Multisite Evaluation and Validation of a Sensitive Diagnostic and Screening System for Spinal Muscular Atrophy that Reports SMN1 and SMN2 Copy Number, along with Disease Modifier and Gene Duplication Variants

Milligan, John N.; Larson, Jessica L.; Filipovic-Sadic, Stela; Laosinchai-Wolf, Walairat; Huang, Ya-Wen; Ko, Tsang-Ming; Abbott, Kristin M.; Lemmink, Henny H.; Toivonen, Minna; Schleutker, Johanna

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*
Publisher's PDF, also known as Version of record

*Citation for published version (APA):*
Milligan, J. N., Larson, J. L., Filipovic-Sadic, S., Laosinchai-Wolf, W., Huang, Y-W., Ko, T-M., Abbott, K. M., Lemmink, H. H., Toivonen, M., Schleutker, J., Gentile, C., Van Deerlin, V. M., Zhu, H., & Latham, G. J. (2021). Multisite Evaluation and Validation of a Sensitive Diagnostic and Screening System for Spinal Muscular Atrophy that Reports SMN1 and SMN2 Copy Number, along with Disease Modifier and Gene Duplication Variants. *Journal of molecular diagnostics*, *23*(6), 753-764. https://doi.org/10.1016/j.jmoldx.2021.03.004

# Multisite Evaluation and Validation of a Sensitive Diagnostic and Screening System for Spinal Muscular Atrophy that Reports *SMN1* and *SMN2* Copy Number, along with Disease Modifier and Gene Duplication Variants

Check for updates

John N. Milligan,* Jessica L. Larson,* Stela Filipovic-Sadic,* Walairat Laosinchai-Wolf,* Ya-Wen Huang,[†] Tsang-Ming Ko,[†] Kristin M. Abbott,[‡] Henny H. Lemmink,[‡] Minna Toivonen,[§] Johanna Schleutker,[§¶] Caren Gentile,[‖] Vivianna M. Van Deerlin,[‖] Huiping Zhu,* and Gary J. Latham*

*From Research and Development, Asuragen Inc.,* Austin, Texas; the GenePhile Bioscience Laboratory,[†] Ko's Obstetrics and Gynecology Clinic, Taipei City, Taiwan; the Department of Genetics,[‡] University Medical Center Groningen, Groningen, the Netherlands; Department of Medical Genetics, Genomics, Laboratory Division, Turku University Hospital,[§] Turku, Finland; Institute of Biomedicine, University of Turku,[¶] Turun yliopisto, Finland; and the Hospital of the University of Pennsylvania,[‖] Philadelphia, Pennsylvania*

Spinal muscular atrophy is a severe autosomal recessive disease caused by disruptions in the *SMN1* gene. The nearly identical *SMN2* gene copy number is associated with disease severity. *SMN1* duplication markers, such as c.*3+80T>G and c.*211_*212del, can assess residual carrier risk. An *SMN2* disease modifier (c.859G>C) can help inform prognostic outcomes. The emergence of multiple precision gene therapies for spinal muscular atrophy requires accurate and rapid detection of *SMN1* and *SMN2* copy numbers to enable early treatment and optimal patient outcomes. We developed and evaluated a single-tube PCR/capillary electrophoresis assay system that quantifies *SMN1/2* copy numbers and genotypes three additional clinically relevant variants. Analytical validation was performed with human cell lines and whole blood representing varying *SMN1/2* copies on four capillary electrophoresis instrument models. In addition, four independent laboratories used the assay to test 468 residual clinical genomic DNA samples. The results were ≥98.3% concordant with consensus *SMN1/2* exon 7 copy numbers, determined using multiplex ligation-dependent probe amplification and droplet digital PCR, and were 100% concordant with Sanger sequencing for the three variants. Furthermore, copy number values were 98.6% (*SMN1*) and 97.1% (*SMN2*) concordant to each laboratory's own reference results. *(J Mol Diagn 2021, 23: 753–764; https://doi.org/10.1016/j.jmoldx.2021.03.004)*

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by the loss of survival motor neuron 1 (*SMN1*) and is characterized by progressive proximal muscle weakness and atrophy, resulting from degeneration of the α motor neurons.[1,2] Affected individuals lose cells within the anterior horn gray matter of the spinal cord and experience widespread skeletal muscle atrophy, respiratory failure, and death.[3] Clinical SMA type is determined by age of onset and severity of muscle weakness. SMA occurs in approximately 1 in 6000 to 10,000 live births worldwide,

and is one of the leading inherited causes of infant death.[4–8] Nondiseased individuals have at least one functional copy of the *SMN1* gene, and most individuals have one on each

chromosome (1 + 1). Approximately 95% of SMA cases are due to a deletion in both chromosomes of exon 7 in the *SMN1* gene (0 + 0). Nearly all of the remaining 5% of cases are compound heterozygotes, with a pathogenic point mutation in *SMN1* on one chromosome and an *SMN1* deletion on the other ($1^d$ + 0). Both *SMN1* and its paralog, *SMN2*, encode the same protein, SMN, and can have a range of copy number combinations.[9,10] *SMN2* has >99.9% sequence identity to *SMN1*, and the only coding-region base difference, NM_000344.3:c.840C>T (Single Nucleotide Polymorphism database, *https://www.ncbi.nlm.nih.gov/snp*, accession number rs1164325688, build 154, last accessed March 17, 2021) in exon 7, results in inefficient *SMN2* exon 7 splicing and predominantly nonfunctional protein products.[11] There is an inverse relationship between disease severity and *SMN2* copy number. Furthermore, a single-nucleotide polymorphism, *SMN2* NM_017411.3:c. 859G>C (dbSNP, rs121909192, build 154, last accessed March 17, 2021), has recently been associated with a less severe SMA phenotype[12–16]; this variant results in a new exonic splicing enhancer, thereby increasing the number of full-length transcripts and functional SMN.

Advances in characterizing the genetic landscape of SMA have enabled rapid development of targeted therapy by the medical community in the recent years. For example, nusinersen (Spinraza, Biogen, Cambridge, MA) is an antisense oligonucleotide that modulates alternate splicing of *SMN2*,[3,17,18] whereas onasemnogene abeparvovec-xioi (Zolgensma, Novartis, Chicago, IL) is a gene replacement therapy[19,20] and risdiplam (Evrysdi, Roche, Basel, Switzerland) is a small-molecule *SMN2* splicing modifier.[21–23] All three therapies prevent development or delay the progression of SMA symptoms. These treatments have driven a demand for increasingly more reliable, rapid, and cheaper molecular diagnostics, particularly those that can be used presymptomatically when the therapies are most effective.[24]

Because of the high global incidence, serious clinical implications, and newly available treatments, the American College of Obstetricians and Gynecologists recommends DNA screening for SMA for all couples seeking preconception or prenatal care, independent of family history.[25] The *SMN1* deletion carrier rate is approximately 1 of 50 individuals for the typical carrier genotype (1 + 0), ranging from 1.4% to 2.2% among different ethnic groups.[8] Detection rates range from 71% to 95% across different populations because of the inability to identify silent carriers [ie, individuals who have two copies of *SMN1* in *cis* on one chromosome and a deletion on the other (2 + 0)]; these carriers are indistinguishable from typical genotypes (1 + 1) based on *SMN1* copy number alone. Two variants, NM_000344.3:c.*3+80T>G (alias g.27134T>G; dbSNP, rs143838139, build 154, last accessed March 17, 2021) and NM_000344.3: c.*211_*212del (alias g.27706_27707delAT; dbSNP, rs200800214, build 154, last accessed March 17, 2021), are associated with an *SMN1* duplication haplotype block in some ethnicities.[26] As a result of this linkage, these markers can flag silent carriers, increase carrier detection rates from 90% to 94% in Ashkenazi Jewish populations, and inform residual carrier risk in all ethnicities examined to date, including Africans/African Americans, Europeans, Asians, and Hispanics.[26–28]

