Asuragen®

AmplideX®

SMA Plus Kit

Instructions for Use

For In Vitro Diagnostic Use

For US Export Only

CE IVD

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Σ 100

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Intended Use

The AmplideX® SMA Plus Kit is an *in vitro* nucleic acid amplification kit intended to aid in the screening of carriers of and diagnosis of spinal muscular atrophy (SMA). The kit generates copy numbers of exon 7 of both *SMN1* and *SMN2* reported as 0, 1, 2, 3, or ≥ 4 genomic copies. The kit is designed for PCR with extracted genomic DNA from human whole blood performed on standard laboratory-validated thermal cyclers, followed by resolution on a general laboratory-validated genetic analyzer or capillary electrophoresis (CE) platform.

Additionally, the kit assesses chimeric genes with both *SMN1* and *SMN2* sequences, variants *SMN1* c.*3+80T>G and *SMN1* c.*211_*212del associated with gene duplication, and variant *SMN2* c.859G>C associated with disease severity.

Limitations

- This kit has been validated for use with genomic DNA (gDNA) isolated from whole blood collected in EDTA tubes and processed within 14 days.
- This kit is designed to quantify exon 7 of the *SMN1* and *SMN2* genes. Nonsense, frameshift, or missense mutations are not detected.
- This kit is designed to measure the number of genomic copies of exon 7 in *SMN1* and *SMN2*. Specimens with two copies of *SMN1* on one chromosome and zero copies on the other (2+0 or silent carriers) may be distinguished from specimens with one genomic *SMN1* copy on each chromosome (1+1) based on the genotype of the gene duplication variants in some populations (Luo *et al.* 2014; Alías *et al*. 2018).
- This kit detects the presence of three variants related to *SMN1* and *SMN2* gene structure and function, including the gene duplication markers c.*3+80T>G and c.*211_*212del (Luo *et al.* 2014), and the disease modifier c.859G>C (Vezain *et al.* 2010, Prior *et al.* 2009). Variants detected by this kit are not gene-specific; variants present in either the *SMN1* or *SMN2* gene will be reported.
- The binding sites for the primers contained in this kit are free of polymorphic sites with minor allele frequencies (MAF) above 0.005 (The Single Nucleotide Polymorphism database dbSNP build 152); however, very rare polymorphisms located within the primer binding sites may affect *SMN1* and *SMN2* copy number quantification (Prior *et al.* 2011).

Warnings and Precautions

- Use appropriate personal protective equipment when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910.1030, CLSI M29, or other applicable guidance when handling human specimens.
- DNase contamination can cause degradation of DNA specimens or PCR products. Use nuclease-free filter pipette tips and nuclease-free tubes. Clean bench surfaces before beginning work.
- PCR carryover contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Prior to use, ensure that pipettes, thermal cyclers, and CE instruments are maintained, calibrated, and validated according to the manufacturer's instructions.

- **WARNING!** CHEMICAL HAZARD. Hi-Di™ Formamide. Causes eye, skin, and respiratory tract irritation. Possible developmental and birth defect hazard. Avoid breathing vapor. Use with adequate ventilation. Storage recommended based on manufacturer's requirements.
- Substances that may interfere with the PCR amplification of gDNA include certain drug compounds and heparin. Highly lipemic specimens, hemolyzed specimens, icteric specimens, or specimens with proteinemia should not be used.
- Do not pool components from different reagent batches, lots, or other kits.
- Do not use reagents after the labeled expiration date.
- Do not interchange the reagent tube caps; this may cause cross-contamination or degradation of reagents.
- The reagents have been verified for up to 8 total uses through volumetric and freeze-thaw studies. Additional uses are not recommended.
- Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible results. The PCR master mix is viscous and can accumulate within the pipette tip.

Summary and Explanation

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by loss of survival motor neuron 1 (*SMN1*) gene function, and is the primary genetic cause of infant death (Stabley *et al.* 2015). SMA is often divided into "types" based on age of onset and maximum motor milestone achievement, with a gradient of phenotypes ranging from never sitting unassisted, with onset prior to six months of age, to adult-onset mild muscular weakness (Prior *et al.* 2011; Glascock *et al*. 2018). Most SMA patients are classified into three types in order of decreasing severity: type 1 (~60%), type 2 (~30%), and type 3 (~10%; Glascock *et al*. 2018). Rarer SMA types, such as type 0 and type 4, are also known to exist (Prior *et al.* 2011; Glascock *et al*. 2018).

SMA has an incidence of ~1/10,000 live births and a carrier rate of ~1/50. *SMN1* exon 7 is absent in ~96% of patients with SMA, whereas most unaffected individuals have two or more functional genomic *SMN1* copies (Mercuri *et al.* 2018). Additionally, ~3–4% of patients are compound heterozygotes, with an *SMN1* exon 7 deletion on one chromosome and a point mutation in *SMN1* on the other chromosome (Mercuri *et al.* 2018). SMA carriers lack a functional *SMN1* copy on a single chromosome and frequently have one functional *SMN1* copy on the other (1+0), though a *cis* carrier genotype (2+0), commonly referred to as a silent carrier, is also known (Prior *et al.* 2011). The detection rate of SMA carriers using *SMN1* copy number alone varies from ~71% to 95% depending on ethnicity, as silent carriers cannot be resolved from 1+1 individuals solely based on copy number (Hendrickson *et al.* 2009). *SMN1* copy number is typically detected at exon 7, where a single exonic nucleotide (c.840C) distinguishes it from the highly homologous gene *SMN2* (c.840T, Prior *et al.* 2011; Stabley *et al.* 2015). Additionally, variants c.*3+80T>G and c.*211_*212del in *SMN1* are associated with *SMN1* duplication in many ethnic groups, and inform risk of silent carrier *SMN1* genotypes (2+0) to varying degree depending on ethnicity (Luo *et al.* 2014; Alías *et al*. 2018).

SMA phenotype severity inversely correlates with *SMN2* copy number, though copy number alone is not sufficient for predicting disease progression (Prior *et al.* 2011; Glascock *et al*. 2018). In *SMN2*, the single nucleotide difference relative to *SMN1* in exon 7 disrupts a splice enhancer that decreases the number of exon 7-containing mRNAs to 10–20%, resulting in a significantly reduced amount of functional SMN protein (Stabley *et al.* 2015). Due to complete homology with the *SMN1*-associated SMN protein sequence, *SMN2*-generated SMN protein levels offer a compensatory effect. Thus, *SMN2* copy number is

associated with severity of the disease, whereas *SMN1* copy number is associated with molecular SMA diagnosis and carrier status (Prior *et al.* 2011; Mercuri *et al*. 2018). Accurate and timely quantification of *SMN2* is also important to determine treatment eligibility for modern SMA therapies, which rely on *SMN2* and are highly effective with early intervention (Mercuri *et al.* 2018; Glascock *et al*. 2018; Glascock *et al*. 2020). In addition to *SMN1* and *SMN2* copy numbers, several mutations can inform disease prognosis. For instance, detection of c.859G>C in *SMN2* is linked to improved splicing efficiency of *SMN2*, leading to reduced disease severity (Vezain *et al.* 2010, Prior *et al.* 2009).

Principle of the Procedure

The AmplideX® SMA Plus Kit is a multiplexed PCR assay used to amplify exon 7 of the *SMN1* and *SMN2* genes along with an endogenous control (EC) gene from purified gDNA. The kit is also designed to detect certain *SMN1*-to-*SMN2* and *SMN2*-to-*SMN1* gene conversions by comparing sequence identity of exon 7 and intron 7. When present, the conversion peaks are detected as unique peaks in the CE trace and referred herein as hybrid peaks. The 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. For more information, see the Technical Note titled, "Gene Conversions and Hybrid Peak Detection in AmplideX PCR/CE *SMN1/2* Kit" (contact support@asuragen.com).

In addition, the kit determines the presence of the c.*3+80T>G and c.*211_*212del alleles associated with gene duplication (Luo *et al.* 2014) and the c.859G>C allele associated with improved *SMN2* splicing (Vezain *et al.* 2010, Prior *et al.* 2009). Fluorescently-labeled amplicons are resolved by CE and categorized, based on size in base pairs (bp), as EC, *SMN1*, *SMN2*, *SMN1* hybrid, and/or *SMN2* hybrid, with the gene name indicating exon 7 status (i.e., c.840C for *SMN1* and c.840T for *SMN2*), as well as the alleles of each of the three variant markers. Due to multiple different names being used for gene duplication variants, the following table aligns the naming conventions of the gene duplication variants from multiple sources. The HGVS standard nomenclature is used in this document.

Gene duplication variant	Location	HGVS standard nomenclature	Luo *et al.* 2014	dbSNP ID (build 152)
Marker 1	Intron 7	c.*3+80T>G	g.27134T>G	rs143838139
Marker 2	Exon 8	c.*211_*212del	g.27706_27707delAT	rs200800214

The AmplideX SMA Plus Kit requires a calibrator and control in each batch run to normalize area ratios and verify performance of the calibrator, respectively. Optionally, the Diluent included in the kit may be used as a no-template negative control (NTC).

The AmplideX PCR/CE Reporter used in conjunction with the AmplideX PCR/CE SMN Plus Analysis Module is an all-in-one data analysis and visualization tool included with this kit. This software tool automatically performs batch and sample level QC, calculates peak areas, quantifies exon 7 *SMN1* and *SMN2* copy number, and determines variant status directly from FSA electropherogram files.

PCR Methods

The kit includes reagents for multiplexed PCR to produce EC, *SMN1*, *SMN2*, hybrid, and variant amplicons. After PCR amplification, the HEX-labeled fluorescent products are analyzed by CE to detect all peaks by size using the included ROX 1000 Size Ladder. Sample electropherograms typically include six peaks (i.e., EC, *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, c.859C) that indicate presence of the variants detected by this kit (**Figure 1**). Fewer than six peaks may also be present in specimens with 0 copies of either *SMN1* and/or *SMN2*.

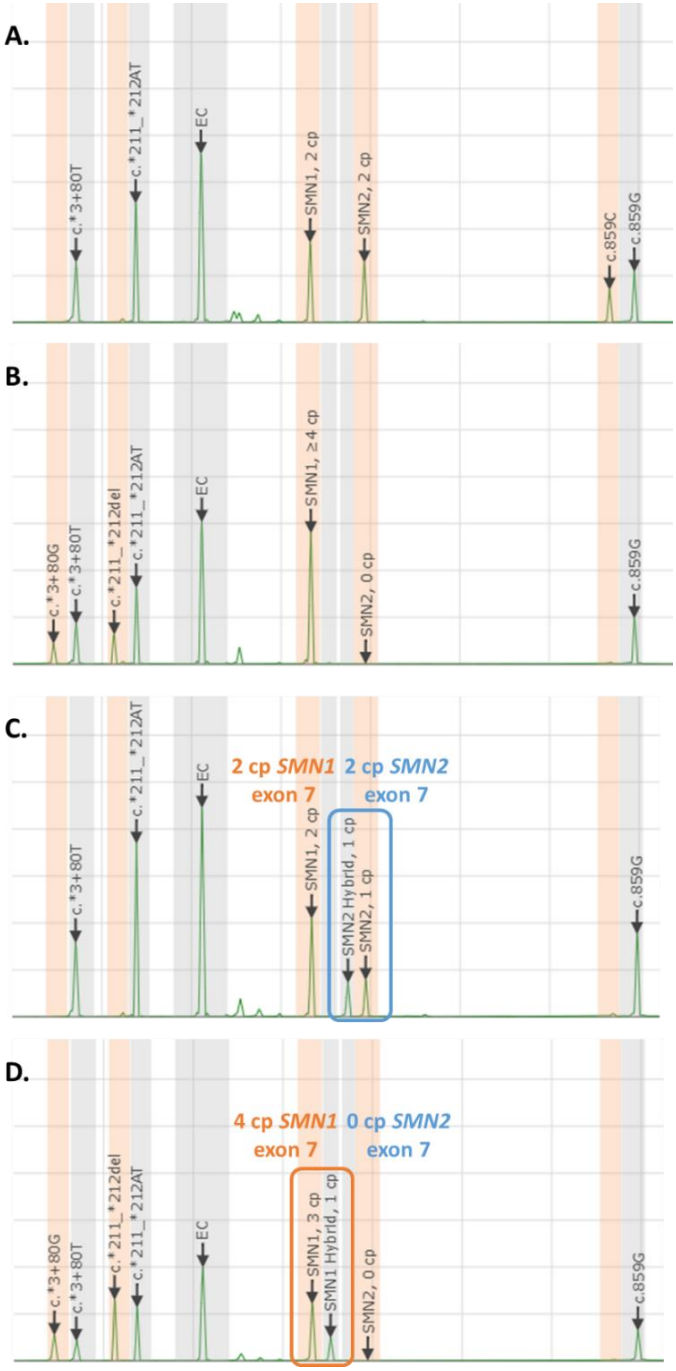

Figure 1. Example AmplideX® SMA Plus Kit electropherogram. **A.** A 2,2 genotype (2 copies *SMN1*, 2 copies *SMN2*) with variant c.859C. **B.** A 4,0 genotype (4 copies *SMN1*, 0 copies *SMN2*) with variants c.*3+80G and c.*211_*212del. **C.** A 2,2 genotype (2 copies *SMN1*, 2 copies *SMN2*) which includes 1 copy of *SMN2* and 1 copy of an *SMN2* hybrid, indicative of an *SMN1*-to-*SMN2* gene conversion at exon 7. **D.** A 4,0 genotype (4 copies *SMN1*, 0 copies *SMN2*) which includes 3 copies of *SMN1* and 1 copy of an *SMN1* hybrid, indicative of an *SMN2*-to-*SMN1* gene conversion at exon 7 that also includes variants c.*3+80G and c.*211_*212del.

