ELECTRONIC LETTER

Robust fragile X $(CGG)_n$ genotype classification using a methylation specific triple PCR assay

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Fragile X syndrome (MIM No 3009550) is the most common inherited mental retardation disorder, affecting approximately 1 in 4000 males and 1 in 8000 females.[1] Its name was derived from the observation of a fragile site on chromosome Xq27.3, designated FRAXA (fragile site, X chromosome, A site). This syndrome is caused by mutations in the *fragile X mental retardation-1* gene (*FMR1*), more than 95% of which involve hyperexpansion and hypermethylation of a polymorphic CGG trinucleotide repeat in the 5′ untranslated region (5′UTR) of the gene.[1–4]

Among normal individuals, the number of CGG repeats ranges between 6 and 55. In most affected patients, the CGG repeats are massively expanded to over 200 repeats, and the gene becomes methylated at CpG islands and is silenced. Individuals with CGG repeats in the premutation range of 55 to 200 repeats are clinically unaffected, but these repeats are likely to be unstable during transmission to the next generation.[1 2] This instability depends on the size of the premutation allele and is much more pronounced during maternal transmission. The larger the premutation alleles, the more likely they will be to expand to full mutations.[1 5 6]

Numerous diagnostic methods have been developed for fragile X syndrome, including cytogenetic, Southern blot, polymerase chain reaction (PCR), methylation specific PCR (ms-PCR), reverse transcription PCR (RT-PCR), and immunohistochemical analyses.[7–10] The most commonly used molecular methods for the diagnosis of fragile X syndrome are Southern blot and PCR/ms-PCR analyses. The major disadvantage of the Southern blot method is its difficulty in distinguishing between large normal and small premutation alleles, while current PCR/ms-PCR techniques have poor sensitivity for detecting large premutation and full mutation alleles, especially in females.[7–10] Although the combination of Southern and PCR methods enables reliable diagnosis of fragile X syndrome, the whole procedure is tedious and time consuming.

We have developed an alternative molecular diagnostic test for fragile X syndrome based on methylation specific PCR which reliably discriminates between normal, premutation, and full mutation affected males and females. After sodium bisulphite treatment, one specific PCR reaction detects all non-methylated allele sizes (normal and premutation), while two PCR reactions are used to classify the methylated allele(s) (normal, premutation, and full mutation). Using this triple ms-PCR strategy, we accurately classified the fragile X status in all 44 male and 45 female DNA samples that were tested.

Key points

- A rapid and reliable methylation specific polymerase chain reaction (PCR) system is described for the molecular diagnosis of fragile X syndrome in both males and females. This is achieved through a combination of specific amplification across the non-methylated and methylated CGG repeats of the *FMR1* gene, as well as amplification that is primed from within the methylated repeat itself.
- In addition to all normal and premutation males and females, all full mutation males and females were also accurately classified using this triple ms-PCR assay, regardless of the size of their expanded full mutation alleles. The assay produced concordant results on all 89 genomic DNA samples where the *FMR1* $(CGG)_n$ genotypes had previously been determined by Southern blot analysis or standard PCR methods.
- This method for fragile X syndrome testing provides a suitable alternative to Southern blot analysis which is less time consuming and more amenable to the clinical testing environment.

METHODS

DNA samples

Initial assay optimisation was undertaken on the following eight lymphoblastoid cell lines: GM07175 (normal female with 30 and 23 CGG repeat alleles), GM06907 (premutation female with 95–140 and 23 CGG repeat alleles), GM06896 (premutation female with 85 and 29 CGG repeat alleles), GM07537 (full mutation female with >200 and 29 CGG repeat alleles), GM06890 (normal male with 30 repeat allele), GM06891 (premutation male with 117 CGG repeat allele), GM06892 (premutation male with 80–85 CGG repeat allele), and GM06852 (full mutation male with >200 repeat allele), which were obtained from the Coriell mutant cell repository in Camden, New Jersey, USA. Assay validation was done on 81 previously genotyped archival patient DNAs and normal DNAs from the DNA diagnostic laboratories of Johns Hopkins Hospital, KK Women's and Children's Hospital, and National University Hospital (Singapore). The study protocol was approved by the institutional review board of the National University Hospital.

Sodium bisulphite treatment

Methylated and non-methylated *FMR1* alleles were differentially modified in the presence of sodium bisulphite according to Clark *et al* (1994),[11] but with modifications. Briefly, NaOH was added to 1 μg DNA to a final concentration of 0.3 M in a final volume of 5 μl. After a 15 minute incubation at 55℃, 75 μl of fresh and prewarmed sodium bisulphite solution was added. The latter was prepared by dissolving 0.6 g of sodium bisulphite in 45 μl of 10 M NaOH and 960 μl of dH_2O. The deamination mixture was incubated at 55℃ for five hours, then purified over a GFX™ column (Amersham Biosciences) and eluted in 50 μl Tris-EDTA, pH 8. The purified deaminated DNA was then desulphonated by the addition of 50 μl of

Abbreviations: FM, full mutation; NL, normal mutation; PM, premutation

Table 1 Primers used in specific amplification of sodium bisulphite treated non-methylated and methylated *FMR1* alleles