PCR-based methods and next-generation sequencing (NGS) are commonly used in SMA molecular diagnosis and carrier screening, but both have limitations. For example, multiplex ligation-dependent probe amplification (MLPA) is often used to determine *SMN1/2* copy number changes and query silent carrier markers for SMA diagnosis and carrier screening,[29] but it has a complex 2-day workflow that can be challenging to execute and requires screening and selection of multiple calibrator samples for analysis. Furthermore, some reports have questioned the accuracy and consistency of copy number values from MLPA.[30] Other studies have shown generally consistent results from MLPA, suggesting that reliable analyses may depend on the isolation method, calibration selection, or other laboratory-specific factors.[31,32] Duplex PCR melting analysis has been recommended for carrier screening, but it can only discriminate one *SMN1* copy from two or more *SMN1* copies.[33] Droplet digital PCR (ddPCR) can determine *SMN1* and *SMN2* copy numbers with resolution similar to other methods but has limited multiplexing capacity and requires a separate assay to detect silent carrier markers.[34] Some specialty clinical laboratories offer SMA carrier screening using NGS,[28,35–40] yet this already-complex method is further complicated by the high homology between *SMN1* and *SMN2* genes and frequent occurrence of hybrid genes that confound standard bioinformatics analyses and cause inaccuracies in quantifying the copy numbers of each gene.[28,38,41]

Herein, we describe the design, workflow, and performance evaluation of a sensitive, streamlined, and rapid PCR/capillary electrophoresis (CE) assay system. This single-tube assay quantifies from zero to four or more *SMN1* and *SMN2* copies, detects *SMN1/2* chimeras, and genotypes *SMN1* c.*3+80T>G and *SMN1* c.*211_*212del gene duplication variants along with *SMN2* c.859G>C, associated with SMA disease severity. The system was developed with a copy-number calibrator and control, and analysis software that integrates calibration, control results, and sample-specific data to produce copy-number and variant genotype results (Figure 1).

## Materials and Methods

### Samples

All human-derived, residual clinical specimens were de-identified for use in these studies. Each participating laboratory obtained informed consent from participants and/or had the required regulatory approvals for evaluating clinical specimens. Genomic DNA (gDNA) was isolated from peripheral blood or amniotic cells using 13 different isolation kits across typical laboratory methods, including salting out/precipitation-based, silica resin/column-based, and

functionalized magnetic bead methods (Supplemental Table S1). Cell-line gDNA was purified using a precipitation-based method (Qiagen, Hilden, Germany; Autopure LS) and obtained from Coriell Institute for Medical Research (Camden, NJ). gDNA quantity (ng/μL) and quality (A260/A280) were assessed using spectrophotometry. gDNA samples were diluted in nuclease-free water to the target concentration for analysis using the assay.

## Study Design

The performance of the assay was evaluated with analytical validation studies designed and conducted according to Clinical and Laboratory Standards Institute guidelines[42,43] as well as a multilaboratory evaluation study at four clinical laboratories worldwide. In all studies, gDNA samples were amplified in a single-tube PCR using kitted reagents [AmplideX SMA Plus catalog numbers A00055 and A00056 (CE-in vitro diagnostic) or AmplideX PCR/CE SMN1/2 Plus

catalog numbers A00050 and A00054 (research use only)] (Asuragen Inc., Austin, TX) following the manufacturer's instructions for use (see also Assay Workflow). The primary difference between these products is their regulatory status; the reagents and analysis software are identical for both kits.

## Assay Workflow

The AmplideX PCR/CE SMN1/2 Plus and SMA Plus kits include reagents for multiplexed PCR to produce endogenous control, SMN1, SMN2, hybrid, and variant amplicons. These amplicons are dye tagged and designed with distinct fragment sizes codified to each targeted sequence, variant, and variant type. For example, SMN1 and SMN2 amplification across exon 7 and intron 7 generates products that are distinguished by electrophoretic mobility, whereas their copy numbers are determined by quantifying the signal intensity of the corresponding peaks relative to other assay information. After PCR amplification, HEX-labeled
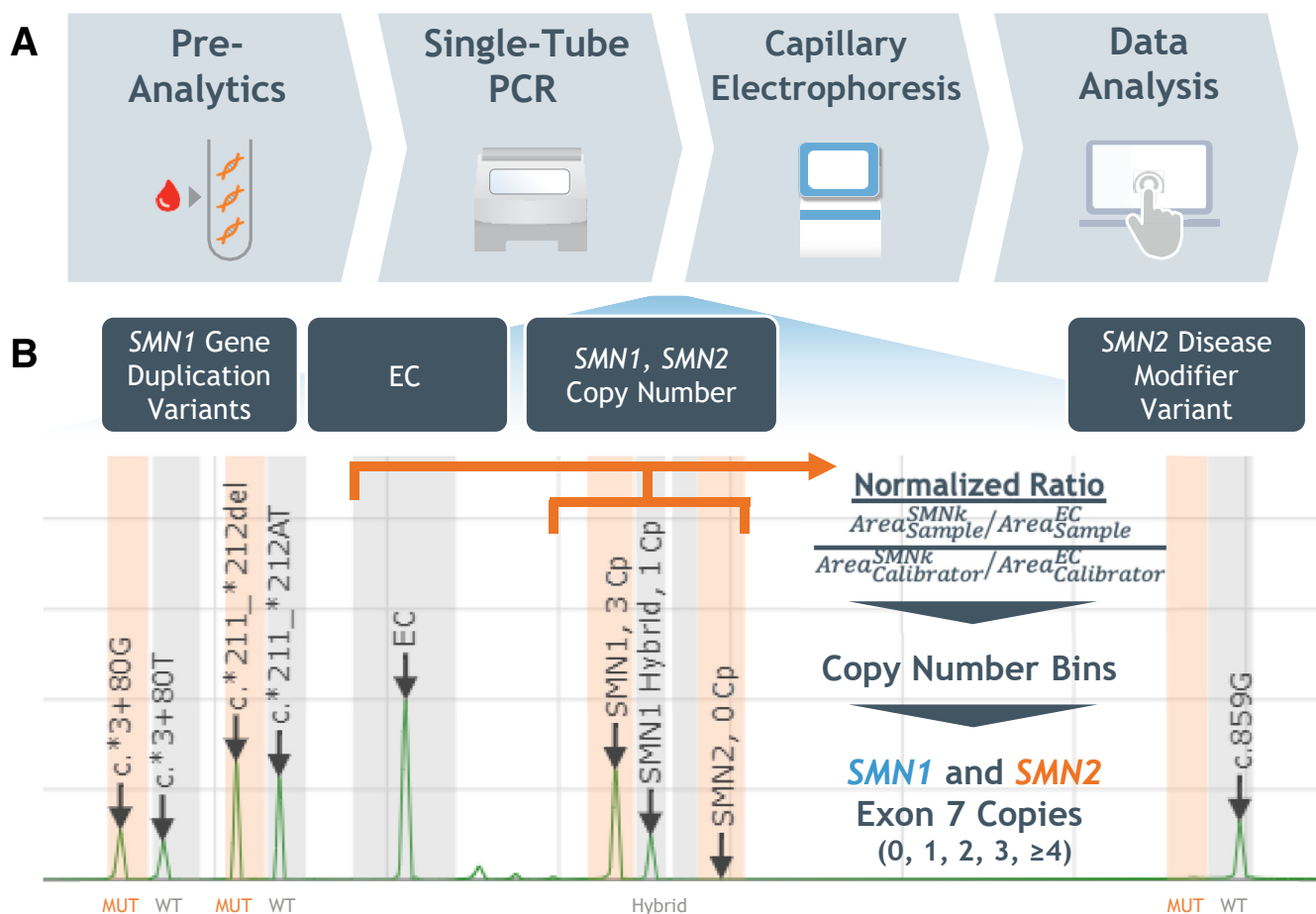


**Figure 1** The SMN1/2 PCR/capillary electrophoresis assay integrates amplification and analysis with a calibrator. **A:** Summary of the assay workflow from blood sample to report of results. **B:** Example electropherogram generated from assay interpretive software. Gene-specific peak area ratios relative to endogenous control (EC) are normalized to a calibrator included in each run to produce normalized ratios, which are binned to determine copy number (see *Materials and Methods*). Total exon 7 copy number for SMN1 and SMN2 is automatically calculated by summing the copy numbers from both the gene-specific and the hybrid gene peaks when present. Hybrid peaks indicate gene conversion between SMN1 and SMN2. In the example above, the total SMN1 exon 7 copy number is four copies, with one copy corresponding to the SMN1 hybrid peak (ie, SMN2 > SMN1 conversion event in exon 7). Where present, mutant (MUT) peaks indicate presence of SMN1 gene duplication (**left side**) and SMN2 disease modifier (**right side**) mutations. In the above example, both gene duplication modifiers are detected, and wild-type (WT) peaks are detected for all three variants. Cp, copies; k, {1,2}.

fluorescent products are analyzed by CE to detect all peaks by size using the included ROX 1000 Size Ladder (Asuragen Inc., Austin, TX). Sample electropherograms typically include six peaks (ie, endogenous control, SMN1 copy, SMN2 copy, c.*3+80T, c.*211_*212AT, and c.859G), but up to 11 peaks are possible, including hybrid SMN1 or SMN2 peaks that indicate gene conversions and variant peaks (c.*3+80G, c.*211_*212del, and c.859C) that indicate presence of the variants detected by this kit (Figure 1).

