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Performance Evaluation of Two Methods Using Commercially Available Reagents for PCR-Based Detection of *FMR1* Mutation

AQ: 10

AQ: au Jane S. Juusola,* Paula Anderson,* Fernanda Sabato,* David S. Wilkinson,* Arti Pandya,*† and Andrea Ferreira-Gonzalez*

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The current workflow for clinical Fragile X testing is time consuming and labor intensive. Recently developed PCR-based methods simplify workflow, amplify full mutation alleles, and improve sensitivity for detecting low-level mosaicism. We evaluated the performance characteristics and workflow of two methods using commercially available reagents for determining *FMR1* mutation status. We also tested each method's ability to detect mosaicism (range, 100% to 1% for males; 50% to 1% for females). One method used reagents from Asuragen (AmplideX *FMR1* PCR, research use only). The second method used analyte specific reagents from Abbott Molecular, including *FMR1* Primer 1 (for repeat sizing) and *FMR1* Primer 2 (for screening of expanded alleles). Each reaction was evaluated for accuracy, precision, correlation with previous results, and workflow. Both methods performed equally well in accuracy and precision studies using NIST standards and previously characterized Coriell samples. Both methods showed 100% concordance with results from a previous consensus study and for previously analyzed patient samples. The Asuragen reagents were able to detect full mutation mosaicism down to 5% and premutation mosaicism to 1%. The Abbott Molecular Primer 2 reagents were able to detect both full mutation and pre-mutation mosaicism down to 25%. Both PCR-based methods for the determination of *FMR1* mutation status performed well, with expected results in their final diagnoses, and differed significantly only in their workflow. ***(J Mol Diagn 2012, xx:xxx; http://dx.doi.org/10.1016/j.jmoldx.2012.03.005)***

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and is caused by an expansion of the CGG repeat region in the 5′ untranslated region of the *FMR1* gene on chromosome Xq27.3. Expansion of the repeats to full mutation range results in hypermethylation of the *FMR1* promoter and prevents the production of *FMR1* mRNA and protein. Other loss-of-function mutations (ie, point mutations, deletions) can also cause FXS.[1–3]

Prevalence of Fragile X is estimated to be 1 in 4000 males and 1 in 5000 to 8000 females. Indications for testing the repeat region of the *FMR1* gene include family history of FXS or undiagnosed intellectual disability; individuals with intellectual disability, developmental delay, or autism; women with fertility problems with elevated follicle-stimulating hormone levels; and men and women with intention tremor and cerebellar ataxia.[4]

Current guidelines define normal alleles as 6 to 44 repeats, intermediate/gray-zone alleles as 45 to 54 alleles, premutation alleles as 55 to 200 repeats, and full mutation alleles as >200 repeats. The categories signify the likelihood of expansion from one generation to the next.[5] Premutation alleles are unstable at meiosis and have an increased risk of expansion to full mutation in the next generation.[6] The risk of expansion is dependent on the size of the premutation. The smallest known premutation allele that expanded to a full mutation in one generation was 56 repeats.[7] In the same family, a large intermediate allele (52 repeats) expanded to full mutation within two generations.[7] Premutation alleles are associated with Fragile X–associated tremor and ataxia syndrome and premature ovarian insufficiency, but also with autism, attention deficit/hyperactivity disorder, and learning disabilities.[8] Full mutation alleles are associated with autism, intellectual disability, and dysmorphic features.[9] FXS is an X-linked dominant disorder, and symptoms are usually milder in affected females.

Among individuals with a Fragile X full mutation, many are found with mosaicism for different-sized repeats in different cells. Size mosaicism is commonly seen as a smear in the full mutation range on the Southern blot.

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Table 1. Accuracy Testing

	Expected	Asuragen		Abbott 2 screening		Abbott 1 sizing	
NIST-A	20	20		19	(−1)	21	(+1)
NIST-B	30	30		30		31	(+1)
NIST-C	41	41		40		42	(+1)
NIST-D	51	51		51		52	(+1)
NIST-E	60	60		60		60	
NIST-F	73	73		73		73	
NIST-G	88/89, 93	88, 93		87, nd	(−1, nd)	87	(−1, nd)
NIST-H	96	97	(+1)	96		96	
NIST-I	118	121	(+3)	120	(+2)	119	(+1)
NA07174	30	30		31	(+1)	31	(+1)
CD00014	56	56		56		56	
NA06892	93	93		92	(−1)	92	(−1)
NA06906	96	101	(+5)	99	(+3)	100	(+4)
NA06891	118	120	(+2)	exp	(nd)	120	(+2)

nd, not detected.

However, size mosaicism with premutation and full mutation alleles has also been reported, as well as methylation mosaicism.[10] Although phenotypic variability may reflect the degree of mosaicism for unmethylated alleles, the role of mosaicism in the clinical presentation of the patient is not clear.[10–13] In a few cases, a mosaic pattern has been reported in patients in whom a full mutation allele coexists with a normal-sized allele.[12,13] The overall incidence of mosaicism is difficult to estimate because the ability to detect mosaicism may be an inherent limitation of current methodologies, and may vary from one laboratory to another, but has been reported to range from 12% to 41% for full mutation–premutation mosaicism.[10,11]

The current workflow in many diagnostic laboratories includes Southern blot analysis for determining mutation status (normal, premutation, full mutation) and methylation status of the *FMR1* promoter, along with a PCR-based assay for determining repeat number in the normal, intermediate, and low premutation range (typically <110 repeats). Large premutation and full mutation alleles generally cannot be detected by PCR alone, which makes interpreting certain sample types (homozygous normal females and mosaic specimens) difficult without results from Southern blot analysis. Conversely, Southern blot analysis does not accurately size alleles in the normal, intermediate, and low premutation range, and may be limited by the hybridization conditions in its ability to detect mosaicism.[10] Therefore, PCR amplification combined with Southern blot analysis has been necessary for accurate CGG repeat detection and sizing. However, this type of testing is time consuming, labor intensive, and not amenable to high-throughput testing. Recently developed triplet-primed PCR-based methods have been designed to simplify workflow, detect full mutation alleles, and improve sensitivity for detecting low-level mosaicism.[14–18]

In this paper, we describe the evaluation of two methods using commercially available reagents labeled for research use only (RUO) and analyte specific reagents (ASR) for determining *FMR1* mutation status in our laboratory.

Materials and Methods

Samples

The National Institute of Standards and Technology (NIST) Fragile X Human DNA Triplet Repeat Standard (SRM2399; NIST A-I) and previously NIST-sequenced DNA samples from five Coriell FX cell lines (NA07174, CD00014, NA06892, NA06906, and NA06891; Coriell Institute for Medical Research, Camden, NJ) were used to assess accuracy of the two methods (Table 1). Twenty-two other control DNA samples from Coriell were used in this study, including 16 samples that had been previously used in a consortium study of nine clinical laboratories (Tables 2 and 3).[19] According to the company website, Coriell genomic DNA is purified from fresh blood or immortalized lymphocytes with the Gentra Autopure method using the Qiagen Autopure instrument according to manufacturer's instructions (Qiagen, Valencia, CA).

