Multi-site International Validation of a PCR-only Workflow for Fragile X Analysis

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SUMMARY

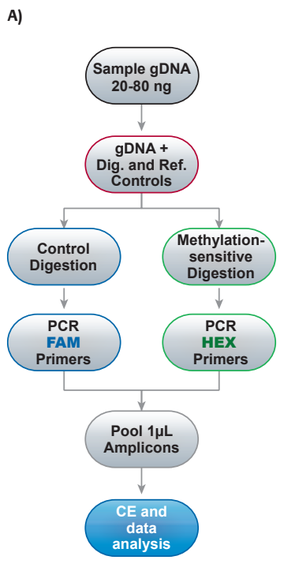
- Determination of methylation status in the *FMR1* gene has mainly relied on Southern blot (SB) analysis, which is laborious, low throughput, low resolution, and requires large quantities of genomic DNA.
- An alternative method, AmplideX® *FMR1* mPCR, was evaluated in 2 European labs using 76 residual clinical samples.
- AmplideX *FMR1* mPCR was concordant with SB and enabled a standardized PCR-only workflow for accurate and sensitive determination of CGG sizing and methylation status.

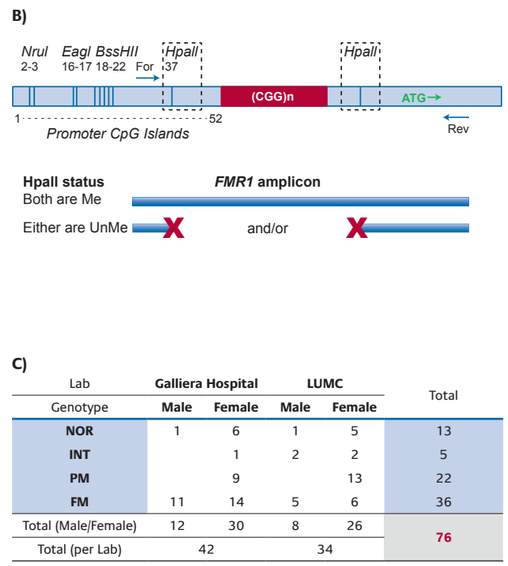
INTRODUCTION

The increasing association of a wide variety of developmental, mental and reproductive criteria for fragile X testing relies on the accurate assessment of methylation status of the fragile X mental retardation-1 (*FMR1*, NM_002024.4) gene. Excessive CGG repeat expansion is directly linked with hypermethylation and consequent silencing of the *FMR1* gene.[1] Methylation of full mutation expansions (>200 CGG), however, can be incomplete, and less severe phenotypes may be associated with methylation mosaicism.[2] Recent advances in targeted therapies for fragile X have shown a predictive response based on the degree of *FMR1* methylation[3] further driving the need for accurate and standardized testing methods. While Southern blot (SB) has been the gold standard for methylation analysis, the method is cumbersome, requires large quantities of DNA, has variable performance between laboratories and lacks sensitivity for low abundance mosaicism. Herein, we demonstrate a simplified PCR-only workflow for *FMR1* methylation analysis and compare results to SB across a range of challenging clinical samples obtained from two European laboratories.

MATERIALS AND METHODS

A set of 76 archived residual DNA samples were analyzed using AmplideX *FMR1* mPCR, a method for determining the CGG repeat length and methylation status using restriction digestion, dye-labeled PCR primers, and two-color capillary electrophoresis (Figure 1). The PCR included digestion and DNA reference controls. Differential PCR results were obtained comparing digested DNA (unmethylated) amplified using HEX-labeled primers and control digested DNA (total) amplified using FAM-labeled primers. The percent methylation was calculated as the ratio of HEX-to-FAM peak heights normalized to the signals of the reference control peaks. The results were correlated to SB analysis for detection of full mutation alleles and size and methylation mosaicism.