The assay workflow includes PCR master mix setup, thermal cycling, and analysis using CE and an assay-specific analysis module in the AmplideX PCR/CE Reporter software version 3.0.2. For this process, purified gDNA derived from human cell lines or whole blood is added to a PCR well containing a master mix of 2× PCR Mix (Asuragen Inc.) and SMN1/2 Plus HEX Primer Mix (Asuragen Inc.) in a final reaction volume of 15 μL. After approximately 1 hour of thermal cycling, PCR products are added to a second master mix composed of Hi-Di Formamide (Thermo Fisher Scientific, Waltham, MA) and ROX 1000 Size Ladder. Following denaturation, amplicons are resolved on an Applied Biosystems Genetic Analyzer (eg, 3130, 3730, or 3500 series, or SeqStudio; Thermo Fisher Scientific). The resulting electropherograms are analyzed with the AmplideX PCR/CE SMN Plus Analysis Module (Asuragen Inc.) using the SMN Plus Analysis Type to generate normalized area ratios that are automatically interpreted as 0, 1, 2, 3, or ≥4 exon 7 copies for both SMN1 and SMN2 (Figure 1). Variant status for the c.*3+80T>G, c*211_*212del, and c.859G>C variants is reported as positive or negative (qualitative). The gene duplication variants may adjust carrier risk when two copies of SMN1 are also present.[26,27] The c.859G>C variant is relevant in cases where SMN1 is not detected; it may indicate less severe disease and is disproportionately represented in patients with type III SMA and two SMN2 copies.[9] For 24 samples analyzed on a 3500xL genetic analyzer, the total hands-on time is 60 minutes, and the total assay time is 3 hours 15 minutes, including analysis. A batch of 24 samples can be genotyped using the automated AmplideX Reporter software in about 5 minutes.

The workflow requires a calibrator and control, which must be tested in singleton in every batch run for data analysis and quality control. The calibrator normalizes all sample results generated using the kit. The control acts as an internal quality control to confirm that normalization to the calibrator is functioning properly in the batch run. The SMN Calibrator and SMN Control (Asuragen Inc.) included in the kit were used with the DNA isolation methods indicated in Supplemental Table S1 (default calibration type). Additional isolation methods were tested with user-defined calibration (UDC), which utilizes a calibrator and control purified using the same DNA isolation workflow as the tested samples (Supplemental Table S1, user-defined calibration type).

## Analytical Validation

The accuracy study utilized 134 gDNA samples isolated from 116 unique specimens consisting of whole blood (n = 119) or human cell lines (n = 15) with independent determinations of 0, 1, 2, 3, or ≥4 SMN1 and SMN2 copies. Specimens also included the c.*3+80T>G variant (n = 24), the c*211_*212del variant (n = 28), and the c.859G>C variant (n = 1). gDNA was isolated with one of the seven unique isolation methods (Supplemental Table S1), including silica column (n = 20), functionalized magnetic bead (n = 76), or precipitation-based purification methods (n = 38). gDNA isolations were executed in accordance with manufacturer's protocols. All samples were tested in singleton using an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific). Samples were tested using a gDNA input of 20 to 80 ng per reaction.

To determine reference values for SMN1 and SMN2 exon 7 copy numbers, all gDNA samples were tested using both an MLPA method (MRC Holland, Amsterdam, the Netherlands; SALSA MLPA assays Probemix P021 and P460), termed method A; as well as a verified ddPCR-based method (Bio-Rad, Hercules; SMN1 Copy Number Determination Kit, catalog number 1863500, and SMN2 Copy Number Determination Kit, catalog number 1863503), referred to as method B. For method A, a subset of samples was provided as isolated gDNA with previously generated results, whereas samples without prior results were tested at a single external site. Consensus values in agreement between both reference methods were calculated and used as reference values for assessment of accuracy, with ambiguous or discordant results (in either reference method) excluded from analysis. In total, 9 samples for SMN1 and 10 samples for SMN2 were excluded because of reference ambiguity. All samples were tested with Sanger sequencing to determine variant status of the gene duplication and disease modifier markers, as well as the gene conversion status, as determined by SMN1/SMN2 sequence identity at exon 7 and intron 7. Fourteen samples were excluded from c.*211_*212del analysis because of ambiguous sequencing results.

A single-site precision study was performed by testing nine gDNA samples in duplicate in each of 20 runs, with two operators running one PCR independently per day over 10 nonconsecutive days, using three reagent lots, for a total of 40 observations per sample. CE was resolved using an Applied Biosystems 3500xL Genetic Analyzer. In sum, 360 sample measurements were collected across 20 PCR runs.

The DNA input range was assessed using six unique cell line gDNA samples and two unique blood gDNA samples. Each sample was tested in duplicate at 10, 20, 50, 60, 70, and 80 ng input for each of two reagent lots. A single operator performed all three PCR runs on the same Applied Biosystems Veriti thermal cycler (Thermo Fisher Scientific, Waltham, MA). All PCR products were subsequently analyzed on four CE platforms (Applied Biosystems 3500xL, 3730xl, 3130xl, and SeqStudio).

## Multisite Evaluation

A total of 468 gDNA samples, purified from whole blood (n = 449) or amniotic cells (n = 19), with varying SMN1/2

**Table 1**   Analytical Validation of Accuracy Compared with Reference Methods

| Reference method | Exon 7 concordance, *n*/total (overall % agreement) | |
| --- | --- | --- |
| | *SMN1* | *SMN2* |
| Method A and method B (*n* = 125 *SMN1*, *n* = 124 *SMN2*) | 121/122 (99.2) | 119/121 (98.3) |
| Method A (*n* = 132 *SMN1*, *n* = 132 *SMN2*) | 126/128 (98.4) | 126/128 (98.4) |
| Method B (*n* = 126 *SMN1*, *n* = 124 *SMN2*) | 122/123 (99.2) | 119/121 (98.3) |

Summary of results generated with the AmplideX SMA Plus kit (PCR/capillary electrophoresis) compared with method A (multiplex ligation-dependent probe amplification), method B (droplet digital PCR), and consensus results (method A and method B). Percentage agreement indicated for total exon 7 copy of both *SMN1* and *SMN2*. Percentage agreement with variant calls compared with Sanger sequencing was as follows: c.*3+80T>G, 100% (132/132); c.*211_*212del, 100% (118/118); and c.859G>C, 100% (132/132).

copies and variant genotypes were tested at four independent clinical and/or research laboratories.

Site A (GenePhile Bioscience Laboratory, Taipei City, Taiwan) tested gDNA isolated from 273 whole blood samples, 13 amniotic fluid, and 6 amniotic cell cultures using the MagCore Genomic DNA Large Volume Whole Blood Kit on a MagCore extractor system H16 (RBC Bioscience Corp., New Taipei City, Taiwan). Fragment size analysis was performed on a 3730*xl* Genetic Analyzer with a 50-cm 48-capillary array. All samples were previously tested using the SALSA MLPA assays Probemix (MRC Holland Amsterdam, the Netherlands) P021-A2 or P021-B1 to obtain *SMN1* and *SMN2* copy numbers.

Site B (Turku University Hospital, Turku, Finland) tested 48 blood-derived gDNA samples isolated using the Illustra Nucleon BACC kit. Fragment size analysis was performed on a 3500xL Genetic Analyzer with a 50-cm capillary array. All samples were previously tested using the SALSA MLPA assays Probemix P021-A2 or P021-B1 to determine *SMN1* copy number values.

Site C (University Medical Center Groningen, Groningen, the Netherlands) tested 108 blood-derived gDNA samples obtained via four different isolation methods: hand isolation (salt precipitation, *n* = 27), Hamilton with Reliaprep (Promega, Madison, WI) (MagBead, *n* = 27), Maxwell with Reliaprep (MagBead, *n* = 26), and an integrated chemagen—Perkin Elmer DNA extraction platform (PerkinElmer, Waltham, MA) (MagBead, *n* = 28). Fragment size analysis was performed on a 3730xl Genetic Analyzer with a 50-cm capillary array. All samples were previously tested using the SALSA MLPA assays Probemix P021-A2 or P021-B1 to obtain *SMN1* and *SMN2* copy numbers.