A total of 40 residual patient samples previously tested for Fragile X were also analyzed. These samples were de-identified and given a new number (VCU##). The DNA had been extracted from whole blood with an organic extraction method using phenol:chloroform and isopropanol precipitation, and was tested using both Southern blot analysis and a laboratory-developed PCR-based test.[20] The de-identified residual patient samples included 9 male samples with repeats in the normal range, 7 normal female, 3 gray-zone/intermediate male, 4 premutation male, 7 premutation female, 5 full mutation male, and 5 full mutation female samples (Table 3).

Six Coriell DNA samples (NA20232, NA06892, NA04025, NA20234, NA06903, and NA05847) representing gray-zone/intermediate, premutation, and full mutation alleles for both males and females were used to ascertain precision. Each sample was run in duplicate on three separate days (six data points per sample). Overall, 22 normal, 10 gray-zone/intermediate, 27 premutation, and 17 full mutation samples were tested.

Artificial mosaic samples were prepared by diluting DNA with expanded alleles with DNA containing normal-sized alleles. Premutation male DNA with 118 repeats

Table 2. Consensus Sample Analysis

Sample ID	Sex	Genotype	Consensus length	Asuragen	Abbott 2 screening	Abbott 1 sizing
NA20230	M	INT	53	54	54	54
NA20232	M	INT	46	46	46	46
NA20234	F	INT	31, 46	31, 46	32, 46	31, 46
NA20235	F	INT	29, 45	29, 45	29, 45	30, 45
NA20236	F	INT	31, 53	31, 54	31, 54	31, 54
NA07538	F	NOR	29, 29	29	30	30
NA20238	F	NOR	29, 30	30, 31	29	30, 31
NA20243	F	NOR	29, 41	29, 41	29, 41	29, 41
NA20244	M	NOR	41	41	41	41
NA20231	M	PRE	76	78	78	77
NA20233	M	PRE	117	119	119	118
NA20237	M	PRE	100–104*	100, 137	100	99, 135
NA20240	F	PRE	30, 80	31, 82	30, 81	31, 81
NA20241	F	PRE	29, 93–110*	30, 91	29, exp	29, 90
NA20242	F	PRE	30, 73	30, 74	31, 74	30, 73
NA20239	F	PRE/FULL	20, 183–193*	21, 200	21, exp	21, 202

AQ: 5 Exp, expansion present; F, female; FULL, full mutation; INT, grey-zone/intermediate; M, male; NOR, normal alleles; PRE, permutation mutation.
*No consensus was reached.

(NA06891) was diluted with normal male DNA with 30 repeats (NA07174) to prepare samples with 100%, 90%, 75%, 50%, 25%, 10%, 5%, and 1% of the expanded allele. The same dilutions were made with full mutation male DNA with 645 repeats (NA04025). Premutation female DNA with 30/100 repeats (NA20242) was diluted with normal female DNA with 29/30 repeats (NA20238) to prepare samples with 50%, 25%, 10%, 5%, and 1% of the expanded allele. The same dilutions were made with full mutation female DNA with 29/>200 repeats (NA07537).

AQ: 1

Asuragen AmplideX FMR1 CGG-Primed PCR

Samples were PCR-amplified using AmplideX FMR1 PCR reagents (RUO) by preparing a master mix with 11.45 μL of GC-rich AMP buffer, 0.5 μL of FAM-labeled *FMR1* forward and reverse primers, 0.5 μL of *FMR1* CGG primers, 0.5 μL of diluent, and 0.05 μL of GC-rich polymerase mix from Asuragen (Austin, TX).[15] Two microliters of DNA (30 ng/μL) were used for each reaction. Samples were amplified with an initial denaturation step of 95°C for 5 minutes, followed by 10 cycles of 97°C for 35 seconds, 62°C for 35 seconds, and 68°C for 4 minutes, and then 20 cycles of 97°C for 35 seconds, 62°C for 35 seconds, and 68°C for 4 minutes (auto + 20 seconds/cycle). The final extension step was 72°C for 10 minutes.

Abbott Molecular FMR1 Primer 1 (Sizing and Sex) PCR

Samples were PCR amplified using Abbott Molecular *FMR1* Primer 1 reagents by preparing a master mix with 13 μL of High GC PCR buffer, 0.6 μL of Gender Primers (ASR), 0.8 μL of *FMR1* primers (ASR), 1.2 μL of TR PCR Enzyme mix, and 1.4 μL of nuclease-free water from Abbott Molecular (Abbott Park, IL).[19] Three microliters of DNA (10 ng/μL) were used for each reaction. The reagents were thawed on ice, and the PCR reactions were set up on ice. Samples were amplified with 15 cycles of 98.5°C for 10 seconds, 58°C for 1 minute, and 75°C for 6 minutes, followed by 15 cycles of 98.5°C for 10 seconds (auto + 0.1°C/cycle), 56°C for 1 minute, and 75°C for 6 minutes with a final hold at 4°C.

Abbott Molecular *FMR1* Primer 1 PCR products were evaluated using agarose gel electrophoresis. One microliter of loading dye was added to 5 μL of PCR product. The samples were run on a 1.5% agarose gel with Tris/borate/EDTA buffer with 150 V for 60 minutes. Gels were stained with ethidium bromide and visualized with UV light.

Before capillary electrophoresis analysis, the Abbott Molecular *FMR1* Primer 1 PCR products were cleaned by adding 3 μL of Clean Up Enzyme (Abbott Molecular) and 2 μL of PCR product to a 96-well plate. The samples were incubated at 75°C for 10 minutes, followed by a 4°C hold.

Abbott FMR1 Primer 2 (Screening) PCR

Samples were PCR amplified using Abbott Molecular *FMR1* Primer 2 reagents by preparing a master mix with 13 μL of High GC PCR buffer, 0.8 μL of *FMR1* Primers 2 (ASR), 1.2 μL of TR PCR Enzyme mix, and 2 μL of nuclease-free water from Abbott Molecular.[17] Three microliters of DNA (10 ng/μL) were used for each reaction. The reagents were thawed on ice, and PCR reactions were set up on ice. Samples were amplified with 50 cycles of 98.5°C for 30 seconds, 53°C for 30 seconds, and 75°C for 1 minute, with a final hold at 4°C.

