Asuragen®

AmplideX®

PCR/CE *FMR1* Kit

Instructions for Use

CE IVD	
REF	**76008**
Σ	**100**

For Export Only

Asuragen, Inc.
2150 Woodward St.
Suite 100
Austin, TX 78744-1840
USA
+1.512.681.5200

EC REP **Emergo Europe**
Prinsessegracht 20
2514 AP
The Hague, The Netherlands
+31.70.345.8570

Eine gültige Version dieser Gebrauchsanweisungen auf Deutsch kann kostenlos erhalten werden, sich mit Ihrem lokalen Verteiler in Verbindung setzend.

Une version valide de ces instructions d'utilisation en français peut être obtenue gratuitement en contactant votre distributeur local.

Una versión válida de estas instrucciones para uso en español puede ser obtenida gratuitamente al ponerse en contacto con su distribuidor local.

Umaversão válida dessasinstruções de uso emportuguês pode ser obtida gratuitamente ao contacto com o seu representante local.

Una versione valida di queste istruzioni per l'uso in italiano può essere gratuitamente ottenuta contattando il suo distributore locale.

Een geldige versie van de gebruiksaanwijzingen in het Nederlands kan gratis verkregen worden door uw plaatselijke vertegenwoordiger te contacteren.

Table of Contents

Intended Use

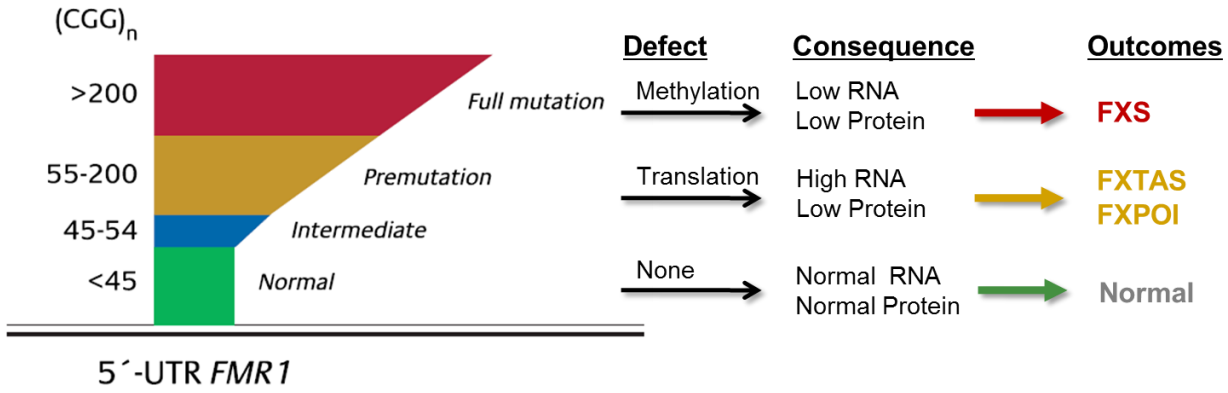
The AmplideX® PCR/CE *FMR1* Kit is an in vitro diagnostic device for professional use in clinical laboratories to amplify and detect the cytosine-guanine-guanine (CGG) repeat region in the 5'-untranslated region of the fragile X mental retardation-1 (*FMR1*) gene. The device is intended as an aid in diagnosis of fragile X syndrome and fragile X associated disorders, e.g. tremor and ataxia syndrome (FX-TAS) and primary ovarian insufficiency (FX-POI), through determination of CGG repeat length up to 200 CGG and detection of alleles greater than 200 CGG. The test consists of a polymerase chain reaction (PCR) of genomic DNA purified from whole blood or buccal cells, followed by fragment sizing on a general laboratory-validated genetic analyzer or capillary electrophoresis platform and conversion of product size to the number of CGG repeats.

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]. Expansion of this region to 200 or more repeats is associated with hypermethylation and resulting inactivation of the *FMR1* gene [2]. Lack of the gene product through hypermethylation results in loss of FMRP, an RNA-binding protein 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], loss of this protein is linked to the fragile X syndrome phenotype, which is characterized by mental retardation, autism, and emotional and psychiatric challenges [5]. The general prevalence of fragile X syndrome is ~1 in 4,000 in males and ~1 in 5,000–8,000 in females. Premutation alleles are linked to fragile X-associated primary ovarian insufficiency (FXPOI) a leading cause of ovarian dysfunction in women [6], and fragile X-associated tremor and ataxia syndrome (FXTAS) [7]. FXTAS, which affects more males than females, is a late-onset disorder characterized by progressive cognitive and behavioral difficulties and intention tremors and ataxia. These premutation disorders are characterized by an increase in mRNA transcription but lower protein levels. The relationship among CGG repeat length, biological consequence and associated phenotype are represented in Figure 1.

Figure 1. Relationship between CGG repeat lengths and corresponding phenotypes

.



Deficits in development, and particularly attention and social communication, have also been noted for children with the *FMR1* premutation allele [8]. Approximately 1 in 250 females and 1 in 800 males carry the *FMR1* premutation. 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: unaffected or normal alleles (<45 CGG), intermediate (45-54 CGG), premutation (55-200 CGG) and full mutation (>200 CGG) [9]. Full mutation alleles can range from 200 to greater than 1000 repeats. The FXS

phenotype is associated with the methylation status of the allele and not necessarily the exact number of repeats exceeding 200 CGG [9]. Premutation carrier females have a risk of having a child with a full mutation expansion. This risk increases with the number of CGG repeats. Above 100 repeats, there is a systematic risk of expansion in the next generation [10] making accurate sizing in this range important. Many *FMR1* alleles contain AGG sequences that are interspersed among the CGG repeats. These AGG "interruptions" confer DNA stability, thereby resulting ina reduced risk of expansion in the next generation [11-13]. The risk profile for mothers with no AGG interrupters is higher than mothers with the same number of repeats but with at least one AGG, and thus fewer consecutive $(CGG)_n$ sequences. For example, the shortest allele to expand to a full mutation in two generations was from a 56 CGG allele that lacked AGG [14].

Fragile X Testing

Most diagnostic testing paradigms for *FMR1* disorders rely 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 has been used to characterize samples with CGG repeat numbers too large to amplify by PCR, and to determine the methylation status of the gene. 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 historically the highly GC-rich character of the fragile X triplet repeat sequence has been refractory to amplification. PCR of premutation and full mutation females is even more challenging due to preferential amplification of the smaller allele [15]. Consequently, the greater than 20% of female specimens that are biologically homozygous had to be reflexed to Southern blot to resolve the potential ambiguity of an unamplified longer allele. This classic workflow for fragile X clinical testing is hands-on, low throughput and not amenable for increases in testing volumes.

The AmplideX® PCR/CE *FMR1* Kit has been designed to allow PCR amplification and detection of CGG repeats for routine *FMR1* analysis reducing the need for Southern blots. The kit is used to determine the number of CGG repeats in the *FMR1* gene using polymerase chain reaction and fragment sizing by CE. The use of the kit provides 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.

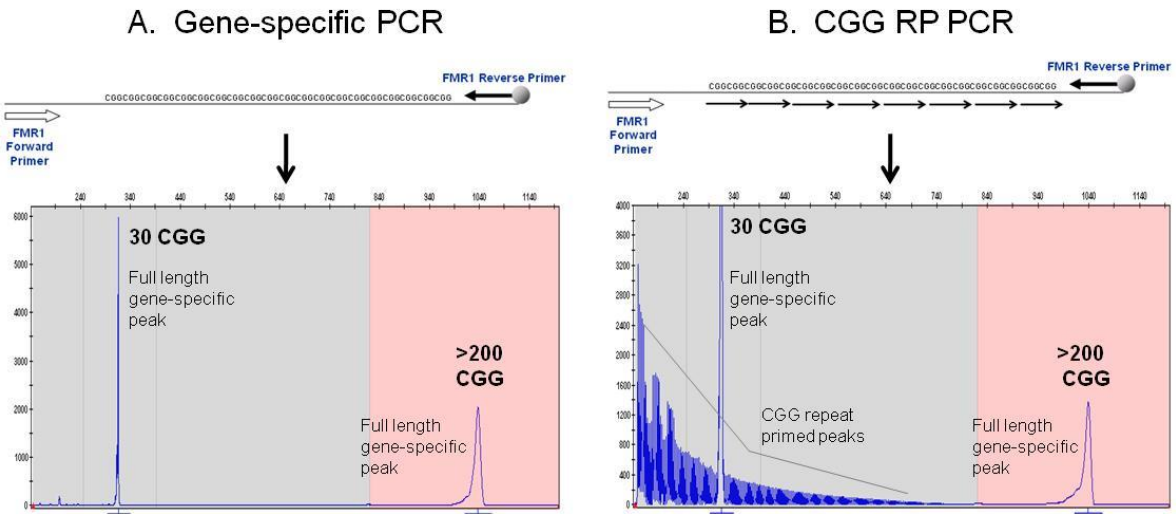
Test Principle

The AmplideX® PCR/CE *FMR1* Kit is based on a three-primer CGG Repeat Primed (RP) PCR from purified genomic DNA and fragment sizing on a validated capillary electrophoresis platform (e.g. Applied Biosystems Genetic Analyzer). The kit includes gene-specific and CGG primers, a polymerase mix and buffer for amplification of the CGG repeat region in the *FMR1* gene and a ROX 1000 Size Ladder for sizing by capillary electrophoresis. Optionally, users may elect to perform a two-primer gene-specific PCR by omitting the CGG primer.The size of the PCR products are converted to the number of CGG repeats using size and mobility conversion factors derived from alleles of known repeat length.

