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STUDY OF MRD

Using LymphoTrack® MiSeq® Assays

Minimal Residual Disease (MRD) testing by Next-Generation Sequencing (NGS) has become an important methodology demonstrating clear potential to optimize therapeutic management of lymphoproliferative diseases.

Several years ago the correlation of MRD status, assessed by multiparameter flow analysis, with longer overall survival was demonstrated for patients with chronic lymphocytic leukemia (JCO 23(13):2884, 2005). More recently, Logan *et al*., (Leukemia 27:1659-1665, 2013) have described NGS approaches to detect and monitor MRD for chronic lymphocytic leukemia (CLL).

Similarly, persistence of MRD has been suggested to be the strongest adverse prognostic factor in acute lymphoblastic leukemia (ALL) and Faham, *et al*. described a deep sequencing method that allows monitoring of treatment response in ALL and other lymphoid malignancies with great sensitivity and precision (Blood 120:5173-5180, 2012).

The LymphoTrack® Assays developed by Invivoscribe are NGS-based targeted deep sequencing assays that can be used to detect virtually all rearrangements within the targeted T-cell receptor (TCR) or immunoglobulin (Ig) antigen receptor loci. Once a specific rearrangement sequence (the clonotype) has been identified in a primary sample, the LymphoTrack® Assays allow for easy tracking of clonal populations at a sensitivity of 10^{-4} or to even lower limits provided sufficient DNA is tested.

1| Sample Preparation

It is recommended that high quality extracted and purified genomic DNA be used. This is especially important when a high quantity of DNA is tested (> 500 ng) as DNA must be free of PCR amplification inhibitors and be quantified with a method specific for double stranded DNA (dsDNA). Invivoscribe recommends a maximum of 2.5 µg of DNA per reaction (5 µL of 500 ng/µL) using the LymphoTrack® Assays. For qualitative assessment, DNA samples may be tested with the Specimen Control Size Ladder master mix from Invivoscribe (Cat#: 2-096-0021 for ABI detection or Cat#: 2-096-0020 for gel detection). This master mix amplifies housekeeping genes producing PCR products of approximately 100, 200, 300, 400, and 600 base pairs and was originally designed by the EuroClonality group as part of the BIOMED-2 concerted action (Leukemia 17:2257-2317, 2003).

2| DNA Input Quantity

The amount of input DNA interrogated and the total number of output sequencing reads are the two critical parameters to consider when performing MRD testing. Routine tracking of clones at a sensitivity of 10^{-4} can be confidently achieved by detecting 1 clonal cell in a background of ten thousand cells by testing using as little as 500 ng of input DNA (**see Figure 2A**).

If sensitivities beyond 10^{-4} are desired, it is important to note that there are approximately 6.5 pg of DNA in each cell. Therefore, to detect 1 clonal cell in the background of a million normal cells at a confidence level of 95% (detection at 10^{-6}) it is necessary to test more than 20 µg (or >20 µg) of genomic DNA. Such a low level of detection, although seemingly attractive, may not be required in most settings, and is beyond the level that is generally accepted to be the threshold indicative of residual disease (10^{-4} - 10^{-5}). Furthermore, obtaining more than 20 µg of DNA (9 replicates of 2.5 µg, **see Table 1**) is often not practical. In Section 5, charts illustrate the relationship between DNA quantities and detection levels when tracking clonal populations with confidence.

Invivoscribe recommends a maximum of 2.5 µg of DNA per reaction (5 µL of 500 ng/µL) using the LymphoTrack® Assays.

3| PCR Plate Set-Up

When setting up your PCR Plate, it is critical to set up reactions in a "staggered" plate format, every other well, **as shown in Figure 1**. This 'staggered' set up may help prevent sample cross-contamination.

4| Sequencing

In any high-throughput sequencing technology that utilizes sample barcodes or identifiers (indices) such as the MiSeq®, the high number of clonotype sequences present in an initial sample may contaminate subsequent samples if some precautions are not taken. To mitigate the chances of such artifacts, the following guidelines should be considered:

- Conduct an Illumina® 'Template Line Wash' with bleach after each MiSeq® run.
- Avoid running subsequent timepoints immediately after a MiSeq® run that contained the initial sample.
- Test follow up samples separately from the initial clonal sample.
- Use different indices for the initial sample and follow up samples (*e.g.*, index 1 for identifying the clonotype sequence and index 2 for a follow up sample from the same subject). Alternatively, unrelated samples may be run between the initial sample and follow up runs on the same instrument as long as a template line wash with bleach is conducted.

