Two-site Evaluation of a Rapid, Multiplexed PCR/CE Assay for Assessment of Spinal Muscular Atrophy *SMN1* and *SMN2* Copy Number Status

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Summary

- Spinal muscular atrophy is a lethal autosomal recessive disease resulting from *SMN1* gene (5q13.2) disruptions.
- The *SMN2* gene can partially compensate for the loss of SMN protein and can modify the severity of the disease.
- We evaluated a streamlined, single-tube AmplideX® PCR/CE prototype assay* for determination of *SMN1* and *SMN2* copies by two laboratory sites (Site 1: Asuragen and Site 2: Rush University Medical Center).
- Both sites successfully genotyped a set of 59 samples, including 3 SMA and 18 carrier samples.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by a mutation in the survival motor neuron 1 gene (*SMN1*) and a primary genetic cause of infant death. The copy number of the highly homologous *SMN2* gene is an important predictor of the severity of SMA, as it has been shown to decrease disease severity in a dose-dependent manner. In recent years, significant progress has been made toward disease modifying treatments for SMA such as the first approved drug, nusinersen (SPINRAZA®), which promotes *SMN2* alternative splicing to enhance the effectiveness of *SMN2* as a functional replacement of *SMN1*. Thus, early detection of SMA along with knowledge of *SMN2* copy number is critical. We developed a single-tube PCR assay (AmplideX® PCR/CE *SMN1*/2) that quantifies *SMN1* and *SMN2* copy number using capillary electrophoresis (CE). Here, we report evaluation of this prototype assay by two laboratories using independently genotyped, residual clinical samples.

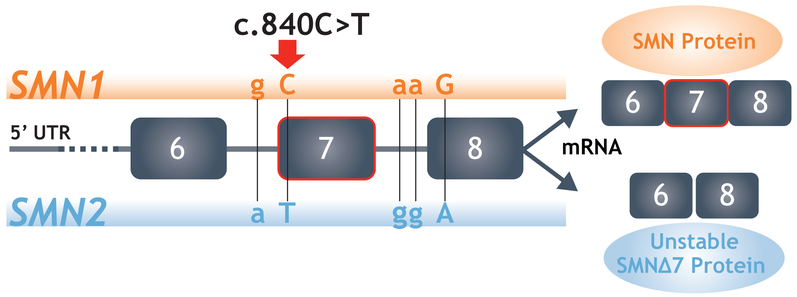

Figure 1. *SMN1* and *SMN2* DNA Sequence Differences. These 2 genes differ by only 5 bases, none of which changes amino acid sequence. The only functional paralogous sequence variant is C to T change in exon 7 (marked by red arrow). While transcriptionally silent, this alteration leads to *SMN2* exon 7 skipping/loss, utilization of an alternative downstream stop codon, and resultant protein degradation.

Materials and Methods

A prototype assay, the AmplideX® PCR/CE *SMN1*/2 Kit, was evaluated at Asuragen (Site 1) and the Rush University Medical Center (Site 2). At each site, PCR products were generated using a Veriti thermal cycler and resolved on a 3500xL Genetic Analyzer (Thermo Fisher Scientific). The electropherogram files were analyzed using GeneMapper® Software 5 (Thermo Fisher Scientific) and the output text file was processed by AmplideX® PCR/CE *SMN1*/2 Macro prototype. Within the macro, the copy number of *SMN1* or *SMN2* was calculated as the peak area ratio of the target gene to an endogenous control, normalized to a calibrator sample. Normalized ratios were further binned into copy number specific bins (separated by gray zones) and results were reported for 0, 1, 2, 3, or ≥4 copies of exon 7.

*Research Use Only. Not for use in diagnostic procedures.

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A common set of 10 cell line samples, 1 residual clinical sample and 2 whole-genome amplified products (derived from samples with *SMN1*/2 hybrid genes) was evaluated in Phase I; a set of 50 residual clinical samples was tested in Phase II (Figure 2). To evaluate accuracy, samples were also characterized using qPCR-based orthogonal assays reporting up to ≥3 *SMN1* or *SMN2* copies (Site 1), and for a subset of samples using a method reporting up to ≥2 copies of *SMN1* (Site 2). In addition, Phase II samples were also tested for *SMN1*-only copies using AmplideX® PCR/CE *SMN1* Kit* (Asuragen, Inc., catalog # 49660) at Site 1.

