Accessible fragment analysis instrumentation allows resolution of challenging genotypes associated with pathogenic repeats, structural variants, SNVs and INDELs

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Summary

- AmplideX® PCR technology allows highly-multiplexed amplification and detection of difficult-to-resolve pathogenic variants, including triplet repeat alleles, hexanucleotide repeat alleles, other STRs, SNVs, INDELs, and CNVs.
- We demonstrate 100% categorical agreement for pathogenic variants in *FMR1* (within repeat sizing tolerance) and 100% agreement for *CFTR, SMN1/2* Plus, and *C9orf72* variants between results generated with each respective AmplideX PCR assay on the 3500 Dx CE instrument and the Spectrum Compact CE System†.
- These results expand the use of AmplideX PCR/CE genetic assays targeting multiple challenging genes and variant classes to the Spectrum Compact, which offers simplified maintenance and operation to reduce CE complexity and low instrument and consumable costs to improve access.

Introduction

Capillary electrophoresis (CE) instruments provide a reliable and robust platform for sequencing and fragment analysis and are already used in a myriad of genetic applications. Advances in the design of these well-proven instruments continue to make them more user friendly for diverse laboratory settings. AmplideX® PCR/CE assays for difficult gene targets such as *C9orf72*, *FMR1*, *SMN1/2*, and *CFTR*, which provide a variety of challenges such as structural variation and GC-rich repeats, are compatible with other established CE platforms. However, enhancements available on the Spectrum Compact may simplify CE analysis and maintenance and reduce instrument and operating costs, enabling broader access to this technology for those interested in these difficult gene targets.

We demonstrate here the detection of a broad range of challenging-to-resolve pathogenic variants across several widely-used PCR/CE assays using the Spectrum Compact CE System. This includes detection of normal, intermediate, premutation, and full mutation *FMR1* triplet repeat alleles, normal, intermediate, and expanded *C9orf72* hexanucleotide repeat alleles, 26 unique pathogenic *CFTR* variants (STRs, SNPs, and INDELs), and 0 to ≥4 copies of *SMN1* or *SMN2* as well as 3 unique variants in *SMN1/2* related to carrier risk or disease prognosis.

Methods

DNA samples were PCR-amplified using AmplideX assays (*C9orf72*†, *FMR1*†,‡, *SMN1/2* Plus†/SMA Plus‡, and *CFTR*†) according to assay instructions, followed by CE on both the Applied Biosystems™ 3500 Genetic Analyzer and the Promega Spectrum Compact CE System‡ (Spectrum) for direct comparison. Injection conditions for the Spectrum instrument used polymer 7 with the following injection conditions for each assay. Conditions may require further optimization.

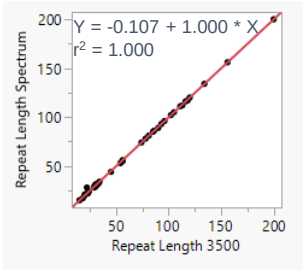
Table 1. Injection Settings for the Spectrum Compact CE System.

Assay	AmplideX Module Version	Run Time	Run Voltage	Injection Time	Injection Voltage
CFTR	2.0.0	1500 sec	13kV	15 sec	1.6kV
SMN1/2 Plus	1.1.5	1500 sec	15kV	20 sec	2.5kV
FMR1	2.0.1, 3.0.5	1500 sec	18kV	20 sec	2.5kV
C9orf72	1.0.1	1500 sec	13kV	15 sec	1.6kV

Samples were chosen 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*, and 0 to ≥4 copies of *SMN1* and *SMN2*, as well as 3 unique variants in *SMN1/2* related to carrier risk or disease prognosis. Samples were derived from commercially available cell lines or residual human blood samples. FSA files were processed and analyzed using AmplideX Reporter Software for *FMR1*, *SMN1/2*, *CFTR* and *C9orf72*; see Table 1 for module versions. QC failures were excluded.

Results

1A.

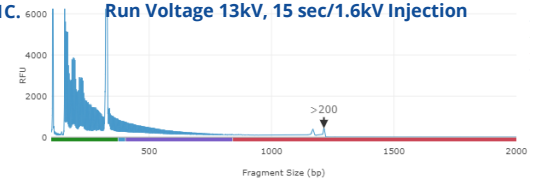


1B.

3500

Spectrum: *FMR1*	Normal	Intermediate	Premutation	Full Mutation
Normal	11	2*	0	0
Intermediate	0	2	0	0
Premutation	0	0	8	0
Full Mutation	0	0	0	8

1C. Run Voltage 13kV, 15 sec/1.6kV Injection



1D. Run Voltage 18kV, 20 sec/2.5kV Injection

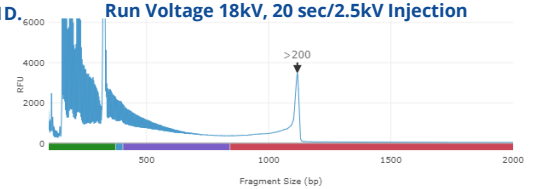
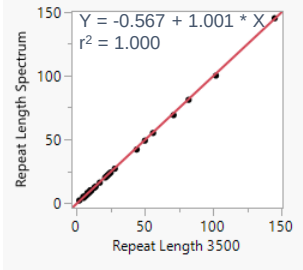


