# Chapter 14

# Triplet-Repeat Primed PCR and Capillary Electrophoresis for Characterizing the Fragile X Mental Retardation 1 CGG Repeat Hyperexpansions

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## Abstract

Fragile X mental retardation 1 (*FMR1*) CGG repeat expansions cause fragile X syndrome—the leading monogenic form of intellectual disability—and increase the risk for fragile X-associated tremor ataxia syndrome and fragile X-associated primary ovarian insufficiency. Southern blot (SB) analysis is the current gold standard test for *FMR1* molecular diagnosis. Several polymerase chain reaction (PCR)-based methods are now available for sizing *FMR1* CGG repeat expansions. These methods offer higher diagnostic sensitivity and specificity compared to SB analysis, significantly reduce the turnaround time and increase throughput. In this chapter, we describe a triplet-repeat primed PCR protocol that employs capillary electrophoresis to resolve the derived amplicon products, enabling precise determination of the *FMR1* genotypes in both males and females and characterization of the CGG repeat structure.

**Key words** Capillary electrophoresis, TP-PCR, *FMR1*, Fragile X syndrome, Repeat expansion, FXTAS, FXPOI

## 1 Introduction

Fragile X syndrome (FXS, OMIM: 300624) is the leading single-gene cause of intellectual disability and autism spectrum disorder [1]. FXS affects ~1 in 5000–8000 females and ~1 in 4000 males [2, 3]. More than 99% of FXS patients have an expanded CGG repeat tract in the 5′-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene [4]. The FXS-causing full-mutation expansions harbor more than 200 CGG repeats, often promoting aberrant methylation of the *FMR1* promoter, transcriptional inactivation, and complete loss of the *FMR1*-encoded fragile X mental retardation protein [5]. Alleles in the premutation size range of 55–200 CGG repeats increase the risk for fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM: 300623) and fragile X-associated primary ovarian insufficiency (FXPOI) [6]. About 1 in 148–209 females and 1 in 290–430 males in the general

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population harbor a premutation allele [7–9]. Premutations are also meiotically unstable, showing greater tendency to expand further in repeat size during mother to child transmission [10]. In general, maternal premutation alleles with ≥90 CGG repeats have 80–100% chance of undergoing full-mutation expansion in the subsequent generation [11]. Allelic instability of premutations with <100 CGG repeats is also modulated by the density of AGG interruptions within the CGG repeat tract [12]. Uninterrupted CGG repeat tracts are prone to the formation of unusual secondary structures that promote strand slippage and addition or deletion of repeat units. Smaller premutations with 56 and 59 uninterrupted CGG repeats have been shown to expand into full-mutations [11, 13]. Absence or lower density of AGG interruptions is a notable feature of expanded *FMR1* alleles. Intermediate or gray zone alleles with 45–54 CGG repeats occur in the general population at a frequency of 1 in 22–66 females and 1 in 42–112 males [14]. Larger intermediate alleles with 50–54 CGG repeats are at higher risk for unstable transmission compared to alleles with 45–49 CGG repeats [15], and intermediate alleles that lack AGG interruptions exhibit higher instability relative to those with one or more AGG interruptions [16]. Alleles in the normal size range of 5–44 CGG repeats, which contain an AGG interruption every 9 or 10 CGG repeats, are stably transmitted across generations.

The recommended gold standard test for *FMR1* CGG repeat expansion detection is Southern blot (SB) analysis. SB allows simultaneous querying of CGG repeat size and methylation status but does not determine the *FMR1* genotypes reliably nor map AGG interruptions. SB is low-throughput and is also labor- and time-intensive. To address these limitations, several polymerase chain reaction (PCR)-based methods have been developed [17–24].

