WHITE PAPER

SeqStudio Genetic Analyzer

The SeqStudio Genetic Analyzer simplifies the analysis of triplet repeat expansions with AmplideX PCR/CE reagents

Abstract

The Applied Biosystems™ SeqStudio™ Genetic Analyzer is our newest tool to provide routine and accessible analysis of DNA using capillary electrophoresis (CE). Fragment sizing with CE is especially compatible with revealing repeat sections of DNA associated with disease. Often, these regions are particularly challenging to amplify and analyze because of high GC content. Therefore, the SeqStudio Genetic Analyzer was evaluated to establish and verify recommended injection and run settings for use with Asuragen AmplideX™ PCR/CE *FMR1* and *C9orf72* reagents, in order to successfully analyze these challenging regions. *FMR1* is the gene that when expanded causes fragile X syndrome. Similarly, *C9orf72* when expanded can cause either frontotemporal dementia, amyotrophic lateral sclerosis (ALS), or both.

Through rigorous testing it was determined that the SeqStudio instrument is capable of effectively resolving PCR products generated using the AmplideX PCR/CE *FMR1* and *C9orf72* reagents under the following run parameters: SeqStudio module = LongFragAnalysis (modified), injection time = 2 sec, injection voltage = 6 kV, run time = 3,300 sec, and run voltage = 6 kV.

Customers wishing to perform evaluation of “dark DNA”—regions of unsequenced repetitive DNA—should use the modified run parameters listed above. Under these conditions, normal allele peaks may result in signal saturation and cause a split-peak profile without impacting the ability to size these smaller alleles. By establishing compatibility with the AmplideX PCR/CE reagents, the tuneable run resolution (ability to modulate sequencing features for better resolution) and the sensitivity demonstrated by the SeqStudio Genetic Analyzer will allow investigators to further their research and understanding of repetitive DNA.

Benefits of the SeqStudio Genetic Analyzer

Flexible

- Perform sequencing and fragment analysis on the same plate
- 4-capillary array minimizes need to batch samples
- Fast turnaround with a run time as short as 30 minutes

Convenient

- Innovative all-in-one cartridge with array, buffer, pump, and polymer included
- Universal polymer for sequencing and fragment analysis
- Easy-to-use touchscreen operation

Easy to use

- Cartridge enables easy tracking of reagent usage
- Individualized cartridges let users easily share an instrument

Connected

- Remote access to instrument controls and data*
- Remote visualization of instrument status and run progress*
- Secure data storage and backup
- Easy data sharing and collaboration

* Available with an Internet connection and Thermo Fisher™ Cloud account.

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Introduction

Although DNA repeat sequences constitute roughly 50% of our genome, technological limitations have historically limited comprehensive investigations of repetitive elements. As a result, much of the known repetitive DNA in human genomes has uncertain or "dark" functions. Furthermore, an estimated 5% of our genomic DNA is thought to be repetitive and both sequence and function are unknown. Recent targeted and genome-wide studies have demonstrated a new appreciation of the genotype–phenotype implications of these dark repeat sequences such as short tandem repeats (STRs). For example, STRs are enriched in regulatory regions, manifest instability and variable length effects, and can impact multiple aspects of gene expression, splicing, and translation. Further, more than 30 Mendelian disorders are linked to STR expansions, including fragile X syndrome, amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), myotonic dystrophy, and Huntington's disease (Figure 1) [1]. AmplideX PCR/CE reagents leverage repeat-primed PCR and CE to report both full-length amplicons that encompass the STR region and repeat-primed products that offer confirmatory peak patterns of pathogenic alleles [2]. This approach has been well established on Applied Biosystems™ 3130, 3500, and 3730 CE platforms and described in over 75 publications since 2010.

CE instruments use electric field strength across a gel polymer and a laser for separation, excitation, and detection of fluorescently labeled nucleic acid fragments. We recently introduced the SeqStudio Genetic Analyzer, designed from the ground up to be easy to use. However, the shorter array and POP-1 gel polymer used by the SeqStudio CE instrument require optimized run settings to match the performance of AmplideX PCR reagents on previous instruments. To this end, a series of experiments were conducted to determine and verify specific run settings for the SeqStudio instrument to align with the performance requirements for the AmplideX PCR/CE reagents. Priority experiments were designed to determine run parameters for long-fragment assays, such as *FMR1* and *C9orf72* from Asuragen, that require sizing resolution exceeding 800 base pairs. The easy-to-use SeqStudio system, together with the repeat-proficient AmplideX PCR reagents, allows genotyping of the elusive GC- and AT-rich repeat expansions.

