Adellgene®

Fragile X

INSTRUCTIONS FOR USE

Kit to determine the number of CGG triplet repeats in the *FMR1* gene using fragment analysis technology

Rev. 03 - 2022/12/01

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50011 – Zaragoza (Spain)

www.bdrdiagnostics.com

CE

Reference No.	UDI-DI	Store:
AD-FMR1-25	8437016942468	from -30°C to -18°C
AD-FMR1-100	8437016942475	

Adellgene®

Fragile X

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1 – Information for safety

Please, read completely these instructions for use and follow them during the use of the current IVD kit.

The IVD kit shall be used by experts with strong experience in DNA analysis and interpretation of genetic results.

Consult the manufacturer if there are doubts involving the description of the assay method. Contact by phone at +34 976 094 603 or email address *customersupport@bdrdiagnostics.com*.

The IVD kit has a limited shelf life. Make sure that the shelf life is not expired before using the kit. Reagents from a kit beyond its expiry date might be degraded, which could impair results. Discard the expired reagents following applicable regulations.

The contamination of the sample or the reagent can render incorrect results. Be careful during the DNA extraction and sample and reagents manipulation.

This kit can be damaged during transport or storage. Do not use the kit in case of suspected damage during transport. Follow carefully the storage conditions described in the label and the IFU.

Ensure that the waste is managed according to local regulations. Incorrect waste management can result in environmental contamination.

The toxicological properties of the kit 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 for the customer by request.

Ensure that this kit is adequate for the requested analysis by the clinician.

2 – Intended Use

Adellgene® Fragile X is a semi-automated *in vitro* diagnostic kit designed for use in clinical laboratories for the amplification and quantitative determination of CGG triplet repeats (cytosine-guanine-guanine) in the 5' untranslated region of gene for fragile X mental retardation ("Fragile X mental retardation-1": *FMR1*). It aims to aid diagnosis of clinical disease associated with Fragile X syndrome (e.g.: mental retardation, primary ovarian failure, tremors / ataxia, etc...).

The technology is based on the polymerase chain reaction (PCR) amplification of genomic DNA extracted from peripheral blood, followed by fluorescence analysis of the size of the PCR fragments obtained by genetic analyzer.

Patients who can benefit from this determination are those referred by a specialist. The intended user of the kit is technical personnel trained to carry out the protocol and the interpretation of results described in this document.

3 – Summary and explanation

Fragile X Syndrome (FXS, OMIM #300624) is an X-linked disease mainly based on the genomic expansion of the CGG (cytosine-guanine-guanine) triplet of nucleotides, located in the 5' untranslated region of the Fragile X Mental Retardation 1 gene (*FMR1*) on chromosome Xq27, and aberrant methylation status of the promoter of the gene (Fig. 1)[1].

Four categories can be established based on the number of triplet repeats:

- Healthy alleles: less than 45 CGG repeats without methylation of the promoter of the gene. The most common alleles contain 29 or 30 repeats[1].
- Intermediate or grey-zone alleles: from 45 to 54 repeats. Alleles in this range are not associated with FXS and can be considered normal. Intermediate alleles have not been observed to expand to a full mutation in one generation, although very rare cases of expansion to a premutation have been described[1,2].
- Premutation alleles: from 55 to 200 CGG repeats, without aberrant methylation. 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) whose severity depends on the methylation state of the promoter[3,4]. It is estimated that the prevalence in the general population is 1 in every 130-250 females and 1 in every 250-810 males[5]. Moreover, premutation alleles are unstable and may increase in size during maternal transmission to the offspring, resulting in a full mutation[6].
- Full mutation alleles: over 200 CGG repeats, with aberrant hypermethylation of the promoter of the gene. This genotype leads to the suppression of gene expression in the human brain, which is associated with mental retardation, autism, and mental and emotional changes. Patients with expanded alleles show a striking phenotype consisting of large ears and an elongated face[1]. The severity of cognitive impairment in patients with FXS is not associated with the magnitude of the full mutation but varies with the state of methylation[7].

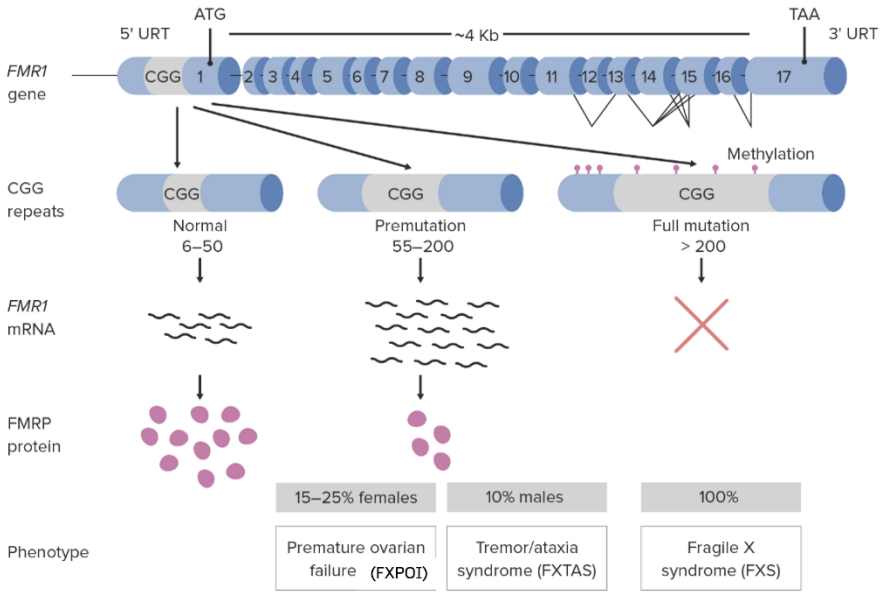


Figure 1. Depiction of the types of FMR1 gene and their production depending on the degree of mutation. Extracted from [11].

Many *FMR1* alleles contain interspersed AGG sequences between the CGG repetitions. It is thought that these inserts may confer stability to the DNA and reduce the risk of triplet repeats expansion in the next generation. Therefore, the likelihood of CGG repetitions expansion in the offspring is higher when the mother does not present AGG insertions in the *FMR1* allele[6,8].

4 – Procedure principles

The method used by the Adellgene® Fragile X kit is based on specific amplification of the 5' untranslated region of the *FMR1* gene, which contains the CGG triplet repeats, by TP-PCR (Triplet-Repeat Primed PCR), from genomic DNA.

The kit includes several primers, one of which is marked with the FAM fluorophore. The PCR products are separated by capillary electrophoresis (CE) and then detected in a genetic analyzer. Later, the size of PCR fragments is converted into number of CGG repeats, using mobility and size correction factors.

