Background: Fragile X syndrome (FXS), the most common cause of inherited mental impairment, is most commonly related to hyperexpansion and hypermethylation of a polymorphic CGG trinucleotide repeat in the 5’ untranslated region of the FMR1 gene. Southern blot analysis is the most commonly used method for molecular diagnosis of FXS. We describe a simplified strategy based on fluorescent methylation-specific PCR (ms-PCR) and GeneScan™ analysis for molecular diagnosis of fragile X syndrome.

Methods: We used sodium bisulfite treatment to selectively modify genomic DNA from fragile X and normal lymphoblastoid cell lines and from patients. We then performed ms-PCR amplification using fluorescently-labeled primers complementary to modified methylated or unmethylated DNA. Amplification products were resolved by capillary electrophoresis. FMR1 mutational status was determined by a combination of fluorescent peak sizes and patterns on the GeneScan electropherogram.

Results: DNA samples from male and female persons with known NL, PM, and FM FMR1 CGG repeats were analyzed. Each FMR1 genotype produced a unique GeneScan electropherogram pattern, thus providing a way to identify the various disease states. The number of CGG repeats in all NL and PM alleles were determined accurately. Analysis by both the new assay and Southern blot of a family segregating with FXS showed complete concordance between both methods.

Conclusions: This simplified molecular diagnostic test, based on fluorescent methylation-specific PCR, may be a suitable alternative or complement to Southern blot analysis for the diagnosis of FXS.

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pends on reliable molecular confirmation of expansion and methylation/silencing of the \textit{FMR1} gene. The most common method of FXS molecular diagnosis is Southern blot analysis supplemented by direct PCR amplification of the \textit{FMR1} CGG repeat (6).

Although Southern blot analysis detects large expansion mutations reliably, small premutation alleles cannot be distinguished from NL alleles in the high end of the range. Conversely, some large premutation and all full mutation alleles cannot be detected reliably by current PCR-based methods. Combining Southern analysis with PCR resolves most of the deficiencies of either method. Even so, this combination allows precise sizing only of alleles in the NL size range. Furthermore, the procedure is highly labor-intensive and time-consuming.

We previously developed an alternative PCR-based FXS classification strategy that took advantage of the differential susceptibility of methylated and nonmethylated DNA to modification by sodium bisulfite (7). The assay involved 3 methylation-specific PCR (ms-PCR) amplifications and separate analyses on agarose gels, which did not allow for precise sizing of CGG repeats. We now describe a fluorescent ms-PCR assay for FXS that classifies NL, premutation, and full mutation affected males and females according to their unique GeneScan™ electropherogram patterns.

**Materials and Methods**

**DNA SAMPLES**

We performed initial assay optimization on DNA extracted from 7 lymphoblastoid cell lines obtained from the Coriell Mutant Cell Repository in Camden, NJ, and peripheral blood leukocytes from an anonymous control female. The \textit{FMR1} genotypes of these DNA samples were previously determined with other molecular methods (Southern and/or PCR). The study was approved by the institutional review board of the National University Hospital (Singapore). We also used both the newly developed assay and Southern blot to perform a prospective analysis of an 8-member family segregating with FXS (8). All participants gave written informed consent.

**GENOMIC DNA MODIFICATION**

We treated DNA samples with sodium bisulfite, using a modified protocol of Zhou et al. (7). NaOH was added to 1 µg DNA to a final concentration of 0.3 mol/L in a final volume of 5 µL. After 15 min incubation at 55 °C, we added 75 µL of fresh, prewarmed sodium bisulfite solution (prepared by dissolving 0.6 g of sodium bisulfite in 45 µL of 10 mol/L NaOH and 960 µL of dH2O). This deamination mixture was incubated at 55 °C for 1 h, then purified using a QIAEX II Gel Extraction Kit (Qiagen) according to manufacturer instructions and eluted in 50 µL Tris-EDTA pH 8.0. The eluted DNA was desulfonated by incubation with 50µL 0.2 mol/L NaOH at 37 °C for 15 min. The mixture was neutralized with 50 µL 0.2 mol/L Tricine and precipitated by adding 1 µL (20 µg) glycogen (Invitrogen), 15 µL 3 mol/L sodium acetate and 375 µL of absolute ethanol, followed by a 20 min incubation at 4 °C. After centrifugation at 13 600 × g for 20 min, the DNA pellet was washed with 0.5 mL of 70% ethanol, air dried for ~10–15 min, then redissolved in 50 µL Tris-EDTA pH 8.0 and stored at −20 °C until further use. The genomic modification process converts all deoxycytidine residues to deoxyuridine. On methylated alleles, however, deoxyctydine residues of Cpg dinucleotides are methylated and are resistant to conversion. This differential susceptibility to conversion leads to methylated and nonmethylated \textit{FMR1} alleles with distinct differences in nucleotide sequence.

