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Fragile X Screening

INSTRUCTIONS FOR USE

Rv 06 / 2019-05-27

Kit for the determination, by fluorescent fragment analysis, of the presence of healthy and premutated alleles of the FMR1 gene of Fragile X Syndrome

CE

Catalog No. AD-FX-48/16

Store: Box 1 from -30ºC to -18ºC

Box 2 from 20ºC to 25ºC

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FRAGILE X SCREENING

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Intended Use

Adellgene Fragile X Screening is a diagnostic kit designed for use in laboratories, which detects the number of CGG triplet repeats (cytosine-guanine-guanine) in the 5' untranslated region of the gene for fragile X mental retardation ("Fragile X mental retardation-1": FMR1). It aims to aid diagnosis of the clinical disease associated with Fragile X syndrome, for example, mental retardation, primary ovarian failure, and tremors / ataxia. This kit can be used for the determination of healthy and premutated individuals, who have 10 to 50 and 50 to 200 CGG triplet repeats, respectively (see section 'Summary and explanation'). Individuals with more than 200 CGG triplet repeats (see section 'Summary and explanation') need to be analyzed using other appropriate technique. The technology is based on the polymerase chain reaction (PCR) amplification of genomic DNA, extracted and purified from peripheral blood, followed by fluorescence analysis of the size of the PCR fragments obtained by a genetic analyzer and conversion of that size into the relevant number of CGG repeats.

Summary and explanation

Fragile X Syndrome (FXS, OMIM # 309550) is an X-linked disease that is primarily based on the genomic expansion of a triplet of nucleotides (CGG), and aberrant methylation of the promoter region [1]. The expansion is located in the 5' untranslated region of the FMR1 gene ("Fragile X Mental Retardation 1) which is situated in the Xq27 region (Fig. 1) [1].Depending on the number of repetitions of this triplet, three categories can be established:

a) Number of repetitions from 6 to 45 or 55 [2, 3] and without methylation in the promoter of the gene, defines individuals with healthy alleles.

b) Number of repeats from 45 or 55 to 200 and often without aberrant gene methylation, defines the category of individuals with premutations (PM). In this case individuals are asymptomatic for disorders associated with FXS but can be associated with two clinical disorders: fragile X syndrome-associated tremor/ataxia (FXTAS) and fragile X-associated with primary ovarian insufficiency (FXPOI) [4, 5], the severity of which depends on the methylation state of the promoter [6, 7]. It is estimated that the incidence in the general population is 1 in every 130-250 females and 1 in every 250-810 males [8].

c) Number of repetitions over 200 and with aberrant methylation of the gene promoter defines individuals with full mutations, which results in the elimination of gene expression in the human brain [9], and is associated with mental retardation, autism, and mental and emotional changes. It has a striking phenotype consisting of large ears and a prominent jaw, with an incidence of 1 in 4000 males and 1 in 8000 females [10]. However, there are some rare cases where large unmethylated expansions are present in normal patients [11] (Fig. 1). Moreover, CGG repeats in premutated alleles are unstable and may increase in size from generation to generation by maternal transmission, resulting in a generation with a fully mutated allele [12].

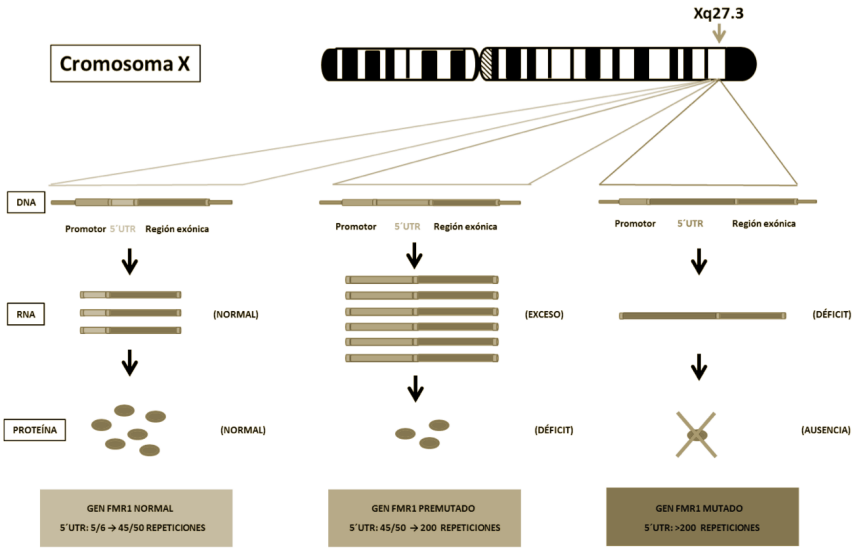

Figure 1. Representation of the FMR1 gene types and their production depending on the degree of mutation.

