

Qualitative assessment of *FMR1* (CGG)_n triplet repeat status in normal, intermediate, premutation, full mutation, and mosaic carriers in both sexes: Implications for fragile X syndrome carrier and newborn screening

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Purpose: Fragile X syndrome is caused by expansion and subsequent methylation of a CGG trinucleotide repeat in the *FMR1* 5'-untranslated region. Southern blot analysis is typically required to determine expansion size for triplet repeat lengths >200. We describe a triplet-primed polymerase chain reaction-based method using automated capillary electrophoresis detection for qualitative assessment of expanded CGG repeats. **Methods:** The assay uses triplet-primed polymerase chain reaction in combination with GC-melting reagents and substitution of 7-deaza-2-deoxyGTP for dGTP. Amplicons are resolved by capillary electrophoresis. **Results:** A distinctive pattern of tapering or "stutter" polymerase chain reaction amplification was evident on capillary electrophoresis in male and female patients harboring all expanded allele lengths examined (up to 2000 CGG repeats) and could be used to differentiate normal, intermediate, premutation, and full mutation alleles. Full mutation alleles exhibited an additional late-migrating amplicon on capillary electrophoresis. Mixing experiments demonstrated sensitivity as low as 1% for detection of the full mutation allele. In a 1275-sample concordance study against our existing polymerase chain reaction platform (with Southern blot analysis for repeat lengths ≥ 55), the triplet-primed polymerase chain reaction method exhibited 100% concordance for normal, intermediate, expanded, and full mutation alleles. This method also detected the full mutation alleles in DNA isolated from blood spots. **Conclusion:** This assay provides an accurate assessment of *FMR1* repeat status and holds promise for use in carrier and newborn screening. The method distinguishes normal homozygous females from full mutation carrying females. Although the method is not useful for accurate sizing, it supplements the classic polymerase chain reaction method and results in significant reduction in the number of Southern blot analyses required to be performed in the laboratory to accurately assess the *FMR1* genotype in all individuals. *Genet Med* 2010;12(3):162–173.

Key Words: fragile X, *FMR1* gene, CGG repeat, carrier screen, newborn screen

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Fragile X syndrome (FXS, OMIM 300624) is the most common inherited mental retardation syndrome, affecting ~1:4000 men and ~1:8000 women.¹ The disease is caused by the expansion of a trinucleotide CGG repeat in the 5'-untranslated region of the *FMR1* gene. Methylation of the expanded CGG tract leads to silencing of expression of the *FMR1* gene. The American College of Medical Genetics^{2,3} defines a normal repeat length as between 5 and 44. Intermediate alleles of between 45 and 54 repeats almost never expand to full mutations in a single meiosis. Premutation alleles are defined as 55–200 CGG. Premutation alleles of <90 repeats have a variable risk for expansion to full mutations, whereas larger premutation alleles almost always expand when inherited through a female. Full mutation alleles are defined as >200 repeats. FXS is an X-linked dominant disease. Symptoms are usually milder in affected females and may consist only of attention deficit disorder or personality disorder. Men with FXS have profoundly delayed speech, mild to moderate mental retardation, and distinctive physical and behavioral traits. FXS is diagnosed more frequently in males than females. Thirty to 40% of male carriers of a premutation allele will suffer from fragile X-associated tremor and ataxia (FXTAS) by age 50 years,^{4,5} whereas 20% percent of female premutation carriers are affected with premature ovarian insufficiency (POI).⁶

Several studies have examined the carrier frequency of expanded alleles (≥ 55 repeats) in various populations. Observed carrier frequencies for FXS vary by population with 1:113 in Israel,⁷ 1:259 in Quebec, Canada,⁸ and 1:317–1:382 in the United States.^{9,10} Because FXS is an X-linked disorder, every woman found to be a carrier is at risk for having affected offspring regardless of the genotype of her husband. In contrast, for recessive diseases, only couples in which both parents are carriers are at risk. Therefore, population-based carrier screening for FXS would yield a greater number of at-risk couples than cystic fibrosis in Caucasians (1:784 couples), Tay Sachs Disease in Ashkenazi Jewish couples (1:900 couples), or in diseases with the highest incidences where both the American College of Medical Genetics and American College of Obstetrics and Gynecology have recommended population-based carrier screening. Taking the lowest carrier frequency for FXS or 1:382 in the United States would yield approximately twice the number of at-risk Caucasian couples than cystic fibrosis and thrice the number of at-risk Ashkenazi Jewish couples for Tay Sachs disease. Interest in population-based carrier screening for FXS was shown and debated,^{11–13} and efforts to educate the public about FXS were conducted.¹⁴ We have experienced an increased demand for population-based carrier detection for

FXS.¹⁵ Surveys have established that there is interest in prenatal screening and newborn screening for FXS.^{16–18} Despite educational campaigns, FXS remains difficult to diagnose for the majority of pediatricians. Kemper and Bailey¹⁸ demonstrated that only half of the doctors surveyed were aware that FXS could affect females and that only 28% of the surveyed doctors knew that premutation carriers might have symptoms of POI or FXTAS.

A recent study¹⁹ has shown that the age of diagnosis remains >3 years of age. Often, another sibling has been born by the time a proband with FXS is diagnosed. Newborn screening could lead to diagnosis in the newborn period and allow early intervention and family planning for future pregnancies. Population-based prenatal carrier detection could be cost-efficient²⁰ and avoid the birth of affected children. Until recently, however, the technical difficulties of performing newborn screening²⁰ and population-based carrier detection precluded offering them to the general population. Large expansions are refractory to polymerase chain reaction (PCR) amplifications. Thus, strategies for newborn screening were limited to screening only males and suffered from high false-positive rates,^{21–23} whereas strategies for population-based carrier screening for women required Southern blot analysis in addition to the PCR for each individual.

Recent advancements have overcome the technical limitations of FXS analysis. Recent studies have described new methods or improved on existing methods for the qualitative detection of expanded CGG repeats.^{21–24} We devised a method of capillary Southern analysis²⁵ that was capable of identifying all female carriers of FXS. However, this technique was not suitable for newborn screening.

