A PCR-only Workflow for *FMR1* Analysis that Amplifies Fragile X Full Mutation Alleles, Definitively Resolves Allele Zygosity, and Accurately Assesses Methylation Status.

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SUMMARY

- Fragile X syndrome (FXS) is associated with an expansion of a CGG trinucleotide repeat in the 5' untranslated region of the fragile X mental retardation (*FMR1*) gene, promoter hypermethylation, and gene silencing.
- Molecular characterization of FXS currently requires both PCR and Southern blot methods. A rapid and high throughput workflow is needed that can ideally replace the requirement for low throughput and onerous Southern blot analyses.
- A PCR-only workflow is described here that correctly reports all categories of *FMR1* alleles, including full mutations (fm) with at least 1300 CGG repeats, and accurately assesses the methylation status of each amplified allele.
- These PCR technologies definitively identify female heterozygous samples, and provide enhanced resolution of allele-specific methylation patterns, revealing patterns of skewed methylation that are missed by Southern blot analysis.
- Using this PCR-only approach, 103 samples, including 33 full mutations and 31 premutations, were each accurately sized and the methylation status correctly determined as referenced to Southern blot analysis.

MATERIALS AND METHODS

Genomic DNA (gDNA) samples were obtained from either the Coriell Cell Repositories (cell lines), or the M.I.N.D. Institute (clinical specimens). DNA was amplified, detected, and analyzed following CGG Repeat Primed (RP) *FMR1* PCR using the procedures outlined in Asuragen's RUO* kit. HpaII-digested or non-HpaII treated gDNA were amplified with FAM-labeled or HEX-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 percentage of methylation by allele was calculated from the ratio of peak intensities of PCR amplicons produced from digested and undigested gDNA. Results were compared with Southern blotting of 7-10 µg of gDNA, as performed by the M.I.N.D. Institute.

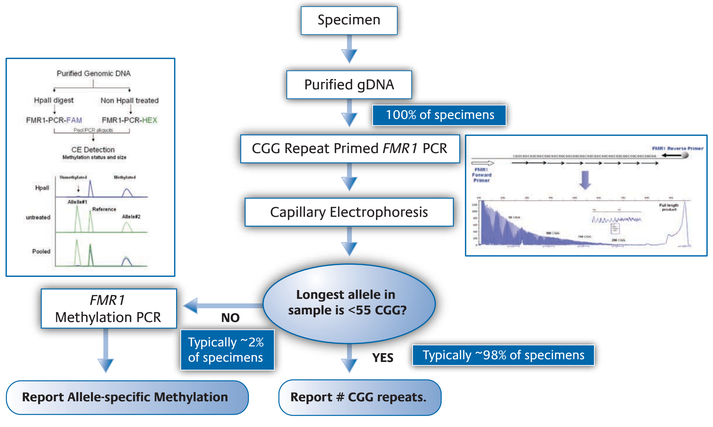


Fig. 1. Workflow for CGG Repeat Quantification and Allele-specific Methylation Assessment using Novel *FMR1* PCR Reagents. Purified DNA from blinded whole blood samples was input into CGG RP *FMR1* RUO* PCR to assess the repeat size. Those samples with >54 CGG repeats were then interrogated with methylation *FMR1* PCR (mPCR), and the results compared with Southern blot analysis performed at the M.I.N.D. Institute. Note that published statistics report ~2% of samples comprise pre- or full mutation alleles, although in this study a much higher proportion of expanded alleles was characterized.

*Research Use Only, Not Intended for Use in Diagnostic Procedures

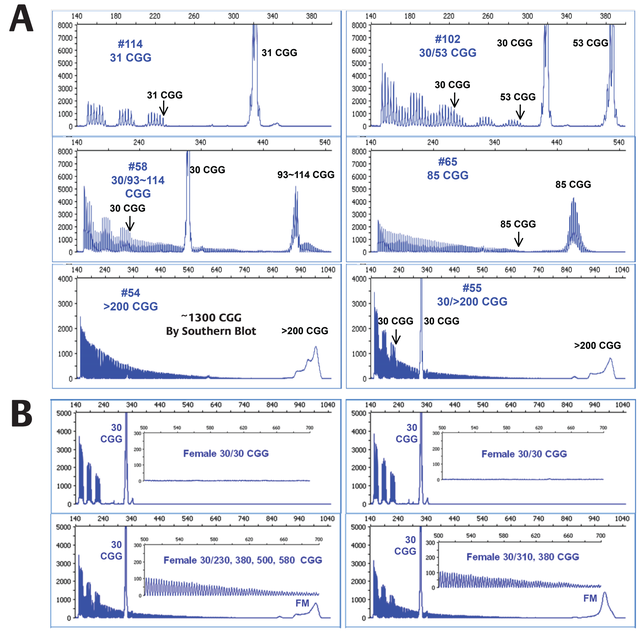
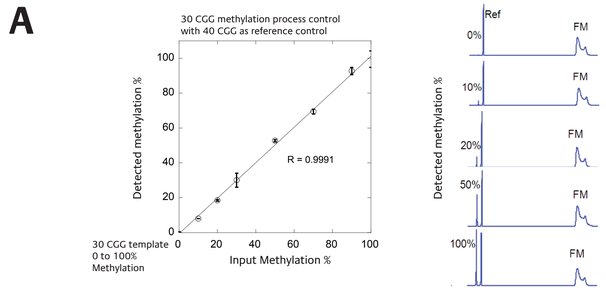


Fig. 2. CGG RP PCR Identifies All Categories of *FMR1* Alleles including Alleles up to at least 1300 CGG, and Reconciles Zygosity in Female Samples.
A) Representative electropherograms of normal, premutation, and full mutation (fm) alleles from clinical samples. **B)** The repeat primed data "signature" of heterozygous samples permitted unambiguous differentiation of heterozygous and homozygous samples.



