A Novel FMR1 PCR Method for the Routine Detection of Low Abundance Expanded Alleles and Full Mutations in Fragile X Syndrome

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BACKGROUND: Fragile X syndrome (FXS) is a trinucleotide-repeat disease caused by the expansion of CGG sequences in the 5’ untranslated region of the FMR1 (fragile X mental retardation 1) gene. Molecular diagnoses of FXS and other emerging FMR1 disorders typically rely on 2 tests, PCR and Southern blotting; however, performance or throughput limitations of these methods currently constrain routine testing.

METHODS: We evaluated a novel FMR1 gene–specific PCR technology with DNA templates from 20 cell lines and 146 blinded clinical samples. The CGG repeat number was determined by fragment sizing of PCR amplicons with capillary electrophoresis, and results were compared with those for FMR1 Southern blotting analyses with the same samples.

RESULTS: The FMR1 PCR accurately detected full-mutation alleles up to at least 1300 CGG repeats and consisting of >99% GC character. All categories of alleles detected by Southern blotting, including 66 samples with full mutations, were also identified by the FMR1 PCR for each of the 146 clinical samples. Because all full mutation alleles in samples from heterozygous females were detected by the PCR, allele zygosity was reconciled in every case. The PCR reagents also detected a 1% mass fraction of a 940-CGG allele in a background of 99% 23-CGG allele—a roughly 5-fold greater sensitivity than obtained with Southern blotting.

CONCLUSIONS: The novel PCR technology can accurately categorize the spectrum of FMR1 alleles, including alleles previously considered too large to amplify; reproducibly detect low abundance full mutation alleles; and correctly infer homozygosity in female samples, thus greatly reducing the need for sample reflexing to Southern blotting.

Fragile X syndrome (FXS),4 the most common form of inherited intellectual impairment and known genetic cause of autism, was one of the first human diseases to be linked to an expansion of triplet nucleotide repeats (1–4). FXS is caused by expansions of the CGG repeat sequence located in the 5’ untranslated region of the FMR1 (fragile X mental retardation 1) gene (2). Individuals with “normal” (<45 CGG repeats) or intermediate (45–54 CGG repeats) FMR1 alleles are currently thought to be asymptomatic for disorders associated with the FMR1 gene; however, individuals who are carriers of a premutation allele (55–200 CGG repeats) can develop fragile X–associated tremor/ataxia syndrome (5) or fragile X–associated primary ovarian insufficiency (6–8), whereas individuals with the FMR1 full mutation (>200 CGG repeats) typically have FXS (9). As many as 1.5 × 10⁶ individuals in the US are thought to be at risk for at least one FMR1 disorder (10). Thus, these diseases are clinically important and affect a broad range of populations and ages. Currently, most diagnostic-testing paradigms for FMR1 disorders rely on the PCR with size resolution by capillary electrophoresis (CE), agarose gel electrophoresis (AGE), or PAGE for size resolution for the detection of up to 100–150 CGG repeats. FMR1 Southern blot analysis is used both to characterize samples with numbers of CGG repeats too large to amplify by the PCR and to determine the methylation status of the gene (11). Unfortunately, this work flow is costly, is time and labor intensive, and requires large amounts of genomic DNA (gDNA), making it unsuitable for higher testing volumes or population screening. The
PCR has the potential to address each of these limitations, yet the highly GC-rich character of the fragile X triplet-repeat sequence historically has been refractory to amplification. Such PCR innovations as the use of osmolyte adjuvants, modified nucleotides, and specific cycling conditions have improved detection up to approximately 300–500 CGG repeats (12, 13), yet even this performance would fail to detect many, if not most, full mutation alleles (14). Importantly, PCR analysis of samples from premutation and full-mutation females has been much less successful because of the preferential amplification of the smaller allele (12). Consequently, the >20% of female samples that are homozygous must be reflexed to Southern blotting analysis to resolve the potential ambiguity of an unamplified longer allele.

We describe the performance of a novel gene-specific FMR1 PCR technology that can resolve many of the technological challenges that now limit routine fragile X testing. This method reproducibly amplified alleles with >1000 CGG repeats and demonstrated excellent concordance with Southern blotting in an assessment of clinical samples with FMR1 alleles that spanned the entire range of CGG repeats. The consistency and sensitivity of the reagents to detect premutation and full mutation alleles, including mosaic species that may be present in only a few percent of cells, also resolved ambiguities in identifying samples from homozygous females that can confound conventional FMR1 PCR assays. Reproducible detection of full mutation alleles by the PCR has implications for the broader adoption of FMR1 analysis.