Primer	5′→3′ sequence	GenBank ID: nucleotides	Concentration	Amplicon size*
Non-methylated allele PCR (non-Met-PCR)				
Non-Met-F	AAACACTCAACTCCAT TTCAATTTCACTTCCA	L29074: 13708→13739	0.2 μM	168 bp+3n
Non-Met-R	GAGTTTGTTTTTGAGA GGTGGGTTGTGGGT	L29074: 13935→13906	0.2 μM	
Methylated allele PCR (Met-PCR)				
Met-F	CCGCCTCTAAACGAA CGACGAACCGACG	L29074: 13748→13775	0.2 μM	108 bp+3n
Met-R	GGTTGCGGGCGTTC GAGGTTTAGTCGTC	L29074: 13915→13888	0.2 μM	
Methylated allele triplet primed PCR (mTP-PCR)				
mTP-F	GCCGCTACCAAAAAA CGTACGACAACGCG	L29074: 13806→13834	0.2 μM	Not applicable
mTP-R	TACCGATACGCATCCC AGTTTGTCAGC$(TCG)_8$	Not applicable	0.02 μM	
Tail-R	TACCGATACGCAT CCCAGTTTGTCAGC	Not applicable	0.2 μM	

*n, number of trinucleotide repeats.
bp, base pair; PCR, polymerase chain reaction.

0.2 M NaOH and incubation at 37℃ for 15 minutes. After neutralisation with 50 μl of 0.2 M Tricine, the mixture was purified over a second GFX™ column, eluted in 50 μl Tris-EDTA, pH 8, and stored at −20℃ until use. The sodium bisulphite treatment results in methylated and non-methylated *FMR1* alleles with distinct differences in nucleotide sequence (fig 1A).

Methylation specific PCR

Three sets of primers were designed to amplify from the antisense strand of bisulphite modified DNA, one set targeting the non-methylated allele, and the other two sets targeting the methylated allele (table 1; fig 1B).

Specific amplification and sizing of the non-methylated *FMR1* repeat, designated as ''non-Met-PCR'', was accomplished with primers non-Met-F and non-Met-R. Amplification was carried out in a 50 μl volume containing 0.2 μM of each primer, 0.2 mM dNTPs, 2.5 U HotStarTaq™ DNA polymerase (Qiagen), 0.5×Q solution (Qiagen), 1×supplied PCR buffer (including 1.5 mM $MgCl_2$), and 7 μl (calculated at ~100 ng) of bisulphite modified DNA. An initial denaturation at 95℃ for 15 minutes was followed by 40 cycles of 98℃ for one minute, 62℃ for one minute, and 72℃ for two minutes, followed by a final extension at 72℃ for 10 minutes. This reaction is expected to detect all non-methylated normal and premutation *FMR1* repeats (fig 1C, upper panel).

Two different PCR reactions were carried out to detect the methylated allele. The first reaction, designated as ''Met-PCR'', used primers Met-F and Met-R to amplify across the methylated *FMR1* repeat. Reaction conditions were similar to that for the non-Met-PCR, except that 1.5×Q solution was used instead. This reaction is expected to determine all normal and premutation methylated allele sizes, as well as full mutation alleles up to~350 repeats (fig 1C, middle panel).

The second methylated allele PCR reaction, designated ''mTP-PCR'', is an adaptation of the triplet primed PCR (TP-PCR) strategy first described by Warner *et al*.[12] In mTP-PCR, primers mTP-F, mTP-R, and Tail-R were used to amplify from within the methylated repeat (table 1; fig 1B). Reaction conditions were similar to that for Met-PCR, except that each reaction contained 0.2 μM each of primers mTP-F and Tail-R, 0.02 μM of primer mTP-R, and 14 μl (~200 ng) of the bisulphite modified DNA. In the presence of either a methylated premutation or full mutation allele, an mTP-PCR product smear extending upwards beyond 300 base pairs (bp) is expected (fig 1C, lower panel). This product is referred to as a pre/full mutation (PFM) smear. In normal female samples, smearing below 300 bp (NL smear) is expected and represents product from the normal *FMR1* allele on an inactive X chromosome.

A 15 μl aliquot of each non-Met-PCR, Met-PCR, and mTP-PCR product was analysed by electrophoresis through a 1.5% agarose gel at 6 V/cm, stained with ethidium bromide for 30 minutes, and photographed over an ultraviolet transilluminator. The triple PCR assay will generate distinct banding and smear patterns depending on *FMR1* $(CGG)_n$ genotype, and anticipated results for various genotypes are illustrated schematically in fig 1C. Throughout the results and discussion sections of this paper, NL, PM, and FM are used to indicate normal, premutation, and full mutation, respectively.

RESULTS

Initial assay optimisation studies were undertaken using DNA obtained from four male and four female cell lines from the Coriell cell repository. For each cell line, the products of the non-Met-PCR, Met-PCR, and mTP-PCR were easily visualised by ethidium bromide staining (fig 2A), and the *FMR1* $(CGG)_n$ genotype classifications obtained were consistent with their known fragile X status (table 2). For example, in NL male 1 (cell line GM06890), the non-Met-PCR reaction produced the expected single non-methylated NL allele, while the Met-PCR and mTP-PCR reactions were negative (fig 2A). The assay easily detected the non-methylated PM alleles from both PM male 1 (GM06891) and male 2 (GM06892), as well as the methylated FM allele from the FM male 1 (GM06852). Using mTP-PCR analysis, a pre/full mutation (PFM) smear was observed only from FM male 1 (GM06852) among the male cell lines. A PFM smear is defined as a smear of DNA amplification product that extends upwards beyond 300 bp and is characteristic of all full mutation alleles and most methylated premutation alleles.

