Asuragen®

AmplideX®

PCR/CE *SMN1/2* Plus Kit

Protocol Guide

Research Use Only. Not for use in diagnostic procedures.

REF A00050

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REF A00054

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Purpose

The AmplideX® PCR/CE *SMN1/2* Plus Kit (RUO) is an *in vitro* nucleic acid amplification kit for determination of exon 7 copy number and the genotype status of relevant variants in the *SMN1* and *SMN2* genes. The kit generates exon 7 copy numbers for both *SMN1* and *SMN2* reported as 0, 1, 2, 3, or ≥ 4 genomic copies, and variant status. These variants include *SMN1* c.*3+80T>G and *SMN1* c.*211_*212del, the gene duplication variants, as well as the disease modifier *SMN2* c.859G>C. The kit is designed for PCR on extracted genomic DNA (gDNA) 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.

The AmplideX PCR/CE *SMN1/2* Plus Kit (RUO) is for research use only and not for use in diagnostic procedures.

Limitations

- Blood samples processed into gDNA for use in this product must be collected using K_2EDTA-treated blood collection tubes and processed into gDNA 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*. Samples with two copies of *SMN1* on one chromosome and zero copies on the other (2+0 or silent carriers) may be distinguished from samples 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).
- This kit detects the presence of three variants related to *SMN1* and *SMN2* gene structure and function, including 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).
- Some DNA purification methods may not be compatible with the default workflow and analysis settings in the software as described in this protocol. For more information, see the **Pre-Analytical Steps** section.

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 samples.
- DNase contamination can cause degradation of DNA samples 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 samples, hemolyzed samples, icteric samples, or samples 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.
- 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.

Principle of the Procedure

The AmplideX® PCR/CE *SMN1/2* 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 table below aligns the naming conventions of the gene duplication variants from multiple sources. The HGVS standard nomenclature is used in this protocol guide.

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 PCR/CE *SMN1/2* Plus Kit includes an SMN Calibrator and SMN Control, which are required in each batch run to normalize area ratios and verify performance of the calibrator, respectively. Optionally, use the Diluent as a no-template negative control (NTC).

The AmplideX® PCR/CE Reporter used in conjunction with the AmplideX PCR/CE SMN 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.

Background Information

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 has an incidence of ~1/10,000 live births and a carrier rate of ~1/50. *SMN1* exon 7 is absent in ~95% of patients with SMA, whereas normal individuals typically have two or more functional genomic *SMN1* copies (Prior *et al.* 2011). *SMN1* copy number is typically detected at exon 7, where a single nucleotide (c.840C) distinguishes it from the highly homologous gene *SMN2* (c.840T, Prior *et al.* 2011; Stabley *et al.* 2015). 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; Stabley *et al.* 2015). SMA carriers lack a functional *SMN1* copy on a single chromosome and often have one functional *SMN1* copy (1+0), though a *cis* carrier genotype (2+0) is also known (Prior *et al.* 2011).

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*, *SMN2.* 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 samples with 0 copies of either *SMN1* and/or *SMN2*.

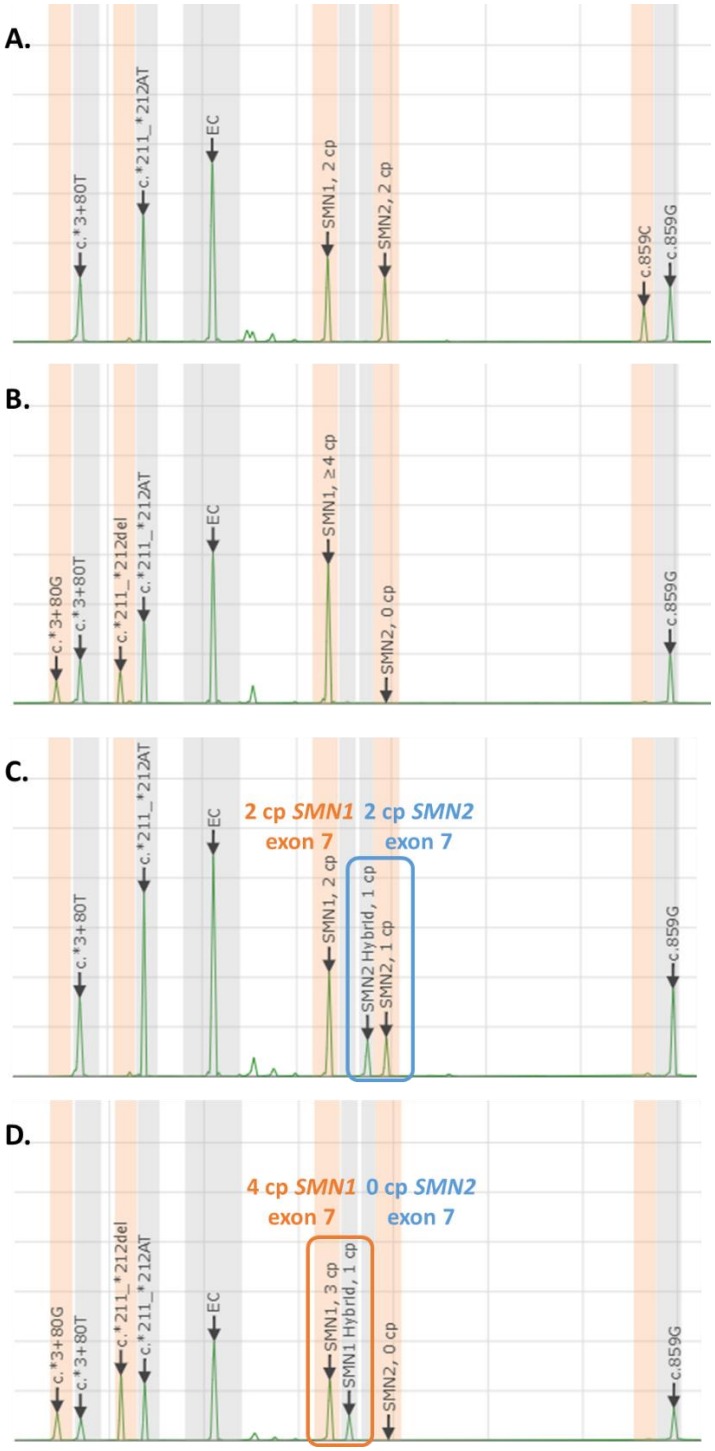

Figure 1. Example AmplideX® PCR/CE *SMN1/2* Plus Kit electropherogram. **A.** A normal 2,2 genotype (2 copies *SMN1*, 2 copies *SMN2*) with variant c.859C. **B.** A 4,0 genotype (4 copies *SMN1*, 0 copies *SMN2*) with variants c.*3+80G, c.*211_*212del. **C.** A 2,2 genotype (2 copies *SMN1*, 2 copies *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, c.*211_*212del.

