

# Reliable and Sensitive Detection of Fragile X (Expanded) Alleles in Clinical Prenatal DNA Samples with a Fast Turnaround Time

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**This study evaluated a large set of blinded, previously analyzed prenatal DNA samples with a novel, CGG triplet-repeat primed (TP)-PCR assay (Amplidex FMR1 PCR Kit; Asuragen, Austin, TX). This cohort of 67 fetal DNAs contained 18 full mutations (270 to 1100 repeats, including 1 mosaic), 12 premutations (59 to 150 repeats), 9 intermediate mutations (54 to 58 repeats), and 28 normal samples (17 to 50 repeats, including 3 homozygous female samples). TP-PCR accurately identified *FMR1* genotypes, ranging from normal to full-mutation alleles, with a 100% specificity (95% CI, 85.0% to 100%) and a 97.4% sensitivity (95% CI, 84.9% to 99.9%) in comparison with Southern blot analysis results. Exact sizing was possible for a spectrum of normal, intermediate, and premutation (up to 150 repeats) alleles, but CGG repeat numbers >200 are only identified as full mutations. All homozygous alleles were correctly resolved. The assay is also able to reproducibly detect a 2.5% premutation and a 3% full-mutation mosaicism in a normal male background, but a large premutation in a full male mutation background was masked when the amount of the latter was >5%. Implementation of this TP-PCR will significantly reduce reflex testing using Southern blot analyses. Additional testing with methylation-informative techniques might still be needed for a few cases with (large) premutations or full mutations. (*J Mol Diagn* 2012, 14:560–568; <http://dx.doi.org/10.1016/j.jmoldx.2012.05.003>)**

Fragile X syndrome (FXS; Online Mendelian Inheritance of Man 309550) is the most common form of X-linked inherited intellectual disability, with an estimated prevalence of 1 in 4000 for male and 1 in 7000 to 8000 for female individuals.<sup>1</sup> FXS is associated with moderate to

severe intellectual and social impairment, anxiety, attention-deficit/hyperactivity disorder, and autism. The phenotypic presentation is variable, but characteristic physical features include a prominent forehead, a long narrow face, protruding ears, and macroorchidism in adolescent and adult males<sup>2</sup>; 50% to 60% of affected females will have mild to moderate intellectual disability.<sup>3</sup>

At the molecular level, FXS is almost exclusively characterized by an expansion of a (CGG)<sub>n</sub> trinucleotide repeat, located in the 5'-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene encoding the FMRP RNA binding protein.<sup>4–6</sup> The disorder occurs in most ethnic populations, but prevalence may vary from group to group. The frequency of premutation carriers in the general population varies between 1 in 251 and 1 in 813 in males and between 1 in 113 and 1 in 259 in females.<sup>7–12</sup>

The CGG repeat lengths can be divided into four diagnostic categories, according to the European Molecular Genetics Quality Network *FMR1* guidelines 2006 ([http://www.emqn.org/emqn/digitalAssets/0/233\\_EMQN\\_guidelines\\_FRAX\\_2006.pdf](http://www.emqn.org/emqn/digitalAssets/0/233_EMQN_guidelines_FRAX_2006.pdf), last accessed April 10, 2012). The normal form of the CGG repeat is highly polymorphic in the general population and contains 6 to approximately 50 trinucleotide repeats, with 29 to 30 being the most prevalent alleles.<sup>7</sup> Intermediate trinucleotide lengths range between 51 and 58 repeats and are slightly unstable on transmission from generation to generation but rarely jump to full expansions over a single meiosis.<sup>13</sup> Many *FMR1* alleles contain AGG sequences that are interspersed among the CGG triplets. These AGG interruptions are believed to confer stability and to reduce the risk of expansion.<sup>14</sup> Patients with FXS carry >200 repeats, also referred to as full-mutation (FM) alleles. Consequently, the CGG repeat and the upstream promoter region are, generally, hypermethylated, leading to transcriptional silencing of the *FMR1* gene and absence or reduction of the RNA binding protein FMRP. Unmethy-

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lated expansions of 59 to 200 repeats, called premutations (PMs), are at a high risk of expansion into a full mutation on maternal transmission over one or more generations.<sup>15</sup> The risk for expansion from a female PM carrier to an FM increases with repeat length. Small expansions do not cause FXS, but carriers have an additional risk of developing FXS-associated disorders. Fragile X tremor/ataxia syndrome (Online Mendelian Inheritance of Man 300623) is a late-onset (>50 years) neurodegenerative disorder.<sup>16–18</sup> In addition, 15% to 20% of the female PM carriers experience primary ovarian insufficiency, also called premature ovarian failure.<sup>19,20</sup> Distinct mechanisms lead to FXS and fragile X tremor/ataxia syndrome.