**METHYLATION-SPECIFIC PCR**

Bisulfite-modified DNA was subjected to methylation-specific PCR according to a modified protocol of Zhou et al. (7). Two different PCR reactions were performed on the methylated allele (Table 1; Fig. 1A). The mTP-PCR amplification, which is an adaptation of the triplet-primed PCR (TP-PCR) strategy of Warner et al. (9), was performed with 3 primers, a \textit{Ned}-labeled forward primer upstream of the repeat, an unlabeled tailed reverse primer annealing within the modified methylated repeat, and a second unlabeled reverse primer specific to the tailed segment of the first reverse primer. This reaction detects

**Table 1. Primers used in specific amplification of sodium bisulfite–treated methylated and non-methylated \textit{FMR1} alleles.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′→3′ sequence</th>
<th>GenBank ID: nucleotides</th>
<th>Concentration, µmol/L</th>
<th>Amplicon Size a</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTP-PCR</td>
<td></td>
<td>L29074: 13806→13834</td>
<td>0.2</td>
<td>NA</td>
</tr>
<tr>
<td>Ned-mTPF</td>
<td>NedGCGGCTACAAAAACGTACGCAACGGG</td>
<td>n.a.</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>mTPR</td>
<td>TACCGATACGCATCCCCAGTTGTCAGC(TG)₉</td>
<td>n.a.</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>TailR</td>
<td>TACCGATACGCATCCCCAGTTGTCAGC</td>
<td>n.a.</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Met-PCR</td>
<td></td>
<td>L29074: 13748→13775</td>
<td>0.2</td>
<td>108 bp + 3n</td>
</tr>
<tr>
<td>Fam-Met1F</td>
<td>Fam-CGCCCTCTAAGACAGGACGAACCGACG</td>
<td>L29074: 13915→13888</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>GGTGCCGGCGCTGAGTTTATGCAGTC</td>
<td>L29074: 13708→13741</td>
<td>0.2</td>
<td>168 bp + 3n</td>
</tr>
<tr>
<td>nonMet-PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hex-nonMetF</td>
<td>HexAAACACTCAACCCTTCAATTTTTTCTTCTCAAT</td>
<td>L29074: 13935→13906</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>nonMetR</td>
<td>GAGTTTGTGGTTAGGTTTTGTTG</td>
<td>L29074: 13834→13834</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

* a, the number of trinucleotide repeats; NA, not applicable.
Fig. 1. Schematic illustration of fluorescent ms-PCR assay and expected results from normal, premutation, and full mutation males and females. (A), relative annealing positions of mTP-PCR, Met-PCR, and nonMet-PCR primers on sodium bisulfite–modified nonmethylated and methylated DNA templates. (B), expected GeneScan results generated by mTP-PCR (black patterns, left panels), Met-PCR (blue peaks, center panels), and nonMet-PCR (green peaks, right panels).
the presence of all methylated alleles and produces a specific GeneScan pattern in the presence of a premutation or full mutation.

The second methylated allele amplification, designated as Met-PCR, used a Fam-labeled forward primer and an unlabeled reverse primer to amplify across the methylated FMR1 repeat. This reaction detects and accurately sizes all NL and premutation methylated alleles. In combination, the Met-PCR and mTP-PCR results enable unambiguous discrimination between methylated NL, premutation, and full mutation alleles.

Amplification of the nonmethylated FMR1 trinucleotide repeat (nonMet-PCR) was achieved with a Hex-labeled forward primer and an unlabeled reverse primer flanking the bisulfite-modified nonmethylated FMR1 repeat. This reaction detects and accurately sizes all nonmethylated alleles.