Workflow

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. For this process, purified gDNA derived from human cell lines or whole blood is added to a PCR well containing a master mix of 2X PCR Mix and SMN1/2 Plus HEX Primer Mix in a final reaction volume of 15 µL. After ~1 hour of thermal cycling, PCR products are added to a second master mix comprised of Hi-Di™ Formamide and ROX 1000 Size Ladder. Following denaturation, amplicons are resolved on an Applied Biosystems® Genetic Analyzer (e.g., 3130, 3730, 3500 series, or SeqStudio™). See **Figure 2** for a schematic of the workflow.

After resolving the PCR products by CE, the resulting electropherograms are analyzed using the AmplideX PCR/CE SMN Plus Analysis Module 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.

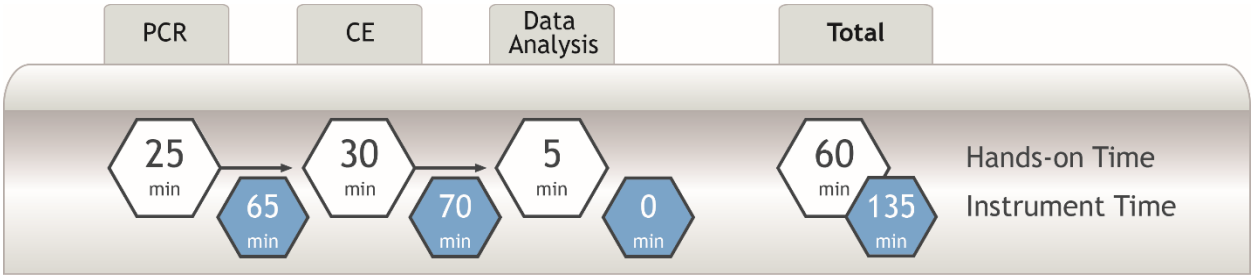

Figure 2. The workflow is streamlined from sample-to-answer and can be performed in less than 4 hours with ~60 minutes of total hands-on time. CE instrument time is for a single injection, or 24 specimens using an Applied Biosystems 3500xL Genetic Analyzer.

Use of Calibrator and Control

The kit 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. For details on calibrator and control specifications, refer to the **Quality Control Procedures** section of this protocol.

The SMN Calibrator and SMN Control included in the kit have been validated for use with the DNA isolation methods listed in the **Required but Not Provided** section, which include both silica column and precipitation-based purification technologies. Additional workflows have been validated for use with user-defined calibration (UDC), which utilizes a calibrator and control purified using the same DNA isolation workflow as the tested specimens. For more information, refer to the **Pre-Analytical Steps** section of this protocol.

Use of Batch Run Controls

Use of a negative control (i.e., NTC) is optional. If an NTC is desired, the Diluent included in the kit may be used in place of sample DNA.

If desired, human cell line-derived samples with confirmed *SMN1* and *SMN2* copy numbers may be purchased from the Coriell Institute for Medical Research to include as additional controls in the assay (**Table 1**). These samples are not required for proper functionality of the assay; only the calibrator and control are required for each batch run.

Note: Since the source material and purification methods used for Coriell cell line DNA are different than standard specimens processed by the laboratory, copy number performance of these samples may be impacted (e.g., increased precision QC failures) when compared to whole blood specimens. Coriell cell line DNA may also show variable results (e.g., normalized ratios) between different lots.

Table 1. Human cell line-derived samples with confirmed *SMN1* and *SMN2* exon 7 copy numbers and variant status.

DNA ID	Expected *SMN1* copies	Expected *SMN2* copies	c.*3+80T>G	c.*211_*212 del	c.859G>C
NA00232	0	2	Negative	Negative	Negative
NA03813	0	3	Negative	Negative	Negative
NA03814	1	≥ 4	Negative	Negative	Negative
NA03815	1	1	Negative	Negative	Negative
NA17117	3	0	Positive	Positive	Negative
NA18517	≥ 4[a]	0	Positive	Positive	Negative
NA19026	≥ 4	1	Positive	Positive	Negative
NA20888	2	2	Negative	Negative	Positive
NA23255	0	3	Negative	Negative	Negative
NA23688	1	2	Negative	Negative	Negative
NA18641	2	2[b]	Negative	Negative	Negative

[a] 2 copies of *SMN1* and 2 copies of *SMN1* hybrid, for a total of ≥ 4 *SMN1* exon 7 copies
[b] 1 copy of *SMN2* and 1 copy of *SMN2* hybrid, for a total of 2 *SMN2* exon 7 copies

Reagents Supplied with the Kit

Item #	Description	Storage Temp
145592	2X PCR Mix	-15 to -30 °C
A00052*	SMN1/2 Plus HEX Primer Mix	-15 to -30 °C
145594	SMN Calibrator	-15 to -30 °C
145595	SMN Control	-15 to -30 °C
145427*	ROX 1000 Size Ladder	-15 to -30 °C
145388	Diluent	-15 to -30 °C

* One kit component tube included in Kit A00055; two kit component tubes included in Kit A00056

Required but Not Provided

Item numbers, where provided, may vary by region. Please contact Asuragen® Technical Support for assistance.

- Reagents for DNA isolation are not included in the kit. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high quality, intact DNA such as the QIAamp® DNA Blood Mini, Midi, or Maxi Kits (QIAGEN®, column purification), FlexiGene DNA Kit (QIAGEN®, precipitation purification), or Wizard® Genomic DNA Purification Kit (Promega®, precipitation purification). Refer to additional notes in the **Pre-Analytical Steps** section.
    - For other purification methods, two user-provided DNA samples for use as a calibrator and control extracted with the same isolation method as the specimens to be tested are required. Refer to the **Pre-Analytical Steps** section.
- General laboratory equipment and workspace to perform PCR and CE procedures
- Centrifuge capable of spinning 96-well plates
- Micro-centrifuge or benchtop centrifuge capable of spinning reagent tubes: Eppendorf® 5810 or equivalent
- Vortex mixer
- Pipettes with an accuracy range between 0.2–2 µL, 1–10 µL, 2–20 µL, 20–200 µL, and 100–1000 µL
- Multi-channel pipette unit capable of pipetting 1–10 µL
- 96-well thermal cycler: Applied Biosystems® Veriti™ 96-well Thermal Cycler, Applied Biosystems GeneAmp® PCR System 9700 (gold block), Bio-Rad® C1000 Touch™ Thermal Cycler (96-well Fast Reaction Module)

  Note: For best results, thermal cyclers should have a ramp rate of ≥ 2.6°C/sec
- 96-well PCR semi-skirted plates for use with the Applied Biosystems Veriti 96-well, GeneAmp® 9700 (gold block), and Applied Biosystems Genetic Analyzers: Phenix Research Products P/N MPS-3580, or equivalent
- Adhesive Foil Seals or Plastic Seals for use with the Applied Biosystems Veriti 96-well and GeneAmp® 9700 (gold block) Thermal Cyclers: Beckman Coulter® P/N 538619, VWR™ P/N 89134-428, or equivalent

- 96-well PCR fully-skirted low-profile plates for use with the Bio-Rad® C1000 Touch™ Thermal Cycler: Bio-Rad P/N HSP9631, or equivalent
- Microseal® plate seals for use with the Bio-Rad C1000 Touch Thermal Cycler: Bio-Rad P/N MSB1001 or MSC1001

  Note: In order to prevent evaporation from plate wells during cycling, other PCR plate seals are not recommended for use with the Bio-Rad C1000 Touch Thermal Cycler
- MicroAmp™ Optical Film Compression Pad for use with Applied Biosystems® thermal cyclers: Applied Biosystems P/N 4312639, or equivalent
- Applied Biosystems Genetic Analyzers running POP-7™ polymer (3130, 3500 or 3730 series) or POP-1™ polymer (SeqStudio™)
    - 3130 Series:
        - POP-7 Polymer: Applied Biosystems P/N 4363785, or equivalent
        - Running Buffer: Applied Biosystems P/N 402824
    - 3500 Series:
        - POP-7 Polymer: Applied Biosystems P/N 4393708, or equivalent
        - Anode Buffer: Applied Biosystems P/N 4393925, or equivalent
        - Cathode Buffer: Applied Biosystems P/N 4408258, or equivalent
    - 3730 Series:
        - POP-7 Polymer: Applied Biosystems P/N 4363935, or equivalent
        - Running Buffer (10X): Applied Biosystems P/N 4335613
    - SeqStudio:
        - Cathode Buffer: Applied Biosystems P/N A33401
        - Cartridge: Applied Biosystems P/N A33671 or A41331 (V2)
- Hi-Di™ Formamide: Applied Biosystems P/N 4311320, or equivalent
- DS-30 Matrix Standard Kit (Dye Set D): Applied Biosystems P/N 4345827

Number of Reactions

- The provided reagents are sufficient for up to 50 reactions (A00055) or 100 reactions (A00056), including master mix overage.
- Master mixes can be prepared for the appropriate number of specimens with a recommended total number of at least 5 reactions per run. We recommend 15% overage for PCR master mixes and 10% overage for CE master mixes.

Reagent Stability

The reagents are stable through the labeled date when stored under the specified conditions.

Storage & Handling

- Store frozen reagents in a non-frost-free freezer protected from light at -15 to -30 °C.
- The reagents have been validated for up to 8 total uses through freeze-thaw studies. Additional uses are not recommended.
- Completely thaw reagents at room temperature before use (at least 30 minutes). Vortex all reagents after thawing.
- Prior to opening, briefly centrifuge each component to collect the solutions at the bottom of the vials.
- Assay setup should be performed at room temperature (approximate range of 18 to 25 °C). Individual kit components are stable for up to 45 minutes per thawing event at room temperature. Once assembled, master mixes are stable for up to one hour at room temperature prior to aliquoting onto the sample plate.

Procedural Steps

Pre-Analytical Steps

Human genomic DNA (gDNA) extracted from K_2EDTA-treated whole human blood via laboratory-validated DNA isolation methodologies is compatible with the AmplideX® SMA Plus Kit. It is recommended that whole blood specimens are stored at 2 to 8°C prior to processing within 14 days of collection.

It is essential to use a validated isolation method to ensure that DNA is consistently of high quality and purity. Commercially available silica column and precipitation purification technologies listed in the **Number of Reactions** section are validated for use using the default software settings and SMN Calibrator and SMN Control included in the kit (default workflow). Additional technologies utilizing automated and manual magnetic bead-based DNA isolation methods have also been validated for use using the user-defined calibration (UDC) workflow. While the default workflow may be compatible with other isolation methods not listed such as magnetic bead-based methods, Asuragen recommends using the UDC workflow as validated for these methods. For more details, see the **User-Defined Calibration** section. Each DNA isolation method used with the AmplideX SMA Plus Kit should be independently validated with a representative sample set of at least 10 to 20 specimens prior to use.

DNA amount and purity should be quantified using a spectrophotometer (e.g., Thermo Scientific™ NanoDrop™). Sample requirements are as follows:

- $A_{260}/A_{280} = 1.8 \pm 0.3$
- 2 µL of DNA per PCR reaction
- DNA Input for Applied Biosystems® Genetic Analyzers:

Instrument	DNA Input Range	DNA Input Recommendation
3130	20 to 60 ng	20 ng
3500, 3730, or SeqStudio™	20 to 70 ng	20 ng

Additionally, before beginning CE, ensure the calibrated instrument detects HEX™ and ROX™ using the DS-30 Matrix Standard Kit (Dye Set D, Applied Biosystems®) according to the manufacturer instructions. For further details on setting up the genetic analyzer instrument, refer to the **Capillary Electrophoresis Using a Genetic Analyzer** section.

User-Defined Calibration

User-defined calibration (UDC) is an alternative workflow validated to support DNA isolation methods not listed in the **Required but Not Provided** section, including automated and manual magnetic bead-based workflows. For this approach, users select a calibrator and control extracted using the same DNA isolation workflow as the specimens to be tested, and include them in each PCR batch instead of the SMN Calibrator and SMN Control included in the kit. The following pre-analytical steps are required prior to beginning specimen testing with the UDC workflow:

- Adjustment of copy number bins in the AmplideX® PCR/CE Reporter software
- Selection of appropriate calibrator and control reference samples

Adjusting Copy Number Bins for User-Defined Calibration

Before analyzing data with the UDC workflow, adjust the copy number bins to the UDC bin values indicated in **Table 2** for each copy number bin for both *SMN1* and *SMN2*. No adjustments are necessary for the hybrid bin values. Refer to the AmplideX PCR/CE Reporter SMN Plus Analysis Module Software User Guide (00002540) for instructions on adjusting the copy number bins. Bin values used for each analysis are reported in the CSV and PDF output generated from AmplideX PCR/CE Reporter. **Note:** After the copy number bins are updated and saved, these values will be preserved for subsequent analyses.

