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Interpreting Variants with the AmplideX® PCR/CE *SMN1/2* Plus and SMA Plus Kits*†

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OVERVIEW

The AmplideX® PCR/CE *SMN1/2* Plus* and AmplideX SMA Plus† kits are *in vitro* nucleic acid amplification kits for the determination of *SMN1* and *SMN2* exon 7 copy number[1,2]. Both kits are PCR assays that amplify dis-tinctive *SMN1* and *SMN2* gene regions and an endogenous control (EC) gene from purified genomic DNA in a single reaction for up to 94 samples per run. Fluorescently-labeled *SMN1*- and *SMN2*-specific amplicons are resolved by capillary electrophoresis (CE) and referenced to co-amplified EC gene products to determine copy numbers. The kits resolve *SMN1* and *SMN2* exon 7 copy numbers and identify hybrid peaks associated with chimeric genes resulting from gene conversion[3]. Additionally, they determine the status of three im-portant polymorphism variants, including two (c.*3+80T>G and c.*211_*212del) associated with *SMN1* gene duplication[4] and one (c.859G>C) associated with reduced disease severity due to improved *SMN2* splicing[5,6].

In this technical note, we discuss the latest clinical research on the variants detected by this kit, including how they relate to the underlying genetics and outcomes for spinal muscular atrophy disease prognosis and carrier risk.

SPINAL MUSCULAR ATROPHY

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by loss of survival motor neuron 1 (*SMN1*) gene function, and is the primary genetic cause of infant death[7]. SMA is often divided into "types" based on age of onset and maximum motor milestone achievement, with a gradient of phenotypes ranging from never sitting unassisted, with onset prior to six months of age, to adult-onset mild muscular weakness[8,9]. Most SMA patients are classified into three types in order of decreasing severity: type 1 (~60%), type 2 (~30%), and type 3 (~10%)[8]. Rarer SMA types, such as type 0 and type 4, also exist[8,9].

SMA has an incidence of ~1/10,000 live births and a carrier rate of ~1/50. *SMN1* exon 7 is absent in ~96% of patients with SMA, whereas most unaffected individuals have two or more functional genomic *SMN1* copies[10]. Additionally, ~3–4% of patients are compound heterozygotes, with an *SMN1* exon 7 deletion on one chromosome and a point mutation in *SMN1* on the other chromosome[10]. SMA carriers lack a functional *SMN1* copy on a single chromosome and frequently have one functional *SMN1* copy on the other (1+0), though a *cis* carrier genotype (2+0), commonly referred to as a silent carrier, is also known[9]. In one study examining a large North American population, the detection rate of SMA carriers using *SMN1* copy number alone varied from ~71% to 95% depending on ethnicity, as silent carriers cannot be resolved from wild-type (1+1) individuals solely based on copy number[11]. *SMN1* copy number is typically detected at exon 7, where a single exonic nucleotide (c.840C) distinguishes it from the highly homologous gene *SMN2* (c.840T)[7,9]. Recent studies have shown that variants c.*3+80T>G and c.*211_*212del in *SMN1* are associated with *SMN1* duplication in many ethnic groups, and inform risk of silent carrier *SMN1* genotypes (2+0) to varying degree depending on ethnicity[4,12].

SMA phenotype severity inversely correlates with *SMN2* copy number, though copy number alone is not sufficient for predicting disease progression[8,9]. In *SMN2*, the single nucleotide difference relative to *SMN1* in exon 7 disrupts a splice enhancer that decreases the number of exon 7-containing mRNAs to 10–20%, resulting in a significantly reduced amount of functional SMN protein[7]. Due to complete homology with the

SMN1-associated SMN protein sequence, *SMN2*-generated SMN protein levels offer a compensatory effect. Thus, *SMN2* copy number is associated with severity of the disease, whereas *SMN1* copy number is associated with molecular SMA diagnosis and carrier status[9,10]. In addition to *SMN1* and *SMN2* copy numbers, several mutations are known to be disease modifiers. For instance, c.859G>C in *SMN2* is linked to improved splicing efficiency of *SMN2*, leading to reduced disease severity[5,6].

GENETICS OF SMA CARRIERS

An SMA carrier is an asymptomatic individual lacking a functional copy of *SMN1* on one chromosome. Most SMA carriers have an exon 7 deletion in *SMN1* on one chromosome and one functional *SMN1* copy on the other (1+0), representing a heterozygous deletion. Silent carriers, in contrast, have a (2+0) genotype whereas others may have a loss of function mutation in *SMN1* on one chromosome and two *SMN1* exon 7 copies (1[d]+1) or rarer genotypes with higher *SMN1* exon 7 copy numbers (1[d]+2, 3+0)[9]. Because of this, the detection rate of SMA carriers using *SMN1* copy number alone to detect (1+0) genotypes varies from ~71% up to 95% depending on ethnicity due to differences in the frequency of gene duplication events and loss of function mutations[11]. Thus, there is a potential for up to ~30% false negatives for carrier status using typical methods.

