Protocol

For Research Use Only. *Not for use in diagnostic procedures.*
REF **49442**
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Background Information

Fragile X syndrome (FXS) is a trinucleotide repeat disorder 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 FMR1 protein is a RNA-binding protein that acts as a global regulator of translation in neurons and is important for synaptic plasticity [2]. Because of its key role in neural development and RNA transport, stability, and translation [3], this gene is implicated in a number of fragile X-related disorders [4].

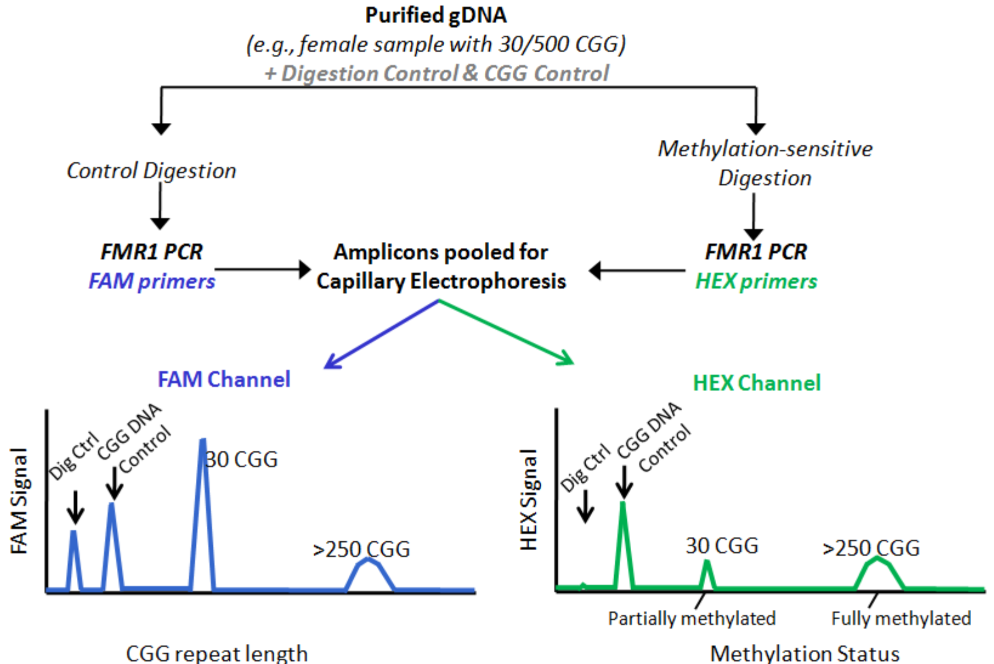
Risk assessment and clinical interpretation of FXS and related disorders are defined by the number of CGG repeats and methylation status of the gene. Based on the number of CGG repeats it is possible to distinguish four types of alleles: unaffected or normal alleles (<45 CGG), intermediate (45-54 CGG), premutation (55-200 CGG) and full mutation (>200 CGG). Individuals with full mutations (>200 CGG repeats) often present classic FXS, characterized by mental retardation, autism, and emotional and psychiatric challenges [4] due to hypermethylation of the expanded repeats in the FMR1 gene. Moreover, the severity of the phenotype may be associated with methylation status of the expanded allele and not just the number of CGG repeats [5, 6].

Most testing paradigms for *FMR1* disorders rely on a combination of PCR with size resolution by capillary electrophoresis and *FMR1* Southern blot analysis which is used to determine the methylation status of the gene [8]. Unfortunately, this workflow is costly, time- and labor-intensive, and requires large amounts of genomic DNA, and the data quality can be ambiguous to interpret. The AmplideX® mPCR *FMR1* assay provides a fast, simple solution to overcome these issues. It enables the researcher to amplify and detect the number of the CGG repeats in the *FMR1* gene and its associated methylation status in the 5'-untranslated region, without any requirement for Southern blot analysis [10].