Capillary Electrophoresis

Asuragen AmplideX FMR1

The Asuragen AmplideX *FMR1* PCR products were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using POP-7 polymer (Applied Biosystems) with a 50-cm capillary. Samples were prepared for analysis by mixing 2 μL of PCR product with 2 μL of ROX 1000 Size Standard (Asuragen) and 11 μL of Hi-Di Formamide (Applied Biosystems). These samples were heat denatured at 95°C for 2 minutes and cooled to 4°C. The run conditions included injection voltage/time of

Table 3. Patient and Additional Coriell DNA Sample Analysis

	Expected				Asuragen			
	Sex	Genotype	A1	A2	Genotype	A1	A2	A3
VCU13	F	NOR	31	31	NOR	31	31	
VCU15	F	NOR	22	30	NOR	23	30	
VCU16	F	NOR	29	31	NOR	29	31	
VCU17	F	NOR	30	43	NOR	30	44	
VCU18	F	NOR	30	30	NOR	30	30	
VCU19	F	NOR	30	43	NOR	31	43	
VCU20	F	NOR	31	35	NOR	31	35	
VCU14	M	NOR	18		NOR	20		
VCU21	M	NOR	18		NOR	20		
VCU22	M	NOR	24		NOR	24		
VCU23	M	NOR	31		NOR	31		
VCU24	M	NOR	20		NOR	20		
VCU25	M	NOR	29		NOR	30		
VCU26	M	NOR	41		NOR	41		
VCU27	M (XXY)	NOR	29	29	NOR	31	31	
VCU28	M	NOR	20		NOR	21		
NA13664	F	INT	28	49	INT	30	52	
VCU30	M	INT	50		INT	51		
VCU31	M	INT	48		INT	49		
VCU42	M	INT	52		INT	53		
VCU32	F	PRE	31	90	PRE	29	91	
VCU33	F	PRE	19	80	PRE	24	84	
VCU35	F	PRE	31	79	PRE	24	79	
VCU36	F	PRE	34	121	PRE	26	123	
VCU37	F	PRE	34	112	PRE	31	115	
VCU38	F	PRE	30	82	PRE	30	85	
VCU39	F	PRE	42	63	PRE	42	66	
VCU29	M	PRE	56		PRE	56		
VCU34	M	PRE	84		PRE	85		
VCU40	M	PRE	88		PRE	93		
VCU41	M	PRE	130		PRE	138		
VCU43	F	FULL	35	FULL	FULLm	33	162	>200
VCU44	F	FULL	32	FULL	FULL	31	>200	
VCU45	F	FULL	30	FULL	FULL	30	>200	
VCU46	F	FULL	24	FULL	FULL	23	>200	
VCU48	F	FULL	30	FULL	FULL	30	>200	
NA05847	F	FULL	21	650	FULL	20	>200	
NA07537	F	FULL	29	FULL	FULL	29	>200	
VCU47	M	FULL	FULL		FULL	>200		
VCU49	M	FULL	FULL		FULL	>200		
VCU50	M	FULL	FULL		FULL	>200		
VCU51	M	FULL	FULL		FULL	>200	>200	
VCU52	M	FULL	FULL		FULL	>200		
NA04025	M	FULL	645		FULL	>200		
NA07862	M	FULL	501–550		FULL	>200		
NA09237	M	FULL	931–940		FULL	>200		

(table continues)

AQ:6

AQ:7,8 EXP, Expansion genotype; F, female; FULL, full mutation; INT, grey-zone/intermediate; M, male; NOR, normal alleles; PRE, permutation mutation.

2.5 kV/15 seconds and run voltage/time of 15 kV/4200 seconds; all other settings were default for the POP-7/50-cm capillary.

Abbott Molecular FMR1 Primer 1 (Sizing and Sex)

The Abbott Molecular *FMR1* Primer 1 PCR products were analyzed on an ABI 3130xl Genetic Analyzer using POP-6 polymer (Applied Biosystems) with a 50-cm capillary. Samples were prepared for analysis by adding 3 μL of ROX 1000 Size Standard and 17 μL of Hi-Di Formamide to the 5 μL of cleaned-up PCR product. The samples were heat denatured at 93°C for 30 seconds followed by a 25°C hold. Each sample was injected and analyzed with two different run settings, targeting small and large fragments. For small fragments, the run conditions included injection voltage/time of 10.0 kV/1 second and run voltage/time of 15 kV/6000 seconds. For large fragments, the run conditions included injection voltage/time of 8.0 kV/22 seconds and run voltage/time of 15 kV/6800 seconds; all other settings were default for the POP-6/50-cm capillary.

Abbott Molecular FMR1 Primer 2 (Screening)

The Abbott Molecular *FMR1* Primer 2 PCR products were analyzed on an ABI 3130xl Genetic Analyzer using POP-6 polymer with a 50-cm capillary. Samples were prepared

Table 3. *Continued*

Abbott 2 screening			Abbott 1 sizing				
Genotype	A1	A2	Sex	Genotype	A1	A2	Smear on gel
NOR	30	31	XX	NOR	30	31	No
NOR	23	31	XX	NOR	23	30	No
NOR	30	31	XX	NOR	29	31	No
NOR	30	44	XX	NOR	30	44	No
NOR	31		XX	NOR	30	30	No
NOR	31	43	XX	NOR	31	43	No
NOR	32	35	XX	NOR	32	36	No
NOR	21		XY	NOR	21		No
NOR	21		XY	NOR	21		No
NOR	25		XY	NOR	25		No
NOR	32		XY	NOR	31		No
NOR	21		XY	NOR	21		No
NOR	29		XY	NOR	30		No
NOR	41		XY	NOR	41		No
NOR	31		XY	NOR	30	31	No
NOR	21		XY	NOR	21		No
INT	31	52	XX	INT	30	52	No
INT	51		XY	INT	51		No
INT	49		XY	INT	49		No
INT	53		XY	INT	53		No
PRE	29	90	XX	PRE	29	90	No
PRE	24	83	XX	PRE	24	83	No
PRE	24	78	XX	PRE	24	78	No
EXP	27		XX	PRE	26	120	No
EXP	31		XX	PRE	31	113	No
PRE	30	84	XX	PRE	30	84	No
PRE	42	66	XX	PRE	42	65	No
PRE	56		XY	PRE	56		Yes
PRE	83		XY	PRE	83		No
PRE	92		XY	PRE	92		No
EXP			XY	PRE	136		No
EXP	34		XX	FULL	33		Yes
EXP	31		XX	FULL	30		Yes
EXP	29		XX	FULL	29		Yes
EXP	24		XX	FULL	24		Yes
EXP	30		XX	FULL	30		Yes
EXP	21		XX	FULL	21		Yes
EXP	30		XX	FULL	29		Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes
EXP			XY	FULL			Yes

for analysis by mixing 2 μL of PCR product with 2 μL of ROX 1000 Size Standard and 20 μL of Hi-Di Formamide. These samples were heat denatured at 95°C for 2 minutes, followed by a 25°C hold. The run conditions included injection voltage/time of 8.0 kV/8 seconds and run voltage/time of 15 kV/5000 seconds; all other settings were default for the POP-6/50-cm capillary.