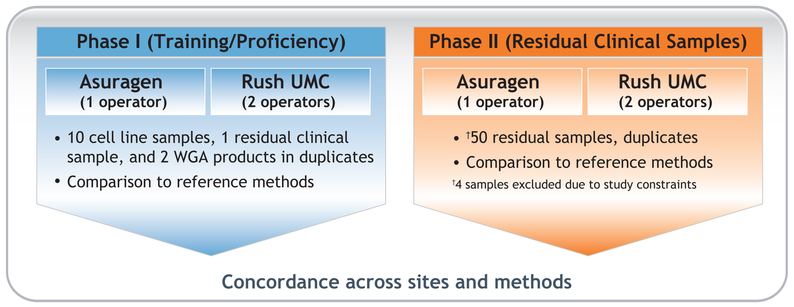


Figure 2. Study Design. Study was executed in 2 stages; Phase I focused on training/proficiency and it utilized mainly cell line samples, while Phase II focused on testing a set of residual clinical samples.

Results

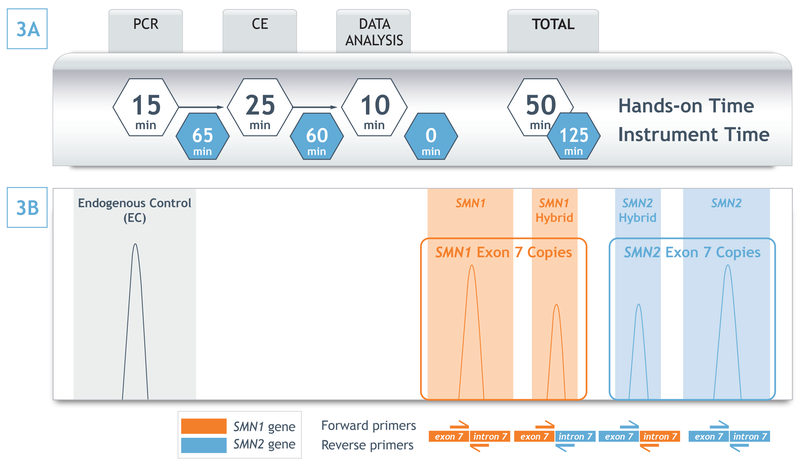


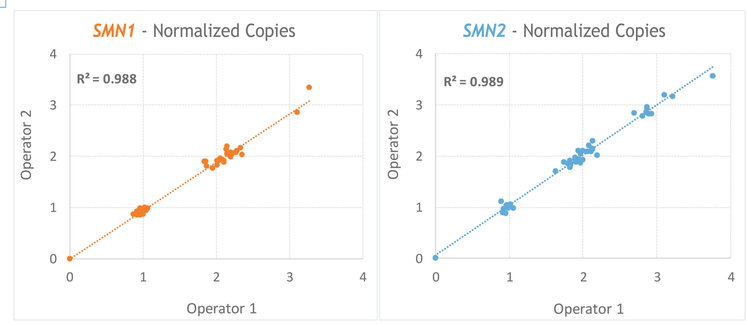
Figure 3. Workflow and Electropherogram Output. A) The workflow is streamlined from sample-to-answer and can be performed in less than 3 hours with ~50 minutes of total hands-on time. **B)** The resultant PCR amplicons are categorized based on size in base pairs, as one or more of the following: EC, *SMN1*, *SMN2*, and/or *SMN1* or *SMN2* hybrid. When present, hybrid peaks indicate exon 7/intron 7 sequence mismatch due to gene conversion. Aggregate exon 7 status is used in the final copy number calculation.

Table 1. Phase I Results for Site 2 Training Runs (Normalized, Pre-Binned Copy Numbers). All results matched expected copy numbers, generated using a commercially available qPCR-based assay. WGA products (P271 and K85) were generated from primary blood gDNA and represented gene conversions as confirmed by phased long-read sequencing.