Test Principle

The AmplideX® mPCR *FMR1* assay is based on a rapid, sensitive and robust GC-rich amplification technology developed by Asuragen. The test principle relies on pretreatment of DNA with a methylation-sensitive restriction enzyme, PCR, fragment sizing and comparison to a control aliquot of the same DNA sample. The principles of the workflow and two-color PCR are shown in Figure 1.

Figure 1. AmplideX® mPCR *FMR1* Assay Workflow.



Genomic DNA purified from whole blood is first premixed with DNA control mix, including the Digestion Control and CGG Control. Each aliquot is then subjected to methylation-sensitive DNase or control enzyme treatment. As a result, only the methylated alleles are retained in the digested reaction and carried through the following reactions, while the control reaction reflects full spectrum of the sample alleles. The digestion control in DNA control confirms the success of the digestion reaction.

After the enzyme treatment, each aliquot is directly subjected to PCR amplification of the *FMR1* gene using dye-tagged primers that codify digested and control gDNA. The digestion reaction is amplified with HEX-labeled *FMR1* primers, while the control reaction with FAM-labeled *FMR*1 primers. A CGG DNA control provides a reference (REF) peak and is co-amplified with each sample. This control is used to determine any systematic variability in the amplification efficiency across wells and subsequently normalizes the signal in each channel for the methylation percentage calculation.

After thermal cycling, unpurified PCR products are pooled from each paired reaction and analyzed directly on a validated capillary electrophoresis (CE) platform (e.g. Applied Biosystems Genetic Analyzer).

After CE analysis, the electropherograms are analyzed from control reaction (FAM) and the corresponding digestion reaction (HEX) to identify full length product peaks and the associated methylation percentages. The methylation percentage for each allele is determined from the ratio of amplicon peak height in HEX relative to FAM, after normalization by the corresponding CGG control (REF peak) within the same channel. Please refer to the sections titled “Software Procedure” and “Data Interpretation” for more information.

Kit Components

Reagents Supplied with this Kit

The AmplideX® mPCR *FMR1* provides all the components for digestion and PCR, and a ROX-labeled sizing ladder for capillary electrophoresis.

Table 1. AmplideX® mPCR *FMR1* (P/N 49442).

Item #	Description	Volume	Storage Temp
145213	mPCR Digestion Buffer	178 µL	-15 to -30°C
145214	mPCR Control Enzyme (FAM)	8 µL	-15 to -30°C
145215	mPCR Digestion Enzyme (HEX)	8 µL	-15 to -30°C
145232	mPCR DNA Control Mix	48 µL	-15 to -30°C
145217	mPCR GC-Rich Amp Buffer	960 µL	-15 to -30°C
145218	mPCR GC-Rich Polymerase Mix	5 µL	-15 to -30°C
145220	mPCR HEX Primers	46 µL	-15 to -30°C
145221	mPCR FAM Primers	46 µL	-15 to -30°C
145222	mPCR ROX 1000 Size Ladder	48 µL	-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 24 PCR reactions and 24 subsequent CE analyses.
- The reagents have been verified for use up to 4 freeze-thaw cycles. Additional cycles are not recommended.

Reagent Stability

- The product will maintain performance for 18 months from the manufacturing date printed on the label when stored and handled under the specified conditions.

Calibrators and/or Controls

- A ROX-labeled size ladder is included in the kit for use with calibrating size in base pairs using capillary electrophoresis.