The conventional flanking or repeat-spanning PCRs employ two locus-specific primers that anneal to the sequences flanking the *FMR1* CGG repeat tract and amplify across the repeat region. The derived amplicons can be sized by slab-gel electrophoresis alongside a DNA ladder containing fragments of known sizes (in base pairs or bp). A more robust approach is to perform PCR with a fluorophore-tagged primer and analyze the generated amplicons by capillary electrophoresis (CE). In contrast to gel electrophoresis, CE facilitates accurate repeat size estimation and resolution of alleles that differ by one or few repeat units. Some CE-based flanking PCR methods are restricted to the analysis of alleles in the normal to premutation or small full-mutation size range and perform poorly at differentiating normal homozygous females (with two *FMR1* alleles of identical CGG repeat size) from heterozygous females with a normal and a non-amplifiable full-mutation expansion [25]. Preferential amplification of the smallest of the two *FMR1* alleles in females and the inability to reliably detect large GC-rich FM expansions hinder the clinical diagnostic

application of flanking PCRs. Furthermore, flanking PCRs cannot map AGG interruptions.

In 1996, Warner et al. developed a unique approach, referred to as triplet repeat primed PCR (TRP-PCR or TP-PCR) to detect the CAG repeat expansion mutation associated with myotonic dystrophy [26]. This method has now been adapted for the *FMR1* CGG repeat locus [17, 20–24, 27]. TP-PCR assays, in general, employ three primers—a locus-specific primer that anneals to the sequence either upstream or downstream of the repeats; a triplet-primed (TP) primer with a 3′ sequence that hybridizes within the repeat region and generates a heterogenous pool of amplicon fragments that differ in size from each other by a repeat unit; and a tail primer that has the same sequence as the 5′ overhanging sequence of the TP primer, which promotes further amplification of the fragments generated by the annealing and extension of the TP primer. Samples with expansions produce a highly heterogeneous mixture of TP-PCR amplicon fragments that appear as a "smear" upon agarose gel electrophoresis. Although the observation of a smear on agarose gel would suggest the presence of an expansion, neither the *FMR1* genotype nor its size can be determined.

CE analysis of expanded TP-PCR amplicons, on the other hand, offers superior resolution by generating a characteristic stutter pattern wherein the amplicon peaks exhibit decreasing fluorescence intensity with increasing fragment length. Each amplicon peak represents a repeat unit and the number of CGG repeats in a sample can be estimated by simply counting the number of peaks in the TP-PCR electropherogram. Combining TP-PCR with CE significantly enhances our ability to reliably detect expanded *FMR1* alleles, size alleles in the normal, intermediate, premutation, and small full-mutation size range, and resolve zygosity in females. Unlike flanking PCRs, which may fail when an expansion is too large to be amplified, TP-PCR assays will almost always detect an expansion regardless of its actual length. The number and pattern of AGG interruptions within the CGG repeat tract can also be deciphered from the TP-PCR electropherogram pattern. Therefore, CE-based TP-PCR methods are ideal for first-tier evaluation of *FMR1* CGG repeat size, and SB and other methylation-specific PCR assays [18, 22] can be reserved for confirmatory analysis of repeat length and/or methylation status in samples with expanded premutation and full-mutation alleles.

In this chapter, we describe an *FMR1* TP-PCR and CE assay [21] that facilitates accurate CGG repeat size determination in samples with normal, intermediate, and premutation alleles, reliable detection of full-mutation expansions, and assessment of CGG repeat structure in both males and females.

## 2 Materials

Our TP-PCR and CE protocol for *FMR1* CGG repeat analysis requires the following reagents, consumables, and instruments:

### 2.1 FMR1 TP-PCR Assay

1. 5 U HotStarTaq DNA polymerase.
2. Buffer supplied with HotStarTaq DNA polymerase.
3. 2.5× Q-solution: Add 7.5 μL of 5× Q solution (Qiagen, Hilden, Germany) to each TP-PCR assay reaction.
4. Deoxyribonucleotide triphosphates (dNTPs): 0.85 mM of dGTP and dCTP, and 0.17 mM of dATP and dTTP.
5. TP-PCR primers (Sequences are provided in Table 1): 0.60 μM of 6-carboxyfluorescein or *Fam*-labeled F primer, 0.60 μM of Tail primer, and 0.60 nM of TP primer.
6. 5 U Taq Extender™ PCR Additive.
7. 1× Tris-EDTA (TE) buffer.
8. Genomic DNA (diluted to 100 ng/μL with 1× TE buffer).
9. HPLC grade water.