Exceptionally, FXS can be caused by a deletion or a point mutation in the *FMR1* gene and not by a CGG expansion.<sup>21,22</sup>

Thus, molecular diagnosis of FXS relies on an accurate and efficient determination of the number of the triplet repeat elements in the promoter region. Routine molecular analysis includes PCR amplification, with primers flanking the CGG repeat, and Southern blot (SB) analysis of the alleles, including examination of their methylation status. The SB analysis is required to detect larger alleles not amplified by PCR and to resolve zygosity in female samples in which there may be any ambiguity about a missed FM allele. SB analysis requires a larger quantity of DNA than PCR, which, for a prenatal analysis, may require culturing cells and a consequent long delay in obtaining and reporting results.

In this study, we evaluated a commercialized PCR-based assay (Asuragen, Austin, TX) using a large set of archived prenatal DNA samples that were previously analyzed for FXS with conventional PCR and SB analysis in our Center for Medical Genetics clinical laboratory (UZ Brussel, Brussels, Belgium). We also evaluated the analytical sensitivity of the new assay using mixtures of DNA with normal, PM, and FM alleles to model mosaicism.

## Materials and Methods

### DNA Samples

This is a retrospective study on archived prenatal DNA samples from women carrying a PM or FM *FMR1* allele. All analyzed samples were derived from different pregnant women, except six PM carrier women who demanded a prenatal diagnosis for FXS in subsequent (between two and four) pregnancies, whereas three others carried twins.

The DNA of all prenatal samples (purified fresh chorionic villi and amniotic cells) and control commercial Epstein-Barr virus cell lines was manually extracted by an in-house classic phenol-chloroform and ethanol precipitation protocol and (long-term) stored at 4°C until use.

Genomic DNA specimens from the Coriell cell lines (Coriell Cell Repositories, Camden, NJ), with sample identification numbers NA20239 (20 and approximately 199 CGGs), NA07541 (29 and 31 CGGs), NA20230 (54 CGGs), NA06891 (119 CGGs), and NA09237 (approx-

imately 940 CGGs), were used for training and/or as positive control templates.

DNA from peripheral blood leukocytes (from 5 to 7 mL whole blood) was prepared with magnetic bead chemistry [Perkin Elmer (Chemagen), Zaventem, Belgium] on an automated Magnetic Separation Module I robot [Perkin Elmer (Chemagen)].

All archived DNA samples were requantified with a Nanodrop spectrophotometer (Thermo Scientific, Erembodegem-Aalst, Belgium) before use. DNA was diluted to 25 ng/μL in H<sub>2</sub>O before PCR amplification.

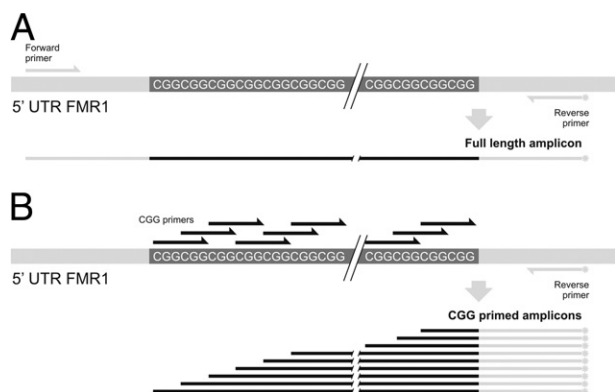
To evaluate the analytical sensitivity of the assay to mosaicism, different mock sample sets were prepared and analyzed systematically. A first DNA sample set was prepared by dilution of the DNAs of a prenatal FM male with a prenatal normal male sample. The percentage mass of the full-length DNA varied from 0% to 10%, with intervals at 0.5%, 1%, 2%, 3%, 5%, and 10%. Two other mock mosaic PM and FM sample sets were prepared by admixing various proportions of a normal with a PM or a PM with an FM allele (all male) sample in a range of 1.25% to 20%, with intervals at 1.25%, 2.5%, 5%, 10%, and 20% of the PM or FM, respectively. Both of these sample sets were completed with genomic DNA extracted from leukocytes, because only samples of a normal and a PM template from amniotic cells and a PM and FM template from chorionic villi were available for investigation. All mosaic sample experiments were performed in quadruplicate.

### SB Analysis

DNA (5 to 7 μg) was digested overnight with HindIII (EcoRI was used in former experiments) and the methylation-sensitive restriction enzyme BssHII before electrophoresis on a 0.8% agarose gel in electrophoresis buffer (90 mmol/L Tris phosphate, 20 mmol/L EDTA, and 1.30% orthophosphoric acid, pH 8.0). After transfer, the charged nylon membrane (Roche Diagnostics, Vilvoorde, Belgium) was incubated with a Dig-11-dUTP-labeled *FMR1* probe (PCR Dig Synthesis Kit; Roche Diagnostics) and processed according to the manufacturer (Dig Wash & Block buffer set; Roche Diagnostics).