The assay was also able to classify accurately the *FMR1* $(CGG)_n$ genotypes of the female cell lines. For example, NL female 1 (GM07175) showed an NL non-methylated allele

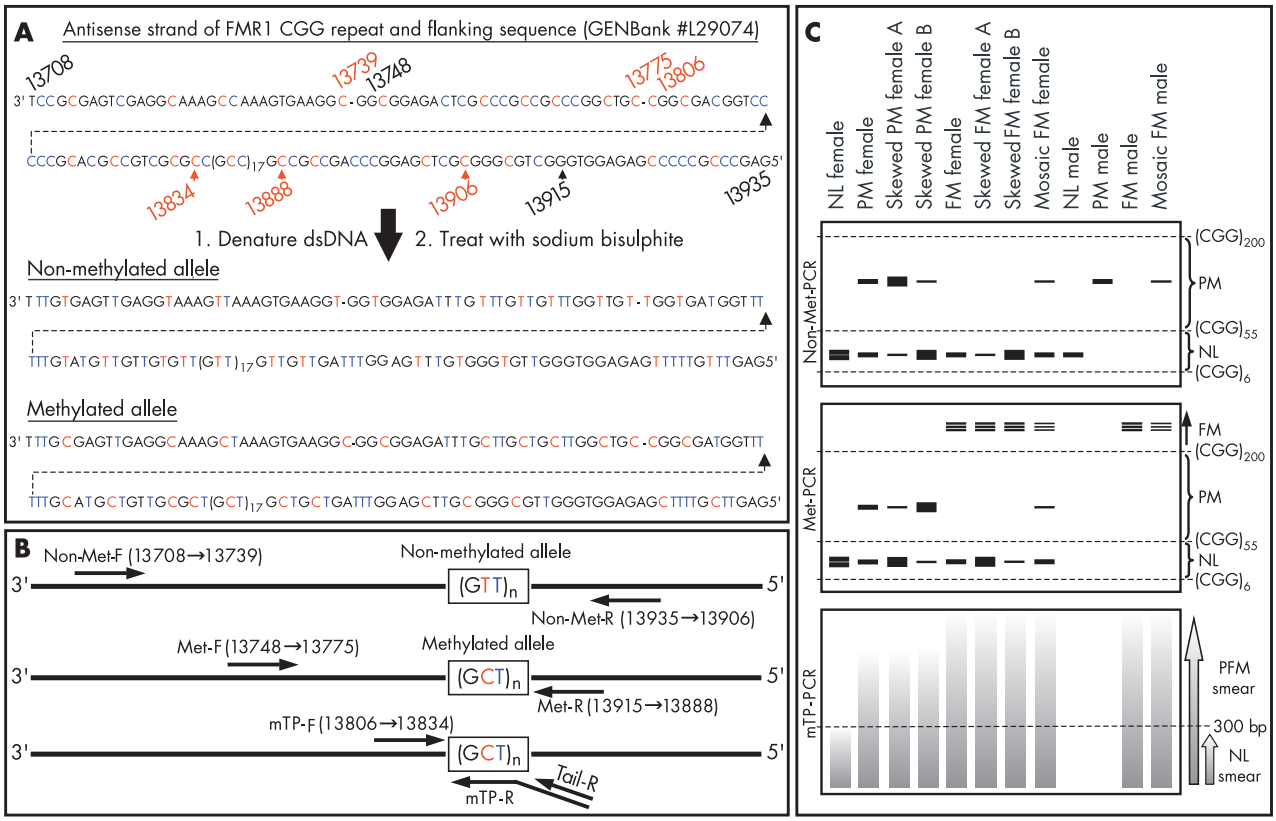

Figure 1 *FMR1* $(CGG)_n$ methylation specific triple polymerase chain reaction (PCR) strategy. (A) Sodium bisulphite modification of non-methylated and methylated *FMR1* alleles. On non-methylated alleles, all dC residues of the CGG repeat and flanking sequences (red and blue) are converted to dU in the presence of sodium bisulphite, followed by replacement with dTs upon subsequent allele specific PCR. On methylated alleles, however, all dC residues of CpG dinucleotides (red coloured dCs) are methylated and remain unconverted. (B) Allele specific PCR amplification after sodium bisulphite modification. Primers were designed to amplify specifically from the bisulphite modified antisense strand of either the non-methylated or the methylated allele. Primers non-Met-F and non-Met-R will amplify across all non-methylated NL and PM alleles, while primers Met-F and Met-R will amplify across all methylated NL and PM alleles, as well as FM alleles of up to ~350 repeats. FM alleles that are too large to be amplified using met-F/R are detected by methylated allele triplet primed PCR (mTP-PCR) using primers mTP-F, mTP-R, and Tail-R. Five digit numbers in panels A and B indicate positions of nucleotides in GenBank entry No 29074. (C) Schematic illustration of ethidium bromide staining patterns after agarose gel electrophoresis of non-Met-PCR (top panel), Met-PCR (middle panel), and mTP-PCR products (lower panel) from males and females of various *FMR1* $(CGG)_n$ genotype classes. Skewed PM or FM female A, skewed X inactivation where an excess of cells have the NL allele on the inactive X chromosome; skewed PM or FM female B, skewed X inactivation where an excess of cells have the NL allele on the active X chromosome.

and an NL methylated allele after non-Met-PCR and Met-PCR analyses, respectively, and a short smear not exceeding 300 bp (NL smear) was observed by mTP-PCR analysis. The NL and PM alleles in PM female 1 (GM06907) and female 2 (GM06896) were also easily detected using this triple PCR assay, as were the NL and FM alleles in FM female 1 (GM07537). Interestingly, the results also revealed evidence of non-random or skewed X inactivation in these cell lines, with the majority of cells carrying the NL allele on the active X and the PM and FM alleles on the inactive X. We confirmed the skewed X inactivation in these samples by Southern blot analysis (data not shown), and have quantified the degree of skewing in all four female cell lines using the HUMARA assay[13 14] (table 2). As expected for the PM female and FM female cell lines, mTP-PCR analysis detected PFM smears in all three samples.