Data Analysis

Capillary electrophoresis data were analyzed on GeneMapper 4.0 software (Applied Biosystems). Asuragen provided panels for the software as well as a macro to analyze the data. We developed panels for displaying and analyzing the Abbott Molecular data.

Interpretation of Results

Figure 1 shows a full mutation female sample (NA05847) amplified with the Asuragen AmplideX *FMR1* reagents (Figure 1A) and the two Abbott Molecular *FMR1* Primer sets (Figure 1, B–D). Samples tested with the Asuragen reagents were analyzed with GeneMapper 4.0 and an Excel-based macro, which calculated repeat sizes and assigned a genotype (Figure 1A and Table 3). The formula used by the macro (2.946x + 230.2) was generated using a process control on the 3130xl instrument at the beginning of the validation.[14]

Using the Abbott Molecular reagents, a sample is first analyzed with the *FMR1* Primer 2 reaction (Figure 1B), which is CGG primed and gives a ladder motif in the presence of an expanded allele (premutation or full mu-

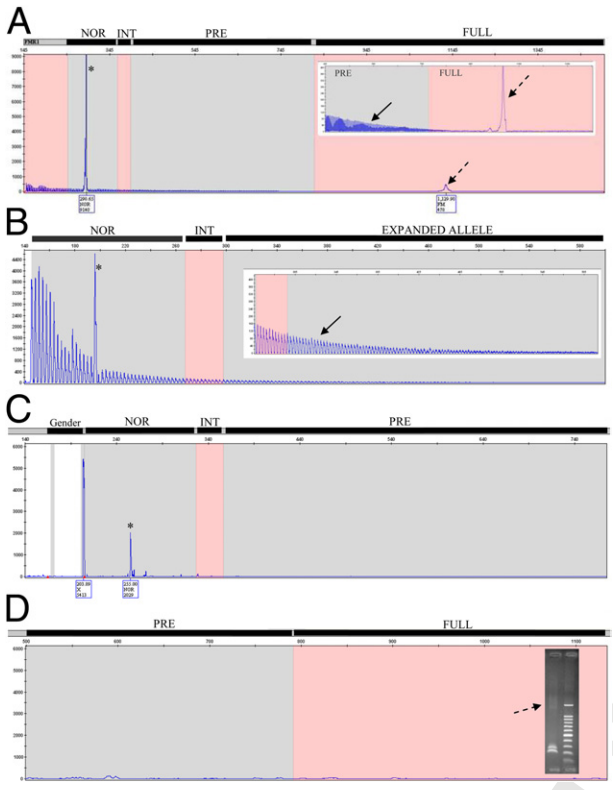

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Figure 1. Interpretation and comparison of results using different reagents. A full mutation female sample (NA05847) was tested with the Asuragen AmplideX *FMR1* reagents (**A**) and the two Abbott Molecular *FMR1* Primer Sets (**B–D**). **A:** With the Asuragen reagents, the full mutation female shows a peak in the normal repeat range (NOR; 290 bp; 21 repeats; **asterisk**) and a peak in the full mutation range (FM/FULL; 1129 bp; >200 repeats; **dashed arrow**). There are also triplet-repeat specific products that are visible as a ladder motif (**solid arrow**) in the zoomed-in electropherogram (**inset**). **B:** With the Abbott Molecular *FMR1* Primer 2 reaction (screening), the full mutation female sample shows a normal allele peak (**asterisk**) and the triplet-repeat specific products (**solid arrow**), which are indicative of an expanded allele. **C:** The Abbott Molecular *FMR1* Primer 1 reaction shows a sex-specific peak for the X chromosome (203 bp), with an absence of the Y chromosome peak (170 bp), and a peak in the normal repeat range (255 bp; 21 repeats, **asterisk**). **D:** This panel shows that no large fragments (in the 500- to 1500-bp range) were visible on the electropherogram, but a smear that is >1000 bases is visible on the agarose gel (**dashed arrow**). INT, intermediate.

tation). Repeat lengths can be calculated for normal, intermediate, and some premutation alleles [formula: repeat number = (peak size −134)/3]. The Abbott Molecular *FMR1* Primer 1 reaction can be used to amplify and size normal, intermediate, and some premutation alleles [formula: repeat number = (peak size − 193)/3]. This reaction will also reveal sex (Y chromosome, 170 bp; X chromosome, 203 bp) (Figure 1, C and D). The PCR products are analyzed with both agarose gel electrophoresis and capillary electrophoresis. Unlike capillary electrophoresis, agarose gel electrophoresis will usually detect an expanded allele as a distinct band or a smear on the gel (Figure 1D, gel). Any sample with an expansion should be reflexed to Southern blot analysis.

Results

Accuracy

To ascertain accuracy, standards with certifiable repeat numbers were tested with all three reactions. The NIST Fragile X Human DNA Triplet Repeat Standard and DNA from previously NIST-sequenced Coriell samples have been previously used to validate various platforms.[19] Table 1 shows results for all three reactions. Even the Abbott Molecular Primer 2 reaction, which is designed for detecting expanded alleles, not for sizing repeats, performed equally well in accuracy (±1 repeat) for repeat lengths <96 repeats. Repeat lengths >96 repeats had a wider variance (−1 to +5 repeats) compared with the expected values.

Concordance

Sixteen Coriell DNA samples from a consortium study of nine clinical laboratories were used for concordance studies.[19] The results from the published consortium study were compared with results from the three PCR reactions. Normal and intermediate alleles were sized accurately within one repeat, and the premutation alleles were sized to within one to three repeats (Table 2). The greatest difference between the results of the consortium study and this study was seen in samples for which no consensus was reached in the previous study (NA20237, NA20241, and NA20239).[19]

To assess performance of the two methods on patient samples, 40 de-identified residual samples were evaluated with each set of reagents. These samples had been previously analyzed with an in-house laboratory-developed test and represented each allele type. All three reactions demonstrated 100% concordance (95% confidence interval: 97% to 100%) with the previous genotyping results (Table 3). There were some minor differences in repeat numbers compared to the laboratory-developed test assay, with strong consensus among the methods being tested. For the Abbott Molecular *FMR1* Primer 2 reaction, when there was no definite peak that could be used for sizing, but there was a ladder motif present, the genotype was assigned as “EXP” for “expansion.”

Precision

Precision studies were performed on six Coriell DNA samples representing gray-zone/intermediate, premutation, and full mutation alleles for both males and females. Each sample was run in duplicate on three separate days (six data points per sample) to obtain data for intra-assay and interassay precision. The intra-assay variability for all three reactions was ±1 bp (<1 repeat) for all genotypes except the full mutation, which cannot be accurately sized with either method (data not shown). Table 4 shows T4 that the reproducibility of results for the two sizing reactions (Asuragen and Abbott Molecular *FMR1* Primer 1) is comparable (±0.04 to 0.12 repeats SD from mean). Even the Abbott Molecular *FMR1* Primer 2 reaction showed similar reproducibility (±0.13 to 0.18) with the exception of the larger allele in the female gray-zone sample (±1.04).