C)

Lab	Galliera Hospital		LUMC		Total
Genotype	Male	Female	Male	Female	
NOR	1	6	1	5	13
INT		1	2	2	5
PM		9		13	22
FM	11	14	5	6	36
Total (Male/Female)	12	30	8	26	76
Total (per Lab)	42		34		

Figure 1. Methylation PCR (mPCR) workflow, methodology and sample set description. A) DNA samples were mixed with procedural controls and treated with separate methylation-sensitive restriction and control reactions followed by PCR using different dye labels. Amplicons were pooled and sized using capillary electrophoresis. **B)** Restriction digestion Hpa II sites. Methylation at both sites was required for amplification. **C)** Archived clinical samples were obtained from and tested using mPCR in 2 different laboratories. These samples were obtained over a 10-20 year period and represented a range of normal and challenging *FMR1* genotypes.

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RESULTS

Sizing resolution and sensitivity

The analysis of a pooled process control comprised of alleles with 18, 30, 32, 56, 85, 116 and >200 CGG highlighted capabilites to quantify size and methylation mosaicism (Figure 2A). mPCR 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 sample (Figure 2B).

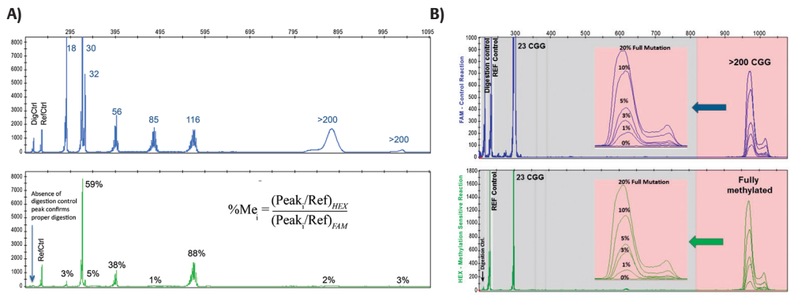

Figure 2. Examples of multi-allele controls for assessing mPCR CGG sizing and analytical sensitivity of a methylated full mutation. A) AmplideX *FMR1* PCR Control is a pooled gDNA control with peaks in normal (NOR, <45 CGG), premutation (PM, 55-200 CGG) and full mutation (FM, >200 CGG) ranges. **B)** Sensitivity by testing artificial mosaic mixture of 2 male cell line gDNA: prepared cell line mixtures contained various amounts of NA04025 (male full mutation, 645 CGG) mixed into a background of NA06895 (normal male, 23 CGG). Amplification and detection were demonstrated with as little as 800 pg of a full mutation allele in a total of 80 ng DNA mixture.

Example mPCR electropherograms for clinical samples

Results were matched to SB for all 76 clinical samples. Example comparisons between mPCR and SB demonstrate higher resolution in the PM range and higher sensitivity in the FM range over SB analysis (Figure 3). mPCR can help flag the identification of sex chromosome aneuploidies and enable the analysis of novel sample types (Figure 4).

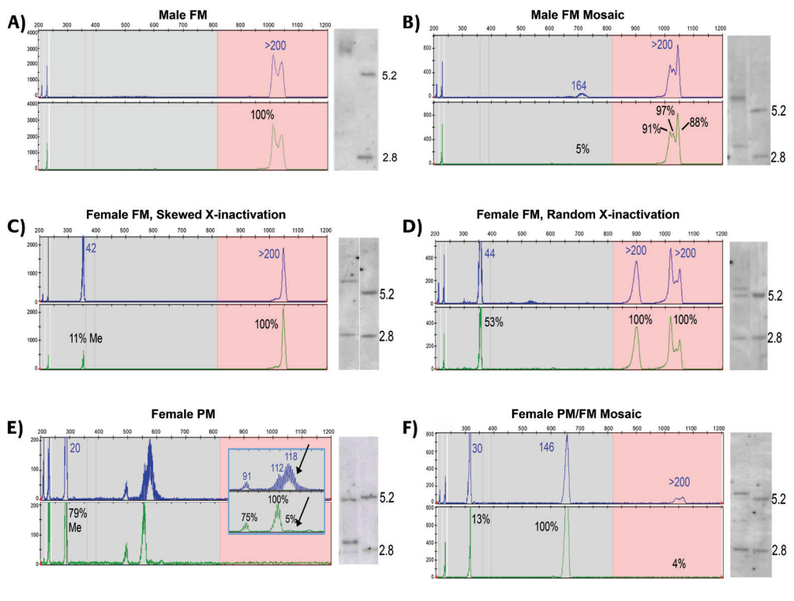

Figure 3. mPCR electropherograms and SB images for male full mutation, female full mutation, female premutation and mosaic samples. A) Male, fully methylated FM allele. **B)** Male with FM/PM mosaicism. **C)** Female, FM with skewed X inactivation. **D)** Female, FM with random X inactivation. **E)** Female, PM with specific pattern of size and methylation mosaicism obscured using SB. **F)** Female sample showing higher resolution and sensitivity of detection for a FM allele not detected using SB. SB images include a normal female sample to reference the 2.8 and 5.2 kb bands respectively.