Reagents Required but not Provided

- Reagents for DNA isolation are not included in the kit. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high quality, intact DNA.
- Hi-Di Formamide: Applied Biosystems, #4311320 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 MasterCycler
- Centrifuge capable of spinning 96-well plates
- Vortex
- Microcentrifuge
- 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: ABgene® #AB-0900 or equivalent
- PCR Plate Seals: ABgene® #AB-0558, Phenix LMT-0028 or equivalent
- PCR Compression Pad: Applied Biosystems #4312639 or equivalent
- ABI Genetic Analyzers running POP-7 polymer (3130, 3730 or 3500 series only)
- Dye set calibrators DS-30 or DS-31: Applied Biosystems #4345827, #4345829, or equivalent
- GeneMapper 4.0/4.1 software or equivalent

Warnings and Precautions

- Use proper personal protective equipment. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910:1030, CLSI M29, or other applicable guidance when handling human samples.
- **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.
- Substances that may interfere with the reagents include certain drug compounds and heparin. Highly lipemic samples, hemolyzed samples, icteric samples, or samples with proteinemia should not be used.
- 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.
- Use laboratory-validated methods and platforms.
- Prior to use, ensure that all instruments are calibrated according to the manufacturer's instructions.
- Safety data sheets (SDSs) are available upon request. Contact Asuragen or your local distributor.

Pre-Analytical Steps

Genomic DNA extracted via common sample preparation methodologies from whole blood collected in EDTA is compatible with the AmplideX® mPCR *FMR1*. 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.

AmplideX® mPCR *FMR1* Protocol

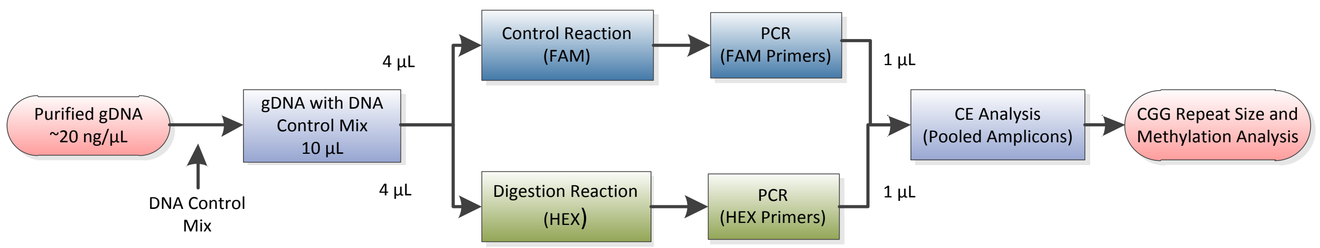
Overview

The test protocol involves four key sets of procedures:

1. Restriction enzyme digestion
2. PCR master mix setup and thermal cycling
3. Capillary electrophoresis for fragment sizing
4. Data analysis for repeat length and methylation status

An overview of the *AmplideX® mPCR FMR1* workflow is shown in Figure 2. Each DNA sample is pre-diluted to 20 ng/µL and a total 160 ng of gDNA is used in a single test. 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.

Figure 2. The AmplideX® mPCR *FMR1* Procedural Workflow.



The workflow should proceed in a uni-directional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area.

Restriction Enzyme Digestion

1. Thaw the following reagents at least 10 min at room temperature, including Digestion Buffer and DNA Control Mix. Place Control Enzyme (FAM) and Digestion Enzyme (HEX) on ice.
2. Thoroughly vortex all the tubes (3-5 times pulse vortexing), except the enzymes. Quick spin all the tubes to collect the reagents at the bottom.
3. For each DNA sample, prepare the sample/control mixture by adding the components in the following order. Thoroughly vortex to ensure sufficient mixing (3-5 times pulse vortexing).

DNA Sample	8 µL
DNA Control Mix	2 µL
Total Volume per Sample	**10 µL**

4. Prepare FAM control and HEX digestion reaction master mixes in separate tubes, as specified in Table 2. Thoroughly vortex master mix (3-5 times pulse vortexing).

Table 2. Restriction Enzyme Digestion Master Mix Setup.

Component	Control (FAM)	Digestion (HEX)
Digestion Buffer	3.7	3.7
Control Enzyme (FAM)	0.3	-
Digestion Enzyme (HEX)	-	0.3
Total Volume (µL)	**4.0**	**4.0**

5. For each DNA sample, dispense 4.0 µL FAM control master mix and HEX digestion master mix individually to separate wells of a PCR plate or strip tubes. Spin down briefly to collect the liquid at the bottom.
6. Aliquot 4.0 µL of each DNA sample/control mix to both the FAM control mix and HEX digestion mix for each sample. Pipette up and down 5-6 times to ensure adequate mixing.