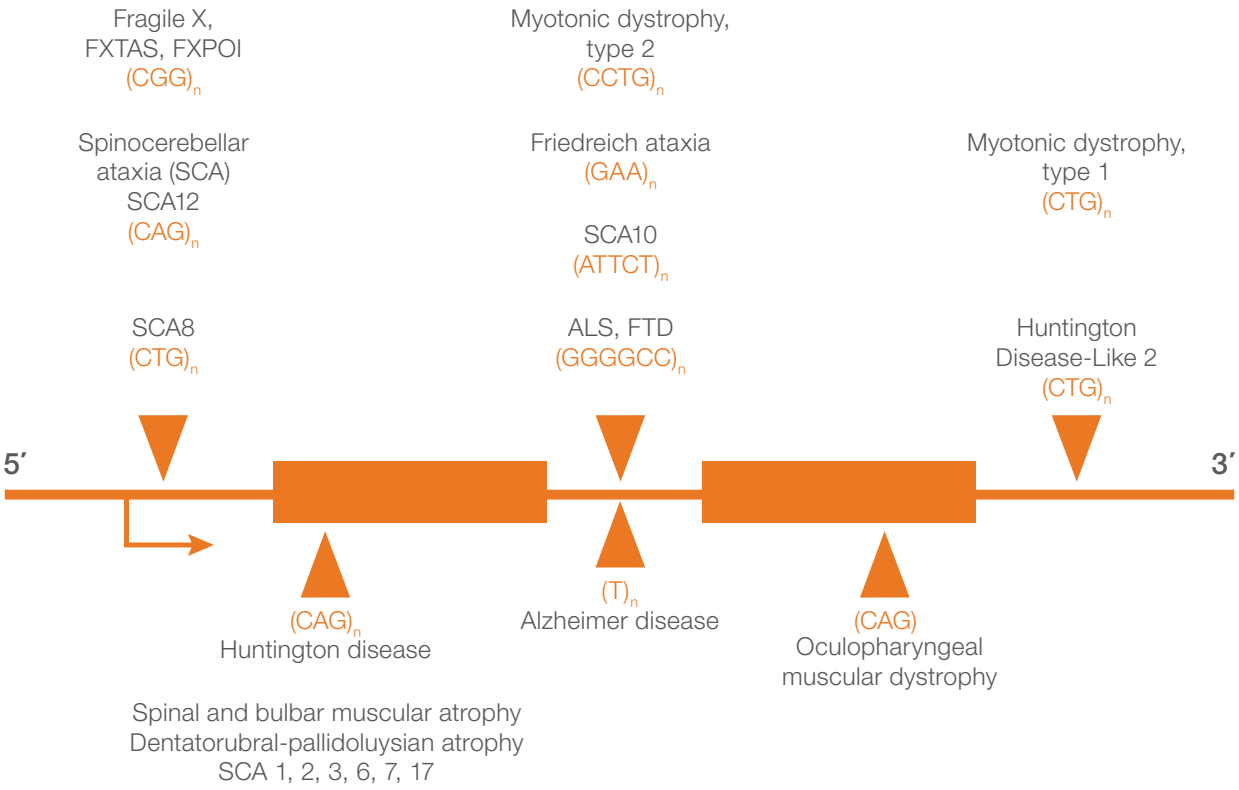

Figure 1. Locations and sequences of repeat regions known to cause various human diseases.

Methods

Several sample panels were used to verify *FMR1* and *C9orf72* assay performance at multiple sites with an optimized set of SeqStudio instrument run parameters, summarized in Panels A–E below. These samples represent a range of challenging genotypes and detection modes, lending credence to the robustness of the SeqStudio Genetic Analyzer.

Panel A: For replicates of samples covering many repeat sizes to evaluate method variability.