Materials and Methods

**CLINICAL AND CELL LINE DNA SAMPLES**

Blood samples were obtained from individuals evaluated at the M.I.N.D. Institute Clinic after they had provided informed consent and in accordance with an approved Institutional Review Board protocol. gDNA was isolated from peripheral blood leukocytes (5 mL of whole blood) with standard methods (Genta Pure-gene Blood Kit; Qiagen). Only the code number was known to the technician who handled the samples. A total of 146 coded samples were sent to Asuragen for PCR analysis. All cell line DNA samples were obtained from Coriell Cell Repositories (Coriell Institute for Medical Research). Clinical and cell line DNA samples were quantified with a NanoDrop spectrophotometer (Thermo Scientific) and diluted to 20 ng/μL in 10 mmol/L Tris, 0.5 mmol/L EDTA, pH 8.8, prior to the PCR.

All PCR sample batches included at least one pooled cell line “process control.” The process control was generated from 4 cell line samples—NA20239 (10 ng/μL), NA07541 (5 ng/μL), NA20230 (12 ng/μL), and NA06891 (10 ng/μL)—that were admixed in deionized water. The use of this control produced 6 PCR product peaks corresponding to 20, 29, 31, 54, 119, and 199 CGG repeats that could be detected in a single capillary. Allele amplicons for 29, 54, and 119 CGG repeats were directly verified by DNA sequencing, and amplicons for the 20- and 31-CGG repeats were matched to the base pair size of sequenced verified alleles. In each of these cases, the deviation from the sequencing result was less than a single CGG repeat. The repeat length of the 199-CGG allele was inferred for this cell line from the system calibration to the first 5 alleles. Finally, the reproducibility of detection for each of the 6 process control alleles produced an SD of less than a single CGG repeat across 12 independent CE runs.

To evaluate the analytical sensitivity of PCR and Southern blotting, we prepared a mock female heterozygous control sample by admixing the DNA isolated from samples of 2 cell lines, NA06895 (23 CGG repeats) and NA09237 (940 CGG repeats). These admixtures retained the same mass input in each case, 7 μg for Southern blotting and 40 ng for the PCR, while the percentage mass of the 940-CGG allele was varied from 1% to 100%.

**GENE-SPECIFIC FMR1 PCR**

Samples were prepared for the PCR with a master mix from Asuragen containing 11.45 μL GC-Rich AMP Buffer, 1.5 μL of 6-carboxyfluorescein (FAM)-labeled FMR1 primers, and 0.05 μL GC-Rich Polymerase Mix. The primers were FMR1 Forward (TCA GGC GCT CAG CTC CGT TTC GGT TTC A) and FMR1 Reverse (FAM-AAG CGC CAT TGG AGC CCC GCA CTT CC). The master mix was vortex-mixed before dispensing into a microtiter plate (96 or 384 wells; Phenix Research Products). Aliquots of the DNA sample, typically 2 μL at 20 ng/μL, were transferred to the plate. Sealed plates (ABGene Aluminum; Phenix Research Products) were vortex-mixed, centrifuged, and transferred to a thermal cycler (ABI 9700; Applied Biosystems). Samples were amplified with an initial heat-denaturation step of 98 °C for 5 min; 25 cycles of 97 °C for 35 s, 62 °C for 35 s, and 72 °C for 4 min; and a final of extension at 72 °C for 10 min. After the PCR, samples were stored protected from light at −15 °C to −30 °C before analysis by either AGE or CE. A schematic for the technology and work flow is shown in Fig. 1.

**AGAROSE GEL ELECTROPHORESIS**

We combined 6 μL of the PCR reaction with 3 μL of 3× AGE loading dye (150 g/L glycerol, 2.5 g/L bromophenol blue; both from Sigma–Aldrich); the entire 9-μL volume was loaded on a 17.5-g/L agarose gel. Gels
were stained with SYBR Gold Nucleic Acid Gel Stain (10,000×; Invitrogen) and imaged by UV light with a FluorChem 8800 imaging detection system (Alpha Innotech).

