Accurate Single-Tube Quantification of *SMN1* and *SMN2* Copy Numbers Using a Rapid and Streamlined PCR/CE Assay Evaluated at Two Different Laboratories

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Summary

- Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disease and a leading genetic cause of infant mortality.
- The severity of SMA is often modulated by the copy number of the paralogous *SMN2* gene.
- We report a two-site evaluation of a single-tube *SMN1/2* PCR/CE assay that quantifies *SMN1* and *SMN2* copy number (Site 1: Centro Hospitalar Porto, Site 2: Asuragen).
- Across both sites, the *SMN1*/2 PCR/CE assay accurately genotyped 60 samples, including 15 SMA and 13 carrier samples, using a rapid and simple workflow.

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disease and a leading genetic cause of infant mortality. SMA is commonly caused by homozygous exon 7 deletions in the survival motor neuron 1 gene (*SMN1*). The severity of SMA is modulated by the copy number of the paralogous *SMN2* gene. With significant progress made toward disease modifying treatments for SMA, such as the first approved drug nusinersen (SPINRAZA®), early detection of SMA along with knowledge of *SMN2* copy number is critical. Here, we report the two-site evaluation of a single-tube assay that quantifies *SMN1* and *SMN2* copy number.

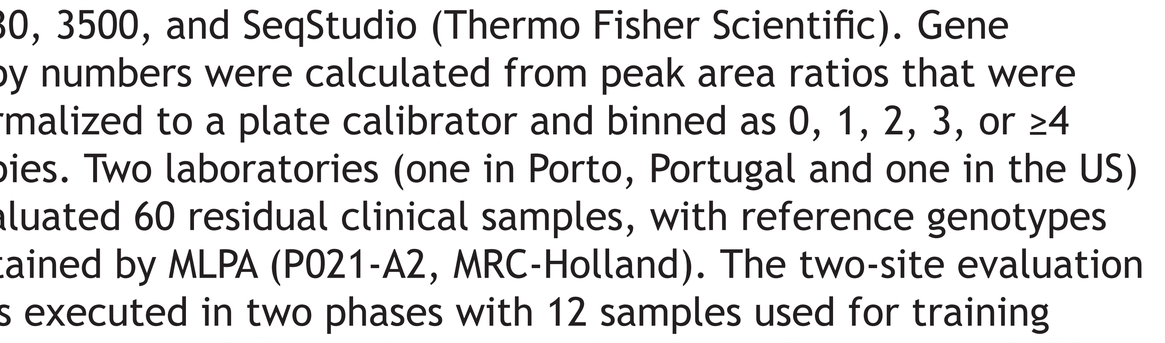
Materials and Methods

A prototype *SMN1*/2 single-tube PCR was developed from AmplideX® PCR/CE *SMN1* reagents* (Asuragen). Amplicons generated from whole blood genomic DNA were resolved by capillary electrophoresis (CE) on 3 different genetic analyzers: 3130, 3500, and SeqStudio (Thermo Fisher Scientific). Gene copy numbers were calculated from peak area ratios that were normalized to a plate calibrator and binned as 0, 1, 2, 3, or ≥4 copies. Two laboratories (one in Porto, Portugal and one in the US) evaluated 60 residual clinical samples, with reference genotypes obtained by MLPA (P021-A2, MRC-Holland). The two-site evaluation was executed in two phases with 12 samples used for training (Phase 1) and 48 for clinical evaluation (Phase 2) using the 3130 genetic analyzer. In addition, 60 samples, including a subset of 16 samples from the initial study, were used to compare the 3500 and SeqStudio CE instruments at Site 2. Data were analyzed using GeneMapper Software (Thermo Fisher Scientific) and copy numbers were calculated using AmplideX PCR/CE *SMN1*/2 Macro (Asuragen).

1A

PCR Cycles	CE Instrument, Capillary Length	Injection	Pre-Run	Run	Oven Temp
27	3130xl, 36 cm	2.5 kV, 20 s	15 kV, 900 s	15 kV, 1500 s	60°C
25	3500xL, 50 cm	2.5 kV, 20 s	15 kV, 900 s	19.5 kV, 2100 s	60°C
25	SeqStudio	6 kV, 2 s	13 kV, 180 s	6 kV, 3000 s	60°C

1B



Normalized Ratio		Copy Number
SMN1	*SMN2*	
≤0.200	≤0.200	0
0.300-0.600	0.305-0.615	1
0.700-1.125	0.700-1.070	2
1.170-1.570	1.095-1.650	3
≥1.615	≥1.675	≥4

Figure 1. PCR/CE Assay and Data Analysis Workflow. A) PCR cycles and injection conditions recommended for specific CE instruments. **B)** Data analysis workflow. Normalized ratios are calculated for each peak by dividing peak area by endogenous control peak area and normalizing to the same ratio derived from the calibrator sample (included in each batch). Normalized ratios are then placed within copy number bins, and results are reported as 0, 1, 2, 3, or ≥4 copies.