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The severity of cognitive impairment in patients with FXS is not associated with the magnitude of the full mutation [13] but varies with the state of methylation [14, 15].

Besides the number of CGG triplet repeats, many FMR1 alleles contain AGG sequences that are inserted between the CGG triplets. It is thought that these inserts may confer stability to the DNA and reduce the risk of expansion in the next generation. The possibility that mothers without AGG insertions may give rise to CGG triplet extensions in the next generation is greater than those with at least one AGG insertion [12, 16-18].

One of the most common methods of molecular diagnosis of fragile X syndrome is the Southern blot of genomic DNA, digested with the restriction endonucleases EcoRI and EagI, and hybridized with a specific probe [19, 20]. Although this method allows the measurement of the quantity of the CGG expansions and determines the methylation status, it has considerable drawbacks. This method requires large amounts of DNA, is labor intensive and time consuming, which makes it unsuitable for larger volumes of tests or whole population screening. Moreover, it does not allow determination of the methylation process in the majority of DNA samples extracted from chorionic villi (CVS), this is because the DNA has low sensitivity and the methylation status is not (or not fully) present between the eighth and tenth week of pregnancy [21]. The current trend is to perform the specific amplification of the region containing the CGG triplet expansion by PCR and with specific primers.

The PCR has the potential to address each of these limitations; however, the highly GC-rich triplet repeat sequence in the fragile X syndrome has historically been refractory to the amplification process. A number of variations within the amplification process have been devised, which involves the use of osmotic adjuvants, modified nucleotides and specific conditions of the PCR process [12, 22].

The "ADELLGENE Fragile X Screening" kit is optimized to carry out rapid screening for the detection of approximately 10-200 CGG triplet repeats. Screening directly discards the men with normal (<50 repeats) or premutated alleles (50-200), and females who have two healthy alleles (<50) of different size, one healthy (<50) and one premutated (50-200), or two premutated alleles (50-200) of different size. It is based on the analysis of fragments generated with fluorescence primers and capillary electrophoresis in a genetic analyzer; with the appropriate software for detection and interpretation. Those cases of males without any signal and females with only one peak corresponding to a normal or premutated number of repeats, must be tested using other appropriate technique.

Principles of the procedure

The detection method employed by the "Adellgene Fragile X Screening" kit is based on the specific amplification, from purified genomic DNA, of the fragment containing the gene region FMR1. The CGG repeats are amplified with 2 primers, one of which is labeled with a fluorophore for subsequent detection in a DNA fragment analyzer. The kit includes primers, polymerase "mix" and a specific buffer for the amplification of GC-rich regions. The use of ROX 1000™ tagged fragments is recommended. The size of the PCR products are converted into the number of CGG triplet repeats using conversion factors of mobility and size.

Kit contents

Reference AD-FX-16 (16 tests)

BOX 1 of 2:

- AD-FX-PM: Primer Mix (PM). 1 vial x 87 µL
- AD-FX-POM: Polymerase Mix (POM). 1 vial x 355 µL

BOX 2 of 2:

- AD-PUR-16: Purification reagents for 16 tests. 1 unit.
  - Capture buffer: 1 vial x 9.8 mL
  - Wash buffer: 1 vial x 2.0 mL (see how to prepare in section Procedure / D)
  - Elution buffer: 1 vial x 300 µL
  - Collection tubes: 17 tubes
  - Microcolumns: 17 columns

Reference AD-FX-48 (48 tests)

- AD-FX-PM: Primer Mix (PM). 3 vials x 87 µL
- AD-FX-POM: Polymerase Mix (POM). 3 vials x 355 µL

BOX 2 of 2:

- AD-PUR-16: Purification reagents for 16 tests. 3 units.
  - Capture buffer: 3 vial x 9.8 mL
  - Wash buffer: 1 vial x 2.0 mL (see how to prepare in section Procedure / D)
  - Elution buffer: 3 vial x 300 µL
  - Collection tubes: 51 tubes
  - Microcolumns: 51 columns

Kit storage

- Box 1 should be stored from -30ºC to -18ºC. Box 2 should be stored from 20ºC to 25ºC. Reagents are stable at these temperatures until their expiration date as indicated on the package.
- Allow Primer Mix vial to reach room temperature before use. Gently shake all reagents, except AD-FX-POM, after thawing.
- Before opening the reagent tubes, perform a gentle centrifugation of each component so that the reagents are deposited at the bottom of the tube and not loose on the walls.
- The test should be carried out keeping reagents on ice or a cold block.
- Do not make more than 3 cycles of freeze / thawing of the vials of Primer Mix (AD-FX-PM) and Polymerase Mix (AD-FX-POM), as this may reduce the sensitivity of the test and alter the results.
- Due to the photo-sensitive nature of the reagent AD-FX-PM, avoid continued exposure to light.

