Verification of a Single-tube PCR/CE Kit for *SMN1/2* Copy Number and Variants Associated with Gene Duplication and SMA Disease Severity

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Summary

- The AmplideX® PCR/CE *SMN1/2* Plus Kit evaluates exon 7 copy number of *SMN1* and *SMN2*, and variant status of gene duplication and disease modifier markers.
- This kit design was verified on whole blood DNA from multiple purification methods across an input range of 20 to 80 ng, and tested across operators, thermal cyclers, and genetic analyzers.
- Integrated AmplideX Reporter software automates quality control checks, peak detection and area integration, and provides a detailed sample genotype report.
- The AmplideX PCR/CE *SMN1/2* Plus Kit is a single-tube, streamlined workflow with robust, accurate, and highly specific performance.

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disease that results from mutation of the survival motor neuron 1 gene (*SMN1*). SMA molecular assessment is complicated by high homology between *SMN1* and *SMN2*, and only differ at their 3' ends by five nucleotides. Approximately 95% of SMA cases are caused by a deletion of exon 7 in both alleles of the *SMN1* gene, the result of a single functional base change in *SMN1* exon 7 (c.840C>T) that disrupts a splicing enhancer element. Of particular concern is the "silent carrier" population that have *SMN1* gene duplication on one chromosome and a deletion on the other (2+0 genotype). Two *SMN1* variants, c.*3+80T>G and c.*211_*212del, are found in linkage disequilibrium with a *SMN1* duplication haplotype and can help identify "silent carriers". In addition, SMA severity is directly influenced by the ability of *SMN2* to produce functional SMN protein. An *SMN2* variant, c.859G>C, improves inclusion of *SMN2* exon 7 in the full length transcript, and is associated with reduced disease severity[1].

Here we describe the analytical performance of a robust, rapid, and accurate PCR/CE assay and analysis tool that can resolve 0 to ≥4 exon 7 copies of *SMN1* and *SMN2*, as well as variant status of gene duplication and disease modifier markers.

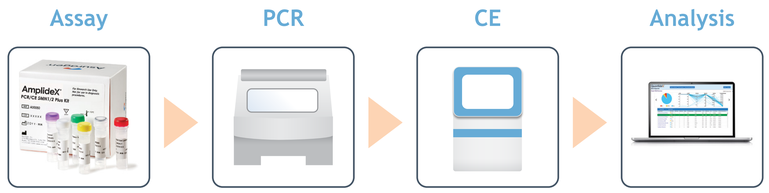

Figure 1. Assay Workflow. The simplicity of the AmplideX technology comes complete with all reagents needed for preparing both PCR and CE, and an automated analysis tool that dramatically improves analysis time and provides copy number quantification and variant detection from a single reaction.

Materials and Methods

Fifty-seven unique, residual clinical DNA samples, purified by column, precipitation, or magnetic bead-based methods, including 8 cell line DNAs (Coriell Institute for Medical Research) were tested to assess the AmplideX PCR/CE *SMN1/2* Plus Kit performance. Samples were evaluated for accuracy, precision, specificity, and DNA input range. PCR products were generated using an ABI Veriti thermal cycler and resolved on the Applied Biosystems™ 3500xL, 3130xl, 3730xl, and SeqStudio™ Genetic Analyzers.

Reference genotypes were determined by MLPA, a qPCR-based method, and the AmplideX PCR/CE *SMN1/2* Kit for *SMN1* and *SMN2* copy number, and Sanger Sequencing for markers associated with gene duplication and disease modification.

FSA files were analyzed using the AmplideX PCR/CE Reporter and AmplideX PCR/CE SMN analysis module. *SMN1* and *SMN2* exon 7 copy number was calculated as the peak area ratio of the target gene to an endogenous control (EC), and then normalized to the SMN Calibrator. The resultant normalized ratios were binned into copy number specific bins and results were reported as 0, 1, 2, 3, ≥4 exon 7 copies of *SMN1* and *SMN2*. In addition, gene duplication and disease modifier status was reported as positive or negative.

