AmplideX® PCR/CE *FMR1* Reagents

Protocol Guide

For Research Use Only.
Not for Use in Diagnostic Procedures.

REF	**49402**
Σ	**100**

Asuragen, Inc.
2170 Woodward St.
Austin, TX 78744-1840
USA
+1.512.681.5200

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Background Information

Fragile X syndrome (FXS) is a trinucleotide repeat disease caused predominantly by the expansion of CGG sequences in the 5' untranslated region of the Fragile X Mental Retardation 1 (*FMR1*, NM_002024.4) gene [1]. The number of CGG repeats is associated with a constellation of disorders that can affect patients both young and old [2]. Expansion to 200 repeats or more results in the inactivation of the *FMR1* gene through the methylation of the CGG repeats and upstream CpG islands. Loss of the *FMR1* protein affects an RNA-binding protein called FMRP that acts as a global regulator of translation in neurons and is important for synaptic plasticity [3]. Because of its key role in neural development and RNA transport [4], this gene is implicated in a number of fragile X-related disorders.

Individuals with full mutations (>200 CGG repeats) often present classic FXS, characterized by mental retardation, autism, and emotional and psychiatric challenges [5]. Premutation carriers (55-200 or 59-200 CGG) are known to be at risk for fragile X-associated primary ovarian insufficiency (FXPOI) a leading cause of ovarian dysfunction in women [5], and fragile X-associated tremor and ataxia syndrome (FXTAS) which is primarily associated with parkinsonism and dementia in male premutation carriers over the age of 50. Premutation phenotypes are associated with excess messenger RNA and consequent RNA toxicity and dysregulation of numerous proteins [6]. Deficits in development, and particularly attention and social communication, have also been noted for many children with the *FMR1* premutation alleles [7]. Thus, fragile X syndrome and associated disorders impact a broad range of individuals of all ages across multiple mental and health conditions.

Risk Assessment

Risk assessment and clinical interpretation of FXS and related disorders are defined by the number of CGG repeats and methylation status of the gene. Based on the number of CGG repeats it is possible to distinguish four types of alleles: unaffected or normal alleles, intermediate (or gray zone), premutation and full mutation (>200 CGG). The relationship among CGG repeat length, defect and phenotype are represented in Figure 1.

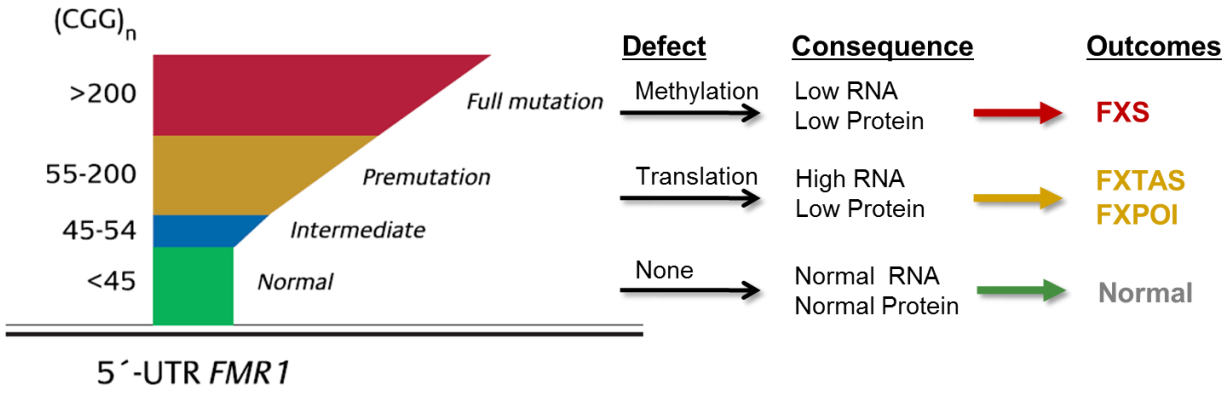


Figure 1. Relationship between CGG repeat lengths and corresponding phenotypes. Repeat sizes fall into four categories; normal (green), intermediate (blue), premutation (orange) and full mutation (red). The specific ranges of repeats associated with these lengths are listed in Table 1.

Full mutation alleles can range from 200 to greater than 1000 repeats. Above 200 repeats, the FXS phenotype is associated with the methylation status of the allele and not necessarily the exact number of repeats exceeding 200 CGG [8]. In premutation alleles (~60 to 200 repeats), the risk of expansion to a full mutation increases with size. Above 100 repeats, there is a systematic risk of expansion in the next generation [9]. The CGG repeat length cutoffs between normal, intermediate and premutation alleles are associated with the smallest repeat lengths known to expand to a full mutation. Current cutoffs according to the American College of Medical Genetics and the European Society for Human Genetics are listed in Table 1 [10, 11].

Table 1. Genotype boundaries for various CGG repeat lengths in the *FMR1* gene according to region.

Genotype Category	ACMG Guidelines	ESHG Guidelines
Normal	<44	<50
Intermediate	45-54	50-58
Premutation	55-200	59-200
Full mutation	>200	>200

In addition to risks associated with the total CGG repeat length, many *FMR1* alleles contain AGG sequences that are interspersed among the CGG repeats. These AGG "interruptions" are thought to confer DNA stability and to reduce the risk of expansion in the next generation [12-15]. The risk profile for mothers with no AGG interrupters may be higher than mothers with the same number of repeats but with at least one AGG, and thus fewer consecutive $(CGG)_n$ sequences.

Fragile X Testing

The analysis of the CGG repeats in the *FMR1* gene typically relies on PCR with size resolution by capillary electrophoresis (CE), or agarose or polyacrylamide gel electrophoresis to detect up to 100-150 CGG repeats. *FMR1* Southern blot analysis is used to characterize samples with CGG repeat numbers too large to amplify by PCR, and to determine the methylation status of the gene [16]. Unfortunately, this workflow is costly, time- and labor-intensive, and requires large amounts of genomic DNA, and is thus unsuitable for higher testing volumes or population screening. PCR can potentially address each of these limitations, yet the highly GC-rich character of the fragile X triplet repeat sequence historically has been refractory to amplification. PCR of premutation and full mutation females is even more challenging due to preferential amplification of the smaller allele [17]. Consequently, the greater than 20% of female specimens that are biologically homozygous must be reflexed to Southern blot to resolve the potential ambiguity of an unamplified longer allele. As a result, many, if not all, samples are reflexed to the more cumbersome Southern blot. Thus, the current workflow for *FMR1* analysis is hands-on, low throughput, and costly, and cannot readily support increasing needs for research.

The AmplideX® PCR/CE *FMR1* Reagents (RUO) were designed to provide accurate sizing of alleles up to 200 CGG, identification of full mutation alleles >200 CGG and a characteristic product peak profile that resolves zygosity in female samples.

Test Principle

The AmplideX® PCR/CE *FMR1* Reagents (RUO) use a three-primer CGG Repeat Primed (RP) PCR from purified genomic DNA and fragment sizing on an Applied Biosystems Genetic Analyzer. The PCR reagents include gene-specific and CGG primers, a polymerase mix buffer for amplification of the CGG repeat region in the *FMR1* gene and a ROX 1000 Size Ladder for sizing by capillary electrophoresis and a diluent. The size of the PCR products are converted to the number of CGG repeats using size and mobility conversion factors. Optionally, users may elect to perform a two-primer gene-specific PCR by omitting the CGG primer.

PCR Methods

The box includes reagents to perform CGG RP PCR with an option for gene-specific PCR (Figure 2). Gene-specific PCR uses two primers that span the CGG repeat region. PCR products from the gene-specific primers represent full length alleles (Fig. 2A). CGG RP PCR is primarily distinguished from the more conventional two-primer, gene-specific PCR by the addition of third PCR primer that is complementary to the *FMR1* triplet repeat region. The resulting electropherogram includes the full length PCR products generated from the primers that span the CGG repeat region and CGG repeat primed peaks (Fig. 2B). The full length gene-specific peaks are similar between the two methods. The CGG RP PCR products correspond to individual PCR amplicons from each combination of the repeat primer with the reverse primer. These RP peaks are separated by 3 bp, as expected. The profile of peaks provides confirmatory information about a sample including zygosity and presence of interspersed AGG.

