adellgene

ADELLGENE FRIEDREICH's ATAXIA

INSTRUCTIONS FOR USE

Rev. 05 - 2019/05/27

Kit for the determination by fluorescent fragment analysis of the presence of healthy, premutated and mutated alleles of the FXN gene of Friedreich's ataxia

CE

Catalog No. AD-FA-16

Storage: Box 1 from -30ºC to -18ºC

Box 2 from 20ºC to 25ºC

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1-Intended Use

Adellgene Friedreich's Ataxia is a kit for In Vitro Diagnostic designed for detecting the number of repetitions of GAA in the first intron of the gene encoding frataxin (FXN) gene located in chromosome 9 resulting in Friedreich's ataxia disease. It aims to aid clinical diagnosis associated with clinical findings in Friedreich's ataxia that span from mild to severe symptoms.

The use of this kit is the determination of healthy individuals who have between 5 to 30 GAA repeats, patients with mild phenotype (30-49 repeats), and severe (50-1300). The technology is based on the amplification chain reaction (PCR) of genomic DNA extracted and purified from peripheral blood followed by fluorescence analysis of the size of the PCR fragments obtained by genetic analyzer and conversion of that size in the number of GAA repeats. The kit consists of two Primer Mixes (PM): Primer Mix 1 for amplification of all samples, detecting heterozygous and possible homozygous alleles with a size less than 200 GAA repeats; and Primer Mix 2 for confirmation of the homozygous samples and the detection of false homozygous for the occurrence of a higher range allele obtainable with PM1.

2-Summary and explanation

Friedreich's ataxia (1) is the most common hereditary ataxia, with an estimated prevalence of 1 in 50000 and a deduced carrier frequency of 1 in 120 in Europe populations (2). FRDA is an autosomal recessive degenerative disease characterized by a progressive gait and limb ataxia, a lack of tendon reflexes in the legs, loss of position sense, dysarthria, and pyramidal weakness of the legs (3). Hypertrophic cardiomyopathy is found in almost all patients (4). Diabetes mellitus is seen in about 10% of the patients (5). The age of onset is usually around the puberty and almost always before age 25 and there is no treatment to slow the progression of the disease.

The most common DNA abnormality associated with Friedreich's ataxia (FRDA; 6) is the expansion of a GAA triplet repeat polymorphism localized within an Alu sequence in the first intron of the gene encoding frataxin (FXN), on chromosome 9q13.

About 98% of individuals with FRDA are homozygous for an expansion of a GAA trinucleotide repeat sequence within the first intron of the FXN gene. The remaining individuals are compound heterozygotes for a GAA expansion and a point mutation. Pathogenic GAA expansion alleles are in the size range of 50 to >1300 repeats with three different intervals, healthy (between 5-30 repeats), with mild symptoms (30-49 repeats) and with severe symptoms (50-1300 repeats) (see Table 1).

Clinical condition	Phenotype	GAA Repeat Size
Healthy	Normal	5 to 30
Mild	Premutation	30 to ~49
Severe	Full mutation	~50 to ~1300

Table 1. Correlation of phenotype and GAA Repeat Length in Friedreich's ataxia

The presence of a GAA repeat expansion results in the inhibition of FXN gene expression, reduced levels of full-length FXN transcript, and an insufficiency. The formation of a persistent RNA/DNA hybrid during transcription of the GAA repeat sequence appears to impede transcription elongation (7). It is now apparent that the GAA repeat expansion also generates a heterochromatin-mediated gene silencing effect (8, 9). Age-dependent and tissue specific somatic instability of the GAA repeat expansion may also be a determinant of the progressive pathology of FRDA (10-12).

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Repeats in normal chromosomes are stable when transmitted from parent to offspring and of equal size in all tissues (6,13,14), while hyperexpanded, disease-associated repeats show meiotic as well as mitotic instability (14-16). Analysis of FRDA families (6, 15) has shown that maternally transmitted expansions contract or expand with equal frequency, while paternal transmission almost always results in size contraction (15). Mitotic instability has been demonstrated in different cell types from the same patient (16). In particular, most brain regions show a very complex pattern of allele sizes, indicating extensive heterogeneity (14). Hyperexpansion of the GAA repeat leads to suppression of frataxin gene expression, probably through a directional blockade of transcriptional elongation resulting from the formation of a non-B DNA structure (17).