Assay	Sample ID	Repeat alleles	Genotype
FMR1	ASGN-001	20	NOR
FMR1	ASGN-002	29, 30	NOR
FMR1	ASGN-005	29, 45	INT
FMR1	ASGN-007	54	INT
FMR1	ASGN-009	30, 56	PM
FMR1	ASGN-011	59,77	PM
FMR1	ASGN-013	91	PM
FMR1	ASGN-016	18, 115	PM
FMR1	ASGN-018	20, 197, 200	FM
FMR1	ASGN-021	30, 200	FM
FMR1	ASGN-023	200	FM
FMR1	ASGN-101	35, 93	PM
FMR1	ASGN-102	30, 80	PM
FMR1	ASGN-103	30, 55	PM
FMR1	ASGN-104	24, 200	FM
FMR1	ASGN-105	24, 200	FM
FMR1	ASGN-106	30, 57	PM
FMR1	ASGN-108	29, 30	NOR
FMR1	ASGN-109	30	NOR
FMR1	ASGN-110	30, 31	NOR
FMR1	ASGN-111	29, 50	INT
FMR1	ASGN-112	29, 30	NOR
FMR1	ASGN-113	30, 103	PM
FMR1	ASGN-114	33	NOR
FMR1	ASGN-115	29	NOR
FMR1	ASGN-116	35	NOR
FMR1	ASGN-117	26, 30	NOR
FMR1	ASGN-118	20, 30	NOR
FMR1	ASGN-119	20	NOR
FMR1	ASGN-120	29	NOR
FMR1	ASGN-121	30	NOR
FMR1	ASGN-122	29	NOR
FMR1	ASGN-123	30	NOR
FMR1	ASGN-124	30, 94	PM
FMR1	ASGN-125	182, 200	FM

Panel D: For single-repeat allele resolution.

Assay	Sample ID	Repeat alleles	Genotype
FMR1	ASGN-002	29, 30	NOR
FMR1	ASGN-108	29, 30	NOR
FMR1	ASGN-110	30, 31	NOR
FMR1	ASGN-112	29, 30	NOR
FMR1	AG003	39, 40	NOR
FMR1	RU009	30, 32	NOR
FMR1	RU002	30, 30	NOR

Panel B: For assay sensitivity to a range of PCR inputs.

Assay	Sample ID	Repeat alleles	Genotype	PCR input (ng)
C9orf72	ND06769-40ng	13, 44, Exp	Exp	40
C9orf72	ND06769-20ng	13, 44, Exp	Exp	20
C9orf72	ND06769-10ng	13, 44, Exp	Exp	10
C9orf72	ND06769-05ng	13, 44, Exp	Exp	5
C9orf72	ND06769-01ng	13, 44, Exp	Exp	1
FMR1	RU003 40ng	200	FM	40
FMR1	RU003 20ng	200	FM	20
FMR1	RU003 10ng	200	FM	10
FMR1	RU003 05ng	200	FM	5
FMR1	RU003 01ng	200	FM	1
FMR1	PC 40ng	18, 30, 32, 56, 85, 116, 200	FM	40
FMR1	PC 20ng	18, 30, 32, 56, 85, 116, 200	FM	20
FMR1	PC 10ng	18, 30, 32, 56, 85, 116, 200	FM	10
FMR1	PC 05ng	18, 30, 32, 56, 85, 116, 200	FM	5
FMR1	PC 01ng	18, 30, 32, 56, 85, 116, 200	FM	1

Panel C: For assay sensitivity to mosaics and minor alleles. Pooled samples were generated by mixing DNA from two known samples at various allele ratios.

Assay	Sample ID	Major allele repeat	Minor allele repeat	Minor allele (%)	Genotype	Sample type
C9orf72	C9Exp-01%	12, 14	9, Exp	1%	Exp	Pool
C9orf72	C9Exp-05%	12, 14	9, Exp	5%	Exp	Pool
C9orf72	C9Exp-10%	12, 14	9, Exp	10%	Exp	Pool
C9orf72	C9Exp-20%	12, 14	9, Exp	20%	Exp	Pool
C9orf72	C9Exp-25%	12, 14	9, Exp	25%	Exp	Pool
FMR1	FXFM-01%	23	200	1%	FM	Pool
FMR1	FXFM-05%	23	200	5%	FM	Pool
FMR1	FXFM-10%	23	200	10%	FM	Pool
FMR1	FXFM-20%	23	200	20%	FM	Pool
FMR1	AG395	23, 47	63		PM	Clinical
FMR1	AG308	20, 82, 86	70		PM	Clinical
FMR1	AG557	20, 160	200		FM	Clinical
FMR1	RU005	23	113, 118		PM	Clinical
FMR1	RU008	86	200		FM	Clinical

Panel E: For borderline cases for genotyping accuracy.

