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Assays for determining repeat number, methylation status and AGG interruptions in the Fragile X-related disorders

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Summary/Abstract

Assays for CGG•CCG-repeat number, AGG interruption status and the extent of DNA methylation are vital for both diagnosis of the Fragile X-related disorders and for basic research into disease mechanisms. We describe here assays that we use in our laboratory to assess these parameters. Our assays are PCR-based and include one for repeat size that can also be used to assess the extent of methylation and a related assay that allows the AGG interruption pattern to be reliably determined even in women. A second more quantitative methylation assay is also described. We also describe our method for cloning of repeats to generate the reference standards necessary for the accurate determination of repeat number and AGG interruption status.

Keywords

Repeat PCR; AGG PCR assay; Quantitative Methylation assay; *FMR1* ; qPCR; AGG interruptions; *FMR1*-related disorders (*FMR1* disorders); FX-associated tremor and ataxia syndrome (FXTAS); FX-associated primary ovarian insufficiency (FXPOI); Fragile X syndrome (FXS); Repeat PCR assay; Methylation assays; AGG assays

1 Introduction

The mutation responsible for the Fragile X-related disorders is an expansion of a CGG•CCG-repeat tract in exon 1 of the X-linked gene, *FMR1* (1). As discussed elsewhere in this volume, the symptoms of these disorders are related to the repeat number, with alleles having 55–200 repeats conferring risk of Fragile X-associated tremor/ataxia and Fragile X-associated primary ovarian insufficiency and alleles with >200 repeats resulting in Fragile X syndrome. However, the repeat tract is difficult to amplify by PCR likely because of the secondary structures formed by the repeats (2). This difficulty is compounded by the mosaicism that is often present in affected individuals (3). The risk of further expansion on maternal transmission is related to both the repeat number and the number of AGG interruptions. Thus, it is important to be able to reliably determine this second parameter, particularly in females (4–7). However, the presence of a second X chromosome can make

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doing so quite challenging. Finally, expansion beyond 200 repeats results in repeat-mediated gene silencing, the extent of which affects symptom severity. Methylation mosaicism is also present in many individuals (3). Thus, quantitative assays for DNA methylation are also an important tool in the diagnostic toolbox. The ability to accurately measure repeat number and methylation status is also important in a research setting since the cell lines that are frequently used can sometimes show both variations in repeat number and the extent of DNA methylation (8, 9). We describe here the PCR methods that we use in our laboratory to determine all the parameters necessary for a complete genetic workup or thorough laboratory study, including an assay that can be used to unambiguously ascertain the number of AGG interruptions even in women and a quantitative assay for the methylation status that is useful even in the case of extensive methylation mosaicism. We also describe how to generate suitable standards for the accurate determination of both the repeat number and the AGG interruption status.

2 Materials

2.1 Repeat PCR assay

1. 10 × PCR buffer: 500mM Tris.HCl pH 9.0, 15 mM $MgCl_2$, 220mM $(NH_4)_2SO_4$ (see Note 1), 2% Triton X-100. Store at room temperature.
2. 5 M betaine: Weigh 33.8g betaine monohydrate and add water to give a final volume of 50 ml. Filter through a 0.2 μm filter to sterilize. Store at 4°C.
3. DMSO. Store at room temperature in the dark.
4. dNTP solution: 10 mM each (see Note 2)
5. Primers:
   a. Not_FraxC (5’-**AGTTCAGCGGCCGC**GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3’),
   b. Not_FraxR4 (5’-**CAAGTCGCGGCCGC**CTTGTAGAAAGCGCCATTGGAGCCCCGCA-3’).

   Prepare working stocks of 10 μm of each primer in water (see Note 3). For fragment analysis by capillary electrophoresis Not_FraxR4 should be labeled with a fluorophore.
6. Thermostable polymerase: Q5® Hot Start polymerase (New England Biolabs; see Note 4).
7. Restriction enzymes: HindIII and HpaII. Store at −20°C (see Note 5).
8. Genomic DNA: High molecular weight DNA prepared from cells by the salting out method (10) or from saliva using Oragene reagents (DNA Genotek; see Note 6).
9. CGG-repeat size markers for capillary electrophoresis (see Note 7).