Table 4. Precision Analysis

		Asuragen			Abbott 2 screening			Abbott 1 sizing		
	Expected	Ave	SD	CV	Ave	SD	CV	Ave	SD	CV
Male										
Grey zone (NA20232)										
Allele 1	46	46	0.11	0.25	46	0.16	0.35	46	0.12	0.25
Premutation (NA06892)										
Allele 2	93	93	0.12	0.13	92	0.18	0.19	92	0.04	0.05
Full mutation (NA04025)										
Allele 3	645	>200	na	na		na	na	>200	n/a	n/a
Female										
Grey zone (NA20234)										
Allele 1	31	31	0.05	0.17	32	0.18	0.56	31	0.07	0.22
Allele 2	46	46	0.06	0.13	47	1.04	2.22	46	0.06	0.14
Premutation (NA06903)										
Allele 1	30	31	0.05	0.17	31	0.15	0.48	31	0.06	0.19
Allele 2	73	74	0.06	0.08	74	0.14	0.19	74	0.05	0.07
Full mutation (NA05847)										
Allele 1	21	21	0.07	0.33	21	0.13	0.61	21	0.05	0.24
Allele 2	650	>200	na	na		na	na	>200	na	na

AVE, average; CV, coefficient of variation; na, not applicable.

Mosaicism

The ability to detect mosaicism was assessed with both male and female premutation and full mutation samples. Dilutions were made with a range of expanded allele DNA and normal allele DNA. The Asuragen AmplideX *FMR1* reagents were able to detect full mutation mosaicism down to 5% (Figure 2A) and premutation mosaicism to 1% (Figure 2B). The Abbott Molecular *FMR1* Primer 2 reagents were able to detect both full mutation (Figure 2C) and premutation mosaicism (Figure 2D) down to 25%.

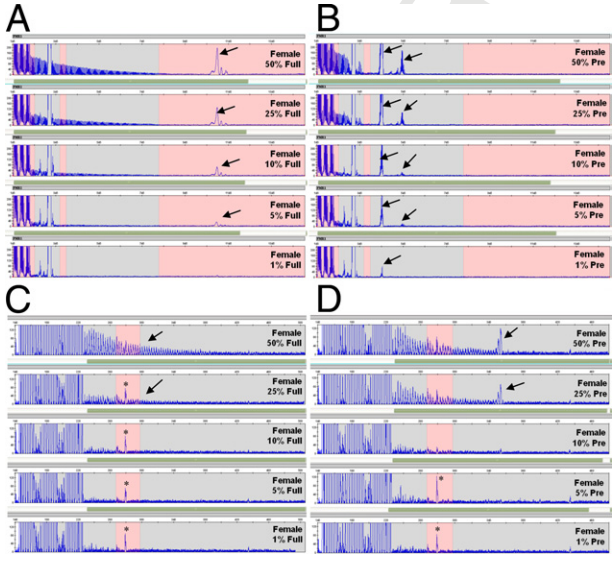


Figure 2. Detection of mosaicism. DNA from a normal female sample was mixed with DNA from a premutation (Pre) or full mutation (Full) female sample to give a final concentration of 50% to 0% expanded allele. The Asuragen AmplideX *FMR1* reagents were able to detect full mutation mosaicism down to 5% (**A**) and premutation mosaicism to 1% (**B**). The Abbott Molecular *FMR1* Primer 2 reaction was able to detect both full mutation (**C**) and premutation mosaicism (**D**) down to 25%. A triplet-repeat–specific ladder motif is visible into the premutation region (second **gray area** in A and B, **right-most gray area** in C and D). The **asterisks** represent a nonspecific product that is present in some samples.

Homozygous Female

One of the most challenging results to interpret is a female sample with a single PCR product. Both the Asuragen (Figure 3A) and Abbott Molecular reagents (Figure 3, B–D) were able to reliably detect the homozygous peak and show the absence of an expansion pattern in female samples that were homozygous for a normal allele.

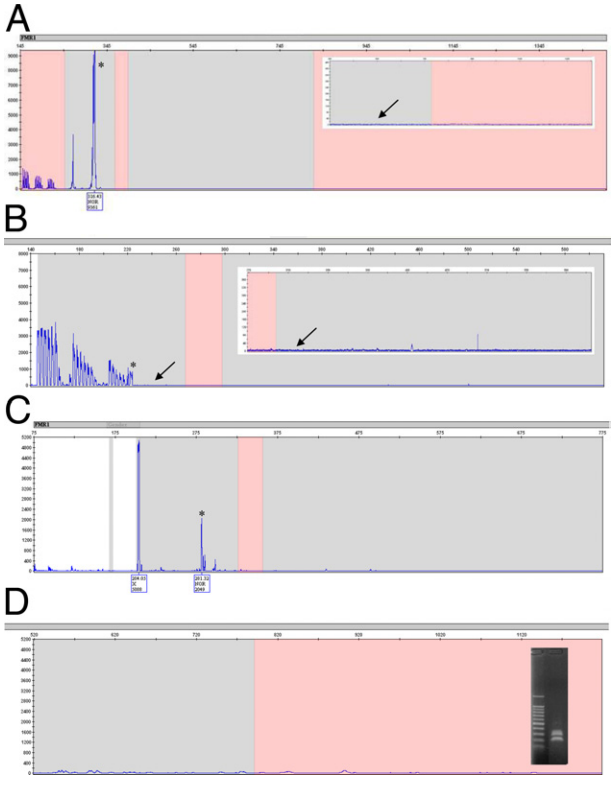
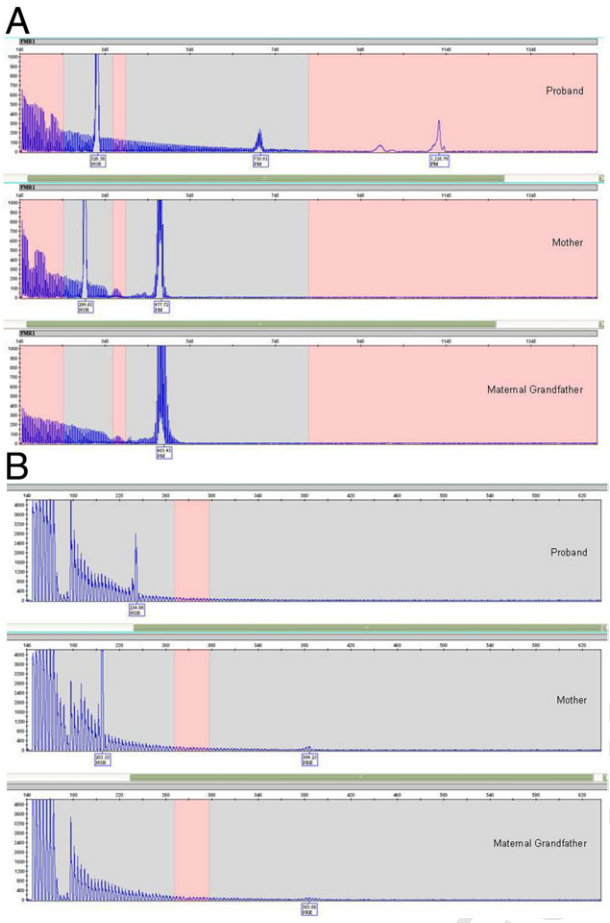