Assay	Sample ID	Repeat alleles	Genotype
FMR1	AG033	30, 46	INT
FMR1	AG036	30, 44	NOR
FMR1	AG306	46	INT
FMR1	AG366	29, 53	INT
FMR1	ASGN-005	29, 45	INT
FMR1	ASGN-007	54	INT
FMR1	ASGN-009	30, 56	PM
FMR1	ASGN-011	59, 77	PM
FMR1	ASGN-018	20, 197, 200	FM
FMR1	ASGN-103	30, 55	PM
FMR1	ASGN-106	30, 57	PM
FMR1	ASGN-125	182, 200	FM
FMR1	FMR1-SC	30, 56, 200	FM
FMR1	RU004	85	PM
FMR1	RU006	44, 64	PM

Following amplification, PCR products were combined with the ROX 1000 Size Ladder (Asuragen) and Applied Biosystems™ Hi-Di™ Formamide. This mix was denatured at 95°C for 2 min in a thermal cycler followed by incubation at 4°C until the sample was ready for analysis. Heat-denatured samples were then loaded onto a SeqStudio Genetic Analyzer (4-capillary array, 28 cm, POP-1 polymer). Operation of the SeqStudio Genetic Analyzer was carried out according to the SeqStudio Genetic Analyzer Instrument and Software v1.1 User Guide Rev A.0. Run setup was managed through the Applied Biosystems™ SeqStudio™ Plate Manager app located on the Thermo Fisher Cloud server. A series of CE runs were conducted on the SeqStudio instrument using variations on four run parameters: injection time, injection voltage, run time, and run voltage.

Unless otherwise noted, the majority of runs, including precision and sensitivity studies, were conducted under the optimized parameters listed in Table 1.

Table 1. SeqStudio instrument run settings for AmplideX PCR/CE *FMR1* and *C9orf72* reagents.

Modified SeqStudio run module	Injection time (sec)	Injection voltage (kV)	Run time (sec)	Run voltage (kV)
LongFragAnalysis	2	6	3,300	6

Results from the SeqStudio instrument were compared to the results from the same PCR products (Panels A–F) on the 3500xL Genetic Analyzer (36 cm, 24-capillary array, POP™-7 polymer) using the manufacturer's recommended settings.

Analysis and acceptance criteria

Fragment sizing analysis of the *FMR1* or *C9orf72* products required the use of Applied Biosystems™ GeneMapper™ 5 Software (version 5.1) or SoftGenetics™ GeneMarker™ software (version 2.7.0). Files (*.fsa) were imported into GeneMapper 5 Software and processed to acquire allele base pair sizing and signal results. For both *FMR1* and *C9orf72* assays, the allele sizes were determined by comparison to the ROX 1000 sizing ladder peaks. Repeat sizing of sample alleles was manually derived using mobility and correction factors determined from both the *FMR1* and *C9orf72* positive control samples, using the manufacturer's methods. Acceptable run performance was established via specific criteria, including precision, accuracy and resolution, input sensitivity, and analytical sensitivity.

Results

The AmplideX PCR/CE *FMR1* and *C9orf72* reagents were optimized on the SeqStudio instrument for the following:

- **Preferred module**—FragAnalysis or LongFragAnalysis modules had a comparable impact on signal intensity. However, while higher signal intensities were obtained using the FragAnalysis module (approximately 50% greater in signal intensity), a split-peak profile was observed for signal-saturated gene-specific peaks. The commonality of these peaks for both *FMR1* and *C9orf72* warranted use of LongFragAnalysis as the base module, using 2 sec injections, 6 kV injection and run voltages, and a run time of 3,300 sec (55 min). These conditions yielded a profile similar to that of the 3500xL instrument and were used in the subsequent analysis of precision and sensitivity (Figure 2).
- **Precision**—Expected repeat alleles of the *FMR1* and *C9orf72* controls were evaluated for base pair call precision. The base pair size averages were then used to generate instrument-specific mobility (m_o) and correction (C_o) factors for conversion of base pair allele sizes to allele repeat numbers. The resulting 448 *FMR1* and 126 *C9orf72* allele repeat numbers were plotted against expected values, as shown in Figure 3. Excellent overall concordance was observed. Results were similar for a matched set of 24 samples across three separate laboratories using distinct instruments (data not shown).
- **Resolution and sizing**—Consistent detection of expected n and n + 1 alleles demonstrates single-repeat assay resolution. Genotype and allele repeat lengths were 100% concordant with expected values (Figure 3).
- **DNA input**—A benchmark for the performance of the AmplideX reagents is compatibility with a range of DNA input and sensitivity for full mutation detection at low DNA input (~1–5 ng).