Each amplification reaction was performed in a 50 μL volume containing the respective primers at concentrations as indicated in Table 1; 0.2 mmol/L of each deoxynucleotide triphosphate, 2.5 U HotStarTaq™ DNA polymerase (Qiagen), Q-solution (Qiagen) at a concentration of either 0.5× (for nonMet-PCR) or 1.5× (for mTP-PCR and Met-PCR), 1× supplied PCR buffer (including 1.5 mmol/L MgCl₂), and 5 μL of the bisulfite-modified DNA. An enzyme activation step at 95 °C for 15 min was followed by 40 cycles of 98 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

CAPILLARY ELECTROPHORESIS AND GENESCAN ANALYSIS
Amplification products from the mTP-PCR, Met-PCR, and nonMet-PCR reactions were combined in a ratio of 2:1:1 μL and mixed with 1 μL of MapMarker ROX-1000 size marker (Bioventures) and 9 μL of HiDi Formamide (Applied Biosystems). With an injection time of 22 seconds at 1 kilovolt, we loaded the mixture onto an ABI 3100 Genetic Analyzer (Applied Biosystems) for capillary electrophoresis for 40 min to detect NL and premutation alleles. We analyzed electropherograms with the GeneScan version 3.7.1 application software (Applied Biosystems) according to manufacturer instructions.

Results
Schematic illustration of the expected GeneScan electropherogram results after ms-PCR of an NL male, a premutation male, a full mutation male, an NL female, a premutation female, and a full mutation female are presented in Fig. 1B. The mTP-PCR, Met-PCR, and nonMet-PCR traces are shown in separate panels to illustrate the expected products of each amplification regimen for each of the 6 samples.

NL males have only 1 X chromosome and thus carry only 1 NL, functional (and therefore nonmethylated) FMR1 allele. Therefore, only the nonMet-PCR will yield an amplification product in the NL size range (Fig. 1B, NL male row, right panel). Likewise, a premutation male carries only the premutation FMR1 allele, which is also nonmethylated, and thus a premutation size amplification product (PM allele) is expected from the nonMet-PCR reaction only (Fig. 1B, premutation male row, right panel). In contrast, FXS-affected males carry a methylated full mutation allele that is too large to be amplified by Met-PCR. Also, no product is expected from the nonMet-PCR reaction.

However, the mTP-PCR reaction will yield a characteristic pre/full mutation (PFM) pattern on the Ned fluorescence detection channel (Fig. 1B, full mutation male row, left panel). This PFM pattern consists of a continuous series of peaks of 3 bp difference that show an inverse correlation between peak height (i.e., amplification yield) and fragment size, and is generated from the long uninterrupted stretch of CGG repeats present in a PM or FM allele. Where there is an AGG interruption at the 5′ end of the CGG repeat stretch, a discrete cluster of 2–3 peaks ~24 bp to the left of the peak ladder can also be observed. This pattern occurs because the mTPR primer carries 8 5′-TCG-3′ repeats and will thus anneal only to the bisulfite-modified methylated FMR1 repeat wholly within the 9 or 10 5′-CGA-3′ repeat stretch 5′ of the interruption. At the AGG interruption site, the bisulfite treatment will ultimately produce a 5′-AAA-3′ on the complementary strand of the converted antisense strand. Because the mTPR primer cannot properly anneal to any 8-repeat stretch that includes the interruption, no PCR product is formed. The result is that the mTP-PCR trace will contain an ~24 bp clear zone equivalent to the absence of 8 amplification peaks.

Most full mutation alleles involve greatly expanded repeats that the Met-PCR assay cannot efficiently detect. However, the presence of the mTP-PCR PFM pattern is itself diagnostic of full mutation–affected status in males, and further detection of an Met-PCR full-variation allele is not necessary. Females carry 2 X chromosomes, one of which is randomly X-inactivated under routine circumstances. FMR1 alleles on an inactive X chromosome are methylated. Therefore, an NL female with 2 NL FMR1 alleles of different CGG repeat sizes will be expected to yield 2 NL allele peaks after both Met-PCR and nonMet-PCR (Fig. 1B, NL female row, center and right panels). Where both NL alleles are of the same repeat size, only one NL allele peak will be observed after each PCR.