Critical! Accurate pipetting and thorough mixing of each reaction is the key to ensuring appropriate methylation ratios for each allele.

7. Seal the tubes or plate with an adhesive film seal.
8. Vortex briefly and spin down at 2,000 rpm for 10-30 seconds.
9. Incubate at 37°C for 2 hours followed by 4 °C using the heated lid options if available.
   When the reaction reaches 4°C, proceed to methylation PCR within the same day. Do not store or freeze reactions before proceeding to PCR.

PCR Master Mix Setup and Thermal Cycling

1. Thaw the following reagents including mPCR GC-Rich Amp buffer and mPCR FAM- and HEX-Primers at least 10 minutes at room temperature. Place GC-Rich Polymerase Mix on ice.

   Note: The mPCR GC-Rich Amp Buffer may be occluded or have observable precipitation when cold; this buffer should be equilibrated at room temperature prior to PCR set-up.

2. Thoroughly vortex all tubes (3-5 times pulse vortexing) except Polymerase Mix. Quick spin all the tubes to collect the reagents at the bottom.
3. To prepare one of each FAM and HEX PCR master mixes, using the respective primers, add the appropriate components to a 1.5 mL microfuge tube in the exact order specified in Table 3.

   Table 3. mPCR Master Mix Setup.

Component	FAM-PCR Master Mix	HEX-PCR Master Mix
mPCR GC-Rich Amp Buffer	20	20
GC-Rich Polymerase Mix	0.1	0.1
mPCR FAM-Primers	1.9	N/A
mPCR HEX-Primers	N/A	1.9
Total Volume (µL)	**22**	**22**

   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 master mix.

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

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

5. For each DNA sample, dispense 22 µL of FAM-PCR master mix to the FAM control reaction tubes and dispense 22 µL of HEX-PCR master mix to the HEX digestion reaction tubes from enzyme digestion step.

   Note: Use a repeater pipettor, if available.

   Critical! **To minimize variability,** the FAM and HEX PCR reaction mixtures should be treated (vortexed, centrifuged, etc.) as identically as possible throughout the procedure.

6. Seal the plate with an adhesive film seal.
7. Gently vortex the plate (3-5 times pulse vortexing) to ensure mixing and centrifuge the plate to remove bubbles (1 min at 1600 rcf).

   Important! Ensure that all bubbles are removed from the wells.

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

mPCR Thermal Cycling	
Description	**Duration**
1 hold	95°C for 5 min
27 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

9. **Potential Stopping Point**. The PCR products can be transferred for CE analysis immediately following amplification. If required, the PCR products can be stored at 4°C overnight or at -15 to -30°C up to 30 days until analyzed.

Capillary Electrophoresis with POP-7

1. Thaw the Hi-Di formamide and ROX 1000 Size Ladder for 10 min at room temperature. Thoroughly vortex (up to 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 **1 µL of FAM-PCR** products to each well, and then transfer **1 µL of HEX-PCR** products to the well with the matching FAM-labeled PCR product. A multi-channel pipette is recommended for transfer.
6. Seal the plate with an adhesive film seal. Centrifuge at 2,000 rpm for 10-30 seconds to remove bubbles and transfer to a thermal cycler.
7. **Potential Stopping Point**. Denature for 2 min at 95°C followed by 2 min at 4°C. Alternatively, the products may be stored on ice for up to 12 hours and protected from light after the denaturation step.
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 FAM, HEX 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 Condition	Run Condition
3130, 3130*xl*	36 cm	2.0 kV, 40 s	15.0 kV, 2400 s
3730, 3730*xl*	50 cm	2.0 kV, 40 s	15.0 kV, 4200 s
3500, 3500xL	50 cm	2.0 kV, 40 s	19.5 kV, 2400 s

9. After the run, the data may be analyzed for CGG repeat length by amplicon size conversion and for methylation percentage by the ratio of normalized peak heights for each allele PCR product from HEX and FAM channels.