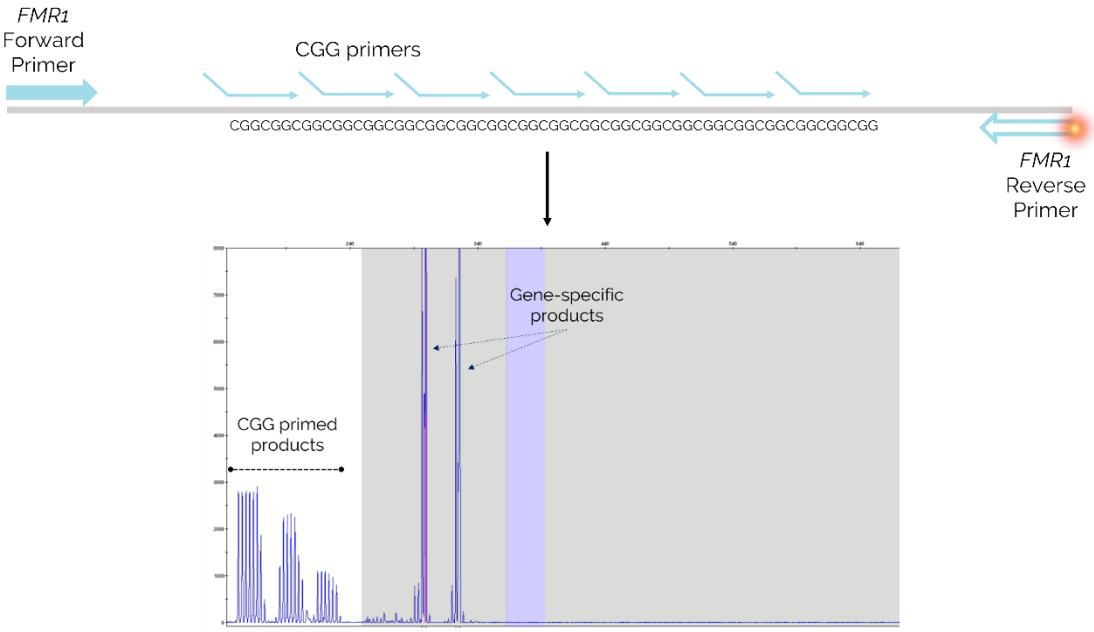


Figure 2. Procedure principles of Adellgene® Fragile X kit.

TP-PCR allows rapid detection of PCR products by adding a primer that binds within the triplet-repeat region. This repeat primer overlaps the CGG repeat sequence and produces multiple amplicons, each one with a difference in length of 3 bp. The profile of CGG primed peaks can provide information about the sample, such as zygosity, AGG interruptions profile and even the number of repeats, adding sizing confirmation.

The TP-PCR increases the amount of full-length product from the largest-repeats allele and enables accurate sizing of fragments up to 200 CGG repeats. This technique also reduces the risk of PCR allele dropout, as the presence of longer alleles is always perceptible regardless of whether full-length products of such alleles are detected.

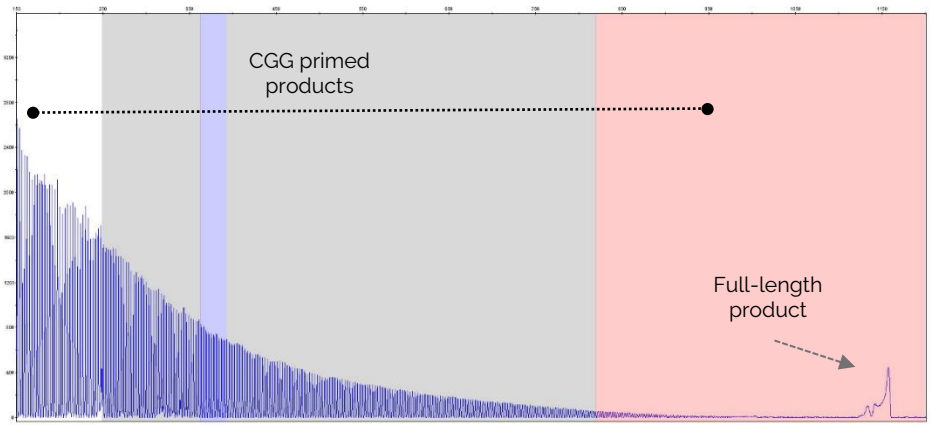


Figure 3. Full mutation male, with >200 CGG repeats

During CE, fragments are separated by size and then detected. The separation matrix for CE is a polymer with a resolution limit of up to 200 CGG. Due to this intrinsic limitation of the technique, alleles beyond 200 repeats can be detected but not precisely sized, and so these alleles are identified as >200 CGG.

5 – Kit contents

➔ Reference AD-FMR1-25 (25 tests):

- AD-FMR1-PM: Primer Mix. 1 vial, 25 µL
- AD-FMR1-POM: Polymerase Mix. 1 vial, 12.5 µL
- AD-FMR1-GC: GC-Rich Buffer. 1 vial, 300 µL
- AD-FMR1-SL: ROX 1000 Size Ladder. 1 vial, 50 µL

➔ Reference AD-FMR1-100 (100 tests):

- AD-FMR1-PML: Primer Mix. 1 vial, 100 µL
- AD-FMR1-POML: Polymerase Mix. 1 vial, 50 µL
- AD-FMR1-GCL: GC-Rich Buffer. 1 vial, 1.2 mL
- AD-FMR1-SLL: ROX 1000 Size Ladder, 1 vial, 200 µL

6 – Kit storage

All the components of the kit must be stored between -30°C and -18°C upon reception. Under these conditions, they are stable up to their expiry date indicated on the label.

Do not perform more than 2 freeze/thaw cycles to the kit vials as this may reduce the sensitivity of the assay and affect the results. If assays are to be performed with few samples, it is recommended to use aliquots of the reagents to avoid exceeding the recommended number of freeze/thaw cycles.

Due to the photosensitive nature of the reagents, avoid continuous exposure to light.

