A Sensitive and High Throughput PCR Method that Accurately Sizes *FMR1* Alleles, Reproducibly Detects Fragile X Full Mutation Expansions, and Determines *FMR1* Methylation Status

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SUMMARY

- Fragile X Syndrome (FXS) is a triplet repeat disorder caused by CGG expansions in the 5' untranslated region of the fragile X mental retardation (*FMR1*) gene and subsequent gene silencing.
- We previously described a novel PCR-only technology* (mPCR-CE) for comprehensive *FMR1* assessment using methylation-sensitive restriction enzyme digestion followed by long read PCR that produces concordant results with Southern blot analyses.
- We have optimized mPCR-CE* by incorporating more informative controls, better balancing the relative signals from the dye-tagged PCR products, and improving the overall workflow.
- mPCR-CE* offers high analytical sensitivity and accurately and reproducibly determines the methylation status of *FMR1* alleles across all categories of CGG expansions. As a result, this PCR-based method is better suited to high volume, routine molecular assessments than Southern blot analysis.

MATERIALS AND METHODS

Genomic DNA samples were obtained from either the Coriell Cell Repositories (cell lines), or the M.I.N.D. Institute (clinical specimens). HpaII-digested or non-HpaII treated gDNA, along with a spike-in digestion control and "0 CGG" reference, were amplified with HEX-labeled or FAM-labeled primers in separate reactions using a PCR procedure developed at Asuragen. Equal volumes of the two PCR reactions were then combined and analyzed by capillary electrophoresis (CE). The allele-specific methylation fraction was calculated from the ratio of peak intensities of PCR amplicons produced from digested and undigested genomic DNA (gDNA). Results were compared with Southern blotting of 7-10 µg of gDNA, as performed by the M.I.N.D. Institute (clinical samples), or by Asuragen, Inc. (cell line samples)

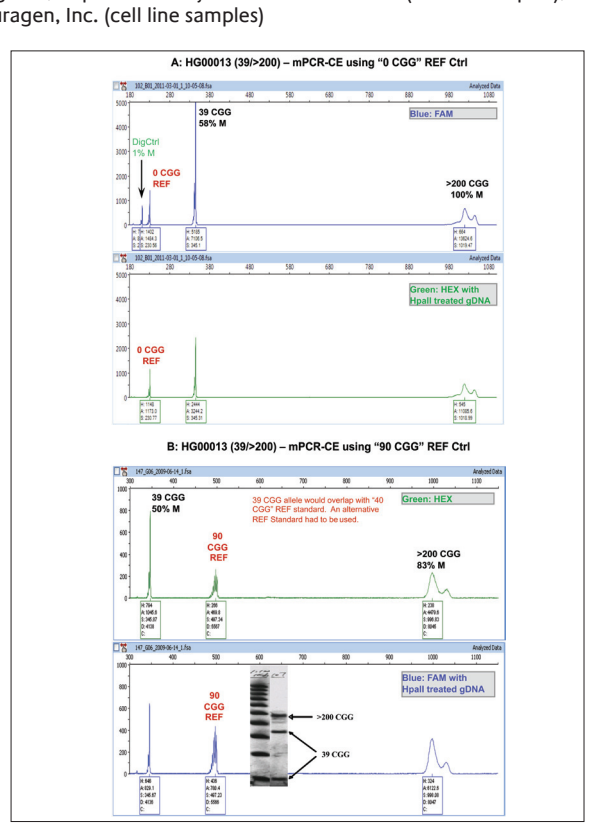


Fig. 1. A "0 CGG" reference control eliminates the overlap of sample *FMR1* alleles with the previously developed "40 CGG" reference. The advantage of using "0 CGG" reference control is shown with clinical sample HG00013 (39/>200 CGG). This sample has a normal *FMR1* allele that co-migrates with the previously developed reference. In that particular case, an alternative control that migrated at 90 CGG had to be used in a repeated PCR assay. The "0 CGG" reference eliminates any possible overlap with a biological *FMR1* allele.

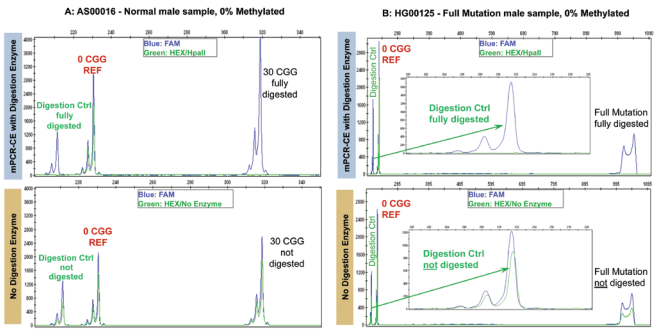
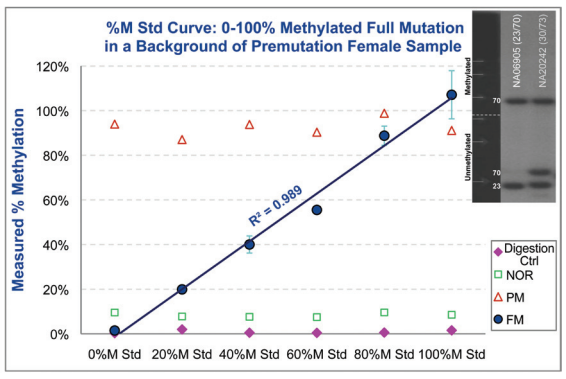


Fig. 2. An external assay control confirms the methylation-specific digestion of sample genomic DNA. Inclusion of the digestion control reveals only ~1% methylation (that is, ~99% loss of signal) when the methylation-sensitive HpaII enzyme is added, but retention of the corresponding peak when the enzyme treatment is omitted. As expected, the unmethylated biological allele (NOR allele in AS00016, and FM allele in HG00125), presents as methylated in the absence of HpaII enzyme.