Figure 3. .Homozygous normal female sample analysis. A homozygous normal female sample (29 repeats) analyzed with the Asuragen AmplideX *FMR1* reagents (**A**) and the two Abbott Molecular *FMR1* Primer Sets (**B** and **D**). Both methods show a peak in the normal repeat range (NOR; **asterisk**) and an absence of the ladder motif following that peak (**arrow**).



COLOR

Figure 4. Interesting case with electropherograms for a female proband with a normal allele (NOR), a full mutation allele (FM), and mosaicism for a premutation (PRE; 35/162/>200 repeats), her mother with a premutation (19/80 repeats), and maternal grandfather with a premutation (84 repeats). **A:** The Asuragen AmplideX *FMR1* reaction amplifies peaks for each allele and the triplet-repeat–specific products indicative of an expanded allele. **B:** The Abbott Molecular Primer 2 reaction shows the presence of expanded alleles in each family member as a triplet-repeat ladder motif.

Interesting Case

We evaluated three generations of a family that previously proved challenging to analyze with our laboratory-developed assay. The trio included a female proband (VCU43: full mutation, 35/~560 to 600 repeats), her mother (VCU33: premutation, 19/80 repeats), and the maternal grandfather (VCU34: premutation, 84 repeats) (Table 3). Our in-house laboratory-developed method can successfully amplify alleles up to 110 repeats, but had difficulty amplifying the 80-repeat premutation allele in the mother in the presence of the uncommonly small 19-repeat allele. The Asuragen AmplideX *FMR1* reaction was able to correctly genotype each sample and amplify each allele in all three patients (Figure 4A). It was also able to detect a 162-repeat mosaic allele in the proband, in addition to her normal and full mutation alleles. The Abbott Molecular *FMR1* Primer 2 reaction was able to detect an expansion in all three samples and correctly size the normal and premutation alleles (Figure 4B).

F4

Workflow

We evaluated the workflow of each method. The method using Asuragen AmplideX *FMR1* reagents includes PCR followed by a single injection and analysis with capillary electrophoresis, with total processing time of approximately 9 hours from PCR setup to interpretation, including 3 hours of hands-on time (Table 5). The method using Abbott Molecular reagents includes two PCR reactions. The PCR product from the *FMR1* Primer 2 reaction (screening) can be analyzed directly by capillary electrophoresis, whereas the PCR product for the *FMR1* Primer 1 reaction (sex/sizing) is first checked by agarose gel electrophoresis, enabling visualization of full mutation products as smears, followed by post-PCR clean-up and two injections on capillary electrophoresis (one for short and one for long fragments). The total time required for testing was approximately 21 hours 45 minutes, including 7 hours total hands-on time (Table 5).

T5

AQ: 2

Discussion

Recently developed novel methods for Fragile X testing are designed to simplify workflow and detect full mutation alleles.[14–18] We evaluated two methods using commercially available reagents for PCR-based analysis of *FMR1*

Table 5. Workflow Data

	Asuragen	Abbott 2 screening	Abbott 1 sizing	
PCR set-up	60 minutes	60 minutes	30 minutes	
PCR	4 hours 30 minutes	2 hours 25 minutes	4 hours 30 minutes	
Post-PCR processing	0 minutes	0 minutes	Gel set-up	1 hour 30 minutes
			Gel	1 hour 30 minutes
			Set-up	15 minutes
			Clean up	30 minutes
CE set-up	30 minutes	30 minutes	30 minutes	
CE runtime	1 hour 26 minutes	1 hour 15 minutes	Injection 1 (small)	1 hour 40 minutes
			Injection 2 (large)	1 hour 55 minutes
Analysis and interpretation	1 hour 30 minutes	45 minutes	3 hours 0 minutes	
Total runtime	5 hours 56 minutes	3 hours 40 minutes	10 hours 5 minutes	
Total hands-on time	3 hours 0 minutes	2 hours 15 minutes	5 hours 45 minutes	
Total time	8 hours 56 minutes	5 hours 55 minutes	15 hours 50 minutes	
			21 hours 45 minutes	

CE, capillary electrophoresis.

mutation status using 36 known Coriell DNA samples and NIST standards, and 40 previously tested anonymized patient samples. In all, we tested 22 samples with repeats in the normal range, 10 in the gray-zone/intermediate range, 27 in the premutation range, and 17 in the full mutation range. We also tested each method's ability to detect mosaicism (ranges, 100% to 1% for males; 50% to 1% for females). One method included the AmplideX *FMR1* PCR reagents from Asuragen, which were labeled RUO at the time of testing. Only the CGG repeat-primed reaction was tested. The second method used a set of ASRs from Abbott Molecular, and consisted of the *FMR1* Primer 1 reaction (for sex determination and repeat sizing) and the *FMR1* Primer 2 reaction (for screening of expanded alleles). The *FMR1* Primer 2 reaction is CGG repeat-primed. Each reaction was evaluated for accuracy, precision, correlation with previous results, ability to detect mosaicism, and workflow. Although the reaction conditions for each method have been previously published, the performance of the assays reflects, at least in part, the conditions developed and optimized in our laboratory.[15,17,19]

Both methods performed equally well in accuracy (±1 repeat) compared to NIST standards and Coriell samples (Table 1), and demonstrated 100% concordance with expected results for samples from a previous consensus study (Table 2), as well as with results for patient samples previously analyzed with a laboratory-developed test (Table 3). Precision studies showed similar results for both methods (±0.04 to 0.12 repeats SD from mean) (Table 4).