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Materials required but not supplied

Isolation and Purification Reagents

- Reagents for DNA isolation and purification are not included. The DNA can be extracted by any method validated in the laboratory to ensure high quality and intact DNA
- Ethanol absolute

Capillary Electrophoresis Reagents (recommended)

- Genetic Analyzer for polymer POP-7 (ABI: 3130, 3730 or 3500)
- POP-7 polymer (ABI, Ref: 4363785 or equivalent)
- High purity formamide (Hi-Di Formamide; ABI, Ref.: 4311320 or equivalent)
- Calibrators for fluorophores FAM and ROX (ABI, Ref.: 4345827, 4345829 or equivalent)
- Size standard marker ROX 1000™ (ABI, Ref.: 401098; Eurogentec, Ref.: MW-0195-80ROX)

Supplies and equipment

- General equipment of a laboratory dedicated exclusively to performing the PCR
- Thermocycler (ABI, 9700, Veriti or equivalent)
- Centrifuge for 96-well plates
- Shaker (Vortex)
- Micro-centrifuge
- Pipettes (P1000, P200, P20 and P2) and filter tips for specific pipette
- Multichannel pipette capable of dispensing of 1-10 µl
- PCR plates of 96 wells (optional)
- PCR Plate Sealer (optional)

Positive Control

- The recommended WHO standard for Fragile-X syndrome or any cell line whose DNA corresponds with a validated sample

Sample collection and preparation

- All biological and blood samples should be treated as potentially infectious. When handling samples, basic or 'universal' precautions must be observed. Any sample handling should be done with appropriate personal protection such as goggles, gloves and appropriate clothing.
- This test should only be performed with whole blood samples treated with EDTA or ACD anticoagulants. Heparin can interfere with PCR and should not be used in this procedure. No hyperlipemic, hemolyzed, jaundiced or proteinemia blood samples should be used.
- Contamination by DNAses can produce DNA degradation, so filter pipette tips and DNAse-free tubes should be used. Take care with handling, pipetting and using devices, to avoid PCR failure.
- Do not use components from different lots. Do not use reagents beyond the expiration date.
- Before using the kit, ensure that the equipment (thermal cycler, genetic analyzer etc.) has been calibrated according to the manufacturer`s instructions.
- The EtBr is potentially carcinogenic, therefore, avoid inhalation of vapors and use appropriate ways of handling and disposal (ventilation, toxic fume hood, etc.).

Caution

Kit toxicological properties have not been studied in depth so it is recommended to avoid contact with skin and mucous membranes. Do not ingest. Manual Safety Data Sheets (MSDS) are available to the customer.

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Procedure

A. DNA extraction

Extraction of genomic DNA by standard methods from whole blood collected with EDTA is compatible with the use of this kit. It is recommended to perform the evaluation of the quality and quantity of DNA obtained by measurements of absorbance at 260nm (OD260; concentration) and the ratio of the measurements at 260 and 280 nm (OD; purity). The DNA obtained can be stored at -20°C until use. The appropriate amount of DNA in each PCR reaction is about 30 ng (i.e.1 µ of DNA to 30 ng/µl).

B. Preparation and conditions of the PCR reaction

Precautions

- Thaw all kit components before starting the test, mix, and centrifuge.
- Work on ice or on a cold block.
- The PCR should be prepared in the pre-PCR area with all precautions discussed above.
- Use only filter tips and 1.5mL autoclaved tubes.
- Always wear gloves and a laboratory coat.

1. Mix preparation of the Primer Mix and Polymerase Mix for n+1 samples:

Reagent	Vol. for each sample (ul)
AD-FX-PM	4.7
AD-FX POM	19.3
Total volume for each reactions	**24**

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- Shake the sample gently 3-5 times before distributing into the PCR tubes, to ensure complete homogenization.
- Perform a pulse centrifugation to ensure that there is no sample on the tube walls, or bubbles.

2. Pipette 24 µl of this mixture into an optical plate or sterile tube and add the required volume of DNA, or negative control in the case of the contamination control well, to reach 30 ng (i.e. 1 µl of DNA to 30 ng/µl).

3. Seal the plate, or tube, with appropriate coverslips (and perform a pulse centrifugation to ensure thorough mixing and the removal of bubbles.

4. Place the plate in the thermocycler and perform the PCR reaction.

	Number of cycles	Temperature (ºC)	Time (mm:ss)
DENATURATION	1	98	05:00
CYCLES	10	97	00:35
		62	00:35
		68	04:00
CYCLES	20	97	00:35
		64	00:35
		68	06:00+30 sec additional cycle
FINAL EXTENSION	1	72	30:00
COOLING	1	4	∞

C. Confirmation of the amplification products

Confirmation of the amplification products can be made through appropriate systems such as horizontal electrophoresis in agarose gels. To perform a horizontal electrophoresis prepare an agarose gel at a concentration of 1-1.2% w/v, validated according to the laboratory protocol, and analyze 2µl of each amplification to certify that the PCR was successful.