Workflow

The assay workflow includes PCR master mix setup, thermal cycling, and analysis using CE. For this process, first add purified gDNA derived from human cell lines or whole blood 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, add PCR products to a second master mix comprised of Hi-Di™ Formamide and ROX 1000 Size Ladder. Following denaturation, resolve amplicons 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, analyze the resulting electropherograms on the AmplideX® PCR/CE SMN Analysis Module using the *SMN1/2* Plus Analysis Type to generate normalized area ratios that are automatically interpreted as 0, 1, 2, 3, or ≥ 4 exon 7 copies.

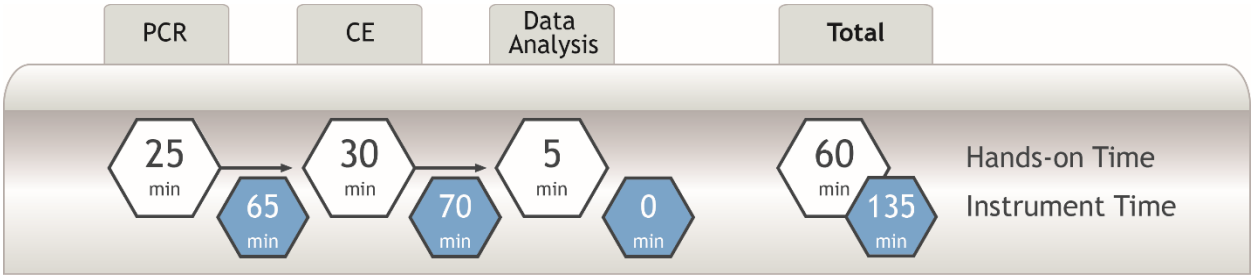

Figure 2. The workflow is streamlined from sample-to-answer and can be performed in less than 3 hours with ~60 minutes of total hands-on time. CE instrument time is for a single injection, or 24 samples using an Applied Biosystems 3500xL Genetic Analyzer.

Use of Calibrator and Control

The kit includes a calibrator (SMN Calibrator) and control (SMN Control), which must be tested in singleton in every batch run for data analysis and quality control. The SMN Calibrator normalizes all sample results generated using the kit. The SMN Control acts as an internal quality control to confirm that normalization to the SMN Calibrator is functioning properly in the batch run. For details on the calibrator and control included in the kit, refer to the **Quality Control Procedures** 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 should be used in place of sample DNA.

If desired, human cell line-derived samples with confirmed *SMN1* and *SMN2* copy numbers and 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 SMN Calibrator and SMN Control included in the kit are required for each batch run.

Note: Since the source material and purification methods used for Coriell cell line DNA are different than standard samples processed by the laboratory, copy number performance of these samples may be impacted (e.g., increased precision QC failures) when compared to whole blood samples. 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* 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*	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†	Negative	Negative	Negative

* 2 copies of *SMN1* and 2 copies of *SMN1* hybrid, for a total of ≥ 4 *SMN1* exon 7 copies
† 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

* Two kit component tubes included in Kit A00054

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 Kit (QIAGEN®) or similar kit. See additional notes in 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
- 96-well PCR fully-skirted plates for use with the Bio-Rad C1000 Touch Thermal Cycler: Bio-Rad, P/N HSP9631 or equivalent
- Adhesive Foil Seals (Beckman Coulter® P/N 538619, or equivalent) or Plastic Seals (VWR P/N 89134-428, or equivalent)
- 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™)
    - SeqStudio:
        - Cathode Buffer: Applied Biosystems P/N A33401
        - Cartridge: Applied Biosystems P/N A33671 or A41331 (V2)
    - 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
- 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 (A00050) or 100 reactions (A00054), including master mix overage.
- The kit supports up to 8 freeze-thaw cycles.
- Master mixes can be prepared for the appropriate number of samples 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.
- 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 gDNA samples prepared from K_2EDTA-treated whole human blood (fresh or stored at 2 to 8 °C for up to 14 days) and collected via laboratory-validated procedures may be isolated using various extraction and purification methods (precipitation, silica column, or functionalized magnetic beads).

It is essential to use a validated isolation method to ensure that DNA is consistently of high quality and purity. Commercially available silica column, precipitation, and magnetic bead purification technologies have all been tested and are generally compatible, as are many automated DNA purification methods. However, some DNA purification methods may benefit from refinements to the workflow and/or software analysis settings to achieve optimal performance. For more information, contact support@asuragen.com.

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 80 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.

Overview of the AmplideX PCR/CE *SMN1/2* 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

1. Thaw the 2X PCR Mix, SMN1/2 Plus HEX Primer Mix, SMN Calibrator, SMN 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 sample to be tested, including the SMN Calibrator and SMN 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
Sample, SMN Calibrator, SMN 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 the appropriate DNA sample 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 SMN Calibrator and the SMN 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

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 samples 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 QC Information section of the AmplideX® PCR/CE SMN Analysis Module Software User Guide. For more information, contact Technical Support.

Data Analysis

The AmplideX® PCR/CE *SMN1/2* 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 *SMN1/2* 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* 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 $Area_{FSA}^{SMN\#}$ 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 2** and **Table 3**.

Table 2. 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 3. Default *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

AmplideX PCR/CE Reporter SMN Analysis Module

The AmplideX® PCR/CE SMN Analysis Module provides the *SMN1/2* Plus Analysis Type for data analysis and interpretation. The software is available at https://asuragen.com/myasuragen. The AmplideX PCR/CE Reporter Software User Guide (00002406) and SMN Analysis Module User Guide (00002468) provides detailed instructions for using the software and are provided with the download package.