**CAPILLARY ELECTROPHORESIS**

CE offers single-repeat resolution, which is much higher than that of slab gel electrophoresis, and thus is the platform of choice for providing accurate quantification of repeats, particularly for samples with borderline numbers of CGG repeats (e.g., 45 or 55 repeats). A 3130xl Genetic Analyzer (Applied Biosystems) running POP-7 polymer (Applied Biosystems) with 36-cm capillaries was used for all experiments. Samples were prepared for CE analysis by mixing 2 μL of the unpurified PCR product with 11 μL of Hi-Di Formamide (Applied Biosystems) and 2 μL of the ROX 1000 Size Ladder (Asuragen). Samples thus prepared were denatured at 95 °C for 2 min and then cooled at 4 °C for at least 2 min. Except where noted, applied voltages for all injections were 2.5 kV for 20 s with a 40 min run at 15 kV.

**DATA ANALYSIS**

PCR products analyzed by AGE were sized relative to the molecular size ladder up to about 1500 CGG repeats. PCR products detected by CE were analyzed with GeneMapper software (version 4.0; Applied Biosystems). The peak size in base pairs was converted to the number of CGG repeats by referencing the base pair size of the process control alleles to the base pair size of the sample’s product peaks. Indications of genotype followed the American College of Medical Genetics guidelines for “normal” (<45 CGG repeats), intermediate (45–54 CGG repeats), premutation (55–200 CGG repeats), and full mutation (>200 CGG repeats) alleles (9, 15). The full mutation mosaic category was used only for samples containing both a premutation allele and a full mutation allele.

**SOUTHERN BLOTTING**

For Southern blot analysis, 7–10 μg of isolated DNA was digested with EcoRI and NruI and separated on an 8-g/L agarose gel containing Tris-acetate-EDTA buffer (40 mmol/L Tris-acetate and 1 mmol/L EDTA). After DNA transfer, the membranes were hybridized with a FMR1-specific genomic probe, StB123. Additional details of the method are as previously described (16).

**Results**

Because FMR1 disorders such as FXS, fragile X–associated primary ovarian insufficiency, and fragile X–associated...
tremor/ataxia syndrome are associated with the number of triplet repeats in the 5' untranslated region of the gene, DNA-based assays that interrogate the length of the CGG tract are the methods of choice for molecular testing. Although procedures such as Southern blotting and DNA sequencing can enumerate the repeat segment, these approaches are primarily limited by the number or accuracy of repeat quantification, the amount of gDNA material that is required, or workflow considerations that are incompatible with high-throughput procedures (17). For these reasons, the PCR is the preferred molecular technique. The goal of this study was to characterize a novel set of gene-specific PCR reagents with both cell lines and clinical DNA samples and reference the results to Southern blotting results as a first step in the development of a PCR-only technology for FMR1 analysis.

To establish the performance of the gene-specific FMR1 reagents with defined DNA templates, we amplified a collection of cell line gDNAs from Coriell Cell Repositories. Products for samples from both males and females were characterized by both AGE (Fig. 2A) and CE (see Fig. 1 and Table 1 in the Data Supplement). The DNA templates included several gDNA materials previously assessed by sequencing and/or consensus CGG repeat sizing (18). As shown in Fig. 2, the FMR1 reagents amplified cell line templates with CGG repeat numbers spanning all allele categories, from normal to full mutation. Templates with up to approximately 1000 repeats were readily detected by both AGE and CE (see Fig. 2A, lane 6, and Fig. 5; also see Fig. 1 in the online Data Supplement). In each case, the numbers of inferred CGG repeats were consistent with those of the reference method (see Table 1 in the online Data Supplement).