PCR Methods

The kit 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 (Figure 2A). CGG RP PCR is primarily distinguished from the more conventional two-primer, gene-specific PCR by the addition of a 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 (Figure 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 [16].

Figure 2. *FMR1* PCR Methodologies highlighting the features of the gene-specific, 2-primer, and CGG Repeat Primed (RP), 3-primer, PCR methods.

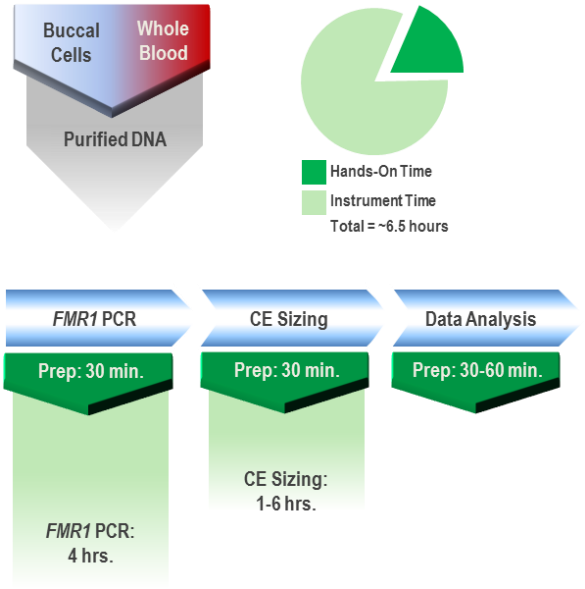


The CGG RP PCR repeat profile can telegraph the presence of longer alleles in the amplification, irrespective of whether such alleles are detected as full length products [16]. Consequently, the risk of PCR dropout of the longer allele is reduced. The full length gene-specific product peaks may be converted 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 4.5 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 a validated capillary electrophoresis platform (e.g. 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/CE *FMR1* Kit 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 [16]. Consequently, *FMR1* PCR products exceeding 200 CGG are identified categorically as >200 CGG.

Kit Components

Reagents Supplied with this Kit

The PCR reagents and ROX 1000 Size Ladder supplied with the kit are listed in Table 1.

Table 1. AmplideX® PCR/CE *FMR1* Kit Components (P/N 76008)

Item #	Description	Storage Temp
145185	*FMR1* F,R FAM-Primers	-15 to -30°C
145184	*FMR1* CGG Primer	-15 to -30°C
145186	GC-Rich Amp Buffer	-15 to -30°C
145187	GC-Rich Polymerase Mix	-15 to -30°C
145188	ROX 1000 Size Ladder	-15 to -30°C
145183	Diluent	-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 to 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 with 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 minimum of 16 reactions per batch.

Reagent Stability

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

Calibrators and/or Controls

- Calibrators and/or Controls are not provided in the kit, but are recommended to be used in every run.
- The Diluent provided with the kit may be used as a negative no-template control.
- AmplideX® *FMR1* Controls (sold separately), the Fragile X Syndrome Genetic Reference Panel (NIBSC, 08/158) or other commercially available cell line DNA [17] may be used as positive controls.

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.

Consumables & Equipment Required but not Provided

- General laboratory equipment and workspace to perform PCR
- Thermalcycler: ABI 9700, ABI Veriti (run in 9700-max mode), or MJ Research PTC-225
- 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: ABgene® #AB-0900 or equivalent
- PCR Plate Seals: ABgene® #AB-0558, Phenix LMT-0028 or equivalent
- PCR Compression Pad: ThermoFisher # 4312639 or equivalent
- Laboratory-validated capillary electrophoresis platforms and materials
- ABI Genetic Analyzers running POP-7 polymer (e.g. 310, 3130, 3730 or 3500 series)
- POP-7 Polymer: ThermoFisher, #4363785 or equivalent
- Hi-Di Formamide: ThermoFisher, #4311320 or equivalent
- Dye set calibrators for FAM and ROX, DS-30 or DS-31 dye set: ThermoFisher #4345827, #4345829, or equivalent
- Data analysis software, GeneMapper 4.0/4.1, GeneMarker® v.2.6.0 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 Kit 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 your local distributor.

Pre-Analytical Steps

Genomic DNA extracted via common sample preparation methodologies from either whole blood collected in EDTA or buccal cells is compatible with the AmplideX® PCR/CE *FMR1* Kit. 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* Kit 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. No more than 4 freeze-thaw cycles are 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 GC-Rich Polymerase Mix, for approximately 10 minutes at room temperature. Place GC-Rich Polymerase Mix on ice. Briefly vortex all tubes (3-5x pulse vortexing) except GC-Rich Polymerase Mix. Spin down all tubes prior to use.

   Notes: 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
Total Volume per Reaction	**13.00 µL**	**13.00 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 from Table 4:

Table 4. Thermal-cycler Protocols

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.

Fragment sizing with capillary electrophoresis platforms

For CGG repeat size analysis, several alternative microfluidic and capillary electrophoresis systems may be used, e.g. ABI Genetic Analyzers. Please refer to the specific instument manuals or contact technical support at: support@asuragen.com, for further platfrom specific information. The following instructions are specific for Genetic Analyzers running POP-7 polymer.

1. Thaw the Hi-Di™ Formamide and ROX 1000 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 Hi-Di™ 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 5.

   Table 5. 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
PRISM®310	36 cm	2.5kV, 15 s	2400 s
3130, 3130*xl*	36 cm	2.5 kV, 20 s	2400 s
3730, 3730*xl*	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 Interpretation. GeneMapper 4.0/4.1, GeneMarker® v.2.6.0, or equivalent software is used for fragment sizing analysis of gene-specific or CGG RP PCR data. Please refer to the Software Procedure (Page 13) for detailed instructions.

Control Procedure

Positive and negative controls are to be included in every run. The Diluent provided with the kit may be used as a negative no-template control. Genomic DNA extracted from well-characterized cell lines may be used for positive controls. Pooled genomic DNA controls can be purchased separately from Asuragen (AmplideX® PCR/CE *FMR1* Control, P/N 49513). Alternatively, cell lines or corresponding purified genomic DNA can be obtained from the Coriell Cell Repositories [17]. 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 [18]. Representative examples with these materials are shown in the Data Interpretation section below. The positive and negative controls need to be in the specified range for data acceptance.

1. Ensure that negative control included in the batch run meets specification.
2. Ensure positive controls meet specifications. See Examples of Controls in Data Interpretation, Part II (Page 17) for more information.

