Low Cost Electrophoresis Systems Detect and Resolve All Categories of Fragile X Gene Expansions Amplified Using AmplideX® *FMR1* PCR Reagents

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SUMMARY

- Low cost electrophoresis platforms can increase accessibility to more routine fragile X screening and diagnostic tests.
- Two such platforms, the Agilent 2100 Bioanalyzer and the Lonza FlashGel™, were compared with the Life Technologies 3500xL for CGG repeat size resolution, detection sensitivity, reproducibility, and categorical agreement using AmplideX® *FMR1* PCR reagents.
- The AmplideX *FMR1* Process Control enabled the calibration of repeat length across the three electrophoresis platforms.
- *FMR1* CGG repeat detection can be achieved on alternative platforms with trade-offs in genotype categorization and sensitivity compared to the 3500xL.

INTRODUCTION

The fragile X gene, *FMR1*, codes for an RNA-binding protein that regulates the translation of hundreds of genes. Expansion of the CGG repeats in the 5' untranslated region of *FMR1* modulates RNA and protein levels such that clinical phenotypes become apparent in both premutation carriers (55-200 repeats) and full mutation individuals (>200 repeats). These phenotypes include intellectual disability, autism, anxiety, seizures, ADHD, infertility, ataxia, and parkinsonism.[1] Accurate measurement of the number of *FMR1* CGG repeats can help elucidate the risk of developing the stated diseases and disorders. Current testing for expansion is limited to labor intensive Southern blot analysis or less accessible capillary electrophoresis systems. Broader access to *FMR1* genetic technologies may support routine screening and molecular diagnostics. In this poster, we characterize detection of normal and expanded fragile X alleles across three electrophoresis platforms, including two low cost systems.

MATERIALS AND METHODS

The sizing resolution and analytical sensitivity of amplicons produced using AmplideX *FMR1* PCR reagents were compared across three platforms: the 3500xL Genetic Analyzer (Life Technologies), the 2100 Bioanalyzer (Agilent Technologies), and the FlashGel™ DNA System (Lonza). Samples were a combination of 97 blinded clinical specimens obtained from the New York State Institute of Basic Research and from de-identified internal donors (Table 1). Gene-specific PCR products of DNA samples were generated using AmplideX *FMR1* PCR reagents.[2] Unpurified aliquots were run on the different platforms. The AmplideX *FMR1* Process Control (Asuragen), a pooled cell line control with alleles corresponding to 18, 30, 32, 56, 85, 116 and >200 CGG, was used to convert size in base pairs to CGG repeats (Figure 1).

Table 1. Genotype distribution of residual clinical samples used to characterize *FMR1* repeat sizing on different electrophoresis platforms.

Genotype	Male	Female	Total
Normal	14	8	**22**
Intermediate	0	9	**9**
Premutation	0	31	**31**
Full Mutation	18	17	**35**
	32	**65**	**97**

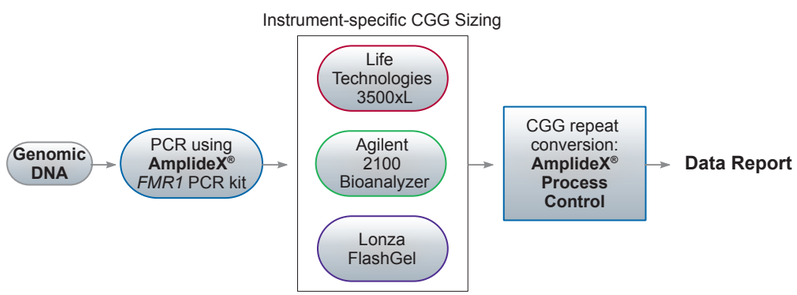

Figure 1. Workflow of clinical sample testing. AmplideX PCR products were amplified and analyzed on three electrophoresis platforms, then calibrated using the AmplideX *FMR1* Process Control.