2.2 AGG PCR assay

1. 10 × AGG PCR buffer: 500 mM Tris.HCl pH 9.0, 17.5 mM $MgCl_2$, 220 mM $(NH_4)_2SO_4$ (see Note 1). Store at room temperature.
2. 5 M betaine: Weigh 33.8g betaine monohydrate and add water to give a final volume of 50 ml. Filter through a 0.2 μM filter to sterilize. Store at 4°C.
3. DMSO. Store at room temperature in the dark.
4. dNTP solution: 10 mM each (see Note 2)
5. Primers:
   a. Not_FraxC (5'-FAM-**AGTTCAGCGGCCGC**GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3'),
   b. Not_FraxR4 (5'-HEX-**CAAGTCGCGGCCGC**CTTGTAGAAAGCGCCATTGGAGCCCCGCA-3'),
   c. Not_PsdR4 (5'-NED-**CAAGTCGCGGCCGC**AGCCGCGAGAAATGCCTCCTGCGCAATGT-3'),
   d. A-primer (5'-**AGCGTCTACTGTCTCGGCACTGT**CGGCGGCGGA-3'),
   e. T-primer (5'-**AGCGTCTACTGTCTCGGCACTTGC**CCGCCGCCGCCT-3').

   Prepare working stocks of 10 μm of each primer in water (see Note 3). If Not_PsdR4 is to be incorporated into the reaction as a positive control, make a combined working stock with Not_FraxR4 with both at 10 μM each.
6. Thermostable polymerase: KAPA2G Robust Hot Start (KAPA Biosystems; see Note 8).
7. Restriction enzyme: HindIII. Store at −20°C (see Note 5).
8. Genomic DNA: High molecular weight DNA prepared from cells by the salting out method (10) or from saliva using Oragene reagents (see Note 6).
9. CGG-repeat size markers carrying known AGG interruption patterns (see Note 7).

2.3 Quantitative methylation assay

1. Restriction enzyme: HpaII. Store at −20°C
2. Genomic DNA: High molecular weight DNA prepared from cells by the salting out method (10) or from saliva using Oragene reagents (see Note 6).
3. Primers:

a. FMR1 ex1 F (5’-GAACAGCGTTGATCACGTGAC-3’)

b. FMR1 ex1 R (5’-GTGAAACCGAAACGGAGCTGA-3’)

c. GAPDH ex1 F (5’-TCGACAGTCAGCCGCATCT-3’)

d. GAPDH int1 R (5’-CTAGCCTCCCGGGTTTCTCT-3’)

Prepare working stocks of 10 μm of each primer in water (see Note 3).

4. Power SYBR Green Master Mix (ThermoFisher Scientific)

2.4 Generation of reference standards

1. 10 × PCR buffer: 500mM Tris.HCl pH 9.0, 15 mM $MgCl_2$, 220 mM $(NH_4)_2SO_4$ (see Note 1), 2% Triton X-100. Store at room temperature.
2. 5 M betaine: Weigh 33.8g betaine monohydrate and add water to give a final volume of 50 ml. Filter through a 0.2 μM filter to sterilize. Store at 4°C.
3. DMSO. Store at room temperature in the dark.
4. dNTP solution: 10 mM each (see Note 2)
5. Primers: Gb_FraxC (5’-**CTGGAGCAATTCCGGCGCGCC**GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3’), Gb_FraxR4 (5’-**CTCGCCCTTGCTCACCATGG**GAACATCCTTTACAAATGCCTTGTAGAAAGCGCCATTGGAGCCCCGCA-3’).

   612F (5’- ATAAGCTTTAGGCGTGTACGG-3’)

   1311R (5’- CGCTGAACTTGTGGCCGTTTA-3’)

   Prepare working stocks of 10 μm of each primer in water (see Note 3).
6. Thermostable polymerase: Q5® Hot Start (New England Biolabs).
7. Restriction enzymes: HindIII. Store at −20°C (see Note 5).
8. Genomic DNA: High molecular weight DNA prepared from cells by the salting out method (10) or from saliva using Oragene reagents (see Note 6).
9. Addgene plasmid 99255 (see Note 7).