Calibration Procedure

<u>Calibration procedure used to derive the size and mobility correction factors</u>

The AmplideX® mPCR *FMR1* incorporates two correction factors for conversion of size in base pairs to the number of CGG repeats for each allele. 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. These factors are derived using the size in base pairs of allele amplicons from a pooled cell line control. This pooled control includes alleles previously matched to DNA sequencing [9]. It can also be used 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 5 below.

Table 5. Pooled control formulation using 4 cell line DNA from the Coriell Cell Repository (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

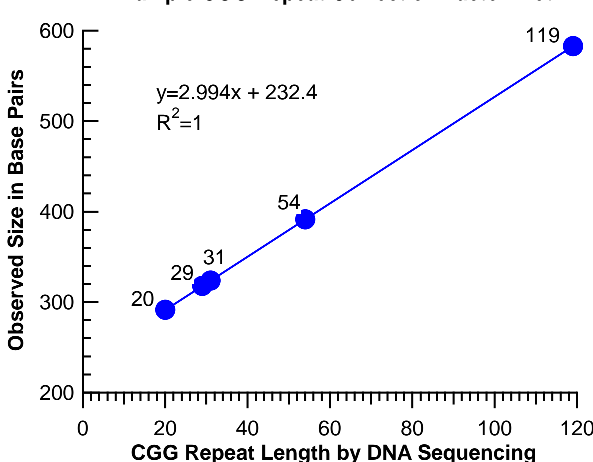
Terms for c_0 and m_0 are derived from a linear fit of expected CGG repeat length and base pair size for the first five peaks, 20, 29, 31, 54 and 119 CGG repeats, of the pooled control allele amplicons from FAM channel. The 199 CGG allele is not used for this plot as this allele length could not be verified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

1. Analyze the pooled control with the AmplideX® mPCR *FMR1* and determine the measured size in base pairs for each of the first five amplicon peaks from FAM channel. It is recommended to calculate the average base pair size for each peak from at least 2 independent runs.

Table 6. Example data comparing expected repeat length and observed size.

Repeat Length (CGG)	Observed Size [11]
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.

Note: If you have previously obtained the factors using the AmplideX® *FMR1* PCR reagents, these values also can be applied to the mPCR assay.