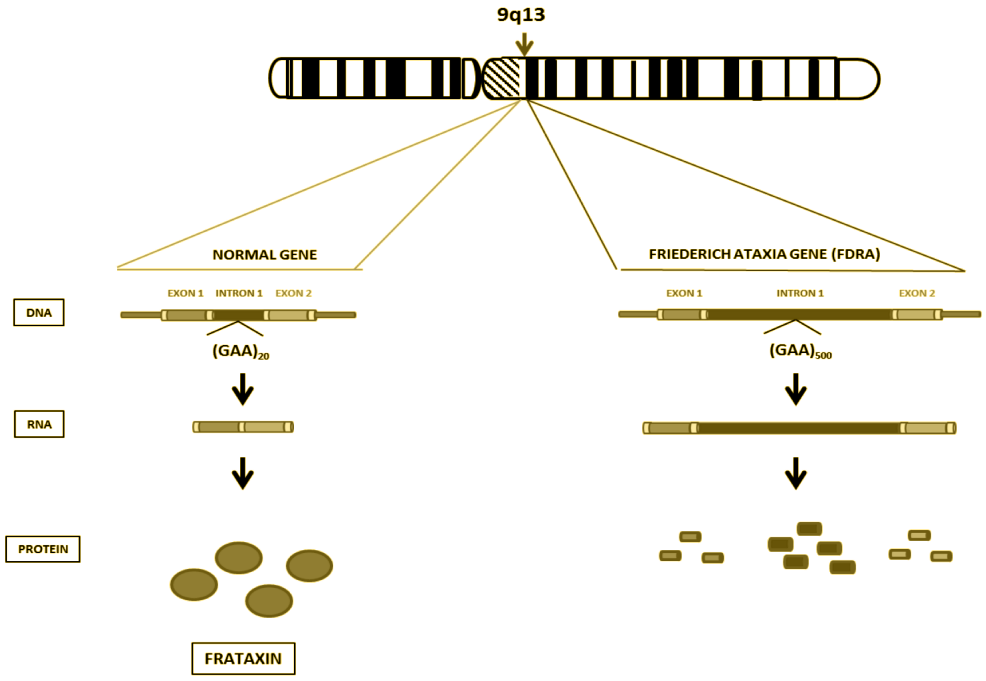

Figure 1. Representation of the FXN gene types and their production depending on the degree of mutation.

Such a loss-of-function pathogenetic mechanism fits the model for a recessive disease. Furthermore, the residual amount of frataxin mRNA and protein is inversely proportional to expansion sizes (Campuzano et al., submitted). This graded effect provides a biological basis for the correlation between expansion sizes and phenotypic features, including age at onset as well as severity and extent of disease involvement, that has been determined in several studies (16, 18, 19). The underlying process by which hyperexpansion of normal alleles occurs in FRDA remains unknown. The finding of strong linkage disequilibrium between FRDA and neighboring markers (20-22) suggests that either a few, possibly even a single, ancestral events gave rise to the FRDA expansion, or that there are recurrent expansions in alleles at risk (23). The second hypothesis is supported by

the observation of a de novo hyperexpansion of a premutated FRDA allele, which we report here. In addition, to obtain clues about possible mechanisms responsible for the expansions and polymorphisms of normal alleles, we describe the characteristics of the Alu sequence containing the GAA triplet repeat polymorphism, and the size distribution and structure of normal alleles.

Adellgene Friedreich's Ataxia PM1 is optimized for the detection of about 200 GAA repeats so as to quantify the number of repeats of heterozygous healthy individuals (5-30 repeats), heterozygous mild patients (30-49 repeats), and heterozygous severe patients (50-1300, but only until 200 repeats). PM2 is designed to perform a TP-PCR on the samples which result in only one peak with PM1, confirming this way the homocigosity or the presence of a mutated allele. Both determinations are based on the analysis of fragments generated with fluorescent primers and capillary electrophoresis in a genetic analyzer, and the subsequent analysis with appropriate software for detection and interpretation.

3-Principles of the procedure

The detection method employed by the Adellgene Friedreich's Ataxia kit is based on the specific amplification from genomic DNA of the first intron of the gene encoding frataxin (FXN), which containing the GAA repeats. Primer Mix 1 includes 2 primers, one of which is labeled with a fluorophore for subsequent detection in a DNA fragment analyzer. The size of PCR products are converted into the number of GAA triplet repeats using conversion factors of mobility and size. The use of ROX1000 size marker is recommended. Primer Mix 2 includes a set of primers to do a triplet primed repeat PCR to test all the repeats.