We currently use a laboratory-developed Southern blot analysis method to determine Fragile X mutation status and methylation status, and a laboratory-developed PCR assay to determine repeat size in every sample we test. One advantage of the new PCR-based methods is the reduction in the need for Southern blot analysis for nearly all samples, because both repeat length and Fragile X mutation status can be ascertained. A sample may be reflexed to Southern blot analysis only if methylation status is needed. This approach is estimated to reduce the number of samples analyzed by Southern blot by at least 98%, making the PCR-based assays amenable to more high-throughput testing, such as for carrier or newborn screening.[21–24] The feasibility of using the Abbott Molecular reagents with DNA extracted from dried-blood spots has been previously shown.[17] Even reaching out to other non-genetics specialties to increase test volume, particularly from patients with premature ovarian insufficiency or Fragile X–associated tremor and ataxia syndrome, can be easily managed with the PCR–capillary electrophoresis platforms. Since the Asuragen AmplideX *FMR1* and the Abbott Molecular *FMR1* reagents do not reveal methylation status, samples with premutation and full mutation alleles should be reflexed to Southern blot or PCR-based methylation analysis.[25]

One of the most challenging results to interpret is a female sample with a single PCR product. At least 25% of female samples appear to be homozygous for a normal allele and cannot be distinguished from a heterozygous sample with one unamplifiable (and possibly expanded) allele using conventional PCR methods. Good laboratory practice would dictate that these samples be analyzed by Southern blot to detect either two normal alleles or any expanded allele. One advantage of CGG repeat-primed PCR methods over conventional PCR is that they reveal the presence of expanded alleles by the presence of a ladder motif. Both the Asuragen AmplideX *FMR1* and Abbott Molecular *FMR1* methods showed a ladder motif with every premutation or full mutation sample (Tables 1–3). The Asuragen AmplideX *FMR1* reaction also showed a final terminating peak for that ladder motif, as appropriate, either in the premutation or full mutation range. Conversely, for each homozygous normal sample we tested (NA07538, VCU13, VCU18, and VCU27), both methods amplified peaks in the normal range and did not show the ladder motif. Figure 3 shows a homozygous normal female sample (NA07538) in which both the Asuragen AmplideX *FMR1* (Figure 3A) and Abbott Molecular *FMR1* methods (Figure 3, B–D) correctly identified a normal allele without an expansion pattern. The consistency of obtaining the peak and ladder motif together for every expanded sample and the absence of the expansion pattern for every normal or gray-zone sample builds confidence in the final result and significantly reduces the need for Southern blot analysis in cases of apparent homozygous samples.

Mosaicism, or the coexistence of full mutation alleles with premutation or normal alleles, is estimated to be present in 15% to 20% of individuals with *FMR1* mutations.[10,11,26] Mosaicism for methylated full mutation and unmethylated premutation alleles has been reported,[10,11] as well as mosaicism for full mutation and normal-sized alleles.[12,13] Typically, the mothers of these individuals are premutation carriers, and mosaicism with full mutation and normal alleles is most likely due to postzygotic regression by deletion rather than a lack of expansion of the premutation allele.[12,13] Traditionally, these genotypes have been difficult to detect with PCR-based methods alone, and required the use of Southern blot analysis. It appears that CGG repeat-primed methods are better able to detect mosaicism,[16] probably because they do not necessarily rely on the amplification of a full mutation allele, although many are capable of amplifying a full mutation product, but rely instead on the detection of the ladder motif that signals the presence of an expanded allele. We were able to detect mosaicism for premutation and full mutation alleles with both methods. The Abbott Molecular *FMR1* Primer 2 reaction detected mosaicism to 25% with the presence of the ladder motif. The Asuragen AmplideX *FMR1* reaction was able to detect full mutation and premutation mosaicism to 5% and 1%, respectively, with the presence of a ladder motif and terminating premutation or full mutation peak. Previous studies have detected mosaicism with the Abbott Molecular *FMR1* Primer 2 reagents to 12.5%, which is within one dilution of our study,[17] and to <5% with the Asuragen AmplideX *FMR1* reagents, which is consistent with our study.[14] Although the clinical significance of this difference is not known, it is expected that the increased sensitivity in detecting mosaicism will enable the identification of minor component (low-percentage) alleles that could ex-

pand in offspring or explain a variable phenotype of a patient.[10–13]

The most significant difference between the two methods is the workflow. The method using Asuragen AmplideX *FMR1* PCR reagents (RUO) includes PCR followed by a single injection and analysis with capillary electrophoresis. The data analysis for the Asuragen reagents involves analysis and visualization with GeneMapper, followed by running a macro to provide repeat number and diagnosis. The total time required to run the assay is significantly less than the method using the Abbott Molecular *FMR1* reagents. Absolute sizing of repeat numbers in full mutation alleles is not possible with the Asuragen AmplideX *FMR1* PCR reagents due to limitations with capillary electrophoresis. However, full mutation alleles can be clearly visualized as terminating peaks in the full mutation region of the electropherogram (>800 bp), because of the presence of both a forward and a reverse primer in the reaction in addition to the CGG-repeat primer.[15] Full mutation alleles are identified as >200 repeats. The master mix must be set up at room temperature and vortexed vigorously due to the viscosity of the PCR buffer. One disadvantage of the Asuragen reagents is that, depending on a laboratory's Fragile X sample volume, the master mix may need to be prepared for more samples than are being run (at least eight) because of the small volume of enzyme (0.05 μL) required for each reaction. Also, the assay is very sensitive to any extra enzyme, so care must be taken to make sure no droplets of enzyme are present on the outside of the tip.

The method using Abbott Molecular *FMR1* reagents (ASR) include two separate PCR reactions. Both *FMR1* Primer 1 and 2 reactions must be set up on ice or a cold block. The PCR product from *FMR1* Primer 2 (screening) can be analyzed directly by capillary electrophoresis, whereas the PCR product for *FMR1* Primer 1 (sizing) is first checked by agarose gel electrophoresis, followed by post-PCR clean-up and two injections on capillary electrophoresis (one for short and one for long fragments). Our experience was that the second injection for long fragments was only useful for increasing the peak height of the gray zone and premutation alleles, but did not aid in the ability to see the full mutation alleles. Additional assay optimization may be able to improve the ability to amplify and visualize full mutation alleles. The agarose gel is a helpful check for the presence of PCR products before proceeding with the enzymatic clean-up and capillary electrophoresis steps, and can also aid in visualizing a smear or band that represents the full mutation allele. One unique feature of the Abbott Molecular *FMR1* Primer 1 reaction is its incorporation of primers for sex identification, which the other reactions do not have. However, although the Abbott Molecular Gender Primers can identify the presence of X and Y chromosome material, they cannot detect the dose of each and therefore cannot be used to differentiate a homozygous female sample from a full mutation female sample or identify other sex chromosome dosage abnormalities (Table 3, sample VCU 27). As such, results from the Abbott Molecular *FMR1* Primer 2 reaction and conventional karyotyping can be used to complement the results from the *FMR1* Primer 1 reaction for both male and female samples.

Overall, both methods that we evaluated were able to correctly genotype the samples tested (100% correlation). Each method has the ability to reduce the number of samples analyzed by Southern blot, because all normal and intermediate alleles, as well as expanded alleles, can be identified with confidence. The most significant differences between the two methods were their ability to detect mosaicism and the workflow.