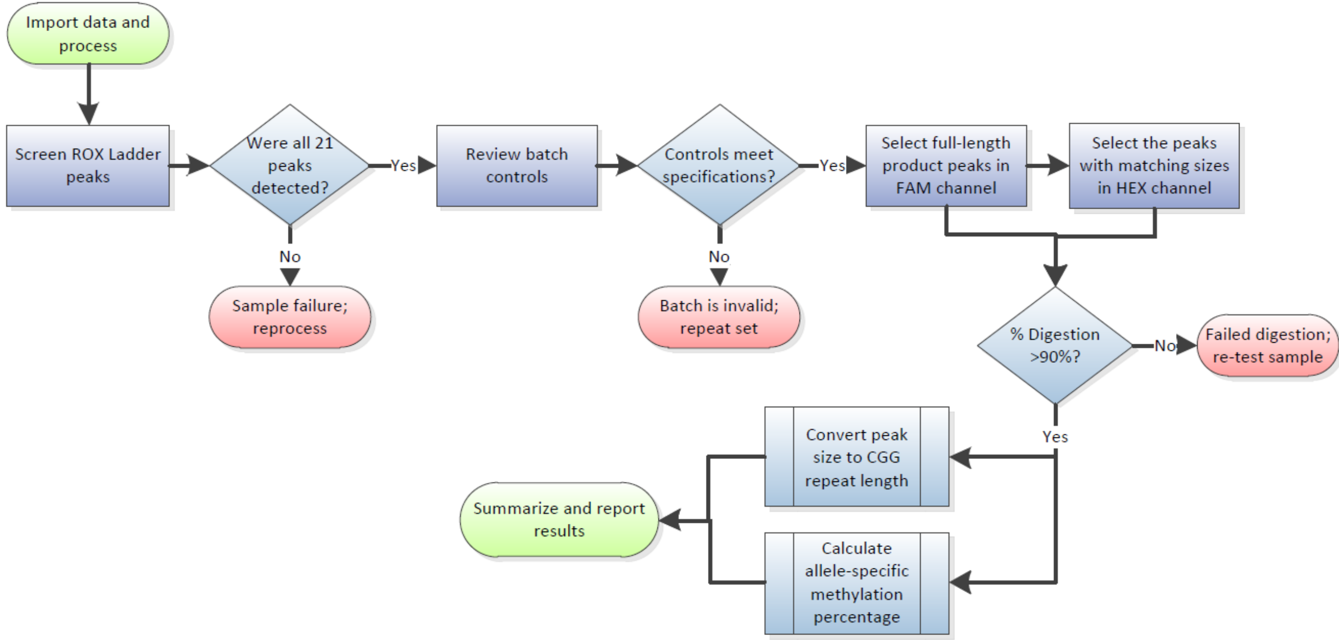
Control Procedure

Positive and negative DNA controls are not provided. 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 [12] and the National Institute of Biological Standards and Controls (NIBSC) [13]. A Digestion Control and CGG DNA Control are premixed with every sample as a component of analysis.

Software Procedure

GeneMapper 4.0/4.1 or equivalent software can be used for fragment sizing analysis of methylation PCR data. It involves a series of steps to obtain the size of full length product peaks in the FAM channel, and derive the methylation percentage from the matching peak heights selected in both FAM and HEX channels. 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 3.

Figure 3. 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
2. Qualify the run by screening the ROX Ladder Peaks and reviewing batch controls
3. Select the peaks in FAM and the matching peaks in the HEX channels. Selection of matching peaks in both the FAM and HEX channels is used for calculating percent methylation.
4. Genotype tables are exported as .txt files under the table setting established for AmplideX® mPCR *FMR1*.
5. The .txt file can be imported in Microsoft Excel and used to calculate the CGG repeat length and allele-specific methylation percentage.

Data Interpretation

Overview

Proper data interpretation requires three steps:

1. Calculation of CGG repeat length
2. Calculation of allele-specific methylation percentage
3. Interpretation and Reporting of test results

Part I - Calculation of CGG Repeat Length

Convert peak size to CGG repeat length

The CGG repeat length of the allele peak is converted from base pairs by subtracting the corrected size of the non-CGG repeat region of the amplicon and dividing by the relative size of the CGG repeat region according to the following equation:

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

where $Peak_i$ is the size in base pairs of a given product peak; c_0 is the size correction factor; and m_0 is the mobility correction factor for each CGG repeat. Laboratory-specific correction factors or correction factors for different instruments, capillary length, polymer type or run conditions may be determined following the "Calibration Procedure" section on Page 9.

Part II – Calculation of Allele-Specific Methylation Percentage

1. For each sample, the effectiveness of the methylation-sensitive enzyme digestion is reported as the percentage of digestion control being digested. It is calculated as the ratio of digestion control amplicon peak height between the digested (HEX) and undigested samples (FAM) after normalized to the CGG control amplicon peak height, as shown in Equation 2. A value of 90% or more indicates appropriate performance of the digestion reaction. Otherwise, the sample should be re-tested.

$$\%Digestion = 100 \times (1 - \frac{[Peak_{Dig}/Peak_{REF}]_{HEX}}{[Peak_{Dig}/Peak_{REF}]_{FAM}})$$

   where $Peak_{Dig}$ is the peak height of remaining digestion control amplicon peak in the corresponding FAM or HEX channel and $Peak_{REF}$ is the peak height of CGG control amplicon peak within the matched channel.