It is recommended to use a positive control with a control sample which the number of repetitions is known (see section 6).

4-Kit contents

Reference AD-FA-16

Box 1 of 2

- AD-FA-PM1: Primer Mix 1. 1 vial x 79 µL
- AD-FA-POM1: Polymerase Mix 1. 1 vial x 344 µL
- AD-FA-PM2: Primer Mix 2. 1 vial x 162 µL
- AD-FA-POM2: Polymerase Mix 2. 1 vial x 344 µL

Box 2 of 2:

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

5-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 the reagents (except AD-FA-POM1 and AD-FA-POM2) to reach room temperature before use. Shake gently all reagents except AD-FA-POM1 and AD-FA-POM2 after thawing.
- Before to open the reagents tubes, perform a gentle centrifugation of each component so that the reagents are deposited on the bottom of the tube and no loose by the walls thereof.
- The test should be done keeping reagents on ice or cold block.
- Do not make more than 3 cycles of freeze / thawing to the vials of Primer Mix (AD-FA-PM1 and AD-FA-PM2), and Polymerase Mix (AD-FA-POM1 and AD-FA-POM2) as this may reduce the sensitivity of the test and alter the results.
- Due to the photo-sensitive nature of the reagents AD-FA-PM1 and AD-FA-PM2, avoid continued exposure to light.

6-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 that the DNA is intact.
- 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 or equivalent)
- Size standard marker ROX1000™ (ABI, Ref.: 401098; Eurogentec, Ref.: MW-0195-80ROX)

Supplies and equipment

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

Positive Control

- The recommended WHO standard for Friedreich's ataxia or any cell line whose DNA corresponds with validated sample.

7-Sample collection and preparation

- All biological and blood samples should be treated as potentially infectious. When handling, 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
- DNAses contamination can produce DNA degradation, so should be used with filter pipette tips and DNAse-free tubes. To take care with handling, pipetting and working systems to avoid PCR failure.
- Do not use components from different lots. Do not use reagents beyond the expiration date.
- Before to use the kit, to ensure that the equipment (thermal cycler, genetic analyzer, ...) has been calibrated according to the manufacturers´ instructions.

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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8-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 40-50 ng (e.g.: 1µl of DNA to 40-50 ng/µl).

B. Preparation and conditions of the PCR reaction

Precautions

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

1. Mix preparation of the Primer Mix and Polymerase Mix for n+1 samples. Use components 1 for the analysis of regular samples. Use components 2 with the purpose of confirming the samples showing only one amplification peak with test 1 (see sections 1 and 2).

Reagent	Vol. for each sample (ul)
AD-FA-PM1	4.3
AD-FA-POM1	18.7
Total volume for each reactions	**23**

Reagent	Vol. for each sample (ul)
AD-FA-PM2	8.8
AD-FA-POM2	18.7
Total volume for each reactions	**27.5**

- To shake the sample gently 3-5 times before distribution in PCR tubes ensuring of the complete homogenization
- To perform a pulse centrifugation to ensure that there is no sample in the tube walls or bubbles.

NOTE: Excess AD-FA-PM1/2 could inhibit the PCR reaction

2. To pipette 23 and 27.5 uL of this mixtures, respectively, on a plate or sterile tube and add DNA volume subsequently needed to get 45-50 ng (p.e.: 1 µl of DNA to 45-50 ng/µl) or negative control in the case of contamination control well. In the case of low DNA concentration, a higher volume of DNA could be added independently of the increase of the total volume of the PCR reaction.

3. To seal the plate with coverslips (including with plates) or the tube and perform a pulse centrifugation to ensure mixing and there are no bubbles.

4. To place the plate in the thermo cycler and perform the PCR reaction.

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	Number of cycles	Temperature (ºC)	Time (mm:ss)
DENATURATION	1	98	05:00
CYCLES	35	97	00:45
		64	00:45
		68	03:00+ 00:10SEC/CY
FINAL EXTENSION	1	72	30:00
COOLING	1	4	∞

C. Confirmation of the amplification products

Confirmation of the amplification products could be made through an appropriate system as the horizontal electrophoresis in agarose gels. To prepare an agarose gel at a concentration of 1-1.2% w / v validated according to the lab protocol and analyze 2ul of each amplified to certify that the PCR was successful.