5| Relationship Between DNA Input, Read Frequency & Level of Confidence

The following figure diagrams depict the level of confidence for detecting a clonotype (detected with at least 5 reads) at various DNA input quantities and replicates as a function of the number of sequencing reads obtained.

FIGURE 2A. CONFIDENCE LEVELS FOR CLONOTYPE DETECTION AT 10^{-4} TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH

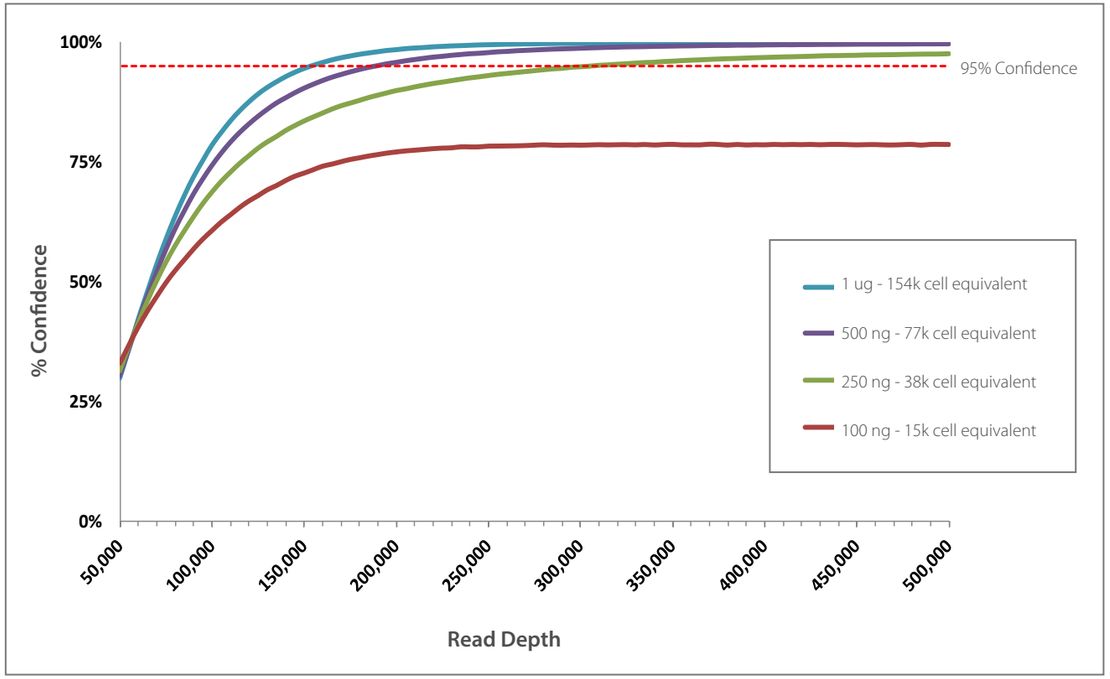


FIGURE 2B. CONFIDENCE LEVELS FOR CLONOTYPE DETECTION AT 10^{-5} TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH

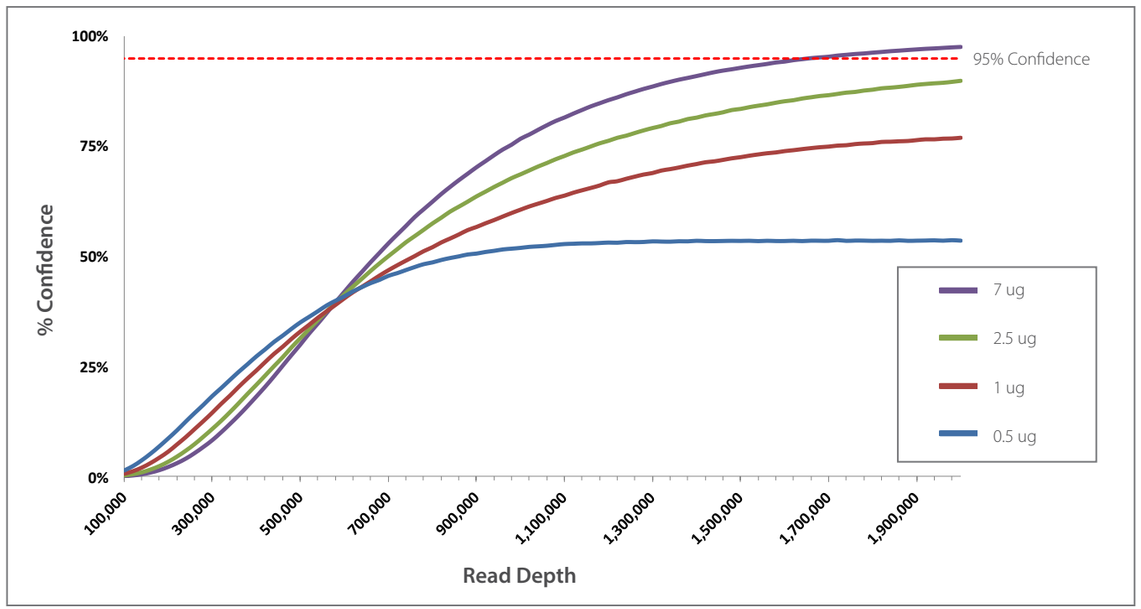