Table 2. User-Defined Calibration (UDC) copy number bins settings.

Copy Number	*SMN1*		Hybrid		*SMN2*	
	Lower	Upper	Lower	Upper	Lower	Upper
0	0.000	0.200	0.000	0.250	0.000	0.200
1	0.350	0.650	0.250	0.750	0.350	0.650
2	0.750	1.200	> 0.750	-	0.750	1.200
3	1.250	1.650	-	-	1.250	1.650
≥ 4	> 1.750	-	-	-	> 1.750	-

Selecting Calibrator and Control Reference Samples

For both the calibrator and control, the UDC workflow requires a unique wild-type reference DNA sample collected, handled, and extracted with the same procedures and DNA isolation workflows as the specimens to be tested. An appropriate wild-type reference sample is defined as having 2 copies of *SMN1* exon 7, 2 copies *SMN2* exon 7, no hybrid peaks, and negative status for the c.*3+80G, c.*211_*212del, and c.859C variants measured by the AmplideX® SMA Plus Kit. Selection of two appropriate reference samples to use as the calibrator and control is vital for accurate copy number quantification. In order to identify appropriate reference samples, follow these steps:

1. Test 20 to 30 DNA samples from healthy individuals along with one reaction of the SMN Calibrator and SMN Control included in the kit according to the **Procedural Steps** section.
2. Analyze the batch using the AmplideX PCR/CE Reporter with the SMN Plus Analysis Type
   a. Prior to analysis, adjust the copy number bins for UDC as indicated in **Table 2**.
   b. After uploading FSA files for analysis, select CAL and CON for the SMN Calibrator and SMN Control, respectively, on the **Annotate** tab.
3. Following analysis, identify DNA samples that have the appropriate wild-type genotype and calculate the mean normalized ratio for both *SMN1* and *SMN2* for the wild-type samples.
4. Identify two reference samples with *SMN1* and *SMN2* normalized ratios that are within 10% of the mean. **Note:** Within a given DNA isolation method, most wild-type DNA samples will fall within 10% of the mean normalized ratio values for *SMN1* and *SMN2.*

Approximately 40–45% of DNA samples from the broader population are expected to have a compatible genotype for use as a calibrator or control with the UDC workflow, though this can vary by ethnicity. Once appropriate reference samples are identified for the calibrator and control, measure the DNA concentrations using a spectrophotometer (e.g., Thermo Scientific™ NanoDrop™). Dilute the DNA to a target concentration of 10 ng/µL using the Diluent included in the kit, such that 20 ng of DNA input will be included when following the protocol. Store and handle the UD calibrator and control reference samples in accordance with the instructions provided in this guide for other kit components. If desired, aliquot the UD calibrator and control reference samples to minimize freeze-thaw cycles.

Overview of the AmplideX SMA Plus Kit Protocol

The test protocol involves three key sets of procedures:

1. PCR master mix setup and thermal cycling
2. Capillary electrophoresis using a genetic analyzer
3. Fragment analysis (PCR/CE)

The protocol is written for a single reaction; master mixes can be prepared for the appropriate number of reactions at each step of the protocol. The provided reagents are sufficient for up to 50 or 100 reactions performed in up to 8 independent batches, including 15% overage for PCR master mix formulations and 10% overage for CE injection master mix formulations.

The workflow should proceed in a unidirectional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize the risk of amplicon contamination.

PCR Master Mix Setup and Thermal Cycling

Note: Before beginning, review the **Pre-Analytical Steps** section to identify the appropriate Calibrator and Control for your workflow

1. Thaw the 2X PCR Mix, SMN1/2 Plus HEX Primer Mix, Calibrator, Control, and Diluent (if NTC is to be included) at room temperature until each component is a uniform liquid (up to 30 minutes depending on volume). Vortex all tubes for approximately 5 seconds at maximum speed and briefly centrifuge to ensure contents are at the bottom of the tube.
2. Assemble the reagents in the order listed according to the PCR Master Mix in the following table. Volumes indicated are for a single reaction and do not include the recommended overage. We recommend creating a master mix with 15% overage for each reagent (volume/reaction * number of reactions * 1.15 for each reagent, rounded to nearest 0.1 µL). Prepare sufficient volume of PCR master mix for each DNA specimen to be tested, including the calibrator and control. Inclusion of a reaction with diluent in place of DNA as an NTC is optional. **Note:** The 2X PCR Mix is viscous; therefore, retract pipette piston slowly to acquire the desired solution.

PCR Master Mix

Reagent	Volume/reaction (µL)
2X PCR Mix	7.5
SMN1/2 Plus HEX Primer Mix	5.5
DNA specimen, calibrator, control, or NTC*	2.0*
Total Volume	**15.0**

* Do not add to bulk master mix

3. Thoroughly vortex the PCR Master Mix for approximately 5 seconds at maximum speed and briefly centrifuge prior to aliquoting to the PCR plate. **Note**: The Master Mix must be vortexed prior to dispensing to ensure adequate mixing of all reagents.
4. Dispense 13.0 µL of the PCR Master Mix to each well or tube. Use a repeater pipette if available.
5. Add 2.0 µL of DNA (specimen, calibrator, or control) to each well (see **Pre-Analytical Steps** for sample input requirements). Pipette up and down at least three times to ensure adequate mixing. **Note**: A single reaction of both the calibrator and the control must be included in each batch run and is required for analysis by the AmplideX® PCR/CE Reporter.
6. Seal the plate with an adhesive film seal (plastic or foil); ensure that all the wells and the plate edges are properly sealed. **Note**: When using foil seals, use of a roller (Beckman Coulter P/N 538618 or equivalent) is recommended to prevent evaporation during thermal cycling.
7. Gently vortex the plate.
8. Centrifuge the plate to remove bubbles (1 minute at 1600 x g). **Note**: Ensure all bubbles are removed from the bottom of the plate wells.
9. Transfer the sealed PCR plate to a preprogrammed supported thermal cycler, and run the following cycling protocol setting the reaction volume to 15 µL. **Note:** Use default ramp rates for all thermal cyclers. Thermal cyclers with maximum ramp rates < 2.6 °C/s are not recommended.

Temperature	Time	Cycling
94 °C	2 minutes	1 hold
94 °C	30 seconds	25 cycles
52 °C	30 seconds	
72 °C	30 seconds	
72 °C	5 minutes	1 hold
4 °C	Hold	1 hold

Note: For best results, thermal cyclers should have a ramp rate of ≥ 2.6°C/sec.

10. Transfer PCR products for CE analysis or store at -15 to -30 °C until analyzed. **Note**: Once the PCR cycling is complete, PCR products are stable for up to 48 hours at 2 to 8 °C. When stored in a freezer, PCR products are stable at -15 to -30 °C for at least 1 month. Storage of amplicons at ambient temperature is not recommended.

Capillary Electrophoresis Using a Genetic Analyzer

1. Thaw the Hi-Di™ Formamide and ROX 1000 Size Ladder at room temperature until each component is a uniform liquid.
2. Thoroughly vortex (max speed for 5 to 10 seconds) and briefly centrifuge to collect the contents.
3. Prepare a CE master mix solution by adding components in the order listed according to the CE Master Mix table. Volumes indicated are for a single reaction and do not include the recommended overage. We recommend creating a master mix with 10% overage for each reagent (volume/reaction * number of reactions * 1.10 for each reagent, rounded to nearest 0.1 µL).

CE Master Mix

Reagent	Per Reaction (µL) (SeqStudio, 3500 series)	Per Reaction (µL) (3130, 3730 series)
Hi-Di Formamide	11.0	8.0
ROX 1000 Size Ladder	2.0	1.0
PCR Product*	2.0*	1.0*
Total Volume	**15.0**	**10.0**

* Do not add to bulk master mix

4. Mix all added reagents (vortex at max speed for 5 seconds), and briefly centrifuge to collect the contents.
5. Aliquot 13.0 µL (if using a SeqStudio™ or 3500 series Genetic Analyzer) or 9.0 µL (if using a 3130 or 3730 series Genetic Analyzer) of CE Master Mix to each well of a new CE analysis plate compatible with the genetic analyzer. **Note**: Match layout of the CE plate to the injection configuration of the genetic analyzer in appropriate groups of 4, 8, 16, 24, or 48 capillaries depending upon CE model (e.g., A1–H3, A4–H6, A10–H12 for 3500xL). If running less than the number of samples for any injection group, fill empty wells subject to injection with 15.0 µL (SeqStudio or 3500 series) or 10.0 µL (3130 or 3730 series) of Hi-Di Formamide.

6. Prior to transfer, centrifuge the plate containing PCR products to collect the contents at bottom of wells and prevent contamination when opening (1 minute at 1600 x g).
7. Transfer the PCR products to the CE plate (2.0 µL if using the SeqStudio or 3500 series or 1.0 µL if using the 3130 or 3730 series), pipetting up and down at least 3 times to mix. **Note**: A multi-channel pipette is recommended for this transfer, especially for multiple columns of PCR products.
8. Seal the plate with an adhesive film seal ensuring that all the wells and the plate edges are properly sealed.
9. Gently vortex the plate.
10. Centrifuge the plate to collect the contents at bottom of wells (1 minute at 1600 x g).
11. Denature the CE plate on a thermal cycler for 2 minutes at 95 °C followed by a 4 °C hold for at least 2 minutes. Centrifuge the plate to collect the contents at bottom of wells (1 minute at 1600 x g), ensuring that no bubbles are present within plate wells. After the denaturation step, store the plate on ice and protected from light. **Note**: The amplicons must be denatured prior to CE analysis and may be run up to 48 hours after denaturing; instrument deck time stability has been verified up to 48 hours.
12. Prepare the genetic analyzer for data acquisition according to the manufacturer instructions. Final injection and run conditions must be validated by the end user and may differ between instruments. **Note: Per the manufacturer instructions, instrument buffers and polymer must be at room temperature before installing on the instrument.**
    The following considerations apply:
    i. Calibrate the instrument for the detection of both HEX™ and ROX™ fluorescent dyes using the DS-30 Matrix Standard Kit.
    ii. Adjust the injection conditions and run time according to the particular instrument configuration. The following table lists recommended starting values.
    iii. For the 3130 and 3730 series Genetic Analyzer, the 10X running buffer must be diluted to 1X with high quality purified water per manufacturer recommendations prior to installing on the instrument. For best results, dilute the running buffer immediately prior to use. Once installed on the instrument, diluted 1X running buffer is stable for up to 24 hours. Diluted 1X running buffer may be stored for up to 7 days at either room temperature or 2 to 8 °C. **Note: 1X running buffer must be at room temperature prior to installation on the instrument.**
    iv. Genetic analyzer performance can be affected by fluctuations in room temperature according to the manufacturer. Ensure that the room temperature is compliant with manufacturer recommendations before and during use. For best results, we recommend a room temperature of 18 to 30 °C with a maximum change of less than 2 °C per 24 hours.

The following instrument default run modules can be used as templates:

- 3130, 3130*xl*: FragmentAnalysis36_POP7
- 3500, 3500xL: FragmentAnalysis50_POP7, FragmentAnalysis50_POP7xl, FragmentAnalysis36_POP7, FragmentAnalysis36_POP7xl
- 3730, 3730*xl*: GeneMapper50_POP7, GeneMapper36_POP7
- SeqStudio™: FragAnalysis

Adjust the injection conditions in default run module templates as follows:

Instrument	Capillary Length	Injection	Pre-Run	Run	Oven Temperature
3130, 3130*xl*	36 cm	3.5 kV, 35 s	15 kV*, 900 s	15 kV*, 1500 s	60 °C*
3500, 3500xL	50 cm	2.5 kV, 20 s	15 kV*, 900 s	19.5 kV*, 2100 s	60 °C*
3500, 3500xL	36 cm	2.5 kV, 20 s	15 kV*, 900 s	15 kV*, 1500 s	60 °C*
3730, 3730*xl*	50 cm	2.5 kV, 10 s	15 kV*, 900 s	15 kV*, 2900 s	63 °C*
3730, 3730*xl*	36 cm	2.5 kV, 10 s	15 kV*, 900 s	15 kV*, 1500 s	63 °C
SeqStudio	28 cm	6.0 kV, 2 s	13 kV*, 180 s*	6.0 kV, 3000 s	60 °C*

* indicates settings that are identical to default instrument settings for the given capillary length and polymer type

Note: SeqStudio users may experience a high number of QC flags relating to the size standard identified by the instrument after a run using default settings. These instrument size standard flags are only visible on the SeqStudio instrument interface, and do not impact downstream analysis or indicate an issue with the run. For size standard quality evaluation, refer to the **Samples** section of the Quality Control Procedures. For more information, contact Technical Support.