Table 1. Residual SMA carrier risk estimates by ethnicity based on *SMN1* copy number and gene duplication variant status.

		Residual Risk Estimates			
Ethnicity	**Carrier Frequency**	**2 copies *SMN1* exon 7[f]**	**3 copies *SMN1* exon 7[f]**	**2 copies *SMN1*, variant status "Negative" [g]**	**2 copies *SMN1*, variant status "Positive" [g]**
Ashkenazi Jewish	1:56[a]	1:514[a]	1:5899[a]	1:580[b]	SMA Carrier[b]
Asian	1:50[a]	1:719[a]	1:5185[a]	1:779[c]	1:57[c]
African American/ Black	1:71[a]	1:132[a]	1:6997[a]	1:375[d]	1:39[d]
Caucasian/European	1:45[a]	1:604[a]	1:4719[a]	1:814[c]	1:12[c]
Hispanic	1:83[a]	1:641[a]	1:7574[a]	1:906[d]	1:99[d]
Spanish	1:40[e]	1:781[e]	Not Reported	1:888[e]	SMA Carrier[e]
Israeli Jewish	1:38[a]	1:450[a]	1:4004[a]	Not Reported	Not Reported
Asian Indian	1:50[a]	1:428[a]	1:5252[a]	Not Reported	Not Reported
Iranian	1:16[a]	1:96[a]	1:1604[a]	Not Reported	Not Reported

[a]According to MacDonald *et al.* (2014).
[b]According to Luo *et al.* (2014). Values rounded to nearest integer.
[c]According to Chen *et al.* (2020). For Asian ethnicity, includes South Asians and East Asians.
[d]According to Feng *et al.* (2018).
[e]According to Alías *et al.* (2018).
[f]Residual risk values based on *SMN1* copy number alone
[g]Residual risk values based on *SMN1* copy number of 2 and *SMN1* c.*3+80T>G and c.*211_*212del status

Residual carrier risk estimations based on *SMN1* copy number alone have been calculated for many ethnicities by compiling results across multiple studies and ethnicities (**Table 1**, first four columns)[13]. While gene conversion is known to occur and is also one potential cause for the silent carrier (2+0) genotype[9], the clinical significance of gene conversions is not fully understood. Since the total *SMN1* copy number is used to assess carrier risk, limitations of such testing should be described and reported[9].

In addition to *SMN1* copy number, research has shown that the presence of *SMN1* gene duplication variants c.*3+80T>G in intron 7 and c.*211_*212del in exon 8 can be indicative of the silent carrier (2+0) genotype

in some ethnicities[4,12]. Typically, these variants co-occur[4]; however, individuals with only one of the variants have been identified[12]. Detection of either c.*3+80T>G or c.*211_*212del alone is considered indicative of *SMN1* gene duplication[4].

In response to characterization of the *SMN1* gene duplication variants across multiple ethnicities, guidelines have been updated to reflect that these variants improve residual risk estimates[14]. **Table 1** (last two columns) summarizes these results across several studies. The impact of these variants has not been evaluated in all ethnicities, and some studies show varying residual risk levels within an ethnicity[4,15,16]. This is likely due to the broad range of ethnic backgrounds included in each category. Consequently, the numbers shown here represent risk estimations from studies with the largest number of individuals analyzed for each ethnicity.

Importantly, absence of these duplication variants does not rule out the possibility of a carrier (2+0) genotype. Their presence also does not diagnose silent carriers in most ethnicities. However, resolution of *SMN1* gene duplication variants modifies the residual risk of SMA carrier status in all ethnicities studied to date (**Table 1**). Therefore, co-occurrence of these variants with two copies of *SMN1* indicate "increased carrier risk", while absence of the variants with two copies of *SMN1* indicates "reduced carrier risk" compared to using *SMN1* copy number alone, regardless of ethnicity[4,12,14-16].

GENETICS OF SMA PROGNOSIS

While *SMN2* exon 7 copy number is not used in a diagnosis of SMA, guidelines recommend that results are reported and assessed to inform prognosis and treatment decisions[8,10]. *SMN2* copy number is strongly correlated with SMA type, but copy number alone is not sufficient to predict SMA type in all cases[10]. These limitations should be communicated when assessing *SMN2* copy number results.

