Analytical and Clinical Validation of a PCR/CE Assay System for the Diagnosis of Fragile X Syndrome and Carrier Screening

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Summary

- Fragile X syndrome (FXS) is an inherited repeat disorder and the most common known genetic cause of autism. About 1 in 150 women are fragile X carriers.
- The AmplideX® Fragile X Dx and Carrier Screen Kit is the first and only genetic test for FXS and related disorders authorized by the FDA as an aid in diagnosis and for carrier screening in adults.
- The Kit demonstrates accurate, sensitive, and precise determination of genotype category for CGG repeat expansions in the *FMR1* gene.
- Analytical validation studies for the test included between-site and mosaicism precision, lot-to-lot reproducibility, limit of detection, DNA input, thermal cycler and extraction equivalence, and stability among others.
- A three-site clinical validation study demonstrated >95% diagnostic accuracy for the test.

Introduction

Fragile X syndrome (FXS) is an inherited genetic condition that is a leading cause of mental and cognitive disabilities. It is characterized by an expanded cytosine-guanine-guanine (CGG) nucleotide repeat (≥200 CGG) in the 5' untranslated region of the *FMR1* gene.[1] This expansion can lead to a variety of consequences depending on the length of the CGG expansion, including the development of FXS for individuals with a full mutation (Figure 1). Individuals with a premutation expansion (55-199 CGG) generally do not develop FXS, but are at risk of developing fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-associated primary ovarian insufficiency (FXPOI). Estimated frequencies in the US population are 1:3335 for the full mutation, 1:178 for female premutation carrier, and 1:4848 and 1:3560 for FXTAS and FXPOI, respectively.[2,3,4]

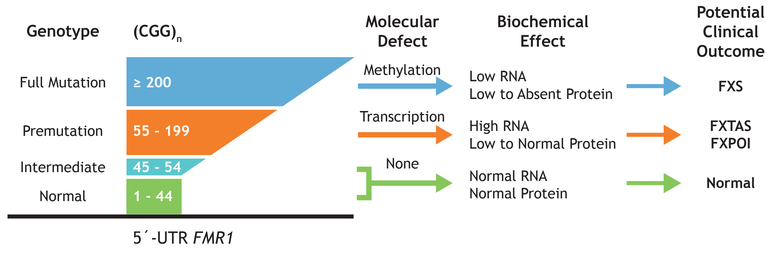

Figure 1. ***FMR1*** **CGG Repeat Length Ranges and Their Corresponding Genotypes and Outcomes.**

Materials and Methods

The AmplideX Fragile X Dx & Carrier Screen Kit quantifies the number of CGG repeats in the *FMR1* alleles in a purified genomic DNA sample using PCR with gene-specific and triplet repeat primers followed by size resolution with capillary electrophoresis (CE). AmplideX® Fragile X Reporter software provides automated analysis of the resulting electropherogram to accurately identify and size *FMR1* alleles (Figure 2) and categorize the genotype sample as either a normal, intermediate, premutation, or full mutation. Additionally, the kit can resolve zygosity in female samples and identify a relatively low level of size mosaicism. Results can be obtained in as few as seven hours with only 60 minutes of operator hands-on time (Figure 3).

All studies used the Applied Biosystems® 3500 Dx Series Genetic Analyzers and reagents (Thermo Fisher Scientific), including POP-7 and Dye Set D (DS-30). Multiple study-specific sample panels consisting of clinical and/or contrived samples (cells spiked into leukocyte-depleted blood) were used to represent all *FMR1* genotype categories. Single-site precision (repeatability), analytical sensitivity and specificity, and limit of detection (LoD) studies, among others, were designed and executed consistent with CLSI guidance. Three external laboratories (UC Davis MIND Institute, Rush University Medical Center, and New York State Institute for Basic Research) performed clinical validation studies using archived clinical specimens. Diagnostic performance and carrier screening performance was assessed in comparison to reference methods (Southern blot analysis and *FMR1* dual-PCR, respectively). The dual-PCR method employs two alternative primer pairs to detect *FMR1* that differ from those in the AmplideX Fragile X Dx & Carrier Screen kit.