2A

2B

Samples												
Sample	Well	*SMN1* Normalized Ratio	*SMN1* Exon 7 Copy Number	c.*3+80T>G	c.*211_*212del	*SMN2* Normalized Ratio	*SMN2* Exon 7 Copy Number	c.859G>C	Hybrid Peak	QC	Rerun
Sample44-EXP1-PCR01-CE01-3500-D01-2019-08-26-17-31-05-01	D01	1.032	3	Negative	Negative	0.519	1	Negative	SMN1	PASS	0

2C

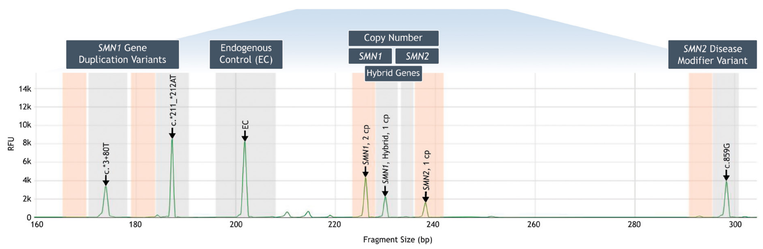

Figure 2. Total-Assay-Time and Electropherogram Output. A) The workflow is streamlined from sample-to-result in less than 4 hours with 1 hour of hands-on time. **B)** The AmplideX PCR/CE Reporter interface displays *SMN1* and *SMN2* normalized ratios, exon 7 copy number, and positive/negative detection of gene duplication and disease modifier variants. Results are easily exported as PDF and CSV files. **C)** Fluorescently-labeled PCR amplicons are characterized by size (in base pairs) on ABI Genetic Analyzers as one of the following: EC, *SMN1*, *SMN2*, *SMN1* or *SMN2* gene conversion, gene duplication, or disease modifier.

Results

1A

	Expected *SMN1* Copy						
Predicted *SMN1* Copy	0	1	2	3	4	Sum
0	30	0	0	0	0	30
1	0	82	0	0	0	82
2	0	0	253	0	0	253
3	0	0	0	87	0	87
4	0	0	0	5	64	69
PR	0	0	2	4	1	7
Sum	30	82	255	96	65	528

1B

	Expected *SMN2* Copy						
Predicted *SMN2* Copy	0	1	2	3	4	Sum
0	137	0	0	0	0	137
1	0	113	0	0	0	113
2	0	0	138	0	0	138
3	0	0	0	97	0	97
4	0	0	0	1	29	30
PR	0	0	5	8	0	13
Sum	137	113	143	106	29	528

Table 1. Accuracy Study. The accuracy panel consisted of eighty DNA samples purified from residual clinical whole blood by column, precipitation, or magnetic bead-based methods, 4 samples from an unknown source, and 6 Coriell cell line DNA samples were analyzed in singleton on 6 ABI Genetic Analyzer configurations. Reference method exon 7 copy number was determined by an external MLPA assay, a qPCR-based method, or the AmplideX PCR/CE *SMN1/2* Kit. Agreement with expected copy number was determined for **A)** *SMN1* and **B)** *SMN2*. 0 and 1 copy *SMN1* samples exhibited perfect concordance with expected copy number (red box). Precision (PR) QC failures were excluded from analysis.

Platform	Capillary Length (cm)	*SMN1*	*SMN2*	c.*3+80T>G	c.*211_*212del	c.859G>C
3130	36	261/266	256/262	268/268	268/268	268/268
3500	36	261/263	257/259	269/269	269/269	269/269
	50	259/262	254/259	268/268	268/268	268/268
3730	36	258/261	253/261	268/268	268/268	268/268
	50	247/252	248/254	263/263	263/263	263/263
SeqStudio	28	258/259	254/259	269/269	269/269	269/269
Total		98.8% (1544/1563)	97.9% (1522/1554)	100% (1605/1605)	100% (1605/1605)	100% (1605/1605)

Table 2. Overall Assay Agreement. Across all studies performed, the *SMN1*, *SMN2*, gene duplication (c.*3+80T>G and c.*211_*212del), and disease modifier (c.859G>C) results were ≥96.9% agreement per configuration with the reference method. QC failures were removed from analysis.