7 – Materials required but not supplied

➔ Reagents

- Isolation and purification reagents for DNA extraction are not included. DNA can be extracted using any laboratory-validated extraction system that allows the recommended concentrations and levels of DNA purity for this test (see the "Specific operation data" section)
- Controls are not included but are recommended to be used in every run. The Fragile X Syndrome Genetic Reference Panel (NIBSC, 08/158)[9] may be used as a positive control, as well as other well-characterized commercially available cell line DNA[10].
- DNAse-free distilled water
- POP-7™ polymer: ref: 4363785, Applied Biosystems™; or equivalent
- Highly deionized (Hi-Di) formamide: ref.: 4311320, Applied Biosystems™; or equivalent
- Dye set calibrators for FAM and ROX: ref.: 4345827, 4345829: DS-30/31 Matrix Standard Kit (Dye Set D), Applied Biosystems™; or equivalent

➔ Equipment

- General laboratory equipment dedicated to PCR techniques
- Thermal cycler
- Genetic analyzer running POP-7™/POP-1™ polymer:
  - 3130/3130xl Genetic Analyzer, Applied Biosystems™
  - 3730/3730xl Genetic Analyzer, Applied Biosystems™
  - 3500/3500xl Genetic Analyzer, Applied Biosystems™
  - SeqStudio™ Genetic Analyzer, Applied Biosystems™
- PCR plate/tube centrifuge
- Vortex mixer
- Pipettes (P1000, P200, P20 and P2) and specific filter tips for each pipette
- PCR plates/tubes with their corresponding covers/caps
- Data analysis software: GeneMapper™ software or equivalent

8 – Sample collection and preparation

Samples must be collected in line with the instructions for use of the collection device (not included with the kit) and under national and international guidelines.

The present test should only be performed with DNA extracted from whole blood samples preserved with anticoagulant agents such as EDTA or citrate. Heparin can interfere with the PCR process and should be avoided.

The technique is compatible with several DNA extraction methods. Before delivering results with a diagnostic purpose, a validation assay with such extraction method should be done.

CAUTION!

All biological and blood samples must be treated as potentially infectious. When handling them, take the corresponding basic and universal precautions.

9 – Usage procedures

➔ DNA extraction

This kit is compatible with any standard DNA extraction method from whole blood preserved with EDTA or citrate. It is recommended to evaluate the purified DNA for concentration (OD260) and purity (OD260/280). The DNA obtained should be stored at -20°C until used.

The recommended amount of DNA is 20–80 ng (2 µl of DNA at 10-40 ng/µl), with a OD260/280 ratio ~1.8.

➔ PCR set up

CAUTION!

- Define pre- and post-PCR work areas that should be kept separated to reduce the risk of contamination. Use filter tips and autoclaved or sterile tubes.
- Use laboratory coat and disposable gloves.
- Allow all kit components to fully thaw before using. Polymerase Mix should be kept on ice during the whole procedure.

1. Thaw the Primer Mix and GC-Rich Buffer at room temperature and vortex. Place the Polymerase Mix on ice and mix carefully when fully thawed. Centrifuge briefly to collect the volume at the bottom of the tubes.
2. Prepare the reaction mix for n+1 samples, using the quantities indicated in the following table:

Reagent	Volume per sample (µL)
Primer Mix	1.0
GC-Rich Buffer	12.0
Polymerase Mix	0.5
Distilled water	4.5
Total volume	**18.0**

Vortex thoroughly and centrifuge to ensure all volume settles to the bottom of the tube.

3. Pipette 18 µL of this mix into the PCR plate/tubes.
4. Add into each well 2 µL of DNA (recommended concentration 10-40 ng/µL), to reach a final volume of 20 µL.
5. Seal the plate/tubes using the appropriate sealer and centrifuge briefly to ensure that all the volume settles to the bottom of the well. If possible, make sure there are no bubbles in the wells.
6. Place the plate/tubes in the thermal cycler and set up the thermal cycler amplification program as described in the following section.

➔ Thermal cycler configuration

1. Set up the appropriate amplification profile, and start the run:

	Cycles	Temperature (°C)	Time (mm:ss)
Denaturation	1	98	05:00
Cycles	10	97	00:35
		62	00:35
		68	04:00
Cycles	20	97	00:35
		62	00:35
		68	04:00 + **00:20** each additional cycle
Final extension	1	72	10:00
Cooling	1	4	∞

2. After the run, continue with sample preparation for the capillary electrophoresis or store the PCR product at -20°C.

➔ Sample preparation for capillary electrophoresis

CAUTION!

CHEMICAL HAZARD. Formamide should be handled with extreme caution as contact can cause eye irritation and burns, skin irritation, rash, and reproductive damage.

1. Thaw formamide and ROX 1000 Size Ladder. Vortex and spin the tubes before use.
2. Prepare the following mix adding the components specified in the table below for n+1 samples:

Reagent	Volume per sample (µL)
Hi-Di™ Formamide	8.0
ROX 1000 Size Ladder	1.0
Total volume	**9.0**

Mix the solution and spin down to collect.

3. Dispense 9 µL of the mix to each well of the CE plate.
4. Transfer 1 µL of the PCR product to the plate and mix by pipetting 2-3 times.
5. Seal the plate, vortex, and centrifuge briefly to remove bubbles.
6. Transfer the plate to the thermal cycler and set the program below:

	Cycles	Temperature (°C)	Time (mm:ss)
Denaturation	1	95	02:00
Cooling	1	4	∞

7. After the run, keep the plate on ice and protected from light until the injection on the genetic analyzer. Samples may be injected up to 24h after this step.

➔ Genetic analyzer set up

1. Follow the manufacturers' directions to set the data acquisition in the genetic analyzer. Ensure the instrument is calibrated for the detection of ROX and FAM dyes.
2. Use the default run module for Long Fragment Analysis for the specific polymer and capillary length of the instrument as base protocol.
3. Adjust injection conditions and run time according to recommended starting values listed in the table below.

Genetic Analyzer	Capillary length	Injection voltage	Injection time	Run time
3130 /3130xl	36 cm	2.5 kV	20s	2400s
3730/3730xl	50 cm	2.5 kV	20s	4000s
3500/3500xl	50 cm	2.5 kV	20s	4000s
SeqStudio™	28 cm	1.2 kV	7s	6200s

NOTE: The module of the analyzer may differ between instruments, and final run and injection conditions should be validated by the user.

➔ Disposal

Waste products shall be managed according to local regulations.

10 – Results and interpretation

The Adellgene® Fragile X kit is a quantitative technique used to determine the number of CGG triplet repeats in the 5'UTR of the FMR1 gene. The kit allows the detection of all kinds of alleles (normal, intermediate, premutant and mutant alleles) and quantification of expansions up to 200 CGG repeats.

The determination of these alleles is challenging due to its high concentration of GCs, which creates a strong secondary structure that prevents replication of this region even when using Taq polymerase at high temperatures. The Adellgene® Fragile X kit includes a series of reagents that allow the amplification of this region and the determination of the number of repeats, enabling the differentiation between wild-type, premutation and expanded individuals.

➔ Electropherograms interpretation

Once the CE run is finished, results are to be imported in the analysis software, such as GeneMapper™ or equivalent.

