation Sequencing**

T. Looney[1], N. Van Wietmarschen[2], L. LaMarca[2], K. Gouin[2], A. Decker[2], S. Shore[2], D. Witters[2], R. Shultzaberger[2], M. Fabani[2], E. Glezer[2]
[1]Singular Genomics, Austin, TX; [2]Singular Genomics, San Diego, CA.

Introduction: Next-generation sequencing has become a foundational tool for both biological research and *in-vitro* diagnostics, particularly in oncology, immunology, and detection of genetic disorders. Despite its success, there is a need for new DNA sequencing platforms that combine high accuracy, speed, and flexible throughput to provide timely results and cost-effective operations for research and clinical applications. Here we evaluate the performance of the novel Singular Genomics G4 platform for rapid sequencing-by-synthesis (SBS). We demonstrate its utility for whole-genome sequencing (WGS) and for bulk and single-cell RNA-Seq. **Methods:** The G4 platform utilizes novel SBS chemistry with optimized optics, fluidics, and image processing to deliver up to four 30x human whole genomes in less than 19 hours. The platform supports paired 2x150bp reads and dual indices for sample multiplexing. To maximize flexibility, up to four flow cells of two types (F2: 150M and F3: 300M reads) may be loaded at runtime. To assess performance, we prepared libraries for bulk RNA-Seq (Universal Human Reference, with External RNA Controls Consortium [ERCC] spike-in), scRNA-Seq (PBMC prepared via the 10x Chromium 3' scRNA-Seq kit), and human WGS (PCR-free preparation from NA12878). Sequencing was carried out using the G4 platform and standard reagents. **Results:** Bulk RNA-Seq of UHR libraries (2x100) yielded high-quality data, with ≥86% bases at Q30 for Reads 1 and 2. Transcript counts for technical replicates were highly correlated (R^2=0.99) as were ERCC observed versus expected counts (R^2 = 0.91). Moreover, transcript counts and genes detected were highly concordant to equivalent libraries sequenced on the Illumina NextSeq platform. Similarly, data from PBMC scRNA-Seq (28x91) showed high technical reproducibility (R^2=0.99) and high concordance to libraries sequenced on NovaSeq as determined by UMAP clustering and the estimated frequency of key immune cell populations. Finally, we achieved high-quality 2x150bp sequencing of NA12878, with ≥88% bases at Q30 for Reads 1 and 2, equivalent to single-pass read accuracies ≥99.8%. At 40x coverage, we achieved high precision and sensitivity across all variant types within high confidence regions, resulting in an SNP F1 of 99.4% and an indel F1 of 96.8%, as determined by hap.py. **Conclusions:** The G4 platform delivers high accuracy and technical reproducibility over a range of applications, with a faster turnaround than traditional reversible terminated nucleotide sequencing systems. Notably, the error profile of the G4 platform closely matches that of Illumina platforms, yielding highly correlated results for RNA expression profiling and WGS. We expect the rapid turnaround and flexible throughput will be especially relevant for future translational and clinical research.

TT025. **Reference Samples from Human Plasma-Derived cfDNA for Use in Organ Transplant Surveillance**

F. Raymond, C. Raymond
Ripple Biosolutions, Seattle, WA.

Introduction: The use of non-invasive methods to accurately detect allograft rejection has become more widespread over the last several years. One such approach is the analysis of cell-free DNA (cfDNA) in the plasma of a transplant recipient; cfDNA in these patients originates from both the recipient's native tissues and from the transplanted donor organ. These assays measure the allele fraction of the donor cfDNA as a percentage of that from the recipient. Several studies have demonstrated that an increase in donor-derived cfDNA correlates with acute graft rejection. The use of reference standards in these assays enables optimal and consistent clinical lab performance. Here we describe reference standards derived from human cfDNA for use in organ transplant surveillance. **Methods:** In this pilot study, reference standards were created from bulk cfDNA that was purified from approximately 800 mL plasma samples from each of two unrelated

human donors. Genotyping at 1,369 common SNP loci revealed 356 polymorphic sites where one sample had homozygous alleles and the other had heterozygous or opposite allele homozygous markers. These cfDNAs had the size distribution and amplifiable Alu content anticipated for high-quality human cfDNA. The cfDNAs were designated as either "background" or "spike-in" and were blended at seven different ratios. A portion of each blended sample was sequenced using targeted hybrid capture next-generation sequencing (NGS) followed by quantitative bioinformatic analysis of minor allele frequencies (MAFs). The observed MAFs were adjusted to account for heterozygous polymorphisms versus homozygous polymorphic markers and the aggregate data were used to calculate the average genomic contribution of the spiked-in samples. **Results:** Seven reference standards were produced. The mean spike-in percentages and 99% confidence intervals (CI) of the mean were 0.55 ± 0.03, 1.10 ± 0.07, 1.82 ± 0.13, 2.55 ± 0.21, 7.64 ± 0.58, 14.7 ± 0.7, and 25.4 ± 0.9. In all cases the coefficient of variance for the 99% CI was less than 10%. **Conclusions:** Coupling of large-scale cfDNA purification with quantitative NGS-based genotyping is a plausible and cost-effective method to produce high-quality reference standards that can be used to calibrate non-invasive transplant surveillance assays.

TT026. **Analyzing Pre-analytical Factors Impacting Saliva Liquid Biopsy cfDNA Recovery**

B. Salfer, L. Zhang, F. Li, D. Wong
University of California, Los Angeles (UCLA), Los Angeles, CA.

Introduction: There is abundant literature regarding plasma liquid biopsy and cell-free DNA (cfDNA) recovery, but saliva liquid biopsy is lacking research in many regards, including the impact of pre-analytical factors. For practical clinical use of saliva in cancer liquid biopsy detection, we must first understand the impacts of pre-analytical factors on cfDNA recovery. Different saliva collection devices are available, some including preservatives and/or some with filters. Another important pre-analytical factor is the stability of salivary cfDNA between sample collection and cfDNA extraction with different conditions. **Methods:** In this study, we tested the efficacy of three different saliva collection devices/procedures and the effect of adding different preservatives to collected saliva before processing and DNA extraction. We performed DNA extraction at different time points and storage temperatures to determine endogenous cfDNA degradation after saliva collection. For each experiment, saliva was collected from members in the lab and was pooled and vortexed. Triplicate aliquots of 0.5mL of saliva were made from the pooled saliva. Prior to cfDNA extraction, aliquots were centrifuged at 2,600g and 4°C for 15 minutes. The cfDNA was extracted upon initial collection and one, four, and seven days after collection using the Qiagen Circulating Nucleic Acid Kit, following the miRNA protocol. Extracted cfDNA was stored at -20°C until endogenous *EGFR* (exon 20) quantification was performed using ddPCR. Statistical analysis was performed using paired Student's t-test in Microsoft Excel where $p < 0.05$ was deemed significant. **Results:** Our initial recovery analyzing cfDNA between our SOP method (Falcon tube without preservative) and other collection methods after extraction on day 0 showed there was a 91% and 57% decrease in the recovery with Oasis saliva collection device and Novosanis' UAS preservative, respectively. No significant decrease was observed with EDTA and Streck Urine Preserve. The SOP method showed a steady decrease in endogenous *EGFR* targets recovered as time progressed, and only 2% was detectable after seven days. Surprisingly, EDTA at 5mM, 10mM, and 20mM concentrations remarkably increased cfDNA recovery. Streck Urine Preserve also increased cfDNA recovery by 114% after seven days compared with day 0. Novosanis' UAS preservative showed a 27% decrease in the recovery over seven days. **Conclusions:** We have concluded that the Oasis collector is not ideal for clinical usage because of the low cfDNA recovery compared to the SOP method. Additionally, the Spectrum device is not suitable because the included preservative lyses cells and the genomic DNA will contaminate cfDNA. We also have concluded that the UAS preservative best preserves cfDNA in our test; however, the initial reduction is concerning.

TT027. **A Single-Tube NGS Assay for Simultaneous Detection of DNA and RNA Biomarkers as a Comprehensive Solution for Clinical Management and Guiding Therapeutic Intervention of Ph-like ALL Patients**

L. Zelinger[1], A. Ghosal[1], F. Alarcon[1], S. Koo[1], S. Jung[1], B. Thomas[3], H. Nam[1], F. Lopez Diaz[3], F. Ye[3], V. Funari[1]
[1]NeoGenomics, Aliso Viejo, CA; [2]NeoGenomics, Houston, TX; [3]NeoGenomics, Carlsbad, CA.

Introduction: Accurate molecular characterization of acute lymphoblastic leukemia (ALL) subtypes is crucial for clinical management and guiding therapeutic intervention. More than 20 B-cell acute lymphoblastic leukemia (B-ALL) subtypes are defined by genetic variants and changes in gene expression. Upregulation of *CRLF2* correlates with poor prognosis in 25%-30% of Ph-like (PhL) ALL. Upregulation of *CRLF2* can be caused by gene fusion (e.g., *IGH-CRLF2*, *P2RY8-CRLF2* or *CSF2RA-CRLF2*) or mutations in *CRLF2*, *IKZF1*. Most panel-based assays screen either DNA or RNA, requiring higher input material and multiple workflows leading to higher cost and processing time. We report a single tube next-generation sequencing (NGS) panel that uses total nucleic acid as input for simultaneous screening of DNA and RNA, providing a comprehensive genetic profile for diagnosis, and therapeutic guidance of PhL ALL patients. **Methods:** Bone marrow (BM) and peripheral blood (PB) from ALL patients, healthy donors, synthetic controls, and cell lines with known fusions were used to establish sensitivity, specificity, accuracy, reproducibility, and repeatability. Libraries were prepared using a custom Qiagen Multimodal NGS panel targeting 297 DNA targets and 213 RNA fusions. Enriched amplicon libraries were sequenced with unique dual indices on Illumina NovaSeq 6000. Data was analyzed on in-house bioinformatic pipeline. Variants were confirmed by NGS assays (DNA), and Archer Panel/RT-Sanger assay (RNA). Expression values were normalized to *GUSB* and trained to account for variance seen in poor-quality samples. Baseline *CRLF2* expression was established using non-ALL samples, and qRT-PCR assay was developed for validation. After launch in a clinical setting, samples were segregated into PhL ALL or Ph+ ALL. For Ph+ ALL a CLIA validated qRT-PCR was used to detect *BCR:ABL1* fusion. **Results:** *CRLF2* was upregulated in PhL ALL cases compared to chronic lymphoblastic leukemia. Findings were confirmed by qRT-PCR with >95% concordance. In clinical data (n=139), *CRLF2* expression segregated Ph+ (*BCR:ABL1*) and PhL (*BCR:ABL1* neg and *CRLF2* upregulated) samples. Nineteen samples had *BCR:ABL1* fusion and upregulation of *CRLF2*. Eight samples were Ph neg but overexpressed *CRLF2,* which could be due to mutations/gene fusions of *IKZF1* detected in these samples. Samples overexpressing *CRLF2* also showed significant ($P < 0.01$) upregulation of *ARHGEF12*, *FOX04*, *ABL1*, *FLT3*, *MAFB*, *LMNA* genes and gene fusion events (*IGH-CRLF2*, *P2RY8-CRLF2*, *IKZF1-LINC00649*) and mutations (SNV and CNV in *IKZF1*). **Conclusions:** We demonstrate the use of a single-tube multimodal NGS assay for comprehensive genomics profiling that simultaneously screens DNA and RNA for expression and variants. It is a powerful and cost-effective tool to help classify PhL B-ALL subgroup for clinical management and guiding therapeutic intervention.

TT028. **Mosaic Structural Variation Detection with Optical Genome Mapping: Lower Limit of Detection Study**

N. Sahajpal[1], R. Kolhe[2], A. Mondal[1]
[1]Augusta University, Augusta, GA; [2]Augusta University, Martinez, GA.

Introduction: Optical genome mapping (OGM) has emerged as a next-generation cytogenomic technology that can detect all classes of SVs at a higher resolution than the SOC techniques. Recently, the technology has gained enormous traction and has been evaluated in several settings, including prenatal, postnatal, hematological neoplasms, and solid tumors. However, the literature lacks any report evaluating the lower limit of detection of the platform for these

applications. The present work aimed to evaluate the limit of detection (LoD) of the platform for both constitutional and somatic workflows. **Methods:** The OGM data can be processed using two pipelines, the *de novo* assembly pipeline, and the rare variant pipeline. The *de novo* assembly pipeline enables the detection of germline SVs and CNVs and is not designed for the detection of low mosaic events, whereas the rare variant pipeline (RVP) compares individual molecules to a reference assembly enabling the detection of low mosaic events. To estimate the LoD of the OGM platform, the samples were analyzed through both the *de novo* assembly pipeline and the rare variant pipeline with a genome coverage of ~160x for constitutional application, and with the RVP pipeline at ~400x for somatic application. The LoD was assessed for aneuploidy, translocation, interstitial deletion, and duplication. The LoD studies were performed for each variant class by diluting a sample with known variant and allele fraction (reported by SOC method) and diluting it with wild-type DNA to provide the following allele fractions: 25%, 16.6%, 12.5%, 10%, and 5%. The samples at 12.5%, 10%, and 5% allele fractions (theoretical LoD range) were run in triplicate to confirm the LoD. **Results:** For constitutional application, with the *de novo* genome assembly pipeline, aneuploidy was detected down to 10% allele fraction, translocation to 12.5% allele fraction, and interstitial deletion and duplication to 16.6% allele fractions. With the rare variant pipeline, the interstitial deletion and duplication were detected consistently from 25% down to 5% allele fraction, whereas translocation and aneuploidy were detected down to 10% allele fraction. For tumor analysis, the RVP pipeline detected all SV classes down to a 5% allele fraction. **Conclusions:** This is the first study to formally assess the LoD, and establishes the coverage and pipeline analysis required to achieve the low mosaic events down to a 5% allele fraction. This demonstrates the ability of the OGM technology to detect low mosaic events for both constitutional and somatic applications.

TT029. **Using Quality-Control Monitoring of COVID Testing to Drive Laboratory Operational Decision Making: Lessons Learned for Future Automation of Molecular Diagnostic Testing**

R. Henkhaus, H. Fehling
Clinical Reference Laboratory, Lenexa, KS.

Introduction: Quality-control monitoring has traditionally been an integral part of laboratory operations to ensure that accurate results are provided from the lab to the patient or referring physician. Although this remains a primary goal, practices adopted for molecular diagnostic (MDx) testing for SARS-CoV-2 have shown that QC monitoring can guide operational decisions by identifying impacts on turnaround time, report integrity, supply usage, and workflow management. Although monitoring and reviewing quality statistics are regulatory requirements, they can also stimulate and guide communication among departments that may not have conventional communication structures in place. Affected departments include instrumentation specialists, laboratory management, purchasing (supply management), facilities, and QC personnel. By establishing QC checkpoints along the MDx method workflow, problems can be caught early, and efficiencies can be optimized on an ongoing basis. **Methods:** The QC practices consist of monitoring for high-positive sample carryover on PCR plates, analyzing positive pool results, and tracking individual sample and plate failures. Statistical analysis is performed with Microsoft Excel and Power BI. **Results:** To detect carryover from samples with a high concentration of viral RNA (i.e., hot-well), positive samples adjacent to hot-well samples were repeated, with 23% of those samples reporting as negative. Statistical analysis led to an empirically determined cut-off value of $C_t \geq 30$ for repeat analysis of adjacent samples, because 98.3% of samples with $C_t < 30$ repeated as positive. Mapping the location of repeat testing showed that the wells most at risk for carryover were the wells within the same row, directly to the left or right of the hot-well. Analysis of the five-pooled sample workflow determined that pooling is operationally inefficient at a positivity rate >10%. Monitoring of failed plates and unamplified wells (i.e., drop-outs) can reveal significant problems with instrumentation. When the percentage of failed plates and drop-out wells for a particular instrument exceed what is typically observed (8.3% per plate and 2.7% per well), issues like channel failures, liquid handling errors, and influential factors such as ambient humidity can be identified and addressed. **Conclusions:** Integrating MDx QC monitoring practices into operational decision-making leads to positive outcomes including faster turnaround times, efficient supply management, better responsiveness in the use and maintenance of instruments, and more accurate reporting. Though these practices are not regulatory requirements, we have found them to be indispensable for the continued success of our high-throughput MDx laboratory. These principles are widely applicable to MDx methods performed in micro-titer plate-based assays.