Additionally, the c.859G>C variant is a positive disease modifier associated with reduced disease severity and improved prognosis[5,6,8]. Evidence suggests that c.859G>C improves *SMN2* splicing, exon 7 inclusion, and full-length SMN protein production, leading to improved phenotypic outcomes[5,6]. For instance, while 90% of individuals with SMA and two copies of *SMN2* exon 7 typically have SMA type 1, individuals with SMA that have two copies of *SMN2* exon 7 and the c.859G>C variant typically have SMA type 2 or type 3, with no known cases of SMA type 1 in individuals with this genotype[5,6,17].

Table 2. Likely SMA prognosis based on *SMN2* copy number and variant status.

SMA diagnosis	*SMN2* Copy Number	c.859G>C Variant Status	Prognostic Information
SMA	1	Negative	Probable Type 0[a]
SMA	2	Negative	Probable Type 1[a]
SMA	2	Positive	Probable Types 2/3[b]
SMA	3	Negative	Probable Types 2/3[a]
SMA	3	Positive	Probable Type 3[c]
SMA	≥ 4	Negative	Probable Types 3/4[a]

[a]According to Glascock et al (2018).
[b]Based on results from Calucho *et al.* (2018), Vezain *et al.* (2010), Prior *et al.* (2009).
[c]Based on results from Calucho *et al.* (2018).

While the impact of the c.859G>C variant in individuals with SMA that have *SMN2* copy numbers other than two is presumably similar, reports in the literature are limited. However, evidence does suggest that co-occurrence of c.859G>C with three *SMN2* copies leads to the milder Type 3, rather than probable Type 2/Type 3, as indicated by *SMN2* copy number alone[17]. Impact of this variant on other *SMN2* copy numbers is unknown, but presumably indicative of improved prognosis relative to copy number alone. Such instances

are presumably rare, given the frequencies of both the mutation and other *SMN2* copy numbers among individuals with SMA. Therefore, a positive result for c.859G>C may be interpreted generally as "positive disease modifier detected; reduced severity/improved prognosis relative to typical presentation based on *SMN2* copy number genotype."

A summary of likely SMA types based on *SMN2* exon 7 copy number from published treatment guidelines is provided in **Table 2**. Prognostic information is relevant only for individuals diagnosed with SMA.

INTERPRETING VARIANTS

In this section, we will summarize when the status of variants in *SMN1* and *SMN2* discussed above are relevant, and how they can be interpreted based on the available literature. Importantly, relevant information for these variants is dependent on the type of testing (diagnosis or carrier testing).

Carrier Testing

For SMA carrier testing, interpretation of the c.*3+80T>G and c.*211_*212del *SMN1* gene duplication variants is not necessary when a typical carrier genotype is identified (1+0, 1 copy *SMN1*). It is most relevant in informing residual risk when two copies of *SMN1*—the most common genotype in many ethnicities—are present[4,12,15,16]. By contrast, interpretation of these variants is unnecessary when 3 or more copies of *SMN1* are present given the extremely low likelihood of being a carrier[13].

Based on this, c.*3+80T>G and c.*211_*212del variant status can be provided and interpreted in instances of 2 *SMN1* copies for carrier testing purposes (see **Table 1**). Continued research is likely to further refine values, and should be reviewed regularly. Examples are provided in **Appendix A** based on available guidelines[9,14]; see also Prior *et al*. 2011 for an example report[9].

Disease Prognosis

For individuals with SMA, interpretation of the c.859G>C variant is relevant only when determining likely disease progression based on the *SMN2* genotype[8]. When the c.859G>C variant is not detected, likely prognosis can be interpreted using *SMN2* copy number alone, noting that correlation between genotype and phenotype is not absolute[8-10]. When the variant is detected in diagnosed individuals with two or three *SMN2* copies, probable SMA type can be directly inferred[5,6,17]. While c.859G>C has not been identified in individuals with SMA that have other *SMN2* copy numbers, improved prognosis relative to probable type based on copy number alone may be inferred[5,6,8].

Based on this, c.859G>C variant status may be reported with *SMN2* copy number and used to infer likely SMA Type for individuals diagnosed with SMA (see **Table 2**). While the c.859G>C mutation accounts for many atypical cases, research on SMA disease modifiers is ongoing, and should be reviewed regularly. Examples are provided in **Appendix B** based on available guidelines[8-10].