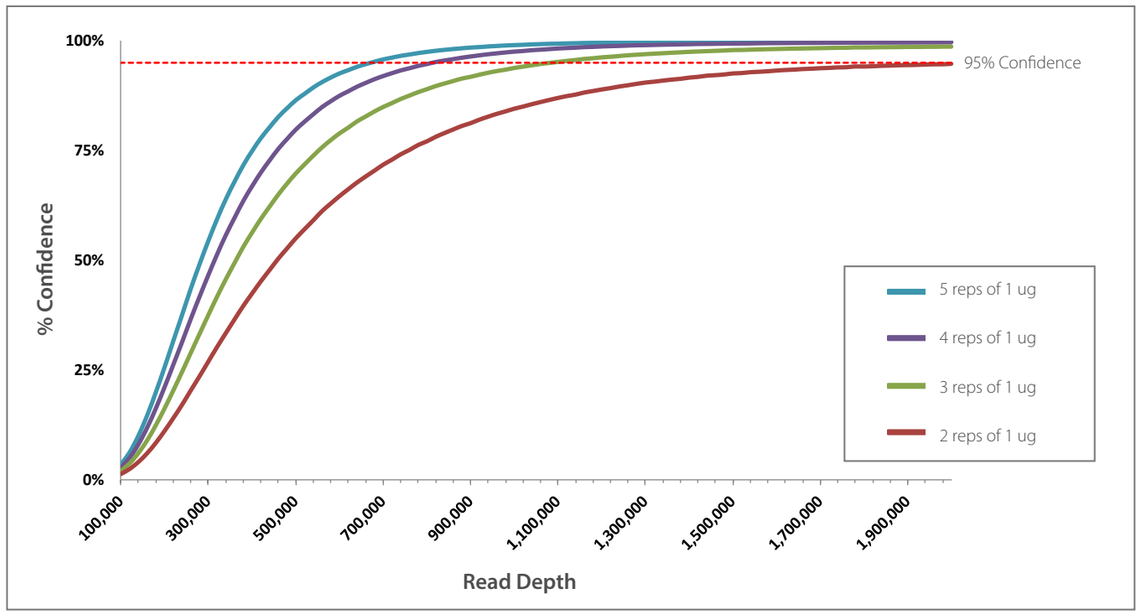


FIGURE 2C. CONFIDENCE LEVELS FOR CLONOTYPE DETECTION AT 10^{-5} AT MULTIPLE REPLICATES OF 1 MG DNA INPUT, AS A FUNCTION OF READ DEPTH



General guidance for obtaining $1x10^{-4}$, $1x10^{-5}$, and $1x10^{-6}$ is provided in Table 1 considering a 2 x 250 bp MiSeq® run (total of 500 sequencing cycles) using either the v2 or v3 chemistries, depending upon the required read depth and total number of samples. Note that the 500 cycle MiSeq® Reagent Kit v2 (Illumina® Cat#: MS-102-2003), and the 600 cycle MiSeq® Reagent Kit v3 (Illumina® Cat#: MS-102-3003) will produce up to 15 and 25 million reads, respectively.