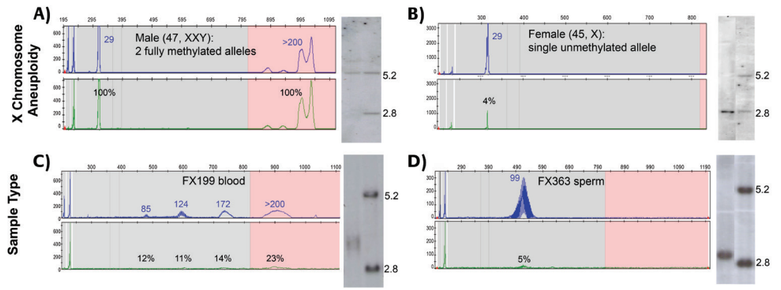

Figure 4. mPCR electropherograms and SB images for X chromosome aneuploidy and matched sources of DNA. A) Klinefelter's syndrome male (47, XXY) with skewed X inactivation: the FM allele is 100% methylated; the normal allele is also fully methylated (100% Me) because of X inactivation skewing, consistently with the presence of the 5.2 kb seen by SB. **B)** Turner syndrome female (45, X): the X monosomy is detected as a single unmethylated peak consistent with the only 2.8 kb band seen by SB. **C)** Matched blood and **D)** sperm sample for a male with full mutation allele in the blood but premutation in the sperm. SB images include a normal female sample to reference the 2.8 and 5.2 kb bands respectively.

Concordance between mPCR and SB

The following criteria were used to determine the concordance of mPCR and SB analysis:

1) All alleles detectable by SB analysis were compared with mPCR with the exception of select normal alleles that produced a saturating signal by mPCR and were not re-injected to bring this signal into the range of accurate quantification.
2) Samples were called concordant when agreement among the categorical methylation calls was achieved. Discordant calls were noted when there was a clear discrepancy between the independent interpretation of the SB compared to the quantitative mPCR methylation fraction.

Table 1: Comparison of methylation concordance by allele between SB analysis and mPCR (n=115)

Methylation Concordance by Allele		AmplideX® *FMR1* mPCR			
		Fully Methylated	Partially Methylated	Unmethylated	Total
Southern Blot	Fully Methylated	37	*1	0	**38**
	Partially Methylated	0	64	0	**64**
	Unmethylated	0	0	13	**13**
	Total	**37**	**65**	**13**	**115**

*In one sample (GH-FX630/10) mPCR yielded partial methylation on a full mutation allele indicated as fully methylated using SB analysis; however, the SB had low intensity bands not allowing partial methylation of this allele to be ruled out.

CONCLUSION

- mPCR enables superior size resolution and analytical sensitivity for size and methylation mosaicism compared to SB while providing 99% categorical concordance across 76 residual clinical samples.
- The 50- to 100-fold reduced DNA input required by mPCR compared to SB enables the analysis of alternative clinical specimens to whole blood with potential benefits for sample procurement, sample management, and/or genotype/phenotype associations.
- mPCR provides a standardized procedure for *FMR1* methylation analysis that can harmonize results across different laboratories.

Acknowledgements

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References

1. Hagerman RJ, Hagerman PJ: Testing for fragile X gene mutations throughout the life span. JAMA 2008, 300:2419e2421
2. Rousseau F, Heitz D, Tarleton J, MacPherson J, Malmgren H, Dahl N, et al: A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases. Am J Hum Genet 1994, :225e237
3. Jacquemont S, Curie A, des Portes V, Torrioli MG, Berry-Kravis E, Hagerman RJ, et al: Epigenetic modification of the FMR1 gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056. Sci Transl Med 2011, 3:64ra1