1. Import the .fsa files into the software and analyse the data according to the analysis methods, panels and size standard settings established for the Adellgene® Fragile X kit. These files are available at the website *https://www.bdrdiagnostics.com/* or by contacting our technical support department at *customersupport@bdrdiagnostics.com*. Alternatively, results may also be analysed with general default methods.
2. Review ROX 1000 size ladder peaks for every sample, including controls. All 21 peaks should be detected and labelled correctly, as shown in the figure below. The sizes of the ROX 1000 ladder peaks are: 79, 90, 105, 131, 151, 182, 201, 254, 306, 337, 362, 425, 486, 509, 560, 598, 674, 739, 799, 902, 1007 bp.

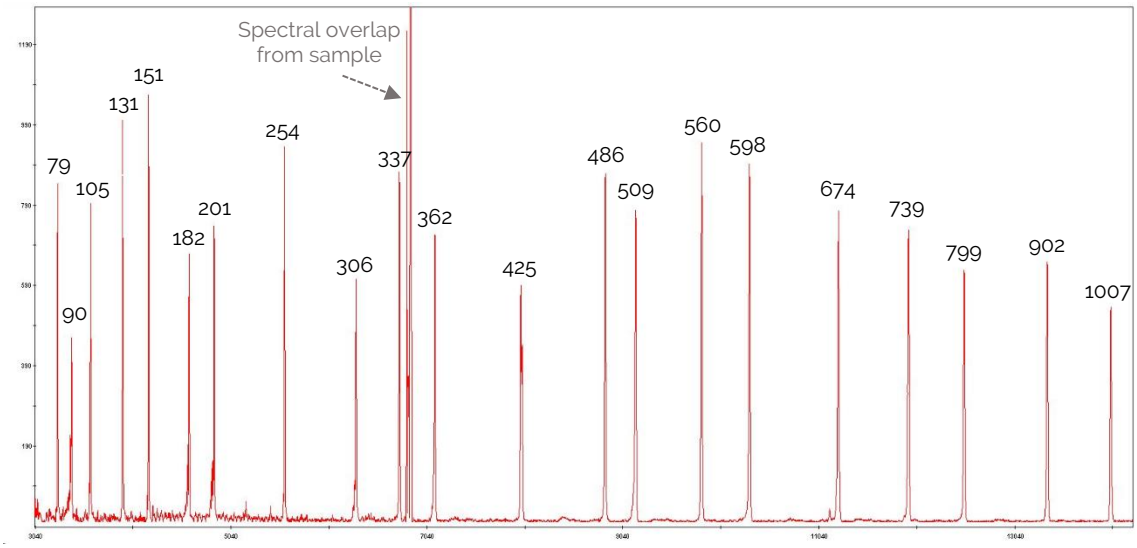


Figure 4. ROX1000 size ladder peaks

A spectral overlap from the PCR product may be seen in the ROX channel, appearing among the size standard peaks. This peak should not interfere with the sizing algorithm in the analysis software, otherwise, the user should deselect this peak and correct the calling. Samples without a correct labelled ladder should be excluded from further analysis.

3. Check control results, if included. The negative control should not report any peaks in the FAM channel in the electropherogram, and positive controls should meet the specifications. If control results are not correct, samples should be reanalysed.

4. Check the samples' results and determine the size of the gene-specific fragments.

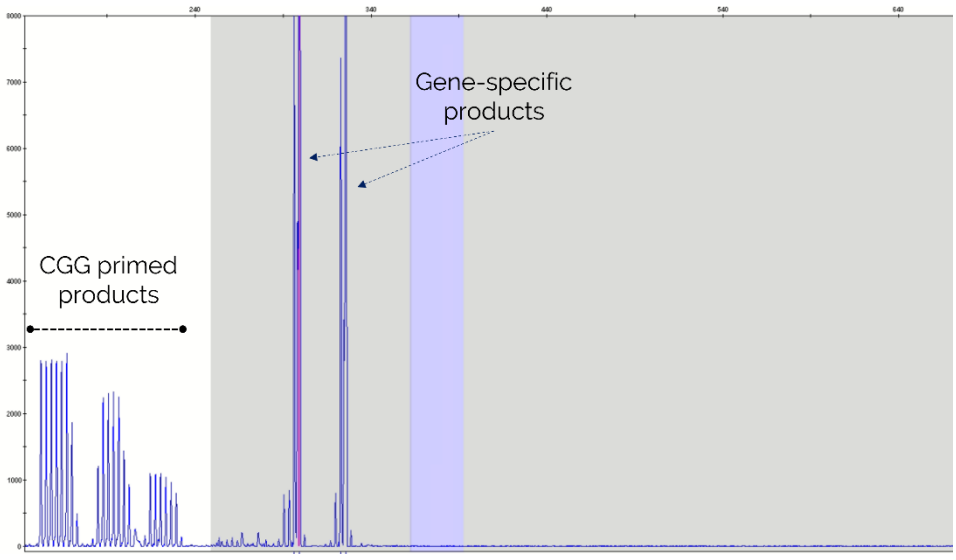


Figure 5. Electropherogram identifying the gene-specific products.

To detect gene-specific product peaks corresponding to the full size of the alleles in the electropherograms, deselect the CGG ladder peaks so only the gene-specific amplification product will stay selected. See Figure 6 for an example. These CGG peaks are the product of the repeat primer annealing within the region of repeats, producing multiple amplicons.

Table 1 shows the guidelines to identify the main target peaks among the rest of the product peaks.

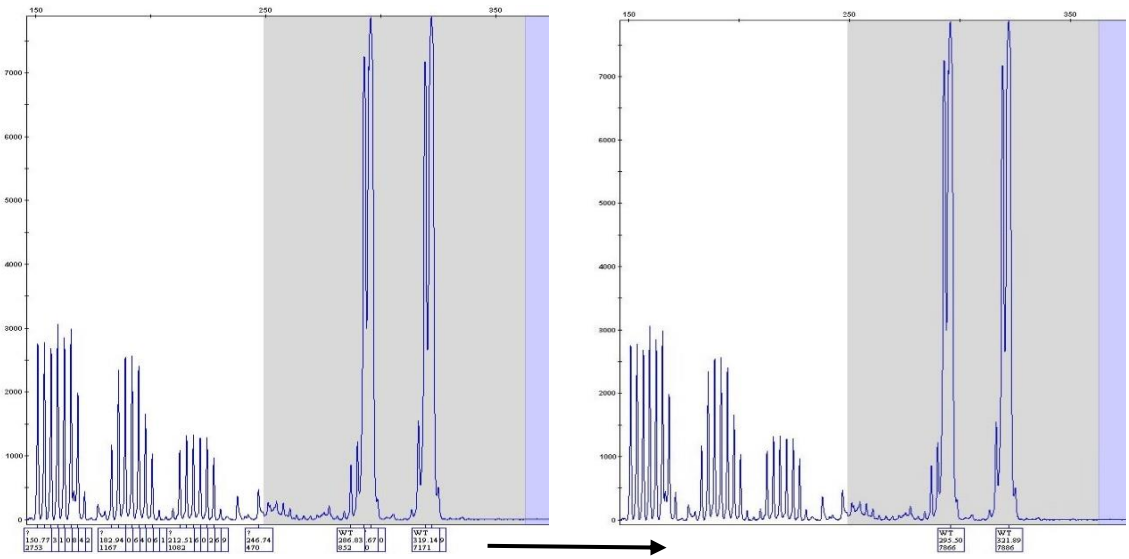


Figure 6. Electropherograms showing the deselection of the non gene-specific peaks.