TABLE 1. GENERAL GUIDANCE

95% PROBABILITY OF **DETECTING 5 READS** OF THE TARGET SEQUENCE				
SENSITIVITY	DNA PER REPLICATE	# REPLICATES	READ DEPTH PER REPLICATE	# OF DIFFERENT SAMPLES FOR CLONOTYPE TRACKING PER RUN
$1x10^{-4}$	0.250 µg 0.500 µg	1 replicate of 0.250 µg 1 replicate of 0.500 µg	>310,000 >190,000	21 samples per run plus 3 controls
$1x10^{-5}$	1 µg	3 replicates of 1 µg each 4 replicates of 1 µg each	>1,080,000 >820,000	7 samples per run* plus 3 controls or 5 samples per run plus 3 controls
$1x10^{-6}$	2.5 µg	9 replicates of 2.5 µg each	>4,000,000	1 sample over 2 runs**

Note: A replicate is an independent PCR reaction with input DNA from the same subject.

*25,000,000 total reads (for the v3 600 cycle flow cell) divided by ~1,000,000 required reads per sample ~24 samples; utilizing the 24 indices provided in the LymphoTrack® MiSeq® Assay Panels and including 3 controls = 21 indices remaining for samples; requiring 3-4 replicates to obtain 95% probability of detecting 5 target reads allows 5-7 independent clonal tracking samples to be run on the same flow cell.

** To obtain a 95% probability of detecting 5 reads of the target sequence (if the total number of reads is at least 24,000,000) requires 9 replicates of 2.5 µg each of input DNA and a minimum of 2 flow cells to assess a sample for the presence of a clonal sequence in a subsequent sample. Adding the 3 controls to the flow cell may reduce the number of sample reads below the required read depth thus reducing the 95% confidence.

It is recommended that the following three controls be included in every run:

1. A positive control at 10^{-4} concentration

2. A negative control devoid of the clonotype sequence

3. A no template control (NTC) with water in place of the DNA sample in the PCR reaction.

Invivoscribe LymphoTrack® MiSeq® Assay panels are provided with 24 different indices thus allowing 21 different subject samples to be run along with 3 controls on a single flow cell when tracking clonotype sequences to a sensitivity of 10^{-4}.

6 Invivoscribe MRD Software Application

Invivoscribe has developed a LymphoTrack® MRD Data Analysis Tool (MRDDAT) to facilitate the tracking of the clonotype DNA sequences in follow up samples. This application not only identifies the clonotype and the degree of miss-matches (0, 1, 2), but also calculates the read frequency and the degree of confidence that the clonotype is present at 10^{-3}, 10^{-4}, and 10^{-5} levels. **Examples of MRD Software Input and Output are shown in Figures 3A, 3B, and 3C.**