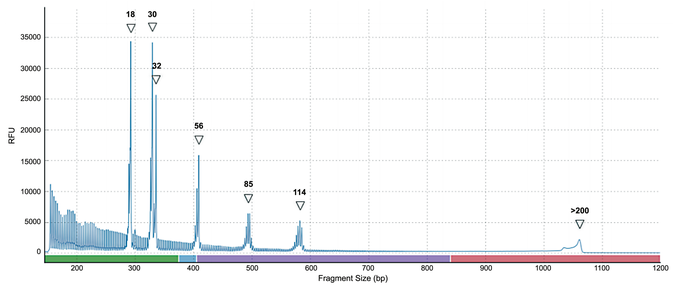

Figure 2. Positive Control (PC) Electropherogram from AmplideX Fragile X Dx & Carrier Screen Kit. PC includes alleles at 18 ±1, 30 ±1, 32 ±1, 56 ±1, 85 ±3, 114 ±3, and > 200 CGG repeats, and yields a full mutation genotype.

Figure 3. Workflow for the AmplideX PCR/CE Fragile X Dx & Carrier Screen Kit. An overview of the workflow, highlighting the rapid turnaround time of about 7 hrs from gDNA to answer. Total hands on time is 60 mins for 24 samples with one CE injection.

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Results

CGG Repeat Length Measurement Precision

Sample ID	Mode Expected CGG Length (± Target Precision)	Reported CGG Length	Number of Replicates at That Size	Total Number of Replicates	% within Target Precision Range
ASGN-109	30 ± 1	29	4	200	100.0
		30	196		
ASGN-112	29 ± 1	28	2	214	100.0
		29	212		
	30 ± 1	29	2	214	100.0
		30	212		
	Contamination/artifact*	114	1	1	0.5
ASGN-101	35 ± 1	35	212	212	100.0
	92 ± 3	92	187	212	100
		93	24		
		94	1		
	Mosaic result*	96	10	22	10.4
		97	12		
	Contamination/artifact*	30	1	3	1.4
		55	1		
		79	1		
ASGN-005	29 ± 1	29	209	209	100.0
	45 ± 1	45	209		
ASGN-111	29 ± 1	29	213	213	100.0
	50 ± 1	50	208	213	100.0
		51	5		
ASGN-103	30 ± 1	30	213	213	100.0
	55 ± 1	55	207	213	100.0
		56	6		
ASGN-113	30 ± 1	30	208	208	100.0
	102 ± 3	101	5	208	100.0
		102	123		
		103	70		
		104	9		
		105	1		
	Mosaic result*	87	25	45	21.6
		88	20		
ASGN-016	18 ± 1	17	21	213	100.0
		18	192		
	113 ± 3	113	176	213	100.0
		114	32		
		115	4		
		116	1		
	Contamination/artifact*	> 200	2	2	9.4
ASGN-018	20 ± 1	19	3	212	100.0
		20	209		
	196 ± 5% (10 CGG)	195	19	212	100.0
		196	112		
		197	60		
		198	17		
		199	3		
		> 200	1		
	Mosaic allele*	178	2	131	52.8
		179	6		
		180	5		
		181	5		
		182	1		
		> 200	112		
ASGN-023	> 200	> 200	212	212	100.0
ASGN-104	24 ± 1	23	1	210	100.0
		24	209		
	> 200	> 200	210	210	100.0

Table 1. Single-Site Repeatability Generated 2316 Data Points and Achieved CGG Repeat Precision Ranges of ±1 for Repeats ≤ 70, ±3 for ≥ 71 and ≤ 120, and ±5% for ≥ 121 and < 200. Eleven-member test panel with various genotypes covering all the *FMR1* genotype categories assessed for CGG repeat length. The study design included 2375 measurements across three kit lots, three CE instruments, three operators, and 12 days, of which 2316 valid data points were generated. For all replicates passing QC criteria, 100% fell within the target precision ranges for their associated repeat length(s). *Potential mosaic alleles were confirmed with *FMR1* dual-PCR. Mosaic results may be below the limit of detection for mosaicism. Contamination/artifact calls were additional alleles that did not produce CE peaks in all three assays (AmplideX, dual-PCR).