Adjustable Copy Number Bins

The default *SMN1*, *SMN2*, and hybrid peak copy number bins in the *SMN1/2* Plus Analysis Type of the AmplideX PCR/CE SMN Analysis Module are a result of extensive assay verification on whole blood and cell-line gDNA. These copy number bins should not require adjustment for the supported instrument configurations, DNA purification methods, and sample types. However, bin values may be adjusted to accommodate non-standard configurations or to change or remove gray zones between sample bins that result in QC failures in the output. Modification of these values is solely at the discretion of the end user. 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 Analysis Module Software User Guide (00002468).

Quality Control Procedures

Calibrator

The SMN Calibrator included in the kit is used to normalize all sample peak area results. The following formula determines the (non-normalized) area ratio for the SMN Calibrator (where *k = {1, 2}*, corresponding to *SMN1* and *SMN2)*:

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

The SMN 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 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 SMN Calibrator sample is outside of the specified range in the following table, 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 Sample and Control QC failure for all samples in the CSV and PDF reports. This alerts the user of a deviation and the batch run is invalid. A valid SMN 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. For further details, refer to the **Troubleshooting** section and the AmplideX PCR/CE SMN Analysis Module Software User Guide (00002468).

Gene	Calibrator Area Ratio (non-normalized)	Expected Exon 7 Copies
SMN1	0.256–0.614	2 copies
SMN2	0.238–0.453	2 copies

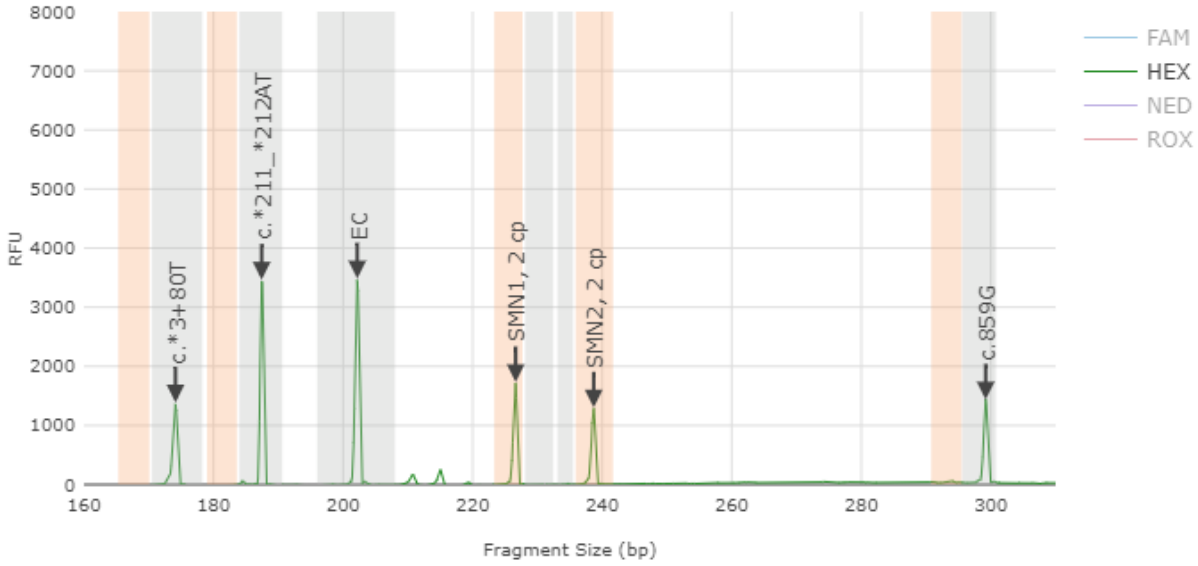

Figure 3. Example of a valid SMN Calibrator CE result from AmplideX® PCR/CE Reporter

Control

The SMN Control included in the kit demonstrates that normalization to the SMN Calibrator is functioning properly in the batch run. The normalized ratio of the SMN Control is based on the formula in the **Data Analysis** section.

The SMN 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 the following table, 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 Sample and Control QC failure for all samples in the CSV and PDF reports. This alerts the user of a deviation and the batch run is invalid. A valid SMN 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. For further details, refer to the Troubleshooting section and the AmplideX PCR/CE SMN Analysis Module Software User Guide (00002468).

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

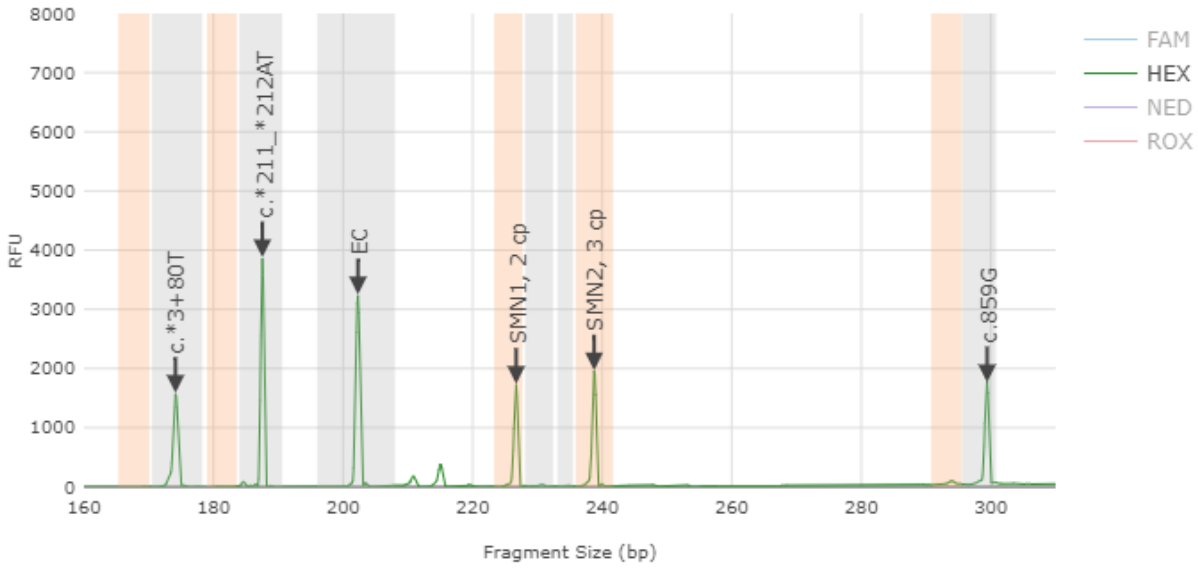

Figure 4. Example of valid SMN Control CE trace from AmplideX® PCR/CE Reporter

No Template Control (NTC)

Use of a negative control (i.e., NTC) is optional. If an NTC is desired, the kit Diluent should 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.