- **Accuracy**—All samples run on the SeqStudio Genetic Analyzer maintained high allele sizing accuracy and precision across all mass inputs. Accuracy was 100% for all called alleles, with no allele signal intensities falling below 100 RFU (Figure 2).
- **Mosaicism**—A series of full mutation mosaic alleles in *C9orf72* and *FMR1* were prepared and analyzed to assess detection sensitivity of the SeqStudio Genetic Analyzer. These mosaic alleles are a challenge to amplify by PCR because of the longer repeats in the presence of shorter alleles at a higher mass fraction. Further, detection of these alleles may be hindered by preferential loading of low molecular weight DNA amplicons under electrokinetic injection. A target performance of 5% full mutation mosaic alleles was observed for both repeat categories. The lowest level of consistent minor allele detection with a minimum 100 RFU signal occurred at 5% but not 1% (levels between these two values were not tested).

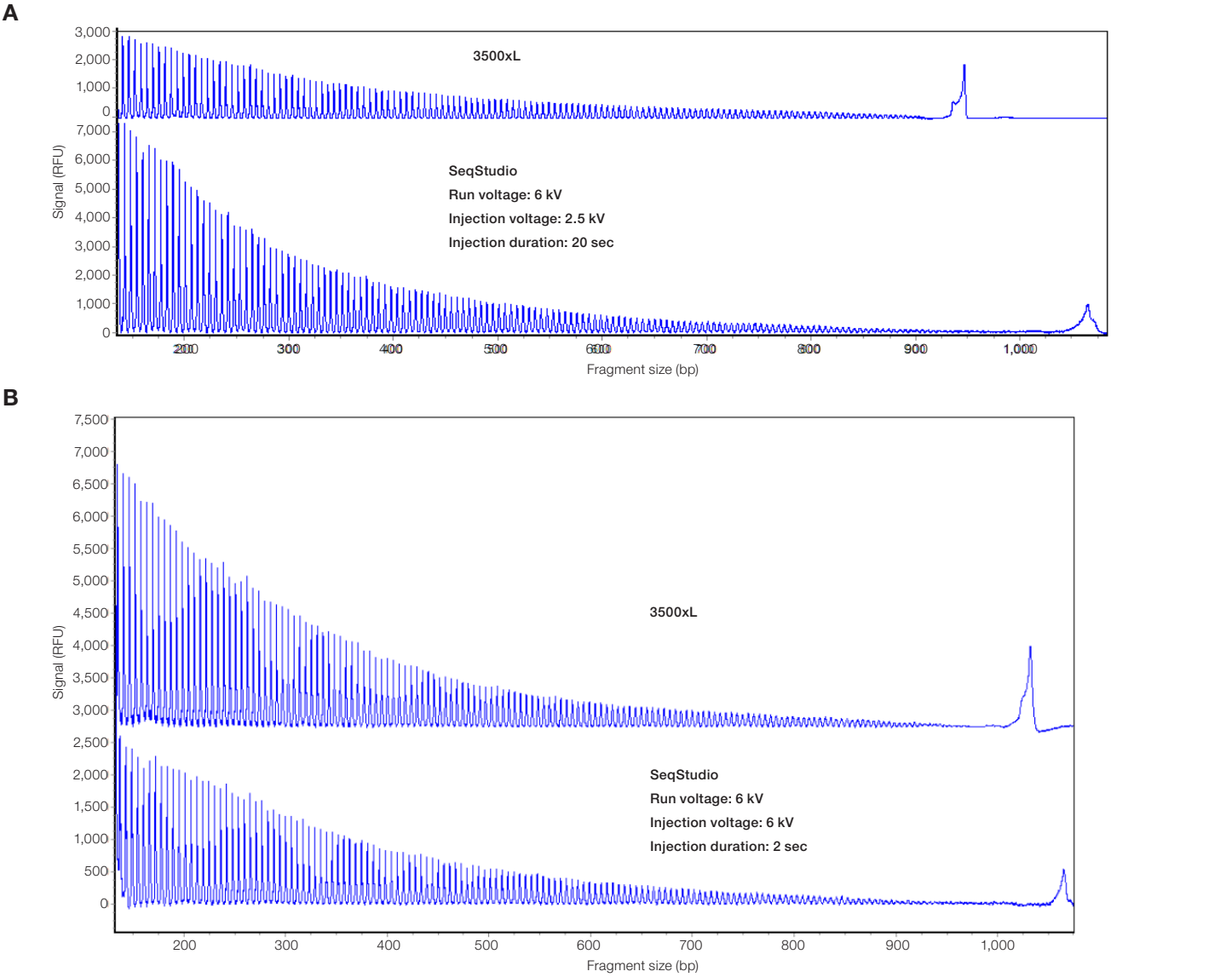

Figure 2. Electropherogram images generated by GeneMapper software. Electropherograms of **(A)** an *FMR1* full mutation on the 3500xL instrument vs. the same sample on the SeqStudio instrument, and **(B)** a *C9orf72* expansion allele on the 3500xL instrument vs. the same sample on the SeqStudio instrument.

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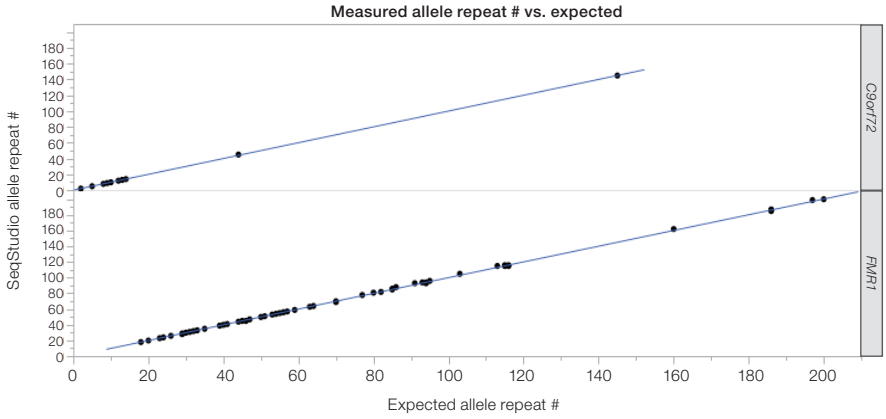

C9orf72 summary of fit	
R^2	0.99998
R^2 adj.	0.99998
RMS error	0.2725
Mean of response	45.48
N	126
***FMR1* summary of fit**	
R^2	0.99995
R^2 adj.	0.99995
RMS error	0.4729
Mean of response	72.82
N	448

Figure 3. Concordant sizing was obtained across relevant ranges of *FMR1* and *C9orf72* repeats. Plots match the SeqStudio instrument's observed repeat length—using instrument-specific size correction and repeat mobility factors—relative to expected values.

Discussion and conclusions