Data Analysis

The AmplideX® SMA Plus Kit produces electropherogram data that is converted into sample-specific EC, *SMN1, SMN2*, and associated hybrid CE peak areas using the AmplideX PCR/CE Reporter with the SMN Plus Analysis Type utilizing a ratio scaling and conversion method. Normalized ratios for each peak are converted to integer copy numbers and exon 7 copy numbers for *SMN1* and *SMN2* and are determined automatically as the sum of gene-specific and hybrid peak integer copy numbers. Hybrid peaks are present when a gene conversion event affects gene-specific priming in the assay at either exon 7 or intron 7 in *SMN1* or *SMN2*; exon 7 c.840 status assigns the hybrid peak to *SMN1* or *SMN2*.

Normalized ratios of *SMN1*, *SMN2*, and hybrid genes are automatically calculated in the software via Equations 1–4, where $Area_{FSA}^{SMNk}$ corresponds to the peak area for *SMNk*, *k = {1, 2}*, and *FSA* indicates the FSA origin (Sample or Calibrator):

$$SMN1\ Normalized\ Ratio_{Sample} = \frac{Area_{Sample}^{SMN1}/Area_{Sample}^{EC}}{Area_{Calibrator}^{SMN1}/Area_{Calibrator}^{EC}}$$

Equation 1

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

Equation 2

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

Equation 3

$$SMN2\ Normalized\ Ratio_{Sample} = \frac{Area_{Sample}^{SMN2}/Area_{Sample}^{EC}}{Area_{Calibrator}^{SMN2}/Area_{Calibrator}^{EC}}$$

Equation 4

Normalized ratios are then allocated into copy number bins as described in **Table 3** and **Table 4** (default workflow) or in **Table 4** and **Table 5** (UDC workflow). For workflow details, see the **Pre-Analytical Steps** section.

Table 3. Default *SMN1* and *SMN2* copy number bins

	Normalized Ratio	
Copy Number Bin	***SMN1***	***SMN2***
0	≤ 0.185	≤ 0.240
1	0.236–0.593	0.290–0.612
2	0.643–1.118	0.663–1.079
3	1.193–1.552	1.154–1.520
≥ 4	> 1.627	> 1.595

Table 4. Default and user-defined calibration *SMN1* Hybrid and *SMN2* Hybrid copy number bins

Hybrid Copy Number Bin	Normalized Ratio
0	≤ 0.250
1	0.250–0.750
≥ 2	> 0.750

Table 5. User-defined calibration *SMN1* and *SMN2* copy number bins

	Normalized Ratio	
Copy Number Bin	***SMN1***	***SMN2***
0	≤ 0.200	≤ 0.200
1	0.350–0.650	0.350–0.650
2	0.750–1.200	0.750–1.200
3	1.250–1.650	1.250–1.650
≥ 4	> 1.750	> 1.750

AmplideX PCR/CE Reporter SMN Plus Analysis Module

The AmplideX® PCR/CE SMN Plus Analysis Module provides the SMN Plus Analysis Type for data analysis and interpretation. The software, AmplideX PCR/CE Reporter Software User Guide (00002406), and AmplideX PCR/CE SMN Plus Analysis Module Software User Guide (00002540) are available for download at https://asuragen.com/myasuragen.

The analysis steps in the software differ between the default workflow (utilizing the SMN Calibrator and SMN Control included in the kit) and the UDC workflow (utilizing reference samples extracted with laboratory-validated DNA isolation workflows as calibrator and control). For more information, refer to the **Procedural Steps** section.

When using the default workflow, follow these steps:

- No changes are required to the default software settings prior to analysis.
- After uploading FSA files for analysis, select CAL and CON for the SMN Calibrator and SMN Control, respectively, on the **Annotate** tab.

When using the UDC workflow, follow these steps:

- Prior to analysis, adjust the copy number bins for UDC and identify appropriate calibrator and control reference samples as indicated in the **User-Defined Calibration** section.
- After uploading FSA files for analysis, select UDCAL and UDCON for the user-defined calibrator and control, respectively, on the **Annotate** tab.

Adjustable Copy Number Bins

The *SMN1*, *SMN2*, and hybrid peak copy number bins described in the **Data Analysis** section are a result of extensive assay verification and validation on whole blood and cell-line gDNA. These copy number bins should not require further adjustment for the supported instrument configurations, DNA purification methods, and sample types.

For more information on compatible purification methods, see the **Pre-Analytical Steps** section. For details on how to adjust the copy number bins, refer to the AmplideX® PCR/CE SMN Plus Analysis Module Software User Guide (00002540), the **User-Defined Calibration** section. **Note:** When using UDC workflow, adjust bins prior to analyzing data.

Quality Control Procedures

Calibrator

A calibrator tested in singleton is required in each batch run to normalize all sample peak area results. The following formula determines the (non-normalized) area ratio for the calibrator (where *k = {1, 2}*, corresponding to *SMN1* and *SMN2)*:

$$SMNk\ Area\ Ratio_{Calibrator} = Area^{SMNk}_{Calibrator} / Area^{EC}_{Calibrator}$$

Equation 5

The calibrator sample is included in the CSV and PDF reports with a normalized area ratio of 1.000 (i.e., normalized to itself) in the *SMN1* and *SMN2* Normalized Ratio column. The non-normalized (i.e., raw) area ratios are indicated in the PDF report in the Batch Details section next to the labels *SMN1* Calibrator Ratio and *SMN2* Calibrator Ratio, and are also indicated in the Results view within the software in the calibrator section under the headings *SMN1* Raw Ratio and *SMN2* Raw Ratio. If the Calibrator Raw Ratio of *SMN1* or *SMN2* for the calibrator sample is outside of the specified range in **Table 6**, the QC column for the calibrator sample will display PR (Precision QC) and CF (Control QC) in the Results view of the software, and will indicate a Control QC failure for all samples, calibrator, and control in the CSV and PDF reports and in the Results view of the software. Additionally, failures may occur due to the ROX QC, Signal Magnitude QC, or Saturation QC (see the **Samples** section for details), or if *SMN1* or *SMN2* copy number or variant status is incorrect. The expected variant status is negative for the c.*3+80G, c.*211_*212del, and c.859C variants.

These QC failures alert the user of a deviation and the batch run is invalid. A valid calibrator will display PASS in the QC column in the Results view of the software and in the Sample and Control QC sections of the CSV and PDF reports for the calibrator. If PM (Peak Morphology QC) is displayed, the run may be considered valid following review of the CE trace as described in the **Samples** section. For further details, refer to the **Troubleshooting** section and the AmplideX PCR/CE SMN Plus Analysis Module Software User Guide (00002540).

Table 6. Calibrator raw area ratio specifications. Specifications depicted are identical between the default and UDC workflows (see the **Procedural Steps** section).

Gene	Calibrator Raw Ratio (non-normalized)	Expected Exon 7 Copies
SMN1	0.195–0.776	2 copies
SMN2	0.173–0.573	2 copies

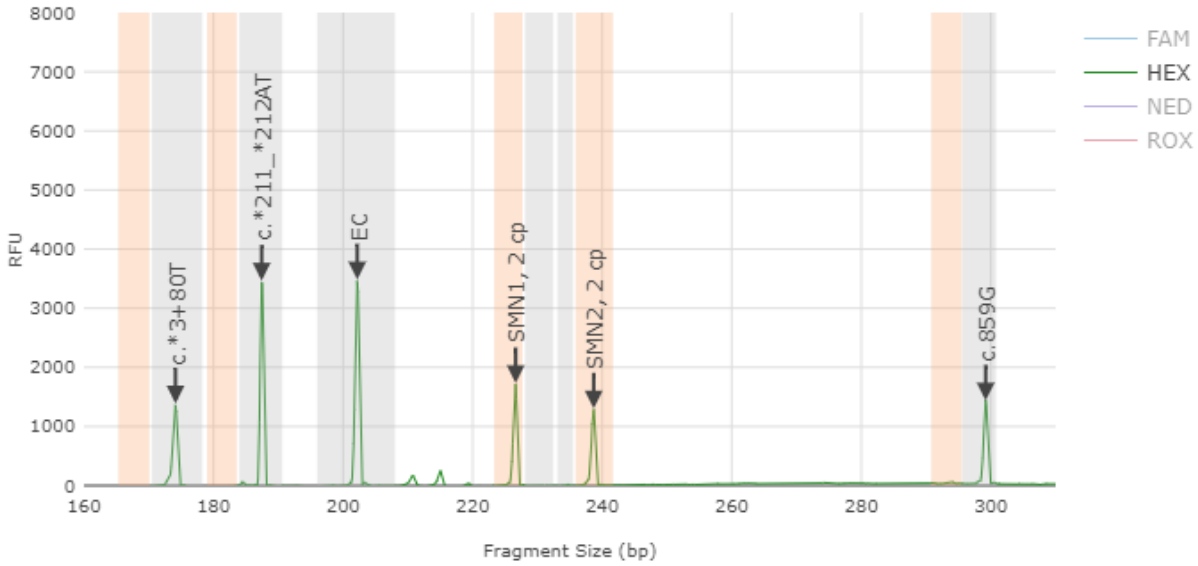

Figure 3. Example of a valid SMN Calibrator CE result from AmplideX® PCR/CE Reporter.

Control

A control tested in singleton is required in each batch run to demonstrate that normalization to the calibrator is functioning properly. The normalized ratio of the control is based on the formula in the **Data Analysis** section.

The control sample is included in the CSV and PDF reports. If the normalized peak area ratio of *SMN1* or *SMN2* for the SMN Control sample is outside of the specified range in **Table 7** (default workflow) or **Table 8** (UDC workflow), the QC column for the control sample will display PR (Precision QC) and CF (Control QC) in the Results view of the software, and will indicate a Control QC failure for all samples, calibrator, and control in the CSV and PDF reports and in the Results view of the software. Additionally, failures may occur due to the ROX QC, Signal Magnitude QC, or Saturation QC (see the **Samples** section for details), or if *SMN1* or *SMN2* copy number or variant status is incorrect. The expected variant status is negative for the c.*3+80G, c.*211_*212del, and c.859C variants.

These QC failures alert the user of a deviation and the batch run is invalid. A valid control will display PASS in the QC column in the Results view of the software and in the Sample and Control QC sections of the CSV and PDF reports for the control. If PM (Peak Morphology QC) is displayed, the run may be considered valid following review of the CE trace as described in the **Samples** section. For further details,

refer to the **Troubleshooting** section and the AmplideX PCR/CE SMN Plus Analysis Module Software User Guide (00002540).

Table 7. Control normalized ratio specifications. Specifications depicted are for the default workflow (see the **Procedural Steps** section).

Gene	Control Normalized Ratio	Expected Exon 7 Copies
SMN1	0.719–1.219	2 copies
SMN2	1.097–1.768	3 copies

Table 8. Control normalized ratio specifications. Specifications depicted are for the UDC workflow (the **Procedural Steps** section).

Gene	Control Normalized Ratio	Expected Exon 7 Copies
SMN1	0.750–1.200	2 copies
SMN2	0.750–1.200	2 copies

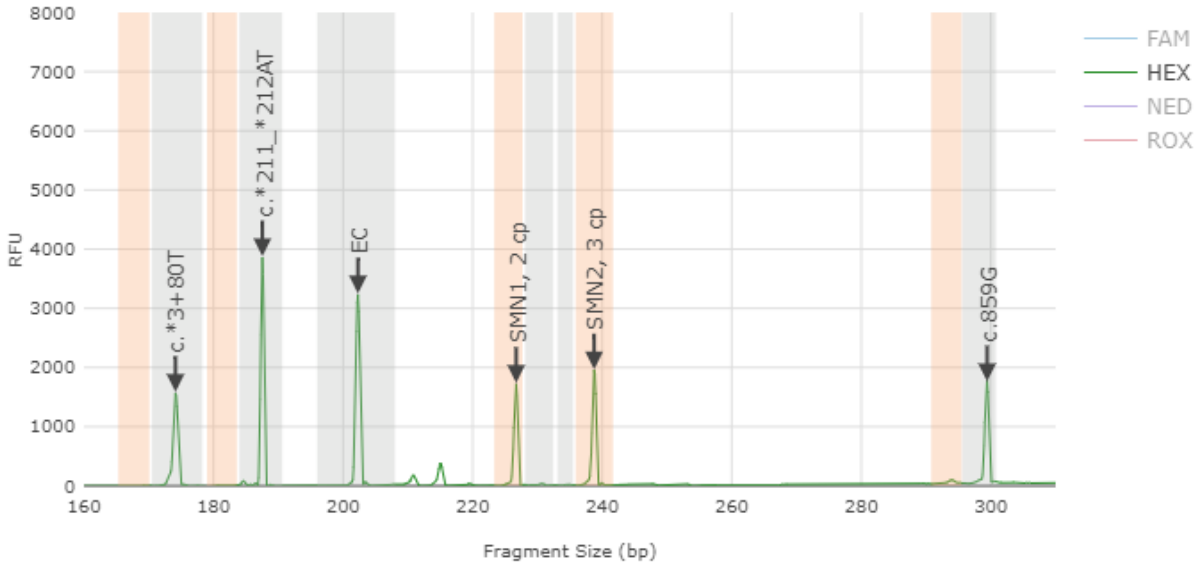

Figure 4. Example of valid SMN Control CE trace from AmplideX® PCR/CE Reporter. **Note:** When using the UDC workflow, the control will have 2 copies of both *SMN1* and *SMN2*.