Training Sample ID	*SMN1* copies			*SMN1* hybrid			*SMN2* hybrid			*SMN2* copies		
	Run1	Run2	Expected	Run1	Run2	Expected	Run1	Run2	Expected	Run1	Run2	Expected
Calibrator	2.0	2.0	2							2.0	2.0	2
Control	2.0	2.1	2							3.1	3.3	≥3
NTC	ND	ND	0							ND	ND	0
8804 (residual clinical)	1.0	1.0	1							3.0	3.1	NA
TR01	2.0	1.9	2							2.1	2.0	2
TR02	2.0	1.9	2							3.2	3.1	≥3
TR03	2.1	2.0	2							2.1	2.0	2
TR04	2.1	2.0	2							2.2	2.1	2
TR05	2.0	1.9	2							2.1	2.0	2
TR06	2.0	2.0	2							1.0	1.0	1
TR07	2.0	1.9	2							1.0	1.0	1
TR08	2.0	1.9	2							1.0	1.0	1
TR09	1.9	1.9	2							2.9	3.1	≥3
TR10	2.0	1.9	2							3.1	3.1	≥3
WGA_P271	2.2	2.0	2	1.0	1.0	1				2.0	2.0	2
WGA_K85	1.7	1.7	2				0.8	0.9	1	0.0	0.0	0

NTC: No Template Control; ND: Not Detected; NA: Not Available

4A Operator-to-Operator correlations for *SMN1* and *SMN2*



4B Site-to-Site correlations for *SMN1* and *SMN2*

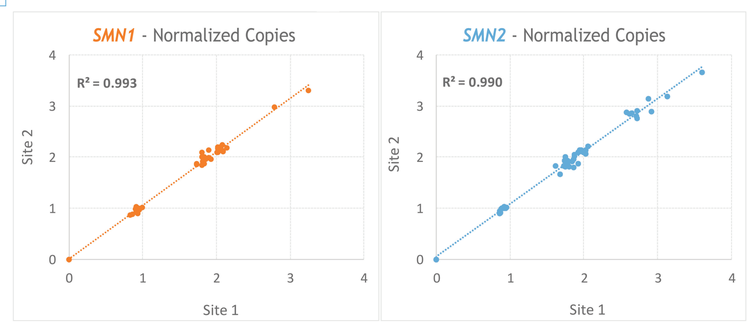


Figure 4. Correlation Plots of Average Normalized Copy Numbers (Copy Numbers Prior to Binning) for Phase II Samples. A) Operator-to-Operator comparison (performed at Site 2) and **B)** Site-to-Site comparison showing correlation coefficient of >0.98 for both *SMN1* and *SMN2*.

Table 2. Phase II Results. Copy-number calls for *SMN1* were 100% concordant across both sites, and with Reference methods 1 and 2, for the 46 residual clinical samples that were assessed post-training. *SMN2* calls were concordant for 45/46 (98%) samples between the two sites, for 45/46 (98%) samples between Site 1 and Reference method 2, and for 44/46 (96%) samples between Site 2 and Reference method 2.

Phase II results		0 copy	1 copy	2 copy	3 copy	≥4 copy
SMN1	Site 1	3	17	24	1	1
	Site 2	3	17	24	1	1
	‡Reference 1	3	17	24	1	1
	§Reference 2	3	17	24	2	
SMN2	Site 1	2	10	24	9	1
	Site 2	2	10	23	10	1
	§Reference 2	2	10	25	9	

‡AmplideX® PCR/CE *SMN1* Kit (catalog # 49660)
§qPCR-based orthogonal reference method (reports 0, 1, 2 and ≥3 copies of *SMN1* or *SMN2*)

Conclusions

- The prototype AmplideX PCR/CE *SMN1*/2 assay has a rapid workflow, requiring only 3 hours from sample-to-answer.
- Assay accurately quantifies gene copy numbers in cell-line and blood-derived samples, reporting 0, 1, 2, 3, or ≥4 gene copies for both *SMN1* and *SMN2*.
- Copy number calls for *SMN1* were 100% concordant across both sites and with the 2 reference methods; *SMN2* copy number calls were concordant for 98% samples across the sites and 96-98% across different methods and sites.

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