D. Preparation of the samples for capillary electrophoresis

1. Purification of the PCR reaction

WARNING

Perform this step only in case of being processing components 2 amplification products. If you are analyzing amplicons resulting from components 1, go to next section D.2.

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- Use Box 2 of the kit (Purification reagents). To use a PCR volume of 18 µl to obtain a purified PCR volume of 18 µl. These volumes are restricted to obtain a good result in the fragment analysis.

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.

a. Add 500 ul Capture Buffer to the sample (to use 18 ul of PCR volume). If necessary, transfer the PCR product to a bigger tube.

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 Microcolumn 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 Microcolumn back inside the Collection Tube.

i. Add 500 ul Wash buffer to the Microcolumn.

j. Spin the assembled column and Collection Tube at 16000 g for 30 seconds.

k. Discard the Collection Tube and transfer the Microcolumn to a fresh DNase-free 1.5 ml microcentrifuge tube (provided by user).

l. Add 18 ul Elution Buffer to the center of the membrane in the assembled Microcolumn and sample collection tube.

m. Incubate the assembled Microcolumn 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 the general protocol.

2. Preparation of Samples for DNA analyzer

The following mixture must be performed with the PCR product:

Reagent	Vol. for each sample (µl)
Reaction 1 PCR purified product	2 *
Hi-Di-Formamide	10
Marker ROX1000™	0.5
Total volume for each reactions	**12.5**

** It is recommended to use 2-3ul the PCR product for the 3130/3130XL and 1-2 ul for the 3730xl DNA analyzer, or to check the optimal volume between 1 or 3 ul to charge in the DNA analyzer.*

Reagent	Vol. for each sample (µl)
Reaction 2 PCR purified product	1 *
Hi-Di-Formamide	10
Marker ROX1000™	0.5
Total volume for each reactions	**11.5**

** It is recommended to use from 1 ul to 2ul for the 3130/3130XL and from ½ to 1ul for the 3730xl DNA analyzer or to check the optimal volume between 1/2 or 2 ul to charge in the DNA analyzer for the purified product.*

- To perform a pulse of centrifugation (3-5 times) to ensure mixing of the reagents and transfer 2 µl the mixture to the corresponding plate for capillary electrophoresis

- To seal the plate, shake and centrifuge to remove bubbles and transfer to the thermal cycler.
- To denature 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 with a control sample which the number of repetitions is known (see section 6).

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 uAmps	5.0 uAmps
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

* *In both cases is recommended the use of polymer POP7*

9-Results and interpretation

1. Component 1 test

Adellgene Friedreich's Ataxia component 1 is a quantitative technique for identifying the number of GAA triplet repeats between 5 and at least 200 (see section 2).

The kit includes a serie of reagents that provide amplification of this region and allows determining the number of repetitions to establish the healthy and unhealthy individuals. Moreover, samples that give only one peak will have to go to a later study by the components 2 of kit to assure or discard the homozygosis of the sample.

Using the reagents supplied in this kit, and in the conditions described in the previous section (8.D.3), if the sample has a number of 5 GAA repeats, the size of the amplified fragment will be of 301 bases (Table 1). All other number of repetitions obtained with this component of the kit may be tabulated based on this size. The introduction of fragments marker allows to the genetic analyzer software gives us the size of the amplified directly and therefore we can get the number of GAA triplet repeats. It is not necessary to use any passive reference.

In any case the use of a control sample with a known number of GAA repeats, determined by sequencing, is strongly recommended. The conversion of the fragment size into number of repeats of samples of the batch, must be done extrapolating the information of the control sample.

In Figures 2, 3, and 4 are expressed fragment analysis graphs of components 1 results for a homozygous healthy individual (Fig.2), heterozygous healthy individual (Fig.3), and unhealthy individual (Fig.4).