No Template Control (NTC)

Use of a negative control (i.e., an NTC) is optional. If an NTC is desired, the kit Diluent may be used instead of the same volume of DNA. In the CSV and PDF reports, an NTC sample is displayed in the list of samples tested. A properly functioning NTC exhibits a flat CE electropherogram and has a QC status of LS (Low Signal) in Results view of the software. In the exported reports, the NTC will display FAIL in

the Signal Magnitude QC and Sample QC columns of the CSV report and will display FAIL in the Sample QC section of the PDF report for the relevant sample.

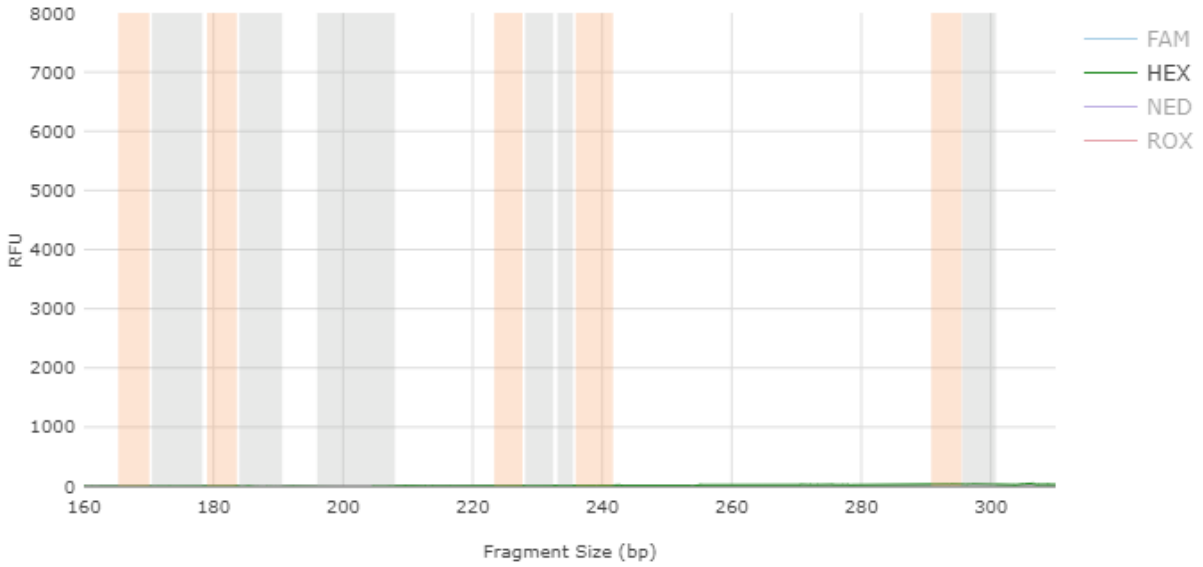

Figure 5. Example of valid NTC CE trace from AmplideX® PCR/CE Reporter

When included, if the NTC passes the Signal Magnitude QC by producing a detectable EC peak, the batch run may be considered invalid. However, since the NTC is not required, the operator or laboratory will need to determine how to use this information for identifying batch run failures.

Samples

The AmplideX PCR/CE Reporter with the SMN Plus Analysis Module performs QC checks on all samples to ensure quality results. A precision check (Precision QC) ensures that the normalized ratio for each copy number peak in each sample falls within one of the expected (**Table 3** and **Table 4)** or user-defined (**Table 4 and Table 5)** copy number bins. The software also performs QC checks for the ROX ladder (ROX QC), minimum control peak heights (Signal Magnitude QC), signal saturation (Saturation QC), and peak shape (Peak Morphology QC). If a sample fails the ROX, Signal Magnitude, or Saturation QC, it will not produce a copy number for *SMN1* or *SMN2*, indicating QC failure. Further, variant status is not reported for samples failing the ROX or Signal Magnitude QCs. Samples that fail the Precision QC will still display the copy number for the gene that passes this QC (e.g., if the *SMN1* normalized ratio falls in between bins but the *SMN2* ratio is within a bin, the *SMN2* copy number will be reported). If a sample fails the Peak Morphology QC, all results are displayed, but the sample will be flagged as at risk and should be reviewed prior to interpretation as described. For more details, see the following bullet points. QC failures are summarized in **Table 9**.

In addition to these built-in QC checks, we recommend inspecting sample CE traces for samples reported as 0 copies of *SMN1* and/or *SMN2* for peaks outside of the expected peak size ranges that exhibit comparable peak height and shape to assay-specific peaks. If such a peak is present, it may indicate a rare insertion or deletion event. A corresponding shift in one or more of the allele-specific wild type or variant peaks detected by this assay (c.*3+80T>G, c.*211_*212del, c.859G>C) may also be observed. Known insertions and deletions all have MAF less than or equal to 0.00006 (dbSNP build 152).

- The **ROX QC** is designed to check the ROX 1000 Size Ladder results to prevent incorrect sizing. A failing result will display LD in the QC column in the Results view of the software, indicating a ladder error. In the PDF and CSV reports, the Sample QC section will display FAIL, and the CSV report will also display FAIL in the ROX QC column. A sample with this result will not produce any copy number or variant data. The sample should be re-run with a calibrator and control from the same PCR batch run. For further details, refer to the **Troubleshooting** section.
- The **Signal Magnitude QC** is designed to ensure the EC peak has sufficient signal to noise ratio. A failing result will display LS in the QC column in the Results view of the software, indicating low signal. In the PDF and CSV reports, the Sample QC section will display FAIL, and the CSV report will also display FAIL in the Signal Magnitude QC column. A sample with this result will not produce any copy number or variant data. The sample should be re-run with a calibrator and control from the same PCR batch run. For further details, refer to the **Troubleshooting** section.
- The **Saturation QC** is designed to ensure signal of the EC peak is within the upper bounds of the detection capability of the instrument. A failing result will display SA in the QC column in the Results view of the software, indicating saturated signal in the electropherogram. In the PDF and CSV reports, the Sample QC section will display FAIL, and the CSV report will also display FAIL in the Saturation QC column. The sample should be re-run with a calibrator and control from the same PCR batch run. For further details, refer to the **Troubleshooting** section.
- The **Precision QC** is designed to ensure the normalized ratio of each sample falls within the expected or user-defined copy number bins, as defined in **Table 3**, **Table 4**, and **Table 5**. A failing result indicates the normalized ratio for at least one copy number peak (*SMN1* or *SMN2*) falls in between copy number bins, and will display PR in the QC column and a dash under the corresponding gene copy number column in the Results view of the software, indicating insufficient precision. In the PDF and CSV reports, the Sample QC section will display FAIL and the corresponding gene copy number column will display a dash. The CSV report will also display FAIL in the Precision QC column, and will display QC Failure in all associated copy number columns. In most cases, only the normalized ratio from one gene causes this failure; if the other gene target produces valid normalized ratio results, the exon 7 copy number for that gene will still be reported, along with the variant status for all variants detected by this kit. The sample should be re-run with a calibrator and control from the same PCR batch run. For further details, refer to the **Troubleshooting** section. **Note:** using the settings validated herein for hybrid peak normalized ratio bins (**Table 4**), hybrid gene peaks will not cause this QC failure. However, if gray zones are added to the hybrid bins by adjusting copy number bins, hybrid peaks may also cause PR failures as previously described.
- The **Peak Morphology QC** is designed to ensure that peak shapes are appropriate for accurate quantification of copy numbers. A failing result will display PM in the QC column in the Results view of the software, indicating abnormal peak shapes within the trace. In the PDF and CSV reports, the Sample QC section will display RISK, and the CSV report will also display RISK in the Peak Morphology QC column. However, all copy number and variant status information will still be displayed. If this occurs, carefully review the CE trace for shoulders, background peaks, or other abnormalities by comparing the CE trace to the other samples in the run. Peak shoulders can trigger a Peak Morphology QC warning, but typically do not affect copy number quantification or variant calling, especially when the peak shape is consistent across all samples and controls in a given run. See for examples. If peak morphology is consistent between the flagged sample and other samples, calibrator, and control in the run, the result may be considered valid. If abnormalities are observed compared to other samples, the sample should be re-run with a calibrator and control from the same PCR batch run. For further details, refer to the **Troubleshooting** section.

Table 9. QC failure mode summaries. For more details on these QC checks, refer to the **Troubleshooting** section and the AmplideX® PCR/CE SMN Plus Analysis Module Software User Guide (00002540).

QC	Description	QC Type & Cause	Report	Resolution
ROX QC (LD)	The ROX QC is designed to flag samples that have aberrant sizing ladders and/or mobility correction factors that cannot be accurately determined. These prevent accurate sizing and are typically encountered when unexpected peak artifacts present in the ROX channel are similar in size and shape to expected ROX peaks.	Fail: Ladder is abnormal or missing	No genotype annotation	Rerun sample
Signal Magnitude QC (LS)	The Signal Magnitude QC is designed to flag samples with insufficient signal. The analysis verifies that the endogenous control peak has a sufficient signal-to-noise ratio and will fail samples where a specific magnitude criteria is not met.	Fail: Extremely low or undetected signal	No genotype annotation	Rerun sample
Saturation QC (SA)	The Saturation QC is designed to identify overblown signal in the endogenous control peak, which can cause loss of precision for determining copy number genotypes.	Fail: Peak magnitude is above the limit to accurately quantify peak area	No copy number reported, variant status provided	Rerun sample
Precision QC (PR)	The Precision QC is designed to protect against resolving ambiguous genotypes with peak ratios outside tolerable precision ranges.	Fail: The normalized ratio falls between copy number bins	Copy number provided for targets passing QC, variant status provided	Rerun sample
Control QC (CF)	The Control QC indicates a batch-level failure determined by the failure of one or more control samples (CAL, CON, UDCAL, or UDCON).	Fail: A calibrator or control has the wrong copy number or variant call or fails its ROX, signal magnitude, saturation or precision QC.	No copy number reported, variant status provided	Rerun batch
Peak Morphology QC (PM)	The Peak Morphology QC is designed to identify abnormally shaped peaks for the endogenous control peak (possible split peak structure or unexpected additional peaks in EC region).	Risk: The endogenous control peak is abnormally shaped	Copy number and variant status provided	Review sample

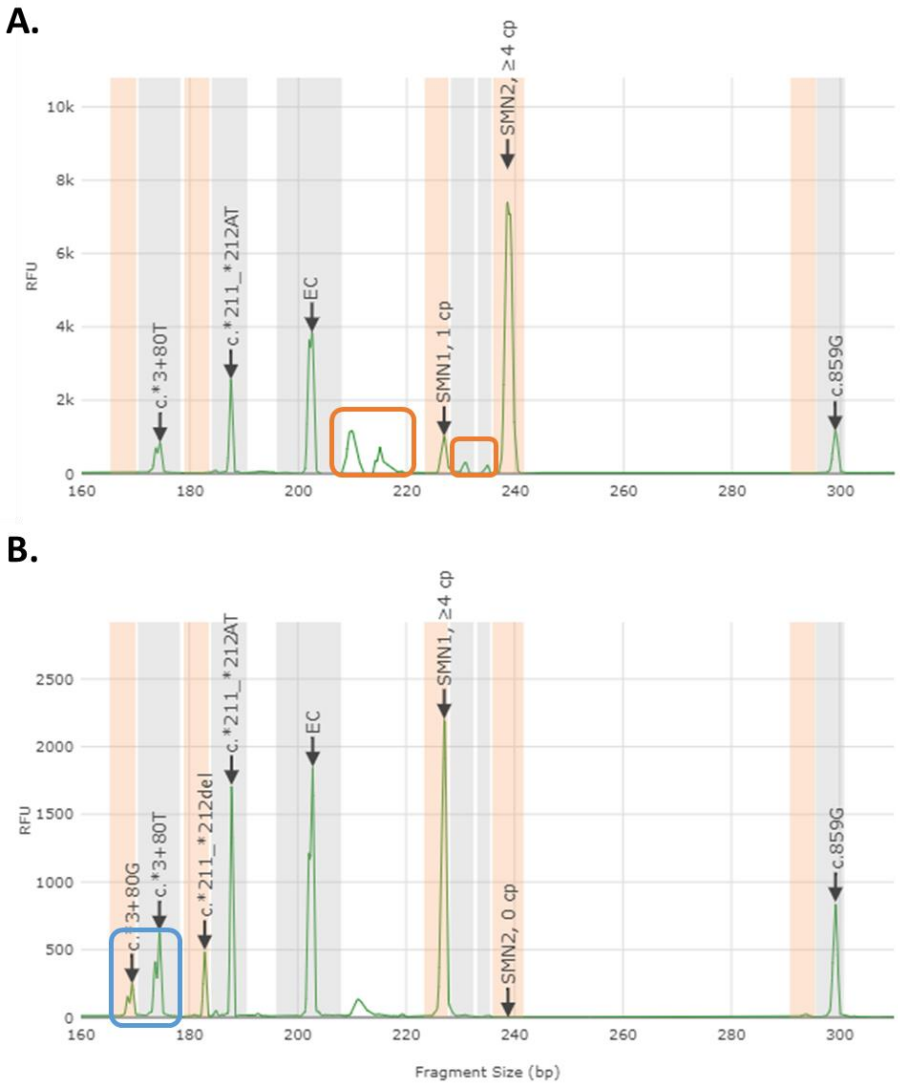

Figure 6. Example of sample CE traces from AmplideX® PCR/CE Reporter with Peak Morphology QC failures. **A.** In this sample, background peaks between the EC and *SMN1* peaks are much higher than other samples in the run, and several background peaks are present between *SMN1* and *SMN2* peaks (orange boxes). Background peaks between the *SMN1* and *SMN2* peaks could lead to false identification of hybrid peaks in extreme cases, though they are quantified correctly in this case. This peak morphology is atypical when compared to other sample traces in the same run, and thus the sample should be rerun to confirm results. **B.** In this sample, background peaks are low compared to the trace above it. N-1 peak shoulders are present in some peaks (blue box), but occur in the same peaks as the trace above it. This profile does not usually affect quantification when consistent across the run. In this case, the sample is correctly quantified and does not need to be rerun.