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D. Preparation of the samples for capillary electrophoresis

1. Purification of the PCR reaction

The first time that Box 2 is used, it is necessary to add 8mL of absolute ethanol to the Wash Buffer vial. Mark the corresponding box on the label of that vial to indicate that the addition has been made.

- Use Box 2 of the kit (Purification reagents). To use a PCR volume of 20 µl to obtain a purified PCR volume of 15 µl. These volumes are restricted to obtain a good result in the fragment analysis.
- Protocol:

a. Add 500 µl of Capture buffer to the sample (use 20 µl of PCR volume).

b. Mix thoroughly.

c. Check that the capture buffer plus sample is yellow or pale orange in color.

d. For each purification that is to be performed, place one micro-column into one collection tube.

e. Centrifuge capture buffer with the sample briefly to collect the liquid in the bottom of the tube.

f. Load the capture buffer with the sample onto the assembled micro-column and collection tube.

g. Spin the assembled column and collection tube at 16000 g for 30 seconds.

h. Discard the flow through by emptying the collection tube. Place the micro-column back inside the collection tube.

i. Add 500 µl of Wash buffer to the micro-column.

j. Spin the assembled column and collection tube at 16000 g for 30 seconds.

k. Discard the collection tube and transfer the micro-column to a fresh DNase-free 1.5 ml microcentrifuge tube.

l. Add 15 µl of Elution buffer to the center of the membrane in the assembled micro-column and sample collection tube.

m. Incubate the assembled micro-column and sample collection tube at room temperature for 1 minute.

n. Spin the assembled column and sample collection tube at 16000 g for 1 minute to recover the purified DNA.

o. Proceed to Preparation of samples for DNA analyzer.

2. Preparation of Samples for DNA analyzer

- The following reaction mixture is performed with the purified PCR:

Reagent	Vol. for each sample (µl)
PCR purified product	2 *
Hi-Di-Formamide **	10
LIZ500™ Maker **	0.3
Total volume for each reactions	**12.3**

** It is recommended to use a range of between 1 to 3 µl of charge volume to optimize the results*

*** These items are not supplied with the kit*

- Perform a pulse of centrifugation (3-5 times) to ensure thorough mixing of the reagents, and transfer 10 µl of the mixture to the corresponding plate for capillary electrophoresis.
- Seal the plate, shake and centrifuge to remove bubbles. Transfer to the thermal cycler.
- Allow sample to denature for 2 minutes at 95°C and transfer to ice, protecting the sample from light until injection into the genetic analyzer. It is recommended to use a positive control, in which the number of repetitions is known (see section 'Materials required but not supplied').

3. Module of DNA Analyzer

The following module of work is recommended for the corresponding DNA analyzer:

	DNA ANALYZERS	
PARAMETERS	3130/3130xl	3730xl
Oven Temperature	60 ºC	63 ºC
Poly Fill Vol.	7300 steps	6500 steps
Current Stability	5.0 µAmps	5.0 µAmps
Pre-Run Voltage	15.0 KVolts	15.0 KVolts
Pre-Run Time	180 sec	180 sec
Injection voltage	3.0 KVolts	1.6 KVolts
Injection time	15 sec	30 sec
Voltage Number of steps	20 nK	20 nK
Voltage Step Interval	15 sec	15 sec
Data Delay Time	60 sec	60 sec
Run voltage	15.0 kVolts	15.0 kVolts
Run time	3000 sec	2200 sec

NOTE: In both cases the use of polymer POP7 is recommended.

Results and interpretation

The Adellgene Fragile X Screening kit is a quantitative technique for identifying alleles of the fragile X syndrome; consisting of a number of CGG triplet repeats between 10 and 200 (see section 'Summary and explanation'). The determination of these alleles is considered difficult because of the high concentration of GC bases, which creates a strong secondary structure that prevents replication of this region, even with the use of Taq polymerase at elevated temperatures. The kit includes a series of reagents that provide amplification of this region and allows general screening to discard samples with healthy and premutated alleles, in males and in heterozygous females.

Moreover, those men that do not give any signal for Fragile X specific amplification and the females that give only one peak, corresponding to a normal or premutated number of repetitions, will have to be further tested by the conventional technique of "Southern Blotting" or other appropiate methodology.

