FMR1 methylation and sizing PCR assays improve sensitivity and efficiency in clinical testing for Fragile X syndrome, revealing some uncommon and unexpected results

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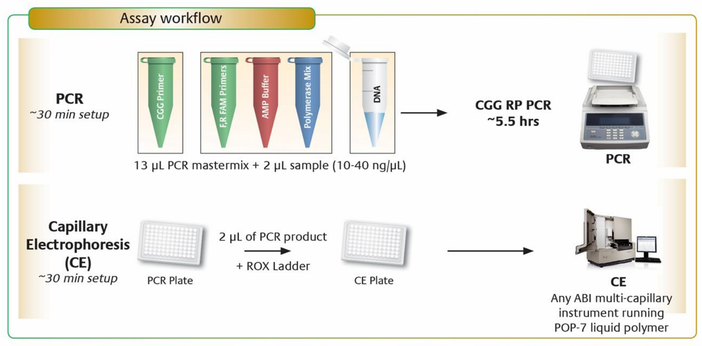
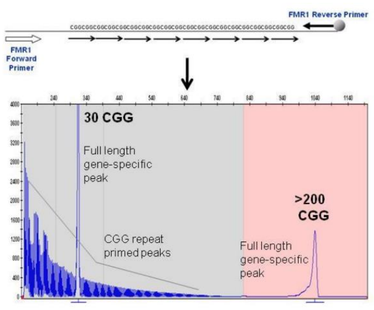
Abstract

Fragile X syndrome is the most recognized cause of autism worldwide, affecting 1 in 4000 males and 1 in 6000 females, and is caused by a CGG triplet repeat expansion in the *FMR1* gene. *FMR1*-related disorders also include fragile X-associated tremor ataxia syndrome and *FMR1*-related primary ovarian insufficiency. The disease mechanism involves aberrant methylation and expression of *FMR1*. A repeat size of 200 CGG repeats or greater is considered a full mutation, causing hyper-methylation of the *FMR1* promoter and transcriptional silencing. Premutations are between 55 and 200 CGG repeats and are generally not associated with abnormal methylation; they typically result in an unmethylated allele in males and partially methylated alleles in females due to X-inactivation, and are associated with increased expression. In our molecular diagnostic laboratory, we have implemented a more sensitive methylation PCR since July 2017, as well as a sizing PCR since November 2015, replacing the traditional Southern blotting and homebrew PCRs, respectively. By implementing these assays, we have been able to improve the efficiency of our workflow, as well as detect size and methylation mosaicism with a greater accuracy than was possible by traditional methods. We have also detected some uncommon and unexpected results, including males and females with size and methylation mosaicism as well as with fully-methylated premutations. Here we describe our experience to date, share some unusual findings, and reaffirm that methylation analysis be performed for any patient with a premutation-sized allele or greater.

AmplideX®Sizing RP FMR1 PCR Assay



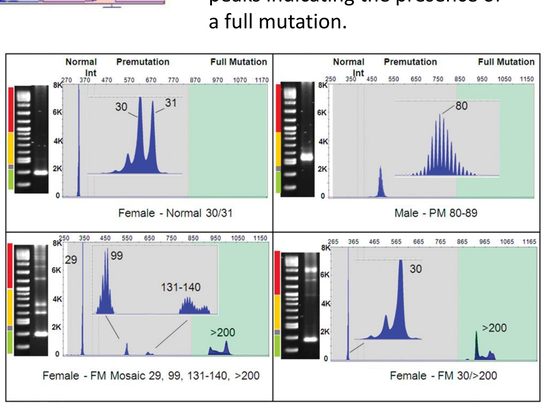
The AmplideX® PCR/CE FMR1 Reagents use a three-primer CGG Repeat Primed (RP) PCR from purified genomic DNA and fragment sizing by capillary electrophoresis. The PCR reagents include gene-specific and CGG primers, a polymerase mix buffer, a diluent, and a ROX 1000 size ladder for sizing by capillary electrophoresis. The size of the PCR products is converted to the number of CGG repeats using size and mobility conversion factors.



