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Equivalent Performance of the Applied Biosystems™ 3500 and SeqStudio™ Flex Genetic Analyzer CE Systems for AmplideX® Gene Variant Analysis

Executive Summary

Genetic assay adoption by clinical research laboratories depends on demonstrated equivalent results when performed on diverse instrumentation platforms employed across testing facilities. The AmplideX® PCR/CE family of genetic tests interrogates genomic DNA for diverse pathogenic gene variants for which accurate analysis can be challenging. The Applied Biosystems® SeqStudio™ Flex† and Applied Biosystems™ 3500 Genetic Analyzer† platforms (3500 CE Systems) demonstrated comparable performance with 100% agreement when used to run four different AmplideX assay kits. The SeqStudio Flex offers new advantages in technical flexibility, remote troubleshooting resources, and connectivity and data-sharing capabilities.

Key Points

- AmplideX® PCR/CE technology allows detection of difficult-to-resolve pathogenic variants, including triplet and hexanucleotide repeats, other STRs, SNVs, INDELs, and CNVs.
- Molecular genetic test adoption by clinical research laboratories necessitates reliable agreement in assay results across diverse instrumentation platforms.
- The Applied Biosystems SeqStudio Flex and 3500 Genetic Analyzers capillary electrophoresis (CE) systems, in combination with the AmplideX PCR/CE family of genetic tests and AmplideX PCR/CE Reporter analysis software, demonstrated 100% agreement for *C9orf72*, *CFTR*, *FMR1*, and *SMN1/2* pathogenic variant identification and/or categorization, within allowable precision tolerances.
- New capabilities of the SeqStudio Flex improve ease-of-use, flexibility, serviceability, and connectivity for improved laboratory efficiency with streamlined data access and sharing.
- The intuitive user-friendly characteristics of the SeqStudio Flex Genetic Analyzer might be attractive to laboratories that perform high-accuracy medium-to-high throughput gene analysis.

Background

Molecular genetic assays demand accurate and reproducible performance when run on the diverse instrumentation platforms used in clinical research laboratories worldwide. The Asuragen AmplideX® PCR/CE family of genetic tests are robust inherited disease testing tools that use capillary electrophoresis (CE) technology to resolve, analyze, and report gene sequence information contained in PCR-amplified fragments derived from subject genomic DNA. The AmplideX PCR/CE family of genetic tests analyzes difficult gene targets including *C9orf72*, *CFTR*, *FMR1*, and *SMN1/2*,[1–4] which present a variety of challenges such as structural variation, pseudogenes, and GC-rich repeats.[5,6]

Advances in CE instrument design continue to improve user-friendliness and expand their suitability for easy adoption and operation in diverse laboratory settings. The SeqStudio Flex Genetic Analyzers (Applied Biosystems/Thermo Fisher Scientific) may permit broader access to affordable CE technology for analyzing difficult gene targets.[7] The SeqStudio Flex unit has an intuitive guided user touchscreen interface that facilitates straightforward operation and maintenance regardless of experience level.

SeqStudio Flex analysis is streamlined by the availability of convenient prefilled consumables, including capillary arrays and anode & cathode buffer tanks. The SeqStudio Flex is compatible with existing Sanger sequencing chemistries and fragment analysis using common STR kits available from Thermo Fisher and other vendors. Importantly, the SeqStudio Flex was purposefully designed with expanded plate-handling capacity to increase throughput, simplified user-friendly assay setup, monitoring and modification, and enhanced connectivity and data-sharing capabilities.

It is essential that a DNA sample yields the same genotype when run across different CE systems that may exist in different laboratories. We compared analytical performance of the Applied Biosystems™ SeqStudio Flex versus the Applied Biosystems 3500 CE instrument[8] (Thermo Fisher Scientific) when interrogating 130 genomic DNA samples using four widely used inherited-disease assays from the AmplideX PCR/CE family of genetic tests. We demonstrate the concordant identification and classification of a broad range of challenging-to-resolve pathogenic variants between the SeqStudio Flex and 3500 CE Systems.