TT030. **Analytical Validation of the *BCR-ABL* p190 Transcripts Using GeneXpert Dx System**

M. Elsayad, A. Azad, D. Fedchenko, D. Goldstein, C. Huang, H. Jin, R. Naeem, G. Wang
Montefiore Medical Center, Bronx, NY.

Introduction: *BCR-ABL1* p190 fusions are one of the oncogenic Philadelphia chromosomes (Ph) translocation products. The p190 "e1a2/e3a2" transcript is detected in approximately 50%-70% of adult acute lymphoblastic leukemia (ALL) and about 90% of childhood ALL. The GeneXpert BCR-ABL Ultra p190 RUO assay is an automated, cartridge-based assay for quantifying BCR-ABL1 p190 transcripts as a ratio of (BCR-ABL1 p190)/ABL1. The assay is performed on a single-use disposable cartridge that contains reaction reagents and host RT-PCR, nested PCR, and analyzed the results with pre-loaded software. The GeneXpert platform allows for workflow simplification, decreased technical performance complexity, and reduced hands-on time. Here we present initial validation of BCR-ABL p190 quantification on peripheral blood and bone marrow samples by this GeneXpert assay. **Methods:** We tested 20 clinical samples from 11 patients in addition to several replicates of titered control material. Clinical samples included 16 from peripheral blood and four samples from bone marrow. The clinical material was assayed by both the Xpert p190 assay as well as the laboratory test of record, the ipsogen BCR-ABL1 mbcr kit (Qiagen, US) on the ViiA7 platform. Performance was assessed by evaluating accuracy and precision inter- and intra-run reproducibility across all the various clinical and contrived sample types. Linearity was established by running serially diluted p190 control across 10 different concentrations. **Results:** Among the 20 clinical samples tested, 10 samples tested negative, and 10 samples tested positive by both methods. All control materials tested at all levels also demonstrated 100% concordance. Additionally, the two methods demonstrated excellent correlation (R^2=0.9982). Standard deviation of all control levels and clinical replicates showed adequate precision at all control levels. It should be noted that the Xpert p190 linear range spanned from 0.006% to 25% with some clinical samples lying outside the dynamic range. **Conclusions:** The Cepheid Xpert p190 RUO assay demonstrated excellent laboratory performance on both clinical and contrived materials as compared to the laboratory test of record. The simplicity of the assay also allows for decreased turnaround, less hands-on time, and fewer required technical skills which may enable increased laboratory efficiencies over conventional methods.

TT031. **Validation of COVIDSeq: An NGS-Based Assay to Identify SARS-CoV-2 Variants**

C. Rogers, C. Tang, L. Cowden, S. Herlihy, K. Rodino
Hospital of the University of Pennsylvania, Philadelphia, PA.

Introduction: SARS-CoV-2 was first detected in December 2019. As viruses replicate, they acquire mutations that can affect viral function and confer competitive advantages. SARS-CoV-2 genomic surveillance efforts benefit global data collection, inform public health policy, and provide insight into mutations that may evade diagnostics or vaccine efficacy. Our institution monitors SARS-CoV-2 variants in the Delaware Valley through an unbiased epidemiologic surveillance program; however, a more rapid and targeted workflow for variant identification was desired for infection control investigation and

diagnostic assay quality assurance. To address these needs, we validated a next-generation sequencing assay for SARS-CoV-2 genomic analysis. **Methods:** One hundred fifty-seven unique residual swab and saliva specimens confirmed to be SARS-CoV-2-positive or -negative by emergency use authorized PCR-based assays were used in this validation. Positive samples were previously sequenced by the Bushman Laboratory at the University of Pennsylvania as part of the Penn Medicine SARS-CoV-2 genomic surveillance initiative. These samples were then run on COVIDSeq to assess accuracy, inter- and intra-assay precision, analytical specificity, analytical sensitivity, and reference range. In addition, to evaluate the impact of two different SARS-CoV-2 variants present in a single sample, different ratios of 20B (B.1.1)- and 20I (B.1.1.7)-positive samples were mixed and sequenced. Only samples passing sequencing QC were analyzed. **Results:** COVIDSeq showed an overall concordance rate of 97% for positive samples and 100% for negative samples as compared to reference sequencing lineage calls. Specificity was 100%, as samples positive for common non-SARS-CoV-2 respiratory viruses showed no cross-reactivity. The limit of detection was approximately 200 viral copies/μL. SARS-CoV-2 was not detected in any of the 43 negative specimens confirming a result of "Not Detected" for the reference range. Samples used to assess precision showed low coefficients of variation (CVs) <1% for %non-N bases but were less precise for median coverage with CVs ranging from 4%-43%. Lastly, mixing studies demonstrated that only mutations present at >50% could be detected, meaning that only the dominant SARS-CoV-2 variant is reported by the assay. **Conclusions:** These data show that the performance of the assay is suitable for SARS-CoV-2 genomic sequencing in lineage determination. The assay is currently utilized for quality assurance in cases of diagnostic test result variability and more rapid institutional infection control investigations.

TT032. **The Use of Internal Controls in RNA-Based Next-Generation Sequencing Panels to Quantitate and Standardize Gene Fusion Detection**

B. Lo[1], J. Bosompem[1], B. Yeung[2], B. Austermiller[3], V. Chirip[1], T. Morrison[3]
[1]The Ottawa Hospital, Ottawa, Canada; [2]Ottawa Hospital Research Institute, Ottawa, Canada; [3]AccuGenomics, Wilmington, NC.

Introduction: Gene fusions and exon skipping variants are relevant biomarkers in treatment decision algorithms for non-small-cell lung cancer and can be detected using next-generation sequencing (NGS) approaches. RNA-based targeted NGS panels present significant advantages, especially in terms of analytical sensitivity; however, they are also challenging to standardize. We present here the use of Internal Standards (IS) added to patient samples that enable the calculation of transcript abundances and the determination of a limit of detection. **Methods:** SNAQ-SEQ ssRNA Internal Standards (IS) were designed for *EML4-ALK*, *MET*-exon 14 skipping (*MET*-EX14) and *TMP3-NTRK1* native templates (NT); these standards included unique base changes flanking the fusion for bioinformatic separation. The IS mixture (110 copies each) was added to three patient RNA samples positive for *ELM4-ALK*, *MET*-EX14, or *IRF2BP2-NTRK1* fusions, and an NTC, sequenced using the Oncomine Comprehensive Assay v3 panel (Thermo Fisher Scientific), with fusion reads extracted using a modified analysis workflow of Ion Reporter software (version 5.16). IS reads were used to determine if SNAQ-SEQ approach could provide a useful limit control and NT:IS ratio was used to demonstrate NT fusion abundance. **Results:** All IS fusions were detected in each sample; the *ELM4-ALK*, *MET*-EX14 NT reads were 10-fold greater. No *TMP3-NTRK1* NT reads indicate less than 110 starting copies. NT:IS ratio estimated *EML4-ALK* and *MET*-EX14 abundance as 7,800 and 16,000 copies, respectively. With one exception, SNAQ estimated all other native fusions had sub 1 copy abundance (median 0.7, range 0.003-0.5) which suggests these are background noise. *MET*-EX14 fusion in the *EML4-ALK* sample had 14 copies, similar to limit of detection levels found in other quantitative nucleic acid detection technologies. **Conclusions:** The samples containing low IS all had strong positive IS fusion read counts, indicating that ≥110 NT fusions should have been detected. The native *ELM4-ALK* and *MET*-EX14 fusions had >70-fold higher abundance than the IS spike-in. SNAQ-SEQ IS ability to provide standardized abundance measurement could eliminate the less accurate read-based thresholds, and instead allow NGS platforms to use established reporting range analytic validation like other quantitative RNA technologies.

TT033. **Analytical Validation of a 33-Gene Pan-Cancer Circulating Tumor DNA-Based Next-Generation Sequencing Assay**

A. Schneider[1], M. Campion[2], C. Hofich[2], D. Milosevic[2], J. Voss[2], B. Pitel[2], L. Tillmans[2], A. Ward[2], L. Peterson[2], T. Stier[2], J. Swank[2], J. Peng[2], K. Halling[2], B. Kipp[2], Z. Niu[2], G. Zheng[2]
[1]Mayo Clinic, Stewartville, MN; [2]Mayo Clinic, Rochester, MN.

Introduction: Circulating tumor DNA (ctDNA) enables non-invasive diagnostic assays for precision medicine, providing similar comprehensive molecular mutation profile information as formalin-fixed, paraffin-embedded (FFPE) tumor specimens. In this study, we validated a 33-gene pan-cancer ctDNA next-generation sequencing (NGS) assay using the Personal Genome Diagnostics (PGDx) elio plasma resolve assay. **Methods:** Forty clinical plasma samples, 10 healthy donor plasma samples, 20 contrived samples using DNA derived from FFPE tumor tissue, and three commercial reference materials were used in the study. To prepare contrived samples, FFPE DNA was fragmented to 150 bps in size to mimic ctDNA, and then spiked into normal donor plasma ctDNA to obtain desired variant allele frequencies (VAF). ctDNA was extracted from whole blood using the KingFisher Presto Nimbus automated platform. Genomic libraries were prepared using 10 to 40 ng of ctDNA with the PGDx elio plasma resolve kit (Baltimore, MD). The sequencing was performed on an Illumina NextSeq550. PGDx's machine-learning-based software performs alignment, variant calling, and filtering. The alterations detected include single nucleotide variants (SNVs), small deletions and insertions (indels), amplifications, translocations, and microsatellite instability (MSI) status. Detected DNA variants were compared to orthogonal NGS testing results, droplet digital PCR (ddPCR) for amplifications, or 2-Step PCR for SNVs and indels. **Results:** Both intra-run and inter-run reproducibility studies showed >99% concordance across different types of alterations. Limit of detection (LoD) for DNA input evaluated by using SeraCare ctDNA complete reference material was 10 ng. The assay showed an LoD of at least 0.5% VAF for hotspot SNVs, and 1.0% for non-hotspot SNVs; 2.5% for indels, 1.2- to 1.6-fold change for gene amplification depending on specific genes and the number of single nucleotide polymorphisms (SNPs) available to assess allelic imbalance, 2.0% for MSI status detection, and two supporting reads for fusion detection. Hotspot mutation reportable range was as low as 0.1% VAF with a specificity of 100% between 0.1% to 0.5% VAF. The sensitivity for SNVs and indels was 97% (97/100), specificity was 100% (123/123), and overall concordance was 99% (220/223). Concordance for gene amplification was 95% (20/21), 100% for translocations (17/17), 88% for MSI status (30/34), and 100% for MSI status on non-endometrial cancer cases (29/29). **Conclusions:** This 33-gene ctDNA NGS assay shows high reproducibility, accuracy, and sensitivity, which enable the assay to be applied clinically in cancer mutation profiling to aid clinical decision making for oncology patients.

TT034. **Isotachophoresis Extraction and Purification of Nucleic Acid from FFPE Samples**

R. Barney[1], W. Khan[1], L. Thompson[1], T. Gallagher[1], K. Hershberger[2], M. Morrisson[3], E. LaRochelle[3], G. Tsongalis[1]
[1]Dartmouth-Hitchcock Medical Center, Lebanon, NH; [2]Virginia Commonwealth University, Richmond, VA; [3]Dartmouth, Hanover, NH.

Introduction: Extraction of nucleic acids from formalin-fixed, paraffin-embedded (FFPE) tissue is commonly performed for various molecular pathology applications. Column-based extraction is a well-known workflow for FFPE tissue processing but remains time-consuming and requires a large amount of tissue input for downstream applications.

We have validated an approach that uses a novel charge-based extraction to isolate nucleic acids from FFPE tissues at scale. The process by which this occurs is known as isotachophoresis (ITP). ITP separates charged analytes based on ionic mobility. Here we demonstrate the utility of ITP extraction on FFPE tissue and compare it to column-based extractions. **Methods:** Nucleic acids (DNA and RNA) were extracted from FFPE tissue with a range of tumor content from 10% to 90%. More than 650 samples were extracted with ITP, and 60 were applied to various downstream molecular assays and analyzed against previously reported data. All samples had been previously extracted with the AllPrep (QIAGEN) column method. To test the robustness of the process, samples that were identified as insufficient quality in our current workflow were also assessed with ITP. ITP extractions were collected in a final elution volume of 50 µL. Quantification of nucleic acids was evaluated using Qubit fluorometric quantification (Thermo Fisher) and/or TapeStation automated electrophoresis (Agilent) to check the quality and size distributions of the extractions. **Results:** Compared to our standard column method, all ITP extractions performed comparably. The ITP DNA yields ranged from 6.1 to 12,000 nanograms (ng), whereas total RNA yields ranged from 200 to 7,100 ng. Samples processed in replicate, and from a spectrum of tumor contents (<20%, 20%-40%, 40%-60%, >60%), revealed reproducible findings. Previously reported small gene-sequence variants, copy number aberrations, and cycle threshold values from routine laboratory testing workflows were recapitulated**.** Of note, paired samples tested by column-based FFPE and ITP extractions for next-generation sequencing of a solid tumor panel (n = 170 genes) exhibited similar variant allele frequency outputs for the minor allele. These ranged from 0.376-0.528 for column-based extractions and 0.392-0.512 for ITP. The average sequence depth ranged from 677-1,212 and 657-4,910-fold coverage between spin column and ITP extractions, respectively. **Conclusions:** The ITP methodology has allowed us to multiplex up to 16 samples simultaneously in less than two hours. We have demonstrated similar results and quality parameters for FFPE-extracted samples relative to our current procedure with minimal hands-on time. This novel ITP-based extraction of FFPE tissue provides a streamlined approach to extracting nucleic acids from FFPE tissues.