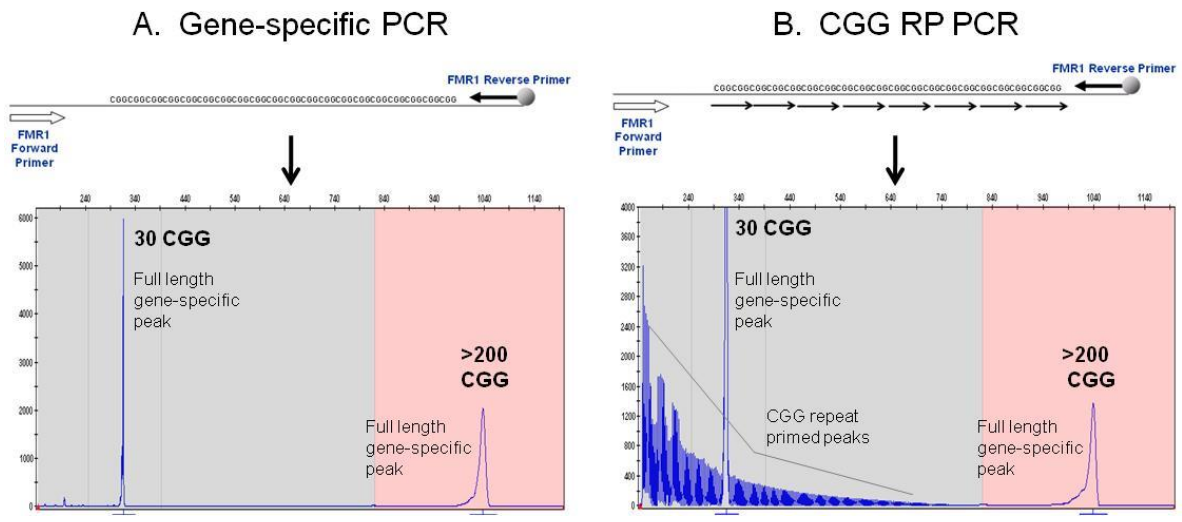


Figure 2. *FMR1* PCR Methodologies highlighting the features of the 2- and 3-primer PCR systems.

The CGG RP PCR repeat profile can telegraph the presence of longer alleles in the amplification, irrespective of whether such alleles are detected as full length products. Consequently, the risk of PCR dropout of the longer allele is reduced. The full length gene-specific product peaks may be converted from size in base pairs to the number of CGG repeats using predefined conversion factors. The peak profile can theoretically provide very accurate $(CGG)_n$ repeat quantification by counting the number of CGG repeat primed amplicon peaks up to ~200 CGG, providing complementary sizing confirmation.

Workflow

The workflow for the test includes PCR master mix setup, thermal cycling and analysis using capillary electrophoresis. Genomic DNA is added to a master mix containing GC-Rich Amp Buffer, GC-Rich Polymerase Mix, and the *FMR1* F,R FAM-Primers for gene-specific PCR. The *FMR1* CGG Primer is also added to the master mix to enable CGG RP PCR. After thermal cycling, approximately 6 hours, unpurified PCR products are directly mixed with Hi-Di Formamide and the ROX 1000 Size Ladder. After denaturing the products, the amplicons are sized on any Applied Biosystems Genetic Analyzer running POP-7 polymer. A schematic of the workflow is shown in Figure 3.

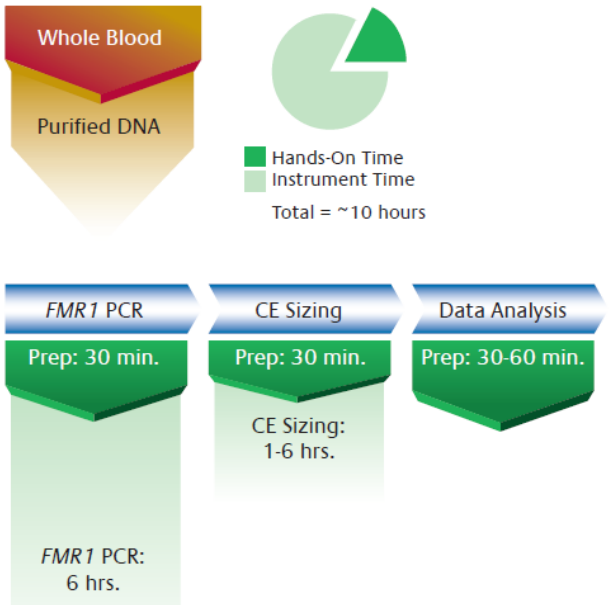


Figure 3. Overview of the AmplideX™ PCR Reagents Workflow showing key steps and time estimates for each step (thermal cycling for gene-specific PCR is 3.5 hours). The CE analysis protocol requires approximately a 1 hour run time for each set of 4 to 96 samples per injection depending on the model used.

After CE analysis, the electropherograms are analyzed to identify full length gene-specific product peaks. Up to approximately 200 CGG, peaks are detected within the linear range of the instrument. These peaks are converted from size in base pairs to the number of CGG repeats using correction factors derived for that instrument configuration. Beyond 200 CGG, the size of the PCR product exceeds the resolving threshold of the POP-7 gel polymer. PCR fragments exceeding this threshold have an equivalent migration rate independent of product size [18]. Thus, *FMR1* PCR products exceeding 200 CGG are identified categorically as >200 CGG. In

addition to sizing information, qualitative trace features such as zygosity and presence of interspersed AGG may be derived from the CGG RP PCR products (Chen et al. 2010).

Use of Controls

Positive and negative controls are recommended in every run. The Diluent provided within the reagent set may be used as a negative no-template control. Genomic DNA extracted from well-characterized cell lines may be used for positive controls. Cell lines or corresponding purified genomic DNA can be obtained from various repositories such as the CCR [19]. In addition, a reference material panel endorsed by the European Society of Human Genetics and approved as an International Standard by the Expert Committee on Biological Standardization at the World Health Organization is commercially available [20]. Representative examples with these materials are shown in the Data Interpretation section below.

Reagents

Reagents Provided with the Kit

Table 1. AmplideX® PCR/CE *FMR1* Reagents Components (RUO)(P/N 49402)

Item #	Item Description	Volume	Storage Temp
145151	*FMR1* F,R FAM-Primers	50 µL	-15 to -30°C
145156	*FMR1* CGG Primer	50 µL	-15 to -30°C
145152	GC-Rich Amp Buffer	1.2 mL	-15 to -30°C
145153	GC-Rich Polymerase Mix	5 µL	-15 to -30°C
145154	ROX 1000 Size Ladder	200 µL	-15 to -30°C
145157	Diluent	1.0 mL	-15 to -30°C

Handling and Storage

- Store the reagents in a non-frost-free freezer in the dark at -15 to -30 °C.
- Allow reagents (except GC-Rich Polymerase Mix) to thaw at room temperature before use. Vortex all reagents (except GC-Rich Polymerase Mix) after thawing.
- Prior to opening, briefly centrifuge each component to collect the solutions at the bottom of the vials.
- Assay setup should be performed at room temperature (approximate range of 18-25 °C).

Number of Reactions

- The provided reagents are sufficient for up to 100 reactions, gene-specific PCR or CGG RP PCR, and 100 subsequent CE analyses.
- The reagents have been verified for use up to four freeze-thaw cycles. Additional cycles are not recommended.
- Master mixes can be prepared for the appropriate number of samples with a recommended total number of at least 16 reactions per run.

Reagent Stability

- The product will maintain performance through the expiration date printed on the label when stored under the specified conditions.