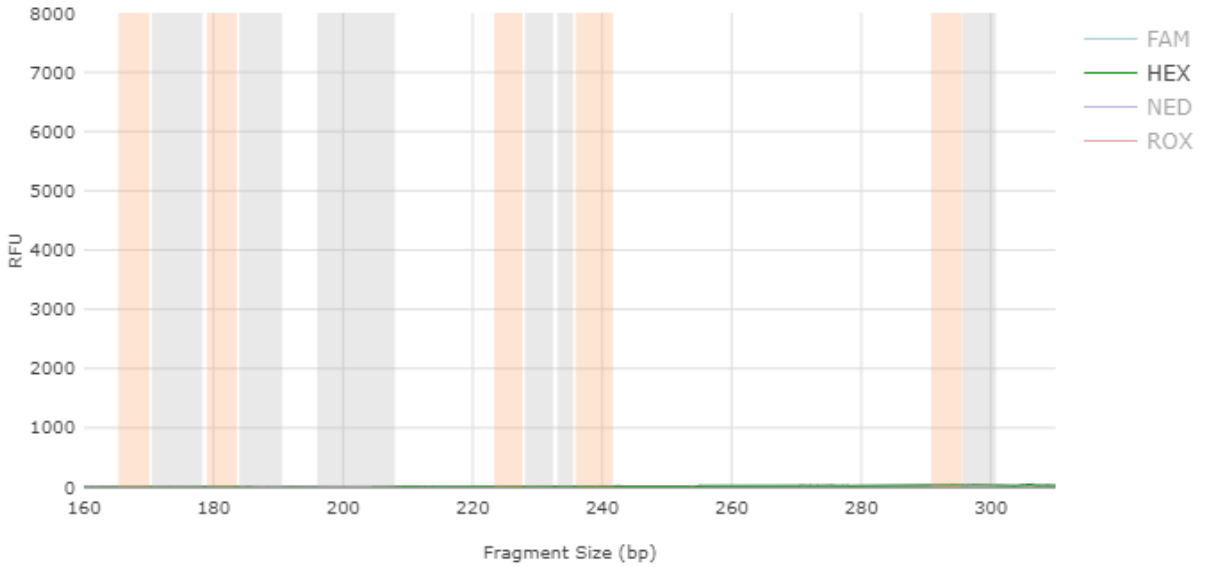

Figure 5. Example of valid NTC CE trace from AmplideX PCR/CE Reporter

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, it is up to the operator or laboratory to determine how to use this information for identifying batch run failures.

Samples

The AmplideX® PCR/CE Reporter with the SMN Analysis Module performs QC checks on all samples to ensure quality results. A precision check (Precision QC) ensures that the normalized ratio of each sample falls within one of the expected (**Table 2** and **Table 3)** or user-defined 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, Saturation, or Precision QC, it will not produce a copy number for *SMN1* or *SMN2*, indicating QC failure. If a sample fails the Peak Morphology QC, it will display all results including copy number, but will indicate the sample is at risk.

In addition to these built-in QC checks, we recommend inspecting samples containing 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. 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. 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 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 2** and **Table 3**. A failing result indicates the normalized ratio for either *SMN1, SMN2,* or both falls in between copy number bins, and will display PR in the QC 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 CSV report will also display FAIL in the Precision 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. **Note:** While copy number will not be reported for either *SMN1* or *SMN2* in the event of a QC failure, the Precision QC may only be out of range for one of the genes. In this case, copy number may optionally be interpreted for the other gene by comparing the normalized ratio to the appropriate bins. For bin values, see **Table 2** in the Data Analysis section.
- The Peak Morphology QC is designed to ensure that peak shapes are sufficient 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. 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. Typically, peak shoulders do not affect copy number quantification, especially when the peak shape is consistent across all samples and controls in a given run. See **Figure 6** for examples. 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.

For more details on these QC checks, refer to the **Troubleshooting** section and the SMN Analysis Module Software User Guide (00002468).