Site D (Hospital of the University of Pennsylvania, Philadelphia, PA) tested 20 samples isolated using QIAsymphony Blood Kit (Qiagen, Hilden, Germany) (*n* = 16) or QIAcube Blood Mini Kit (Qiagen) (*n* = 4). Fragment size analysis was performed on a 3500xL Genetic Analyzer with a 50-cm capillary. *SMN1* and *SMN2* copy number values were previously determined using either MLPA (P460 or P060) or a laboratory development test that includes PCR, restriction enzyme digestion, and capillary electrophoresis steps.

## Data Analysis

Raw electrophoresis data (fragment analysis data [FSA] files) were directly analyzed by the AmplideX PCR/CE SMN Plus analysis module version 1.0.5 of AmplideX Reporter version 3.0.2 (Asuragen), according to the *Software User Guide*. The software's SMN Plus analysis module determines *SMN1* and *SMN2* copy numbers utilizing a ratio scaling and conversion method. For both genes, peak area, $Area_{FSA}^{SMNk}$, where $k = \{1, 2\}$, and *FSA* indicates the FSA origin (sample or calibrator), is calculated and compared with that of an endogenous control (EC) peak, $Area_{FSA}^{EC}$. This raw ratio is then scaled by the corresponding calibrator ratio to obtain a normalized ratio:

$$SMNk\ Normalized\ Ratio_{Sample} = \frac{Area_{Sample}^{SMNk} / Area_{Sample}^{EC}}{Area_{Calibrator}^{SMNk} / Area_{Calibrator}^{EC}} \quad (1)$$

Chimeric, or hybrid gene peaks, are normalized in a similar way:

$$SMNk\ Hybrid\ Normalized\ Ratio_{Sample} = \frac{Area_{Sample}^{SMNk\ Hybrid} / Area_{Sample}^{EC}}{((Area_{Calibrator}^{SMN1} / Area_{Calibrator}^{EC} + Area_{Calibrator}^{SMN2} / Area_{Calibrator}^{EC}) / 2)} \quad (2)$$

Normalized ratios for each peak are converted to integer copy numbers based on corresponding bins established by the manufacturer, and exon 7 copy numbers for *SMN1* and *SMN2* are automatically calculated as the sum of the gene-specific and hybrid peak integer copy numbers. Of note, hybrid peaks are identified by exon 7 status, with *SMN1* hybrid peaks indicating *SMN2*-to-*SMN1* gene conversion and *SMN2* hybrid peaks indicating *SMN1*-to-*SMN2* gene conversion. The copy number bins were developed after extensive testing of >2000 observations.

Depending on the gDNA isolation methods (Supplemental Table S1), samples used either a kit-provided calibrator for these calculations or a UDC extracted from the same gDNA isolation workflow.

The presence of three variants of interest (*SMN1* c.*3+80T>G, *SMN1* c.*211_*212del, and *SMN2* c.859G>C)

was qualitatively assessed on the basis of automated calls for corresponding peaks in designated trace windows.

All statistical analyses were performed with custom analysis scripts in R version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) or JMP version 14.0.0 (SAS Institute, Cary, NC). Samples with quality control failures were excluded from percentage agreement calculations.

## Results

### Accuracy

The accuracy of the AmplideX assay was assessed by comparing *SMN1* and *SMN2* exon 7 copy numbers with a validated MLPA-based method (method A) and a verified ddPCR-based method (method B). Consensus values for both methods were determined and used as a reference. Concordance with each method was also assessed. Agreement for *SMN1* c.*3+80T>G, *SMN1* c.*211_*212del, and *SMN2* c.859G>C variants was assessed using Sanger sequencing.

Across all valid measurements, *SMN1* and *SMN2* copy numbers produced by the assay were 98.3% to 99.2% concordant with consensus values (Table 1). Results were similar when compared with each reference method alone [*SMN1*: 98.4% (95% CI, 94.5%−99.6%) for method A and 99.2% (95% CI, 95.5%−99.9%) for method B; *SMN2*: 98.4% (95% CI, 94.5%−99.6%) for method A and 98.3% (95% CI, 94.2%−99.5%) for method B]. All 15 samples with 0 *SMN1* copies, indicative of SMA, and all 16 samples with 1 *SMN1* copy, consistent with an SMA carrier, were concordant between the assay and the consensus reference result (Table 2). Results for *SMN2* copy numbers were similar (Table 3). In addition, presence or absence of variants c.*3+80T>G, c.*211_*212del, and c.859G>C was 100% concordant with Sanger sequencing results. Gene conversions identified by the assay were also concordant with Sanger sequencing results for both *SMN1* hybrids (8/8) and *SMN2* hybrids (2/2).

### Intralaboratory Precision

To assess the within-laboratory precision of the assay, a genetically diverse cohort of nine samples across multiple sources of variability, including replicate, day, reagent lot, and operator, for a total of 40 observations per sample were observed. For *SMN1*, the SD of the normalized ratio per sample across all conditions ranged from 0.000 to 0.159, with a defined %CV range of 4.5% to 7.9% (Table 4 and Supplemental Table S2). For *SMN2*, the corresponding SD across all conditions ranged from 0.000 to 0.239, with the defined %CV range from 5.4% to 8.4% (Table 4 and Supplemental Table S2). Furthermore, 99.7% (357/358) and 99.4% (344/346) of the copy number calls for *SMN1* and *SMN2*, respectively, agreed across all conditions (Table 4).

**Table 2**  *SMN1* Exon 7 Accuracy Compared with Reference Methods by Copy Number

| Variable | | Consensus *SMN1* copy number (MLPA + ddPCR) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | Sum |
| Measured *SMN1* copy number (PCR/CE) | 0 | 15 | 0 | 0 | 0 | 0 | 15 |
| | 1 | 0 | 16 | 0 | 0 | 0 | 16 |
| | 2 | 0 | 0 | 60 | 1 | 0 | 61 |
| | 3 | 0 | 0 | 0 | 20 | 0 | 20 |
| | 4 | 0 | 0 | 0 | 0 | 10 | 10 |
| | Sum | 15 | 16 | 60 | 21 | 10 | 122 |

Contingency table comparing measured *SMN1* copy number with the AmplideX SMA Plus kit (rows) versus consensus reference copy number from MLPA and ddPCR (columns).

CE, capillary electrophoresis; ddPCR, droplet digital PCR; MLPA, multiplex ligation-dependent probe amplification.

For the gene duplication and disease modifier variants, 100% (279/279) of calls were concordant with the expected results across all conditions. Although sample 8 had the highest %CV for both genes, all normalized ratios of this sample across all conditions were binned correctly, and normal CE traces were observed.

Analysis of variance was performed separately for *SMN1* and *SMN2* to estimate sources of variance. Across all samples, the largest contributor to variance was residual, and the smallest contributor was reagent lot (Supplemental Table S2). These data suggest that the assay precision is sufficient to produce consistent copy numbers across several common sources of measurement variability.

### Effect of DNA Input

A DNA input range of 10 to 80 ng per reaction was measured on 3500xL, 3730*xl*, and SeqStudio CE instruments, and 10 to 70 ng per reaction on 3130*xl* with eight unique samples, including six cell lines. *SMN1* copy number agreement ranged from 96.8% to 100%, with 99.2% (711/717) overall agreement (Table 5). The six discordant results were all observed on the 3130*xl* instrument, and three of these were derived from a single sample at 10 ng total input (2 μL sample at concentration 5 ng/μL), which is outside of the recommended assay input range for that specific CE instrument. For *SMN2*, agreement ranged from 98.3% to 100%, with 99.2% (712/718) overall agreement (Table 5). Here again, the six discordant measurements were all observed on the 3130*xl*, and all were from a single sample. Two of these six measurements were taken from inputs outside the intended input range of the assay. Finally, all three variants were perfectly called across all inputs (735/735).

### Multisite Evaluation

The four external laboratories tested 468 unique residual clinical samples. A total of 433 valid measurements for

**Table 3** *SMN2* Exon 7 Accuracy Compared with Reference Methods by Copy Number

| Variable | Consensus *SMN2* copy number (MLPA + ddPCR) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | Sum |
| Measured *SMN2* | | | | | | |
| copy number (PCR/CE)   0 | 25 | 0 | 0 | 0 | 0 | 25 |
| 1 | 0 | 40 | 0 | 0 | 0 | 40 |
| 2 | 0 | 0 | 33 | 0 | 0 | 33 |
| 3 | 0 | 0 | 1 | 15 | 0 | 16 |
| 4 | 0 | 0 | 0 | 1 | 6 | 7 |
| Sum | 25 | 40 | 34 | 16 | 6 | 121 |

Contingency table comparing measured *SMN2* copy number with the AmplideX SMA Plus kit (rows) versus consensus reference copy number from MLPA and ddPCR (columns).