Several investigators have published PCR-based methods for detecting full CGG expansions. The first PCR assay to successfully amplify the full CGG expansion was described by Fu et al.²⁶ The method used 7-deaza-2-deoxyGTP in addition to dATP, dCTP, dTTP, and dGTP and amplicons internally labeled with ³²P-dCTP. This approach could detect up to 200 CGG repeats. Since then, attempts by various laboratories to improve on and automate this original method to detect larger

CGG expansions or detect methylation status of CGG repeats was developed. Pergolizzi and coworkers^{27–31} used deaza-dGTP in PCR in place of dGTP, however, because amplicons with deaza-dGTP cannot be stained by ethidium bromide, this method requires Southern blot analysis of amplicons, thus limiting its utility. The use of triplet-primed PCR (TP-PCR) for the detection of CGG expansion in *FMRI* was initially described by Daniels et al.,³² who coined the term “repeat primer PCR.” This technique involved nested PCR, using a reverse PCR primer having seven CCG repeats mixed with the forward primer of Fu et al.,²⁶ to identify full mutations from as little as 20–40 buccal cells. The method showed 84% sensitivity of detection of full mutations.³² Other approaches for the detection of expansions, methylation status, or expression of *FMRI* gene included the use of antibodies against FMR1 protein,³³ interrogation of upstream regions to interpret methylation status of the CGG repeats in the *FMRI* gene,^{34,35} methylation-specific PCR,³⁶ or methylation-specific PCR incorporating TP-PCR for identification of methylated CGG alleles in the *FMRI* gene.^{37,38} Recently, a modified Southern analysis method was used for rapid qualitative population-based screening of *FMRI* gene.²⁵ Two recent enhancements to the classic PCR methods were developed as shown using a combination of betaine and TP-PCR.^{21,22}

By using the wealth of knowledge in the literature, we were able to design a simple, rapid PCR-based method with capillary electrophoresis for the qualitative detection of expanded CGG repeats in the *FMRI* gene. The method we describe is a single-tube assay that detects expansions and full mutations in both females and males, including mosaics. The method complements the classic PCR method by reducing the number of Southern blot analyses required to be performed as the method can be used to distinguish normal homozygous females from full mutation carrier females (as shown in Fig. 1). The method is robust, accurate, and appropriate for both prenatal population-based carrier screening and newborn screening programs.

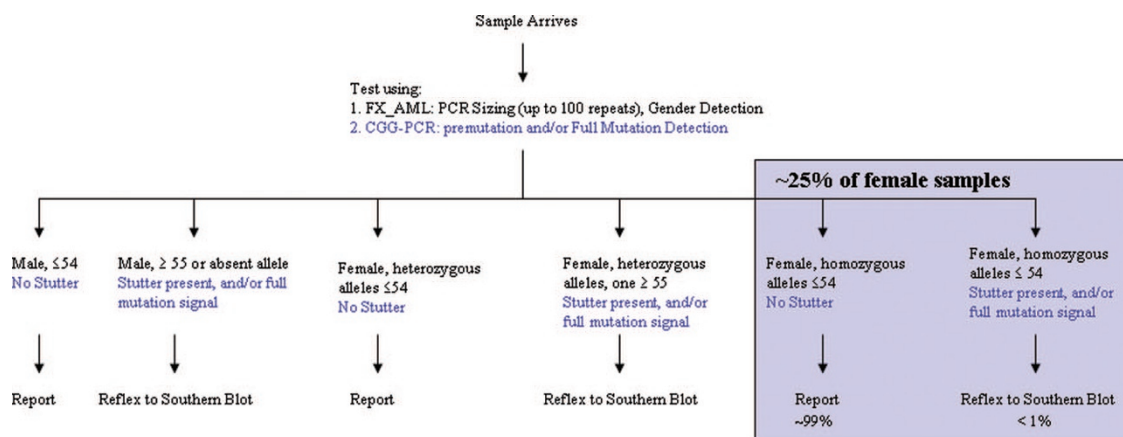


Fig. 1. Testing algorithm for samples submitted for fragile X testing. Samples received will be tested using two PCR reactions. Reaction 1 (in black) is for sizing normal, intermediate and small permutations and for gender determination,²⁵ whereas Reaction 2 (CGG-PCR in blue) will detect the presence of expanded alleles and full mutations, without sizing. Samples showing evidence of stutter or full mutation amplification will be reflexed to Southern blot analysis for methylation status and sizing. As shown in the figure, the incorporation of the triplet-primed PCR method described here significantly reduces the number of Southern blot analyses performed in the laboratory as the method clearly distinguishes normal homozygous females, which in our experience represent ~99% of all female samples,²⁵ from full mutation carriers.

MATERIALS AND METHODS

Clinical samples

Residual archived DNA from patient samples already processed for fragile X analysis using our existing assays were de-identified and used in the development of the new method. DNA was extracted using the Qiagen M96 robot and reagents, Qiagen Genra robot and reagents, and Qiagen 9604 reagents (Qiagen, Carlsbad, CA).

Assay conditions

The PCR step of this assay uses the 7-deaza-2-deoxy guanosine triphosphate (deaza-dGTP) in place of dGTP, a combination of Q-solution and dimethyl sulfoxide (DMSO) in the PCR mix, and an optimized cycling program. The Roche FastStarTaq kit (Roche Applied Science, Indianapolis, IN) reagents were used in the PCR mix. The PCR mix contained 1× PCR buffer; 2 mM MgCl₂; 6% DMSO (Sigma); 1.7% Q solution (Qiagen); 0.2 mM each of deaza-dGTP, dATP, dCTP, and dTTP; 1 unit of FST polymerase; and 0.6 μM each of the forward primer (FMR1F), reverse primer 1 (FMR1R) or 2 (FMR1CCGR), and M13 reverse linker primer:

FMR1F: TGAAAAACGACGGCCAGTGCTCAGCTCCG-TTTCGGTTTCACTTCCGGT;

FMR1R: CAGGAAACAGCTATGACCCTCGAGGC-CCAGCCGCCGCCGCC;

FMR1CCGR: CAGGAAACAGCTATGACCCGCCGCC-CCGCC; and

M13 reverse linker primer: CAGGAAACAGCTATGACC.

The underlined sequence of FMR1F is the C primer of Fu et al.²⁶; the underlined sequence of FMR1R is from bases 13882–13904 of GenBank accession number L29074.1, corresponding to the junction sequence of the 3'-end of the CGG repeat tract and flanking sequence; and the underlined sequence of FMR1CCGR represents almost 4 units of reverse CCG repeats that hybridize randomly across the CGG repeat tract (TP-PCR). The M13 reverse linker primer corresponds to the linker sequence of reverse primers and helps to boost the signals of the fragments amplified. Three microliters of genomic DNA (10–150 ng totally) was added for a final PCR reaction volume of 15 μL.

The cycling program, performed on an ABI 9700 thermal cycler, was started by incubating the mix at 98°C for 10 minutes to activate the polymerase, followed by 10 cycles of denaturation at 97°C for 35 seconds, annealing at 64°C for 2 minutes, and extension at 68°C for 8 minutes, followed by 25 cycles of denaturation at 97°C for 35 seconds, annealing at 64°C for 2 minutes, and extension at 68°C for 8 minutes 20 seconds (with 20 seconds extension each additional cycle). For all 35 cycles, the ramp rate was adjusted to 64% for the denaturation and extension steps (ramping up at 0.5°/second), and to 25% for the annealing step (ramping down at 0.5°/second).