Calibration Procedure

Calibration procedure used to derive the size and mobility correction factors

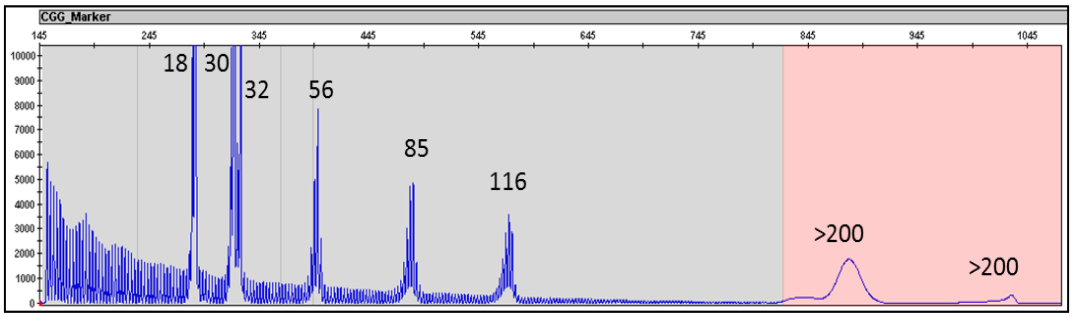
After capillary electrophoresis, the size of the target amplicon is derived from comparison to a co-injected size standard, e.g. ROX 1000 Size Ladder. However, PCR products of triplet repeat regions have an anomalously faster migration than the generic DNA of the size standard [19-21]. This higher migration, 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* Kit 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:

$$CGG_i = \frac{Peak_i - c_0}{m_0}$$ **Equation 1.**

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. 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.

Asuragen offers a commercially available pooled genomic DNA control (see http://asuragen.com). This control is provided as a well characterized, ready to use admixture of 7 *FMR1* alleles spanning the entire CGG repeat size range shown as an example electropherogram in Figure 4.

Figure 4. AmplideX® PCR/CE *FMR1* Control (P/N 49513)



Values for c_0 and m_0 are derived from a linear fit of expected CGG repeat length and base pair size for the first six peaks, 18, 30, 32, 56, 85 and 116 CGG repeats, of the pooled control allele amplicons. The >200 CGG allele is not used for this plot as this allele length exceeds the linear range. To calculate specific conversion factors follow these steps:

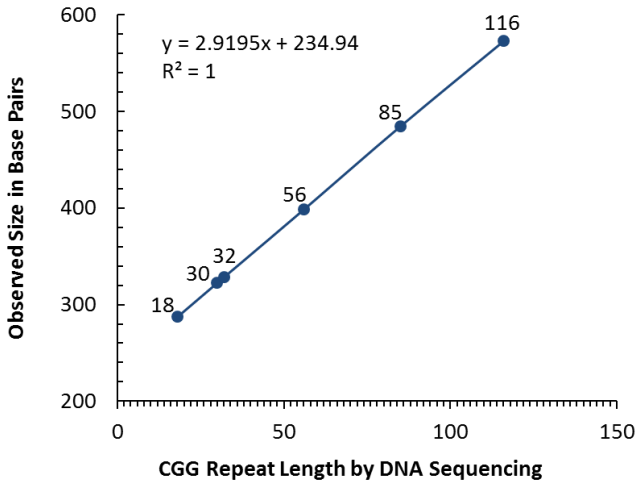
1. Analyze the pooled control with the AmplideX® PCR/CE *FMR1* Kit and determine the measured size in base pairs for each of the first six amplicon peaks as shown in Table 6. It is recommended to calculate the average base pair size for each peak from at least 2 independent runs.

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

Repeat Length	Observed Size
18	287.21bp
30	322.43bp
32	328.28bp
56	398.45bp
85	484.49bp
116	572.68bp

2. Calculate the slope (m_0) and intercept (c_0) of the correlated data in Excel or comparable program as shown in Figure 5.

Figure 5. Example CGG Repeat Correction Factor Plot



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=234.94 and m_0=2.9195.

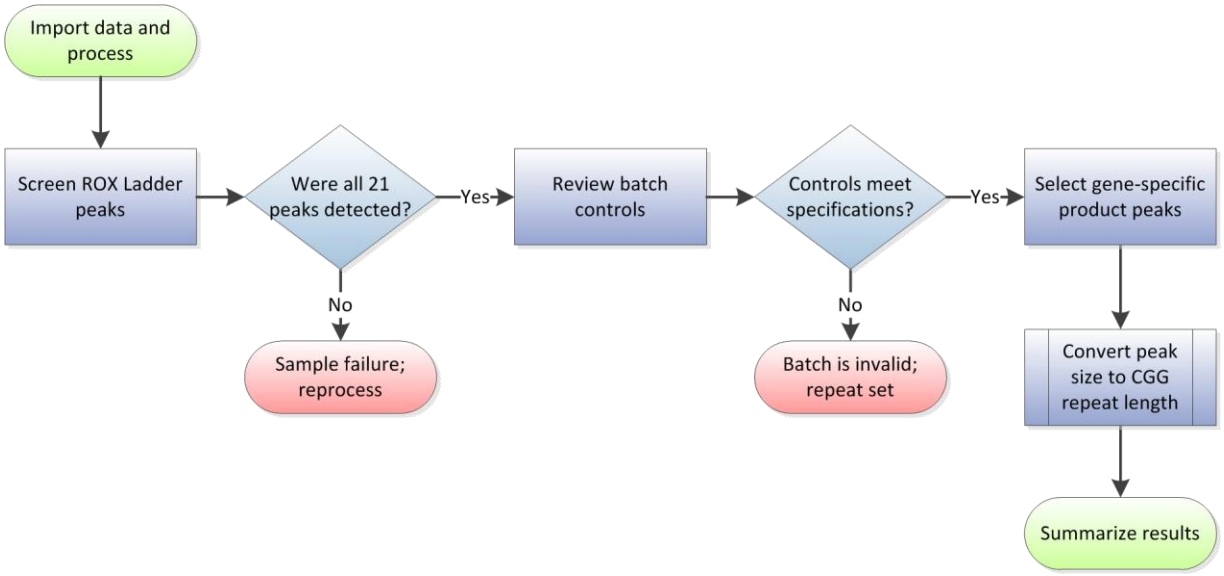
To verify the derived correction factors, the operator may test the WHO International Standard, Fragile X Syndrome, Genetic Reference Panel (NIBSC, 08/158) or other commercially available cell line DNA standards.

3. Use the m_0 and c_0 values to calculate CGG repeat length for samples using the formula from Equation 1.

Software Procedure

GeneMapper 4.0/4.1, GeneMarker® v.2.6.0 or other equivalent software can be used for fragment sizing analysis of gene-specific or CGG RP PCR data. A series of steps are followed 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. An overview of the fragment sizing analysis workflow is shown in Figure .

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



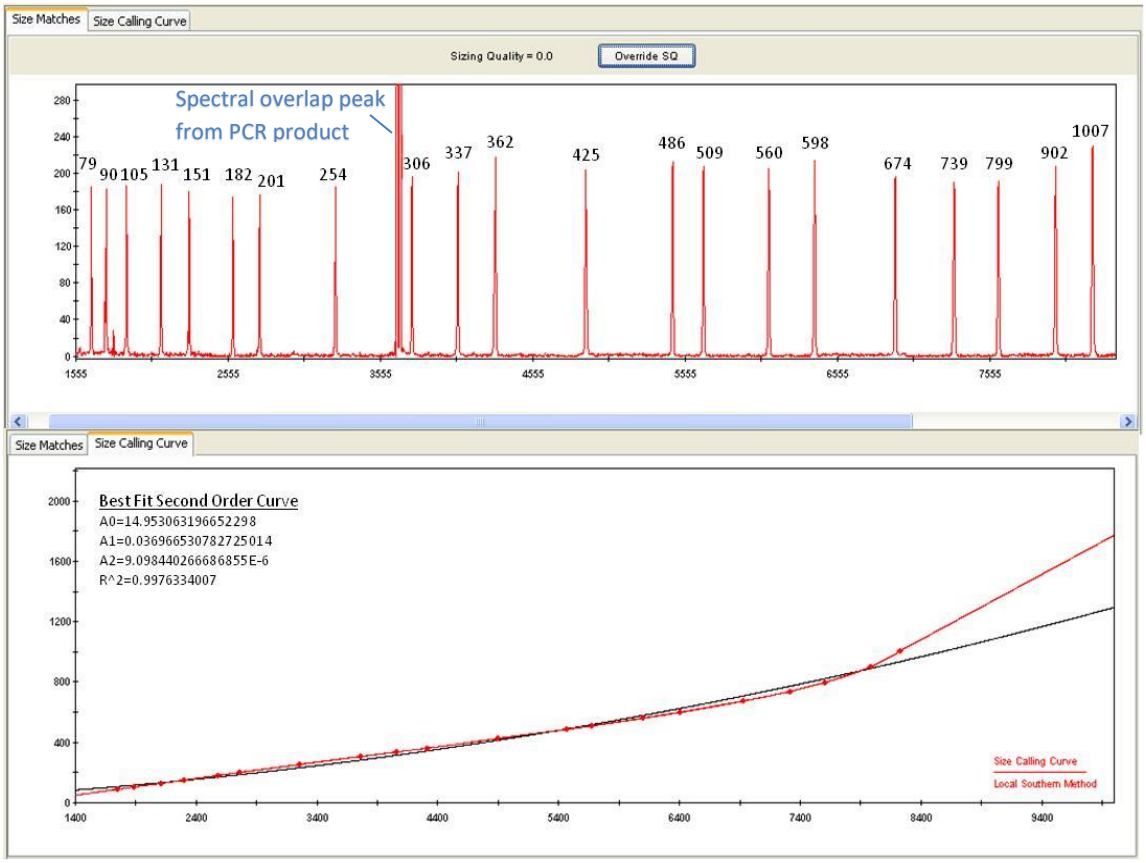
The terms used for analysis below refer to GeneMapper 4.0/4.1 features. When using GeneMarker® v.2.6.0, for data analysis, please refer to the user manual or contact GeneMarker® technical support team at: support@softgenetics.com for more information.