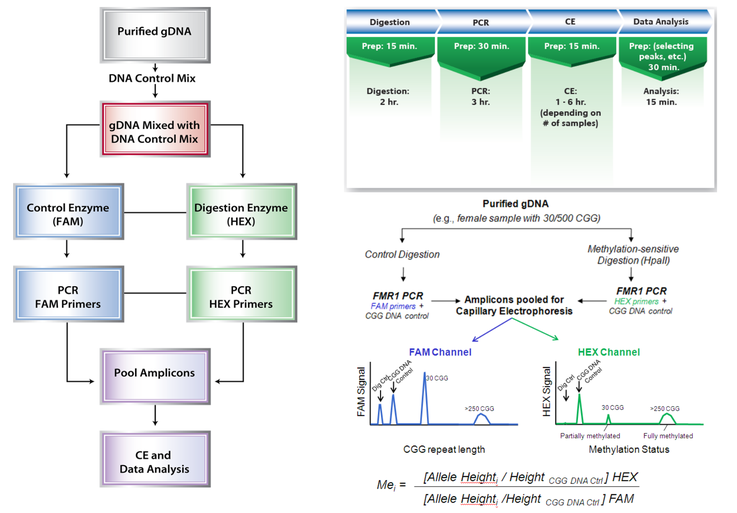
CGG repeat-primed PCR is shown, including the relative locations of the forward, reverse, and internal primers.

Example results are shown, indicating a normal allele with 30 CGG repeats, and a full mutation allele with greater than 200 CGG repeats. Note the "ladder pattern" of CGG repeat primed peaks indicating the presence of a full mutation.

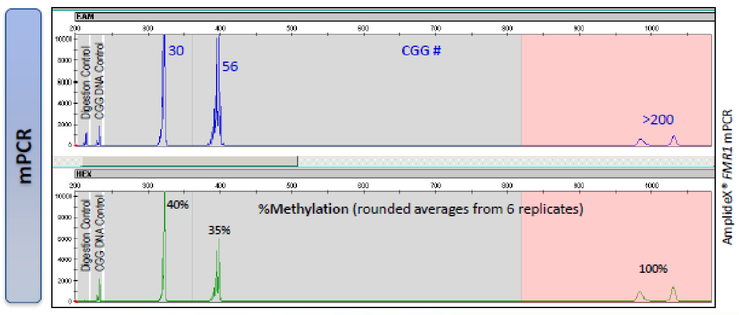
Shown are examples of different-sized repeats and the corresponding peak patterns. **(Upper left)** normal-sized alleles, **(upper right)** premutation allele, **(lower left)** mosaic normal allele, premutations, and full mutations, **(lower right)** mosaic normal and full mutation.



AmplideX® FMR1 mPCR Assay



The AmplideX® mPCR workflow is shown above. Control (FAM) and enzyme (HEX) digestions are set up in parallel, pooled, separated by capillary electrophoresis, and analyzed. The mPCR assay is designed to complement the AmplideX ® FMR1 sizing RP PCR.



Typical results for the mPCR assay are shown. In the example, allele sizes of 30, 56, and two expansions greater than 200 CGG repeats are present. The normal and intermediate-sized alleles are partially methylated, and the full-mutation alleles are fully methylated. Methylation levels greater than 80% are considered fully methylated.

Workflow Comparison

- We switched from using a combination of a homebrew sizing PCR plus a screening PCR (Abbott Molecular) to a single sizing PCR by Asuragen, thus improving the efficiency of our workflow.
- The Asuragen sizing PCR is highly sensitive and can detect alleles down to 1% allele fraction, more sensitive than previous assays.
- We had previously performed traditional Southern blotting for sizing expanded alleles, requiring ≥ one week from set up to result.
- Replacing Southern blotting with the Asuragen mPCR further streamlined our workflow, increased sensitivity, and reduced our bench time by at least one week for expanded samples.