Reagents Required but not Provided

- Reagents for DNA isolation are not included. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high quality, intact DNA.

Capillary Electrophoresis Materials Required but not Provided

- ABI Genetic Analyzers running POP-7 polymer (e.g. 3130, 3730 or 3500 series).
- POP-7 Polymer: Applied Biosystems, #4363785 or equivalent
- Hi-Di Formamide: Applied Biosystems, #4311320 or equivalent
- Dye set calibrators for FAM and ROX, DS-30 or DS-31 dye set: Applied Biosystems #4345827, #4345829, or equivalent

Consumables & Equipment Required but not Provided

- General laboratory equipment and workspace to perform PCR
- Thermalcycler: ABI 9700, ABI Veriti (run in 9700-max mode), MJ Research PTC-200, or Eppendorf Mastercyler
- Centrifuge capable of spinning 96-well plates
- Vortex
- Micro-centrifuge
- Pipettes: Units with an accuracy range between 0.2-2 µL, 1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL
- Multi-channel pipette unit capable of pipetting 1-10 µL
- 96-Well PCR Plates: AB Gene #AB-0900 or equivalent
- PCR Plate Seals: AB Gene #AB-0558, Phenix LMT-0028 or equivalent
- PCR Compression Pad: Applied Biosystems # 4312639 or equivalent

Positive Controls Recommended but not Provided

- WHO International Standard, Fragile X Syndrome, Genetic Reference Panel (NIBSC, 08/158) or other commercially available cell line DNA standards.

Warnings and Precautions

- Use proper personal protective equipment. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials. Use nuclease-free lab ware (e.g., pipettes, pipettes tips, reaction vials).
- **WARNING! CHEMICAL HAZARD.** Hi-Di™ Formamide. Causes eye, skin, and respiratory tract irritation. Possible developmental and birth defect hazard. Avoid breathing vapor. Use with adequate ventilation.
- Follow Universal Precautions when handling human samples.
- Substances that may interfere with the PCR of DNA include certain drug compounds and heparin. Highly lipemic samples, hemolyzed samples, icteric samples, or samples with proteinemia should not be used.
- DNase contamination can cause degradation of the DNA samples. Use nuclease-free filter pipette tips and nuclease-free tubes.
- PCR carry-over contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Do not pool components from different reagent lots. Do not use reagents after the labeled expiration date.
- Do not interchange the reagent tube caps which may cause cross-contamination or degradation of reagents.
- Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible results. Ensure even distribution of master mix which is viscous and can accumulate within the pipette tip.
- Prior to use, ensure that the Genetic Analyzer is calibrated according to the manufacturer's instructions.

Caution: The toxicological properties of the PCR reagents have not been fully investigated. Avoid contact with skin and mucous membranes. Do not ingest. Safety Data Sheets are available upon request.

Pre-Analytical Steps

Genomic DNA extracted via common sample preparation methodologies from whole blood collected in EDTA is compatible with the AmplideX® PCR/CE *FMR1* Reagents (RUO). It is recommended that the purified genomic DNA be evaluated for concentration (OD260) and purity (OD260/280) and to store DNA samples below -15°C. Input 20 – 80 ng into each reaction (2 µL of DNA at 10 – 40 ng/µL).

AmplideX® PCR/CE *FMR1* Reagents (RUO) Protocol

The test protocol involves three key sets of procedures:

1. PCR master mix setup and thermal cycling
2. Capillary electrophoresis
3. Fragment sizing analysis

The instructions below are written for the preparation and analysis of gene-specific PCR or CGG RP PCR products. There are only two differences in the protocol: preparation of the PCR master mixes with or without the *FMR1* CGG Primer and different cycling conditions. The protocol is written for a single reaction; master mixes can be prepared for the appropriate number of reactions at each step of the protocol. The provided reagents are sufficient for up to 100 reactions performed in up to 4 independent batches including 10% overage for the preparation of additional reactions (e.g., 1 run with 100 reactions or 4 runs with 25 reactions). The minimum number of reactions per batch is 16 and no more than 4 freeze-thaw cycles is supported. Examples of recommended overage for a given batch size are provided in Table 2.

Table 2. Examples of PCR Master Mix Setup.

Sample Batch Size	Recommended Overage
16	+2
25	+2
50	+5
100	+10

The workflow should proceed in a uni-directional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize risk of amplicon contamination.

PCR Master Mix Setup and Thermal Cycling

1. Thaw all reagents except Polymerase Mix ~10 minutes at room temperature. Place GC-Rich Polymerase Mix on ice. Briefly vortex all tubes (3-5x pulse vortexing) except Polymerase Mix.

   Note: GC-Rich Polymerase Mix should be stored on ice at all times. The GC-Rich Amp Buffer may be occluded or have observable precipitation when cold. After completely thawing the tube, vortex to ensure mixing.

2. Add the appropriate components to a 1.5 mL microfuge tube in the exact order specified in Table 3.

Table 3. PCR Master Mix Setup.

Component	Gene-specific PCR	CGG RP PCR
GC-Rich Amp Buffer	11.45 µL	11.45 µL
FMR1 F,R FAM-Primers	0.50 µL	0.50 µL
FMR1 CGG Primer	0 µL	0.50 µL
Diluent	1.00 µL	0.50 µL
GC-Rich Polymerase Mix	0.05 µL	0.05 µL
DNA Sample	2.00 µL	2.00 µL
Total Volume per Reaction	**15.00 µL**	**15.00 µL**

Note: The GC-Rich Amp buffer is viscous; retract piston slowly to acquire solution.

Important! Excess GC-Rich Polymerase Mix may inhibit the reaction. Ensure that there are no additional droplets on the pipette tip prior to dispensing to the mastermix.

3. Thoroughly vortex master mix (3-5 times pulse vortexing) prior to aliquoting to PCR plate or strip-tubes.

Critical! The mastermix must be vortexed prior to dispensing to ensure adequate mixing of all reagents.

4. Dispense 13.0 µL master mix to each well or tube. Use a Repeater pipettor, if available. Switch pipette tip at the start of every column of the plate if using a standard pipettor.
5. Add 2.0 µL of the appropriate DNA sample to each well. Pipette up/down at least twice to ensure adequate mixing.
6. Seal the plate with an adhesive film seal; ensure that all of the wells and plate edges are well sealed.
7. Gently vortex the plate.
8. Centrifuge the plate to remove bubbles (1 min at 1600 rcf).

Important! Ensure all bubbles are removed from the wells.

9. Transfer the sealed PCR plate to a preprogrammed thermal cycler and run the appropriate cycling protocol:

Gene-specific PCR	
Description	**Duration**
1 hold	98°C for 5 min
25 Cycles	97°C for 35 sec
	62°C for 35 sec
	72°C for 4 min
1 hold	72°C for 10 min
1 hold	4°C forever

CGG RP PCR	
Description	**Duration**
1 hold	95°C for 5 min
10 Cycles	97°C for 35 sec
	62°C for 35 sec
	68°C for 4 min
20 Cycles	97°C for 35 sec
	62°C for 35 sec
	68°C for 4 min + **20s/cycle***
1 hold	72°C for 10 min
1 hold	4°C forever

*Follow the instruction manual of the thermal cycler to add 20 seconds extension time per cycle for this step.

10. Transfer PCR products for CE analysis or store at -15 to -30 °C until analyzed. PCR product stability at -15 to -30 °C has been verified for up to 10 days storage.

Capillary Electrophoresis POP-7

1) Thaw the formamide and ROX1000 Size Ladder at room temperature. Thoroughly vortex (15 seconds) and spin tubes before use.

2) Prepare a master mix solution by adding components in the order listed:

Hi-Di Formamide	11 µL
ROX 1000 Size Ladder	2 µL
Total Volume per well	**13 µL**

3) Mix all added reagents (by pulse vortexing 3-5 times), and spin down briefly to collect.

4) Aliquot 13.0 µL of Formamide/ROX solution to each well of a new CE analysis plate.