The concentration and volume per reaction of all TP-PCR reagents are shown in Table 2.

### 2.2 CE Analysis

1. HiDi™ Formamide.
2. POP-7™ polymer.
3. CE running buffer: prepare 50 mL of CE running buffer by adding 45 mL of distilled water to 5 mL of 10× CE running buffer. Prepare the CE running buffer freshly before use.
4. MapMarker1000®.

### 2.3 Surface Decontaminants

1. 70% ethanol.
2. DNA away.

### 2.4 Disposables

1. 0.6 and 1.5 mL microcentrifuge tubes.
2. 36 cm 16-capillary array.

**Table 1**
**TP-PCR primer sequence**

| Primer ID | Primer sequence (5′–3′) | Primer length (bp) |
|---|---|---|
| *Fam*-F [28] | *FAM*-AGCCCCGCACTTCCACCACCAGCTCCTCCA | 30 |
| TP | TGCTCTGGACCCTGAAGTGTGCCGTTGATA$(CGG)_5$ | 45 |
| Tail | TGCTCTGGACCCTGAAGTGTGCCGTTGATA | 30 |

**Table 2**
**TP-PCR master mix**

| Components (Concentration) | Final concentration | Volume per reaction |
|---|---|---|
| HPLC grade water | – | 0.90 µL |
| PCR Buffer (10×) | 1× | 1.50 µL |
| Q-solution (5×) | 2.5× | 7.50 µL |
| *Primers* | | |
| *Fam*-F (25 µM) | 0.60 µM | 0.36 µL |
| TP (25 nM) | 0.60 nM | 0.36 µL |
| Tail (25 µM) | 0.60 µM | 0.36 µL |
| *dNTPs* | | |
| dGTP (50 mM) | 0.85 mM | 0.26 µL |
| dCTP (50 mM) | 0.85 mM | 0.26 µL |
| dATP (10 mM) | 0.17 mM | 0.26 µL |
| dTTP (10 mM) | 0.17 mM | 0.26 µL |
| HotStarTaq DNA polymerase (5 U/µL) | 5 U | 1.0 µL |
| Taq Extender Additive (5 U/µL) | 5 U | 1.0 µL |
| Genomic DNA (100 ng/µL) | 100 ng | 1.0 µL |

3. PCR tubes.
4. MicroAmp Optical 96-well reaction plate.
5. 1–10, 2–20, 20–200, and 200–1000 µL aerosol barrier pipette tips.

### 2.5 Instruments

1. Thermal cycler: GeneAmp® PCR System 9700 (Applied Biosystems).
2. Vortex mixer.
3. 1–1000 µL micropipettes.
4. Microplate centrifuge.
5. Microcentrifuge to spin down 0.6 and 1.5 mL tubes.
6. CE instrument: 3130*xl* Genetic Analyzer (Applied Biosystems).
7. GeneMapper® or Peak Scanner™ software for TP-PCR electropherogram analysis.