The SeqStudio Genetic Analyzer is available at an affordable cost, has a small footprint, and has an updated user interface for routine fragment sizing using CE. Characterization of the run parameters specific to the capillary length and POP-1 polymer were evaluated and verified in order to support adoption of this system by new users, customers migrating from older platforms such as the 310 or 3130 instruments, or current and new customers of AmplideX PCR/CE reagents.

Run parameters of a 2 sec injection, 6 kV injection and run voltage, and run time of 55 min under the LongFragAnalysis run module maintained the expected assay performance of the AmplideX PCR/CE *FMR1* and *C9orf72* reagents. By using these optimized run parameters, the SeqStudio Genetic Analyzer can be made compatible with AmplideX PCR/CE *FMR1* and *C9orf72* kits.

The running conditions described herein require adjustment of the default parameters on the SeqStudio Genetic Analyzer. For instructions on how to do this, see the SeqStudio Genetic Analyzer user guide or contact your local field application specialist (FAS).

Researchers can elucidate the relevance of repetitive DNA sequences using the combination of the SeqStudio platform and the high-performance AmplideX kit reagents to further characterize pathogenic DNA sequences—including those involved in myotonic dystrophy, Huntington disease, and many other diseases. The growing options for CE analysis with the launch of the SeqStudio system can also help scientists make important discoveries on emerging short tandem repeats that have eluded resolution by conventional PCR.

For more information, visit **thermofisher.com/blog/behindthebench/shedding-light-on-missing-heritability-seqstudio-fragment-analysis-for-neurological-disease-research/**

Visit **thermofisher.com/seqstudio** and **asuragen.com**

References

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2. Chen L, Hadd A, Sah S et al. (2010) An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis. *J Mol Diag* 12(5):589–600.
3. PC-3813v1 AmplideX PCR/CE C9orf72 protocol guide
4. PC-0170v2 AmplideX PCR/CE FMR1 protocol guide

Find out more at **thermofisher.com/seqstudio**

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