CE, capillary electrophoresis; ddPCR, droplet digital PCR; MLPA, multiplex ligation-dependent probe amplification.

*SMN1* and 382 valid measurements for *SMN2* were compared with each laboratory's reference method following exclusions for site B (which only tested for *SMN1* copy number) and 73 indeterminate copy number calls (*n* = 35 for *SMN1* and *n* = 38 for *SMN2*). Across all sites, the assay copy number result was 98.6% and 97.1% concordant with reference for *SMN1* and *SMN2*, respectively (Table 6). For *SMN1*, there were six discordant calls (Table 7), including three for expected carriers and one false-positive carrier call that were all from site A (Supplemental Figure S1A). Eleven discordant *SMN2* copy number calls were observed, all of which were expected to be two or three copies (Table 8 and Supplemental Figure S1B).

Each laboratory site compared results generated by the assay with its own validated reference methods. Site A tested 273 blood gDNA, 6 amniotic fluid cell gDNA, and 13 amniotic fluid gDNA samples. Copy number calls for the assay were 98.2% (274/279) and 96.8% (270/279)

concordant with MLPA P21 Kit reference results for *SMN1* and *SMN2*, respectively (Table 6). The concordance was 98.1% (257/262) for *SMN1* and 97.3% (254/261) for *SMN2* for all blood gDNA samples. Site B tested *SMN1* in a total of 48 samples. All samples were successfully called by the assay without quality control failure, and the results were 100% concordant with MLPA P21 Kit reference results. Site C tested 108 samples. The kit copy number call was 98.9% concordant with reference results for both *SMN1* (88/89) and *SMN2* (87/88). Site D tested 20 samples, 16 with reference *SMN1* and *SMN2* copy number results and 4 with *SMN1* results only. Copy number calls by the evaluated assay were 100% (17/17) and 93.3% (14/15) concordant with the reference results for *SMN1* and *SMN2*, respectively.

## Discussion

With the recent availability of groundbreaking targeted therapies for SMA patients, there is a growing need for more rapid and accurate tests.[30,31,44] The MLPA reference method frequently used to assess *SMN1* and *SMN2* copy number has been shown to lack interlaboratory reproducibility for *SMN2* copy numbers in some cases, including a recent study where 45% of samples produced discordant MLPA results for *SMN2*.[30] In response, others have re-affirmed these results, suggesting that more reliable methods are necessary to determine *SMN2* copy number given the impact of this information on treatment decisions.[45−47] In contrast, a recent study showed disagreement in only 3% of MLPA *SMN2* copy numbers, suggesting that variability may be due to more complex factors, such as laboratory, isolation method, analysis method, kit version, or calibrator sample selection.[31] Although recent guidelines provide a framework for retesting of *SMN2* copy numbers and further investigation of relevant disease modifiers in cases where *SMN2* copy numbers and clinical outcomes do not align, accurate

**Table 4** Analytical Validation of Within-Laboratory Precision

| Sample | Sample type | *SMN1* copies | *SMN1* copy number agreement, % (*n*/total) | *SMN1* %CV (NR) | *SMN2* copies | *SMN2* copy number agreement, % (*n*/total) | *SMN2* %CV (NR) |
|---|---|---|---|---|---|---|---|
| 1 | Blood | 0 | 100 (40/40) | NA | 2 | 100 (40/40) | 8.0 |
| 2 | Blood | 1* | 97.5 (39/40) | 5.8 | 3 | 96.8 (30/31) | 5.8 |
| 3 | Blood | 1 | 100 (40/40) | 6.3 | 4 | 100 (38/38) | 5.8 |
| 4 | Blood | 4 | 100 (40/40) | 4.5 | 1 | 100 (40/40) | 6.3 |
| 5 | Cell line | 0 | 100 (40/40) | NA | 2 | 100 (40/40) | 5.4 |
| 6 | Cell line | 1 | 100 (40/40) | 6.7 | 3 | 97.4 (37/38) | 6.9 |
| 7 | Cell line | 3 | 100 (38/38) | 5.6 | 0 | 100 (39/39) | NA |
| 8 | Cell line | 2 | 100 (40/40) | 7.9 | 2 | 100 (40/40) | 8.4 |
| 9 | Cell line | 2 | 100 (40/40) | 6.9 | 2 | 100 (40/40) | 5.8 |
| Total | | | 99.7 (357/358) | | | 99.4 (344/346) | |

Summary of results generated with the AmplideX SMA Plus kit across 20 runs, including multiple reagent lots and operators. For each sample, expected copy number, percentage agreement, and %CV of normalized ratios across all replicate measurements are indicated for both *SMN1* and *SMN2*. Percentage agreement with variant calls compared with Sanger sequencing was as follows: c.*3+80T>G, 100% (279/279); c.*211_*212del, 100% (279/279); and c.859G>C, 100% (279/279).

*Copy number, percentage agreement, and %CV based on *SMN1* hybrid.

NA, not applicable; NR, normalized ratio.

**Table 5** Analytical Validation of DNA Input Range

| DNA input, ng | SMN1 copy number agreement, % (n/total) | SMN2 copy number agreement, % (n/total) |
|---|---|---|
| 10 | 96.8 (121/125) | 100 (123/123) |
| 20 | 100 (128/128) | 100 (128/128) |
| 50 | 99.2 (126/127) | 98.4 (124/126) |
| 60 | 99.2 (123/124) | 98.4 (124/126) |
| 70 | 100 (124/124) | 98.3 (118/120) |
| 80 | 100 (89/89) | 100 (95/95) |
| Total | 99.2 (711/717) | 99.2 (712/718) |

Summary of results generated with the AmplideX SMA Plus kit with a range of DNA inputs across four unique capillary electrophoresis instrument models. Percentage agreement indicated for total exon 7 copy of both SMN1 and SMN2 across all sample measurements at each given total DNA input. Percentage agreement with variant calls compared with Sanger sequencing was as follows: c.*3+80T>G, 100% (735/735); c.*211_*212del, 100% (735/735); and c.859G>C, 100% (735/735).

quantification of SMN2 copy number is nevertheless critical, as it can impact treatment eligibility.[44,48]

Furthermore, although the cost of the MLPA workflow is relatively low (Supplemental Table S3), MLPA can take several working days to complete, limiting throughput and contributing to long turn-around times. NGS offers an alternative for determining SMN1 copy numbers, increasingly as part of large carrier screening panels; however, NGS also involves cumbersome, multiday workflows. Furthermore, NGS coverage of SMN1 is problematic because of the relatively close proximity of the highly homologous SMN2 gene[28,39,49] and thus copy-number quantification requires sophisticated analysis pipelines and generates results that may require confirmation.[28,39] Other methods, such as ddPCR and real-time quantitative PCR, offer rapid alternatives, but experience other limitations compared with some other methods, such as higher costs and specialized equipment (ddPCR) or the need for replication for accurate results (real-time quantitative PCR).[48]

In this study, we characterized a novel PCR/CE-based assay that can be used for both SMA diagnostics and carrier screening, and it generates reliable and rapid results without complex informatics. The analytical validation and external evaluation of the assay demonstrated consistency and accuracy across multiple sites and different reference methods. For example, SMN1 and SMN2 copy numbers were compared with both MLPA and ddPCR reference assays among 134 samples isolated from 116 unique specimens using seven

isolation methods, including magnetic bead, column, and precipitation workflows. Copy number accuracy was 99.2% for SMN1 and 98.3% for SMN2, including 100% accuracy for samples indicative of SMA (zero SMN1 copies) and of typical carriers (one SMN1 copy). Results from four additional site evaluations using >400 unique samples were similar when compared with their laboratory-validated method, with 98.6% overall agreement for SMN1 and 97.1% overall agreement for SMN2. These studies included eight unique gDNA isolation methods, including five methods not tested during analytical validation. Despite using different reference methods, isolation methods, and thermal cycler and CE instruments, agreement was similar across laboratories. This result demonstrates that the assay reports accurate SMN1 and SMN2 copy numbers for multiple workflows.