Using the reagents supplied in this kit, if the sample has 30 CGG repeats, the size of the amplified fragment will be 320 bases. All further numbers of repetitions, obtained with this kit, may be tabulated based on this size (see Table 1). This table has been devised from the results obtained with the kit in a 3730xl Genetic Analyzer (Applied Biosystems), using a POP7 polymer. For a better adjustment of the repetitions, the user must include a control sample with a known number of repetitions in each batch of analysis, in order to normalize the peak mobility in particular electrophoresis conditions (polymer, genetic analyzer, run conditions, etc.). The introduction of fragment markers allows the genetic analyzer software to give us the size of the amplification directly; therefore we can establish the number of CGG triplet repeats. It is not necessary to use any passive reference. Figures 2-7 represent fragment analysis graphs for a healthy male (Fig. 2), an unhealthy male (Fig. 3), a heterozygous healthy female (Fig.4), one homozygous healthy female (Fig. 5) and two heterozygous healthy/premutated females (Fig. 6, 7).

Samples of an unhealthy male and homozygous female would be further analyzed with the kit "Adellegene Fragile X Confirmatory kit" or classical techniques of Southern blotting. This would determine the number of triplets in the case of an unhealthy male and a homozygous female, to confirm whether it is truely a healthy homozygous female, or a heterozygous female with one healthy or premutated allele, and one unhealthy allele.

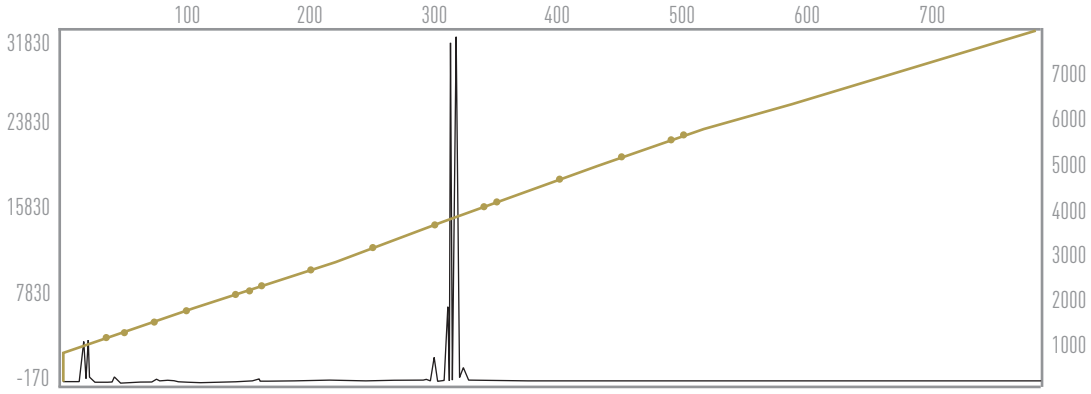

Figure 2. Healthy male (26 CGG repeats). The dots on the line indicate the fragments corresponding to the marker 500 ROX™.

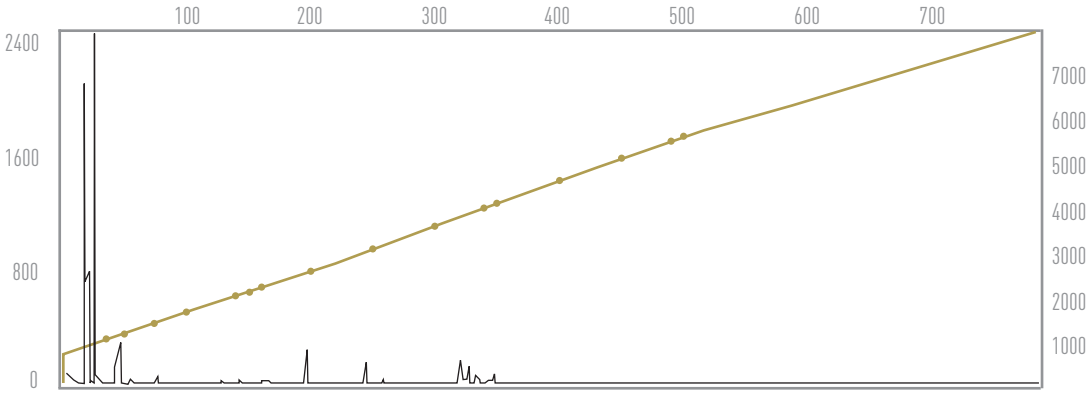

Figure 3. Unhealthy male (>200 CGG repeats). Confirmation is needed. The dots on the line indicate the fragments corresponding to the marker 500 ROX™.