Methods

Overview:
Genomic DNA samples were PCR-amplified using the AmplideX PCR/CE family of genetic tests (*C9orf72*†, *CFTR*†, *FMR1*†,‡, and *SMN1/2* Plus†/*SMA* Plus‡), followed by CE resolution on both the SeqStudio Flex CE System† (SeqStudio Flex) and the Applied Biosystems 3500 Genetic Analyzer for direct comparison. Final assay results were obtained via automated analysis of CE electropherogram outputs with AmplideX Reporter.

Samples:
Approximately 130 DNA samples were obtained from peripheral venous blood, commercially available cell lines, and plasmid mixes (*CFTR* only). Samples were selected to cover all genotype categories including normal, intermediate, premutation and full mutation for *FMR1*; normal, intermediate and expanded for *C9orf72*; SNP, STR and INDEL variants for *CFTR*; 0 to ≥4 copy number variants for *SMN1* and *SMN2*, and 3 unique variants of *SMN1/2* related to carrier risk or disease prognosis.

PCR/CE and Analysis:
Genomic DNA samples were amplified by PCR using AmplideX reagents, per assay instructions, followed by CE on both the 3500 and the SeqStudio Flex systems for direct comparison. Notable characteristics of both CE instruments are presented in Table 1. In preliminary experiments, the run parameters for both CE units were optimized to obtain expected peak morphology and resolution for each assay.[9] Injection and run conditions for the SeqStudio Flex instrument were identical to those used with the 3500 (Table 2). Spatial and spectral calibrations were performed in accordance with the respective operating manual.[10,11] Both instruments were calibrated using a DS-30 matrix dye standard per assay instructions.[12] Final assay results were generated via analysis of CE electropherograms with AmplideX Reporter Software for *FMR1*, *SMN1/2*, and *CFTR*. GeneMapper® 6.1 software and a sizing macro were used to process and analyze samples for *C9orf72*. Runs that were identified as QC failures by AmplideX Reporter were noted but excluded from analysis.

TABLE // 01

Characteristics of the SeqStudio Flex and Applied Biosystems 3500 CE Instruments

	CE System	
Parameter	**SeqStudio Flex**	**3500**
		
	Instrument Specifications	
Capillaries, n	8 standard, 24 available	8 standard, 24 available
Array Length	36 and 50 cm	36 and 50 cm
Sample Capacity, n	32 (strips), 96- and 384 well plates	32 (strips), 96- and 384 well plates
Plate Capacity, n	4 plates	2 plates
Throughput Level	Medium	Medium
Polymers Available	POP-4, POP-6, POP-7	POP-4, POP-6, POP-7
Resolution	1 bp	1 bp
Footprint	70 cm W x 68 cm D (4725 cm^2)	61 cm W x 61 cm D (3721 cm^2)
Height	87 cm	72 cm
Weight	115 kg	82 kg
	Workflow Features	
Controller	Integrated touchscreen	External PC (vendor supplied)
Continual Plate Loading	Yes	No
Sample Access	Can reprioritize w/o stopping run	Must stop to reprioritize
Remote Run Monitoring*	Yes	No
Remote Data Sharing	Yes	No
Amazon Alexa™ Voice Control	Yes	No
Connectivity	USB, Ethernet ports, Wi-Fi dongle	Ethernet port
Integrated Remote Troubleshooting Tools	Yes	No
Consumables Tracking	RFID	RFID

*Remote monitoring via mobile phone, tablet, laptop, or remote desktop.

TABLE // 02

Identical optimized AmplideX run parameters for the SeqStudio Flex and 3500 CE systems. Parameters are shown for 50 cm capillary lengths. For 36 cm capillary lengths, consult the relevant protocol guide. We assessed kit performance on the SeqStudio Flex with the off-scale recovery (OSR) feature turned off.

AmplideX Assay	Analysis Module Version	Run Time	Run Voltage	Injection Time	Injection Voltage
CFTR	3.0.4	2100 s	19.5 kV	20 s	2.5 kV
SMN1/2 Plus	1.1.5	2100 s	19.5 kV	20 s	2.5 kV
FMR1	2.0.1, 3.0.5	2400 s	19.5 kV	20 s	2.5 kV
C9orf72	1.0.1	2400 s	19.5 kV	20 s	2.5 kV

POP-7 (ThermoFisher #4393714) was used in all runs, on both instruments.