### Assay Findings

The Amplidex *FMR1* PCR kit (Asuragen) has been designed to identify the full range of *FMR1* CGG expansions (including PMs and FMs) by PCR and capillary electrophoresis (CE). The assay allows two types of tests: a gene-specific PCR and a triplet-repeat primed (TP)-PCR. The TP-PCR assay contains a third chimeric primer in combination with the gene-specific forward and reverse primer pair set, hybridizing randomly within the CGG repeat region and generating numerous amplicons of various sizes in addition to the gene-specific allele fragment(s). Figure 1 shows a schematic diagram of the primer locations. The concept of the method has already been described in detail, and the gene-specific primers were previously reported.<sup>23,24</sup> All amplicons are analyzed in one single CE trace. The full-length PCR products allow



**Figure 1.** Schematic representation of the primer locations of the gene-specific PCR (A) and TP-PCR assay (B) in the 5'-untranslated region of the *FMR1* gene. The forward and reverse primers amplify the full-length amplicon according to the number of triplet repeats. The TP-PCR assay contains a third chimeric primer in combination with the gene-specific forward and reverse primer pair set, hybridizing randomly within the CGG repeat region and generating numerous amplicons of various sizes in addition to the gene-specific allele fragment(s).

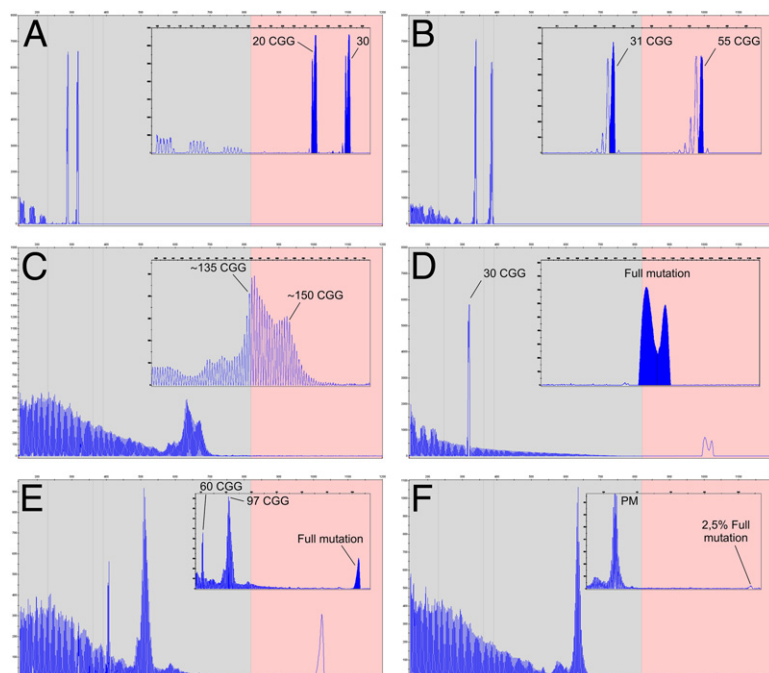
accurate sizing of any allele less than approximately 200 repeats, whereas larger alleles appear as a characteristic peak located at approximately 850 to 1100 bp. The CGG TP-PCR amplicons yield a unique typical profile of individual peaks that can be used to resolve zygosity and flag the presence of any expanded (large) allele. The assay is also able to identify AGG interruptions, as determined in signal intensity at specific increments within the individual CGG repeat primed peaks, as seen in Figure 2.<sup>24</sup> However, this information is not routinely used to provide risk assessment for expansions in a routine clinical diagnostic setting. The TP-PCR was used for all experiments.

The TP-PCR assay and CE were performed according to the manufacturer's instructions, but an adjustment was

made regarding the volumes of reagents. The original prescribed total volume was reduced to half (7.5  $\mu$ L) with respect to the molarities of the reagents in combination with a DNA input of 25 ng. PCR amplification was performed on a Veriti thermal cycler (Life Technologies, Merelbeke, Belgium).

Each amplicon, 2  $\mu$ L, was mixed with the same amount of Rox 1000 standard (included in the kit) and supplemented with 11  $\mu$ L of Hi-Di formamide (Life Technologies). All amplicons were denatured by heat incubation at 97°C (thermal cycler) for 5 minutes and immediately cooled at 0°C (ice-H<sub>2</sub>O) for at least 3 minutes, before loading onto an ABI3130 Genetic Analyzer (Life Technologies) for CE, using a POP-7 polymer (Life Technologies) on a 36-cm array with a 2.5-kV injection for 20 seconds and a 40-minute run at 15 kV.

In an in-house allele-sizing PCR assay, 50 ng of genomic DNA was amplified in 15  $\mu$ L of reaction mix containing one times PCR buffer 2 Expanded Long Template PCR system (Roche Diagnostics, Vilvoorde, Belgium); 12% dimethylsulfoxide (Merck, Overijse, Belgium); 0.5 mmol/L each of deoxy-ATP, deoxy-TTP, and deoxy-CTP (Life Technologies); 0.125 mmol/L deoxy-GTP (Life Technologies); 0.375 mmol/L 7-deaza-2-deoxy-GTP (GE Healthcare, Diegem, Belgium), 0.5 U Expanded Long Template DNA polymerase, and 0.3  $\mu$ mol/L each of the forward (CY5-labeled) primer c and reverse primer f.<sup>4</sup> Samples were amplified in a 2400 thermal cycler (Life Technologies) after an initial heat denaturation step of 95°C for 5 minutes, followed by 35 cycles of 96°C for 45 seconds, 62°C for 30 seconds, and 72°C for 2 minutes and a final extension step of 72°C for 10 minutes. Amplicons were resolved by PAGE on the ALF Express Genetic Analyzer system (Pharmacia, Uppsala, Sweden).