2A

Genotype Agreement over DNA Input Range

Input (ng)	Genotype Agreement/ Replicates	Percent Agreement	Lower CI (%)	Upper CI (%)
160	19/19	100%	83.2	100
80	20/20	100%	83.9	100
40	20/20	100%	83.9	100
20	20/20	100%	83.9	100
10	20/20	100%	83.9	100
1	19/19	100%	83.2	100

2B

Genotype Agreement for 2 Weeks of Blood Storage at 4˚C

Sample	Genotype	Days	Genotype Agreement/ Replicates	Percent Agreement	Lower CI (%)	Upper CI (%)
ASGN-122	Normal	14	17/17	100%	81.57	100
ASGN-111	Intermediate	16	16/16	100%	80.64	100
ASGN-113	Premutation	14	17/17	100%	81.57	100
ASGN-105	Full Mutation	16	17/17	100%	81.57	100

2C

Sample	Category	Genotype Agreement by Thermal Cycler: Replicates	Thermal Cycler 1	Thermal Cycler 2	Thermal Cycler 3	Genotype Agreement by Extraction Method: Replicates	Extraction Method 1	Extraction Method 2	Extraction Method 3
ASGN-002	Normal	48	100%	100%	100%	24	100%	100%	100%
ASGN-007	Intermediate (Premutation)*	48	100%	100%	100%	24	100%	100%	100%
ASGN-013	Premutation	48	100%	100%	100%	24	100%	100%	100%
ASGN-016	Premutation	48	100%	100%	100%	24	100%	100%	100%
ASGN-021	Full Mutation	48	100%	100%	100%	22	100%	100%	100%

Table 2. Measured Genotypes were 100% Concordant with Expected Genotypes in All Studies, Including Input (Range: 1-160 ng gDNA), Whole Blood Stability (Blood Stored at 4˚C for up to 14 Days), Thermal Cycler Equivalence, and DNA Extraction Method. A) Genotype agreement across DNA input range for sample ASGN-105 (Full Mutation). Genotype agreement determined for eight-member panel representing all genotype categories. Each sample tested at six mass inputs (1, 10, 20, 40, 80, and 160 ng) run in duplicate for five total runs. 100% of replicates across all DNA inputs yielded correct genotype. **B)** Genotype agreement across two weeks of whole blood storage at 4˚C for four samples. Genotype agreement determined for 14-member panel representing all genotype categories. DNA was extracted from each sample after various storage times, with three replicates at time 0, and then two replicates at each of the seven remaining extraction time points. 100% of replicates across extraction times yielded correct genotype. **C)** Thermal cycler and extraction equivalence determined using a five-member genomic DNA panel representing all genotype categories. Thermocyclers: (1) ABI Veriti, (2) ABI 9700, and (3) Eppendorf Mastercycler. Extraction Methods: (1) Thermo Fisher Scientific MagMAX (manual magnetic bed), (2) QIAGEN Blood Mini (manual silica spin column), and (3) QIAGEN Gentra PureGene (manual solution precipitation). Genotype concordance was 100% between thermal cyclers and extraction methods across all samples.

Lower Limit of Detection for Mosaic Alleles

Sample	Alleles: Mosaic	Alleles: Major	LoD for Mosaic Allele (% MAF)
1	Intermediate (I)	Normal (N)	2.0
2	Premutation (PM)	Normal	2.0
3	Full Mutation (FM)	Normal	6.1
4	Normal	Premutation	2.0
5	Premutation	Premutation	5.0
6	Full Mutation	Premutation	7.0
7	Normal	Full Mutation	2.0
8	Premutation	Full Mutation	10.0

Table 3. In Lower Limit of Detection (LoD) Studies for Detection of Mosaic Alleles, Limits were Determined for the Following Mosaic/Major Allele Combinations: 2% for I/N, PM/N, N/PM, and N/FM, 5% for PM/PM, 6.1% for FM/N, 7% for FM/PM, and 10% for PM/FM. Mosaic/major allele combinations were generated by combining various amounts of DNA of known gender, genotype, and expected allele sizes for a mosaic allele (0.01 to 10 mass percent).