ABI-3130xl CE

Sample	Allele	Average (%)	Stdev (%)	CV	95% CI
AS10017	30	74	5.3	7%	70.4 - 77.7
AS10017	32	25	2.0	8%	23.4 - 26.2
NA07862 (550)	~550 CGG	101	10.0	10%	94.5 - 108.4
NA09237 (940)	~940 CGG	97	15.7	16%	86.5 - 108.3

ABI-3500xl CE

Sample	Allele	Average (%)	Stdev (%)	CV	95% CI
AS10017	30	76	5.7	8%	71.8 - 79.7
AS10017	32	27	2.1	8%	25.9 - 28.8
NA07862	~550 CGG	101	8.0	8%	95.7 - 106.8
NA09237	~940 CGG	97	12.0	12%	88.6 - 105.3

Fig. 3. Methylation PCR Accurately and Reproducibly Measures the Proportion of Methylated DNA Templates. A) mPCR quantitatively recovers the known fraction of methylated DNA, as assessed from mixtures of methylated DNA standards titrated with unmethylated DNA. A background of fully methylated 645 CGG full mutation (FM), included in each titration standard, was also accurately interpreted as completely methylated in each case. **B)** The methylation fraction determined by mPCR was calculated with a CV<20% for 8 replicates of both a normal clinical sample, and two full mutation samples, across two different ABI CE platforms.

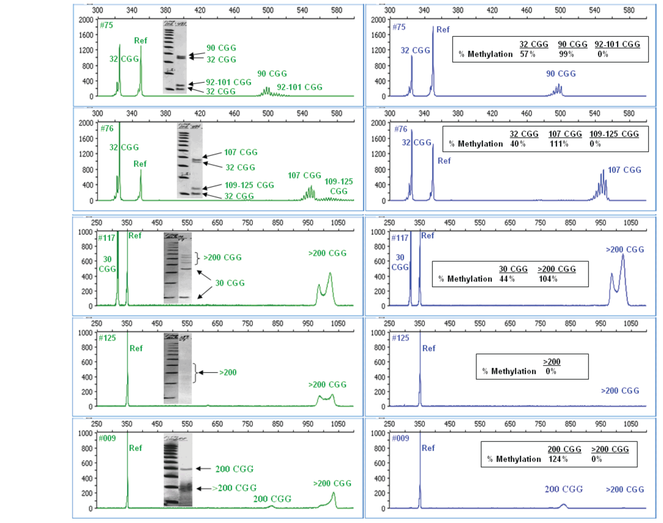


Fig. 4. Novel *FMR1* Methylation Patterns Masked by Low Resolution Southern Blot Analysis are Revealed by High Resolution Methylation PCR. Allele-specific methylation patterns determined from mPCR were qualitatively consistent with Southern blot results, but the higher resolution of the CE approach provided enhanced molecular detail. For example, 16 of 19 female carriers revealed a common pattern lost on Southern blots: An unmethylated premutation mosaic allele(s) in combination with a slightly smaller - but highly methylated - premutation allele(s). Three full mutation samples are also shown.

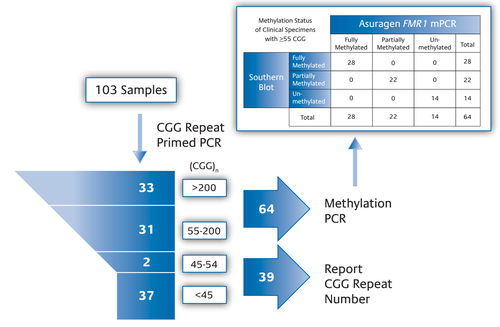


Fig. 5. A PCR-only Workflow for *FMR1* Analysis that Eliminates the Requirement for Southern Blot Analysis. The combination of CGG RP PCR and mPCR correctly categorized all samples, and enabled accurate methylation assessments from 33 full mutation and 31 premutation samples in concordance with Southern blot analysis.

CONCLUSIONS

- CGG Repeat Primed PCR correctly categorized all alleles from 103 blinded clinical samples, including expanded alleles with several hundred to at least 1300 CGG.
- mPCR accurately recovered the expected methylation fraction from standards comprised of 0-100% methylation.
- mPCR results were reproducible across two different ABI CE platforms.
- The two PCR technologies were robust, and no failed reactions across 103 clinical specimens were observed with either CGG RP PCR or mPCR.
- All mPCR results (including NOR, INT, PM and FM) were consistent with qualitative methylation assessments from Southern blotting.
- mPCR revealed novel allele-specific methylation patterns at single triplet repeat resolution that were masked on Southern blots.
- mPCR may be combined with Asuragen's existing RUO* *FMR1* to enable comprehensive *FMR1* analysis that does not require Southern blotting.