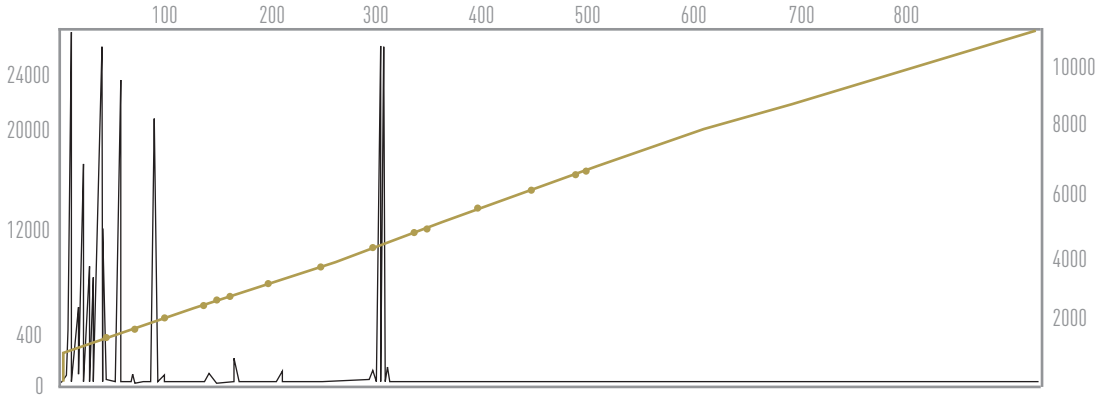

Figure 2. Homozygous healthy individual (3 rep, 3rep)

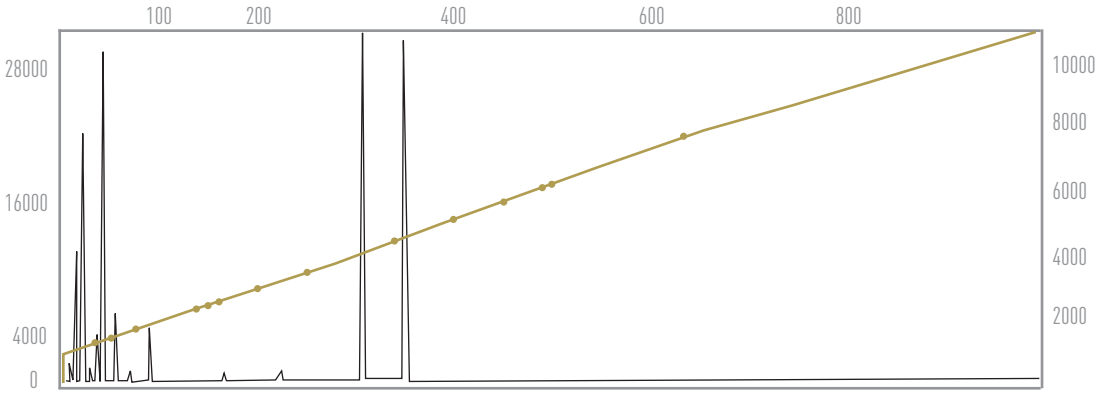

Figure 3. Heterozygous healthy individual (3 rep, 16 rep)

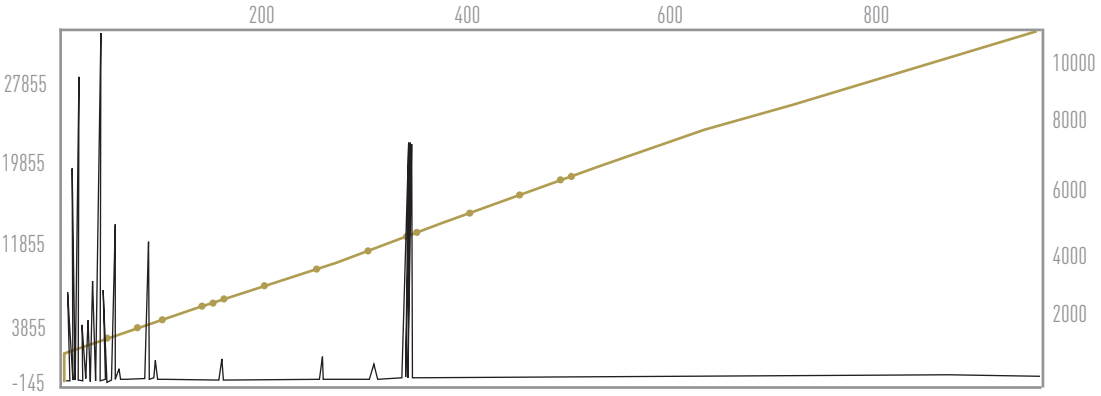

Figure 4. Heterozygous carrier individual (15 rep, >200 rep (expanded))

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Table 1. Correlation between the size fragments and the number of GAA repeats