Important! Samples must be matched to the injection configuration of the Genetic Analyzer (e.g. A1-H2, A3-H4…A11-H12) in appropriate groups of 8, 16 or 24 capillaries. If running less than the number of samples for any injection group, fill empty wells subject to injection with 15µL of Hi-Di Formamide.

5) Transfer 2 µL of PCR products to the CE plate, pipetting up and down 2 to 3 times to mix. A multi-channel pipette is recommended for transfer.

6) Seal the plate, vortex, centrifuge to remove bubbles and transfer to a thermal cycler.

7) Denature for 2 min at 95°C followed by 4 °C until ready for injection on the CE instrument. Alternatively, the products may be stored on ice and protected from light after the denaturation step.

Critical! The samples must be denatured prior to CE analysis.

Note: Samples may be run up to 24 hours after denaturation.

8) Prepare Genetic Analyzer for data acquisition according to manufacturer's directions. Final injection and run conditions must be validated by the end user and may differ between instruments. The following considerations apply:

- The instrument must be calibrated for the detection of both FAM and ROX fluorescent dyes.
- Use the factory installed Fragment Analysis Protocol for POP-7 polymer and capillary length for your instrument as a base protocol.
- Adjust the injection conditions and run time according to the particular instrument configuration and capillary length. Recommended starting values are listed in Table 4.

Table 4. Injection and Run Time adjustments to the default Fragment Analysis Protocols for different instrument classes and capillary lengths running POP-7 polymer.

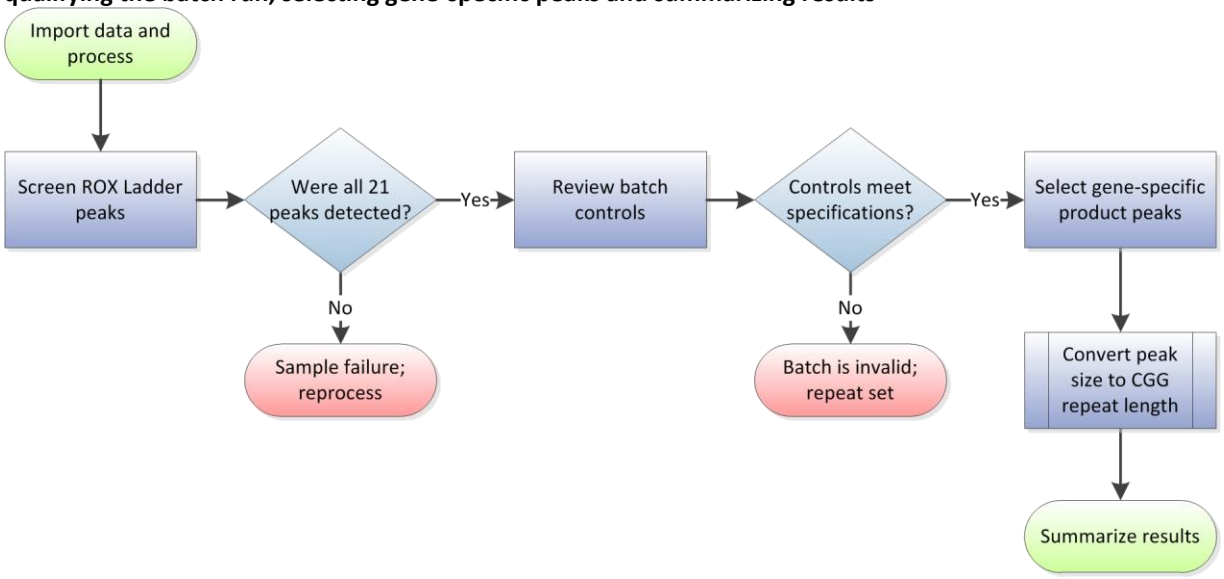
Instrument	Capillary Length	Injection	Run Time
3130,3130*xl*	36 cm	2.5 kV, 20 s	2400 s
3730, 3730xL	50 cm	2.5 kV, 20 s	4200 s
3500, 3500xL	50 cm	2.5 kV, 20 s	2400 s

9) After the run, the data may be analyzed for amplicon size and conversion to CGG repeat length.

Fragment Sizing Analysis

Fragment sizing analysis of gene-specific or CGG RP PCR data involves a series of steps to obtain the size of full length product peaks and identify features in the run for interpretation of the data. These results are converted to CGG repeat length as described in Data Analysis. The terms used for analysis refer to GeneMapper 4.0/4.1 features. An overview of the fragment sizing analysis workflow is shown in Figure 4.

Figure 6. Schematic workflow for data analysis including sample file processing, scoring the ladder peaks, qualifying the batch run, selecting gene-specific peaks and summarizing results



1. Import data and process

   a. Import the *.fsa files into GeneMapper®.

   b. Process files according to the methods, panels and size standard settings established for *FMR1* PCR product analysis.

2. Qualify the run

   b. Screen ROX Ladder Peaks.
   Review Size Matches and Size Calling Curve of the ROX 1000 Size Ladder for all samples. Identify any irregularities in the fit or any missing peaks for the Ladder.

   Critical! Samples without a properly called ladder must be excluded from further analysis.