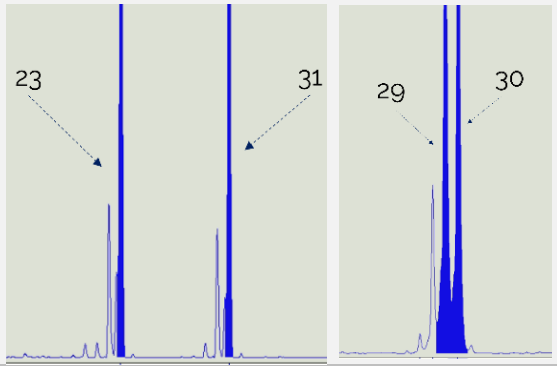
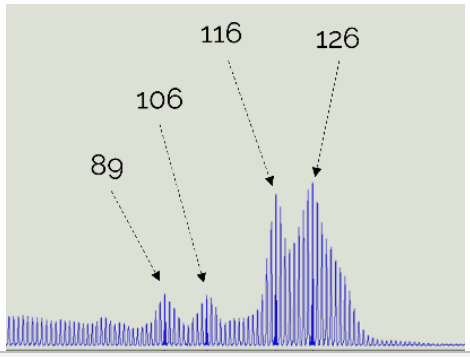
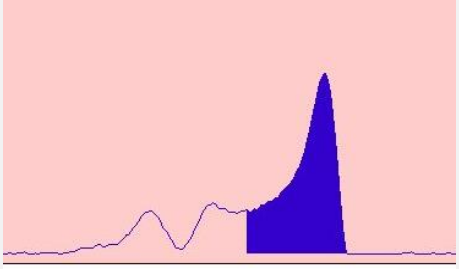
Allele ranges	Guidelines to select main peaks	Examples
Normal and intermediate alleles	Select the highest peak of a single allele group. In the case of heterozygous alleles with one CGG repeat difference (e.g. 29/30), two allele groups may not be fully-separated. In this case, the two highest peaks should be selected.	
Premutation alleles	Select the highest peak of the peak population. In the case of alleles with several peak populations, select the highest peak of each group.	
Mutant alleles	Select the component of the peak group with the highest peak. All peaks in the mutant range are identified as >200 CGG.	

Table 1. Guidelines to identify the main peaks in the electropherograms.

➔ Calculation of the number of CGG repeats

To calculate the number of repeats corresponding to each allele, the equation given next must be applied:

$$No.\,of\ triplet\ repeats = \frac{(Peak\ size - C_0)}{m_0}$$

Where c_0 is the size correction factor, m_0 is the mobility correction factor and the peak size is expressed in base pairs.

Correction factors can be determined following the procedure described in the "Standardisation process" section. These values may vary from laboratory to laboratory, consider using positive controls in order to verify the accuracy of the number of repeats estimation when using the kit for the first time.

The theoretical values for these correction factors are the following:

c_0	230
m_0	3

NOTE: To ensure an accurate estimation of the number of CGG repeats, calculation of specific correction factors and the use of a positive control is strongly recommended.

➔ Standardisation process

To standardise the fragments' mobility in the particular conditions of electrophoresis in which the experiment is carried out (polymer, genetic analyzer, etc.), two correction factors are used in the calculation of the number of CGG repeats: the size correction factor (C_0) and the specific mobility correction factor (m_0).

$$No.\,of\ triplet\ repeats = \frac{(Peak\ size - C_0)}{m_0}$$

C_0 and m_0 values can be calculated using a pooled DNA control. This control should be a mixture of several PCR products including *FMR1* alleles with a known number of triplet repeats, ranging through the peaks' size range (<200 repetitions). C_0 and m_0 values are determined from a linear fit of the number of repeats and fragment size for these alleles.

This pooled DNA control can be prepared by mixing PCR amplicons. See references commercially available below.

Catalog number	CGG repeats by Adellgene® Fragile X kit.
NA20235	29, 45
NA20236	31, 54
NA06891	118
NA20239	20, 199

Table 2 Reference DNA templates to prepare the pooled DNA control. For more information, see https://www.coriell.org/

To calculate the values of the correction factors to be used in the experiment, proceed as follows:

1. Analyse the pooled DNA control in the run and calculate the peaks' size for each of the *FMR1* alleles. It is recommended to determine the average size in base pairs of the alleles from two separate runs.

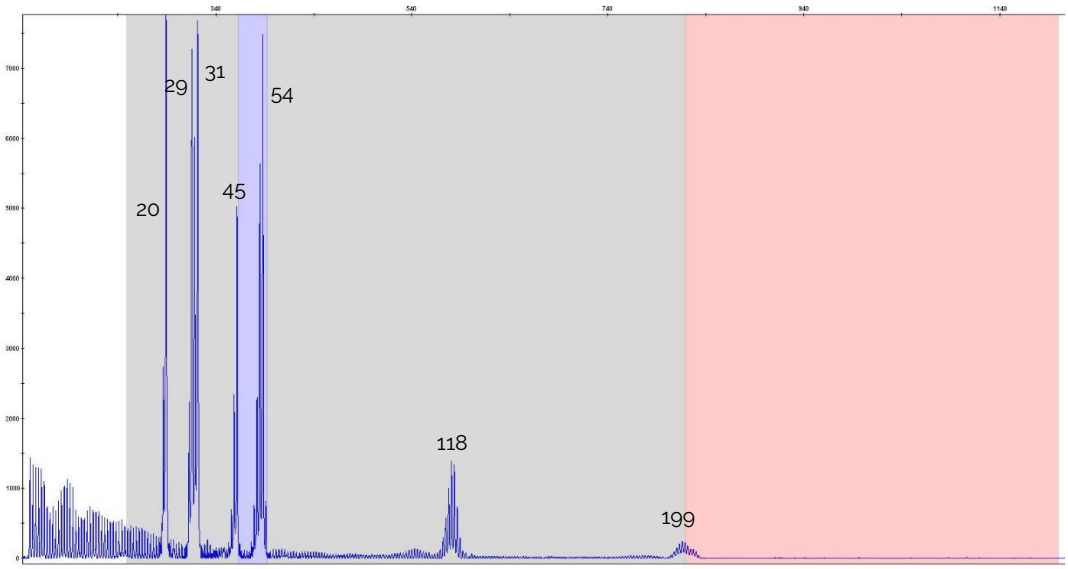
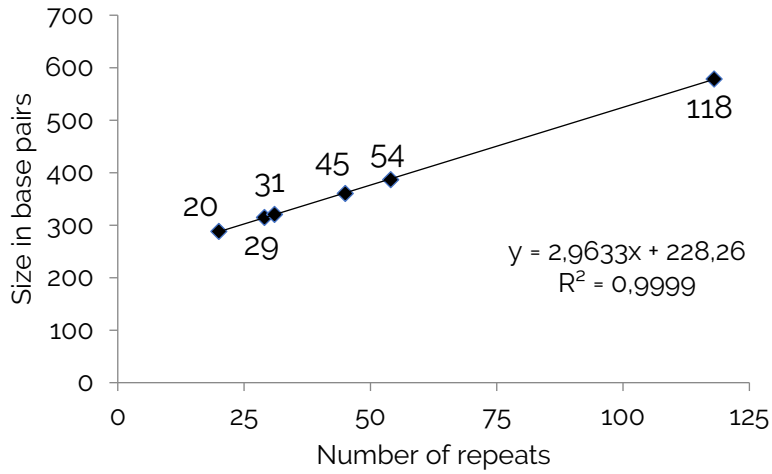