Interpretation of Results

Specimen results should be interpreted according to applicable clinical practice guidelines. For example, see:

- "Diagnosis and management of spinal muscular atrophy: Part 1: Recommendations for diagnosis, rehabilitation, orthopedic and nutritional care" (Mercuri *et al.* 2018)
- "Technical standards and guidelines for spinal muscular atrophy testing" (Prior *et al.* 2011)
- "Best practice guidelines for molecular analysis in spinal muscular atrophy" (Scheffer *et al.* 2001)

For both diagnosis and carrier determination, *SMN1* exon 7 copy number is the primary relevant genetic information, while *SMN2* exon 7 copy number is relevant for patient prognosis and treatment decisions, but not carrier testing (Glascock *et al.* 2018, Mercuri *et al.* 2018, Prior *et al.* 2011, Sheffer *et al.* 2001). For more details on the relevant information for diagnosis and carrier status interpretation, see the appropriate following sections.

While *SMN1* and *SMN2* exon 7 copy numbers are broadly considered the most critical clinical information for molecular SMA testing, several point mutations detected by this kit may further inform residual carrier risk and disease prognosis in some cases. Clinical evidence suggests that the presence of *SMN1* gene duplication variants c.*3+80T>G and c.*211_*212del detected by this kit can indicate silent carriers and modify residual carrier risk in some ethnicities (Luo *et al.* 2014; Alías *et al.* 2018). Further, the c.859G>C mutation detected by this kit is linked to improved splicing efficiency of *SMN2*, leading to reduced disease severity (Glascock *et al.* 2018, Vezain *et al.* 2010, Prior *et al.* 2009). The impact of the variants detected by this kit is an area of ongoing investigation in the literature and guidelines; any interpretation based on the presence of these variants should be informed by the most recent applicable guidelines and literature available.

This kit also detects the presence of gene conversions between *SMN1* and *SMN2* using gene-specific nucleotides in exon 7 and intron 7, herein referred to as hybrid genes. While gene conversion is known to occur and is one potential cause for the silent carrier 2+0 genotype (Prior *et al.* 2011), the clinical significance of these hybrid genes is not fully understood. For the purposes of diagnosis and carrier testing, only the total *SMN1* and *SMN2* exon 7 copy numbers reported by this kit should be considered.

Interpretation for Diagnosis

Most SMA patients (~96%) have 0 copies of *SMN1* exon 7 (0+0), representing a homozygous exon 7 deletion (Mercuri *et al.* 2018), which are detected by this kit. Approximately 3–4% of SMA patients have a mutation in *SMN1* leading to loss of function in one *SMN1* copy, and a deletion of exon 7 in the other copy of *SMN1* (1^{d}+0), representing a compound heterozygote (Mercuri *et al.* 2018, Prior *et al.* 2011). These loss of function mutations are not detected by this kit; 1^{d}+0 genotypes will present as 1 copy of *SMN1* exon 7. In these cases, guidelines recommend sequencing of *SMN1* to identify mutations when clinical signs of SMA are present (Mercuri *et al.* 2018). Though extremely rare and likely due to consanguinity, it is also possible for SMA patients to have 2 copies of *SMN1* exon 7 with loss of function mutations present in both copies of *SMN1* (1^{d}+1^{d}), which are also not detected by this kit (Mercuri *et al.* 2018, Prior *et al.* 2011). Refer to **Table 10** for details on the interpretation of *SMN1* exon 7 copy numbers for disease diagnosis. Interpretations are based on clinical suspicion of SMA prior to diagnostic testing.

Table 10. Interpretation of results for diagnosis based on best practices

***SMN1* Exon 7 Copy Number**[a]	**Interpretation**[b]	**Additional Information**[b]
0	Confirmed SMA	Definitive diagnosis of SMA
1	SMA possible	Possible compound heterozygote; sequencing of *SMN1* is recommended to identify subtle mutations if SMA is suspected
≥ 2	SMA unlikely	Though highly unlikely, it is possible for both copies of *SMN1* to possess loss of function mutations; sequencing of *SMN1* is recommended in cases of striking consistency with SMA phenotype or consanguinity

[a] Generated by the test.

[b] According to Mercuri *et al* (2018). Interpretations are based on clinical suspicion of SMA prior to diagnostic testing.

Interpretation for Prognosis

While *SMN2* exon 7 copy number is not essential for diagnosis of SMA, guidelines recommend that results are reported and assessed to inform prognosis and treatment decisions (Glascock *et al.* 2018, Mercuri *et al.* 2018). Though *SMN2* copy number is strongly correlated with SMA type, there are exceptions, and copy number alone is not sufficient to predict SMA type in all cases (Mercuri *et al.* 2018). These limitations should be communicated when reporting *SMN2* copy number results.

Additionally, the c.859G>C variant detected by this kit is a positive disease modifier associated with reduced disease severity and improved prognosis (Glascock *et al.* 2018, Vezain *et al.* 2010, Prior *et al.* 2009). Evidence suggests that c.859G>C improves *SMN2* splicing, exon 7 inclusion, and full length SMN protein production, leading to improved phenotypic outcomes (Vezain *et al.* 2010, Prior *et al.* 2009). For instance, while 90% of patients with 2 copies of *SMN2* exon 7 typically have SMA type 1, patients with 2 copies of *SMN2* exon 7 and the c.859G>C variant typically have SMA type 2 or type 3, with no known cases of SMA type 1 in patients with this genotype (Calucho *et al*. 2018, Vezain *et al.* 2010, Prior *et al.* 2009). While the impact of the c.859G>C variant in patients with *SMN2* copy numbers other than 2 is presumably similar, there are currently no reports in the literature on co-occurrence of the variant with other *SMN2* copy number genotypes in SMA patients. Based on this evidence, a positive result for

c.859G>C may be interpreted as “positive disease modifier detected; reduced severity/improved prognosis relative to typical presentation based on *SMN2* copy number genotype.”

A summary of likely SMA types based on *SMN2* exon 7 copy number from published treatment guidelines is provided in **Table 11**. Prognostic information is relevant only in cases of SMA diagnosis. When reporting *SMN2* copy number, it is imperative to communicate that the relationship between *SMN2* copy number and disease severity is not absolute (Prior *et al*. 2011, Mercuri *et al.* 2018). Recommendations for treatment based on *SMN2* copy number are evolving and may be variable depending upon region. Interpretation should follow all local regulatory and clinical guidelines.

Table 11. Interpretation of results for likely SMA prognosis based on best practices.

SMA diagnosis (Table 10)	*SMN2* Exon 7 Copy Number[a]	Prognostic Information[b]
SMA	1	Probable Type 0
SMA	2	Probable Type 1[c]
SMA	3	Probable Types 2/3
SMA	≥ 4	Probable Types 3/4

[a] Generated by the test.
[b] According to Glascock *et al* (2018).
[c] If c.859G>C is positive and *SMN2* exon 7 copy number is 2, Probable Types 2/3 (Calucho *et al*. 2018, Vezain *et al.* 2010, Prior *et al.* 2009).

Interpretation for Carrier Testing

An SMA carrier is an asymptomatic individual lacking a functional copy of *SMN1* on one chromosome. Most SMA carriers have an exon 7 deletion in *SMN1* on one chromosome and one functional *SMN1* copy on the other (1+0), representing a heterozygous deletion (Prior *et al.* 2011). A cis carrier genotype (2+0), commonly referred to as a silent carrier, is also known (Prior *et al.* 2011). Additionally, carriers may instead have a loss of function mutation in *SMN1* on one chromosome and two *SMN1* exon 7 copies (1^{d}+1), or rarer genotypes with higher *SMN1* exon 7 copy numbers (1^{d}+2, 3+0). Because of this, the detection rate of SMA carriers using *SMN1* copy number alone to detect 1+0 genotypes varies from ~71% up to 95% depending on ethnicity due to differences in the frequency of gene duplication events and loss of function mutations (Hendrickson *et al.* 2009). Thus, the potential for false negative results should be communicated when reporting results. Based on this, residual carrier risk estimations have been calculated for many ethnicities (MacDonald *et al.* 2014), and should be used where available (Prior *et al.* 2011).

In addition to *SMN1* copy number, the presence of *SMN1* gene duplication variants c.*3+80T>G and c.*211_*212del detected by this kit can be indicative of the silent carrier 2+0 genotype in some ethnicities (Luo *et al.* 2014; Alías *et al.* 2018). Typically, these variants co-occur (Luo *et al.* 2014); however, individuals with only one variant are known to occur (Alías *et al.* 2018). For evaluation of these variants as indicators of silent carrier status and residual risk evaluation, a result of “positive” for either c.*3+80T>G or c.*211_*212del should be considered positive for gene duplication variants. Importantly, a negative result for these variants does not rule out the possibility of an individual having a silent carrier 2+0 genotype, and a positive result does not definitively identify silent carriers in all ethnicities. Additionally, the impact of

these variants has not been evaluated in all ethnicities. In specimens of unknown ethnicity or in ethnicities where variant status is of unknown significance for residual risk, a result of 2 *SMN1* exon 7 copies and positive for either gene duplication variant may be considered “increased silent carrier risk”.

Refer to **Table 12** for details on the interpretation of *SMN1* exon 7 copy numbers for carrier status determination. Refer to **Table 13** for carrier frequency and residual risk estimations by ethnicity based on current literature reports, including for impact of *SMN1* gene duplication variants on residual risk where known. Interpretation should follow all local regulatory and clinical guidelines.

Table 12. Interpretation of results for carrier testing based on best practices

***SMN1* Exon 7 Copy Number**[a]	**Interpretation**[b]	**Additional Information**
1	SMA Carrier	Definitive carrier of SMA
2	Negative - Reduced Carrier Risk	Residual carrier risk varies from 1:96 to 1:781 depending on ethnicity based on copy number alone[c]; *SMN1* gene duplication variant detection may identify silent carriers and modify residual risk numbers[d]; see **Table 13**
≥ 3	Negative - Significantly Reduced Carrier Risk	Residual risk varies from 1:1604 to 1:7574 depending on ethnicity based on copy number alone[c]; see **Table 13**

[a] Generated by the test.
[b] According to Prior *et al*. (2011).
[c] According to MacDonald *et al*. (2014).
[d] According to Luo *et al*. (2014), Alías *et al*. (2018).

Table 13. Residual carrier risk estimates by ethnicity for negative carrier status results (see **Table 12**). Variants refer to *SMN1* gene duplication variants c.*3+80T>G and c.*211_*212del detected by this kit.