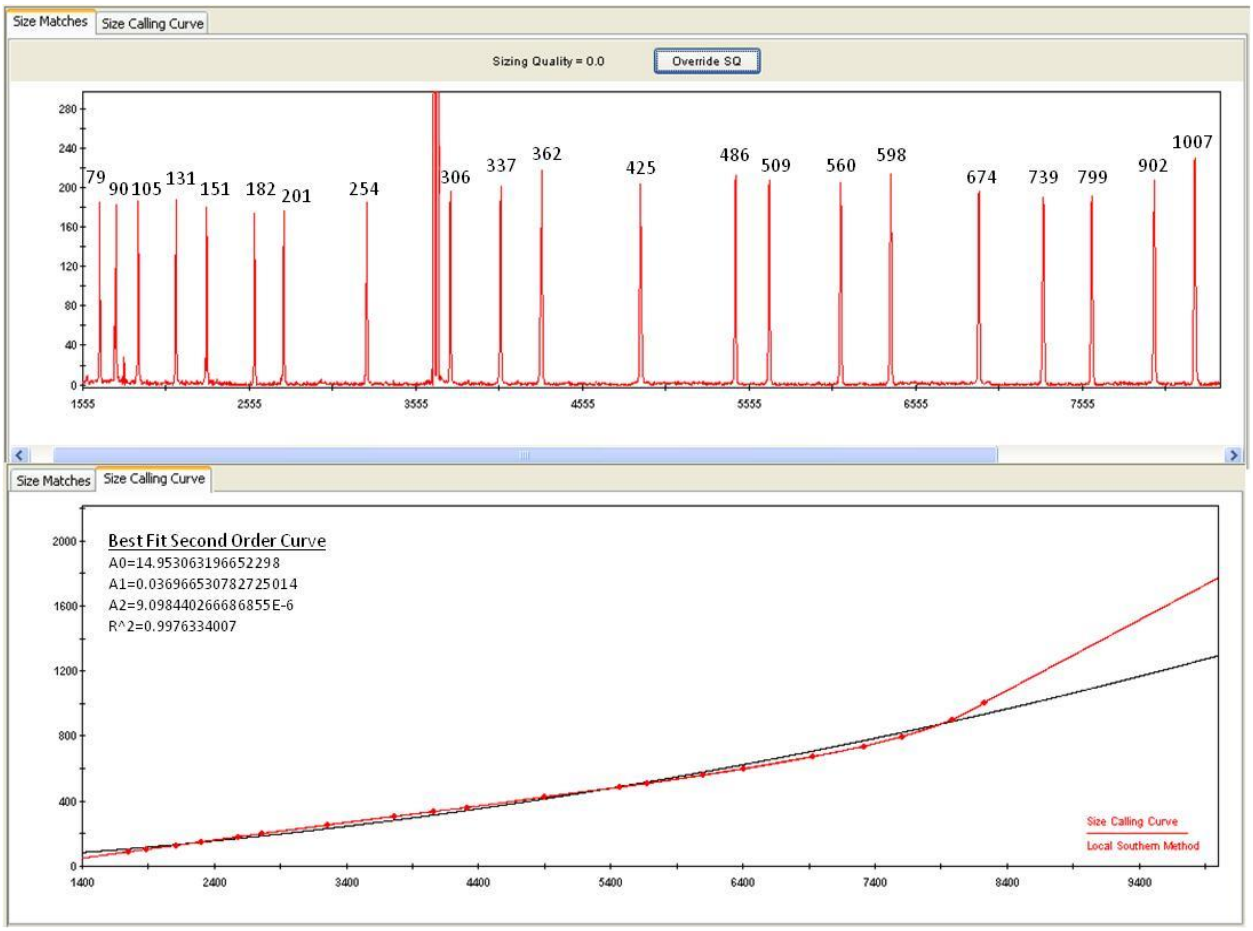


Figure 5. ROX 1000 Size Matches and Size Calling Curve. The SizeMatch view highlights the 21 peaks of the ladder and includes an example spectral pull up peak from the FAM labeled target peak that should be ignored in the ROX channel.

Note: A spectral pull up peak from the FAM channel may be observed. This peak will generally not interfere with the sizing of the ladder. Doublet peaks may be observed, e.g. 90 and 151, which do not interfere with ladder function. An example ROX 1000 Size Ladder size match and calling curve is shown in Figure 5.

c. Review Batch Controls

i) Ensure that negative control included in the batch run meet specifications.

ii) Ensure positive controls meet specifications. See Examples of Controls in Data Interpretation and Appendix A for more information.

3. Select gene-specific target peaks.

a. The electropherogram traces are reviewed for peak selection criteria. For analysis of CGG RP PCR products, the multiplicity of CGG RP peaks is deselected in order to simplify conversion of the full length gene-specific PCR product peak to CGG repeat length. An example of this process is highlighted below for a female premutation allele.

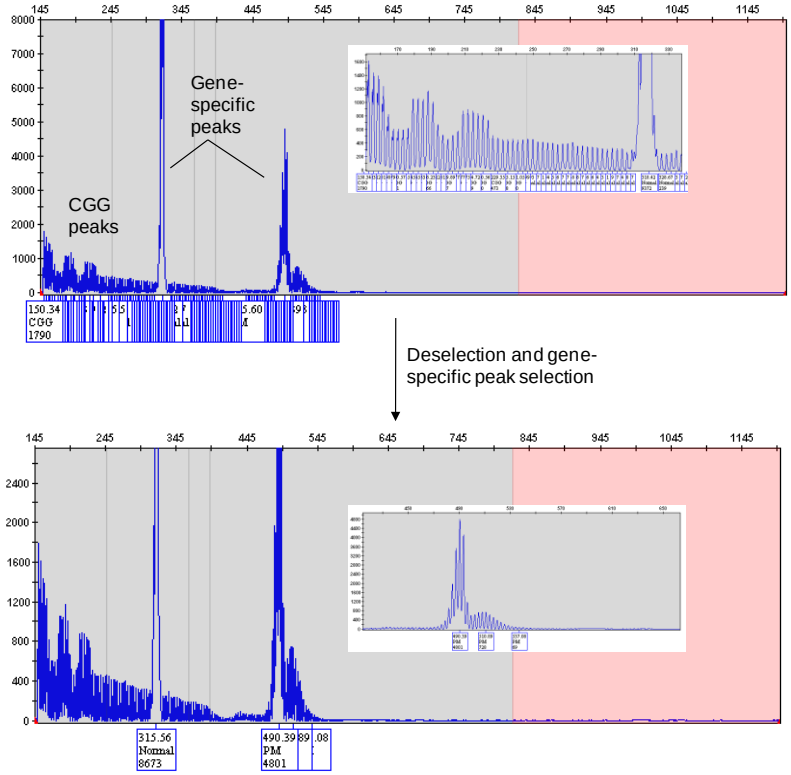


Figure 6. Example electropherogram with default analysis settings (top) and only gene-specific full length peaks selected from the CGG RP PCR electropherogram (bottom).

NOTE: If processing the results of a gene-specific PCR, only the gene-specific peaks will be present and only these peaks need to be selected. Deselection will not be required.

b. Deselect all peaks, then select gene-specific full length product peaks. In general, peaks exceeding an instrument-specific cutoff are automatically selected. Minor or low intensity peaks may be manually selected according to the guidelines in Table 5.

Table 5. Manufacturer default signal intensity cutoffs and low peak ranges for different CE instrument configurations.

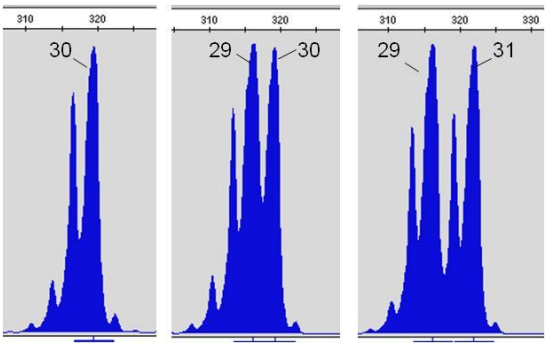
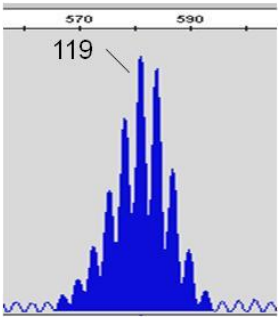
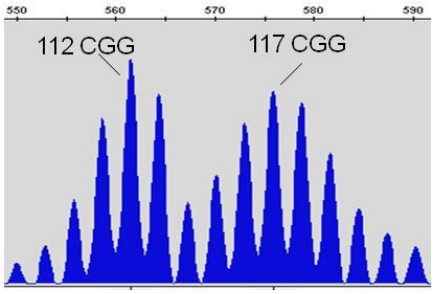
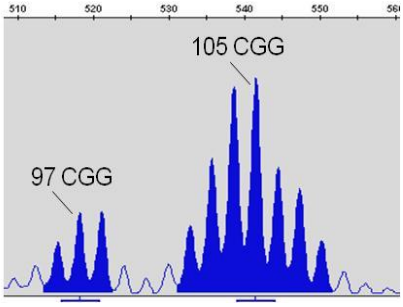
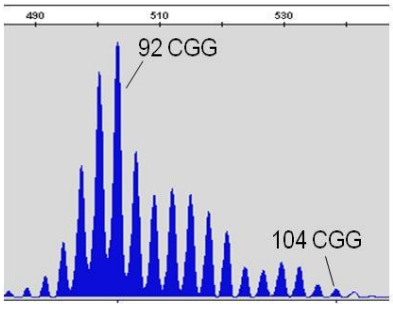
Instrument	Cutoff (rfu)	Low Peak Range (rfu)
3130, 3130xl	50	10-49
3730, 3730xl	175	50-174
3500, 3500xl	175	50-174

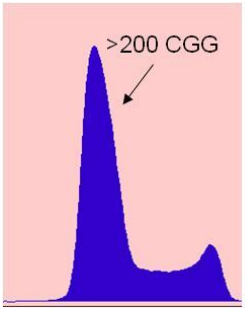
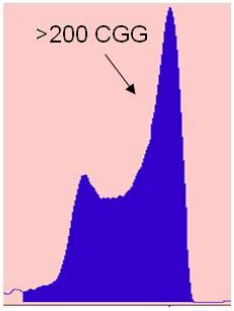
c. After deselecting peaks in all samples, identify gene-specific full length product peaks for each region of the electropherogram using the guidelines listed in Table 6.

   i. Lower signal intensity peaks may be identified that are in the low peak range.

   ii. Remove extra peaks from sample or non-CGG repeat morphology peaks.