For each of the eight cell lines, the CGG repeats of the non-methylated and methylated alleles were calculated from their respective non-Met-PCR and Met-PCR fragments according to the formula in table 1. The calculated CGG repeats were concordant between the two PCRs and were in good agreement with the approximate CGG repeat sizes inferred from the Southern blot results (table 2). PCR products of FM alleles, however, could not be accurately sized beyond 1 kb on this gel system. Such alleles were labelled as having >295 CGG repeats, which is the literal conversion from a 1 kb methylated fragment rounded off to the nearest five CGG repeats.

We further evaluated the assay on peripheral blood leucocyte DNAs of known *FMR1* $(CGG)_n$ genotype classification. These samples were selected to represent the widest spectrum of NL, PM, and FM *FMR1* genotypes that we have previously analysed. The triple PCR assay of non-Met-PCR, Met-PCR, and mTP-PCR reactions was able to classify the *FMR1* $(CGG)_n$ genotype of each male and female sample accurately (table 3). All non-methylated and methylated NL and PM alleles in males and females could be clearly detected (fig 2B). All samples carrying methylated PM or FM alleles generated a PFM smear by mTP-PCR, while NL males and PM males did not produce a smear, and NL females displayed only an NL smear.

This evaluation allowed us to delineate the upper limit of detection of FM alleles by Met-PCR analysis at ~350 repeats. Taking FM female 4 (FX0025) and FM male 3 (FX0012) as examples, the Met-PCR reaction failed to generate an amplification product from either sample (fig 2B, middle panel), both of whose FM alleles exceed 500 repeats as determined by Southern analysis (table 3). The mTP-PCR reaction, however, produced a PFM smear in both samples, thus ensuring that the FM alleles in both samples were not