2. For each allele in the sample, the methylation percentage is calculated as a ratio of peak heights between the digested (HEX) and undigested samples (FAM) normalized to the CGG control amplicon peak height, as shown in Equation 3.

$$\%Me_i = 100 \times \frac{[Peak_i/Peak_{REF}]_{HEX}}{[Peak_i/Peak_{REF}]_{FAM}}$$

   where $Peak_i$ is the peak height of a given product peak in the corresponding FAM or HEX channel and $Peak_{REF}$ is the peak height of the CGG control amplicon peak within the matched channel.

   a) For allele peaks with signal intensity beyond the CE instrument saturation limit, accurate determination of methylation percentages must be derived from a shorter injection of the sample, for example at 2.0 kV for 5-10 seconds (Table 7).

Table 7. Default signal intensity saturation limit for different CE instrument configurations.

Instrument	Saturation Limit (rfu)
3130, 3130*xl*	6,000
3730, 3730*xl*	24,000
3500, 3500xL	24,000

b) For allele peaks with low signal intensity in FAM (below the CE instrument cut-off, the high baseline signal in HEX could result in an artificially inflated methylation percentage. To avoid this issue, the average baseline signal may be subtracted from both FAM and HEX channels prior to the calculation of methylation percentage.

Part III – Interpretation of Results

CGG Repeat Length

Alleles are reported with a whole integer number of repeats associated with a specific genotype category, namely normal, intermediate, premutation, full mutation, or full mutation mosaic (ie, both FM and PM represented in electropherogram) [7]. The reportable range of the mPCR assay on CE is 5-200 repeats; above 200 repeats all alleles are identified as >200 CGG.

Report alleles sizes assigned according to the reference range. In samples with multiple allele peaks, the longest allele peak is reported. The genotype may be assigned according to regional specific guidelines as shown in Table 8 [7, 14, 15].

Table 8. 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

Allele-Specific Methylation Status

Methylation percentages that are nominally calculated in excess of 100% are scaled to 100%. For comparison to Southern blot analysis, these values can be binned into categories of un-, partially, or fully methylated alleles. The recommended default ranges for these categories relative to the percent methylation are shown in Table 9. These ranges may be refined by the individual research laboratory.

Table 9. Suggested methylation boundaries for specific categories (un-, partially, and fully methylated).

Methylation Category	Methylation Percentage
Unmethylated	≤20%
Partially methylated	20-80%
Fully methylated	≥80%

Due to the higher resolution capabilities of CE, a single “allele” on Southern blot may be represented as a range of peak sizes and different methylation states by the mPCR assay. This effect is particularly evident in female premutation alleles [10]. In full mutation alleles, the combined values across different peaks may aid in the overall interpretation of the methylation status.

Notice to Purchaser

1. This product is intended for research use only. It is not intended for diagnostic use.
2. This product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen.
3. AmplideX® is a registered trademark of Asuragen, Inc.
4. This product is covered by US Patent Applications 20100209970, 20100243451, 20120107824 and other foreign patents pending, which patents are licensed to Asuragen, Inc. by EPICENTRE Technologies Corporation, 726 Post Road, Madison, WI 53713, U.S.A.
5. The purchase of this product conveys to the buyer a limited, non-exclusive, non-transferable right under those patents and/or patent applications to use the purchased product for performing molecular tests targeting the *FMR1* gene and/or the *FMR2* gene. No other license is granted to the buyer whether expressly, by implication, by estoppel or otherwise.
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Appendix A: Glossary of Symbols

Symbol	Description
REF	Catalog number
LOT	Batch code
Σ	Contains sufficient for <n> tests
	Consult instructions before use
	Temperature limitation
	Use by
	Date of manufacture
	Manufactured by
	Irritant

PC-0191v2