In addition, mTP-PCR will produce a specific NL pattern on the Ned fluorescence detection channel, which is different from the PFM pattern (Fig. 1B, NL female row, left panel). This NL pattern consists of 3 discrete clusters of 2–3 peaks each, with the clusters separated by 2 clear stretches of ~24 bp. This amplification pattern arises because the NL allele usually consists of 29 to 30 CGG repeats interrupted by 2 AGG trinucleotides, in a 9 + 9 + 9 or 10 + 9 + 9 configuration (the numerals indicating the number of CGG repeats and the “+” symbol indicating the position of the AGG interruption). As a result of the
AGG interruptions, the mTPR primer, which contains a complementary sequence to anneal to 8 uninterrupted repeats, can anneal wholly only within the 9 or 10 CGG repeat segments. Whenever the primer partially overlaps the AGG interruption, it will not anneal properly, and therefore no amplification product is formed. The result is the distinctive pattern of 3 discrete clusters of 2 to 3 amplification peaks separated by 2 clear zones of ~24 bp, each zone equivalent to the absence of 8 amplification peaks.

The first cluster of 2 or 3 peaks comes from the primer annealing within the 5'-most 9 or 10 repeat stretch, respectively. The next cluster of 2 peaks comes from the primer annealing within the middle 9 repeat stretch, and the last cluster of 2 peaks comes from the primer annealing within the 3'-most 9 repeat stretch. The 3 peak clusters are separated by 2 clear zones where there are no peaks, consistent with the primer not annealing if it partially overlaps the AGG interruption.

In a premutation female, mTP-PCR is expected to produce a PFM pattern from the methylated/X-inactivated premutation allele (Fig. 1B, premutation female row, left panel). Furthermore, an NL and a PM allele product are expected after both Met-PCR and nonMet-PCR (Fig. 1B, premutation female row, center and right panels).

In a full mutation–affected female, a PFM pattern is observed after mTP-PCR (Fig. 1B, full mutation female row, left panel). Additionally, only an NL allele is expected after both Met-PCR and nonMet-PCR, the full mutation allele being too large to be detected (Fig. 1B, full mutation female row, center and right panels).

Fig. 2 shows actual ms-PCR GeneScan tracings from 8 genotype-known DNA samples representing a broad spectrum of FMR1 CGG alleles. After ms-PCR, the Ned-labeled mTP-PCR products, Fam-labeled Met-PCR products, and Hex-labeled nonMet-PCR products were combined in a single capillary electrophoresis run. The Ned-, Fam-, and Hex-labeled products appear as black, blue, and green peaks and tracings, respectively, on the electropherogram. Sizes (in base pairs) of NL and PM alleles were accurately determined with the aid of the Rox-labeled internal size calibrator, represented by the red peaks, and converted to actual repeat numbers according to the formulae in Table 1.

In the NL male sample, as expected, there were no mTP-PCR or Met-PCR products, whereas the NL allele was easily detected as a green peak after nonMet-PCR (Fig. 2, NL male). As predicted again, no products were detected from the 2 premutation male samples after the mTP-PCR and Met-PCR reactions (Fig. 2, premutation males #1 and #2). However, the different PM alleles from the 2 samples were successfully detected and sized after nonMet-PCR. For premutation male #1 (Coriell lymphoblastoid cell line GM06891), our previous analysis using nonfluorescent ms-PCR and agarose gel electrophoresis also detected 2 PM alleles, with the intensity of the larger (~155 repeat) band being much stronger than that of the smaller (~100 repeat) band (7). The smaller PM allele is thus likely a result of somatic instability and contraction of the larger allele related to long-term culture of this lymphoblastoid cell line. For the FXS full mutation–affected male, the presence of the PFM pattern after mTP-PCR indicated the presence of his methylated full mutation allele, confirming his FXS affected status (Fig. 2, full mutation male).

Similarly, the fragile X status of the female DNA samples was accurately classified. In the NL female DNA sample, the mTP-PCR reaction produced the expected NL pattern and not the PFM pattern, confirming her NL unaffected status (Fig. 2, NL female; see left inset). In addition, both the Met-PCR and nonMet-PCR reactions detected 2 NL alleles differing by a single trinucleotide repeat, respectively (Fig. 2, NL female, see right inset). For both premutation female samples, the NL and PM alleles were also easily detected, although the peak heights of the PM alleles were lower than those of the NL alleles (Fig. 2, premutation females #1 and #2). The presence of the mTP-PCR PFM pattern also confirmed their premutation status.

