Development of External Controls for SMA Carrier Screening to Accurately Monitor *SMN1*/*SMN2* Copy Number Variation and Disease Modifier Variants

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Introduction

Spinal muscular atrophy (SMA) is a devastating neuromuscular disorder characterized by loss of motor neurons and muscle atrophy, most often during infancy or adolescence. SMA is caused by low levels of the survival motor neuron protein (SMN) due to inactivating mutations in the encoding gene *SMN1*. Diagnostic testing is primarily associated with determination of *SMN1* and *SMN2* copy numbers. Gene therapy treatments resulting in an increase in SMN proteins have become available, however early diagnosis of SMA is critical for providing treatment before the onset of symptoms.

With a carrier frequency of 1 in 40-50 and an estimated incidence of 1 in 10,000 live births, SMA is the second most common autosomal recessive disorder. Population-based SMA carrier screening identifies carrier couples that may pass on this genetic disorder to their offspring. This allows the carriers to make informed reproductive choices or prepare for immediate treatment for an affected child, with treatment strategies including Nusinersen (Spinraza), Onasemnogene abeparvovec (Zolgensma), or Risdiplam (Evrysdi).

A robust and reliable assay for SMA carriers screening diagnostics requires the ability to annotate test results with accurate, precise and reproducible data, thus the need for a comprehensive control to accurately monitor both *SMN1* and *SMN2* copy numbers, as well as important disease modifying variants, is critical. A panel of plasmid-generated controls containing important SMA genetic markers within all exons and intronic junctions of *SMN1* and *SMN2* genes has been developed and verified across various carrier screening tests.

Materials and Methods

A panel of four clinically relevant, synthetic plasmid-based controls were designed and developed by MMQCI for detecting *SMN1* and *SMN2* copy numbers to represent SMA, SMA Carrier, SMA Silent Carrier and Normal status. The plasmid controls were quantified by UV spectrophotometry, suspended in a proprietary liquid stabilizer, and subsequently copy number levels were normalized to a reference plasmid. This control panel consists of 4 vials with different SMA genotypes. SMA Control A (WT) contains 2 copies of *SMN1* and 2 copies of *SMN2*. SMA Control B (SMA Carrier) contains 1 copy of *SMN1* and 3 copies of *SMN2*. SMA Control C (SMA Silent Carrier) contains 3 copies of *SMN1* and 1 copy of *SMN2*. SMA Control D (SMA) contains 0 copies of *SMN1* exon 7 and 2 copies of *SMN2*. Several testing methods were used to confirm accurate copy number variations (CNVs) and to assess performance including ddPCR *SMN1* and *SMN2* Copy Number Determination kits (BioRad, Hercules, CA), AmplideX PCR SMN1/2 Plus kit (Asuragen, Austin, TX), and MRC Holland (Amsterdam, Netherlands) MLPA assay utilizing Probemix P021 SMA, Probemix P060 SMA Carrier Assay and Probemix 460 SMA (Silent) Carrier. To represent a "patient" sample, whole blood was tested alongside the appropriate controls for comparison. To assess variability from vial to vial across at least three lots, each sample was tested using the *SMN1/SMN2* ddPCR-based assay from Bio-Rad.

A schematic workflow for testing is shown in **Figure 1**; a table representing the expected results for each Control component is shown in **Table 1**.

Figure 1. Schematic of Methods for Testing

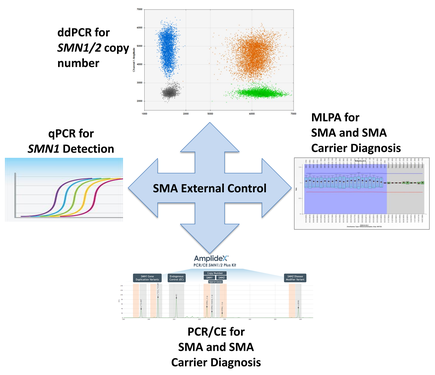

Table 1. Expected Results for SMA Controls

Control Component	*SMN1*, *SMN2* Copies	SMA Status
SMA Control A (2,2)	2 *SMN1* 2 *SMN2*	WT
SMA Control B (1,3)	1 *SMN1* 3 *SMN2*	SMA Carrier
SMA Control C (3,1)	3 *SMN1* 1 *SMN2*	SMA Silent Carrier g.27134T>G g.27706-27707delAT
SMA Control D (0,2)	0 *SMN1* 2 *SMN2*	SMA