**Figure 2.** TP-PCR data of the four different categories: a heterozygous prenatal female DNA sample with two normal alleles (20 or 30 repeats) (A), a heterozygous prenatal female DNA sample with a normal (31 repeats) and an intermediate (55 repeats) allele (B), a prenatal male DNA sample with a PM (approximately 130 to 150 repeats) allele (C), and a heterozygous prenatal female DNA sample with a normal (30 repeats) and an FM allele (D). TP-PCR data of a male clinical mosaic PM/FM DNA sample (E) and a mock mosaic PM/FM DNA sample (F). The pink background indicates the full mutation region, whereas the gray background indicates a normal, intermediate, or premutation region. This color convention facilitates visual confirmation of affected individuals.

## Data Analysis

Indications of genotyping followed the guidelines endorsed by the European Molecular Genetics Quality Network for normal (6 to 50 repeats), intermediate (51 to 58 repeats), PM (59 to 200 repeats), and FM (>200 repeats) *FMR1* alleles ([http://www.emqn.org/emqn/digitalAssets/0/233\\_EMQN\\_guidelines\\_FRAX\\_2006.pdf](http://www.emqn.org/emqn/digitalAssets/0/233_EMQN_guidelines_FRAX_2006.pdf), last accessed April 10, 2012). Thresholds for the different categories of normal, intermediate, and PM alleles are arbitrary and slightly deviating in comparison to the guidelines issued by the American College of Medical Genetics.<sup>1,25</sup>

All amplicons were evaluated on an ABI3130XL Genetic Analyzer. The length findings were derived from the size of the PCR product calibrated to a ROX 1000 Size Ladder (included in the kit; Asuragen) co-injected with every sample. The size of the PCR product was converted to CGG repeats using characterized cell line DNA from the Coriell Cell Repositories for alleles representing 20 or 199, 29 or 31, 54, and 119 CGGs as positive control reference material (sample training set; Asuragen) with sizes established by either DNA sequencing or consensus.<sup>24,26</sup> The repeat lengths of the gene-specific amplicons were calculated and converted to repeat sizes for all normal, intermediate, and PM alleles. Asuragen supplies an MS Excel-based data analysis macro, using files exported from GeneMapper version 4.1 (Life Technologies), that semiautomates the process of calculating repeat sizes.

Data on sizing of CGG repeats obtained by both methods (the in-house and the TP-PCR methods) were assessed by the Bland-Altman graphical approach (Microsoft Office Excel 2003). If measurement methods are comparable (unbiased), the differences should be small, centered around 0, and show no systematic variation on the Bland-Altman plot.

All aspects of this study (re-analysis of archived prenatal DNA samples) were approved by the Institutional Review Board of the Center for Medical Genetics, UZ Brussel.

## Results

### Prenatal and Control DNA Samples

A large set of archived prenatal DNA samples ( $n = 67$ ) of both male and female fetuses, with a variety of CGG allele repeat numbers in the *FMR1* gene, were analyzed with the novel TP assay from Asuragen. All samples were previously characterized by both conventional in-house PCR and SB protocols.

In this set of clinical samples, 56 were procured from chorionic villi, whereas 11 samples resulted from cultured amniotic cells. The sex of all fetuses was known: 33 samples were female, and 34 samples were male.

Across this full set of 67 prenatal DNA samples, 28 were normal, 9 were intermediate, and 12 were PM templates. In addition, SB analysis identified 17 FM and 1 mosaic PM-FM sample. The intermediate allele sizes ranged from 51 to 58 repeats, the PM allele sizes ranged from 59 to 160 repeats, and the FM allele sizes ranged from >200 to 1100 repeat elements. The genotypes of all samples are summarized in Table 1. The

**Table 1.** Summary of Prenatal DNA Samples Tested in This Study

Variable	No. of samples	M/F ratio
Total no.	67	34:33
Normal <i>FMR1</i> genotype	28	16:12
Intermediate <i>FMR1</i> genotype	9	6:3
Premutation <i>FMR1</i> genotype	12	5:7
Full-mutation <i>FMR1</i> genotype (including a mosaic PM/FM)	18	7:11
Homozygous females	3	0:3
Chorionic villi	56	28:28
Amniotic cells	11	6:5

Comparison of TP-PCR results with SB analysis	TP-PCR Observed	
	FM, >200 CGGs	Not FM, <200 CGGs
SB analysis FM, >200 CGGs	17	1*
SB analysis Not FM, <200 CGGs	0	49

\*The Discussion explains the discrepancy. F, female; M, male.

CGG repeat length was tabulated for each clinical sample and compared with the results of previously determined in-house PCR and SB analysis in Table 2. The sensitivity and specificity of the Amplidex FMR1 PCR system was calculated. A 100% specificity (95% CI, 85.0% to 100%) and a 97.4% sensitivity (95% CI, 84.9% to 99.9%), in comparison with the SB analysis results of this cohort of 67 samples, were achieved.