Table 6. Peak selection guidelines based on size range and features. Example traces are listed in Data Interpretation.

If the peak size is in this range	**Or** has these features…	**Then** follow these guidelines for peaks exceeding the signal cutoff for the instrument	**Using** the following as examples.
245-400 bp	Normal and Intermediate alleles	Select the highest peak, generally the right-most peak in this size range. There may be multiple peaks in the normal range. Confirm selection of all peaks (e.g. 30 or 29,30 or 29,31 CGG).	
~400-820 bp	Premutation alleles with a single peak population less than 8 peaks from center to end.	Select the highest peak, generally the center peak for multi-peak alleles or peaks in this size range (e.g. 119 CGG).	
	Premutation alleles with complex distributions of peaks.	Select center peaks of two allele groups. If the peaks between the alleles exceed the signal cutoff, identify both groups separated by a dash "-" (e.g. 112-117 CGG).	
		If the peaks between two allele groups are less than 50 rfu (or other cutoff), the alleles can be identified with a comma as distinct alleles (e.g. 97, 105 CGG).	
		Select center-peak and the last-peak >50 rfu (or other cutoff) for alleles with more than 8 peaks from center to end (e.g. 92-104 CGG).	

> 820 bp	Full mutation alleles less than approximately 1000 bp that may be resolved from larger full mutation peaks and full mutation alleles.	Select only the component of the peak group containing the highest peak. Deselect other peaks within that group. Identify peak as >200 CGG.	 

4. Convert peak size to CGG repeat length

After capillary electrophoresis, the size of the target amplicon is derived from comparison to a co-injected size standard, e.g. ROX 1000 Size Ladder. However, PCR products of triplet repeat regions have an anomalously faster migration than the generic DNA of the size standard [21, 22]. This higher migration, attributed to the structure of GC-rich DNA, may result in underreporting of repeat length without the use of an appropriate correction factor. The AmplideX® PCR/CE *FMR1* Reagents (RUO) incorporates two correction factors for conversion of size in base pairs to the number of CGG repeats for each allele. The size of each peak may be converted to repeat length according to Equation 1:

$$CGG_i = \frac{Peak_i - c_0}{m_0}$$

Where: $Peak_i$ is the size in base pairs of a given product peak; c_0 is the a size correction factor; and m_0 is the mobility correction factor for each CGG repeat. The size correction factor represents the common region of DNA included in the primers but omitting the CGG repeats. The mobility factor accounts for the apparent mobility of each repeat unit. CGG containing amplicons will have slightly different correction factors depending on the specific run conditions and instrument configuration used. Correction factors for supported configurations are listed in Table 7.

Table 7. Size and mobility correction factors for standard instrument configurations.

Configuration		c_0	m_0
3130, 3130*xl*	36 cm	229.4	2.965
3730, 3730xL	50 cm	231.9	2.937
3500, 3500xL,	50 cm	232.6	2.962

Correction factors for other instrument, capillary length, polymer type or run conditions are not supported but may be determined using the procedures described in Appendix A.

Data Interpretation

Alleles are reported as whole-integer repeats associated with a specific genotype category: normal, intermediate premutation and full mutation and full mutation mosaic. The reportable range is 5-200 repeats; above 200 repeats all alleles are identified as >200 CGG.

Report the sample indication for alleles assigned according the reference range. In samples with multiple alleles, the indication of the longest allele is the reported one. Samples with both premutation and full mutation alleles are identified as full mutation mosaics [23]. The genotype may be assigned according to specific guidelines as shown in Table 1.

Example CGG RP PCR Results for Normal, Premutation and Full mutation alleles

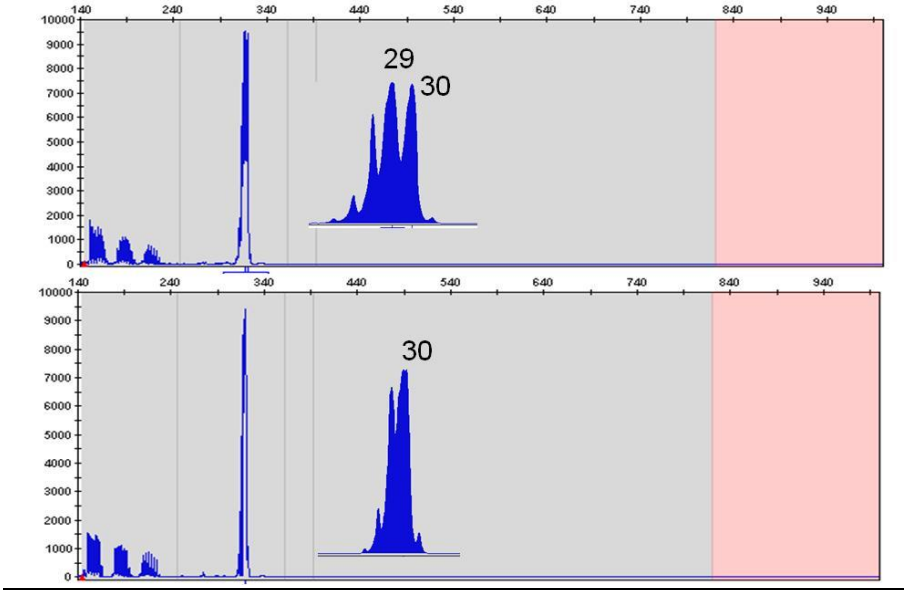


Figure 7. Normal allele examples showing a 29,30 CGG compared to a 30 CGG.

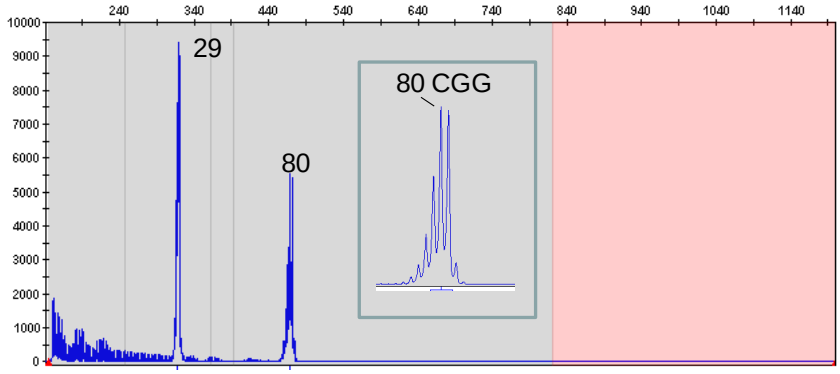


Figure 8. Example premutation allele. The highest peak in the sample is selected to represent the 80 CGG allele.

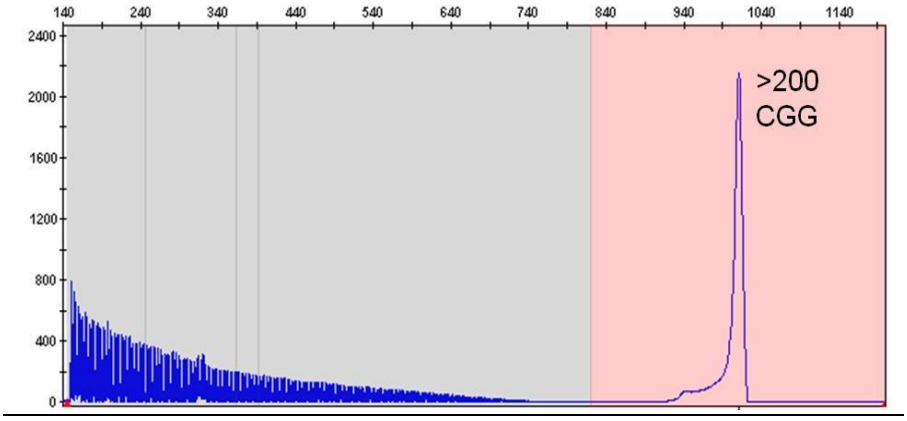


Figure 9. Example full mutation allele; male sample. The full length product peak exceeds 200 CGG and is identified as >200 CGG. Individual CGG repeat product peaks can be identified in the sample trace.