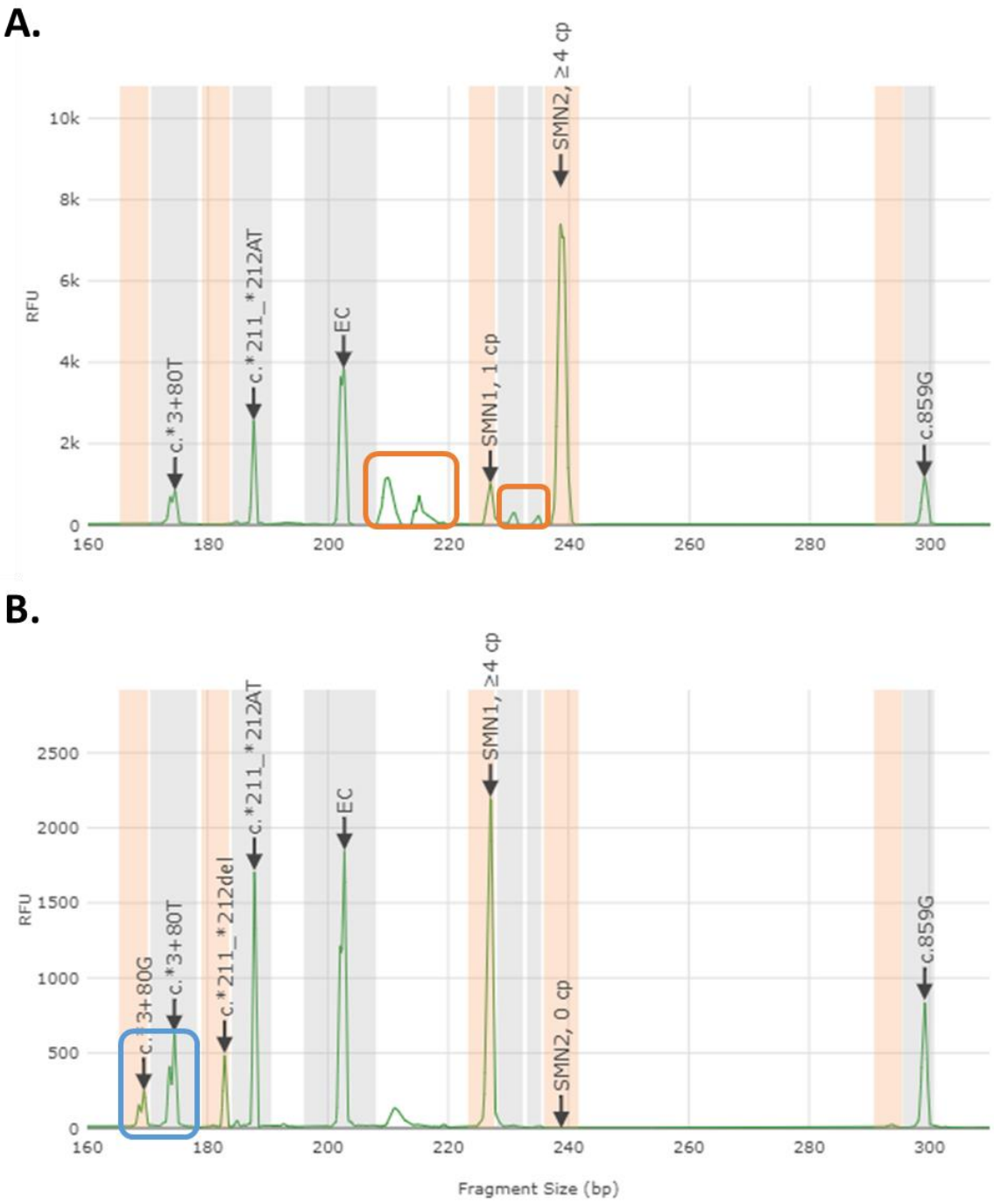

Figure 6. Example of Sample CE traces from AmplideX® PCR/CE Reporter with Peak Morphology QC failures. **A.** In this sample, background peaks between the EC and *SMN1* peaks are much higher than other samples in the run, and several background peaks are present between *SMN1* and *SMN2* peaks (orange 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). This profile does not usually affect quantification. In this case, the sample is correctly quantified and does not need to be rerun.

Troubleshooting

Observation	Potential Cause	Action
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 SMN Calibrator and SMN Control). • Check the raw CE data of the ROX ladder and sample for issues (e.g., extra and/or atypical peaks). • If failure is due to issues above or SMN 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 samples, calibrator, and control.
Sample, 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.

Observation	Potential Cause	Action
Sample, calibrator, or control fails Signal Magnitude QC criteria (LS in QC column)	DNA input is below mass input range (< 20 ng), sample 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 sample 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 samples with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare a fresh CE plate). • Check 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 samples, 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.
Sample, 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 sample for issues (e.g., extra peaks). • If failure is due to issues above, repeat CE plate injection for affected samples with corresponding batch calibrator and control if ≤ 48 hours have passed since preparation (otherwise, prepare fresh CE plate). • Check the sample concentration to ensure it is within assay range; note that some contaminants can affect quantitation of DNA concentration. • Dilute the sample with Diluent included in the kit; if the sample 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 samples, calibrator, and control.

Observation	Potential Cause	Action
Sample, 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 SMN Calibrator and SMN Control). • 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). • 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 sample to other samples from the same purification method may help identify potential contamination issues. • If a concentration or contamination issue is suspected, dilute the sample with Diluent included in the kit to ~10 ng/µL such that the DNA input is 20 ng, then repeat PCR with all affected samples, 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 samples, calibrator, and control.

Observation	Potential Cause	Action

Sample, 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 samples from the run have similar morphology; if so, no action is needed. See **Table 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 samples 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.

Observation	Potential Cause	Action
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 SMN Calibrator and SMN Control). • 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. In Asuragen's experience, this has been most often observed with fully automated magnetic bead-based procedures, though not all laboratory workflows with such methods are affected. If an issue is suspected with your purification method or you are purifying DNA from sources other than blood, contact support@asuragen.com for assistance.
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.

Analytical Assessment

Method Comparison

In order to assess performance of the AmplideX® PCR/CE *SMN1/2* Plus Kit, 90 gDNA samples purified from human blood using silica columns (n=28), functionalized magnetic beads (n=14), precipitation-based purification methods (n=44), or unknown methods (n=4) were tested to determine *SMN1* and *SMN2* exon 7 copy number concordance compared to three comparator methods. Seventy-four samples were tested using a qPCR-based method (Method A), and 86 samples were tested externally using an MLPA-based method (Method B). Additionally, all 90 samples were tested with the AmplideX PCR/CE *SMN1/2* Kit (catalog # A00001) to determine concordance between products, as well as with Sanger sequencing to determine variant status of the gene duplication and disease modifier markers. The sample set included samples with the c.*3+80T>G variant (n=8), the c*211_212del variant (n=10), and the c.859G>C variant (n=3). This sample set also included both *SMN1*-to-*SMN2* and *SMN2*-to-*SMN1* gene conversions as determined by *SMN1/SMN2* sequence identity at exon 7 and intron 7.

All 90 samples were tested in singleton using the AmplideX PCR/CE *SMN1/2* Plus Kit and analyzed using each of the following Applied Biosystems® Genetic Analyzers: 3500xL (50 cm and 36 cm capillary lengths), SeqStudio™ (28 cm capillary length), 3130*xl* (36 cm capillary length) and 3730*xl* (with 48-capillary configuration, 50 cm and 36 cm capillary lengths). 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. For Method A and Method B, copy numbers were binned as 0, 1, 2, or ≥ 3 for comparison to the AmplideX PCR/CE *SMN1/2* Plus Kit. Samples with QC failures in either the comparator method or the AmplideX PCR/CE *SMN1/2* Plus Kit were excluded from analysis.