Two microliters of the PCR product (sometimes diluted 1:5 or 1:10 in H₂O) were mixed with HiDi Formamide and Rox 1000 size standard (Celera), and samples were injected on an ABI 3730 DNA Analyzer equipped with a 36-cm capillary loaded with Pop-7 polymer (Applied Biosystems) at 3 V for 7 seconds. Data were analyzed using GeneMapper software (Applied Biosystems). The Rox 1000 size standard, although not used for accurate sizing, is helpful in locating full mutation peaks, which are usually located around 1050 bases on the electropherogram. Rox 600 (Applied Biosystems) or MapMarker1000 (Bioventures, Inc., Murfreesboro, TN) can also be used. Although full mutations ≥200 repeats will have a com-

position of at least 770 bp using our primers, all full mutation alleles examined appear at around 1050 bases. That is because of the limitation of the capillary. We used a 50-cm capillary as well to determine whether we can size better the full mutation peaks, but all full mutation peaks appeared around the 1050 bases.

A panel of anonymized, previously genotyped samples were used for initial development of the PCR method. The panel included a homozygous normal female (28/28 repeats), a heterozygous normal female (18/21), a premutation female (31/64), full mutation females (19/450 and 29/530), a normal male (28), a premutation male (74), and mosaic full mutation male (105/350). Once conditions for the PCR were established, a larger set of 47 anonymized, previously genotyped samples (Table 1), weighted toward full mutation carriers (male or female) was used to test the robustness of the assay.

To evaluate assay sensitivity to mosaicism, we made three DNA sets using archived, previously genotyped samples, each set harboring two DNA samples mixed at different ratios. The first set contained DNA samples from two males, one from a normal male with 28 CGG repeats, and one from affected male with 850 CGG repeats; set B contained two males, a normal male (28 CGG) and affected male (853); and set C contained two female samples, a normal (28/28) and an affected female (19/450).

Detection from blood spots

Whole blood from previously genotyped, residual samples was spotted on blood cards. Blood spots were generated using the BSD1000 GenePunch instrument (BSD Robotics, Australia). DNA was extracted using BioSprint 96 DNA Blood Kit reagents from Qiagen.

RESULTS

Assay development

The concept of the new method is similar to the TP-PCR methods initially described for fragile X³² and then applied for other triplet repeat-containing genes.^{39,40} Figure 2 shows the location of FMR1F and FMR1R primers and the concept of the method. Two reverse primers were tested, each harboring almost four CCG-units; the difference between FMR1R and FMR1CCGR is that the FMR1R primer included *FMR1* sequences downstream of the CGG repeat tract, with the concept that presence of both triplet CCG sequences and downstream sequences might facilitate unwinding of the CGG secondary structure at the junction point and could also provide better specificity for *FMR1* sequences. The FMR1CCGR primer contains only triplet CGG repeats in addition to the linker. Daniels et al.³² used a 7-CCG repeat primer, whereas our triplet primer contains almost four CCG units. FMR1R contains both CGG triplets in addition to *FMR1* gene-specific nonrepeat sequences. Once the initial round of PCR is complete, longer templates are generated with more CCG units that can serve themselves as primers and templates for further cycling rounds (Fig. 2).

We initially attempted to devise a PCR mix for amplification of all normal, premutation, and full mutations of the CGG tract by formulating various combinations of mixes using Roche Expand polymerase PCR mix buffer 2 or 3 and Roche High-GC PCR mix, and incorporating various additives at different concentrations including Roche Q-solution, DMSO, HiDi formamide, Tween-20, or combinations thereof. We first settled on the use of Roche Expand kit without the addition of deaza-dGTP. We then tested the use of the Roche FastStarTaq kit

Table 1 Panel of previously *FMR1*-genotyped samples used to test performance of the triplet-primed PCR/capillary electrophoresis-based assay

Sample	Previous genotype call	Previous result	Notes from Southern blot analysis	Classification by triplet-primed PCR/CE
1	23/800	FF		FF
2	29/590	FF		FF
3	687	FM		FM
4	19/88	PF		PF
5	104	PM		PM
6	82	PM		PM
7	28/400	FF		FF
8	506–1006	FM		FM
9	29/286	FF		FF
10	28/98	PF		PF
11	660	FM		FM
12	400	FM		FM
13	29/29	HF		No expansion
14	696	FM		FM
15	67/500	FM		FM
16	28/29	HetF		No expansion
17	78/2000	FM	78 (60%, unmethylated)/2000 (40%, methylated)	FM
18	45	IM		No expansion
19	QC blank	No amplification		No amplification
20	23/400–1250	FF		FF
21	650	FM		FM
22	9850	FM		FM
23	45/600	FF		FF
24	30/550	FF		FF
25	30/49	IF		No expansion
26	713	FM		FM
27	500	FM		FM
28	400	FM		FM
29	30/520	FF		FF
30	19/19	HF		No expansion
31	500	FM		FM
32	30/30	HF		No expansion
33	32/73	PF		PF
34	31/480	FF		FF
35	170–240	FM		FM
36	400	FM		FM
37	600	FM		FM
38	200–500	FM	200 (10%, unmethylated)/200–500 (90%, methylated)	FM

(Continued)

Table 1 Continued

Sample	Previous genotype call	Previous result	Notes from Southern blot analysis	Classification by triplet-primed PCR/CE
39	420	FM		FM
40	30/74	PF		PF
41	853	FM		FM
42	30/313	FF		FF
43	606	FM		FM
44	433	FM		FM
45	140/(200–400)	FF	140 (5–10%, unmethylated)/200–400 (90–95%, methylated)	FF
46	300–500	FM		FM
47	590–1143	FM		FM
48	666	FM		FM

No expansion includes normal and intermediate alleles.

HF, normal homozygous female; HetF, normal heterozygous female; IF, intermediate female; PF, premutation female; FF, full mutation female; NM, normal male; IM, intermediate male; PM, premutation male; FM, full mutation male; CE, capillary electrophoresis; PCR, polymerase chain reaction.