Results

CE System Comparison:
The SeqStudio Flex Genetic Analyzer has a footprint that is 27% larger than the 3500, though the depth of both instruments (27″ and 24″, respectively; **Table 1**) is easily accommodated by standard laboratory counter tops. The SeqStudio Flex and 3500 require vertical clearances of approximately 34″ and 28″ above the workstation surface, respectively. The SeqStudio Flex is controlled using an integrated touchscreen linked to a 500 GB internal solid-state hard drive (SSD) so, unlike the 3500, does not require an auxiliary PC connection for instrument operation. The SeqStudio Flex uses the same 96-well and 384-well plates as the 3500, but has twice the plate-handling capacity (n=4) than the 3500 (n=2). Both systems alternatively accommodate up to 32 samples in 8-tube strips. This means that while the SeqStudio Flex has the ability to run smaller batches, it can also accommodate higher throughput for larger-volume labs compared to the 3500. Improved system connectivity capabilities simplify data access and sharing to facilitate collaborative analyses and enhance laboratory productivity.

Operation of the SeqStudio Flex has been streamlined to enhance user-friendliness. One-touch start-up, autocalibration, and easy on-board navigation through troubleshooting and maintenance menus together minimize start-up time. In terms of instrument maintenance, replacement of consumables and capillary arrays on the SeqStudio Flex is straightforward, and the rigid structure of the capillary makes it less prone to accidental damage during installation compared to older CE instrument models. Beyond consumable replacement, instrument maintenance requirements are minimal. Additionally, injections can be reviewed real-time as needed and re-prioritized as needed, enabling adjustments based on laboratory needs. These features make the SeqStudio Flex accessible for new users less familiar with CE instruments and simplify training and maintenance for experienced labs.

Assay Run Time:
Optimized electrophoresis run times (**Table 2**) on both the SeqStudio Flex and 3500 instruments were 2100 s (35 min) for *CFTR* and *SMN1/2* AmplideX kits, and 2400 s (40 min) for the *C9orf 72* and *FMR1* AmplideX kits.

***C9orf72* Overview:**
Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are proteinopathic neurodegenerative diseases linked to aberrantly expanded hexanucleotide repeat GGGGCC (G4C2) sequences in a non-coding region of the *C9orf72* (*chromosome 9 open reading frame 72*) gene.[13] Normal individuals carry 2–10 G4C2 repeats.

***C9orf72* Genotype Categorization:**
The AmplideX *C9orf72* PCR/CE Kit† categorizes DNA samples into three groups (Normal, Intermediate, and Expanded) based on the number of G4C2 repeats (≤19, 20–30, and >30, respectively) detected in *C9orf72*.[1] The assay's upper sizing limit is 145 repeats. Through 48 total repeat category calls made using 20 unique samples, the SeqStudio Flex showed 100% agreement with the 3500 output (**Table 3**).

TABLE // 03

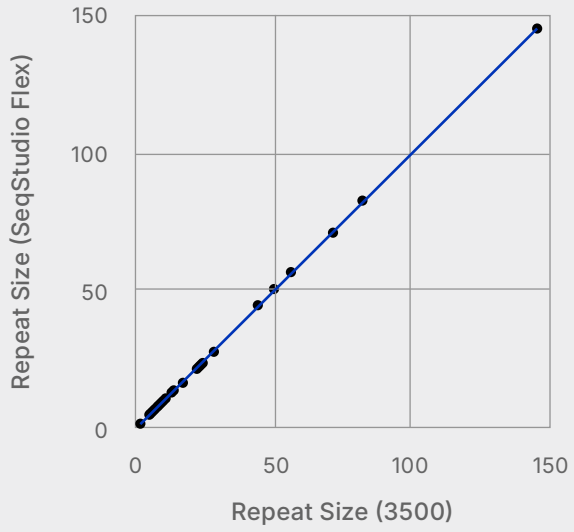
C9orf72 genotype category agreement between the SeqStudio Flex and 3500 CE systems.