GAA repeats	Size fragment	GAA repeats	Size fragment
5	301	105	601
10	316	110	616
15	331	115	631
20	346	120	646
25	361	125	661
30	376	130	676
35	391	135	691
40	406	140	706
45	421	145	721
50	436	150	736
55	451	155	751
60	466	160	766
65	481	165	781
70	496	170	796
75	511	175	811
80	526	180	826
85	541	185	841
90	556	190	856
95	571	195	871
100	586	200	886

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

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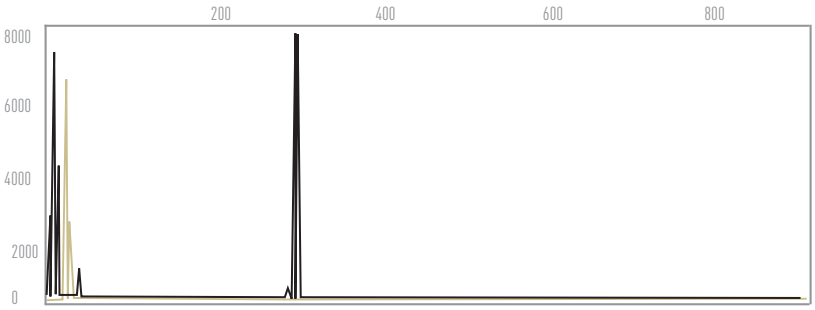
2. Component 2 test

Adellgene Friederich Ataxia component 2 is a complement technique for confirming the number of GAA triplet repeats obtained from the test 1. In the case of one sample have only one peak from PM1 reagents, we should assure or discard the homozygosis of this sample using the components 2.

The resulting electropherogram of components 2 show the full length PCR products generated from the GAA repeat primed peaks. These peaks are separated by 3 bp. The profile of peaks indicate the information about the sample: only one peak correspond to an allele ≤8 GAA; group of peaks starting approx. at 170 bp with no declination pattern correspond to a normal length allele; decreasing peak's saw along the electropherogram correspond to a >200 GAA repeats.

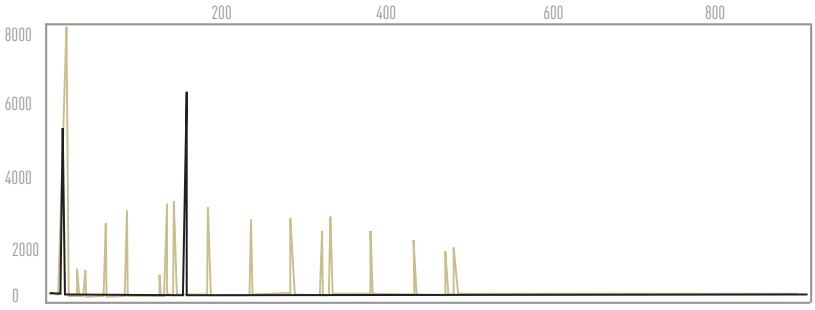
In Figures 5, 6 and 7 are expressed fragment analysis graphs of components 1 and 2 resulting for a homozygous healthy individual (Fig.5), heterozygous healthy individual (Fig.6) and heterozygous unhealthy individual (Fig.7).

Figure 5. Homozygous healthy individual (8 rep, 8 rep)



Component 1 results

Component 2 results



NOTE: Alleles with a number of GAA repeats ≤8 will show a pattern of only 1 peak in component 2 electropherogram.

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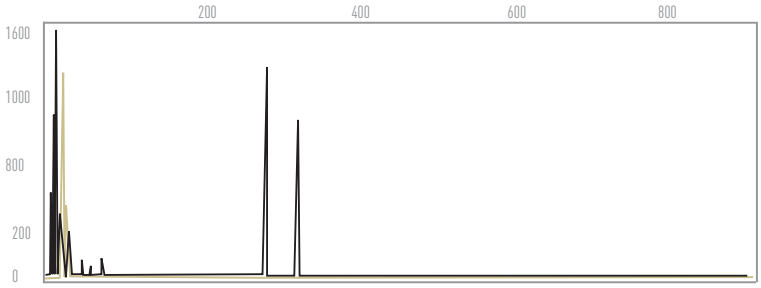
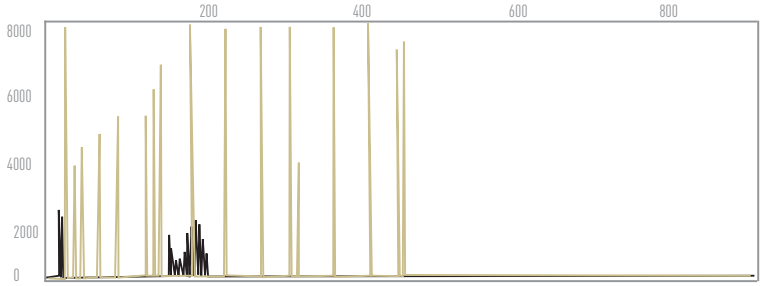
Figure 6. Heterozygous healthy individual (10 rep, 24 rep)