Figure 7. Pooled DNA control electropherogram

2. In Excel or similar program, create a table with the fragments' size and the number of triplet repeats of each allele, and insert a scatter chart. Perform a linear regression obtaining the corresponding equation to the linear fit (Graph 1).



Graph 1. Pooled DNA control plot

From the equation of this regression fit, the correction factors are determined: C_0 corresponds to the intercept of the linear fit, whereas m_0 corresponds to the slope of the line. In the graph shown, C_0 = 228.26 and m_0 = 2.9633.

The user may test the veracity of the correction factors with positive controls such as the Fragile X Syndrome Genetic Reference Panel (NIBSC, 08/158) and other well-characterized commercially available cell line DNA[10].

➔ Electropherogram results

Examples of electropherogram results obtained with this kit are shown in the following images: healthy heterozygous female (Figure 8), heterozygous female with a healthy allele and another premutation or expanded allele (Figures 9-10) and an expanded male (Figure 11).

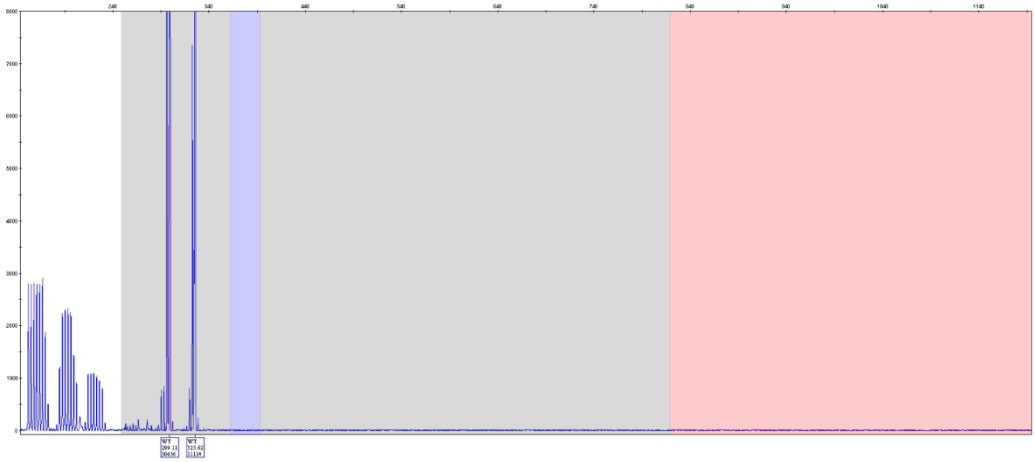


Figure 8. Healthy heterozygous female, 22/31 CGG.

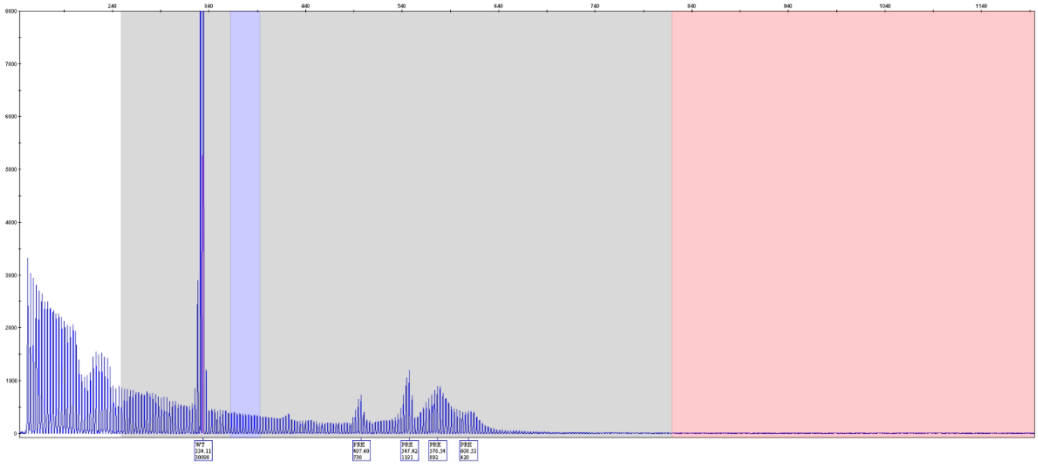


Figure 9. Heterozygous female with a premutation allele in mosaic, 35/89, 106, 117, 128 CGG