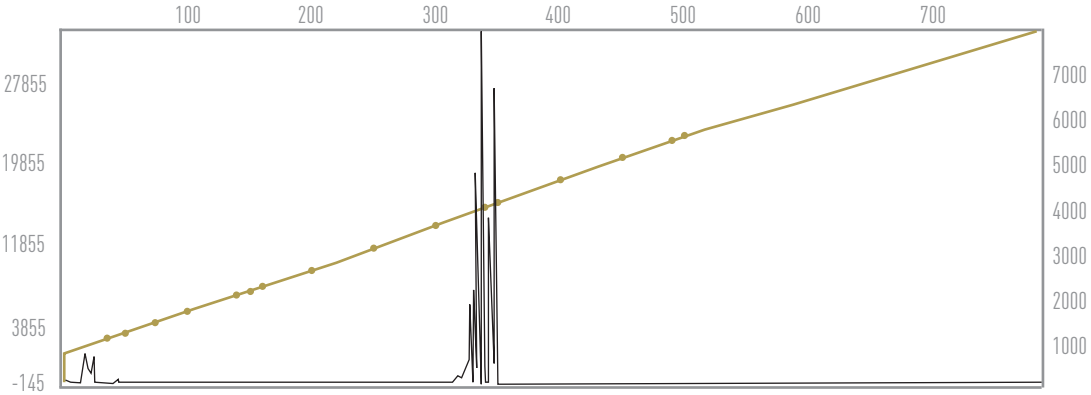

Figure 4. Healthy heterozygous female (31/35 CGG repeats). The dots on the line indicate the fragments corresponding to the marker 500 ROX™.

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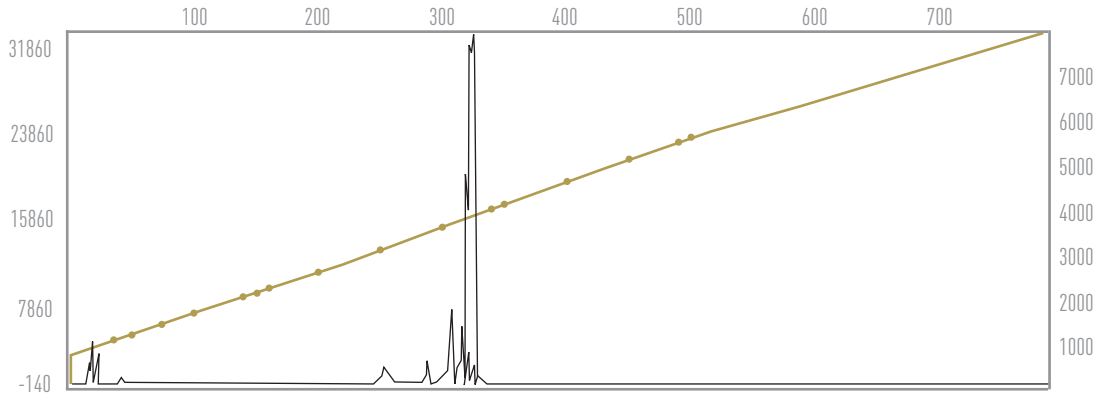

Figure 5. Healthy homozygous or unhealthy heterozygous female (29 CGG repeats). Confirmation is needed. The dots on the line indicate the fragments corresponding to the marker 500 ROX™.

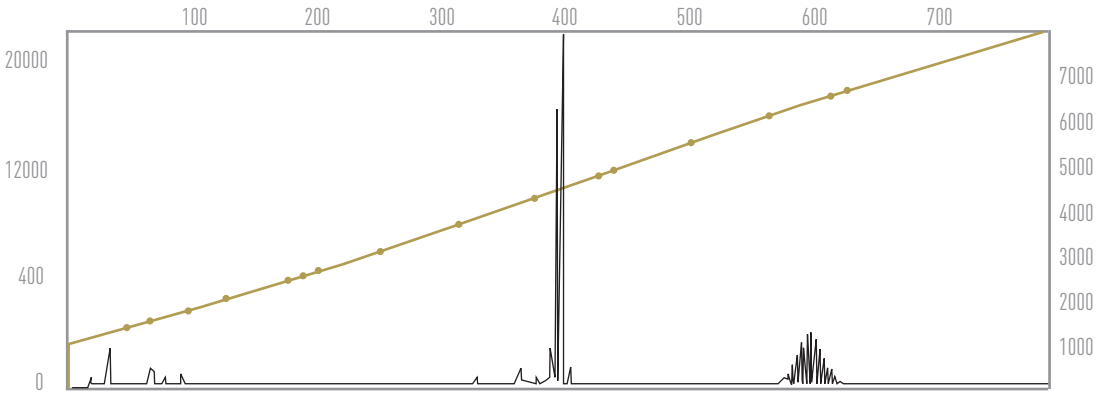

Figure 6. Healthy and premutated heterozygous female (29/81 CGG repeats). The dots on the line indicate the fragments corresponding to the marker 500 ROX™.