TT035. **Developing a Copy Number Variation ddPCR Assay for FFPE Samples**

R. Barney, E. Hughes, G. Tsongalis, A. Sriharan
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: Droplet digital PCR (ddPCR) is a sensitive technique that provides absolute quantification of nucleic acids and can also be used to determine copy number variations (CNV). ddPCR determines the copy number of a target gene by using a reference gene locus that is expected to not be amplified or changed in any way. Due to this, it is important to choose a reference gene that is relatively stable within the genome, to provide accurate copy number calls of your gene of interest. This is especially important when interrogating CNV in degraded DNA such as that derived from formalin-fixed, paraffin-embedded (FFPE). Here we describe how we optimized a method for selecting optimal reference genes for use in a ddPCR CNV assay for small, dermatological cancer patient FFPE samples. **Methods:** Five target genes included *CDKN2A, RREB1, MYC*, *MYB*, and *CCND1*. A literature search revealed genomic locations of low copy number frequency in melanoma from which 10 reference genes were selected (Ref gene A-J). Patient samples were run on chromosomal microarray (CMA, OncoScan, Affymetrix) to find samples with no CNVs in any of the target and reference genes. DNA from up to six of these patient samples was then pooled and 10 ng of pooled DNA was added to individual primer/probe reaction mixes for each target/reference gene combination for ddPCR (Bio-Rad). **Results:** We found that despite CMA confirmation of diploid copy numbers (CN) for both target and reference genes in patient specimens, the data generated by ddPCR were variable for CN estimate for each target/reference gene pair, likely due to the degraded and impure nature of FFPE DNA. CN ranges varied by target with a range from 1.2 to 4.31 across all reference genes. But, importantly, from the 10 reference genes investigated, we found that at least four or five reference genes consistently resulted in target gene CN estimates close to the diploid number of 2; however, the identity of this group of reference genes was different for each target gene. For example, we found only two reference genes worked well for *CDKN2A, RREB1, MYC*, and *MYB* but not *CCND1*, whereas some other reference genes appeared to work well with only *CCND1*. The selected target/reference gene combinations were then further tested on individual patient specimens, also assessed by CMA, to check assay performance accuracy. **Conclusions:** In small FFPE samples, judicious selection of reference genes can significantly optimize CNV data obtained by ddPCR. CNVs can be detected in small FFPE samples using ddPCR. Replicating CMA copy number call in ddPCR depends on the specific reference genes used. To reduce artifact effects, pooling samples with no known CNV should be used to determine optimal target/reference gene pairs. Choosing multiple reference genes per targ*et* also minimize effects from reference gene mutations.

TT036. **Workflow Assessment for the Oncomine Myeloid Next-Generation Sequencing Panel**

B. Houde, K. Godwin, S. Deharvengt, D. Green, K. Winnick, G. Tsongalis, E. Loo
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Introduction: The WHO classification recognizes about seven myeloid-lineage hematologic neoplasm categories with more than 50 distinct entities, collectively referred to as myeloid neoplasms. The mutational profile underlying a particular myeloid neoplasm often has diagnostic, therapeutic, and/or prognostic implications. Sequencing characterization of these neoplasms has become the standard of care, resulting in a constant stream of cases to our molecular lab. Here we describe the workflow for the 40-gene DNA-panel portion of the Oncomine Myeloid Research Assay as a more automated mutation profiling alternative. **Methods:** We performed a workflow comparison between two myeloid gene sequencing panels deployed for clinical use in our laboratory. From 2015-2021, our laboratory used a non-automated 54-gene next-generation sequencing panel on the MiSeq platform. In 2021, we started using the Thermo Fisher Oncomine Myeloid assay using the Ion Chef and Gene Studio S5 systems, which include some automated steps. Sequencing was done on both platforms over a two- to three-day span, but technologist workflow differences were evaluated and are outlined below. **Results:** The sequencing panel initially used in our laboratory required significant hands-on technical time for library preparation, and normalization, library pooling, and sample loading. Library pools sequenced on the Illumina MiSeq also require a post-run wash, monthly maintenance, and monthly bleach washes. Total hands-on time accumulates to two working days, leading to difficulties in maintaining technologist proficiency and adequate staffing. Library preparation for the Oncomine Myeloid assay is fully automated. Library preparation, library templating, and chip loading are performed on the Ion Chef, whereas sequencing is performed on the Ion S5 system. The S5 requires a post-run wash that is performed by the instrument. Total technologist hands-on time for all steps accumulates to approximately 1.5 hours. Sequencing data are obtained faster and with a more consistent coverage with the Thermo Fisher assay. The MiSeq assay failures were at a coverage level. **Conclusions:** Automated sequencing systems such as the Ion Chef and S5 systems, utilized by the Oncomine Myeloid assay, assist in processing samples quickly and effectively. Greater system automation made it easier for technologists to maintain proficiency and facilitated additional staff coverage for the assay due to lower amount of hands-on time.

TT037. **Reduction of Artifact Chimeric NGS Reads in DNA Extracted from FFPE Tissue Using S1 Nuclease**

C. Artymiuk, S. Basu, J. Balan, X. Wu, M. Dina, R. He, D. Viswanatha
Mayo Clinic, Rochester, MN.

Introduction: A chimeric read in next-generation sequencing (NGS) is a read that aligns to two distinct portions of the genome without large overlap. Chimeric reads are normally caused by structural variants (SVs). However, artifact chimeric reads (ACRs) are produced by non-biological processes. One proposed mechanism of an ACR is through the binding of ssDNA fragments which are enriched in DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue (Haile, *et al.*, 2019). This artifact can be erroneously called as a mutation by NGS variant callers that rely on chimeric reads to identify true SVs. Therefore, we sought to reduce the prevalence of ACRs by digesting FFPE-derived ssDNA prior to library preparation using an S1 nuclease. **Methods:** Five FFPE samples were extracted for DNA using a lab-modified version of the Maxwell RSC DNA FFPE Kit (Promega). The DNA was split equally into two aliquots. One aliquot was treated with 10U of S1 nuclease (Thermo Fisher Scientific) for 30 mins ambient followed by column purification (Zymo). The other aliquot was left untreated. Both aliquots were prepared for NGS, sequenced on the Illumina NovaSeq 6000, and analyzed using an internal bioinformatics pipeline. Variant calling was performed using TNhaplotyper2 (Sentieon) for small SNV and indel variants and Manta (Chen, *et al.*, 2016) and Pindel (Ye, *et al.,* 2009) for SVs. ACRs were enumerated by the R script FilterFFPE using default arguments (Wei, *et al.,* 2021). **Results:** The number of total unique read pairs demonstrated an up to fourfold increase (1.6 to 3.9) in the S1 nuclease-treated samples. ACRs in the S1 nuclease-treated group were significantly reduced compared to the untreated group (0.2% ± 0.04%, range 0.1% to 0.2% vs. 1.6% ± 0.8%, range 1.2% to 2.9%, p=0.01). This improvement was also reflected by an 84% to 99% reduction in SV calls made by Manta and Pindel and 100% reduction of read-orientation biased calls made by TNhaplotyper2. **Conclusions:** Total DNA extracted from FFPE tissue contains variably abundant ssDNA that results in ACRs. Treatment of DNA with an S1 nuclease enzyme degrades ssDNA resulting in a reduction of chimeric dsDNA fragments during library preparation. The number of ACRs by NGS is dramatically reduced resulting in fewer errant variant calls by secondary pipeline analysis. Sequencing efficiency is further improved in the S1 nuclease-treated samples in part due to a more accurate quantitation and pooling of libraries that do not contain artificial chimeric dsDNA fragments. Clinical NGS testing using FFPE samples should consider the possibility for ssDNA ACRs, which can be mitigated using simple pre-library S1 nuclease DNA treatment, thereby significantly improving NGS quality.

TT038. **Extracting the Buffy Coat Layer of Hypocellular Bone Marrow Aspirates to Increase Nucleic Acid Yield in a Next-Generation Sequencing Hematologic Malignancy Panel**

B. Mahoney[1], K. Hampel[2], D. Gerrard[2], M. Cameron[2], E. Markworth[2], M. Lebeau[2], A. Ostafin[2], J. Shearer[2], N. Sidiropoulos[2], J. Conant[2]
[1]University of Vermont Medical Center, Essex Junction, VT; [2]University of Vermont Medical Center, Burlington, VT.

Introduction: Bone marrow aspirates are the “gold standard” for next-generation sequencing (NGS) detection of somatic gene alterations in hematologic malignancies, particularly myeloid neoplasms. However, a subset of aspirates are markedly hypocellular, yielding an insufficient amount of nucleic acid to proceed with testing. This results in cancellation of the test and necessitates the need to obtain an alternative sample, delaying clinical care. These bone marrows are frequently from acutely ill patients, in which making timely treatment decisions is imperative. Here, we report our initial results on a method to increase nucleic acid yield: extracting total nucleic acid (TNAc) from the buffy coat layer (BCL) of bone marrow aspirates. **Methods:** TNAc was extracted from nine hypocellular bone marrow aspirates collected in EDTA using the Qiagen QIAamp DNA Blood Mini Kit. BCL was obtained from an aliquot of each aspirate and TNAc was extracted in the same manner. Extracted DNA and RNA were quantified using Invitrogen’s Qubit Fluorometry assays. NGS libraries were produced using a custom hematologic malignancy panel, GenePanel Heme, designed to target up to 151 clinically relevant genes across a spectrum of hematologic malignancies. It leverages ArcherDx VariantPlex and FusionPlex methodologies to detect single nucleotide variants (SNVs), insertion/deletions (indels), and internal tandem duplications (ITDs) from DNA and fusions from RNA. Target-enriched libraries produced by GenePanel Heme were sequenced using an Illumina NextSeq550. FASTQ files were analyzed using the Archer Analysis platform, then uploaded to PierianDx’s Clinical Genomics Workspace software platform for variant calling and review. **Results:** The yield of DNA and RNA total mass increased by 282% and 58%, respectively, in BCL extraction compared to the corresponding bone marrow aspirate. We observed 100% concordance with respect to variant calling. The cohort of samples analyzed to date contains 39 DNA variants (32 SNVs, six indels, and one *FLT3*-ITD). The concordance in variant allele frequency (VAF) for each variant between the bone marrow aspirate and BCL samples was high (y= 1.0094x + 0.1948; R^2 = 0.9913). **Conclusions:** The data indicate that extracting TNAc from the BCL of hypocellular bone marrow aspirates increases nucleic acid yield and produces comparable high-quality sequencing results to standard aspirates, with no significant difference in variant calls or VAF. This proof-of-concept demonstrates BCL can be used as an alternative specimen option for NGS-based hematologic assays. Using BCL for hypocellular aspirates can reduce the number of canceled tests due to suboptimal nucleic acid quantity and minimize delay in clinical care.

TT039. **Innovative Method for Calibration and Quantification of Cell-Free DNA Measurements via Next-Generation Sequencing**

D. Hoerres, Q. Dai, S. Elmore, S. Sheth, G. Gupta, S. Kumar, M. Gulley
University of North Carolina School of Medicine, Chapel Hill, NC.

Introduction: Monitoring disease burden depends on accurate quantification of disease markers. Next-generation sequencing (NGS) technology is increasingly used for non-invasive monitoring, but plasma cell-free DNA (cfDNA) levels are often reported in misleading, relative units of measurement (e.g., allele fraction) that are confounded by non-disease factors. Absolute quantification of targets in "copies per mL" via droplet digital PCR (ddPCR) overcomes this problem; however, ddPCR assays are limited in scale and scope compared to NGS. To address this, we developed a novel strategy to report NGS values in "copies per mL" using synthetic oligonucleotide spikes and a normalization strategy alongside an assay calibration method to report absolute concentrations of target cfDNA targets by NGS, using the Epstein Barr virus (EBV) genome as a model target. **Methods:** In this study we refined our NGS protocol to calculate absolute analyte concentrations by first adjusting read counts for assay efficiency, as judged by recovery of synthetic normalizer DNAs spiked into plasma, and then calibrating NGS values against ddPCR. As a model system, we developed two ddPCR assays to measure highly conserved segments within the EBV genome: *BALF4* and *BMRF1*. In patient plasma samples (n=12) and mock plasmas (n=12), NGS and the two ddPCRs were applied to measure EBV load. For NGS results, EBV viral load was calculated two ways: in copies/million human reads, and in relative copies/mL of plasma after normalizing for assay efficiency. Relative NGS values were then converted to absolute values ("copies/mL") using the linear relationship between NGS and ddPCR values to calibrate NGS concentrations. **Results:** The two ddPCR assays yielded EBV loads that were strongly correlated with each other across a wide range of EBV loads (R^2=0.99). NGS was equally sensitive as ddPCR, with improved linearity when NGS values were normalized for spiked DNA read counts (R^2=0.95 for normalized vs. 0.91 for raw read concentrations). Linearity permitted NGS values to be calibrated against each of the ddPCR assays, achieving equivalent concentrations in copies per mL of plasma (R^2=0.95 *BMRF1*, 0.96 *BALF4*). **Conclusions:** We propose a novel strategy for calibrating

NGS assays using spiked oligonucleotide normalizers to improve precision of measurement, and calibration against ddPCR to optimize accuracy of analyte concentrations reported in "copies per mL." We also demonstrate NGS sensitivity and linearity are comparable to ddPCR across a wide dynamic range. This innovation aims to overcome biologic and pre-analytic variables hindering traditional NGS strategies for quantifying and monitoring disease burden.

TT040. **Utilizing Data from Fusion Transcript Analysis by Massively Parallel Sequencing beyond Validated Gene Fusion Reporting**

A. Good[1], S. Priore[2], J. Roth[2], G. Yang[2]
[1]University of Pennsylvania, Springfield, PA; [2]University of Pennsylvania, Philadelphia, PA.

Introduction: Fusion transcript analysis by massively parallel sequencing (MPS) detects gene fusions and novel isoforms with single nucleotide resolution. Although the reporting of these specific findings encompasses the validated usage of this methodology in our clinical laboratory, further interrogation of fusion transcript sequencing data may provide additional diagnostic benefit. Here we present a series of cases from our institution that highlight our experience in using data from a fusion transcript panel beyond detection of gene fusions, including to rule out contamination, exclude low-level variants as likely artifacts, and suggest additional testing to investigate nonspecific fusion variant calls. **Methods:** Select cases from our clinical oncology MPS laboratory were chosen based on the use of data from a fusion transcript panel beyond the reporting of gene fusions. Each case was reviewed to evaluate the impact on variant reporting or the recommendation for additional testing. Immunohistochemical studies were performed in one case to further investigate atypical variant calls in the fusion panel. **Results:** Four cases were selected to highlight scenarios in which analysis of fusion transcript data provided benefit beyond detection of fusion transcripts. In one case, concern that an *EGFR* deletion detected on a gene sequencing panel could be caused by sample contamination was mitigated by detecting the same *EGFR* deletion in the concurrent fusion transcript panel, which allowed for confident variant reporting. In two cases, an oncogenic isoform was detected on the fusion panel but at levels significantly below our cut-off for reporting. Closer examination of the data demonstrated numerous nonspecific splicing and fusion variants, a phenomenon we repeatedly see when a gene is overexpressed at high levels due to copy number gain, and thus the variant was not reported. Lastly, a lung cancer case demonstrated numerous nonspecific *CALCA* fusion transcript calls, suggesting the unexpected overexpression of calcitonin in the patient's tumor. Comparison of *CALCA* RNA levels across samples on the sequencing run suggested a marked overexpression in the specimen. Immunohistochemical stains for calcitonin were performed to investigate this finding. **Conclusions:** Data generated from fusion transcript panels contain a wealth of information that can provide insights beyond detecting gene fusions. These cases highlight scenarios in which additional analysis provided clinical benefit by increasing confidence in variant reporting or uncovering unusual variant patterns with suggested follow-up testing. Thus, investigation of orthogonal data proves useful for a variety of clinical benefits and is applicable across many different laboratory assays.