SeqStudio Flex \ 3500	Normal	Intermediate	Expanded
Normal	6	0	0
Intermediate	0	10	0
Expanded	0	0	32

***C9orf72* G4C2 Repeat Sizing:**
Because the *C9orf72*-associated disease phenotype is aligned with the number of G4C2 repeat sequences present,[13] accurate repeat size determination is essential. There was excellent overall concordance in repeat size determination between data generated on the SeqStudio Flex and the 3500 (**Figure 1**).

FIGURE // 01

Agreement in *C9orf72* hexanucleotide G4C2 repeat sizing between the SeqStudio Flex and 3500 CE systems. Logistic regression shows high concordance between repeat size determined by both CE platforms (N=48), with r^2=1.000 and y=1.000(x)+0.059. Identified full mutations with repeat sizes greater than the assay sizing limit (145 repeats) were assigned value=145 for logistic regression.



***CFTR* Overview:**
Cystic fibrosis is a potentially lethal autosomal recessive disease characterized by severe impairment of the respiratory system and digestive tract.[14] More than 400 pathogenic variants of CF transmembrane conductance regulator (CFTR) gene are known.[15] Carrier frequency in the U.S. for CF-associated *CFTR* variants ranges from ≈1:30 in non-Hispanic whites to ≈1:90 in Asians.[16] The AmplideX *CFTR* PCR/CE Kit† evaluates 65 disease causing *CFTR* variants, providing 92% coverage for the U.S. populace and an estimated 95% coverage worldwide.[2,17]

Unique samples for *CFTR* analysis included 8 whole blood specimens, 32 cell line specimens, and 4 plasmid mixes which together covered all 65 kit-assessed pathogenic variants. A total of 63 sample measurements were collected across 44 unique samples. PCR was performed on the Veriti (Applied Biosystems) thermal cycler.

***CFTR* Variant-Level Genotyping:**
CFTR variant-level metrics were in exact agreement between the SeqStudio Flex and 3500 CE systems (**Table 4** and **Table 5**) Each sample measurement generated 63 unique variant calls. PolyT/TG agreement was evaluated separately, and all results were 100% concordant.

TABLE // 04

CFTR genotype category agreement between the SeqStudio Flex and 3500 CE systems.

SeqStudio Flex \ 3500	# *CFTR* Variants	0	1	≥2
	0	29	0	0
	1	0	16	0
	≥2	0	0	18

TABLE // 05

CFTR variant-level metrics on SeqStudio Flex versus 3500 CE systems.

Measure	N	Percent Concordant
PPV	402	100%
PPA	402	100%
NPA	2819	100%
OPA	3171	100%
PA_z	3171	100%
PA_{TTG}	72	100%

Abbreviations: PPV = positive predictive value, PPA = positive percent agreement, NPA = negative percent agreement, OPA = overall percent agreement, PA_z = zygosity agreement, PA_{TTG} = percent agreement PolyT/TG.

***FMR1*/Fragile X Syndrome Overview:**
Fragile X syndrome is an inherited CNS disorder caused by an expansion of the CGG triplet repeat within the *FMR1* (*fragile X messenger ribonucleoprotein 1*) gene on the X chromosome, which causes methylation-dependent *FMR1* promoter silencing.[18] This repeat size variation results in a deficiency of the downstream protein (FMRP) that is responsible for normal synaptic development and architecture. Normally, there are between 5–44 CGG repeats; fragile X syndrome occurs with >200 repeats.[18] Triplet copy numbers ranging between 45–54 repeats are considered an 'intermediate' grey zone, and 55–200 repeats are considered 'premutation'; the risk of women having children with fragile X syndrome or associated disorders rises with increased repeat sizing.[19] The AmplideX *FMR1* PCR/CE Kit*† reagents are designed to identify and accurately size CGG repeat alleles up to 200 repeats, identify all allele expansions including low-abundance full mutation size mosaics up to 1300 CGG repeats, and accurately resolve female *FMR1* zygosity.[3]

Samples for *FMR1* analysis included 35 cell line samples and two controls, each run 1–9 times using both CE platforms.