Across all valid measurements, results were concordant with reference methods and were consistent across all instrument platforms using the AmplideX PCR/CE *SMN1/2* Plus Kit (**Table 4**). Compared to Methods A and B, concordance was higher for *SMN1* than for *SMN2*. For samples with a reference method result of 0 *SMN1* copies, 4/4 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method A, and 5/5 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method B. For samples with a reference method result of 1 *SMN1* copy, 14/14 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method A, and 13/14 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method B.

For samples with a reference method result of ≥ 3 *SMN2* copies, 20/20 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method A, and 14/14 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method B. However, some samples with a reference method result of 2 *SMN2* copies had an AmplideX PCR/CE *SMN1/2* Plus Kit result of 3 or ≥ 4 *SMN2* copies (n=1 Method A, n=7 Method B). Thus, most discrepancies were due to reference method results of 2 copies that measured as 3 or ≥ 4 copies using the AmplideX PCR/CE *SMN1/2* Plus Kit, as all reference method results of ≥ 3 copies from Method A or Method B were quantified correctly. Notably, discrepancies in *SMN2* copy number with MLPA (Method B) have been reported upon sample retesting (Schorling *et al.* 2019). For samples with a reference method result of 2 *SMN2* copies, 20/21 were concordant between the AmplideX PCR/CE *SMN1/2* Plus Kit and Method A.

Both *SMN1* and *SMN2* copy number results were concordant between the AmplideX PCR/CE *SMN1/2* Plus and AmplideX PCR/CE *SMN1/2* Kits. 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 5**).

Taken together, these data demonstrate that the AmplideX® PCR/CE *SMN1/2* Plus Kit is highly concordant with multiple reference methods across the full range of copy numbers for both *SMN1* and *SMN2* (**Table 6**) using different sample purification methods and including samples with *SMN1*-to-*SMN2* and *SMN2*-to-*SMN1* gene conversions.

Table 4. *SMN1* and *SMN2* copy number concordance of the AmplideX PCR/CE *SMN1/2* Plus Kit. The row labeled Method A or Method B indicates concordance with at least one of the reference methods.

Reference Method	Instrument, Capillary Length	Exon 7 Concordance, Overall Percent Agreement	
		SMN1	***SMN2***
Method A or Method B (n=88 *SMN1*) (n=89 *SMN2*)	**3130, 36 cm**	87/88, 98.9%	82/86, 95.3%
	3730, 50 cm	79/80, 98.8%	78/82, 95.1%
	3730, 36 cm	86/87, 98.9%	83/87, 95.4%
	3500, 50 cm	85/86, 98.8%	82/86, 95.3%
	3500, 36 cm	86/87, 98.9%	82/86, 95.3%
	SeqStudio, 28 cm	86/87, 98.9%	84/88, 95.5%
Method A (n=74 *SMN1*) (n=74 *SMN2*)	**3130, 36 cm**	74/74, 100%	67/71, 94.4%
	3730, 50 cm	69/69, 100%	65/69, 94.2%
	3730, 36 cm	73/73, 100%	68/72, 94.4%
	3500, 50 cm	73/73, 100%	67/71, 94.4%
	3500, 36 cm	73/73, 100%	67/71, 94.4%
	SeqStudio, 28 cm	73/73, 100%	69/73, 94.5%
Method B (n=85 *SMN1*) (n=84 *SMN2*)	**3130, 36 cm**	83/85, 97.6%	72/81, 88.9%
	3730, 50 cm	76/78, 97.4%	69/78, 88.5%
	3730, 36 cm	82/84, 97.6%	72/82, 87.8%
	3500, 50 cm	81/83, 97.6%	72/81, 88.9%
	3500, 36 cm	83/85, 97.6%	72/81, 88.9%
	SeqStudio, 28 cm	83/85, 97.6%	73/83, 88.0%
AmplideX PCR/CE *SMN1/2* Kit (n=89 *SMN1*) (n=89 *SMN2*)	**3130, 36 cm**	88/89, 98.9%	85/86, 98.8%
	3730, 50 cm	80/81, 98.8%	82/82, 100%
	3730, 36 cm	87/88, 98.9%	87/87, 100%
	3500, 50 cm	86/87, 98.9%	86/86, 100%
	3500, 36 cm	87/88, 98.9%	86/86, 100%
	SeqStudio, 28 cm	88/88, 100%	88/88, 100%

Table 5. Concordance of variants c.*3+80T>G, c.*211_*212del, and c.859G>C between Sanger sequencing and the AmplideX® PCR/CE *SMN1/2* Plus Kit.

Variant	Instrument, Capillary Length	Concordance, Overall Percent Agreement
c.*3+80T>G	**3130, 36 cm**	90/90, 100%
	3730, 50 cm	84/84, 100%
	3730, 36 cm	90/90, 100%
	3500, 50 cm	90/90, 100%
	3500, 36 cm	90/90, 100%
	SeqStudio, 28 cm	90/90, 100%
c.*211_*212del	**3130, 36 cm**	90/90, 100%
	3730, 50 cm	84/84, 100%
	3730, 36 cm	90/90, 100%
	3500, 50 cm	90/90, 100%
	3500, 36 cm	90/90, 100%
	SeqStudio, 28 cm	90/90, 100%
c.859G>C	**3130, 36 cm**	90/90, 100%
	3730, 50 cm	84/84, 100%
	3730, 36 cm	90/90, 100%
	3500, 50 cm	90/90, 100%
	3500, 36 cm	90/90, 100%
	SeqStudio, 28 cm	90/90, 100%

Table 6. *SMN1* and *SMN2* copy number distribution of samples included in the method comparison study as measured by the AmplideX PCR/CE *SMN1/2* Kit. Copy numbers indicated are exon 7 copy numbers.