TT041. **Spatial Transcriptomic Analysis of FFPE Sections Mounted on Standard Glass Slides**

N. Mikhaiel[1], H. Singh[2], A. Santiago[2], L. Gutgesell[2], A. Patel[2], D. Sukovich[2], A. Tentori[2]
[1]10x Genomics, Dublin, CA; [2]10x Genomics, Pleasanton, CA.

Introduction: Visium CytAssist is a compact, benchtop instrument that allows automated transfer of analytes from pre-mounted tissue sections on standard slides to the Visium Gene Expression slide, enabling flexibility in profiling a broad spectrum of samples. Using standard histology methods, tissue sections can be reviewed based on the section integrity, morphology, or pathological landmarks of interest before selecting target regions for analyte transfer. **Methods:** Different types of formalin-fixed, paraffin-embedded (FFPE) tissues were leveraged to demonstrate the accuracy and precision of analyte transfer on the Visium CytAssist. Hematoxylin and eosin (H&E) or immunofluorescently stained tissue sections were imaged to select the region of interest to be targeted for whole-transcriptome analysis. Following decrosslinking and probe hybridization, the samples were prepared for analyte transfer. The alignment guides within the instrument were used to target different regions of the tissue to be transferred to the Visium slides with 6.5 x 6.5 mm or 11 x 11 mm capture areas. The captured analytes were used in the downstream genomics workflow to generate sequencing-ready libraries from each tissue section. **Results:** Two FFPE sections from the human lymph nodes with reactive follicular hyperplasia, H&E stained ~6 months ago, and stored at room temperature showed clusters of Ki67 antigen+ cells in the lymph follicles. In the same section, ligated paired probes transferred using Visium CytAssist detected on average 2,718 genes/spot at the sequencing depth of 10k reads per spot. Ki67 RNA expression correlated with the Ki67 protein expression. Unsupervised clustering using transcriptomes segregated Ki67+ cells in the lymph follicles into a distinct cluster, suggesting that Visium CytAssist enabled accurate capture of the biological cell states in the archived sections. In another scenario, transcriptome analysis from recently placed two FFPE sections from a glioblastoma sample identified a large mass of cells expressing high levels of oncogenes *MYCC*, *MYCN,* and cell cycle-related genes *Ki67* and *CCNB1*. In addition, Visium CytAssist enabled identification of a smaller mass of cells which expressed high levels of *MYCC* and weak Ki67. Both tumors segregated as distinct clusters; however, the bigger tumor contained a small population of cells that clustered with the cells of the smaller tumor, indicative of cells which are precursors to the smaller tumor. **Conclusions:** The data highlight that Visium CytAssist can retrieve transcriptome information from archived FFPE sections and accurately identify molecular changes in a spatial context. Visium CytAssist expands sample access to archived tissue sections, tissue block or region screening, and flexible tissue placement for gaining broader spatial insights.

TT042. **Evaluation of the Effect of Select Pre-analytic Variables for Cell Blocks and Needle Core Biopsy on Next-Generation Sequencing DNA and RNA Quality Metrics**

K. Behling, H. Gungor, K. Hunter, G. Zhu, T. Edmonston
Cooper University Hospital, Camden, NJ.

Introduction: Cell blocks (CB) and small-needle core biopsies (SNCB) may be the only available specimens for next-generation sequencing (NGS) in patients with advanced malignancies. These specimens may undergo extended fixation prior to embedding due to procurement at satellite locations. They also may be contaminated with heme-containing blood, a known PCR inhibitor. Here, we examined the effects of fixation time and presence of blood on NGS DNA/RNA quality metrics. **Methods:** Fixation times, DNA/RNA library preparation yields for the TST170 assay (Illumina, San Diego, CA), insert sizes, and NGS quality measures for 102 specimens, including 36 SNCBs and 66 CBs, were analyzed. Presence of more than 25% blood was estimated on flanking hematoxylin and eosin stained slides. Statistical analyses were performed using one-way Anova and Independent T-tests, and statistical significance was set at $p < 0.05$. **Results:** Fixation times for both CBs and SNCBs ranged between 1 and 4+ days depending on the day of week and time that specimens were received. Most specimens with delayed processing were held in formalin and a smaller number was held in Cytolyte but details about fixation prior to embedding could not be ascertained retrospectively. Longer fixation times resulted in statistically significant lower DNA and RNA insert size in both CBs and SNCBs and RNA library preparation yield in SNCBs. Increased fixation time was also associated with statistically significant poorer RNA quality metrics for NGS (CB and SNCB: percent mapped reads; SNCB: decreased total and unique reads) and poorer DNA quality metrics (SNCB: decreased percent mapped and unique on target reads). In SNCBs, presence of blood was associated with statistically significant poorer DNA (lower total reads and average

coverage) and RNA (lower unique reads) quality metrics whereas the opposite was seen in CBs where presence of blood was associated with statistically significant larger DNA/RNA insert sizes and better RNA (higher total reads) and DNA (higher total and percent unique on target reads) quality metrics. For both CBs and SNCBs, most quality parameters were within the reference range except for RNA total reads and RNA percent mapped reads for specimens with fixation of 4+ days and RNA percent mapped reads for specimens with >25% blood (not statistically significant). **Conclusions:** Pre-analytic variables including time in formalin and cytolyte fixative and presence of >25% blood may affect DNA and RNA NGS quality metrics and should be considered, both when choosing specimens and setting parameters for CB and SNCB specimen processing. Despite this, most specimens will still pass quality control (QC) but fixation of 4+ days and presence of >25% blood might affect RNA quality and result in QC failure.

TT043. **Reverse Transcription Cycling Achieves Greater Than 100% cDNA Conversion with Implications for Ultra-High Sensitivity of Clinically Actionable, Oncogenic RNA Mutations**

M. Church, L. Chen, S. Gokul, G. Latham, R. Rinehart, S. Sadic
Asuragen, a Bio-Techne Brand, Austin, TX.

Introduction: RNA biomarkers are present at low copy numbers in many specimens, especially liquid biopsies and formalin-fixed, paraffin-embedded (FFPE) samples. Although RT-PCR is the method of choice for RNA detection, particularly for clinical applications, reverse transcription (RT) is a stoichiometric process. As a result, low-copy RNA templates have "one shot" to be commuted into complementary DNA (cDNA). If this conversion is incomplete, then information about the RNA molecule is irrevocably lost. To address this limitation, we developed a novel technology that combines single-enzyme RT-PCR with high-temperature RT that preserves RNA intactness and cycles cDNA synthesis. The ability to generate multiple copies of cDNA from a single copy of RNA offers "multiple shots on target" to both capture RNA-level information and amplify it. Here, we describe this RT cycling method, which relies on optimized buffer and cycling conditions and thermostable RNA-dependent DNA polymerases. **Methods:** We evaluated multiple thermostable polymerases, including HawkZ05 Fast polymerase (Roche), for their ability to amplify RNA targets derived from cell lines, synthetic materials and non-small cell lung cancer FFPE biopsies. RT cycling reactions were performed using mixtures of *MET* exon 14 skipped mutant RNA in wild-type sample RNA with a single gene-specific primer. Digital PCR (QX200, Bio-Rad) was used to calculate the number of cDNA copies across cycle numbers. **Results:** We consistently observed greater than 100% cDNA synthesis after optimizing the choice of polymerase, buffer, and cycling regime. For example, *MET* exon 14 cDNA yield increased linearly with cycle number up to 20 cycles, resulting in 15-fold amplification. Beyond 20 cycles, additional amplification occurred but linearity was lost. In addition, the variant fraction was preserved up to at least 20 RT cycles in low-quality FFPE RNA using mutant inputs ranging from 280 pg to 5.6 ng. Studies are underway to characterize RNA variant detection in liquid biopsies. **Conclusions:** This study demonstrates that optimized reagent and cycling conditions both maintain RNA intactness and enable linear amplification over multiple rounds of cDNA synthesis even when using low-quality FFPE RNA. This innovative approach is well suited for RNA targets with low copy number or poor RT conversion efficiency. The method has potential to improve the analytical sensitivity of RNA detection for many applications, including the quantification of clinically actionable mutations and monitoring of resistance in oncology.

TT044. **Molecular Testing Opportunities on Cytology Effusion Specimens: The Pre-analytic Effects of Various Body Fluid Cytology Preparation Methods on RNA Extraction Quality and Targeted Sequencing**

G. Sura[1], K. Tran[2], C. Fu[2], L. Du[2], M. Marczyk[3], W. Symmans[2]
[1]Houston Methodist, Houston, TX; [2]The University of Texas MD Anderson Cancer Center, Houston, TX; [3]Silesian University of Technology, Gliwice, Poland.

Introduction: RNA sequencing (RNA-Seq) analysis is emerging as a clinical diagnostic method for targeted gene expression signatures. Laboratories typically default to molecular testing on formalin-fixed, paraffin-embedded (FFPE) samples. RNA-Seq-based gene expression profiles are relatively new to the clinical laboratory. We studied the pre-analytic effects of different cytologic preparations of malignant effusions from patients with metastatic breast cancer on extracted RNA quality and performance on targeted RNA-Seq. Our aim was to make initial steps toward developing standard operating procedures for RNA analysis from cytology preparations other than FFPE cell block. **Methods:** We compared RNA extraction methods (Norgen and PicoPure purification kits) and performance on targeted RNA-Seq indexes of 13 malignant effusion specimens among six cytologic preparations (FFPE cell block, cytospin fixed in Carnoy's solution and Papanicolou stained, cytospin fixed in 95% ethanol (EtOH) and Papanicolou stained, cytospin air-dried and Diff-Quik stained, and liquid-based preparations of ThinPrep (Hologic) and SurePath (BD Diagnostics) to optimally prepared fresh frozen (FF) high-quality RNA samples stored in an RNA preservative. **Results:** The highest average quality of extracted RNA was found using the PicoPure method, which overall demonstrated a 24% higher fraction of RNA fragments that are 200 bases or more. Cytospins fixed in Carnoy's solution and in 95% EtOH resulted in the highest-quality RNA, with 59% and 51%, respectively, of RNA molecules exceeding 1,000 bases in length. On RNA-Seq analysis, cytospin slides using Carnoy's solution, 95% EtOH, and Diff-Quik were comparable in terms of gene concordance (CCC=0.829, 0.812, 0.760, respectively) to FF samples, whereas ThinPrep and FFPE had lower concordance (CCC=0.736 and 0.564). SurePath samples failed quality-control metrics and were not included in the analysis. **Conclusions:** Cytology effusion specimens can be a source of high-quality material for RNA extraction and targeted sequencing. Our study results support the use of the PicoPure RNA purification method using cytospin slides fixed in high concentration ethanol preservative and Papanicolou stained as an alternative to the FFPE cell block. This study serves as a benchmark and first step towards the development of evidence-based standard operating procedures for clinical-level implementation of targeted RNA-Seq analysis on routine cytology and for future optimization and standardization of cytology fluid samples for molecular testing.

TT045. **Validation of a Targeted RNA-Based Next-Generation Sequencing Assay for Gene Fusion Detection**

T. Ma[1], J. Liu[2], S. Li[2], T. Gao[2], Z. Tan[2], X. Liu[3], X. Wang[3], C. Wang[3]
[1]Genetron Health (Beijing) Technology, Co. Ltd., Beijing, People's Republic of China; [2]Peking University Cancer Hospital and Institute, Beijing, People's Republic of China; [3]Transmedicine, Genetron Health, Beijing, People's Republic of China.

Introduction: Due to overlapping histological features and limited clinical biopsy specimens, accurate diagnosis of sarcomas has been a great challenge. Gene fusions are driver mutations in soft tissue sarcomas, and identification of translocations has substantially contributed to precision diagnosis of sarcoma. Here, we developed and validated a targeted RNA-based next-generation sequencing assay (FusionCapture) which covers 395 fusion-associated genes to achieve comprehensive fusion detection in sarcoma. **Methods:** Seraseq FFPE Tumor Fusion RNA v4/Seraseq Fusion RNA Mix v4 with 18 known gene fusions and two cell lines H2228 (*ALK-PTPN3* and *EML4-ALK*), and Reh (*ETV6-RUNX1*, *RUNX1-PRDM7*) were used to analyze the limit of detection (LOD). The repeatability and reproducibility were measured using three fusion-positive clinical sarcoma samples.

Results: The LOD of sequencing data was firstly evaluated under different sequencing data size (10, 8, 6, 4, and 2G) using Seraseq FFPE Tumor Fusion RNA v4. All 17 fusions, except for *PAX8-PPARG1* fusion, which is not covered by FusionCapture panel, were detectable accurately even as low as 2G with a minimum of 14 supporting reads, and the number of supporting reads was linearly correlated with the sequencing data size. In addition, a series of dilutions (*EML4-ALK*, from 2,000 copies to 200 copies) of Fusion RNA Mix v4 with RNA isolated from negative cell line GM24385N was performed to evaluate the effect of RNA input on fusion detection. The *EML4-ALK* fusion was identified in all replicates across all dilutions; however, the number of reads did not reach the positive threshold at 200 copies/200ng, so the LOD was defined as 400 copies/200ng. To determine how tumor cell content affects fusion detection, cell line H2228 and Reh were sequentially mixed to generate gradient of tumor cell content (100%:0%, 90%:10%, 80%:20%, 60%:40%, 40%:60%, 20%:80%, 10%:90%, and 0%:100%). All fusions were discovered in all replicates across all tumor cell content, and the number of supporting reads was highly correlated with the cell line content. To verify the repeatability and reproducibility of FusionCapture, three replicates by the same technician or three independent technicians were performed in three fusion-positive clinical sarcoma samples. All known fusions were uniformly detected across the technical replicates, and the CV values were 18.6, 2.5, and 2.5, respectively. Similarly, an overall reproducibility of 100% with CV value below 20 (17.5, 13.1, 6.7) was observed across three independent technicians. **Conclusions:** FusionCapture panel demonstrates excellent analytic performance, providing evidence for potential clinical application in identifying gene fusions in sarcoma.

TT046. **Improving the Success Rate of a Challenging RNA-Based Next-Generation Sequencing (NGS) Fusion Assay for Formalin-Fixed, Paraffin-Embedded (FFPE) Tissue**

K. Champion[1], B. Lemoine[2], K. Alderete[2], J. Graham[2], S. Saquib[2], A. Tigh[2]
[1]Quest Diagnostics, Irving, TX; [2]Quest Diagnostics, Lewisville, TX.