		Residual Risk Estimates			
Ethnicity	**Carrier Frequency**	**2 copies *SMN1* exon 7[d]**	**3 copies *SMN1* exon 7[d]**	**2 copies *SMN1*, variant status "Negative" [e]**	**2 copies *SMN1*, variant status "Positive" [e]**
Ashkenazi Jewish	1:56[a]	1:514[a]	1:5899[a]	1:580[b]	SMA Carrier[b]
Asian	1:50[a]	1:719[a]	1:5185[a]	1:702[b]	SMA Carrier[b]
African American/Black	1:71[a]	1:132[a]	1:6997[a]	1:396[b]	1:34[b]
Caucasian	1:45[a]	1:604[a]	1:4,719[a]	1:769[b]	1:29[b]
Hispanic	1:83[a]	1:641[a]	1:7574[a]	1:1762[b]	1:140[b]
Spanish	1:40[c]	1:781[c]	Not Reported	1:888[c]	SMA Carrier[c]
Israeli Jewish	1:38[a]	1:450[a]	1:4004[a]	Not Reported	Not Reported
Asian Indian	1:50[a]	1:428[a]	1:5252[a]	Not Reported	Not Reported
Iranian	1:16[a]	1:96[a]	1:1604[a]	Not Reported	Not Reported

[a] According to MacDonald *et al*. (2014).
[b] According to Luo *et al.* (2014). Values rounded to nearest integer.
[c] According to Alías *et al*. (2018).
[d] Residual risk values based on *SMN1* copy number alone
[e] Residual risk values based on *SMN1* copy number of 2 and *SMN1* c.*3+80T>G and c.*211_*212del status

Troubleshooting

Observation	Potential Cause	Action
Peaks present in NTC sample **Note:** The NTC is optional and is not required for analysis; expected results are described in the **Quality Control Procedures** section	Contamination of the NTC, improper instrument calibration	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Check the capillary injection number is within manufacturer specifications. • Perform instrument and capillary maintenance as needed. • Repeat the batch run, beginning with PCR; pay special attention to pipetting technique (e.g., aerosolization of samples) and ensure the PCR plate is centrifuged prior to transfer into the CE plate; review the laboratory's contamination control procedures.
Calibrator or control fails QC criteria (CF in QC column)	Issue with CE injection or PCR batch run, improper instrument calibration	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Ensure a compatible CE instrument/capillary length and appropriate thermal cycler settings, CE instrument settings, and CE formulations were used as described in Procedural Steps. • Ensure the proper calibrator and control are used for analysis (1 replicate each of calibrator and control). • After uploading FSA files, ensure that the appropriate annotation is used for the calibrator and control. For the default workflow, use the CAL and CON annotations for the SMN Calibrator and SMN Control included in the kit. For UDC workflow, use the UDCAL and UDCON annotations. See **User-Defined Calibration** section for details. • For the UDC workflow, analysis is completely dependent on selection of appropriate calibrator and control reference samples. Refer to the **User-Defined Calibration** section for details. • Check the raw CE data of the ROX ladder and HEX channel for issues (e.g., extra and/or atypical peaks). • If failure is due to issues above or control results were < 0.05 outside of expected normalized ratio results, repeat CE plate injection if ≤ 48 hours have passed since preparation (otherwise, prepare a fresh CE plate). • If root cause is not identified above, repeat PCR with all specimens, calibrator, and control.

Observation	Potential Cause	Action
Specimen, calibrator, or control fails ROX QC criteria (LD in QC column)	Issue with ROX ladder	• Repeat CE plate injection for affected samples with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare a fresh CE plate). • If failure persists, check raw data for issues indicating loss of resolution, such as declining peak heights in the ROX ladder, or inconsistent migration of ROX ladder peaks between samples. If issues are observed, inject Hi-Di™ only for 5 to 10 injections to clean system. • Capillary age can also affect consistent migration; check the capillary age and number of injections to confirm it is within manufacturer specifications. • Repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section (re-formulate CE Plate) using previously amplified PCR products for all affected samples along with the corresponding batch calibrator and control.
Specimen, calibrator, or control fails Signal Magnitude QC criteria (LS in QC column)	DNA input is below mass input range (< 20 ng), DNA specimen impurity, no DNA input due to mispipetting, aged capillary, improper instrument calibration	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Check the raw CE data of the ROX ladder and HEX channel for issues (e.g., extra peaks). • Capillary age can affect signal height; check the capillary age and number of injections to confirm it is within manufacturer specifications. • If failure is due to issues above, repeat CE plate injection for affected specimens with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare a fresh CE plate). • Check DNA sample concentration and purity to ensure it is within assay range; note that some contaminants can affect quantitation of DNA concentration. • If root cause is not identified above and sample concentration is sufficient, repeat PCR with all affected specimens, calibrator, and control. Pay special attention to pipetting technique, particularly when adding the DNA sample. • Repeat DNA isolation and review procedure and reagents for potential sources of contamination or degradation of nucleic acids during purification workflow.

Observation	Potential Cause	Action
Specimen, calibrator, or control fails Saturation QC criteria (SA in QC column)	DNA input is above mass input range, improper instrument calibration	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Check the raw CE data of the ROX ladder and HEX channel for issues (e.g., extra peaks). • If failure is due to issues above, repeat CE plate injection for affected specimens with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare fresh CE plate). • Check the specimen concentration to ensure it is within assay range; note that some contaminants can affect quantitation of DNA concentration. • Dilute the specimen with Diluent included in the kit; if the specimen was initially within the assay range, dilute to ~10 ng/µL such that the DNA mass input will be 20 ng. • Repeat the PCR with all affected specimens, calibrator, and control.
Widespread saturation or Precision QC Failure, inconsistent copy number results	An incompatible thermal cycler or CE model was used, incorrect thermal cycling or CE protocol used, improper instrument calibration, incompatible purification method	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Ensure proper instrument model and settings are utilized. • Ensure that the proper calibrator and control are used for analysis (1 replicate each of calibrator and control). Ensure the appropriate settings are used during analysis (see **Data Analysis**) • Repeat PCR, or repeat CE plate injection if ≤ 48 hours have passed since preparation (otherwise, prepare fresh CE plate). • If issues persist, it may indicate that the default protocol and bin settings are not compatible with your DNA purification method. Refer to the Pre-Analytical Steps and User-Defined Calibration sections for details on purification methods and calibration approach.

Observation	Potential Cause	Action
Specimen, calibrator, or control fails Precision QC criteria (PR in QC column)	Injection error, incorrect pipetting, insufficient sample purity, sample contamination with PCR inhibitors	• Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Ensure a compatible CE instrument/capillary length and appropriate thermal cycler settings, CE instrument settings, and CE formulations were used as described in **Procedural Steps**. • Ensure the proper calibrator and control are used for analysis (1 replicate each of calibrator and control). Ensure the appropriate settings are used during analysis (see **Data Analysis**) • Repeat CE plate injection for affected specimens with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare a fresh CE plate). • Check sample purity, input amount, and type to ensure compatibility with the assay; note that some contaminants can affect quantitation of DNA concentration and purity. Note: In addition to the 260/280 specifications mentioned in the protocol, comparing the 260/230 ratio of the affected specimen to other specimen from the same purification method may help identify potential contamination issues. • If a concentration or contamination issue is suspected, dilute the specimen with Diluent included in the kit to ~10 ng/µL such that the DNA input is 20 ng, then repeat PCR with all affected specimens, calibrator, and control. Note: If issues continue to be observed, preparing a titration series of multiple DNA concentrations may help identify potential contamination issues. In such cases, the accuracy of the result often improves as the sample is diluted and the influence of contaminants is reduced. • If root cause is not identified above and sample is compatible, repeat PCR with all affected specimen, calibrator, and control.

Observation	Potential Cause	Action

Specimen, calibrator, or control is flagged by Peak Morphology QC criteria (PM in QC column)
 	
OR
 	
Split peaks observed for the EC, *SMN1*, *SMN2*, *SMN1* hybrid, or *SMN2* hybrid peaks
 	
OR
 	
Variable peak morphology observed across EC peaks in a given run (e.g., peak shoulders present in some EC peaks but not others in a given run)
	Instrument capillary issue, improper instrument calibration, run buffer/polymer temperature issue, thermal cycler issue, CE formulation issue, Hi-Di™ Formamide purity/storage issue, room temperature fluctuation	• Peak shoulders or minor peak splitting, indicated by presence of an N-1 peak relative to the primary peak, do not typically affect quantification. If observed in the EC peak, ensure that all EC peaks in the Calibrator, Control, and specimens from the run have similar morphology; if so, no action is needed **Figure 6** for examples.
		• Check if the issue occurs in a specific capillary or well location, is localized to the first injection, or occurs across the entire injection plate; additionally, check the ROX channel to see if the issue persists in all channels.
		• If the issue is localized to the first injection of each run:
		  ○ Confirm the polymer and run buffer(s) were at room temperature before installing on the instrument prior to starting the run; CE run buffers and polymer that are not warmed to room temperature prior to use can produce split peaks.
		  ○ Confirm that the room temperature conforms to manufacturer recommendations for CE instrument operations.
		• If the issue occurs in a specific capillary or well location, this may indicate a capillary or instrument-specific issue. Repeat CE plate injection for affected specimens with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation, changing plate position if desired. For best results, we recommend also repeating CE plate denaturation to resolve this issue. If more than 48 hours have passed, prepare a fresh CE plate. If the issue persists, contact the instrument manufacturer for further troubleshooting support.

Low peak heights across all HEX-labeled assay peaks for all samples, calibrator, and control included in a run	Incorrect thermal cycling, incorrect instrument settings, incompatible instrumentation, incorrect CE formulation, incompatible reagent storage conditions or handling	• Ensure compatible instrumentation and appropriate thermal cycler settings, CE instrument settings, and CE formulations were used as described in **Procedural Steps**. • Ensure that reagents are stored and handled as described in Storage & Handling and **Warnings and Precautions** • Verify that the SMN1/2 Plus HEX Primer Mix has a light pink color. If the liquid is colorless, it may indicate a storage or contamination issue. Contact support@asuragen.com for more information.

Performance Characteristics – Analytical

Note: For the analytical performance assessment, samples were analyzed using the appropriate workflow (default or UDC) as defined above (see Pre-Analytical Steps). DNA isolated from human whole blood with the methods listed in **Required but Not Provided** (QIAamp® DNA Blood Kits, FlexiGene DNA Kit, or Wizard® Genomic DNA Purification Kit) or from human cell lines as obtained from Coriell were analyzed using the default workflow. DNA isolated using other methods (QIAsymphony DSP DNA Kit, Chemagic™ DNA Blood Kits, custom magnetic bead-based methods) were analyzed with the UDC workflow.

Within-lab Precision

To assess the precision of the AmplideX® SMA Plus Kit, nine unique samples consisting of DNA isolated from human cell lines (n=5) or whole blood (n=4) with 0, 1, 2, 3, or ≥ 4 *SMN1* copies and 0, 1, 2, 3, or ≥ 4 *SMN2* copies were tested using 20 ng DNA input per reaction. Samples were isolated using precipitation or automated magnetic bead methods. This sample set included one sample with an *SMN2*-to-*SMN1* gene conversion and one sample with each of the c.*3+80T>G, c.*211_*212del, and c.859G>C variants was also included in the sample set. Additionally, each batch run contained two NTC reactions, with Diluent added in place of DNA.

Testing was performed in duplicate across 20 batch runs using two operators, three reagent lots, and two Applied Biosystems® Veriti™ 96-well thermal cyclers, generating 40 measurements per sample, or 360 sample measurements in total. The study was analyzed on one Applied Biosystems 3500xL Genetic Analyzer. Equivalency between supported CE instruments and capillary lengths was verified previously. For calculations of mean, standard deviation, and %CV values, precision QC failures were included in analysis; other QC failures were excluded from analysis. For copy number agreement, all QC failures were excluded from analysis.

Out of 359 valid measurements generated by the study, the standard deviation across all normalized ratios for *SMN1* and *SMN2* peaks of each sample ranged from 0.000 to 0.114 (**Table 14**). The %CV was similar across all samples, ranging from 4.5 to 8.4%. Additionally, 357/358 valid *SMN1* measurements (99.7%) and 344/346 valid *SMN2* measurements (99.4%) produced concordant copy number results compared to the copy number mode for each sample.

Taken together, these data demonstrate that the AmplideX PCR/CE SMA Plus Kit has sufficient precision to unambiguously resolve *SMN1* and *SMN2* copy numbers spanning the full copy number range of the assay across multiple operators, and thermal cyclers.

Table 14. Precision of the AmplideX® SMA Plus Kit for *SMN1* and *SMN2* copy number. Measurements are reported in Normalized Ratio (NR), as generated by the AmplideX PCR/CE Reporter using equations 1 and 4 in the Data Analysis section. Results are indicated for primary *SMN1* and *SMN2* peaks only (no hybrid peaks).

Sample	Sample Type	*SMN1* Copies*	n	*SMN1* Mean (NR)	*SMN1* SD (NR)	*SMN1* %CV (NR)	*SMN2* Copies*	n	*SMN2* Mean (NR)	*SMN2* SD (NR)	*SMN2* %CV (NR)
1	Blood	0	40	0.000	0.000	NA	2	40	0.971	0.078	8.0
2	Blood	0	40	0.000	0.000	NA	3	40	1.594	0.092	5.8
3	Blood	1	40	0.523	0.033	6.3	4	40	1.963	0.114	5.8
4	Blood	4	40	1.93	0.087	4.5	1	40	0.525	0.033	6.3
5	Cell Line	0	40	0.000	0.000	NA	2	40	0.909	0.049	5.4
6	Cell Line	1	40	0.466	0.031	6.7	3	40	1.312	0.090	6.9
7	Cell Line	3	39	1.318	0.074	5.6	0	39	0.000	0.000	NA
8	Cell Line	2	40	0.879	0.069	7.9	2	40	0.892	0.075	8.4
9	Cell Line	2	40	0.904	0.062	6.9	2	40	0.898	0.052	5.8

* does not include gene conversions (hybrid peaks)

DNA Input

In order to assess the DNA input range of the AmplideX SMA Plus Kit, eight unique samples consisting of DNA isolated from human cell lines (n=6) or whole blood (n=2) with 0, 1, 2, or 3 *SMN1* copies and 0, 1, 2, 3, or ≥ 4 *SMN2* copies were tested in duplicate with six different DNA input amounts from 10, 20, 50, 60, 70, or 80 ng of DNA per PCR (80 ng excluded for 3130*xl*) using two reagent lots, generating 192 sample measurements per CE instrument (160 for 3130*xl*). Samples were tested on the following Applied Biosystems® Genetic Analyzers: 3500xL, SeqStudio™, 3130*xl* and 3730*xl* (with 48-capillary configuration). QC failures were excluded from analysis.