FIGURE 3A: MRD SOFTWARE INPUT SCREEN EXAMPLE

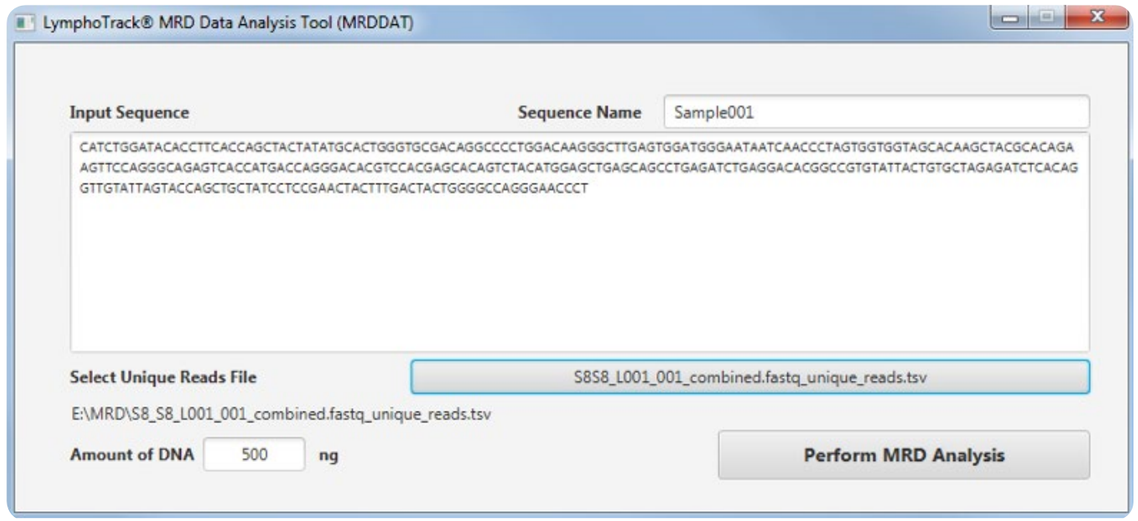


FIGURE 3B: MRD SOFTWARE OUTPUT SCREEN EXAMPLE – SEQUENCE DETECTED

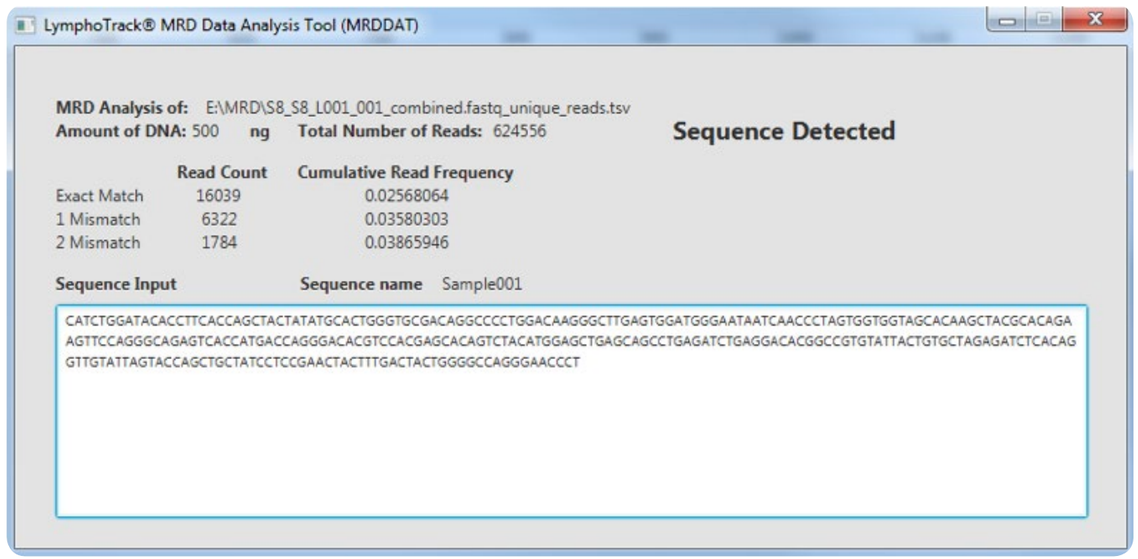
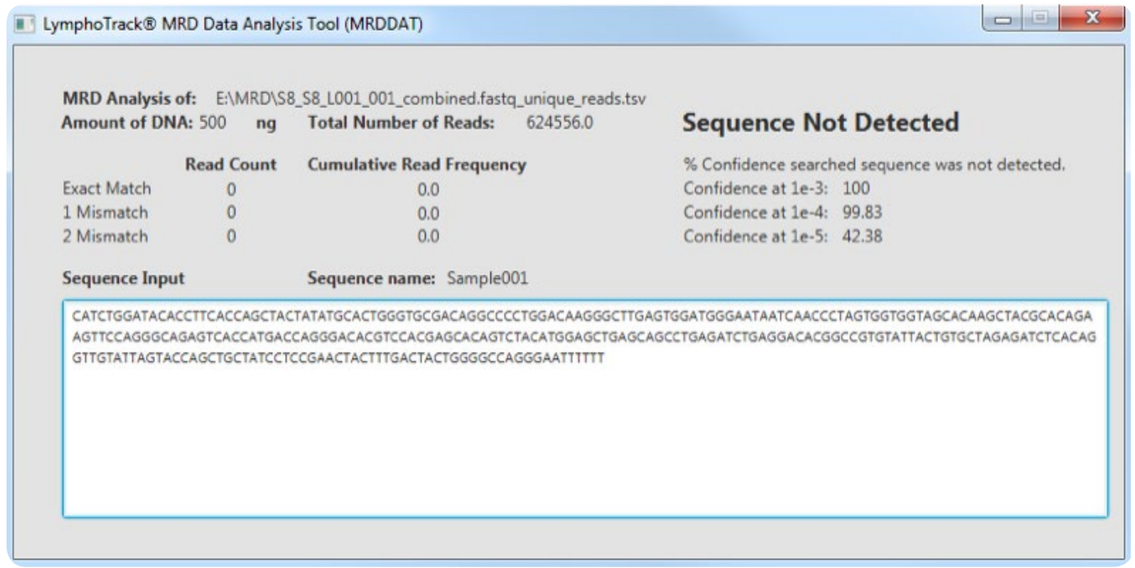