Figure 1. *FMR1* CGG repeat sizing and genotype agreement. A) *FMR1* CGG repeat size correlation plot. B) *FMR1* genotype agreement. Across all measurements agreement was 93.5% (29/31) without known size tolerances. Spectrum = Spectrum Compact. The two discrepancies were at the 45 CGG repeat boundary between normal and intermediate categories per the EMQN Guidelines (<45 vs. 45-54, respectively, see asterisk). The Spectrum results were reported as 44 while the 3500 results were reported as 45. However, when accounting for size tolerance at this repeat range according to EMQN guidelines (+/- 5% of repeat size, ~2 CGG repeats)[1] and performance claims (+/- 1 CGG for <70 CGG) [2], these misses were within precision tolerance around the 45-repeat cutoff (*). C) Electropherogram of Full Mutation with lower run voltage, injection voltage, and injection time. D) Electropherogram of Full Mutation with increased run voltage, injection voltage, and injection time (same sample as C). Conditions shown in D improve pile-up peak height and morphology and are therefore recommended in Table 1. These conditions were used to run all data shown in A and B.

† Research use only. Not for use in diagnostic procedures.
‡ CE-IVD. For US export only.

2A.



2B.

3500

Spectrum: *C9orf72*	Normal	Intermediate	Expanded
Normal	10	0	0
Intermediate	0	7	0
Expanded	0	0	14

Figure 2. *C9orf72* repeat sizing and genotype agreement. A) *C9orf72* G_4C_2 repeat sizing correlation plot. B) *C9orf72* genotype agreement. Across all measurements agreement was 100% (31/31). Spectrum = Spectrum Compact. Sizing differences observed on plot were due to differences in peak detection between the 3500 and Spectrum but did not impact genotype calls (all above 90 repeats).

3A.

3500

Spectrum: *SMN1* Cp#	0	1	2	3	4
0	2	0	0	0	0
1	0	5	0	0	0
2	0	0	10	0	0
3	0	0	0	7	0
4	0	0	0	0	6

3B.

3500

Spectrum: *SMN2* Cp#	0	1	2	3	4
0	7	0	0	0	0
1	0	5	0	0	0
2	0	0	8	0	0
3	0	0	0	3	0
4	0	0	0	0	3

Figure 3. *SMN1* and *SMN2* copy number and variant agreement. A) *SMN1* exon 7 copy number agreement. Across all measurements agreement was 100% (30/30), with no QC failures. The linear correlation between *SMN1* normalized ratios was Y = -0.020+ 1.034 * X (r^2 = 0.995). B) *SMN2* exon 7 copy number agreement. Across all measurements agreement was 100% (26/26), with 3 PR QC failures on the 3500 and 2 on the Spectrum, 1 overlapping on both. PR = precision QC, where results land in equivocal zone between copy number boundaries. The linear correlation between *SMN2* normalized ratios was Y = -0.015+ 1.039 * X (r^2 = 0.997). Agreement for c.*3+80T>G was 96.7% (29/30, one false negative), and for c.*211_*212del and c.859G>C was 100% (30/30). The false negative showed the correct peak upon manual review that was not detected by the software. Spectrum = Spectrum Compact.

4A.

3500

Spectrum: *#CFTR* Variants	0	1	≥2
0	1	0	0
1	0	15	0
≥2	0	0	14

4B.

Measure	N	Percent Agreement
PPV	41	100%
PPA	41	100%
NPA	1,819	100%
OPA	1,860	100%
PA_z	1,860	100%
PA_{TTG}	60	100%

Figure 4. *CFTR* variant and sample genotype agreement. A) Sample-level genotype agreement. Sample genotypes reported by the kit are interpreted as, Wild Type: 0 variants. Heterozygous: 1 variant. Homozygous, Compound Heterozygous, or Multiple: ≥2 variants. Sample-level agreement was 100% (30/30). B) Variant-level agreement metrics. For each metric, the number of expected variant calls per instrument configuration is listed. Each sample measurement generates 62 unique variant calls. PolyT/TG agreement was evaluated separately. 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.

Conclusions

- We demonstrated the feasibility of analyzing five difficult genes—*FMR1, CFTR, SMN1, SMN2*, and *C9orf72*—on the Promega Spectrum Compact
- This study included a challenging sample set with examples of all repeat size categories (*FMR1, C9orf72*), copy numbers (*SMN1, SMN2*) and variants (*CFTR, SMN1, SMN2*) detected by these assays
- Agreement was high across all gene targets, showing analogous performance to the 3500 platform for resolution of STRs, CNVs, SNVs, INDELs, and repeat sizes
- Despite strong performance, some bias was observed on the Spectrum relative to the 3500 for repeat sizing assays and *SMN1/2* normalized ratios. While repeat sizing bias is typically within sizing precision tolerance, *SMN1/2* bias may suggest that bin adjustments could improve precision (PR) QC performance
- Strong performance on this challenging sample set suggests that the Promega Spectrum Compact is compatible with AmplideX PCR/CE assays already used to analyze challenging gene targets

References

1. Biancalana V. et al. (2015) Eur J Hum Genet. 23(4): 417–425.
2. Filipovic-Sadic S. et al. (2010) Clin Chem. 56(3):399-408.

Promega

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