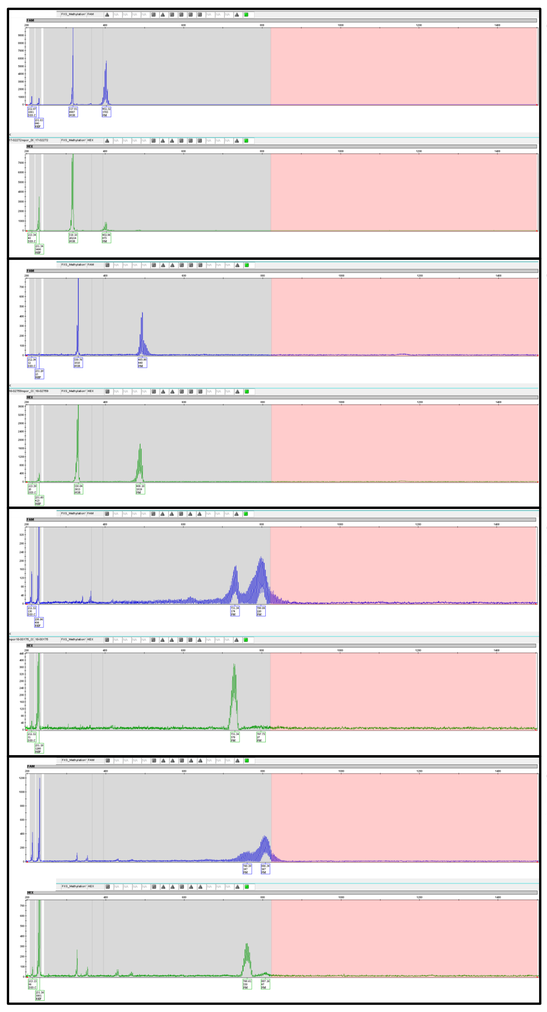
Results

- Since implementing both the Asuragen AmplideX® sizing PCR and the mPCR, we have seen 39 patients with a premutation sized allele or greater.
- Of these patients, 20/39 (51%) had premutations, 16/39 (49%) had full mutations, and 3/39 (8%) had size mosaicism for both premutations and full mutations.
- Of the patients with premutations, 11/20 (55%) had typical results (i.e., no mosaicism, expected methylation pattern), 6/20 (30%) had one premutation allele with an unexpected methylation pattern, and 3/20 (15%) had size mosaicism and methylation mosaicism for multiple premutation alleles.
- Of the patients with full mutations, 13/16 (81%) had typical results (i.e., no mosaicism, expected methylation pattern), and 3/16 (19%) had size mosaicism for multiple full mutation alleles (one of which had methylation mosaicism).

Premutations with Unexpected Methylation

Sample	Sex	Sizing Results
GGC1 ¥	F	58 repeats (unmethylated); 29 repeats (normal allele)
GGC2 ¥*	F	88 repeats (fully methylated); 33 repeats (normal allele)
GGC3	F	96 repeats (fully methylated); 20 repeats (normal allele)
GGC4	F	114 repeats (fully methylated); 23 repeats (normal allele)
GGC5	F	101 repeats (fully methylated); 42 repeats (normal allele)
GGC6	F	87 repeats (fully methylated); 25 repeats (normal allele)
GGC7 ǂ¥*	M	171 repeats (minor, fully methylated) and 193 repeats (major, unmethylated)
GGC8 ǂ¥	M	180 repeats (minor, partially methylated) and 196 repeats (major, unmethylated)
GGC9 ǂ	F	64 repeats (minor, fully methylated) and 72 (major, fully methylated); 29 repeats (normal allele)

ǂ Samples exhibit size mosaisicm for multiple premutation alleles of differing methylation status.
* Mother and son (mother has a fully methylated premutation and the son inherited two large premutations, one fully methylated and one unmethylated.
¥ Representative mPCR data shown below.