3 Methods

3.1 Restriction enzyme digestion of genomic DNA

This protocol is required for both the Repeat PCR and AGG PCR assays.

1. Assemble a 40 μl reaction containing 4 μl of the appropriate 10x PCR buffer (either Repeat PCR buffer or AGG PCR buffer), 600 ng of genomic DNA (see Note 6) and 1 μl of HindIII restriction enzyme (20 U in the case of HindIII-HF from New England Biolabs). Mix well.

2. If the methylation status of the alleles is to be tested (only applicable to the Repeat assay PCR), divide the reaction into 2 × 20 μl aliquots and add 0.5 μl of HpaII (5U in the case of HpaII from New England Biolabs) to one aliquot. Mix well.

3. Incubate overnight at 37°C in either a heating block with a heated lid set at 45°C or in a 37°C air incubator to reduce evaporation (see Note 9).

3.2 Repeat PCR assay

1. Assemble the PCR master mix to give sufficient volume for N + 1 PCR reactions (see Note 10). The following volumes are for 10 PCR reactions (i.e., 9 + 1): 15 μl 10 × PCR buffer, 10 μl 10 μM Not_FraxC, 10 μl 10 μM Not_FraxR4, 100 μl 5M betaine, 4 μl DMSO, 9 μl dNTP solution (see Note 11), and 2 μl Q5® polymerase. Mixing the betaine and 10 × PCR buffer together turns the solution cloudy momentarily.

2. Place 5 μl of the digestion reactions into a PCR tube and then add 15 μl of the PCR master mix, pipetting gently up and down several times to ensure that the solutions are completely mixed.

3. Initiate the PCR program and wait until the block has reached at least 70°C before putting the tubes in. Cycle for: 98°C 3 min, 30 × (98°C 30 sec, 59°C 30 sec, 72°C 210 sec), 72°C 10 min, then hold at 12°C or store at −20°C until ready to analyze.

4. The products can be analyzed by agarose gel electrophoresis. However, ethidium bromide has been reported to alter the mobility of CGG-repeat containing fragments (11), so the gel should be run without ethidium bromide and stained afterwards. An approximate repeat number can be calculated as follows: (Fragment size-270)/3.

5. For more accurate size determinations, the samples can be analyzed by capillary electrophoresis if one of the primers used was fluorescently labeled (see Note 7).

3.3 AGG PCR assay

1. The A-primed assay is used to determine the number of AGG interruptions in each allele. The T-primed assay can be used subsequently for a more accurate determination of the interspersion pattern if needed (see [13] for a more detailed discussion). Assemble the PCR master mix to give sufficient volume for N + 1 PCR reactions (see Note 10). The following volumes are for 10 A-primed PCR reactions without the positive control (ie 9 + 1): 15 μl 10 × AGG PCR buffer, 10 μl 10uM A-primer, 10 μl 10 μM HEX-labeled Not_FraxR4, 100 μl 5M betaine, 4 μl DMSO, 4 μl dNTP solution, 5.5 μl H2O, 1.5 μl KAPA2G polymerase. For 10 A-primed PCR reactions with the positive control: 15 μl 10 × AGG PCR buffer, 10 μl 10 μM A-primer, 10 μl (10 μM HEX-labeled Not_FraxR4 + 10 μM NED-labeled Not_PsdR4), 100 μl 5M betaine, 4 μl DMSO, 4 μl dNTP solution, 1 μl 1pg/μl Addgene plasmid 99257 (see Note 12), 4.5 μl H2O, 1.5 μl KAPA2G polymerase. For 10 T-primed PCR reactions: 15 μl 10 × AGG PCR buffer, 10 μl

10uM T-primer, 10 μl 10 μM FAM-labeled Not_FraxC, 100 μl 5M betaine, 4 μl DMSO, 4 μl dNTP solution, 5.5 μl H_2O, 1.5 μl KAPA2G polymerase.

2. Place 5 μl of the digestion reactions into a PCR tube and then add 15 μl of the PCR master mix, pipetting gently up and down several times to ensure that the solutions are completely mixed (see Note 10).

3. Initiate the PCR program and wait until the block has reached at least 70°C before putting the tubes in. Cycle for: 98°C 3 min, 30 × (98°C 30 sec, 55°C 30 sec, 72°C 210 sec), 72°C 10 min, then hold at 12°C or store at −20°C until ready to visualize.