Introduction: Gene fusions are genetic alterations that occur when parts of two different genes are combined to form a new gene encoding for a chimeric protein. A subset of known fusions has therapeutic significance for the treatment of cancers. Our laboratory currently performs FusionSEQ, an RNA-based next-generation sequencing (NGS) assay that can detect gene fusions from formalin-fixed, paraffin-embedded (FFPE) tissue. Working with RNA from FFPE specimens is challenging because they tend to degrade. Consequently, higher rates of specimen repeats and assay failures than are desirable are often observed. In addition, testing may not always be repeated because many specimens arrive with a limited amount of tissue. In our molecular diagnostic laboratory from May 2021 to April 2022, our assay failure rate was 12.5%, and the repeat rate was 11.6%. In the current study, our objective was to reduce assay failures and repeat rates. **Methods:** RNA was extracted from FFPE tissue using a modified protocol for Omega Bio-Tek's Mag-Bind FFPE DNA/RNA Kit, followed by the addition of an RNA purification step using the Promega's ProNex Size-Selective Purification System. Purified RNA was then processed following our normal library preparation and sequencing procedure. **Results:** A validation study was conducted using 13 clinical FFPE specimens previously tested by FusionSEQ and having enough RNA left for this experiment. Nine out of the 13 samples (69%) were shown to not be adversely affected by the additional purification, meaning that RNA recovery was greater than 90%, and PreSeq (pre-sequencing qPCR) cycle threshold (Ct) was within two cycles of the unpurified RNA. Four out of 13 samples (31%) benefited from the additional purification with RNA recovery greater than 90% and PreSeq Ct at least two cycles earlier than the unpurified RNA. The additional purification step was then implemented into the clinical assay procedure, and the assay failure and repeat rates were tracked for one month following implementation. The assay failure rate was 2.9% (a 76.8% reduction) of the total cases. The assay repeat rate was 6.6% (a 43.1% reduction) of the total cases. **Conclusions:** Implementing an RNA purification step into our RNA-based NGS Fusion assay on FFPE tissue improved the clinical success rate and reduced the repeat rate.

TT047. **Can Wastewater Surveillance Testing Be Performed with FDA EUA SARS-CoV-2 Assays on Automated Testing Platforms?**

S. Sathyanarayana[1], A. Robins[1], D. Toledo[2], P. Shannon[1], T. Gallagher[1], J. Hubbard[3], G. Tsongalis[1], I. Martin[1], J. Lefferts[1]
[1]Dartmouth Health, Lebanon, NH; [2]Broad Institute of MIT and Harvard, Cambridge, MA; [3]QualiTox Laboratories, Pittsburgh, PA.

Introduction: Wastewater-based disease surveillance is a promising approach for tracking SARS-CoV-2 spread in communities. However, conventional methods of wastewater surveillance testing involve time and labor-intense multi-step procedures. In this context, automated testing platforms initially developed for clinical diagnostic testing may offer a more streamlined method with rapid results. This proof-of-principle study aimed to evaluate two automated FDA EUA assays for wastewater surveillance SARS-CoV-2 testing. **Methods:** Twenty-four-hour composite primary influent wastewater samples were collected from a single municipal wastewater treatment facility. The samples were centrifuged for 30 minutes at 4,600xg and supernatant aliquots were frozen. Supernatant samples were tested on two automated testing platforms using FDA EUA assays: 1) the m2000 automated sample preparation and Realtime System (Abbott) using the Abbott-m sample preparation kit (samples tested immediately after centrifugation), and 2) the Alinity m System (Abbott) using SARS-CoV-2 Amp and control kits (samples tested after one freeze-thaw cycle) per the manufacturer's protocol. The AccuPlex SARS-CoV-2 Verification Panel (LGC SeraCare) including three concentrations of whole-genome SARS-CoV-2 viral-based reference material (three, four, and five log copies/mL) was used to generate calibration curve to convert Ct to log copies/mL. To assess specimen stability, we conducted triplicate testing of three additional specimens on both platforms after one freeze-thaw cycle, three freeze-thaw cycles, and 4°C storage for >24 hours. **Results:** Sixteen wastewater samples from December 2020 to January 2021 were selected that tested positive by laboratory-developed manual methods. SARS-CoV-2 viral RNA was successfully detected in 15/16 (93.7%) samples run on the m2000 with an average of 2.25 log copies/mL, whereas 16/16 (100%) samples tested positive on the Alinity with an average of 1.78 log copies/mL. Bland-Altman analysis showed a bias of -0.48 mean difference with an upper limit of 0.25 and a lower limit of -1.22. Specimen stability testing results were as follows (average values of triplicate testing on the Alinity and m2000, respectively): after one freeze-thaw cycle 2.50 log copies/mL and 2.34 log copies/mL; after 4°C storage for >24 hours 2.43 log copies/mL and 2.24 log copies/mL; and after three freeze-thaw cycles 2.15 log copies/mL and 1.60 log copies/mL. **Conclusions:** Our study demonstrates that FDA EUA assays on two automated platforms successfully detected SARS-CoV-2 RNA in centrifuged known positive wastewater samples. The two automated platforms showed concordant results for specimen stability testing with lower recovery after three freeze-thaw cycles. Further study is needed to assess the ability of these assays to perform quantitative testing in the context.

TT048. **Application of Targeted RNA-Based Next-Generation Sequencing in Sarcoma Reclassification**

X. Zhang[1], T. Ma[2], X. Wu[3], X. Liu[4], C. Wang[4]
[1]Liaoning Cancer Hospital and Institute, Shenyang, People's Republic of China; [2]Genetron Health (Beijing) Technology, Co. Ltd., Beijing, People's Republic of China; [3]Ross University School of Medicine, Basseterre, Saint Kitts; [4]Transmedicine, Genetron Health, Beijing, People's Republic of China.

Introduction: Sarcoma represents a heterogeneous group of solid tumors with high pathological similarity, resulting in difficulty to diagnose based on histology alone. Though the integration of molecular detection enables a more precise diagnosis in sarcoma

classification, the clinical application of molecular testing in the classification of sarcoma subtypes needs to be further confirmed. Herein, we evaluated the clinical power of RNA-based next-generation sequencing (NGS) in fusion gene detection to guide precise diagnosis of sarcoma. **Methods:** Tumor tissues from 831 sarcoma patients involving different subtypes were subjected to NGS by FusionCapture (Genetron Health, Beijing), which targets 395 fusion-associated genes in sarcoma. **Results:** In total, 317 patients were found to be fusion-positive after sequencing. According to the clinical pathological diagnosis results, these patients could be divided into two groups: definite subtype group (n = 270) and uncertain subtype group (n = 47). Based on the molecular test results, 18.3% (58/317) of the patients were reclassified to different subtypes. Specifically, in the definite subtype group, 11.5% (31/270) of patients were changed from the initial diagnosis, among which the most pronounced changes occurred in spindle cell tumor (n = 5). These spindle cell tumor patients could be remodified to myofibroblastic tumor (n = 2), Ewing's sarcoma (n = 1), clear cell sarcoma (n = 1) and undifferentiated round cell sarcoma (n = 1), reaching a correction rate of 100%, followed by the osteosarcoma (2/3, 66.7%) and perivascular epithelioid cell tumor (4/7, 57.1%). Comparatively, in the uncertain subtype group, 57.4% (27/47) of patients could be reclassified to a specific sarcoma subtype, including six cases to myofibroblastic tumor (*NAB2 -STAT6*, *EWSR1-ATF1*, *ETV6-NTRK3*, *FUS-CREB3L2*, *ALK-TPM3*), four cases to synovial sarcoma (*SS18-SSX1*), and so on. **Conclusions:** Targeted RNA sequencing can assist in the reclassification of sarcomas with high misdiagnosis rates to improve clinically diagnostic accuracy, which is crucial for treatment decision.

TT049. **Standardized Pre-analytical Stabilization of Human Saliva Prevents Genomic DNA Degradation and Allows for Detection of Rare Tumor Mutations Using dPCR**

F. Kaiser, A. Huxhold, D. Groelz
QIAGEN GmbH, Hilden, Germany.

Introduction: Saliva is an easy-to-use specimen that offers the possibility to examine a wide range of analytes. Collection of saliva samples is non-invasive and may even be performed at home without the involvement of medical personnel. Recent publications show that saliva samples can be used for detection of tumor-specific mutations in lung cancer or oral cancer patients. Additionally, digital PCR (dPCR) represents a promising technique for detection of rare mutations. **Methods:** Saliva was collected from apparently healthy, consented adult donors into PAXgene Saliva Collectors and 15 ml tubes without a stabilization reagent (n = 10). Samples were stored at room temperature or at 4°C. Genomic DNA (gDNA) was extracted from 200 µl of the stabilized and unstabilized saliva samples directly after collection and after one hour, two hours, three hours, four hours, one day, two days, three days, four days, seven days, and 14 days of storage using the QIAGEN QIAamp DNA Mini Kit. The gDNA samples were analyzed using qPCR for human DNA quantity and degradation status with the QIAGEN Investigator Quantiplex Pro RGQ Kit. DNA profile was determined with the Agilent 4200 TapeStation System using Genomic DNA ScreenTape analysis. Artificial DNA including known cancer mutations, such as non-small cell lung cancer mutations *EGFR* exon 19 mutation c.2235_2249del or *EGFR* L858R point mutation in exon 21 (c.2573T >G), was spiked in stabilized saliva samples at calculated mutation rates of 0.5%, 1.0%, 1.5%, and 2.0%. The QIAGEN QIAcuity Digital PCR System was used to detect the respective mutations and determine the mutation rate with the QIAGEN dPCR LNA Mutation Assays in 6k 24-well nanoplates. Experiments were performed according to "ISO 4307:2021-10 Molecular *in vitro* diagnostic examinations – Specifications for pre-examination processes for saliva – Isolated human DNA" to ensure reproducible and reliable results. **Results:** In unstabilized saliva samples, significant gDNA degradation was detected during the first four hours after collection and was continuously increasing. After one day, qPCR target amplification was impaired by severe DNA degradation in unstabilized samples. In contrast, gDNA yielded DNA integrity and consequently qPCR amplification remained stable in samples collected in the PAXgene Saliva Collector. In stabilized samples, spiked-in cancer mutations could be reliably detected by dPCR down to mutation rates of ≥0.5%. **Conclusions:** Storage of saliva samples requires stabilization directly after collection, as gDNA undergoes severe degradation when left unstabilized. More importantly, human saliva samples can be used to detect cancer mutations by dPCR.

TT050. **Novel Low-Bias Small RNA Library Preparation Method Increases Sensitivity of miRNA Detection**

D. Rodriguez, B. Sexton, S. Maguire, N. Guan, G. Naishadham, K. Pinet, R. Fuchs, G. Lohman, B. Langhorst, L. Apone, E. Dimalanta
New England Biolabs, Ipswich, MA.

Introduction: Small RNAs (sRNAs) are key regulators of a wide range of biological processes. A major class of sRNAs, microRNAs (miRNA), have potential as clinical biomarkers. Next-generation sequencing (NGS) has become a powerful tool for the detection and sequence characterization of miRNA and other sRNA. Most NGS library preparation methods for sRNAs rely on ligation of adaptors to the RNA. These methods are challenged by bias in ligation efficiency, which decreases sensitivity to some transcripts and favors others, producing a less accurate representation of the sRNA population in a sample. We have developed an improved sRNA library preparation method that is robust, sensitive, and low-bias. This method features a novel adaptor design, utilizes gel-free size-selection, and works on a wide range of RNA input. **Methods:** Utilizing our novel sRNA library preparation approach, we have generated libraries from both synthetic (miRXplore equimolar pool of 963 synthetic miRNAs) and biological RNA substrates (1 ng, 100 ng and 500 ng of human brain total RNA). For comparison, we made libraries with a commercially available sRNA library preparation method. The libraries were sequenced on Illumina's NextSeq 550 Instrument. Single reads (56 bp) were mapped to miRXplore sequences and the GRCh38 genome with GENCODE v38 annotations using Bowtie2 or STAR. **Results:** Libraries prepared with our sRNA library preparation method and using synthetic controls as input exhibited a strong correlation with the expected abundance levels, indicating a more accurate representation of sRNA abundance. When tested using human brain total RNA as input, our method results in high library yield, high transcript abundance correlation across input amounts and technical replicates, detects an increased number of miRNAs, and has minimal adaptor dimer. **Conclusions:** Our novel sRNA library preparation method works in a wide range of input RNA (1 ng-500 ng) without the need of gel size selection, making processing of samples quicker and more streamlined. Importantly, the method is low-bias, facilitating a more accurate representation of the sRNA population in a sample. We expect this high-sensitivity sRNA library preparation method to facilitate the discovery of sRNAs and improve abundance assessment of sRNAs in clinical and research settings.

TT051. **Development of a Targeted Hybridisation-Based NGS Workflow for Use with Cell-Free DNA**

G. Speight, L. Georgieva, C. Müller, J. Reid, L. Edhouse, J. Holdstock
OGT, Begbroke, United Kingdom.

Introduction: The capture and molecular analysis of cell-free DNA (cfDNA) released from cells under certain pathological conditions, including cancer, provide a non-invasive alternative to currently used practices via use of liquid biopsy samples. The rapid development of next-generation sequencing (NGS) technologies in recent years has led to a significant reduction in sequencing cost with improved accuracy. In liquid biopsy research, NGS can be applied to sequence circulating tumour DNA (ctDNA) and provide molecular characterisation of tumour-specific genomic alterations in DNA released by tumour cells, allowing for non-invasive and real-time monitoring of disease development. The aim of this study is to evaluate a modified version of the OGT Universal NGS Workflow Solution in conjunction with a custom SureSeq targeted panel for use with cfDNA. OGT's Universal Workflow offers a new streamlined hybridisation-based NGS library preparation protocol with reduced hands-on time

and use of unique molecular identifiers (UMIs). **Methods:** We utilised a modified version of the OGT Universal Workflow (comprising Universal Library preparation kit and Universal Hybridisation + Wash kit) together with a SureSeq myPanel Custom panel to assess whether this approach enables library generation and subsequent variant detection in cfDNA samples. We tested commercially available cfDNA standards (Multiplex I cfDNA Reference Standard 5%, Horizon Discovery, and Seraseq ctDNA Complete Reference Material AF 1%, SeraCare) with 10-50 ng DNA input. To mimic different variant frequencies, we made a serial dilution of the reference DNA to create samples with a frequency range of 0.5%-5%. Sequencing was conducted using NextSeq 500/550 (Illumina). Data were analysed using a modified version of OGT's proprietary Interpret software with use of UMIs. **Results:** We have successfully utilised a modified OGT Universal Workflow in combination with a SureSeq myPanel Custom panel to generate high-quality sequencing libraries from cfDNA input range of 10-50 ng. We have achieved high depth of coverage (>1,000x) and uniformity of coverage across the targeted regions in multiplexed samples, allowing for rare variant detection. High concordance of variants was observed with an accurate allele frequency estimation with the lower limit ranging from 0.5% for 50ng DNA input to 1% for 10ng DNA input. **Conclusions:** To detect low-frequency alleles which contribute only a small percentage of the reads at any locus, a highly uniform and sensitive enrichment is required. We have utilised a modified version of OGT Universal NGS Workflow Solution and associated custom panel to generate libraries and reliably detect somatic mutations down to 0.5% variant allele frequency. Our approach confirms OGT's capabilities in the area of cfDNA applications.