***FMR1* Genotype Categorization:**
The AmplideX *FMR1* PCR/CE Kit categorizes DNA samples into four groups (Normal, Intermediate, Premutation, and Full Mutation) based on the number of CGG sequences detected in *FMR1*.[3]

Across 133 total repeat category calls made using 37 unique samples, the SeqStudio Flex showed 98.5% agreement (131/133) with 3500 output (**Table 6**). The two discrepancies were at the 200 CGG repeat boundary between premutation and full mutation categories (one instance each of 198 repeats on the SeqStudio Flex versus 200 repeats on the 3500, and vice versa). These misses were within precision tolerance around the 200-repeat cutoff per American College of Medical Genetics (ACMG) Guidelines.[19] Both discrepancies were within precision allowance when considering ACMG-allowable size tolerances (± 2 SD of repeat size for alleles greater than 100 repeats)[19] and performance claims (±5% of repeat size for repeats >120).[20] In addition to QC failures, 11 samples were excluded from *FMR1* analysis due to high channel crosstalk. This issue was attributed to a defect identified in the SeqStudio Flex data collection software that resulted in application of an incorrect spectral calibration that has since been repaired. This issue was specific to early software versions, and is no longer an issue on the SeqStudio Flex platform.

TABLE // 06

FMR1 genotype category agreement between the SeqStudio Flex and 3500 CE systems.

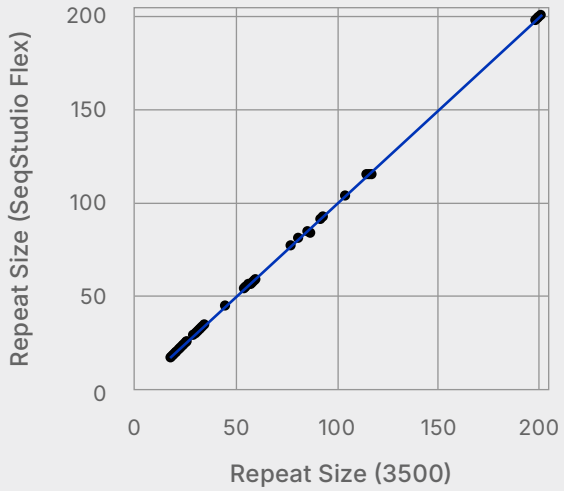
SeqStudio Flex	3500			
FMR1	**Normal**	**Intermediate**	**Premutation**	**Full Mutation**
Normal	61	0	0	0
Intermediate	0	8	0	0
Premutation	0	0	27	1
Full Mutation	0	0	1	33

Both discrepancies were N-2 that occurred at the Premutation/Full mutation boundary (200 CGG repeats). Size differences were within American College of Medical Genetics (ACMG) precision tolerance[19] and Amplidex *FMR1* PCR/CE Kit performance claims.

***FMR1* Repeat Sizing:**
Because the risk of Fragile X and associated disorders is dependent on the number of repeat CGG sequences present in *FMR1*,[19] accurate repeat size determination is essential. There was excellent overall concordance between repeat sizing determined by the SeqStudio Flex and 3500 instruments (**Figure 2**).