Results

SMA External Control Evaluated by ddPCR and qPCR

Table 2. SMA External Control tested using the *SMN1/SMN2* Copy Number Determination Assay (Bio-Rad)[1]. SMA controls tested with *SMN1/SMN2* ddPCR resulted in 100% accurate genotype calls across all tested components. Samples from each lot were tested either in duplicates or triplicates.

					ddPCR			
Control Type	SMN1 Copies	SMN2 Copies	SMA Status	Lot code	Avg SMN1 CNV	StDev	Avg SMN2 CNV	StDev
Coriell (2,2)	2	2	WT	NA00232	2.0	0.0283	1.9	0.0460
Coriell (0,2)	0	2	SMA	NA12878	0.0001	0.0001	2.0	0.0071
SMA Control A (2,2)	2	2	WT	A1	1.9	0.0316	1.9	0.0148
				A2	1.9	0.0219	1.9	0.0118
				A3	1.9	0.0071	1.9	0.0212
SMA Control B (1,3)	1	3	SMA Carrier	B1	1.0	0.0035	3.0	0.0566
				B2	0.9	0.0044	2.9	0.0493
				B3	0.9	0.0240	2.9	0.0212
SMA Control C (3,1)	3	1	SMA Silent Carrier	C1	2.8	0.0283	1.0	0.0170
				C2	2.9	0.0473	1.0	0.0146
				C3	2.7	0.0141	0.9	0.0049
SMA Control D (0,2)	0	2	SMA	D1	0.0003	0.0001	2.1	0.0379
				D2	0.0002	0.0001	1.9	0.0186
				D3	0.0004	0.0001	2.1	0.0141

Table 3. SMA Control A (WT) and Control D (SMA Positive) analyzed using a multi-plex qPCR for *SMN1* and *RPP30* detection[2]. qPCR amplification for detection of human *SMN1* and *RPP30* revealed accurate quantitative results for SMN1 gene target and the reference gene RPP30 in Control A analytes corresponding to WT genotype. Control D assay showed lack of *SMN1* detection corresponding to SMA genotype that identifies the absence of exon 7 in the SMN1 gene. At least three lots of each component were tested. Each lot was run in triplicates by sampling from one vial.

				qPCR					
Control Type	SMN1 Copies	SMN2 Copies	SMA Status	SMN1 Ct	SMN1 Ave Ct	SMN1 STDV	RPP30 Ct	RPP30 Ave Ct	RPP30 STDV
SMA Control A (2,2)	2	2	WT	21.72 21.61 21.69	21.67	0.0569	21.60 21.53 21.54	21.56	0.0379
SMA Control D (0,2)	0	2	SMA	- - -	-	-	21.51 21.51 21.58	21.53	0.0404

SMA External Control Evaluated by MLPA

Table 4. SMA Controls evaluated by SALSA MLPA Probemix P021 SMA assay[3]. Correct ratio reported across 32 MLPA probes, spanning exons 1-8 of *SMN1* and *SMN2* across all 4 control types.

	SALSA MLPA Probemix P021 SMA			
Card Type	SMA Control A (2,2)	SMA Control B (1,3)	SMA Control C (3,1)	SMA Control D (0,2)
SMN1 Copies	2	1	3	0
SMN2 Copies	2	3	1	2
SMA Status	WT	SMA carrier	SMA silent carrier	SMA
Gene-Exon	Ratio	Ratio	Ratio	Ratio
SMN1-7	1.01	0.57	1.44	4%
SMN1-8	1.02	0.90	0.94	0.00
SMN2-7	1.00	1.46	0.59	1.07
SMN2-8	1.00	1.03	0.99	1.05
SMN1&2-Int 6	1.02	0.54	0.56	0.57
SMN1&2-Int 7	1.02	0.98	0.98	0.58
SMN1&2-Int 7	0.99	0.99	1.00	0.57
SMN1&2-Int 7	1.02	0.86	0.86	0.60
SMN1&2-Int 7	0.98	0.98	0.99	0.59
SMN1&2-8	1.00	1.03	1.04	0.60
SMN1&2-8	0.99	1.20	1.02	0.56