Presented at AMP 2013

RESULTS

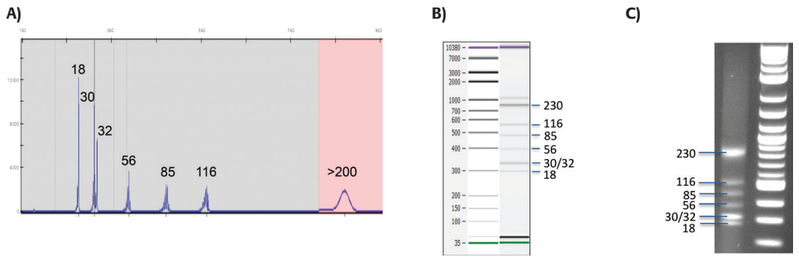

Figure 2. The AmplideX *FMR1* Process Control highlights the capacity of each electrophoresis platform to resolve distinct fragile X genotypes. The process control contains three normal alleles (18, 30, 32), three premutation alleles (56, 85, 116), and one full mutation allele (~230). Results are shown from **A)** 3500xL **B)** 2100 Bioanalyzer and **C)** FlashGel. The full mutation allele was detected as >200 CGG using the 3500xL but sized at ~230 CGG on the other two platforms.

The 3500xL and 2100 Bioanalyzer were further tested for their ability to resolve repeats similar in size: (1) normal alleles, (2) the boundary between intermediate and premutation alleles, and (3) small premutation alleles. Samples were run in individual wells on the same plate/chip, and the resulting electropherograms were overlaid (Figure 3).

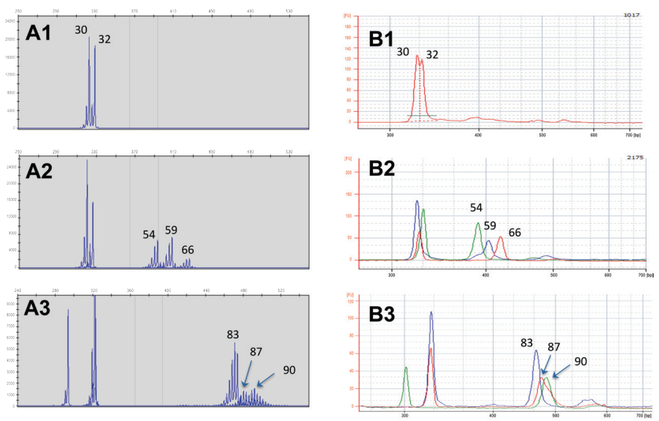

Figure 3. Resolution of the capillary electrophoresis platforms across three allele categories for the 3500xL (left) and 2100 Bioanalyzer (right). Single repeat resolution was observed using the 3500xL. Distinct allele sizes were observed using the 2100 Bioanalyzer with a sizing resolution of 2-3 CGG repeats.

The analytical sensitivity of each platform was assessed by testing samples that contained a 5%, 10%, or 20% mass fraction of a full mutation allele (Figure 4).

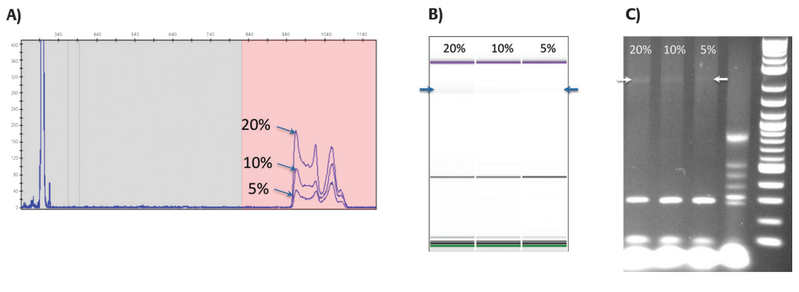

Figure 4. Sensitivity of electrophoresis platforms to detect low abundance full mutation alleles. Titration series of a 5%, 10%, and 20% 940 CGG allele in the background of a 23 CGG allele detected on **A)** 3500xL **B)** 2100 Bioanalyzer and **C)** FlashGel. As low as 5% sensitivity was readily observed using the 3500xL with 5-10% sensitivity for the 2100 Bioanalyzer and FlashGel systems.