In addition to copy number accuracy, the within-laboratory precision of the assay was evaluated with 360 total measurements. Across all samples, copy number results were >99% concordant for both SMN1 (99.7%) and SMN2 (99.4%), and CV was <10% for all samples. Analysis of variance showed that residual variability was the largest contributor to overall variability, indicating that variability contributed by multiple operators, days, and lots was minimal. Overall, these results show robust performance across common sources of assay variability within a laboratory. Although multisite precision was not directly assessed using a single set of samples across all sites as part of this evaluation, similar concordance with the reference

**Table 6** Multisite Evaluation Against Reference Methods

| Site | SMN1 copy number agreement, % (n/total) | SMN2 copy number agreement, % (n/total) |
|---|---|---|
| A | 98.2 (274/279) | 96.8 (270/279) |
| B | 100 (48/48) | NA |
| C | 98.9 (88/89) | 98.9 (87/88) |
| D | 100 (17/17) | 93.3 (14/15) |
| Total | 98.6 (427/433) | 97.1 (371/382) |

Summary of results compared with each laboratory's reference results from multiplex ligation-dependent probe amplification across four independent laboratories. Over 400 unique samples isolated with eight unique isolation methods were tested. Percentage agreement indicated for total exon 7 copy of both SMN1 and SMN2.

NA, not applicable.

**Table 7** Multisite *SMN1* Exon 7 Accuracy Compared with Reference Method by Copy Number

| Variable | *SMN1* copy number (MLPA) | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | Sum |
| Measured *SMN1* copy number (PCR/CE) | 0 | 32 | 0 | 0 | 0 | 0 | 32 |
| | 1 | 0 | 65 | 1 | 0 | 0 | 66 |
| | 2 | 0 | 3 | 261 | 1 | 0 | 265 |
| | 3 | 0 | 0 | 0 | 44 | 0 | 44 |
| | 4 | 0 | 0 | 0 | 1 | 25 | 26 |
| | Sum | 32 | 68 | 262 | 46 | 25 | 433 |

Contingency table comparing measured *SMN1* copy number (rows) versus reference copy number from MLPA (columns) across all sites.

CE, capillary electrophoresis; MLPA, multiplex ligation-dependent probe amplification.

method was observed during single and multilaboratory accuracy testing with unique sample sets and isolation methods, suggesting that performance is likely consistent across laboratories.

Across the accuracy, within-laboratory precision, and DNA input studies, the overall accuracy of the *SMN1* gene duplication markers associated with silent carriers (c.*3+80T>G and c.*211_*212del) and the *SMN2* disease modifier (c.859G>C), as measured by the assay, was 100% compared with sequencing results. This demonstrates robust performance across common sources of assay variability, enabling efficient analysis of relevant markers together with *SMN1* and *SMN2* copy number in a single PCR/CE reaction without compromising accuracy. Many current SMA tests do not yet include these gene duplication variants. In fact, even tests that only assess *SMN1* and *SMN2* copy numbers often do so in independent assays, sometimes using different assay technologies for each gene. With guidelines and literature reviews acknowledging the utility of the gene duplication markers to further resolve residual risk for carrier screening,[8,24,50,51] and of detecting disease modifiers to inform disease phenotypes,[24,52] the information provided by these markers when combined with *SMN1* and *SMN2* copy

**Table 8** Multisite *SMN2* Exon 7 Accuracy Compared with Reference Method by Copy Number

| Variable | *SMN2* copy number (MLPA) | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | Sum |
| Measured *SMN2* copy number (PCR/CE) | 0 | 29 | 0 | 0 | 0 | 0 | 29 |
| | 1 | 0 | 108 | 0 | 0 | 0 | 108 |
| | 2 | 0 | 3 | 170 | 0 | 0 | 173 |
| | 3 | 0 | 0 | 6 | 38 | 0 | 44 |
| | 4 | 0 | 0 | 0 | 2 | 26 | 28 |
| | Sum | 29 | 111 | 176 | 40 | 26 | 382 |

Contingency table comparing measured *SMN2* copy number (rows) versus reference copy number from MLPA (columns) across all sites.

CE, capillary electrophoresis; MLPA, multiplex ligation-dependent probe amplification.

number, particularly from a single PCR, is practical and relevant for both carrier testing and diagnostic applications.

Generally, previous publications suggest that the *SMN1* gene duplication markers c.*3+80T>G and c.*211_*212del detected by this assay are both associated with silent carriers and typically co-occur.[8,24,26,50,51] However, others have noted that c.*211_*212del can occur without c.*3+80T>G in SMA patients with zero copies of *SMN1* due to the presence of hybrid *SMN2-SMN1* genes when exon 8 matches *SMN1* and contains the c.*211_*212del marker.[27] Therefore, presence of these variants must be interpreted carefully in the context of all genotype information.

In addition to default calibration using the calibrator and control included in the kit, the assay design also allows for UDC, where a calibrator and control generated using the same gDNA isolation workflow as the samples to be tested are used. This approach reduces variability associated with gDNA isolation workflows.[29,30,46,47] UDC is recommended for gDNA isolation methods not explicitly validated with the default workflow. For the studies included herein, samples were analyzed using either the default or the UDC calibration methods, as described (Supplemental Table S1). However, automated magnetic bead-based isolation methods tested at sites A and D were analyzed with default calibration during the multilaboratory study and with UDC during analytical validation, and concordance for both *SMN1* and *SMN2* copy numbers was similar. In addition, a comparison between default and UDC calibration at site C during the multisite evaluation utilizing four unique isolation methods showed similar performance for *SMN1* but improved performance for *SMN2* using UDC calibration (Supplemental Tables S4−S7). These results suggest that either the default or the UDC workflow may be appropriate for some laboratory workflows, but UDC can improve results with workflows that have higher variability. Although the number of calibrator and control reactions (one each, or two total) is the same in both workflows, default calibration eliminates the need to screen for appropriate calibrator and controls, which may be beneficial for some laboratories. Because UDC can reduce variability caused by the isolation method, it may also help resolve higher copy numbers of *SMN2* (eg, four versus five copies), which can be beneficial in determining if treatment is appropriate for SMA.[44,52] Where available, addition of control samples isolated with the same method that are known to contain four or five *SMN2* copies may also help resolve higher copy numbers of *SMN2* in cases where results are ambiguous.

Although the *SMN1/2* PCR/CE assay is able to identify gene conversions between *SMN1* and *SMN2* by comparing exon 7 status relative to intron 7 status, gene conversion is not currently clinically significant for either carrier or diagnostic testing, as only exon 7 copy numbers for *SMN1* (carrier and diagnostic) and *SMN2* (diagnostic) are needed for interpretation.[50,52] Therefore, although hybrid genes were confirmed using Sanger sequencing, results for gene

conversions were not assessed independently of overall exon 7 copy number as part of this study. Notably, the mechanism for gene conversion detection with this kit differs from MLPA, which compares exon 7 and exon 8. Thus, identification of gene conversions between these different methods is not directly comparable. As our understanding of the underlying genetics of SMA and their implications on treatment continues to expand, resolution of these gene conversions may prove valuable for further understanding genotype-phenotype connections or residual carrier risk assessments, and should be considered in future clinical investigations.[31] For example, the allele used to distinguish intron 7 status between *SMN1* and *SMN2* with this assay is also a splicing silencer element in *SMN2* not present in *SMN1* that contributes to reduced *SMN2* splicing efficiency,[14,53] suggesting that *SMN2* hybrids detected by this assay may have improved splicing efficiency. In addition, recent studies comparing SMA patients with and without an *SMN* hybrid gene found a less severe clinical phenotype in patients with a hybrid gene, and that individuals with two hybrid genes had higher expression of full-length *SMN2*, suggesting that hybrid genes may improve transcription.[31,54] On the basis of internal screening of nearly 2000 samples, we have found that hybrids detected using the exon 7/intron 7 method incorporated in this assay are similar to other reports,[55] with an overall gene conversion rate of 1.15% (0.93% *SMN1* hybrid, 0.21% *SMN2* hybrid).

In conclusion, the PCR/CE assay system evaluated herein shows robust and accurate quantification of *SMN1* and *SMN2* copy numbers and additional markers relevant to carrier screening and disease severity in a single PCR. More important, our results demonstrate the validity of an all-in-one approach to comprehensive SMA testing, and may help address accuracy and reproducibility concerns observed with other assays, particularly for *SMN2* copy number, which provides important prognostic information and is used for treatment decisions. Furthermore, the total assay time, including setup, PCR, electrophoresis, and automated analysis, is <4 hours, a significant improvement compared with common methods like MLPA (Supplemental Table S3) or NGS, which can take multiple days and require more hands-on time. Finally, the additional information provided by markers indicative of silent carriers and reduced disease severity provides additional information for both carrier screening and diagnostic applications.