Interestingly, in premutation female #2, the absence of a methylated NL allele (absence of a Met-PCR–generated blue peak in the NL size range) and corresponding absence of a nonmethylated PM allele (absence of a nonMet-PCR–generated green peak in the PM size range) are indicative of skewed X-inactivation in this individual, i.e., her NL FMR1 allele is carried predominantly on the active X chromosome and her PM allele is on the inactive X chromosome. In the full mutation female, the clear presence of the PFM pattern after mTP-PCR, together with absence of any PM alleles after Met-PCR and nonMet-PCR, confirm her full mutation affected status (Fig. 2, full mutation female). The fact that the NL allele was detected only by the nonMet-PCR reaction (presence of a green peak in the NL size range) but not by the Met-PCR reaction (absence of a blue peak in the NL size range) indicates the presence of extremely skewed X-inactivation in this individual.

As can be seen, the fluorescent ms-PCR GeneScan peaks and patterns differ among the 8 genotype-known DNA samples, enabling their FMR1 genotypes to be accurately determined.

We then performed a prospective analysis of a family of 8 members (both parents and 6 children) segregating with FXS. We performed Southern blot analysis in parallel on all 8 DNA samples. In the father (patient #1), the presence of only an NL-sized nonMet-PCR (green) peak of 27 repeats indicated that he is unaffected (Fig. 3A, patient 1). The mother (patient #2), however, showed the presence of an mTP-PCR (black) PFM pattern in addition to a 27-repeat NL allele detected by both Met-PCR and nonMet-PCR. Given the absence of a PM allele in the mother, the ms-PCR results indicate that she is an affected full mutation female. Among her offspring, 2 sons (pa-
patients #4 and #6) were also positive for the mTP-PCR PFM pattern, which in males is diagnostic of the presence of the full mutation. These results were consistent with clinical findings of moderate to severe mental impairment and typical facial dysmorphism of FXS. As expected, NL or PM allele peaks were not observed in patient #6. In patient...
Fig. 3. Molecular diagnosis of a family segregating with FXS by fluorescent ms-PCR analysis (A) and Southern blot analysis (B). The mother (patient 2) was determined to be a full mutation female. She passed on her FM allele to 2 of her 5 sons (patients 4 and 6), who are thus full mutation–affected males. Patient 4 is also mosaic for a PM allele. The father, daughter, and remaining 3 sons were determined to be unaffected. The results from both techniques were completely concordant.
#4, a small blue PM allele peak of 149 repeats was detected, suggesting that he may be mosaic for a PM allele. The 3 remaining sons (patients #3, #7, and #8) inherited their mother’s NL FMR1 allele, as demonstrated by the presence of only the 27-repeat nonMet-PCR (green) peaks, and are thus unaffected NL males. The only daughter (patient 5) also did not inherit her mother’s FM allele, as demonstrated by the presence of an mTP-PCR NL pattern, and is thus an unaffected NL female.

In the Southern blot analysis, full mutation–methylated fragments (>5.2 kb) were detected in the mother (patient #2) and 2 sons (patients #4 and #6) (Fig. 3B). The father (patient #1) and remaining sons (patients #3, #7, and #8) showed only the nonmethylated (2.8 kb) NL fragment, whereas only nonmethylated (2.8 kb) and methylated (5.2 kb) NL fragments were observed in the daughter (patient #5). The Southern blot results were thus completely concordant with the results obtained by fluorescent ms-PCR, further validating the diagnostic accuracy of the new assay in accurately determining the fragile X status of all members of this family.

### Discussion

Several PCR-based methods for amplification of FMR1 CGG repeats have been upgraded to automated/semiautomated capillary systems (10–12). However, the high GC content of this region and competition from the normal-sized allele in females has severely limited the utility of conventional PCR methods in detecting the presence of FM and even PM alleles. As a result, although fluorescent PCR-based assays allow highly precise (CGG)n repeat sizing, alleles with >100 repeats remain a challenge to detect (10).