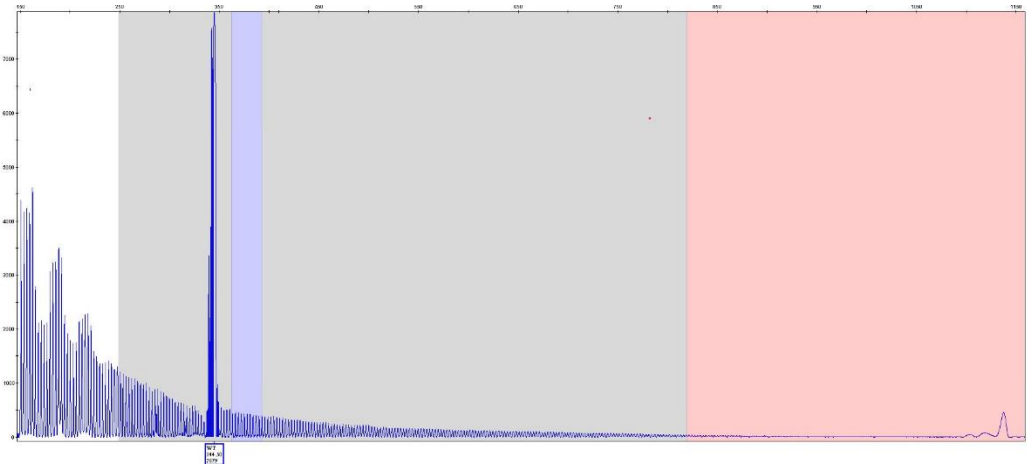


Figure 10. Expanded heterozygous female, 39/>200 CGG

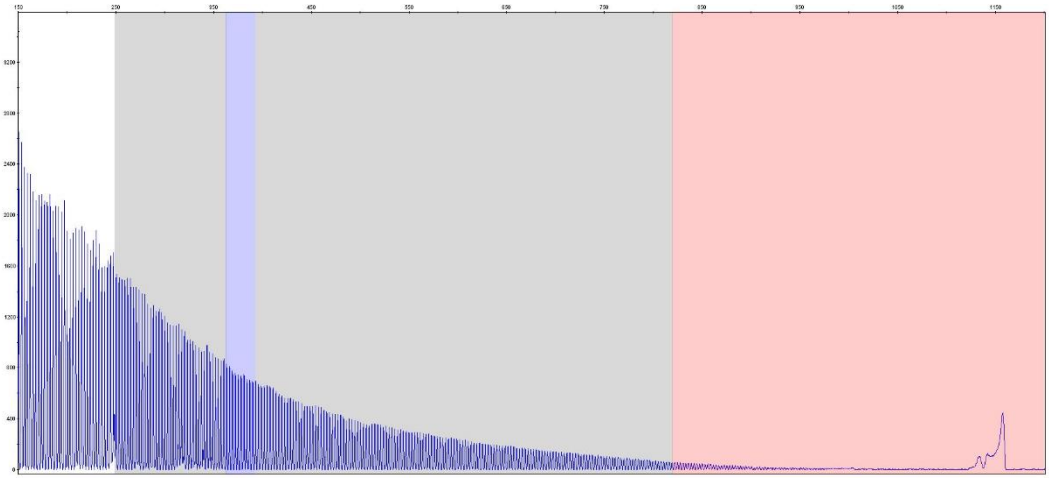


Figure 11. Expanded male, >200 CGG

Primers of the Adellgene® Fragile X kit are specific for CGG repeats and do not hybridize with AGG sequences commonly found within the *FMR1* gene. Accordingly, the signal valleys that appear on the electropherogram correspond to the presence of interspersed AGG regions.

The AGG interruptions are thought to confer DNA stability and diminish the risk of expansion in the offspring[8]. Figure 12 shows a healthy female sample yielding the pattern composed of CGG and AGG triplets as indicated.

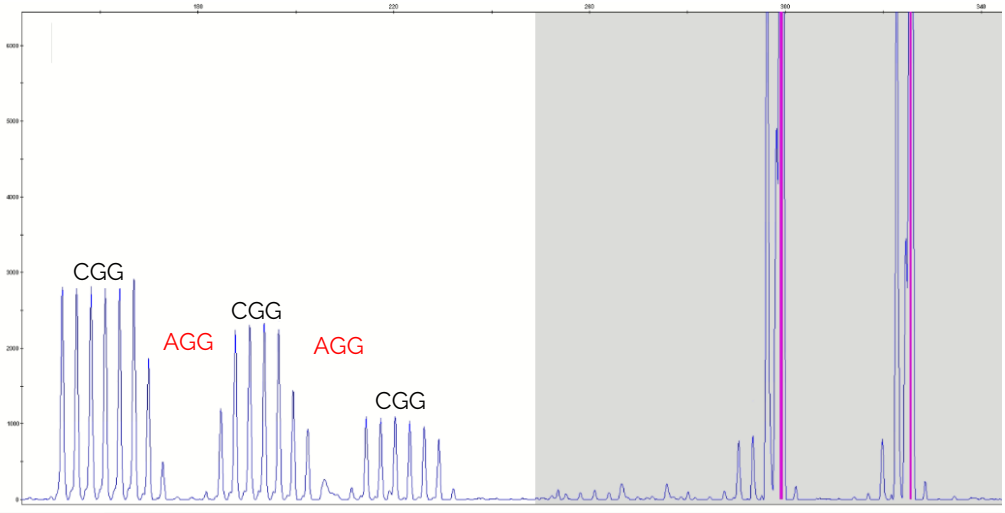


Figure 12. Healthy female sample showing the characteristic pattern composed by CGG primed amplicons and 2 AGG interruptions.

The profile of CGG primed amplicons can provide information about AGG interruptions and allele sequence based on peak counting and signal valleys. Although sometimes it may not be possible to sequence-map the AGG positions based on this profile, the most likely interpretation could be inferred considering the most common AGG haplotypes. Specific resolution of AGG pattern may require additional procedures.

In the case of obtaining saturated peaks in the electropherogram, consider adjusting injection and run parameters. Alternatively, a new injection could be done diluting the PCR products (e.g. 1:10), to set the peak's size more accurately.

11 – Quality control

Given the quantitative nature of this test and to ensure correct detection of the fluorescent signal, the fluorophores FAM and ROX must be calibrated in the genetic analyzer.

In each round of analysis, a negative control should be added to ensure the assay is contaminant free. The use of a positive control is also strongly recommended.

To be able to correctly measure the size of the alleles, the size ladder must be added to all wells. All size ladder peaks must be detected for all samples (including the negative control).

The negative control should not report any peaks in the FAM channel in the electropherogram, and positive controls should meet the specifications. If control results are not correct, samples should be reanalysed.

12 – Specific operation data

➔ Analytical specificity

This kit´s primers are specific to the human Fragile X Mental Retardation gene (*FMR1*) (NC_000023.11) and comprise the CGG repeat region within the 5'UTR of the gene. The specific amplification of this region was verified by DNA sequencing and testing samples from healthy individuals and well-characterised patients. No cases of cross-reactivity with another gene from genomic DNA have been reported.

➔ Analytical sensitivity

To determine the appropriate amount of DNA for this test, tests were carried out analysing five different samples (healthy, premutation and expanded females, and premutation and expanded males), between 10 ng and 200 ng. The assignment of the peak size obtained was independent of the amount of input DNA. The recommended working range is between 20 and 80 ng of DNA.