FIGURE // 02

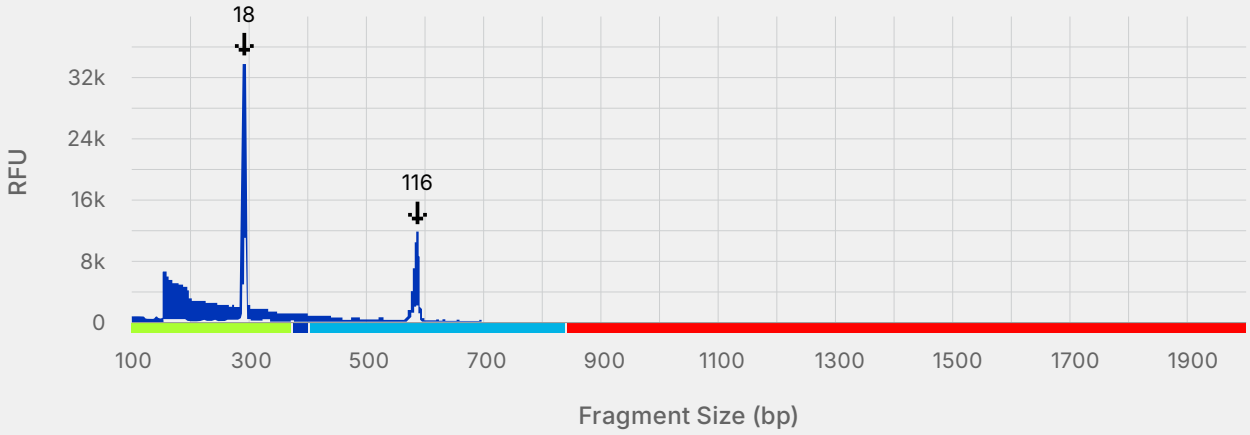
Agreement in *FMR1* trinucleotide CGG repeat size determined by the SeqStudio Flex and 3500 CE systems. Logistic regression shows high concordance between repeat size determined by both CE platforms (N=133), with r^2=0.999 and y=0.999(x)-0.090. Identified full mutations with repeat sizes greater than the assay sizing limit (200 repeats) were assigned value=200 for the logistic regression.



***FMR1* Large Repeat Resolution:**
Excellent resolution of premutation (shown) and full mutation *FMR1* alleles is achievable with the SeqStudio Flex CE System (**Figure 3**).

FIGURE // 03

FMR1 premutation (shown) and full mutation alleles are easily resolved on this SeqStudio Flex CE system as pileup peaks at the expected repeat size. RFU, relative fluorescence units.

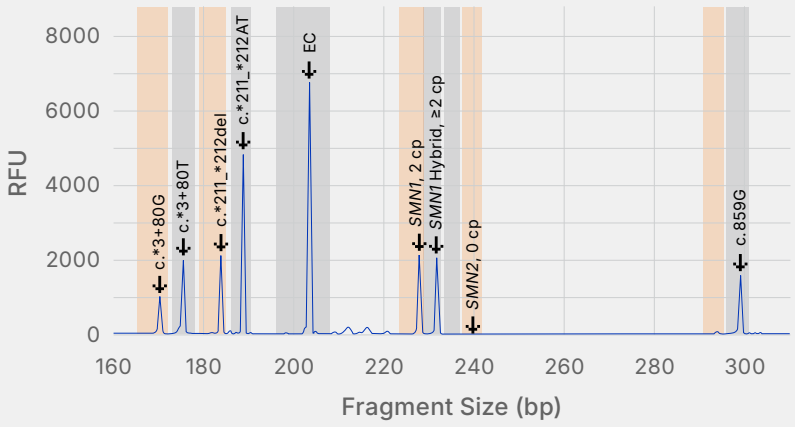


***SMN1/2* Overview:**
Copy number variations in *SMN1* and its paralog *SMN2* (both encoding survival motor neuron protein) are associated with the onset and severity of spinal muscular atrophy, SMA.[21] Carrier risk and disease severity may also be impacted by the presence of *SMN1/2* gene duplication variants and a disease modifier variant in *SMN2*, respectively.[22] The AmplideX PCR/CE *SMN1/2* Plus Kit† identifies copy number variations for both *SMN1* and *SMN2*, quantifies *SMN1* exon 7 copy number, and detects relevant *SMN2* variants and gene hybrids, in a single reaction.[4]

Samples for *SMN1/2* analysis included 28 whole blood samples and four cell line samples, with PCR performed on one thermal cycler (Veriti) for a total of 1–9 runs on both CE platforms. A representative electropherogram of *SMN1/2* resolved on the SeqStudio Flex is shown in **Figure 4**.

FIGURE // 04

Typical *SMN1/2* peak morphology observed on the SeqStudio Flex CE electropherogram. The AmplideX *SMN1/2* Plus Kit (*SMN1* hybrid with gene duplication variants) provided accurate exon 7 copy number categorization and reporting. RFU = relative fluorescence units; EC = endogenous control.



***SMN1* and *SMN2* Exon 7 Copy Number Agreement:**
Overall *SMN1* concordance was excellent at 100% (174/174 calls) between the SeqStudio Flex and 3500 systems (**Table 7**).