TT052. **Development of an NGS-Based Measurable Residual Disease Monitoring Capability Using Hybridisation-Based Target Enrichment**

G. Speight, L. Georgieva, N. Milner, J. Reid, V. Pullabhatla, L. Edhouse

OGT, Begbroke, United Kingdom.

Introduction: Improved methods of disease status monitoring with detection of low-level variants are essential to facilitate early therapeutic interventions and potentially prevent disease recurrence. At present, two different types of methods are used for detection of measurable residual disease (MRD): immunophenotypic based on multiparameter flow cytometry (MFC), and molecular methods which include real-time quantitative polymerase chain reaction (RQ-PCR), digital droplet PCR (ddPCR), or next-generation sequencing (NGS). Each of these methods differs in its applicability, specificity, and sensitivity of detecting MRD. NGS provides a solution allowing the evaluation of multiple genes in a single assay. Together with significant reduction in sequencing cost and improved accuracy, NGS can now be used in monitoring. In this study we aim to evaluate the OGT Universal Workflow together with a SureSeq Custom AML panel for suitability with deep sequencing and rare variant detection required for MRD monitoring. **Methods:** We have tested the OGT Universal NGS Workflow Solution in conjunction with SureSeq Custom AML panel for suitability of detecting variants present at very low frequency. SureSeq Custom AML panel is an 11kb panel that covers 50 hotspot exons in 22 genes relevant to acute myeloid leukaemia (AML). The workflow and the panel were tested using Myeloid Reference Standard (Horizon Discovery). This is a well-characterised control material containing a range of variant types (single nucleotide variants [SNVs], indels, and internal tandem duplications [ITDs]) and frequencies suitable for testing in an AML monitoring assay. To mimic different variant frequencies, we made a serial dilution of the reference DNA to create samples with a frequency range of 1.0%-0.1% variant allele frequency (VAF). Sequencing was conducted using NextSeq (Illumina). Data were analysed using a modified version of OGT's Interpret software. **Results:** We have successfully utilised the OGT Universal Workflow and have achieved a high depth of coverage and excellent uniformity in multiplexed samples permitting rare variant detection. In the initial experiments, suitability of the workflow was confirmed by accurate detection of all variants with frequencies of 1%-7% VAF, and a high concordance of observed versus expected allele frequencies was found. Subsequent experiments achieved a higher depth of coverage (>10,000x) allowing us to achieve 100% accuracy in detection of SNVs, indels and a 300bp ITD, within the range 0.1%-0.7% VAF. **Conclusions:** To detect very low frequency alleles a highly uniform and sensitive enrichment is required. We have utilised the OGT NGS Universal Workflow and SureSeq AML Custom panel to demonstrate reliable and accurate detection of variants down to 0.1% VAF. This provides researchers with the capability to use capture-based NGS technology for MRD monitoring.

TT053. **Converting Targeted Proteomics into Molecular Classification for Non-Small Cell Lung Cancers**

J. Koomen, S. Putty Reddy, A. Alontaga, P. Stewart, G. Nazario, E. Welsh, L. Saeed, L. Darville-Bowleg, B. Fang, S. Eschrich, E. Haura, T. Boyle

Moffitt Cancer Center, Tampa, FL.

Introduction: Mass spectrometry-based targeted proteomics offers unique capabilities in quantification of large biomarker panels, which can screen for different tumor drivers and establish their biological context by assessing different tumor phenotypes for molecular classification. To implement this technology effectively, protein biomarkers (and their ratios) need to be strongly correlated with phenotypes, and communication of these results to pathologists and oncologists must be optimized. Here, we describe navigation between biomarker measurements, integrated evaluation at the pathway level, and simplified scoring to make the results accessible. These steps will further establish unique tools for protein biomarker quantification in biopsies to prioritize treatment options with FDA-approved companion diagnostics, match patients to clinical trials, and measure clinical correlates to understand drug response and resistance. **Methods:** Liquid chromatography-multiple reaction monitoring mass spectrometry assays (n = 97) have been applied to different cohorts of non-small cell lung cancer (NSCLC) tumors, including both frozen and formalin-fixed, paraffin-embedded (FFPE) samples, which have been reviewed by a pathologist. Quantitative results are used to calculate biomarker expression levels (in amol/µg protein digest) as well as selected molar ratios. Data are integrated into pathways and scored to provide simplified metrics for tumor classification. **Results:** Protein quantification from 1 µg of total protein digest was successful for three cohorts: 1) a pilot project of FFPE bronchoscopic LUAD and LSCC biopsies (n = 30), 2) frozen LSCC tumors (n = 108), and 3) FFPE sections of primary and metastatic lesions recovered from patients consented to rapid tissue donation after autopsy. Detectability (%) and maximum-to-minimum ratios were used to assess the utility of each protein biomarker. Ratios were calculated to differentiate LUAD and LSCC, assess EMT, tumor glycolysis, and other phenotypes, which were connected to signatures from our previous proteogenomics project (Stewart, *et al.,* 2019). Data were also used to develop phenotypic scores that can be used for molecular classification and presented to enable navigation among quantitative data, pathways, and molecular classification. **Conclusions:** Targeted proteomics supports molecular classification of NSCLC tumors. These assays complement existing molecular pathology strategies by solving problems associated with quantifying numerous biomarkers using only limited biopsy material. Optimization of communication will improve the utility of targeted proteomics in clinical diagnostics and community uptake of this technique.

TT054. **Rapid Molecular Response Evaluation of *BCR::ABL1* Using a Single-Use Fully Automated Cartridge-Based System**

S. Sathyanarayana, W. Keegan, J. Barbuto, T. Wilson, M. Ells, I. Craven, G. Tsongalis, E. Loo
Dartmouth Health, Lebanon, NH.

Introduction: Chronic myeloid leukemia is a myeloproliferative neoplasm that arises from a translocation between chromosomes 9 and 22, resulting in a *BCR::ABL1* gene fusion. Reverse transcriptase (RT)-based PCR to monitor *BCR::ABL1* transcript levels is needed to guide clinical decision making. The Xpert BCR-ABL Ultra (Cepheid) is an automated cartridge-based assay with onboard sample preparation, RT-PCR, and fluorescent signal detection for the major p210 fusion transcript. We evaluated the performance qualities of this cartridge-based assay against a conventional TaqMan-based qPCR assay. **Methods:** Twenty positive and 20 negative patient peripheral blood samples were prospectively collected and stored at 2°C to 8°C, underwent lysate preparation within 72 hours, and then kept at -80°C until used in test evaluation. Samples were evaluated using the GeneXpert BCR-ABL Ultra (Cepheid) and our current clinically validated assay for *BCR::ABL1* p210, following standard manufacturer protocols. A commercially available quality-control panel, Xpert BCR-ABL IS Panel C130 (Maine Molecular Quality Controls, Inc.), was also tested in seven replicates for linearity, precision, and reproducibility. **Results:** All 20 positive calls on patient samples were concordant between assays, though three positive Cepheid tests were called below limit of detection (<0.0030% IS, >4.52 log-reduction, aka MR). One of 20 negative Cepheid calls was discordant, with the other assay calling a low-positive (MR 4.47). We noticed a mild increase in MR result differences at lower fusion transcript levels, with the Cepheid assay averaging 0.3 higher, about the expected MR 4.5, but with no difference at MR 3. Linearity with patient samples was comparable between assays (R^2= 0.9563, p <0.0001). Bland-Altman's analysis displayed a good correlation between the two methods with a mean difference bias of 0.42, with the upper limit of two standard deviations (SD) of 0.04 and a lower SD of 0.78. Linearity using the MMQCI IS C130 panel displayed excellent correlation between the expected and observed values (R^2=0.9903, p=0.0004). Precision and reproducibility between operators also showed an excellent correlation between the observed and expected values (R^2= 0.9957, p=0.0001). In terms of workflow, the Cepheid assay has a single-day turnaround of 2h 40min (~50min hands-on, 1h 45min machine time). Whereas our present assay needs ~2h 20min hands-on time per sample, it has an overall turnaround time of about a week due to batching. **Conclusions:** In conclusion, we observed slightly higher MR values with the Cepheid assay compared to our current TaqMan-based qPCR assay, but within acceptable range. The Xpert BCR-ABL Ultra assay showed similar performance to our current clinical assay, but had faster turnaround and less hands-on time in our lab.

TT055. **Measuring "Truth": Disparities in RNA Quantitation by Two-Step Reverse Transcription-Droplet Digital PCR (RT-ddPCR)**

S. Pelsue, M. Held, A. Karaczyn, J. Gordon, T. Spenlinhauer
Maine Molecular Quality Controls, Inc., Saco, ME.

Introduction: Digital PCR (dPCR) and droplet digital PCR (ddPCR) are established gold standard technologies for quantification of DNA, and their use in diagnostic assays is increasing. Quantification of RNA by ddPCR likewise offers an attractive approach for disease detection and monitoring, but is less defined in part due to unstandardized reverse transcription (RT) chemistries and inadequate availability of RNA reference standards. In this study, we performed ddPCR on complementary DNA (cDNA) generated from a synthetic *in vitro* transcript (IVT) using a variety of two-step RT kits and found significant differences when compared to IVT quantification by UV-spectroscopy (UV-spec). As the number of cDNA molecules produced in an RT reaction should correspond to the number of RNA copies estimated by UV-spec, the observed discordance between these methods introduces uncertainty as to whether RT-ddPCR can reliably quantify RNA. **Methods:** IVTs were generated, purified, quantified by UV-spec, and serially diluted to a concentration range between $1x10^3$ to $5x10^6$ cps/mL. IVTs were converted to cDNA using 12 different RT kits from nine companies, amplified using the same ddPCR kit and analyzed using the QX200 Droplet Digital PCR System (Bio-Rad). RT kits were compared for quantitative performance by RT-ddPCR and compared to UV-spec. Best performing RT kits were further tested for sensitivity and technical reproducibility. Statistical analyses were performed using JMP Pro, v.15.0.0. **Results:** A dozen RT kits were used to generate cDNAs of an ABL1 IVT and subsequent cDNA quantitations by ddPCR were found to vary considerably when compared to UV-spec of the IVT template. Quantitation by ddPCR of cDNAs derived from some RT kits deviated from UV measurement by more than threefold. Only five of 12 RT kits were within 20% of UV quantitation. Linear regression of the IVT dilution series for each kit showed R^2 >0.99. Reproducibility studies utilizing low levels of ABL1 IVT ($5x10^4$ cps/mL) tested with four RT kits most closely aligned with UV-spec showed %CV between 10%-20%. **Conclusions:** Despite evaluation of identical synthetic transcript, we found remarkable variability among RT kits, which warrants caution for the selection of suitable RT enzymes. Importantly, the general incongruence among RT kits highlights the variability inherent in measuring individual transcripts by RT-ddPCR. Greater consistency is likely to be gained by optimizing RT reaction chemistries specifically for ddPCR. Adoption of conciliatory approaches to integrate RT-ddPCR measurements for future clinical diagnostic and research applications may be necessary to determine absolute quantification.

TT056. **Collaborative Clinical Validation of a Rapid *TERT* Promoter Genotyping Assay**

G. Maron[1], N. Georgantas[2], J. Bradley[2], G. Shankar[2], L. Ritterhouse[2], J. Lennerz[2]
[1]Massachusetts General Hospital, Somerville, MA; [2]Massachusetts General Hospital, Boston, MA.

Introduction: Identification of variants in the telomerase reverse transcription (*TERT*) promoter gene plays a vital diagnostic role in classifying brain lesions and a prognostic role in predicting patient response to certain therapies with mutation status contributing to the integrated WHO diagnosis of glial neoplasms. Next-generation sequencing (NGS) is considered the gold standard for brain tumor genotyping; however, current turnaround times (TAT) are 10-14 days. Here, we outline the multidisciplinary clinical validation efforts between pathology, neuro-oncology and neurosurgery to translate a rapid *TERT* promoter assay into a laboratory developed test for use in a CLIA-certified laboratory. **Methods:** A *TERT* promoter genotyping assay was developed to detect the two most prevalent single-nucleotide variants in the *TERT* promoter region (*TERT* c.124C >T/C228T and *TERT* c.146C >T/C250T), utilizing principles of clamping wild-type allele amplification with PNA oligonucleotides and mutant site detection with LNA-based TaqMan probes. Diagnostic performance metrics were assessed in n=97 samples: n=46 formalin-fixed, paraffin-embedded (FFPE), and n=51 fresh frozen (FX) tumor tissue sections. Specificity was assessed in six FFPE and 11 FX known *TERT*-negative brain tumors. Diagnostic sensitivity and concordance between sample types was assessed in 20 *TERT* C250T and 20 C228T mutant brain tumors; positive specimens were tested in duplicate to assess intra-run reproducibility. Limit of detection (LOD) was determined by serial dilution of known positive samples with genomic DNA, which was performed twice to assess inter-run replicability. **Results:** Diagnostic specificity, sensitivity, and positive and negative predictive values were 100%. LOD by allele fraction was 0.47% in C228T-positive samples and 0.64% in C250T-positive samples. All technical replicates and repeats were reproducible (100% precision). Since launching this CLIA-certified test, n=66 brain tumor specimens have been analyzed. Comparison of 16 test results with *post-hoc* NGS testing showed 94% concordance; rapid *TERT* detected a mutant not observed on NGS. The average clinical TAT (from order to result) since launch is four days (range 0-10 days). **Conclusions:** Performance metrics of this rapid *TERT* assay are indicative of analytical validity. Rate of identification of *TERT* promoter mutations indicates clinical validity, and

in-practice concordance and clinical TAT metrics indicate clinical utility. In adopting the assay, we implemented standard operating procedures, competency, and proficiency assessments in accordance with CLIA. The multidisciplinary effort described here highlights the strength of combining translational research efforts with a clinical test integration program to drive innovative patient care.

TT057. **Spiking Synthetic SNPs for Specimen Tracking as a Quality Improvement Initiative**

B. Broadfoot, B. Drumheller, J. Lewis, Y. Ma, A. Ojo, R. Saade, T. Schneider, G. Smith, L. Zhang
Emory University, Little Rock, AR.