## Acknowledgements

## Supplemental Data

## References

1. Prior TW, Leach ME, Finanger E: Spinal muscular atrophy. In GeneReviews [Internet]. Copyright University of Washington, Seattle Available at, *https://www.ncbi.nlm.nih.gov/sites/books/NBK1352/*; 2000
2. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Le Paslier D, Frézal J, Cohen D, Weissenbach J, Munnich A, Melki J: Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995, 80:155–165
3. Chiriboga CA: Nusinersen for the treatment of spinal muscular atrophy. Expert Rev Neurother 2017, 17:955–962
4. Sugarman EA, Nagan N, Zhu H, Akmaev VR, Zhou Z, Rohlfs EM, Flynn K, Hendrickson BC, Scholl T, Sirko-Osadsa DA, Allitto BA: Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. Eur J Hum Genet 2012, 20:27–32
5. Mostacciuolo ML, Danieli GA, Trevisan C, Muller E, Angelini C: Epidemiology of spinal muscular atrophies in a sample of the Italian population. Neuroepidemiology 1992, 11:34–38
6. Pearn J: Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J Med Genet 1978, 15:409–413
7. Thieme A, Mitulla B, Schulze F, Spiegler AW: Epidemiological data on Werdnig-Hoffmann disease in Germany (West-Thuringen). Hum Genet 1993, 91:295–297
8. Verhaart IEC, Robertson A, Wilson IJ, Aartsma-Rus A, Cameron S, Jones CC, Cook SF, Lochmuller H: Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. Orphanet J Rare Dis 2017, 12:124
9. Calucho M, Bernal S, Alias L, March F, Vencesla A, Rodriguez-Alvarez FJ, Aller E, Fernandez RM, Borrego S, Millan JM, Hernandez-Chico C, Cusco I, Fuentes-Prior P, Tizzano EF: Correlation between SMA type and SMN2 copy number revisited: an analysis of 625 unrelated Spanish patients and a compilation of 2834 reported cases. Neuromuscul Disord 2018, 28:208–215
10. Zhang Y, He J, Zhang Y, Li L, Tang X, Wang L, Guo J, Jin C, Tighe S, Zhang Y, Zhu Y, Zhu B: The analysis of the association between the copy numbers of survival motor neuron gene 2 and neuronal apoptosis inhibitory protein genes and the clinical phenotypes in 40 patients with spinal muscular atrophy: observational study. Medicine 2020, 99:e18809
11. Singh RN, Singh NN: Mechanism of splicing regulation of spinal muscular atrophy genes. Adv Neurobiol 2018, 20:31–61
12. Prior TW, Krainer AR, Hua Y, Swoboda KJ, Snyder PC, Bridgeman SJ, Burghes AH, Kissel JT: A positive modifier of spinal muscular atrophy in the SMN2 gene. Am J Hum Genet 2009, 85: 408–413
13. Bernal S, Alias L, Barcelo MJ, Also-Rallo E, Martinez-Hernandez R, Gamez J, Guillen-Navarro E, Rosell J, Hernando I, Rodriguez-Alvarez FJ, Borrego S, Millan JM, Hernandez-Chico C, Baiget M, Fuentes-Prior P, Tizzano EF: The c.859G>C variant in the SMN2 gene is associated with types II and III SMA and originates from a common ancestor. J Med Genet 2010, 47:640–642
14. Vezain M, Saugier-Veber P, Goina E, Touraine R, Manel V, Toutain A, Fehrenbach S, Frebourg T, Pagani F, Tosi M, Martins A: 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. Hum Mutat 2010, 31: E1110–E1125

15. Wu X, Wang SH, Sun J, Krainer AR, Hua Y, Prior TW: A-44G transition in SMN2 intron 6 protects patients with spinal muscular atrophy. Hum Mol Genet 2017, 26:2768−2780

16. Ruhno C, McGovern VL, Avenarius MR, Snyder PJ, Prior TW, Nery FC, Muhtaseb A, Roggenbuck JS, Kissel JT, Sansone VA, Siranosian JJ, Johnstone AJ, Nwe PH, Zhang RZ, Swoboda KJ, Burghes AHM: Complete sequencing of the SMN2 gene in SMA patients detects SMN gene deletion junctions and variants in SMN2 that modify the SMA phenotype. Hum Genet 2019, 138:241−256

17. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, Chiriboga CA, Saito K, Servais L, Tizzano E, Topaloglu H, Tulinius M, Montes J, Glanzman AM, Bishop K, Zhong ZJ, Gheuens S, Bennett CF, Schneider E, Farwell W, De Vivo DC; ENDEAR Study Group: Nusinersen versus sham control in infantile-onset spinal muscular atrophy. N Engl J Med 2017, 377:1723−1732

18. Pechmann A, Langer T, Schorling D, Stein S, Vogt S, Schara U, Kolbel H, Schwartz O, Hahn A, Giese K, Johannsen J, Denecke J, Weiss C, Theophil M, Kirschner J: Evaluation of children with SMA type 1 under treatment with nusinersen within the expanded access program in Germany. J Neuromuscul Dis 2018, 5:135−143

19. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, Lowes L, Alfano L, Berry K, Church K, Kissel JT, Nagendran S, L'Italien J, Sproule DM, Wells C, Cardenas JA, Heitzer MD, Kaspar A, Corcoran S, Braun L, Likhite S, Miranda C, Meyer K, Foust KD, Burghes AHM, Kaspar BK: Single-dose gene-replacement therapy for spinal muscular atrophy. N Engl J Med 2017, 377:1713−1722

20. Stevens D, Claborn MK, Gildon BL, Kessler TL, Walker C: Ona-semnogene abeparvovec-xioi: gene therapy for spinal muscular atrophy. Ann Pharmacother 2020, 54:1001−1009

21. Poirier A, Weetall M, Heinig K, Bucheli F, Schoenlein K, Alsenz J, Bassett S, Ullah M, Senn C, Ratni H, Naryshkin N, Paushkin S, Mueller L: Risdiplam distributes and increases SMN protein in both the central nervous system and peripheral organs. Pharmacol Res Perspect 2018, 6:e00447

22. Ratni H, Ebeling M, Baird J, Bendels S, Bylund J, Chen KS, Denk N, Feng Z, Green L, Guerard M, Jablonski P, Jacobsen B, Khwaja O, Kletzl H, Ko CP, Kustermann S, Marquet A, Metzger F, Mueller B, Naryshkin NA, Paushkin SV, Pinard E, Poirier A, Reutlinger M, Weetall M, Zeller A, Zhao X, Mueller L: Discovery of risdiplam, a selective survival of motor neuron-2 ( SMN2) gene splicing modifier for the treatment of spinal muscular atrophy (SMA). J Med Chem 2018, 61:6501−6517

23. Sturm S, Gunther A, Jaber B, Jordan P, Al Kotbi N, Parkar N, Cleary Y, Frances N, Bergauer T, Heinig K, Kletzl H, Marquet A, Ratni H, Poirier A, Muller L, Czech C, Khwaja O: A phase 1 healthy male volunteer single escalating dose study of the pharmacokinetics and pharmacodynamics of risdiplam (RG7916, RO7034067), a SMN2 splicing modifier. Br J Clin Pharmacol 2019, 85:181−193

24. Serra-Juhe C, Tizzano EF: Perspectives in genetic counseling for spinal muscular atrophy in the new therapeutic era: early pre-symptomatic intervention and test in minors. Eur J Hum Genet 2019, 27:1774−1782

25. ACOG: American College of Obstetricians and Gynecologists Committee opinion No. 691 summary: carrier screening for genetic conditions. Obstet Gynecol 2017, 129:597−599

26. Luo M, Liu L, Peter I, Zhu J, Scott SA, Zhao G, Eversley C, Kornreich R, Desnick RJ, Edelmann L: An Ashkenazi Jewish SMN1 haplotype specific to duplication alleles improves pan-ethnic carrier screening for spinal muscular atrophy. Genet Med 2014, 16:149−156

27. Alias L, Bernal S, Calucho M, Martinez E, March F, Gallano P, Fuentes-Prior P, Abuli A, Serra-Juhe C, Tizzano EF: Utility of two SMN1 variants to improve spinal muscular atrophy carrier diagnosis and genetic counselling. Eur J Hum Genet 2018, 26:1554−1557