Traceability to Fragile X Standards

Representative electropherograms obtained with the reference material panel endorsed by the European Society of Human Genetics and approved as a WHO International Standard are shown in Figure 10. The panel consists of 5 genomic DNA samples:

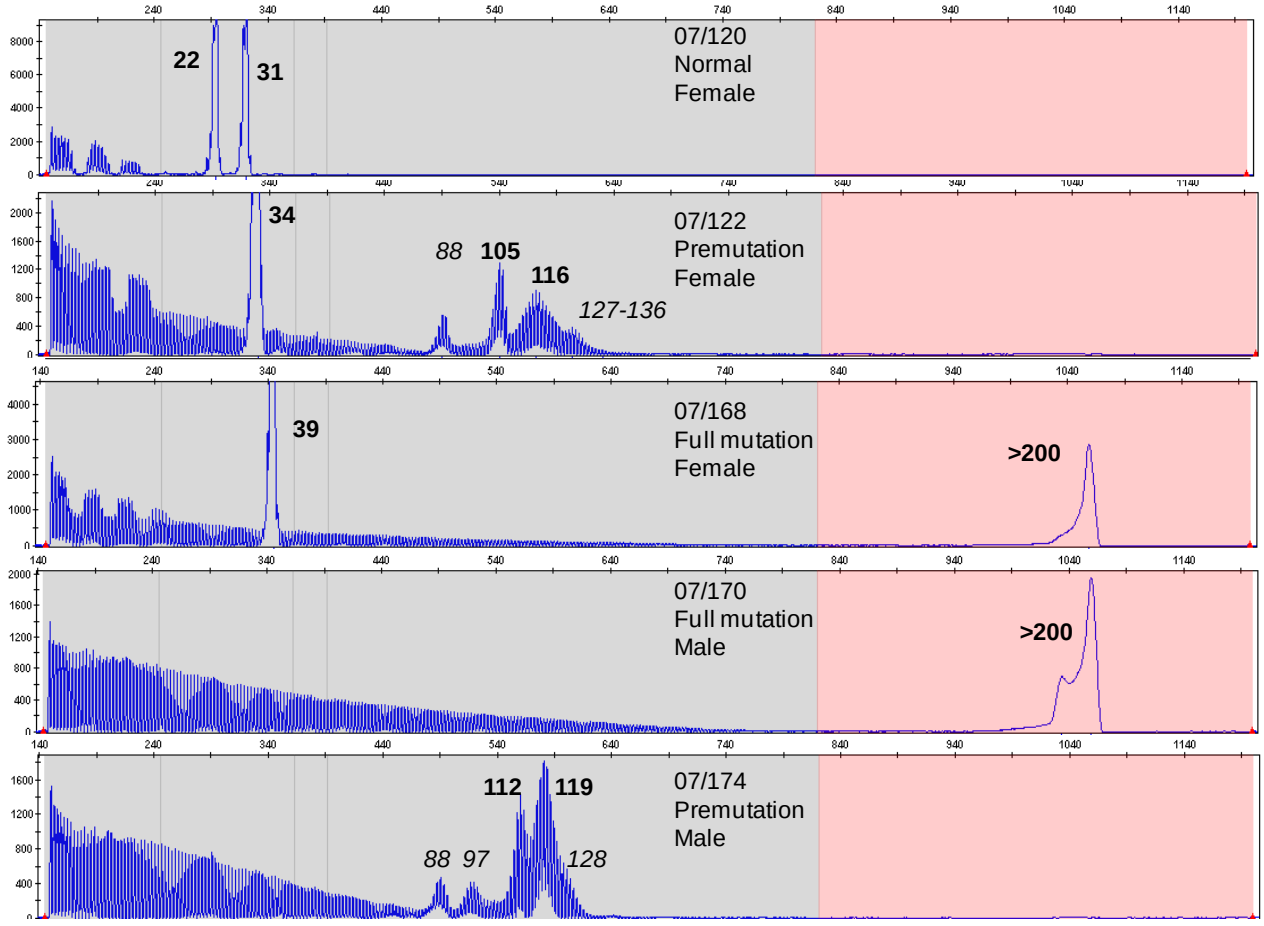


Figure 10. Electropherograms of the WHO International Fragile X Panel (NIBSC #08-158).

A summary of results for the major detected peaks relative to the reported ranges and mean size of these reference materials is shown in Table 8. The results using the AmplideX® PCR/CE *FMR1* Reagents (RUO) were consistent between two sites and published results from 27 European sites using various *FMR1* PCR methodologies.

Table 8. Summary of WHO Fragile X Panel Results (number of CGG repeats) at 2 Sites.

WHO Standards and International Assessment Results (Hawkins et al. 2010)				AmplideX® PCR/CE *FMR1* Reagents Results	
Sample ID	**Sample description**	**Range**	**Mean**	**Site 1**	**Site 2**
07/120	Female, wild-type	19-24 28-33	22 31	22 31	22 31
07/122	Female, premutation	30-36 100-132	33 113	34 105, 116	34 105, 115
07/168	Female, full mutation	33-41 300-401	38 346	39 >200	39 >200
07/170	Male, full mutation	353-960	754	>200	>200
07/174	Male, premutation	97-127	114	112, 119	112, 119

Well-characterized individual cell line DNA samples from the Coriell Cell Repository can also be used as controls [19, 24]. Combinations of cell line DNA samples can generate an information-rich pooled control in one PCR. An example of

electropherogram result for a pooled control sample combining 4 cell line DNA from the CCR is shown in Figure 11. See Appendix A for the formulation of this pool control. These four cell lines provide *FMR1* alleles corresponding to 20, 29, 31, 54, 119 and 199 CGG repeats. The repeat lengths of the first five alleles were verified by Sanger sequencing [25]. Thus the AmplideX® PCR/CE *FMR1* Reagents (RUO) are traceable to the method of DNA sequencing and the Fragile X Panel materials available from NIBSC.

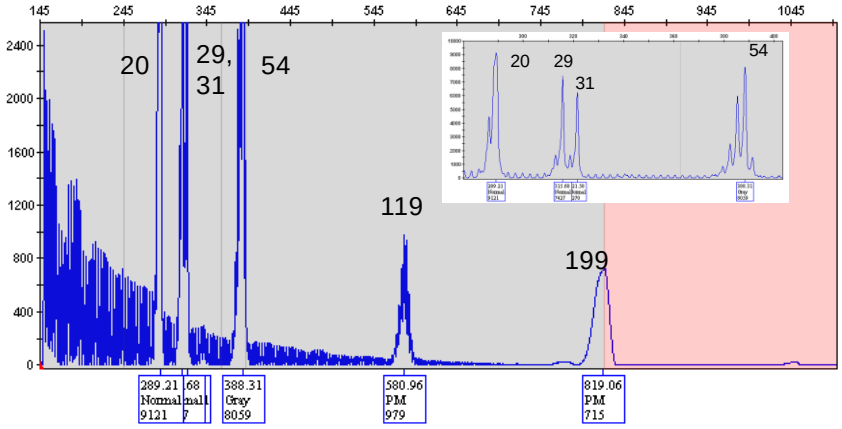


Figure 11. Electropherogram of a pooled control showing the CGG RP PCR results for four cell lines comprising 20, 29, 31, 54, 119 and 199 CGG. The inset plot shows single repeat resolution and signal intensities for the first four alleles.

Resolution of Zygosity.

CGG RP PCR provides a unique signature to resolve homozygous from heterozygous alleles. Example profiles for homozygous and heterozygous alleles are shown in Figure 12. PCR products from homozygous alleles reveal a baseline signal after the CGG RP primed peaks up to the full length product peak, and a baseline resolved signal for the remainder of the electropherogram. Conversely, heterozygous alleles have a characteristic "decay" pattern of CGG RP products that exceed the normal range of CGG repeat lengths along with detection of both the normal and expanded alleles. Moreover, CGG RP products will be generated even if the full length product peak is not detected.