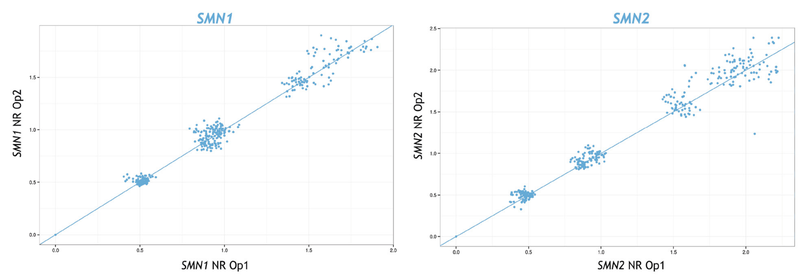

Figure 3. Within-lab Precision Correlation Plots. The within-lab precision study included nine gDNA samples purified from human cell lines or whole blood with 0, 1, 2, 3, or ≥4 *SMN1* copies and 0, 1, 2, 3, or ≥4 *SMN2* copies. Samples were purified using precipitation, functionalized magnetic bead, or silica column-based purification methods and tested in duplicate across eight batch runs using two operators, one reagent lot, and two Applied Biosystems® Veriti™ 96-well thermal cyclers, generating 16 measurements per sample (144 total measurements). Operator-to-operator comparison shows high correlation of normalized ratios (NR) for both *SMN1* and *SMN2*.

Platform	Capillary Length (cm)	*SMN1*	*SMN2*
3130	36	100.0% (35/35)	100.0% (35/35)
3500	36	100.0% (34/34)	100.0% (36/36)
	50	100.0% (35/35)	100.0% (36/36)
3730	36	100.0% (35/35)	100.0% (36/36)
	50	96.9% (31/32)	100.0% (36/36)
SeqStudio	28	100.0% (32/32)	100.0% (35/35)

Table 3. DNA Input Study. One residual clinical DNA sample from whole blood and three Coriell cell line DNA samples were tested in triplicate with three DNA inputs of 20, 40, and 80 ng per PCR (20, 40, and 60 ng for 3130xl). Results support assay DNA input range of 20 to 60 ng per PCR for 3130 Genetic Analyzers, and 20 to 80 ng per PCR for 3500, 3730, and SeqStudio Genetic Analyzers.

Conclusions

- We describe a comprehensive PCR/CE assay verified with 1605 FSA files that exhibits high reproducibility and can rapidly resolve *SMN1* and *SMN2* exon 7 copy numbers and detect variants associated with gene duplication and improved *SMN2* splicing.
- Specificity was 100% for expected 0 copies of *SMN1* and *SMN2*, and expected wild type results for gene duplication (c.*3+80T>G, c.*211_*212del) and disease modifier (c.859G>C) markers.
- The assay accurately quantifies 0, 1, 2, 3, and ≥4 exon 7 copy numbers of *SMN1* and *SMN2* with >95.1% agreement to a reference method across six CE configurations, and 100% variant status agreement with Sanger sequencing.
- Used in conjunction with the AmplideX PCR/CE Reporter software, the AmplideX PCR/CE *SMN1/2* Plus Kit enables sample-to-answer results with 1 hour of hands-on-time and <4 hours from gDNA to answer.

REFERENCES

1. Vezain, M. et al. A rare *SMN2* variant in a previously unrecognized composite splicing regulatory element induces exon 7 inclusion and reduces the clinical severity of Spinal Muscular Atrophy. Human Mutation; 31 (1). E1110-25 (2010).

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