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
   a. 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.

   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 7.

Figure 7. ROX 1000 Size Matches and Size Calling Curve - The Size Match 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.



b. Review Batch Controls

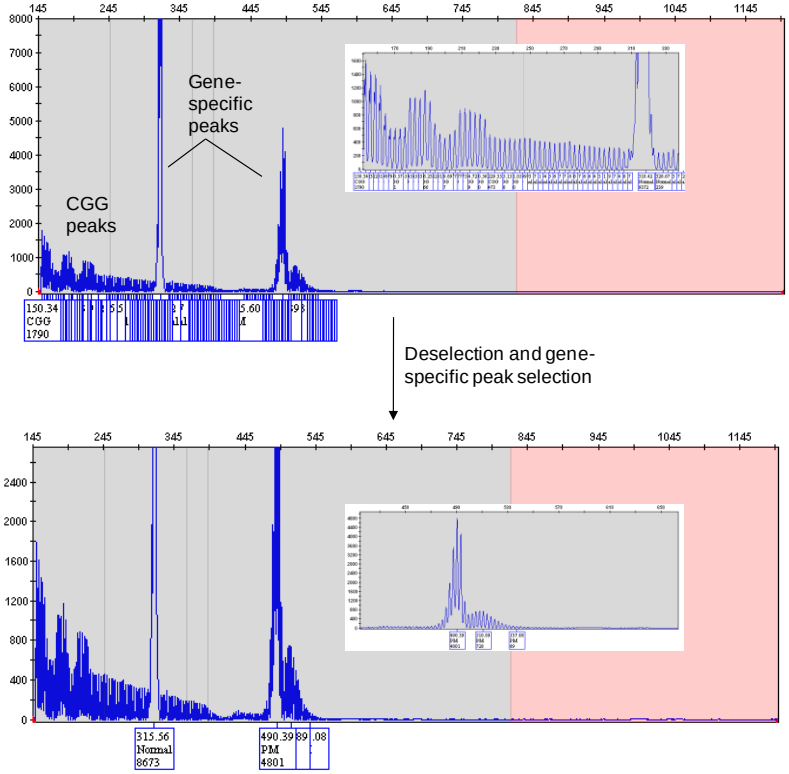
i. Ensure that negative control included in the batch run meets specifications.

ii. Ensure positive controls meet specifications. See Examples of Controls in Data Interpretation, Part II (Page 18) for more information regarding use of positive controls.

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 in Figure 8 for a female premutation allele.

Figure 8. Example electropherogram with default analysis settings (top) and only gene-specific full length peaks selected from the CGG RP PCR electropherogram (bottom)



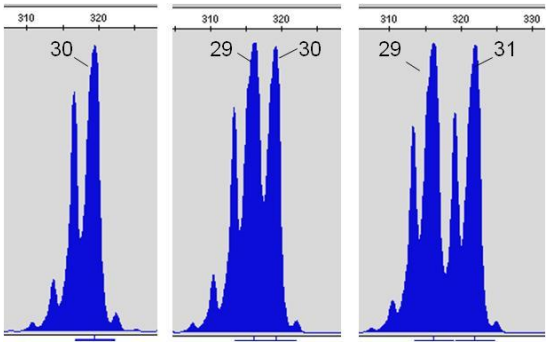
Note: If processing the results of a gene-specific PCR, only the gene-specific peaks will be present and only these peaks need to be selected. Deselection will not be required.

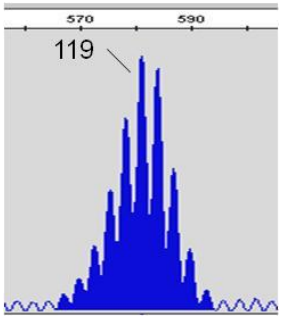
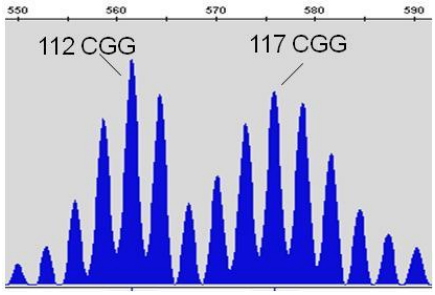
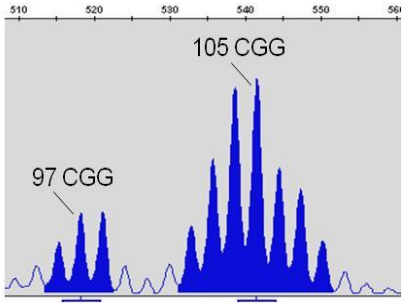
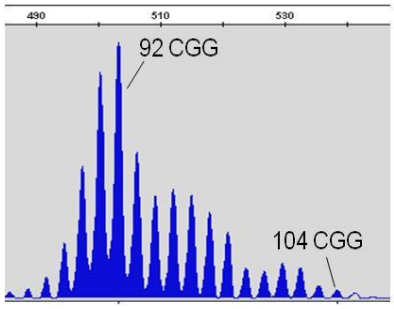
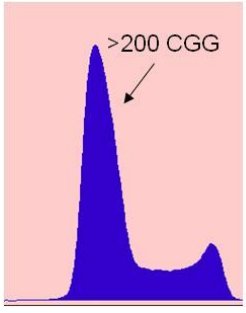
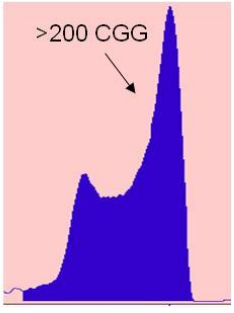
b. After deselecting peaks in all samples, identify gene-specific full length product peaks for each region of the electropherogram using the guidelines listed in Table 7.

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 7. 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).	

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.
~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.	 

a. After selection of gene-specific peaks, review traces for minor or low intensity peaks. These peaks may be manually selected according to the guidelines in Table 8.

Table 8. Manufacturer default signal intensity cutoffs and low peak ranges for different CE instrument configurations

Instrument	Cutoff	Low Peak Range
PRISM®310	50rfu	10-49rfu
3130, 3130*xl*	50rfu	10-49rfu
3730, 3730*xl*	175rfu	50-174rfu
3500, 3500xL	175rfu	50-174rfu

Data Interpretation

Data interpretation contains three parts of information:

1. The calculation of CGG repeat length
2. Interpretation and Reporting of test results
3. Special features of the assay results

Part I - Calculation of CGG Repeat Length

Convert peak size to CGG repeat length

The size of each peak may be converted to repeat length according to equation 2:

$$CGG_i = \frac{Peak_i - c_0}{m_0}$$ **Equation 2.**

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. Correction factors for supported configurations are listed in Table 9.

Table 9. Size and mobility correction factors for some standard instrument configurations

Configuration		c_0	m_0
PRISM®310	36 cm	226.5	2.910
3130, 3130*xl*,	36 cm	229.4	2.965
3730, 3730*xl*,	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 Calibration Procedure (Page 11).

Part II – Interpretation and Reporting of Results

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 size indication for assigned alleles, according to 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 [22]. The genotype may be assigned according to specific guidelines as shown in Table 11 in Medical Decision Points (Page 21).