The results of amplifying a set of cell line gDNA templates from females further underscored the efficiency of the PCR reaction. Historically, PCR-based FMR1 analysis has suffered from biased amplification (12). This bias is exacerbated by the extremely GC-rich sequence context of the triplet-repeat region that favors the more readily amplifiable allele and compounds the difference in product accumulation when both short (i.e., normal) and long (i.e., premutation or http://www.clinchem.org/content/vol56/issue3). The number of CGG repeats for each template was extrapolated from the mobility of the amplicon relative to the size standards for both of the electrophoresis platforms. The DNA templates included several gDNA materials previously assessed by sequencing and/or consensus CGG repeat sizing (18). As shown in Fig. 2, the FMR1 reagents amplified cell line templates with CGG repeat numbers spanning all allele categories, from normal to full mutation. Templates with up to approximately 1000 repeats were readily detected by both AGE and CE (see Fig. 2A, lane 6, and Fig. 5; also see Fig. 1 in the online Data Supplement). In each case, the numbers of inferred CGG repeats were consistent with those of the reference method (see Table 1 in the online Data Supplement).
Alleles with varying numbers of repeats were readily detected in heterozygotes with the FMR1 gene–specific PCR reagents (Fig. 2B). All combinations of a half dozen FMR1 alleles spanning the range of normal to full mutation (i.e., 20, 29, 119, 199, 336, and 645 CGG repeats) could be readily coamplified in the same tube (see lane 3, Fig. 2 in the online Data Supplement).

The FMR1 PCR method was next evaluated with a set of 146 blinded clinical samples provided by the M.I.N.D. Institute at the University of California, Davis, and previously characterized by Southern blot analysis as previously described (16). Comparative Southern blotting and PCR results for a representative set of 17 samples from the larger group of 146 samples are shown in Fig. 3. Although the PCR and Southern blotting methods rely on different sample-processing and detection modalities, the data demonstrate a striking similarity in the pattern distribution and sizes of the FMR1 alleles. For example, results obtained with the 2 methods often mirrored each other in that both methods often represented alleles with similar relative intensities and distribution of product bands, even for expanded alleles (see lanes corresponding to sample nos. 54, 55, 57, 58, 62, and 66). The agreement between the 2 methods in the data, most notably the sample-specific pattern of complex products, suggests that the PCR and Southern blotting methods produce results that are highly consistent with each other and that reflect the true molecular-repeat numbers for patients’ FMR1 alleles.

The gene-specific PCR products were also analyzed by CE (Fig. 4). Consistent with the high resolution of this method, heterozygous alleles that differed by a single CGG repeat were readily differentiated (no. 34, Fig. 4), whereas the limit of resolution for AGE was approximately 5 CGG repeats for alleles in the normal repeat range. With CE, FMR1 alleles could be accurately sized within 1 CGG repeat up to 70 CGG repeats and within 3 CGG repeats to approximately 120 repeats.
(see Table 1 in the online Data Supplement). Full mutation alleles, however, could not be resolved beyond about 250 CGG repeats—just beyond the repeat threshold for a fragile X full mutation—with the CE configuration described. For example, CE of PCR amplicons from sample no. 118, which contained full mutation alleles spanning approximately 375–1200 CGG repeats according to Southern blotting, revealed a peak
mobility and morphology that were similar to those of sample no. 55, which presented alleles of approximately 450–650 CGG repeats by Southern blotting (Fig. 4). Nevertheless, full mutations identified from the CE analysis consistently agreed with category assessments of the same amplicons by AGE or Southern blotting.

Across the full set of 146 samples, 42 normal and 3 intermediate samples were identified by both FMR1 Southern blotting and gene-specific PCR. In addition, the Southern blotting analysis identified 66 full mutations. All 66 of these samples were also detected as full mutations by FMR1 gene–specific PCR (Table 1), as resolved by both AGE and CE. PCR analysis also identified 2 samples with full mutation and premutation alleles that were scored as premutations only by Southern blotting (see below). The remaining samples that were categorized as premutations by Southern blotting were exactly concordant with the results from FMR1 PCR analysis.

The 2 discrepant samples, nos. 22 and 101, revealed not only prominent premutation-size fragments by both Southern blotting and the PCR (see Fig. 3, A and B, in the online Data Supplement) but also low-intensity full mutation amplicons by the PCR when analyzed by CE (see Fig. 3C in the online Data Supplement). Full mutation alleles in other samples that were only faintly visible with Southern blotting were also more clearly detected by PCR/CE, particularly for expanded alleles that spanned a broad size distribution but had “collapsed” through migration in the CE polymer and thus were codetected as a collection of large amplicons with similar electrophoretic mobilities (see no. 125, Fig. 3C in the online Data Supplement). This enhanced detection raised the question of whether the PCR method can be more sensitive than Southern blotting for the detection of low abundance alleles.