28. Chen X, Sanchis-Juan A, French CE, Connell AJ, Delon I, Kingsbury Z, Chawla A, Halpern AL, Taft RJ, BioResource N, Bentley DR, Butchbach MER, Raymond FL, Eberle MA: Spinal muscular atrophy diagnosis and carrier screening from genome sequencing data. Genet Med 2020, 22:945−953

29. Holland MRC: MLPA (Multiplex Ligation-Dependent Probe Amplification) General Protocol for the Detection and Quantification of DNA Sequences. MLPA General Protocol, Instructions for Use MDP Version-007 ed. Amsterdam, The Nethelands: MRC Holland, 2019

30. Schorling DC, Becker J, Pechmann A, Langer T, Wirth B, Kirschner J: Discrepancy in redetermination of SMN2 copy numbers in children with SMA. Neurology 2019, 93:267−269

31. Wadman RI, Jansen MD, Stam M, Wijngaarde CA, Curial CAD, Medic J, Sodaar P, Schouten J, Vijzelaar R, Lemmink HH, van den Berg LH, Groen EJN, van der Pol WL: Intragenic and structural variation in the SMN locus and clinical variability in spinal muscular atrophy. Brain Commun 2020, 2:fcaa075

32. Vill K, Kolbel H, Schwartz O, Blaschek A, Olgemoller B, Harms E, Burggraf S, Roschinger W, Durner J, Glaser D, Nennstiel U, Wirth B, Schara U, Jensen B, Becker M, Hohenfellner K, Muller-Felber W: One year of newborn screening for SMA - results of a German pilot project. J Neuromuscul Dis 2019, 6:503−515

33. Xia Z, Zhou Y, Fu D, Wang Z, Ge Y, Ren J, Guo Q: Carrier screening for spinal muscular atrophy with a simple test based on melting analysis. J Hum Genet 2019, 64:387−396

34. Vidal-Folch N, Gavrilov D, Raymond K, Rinaldo P, Tortorelli S, Matern D, Oglesbee D: Multiplex droplet digital PCR method applicable to newborn screening, carrier status, and assessment of spinal muscular atrophy. Clin Chem 2018, 64:1753−1761

35. Feng Y, Ge X, Meng L, Scull J, Li J, Tian X, Zhang T, Jin W, Cheng H, Wang X, Tokita M, Liu P, Mei H, Wang Y, Li F, Schmitt ES, Zhang WV, Muzny D, Wen S, Chen Z, Yang Y, Beaudet AL, Liu X, Eng CM, Xia F, Wong LJ, Zhang J: The next generation of population-based spinal muscular atrophy carrier screening: comprehensive pan-ethnic SMN1 copy-number and sequence variant analysis by massively parallel sequencing. Genet Med 2017, 19:936−944

36. Larson JL, Silver AJ, Chan D, Borroto C, Spurrier B, Silver LM: Validation of a high resolution NGS method for detecting spinal muscular atrophy carriers among phase 3 participants in the 1000 Genomes Project. BMC Med Genet 2015, 16:100

37. Westemeyer M, Saucier J, Wallace J, Prins SA, Shetty A, Malhotra M, Demko ZP, Eng CM, Weckstein L, Boostanfar R, Rabinowitz M, Benn P, Keen-Kim D, Billings P: Clinical experience with carrier screening in a general population: support for a comprehensive pan-ethnic approach. Genet Med 2020, 22:1320−1328

38. Liu B, Lu Y, Wu B, Yang L, Liu R, Wang H, Dong X, Li G, Qin Q, Zhou W: Survival motor neuron gene copy number analysis by exome sequencing: assisting spinal muscular atrophy diagnosis and carrier screening. J Mol Diagn 2020, 22:619−628

39. Punj S, Akkari Y, Huang J, Yang F, Creason A, Pak C, Potter A, Dorschner MO, Nickerson DA, Robertson PD, Jarvik GP, Amendola LM, Schleit J, Simpson DK, Rope AF, Reiss J, Kauffman T, Gilmore MJ, Himes P, Wilfond B, Goddard KAB, Richards CS: Preconception carrier screening by genome sequencing: results from the clinical laboratory. Am J Hum Genet 2018, 102:1078−1089

40. Hogan GJ, Vysotskaia VS, Beauchamp KA, Seisenberger S, Grauman PV, Haas KR, Hong SH, Jeon D, Kash S, Lai HH, Melroy LM, Theilmann MR, Chu CS, Iori K, Maguire JR, Evans EA, Haque IS, Mar-Heyming R, Kang HP, Muzzey D: Validation of an expanded carrier screen that optimizes sensitivity via full-exon sequencing and panel-wide copy number variant identification. Clin Chem 2018, 64:1063−1073

41. Guo MH, Gregg AR: Estimating yields of prenatal carrier screening and implications for design of expanded carrier screening panels. Genet Med 2019, 21:1940−1947

42. CLSI: Measurement Procedure Comparison and Bias Estimation Using Patient Samples, ed 3. CLSI guideline EP09c. Wayne, PA: Clinical and Laboratory Standards Institute, 2018

43. CLSI: Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline, ed 3. CLSI document EP05-A3. Wayne, PA: Clinical and LaboratoryStandards Institute, 2014

44. Glascock J, Sampson J, Connolly AM, Darras BT, Day JW, Finkel R, Howell RR, Klinger KW, Kuntz N, Prior T, Shieh PB, Crawford TO, Kerr D, Jarecki J: Revised recommendations for the treatment of infants diagnosed with spinal muscular atrophy via newborn screening who have 4 copies of SMN2. J Neuromuscul Dis 2020, 7:97−100

45. Kirschner J, Becker J, Schorling D, Pechmann A, Wirth B: Author response: discrepancy in redetermination of SMN2 copy numbers in children with SMA. Neurology 2020, 95:145

46. Ganesh A, Galetta S: Editors' note: discrepancy in redetermination of SMN2 copy numbers in children with SMA. Neurology 2020, 95:144

47. Dangouloff T, Boemer F, Dideberg V, Caberg JH, Servais L: Reader response: discrepancy in redetermination of SMN2 copy numbers in children with SMA. Neurology 2020, 95:144−145

48. Cusco I, Bernal S, Blasco-Perez L, Calucho M, Alias L, Fuentes-Prior P, Tizzano EF: Practical guidelines to manage discordant situations of SMN2 copy number in patients with spinal muscular atrophy. Neurol Genet 2020, 6:e530

49. Ebbert MTW, Jensen TD, Jansen-West K, Sens JP, Reddy JS, Ridge PG, Kauwe JSK, Belzil V, Pregent L, Carrasquillo MM, Keene D, Larson E, Crane P, Asmann YW, Ertekin-Taner N, Younkin SG, Ross OA, Rademakers R, Petrucelli L, Fryer JD: Systematic analysis of dark and camouflaged genes reveals disease-relevant genes hiding in plain sight. Genome Biol 2019, 20:97

50. Prior TW, Nagan N, Sugarman EA, Batish SD, Braastad C: Technical standards and guidelines for spinal muscular atrophy testing. Genet Med 2011, 13:686−694

51. Prior TW, Nagan N, Sugarman EA, Batish SD, Braastad C: ADDENDUM: technical standards and guidelines for spinal muscular atrophy testing. Genet Med 2016, 18:752

52. Glascock J, Sampson J, Haidet-Phillips A, Connolly A, Darras B, Day J, Finkel R, Howell RR, Klinger K, Kuntz N, Prior T, Shieh PB, Crawford TO, Kerr D, Jarecki J: Treatment algorithm for infants diagnosed with spinal muscular atrophy through newborn screening. J Neuromuscul Dis 2018, 5:145−158

53. Lorson CL, Hahnen E, Androphy EJ, Wirth B: A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A 1999, 96:6307−6311

54. Niba ETE, Nishio H, Wijaya YOS, Lai PS, Tozawa T, Chiyonobu T, Yamadera M, Okamoto K, Awano H, Takeshima Y, Saito T, Shinohara M: Clinical phenotypes of spinal muscular atrophy patients with hybrid SMN gene. Brain Dev 2021, 43:294−302

55. Chen TH, Tzeng CC, Wang CC, Wu SM, Chang JG, Yang SN, Hung CH, Jong YJ: Identification of bidirectional gene conversion between SMN1 and SMN2 by simultaneous analysis of SMN dosage and hybrid genes in a Chinese population. J Neurol Sci 2011, 308: 83−87