Figure 2. Ratio chart of the results of SMA Controls A (WT) and D (SMA Positive) evaluated by SALSA MLPA Probemix P021 SMA assay[3]. A) Control A containing WT *SMN1* and *SMN2*, 2 copies of each gene. **B)** Control D containing 0 copies of exon 7-8 *SMN1* and 2 copies of exon 7 *SMN2*. Red dots display the probe ratios. The error bars are at the 95% confidence ranges. Map view locations are displayed on the x-axis and ratio results on the Y-axis.

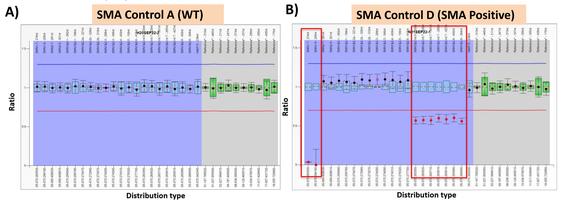

Table 5. SMA External Controls evaluated by SALSA MLPA Probemix P460 SMA (Silent Carrier) assay[3]. Correct copy number variations reported for exon 7 and 8 of *SMN1* and *SMN2*, and accurate detection of the silent carrier risk factor polymorphisms in *SMN1* (g.27134T>G and g.27706-27707delAT).

	SALSA MLPA Probemix P460 SMA (Silent) Carrier			
Card Type	SMA Control A (2,2)	SMA Control B (1,3)	SMA Control C (3,1)	SMA Control D (0,2)
SMN1 Copies	2	1	3	0
SMN2 Copies	2	3	1	2
SMA Status	WT	SMA carrier	SMA silent carrier	SMA
Gene-Exon	Ratio	Ratio	Ratio	Ratio
SMN1-7	1.04	0.57	1.46	0.03
SMN1-8	0.94	0.96	0.94	0.02
SMN2-7	0.96	1.37	0.51	1
SMN1-Intr.7	0	2%	0.60	0
SMN1-8 (MUT)	0	0	0.56	0

SMA External Control Evaluated by MLPA and Amplidex PCR/CE

Figure 3. Ratio chart of the results of SMA Controls A (WT), B (SMA Carrier) , C (SMA Silent Carrier) and D (SMA Positive) evaluated by SALSA MLPA Probemix P060 SMA assay[3]. A) Control A containing WT *SMN1* and *SMN2*, **B)** Control B containing 1 copy of exon 7 *SMN1* and 3 copies of exon 7 *SMN2*, **C)** Control C containing 3 copies of exon 7 *SMN1* and 1 copy of exon 7 *SMN2* and **D)** Control D containing 0 copies of exon 7-8 *SMN1* and 2 copies of exon 7 *SMN2*. Red dots display the probe ratios. The error bars are at the 95% confidence ranges. Map view locations are displayed on the x-axis and ratio results on the Y-axis.

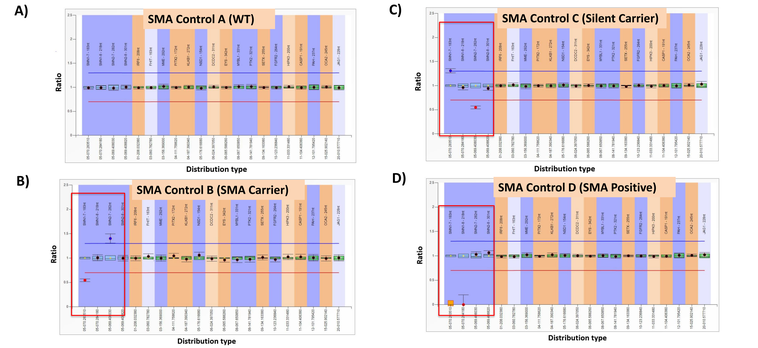

Table 6. SMA Controls evaluated by SALSA MLPA Probemix P060 SMA Carrier assay[3]. Correct copy number variations reported for exons 7 and exon 8 of *SMN1* and *SMN2* across all Control types. Analyses were performed across two to four lots of each component.