- Example CGG RP PCR Results for Normal, Premutation and Full mutation alleles (Figures 9, 10 and 11 respectively)

Figure 9. Normal allele examples showing a 29, 30 CGG compared to a 30 CGG

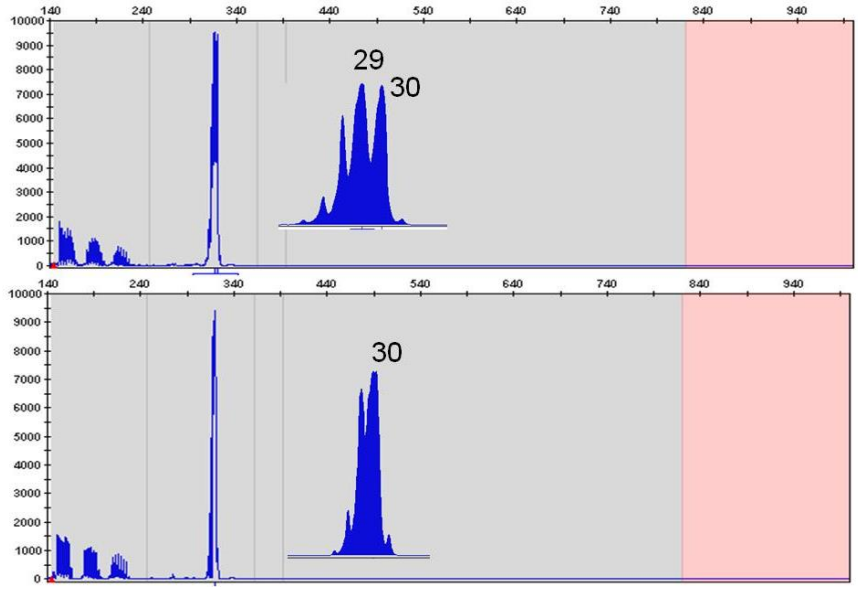


Figure 10. Example premutation allele - The highest peak in the sample is selected to represent the 80 CGG allele.

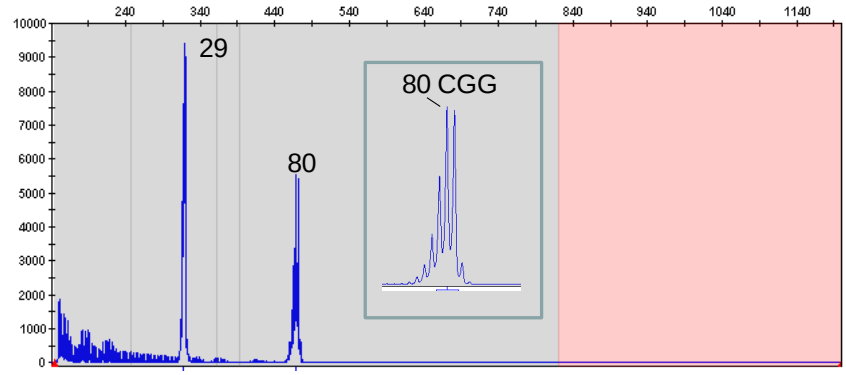
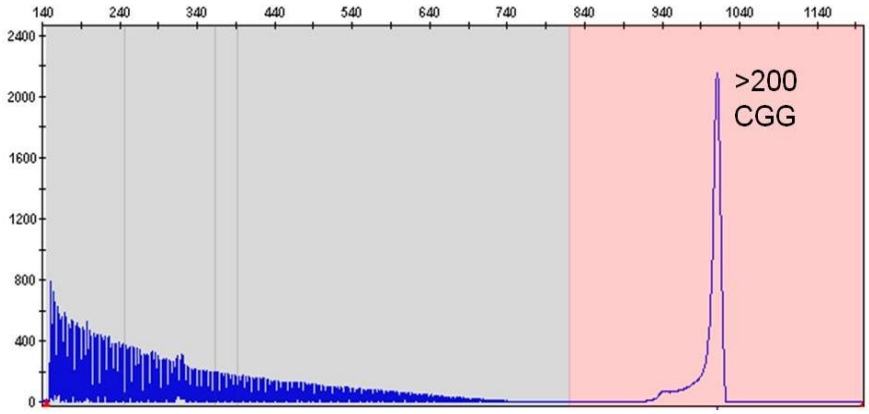


Figure 11. Example full mutation allele; male sample. The full length product peak exceeds 200 CGG and is identified as >200 CGG. Individual CGG repeat product peaks can be identified in the sample trace.



- Traceability to Fragile X standards

Representative electropherograms obtained with the reference material panel endorsed by the European Society of Human Genetics (ESHG) and approved as a WHO International Standard are shown in Figure 12. The panel

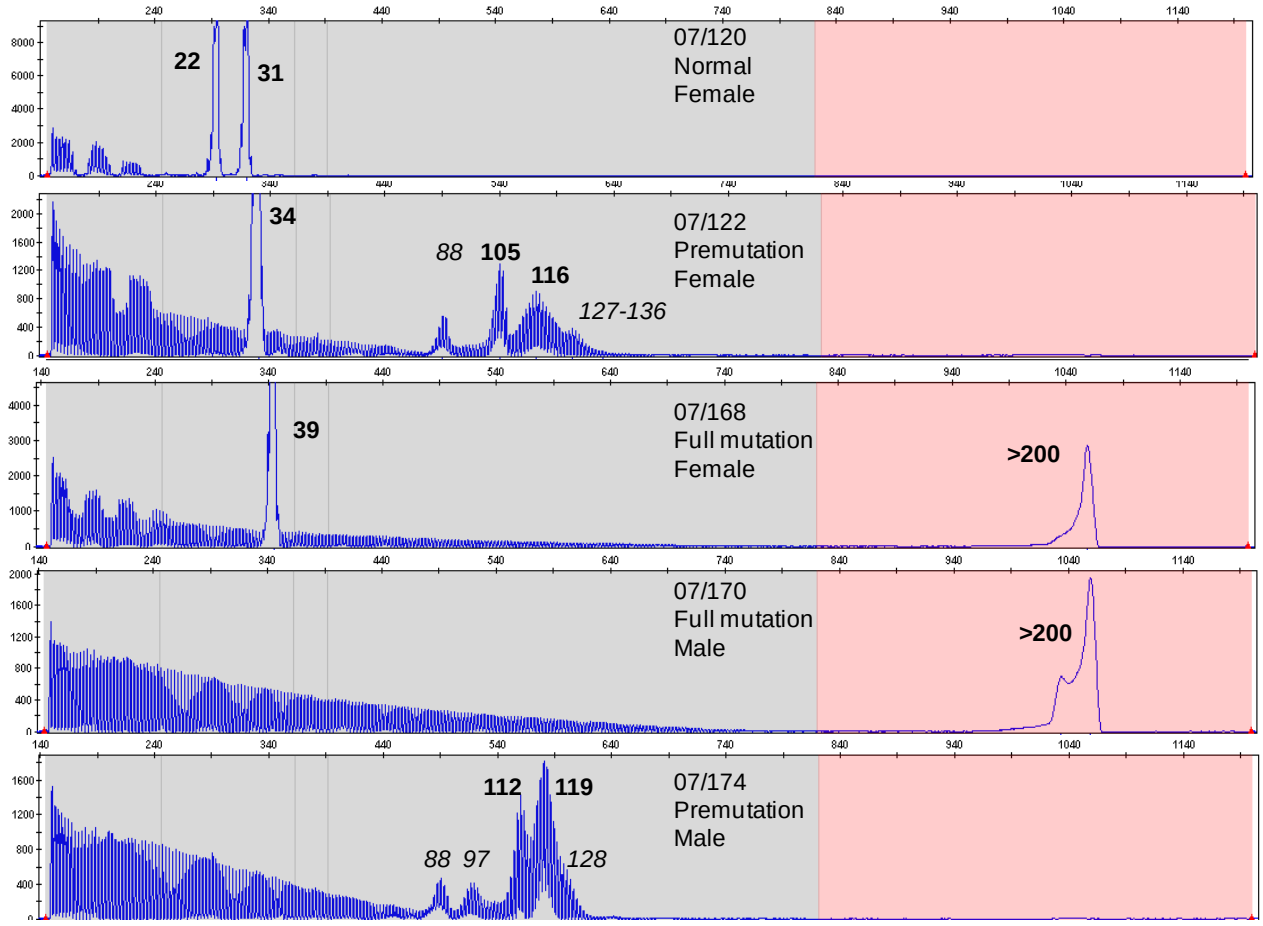
consists of 5 genomic DNA samples. A summary of results for the major detected peaks relative to the reported ranges and mean size of these reference materials is shown in Table 10.