➔ Diagnostic specificity.

Adellgene® Fragile X is a specific test to detect the number of CCG repeats in the 5'UTR of the *FMR1* gene. The kit allows the quantification of healthy and premutation alleles consisting of a number of repeats up to 200 CGG. It also allows the visualisation of expanded alleles (>200 CGG repeats).

Mutations (point mutations, insertions, deletions) at hybridisation points of the amplification primers are possible and may lead to a lack of definition of an allele. Other technologies may be required to resolve the alleles in these cases.

Diagnostic specificity was assessed in one external and one internal study, analysing a total of 34 samples with the following results:

Sample type	No. of analysed samples
2 normal alleles (up to 45 or 55 CGG repeats)	7
1 premutation allele (45 or 55-200 CGG repeats)	18
1 expanded allele (>200 CGG repeats)	9
Total number of samples analysed	**34**

All results matched with prior information available on these samples, which were previously analysed using the routine method of the source laboratory.

➔ Diagnostic sensitivity.

The Adellgene® Fragile X kit can assign a number of CGG triplet repeats up to 200 CGG. This allows the alleles present in the sample of an individual to be classified as healthy (up to 45), intermediate (45-54 CCG), premutation (54-200 CGG) or expanded (>200 CGG).

➔ Accuracy

Interferences

Existing literature has described a number of substances that may be present in peripheral blood and which may potentially interfere with the PCR, inhibiting polymerase's activity. Thus, before proceeding with the test, we recommend assessing DNA's purity. The majority of standard extraction methods allow these substances to be eliminated, for which reason we also recommend validating the extraction method used before proceeding with the test.

Trueness

The precision in the number of calculated repeats was determined by comparing the CGG repeats obtained using Sanger sequencing and Adellgene® Fragile X kit. Thus, an acceptable variability of 1 repeat was established for healthy alleles and 3 for premutation alleles.

According to the EMQN best practice guidelines for the molecular genetic testing and reporting of Fragile X Syndrome, a maximum error of ±5% of the total repeat size is accepted[1], so the Adellgene® Fragile X kit is within the accepted sizing accuracy.

13 – Procedure limitations

- This kit can detect all *FMR1* alleles and quantify CGG repeats up to 200 repeats.
- Mutations or polymorphisms at annealing primer sites are possible and may result in the lack of allele definition. Other technologies may be necessary to resolve these alleles.
- All the recommendations mentioned in this document should be carefully followed. Any performances that do not meet such indications can lead to poor results.
- Do not use the kit if there are any suspicions of possible loss of reactivity, contamination, external box deterioration or any other incidence that might affect the kit's performance.
- All Adellgene® reagent manipulations must be done according to good laboratory practice, being adapted to local regulations.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiration date. Discard expired reagents according to applicable regulations.
- The thermal cycler and genetic analyzer must be calibrated according to the manufacturer´s recommendations and should be used accordingly to the manufacturer's instructions.
- Data and result interpretation should be revised by qualified personnel.
- This product is an auxiliary tool for the diagnosis of patients with suspected disorders related to the *FMR1* gene. Use these results in conjunction with clinical data and results of other tests performed on the patient.

14 – Troubleshooting guide

➔ The negative control shows a positive result

- Contamination of the Primer Mix / Polymerase Mix / the negative control solution
  - Repeat the experiment with new aliquots of the Primer Mix/Polymerase Mix/negative control solution.
- The PCR preparation area is contaminated
  - Clean surfaces, instruments, lab coats, and change consumables and reagents. Repeat the assay.
- Pipetting error
  - Change the pipette tip each time DNA is added to a well. Check that the sample added to the well corresponds to what is written on the worksheet.

➔ Incorrect band size or incorrect number of bands

- Incorrect use of the kit
  - Check the correct kit has been used.
- Incorrect thermal cycling program
  - Check the settings of the thermal cycler.
- Incorrect size standard used in the capillary electrophoresis or wrong detection of the peaks.
  - Check the size standard used is correct.
- Contamination of the PCR
  - Check the negative control of the run.
  - Carry out decontamination protocols and repeat the PCR to identify the source of contamination.
- Incorrect settings used in the genetic analyzer
  - Check the run module and the calibration matrix used.

➔ Weak signal in the electropherograms

- Incorrect settings used in the genetic analyzer
  - Check the run module and the calibration matrix used. Adjust injection and run parameters.
- Low quality or low concentration of DNA input / PCR product
  - Adjust the concentration of DNA to the recommended values for this test.
  - Avoid using whole blood that contains heparin. If necessary, repeat DNA extraction and the PCR.
  - Adjust the amount of PCR product
- Degradation of the kit
  - Confirm the kit has been stored in line with the manufacturer's instructions.
  - Do not freeze/thaw the reagents more than two times.
  - Prepare aliquots of the reagents if necessary.
  - Repeat the analysis with a fresh batch of reagents.

➔ High intensity of fluorescence

- Excess of PCR product
  - Dilute the PCR product.
- Incorrect settings used in the genetic analyzer
  - Check the run module and the calibration matrix used. Adjust injection and run parameters.

➔ High background signal (noisy baseline)

- Contamination of the PCR
  - See above.
- Low quality of PCR product
  - See above.

15 – References

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16 – Notice to purchaser

- This product has been developed for *in vitro* diagnostic purposes.
- When the kit is used within the territory of any Member State of the European Union, should there be any serious incident regarding its use, the user must report it to the manufacturer (*regulatory@bdrdiagnostics.com*) and the Competent Authority of their country and/or the patient´s country. Serious incidents reporting for all other countries must be conducted according to local requirements for each country.
- Blackhills Diagnostic Resources S.L.U. products should not be resold, modified for reselling or be used to manufacture other commercial products without written consent of Blackhills Diagnostic Resources S.L.U.
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17 – Changes control

Version	Description of the modification
Rev. 00	First version of the document
Rev. 01	Correction of errors in the "Procedure" section.
Rev. 02	Correction of writing and translation errors. New sections: *Accuracy*, *Information for safety*, *Changes control* and *Explanation of symbols used on the labels*. Information related to the intended user and intended patient. New document format.
Rev. 03	Correction of writing and translation errors. More information added in the "Procedure principles" and "Results and interpretation" sections. Changes in the recommended genetic analyzer parameters, adding the SeqStudio™ instrument.

18 – Explanation of symbols used on the labels

	*In vitro* diagnostic medical device		Expiration date
	Catalogue number		Contents sufficient for <n> tests
	Lot number		Manufacturer
	Temperature limit		Keep away from sunlight
	Consult electronic Instructions For Use document		
	This product fulfils the requirements of Directive 98/79/EC on *in vitro* diagnostic medical device		