Introduction: Next-generation sequencing (NGS) workflows abstract quality concerns like sample identity and purity behind layers of technical complexity. A sample in the clinical laboratory and its bioinformatic outputs are only related by a file name. As a quality improvement initiative, we investigated redundant mechanisms for determining NGS sample identity and purity that rely on spiking patient samples with synthetic oligonucleotide sequences containing rare, clinically insignificant single-nucleotide polymorphisms (SNPs). The synthetics are introduced early in the wet bench workflow and survive subsequent wet and dry bench processing. The synthetic SNPs are detectable in the final bioinformatic outputs like any other SNP. This is a simple, cost-effective method for identifying patient samples and intra-assay contamination. **Methods:** We identified 46,301 candidate SNPs that met the following requirements: 1) covered by our commercial 500-gene DNA cancer panel, 2) located in an intron, 3) allele frequency (AF) was available in dbSNP, and 4) databases cited by dbSNP reported a reference AF of effectively 100%. Two SNPs in *NAB2* and *NTRK1* with good support were selected. 100-bp double-stranded DNA oligonucleotides containing the SNPs were spiked into two patient samples. *NAB2* (1.0×10^{-5} ng) was added to patient A and *NAB2* and *NTRK1* (5.0×10^{-6} ng each) were added to patient B. The *NTRK1* synthetic was polyacrylamide gel electrophoresis (PAGE)-purified. We inspected the SNP AFs in the variant call file (VCF) outputs of our bioinformatics pipeline. **Results:** At an optimal synthetic input level, our commercial bioinformatics pipeline ran to completion and reported normal quality metrics. The synthetic SNPs were detected in the VCFs of the spiked samples and demonstrated a dose-response relationship between input level and AF (1.0×10^{-5} ng SNP at 80% AF; 5.0×10^{-6} ng SNPs at 35% AF). The PAGE-purified and non-purified synthetics performed similarly. Clinically reported, spiked samples showed identical oncogenic variants with similar AFs (within <1%) to what was detected previously. **Conclusions:** Spiking NGS samples with synthetic SNPs is an effective method for verifying sample identity. The SNPs represent internal qualitative (sample identity verification) and quantitative (presence at expected AF) controls. Analytical interference is minimized by using clinically insignificant SNPs rarely encountered in the wild. We propose using this method with a repertoire of multiplexed SNPs to help track specimens from the wet lab to clinical reporting. We believe this method also has utility in the detection and quantification of intra-assay contamination, which is a major concern in NGS workflows where multiple samples are processed in parallel.

TT058. **Quantification of SARS-CoV-2 for Viral Sequencing Failure Reduction**

D. Green[1], S. Deharvengt[2], K. Winnick[2], P. Shannon[2], J. Sevigny[3], K. Thomas[3], M. Whitfield[4], G. Tsongalis[2], J. Lefferts[2]
[1]Dartmouth-Hitchcock Medical Center, Lebanon, NH; [2]Dartmouth Health, Lebanon, NH; [3]University of New Hampshire, Durham, NH; [4]Dartmouth College, Hanover, NH.

Introduction: Many clinical molecular laboratories have taken on roles in assisting public health efforts during the COVID-19 pandemic by using existing next-generation sequencing (NGS) infrastructure and access to SARS-CoV-2-positive patient samples to perform whole-genome viral sequencing to monitor viral lineage changes over time. During the Omicron wave of the SARS-CoV-2 pandemic, the rate of positive samples exceeded that which could be processed for sequencing. To maximize high-quality data output and reduce expenses, we reviewed the relationship between viral concentration and sequencing quality to create an evidence-based approach for predicting successful sequencing outcomes. **Methods:** PCR-based SARS-CoV-2 testing in a rural academic medical center was performed on the Alinity (Abbott) and m2000 (Abbott) automated testing platforms. As copy number values are specific to an instrument, these values were converted to log copies/ml utilizing the AccuPlex SARS-CoV-2 Verification Panel (LGC SeraCare) to create unique calibration curves with each system. Libraries were prepared for 2,062 residual samples using the ARTIC v3 SARS-CoV-2 kit (ARTIC network) and sequenced on the NovaSeq (Illumina). Lineage calling was preformed using the Pangolin analysis feature of the Illumina DRAGEN COVID Lineage App. A cut-off of 3.0 log copies/ml was established by comparing log copies/ml against the SARS-CoV-2 kmer fraction. After establishing the cut-off, 502 additional samples were sequenced. **Results:** Before implementation of a log copies/ml threshold, Pangolin calls were obtained in 78.3% of samples. After the 3.0 log copies/ml threshold was established the lineage call success rate rose to 88.9%. A chi-square test of independence showed a significant association in failure rate before and after the threshold, X^2 (1, N = 2,564) = 28.695, p = 8.474e-08. **Conclusions:** Establishing a log copy/ml threshold for SARS-CoV-2 sequencing by utilizing NGS post-sequencing quality-control metrics resulted in a significant improvement in ability to obtain lineage calls.

TT059. **Higher Total RNA Yields and Sequenceable RNA Is Attainable Using the nRichDX Revolution Sample Prep System Total Nucleic Acid Isolation Kit**

M. Saidian, J. uriu, J. Saenz, C. Arnold, L. Lee, N. Jafari
nRICHDx, Irvine, CA.

Introduction: The utility of cell-free RNA (cfRNA) as a clinically relevant biomarker in liquid biopsy for early cancer detection is increasing. Compared to circulating tumor DNA which is limited to two copies per cell, there are numerous copies of tissue-specific RNA. However, commercially available kits for the isolation of cfRNA are limited. The nRichDX Revolution Total Nucleic Acid (TNA) Isolation Kit is intended to isolate cell-free nucleic acid (cfNA) from 1 mL to 20 mL of human plasma or urine using a semi-manual workflow. The Revolution TNA Isolation Kit was evaluated by RNA sequencing and compared to the MagMAX Cell-Free TNA Kit and the QIAamp Circulating Nucleic Acid Kit for the isolation of cfRNA from plasma and urine, respectively. **Methods:** Plasma collected in K2EDTA tubes and normal human urine from healthy donors were processed in preparation for cfNA extraction. Half of the 5 mL plasma and urine samples were spiked with 50 ng of RNA with known lung cancer mutations and the rest were not spiked. The samples were extracted using the Revolution TNA Isolation Kit or competitor kits. The extracted samples were analyzed on RNA Pico Bioanalyzer before complementary DNA (cDNA) synthesis using the Clontech SMART-Seq v4 Ultra Low Input RNA Kit. cDNA was adapter-ligated using Illumina Nextera XT DNA Library Prep Kit and sequenced on Illumina NovaSeq 6000 using PE100 chemistry. Raw reads were processed and mapped to the human reference genome to perform differential gene expression analysis. **Results:** The cfRNA quality was assessed using the RNA Pico Bioanalyzer. The Revolution TNA Isolation Kit outperformed MagMAX for plasma-derived cfRNA samples. It was comparable with QIAamp for urine samples. Heat maps of select genes associated with lung cancer show comparable read depth between Revolution TNA Isolation Kit, MagMAX and QIAamp for plasma and urine, respectively. **Conclusions:** The Revolution TNA Isolation Kit extracts cfRNA with comparable yield and quality to competitor kits for plasma and urine. The extracted cfRNA is suitable for downstream RNA sequencing with similar read depth to commercially available kits. In addition, the Revolution TNA Isolation Kit can extract NA from large volumes up to 20 mL, whereas competitor kits are limited to 5 mL. This allows the Revolution TNA

Isolation Kit to recover significantly more cfNA, enabling earlier detection of circulating free nucleic acid cancer markers in blood or urine. Importantly, the yield from a 5 mL Qiagen extraction cannot be scaled 4X to simulate the yield from a single 20 mL extraction. The multiple extractions and subsequent elution pooling steps required for Qiagen significantly diminish the usable cfNA yield. These steps also dramatically increase reagent cost, hands-on time, and ultimately the cost per sample.

TT060- **WITHDRAWN**

TT061. **Next-Generation Cytogenomics: Genome-wide Mapping of Chromosomal Abnormalities in ER+/PR+ Breast Cancer FFPE Tissues**

S. Eacker[1], E. Reister[2], M. Malig[2], B. Nelson[2], M. Wood[2], M. VanDyke[2], J. Braunstein[2], B. Seaton[2], K. Shimp[2], D. Woolston[2], A. Muratov[2], S. Sullivan[2], I. Liachko[2], C. Yeung[2]
[1]Phase Genomics Inc., Seattle, WA; [2]Fred Hutch Cancer Center, Seattle, WA.

Introduction: Cytogenetic analysis is the standard of care in the treatment of hematological malignancies where gross chromosomal rearrangements have both diagnostic and prognostic value. However, in solid tumors cytogenomic analysis is not commonly applied, and cytogenetic methods have limited applicability due to routine storage of samples in formalin-fixed, paraffin-embedded (FFPE) tissue blocks. We have developed OncoTerra, a novel method for high-resolution cytogenomic analysis of FFPE tissues and applied it to a wide range of tumors. This method exploits genomic proximity ligation technology which measures the distance between all loci in the genome simultaneously, using inexpensive short-read sequencing. In hopes of better stratifying risk in a specific solid tumor setting, we explored the landscape of breast cancer-associated chromosome aberrations using OncoTerra. ER+/PR+ tumors are the most common (84%) and treatable forms of breast cancer with an average 90% five-year survival rate. Despite the generally good prognosis in these cases, a significant number of patients with ER+/PR+ breast cancers do not respond well to standard therapy, and stage 4 survival rate drops to 28%. **Methods:** We performed a study of patients with ER+/PR+ tumors and matched normal tissue when available, benchmarking OncoTerra performance on 2 x 5µm curls against matched fresh frozen tissue. In total we analyzed eight breast cancers (including invasive ductal, invasive lobular, and lobular carcinoma *in situ*) and three matched normal samples. **Results:** We found complete concordance between variants observed in FFPE and fresh-frozen matched samples for both cancer and normal tissue. We observed a range of variants including novel inversions in *BRAF*, translocations, large indels, FGF1R amplification, aneuploidy, chromothripsis, and extra-chromosomal circular DNA. No significant variants were observed in normal tissue. **Conclusions:** This study illustrates the use of OncoTerra as a tool to identify genomic variants from FFPE tissue and the potential to stratify patients and identify biomarkers using biopsy specimens collected in the course of standard diagnostic assessment.

TT062. **cfDNA Extraction with the nRichDX Revolution System Can Detect More Low-Frequency Variants in Plasma cfDNA Samples**

N. Jafari, M. Saidian, C. Arnold, L. Lee, J. Saenz, J. Uriu
nRICHDx, Irvine, CA.

Introduction: Liquid biopsies are becoming more frequently used for non-invasive diagnostics and monitoring diseases such as cancer. PCR-based testing is commonly used for routine diagnostics of cancer hotspot mutations; however, targeted next-generation sequencing (NGS) using cell-free DNA (cfDNA) has become one of the most common approaches to assess tumor-specific alterations in the form of circulating tumor DNA (ctDNA). Duplex sequencing is an improved barcoding strategy for tracking double-stranded DNA when unique molecular identifiers are attached to each double-stranded DNA template, followed by the production of low-error consensus sequences to detect low-frequency variants. In this study, we tested these methodologies using the nRichDX extraction method, which can extract cfDNA from large volumes of biological fluids up to 20mL. nRichDX Revolution System demonstrates higher extraction efficiency of cfDNA compared to other methods in the market. **Methods:** Human plasma from healthy donors was collected in K2EDTA tubes and was processed in preparation for cfDNA extraction. Plasma samples at 5mL were extracted using the Revolution Max20 cfDNA Isolation Kit. Similarly, plasma samples at 5 mL were extracted using QIAamp Circulating Nucleic Acid Kit. The quantity of extracted cfDNA was determined by performing tailed amplicon sequencing with MiSeq. The quality of extracted cfDNA was determined using Agilent High Sensitivity D1000 ScreenTape. The presence of *TP53* mutations was analyzed using deep sequencing. Target-specific primers spanning the *TP53* mutations were designed and tagged with Illumina specific-adapter sequences. Amplicon size was 120 bp for the R158L mutation. **Results:** Low-frequency variants (LFVs) of R158L of *TP53* calculated by NGS for the nRichDX system ranged from 60% to 80% and from 50% to 60% for the QIAamp Circulating Nucleic Acid Kit. The LFVs obtained from the nRichDX system were higher than those from QIAamp. **Conclusions:** The nRichDX Revolution Sample Prep System extracts cfDNA and ctDNA with consistently high yields. These data are supported by the results that demonstrate the capability of the nRichDX system to detect more low-frequency variants from the same sample type. This indicated that the nRichDX system could increase the sensitivity of detection of low-frequency mutations in cfDNA. The potential of the nRichDX approach for routine clinical applications will be of great importance for physicians, providing them with a powerful tool to diagnose tumors, monitor tumor dynamics, and evaluate patient responses to targeted therapy.

TT063. **Analytical Validation of a Targeted *CFTR* Laboratory-Developed Test Addressing the Most Prevalent Pathogenic Variants across Diverse Ethnic Groups**

F. Sabato, J. Lynch, E. Barrie, S. Turner, A. Ferreira-Gonzalez
VCU Health, Richmond, VA.

Introduction: Pathogenic variants in the *CFTR* gene are causative of cystic fibrosis (CF). *CFTR* variant testing for carrier screening and diagnostic purposes can be performed using either comprehensive or targeted approaches. Comprehensive approaches involve complex workflow and analysis, and specialized instrumentation. An accurate and simple workflow/analysis assay using a targeted *CFTR* variant panel that addresses genetic diversity across the US population is higher volume routine carrier screening. Commercial reagents are now available that include 65 *CFTR* variants with a 92.2% US population coverage. The analytical performance of a laboratory-developed test (LDT) using that panel was performed and results compared to the GenMark eSensor CF Genotyping Test (eSensor) with a reported population coverage of 86.8%. **Methods:** Accuracy, precision, analytical sensitivity and analytical specificity were evaluated. Selected samples included 23 cell line DNA (Coriell Institute for Medical Research), 12 residual clinical DNA samples from whole blood and two proficiency testing samples. Multiplex PCR was performed with the AmplideX PCR/CE *CFTR* kit (Asuragen) to produce either wild-type or mutant amplicons labeled with either FAM or HEX dyes to determine zygosity across 65 variants in the *CFTR* gene. To avoid sample mix-up, three short tandem repeats (STRs) were also amplified and detected in the NED channel as fingerprinting biomarkers. After PCR, the dye-labeled fluorescent products were resolved by capillary electrophoresis (CE) with POP-7 on the Applied Biosystem 3500xl instrument and associated to expected variant size (bp) using ROX 1,000 size ladder. The resulting electropherograms were processed by the AmplideX PCR/CE *CFTR* Analysis Module v1.1.0 on the AmplideX PCR/CE Reporter v3.0.4 software (Asuragen, Inc.) for peak identification and variant reporting. **Results:** The accuracy of the LDT showed 100% sample genotype and sample variant agreements with the eSensor for the 23 Coriell DNA, 12 clinical samples and two proficiency testing samples. Precision for the most prevalent genotypes

and different variant types showed high reproducibility among runs. Analytical sensitivity studies showed an optimal DNA input concentration of 20 ng/µL. Allelic variations were flagged appropriately by the software when a positive sample with mutations that are adjacent to each other was tested, confirming the analytical specificity of the assay. **Conclusions:** The LDT is an accurate, specific, and reproducible assay, useful for variant detection in diverse populations with a streamlined PCR/CE workflow.