Table 10. Summary of WHO Fragile X Panel Results (number of CGG repeats) at 2 Sites

WHO Standards and International Assessment Results [19]				AmplideX® PCR/CE *FMR1* Kit 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

The results using the AmplideX® PCR/CE *FMR1* Kit were consistent between two sites and published results from 27 European sites using various *FMR*1 PCR methodologies [18]. Thus, the AmplideX® PCR/CE *FMR1* Kit is traceable to the Fragile X Panel of materials available from NIBSC. An example set of Electropherograms for these standards is shown in Figure 12.

Figure 12. Electropherograms of the WHO International Fragile X Panel (NIBSC #08-158)

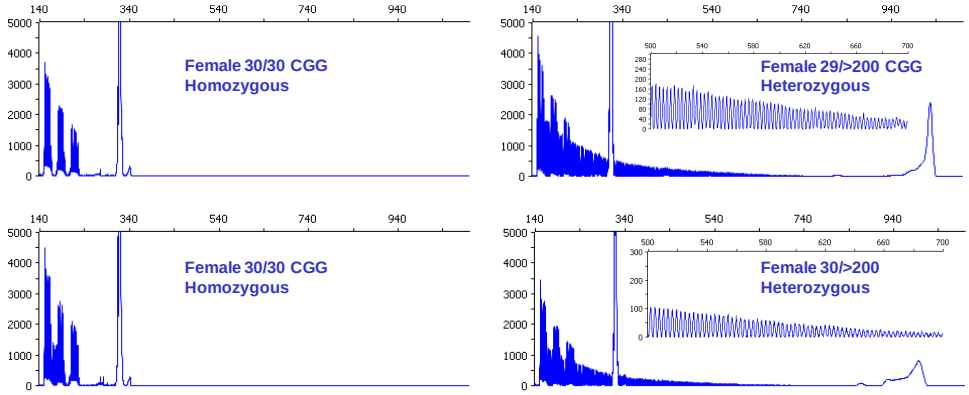


Part III– Special Features

- 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 13. 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.

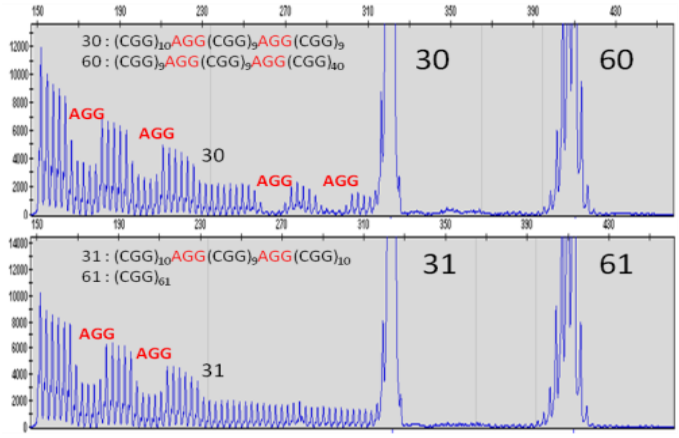
  Figure 13. CGG Repeat Primed *FMR1* PCR reagents provide an unmistakable signature for female samples that resolve zygosity - The inset plots show resolution and signal intensities of the RP PCR products in the 500 to 700 bp range. Each CGG RP amplicon peak is separated by 3 bp.



- 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. Reductions in signal intensity in the CGG RP PCR profile, therefore, correspond to the presence of interspersed AGG [16]. Figure 14 below shows a representative example with 2 samples of nearly equal length, a 30,60 and a 31,61 allele with different AGG profiles.

  Figure 14. Electropherograms comparing a 30,60 CGG sample with AGG on both the 30 and 60 CGG alleles (top) versus a similarly 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 AGGs for the first sample and only 2 AGGs for the second sample. Based on peak counting and on the haplotype inference of a 5'-bias for AGG [24, 25], the 60 CGG allele in the first sample has 2 AGG and the 61 CGG allele in the second sample may have none. Specific resolution of AGG pattern may require additional methods [12].

Limitations of the Examination Procedure

- This test may not detect rare forms of FMRP deficiency not caused by CGG expansion, such as deletion or point mutation, or mental retardation associated with other fragile sites. In these cases, linkage studies, cytogenetic, sequencing and/or assays designed to identify rare mutations and deletions may provide additional important information.
- Expansion of the CGG repeat region in the *FMR1* gene may occur on a tissue specific level, resulting in a tissue specific cryptic mosaic pattern which in rare cases is not detectable in peripheral blood [26, 27]. Therefore, testing of alternative sample types such as buccal cells should be considered.
- The *FMR1* F,R FAM-primers and *FMR1* CGG primers lack polymorphic binding sites as defined by Single Nucleotide Polymorphism database version dbSNP build 131 and Human Genome Build 37.1. However, the presence of rare variants in the *FMR1* gene may result in false results or miscalls. Thus, assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient. This test should not be used alone to diagnose fragile X syndrome or fragile X associated disorders.
- The use of the AmplideX® PCR/CE *FMR1* Kit may identify peaks with lower signal intensity than the allele(s) in a sample. While the presence of these peaks are sample specific, this test is not designed to detect chromosomal aberrations such as trisomy X and/or other aneuploidy types.
- The presence of AGG in female samples can only be inferred using the CGG RP PCR. Changes in signal intensity for longer alleles, confounding interference of the gene-specific peak for the smaller allele and different haplotypes require additional methods to determine the exact AGG haplotype. `

Medical Decision Points

Medical decision points are related to the number of CGG repeats detected. 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 genotype boundaries according to the American College of Medical Genetics (ACMG) and the European Society for Human Genetics (ESHG) are listed in Table 11 [22, 28, 29]. These boundaries may be different in other regions according to country-specific guidelines.

Table 11. 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

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 magnitude of the repeats [8]. In premutation alleles (~55 to 200 repeats), the risk of expansion to a full mutation increases with size.

Performance Characteristics

Analytical

- Analytical Specificity

  The forward and reverse primers target the Human Fragile X Mental Retardation (*FMR1*) gene (NC_000023.10) and span the CGG repeat region within the 5' UTR of the gene (NT_011681.16, Human Chromosome X Contig,

Reference Assembly). Amplification specific to the *FMR1* allele was verified by DNA sequencing and testing of well-characterized cell lines and representative clinical specimens.

- Input Range

  The range of input DNA was first evaluated with 1 to 100 ng of cell line DNA. Full length gene-specific and CGG RP PCR products were observed at all inputs of DNA. The sizing accuracy was unaffected by DNA input. A mosaic sample containing 20% 940 CGG in a background of 80% 23 CGG was then tested in the range of 1 to 100 ng DNA. The recommended range is 20-80 ng of DNA per PCR.

- Analytical Sensitivity

  The detection of a low abundant full mutation allele was determined at 40 ng input using an analytical titration of two clinical samples. A male full mutation clinical sample was admixed with a 31 CGG male normal allele to generate different percentage inputs of the full mutation allele between 0 to 100% keeping the total input of DNA constant at 40 ng. The CGG repeat peaks and full length gene-specific product peaks were detected in as low as a 1% full mutation allele which equated to 400 pg of a full mutation allele in a background of 39.6 ng normal allele. The signal intensity of the full length product peak and CGG RP peaks increased with increasing relative input of the full mutation allele. The detection limit for robust and reproducible detection of a full mutation allele was 5%.

- Sizing Accuracy

  The sizing accuracy is dependent on the size of the amplicon and the peak morphology of gene-specific peaks. All sizes were determined using the highest or center peak for all alleles. The accuracy repeat sizing was determined versus DNA sequencing in the range of 20 to 119 repeats. Between 119 and 200 CGG, the sizing accuracy of the full length gene specific product peak was referenced to the number of individual CGG peaks from the CGG RP PCR products. Above 200 CGG (or ~821 bp), all alleles are identified as >200 CGG.

  Sizing accuracy was matched against published cell line DNA controls using the gene-specific peak and absolute peak counting of the CGG RP PCR products and the WHO International Standard panel of fragile X controls.

- Precision

  1. Repeatability

     The within-run repeatability was evaluated on 2 different CE instruments (3130*xl* and 3500xL) by a single operator using a single thermal cycler. The PCR run consisted of 7 replicates for 6 alleles in the reportable range (20, 29, 31, 54, 119 and 199) and 9 replicates for a full mutation allele (940 CGG) per run. The precision was evaluated after conversion of amplicon size (bp) to CGG repeat (when rounded to whole integer) according to correction factors for size (C_o) and mobility (m_o) specific to either instrument (See Fragment Sizing Analysis Section above, page 11). The maximum difference in measured repeat length was 1 CGG for the 199 allele. These results correspond to 100% agreement between all replicates for the identification of normal, intermediate, or premutation alleles within the accuracy of the method (Table 12).