Results from valid sample measurements showed that DNA inputs from 10 ng to 80 ng of DNA per reaction (10 to 70 ng for 3130*xl*) produced expected copy number results for both *SMN1* and *SMN2* across all instruments (**Table 15**). Additionally, calls for all three variants were 100% concordant for all sample measurements on all instruments. These results support the assay DNA input range of 20 to 60 ng for 3130 Genetic Analyzers, and 20 to 70 ng per PCR for 3500, 3730, and SeqStudio Genetic Analyzers.

Table 15. DNA Input for the AmplideX® SMA Plus Kit. *SMN1* and *SMN2* copy number concordance are indicated.

DNA Input	Exon 7 Copy Number Concordance, Overall Percent Agreement	
	SMN1	***SMN2***
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)**

Analytical Specificity

For the analytical specificity study, data generated from the within-lab precision study, DNA input study, and method comparison study were combined for analysis. For details on these study designs, see the other sections described herein. Inclusivity was not independently evaluated for *SMN1* and *SMN2* copy number, as this was demonstrated in the DNA input and precision studies described in the previous section.

To assess analytical specificity (exclusivity) for *SMN1* and *SMN2* copy number, all valid measurements from samples with 0 *SMN1* copies (279 measurements) and, separately, with 0 *SMN2* copies (156 measurements each) were assessed and compared to consensus reference method values between two independent reference methods (see the **Method Comparison** section), along with 90 NTC measurements where Diluent was added in place of sample DNA during PCR. For samples with 0 *SMN1* copies, 279/279 valid measurements (100%) generated expected copy number results (**Figure 7**). For samples with 0 *SMN2* copies, 156/156 valid measurements (100%) generated expected copy number results (**Figure 8**). Additionally, 90/90 NTC measurements failed to show peak heights above the minimum threshold within the defined peak size bins, generating an expected result of Signal Magnitude QC failure.

To assess analytical specificity (exclusivity) for the c.*3+80T>G, c.*211_*212del, and c.859G>C variants detected by the assay, percent agreement with Sanger sequencing was determined across all instrument configurations with all wild type sample measurements. For all three variants, 100% of sample measurements were in agreement with Sanger sequencing for samples without the variant (c.*3+80T>G: 991/991; c.*211_*212del: 987/987; c.859G>C: 1013/1013).

To assess analytical specificity (inclusivity) for the c.*3+80T>G, c.*211_*212del, and c.859G>C variants detected by the assay, percent agreement with Sanger sequencing was determined across all instrument configurations with all variant positive sample measurements for each variant analyzed independently.

For all three variants, 100% of sample measurements were in agreement with Sanger sequencing for samples with each variant (c.*3+80T>G: 155/155; c.*211_*212del: 159/159; c.859G>C: 133/133). Taken together, these data demonstrate that the AmplideX® SMA Plus Kit has sufficient specificity to determine both *SMN1* and *SMN2* copy numbers within the copy number range of the assay (0, 1, 2, 3, and ≥ 4), as well as to determine variant status for the c.*3+80T>G, c.*211_*212del, and c.859G>C variants.

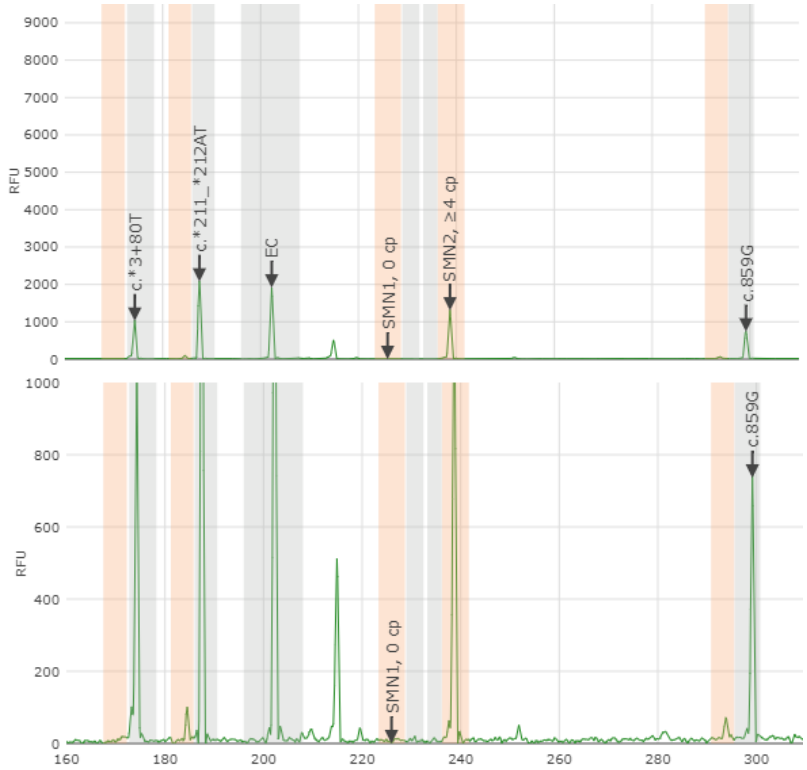

Figure 7. Example electropherogram of a sample with 0 copies of *SMN1* and ≥ 4 copies of *SMN2*. Top panel shows y-axis scaled to ~9,500 RFU; bottom panel shows y-axis scaled to ~1,000 RFU.

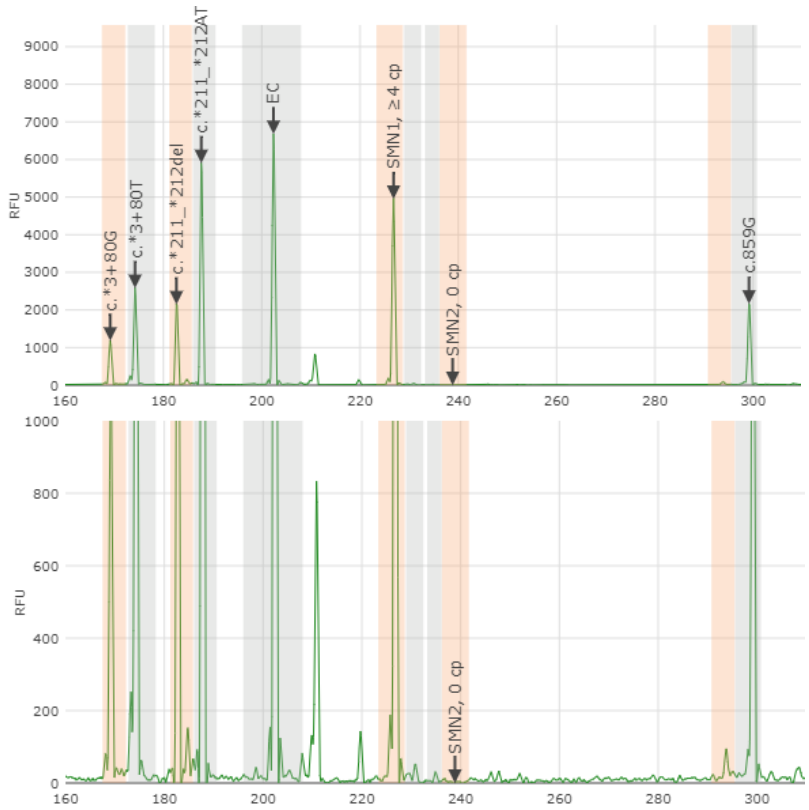

Figure 8. Example electropherogram of a sample with ≥ 4 copies of *SMN1* and 0 copies of *SMN2*. Top panel shows y-axis scaled to ~9,500 RFU; bottom panel shows y-axis scaled to ~1,000 RFU.

Performance Characteristics – Clinical

Note: For the clinical performance assessment, samples were analyzed using the appropriate workflow (default or UDC) as previously defined (see **Pre-Analytical Steps**). DNA isolated from human whole blood with the methods listed in **Required but Not Provided** (QIAamp® DNA Blood Kits, FlexiGene DNA Kit, or Wizard® Genomic DNA Purification Kit) or from human cell lines as obtained from Coriell were analyzed using the default workflow. DNA isolated using other methods (QIAsymphony DSP DNA Kit, Chemagic™ DNA Blood Kits, custom magnetic bead-based methods) were analyzed with the UDC workflow.

Method Comparison

In order to assess performance of the AmplideX® SMA Plus Kit, we tested 134 DNA samples isolated from 116 unique samples consisting of human cell lines (n=15) or whole blood (n=119) with 0, 1, 2, 3 ≥ 4 *SMN1* copies and 0, 1, 2, 3, or ≥ 4 *SMN2* copies. DNA samples were isolated using silica column (n=20), functionalized magnetic bead (n=76), or precipitation-based purification methods (n=38). The sample set included samples with the c.*3+80T>G variant (n=24), the c*211_*212del variant (n=28), and the c.859G>C variant (n=1). This sample set also included both *SMN1*-to-*SMN2* and *SMN2*-to-*SMN1* gene conversions.

To determine reference values for *SMN1* and *SMN2* exon 7 copy numbers, all DNA samples were tested using both a validated MLPA-based method at one of three external sites (Method A) and a verified ddPCR-based method at Asuragen (Method B). For Method A, a subset of samples used in this study

were provided to Asuragen as isolated DNA with previously generated results, while 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, with ambiguous or discordant results in either reference method excluded from analysis. This approach excluded 9 samples for *SMN1* and 10 samples for *SMN2*. Additionally, all samples were tested with Sanger sequencing to determine variant status of the gene duplication and disease modifier markers, as well as gene conversion status as determined by *SMN1/SMN2* sequence identity at exon 7 and intron 7. Of these, 14 samples were excluded from c.*211_*212del analysis due to ambiguous sequencing results.

All 134 samples were tested in singleton using the AmplideX® SMA Plus Kit and analyzed using an Applied Biosystems® 3500xL Genetic Analyzer. Samples were tested using DNA input of 20 to 80 ng per reaction. Exon 7 copy numbers reported for each gene were compared to the reference method to determine concordance. QC failures were excluded from analysis.

Across all valid measurements, results were concordant with both reference methods using the AmplideX SMA Plus Kit for *SMN1* and *SMN2* copy numbers (**Table 16**). For samples with a consensus reference method result of 0 *SMN1* copies indicative of SMA diagnosis, 15/15 were concordant between the AmplideX SMA Plus Kit and Methods A and B (**Table 17**). For samples with a reference method result of 1 *SMN1* copy indicative of SMA carriers, 16/16 were concordant between the AmplideX SMA Plus Kit and Methods A and B (**Table 17**). Additionally, presence or absence of variants c.*3+80T>G, c.*211_*212del, and c.859G>C were 100% concordant with Sanger sequencing results (**Table 19**). Gene conversions identified by the AmplideX SMA Plus Kit were also 100% concordant for both *SMN1* hybrids (8/8) and *SMN2* hybrids (2/2) as compared to Sanger sequencing results.

Taken together, these data demonstrate that the AmplideX SMA Plus Kit is highly concordant with multiple reference methods across the full range of copy numbers for both *SMN1* and *SMN2* (**Table 17** and **Table 18**) using different sample purification methods and including samples with *SMN1*-to-*SMN2* and *SMN2*-to-*SMN1* gene conversions.

Table 16. *SMN1* and *SMN2* copy number concordance of the AmplideX SMA Plus Kit. The row labeled Method A and Method B indicates concordance with consensus results concordant between both reference methods.

Reference Method	**Exon 7 Concordance, Overall Percent 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%

Table 17. *SMN1* exon 7 copy number results as measured by the AmplideX® SMA Kit compared to consensus reference method results. Copy numbers indicated are exon 7 copy numbers.

		Consensus *SMN1* copy					
		0	**1**	**2**	**3**	**4**	**Sum**
***SMN1* copy (Result)**	**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**

Table 18. *SMN2* exon 7 copy number results as measured by the AmplideX SMA Kit compared to consensus reference method results. Copy numbers indicated are exon 7 copy numbers.

		Consensus *SMN2* copy					
		0	**1**	**2**	**3**	**4**	**Sum**
Predicted *SMN2* copy	**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**

Table 19. Concordance of variants c.*3+80T>G, c.*211_*212del, and c.859G>C between Sanger sequencing and the AmplideX SMA Plus Kit.

Variant	Concordance, Overall Percent Agreement
c.*3+80T>G	132/132, 100%
c.*211_*212del	118/118, 100%
c.859G>C	132/132, 100%

Disclaimers

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Glossary of Symbols

Symbol	Description
CE	CE marking
IVD	*In vitro* diagnostic medical device
DI	Device Identifier
REF	Catalog number
LOT	Batch code
	Contains sufficient for <n> tests
	Consult instructions before use
	Temperature limitation
	Use by
	Manufacturer
EC REP	Authorized European representative

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Change History

Revision	Change Description	Effective Date
1	Initial Release	2020-07-07