The FMR1 TP-PCR assay resulted in a multitude of fragments, all differing by only one CGG repeat, with peaks spanning the entire repeat length identifying these long CGG tracts as a stutter. Independent of their real size, FMs were reported as alleles >200 repeats. AGG anchors within the CGG tract were seen as a trough (dip) in the stutter. Typical amplification patterns of the Amplidex FMR1 PCR kit are seen in Figure 2. Results were obtained for all samples with signal intensities meeting expectations for the four different categories of CGG repeat lengths and without any background noises in the PM and FM ranges.

Subsequently, the fragment size (in bp) was converted to the number of CGG repeats, and repeat sizes of the normal, intermediate, and (small) PM alleles were compared with previous data. All sizes were within the limits of  $\pm 3$  CGG repeat difference between the in-house sizing PCR and the Asuragen TP-PCR results. Repeat differences were seen with older samples that were previously analyzed on a PAGE gel (ALF Express Genetic Analyzer) and in one PM sample sized with SB analysis. In addition, this TP-PCR protocol enabled the detection capacity of large premutation fragments compared with our in-house sizing PCR assay, which was limited to a maximum of approximately 120 repeats. A DNA sample as large as approximately 150 repeats was easily amplified and identified as such (Figure 2C).

Of 18 specimens that were scored as FMs by SB analysis, 17 were categorized as FM alleles by analysis using TP-PCR, including one PM/FM mosaic sample (Fig-

**Table 2.** Comparison of In-House (PCR and SB) Analyses and TP-PCR for Sizing of CGG Repeats in *FMRI* Alleles of Clinical Prenatal Samples

Sample no.	Clinical category	In-house results (repeats)	TP-PCR results (repeats)	No. of repeats $\neq$	Sex	Material	
1	Normal alleles	20	20	None	M	AC	
2		20 + 30	20 + 30	None	F	AC	
3		20	20	None	M	CV	
4		26	25	-1	M	CV	
5		28	29	1	M	AC	
6		29	29	None	M	CV	
7		29	29	None	M	CV	
8		29	29	None	M	CV	
9		30	30	None	M	CV	
10		30	30	None	M	CV	
11		31	30	-1	M	CV	
12		21 + 31	20 + 30	-1/-1	F	CV	
13		31	31	None	M	CV	
14		32	31	-1	M	CV	
15		33	33	None	M	CV	
16		33	33	None	M	CV	
17		17 + 31	18 + 31	1/None	F	CV	
18		19 + 31	20 + 31	1/None	F	CV	
19		30	28 + 30	-2/None	F	AC	
20		31	29 + 31	-2/None	F	CV	
21		30	30 + 30	None	F	CV	
22		31	30 + 30	-1	F	CV	
23*		30 + FM	29 + 31	-1/*	F	CV	
24		29 + 32	29 + 32	None	F	CV	
25		29 + 32	29 + 32	None	F	CV	
26		31	30 + 30	-1	F	CV	
27		30 + 40	30 + 40	None	F	CV	
28	49	47	-2	M	CV		
29	50	48	-2	M	AC		
30	Intermediate alleles	38 + 54	37 + 53	-1/-1	F	AC	
31		54	55	1	M	CV	
32		54	55	1	M	CV	
33		55	55	None	M	CV	
34		55	55	None	M	CV	
35		30 + 56	29 + 55	-1/-1	F	CV	
36		32 + 58	31 + 55	-1/-3	F	CV	
37		57	57	None	M	CV	
38		57	57	None	M	CV	
39		PM alleles	20 + 59	20 + 59	None	F	CV
40	29 + 62		29 + 61	None/-1	F	AC	
41	30 + 66		30 + 65	None/-1	F	CV	
42	30 + 67		30 + 66	None/-1	F	CV	
43	31 + 71		31 + 71	None	F	CV	
44	72		72	None	M	AC	
45	31 + 73		30 + 72	-1/-1	F	CV	
46	86		86	None	M	CV	
47	91		93	2	M	AC	
48	100		98	-2	M	CV	
49	29 + 115		29 + 114	None/-1	F	CV	
50	160 <sup>†</sup>		135-150	-10	M	CV	
51	FM alleles		60 + 97 + 800	60 + 95 + >200	None/-2	M	CV
52			29 + 300	29 + >200	None	F	CV
53			28 + 750	29 + >200	1	F	CV
54			30 + 600	30 + >200	None	F	CV
55		31 + 650	30 + >200	-1	F	CV	
56		31 + 300	30 + >200	-1	F	CV	
57		30 + 270	31 + >200	1	F	CV	
58		32 + 750	32 + >200	None	F	CV	
59		32 + 800	32 + >200	None	F	CV	
60		33 + 800	32 + >200	-1	F	AC	
61		32 + 700	33 + >200	1	F	CV	
62		400	>200	—	M	CV	
63		800	>200	—	M	CV	
64		750	>200	—	M	CV	

(table continues)

**Table 2.** *Continued*

Sample no.	Clinical category	In-house results (repeats)	TP-PCR results (repeats)	No. of repeats $\neq$	Sex	Material
65		750	>200	—	M	CV
66		1100	>200	—	M	CV
67		600	>200	—	M	AC
Cs <sup>†</sup>					M/F	EBV cell line

All samples showed a normal 46, XX or 46, XY karyotype on G-banding.