4. Agarose gel electrophoresis is not suitable for the analysis of these reaction products due to priming by the A-primer/T-primer elsewhere in the genome. Thus, capillary electrophoresis with the appropriate reference standards must be used (see Note 7). Note that if the positive control plasmid for the A-primed assay is incorporated as indicated above there will be a NED-labelled fragment present in the “water-only” PCR control.

3.4 Quantitative methylation assay

1. Make a 100 μl volume of genomic DNA at 10 ng/ μl in H_2O.

2. Sonicate to fragment the DNA to 0.5–1 kb in size. This can be done using a Bioruptor (Diagenode) set at medium with cycles of 30 seconds on and 30 seconds off for five minutes (see Note 13). Verify the size by agarose gel electrophoresis of ~100 ng of the sample (see Note 14).

3. Combine 60 μl sonicated DNA (600 ng) with 10 μl 10 x CutSmart® buffer (New England Biolabs) and 25 μl H_2O.

4. Take two 45 μl aliquots and add 5 μl H_2O to one (mock digest) and 1.2 μl HpaII (see Note 14) + 3.8 μl H_2O to the other (to give 12 U HpaII digesting 300 ng DNA). Incubate overnight at 37°C in either a heating block with a heated lid set at 45°C or in a 37°C air incubator to minimize evaporation-related problems. Heat inactivate the HpaII by incubating at 80°C for 20 minutes.

5. Each DNA sample requires 10 PCR replicates for the *FMR1* methylation assay (five undigested and five digested) and 6 PCR replicates for the *GAPDH* digestion control assay (three undigested and three digested). A master mix of ten PCR reactions contains 100 μl Power SYBR Green Mix (2x), 4 μl 10 μM forward primer, 4 μl 10 μM reverse primer and 72 μl H_2O. For the *FMR1* methylation assay the primers are FMR1 ex1 F and FMR1 ex1 R while for the *GAPDH* digestion control the primers are GAPDH ex1 F and GAPDH int1 R. Make sufficient Master Mix to accommodate (No. of samples × No. of replicates) + 1.

6. Aliquot 10 × 18 μl of the *FMR1* methylation Master Mix into 10 wells of a qPCR 96-well plate and add 5 × 2 μl of the undigested DNA and 5 × 2 μl of the digested DNA. Similarly, aliquot 6 × 18 μl of the *GAPDH* digestion control

Master Mix and add 3 × 2 μl of the undigested DNA and 3 × 2 μl of the digested DNA. Repeat for the remainder of the DNA samples.

7. Seal the 96-well plate. Centrifuge at 1000 rpm for 1 minute.

8. Perform the qPCR using the following cycling parameters: 95°C 10 min, 40 × (95°C 15 seconds, 60°C 60 seconds). Analyze the data by setting the Ct threshold to 0.1. Calculate the extent of digestion by taking the average of the Ct values of the undigested *GAPDH* qPCRs and the average of the Ct values of the digested *GAPDH* qPCRs and calculating the ΔCt value ($Ct_{digested} - Ct_{undigested}$). Digestion is complete if the value of $1/(2^{\Delta Ct})$ is ≤ 0.1. Calculate the *FMR1* methylation percentage similarly: ΔCt = (Average $Ct_{digested}$ – average $Ct_{undigested}$). % methylation = $1/(2^{\Delta Ct})$.

3.5 Generation of reference standards

3.5.1 Cloning of repeat sequences for use as reference standards

1. To generate the plasmid backbone use plasmid DNA from Addgene plasmid 99255 and digest 5–10μg with AscI + NcoI to separate the 4.4 kb plasmid backbone from the 350 bp *FMR1* insert. Use agarose gel electrophoresis to separate the two fragments and gel purify the backbone fragment (see Note 15).

2. Digest genomic DNA with HindIII as detailed in Section 3.1. (see Note 16).

3. Perform a preparative scale Repeat PCR by combining 25μl of the digested genomic DNA with 75 μl of the Repeat PCR master mix (see Sections 3.2.1 and 3.2.2) containing Q5® polymerase. Divide into 5 × 20 μl aliquots.