## 3 Methods

### 3.1 TP-PCR Assay

1. Clean the benchtop with 70% ethanol followed by DNA away.
2. Remove the PCR buffer and working stock solutions of primers, dNTPs, and genomic DNAs from −20 °C freezer and thaw them on ice (*see* **Note 1**).
3. Calculate the required volume of each TP-PCR reagent by simply multiplying the values (volume per reaction) in column 3 of Table 2 with the total number of reactions (*see* **Note 2**).
4. Once the PCR buffer and working stock solutions of primers, dNTPs, and genomic DNAs are completely thawed, remove the Q-solution, HotStarTaq DNA polymerase, and Taq Extender Additive from −20 °C freezer and place on ice.
5. Briefly vortex the PCR buffer, primers, dNTPs, and Q-solution and flick the tubes containing HotStarTaq DNA polymerase, Taq Extender Additive, and genomic DNA.
6. Spin down the tubes.
7. Prepare the TP-PCR master mix in a 0.6 or 1.5 mL microcentrifuge tube.
8. First add the Q-solution to the labeled master mix tube, followed by PCR buffer, HPLC grade water, TP-PCR primers, and dNTPs (*see* **Note 3**).
9. Gently mix the contents of the tube by pipetting up and down twice.
10. Add HotStarTaq DNA polymerase and Taq Extender Additive to the master mix.
11. Homogenize the contents by flicking and then briefly spin down the tube.
12. Aliquot 14 μL of the master mix into each labeled PCR tube.
13. Add 1 μL of genomic DNA to the respective PCR tube containing the master mix.
14. Flick the PCR tubes to mix the master mix and genomic DNA and quickly spin down the tubes.
15. Transfer the PCR tubes to the thermal cycler and run the thermal cycling program under the conditions detailed in Table 3.

### 3.2 CE Setup and Analysis

1. Once the thermal cycler enters the hold step, remove the PCR tubes.
2. Spin the tubes briefly and set aside.
3. Thaw HiDi™ Formamide and prepare the HiDi™ Formamide and MapMarker® 1000 mix as shown in Table 4.
4. Vortex briefly and spin down.

**Table 3**
**TP-PCR thermal cycling program**

| Thermal cycling step | Temperature (°C) | Time | Cycle |
|---|---|---|---|
| Initial denaturation | 95.0 | 15 min | 1 |
| Denaturation | 99.0 | 45 s | |
| Annealing | 55.0 | 45 s | 40 |
| Extension | 70.0 | 8 min + 15 s per cycle | |
| Final Extension | 72.0 | 10 min | 1 |
| Hold | 11.0 | ∞ | – |

**Table 4**
**Master mix for CE run**

| Components | Volume per reaction |
|---|---|
| TP-PCR Product | 4.0 μL |
| HiDi™ Formamide | 9.0 μL |
| MapMarker® 1000 | 0.5 μL |

5. Aliquot 9.5 μL of the prepared HiDi™ Formamide/MapMarker® 1000 mix into each well of a MicroAmp Optical 96-well reaction plate.
6. Add 4 μL of the TP-PCR amplicon product into the corresponding wells.
7. Seal the reaction plate with a clean rubber septum.
8. Denature the reaction plate at 95 °C for 5 min.
9. Place on ice until the reaction plate is ready to be loaded into the CE instrument.
10. Set up the CE instrument as per the manufacturer's instructions.
11. Ensure that the CE instrument has sufficient polymer.
12. Replenish the CE reservoirs with fresh buffer and water.
13. In the run module, change the injection time and voltage to 18 sec and 1.2 kV, respectively, and the run time and voltage to 50 min and 15 kV, respectively, with other parameters set to default values.
14. Once the CE run is completed, analyze the generated electropherograms using GeneMapper® or Peak Scanner™ software.