\*There were discrepant results between SB and TP-PCR analyses (as described in *Discussion*).

<sup>†</sup>Repeat number estimated from SB results.

<sup>‡</sup>The following control samples (DNA from cell lines) were used: 20 + approximately 199 (F), approximately 119 (M), and approximately 940 (M). F, female; M, male; AC, amniotic cell; C, control; CV, chorionic villi; EBV, Epstein-Barr virus.

ure 2E). No additional samples were identified as mosaic. However, TP-PCR analysis also identified one DNA sample (1 of 18) as a normal-range amplicon that was previously scored as an FM sample by SB analysis. Visual inspection of the TP-PCR electropherogram for the discrepant sample did not reveal the typical findings for an FM in the CGG stutter pattern or in the full-length peak (data not shown). No further follow-up of this patient was possible.

Heterozygous female prenatal DNA samples, differing by only a single repeat, were clearly resolved and correctly genotyped. Also, all amplification patterns of true homozygous (two normal *FMR1* alleles with the same size) prenatal DNA female samples were effectively and readily distinguished, because they never presented the typical uninterrupted triplet ladder profile seen with heterozygous FM samples.

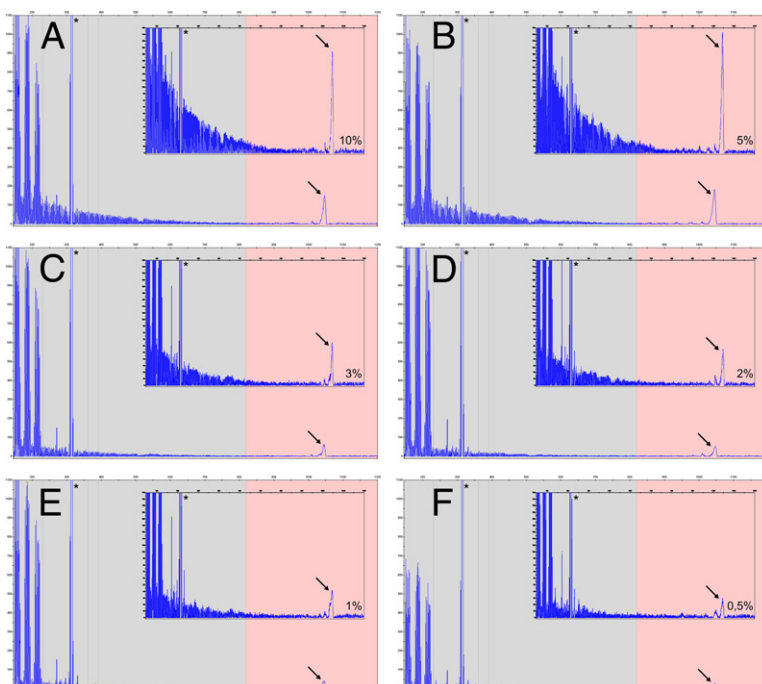
### Mosaic Samples

The sensitivity of the assay to low-level mosaicism was assessed systematically with artificial mosaic samples in

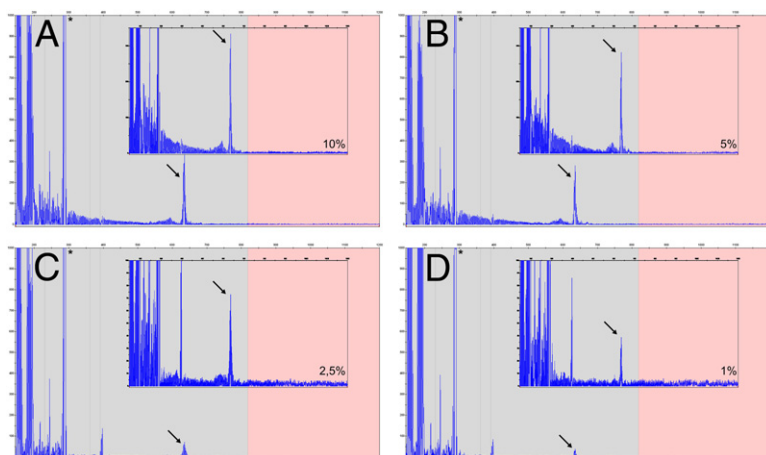
three different experiments by admixing the genomic DNA from the following: i) a normal and a PM allele, ii) a normal and an FM allele, and iii) a PM and an FM allele. Each set harbored two DNA samples mixed at different ratios.