GGC1
Female
1 premutation:
Unmethylated (58)

GGC2
Female, mother of GGC7
1 premutation:
Fully methylated (88)

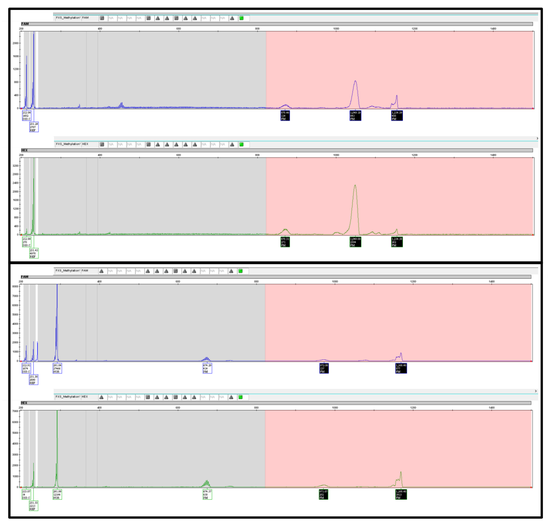
GGC7
Male, son of GGC2
2 premutations:
Fully methylated (171),
Unmethylated (193)

GGC8
Male
2 premutations:
Partially methylated (180),
Unmethylated (196)

Mosaic Premutations/Full Mutations

Sample	Sex	Sizing Results
GGC10 ǂ¥	M	Multiple full mutations >200 repeats (all fully methylated); 76 repeats (premutation, unmethylated); 30 repeats (normal allele)
GGC11 ǂ	M	Multiple full mutations >200 repeats (all fully methylated); 199 repeats (premutation, fully methylated)
GGC12 ǂ¥	F	Two full mutations >200 repeats (both fully methylated); 151 repeats (premutation, fully methylated); 30 repeats (normal allele)

ǂ Samples exhibit size mosaisicm for multiple full mutation alleles and a premutation allele.
¥ Representative mPCR data shown below.



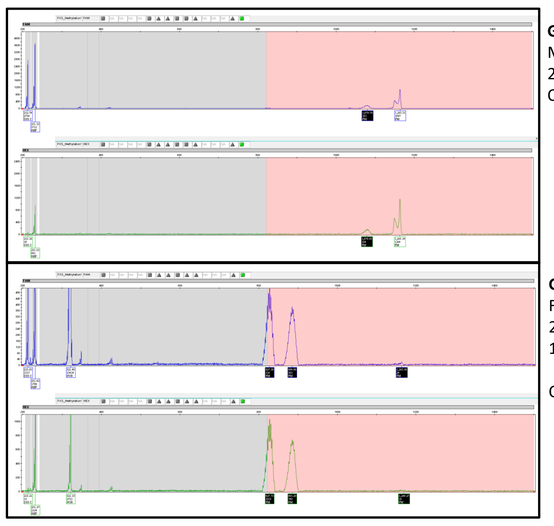
GGC10
Male
3+ methylated full mutations
1 premutation:
Unmethylated (76)

GGC12
Female
2+ methylated full mutations
1 premutation:
Fully methylated (151)

Mosaic Full Mutations

Sample	Sex	Sizing Results
GGC13 ǂ	M	Two full mutations >200 repeats (both fully methylated)
GGC14 ǂ¥	M	Two full mutations >200 repeats (both fully methylated)
GGC15 ǂ¥	F	Three full mutations >200 repeats (two fully methylated, one partially methylated); 30 repeats (normal allele)

ǂ Samples exhibit size mosaisicm for multiple full mutation alleles.
¥ Representative mPCR data shown below.



GGC14
Male
2 methylated full mutations
Others?

GGC15
Female
2 methylated full mutations
1 partially methylated full mutation
Others?

Summary and Conclusions

- The Asuragen AmplideX® assays have enabled us to streamline our workflow to one sizing PCR, and the mPCR eliminates the need for Southern blotting.
- This workflow improvement has decreased our assay time by about one week.
- Since the assays are highly sensitive, we have observed more instances of size and methylation mosaicism than previously, including males and females with multiple alleles, each with varying degrees of methylation.
- We have observed some unexpected results, such as unmethylated premutations, fully methylated premutations, and unmethylated full mutations.
- Therefore, we recommend determining methylation status of all premutation and full mutation alleles.

Acknowledgments

We sincerely thank the clinicians and counselors who have referred patients for clinical testing and the patients and their families for their participation. We also recognize the contributions of our laboratory staff and the clinical team at Greenwood Genetic Center.

Reference

Filipovic-Sadic 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.