FIGURE 1. 'STAGGERED' PCR PLATE SET UP

	1	2	3	4	5	6	7	8	9	10	11	12
A	id01		id09		id18							
B		id05		id13		id22						
C	id02		id10		id19							
D		id06		id14		id23						
E	id03		id11		id20							
F		id07		id15		id25						
G	id04		id12		id21							
H		id08		id16		id27						

FIGURE 3C: MRD SOFTWARE OUTPUT SCREEN EXAMPLE – SEQUENCE NOT DETECTED



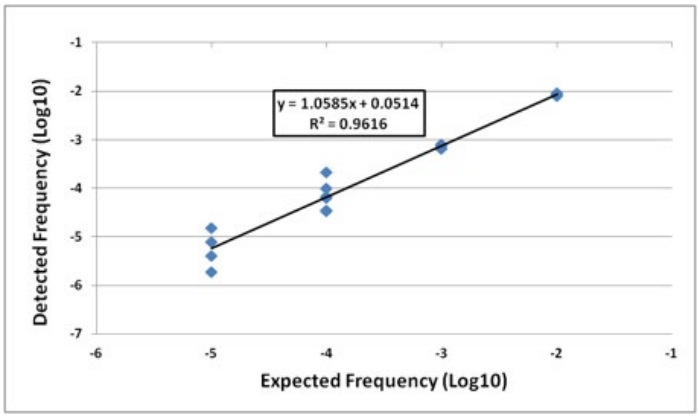
7 Example of Limit of Detection and Linearity for a Dilution Series

DNA from a B-cell line with a known V-J rearrangement was serially diluted into a background of tonsil DNA (abundance of T- and B-cells) and tested with the LymphoTrack® Assays (Input DNA quantity adjusted to 700 ng per dilution point). The LymphoTrack® MRD Data Analysis Tool was then used to detect the known clonal sequence in subsequent samples (**Table 2**). Linearity is shown in **Figure 4**. High concentration samples were run separately from the low concentration samples to avoid cross-contamination as suggested in the guidelines in Section 4.

TABLE 2: DILUTION SERIES

EXPECTED FREQUENCY	DETECTED TOTAL READS	DETECTED CLONOTYPE	
		READS	FREQUENCY
10^{-2}	584618	5415	9.26E-03
10^{-2}	515553	4336	8.41E-03
10^{-2}	524593	4237	8.08E-03
10^{-2}	518593	4586	8.84E-03
10^{-3}	435651	343	7.87E-04
10^{-3}	523460	345	6.59E-04
10^{-3}	342719	252	7.35E-04
10^{-3}	551890	376	6.81E-04
10^{-4}	457377	31	6.78E-05
10^{-4}	508139	18	3.54E-05
10^{-4}	581971	37	6.36E-05
10^{-4}	498037	17	3.41E-05
10^{-4}	445985	45	1.01E-04
10^{-4}	540130	117	2.17E-04
10^{-5}	560826	0	0.00E+00
10^{-5}	502908	4	7.95E-06
10^{-5}	529721	8	1.51E-05
10^{-5}	538617	0	0.00E+00
10^{-5}	526718	1	1.90E-06
10^{-5}	497186	2	4.02E-06

FIGURE 4: EXAMPLE OF A DILUTION SERIES



Ordering Information

Catalog #	Products	Quantity
7-121-0009	LymphoTrack® *IGH* Assay Kit A - MiSeq®	8 indices - 5 sequencing reactions each
7-121-0039	LymphoTrack® *IGH* Assay Panel - MiSeq®	24 indices - 5 sequencing reactions each
7-122-0009	LymphoTrack® *IGK* Assay Kit A - MiSeq®	8 indices - 5 sequencing reactions each
7-122-0019	LymphoTrack® *IGK* Assay Panel - MiSeq®	24 indices - 5 sequencing reactions each
7-227-0019	LymphoTrack® *TRG* Assay Kit A - MiSeq®	8 indices - 5 sequencing reactions each
7-227-0009	LymphoTrack® *TRG* Assay Panel - MiSeq®	24 indices - 5 sequencing reactions each
7-500-0009	LymphoTrack® MiSeq® Software	1 CD complimentary with purchase

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