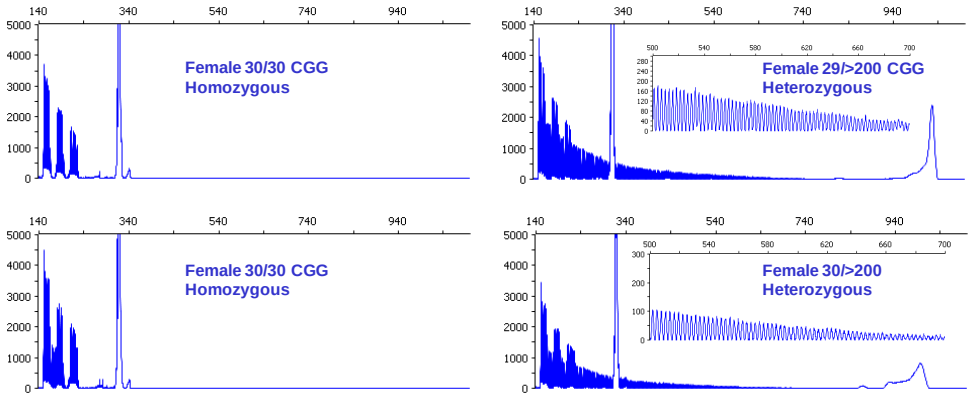


Figure 12. CGG Repeat Primed *FMR1* PCR reagents provide an unmistakable signature for female samples that resolve zygosity. The inset plots show resolution and signal intensities of the RP PCR products in the 500 to 700 bp range. Each CGG RP amplicon peak is separated by 3 bp.

Variation of Signal Intensity in CGG RP PCR Profiles

The *FMR1* CGG Primer is specific for CGG repeats and will not hybridize to AGG sequences commonly found in *FMR1* alleles. Therefore, signal intensity dips in the CGG RP PCR profile correspond to the presence of interspersed AGG. These AGG "interruptions" are thought to confer DNA stability and to reduce the risk of expansion in the next generation [12-15]. Figure 13 below shows a representative example with 2 samples of nearly equal length, a 30,60 and a 31,61 allele.

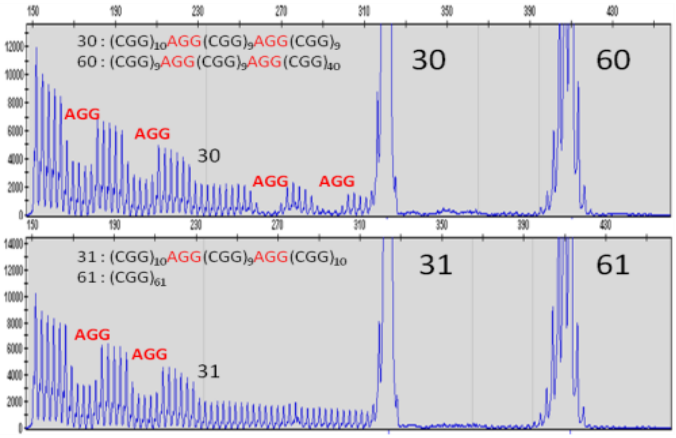


Figure 13. Electropherograms comparing a 30,60 CGG sample with AGG on both the 30 and 60 CGG alleles (top) versus a similiarly sized sample, 31,61 CGG with AGG only on the 31 CGG allele (bottom) The 61 CGG allele lacked AGG.

The CGG RP PCR peak profile indicated 4 AGG for the first sample and only 2 AGG for the second sample. Based on peak counting and on the haplotype inference of a 5'-bias for AGG [26, 27], the exact pattern of CGG repeats and AGG interruptions can be inferred in many cases, even in female samples.

General Disclaimers

- The AmplideX® PCR/CE *FMR1* Reagents (RUO) are produced in the USA and are for Research Use Only. Not for Use in Diagnostic Procedures.
- All instrumentation must be maintained and operated according to manufacturer's instructions.
- In a small number of individuals with FXS, mechanisms other than trinucleotide expansion, such as deletion or point mutation, are responsible for the syndrome. In these cases, linkage studies, cytogenetic, sequencing and/or assays designed to identify rare mutations and deletions may provide important information for relatives.
- This Asuragen product may not be resold, modified for resale, or used to manufacture commercial products without prior written approval of Asuragen.

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Appendix A. Derivation of Size and Mobility Correction Factors

Size (c_0) and mobility (m_0) correction factors are dependent on the instrument, polymer type, capillary length and run conditions used and may vary slightly from laboratory to laboratory. This appendix describes how to determine a laboratory-specific conversion factor using the size in base pairs of allele amplicons from a pooled cell line control. This pooled control can also be used for as a routine run control for use with each PCR batch.

The pooled control is prepared as an admixture of 4 commercially available cell line DNA template (see http://ccr.coriell.org/). Prepare dilutions of each Coriell cell line DNA in 10 mM Tris, 0.5 mM EDTA, pH 8.8 and combine as shown in Table 1 below.

Table 1. Pooled control formulation using 4 cell line DNA from the CCR.

Catalog Number	Catalog Listed Repeats	CGG Repeat Length by AmplideX®	Final Concentration [ng/µL]
NA20239	20, 183-193	20, 199	10
NA07541	29, 31	29, 31	5
NA20230	54	54	12
NA06891	118	119	10

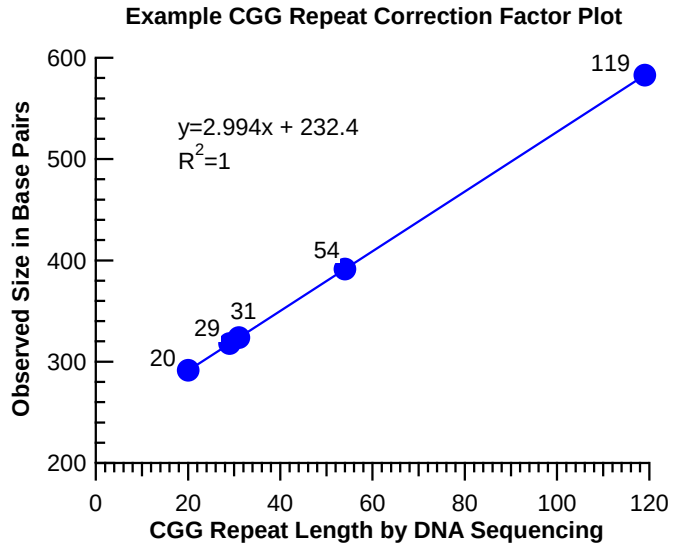
Terms for c_0 and m_0 are derived from a linear fit of expected CGG repeat length and base pair size for the first five peaks, 20, 29, 31, 54 and 119 CGG repeats, of the pooled control allele amplicons. The 199 CGG allele is not used for this plot as this allele length was not verified by Sanger sequencing. To calculate specific conversion factors follow these steps:

1. Analyze the pooled control with the AmplideX® PCR/CE *FMR1* Reagents (RUO) and determine the measured size in base pairs for each of the first five amplicon peaks. It is recommended to calculate the average base pair size for each peak from at least 2 independent runs.

Table 2. Example data comparing expected repeat length and observed size.

Repeat Length	Observed Size [bp]
20	291.43
29	317.78
31	323.65
54	391.30
119	582.87

2. Calculate the slope and intercept of the correlated data in Excel or comparable program.



The intercept of the linear fit corresponds to the correction factor, c_0 and the slope to the mobility factor, m_0. In this example, c_0=232.4 and m_0=2.944.

To verify the derived correction factors, the operator should test the WHO International Standard, Fragile X Syndrome, Genetic Reference Panel (NIBSC, 08/158) or other commercially available cell line DNA standards.

AmplideX® PCR/CE *FMR1* Reagents
PC-0170v2
Effective Date: 2016-02