### 3.3 Interpretation of FMR1 TP-PCR Electropherograms

Figure 1 illustrates the performance of the TP-PCR assay on normal, premutation, and full-mutation samples. For simplification, these schematics do not show the overhanging Tail sequence of the TP primer and the Tail primer itself. As shown, the locus-specific primer, which is tagged with 6-carboxyfluorescein (*Fam*), anneals downstream to the *FMR1* CGG repeat region. The binding efficiency of TP primer within the *FMR1* CGG repeat region depends on the presence or absence of AGG interruptions. The repeat-annealing segment of the TP primer is composed of five CGG repeats. The TP primer anneals to every stretch of 5 CGG repeats and fails to do so when there is an AGG interruption, resulting in the generation of amplicon clusters with discrete gaps or zones of no detectable amplicon peaks. The first amplicon peak is the product generated from the annealing of TP primer to the last five CGG repeat units at the 3′ end of the repeat segment and is counted as five repeats, with the subsequent peaks counted sequentially. The number of 5-peak gaps seen in the electropherogram is equivalent to the number of AGG interruptions within the CGG repeat tract. Therefore, a normal 29-repeat homozygous female having an identical AGG interruption pattern of 9 + 9 + 9 (where 9 refers to the number of CGGs and + indicates an AGG interruption) in both *FMR1* alleles will generate three clusters of amplicons separated by two 5-peak gaps (Panel A). In the case of a premutation female with a 29-repeat normal allele and a 60-repeat premutation allele, we can expect to see overlapping amplicon peaks from both *FMR1* alleles (Panel B). Therefore, the presence of two AGG interruptions within the 29-repeat normal allele is not observed as clear 5-peak gaps but rather as zones of amplicon peaks with reduced fluorescence signal. Both the peak clusters of the normal allele and the stutter pattern of the expanded premutation allele can be clearly visualized. Panel C shows the expected CE profile of a full-mutation female with a 29-repeat normal allele and a full-mutation allele, wherein amplicon peaks from the full-mutation allele are seen extending beyond 200 CGG repeats.

Figure 2 shows the representative CE profiles of male and female samples with normal, premutation, and full-mutation alleles. The electropherograms clearly facilitate the differentiation of normal, premutation, and full-mutation genotypes and enable sizing of normal and premutation alleles. Both full-mutation male and female samples generated amplicon peaks exceeding the full-mutation cutoff of 200 CGG repeats, and in addition displayed a full-length amplicon peak close to 1050 bp (*see* **Notes 4–6**).