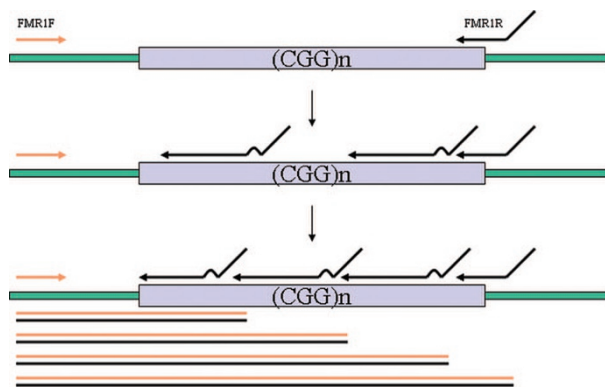


Fig. 2. Schematic representation of the triplet-primed PCR method. Forward and reverse primers are shown. The reverse primer hybridizes at the junction of 3'-end of CGG tract and down stream sequences, but it can also hybridize randomly across the CGG tract. After initial rounds of PCR, extended reverse primers can themselves serve as primers. This results in various PCR product sizes giving the "stutter" pattern on electrophoresis on the ABI 3730.

reagents and found better performance in terms of signal intensity under identical cycling conditions (data not shown). The inclusion of the M13 linker in the reverse primers also helped boost the signal by approximately 30%. The incorporation of ramping in the cycling program, similar to the process we described previously in a different assay,⁴¹ helped further boost signal amplification. Ramping is a PCR cycling parameter that allows the user to control the rate at which the thermocycler moves from one temperature to another. The default ramping parameter is as fast as the machine is capable of changing temperature. Slowing the ramping rate gives more time for primers to bind and rebind to their substrate. In this particular assay, controlling the ramping rate is critical to the robustness of the assay.

Other primers tested include primers containing 7-CCG units, 10-CCG units, and others. All primers showed stutter amplification indicative of presence of premutation or full mutation. All the above assay-optimization experiments and data are not shown but are available on request.

Development of a new triplet-primed PCR for qualitative assessment of FMR1 CGG expansion status

After identifying standard conditions for the PCR mix, we were able to detect a stutter pattern of amplification whenever a premutation or a full mutation was present in a DNA sample. However, because of the complexity of amplifying the CGG tract, we decided to test using deaza-dGTP in the PCR reaction in place of dGTP. Figure 3 shows the effect of addition of deaza-dGTP to the reaction mix of a heterozygote female carrier (29/530). Increasing the concentration of deaza-dGTP not only boosted the signal from stutter amplification but also led to the appearance of a peak that migrated very late on the ABI 3730 capillary and was evident only when a full mutation was present (Figs. 3 and 4). Because the capillary instrument does not rely on ethidium bromide staining to visualize DNA, we are able to detect the presence of a premutation and a full mutation in single PCR reaction containing deaza-dGTP by scrutinizing stutter and full mutation signals. It should be noted that the method does not size alleles correctly. The method, being qualitative, is used as screening method. By using this method, we were able to distinguish homozygous normal females from heterozygous full mutation carrier females in an assay sufficiently robust for population-based carrier screening.

We tested various sizes of CGG triplet repeats by using a panel of eight anonymized, previously genotyped samples. In Figure 4, the gray-shaded area represents the range of normal and intermediate allele CGG repeats numbers (≤ 54), and the pink-shaded area represents expanded CGG repeats (either premutation or full mutation alleles). As shown in Figure 4, normal and intermediate alleles are present only in gray part of the figure, whereas premutation and full mutation alleles (with ≥ 55 CGG repeats) are present in the pink area of the electropherogram that denotes expanded alleles. Normal females and normal

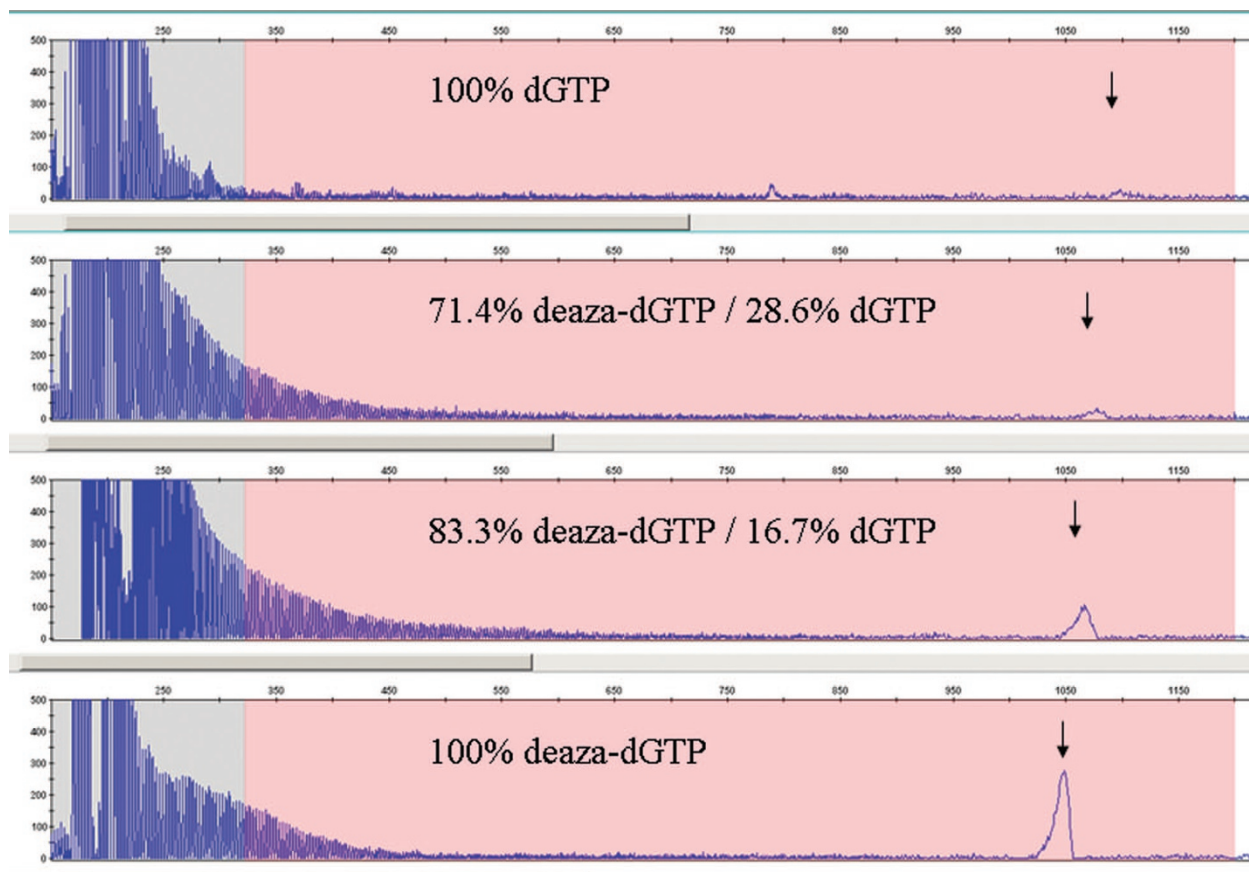


Fig. 3. Effect of deaza-dGTP on full mutation detection. PCR master mixes were formulated with shown concentrations of 7-deaza-dGTP and dGTP. An affected female DNA sample (19/450) was used in the PCR reactions. Normal and intermediate alleles migrate in the gray-highlighted zone of the electropherogram (< 55 CGG repeats), whereas premutation and full mutation alleles are present in the pink-highlighted area of the electropherogram (≥ 55 CGG repeats). As shown, tapering stutter amplification and full mutation signal intensities increase with increasing deaza-dGTP concentration. All electropherograms are zoomed to 500 relative fluorescence units (RFU) to show stutter and full mutation amplification. Full range signal intensity ($> 20,000$ RFU) figures are available on request. The arrow shows the signal for full mutation. Replacing dGTP with deaza-dGTP influences the migration of the amplified full mutation fragment.