CONCLUSIONS

While our understanding of the impact of variants in *SMN1* and *SMN2* on SMA carrier status and disease prognosis continues to evolve, a solid foundation of clinical studies in the literature demonstrates the utility of identifying several key variants in addition to *SMN1* and *SMN2* copy numbers. More specifically, gene duplication variants in *SMN1* can adjust the risk of being a silent carrier and help inform reproductive decisions for couples. Additionally, disease modifier testing can improve prognostic predictions in individuals diagnosed with SMA, explaining some of the discrepancies between observed *SMN2* copy numbers and expected SMA disease progression. The information provided by these variants can benefit laboratories and clinicians interested in providing the most accurate, state-of-the-art information for SMA carrier screening and prognostic predictions.

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Appendix A: Carrier Interpretation Examples

Note: The examples provided here are interpretations based on relevant guidelines[9,14] and literature[4,12,13,15,16]. When interpreting and presenting results, all relevant local guidelines and regulations should be followed.

Genotype:
SMN1 copies: 2
c.*3+80T>G: positive
c.*211_*212del: negative
Reported Ethnicity: Caucasian/European

Interpretation: Increased Carrier Risk
The *SMN1* copy number is two, ruling out a typical carrier genotype (1+0). However, presence of one or more variants indicates increased risk of being a silent carrier. Ethnic-specific risk values based on these results are provided (see **Table 1**, last column). Based on the reported ethnicity, the residual risk of SMA carrier status is 1:12. Genetic counseling is recommended and carrier testing should be made available to other at-risk family members.

Genotype:
SMN1 copies: 2
c.*3+80T>G: negative
c.*211_*212del: negative
Reported Ethnicity: African American

Interpretation: Reduced Carrier Risk
The *SMN1* copy number and variant status indicate reduced, but not eliminated, carrier risk. Ethnic-specific risk values based on these results are provided (see **Table 1**, 2nd to last column). Based on the reported ethnicity, the residual risk of SMA carrier status is 1:375. Genetic counseling is recommended.

Genotype:
SMN1 copies: 3
Reported Ethnicity: Hispanic

Interpretation: Reduced Carrier Risk
The *SMN1* copy number indicates significantly reduced, but not eliminated, carrier risk. Ethnic-specific risk values based on these results are provided (see **Table 1**, Column 4). Based on the reported ethnicity, the residual risk of SMA carrier status is 1:7,574. Genetic counseling is recommended.

Genotype:
SMN1 copies: 1

Interpretation: Carrier
The *SMN1* copy number indicates a carrier of SMA. Genetic counseling is recommended and carrier testing should be made available to other at-risk family members.

Appendix B: SMA Prognosis Examples

Note: The examples provided here are interpretations based on relevant guidelines[8-10] and literature[5, 6,17]. When interpreting and presenting results, all relevant local guidelines and regulations should be followed.

Genotype:
SMN1 copies: 0
SMN2 copies: 2
c.859G>C: positive

Interpretation: SMA (Type 2/3 probable)
The *SMN1* copy number indicates SMA. Whereas most individuals with SMA and two *SMN2* copies present with Type 1 SMA, a disease modifying mutation suggests reduced severity consistent with SMA Type 2/3. While the relationship between *SMN2* copy number and disease outcomes is strongly correlated, it is not absolute, and individual exceptions do occur. Genetic counseling is recommended.

Genotype:
SMN1 copies: 0
SMN2 copies: 3
c.859G>C: positive

Interpretation: SMA (Type 3 probable)
The *SMN1* copy number indicates SMA. Whereas most individuals with SMA and three *SMN2* copies present with Type 2/3 SMA, a disease modifying mutation suggests reduced severity consistent with SMA Type 3. While the relationship between *SMN2* copy number and disease outcomes is strongly correlated, it is not absolute, and individual exceptions do occur. Genetic counseling is recommended.

Genotype:
SMN1 copies: 0
SMN2 copies: ≥4
c.859G>C: positive

Interpretation: SMA (Type 3/4 probable)
The *SMN1* copy number indicates SMA. Whereas most individuals with SMA and four or more *SMN2* copies present with Type 3/4 SMA, a disease modifying mutation suggests reduced severity. While the relationship between *SMN2* copy number and disease outcomes is strongly correlated, it is not absolute, and individual exceptions do occur. Genetic counseling is recommended.

Genotype:
SMN1 copies: 0
SMN2 copies: 2
c.859G>C: negative

Interpretation: SMA (Type 1 probable)
The *SMN1* copy number indicates SMA. Most individuals with SMA and two *SMN2* copies present with Type 1 SMA. While the relationship between *SMN2* copy number and disease outcomes is strongly correlated, it is not absolute, and individual exceptions do occur. Genetic counseling is recommended.

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