References

1. Hammond LS, Macias MM, Tarleton JC, Pai GS: Fragile X syndrome and deletions in FMR1: new case and review of literature. Am J Med Genet 2011, 72:430–434
2. Gronskov K, Brondum-Nielsen K, Dedic A, Hjalgrim H: A nonsense mutation in FMR1 causing fragile X syndrome. Eur J Hum Genet 2011, 19:489–491
3. Coffee B, Ikeda M, Budimirovic DB, Hjelm LN, Kaufmann WE, Warren ST: Mosaic FMR1 deletion causes fragile X syndrome and can lead to molecular misdiagnosis. Am J Med Genet A 2008, 146A:1358–1367
4. Sherman S, Pletcher BA, Driscoll DA: Fragile X syndrome: diagnostic and carrier testing. Genet Med 2005, 7:584–587
5. Spector EB, Kronquist K, Fragile X Molecular Working Group 2005 for the Quality Assurance Committee of the American College of Medical Genetics: Technical Standards and Guidelines for Fragile X Testing: A Revision to the Disease-Specific Supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. 2006 Edition. Available at http://www.acmg.net/Pages/ACMG_Activities/stds-2002/fx.htm. Last accessed December 1, 2011
6. Nolin SL, Brown WT, Glicksman A, Houck GE, Gargano AD, Sullivan A, Biancalana V, Bröndum-Nielsen K, Hjalgrim H, Holinski-Feder E, Kooy F, Longshore J, Macpherson J, Mandel J-L, Matthijs G, Rousseau F, Steinbach P, Väisänen M-L, von Koskull H, Sherman SL: Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles. Am J Med Genet 2003, 72:454–464
7. Fernandez-Carvajal I, Lopez Posadas B, Pan R, Raske C, Hagerman PJ, Tassone F: Expansion of an FMR1 grey-zone allele to a full mutation in two generations. J Mol Diagn 2009, 11:306–310
8. Farzin F, Perry H, Hess D, Loesch D, Cohen J, Bacalman S, Gane L, Tassone F, Hagerman P, Hagerman R: Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. Dev and Behav Peds 2006, 27(2 Suppl):S137–S144
9. Rousseau F, Labelle Y, Bussieres J, Lindsay C: The fragile X mental retardation syndrome 20 years after the FMR1 gene discovery: an expanding universe of knowledge. Clin Biochem Rev 2011, 32:135–162
10. Nolin SL, Glicksman A, Houck GE Jr., Brown WT, Dobkin CS: Mosaicism in fragile X affected males. Am J Med Genet 1994, 15:509–512
11. Rousseau F, Heitz D, Tarleton J, Macpherson J, Malmgren H, Dahl N, Barnicoat A, Mathew C, Mornet E, Tajada I, Maddalena A, Spiegel R, Schinzel A, Marcos JAG, Schorderet DF, Schaap T, Maccioni L, Russo S, Jacobs PA, Schwartz C, Mandel JL: A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases. Am J Med Genet 1994, 55:225–237
12. Orrico A, Galli L, Dotti MT, Plewnia K, Censini S, Federico A: Mosaicism for full mutation and normal-sized allele of the FMR1 gene: a new case. Am J Med Genet 1998, 78:341–344
13. Schmucker B, Seidel J: Mosaicism for a full mutation and a normal size allele in two fragile X males. Am J Med Genet 1999, 84:221–225
14. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, Hagerman PJ, Stenzel TT, Hadd AG, Latham GJ, Tassone F: A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. Clin Chem 2010, 56:399–408
15. Chen L, Hadd AG, Sah S, Filipovic-Sadic S, Krosting J, Sekinger E, Pan R, Hagerman PJ, Stenzel TT, Tassone F, Latham GJ: An infor-

mation-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for Southern blot analysis. J Mol Diagn 2010, 12:589–600

16. Hantash FM, Goos DM, Tsao D, Quan F, Buller-Burckle A, Peng M, Jarvis M, Sun W, Strom CM: 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. Genet Med 2010, 12:162–173
17. Lyon E, Laver T, Yu P, Jama M, Young K, Zoccoli M, Marlowe N: A simple, high-throughput assay for fragile X expanded alleles using triple repeat primed PCR and capillary electrophoresis. J Mol Diagn 2010, 12:505–511
18. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ: A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. J Mol Diagn 2008, 10:43–49
19. Wilson JA, Pratt VM, Phansalkar A, Muralidharan K, Highsmith WE Jr., Beck JC, Bridgeman S, Courtney EM, Epp L, Ferreira-Gonzalez A, Hjelm NL, Holtegaard LM, Jama MA, Jakupciak JP, Johnson MA, Labrousse P, Lyon E, Prior TW, Richards CS, Richie KL, Roa BB, Rohlfs EM, Sellers T, Sherman SL, Siegrist KA, Silverman LM, Wiszniewska J, Kalman LV, Fragile Xperts Working Group of the Association for Molecular Pathology Clinical Practice: Consensus characterization of 16 FMR1 reference materials: a consortium study. J Mol Diagn 2008, 10:2–12
20. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Press, 1989
21. Hantash FM, Goos DM, Crossley B, Anderson B, Zhang K, Sun W, Strom CM: FMR1 premutation carrier frequency in patients undergoing routine population-based carrier screening: insights into the prevalence of fragile X syndrome, fragile X-associated tremor/ataxia syndrome, and fragile X-associated primary ovarian insufficiency in the United States. Genet Med 2011, 12:162–173
22. American College of Obstetricians and Gynecologists Committee on Genetics: Committee Opinion No. 469: carrier screening for fragile X syndrome. Obstet Gynecol 2010, 116:1008–1010
23. Skinner D, Choudhury S, Sideris J, Guarda S, Buansi A, Roche M, Powell C, Bailey DB Jr.: Parents' decisions to screen newborns for FMR1 gene expansions in a pilot research project. Pediatrics 2011, 127:e1455–e1463
24. Fernandez-Carvajal I, Walichiewicz P, Xiaosen X, Pan R, Hagerman PJ, Tassone F: Screening for expanded alleles of the FMR1 gene in blood spots from newborn males in a Spanish population. J Mol Diagn 2009, 11:324–329
25. Chen L, Hadd AG, Sah S, Houghton JF, Filipovic-Sadic S, Zhang W, Hagerman PJ, Tassone F, Latham GJ: High-resolution methylation polymerase chain reaction for fragile X analysis: evidence for novel FMR1 methylation patterns undetected in Southern blot analyses. Genet Med 2011, 13:528–538
26. Saul RA, Tarleton JC: FMR1-Related Disorders. Includes: Fragile X Syndrome, Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS), FMR1-Related Primary Ovarian Insufficiency (POI) In GeneReviews. Copyright University of Washington, Seattle. 1998–2012 Available at http://www.ncbi.nlm.nih.gov/books/NBK1384. Last accessed October 6, 2011