Results

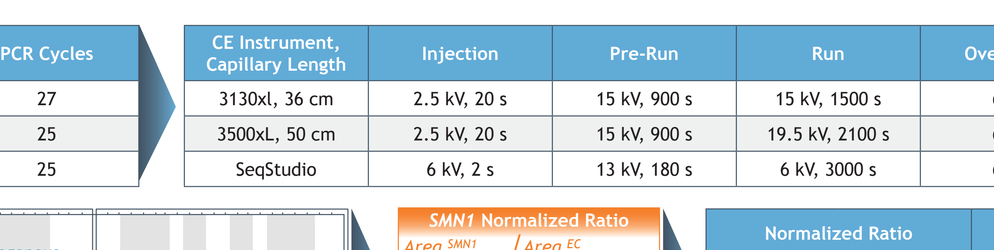


Figure 2. The *SMN1/2* PCR/CE Assay Workflow is Streamlined from Sample-to-Answer. The assay can be performed with a turnaround time of ~3 hours for 1 capillary electrophoresis injection (4-24 samples) with ~50 min of total hands-on time.

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Table 1. Phase 1 Results from Sites 1 and 2 for Normalized, Pre-binned *SMN1* and *SMN2* Copy Numbers. Both sites tested a set of 12 residual clinical samples spanning 0 to ≥4 copies for both *SMN1* and *SMN2* as part of Phase 1 training and proficiency. All results matched expected copy numbers determined using MLPA.

Sample Name	Expected *SMN1* Copies	Site 1	Site 2	Expected *SMN2* Copies	Site 1	Site 2
AS_CHP_I-01	0	0.0	0.0	2	1.9	1.9
AS_CHP_I-02	2	1.9	1.8	1	0.9	0.9
AS_CHP_I-03	2	2.1	1.8	1	1.0	0.9
AS_CHP_I-04	0	0.0	0.0	3	2.8	2.6
AS_CHP_I-05	0	0.0	0.0	3	2.8	2.8
AS_CHP_I-06	4	4.1	3.6	0	0.0	0.0
AS_CHP_I-07	3	2.9	2.6	1	0.9	0.9
AS_CHP_I-08	1	1.1	1.0	1	1.0	1.1
AS_CHP_I-09	1	1.0	0.8	2	1.9	1.8
AS_CHP_I-10	2	2.1	1.8	2	1.9	1.9
AS_CHP_I-11	0	0.0	0.0	4	3.8	3.5
AS_CHP_I-12	1	1.0	0.9	4	4.9	4.6

Phase 1 and 2 Results

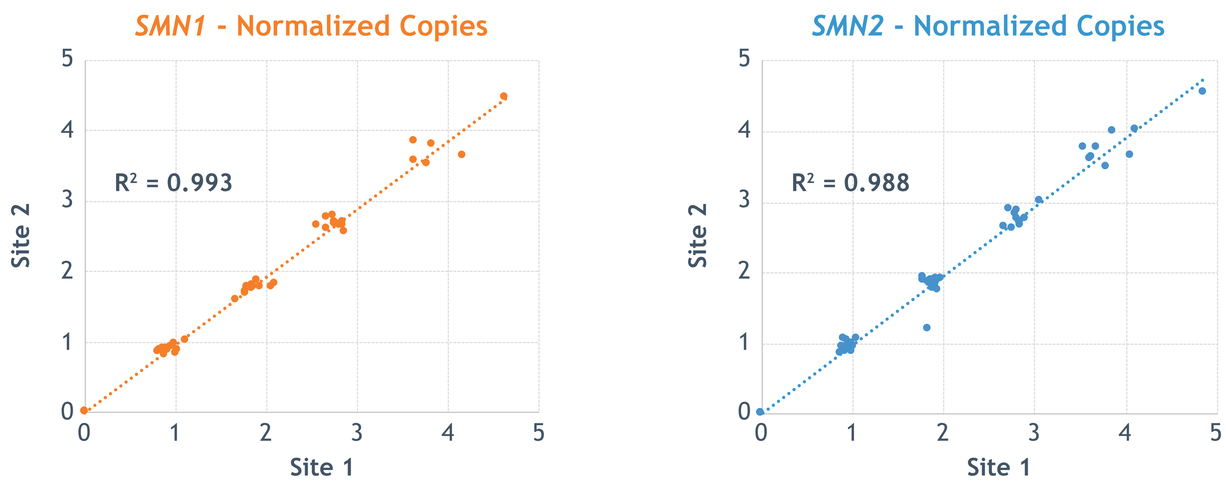

Figure 3. Site-to-Site Comparisons of *SMN1* and *SMN2* Normalized Copy Numbers from Phase 1 and 2 Studies. *SMN1* and *SMN2* plots of averaged, normalized copy numbers for 60 samples tested on the 3130 genetic analyzer. The site-to-site correlation coefficient was >0.98 for both *SMN1* and *SMN2* copy numbers.