males demonstrate alleles only in the gray area of the electropherogram, whereas premutation males and females have alleles in the pink area, with stutter amplification before the actual premutation peak. Full mutation carrying males and females display, in addition to normal allele in females, a tapering stutter amplification followed by a signal at the end of electropherogram, which is present only when a full mutation is present. Most significantly, normal homozygote females can be distinguished from full mutation carrier females using this method (Fig. 4).

To establish assay robustness for the detection of the CGG status, we tested the assay on previously genotyped DNA samples. The individual performing the test was blinded to the status of CGG repeats and the results scored by a second individual also blinded to the previously determined genotype. The panel of 47 anonymized samples contained homozygous females ($N = 3$), normal heterozygous females ($N = 1$), intermediate female ($N = 1$), premutation females ($N = 4$), and full mutation females ($N = 11$), in addition to intermediate male ($N = 1$), premutation male ($N = 2$), and full mutation males ($N = 24$; Table 1). All samples were identified correctly,

demonstrating the capability of the new method in identifying presence of premutations and full mutations in all conditions.

Detection of mosaics and assay sensitivity

One concern for FXS analysis is the ability to detect mosaic alleles of permutation and full mutations that are sometimes identified using sensitive Southern blot analysis. Figure 4 shows the results of a mosaic male with a premutation allele of 105 repeats and a full mutation allele of 350, identified from a blood sample. Table 1 and Figure 5 also show samples with mosaic full mutations including two male samples (samples 15 and 17) and two female samples (samples 2 and 7). Sample 17, with mosaic for 78 and 2000 alleles, showed amplification signal for the longest full mutation, which we have in our archived collection. The signal for the full mutation was weak but detectable. Previous Southern blot analysis of this sample showed the unmethylated premutation allele accounted for 60% of alleles, whereas the methylated full mutation 2000 repeat allele accounted for the rest in this affected male sample. The method also detected mosaic premutations in full mutation carrier fe-

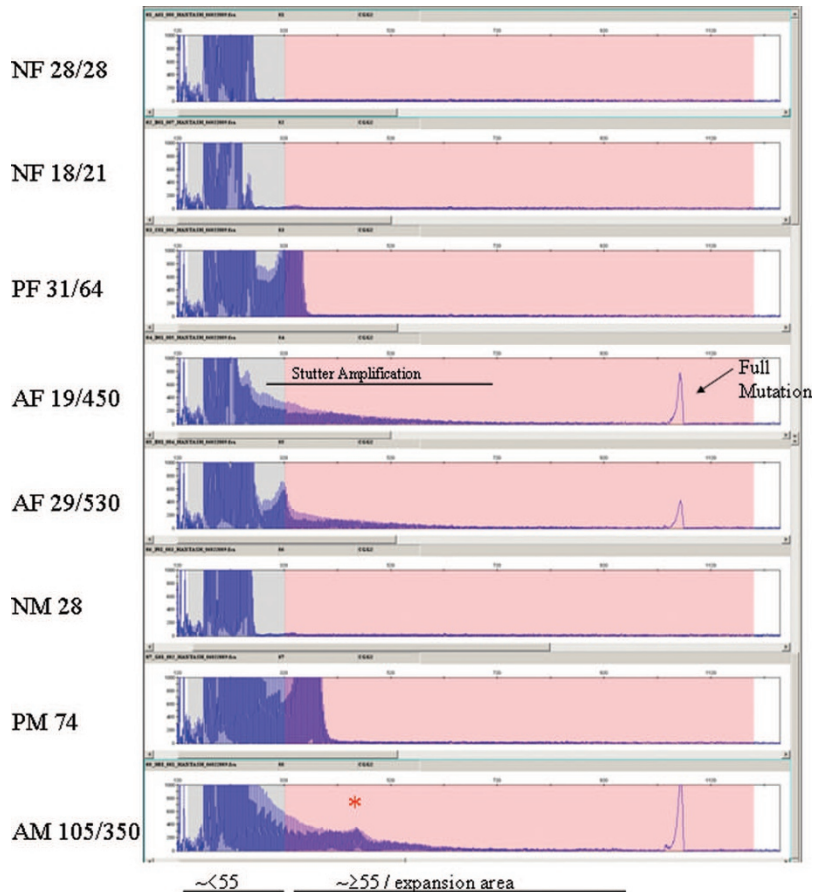


Fig. 4. Example of results obtained from normal, carrier, and affected female and male patients. The status of alleles in each patient is shown. The assay distinguishes between normal females or males, including normal homozygous females, from full mutation carrier females or males. All electropherograms are zoomed to 1,000 RFU to show stutter and full mutation amplification. Full range signal intensity (>20,000 RFU) figures are available on request. The last panel shows a sample from a mosaic male with 105/350 alleles. The 105-repeat allele is marked by an asterisk.

males (Fig. 5). These results show that the new PCR method is able to detect mosaic alleles in both males and females.

We also tested the sensitivity of this assay to low level mosaicism by performing artificial mixing experiments using DNA samples from males and females with normal and full mutations, thereby creating artificial mosaics. We constructed three DNA sets using archived, previously genotyped samples, each set harboring two DNA samples mixed at different ratios; set “A” contained DNA samples from two males, one from a normal male with 28 CGG repeats and one from affected male with 850 CGG repeats; set “B” contained two different male DNAs, a normal male (28 CGG repeats) and affected male (853 CGG repeats), and set “C” contained two female samples, a normal (28/28 repeats) and an affected female (19/450 repeats).