The systematic dilution of a PM or FM male sample in a normal male *FMR1* background allowed the detection of the PM or FM signal in an amount as low as 2.5% or 3%, respectively. All amplifications of this series were performed in quadruplicate. The signal for the FM was reliably detectable, both as the full-length fragment and the typical stutter pattern. At 2%, or even at 1%, mosaic content, a weak FM signal was present, but only in half of the replicates (Figure 3). The dilution signal of a PM in a normal background is illustrated in Figure 4.

The identification of a PM (approximately 150 repeats) male allele in the presence of an FM (approximately 1100 repeats) male proved to be more challenging. Abundance as little as 10% of full-length FM products masked the presence of the PM amplicon. The presence of the PM allele was only detected when the amount of FM



**Figure 3.** Assay sensitivity to low levels of FM alleles by admixing DNA of a normal prenatal sample with DNA from an FM male fetus to give final concentrations of FM content, as indicated in each panel: 10% (A), 5% (B), 3% (C), 2% (D), 1% (E), and 0.5% (F). Repeatable detection of the FM is possible at a mosaic content as low as 3%. **Asterisk**, normal alleles; **arrow**, FM alleles. The pink background indicates the full mutation region, whereas the gray background indicates a normal, intermediate, or premutation region. This color convention facilitates visual confirmation of affected individuals.



**Figure 4.** Assay sensitivity to low levels of PM alleles by admixing DNA of a normal prenatal sample with DNA from a PM male fetus to give final concentrations of FM content as indicated in each panel: 10% (A), 5% (B), 2.5% (C), and 1% (D). Repeatable detection of the PM is possible at a mosaic content as low as 2.5%. **Asterisk**, normal alleles; **arrow**, FM alleles. The pink background indicates the full mutation region, whereas the gray background indicates a normal, intermediate, or premutation region. This color convention facilitates visual confirmation of affected individuals.

template was  $\leq 5\%$  (Figure 2F). In all cases, the FM allele was detected.

The Bland-Altman graphical approach showed a mean difference between methods (bias) of only 2.03. Except for one outlier (as described in *Discussion*), there was no trend, and variability was consistent across the Bland-Altman graph (data not shown).

## Discussion

Molecular diagnosis of FXS relies on determination of the number of CGG repeats in the *FMR1* alleles of the DNA template, and traditionally requires a combination of PCR and methylation-informative SB analysis. Amplification of PM and FM *FMR1* alleles is challenging, and several investigators have described PCR-based methods for amplification of (expanded) *FMR1* alleles since the first publication of Fu et al.<sup>4</sup> However, conventional PCR amplification cannot query long PM and FM alleles because of amplification failure, and is not informative for homozygous repeat alleles or alleles one repeat apart. Although regarded as the gold standard, an additional SB procedure for categorization and sizing is costly and tedious; in addition, it is also laborious and time-consuming, involves large amounts of high-quality DNA, and does not allow exact sizing in the PM range. These conditions are conflicting, especially in a prenatal setting in which large quantities of DNA are not always available and time is limited.

TP-PCR techniques address these limitations efficiently but were routinely only used for other triplet repeat gene targets.<sup>27</sup> Other CGG repeat PCR methods have been described for *FMR1* analysis. However, these methods can only identify the presence of an expanded allele without providing sizing information in the PM or FM range necessary for prenatal sample characterization, or their use is limited to male samples only.<sup>28–31</sup> Asuragen recently developed a simple single-tube TP-PCR protocol, to be used with a sizing PCR, to facilitate *FMR1* gene analysis of all allele repeat lengths (including large PMs and FMs of both male and female samples) in the FXS diagnostic testing workflow. In this retrospective study, we re-analyzed a large set of 67 archived clinical prenatal

DNA samples with a variety of relevant CGG repeat length numbers (including 28 normal, 9 intermediate, 12 PM, and 18 FM samples), along with a set of control samples, to evaluate this novel TP-PCR method in a diagnostic prenatal laboratory setting.

*FMR1* genotypes, ranging from normal over intermediate and PM to FM alleles, were all accurately determined in prenatal and control DNA samples, from both males and females. The Asuragen test amplified and correctly sized expansions up to 150 repeats, the largest available PM allele within our prenatal DNA sample set, compared with a previous upper limit of approximately 120 repeats with an in-house conventional PCR assay. Analyzing extra prenatal DNA samples in this size range would have been of interest, but no additional samples in the large PM range (150 to 200 repeats) were available for testing in our laboratory. This is not surprising, because PM alleles of  $\geq 90$  repeats almost always expand into a full mutation on maternal transmission.<sup>15</sup> A full-length prenatal allele of 1100 repeats was amplified and identified as an FM allele without any problems. Other investigators who have validated this system on DNA samples from leukocytes have also reported efficient amplification of large FM alleles in this size range.<sup>23,24</sup>

This assay performed with 100% specificity (95% CI, 85.0% to 100%) and 97.4% sensitivity (95% CI, 84.9% to 99.9%) in this sample set, compared with their SB analysis results. The results for all FM samples were concordant with corresponding SB analyses, except for one. This prenatal DNA sample, previously characterized with SB analysis as a large expansion on *EcoRI* digestion, was genotyped in the normal range using the novel assay. In TP-PCR analysis, it appeared that the FM allele was missed by amplification, even though the result had neither an expanded full-length peak nor evidence of expansion in the repeat primed PCR peaks by visual inspection of the electropherogram. However, the miscall could be explained by incomplete digestion with *EcoRI* endonuclease before blotting, a problem that was previously encountered and has also been reported in the literature for the *FMR1* locus.<sup>32</sup> Partially digested DNA generates additional fragments that reside at the FM location and could be misdiagnosed as an expanded allele. Unfortu-

nately, this patient was lost for further follow-up. We confirmed our normal result for this DNA using the TP-PCR assay described by Lyon et al.<sup>29</sup> Most likely, the normal result is correct and the SB FM result is because of partial digestion.