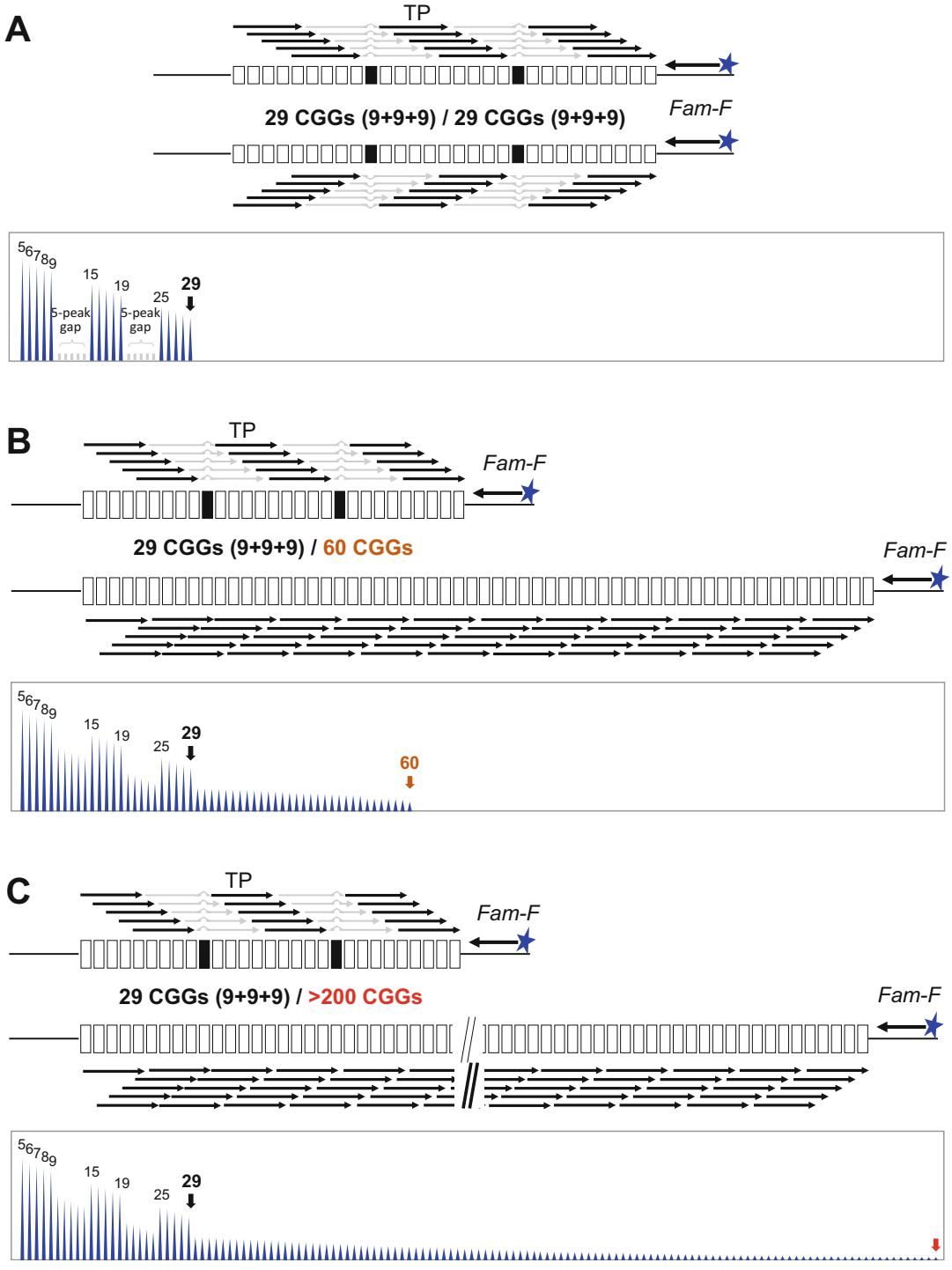


**Fig. 1** Annealing patterns of the FMR1 TP primer and the expected capillary electrophoresis outcomes of normal, premutation, and full-mutation samples. Panels **A**, **B**, and **C** show the annealing patterns of the TP primer and the expected TP-PCR electropherograms of normal, premutation, and full-mutation females, respectively. AGG interruptions within the CGG repeat tract are shown as shaded black rectangles. Black arrows represent the annealing positions of the TP primer, and gray arrows show the positions where the TP primer fails to anneal due to the presence of an AGG interruption. Samples with interrupted normal alleles generate clusters of amplicon peaks separated by gaps, while premutation and full-mutation expansions that lack AGG interruptions generate a stutter of amplicon peaks