TT064. **Ultrasensitive Duplex Sequencing for Diverse Applications on the Singular Genomics G4 Platform**

E. Schmidt, D. Fitzgerald, P. Van, M. Hipp, C. Valentine, J. Salk, J. Higgins
TwinStrand Biosciences, Seattle, WA.

Introduction: Duplex sequencing (DS) is an extremely accurate DNA sequencing technology with broad applications across life science and medicine where detection of low-frequency mutations is critical. DS relies on double-stranded consensus-making to achieve an error rate below one in 10 million. Nearly all DS studies to date have been carried out on Illumina sequencers. The Singular Genomics G4 platform is a newly available instrument with novel chemistry designed to deliver ~2x faster sequencing results. To assess relative platform performance, we benchmarked several TwinStrand DuplexSeq Assays on the Singular G4 platform versus the Illumina NovaSeq 6000. **Methods:** To evaluate data yield over a range of inputs, libraries were seeded with 50-1,000 ng of high-quality human DNA and enriched with a small (2.4 kb) control panel. To evaluate the sensitivity and specificity of mutation detection across a range of variant allele frequencies (VAFs), we prepared synthetic low-frequency mixtures of DNA from cell lines carrying seven cancer-associated *TP53* single-nucleotide variants and indels spiked into DNA derived from a healthy donor (expected VAFs from 6E-3 to 9E-5). Spike-in mixes, as well as pure healthy donor DNA, were sequenced to an average Duplex molecular depth of >50,000x across all exons of *TP53*. To evaluate performance for measuring ultra-low-frequency mutations, 200 ng of DNA from blood of a healthy donor was used to prepare libraries using TwinStrand's DuplexSeq Human Mutagenesis Assay (48 kb panel). **Results:** For input titration experiments, resulting peak Duplex molecular depths ranged from approximately 2,300-47,000x (G4) and 2,800-57,000x (NovaSeq), with the slight differences likely due to variation in experimental conditions. In spike-in experiments, the measured mutation frequencies from both platforms matched expected frequencies, within variation predicted from Poisson sampling statistics. Zero mutant counts at spike-in sites were identified in negative controls; thus, the analytical performance of the assay was 100% sensitivity and 100% specificity for detecting clones to below one in 10,000. In blood from a healthy donor, a total of 50 mutant nucleotides out of 485,440,864 total duplex nucleotides (mutant frequency 1.03E-7 +/-2.5E-8) were identified in the Illumina library, and 70 mutant nucleotides out of 452,802,985 (mutant frequency 1.55E-7 +/- 3.2E-8) in the Singular library. No statistically significant differences in overall mutant frequency or spectrum were observed between the platforms. **Conclusions:** In conclusion, three independent forms of analytical testing of TwinStrand Duplex Sequencing technology across a broad range of DNA input, rare cancer clone detection, and ultra-sensitive mutagenesis applications yielded nearly identical performance on the Singular and Illumina platforms.

TT065. **Expanded Delta PCR: A Streamlined and Sensitive Method for Detection of *BCL2-IGH* Rearrangements, Specifically BioMed2 MBR, 3'MBR, 5'Mcr, Mcr**

S. Fine[1], L. Hutchinson[2], X. Meng[2]
[1]University of Massachusetts, Westborough, MA; [2]University of Massachusetts, Worcester, MA.

Introduction: Chromosomal translocations such as those present in BCL2-positive lymphomas pose PCR detection challenges in the laboratory due to widely distributed breakpoints and a high noise-to-signal ratio requiring nested PCR and specific probes to identify a translocation. This approach increases complexity, turnaround time, and the potential for contamination. We evaluated the use of delta-PCR as a method to overcome these obstacles and simplify laboratory workflow. **Methods:** We adopted the delta-PCR strategy (by Lin, *et al.,* 2011) to modify the Biomed 2 approach to detect *BCL2-IGH* translocations in lymphoma. A two-tube system was created (Tube 1: MBR1 MBR2 mcr1, mcr2, Tube 2: 3'MBR2 3'MBR3 5'mcr mcr0). For each target breakpoint we adapted or designed two forward primers (internal and external) and two fluorescent reverse primers, one for single-step PCR and a second reverse primer for nested PCR. A synthetic control was developed to verify all breakpoints. Capillary gel electrophoresis was used to visualize the size difference observed for internal and external amplicons. **Results:** Seventy-three samples consisting of formalin-fixed, paraffin-embedded, fresh tissue, and bone marrow were tested from patients with or without a history of lymphoma. Relative to traditional PCR/fluorescence *in situ* hybridization, the sensitivity was 93.5% (29/31), and specificity was 97.6% (41/42). Relative to morphological diagnosis, sensitivity was 78.4% (29/37) and specificity 97.2% (35/36). All breakpoints were confirmed by the synthetic control and five of eight were observed in validation samples. Analytical sensitivity in multiplex format was 0.01%-0.1% in single-step PCR. **Conclusions:** Delta PCR is an effective testing strategy to identify translocation breakpoints that aid in the diagnosis of BCL2-positive lymphomas (i.e., follicular lymphoma Grades 1 and 2). Expansion of the *BCL2* breakpoint repertoire increased diagnostic utility. Addition of nested PCR improved minimal residual disease detection. Confirmation of assay validity was simplified by the creation of a comprehensive positive synthetic control.

TT066. **Implementation of Automated NGS to Consolidate Rapid Molecular Testing for NSCLC Delivers Effective Integrated Results in Exchange for Reduced Effort and Cost**

N. Georgantas, J. Lennerz
Massachusetts General Hospital, Boston, MA.

Introduction: Personalized treatment guided by an interpretation of integrated results has become gold-standard practice at leading cancer centers. In accordance, the Massachusetts General Hospital (MGH) Center for Integrated Diagnostics (CID) has established a rapid molecular testing program to expedite testing in settings where the identification of highly actionable somatic variants is likely to have an immediate effect. This is most clearly demonstrated in cases of non-small-cell lung cancer (NSCLC) where the presence of variants in the epidermal growth factor receptor (*EGFR*) region can have a profound effect on treatment course and prognosis. For NSCLC we have (until now) exclusively focused on development of single gene or small panel assays that target specific variants. Although this approach has led to success among patients who exhibit variants in targeted testing, the long turnaround time for traditional next-generation sequencing (NGS) has many waiting for fully integrated results. This, coupled with the complexity of laboratory logistics and costs associated with performing such extensive and diverse testing, has highlighted room for improvement. **Methods:** By implementing a rapid turnaround NGS solution our goal is to drastically improve the turnaround time to provide fully integrated molecular reports while simultaneously lowering costs by reducing complexity and redundancy of the systems currently supporting the legacy testing program. Consolidating multiple tests into one assay that preserves all applicable clinical attributes in a streamlined and automated approach, we aim to ease the burden on laboratory staff of all levels by drastically reducing or eliminating the workload for the following laboratory procedures: accessioning, histology, nucleic acid extraction, assay set-up, result interpretation, report generation, and pathologist sign-out. A micro-cost analysis of laboratory materials (instruments, reagents, and consumables) and labor was performed to assess the financial impact of this consolidation. **Results:** Application of rapid NGS has the following effects on logistical and cost burdens associated with molecular testing in cases of NSCLC: Typical turnaround time to a fully integrated result is reduced from two weeks to 72 hours. By using frozen section (FX)

specimens, results can be available as soon as 36 hours post-resection. Cumulative hands-on time spanning all laboratory staff members involved is reduced by more than 1,000% per sample. Laboratory expenses related to this testing are reduced by more than 300% per patient. **Conclusions:** Despite the initial investment typically associated with launching a new technology, our rapid NGS assay is shown to be a safe, cost-effective, and rapid method for producing accurate, fully integrated molecular results for our patients.

TT067. **An Interlaced Pipeline for Translating NGS-Derived Methylation Biomarkers into Robust Multiplexed qPCR Assays for the Early Detection of Cancer**

M. Dodge, P. Barghouth, B. Printy, T. Gyuris, G. Subramanian, G. Mineo, N. Amidei, E. Bram, C. Schnabel
Nucleix, San Diego, CA.

Introduction: qPCR-based assays hold cost and time advantages over similar next-generation sequencing (NGS)-based approaches, making them the predominant modality for molecular testing in clinical labs. Although NGS technology is valuable for identifying putative markers, translating these findings into multiplex qPCR assays is largely a trial-and-error process. Herein, we report the development of a powerful interlaced (*in-silico/in-vitro*) pipeline for rapid and efficient translation of NGS-derived early-stage cancer methylation biomarkers into simple and robust multiplex qPCR assays (EpiCheck) for highly sensitive quantification of epigenetic changes in cell-free DNA (cfDNA) from blood. **Methods:** More than 100 GC-rich lung cancer markers were previously identified via a proprietary EpiCheck NGS pipeline. These markers were analyzed by bespoke software (PrimerX) to predict optimal qPCR primer and probe candidates, using best practices combined with EpiCheck-specific requirements, such as inclusion of methylation-sensitive restriction enzyme cut sites. All *in-vitro* testing was performed on cfDNA and genomic DNA. For all markers, eight primer pairs were screened for performance and off-targeting using EvaGreen qPCR (QuantStudio 7 Pro), as well as direct product sizing (Fragment Analyzer). Top candidates (n=4) for each target were then assessed for probe-based qPCR performance. The best two primers and probes for each target were then binned *in-silico* by PrimerX into 3-, 4-, or 5-plex qPCR reactions, and tested using dark cycling coupled with NGS for confirmation or iterative redesign. Final multiplex reactions incorporated up to five fluorophores and were validated using clinical and analytical samples. **Results:** The final qPCR assay was highly concordant with NGS-derived data, showing limited translational gaps. More than 75% of initial markers progressed through all development stages into functional multiplex qPCR reactions, with >90% of these targets producing cycle threshold (Ct) values within one cycle of an endogenous control. Clean singleplex amplicon formation and robust Ct values correlated with subsequent multiplex performance. Interestingly, the large number of putative targets counteracted performance loss upon multiplexing, as the PrimerX software had additional flexibility when predicting optimal binning. **Conclusions:** Development of NGS-derived qPCR assays must sustain high-order plexity, quantitativeness, and overall performance integrity to become viable clinical tests. Herein, we have demonstrated that software-driven primer designs and subsequent binning, coupled with an emphasis on robust singleplex performance, significantly accelerates the design process while maintaining high sensitivity. Further incorporation of shallow sequencing informs the original design algorithm, allowing for iterative improvements.

TT068. **Multiplex Gene Expression Profiling Using the Parsortix and HyCEAD Platforms for Analysis of CTCs in Breast Cancer Patients**

D. Englert, W. Cho, D. Englert, M. Farhang Ghahremani, K. Seto, P. Smith
ANGLE Biosciences, Inc., Toronto, Ontario, Canada.

Introduction: Quantitative gene expression profiling of circulating tumor cells (CTCs) is a potential tool for assessing disease status and monitoring treatment response in breast cancer. We developed a Research Use Only workflow including the Parsortix system, followed by HyCEAD (Hybrid Capture Enrichment Amplification and Detection) and Ziplex technologies. The Parsortix system enriches and harvests CTCs from blood, and HyCEAD amplifies short amplicons from up to 100 mRNA targets. Hybridization of amplicons on the Ziplex instrument provides sensitive multiplex gene expression profiling. We have used cancer cell lines and Parsortix harvests from metastatic breast cancer (MBC) patients to assess the sensitivity, linearity, and reproducibility of HyCEAD/Ziplex for the quantification of mRNA targets in CTCs and its ability to discriminate between the different cell lines based on their expression profiles. **Methods:** Primer/probe sets designed for 71 breast cancer-related genes and one white blood cell (WBC) marker were assessed on different concentrations of RNA from four breast cancer and one ovarian cancer cell lines. Cells or total RNA were also spiked into total WBC RNA or into lysates of Parsortix cell harvests derived from healthy volunteer (HV) blood to assess the assay performance. Expression profiles of Parsortix harvests from blood of MBC patients and from HVs were also compared with the assay. **Results:** Using genes with low expression in WBC RNA and/or unspiked Parsortix harvests from HVs, the assay detected and distinguished between the different breast cancer and ovarian cancer cell lines with concentrations as low as 20 pg of total RNA (equivalent to a single cell) in the presence of 1 ng of WBC RNA. Single cultured cells spiked into Parsortix harvests from 10 mL of HV blood were also detected and distinguished from each other. CVs from repeatability experiments were less than 20%. Hierarchical clustering of clinical samples differentiated most MBC patients from HVs.9. **Conclusions:** HyCEAD/Ziplex provided sensitive quantification of expression of 72 genes from 20 pg of total RNA from cultured tumor cell lines or from single cultured tumor cells spiked into lysates from Parsortix harvests of HV blood. The HyCEAD/Ziplex platform enables the identification and quantification of selected genes in the presence of residual nucleated blood cells in Parsortix harvests. The HyCEAD/Ziplex platform is an effective tool for molecular characterization of mRNA in small numbers of tumor cells harvested from blood.

TT069. **Improving Data Quality and Reproducibility with FFPE-Derived RNA Using a Novel, Rapid Whole-Transcriptome Library Preparation Workflow**

J. Walker[1], T. Sanders[1], L. French[2], T. Harrison[1], J. Pavlica[1], K. Reed[1], C. Ross[1], R. Wadsworth[2], B. Kudlow[1]
[1]Watchmaker Genomics, Boulder, CO; [2]Watchmaker Genomics, Cape Town, South Africa.

Introduction: Formalin-fixed, paraffin-embedded (FFPE) samples are an invaluable resource in the oncology space, providing access to a vast library of archived diseased tissue samples paired with relevant donor information. Despite the broad utility of these samples, RNA extracted from FFPE tissue is typically difficult to process due to the presence of residual crosslinks and its degraded nature. Further, these samples often vary widely in performance, as the fixation process, block age, block storage, and extraction method can impart large impacts on resulting template quality. As a result, robust and reproducible RNA sequencing from FFPE-derived RNA remains a challenge with unpredictable and high failure rates. Additionally, workflows that currently accommodate this sample type are often laborious and difficult to automate. To address these needs, we aimed to develop a rapid whole-transcriptome library preparation workflow specifically tailored for processing FFPE material. **Methods:** To streamline the protocol and improve depletion efficiency and specificity, we developed algorithms to computationally design novel depletion probes. To improve sensitivity, we specifically engineered a reverse transcriptase to maximize conversion of RNA to complementary DNA and tailored buffers throughout the workflow to further enhance performance. Cleanup steps were reduced to minimize sample loss, and a novel de-crosslinking step was integrated to improve FFPE performance. With extracted RNA from multiple biorepository-sourced FFPE blocks and a fusion control sample, we compared our solution to commercial products using inputs ranging from 10 ng to 100 ng.

Additionally, a high-quality whole-blood sample was characterized with inputs ranging from 1 ng to 500 ng to ascertain the performance breaking point of the chemistry when template quality was not a factor. **Results:** We observed a significant increase in sensitivity with low input and FFPE samples in comparison to other workflows, as indicated by an increase in the number of unique genes detected and more unique reads supporting a given gene call. Further, Watchmaker libraries showed excellent inter-input correlation and technical replicate reproducibility with respect to both genes identified and their abundance. Analysis of the fusion control sample showed excellent coverage of fusion breakpoints. **Conclusions:** This highly automatable and rapid solution generates libraries in less than five hours and improves on the data quality currently attainable with existing commercial kits, increasing success rates with FFPE-derived samples and improving access to quantitative and clinically relevant data for oncology applications.

To cite the Abstracts published here, please use the following format:
Akgun Y, Solem E, Le L. Rare KIAA1109 Compound Heterozygous Truncating Variants Resulting in Alkuraya-Kučinskas Syndrome. Association for Molecular Pathology 2022 Annual Meeting Abstracts. J Mol Diagn 2022, 24:S1 Abstract G001