Table 2. Site-to-Site Call Concordance and Comparisons with Reference Results. Binned copy-number calls for *SMN1* were 100% concordant across both sites and with a Reference method. *SMN2* calls were concordant for 59/60 (98.3%) samples between the two sites, for 60/60 (100%) samples between Site 1 and the Reference method, and for 59/60 (98.3%) samples between Site 2 and the Reference method.

		0 copy	1 copy	2 copy	3 copy	≥4 copy
SMN1	Site 1	15	13	14	12	6
	Site 2	15	13	14	12	6
	Reference	15	13	14	12	6
SMN2	Site 1	7	14	19	11	9
	Site 2	7	15	18	11	9
	Reference	7	14	19	11	9

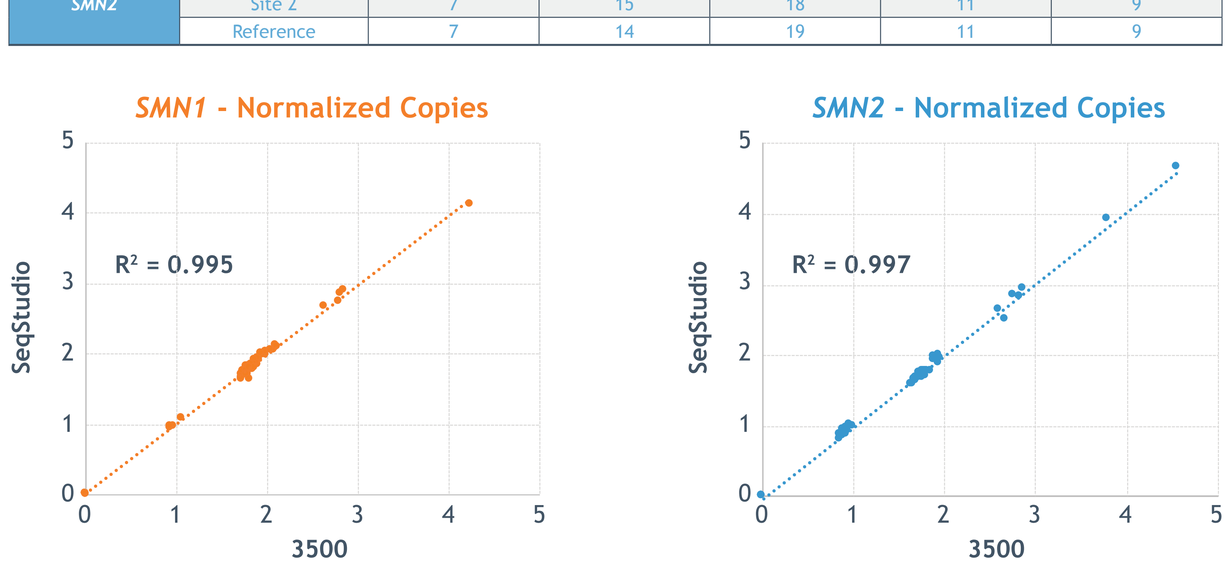

Figure 4. CE Instrument Agreement for Normalized Copy Numbers. *SMN1* and *SMN2* plots of averaged, normalized copy numbers for 60 samples (16/60 samples from Phases 1 & 2 plus 44 additional blood derived samples tested at Site 2 only). 3500 and SeqStudio comparison demonstrated a correlation coefficient of >0.99 for both *SMN1* and *SMN2*. Correlation coefficients were also >0.99 for all pairwise combinations of *SMN1* and *SMN2* copy-number calls using the 3130, 3500, and SeqStudio for 16 common samples.

Conclusions

- The single-tube AmplideX® PCR/CE *SMN1/2* assay* accurately quantifies 0, 1, 2, 3, or ≥4 gene copies for both *SMN1* and *SMN2* from blood specimens using a ~3 hour workflow.
- Copy-number calls for *SMN1* were 100% concordant across two laboratory sites and with a Reference method; *SMN2* copy-number calls were 98% concordant across the sites (using a 3130 CE instrument).
- Accurate copy-number quantification was also observed on the 3500 and SeqStudio genetic analyzers, with instrument-to-instrument correlations of >99%.

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