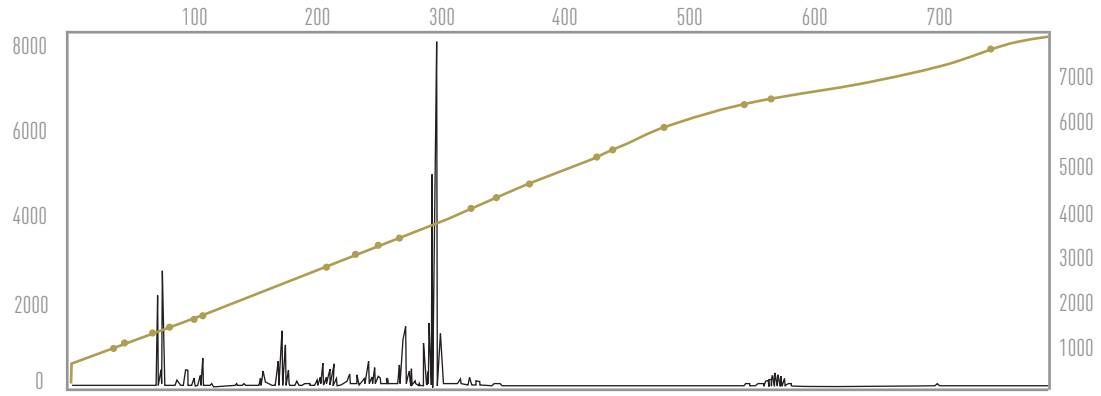

Figure 7. Healthy and premutated heterozygous female (29/162 CGG repeats). The red dots on the line indicate the fragments corresponding to the marker 10000 ROX™.

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CGG repeats	Fragment size (bp)	CGG repeats	Fragment size (bp)
10	260	110	560
15	275	115	575
20	290	120	590
25	305	125	605
30	320	130	620
35	335	135	635
40	350	140	650
45	365	145	665
50	380	150	680
55	395	155	695
60	410	160	710
65	425	165	725
70	440	170	740
75	455	175	755
80	470	180	770
85	485	185	785
90	500	190	800
95	515	195	815
100	530	200	830

Black numbers indicate healthy alleles. Gold numbers indicate premutated alleles.

Important note

This table has been devised from the results obtained with the kit in a 3730xl Genetic Analyzer (Applied Biosystems), using POP7 polymer. For a better adjustment of the repetitions, the user must include a control sample with a known number of repetitions in each batch of analysis, in order to normalize the peak mobility in particular electrophoresis conditions (polymer, genetic analyzer, run conditions, etc.).

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Quality control

Due to the quantitative nature of this test, it is necessary to perform a calibration of the FAM and ROX fluorophores in the genetic analyzer.

Furthermore, as described in the procedure, the addition of the marker fragment ROX™ 1000 is required, for determining the size of the DNA fragments.

A contamination control should be carried out by replacing the DNA for a negative control and a positive control of a known size (see section 'Materials required but not supplied').

The user should consider all precautions, named in section 'Sample collection and preparation' and the limitations in section 'Procedure limitations', of this procedure.

The test should be carried out as recommended in this protocol, as well as with other quality control procedures that comply with local, state, federal and / or certifying agencies.

Specific operation data

1. Analytical specificity and sensitivity

The primers of this kit are specific for the human fragile X mental retardation gene (FMR1) (NC_000023.10) and comprise the CGG repeat region within the 5'UTR of the gene (NT_011681. 16). The specific amplification of this region of the FMR1 gene was verified by DNA sequencing and by assaying samples of healthy individuals and well characterized patients. There is no reported case of cross-reactivity with another gene from genomic DNA.

2. Diagnostic specificity

The ADELLGENE Fragile X Screening kit is a specific assay of the 5´-UTR region of FMR1, to detect healthy and premutated alleles (10-200 CGG repeats) in males and in heterozygous females. If no signals are found in males or only one peak is found in females, the use of other alternative techniques such as Southern Blotting, is necessary to confirm the presence of unhealthy alleles (>200 CGG repeats).

Mutations (point mutations, insertions and deletions) at amplification primer sites are possible and may result in the lack of allele definition. Other technologies could be necessary to resolve this problem. Homozygous results must be confirmed by alternative procedures.

3. Range of:

- DNA concentration

For the best range of DNA volume in each assay, tests were performed between 10 ng and 200 ng with a heterozygous female sample. The size of the peaks obtained was independent of the amount of DNA used. The recommended working amount of DNA is 30 ng.

- Results of the kit

This kit can assign a number of CGG triplet repeats of between 10 and 200 alleles, which is healthy or premutated, respectively. Healthy and premutated alleles have a well-defined peak of less than 200 CGG repeats for males, and two peaks (heterozygous) with less than 200 repeats for females.

The rest, as indicated in section 'Summary and explanation', shall be further studied by classical techniques, such as Southern blotting.

