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

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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. 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 Premutation, and 1:4848 and 1:3560 for FXTAS and FXPOI, respectively.

Conclusions
• The AmplideX Fragile X Dx & 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 assay.
• 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.

Materials and Methods
The AmplideX Fragile X Dx & Carrier Screen Kit quantifies the number of CGG repeats in the FMR1 gene. The kit includes a dual-PCR, respectively). The dual-PCR method employs two alternative primer pairs to detect contamination/artifact calls were additional alleles that did not produce CE peaks in all three assays (AmplideX, dual-PCR). With

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Table 1. Single-Site Repeatability Generated 2316 Data Points and Achieved CGG Repeat Ranges of ± 1 for Repeats ≥ 70, ± 3 for a 71 and a 120, ± 55 for ± 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 76 assays, lots, three instruments, three operators, and 12 days, of which 2316 valid data points were generated. For all replicates passing QC criteria, 100% fell within the precision range for their respective allele sizes. 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).

Table 2. Measured Genotypes were 100% Concordant with Expected Genotypes in All Studies, Including Genotype (Range: 1-160 ng DNA), Whole Blood Stability (Blood Stored at 4°C for up to 14 Days), Thermal Cyclers Efficacy, and 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 inputs (10, 20, 40, 60, 100, and 160 ng) in duplicate for five total runs. 100% of replicates across all DNA inputs yielded correct genotype. B) Genotype agreement determined for two weeks of whole blood storage at 4°C for four samples. Genotype allele 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 remaining extraction time points: 100% of replicates across Extraction times yielded correct genotype. C) Thermal cyclers and extraction equivalence determined using an eight-member panel including all genotype categories. Reagent/Instrument was A3 (ABI Veriti, ABI 9700, and Eppendorf Mastercycler). DNA input, thermal cycler and extraction equivalence determined using an eight-member panel including all genotype categories. Reagent/Instrument was A3 (ABI Veriti, ABI 9700, and Eppendorf Mastercycler). Genotype concordance was 100% between thermal cyclers and extraction methods across all samples.

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

Table 4. Diagnostic Performance of AmplideX Fragile X Dx & Carrier Screen Kit Compared to Southern Blot Analysis for Premutation and Full Mutation Samples. A multi-center clinical validation study was conducted across three US clinical laboratory sites to establish a carrier screening claim. The percent agreement with Southern Blot screening was 100% normal vs. normal and intermediate assessment. PPA, NPA, and OPA were all excellent, 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.

Table 5. Carrier Screening Classification Results and Agreement of AmplideX Fragile X Dx & Carrier Screen Kit Compared to FMNP 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 FMNP dual-PCR Reference Method and the AmplideX Fragile X Dx & Carrier Screen Kit, respectively. The established precision of the assay in this range is ± 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 0.9-2.9%.

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

Figure 2. Positive Control (PC) Electropherogram from AmplideX Fragile X Dx & Carrier Screen Kit. PC includes alleles at 18.1, 30.1, 32.2, 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 hours from DNA to answer. Total hands on time is 60 mins for 24 samples with one CE ejection.

REFERENCES

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