4A

AmplideX Fragile X Dx & Carrier Screen Kit

	Full Mutation Diagnostic - Reference Method (Southern Blot)		
	Positive (≥ 200)	Negative (< 200)	Total
Positive (≥ 200)	67	1	68
Negative (< 200)	3	137	140
Total	70	138	208
	Percent	Lower 95% CI	Upper 95% CI
PPA	95.7	88.1	98.5
NPA	99.3	96.0	99.9
OPA	98.1	95.2	99.0

4B

AmplideX Fragile X Dx & Carrier Screen Kit

	Premutation Diagnostic - Reference Method (Southern Blot)		
	Positive (55-199)	Negative (< 55)	Total
Positive (55-199)	69	2	71
Negative (< 55)	0	67	67
Total	69	69	138
	Percent	Lower 95% CI	Upper 95% CI
PPA	100.0	94.7	100.0
NPA	97.1	90.0	99.2
OPA	98.6	94.9	99.6

Table 4. Diagnostic Performance of AmplideX Fragile X Dx & Carrier Screen Kit Compared to Southern Blot Analysis for A) Full Mutation and B) Premutation Samples. A multi-center clinical validation study was conducted across three US clinical laboratory sites to establish a diagnostic claim. **A)** Full mutation positive vs. negative assessment. The PPA was 95.7% with a 95% confidence interval of 88.1-98.5%. **B)** Premutation vs. normal or intermediate assessment. PPA, NPA and OPA all exceeded 95%, and all two-sided Wilson score 95% confidence intervals were at or above 90%. Note: Southern blot is not expected to have accurate sizing to detect premutation and intermediate mutation alleles.

AmplideX Fragile X Dx & Carrier Screen Kit

	Carrier Screening - Reference Method (*FMR1* Dual-PCR)			
	Premutation/ Full mutation (> 54)	Intermediate (45-54))	Normal (< 45)	Total
Premutation/Full mutation	68	10*	1	79
Intermediate	0	60	0	60
Normal	0	0	68	68
Total	68	70	69	207
Percent Agreement	100.0	85.7*	98.6	
Lower 95% CI	94.7	75.7	92.2	
Upper 95% CI	100.0	92.1	99.7	

Table 5. Carrier Screening Classification Results and Agreement of AmplideX Fragile X Dx & Carrier Screen Kit Compared to *FMR1* Dual-PCR. A multi-center clinical validation study was conducted across three US clinical laboratory sites to establish a carrier screening claim. The percent agreement with the dual-PCR reference method for carrier samples (premutation/full mutation) and normal samples was 100.0% and 98.6%, respectively. *Eight of these 10 samples had allele peaks of 54 or 55 by the validated *FMR1* Dual-PCR Reference Method and the AmplideX Fragile X Dx & Carrier Screen Kit, respectively. The established precision of the assay in this range is ±1 CGG repeat. When taking into account the assay precision and including the eight borderline samples as concordant, the percent agreement for the Intermediate category is 97.1%, 95% CI 90.2-99.2.

Conclusions

- The AmplideX Fragile X Dx and Carrier Screen Kit includes reagents, controls and automated software, and reliably amplifies and genotypes the *FMR1* CGG repeat tract.
- Expanded *FMR1* alleles were reported with a lower limit of detection of 2.0 to 10.0%, depending on the specific admixture.
- Single-site precision across all genotype categories showed 100% agreement at 20 ng input across multiple operators, days, instruments and kit lots.
- Compared to Southern Blot analysis, the overall percent agreement for the test was 98.1% for full mutation and 98.6% for premutation samples. Results were returned in hours using the test rather than several days using Southern blot.

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