Copy Number	***SMN1***	***SMN2***
0	5	23
1	14	19
2	43	24
3	16	18
≥ 4	11	5

Precision

To assess the precision of the AmplideX PCR/CE *SMN1/2* Plus Kit, nine gDNA samples purified from human cell lines or whole blood with 0, 1, 2, 3, or ≥ 4 *SMN1* copies and 0, 1, 2, 3, or ≥ 4 *SMN2* copies were tested using 30 ng DNA input per reaction. Samples were purified using precipitation, functionalized magnetic bead, or silica column-based purification methods. This sample set included one sample with an *SMN1*-to-*SMN2* gene conversion as determined by the AmplideX PCR/CE *SMN1/2* Kit. However, precision was determined for primary *SMN1* and *SMN2* peaks only (identical gene identity at exon 7 and

intron 7), as 8/9 samples have 0 copies of *SMN2* hybrid genes, and all samples have 0 copies of *SMN1* hybrid genes. 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 eight batch runs using two operators, one reagent lot, and two Applied Biosystems® Veriti™ 96-well thermal cyclers, generating 16 measurements per sample, or 144 sample measurements in total. The complete 144-measurement study was performed and analyzed on each of the following Applied Biosystems Genetic Analyzers: 3500xL (50 cm and 36 cm capillary lengths), SeqStudio™ (28 cm capillary length), 3130*xl* (36 cm capillary length), and 3730*xl* (with 48-capillary configuration, 50 cm and 36 cm capillary lengths). Thus, the study generated a total of 864 sample measurements across all instruments, or 96 measurements per sample. QC failures were excluded from analysis.

The standard deviation across all normalized ratios for *SMN1* and *SMN2* peaks of each sample ranged from 0.000 to 0.150 (**Table 7**). The %CV was similar across all samples, ranging from 3.8 to 10.9%. The highest %CV was observed in a sample with 1 copy of *SMN2*, presumably due to the low mean NR, as the standard deviation of this sample was similar to other samples. Some samples had a higher number of QC failures, mostly Precision QC failures. These samples had mean NR values near the edge of the bin boundary, explaining the higher QC failure rate. Impacted samples were purified using precipitation or automated magnetic bead purification methods, suggesting that higher QC failure rate may be associated with certain purification methods.

Taken together, these data demonstrate that the AmplideX® PCR/CE *SMN1/2* 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, thermal cyclers, and CE instrument models.

Table 7. **P**recision of the AmplideX PCR/CE *SMN1/2* Plus Kit. Measurements are reported in Normalized Ratio (NR), as generated by the AmplideX PCR/CE Reporter using equations 1 and 4 above. 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	Cell Line	2	96	0.953	0.073	7.660	1	96	0.477	0.052	10.901
2	Cell Line	1	94	0.522	0.034	6.513	4	93	1.911	0.111	5.808
3	Blood	0	96	0.000	0.000	NA	3	63	1.563	0.093	5.950
4	Blood	0	95	0.000	0.000	NA	4	95	2.070	0.150	7.246
5	Blood	2	96	0.966	0.046	4.762	1	96	0.488	0.030	6.148
6	Blood	3	91	1.447	0.055	3.801	0	96	0.000	0.000	NA
7	Blood	2	96	0.888	0.041	4.617	2	96	0.873	0.042	4.811
8	Blood	1	96	0.503	0.019	3.777	2	94	0.969	0.042	4.334
9	Blood	4	79	1.723	0.101	5.862	0	96	0.000	0.000	NA

* does not include gene conversions (hybrid peaks)

DNA Input

In order to assess the DNA Input range of the AmplideX® PCR/CE *SMN1/2* Plus Kit, four gDNA samples purified from human cell lines or blood with 0, 1, 2, or 3 *SMN1* or *SMN2* exon 7 copies were tested in triplicate with five different DNA input amounts from 10 ng to 100 ng of DNA per PCR (10 ng to 70 ng for 3130*xl*) as quantified by a Thermo Scientific™ NanoDrop™ Spectrophotometer, generating 60 sample measurements per instrument. Samples were tested on the following Applied Biosystems® Genetic Analyzers: 3500xL (50 cm and 36 cm capillary lengths), SeqStudio™ (28 cm capillary length), 3130*xl* (36 cm capillary length) and 3730*xl* (with 48-capillary configuration, 50 cm and 36 cm capillary lengths). QC failures were excluded from analysis.

Results from valid sample measurements showed that DNA inputs from 10 ng to 100 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 8**). One sample within the intended input range produced saturation as indicated by Saturation QC failure. These results support the assay DNA input range of 20 to 60 ng for 3130 Genetic Analyzers, and 20 to 80 ng per PCR for 3500, 3730, and SeqStudio Genetic Analyzers.

Table 8. DNA Input for the AmplideX PCR/CE *SMN1/2* Plus Kit. *SMN1* and *SMN2* copy number concordance are indicated.

Instrument, Capillary Length	Exon 7 Concordance, Overall Percent Agreement	
	SMN1	***SMN2***
3130, 36 cm	60/60, 100%	59/60, 98.3%
3730, 50 cm	51/52, 98.1%	57/57, 100%
3730, 36 cm	57/57, 100%	60/60, 100%
3500, 50 cm	57/57, 100%	60/60, 100%
3500, 36 cm	57/57, 100%	60/60, 100%
SeqStudio, 28 cm	54/54, 100%	59/59, 100%

Analytical Specificity

Analytical Specificity was verified using data from the Precision study, which included 9 samples spanning the full copy number range of the assay for both *SMN1* and *SMN2* tested in duplicate across 8 batch runs per CE platform tested with the following Applied Biosystems® Genetic Analyzers: 3500xL (50 cm and 36 cm capillary lengths), SeqStudio™ (28 cm capillary length), 3130*xl* (36 cm capillary length) and 3730*xl* (with 48-capillary configuration, 50 cm and 36 cm capillary lengths). The study generated a total of 96 measurements per sample. Each batch run also contained two NTC replicates, generating 96 NTC measurements. Within this data, 192 measurements were generated across two samples with 0 *SMN1* copies and 192 measurements were generated across two samples with 0 *SMN2* copies. For full study details, see the **Precision** section.

To assess Analytical Specificity (Inclusivity), samples spanning the full copy number range (0, 1, 2, 3, and ≥ 4) for both *SMN1* and *SMN2* were included in the study. Comparing operator to operator across all valid sample measurements, 403/412 (97.8%) were concordant for *SMN1*, and 382/399 (95.7%) were concordant for *SMN2* (**Table 7**).