	SALSA MLPA Probemix P060 SMA Carrier							
Card Type	SMA Control A (2,2)		SMA Control B (1,3)		SMA Control C (3,1)		SMA Control D (0,2)	
SMN1 Copies	2		1		3		0	
SMN2 Copies	2		3		1		2	
SMA Status	WT		SMA carrier		SMA silent carrier		SMA	
Gene-Exon	Avg Ratio (n=4)	StDev	Avg Ratio (n=3)	StDev	Avg Ratio (n=3)	StDev	Avg Ratio (n=2)	StDev
SMN1-7	1.01	0.0171	0.55	0.0058	1.36	0.0839	3%	0.0071
SMN1-8	0.98	0.0411	0.99	0.0346	0.95	0.0100	0.00	0.0000
SMN2-7	1.00	0.0236	1.42	0.0208	0.56	0.0115	1.04	0.0071
SMN2-8	0.98	0.0377	0.99	0.0173	0.96	0.0265	1.07	0.0141

Table 7. SMA Controls assessed by AmplideX PCR/CE SMN1/2 Plus assay[4]. *SMN1/SMN2* copy number specific PCR/CE-based assay resolved correctly 0 to 3 exon 7 copies of *SMN1* and *SMN2*, as well as variant status of gene duplication and/or gene conversion status in SMA Controls. Analyses were performed in one lot of each component in triplicates.

Control Type	SMA Status	Lot#	*SMN1* copies	*SMN2* copies	AmplideX *SMN1* exon 7 copies	AmplideX *SMN2* exon 7 copies	*SMN1* Gene duplication/ conversion variant
SMA Control A (2,2) 2 *SMN1* 2 *SMN2*	WT	A3	2	2	2	2	N/A
SMA Control B (1,3) 1 *SMN1* 3 *SMN2*	SMA Carrier	B3	1	3	1	3 (with 1 *SMN2* hybrid)	detected
SMA Control C (3,1) 3 *SMN1* 1 *SMN2*	SMA Silent Carrier g.27134T>G g.27706-27707delAT	C3	3	1	3 (with 1 SMN1 hybrid)	1	detected
SMA Control D (0,2) 0 *SMN1* 2 *SMN2*	SMA	D3	0	2	0	2	N/A

Conclusions

- A synthetic plasmid-based SMA control demonstrated reproducible compatibility across multiple platforms assessing *SMN1/SMN2* copy number variation, Bio-Rad ddPCR, MLPA and AmplideX PCR/CE SMN1/2 Plus test methods with 100% correct calls across multiple lots.
- The SMA external control provide the ability to assess routine monitoring of assay determination of copy number of *SMN1* and *SMN2,* and the ability to monitor gene duplication and conversions.
- The SMA external control enables the monitoring of proper detection of clinically relevant variants by SMA and SMA Carrier diagnostic assays.

Acknowledgements

[1]ddPCR performed using *SMN1/SMN2* Copy Number Determination Kits (Bio-Rad, Product Code 1863500/1863503).
[2]*SMN1* and *RPP30* qPCR assays adapted from Taylor JL, Lee FK, Yazdanpanah GK, Staropoli JF, Liu M, Carulli JP, Sun C, Dobrowolski SF, Hannon WH, Vogt RF. Newborn blood spot screening test using multiplexed real-time PCR to simultaneously screen for spinal muscular atrophy and severe combined immunodeficiency. Clin Chem. 2015 Feb;61(2):412-9. doi: 10.1373/clinchem.2014.231019. Epub 2014 Dec 11. PMID: 25502182; PMCID: PMC7906865.
[3]MLPA performed using MRC Holland's SALSA MLPA Probemix P021-B1 SMA, P060-B2 SMA Carrier, and P460-A1 SMA (Silent) Carrier (MRC Holland, Product Code P021-100R, P060-100R, P460-100R).
[4]PCR/CE performed using AmplideX PCR/CE SMN1/2 Plus Kit (Asuragen, Product Code A00050/A00054).