The percent coefficient of variation (CV) was used to measure the instrument precision by repeatedly testing the AmplideX *FMR1* Process Control across multiple runs and operators. For all samples tested, the greatest variation was observed in the smallest repeat size tested, 18 CGG, although the variation was still only 5% or ~1 CGG repeat. The least variation was observed at 56 repeats, further highlighting the effectiveness of the process control to delineate the intermediate/premutation boundary (Figure 5). The sizing accuracy of alleles between the 3500xL and 2100 Bioanalyzer was also compared (Figure 6), utilizing the AmplideX *FMR1* Process Control to standardize across the platforms.

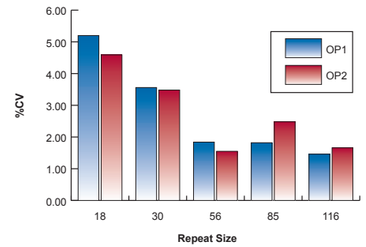

Figure 5. High repeatability from run-to-run and operator-to-operator from AmplideX PCR amplicons analyzed with the 2100 Bioanalyzer. Operator testing was conducted at two different laboratory sites. For OP1 and OP2, n=5 and n=3, respectively.

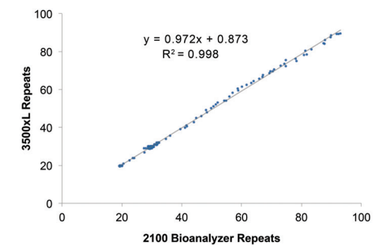

Figure 6. AmplideX PCR products analyzed with the 2100 Bioanalyzer are sized with high accuracy under 100 repeats. In total, 128 alleles were compared, between the 3500xL and 2100 Bioanalzyer.

Strong agreement between the 3500xL and 2100 Bioanalyzer in categorizing fragile X genotype was observed (Table 2A). The only two discrepancies were due to resolution (combining a premutation and full mutation into one peak) and sensitivity (full mutation observed below the signal threshold). For the FlashGel system, 81 of the 97 samples were sized correctly, a success rate of 84% (Table 2B). The majority of samples on this platform that were erroneously categorized were near either the NOR/PM or PM/FM boundary.

2A)

		3500xL				
		NOR	INT	PM	FM	Agreement
2100 Bioanalyzer	NOR	22			1	95/97 = 98%
	INT		9			
	PM			31	1	
	FM				33	

2B)

		3500xL			
		NOR	PM	FM	Agreement
FlashGel	NOR	23	3	3	81/97 = 84%
	PM	8	28	2	
	FM			30	

Table 2. Concordance of AmplideX clinical samples analyzed on low-cost alternative platforms with the 3500xL genetic analyzer. Overall, the **A)** 2100 Bioanalyzer correctly sized 95 of the 97 clinical samples, resulting in an agreement of 98%. The majority of discordant categorical calls on the **B)** FlashGel occurred at the normal and premutation boundary due to lower resolution in this range. Conversely, the FlashGel provided sizing for full mutation alleles that were too large to resolve using CE.

CONCLUSION

- Capital and maintenance cost of genetic analyzer instruments limit the access of molecular assays in some laboratories.
- Using AmplideX PCR reagents, alternative, low cost electrophoresis platforms such as the Agilent 2100 Bioanalyzer can successfully detect and resolve all categories of *FMR1* mutations.
- Both the 2100 Bioanalyzer and FlashGel enabled sensitive detection of a 5-10% full mutation allele, and the Bioanalyzer further achieved a resolution of 2-3 repeats, high repeatability, and sizing agreement (R^2 = 0.998) with the 3500xL genetic analyzer.
- These findings expand platform options for US and international laboratories interested in *FMR1*-based screening and diagnostic testing.

Acknowledgements

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References

1. Monaghan, K. G., Lyon, E., & Spector, E. B. (2013). ACMG Standards and Guidelines for fragile X testing: a revision to the disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics and Genomics. Genetics in Medicine.
2. Filipovic-Sadic, S., et al. (2010). A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. Clinical Chemistry, 56(3), 399-408.