Figure 6 demonstrates the results from these samples artificial mosaic series. For male DNA, the full mutation was detectable at levels of 5% in set “A” and 10% in set “B.” As shown in Figure 6, the intensity of the stutter amplification and the full mutation signals increase as the fraction of full mutation DNA increases. In set “C,” from mixed female samples, the assay is capable of detecting as low as 1% mosaic full mutation content in female DNA that contained alleles of three lengths (19, 28, and 450 repeats), where the smaller, normal alleles would have been favored during PCR. At 1% mosaic content, a

weak signal from a full mutation is present (Fig. 6C), whereas stutter amplification is appearing reliably at 10% content of the full mutation. On average, we were able to detect full mutation expansion, by both the stutter amplification and full mutation signal detection, at levels as low as 5–10% in both males and females.

Concordance study

In developing any new platform for assays with anticipated high volume, our laboratory performs a 1000-sample concordance study to test and compare assay performance with existing platforms.^{25,42–44} We, therefore, evaluated the new method by performing a platform comparison of 1275 consecutive, anonymized whole blood samples submitted for fragile X analysis using our current platform composed of a two-step process: a PCR incorporating gender marker and a reflex to Southern blot analysis for any sample with an expansion length of 45 repeats or above and for any apparent homozygous females. There was 100% concordance with the existing method. As tabulated in Table 2, the series contained a total of six patients (three female and three male) harboring full mutations.

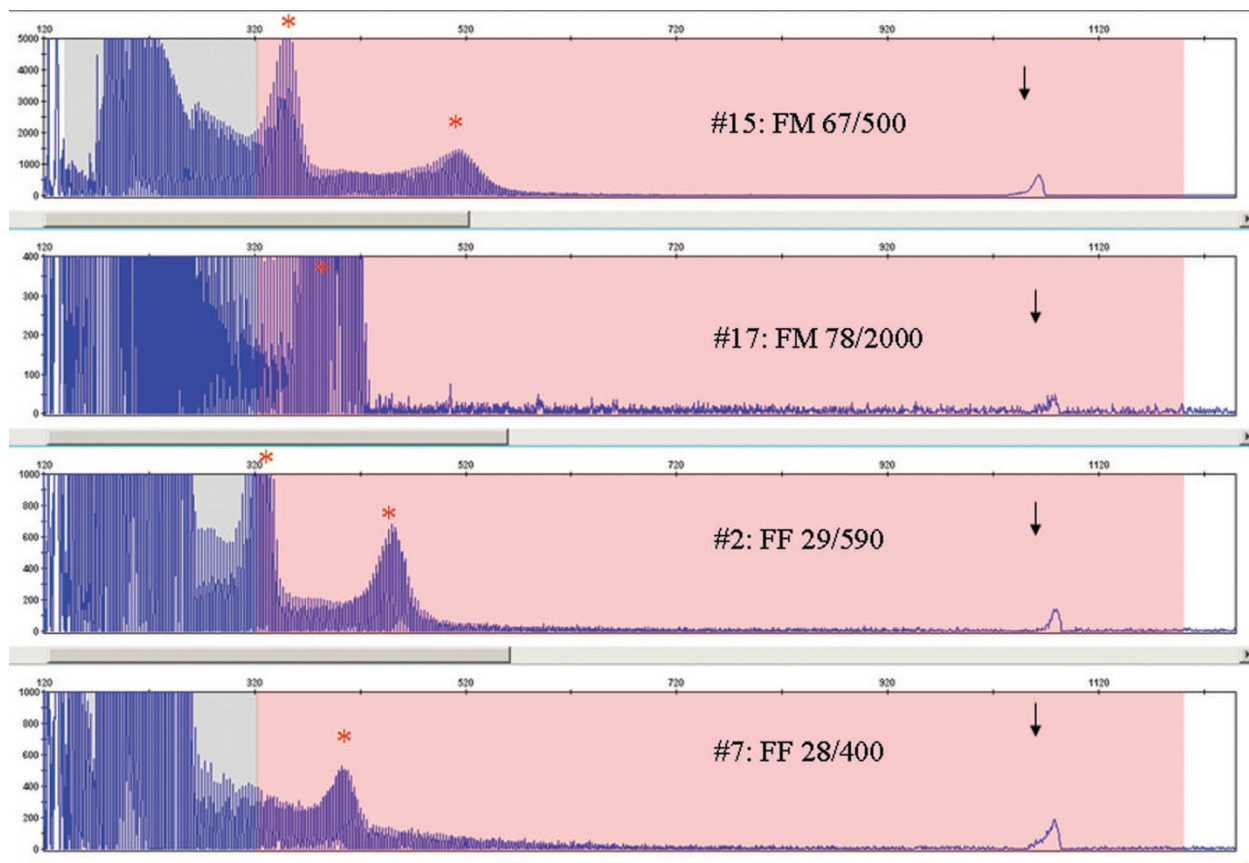


Fig. 5. Detection of mosaic males and females. Samples 15 and 17 from male patients and samples 2 and 7 from female patients (Table 2) with mosaic premutation and full mutations are shown. Sample 17 shows weak signal for full mutation. It is not clear whether the DNA quality or the level of mosaicism of this archived sample affected robustness of amplification of the full mutation, but the signal for full mutation is present, albeit weakly. Other samples show robust stutter and full mutation amplification. Premutations are marked by an asterisk, whereas mutations are marked by an arrow.

Detection from blood spots and saliva

Once we had technically validated the assay on peripheral blood, we attempted two alternate sample types, saliva and newborn blood spots. As we did not have access to prespotted Guthrie cards from FXS patients, we first spotted residual blood from previously genotyped patients on Guthrie cards. Blood spots were punched from each card using BSD1000 GenePunch instrument to generate two punches (3.2 mm each) per patient, and DNA was extracted using Qiagen BioSprint reagents. DNA from blood spots was analyzed by the new PCR method. A total of 37 samples containing the varying *FMR1* CGG expansions in males and females were tested (Table 3). The series contained 14 samples with intermediate repeats, nine samples with pre-mutations, and four samples with full mutations (as determined by Southern blot analysis), one female (20/500 repeats) and three males (300–700; 1250; 1250). Figure 7 shows an example of the results obtained. All the samples were genotyped by the new qualitative method correctly as harboring normal, intermediate, premutation, or full mutations.

DNA prepared from saliva can be successfully amplified in the new assay, but we do not have any samples from affected individuals or carriers, hence, we cannot confirm the ability to detect full mutations and pre-mutations in saliva reliably at this

time. We have no reason to believe that there will be any problems however.