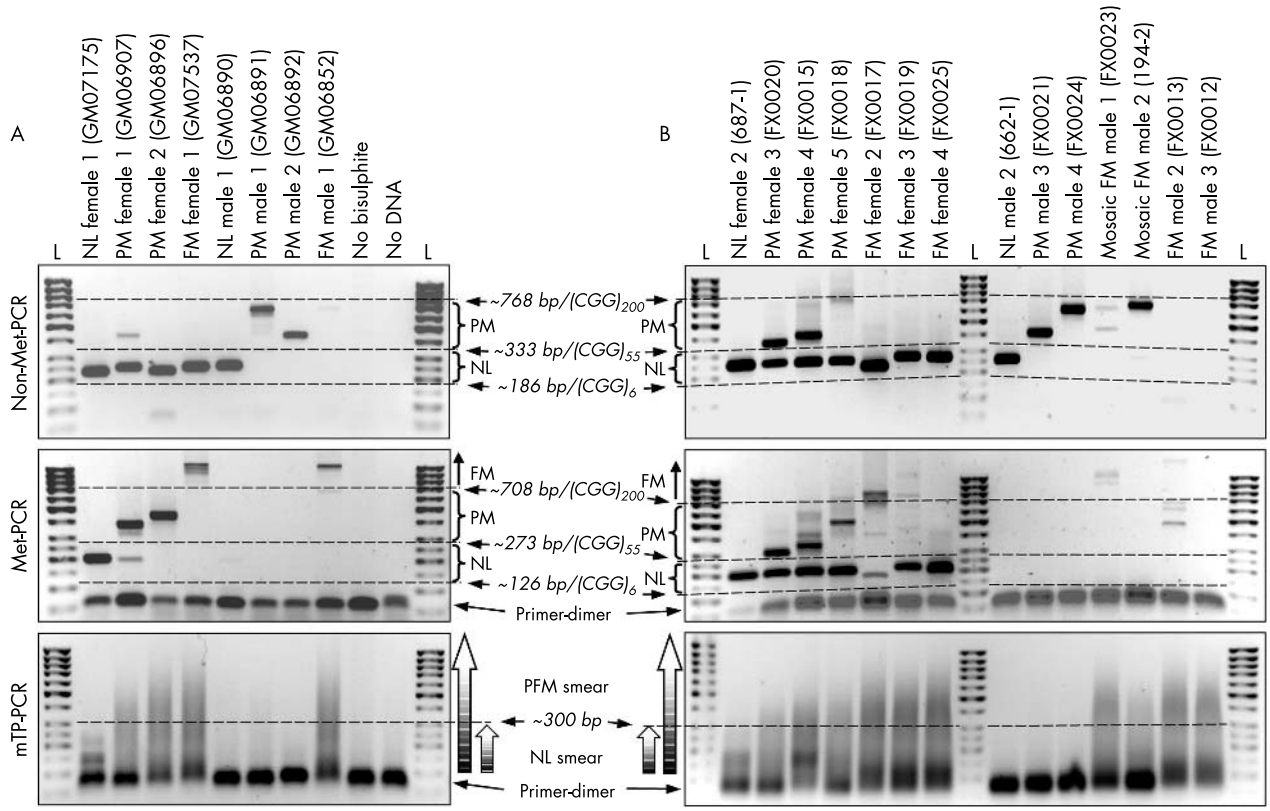

Figure 2 Agarose gel results of methylation specific triple polymerase chain reaction (PCR) of genomic DNA samples. (A) Optimisation on genomic DNA from female and male lymphoblastoid cell lines carrying NL, PM, and/or FM *FMR1* alleles. (B) Validation on leucocyte extracted female and male genomic DNA of known *FMR1* genotype. Top panels, PCR across non-methylated CGG repeat (non-Met-PCR). Middle panels, PCR across methylated CGG repeat (Met-PCR). Bottom panels, triplet primed PCR of methylated allele (mTP-PCR). The ''no bisulphite'' result was derived from a female DNA sample that was not pretreated with sodium bisulphite. L, GeneRuler™ 50 bp DNA ladder (Fermentas).

missed. Although a PFM smear is also generated in the presence of a methylated PM allele, Met-PCR analysis did not detect any methylated PM allele in FM female 4 and FM male 3. Based on our observation that the non-Met-PCR and Met-PCR reactions can reliably detect even the largest PM alleles among our pre-characterised samples, the negative Met-PCR results therefore indicate that the PFM smears of FM female 4 and FM male 3 must have been generated from FM alleles. The combined PCR results thus correctly classified both samples as carrying FM alleles.

To evaluate the specificity of the mTP-PCR reaction in producing a PFM smear only from methylated PM and FM alleles, we carried out mTP-PCR on genomic DNAs from additional NL, PM, and FM males and females. All ''normal'' samples were first confirmed to contain only an NL *FMR1* $(CGG)_n$ allele (for the males), or two NL alleles (for the females) by direct amplification of the CGG repeat[15] and fluorescence detection on an ABI 3100 genetic analyser. The mTP-PCR reaction did not produce any DNA smear in all NL male samples tested (fig 3A), whereas only the short NL

Table 2 Assay optimisation on female and male lymphoblastoid cell lines carrying NL, PM, and/or FM *FMR1 (CGG)n* alleles

Coriell ID/sex	Southern result (kb/CGG rpt*)	Southern classification	Non-Met-PCR result (bp/CGG rpt)†	Met-PCR result (bp/CGG rpt)†	mTP-PCR result	Triple ms-PCR classification	HUMARA assay (% skewing)
GM07175/F	2.9 and 5.2 kb/30 rpt	NL female	260 bp/30 rpt	200 bp/30 rpt	NL smear	NL female	⩾85%
GM06907/F	2.9 and 5.4 kb/30 and 100 rpt	PM female (skewed)‡	260 & 450 bp/30 & 95 rpt	200 & 390 bp/30 & 95 rpt	PFM smear	PM female (skewed)‡	⩾95%
GM06896/F	2.9 and 5.4 kb/30 and 100 rpt	PM female (skewed)‡	260 bp/30 rpt	470 bp/120 rpt	PFM smear	PM female (skewed)‡	⩾99%
GM07537/F	2.9 and 6.3 kb/30 and 400 rpt	FM female (skewed)‡	270 bp/35 rpt	>1000 bp/>295 rpt	PFM smear	FM female (skewed)‡	⩾99%
GM06890/M	2.9 kb/30 rpt	NL male	270 bp/35 rpt	No PCR pdt	No smear	NL male	NA
GM06891/M	3.3 kb/150 rpt	PM male	640 bp/155 rpt	No PCR pdt	No smear	PM male	NA
GM06892/M	3.1 kb/100 rpt	PM male	410 bp/80 rpt	No PCR pdt	No smear	PM male	NA
GM06852/M	6.3 kb/400 rpt	FM male	640 bp/155 rpt	>1000 bp/>295 rpt	PFM smear	Mosaic FM male	NA

*Repeat sizes are based on *EcoRI/NruI* double digests hybridised with probe StB12.3, where a 2.9 kb non-methylated or a 5.2 kb methylated fragment indicates a 30 repeat normal allele. Larger fragments are rounded off to the nearest 50 repeats.
†Rounded off to the nearest 10 bp and five CGG repeats.
‡Majority of cells carry the NL *FMR1* allele on the active X and the PM or FM allele on the inactive X.
F, female; M, male; NA, not applicable; pdt, product; rpt, repeats.

Table 3 Assay validation on peripheral blood DNAs of NL, PM, and/or FM females and males