     Table 12. **Summary of method repeatability for the determination of CGG repeat length**

Instrument	Feature	20 CGG	29 CGG	31 CGG	54 CGG	119 CGG	199 CGG	940 CGG
3130*xl*	Number observations	7	7	7	7	7	7	9
	Percent agreement	100%	100%	100%	100%	100%	100%	100%
	Minimum CGG	20	29	31	54	119	199	>200
	Maximum CGG	20	29	31	54	119	200	
3500xL	Number observations	7	7	7	7	7	7	9
	Percent agreement	100%	100%	100%	100%	100%	100%	100%
	Minimum CGG	20	29	31	54	119	198	>200
	Maximum CGG	20	29	31	54	119	199	

  2. Within-laboratory reproducibility

The within-laboratory method variability was evaluated by testing 7 different alleles representing a clinically relevant range of CGG repeat lengths. Two operators, each using a different thermal cycler, performed PCR reactions on 4 different days over a 7-day period for a total of 8 PCR runs. Each PCR run consisted of 7 replicates for 6 alleles in the reportable range (20, 29, 31, 54, 119 and 199) and 9 replicates for a full mutation allele (940 CGG) for a total of 51 allele-specific PCR products per run. All PCR products were analyzed on 2 different CE instruments (3130*xl* or 3500xL). Thus, a total of 8 x 51 x 2 = 816 allele results were generated. Irrespective of the run, day, operator or instrument, the same CGG repeat length result was obtained for the 20, 29, and 31 alleles. The maximum difference in measured repeat length was 1 CGG for the 54 and 119 alleles, and 2 CGG for the 199 allele. All full mutation alleles were detected as >200 CGG. These results correspond to 100% agreement between all observations for the identification of normal, intermediate, premutation or full mutation alleles within the accuracy of the method (Table 13). An estimate of precision for the corresponding PCR product sizes measured in base pairs by each instrument is also reported in Table 14. Standard deviations were calculated using all the observed data for each allele prior to derivation of CGG repeat lengths using the respective instrument-specific conversion factors (see Part I in Data Interpretation section, page 17).

Table 13. Summary of the within-laboratory method variability for the determination of CGG repeat length

Feature	20 CGG Norm.	29 CGG Norm.	31 CGG Norm.	54 CGG Interm.	119 CGG Premut.	199 CGG Premut.	940 CGG Full Mut.
Number observations	112	112	112	112	112	112	144
Percent agreement	100%	100%	100%	100%	100%	100%	100%
Minimum CGG	20	29	31	53	118	198	>200
Maximum CGG	20	29	31	54	119	200	

Table 14. Estimation of within-laboratory method precision for the measurement of base pair size

Instrument	Feature	20 CGG	29 CGG	31 CGG	54 CGG	119 CGG	199 CGG	940 CGG
3500xL	Number observations	56	56	56	56	56	56	72
	Minimum (bp)	291.42	318.14	324.01	390.75	583.08	820.54	1046.15
	Maximum (bp)	293.28	319.62	325.5	392.46	584.46	824.16	1076.14
	Avg. Size (bp)	292.51	319.01	324.84	391.76	583.91	823.09	1058.67
	Std. Dev.	0.45	0.41	0.41	0.44	0.38	1.26	7.72
	%CV	0.15%	0.13%	0.13%	0.11%	0.07%	0.15%	0.73%
3130*xl*	Number observations	56	56	56	56	56	56	72
	Minimum (bp)	288.95	315.33	321.14	388.04	580.64	818.02	1042.7
	Maximum (bp)	289.79	316.29	322.12	388.96	581.39	822.29	1062.61
	Avg Size (bp)	289.45	315.93	321.77	388.58	581.10	820.53	1051.73
	Std. Dev.	0.20	0.21	0.21	0.22	0.19	1.16	5.36
	%CV	0.07%	0.07%	0.06%	0.06%	0.03%	0.14%	0.51%

3. Inter-laboratory reproducibility

The inter-laboratory reproducibility was evaluated by testing different alleles in the reportable range (20, 29, 31, 54, 119 and 199 CGG) at three different laboratories. At Site 1, the analysis consisted of 7 replicates each on 2 different CE instruments (3130*xl* and 3500xL). Four replicate PCRs were prepared at Site 2 and analyzed on a 3130*xl*. At Site 3, five replicate PCRs were prepared and analyzed using a 3500xL. Thus, a total of 11 replicates for each allele results were generated using a 3500xL, and a total of 12 replicates for each allele were collected using a 3130*xl*. Irrespective of the run, site, or instrument, the same CGG repeat length result was obtained for the 20, 29, 31, 54, and 119 alleles. The only difference in measured repeat length was 1 CGG for the 199 CGG on 3130*xl*, and 2 CGG for the 199 allele on 3500xL; both within the expected range at this

repeat length. These results correspond to 100% agreement between all observations from different laboratories for the identification of normal, intermediate, or premutation alleles (Table 15).

Table 15. Summary of Inter-laboratory method reproducibility for the determination of CGG repeat length

Instrument	Feature	20 CGG	29 CGG	31 CGG	54 CGG	119 CGG	199 CGG
3130*xl*	Number observations	12	12	12	12	12	12
	Percent agreement	100%	100%	100%	100%	100%	100%
	Minimum CGG	20	29	31	54	119	199
	Maximum CGG	20	29	31	54	119	200
3500xL	Number observations	11	11	11	11	11	11
	Percent agreement	100%	100%	100%	100%	100%	100%
	Minimum CGG	20	29	31	54	119	198
	Maximum CGG	20	29	31	54	119	200

- Interference

  Endogenous and/or exogenous interfering substances contained within peripheral blood have been reported in the scientific literature to potentially interfere with PCR methods by inhibiting the DNA polymerase activity [30, 31]. It is therefore necessary to purify the DNA prior to the PCR amplification step. Standard DNA extraction methods have been shown to remove these potentially interfering compounds [32]. The AmplideX® PCR/CE *FMR1* Kit was designed to be compatible with commercially available, laboratory-validated DNA extraction methods. In testing 196 representative clinical samples, no inhibition was observed.

Measuring (Reportable) Range

- The reportable range of repeat length is 5-200 CGG.
- Above 200 CGG all alleles are categorically identified as >200 CGG.

Diagnostic/Clinical

The AmplideX® PCR/CE *FMR1* Kit was evaluated with a set of 196 clinical specimens obtained from two external sites using both gene-specific PCR and CGG RP PCR methods. A set of 146 samples, previously characterized by Southern blot were submitted to Asuragen for analysis. The other 50 samples were tested at an external laboratory. Technologists performing the PCR analysis were blinded to the sample genotypes. The results for both PCR methodologies at each site were identical and are summarized by comparison to Southern blot analysis for each category of genotypes at each site in Table 16. The total sample set consisted of 75 normal alleles, 6 intermediate alleles, 43 premutation alleles, and 72 full mutation alleles, including several alleles with greater than 1000 CGG repeats. The initial pass rate with the AmplideX® PCR/CE *FMR1* Kit was 98.5% (193/196); the remaining 3 samples were successfully amplified on repeat PCR. All samples in the normal, intermediate, premutation and full mutation range were correctly identified with the AmplideX® PCR/CE *FMR1* Kit.

Table 16. Summary of observed sample genotypes by site and methodology compared to Southern blot analysis

Category	Asuragen (PCR)	External (PCR)	Total PCR	Total Southern
NOR	42	33	**75**	**75**
INT	3	3	**6**	**6**
PM	33	8	**41**	**43**
Total FM	68	6	**74**	**72**
Total	146	50	**196**	**196**

In total, 194/196 samples were concordant with Southern blot analysis as measured by the categorical genotype of the longest *FMR1* allele. Two samples with readily detectable premutation alleles by the Kit and Southern blot analysis also presented low intensity full mutation alleles only detectable with the Kit. These results may reflect the increased sensitivity of the PCR-based AmplideX® technology for low abundance full mutation alleles compared to the Southern blot procedure [16, 23]; see also Analytical Sensitivity section above (Page 22).

The test performance for accurate classification of full mutation alleles is summarized in Table 17.