With the new protocol described in this study, NL as well as PM alleles in both males and females can now be accurately sized. By tagging the primers in each of the 3 ms-PCR reactions with a different fluorophore, and pooling the products before capillary electrophoresis, all 3 ms-PCR reaction results can be analyzed simultaneously with mTP-PCR, Met-PCR, and nonMet-PCR results displayed as black, blue, and green traces, respectively.

We have demonstrated the accuracy and sensitivity of this fluorescent ms-PCR assay platform not only in differentiating between 2 NL alleles differing by a single trinucleotide repeat (NL female in Fig. 2), but also in correctly identifying affected males and females although their FM alleles are too large for successful amplification across the expanded repeat (Figs. 2 and 3A). Although mTP-PCR produces the PFM pattern whenever either a PM allele or FM allele is present in a female, a premutation female is clearly distinguished from a full mutation–affected female by the presence of a PM allele peak by both Met-PCR and nonMet-PCR in the premutation female. In males, the presence of the mTP-PCR PFM pattern is diagnostic of a full mutation–affected male. Premutation males do not display the mTP-PCR PFM pattern because their PM allele is nonmethylated.

The lower Met-PCR and nonMet-PCR peak heights of PM alleles compared with NL allele peaks is consistent with the lower amplification efficiency of alleles containing longer trinucleotide repeat tracts. This difference in peak height is especially pronounced in premutation females, given the added “competition” from the NL allele present in these individuals.

It should be noted that the fluorescent ms-PCR assay described here may inaccurately classify mosaic full mutation–affected females, who also carry a PM allele, as unaffected premutation females. This is because the fluorescent ms-PCR results for a premutation female and a mosaic full mutation female are identical. Furthermore, very large premutations approaching 200 repeats may be too large to be detectably amplified by Met-PCR or nonMet-PCR, especially in PM females. Such females may thus be incorrectly classified as full mutation females. Therefore, if there are clinical or genealogical arguments about the fragile X–affected status in a woman, further confirmation by Southern blot analysis may be required. However, full mutation–affected males who are mosaic for a PM allele will not be mistaken as unaffected premutation males. This is because such mosaic males will be positive for the mTP-PCR PFM pattern, which is not observed in pure premutation males.

The fluorescent ms-PCR assay detected the presence of extreme skewed inactivation in premutation female #2 (Fig. 2, GM06896) and the full mutation female (GM07537), consistent with our previous determination that ≥99% of cells in both cell lines carry the NL FMR1 allele on the active X and the PM or FM allele on the inactive X (7). Extreme skewing of X inactivation is uncommon in females; however, the observations here are derived from cell lines, which may have undergone clonal
expansion after long-term culture. Although premutation female #1 (GM06907) was also skewed such that ≥95% of cells carry the NL FMR1 allele on the active X and the PM allele on the inactive X, this skewing was not reflected in our fluorescent ms-PCR results for her (Fig. 2, premutation female #1). Incidentally, our Southern blot results for the FXS family also indicate that the mother has skewed X-inactivation, with a majority of cells carrying the NL FMR1 allele on the active X and the PM allele on the inactive X (Fig. 3B, patient 2). Again, this skewing was not detected by our fluorescent ms-PCR assay. Instead, both premutation female #1 and patient #2 had a methylated (inactive) NL allele blue peak height that was greater than the nonmethylated (active) NL allele green peak height. This observation is most likely the result of the fact that the 6-FAM fluorophore emits a stronger fluorescence compared with HEX. Therefore, this new assay will not accurately detect skewed X-inactivation unless the skewing is extreme. The ability to estimate the degree of X-inactivation in a female full mutation carrier, such as by Southern blot analysis, provides useful additional predictive information about an affected patient’s degree of phenotypic severity.

The fluorescent ms-PCR assay described here requires 5- to 6-fold less patient DNA than Southern blot analysis (1 μg vs 5–6 μg), with a turnaround time from genomic DNA to diagnostic test results of <2 working days compared with a 1–2 week turnaround time for Southern blots. These features and the ability to accurately size NL and PM-sized alleles make this new assay an attractive alternative to the combination Southern blot and direct PCR analysis currently used to detect full mutations and normal-sized alleles in FXS diagnosis.

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**References**