ID/sex	Southern (kb) or PCR (No of repeats*) result	Southern/PCR classification	Non-Met-PCR result (bp/repeats)†	Met-PCR result (bp/repeats)†	mTP-PCR result	Triple ms-PCR classification
687-1/F	30, 31 rpt*	NL female	260 bp/30 rpt	200bp/30 rpt	NL smear	NL female
FX0020/F	2.8, 3.0, 5.2, 5.4 kb	PM female	270 and 360 bp/35 and 65 rpt	200 and 300 bp/30 and 65 rpt	PFM smear	PM female
FX0015/F	2.8, 3.0, 5.2, 5.4 kb	PM female	270 and 410 bp/35 and 80 rpt	200 and 350 bp/30 and 80 rpt	PFM smear	PM female
FX0018/F	2.8, 3.4, 5.2, 5.7 kb	PM female	270 and 700 bp/35 and175 rpt	200 and 550 bp/30 and 145 rpt	PFM smear	PM female
FX0017/F	2.8, 5.2, 6.4 kb	FM female	250 bp/25 rpt	190, 400 and 850 bp/25, 95 and 245 rpt	PFM smear	FM female
FX0019/F	2.8, 5.2, 6.7–8.2 kb	FM female	300 bp/45 rpt	240, 800 and>1000 bp/45, 230 and>295 rpt	PFM smear	FM female
FX0025/F	2.8, 5.2, 7.5–8.0 kb	FM female	300 bp/45 rpt	240 bp/45 rpt	PFM smear	FM female
662–1/M	31 rpt*	NL male	270 bp/35 rpt	no PCR product	no smear	NL male
FX0021/M	3.1 kb	PM male	450 bp/95 rpt	no PCR product	no smear	PM male
FX0024/M	3.3 kb	PM male	650 bp/160 rpt	no PCR product	no smear	PM male
FX0023/M	6.2 kb	FM male	400 and 650 bp/75 and 160 rpt	>1000 bp/>295 rpt	PFM smear	mos FM male
194–2/M	3.3, 6.3, 6.75, 6.3–8.4 kb	mos FM male	700 bp/175 rpt	no PCR product	PFM smear	mos FM male
FX0013/M	6.6–12.0 kb	FM male	no PCR product	500, 600, 700 and >1000 bp/130, 165, 195 and >295 rpt	PFM smear	FM male
FX0012/M	8.8–9.1 kb	FM male	no PCR product	no PCR product	PFM smear	FM male

Southern blot fragments are based on *EcoRI*/*EagI* double digests hybridised with probe StB12.3, where a 30 repeat normal allele is detected as a 2.8 kb non-methylated or a 5.2 kb methylated fragment.
*Number of CGG repeats determined by PCR amplification of unmodified DNA followed by Genescan™ analysis on an ABI 310 (Applied Biosystems, Forster City).
†Rounded off to the nearest 10 bp and 5 CGG repeats.
F, female; M, male; PCR, polymerase chain reaction.

smear was evident in all NL female samples (fig 3B). In contrast, the PFM smears generated from all FM males (fig 3C) and all PM and FM females (fig 3D) were clearly more extensive. Although it is conceivable that the NL smear from a large NL allele (for example, 50 CGGs) would be quite similar to a PFM smear from a small PM allele (say 60 CGGs), the non-Met-PCR and/or Met-PCR results will distinguish between most NL and PM alleles by size, confirmed by sequencing or pedigree analysis if necessary. For those samples where the allele sizes fall within the 50–60 CGGs range, further pedigree or sequencing analysis of the CGG repeat structure is necessary for proper mutation classification.

An algorithm for the classification of the various female and male *FMR1* $(CGG)_n$ genotypes is shown in fig 4, and is intended to simplify interpretation of the agarose gel results obtained from this methylation specific triple PCR assay.

DISCUSSION

More than a decade after the identification of a trinucleotide repeat expansion in the *FMR1* gene as the predominant cause of fragile X mental retardation syndrome, molecular diagnosis of this disorder continues to rely mainly on a combination of PCR and Southern blot analyses. Southern blot analysis is very robust in detecting FM expansions in both males and females. However, it is highly labour intensive and time consuming. Additionally, Southern blots are not ideal for detecting PM alleles at the low end of the size range. PCR amplification across the CGG repeat enables precise sizing, but only of NL and small PM alleles. Larger PM and FM alleles cannot be detected reliably using conventional PCR. Furthermore, PCR does not provide information on the methylation status of the alleles.

More recently, several assays based on ms-PCR have been developed in attempts to address the deficiencies of PCR based methods.[9 10 16] Ms-PCR methods rely on the differential modification of methylated and non-methylated genomic DNA by sodium bisulphite, allowing subsequent specific amplification and detection of methylated and non-methylated alleles.[11] This procedure converts the non-methylated *FMR1* trinucleotide repeat from 100% G:C base pairing (5′-CCG-3′ on the antisense strand) to one with 33% G:C base pairing (5′-TTG-3′ repeat), while the methylated repeat now has ~67% G:C base pairing (5′-TCG-3′ repeat) (fig 1). Thus this conversion not only allows non-methylated alleles to be discriminated from methylated alleles, but also reduces the difficulty of amplifying across the repeats. Even so, larger PM and FM alleles remain refractory to amplification and detection, and are especially difficult to detect in females owing to preferential amplification of their NL alleles.

Weinhausel and Haas (2001) developed a dual PCR methylation specific PCR assay for detection of fragile X syndrome in both males and females.[10] *FMR1* genotypes were identified on the basis of several parameters, including a duplex-PCR of the *FMR1* and *XIST* gene promoters coupled with a duplex PCR across the non-methylated and methylated CGG repeats, followed by densitometric ratio analysis of *FMR1* promoter methylation status normalised against that of the *XIST* promoter. These investigators reported that virtually all categories of *FMR1* genotypes could be detected, although noting that very large PM alleles in female carriers could be missed, thus resulting in occasional difficulties in distinguishing a PM female from an FM female.