To assess Analytical Specificity (Exclusivity), two samples with 0 *SMN1* copies (192 measurements) and two samples with 0 *SMN2* copies (192 measurements each) were assessed independently, along with 96 NTC measurements where Diluent was added in place of sample DNA during PCR. For samples with 0 *SMN1* copies, 191/191 valid measurements (100%) generated expected copy number results for both *SMN1* and *SMN2* (**Figure 7**). For samples with 0 *SMN2* copies, 192/192 valid measurements (100%) generated expected copy number results for both *SMN1* and *SMN2* (**Figure 8**). Additionally, 96/96 NTC measurements were free of 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 (Inclusivity) for the c.*3+80T>G, c.*211_*212del, and c.859G>C variants detected by the assay, percent agreement was determined across all instrument configurations with wild type samples that included each variant (n=96 per variant). For all three variants, 96/96 (100%) of sample measurements were in agreement with Sanger sequencing for samples with the variant.

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 was determined across all instrument configurations with wild type samples that excluded each variant (n=766 per variant). For all three variants, 766/766 (100%) of sample measurements were in agreement with Sanger sequencing for samples without the variant.

Taken together, these data demonstrate that the AmplideX® PCR/CE *SMN1/2* 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.

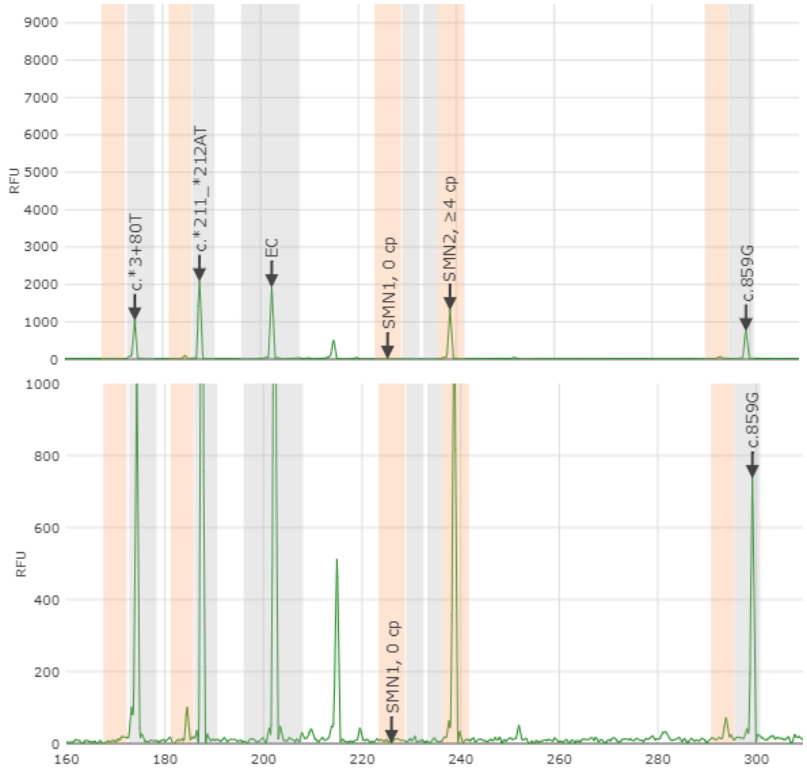

Figure 7. Example electropherogram of a sample with 0 copies of *SMN1* and ≥ 4 copies of *SMN2*. Top panel shows y-axis scaled to ~9,500 RFU; bottom panel shows y-axis scaled to ~1,000 RFU.

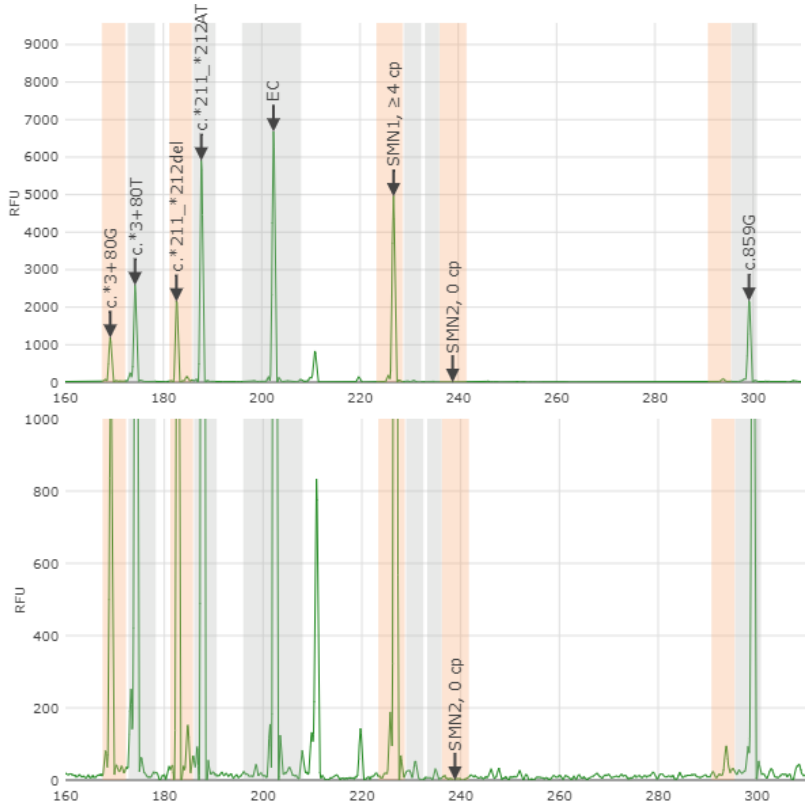

Figure 8. Example electropherogram of a sample with ≥ 4 copies of *SMN1* and 0 copies of *SMN2*. Top panel shows y-axis scaled to ~9,500 RFU; bottom panel shows y-axis scaled to ~1,000 RFU.

Disclaimers

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Glossary of Symbols

Symbol	Description
REF	Catalog number
LOT	Batch code
Σ	Contains sufficient for <n> tests
	Consult instructions before use
	Temperature limitation
	Use by
	Manufacturer

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