	Digestion Ctrl	NOR (23 CGG)	PM (70 CGG)	FM (645 CGG) Ave	FM (645 CGG) StDev
0%M Std	0%	10%	94%	1%	0.2%
20%M Std	2%	8%	87%	20%	0.9%
40%M Std	0%	8%	94%	40%	3.8%
60%M Std	0%	7%	90%	56%	0.2%
80%M Std	1%	10%	99%	89%	4.3%
100%M Std	2%	9%	91%	107%	10.8%
Ave	1%	8%	93%		
StDev	0.7%	1.0%	4.0%		

Fig. 3. mPCR-CE* analysis of a titration series of methylated 645 CGG amplicons, and background cell line DNA (NA06905), accurately recovers the known methylation fractions. The inset graph shows the result from Southern blotting of the NA06905 23/70 CGG DNA, and reveals skewed methylation consistent with the mPCR-CE results.

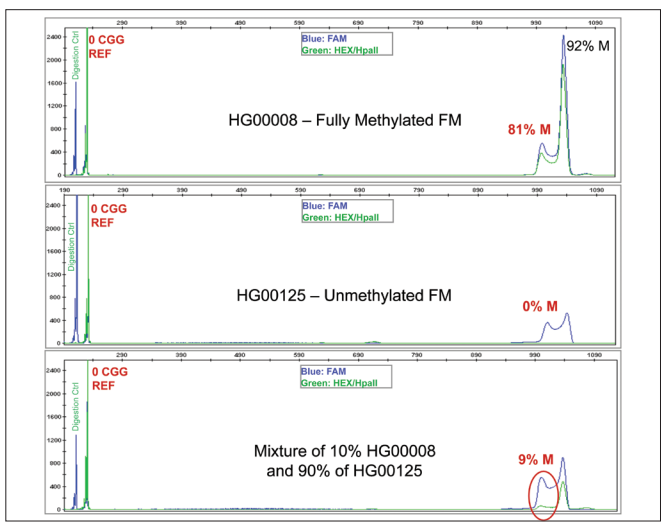


Fig. 4. mPCR-CE* can detect 10% of fully methylated FM in a background of unmethylated FM from a mixture of two human blood DNA samples.

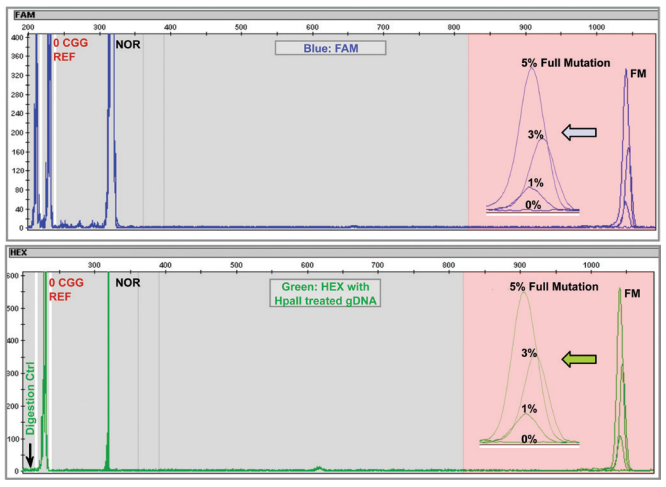


Fig. 5. mPCR-CE* can detect as little as 1% mass fraction of a fully methylated full mutation *FMR1* allele in the background of a 99% mass fraction of a normal allele from two clinical DNA samples. Amplification and detection were demonstrated with as little as 800 pg of a full mutation allele in a total of 80 ng DNA.

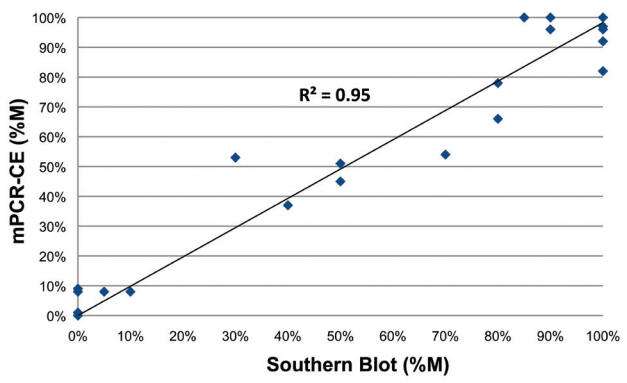


Fig. 6. The quantitative results of mPCR-CE* are highly correlated with those from Southern blot analysis across alleles in 22 human blood samples. Samples included 3 normal, 5 premutation, and 14 full mutations.

CONCLUSIONS

- The mPCR-CE method* was improved to include more informative in-tube controls and optimized signal quantification.
- mPCR-CE* preliminary results were reproducible and quantitative across the full dynamic range of input methylation fractions using a 645 CGG standard, and using well characterized cell line DNA samples.
- mPCR-CE* demonstrated high preliminary analytical sensitivity, including the detection of as little as 1% input fully methylated full mutation, and a 10% methylation mosaic full mutation.
- mPCR-CE* preliminary results were consistent with methylation assessments from Southern blot analysis across 22 clinical samples, including 14 full mutations.
- mPCR-CE* can provide comparable results to Southern blot analysis with superior size resolution and accuracy, 50- to 100-fold less input DNA requirements, and 10-fold less processing time with far greater sample throughput.

Disclosure: AH, LC, SFS, JH, and GJL are employees of Asuragen, Inc.

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