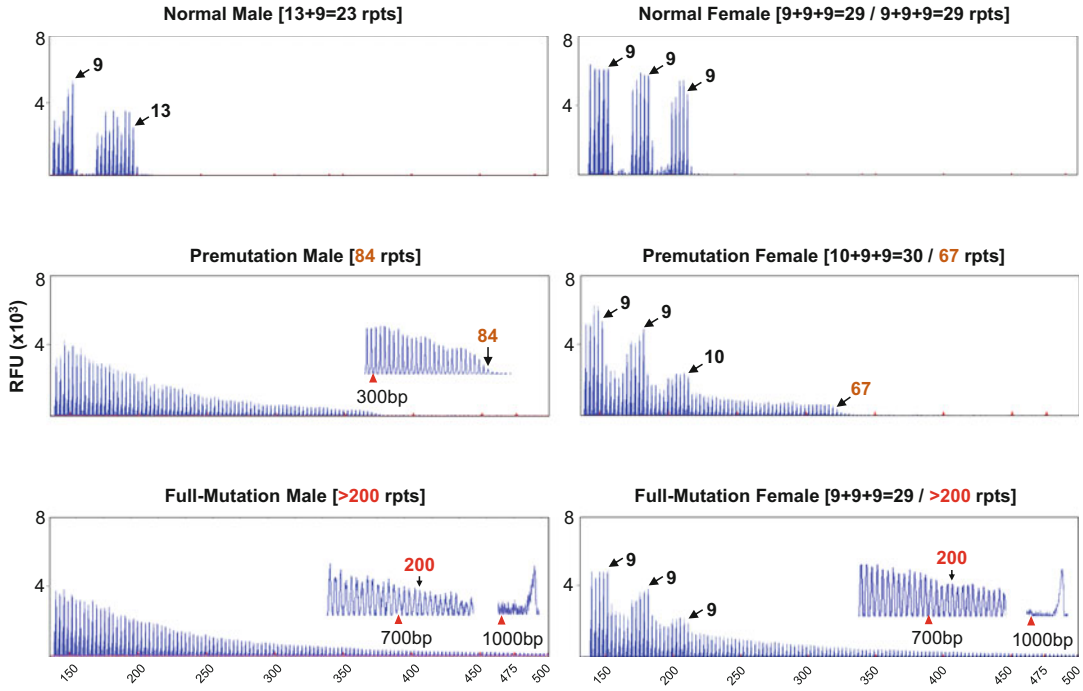


**Fig. 2** Representative Capillary Electropherogram Profiles of Normal, Premutation, and Full-Mutation Samples. X-axis represents fragment length in base pairs or bp and Y-axis represents Relative Fluorescence Units (RFUs). Red peaks in electropherograms are from the ROX-labeled internal size ladder. The CGG repeat size and AGG interruption pattern of *FMR1* alleles are indicated on top of each electropherogram in square brackets. "+" sign indicates the presence of an AGG interruption. The stutter pattern characteristic to expanded *FMR1* alleles are observed in premutation and full-mutation samples. Numbered arrows indicate the number of CGG repeats. Inset panels in premutation and full-mutation samples show the zoomed in view of the amplicon peaks. Full-mutation male and female samples had stutter peaks extending beyond 200 CGG repeats and a full-length peak around 1050 bp

## 4 Notes

1. Repeated freezing and thawing of the reagents may affect the amplification efficiency of TP-PCR. Reduce the number of freeze-thaw cycles by preparing smaller aliquots of primers, dNTPs, and genomic DNAs and storing them in multiple 0.6 mL microcentrifuge tubes.

   Genomic DNA extracted from a wide variety of sources (peripheral blood, lymphoblastoid cell lines, saliva, buccal swab, and dried blood spots) can be tested using the described TP-PCR protocol. However, genomic DNA specimens should be quantified and checked for impurities before running the TP-PCR assay. Presence of impurities in the extracted DNA and using DNA at concentrations below the recommended 100 ng may affect the electropherogram pattern and/or the Relative Fluorescence Units (RFUs) of the generated amplicon peaks. If a significant drop in RFUs or an irregular

electropherogram pattern is observed due to impurities in DNA, perform phenol-chloroform purification of the DNA and repeat the TP-PCR experiment with purified DNA specimen and freshly prepared reagents.

2. We highly recommend including at least two positive controls (fragile X samples with a FM expansion) and a negative control (or blank with water or 1 x TE buffer added instead of genomic DNA) in each TP-PCR experiment. While the positive controls will inform if the assay conditions are optimal for detecting and sizing expanded full-mutation alleles, the negative controls will expose any underlying contaminations.
3. Please ensure that the reagents are used at the recommended final concentrations (column 2 of Table 2). Also note that the ratio of dGTP and dCTP to dATP and dTTP is 5:1 and that the TP primer is supplied at a 1000-fold dilution compared to the *Fam*-F and Tail primers. Pipetting errors that alter the final concentrations of the TP-PCR reagents may affect the electropherogram pattern and the ability of TP-PCR assay to size expanded *FMR1* alleles. Ensure that the pipettes are serviced and calibrated to avoid inaccurate pipetting and suboptimal performance of the TP-PCR assay.
4. Rare deletions and point mutations that account for nearly 1% of the fragile X cases will not be detected by this assay that targets the analysis of *FMR1* CGG repeat expansions.
5. Polymorphism within the annealing position of the locus-specific primer may result in the failure to amplify expanded allele and generate false-negative results.
6. Due to the overlapping amplicon peaks from two *FMR1* alleles in heterozygous females, determination of the exact number and pattern of AGGs in samples with complex interruption patterns can be difficult.

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