To help address this question, we determined the analytical limit of detection for both the PCR and Southern blotting methods after titrating well-defined gDNAs from 2 male cell lines, one containing an FMR1 allele with 940 CGG repeats and the other containing an allele with 23 CGG repeats. Fig. 5 shows that as little as a 1% mass fraction of the 940-CGG template (400 pg, approximately 120 gene copies) was detected by PCR in a background of 99% 23-CGG template.

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**Table 1.** The results of gene-specific FMR1 PCR are concordant with Southern blotting for the detection of FMR1 full mutations.a

<table>
<thead>
<tr>
<th>Fragile X full mutation</th>
<th>Gene-specific FMR1 PCR</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Southern blotting</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>66</td>
</tr>
<tr>
<td>Negative</td>
<td>2b</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
</tr>
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</table>

*a Samples were scored positive if a full mutation allele was detected and negative if not. For the PCR method, all determinations were based on resolution of amplicons by CE rather than by AGE.

*b Two samples showing evidence of not only prominent premutation alleles by both the Southern blotting and PCR methods but also low abundance full mutation alleles by the PCR analysis only.

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**Fig. 5.** Gene-specific FMR1 PCR is 5-fold more sensitive than Southern blotting for the detection of a defined full mutation allele.

A total of 7 μg of gDNA was applied to the Southern blot; 40 ng gDNA was used for the PCR. The limit of detection for the 2 methods, expressed as the percentage of a 940-CGG full mutation allele in a background of excess 23-CGG allele and as revealed in the original autoradiograph or gel image, is marked with an asterisk. L, ladder.
(39.6 ng). In contrast, a 175-fold higher input of gDNA into the Southern blot revealed a limit of detection of about 5% of the 940-CGG allele. Thus, the FMR1 gene-specific PCR is 5-fold more sensitive than Southern blotting (or 5 × 175 = 875-fold more sensitive, given the differences in the inputs of the 940-CGG allele that was analyzed with the 2 methods), at least with these gDNA templates. This result is consistent with the observation that full mutation alleles in clinical samples may be identified by PCR analysis when they cannot be detected by Southern blotting.

Discussion

Inefficient PCR amplification of the 5’ untranslated region of the FMR1 gene has long hindered the development of high-throughput and automation-friendly fragile X molecular diagnostics. Although the literature describes a handful of PCR methodologies that can amplify >200 CGG repeats (12, 19–21), all have been limited to the assessment of smaller full mutations, usually in males only. Indeed, protocols currently used by diagnostics laboratories are commonly restricted to the detection of 100–150 repeats, and full mutations are often suspected by their failure to amplify, rather than by their amplification success (22, 23). Consequently, the workflow for fragile X diagnostics relies on Southern blotting to deliver molecular information not currently achievable with PCR analysis. To overcome these limitations, Asuragen scientists carefully optimized a set of PCR reagents to enable highly efficient amplification of GC-rich DNA. This capability is primarily due to innovations in 3 areas: (a) the gene-specific primers, (b) the amplification buffer for GC-rich templates, and (c) the PCR cycling conditions. For example, >60 distinct primer pairs were screened to identify the optimal pair. More than 1000 different combinations of PCR additives and other buffer components were evaluated with model DNA templates. Key to this effort was the use of conditions that depressed the amplicon melting temperature (Tm) while still supporting primer binding and efficient PCR amplification. The most promising combinations of primers and buffer formulations were then iteratively evaluated with a range of PCR conditions to derive the final reagent set and procedures.

In this report, we have described the performance of these optimized FMR1 PCR reagents with 146 unique clinical samples. The technology can reproducibly amplify full mutations in samples from both males and females, including alleles of up to at least 1300 CGG repeats—several-fold larger than obtained in any other published study (12, 13). Therefore, this technology addresses many of the key problems that have historically limited the utility of FMR1 PCR and thus can greatly reduce the number of samples that must be reflexed to analysis by Southern blotting.