Table 17.Kit performance summary for the detection of full mutation alleles relative to Southern blot analysis. Positive: >200 CGG. Negative: ≤200 CGG.

		AmplideX® PCR		
		Positive	**Negative**	**Total**
Southern Blot	**Positive**	72	0	72
	Negative	2*	122	124
	Total	74	122	196

*These 2 samples presented premutation alleles by both methods and low intensity full mutation alleles detected only by the AmplideX® PCR/CE *FMR1* Kit [23].

The diagnostic sensitivity was 100% [95% CI: 94.9-100%], the diagnostic specificity was 98.4% [95% CI: 94.3-99.6%], and the overall accuracy was 99% [95% CI: 96.4-99.7%].

The AmplideX® PCR/CE *FMR1* Kit was evaluated with a set of 63 matched whole blood and buccal cell clinical specimens, using the CGG RP PCR methods. All buccal samples in the normal, intermediate, premutation and full mutation range were correctly identified with the AmplideX® PCR/CE *FMR1* Kit, providing 100% categorical concordance between the genotypes of both sample types (95% CI [96%-100%]).

The test performance for accurate classification of samples across different sample types is summarized in Table 18.

Table 18. Summary of matched buccal and blood sample genotypes by category

Genotype	Buccal	Blood
NOR	41	41
INT	2	2
PM	3	3
Total FM	17	17
Total	**63**	**63**

Notice to Purchaser

1. This product is intended for in vitro diagnostic use.
2. The AmplideX® PCR/CE *FMR1* Kit CE-marked IVD is produced in the USA and exported only. No reentry into the USA is allowed.
3. This product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen.
4. AmplideX® is a registered trademark of Asuragen, Inc.
5. This product is covered by U.S. Patent No. 6,270,962 and related patents issued or pending, which patents are licensed to Asuragen, Inc. by EPICENTRE Technologies Corporation, 726 Post Road, Madison, WI 53713, U.S.A.
6. 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 diagnostic 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.

7. All instrumentation must be maintained and operated according to manufacturer's instructions.
8. In no event shall Asuragen be liable in any way (whether in contract, tort (including negligence) or otherwise) for any claim arising in connection with or from the use of this Product. Nothing in this document excludes or limits any liability which it is illegal for Asuragen to exclude or limit.

References

1. Verkerk, A.J., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome.* Cell, 1991. **65**(5): p. 905-14.
2. Rousseau, F, et al., *A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases.* Am J Hum Genet. 1994 **55**(2): p. 225-37.
3. Irwin SA, et al, *Dendritic spine structural anomalies in fragile-X mental retardation syndrome*. Cereb Cortex. 2000;**10**: p. 1038–1044.
4. Jin, P., R.S. Alisch, and S.T. Warren, *RNA and microRNAs in fragile X mental retardation.* Nat Cell Biol, 2004. **6**(11): p. 1048-53.
5. Hagerman, R. and P. Hagerman, *Fragile X Syndrome: Diagnosis, Treatment, and Research*. 2002, The Johns Hopkins University Press: Baltimore p. 3-109
6. Sullivan, AK et al, Association of FMR1 repeat size with ovarian dysfunction, Hum Reprod. 2005 **20**(2): p. 402-12.
7. Hagerman RJ and Hagerman PJ, *The Fragile X Premuation: into the phenotypic fold*, Curr Opin Genet Dev, 2002 **12**: p. 278-83.
8. Bailey, DB, et al, *Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey*, Am J Med Genet A. 2008:**146A**(16):2060-9.
9. de Vries, B.B., et al., *Variable FMR1 gene methylation of large expansions leads to variable phenotype in three males from one fragile X family.* J Med Genet, 1996. **33**(12): p. 1007-10.
10. Nolin, S.L., et al., *Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles.* Am J Hum Genet, 2003. **72**(2): p. 454-64.
11. Eichler, E.E., et al., *Length of uninterrupted CGG repeats determines instability in the FMR1 gene.* Nat Genet, 1994. **8**(1): p. 88-94.
12. Nolin S.L., et al. *Fragile X AGG analysis provides new risk predictions for 45-69 repeat alleles.* Am J Med Genet, 2013. **161**(4): p. 771-8.
13. Yrigollen C.M., et al. *AGG interruptions within the maternal FMR1 gene reduce the risk of offspring with fragile X syndrome.* Genet Med, 2012. **14**(8): p. 729-36.
14. Fernandez-Carajval, et al, *Expansion of an FMR1 grey-zone allele to a full mutation in two generations*, J Mol Diagn. 2009 Jul;11(4):306-10.
15. Saluto, A., et al., *An enhanced polymerase chain reaction assay to detect pre- and full mutation alleles of the fragile X mental retardation 1 gene.* J Mol Diagn, 2005. **7**(5): p. 605-12.
16. Chen, L., et al., *An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis.* J Mol Diagn, 2010. **12**(5): p. 589-600.
17. Amos Wilson, J., et al., *Consensus Characterization of 16 FMR1 Reference Materials: A Consortium Study.* J Mol Diagn, 2008. **10**(1): p. 2-12.

18. Hawkins, M., et al., *Preparation and validation of the first WHO international genetic reference panel for Fragile X syndrome.* Eur J Hum Genet, 2010.

19. Ulfelder, K.J. and B.R. McCord, *The separation of DNA by Capillary Electrophoresis*, in *Handbook of Capillary Electrophoresis*, J.P. Landers, Editor. 1997, CRC Press LLC: Salem. p. 347-378.

20. Chastain, P.D., 2nd, et al., *Anomalous rapid electrophoretic mobility of DNA containing triplet repeats associated with human disease genes.* Biochemistry, 1995. **34**(49): p. 16125-31.

21. Kiba, Y., L. Zhang, and Y. Baba, *Anomalously fast migration of triplet-repeat DNA in capillary electrophoresis with linear polymer solution.* Electrophoresis, 2003. **24**(3): p. 452-7.

22. Sherman, S., B.A. Pletcher, and D.A. Driscoll, *Fragile X syndrome: diagnostic and carrier testing.* Genet Med, 2005. **7**(8): p. 584-7.

23. Filipovic-Sadic, S., et al., *A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome.* Clin Chem, 2010. **56**(3): p. 399-408.

24. Poon, P.M., et al., *AGG interspersion analysis of the FMR1 CGG repeats in mental retardation of unspecific cause.* Clin Biochem, 2006. **39**(3): p. 244-8.

25. Eichler, E.E., et al., *Haplotype and interspersion analysis of the FMR1 CGG repeat identifies two different mutational pathways for the origin of the fragile X syndrome.* Hum Mol Genet, 1996. **5**(3): p. 319-30.

26. MacKenzie, J., Sumargo, I. and Taylor, S. *A cryptic full mutation in a male with a classical Fragile X phenotype.* Clin Genet, 2006. 70: 39–42.

27. Maddalena, A., et la., *A fragile X mosaic male with a cryptic full mutation detected in epithelium but not in blood.* Am J Med Genet, 1996. 9;64(2):309-12

28. Monaghan, K.G., Lyon, E, Spector, E.B.. ACMG Standards and Guidelines for fragile X testing: a revision to the disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics and Genomics . Genet Med, 2013. 15, 575–586.

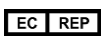
29. Macpherson, J. and H. Sawyer. *Practice Guidelines for Molecular Diagnosis of Fragile X Syndrome.* . Clinical Molecular Genetics Society 2005 [cited 2011 February]; http://www.cmgs.org/BPGs/pdfs%20current%20bpgs/Fragile%20X.pdf].

30. Wiedbrauk, D.L., J.C. Werner, and A.M. Drevon, *Inhibition of PCR by aqueous and vitreous fluids.* J Clin Microbiol, 1995. **33**(10): p. 2643-6.

31. Panaccio, M. and A. Lew, *PCR based diagnosis in the presence of 8% (v/v) blood.* Nucleic Acids Res, 1991. **19**(5): p. 1151.

32. Boom, R., et al., *Rapid and simple method for purification of nucleic acids.* J Clin Microbiol, 1990. **28**(3): p. 495-503.

Appendix A: Glossary of Symbols

Symbol	Description
	Catalog number
	Batch code
	Contains sufficient for <n> tests
	Consult instructions before use
	Temperature limitation
	Use by
	*In vitro* diagnostic medical device
	CE Mark
	Authorized representative in the European Community
	Manufactured by

AmplideX® PCR/CE *FMR1* Kit
PC-0164ENv8
Effective Date: 2018-05-22