Our assay differs from the method of Weinhausel and Haas[10] in two main respects. First, our assay involves three single PCR amplifications instead of two duplex PCR reactions. We chose not to duplex the non-Met-PCR with the Met-PCR after initial trials indicated a reduced detection sensitivity, especially of the large PM and the FM alleles (data not shown). Separate agarose gel analysis of the non-Met-PCR and Met-PCR reactions allows easy identification of non-methylated and methylated alleles and approximate sizing of NL, PM, and FM bands. With a duplex PCR format, agarose gel analysis of the combined non-methylated and methylated PCR products is less straightforward, especially for certain NL and PM female genotypes, owing to the

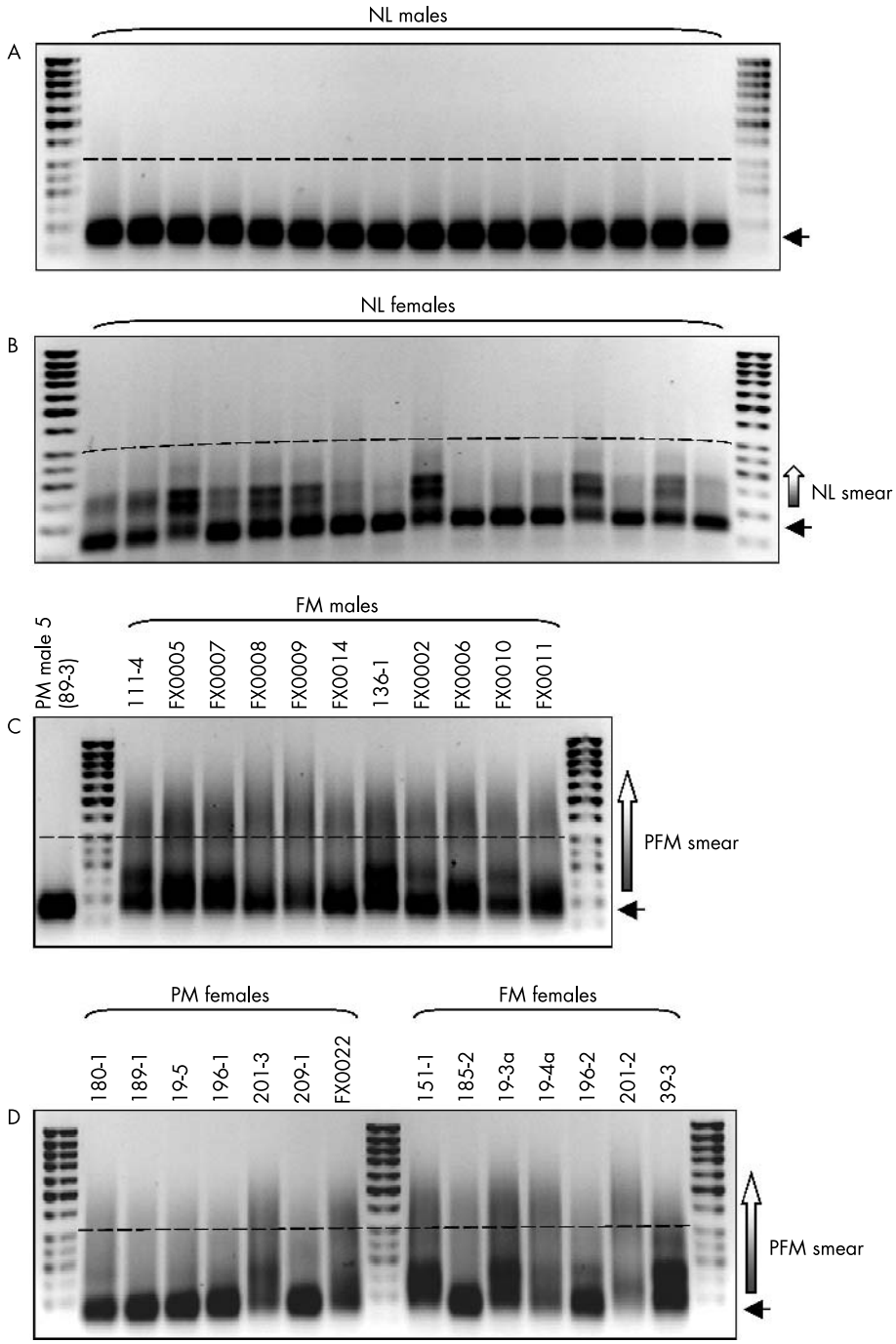

Figure 3 Specificity of a methylated allele, triplet primed, polymerase chain reaction (mTP-PCR) assay for methylated PM and FM *FMR1* alleles. Additional male and female genomic DNAs of known *FMR1* $(CGG)_n$ genotype were analysed by mTP-PCR. (A) Absence of a product smear from NL males. (B) Presence of a short NL smear from NL females. (C) Absence of a product smear from PM male and presence of a PFM smear from FM males. (D) Presence of a PFM smear from both PM females and FM females. Dashed horizontal lines delineate ~300 bp mark, and horizontal arrows indicate positions of primer-dimers. bp, base pairs.

possibility of overlapping non-methylated and methylated fragment sizes or co-migrating non-methylated and methylated fragments of the same size.