The TP-PCR method provides two types of evidence for the presence of FMs, the full-length amplicon and the number of CGG repeats. For this reason, we have confidence that no FMs were actually missed by our analysis.

To further consolidate the TP-PCR results, we decided to test all remaining ( $n = 66$ ) samples of the cohort with the second TP-PCR system.<sup>29</sup> A 100% concordance was achieved between both platforms. A 100% agreement between these two novel, but different, methods and the high consistency with the SB analysis data strongly suggest that the Asuragen results reflect true genotypes.

Although the PCR assay robustly identifies fully expanded mutation alleles, CE only provides resolution up to approximately 200 repeats. Higher than this range, all FM alleles migrated late as an aggregate peak independent of length, located at approximately 850 to 1100 bp, as shown in Figure 2D. Reporting the real size of a full mutation is questionable because it is not associated with the clinical outcome.<sup>33,34</sup> Nevertheless, this information is still often requested by clinicians.

Standard fragile X PCR analysis is not designed to discriminate heterozygotes with alleles one repeat apart from homozygotes or from heterozygotes with a missed large expansion (apparent homozygosity). These indistinguishable samples in the normal allele range usually represent more than a third of all females in the general population and must be processed by SB analysis. However, this novel TP-PCR method offers the possibility of distinguishing these samples unambiguously. Female samples with a single allele in the normal range and the absence of extension TP stutter amplifications can be assumed to be homozygous for the normal allele, thereby strongly reducing or even eliminating the need for additional SB tests.

For only a few prenatal samples, a methylation-sensitive test is required to unequivocally distinguish large PM alleles from small FM alleles (the 180 to 220 repeats range boundary), in which correct clinical interpretation still depends on methylation status. In our laboratory, we restrict reflex SB testing to PM and FM samples and we will be able to reduce the workload for SB analysis by >98% using TP-PCR analysis, a percentage supported by literature data.<sup>30,35,36</sup> Recent advances in methylation PCR protocols may allow determination of the full set of information without SB analysis in the near future.<sup>37</sup>

The assay is also sensitive to identification of mosaicism. Indeed, unique clinical mosaic samples with either a PM or an FM in a normal background were clearly identified. The analytical sensitivity of the assay was systematically addressed in repeatability experiments using artificial prenatal mosaic samples. Low-abundance replicate amplification of an FM male allele in a normal 30-repeat background was reliably possible at 3%. Other investigators reported a limit of detection of 1% for leukocyte samples.<sup>24,30</sup> In our hands, the 2% and 1% signal of prenatal mock samples was weak and only visible in

half (two of four) of the test samples. A 3% allele representation will, most probably, be undetectable in SB analysis images, and the detection of a 3% mosaicism could be achieved with at least a 250-fold lower DNA input than with what SB analysis needs.

In artificial prenatal mosaic samples, it was possible to amplify and clearly detect a PM allele present in a normal template background at a final concentration of only 2.5%. The identification of a PM/FM mosaicism is more complex. Although the presence of a large PM allele did not affect detection of the much longer FM allele, the stutter peaks of the latter largely mask a proper discovery of the PM allele. The PM peak was detectable only in samples with small amounts of FM, as low as 5% and 2.5%.

Enhanced detection of low-abundance alleles might include some challenges. Confirmation analysis with both PCR and SB techniques (limit of detection, >5%<sup>24</sup>) to exclude possible contamination problems is necessary after a partial/full mosaic result. We would also strongly advise new sampling of prenatal biological material, but this is not always feasible, even sometimes for blood samples. In general, the clinical implications of low-abundance *FMR1* expanded alleles are not clear. The results of the investigated material might not be representative of other FXS-involved tissues, because tissue-specific differences are well-known. However, by having the tools to detect them, collecting these data and reporting findings might contribute to the further understanding of this multifaceted and complex disorder.

In summary, this easy one-step single-tube TP-PCR assay is able to accurately size, to classify the CGG elements of the *FMR1* gene unambiguously in one of the four different diagnostic categories, and to support molecular diagnosis with a limited requirement for SB analysis in a diagnostic prenatal setting. It also considerably reduces the time required for sample analysis and scoring. This technique is particularly well suited to the analysis of prenatal DNA samples without the need for culturing the sample or having to delay reporting results until completion of the SB analysis. However, additional techniques are still required to interrogate the methylation status of a sample and to determine the actual size of a full-length allele.

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