4. Initiate the PCR program and place the PCR reactions in the block when it has reached ≥ 70°C. Cycle for 98°C 3 min, 23 × (98°C 30 seconds, 59°C 30 seconds, 72°C 120 seconds), 72°C 10 minutes. (see Note 17)

5. Purify the PCR product using a standard PCR column clean up kit (e.g., Qiagen QiaQuick or NEB Monarch) and elute in the minimum volume of elution buffer. The fragment will be barely visible on an agarose gel.

6. Use a Gibson Assembly kit (e.g., New England Biolabs or Synthetic Genomics) and a ratio of ~5:1 PCR fragment:plasmid backbone to carry out a Gibson Assembly according to the manufacturer's instructions. Electroporate into an *E.coli* strain suitable for the maintenance of repetitive sequences e.g., Endura™ (Lucigen) or NEB® Stable (New England Biolabs).

7. Choose at least 10 colonies and grow 3 ml cultures overnight. Take an aliquot from each culture and make a glycerol stock and then use the remainder to prepare plasmid. Digest the plasmids with AscI + NcoI to identify those containing a correctly sized insert.

8. Verify the clones by sequencing them using primers 612F and 1311R.

9. Generate large quantities of plasmid DNA by inoculating 4 × 100 ml cultures directly from the glycerol stock and harvest while the culture is still in log phase

(<10 hours growth at 37°C). Isolate the plasmid and purify the AscI + NcoI fragment containing the *FMR1* sequence by agarose gel electrophoresis.

3.5.2 Use of reference standards

1. The individual plasmids containing defined repeat numbers or genomic DNA templates of known repeat number and/or interspersion patterns should be amplified using the Repeat PCR protocol for the CGG size ladder or the AGG PCR protocol for the AGG interruption ladder using the same labeled primer as the samples to be analyzed.
2. After purification with a PCR column clean up kit as described above, equivalent amounts of each fragment should be mixed to generate the reference standard.
3. The reference standard should be included in every capillary electrophoresis run. Afterwards a standard curve should be prepared and used to interpolate the repeat number/AGG position.

4 Notes

1. Weigh 14.5 g $(NH_4)_2SO_4$ and add water to give a final volume of 50 ml, giving a 2.2 M solution. Filter through a 0.2 μM filter to sterilize. Store at room temperature. Do not substitute the commercially available “saturated” solutions.
2. Store the dNTPs in small aliquots at −20°C and discard after 3 freeze-thaw cycles.
3. Make a 100 μM master stock of each primer by resuspension in the appropriate volume of 1 x TE. Make up small volume aliquots of 10 μM working stocks and store at −20°C. Discard working stocks after 3 freeze-thaw cycles.
4. The previously published version of the assay (12) used Phusion® polymerase. However, subsequent tests showed that Q5® Hot Start polymerase gives a better fragment yield and eliminates the need to assemble the PCR reactions on ice. Due to their proof-reading activity, these polymerases are not suitable for use in the AGG assay (but also see Note 8).
5. PstI, EcoRI or Bsu36I restriction endonucleases can be substituted for HindIII.
6. Genomic DNA is stored at 4°C in TE buffer at ~100ng/μl. Significantly higher concentrations should not be used due to the difficulty of accurately pipetting small volumes of samples with high viscosity. Since adsorption to plasticware can reduce the amount of DNA in solution, the DNA concentrations should be based on a recent determination, preferably just prior to setting up the digestion reaction.
7. The two strands of the CGG-repeat containing PCR product do not migrate at the same rate and neither migrate at a rate consistent with their true size. Thus, for accurate repeat number determinations, a reference ladder of *FMR1* PCR products containing a known number of repeats and/or AGG interruptions should be used. A set of reference standards for both the Repeat PCR assay and the