Second, our assay does not rely on densitometry and ratio analysis of non-methylated versus methylated promoter PCR fragments to detect FM alleles. We instead used a protocol modified from the triplet primed PCR (TP-PCR) method of Warner *et al*,[12] in order to detect affected males and females with very large FM alleles that could not be amplified successfully using Met-PCR. Our mTP-PCR reaction reliably and specifically detects all PM and FM alleles. Although the mTP-PCR PFM smear generated from a PM female is indistinguishable from that of an FM female, the two genotype classes can be distinguished on the basis of their non-Met-PCR and Met-PCR results. A PM female will display a band in the PM size range in both the non-Met-PCR and Met-PCR gels, whereas an FM female with a large expansion of >350 CGG repeats will not display a band in either the PM or the FM size ranges using either non-Met-PCR or Met-PCR; and an FM female with a smaller expansion of ⩽350 will

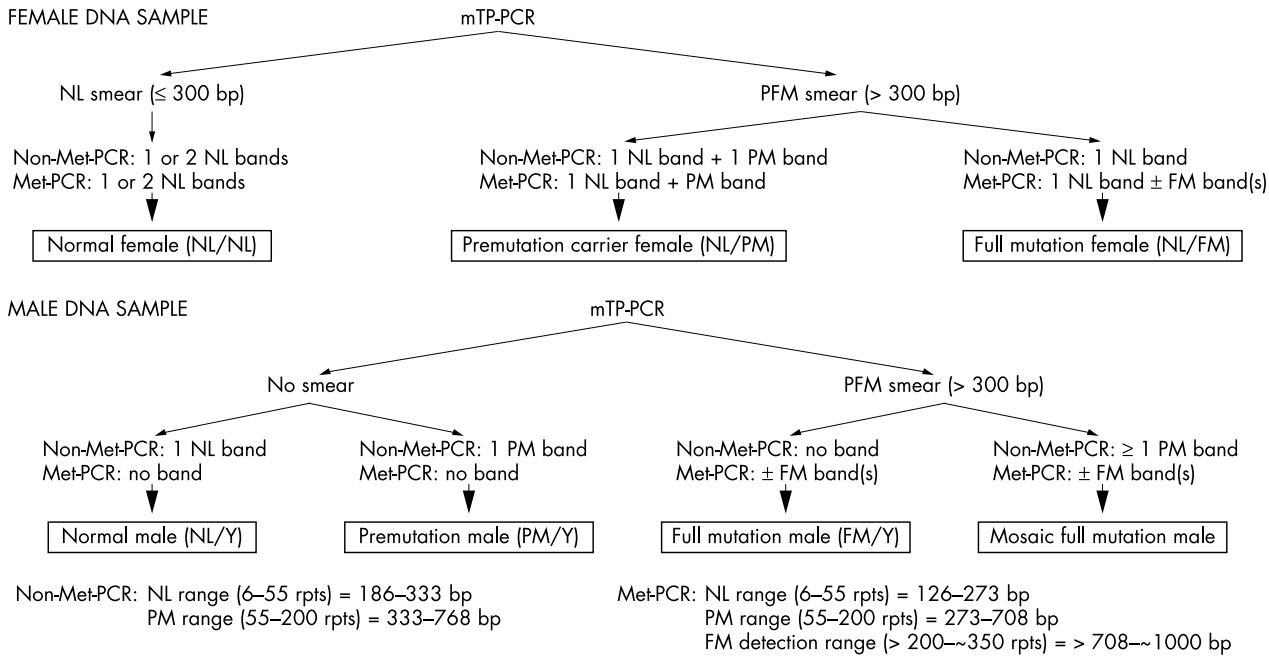

Figure 4 Algorithm for interpretation of triple methylation specific polymerase chain reaction assay results. Met, methylated; mTP-PCR, methylated allele, triplet primed, polymerase chain reaction; non-Met, non-methylated; PFM, pre/full mutation.

display an FM size band in the Met-PCR gel only. The presence of a methylated smear extending beyond 300 bp is thus diagnostic of all FM males, PM females, and FM females. The PFM smear is not observed in NL males or PM males, while NL females have their own characteristic ethidium bromide staining patterns (fig 3).

In the majority of samples tested, the sizes of the non-methylated and methylated alleles, determined from the non-Met-PCR and Met-PCR analyses, respectively, were concordant and were in good agreement with the approximate CGG repeat sizes inferred from the previous Southern blot or direct PCR results (tables 2 and 3). In PM female 5 (FX0018), however, the non-methylated and methylated PM amplicons migrated as apparently different sizes (table 3). We postulate that the observed CGG repeat discrepancy between the non-Met-PCR and Met-PCR products in PM female 5 most probably reflects the combined effect of her very large PM allele (>140 CGGs) coupled with the difference in nucleotide sequence composition between the modified non-methylated and methylated alleles. These two factors result either in anomalous retarded migration of the bisulphite modified non-methylated PM amplicon compared with its methylated counterpart, or conversely in anomalous accelerated migration of the bisulphite modified methylated PM amplicon compared with its non-methylated counterpart. Whether the methylated or non-methylated PM amplicon represents the true CGG repeat length has not been determined, owing to the difficulty in sequencing across such long CGG repeat stretches. The discordant sizes of these samples should not, however, lead to misdiagnosis or misclassification of their *FMR1* genotypes.

It is possible that this assay may misclassify a female mosaic for PM and FM alleles as a constitutive PM female when only the PM allele but not the FM allele is detected by Met-PCR. Such situations may, however, be rare as many mosaic females carry multiple FM alleles including some of less that 350 CGG repeats. If this is the case, the Met-PCR reaction may be able to amplify and detect the smaller FM alleles.

It should be noted that even the gold standard Southern blot analysis is not completely error-proof. For example, some FM affected females harbour highly somatically unstable FM alleles, which sometimes makes them difficult to detect. Detection of such alleles by Southern analysis is obvious only under optimal probe labelling, hybridisation, and wash conditions that maximise signal and minimise background noise. Thus the triple ms-PCR method for fragile X testing demonstrated here provides a suitable alternative to Southern blot analysis which is less time consuming and more amenable to the clinical testing environment.

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