A key feature of the FMR1 PCR technology we have described is the efficiency by which long CGG repeat sequences can be amplified, particularly in samples from females. Full mutation samples from females provide 2 FMR1 alleles, typically one with <40 CGG repeats and one with >200 CGG repeats. Because the shorter allele is amplified much more readily, this template can outcompete the longer allele during the PCR and reduce the yield of the full mutation amplicon that would otherwise be produced if the full mutation allele were amplified in isolation. This imbalance is exacerbated with increasing CGG length because the efficiency of the PCR decreases. The PCR conditions we have described, however, produce very “balanced” PCR product yields (Fig. 2). In fact, as few as about 120 copies (400 pg) of a 940-CGG allele can be detected in a background of a 99-fold excess of a 23-CGG allele (Fig. 5). Moreover, combinations of half a dozen or more alleles, including several full mutation alleles, can be successfully amplified and detected with these reagents (see Fig. 2 in the online Data Supplement). A practical benefit of this capability is the use of a 6-allele process control that spans the sizes of normal alleles to full mutation alleles; this process control was included among the samples evaluated in this study (Fig. 4B).

The performance of the PCR reagents with blinded clinical samples produced an excellent correlation with results produced with Southern blotting. Of particular note is that all 66 full mutations detected by Southern blotting were also detected by the FMR1 PCR. Moreover, a remarkable similarity in the heterogeneous sample-by-sample allele patterns was revealed by comparing the data produced with the 2 methods. In addition, 2 samples with well-defined premutation alleles that were detected by both methods also provided evidence of low abundance full mutation alleles with the PCR, but not with Southern blotting. An analytical titration of full mutation and normal gDNA templates demonstrated that the PCR is 5-fold more sensitive than Southern blotting for detecting the full mutation allele (Fig. 5), even after discounting the 175-fold difference in DNA input. Thus, the PCR can detect at least some full mutation alleles that are below the limit of detection by Southern blotting.

A larger question is this: What are the implications of the detection of such low abundance full mutations? Mosaic alleles are present in a subset of the cell population, and on the basis of the results in Fig. 5, the FMR1 PCR can theoretically detect full mutation alleles in <5% of cells, perhaps in as few as 1% of cells. On the one hand, the lack of FMR1 protein production in such
a cell minority is unlikely to have a large impact on the fragile X phenotype. On the other, \textit{FMR1} testing is performed with a clinically accessible sample (whole blood) that is merely a surrogate for interrogation of the target tissue (brain) that is responsible for the neurologic consequences of FXS. Case studies have demonstrated discrepancies within the same patient in the number of CGG repeats in whole blood compared with such cell types as epidermal cells, which are more closely related in lineage to brain (24–26). Samples presenting detectable full mutation alleles in a subset of blood cells may be worthwhile to reflex test in epidermal cells as a way to begin to assess the molecular implications of such low abundance full mutation alleles. This concept may also be relevant to fragile X–associated primary ovarian insufficiency, an \textit{FMR1} disorder whose biological consequences are realized in cells other than those in whole blood.

The reproducible detection of full mutations by the \textit{FMR1} PCR reagents also has important implications for sample reflexing to Southern blotting. Currently, laboratories either process every clinical sample on Southern blots (because of the inadequacy of most \textit{FMR1} PCR tests) or reflex suspect samples to Southern blotting. Such suspect samples may include samples from males that fail to amplify or samples from females that support only a single PCR product. In the latter case, homozygous samples, which represent >20% of all samples from females, cannot be distinguished from the heterozygous case with one unamplifiable allele. The capabilities of the novel \textit{FMR1} PCR reagents to amplify every full mutation in this study translated to accurate zygoty assessments for all samples. Moreover, the performance of the reagents suggests that only samples that require methylation information need to be reflexed to Southern blotting. Given that many laboratories restrict methylation assessments to premutation and full mutation samples and that these categories represent perhaps 2% of all samples (27), only this small fraction of samples would require reflex testing. Thus, the PCR capabilities we have outlined represent a substantial improvement over current procedures, which reflex approximately 10%–100% of samples to Southern blotting.

In summary, this PCR technology offers a compelling alternative to both Southern blotting and current PCR methodologies for \textit{FMR1} allele sizing. Furthermore, the capability to reproducibly amplify expanded alleles represents a critical first step toward the development of a PCR-only work flow that can support routine \textit{FMR1} analyses.

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**References**

3. Oberle I, Rousseau F, Heitz D, Kretz C, Devys D.