AGG PCR assay can be generated either directly from genomic DNA isolated from patient samples or, more sustainably, from patient cell lines or from cloned and sequence verified plasmid sequences (see section 3.5). We have deposited 2 plasmids, 99255 and 99256, in the Addgene plasmid repository that can be used to prepare reference standards for both the Repeat PCR assay and the AGG PCR assay. Plasmid 99255 (a.k.a. pGLACTE-HT51-10) has the repeat structure: 9-A-9-A-78 and 99256 ((a.k.a. pGLACTE-HT51-13) has the repeat structure: 9-A-9-A-9 (13). Thus, in the AGG PCR assay using the A-primer these two plasmids will produce fragments corresponding to 10, 20, 79 and 89 repeats. In the assay using the T-primer, fragments corresponding to 10 and 20 repeats will be generated (13). Combined with two other plasmids (pGLACTE-23297 and pGLACTE-7306; available on request), these plasmids can also be used as a set of reference standards for the Repeat PCR assay to create a ladder containing 23, 29, 52 and 98 repeats. Genomic DNA containing larger alleles of known size can be used to generate larger reference standards. The reference standards should contain at least four fragments spanning the range of expected repeat sizes that have been amplified using the same labeled primer as the fragments to be analyzed. Note: These plasmids are prone to contraction when grown at 37°C. Upon receipt of the Addgene stab streak it out immediately and verify the insert size and AGG interruption pattern as described in Section 3.5.7.

8. Note that, while in our hands the Repeat PCR assay with Q5® polymerase is more robust, KAPA2G polymerase can also be used for this assay. This has the advantage of requiring a single thermostable polymerase for both assays. Furthermore, since we have found that the Repeat PCR assay with KAPA2G works better when the AGG PCR buffer is used, this further simplifies the workflow since the same buffer can be used for both the Repeat PCR assay and the AGG PCR assay. If capillary electrophoresis is going to be the method of analysis the KAPA2G polymerase in the AGG buffer gives the best fragment resolution, however if agarose gel electrophoresis is to be used and/or considerable mosaicism is expected then Q5 polymerase in the Repeat PCR buffer with enhanced dNTP concentration will be more appropriate.

9. While not absolutely essential, in our experience the best results are obtained if the repeat or AGG PCR assay is carried out on freshly digested template.

10. The solutions involved are both dense (betaine, DMSO) and contain detergent. The volumes being added are relatively small. Thus, it is critical that pipettes are properly calibrated, that care is taken with the pipetting steps, and that the resultant solution is carefully but completely mixed.

11. The previously published version of the assay (12) used a 0.2 mM final dNTP concentration. We have found that increasing the final dNTP concentration to 0.45 mM significantly improves the yield of large alleles even when they are in the presence of smaller alleles. However, the yield of smaller alleles is reduced at this dNTP concentration. At dNTP concentrations even higher than this, the yield of all alleles is reduced. This improvement in yield is only seen using the

Q5/Phusion polymerases and increasing the final dNTP concentration beyond 0.2 mM significantly reduces the final yield for the KAPA2G polymerase in the AGG buffer.

12. Addgene plasmid 99257 (a.k.a. pGLACTE-PseudoHT51 (13)) is used to produce an internal positive PCR control fragment in the A-primed AGG PCR assay. Note: These plasmids are prone to contraction when grown at 37°C. Upon receipt of the Addgene stab streak it out immediately and verify the insert size and AGG interruption pattern as described in Section 3.5.7.

13. During development of the assay we tried HindIII digestion as an alternative to sonication and HaeII and a replacement for HpaII. However, HindIII digestion gave inconsistent results, possibly due to the presence of the CGG-repeat within the fragment that affects the efficacy of denaturation, while HpaII was consistently more reliable than HaeII at producing complete digestion.

14. It is essential that sonication is efficient and that most fragments are less than 1 kb in size. Failure to sonicate effectively will lead to an overestimation of the degree of methylation.

15. Exposure to UV light damages DNA and should be avoided if possible. Large quantities of DNA can be visualized using the visible light stain Crystal Violet (1 μg/ml in 1 x TAE buffer) (14) or, with greater sensitivity, the PrepOne Sapphire Blue Light system (EmbiTec).

16. Long CGG-repeat tracts are difficult to propagate stably in bacteria and are difficult to sequence using conventional sequencing strategies. Thus, the source of CGG repeats for cloning is best limited to genomic DNA with a single allele having 120 repeats or less.

17. The presence of the betaine and DMSO in the PCR reaction vastly increases the PCR error rate so the 23 PCR cycles are a compromise between generating enough product to be useful and minimizing the incorporation of errors.

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