TABLE // 07

SMN1 exon 7 copy number agreement between SeqStudio Flex and 3500 CE systems.

		3500				
SeqStudio Flex	***SMN1* Copy #**	**0**	**1**	**2**	**3**	**4**
	0	27	0	0	0	0
	1	0	27	0	0	0
	2	0	0	73	0	0
	3	0	0	0	20	0
	4	0	0	0	0	27

Overall *SMN2* concordance was also 100% (177/177 calls) between the two CE systems (**Table 8**).

TABLE // 08

SMN2 exon 7 copy number agreement between SeqStudio Flex and 3500 CE systems.

		3500				
SeqStudio Flex	***SMN2* Copy #**	**0**	**1**	**2**	**3**	**4**
	0	27	0	0	0	0
	1	0	35	0	0	0
	2	0	0	64	0	0
	3	0	0	0	24	0
	4	0	0	0	0	27

***SMN1/2* SNP Agreement:**
For *SMN1* SNPs associated with SMA and related pathologies, agreement between the SeqStudio Flex and 3500 systems was exact (**Table 9**). For the *SMN1* duplication markers, agreement was 100% (184/184) for c.*3+80T>G and 100% (184/184) for c.*211_*212del. For the prognostic *SMN2* disease modifier variant c.859G>C, agreement was also 100% (184/184).

TABLE // 09

Agreement in detecting *SMN1* and *SMN2* pathological single-nucleotide polymorphisms.

			3500		
			Negative	Positive	Agreement
SeqStudio Flex	c.*3+80T>G	Negative	131	0	100%
		Positive	0	53	
	c.*211_*212del	Negative	131	0	100%
		Positive	0	53	
	c.859G>C	Negative	159	0	100%
		Positive	0	25	

Summary

The SeqStudio Flex Genetic Analyzer demonstrated 100% concordance with the Applied Biosystems 3500 instrument in reliably identifying and reporting diverse challenging genes in conjunction with the AmplideX PCR/CE family of genetic tests that deliver accurate sample-to-answer results within 4–6 hours. With *C9orf72*, there was 100% genotyping agreement between the SeqStudio Flex and 3500. *CFTR* genotype category agreement was 100% between the SeqStudio Flex and 3500 systems, with a concurrent 100% overall predictive value for accurate variant identification, including zygosity and Poly T/TG status. With *FMR1*, resolution and unadjusted sizing of large repeats was highly concordant between the two CE systems at 98.5% agreement (131/133). The two *FMR1* discrepancies were N-2 calls at the 200-repeat premutation/full mutation category boundary and were within allowable repeat sizing precision tolerance based on ACMG guidelines; thus, agreement in *FMR1* size categorization was effectively 100% between the SeqStudio Flex and 3500 when output accommodated allowable precision. With both *SMN1* and *SMN2*, there was exact agreement (100%) in copy number resolution and variant detection with the SeqStudio Flex and 3500. Overall, the SeqStudio Flex and 3500 systems performed with high precision and in excellent concordance when analyzing diverse and challenging-to-resolve gene targets. Samples included examples of multiple repeat size categories (*C9orf72*, *FMR1*), copy numbers (*SMN1/2*), and unique variants (*CFTR*, *SMN1/2*). New system design upgrades enhance overall user friendliness by providing improved flexibility in run parameter selection and control, simplified maintenance regimens, and advanced connectivity and data-sharing options for optimal laboratory efficiency.

Conclusion

The SeqStudio Flex Genetic Analyzer is a high-precision, affordable, user-friendly CE option that reliably interrogates genomic DNA to identify difficult-to-resolve pathogenic variants in the *C9orf72*, *CFTR*, *FMR1*, and *SMN1/2* genes when coupled with AmplideX PCR/CE family of genetic tests and AmplideX PCR/CE Reporter analysis software. The SeqStudio Flex platform is an appealing option for users who demand reproducibly accurate CE performance with high resolution, require medium-throughput capabilities, and are migrating from older CE models or are contemplating first purchase of an intuitive, reliable, and innovative CE system.

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