DISCUSSION

The method we describe here is a rapid, single-tube method capable of detecting the full spectrum of *FMR1* CGG repeat instability, up to 2000 repeat units, which was the largest sample that we possessed in our archived DNA collection. The method also allowed detection of mosaic males and females from whole blood. The method was also capable of detecting pre-mutations and full mutations from blood spots, which would facilitate newborn screening. The method is capable of rapid turn around time as it does not require Southern blot analysis. We performed an extensive technical validation of the method by testing >1300 samples, with various repeat sizes, and all samples with full mutation alleles, regardless of sex, were correctly identified. This is important, as the true technical impediment for implementing population-based carrier screening or newborn screening using prior methods has been distinguishing truly homozygous females from carrier females.

The method we describe is similar to the nested-repeat primer PCR method developed previously for attempting fragile

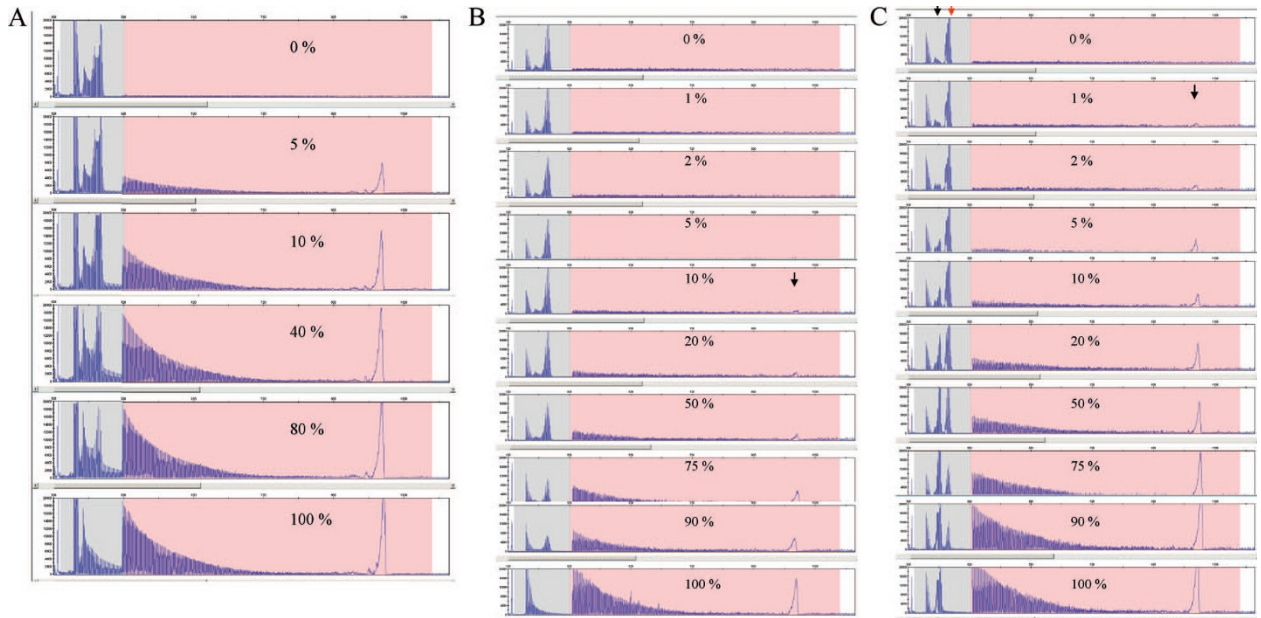


Fig. 6. Assay sensitivity to low levels of full mutations. DNA samples from normal males or females were mixed with affected males or females to give final concentration of full mutation content as shown in each panel. The gray-highlighted region of the electropherograms shows full scale RFU to show normal alleles, whereas the mutant, pink area is shown at 1000 RFU to show mutant and stutter amplification. A, Set A from mixed normal male (28 repeats) and affected male (850 repeats) shows detection of full mutation at 5% “mosaic” content. B, Set B from mixed normal male (28 repeats) and affected male (853 repeats) shows “mosaic” detection from males at 10%. Note the disappearance of the normal 28-repeat allele (black arrow) as the percentage of full mutation is increased. C) Set C from mixed normal homozygous female (28/28 repeats) and affected female (19/450 repeats) shows detection of full mutation in female DNA at 1% “mosaic” content, although the full mutation allele represents only one of the four alleles present in the mix and at very low amounts in the low “mosaic” content. The presence of the 19-repeat allele (black arrow) and 28-repeat allele (red arrow) is shown.

Table 2 Concordance of triplet-primed PCR/capillary electrophoresis method with existing PCR/Southern blot method for determination of *FMR1* trinucleotide expansion status

Classification	N
No expansion (HF, HetF, NM)	1237
Intermediate female	15
Intermediate male	7
Premutation female	10
Premutation male	0
Full mutation female	3
Full mutation male	3

HF, normal homozygous female; HetF, normal heterozygous female; NM, normal male; PCR, polymerase chain reaction.

X screening from single cells.³² In that report, the authors noted that, in samples with full mutations, “the fragile X DNA produce a more complex band pattern, with extra bands of both higher and lower molecular weight.”³² However, in that method, distinguishing full mutation allele from normal one was not very clear using agarose gels because of weaker signal and background fluorescence, which lead to assay sensitivity of 84%

using DNA from 20–40 buccal epithelial cells.³² However, the authors of that report were attempting to design a method for analysis of expansions from single cells for preimplantation genetic diagnosis³² and did not report testing the method on whole bloods. In our method, we used an optimized PCR mix and cycling program without nested amplification. Analysis on capillary electrophoresis instrument using fluorescently labeled amplicons facilitated identifying stutter amplification and full mutation signals using a single PCR tube. We achieved 100% sensitivity, as every sample we tested with full mutation was identified, whether male or female.

Our testing paradigm calls for two PCR reactions per patient run simultaneously (Fig. 1). One reaction, described previously,²⁵ will allow accurate sizing of repeat number in the normal, intermediate, and small premutation zone and, also, gender identification/confirmation. The second PCR reaction is the method described here for identification of presence of larger premutations and full mutations. The combination of two PCR reactions per patient yields results for both males and females, and this would serve the purpose for carrier and/or newborn screening.