4. Accuracy

- Assignment of fragment size

The assignment of the peaks is given by the size of the amplicon obtained and peak morphology. If there are several peaks, it will be considered the highest and the most central peak. The accuracy of the number of repeats was determined by comparing sequenced samples with the sizes obtained with the present kit, comprising a variability of number of repeats ± 1 for healthy alleles and ±3 for premutated alleles.

- General testing

Region 5UTR/FMR1	Samples Tested
Healthy males (1 peak<50 rep.)	14
Heterozygous females (2 peaks<50 rep.)	15
Heterozygous females (1 peak<50 rep.; 1 peak between 50-200 rep.)	4
Real homozygous females	6
False homozygous females *	2
Unhealthy males (>200 rep.)(no peak) *	2
Number of samples tested	**43**

* *The allele >200 repeats was tested by other technologies.*

- Interferences

A number of substances which may be present in the peripheral blood could potentially interfere with the PCR-based methodology; inhibiting the polymerase activity that has been described in the literature. Therefore, it is necessary that the DNA obtained has the required purity to avoid interference. Most standard DNA extraction methods eliminate these substances; therefore it is recommended that the DNA extraction method used in the laboratory is validated.

Procedure limitations

- The method detects all alleles of between 10 and 200 CGG triplet repeats (see section 'Results and interpretation').
- The conditions described for the PCR should be accurately controlled. Deviations from these parameters can lead to poor results.
- All work from ADELLGENE Fragile X Screening kit must be made in accordance with good laboratory practices and in compliance with local regulations, such as the international standard.
- The thermocycler must be calibrated according to manufacturer's recommendations and used within the limits specified by them.
- The DNA analyzer must be calibrated according to the manufacturer's recommendations for the fluorophores, used in compliance with the limits specified by them.
- Do not mix components from other kits and lots.
- Do not use the kit after the expiry date.
- Do not use the kit if loss of reactivity, contamination, deterioration of the container or any other incident that may affect performance, is suspected.
- The interpretation of the data and typing results should be reviewed by qualified personnel.
- Remove expired reagents following applicable regulations.

Troubleshooting guide

PCR negative control is positive

- **Primer mix contamination**
  - Repeat the experiment with new aliquots of Primer mix / Negative Control
  - Handle kit components provided with commonly accepted practices, to avoid contamination
  - Check storage and handling conditions
  - Discard contaminated reagents
- **Contamination in the Pre-PCR area**
  - Confirm that the necessary precautions have been followed in the PCR area
  - Check possible contamination problems in other PCR techniques
  - Confirm the suitability of the consumables used (tubes, pipette tips)
- **Pipetting error**
  - Verify that the sample added always corresponds to the assigned worksheet

Weak or absent signal in the PCR product

- **Poor quality of DNA samples**
  - Repeat the extraction of DNA
- **Samples with very low concentration of DNA**
  - Check the DNA concentration
- **DNA samples with high concentration**
  - Make a preliminary assessment of the extraction system by testing sample dilutions
- **Presence of PCR inhibitors in genomic DNA**
  - Avoid the use of whole blood containing heparin. Re-extract DNA and repeat PCR when possible
- **DNA polymerase not added to master mix or insufficient mixing of PCR mix**
  - Repeat PCR ensuring all components are added and mixed sufficiently

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- **Thermal cycling problems**
  - Check the thermal cycling run parameters and ensure thermal cycler is operating according to manufacturer´s specifications and maintenance
- **No Ethidium bromide (or other alternative DNA colorant) is added**
  - Ensure Ethidium bromide is added to the gel and electrophoresis buffer

Incorrect band size or number of bands

- **Incorrect kit used**
  - Check the appropriate kit is used
- **Incorrect thermal cycling program used**
  - Check thermal cycler parameters
- **PCR contamination**
  - Check the negative control. Proceed with decontamination protocols and repeat PCR to identify the origin of contamination

Weak signal of electropherograms

- **Degradation of the kit**
  - Confirm proper storage of the kit
  - Avoid over 3 cycles of freezing / thawing of the reagents
  - Perform aliquot of fraction reagents as necessary
  - Repeat with fresh batch of reagents
- **Taq polymerase activity lost**
  - Confirm the activity of Taq polymerase
  - Repeat with a new Taq polymerase
- **Weak PCR product**
  - Check gel image and proceed accordingly
- **Insufficient reaction products applied to DNA analyzer**
  - Check analyzer parameters
- **Wrong purification process**
  - Take extreme care in purification process

High fluorescence intensity

- Too much PCR product
  - Check gel image. Dilute PCR product
- Too much product applied to the DNA analyzer
  - Check instrument parameters
- Pipetting error
  - Verify that the volume added in each well is correct

High backgroud (noisy baseline)

- PCR product contaminated
  - See above
- Poor PCR purification
  - Ensure purification process was perfomed following mentioned instrucions

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