For newborn samples showing evidence of full mutation, further follow-up will be recommended, as the amount of available DNA from blood spots will not be sufficient to perform Southern blot analysis. This is similar to the approach of cystic fibrosis newborn screening programs, where a positive immunoreactive trypsinogen test prompts secondary tests of sweat chloride and/or cystic fibrosis transmembrane conductance reg-

Table 3 Samples used for blood spots

Sample	Genotype ^a	Previous result	Classification by triplet-primed PCR/CE ^b
1	29/46	IF	No expansion
2	23/55	PF	PF
3	28/66	PF	PF
4	31/53	IF	No expansion
5	20/500	FF	FF
6	60	PM	PM
7	46	IM	No expansion
8	53	IM	No expansion
9	28/28	HF	No expansion
10	31/45	IF	No expansion
11	30/49	IF	No expansion
12	29/29	HF	No expansion
13	19/54	IF	No expansion
14	30	NM	No expansion
15	34/50	IF	No expansion
16	300–700	FM	FM
17	44/57	PF	PF
18	32/45	IF	No expansion
19	50	IM	No expansion
20	30/51	IF	No expansion
21	29/70	PF	PF
22	1250	FM	FM
23	46	IM	No expansion
24	30/30	HF	No expansion
25	29/78	PF	PF
26	29/29	HF	No expansion
27	30/64	PF	PF
28	1250	FM	FM
29	35/59	PF	PF
30	30/30	HF	No expansion
31	30/66	PF	PF
32	30/30	HF	No expansion
33	29/29	HF	No expansion
34	30/30	HF	No expansion
35	30/30	HF	No expansion
36	32/47	IF	No expansion
37	30/49	IF	No expansion

^aGenotype is listed as the number of triplet repeats on each *FMR1* allele.
^bNo expansion includes normal and intermediate alleles.
 HF, normal homozygous female; IF, intermediate female; PF, premutation female; FF, full mutation female; NM, normal male; IM, intermediate male; PM, premutation male; FM, full mutation male; CE, capillary electrophoresis; PCR, polymerase chain reaction.

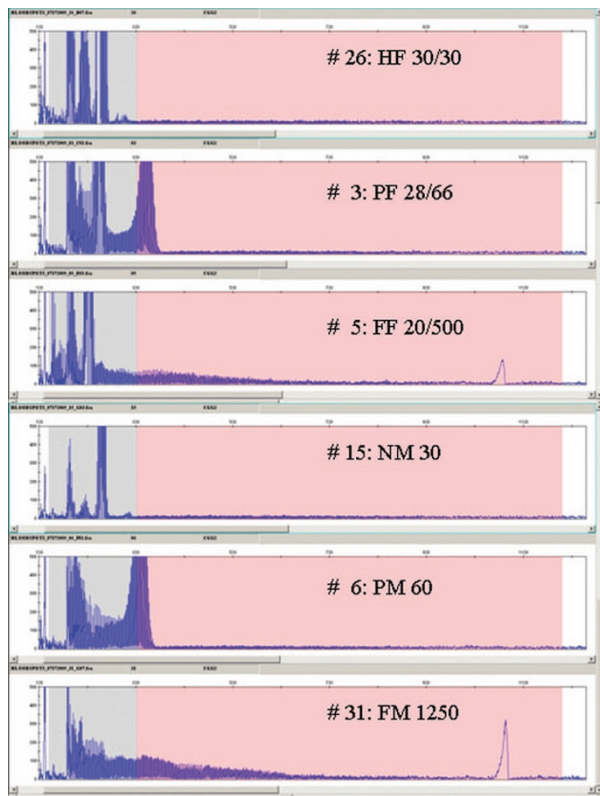


Fig. 7. Detection of normal, premutation, and full mutation carriers from blood spots. Blood samples from samples (given in Table 3) were spotted on filter cards and punched using BSD1000 GenePunch. DNA from two spots was extracted and PCR performed. Number in each panel corresponds to numbers in Table 3. Also shown is allele content in each sample. The new method can qualitatively identify full mutations and distinguish normal females, including homozygous females, from carrier females.

ulator mutation identification. Although a recent report³⁵ described the use of methylation-specific PCR for detection of *FMR1* promoter methylation and FXS incidence in newborns from the state of Georgia, the method, although promising, is laborious, even with pooling of samples, as it relies on the multistep bisulfite conversion of DNA, and the method can miss female samples carrying full mutations, because some full mutation carrying females displayed methylation levels within the range of normal females.³⁵

The method described here is not intended as a stand alone test for diagnostic purposes. Rather, the method is used as a qualitative assessment for repeat status, which would prompt, on a positive finding of a premutation or full mutation, further follow-up of methylation status and accurate sizing. Although the sizing of full mutation has little or no effect on phenotype presentation,⁴⁵ it is important to determine methylation status of the CGG repeats, and therefore Southern blot analysis will be performed. We are developing an alternative method for assessing methylation status without performing a Southern blot using methylation-sensitive restriction digestion followed by PCR using primers flanking the restriction site. Only methylated DNA yields PCR product under these conditions. Because the

actual size of the expanded repeats has little clinical relevance,⁴⁵ a combination of these techniques has the potential to eliminate the need for Southern blotting altogether in fragile X analysis.

Even without the new methylation assay, using the technique described in this article will drastically reduce the number of Southern blots necessary in a FXS screening program. In our laboratory, only approximately 2% of males and 0.5% of females have expanded alleles.¹⁴ Therefore, approximately 99% of Southern blots can be eliminated in a program screening an equal number of males and females.

We have described a method that overcomes the technical block to instituted programs of newborn screening or population-based carrier detection for FXS. Now the medical, ethical, and legal issues can be investigated and discussed as suggested by Bailey et al.⁴⁶ The issues have not changed dramatically since the early 1990s, when carrier/prenatal screening was proposed and debated,^{11–13,47–49} in terms of the incomplete and variable presentation of the FXS phenotype in full mutation carrier females, and of the unpredictable presentation in mosaic males. Another issue is related to stigmatizing carriers of pre-mutations, biases in interaction by peers, or overprotection by parents.⁴⁶

Questions will arise such as would having such information, if newborn screening is implemented, be beneficial for early intervention, or would this cause certain perceived biases against such individuals? Would informing parents of children who happen to harbor the premutation alleles in the *FMRI* benefit or harm the child?

Consenting to prenatal testing in the span of a routine office visit during pregnancy or to newborn screening at hospital admission for delivery will certainly not be sufficient to understand the full impact of such screening. It certainly will be a challenge to educate the public about the complexities of fragile X testing, as the issues involved are not only just related to mental retardation but also have neuropsychologic (memory and function), physical (FXTAS), and reproductive (POI) implications, in addition to involvement of FXS in autism, as was reviewed recently.⁴⁶ Several recent studies have discussed prenatal and newborn screening issues and attitudes,^{17,50} and more studies will be needed if, and when, population and/or newborn screening for FXS is implemented. It is likely that a wide effort to educate the public, during pregnancy at the latest, will be needed to allow enough understanding of the issues, and it might be beneficial to allow voluntary participation in such programs initially until better understanding of implementing such programs is achieved.

In summary, we designed a simple, rapid, high throughput, single-tube assay for the qualitative detection of CGG repeat status in both males and females, suitable for prenatal carrier screening and for newborn screening programs. A recommendation from American College for Medical Genetics or American College of Obstetrics and Gynecology for implementing prenatal screening will likely await further studies of performance of such new tests.

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