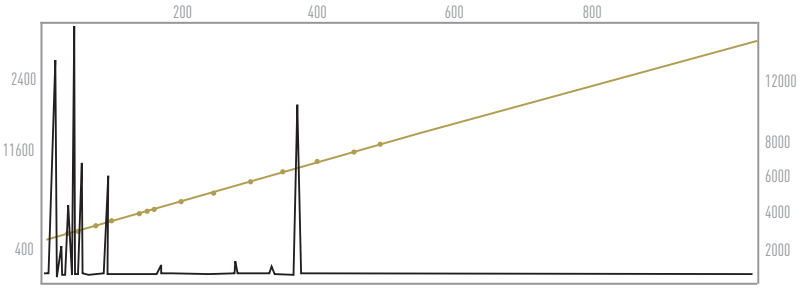
Component 1 results

Component 2 results



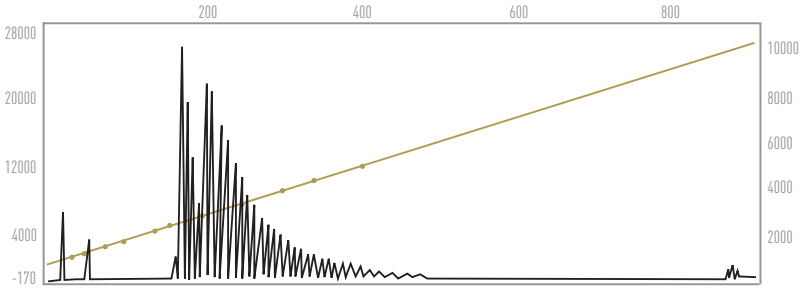
NOTE: Alleles with a number of GAA repeats ≥8 will show a pattern of several peaks in component 2 electropherogram, corresponding to the full length PCR products generated from the GAA repeats primed peaks. These peaks are separated by 3 bp.

Figure 7. Heterozygous carrier individual (15 rep, >200 rep (expanded))



Component 1 results

Component 2 results



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10-Quality control

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

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

A contamination control should carry out by replacing the DNA for a negative control and a positive control of a known size (see section 6).

The user should consider all precautions named in paragraph 7 and the limitations in paragraph 12 of this procedure.

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

11-Specific data of performance

1. Analytical specificity and sensitivity

The primers of this kit are specific of the human Friedreich's ataxia gene FXN and comprise the GAA repeat region within of intron 1 of the gene. The specific amplification of this region of the FXN gene was verified by DNA sequencing and by assaying samples of healthy individuals and patients well characterized. There is no reported case of cross-reactivity with another gene from genomic DNA.

2. Diagnostic specificity

Adellgene Friedreich's Ataxia kit is intron 1 FXN specific assay to detect the number of GAA repeats. Mutations (point mutations, insertions, deletions) at amplification primer sites are possible and may result of the lack of allele definition. Other technologies could be necessaries to resolve the typing.

3. Range of

- DNA concentration

  For the best range of amount of DNA in each assay tests were performed between 5 ng to 200 ng with a heterozygous sample. The assignment of peaks obtained size was independent of the amount of DNA used. The recommended working range is between 40-50 ng of DNA by PCR.

- Results of the kit

  The method of this kit can assign a number of GAA triplet repeats of between 5 and 200 repeat alleles. The use of this kit is the determination of healthy individuals who have between 5 to 30 GAA repeats, patients with mild phenotype (30-49 repeats), and severe (50-1300).

4. Accuracy

- Assignment of fragment size in test 1
- 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 central peak. The accuracy of the number of the repeats was determined comparing sequenced samples with the sizes obtained with the present kit comprising a variability of No. of repeats ± 1 repeat for normal alleles and ±3 repeats for expanded alleles.

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- General testing

Region	Intron 1/FXN
Number of samples tested	68
Number of healthy samples	54
Number of carrier individuals	8
Number of unhealthy individuals	6

- Interferences

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

12-Procedure limitations

- The method quantify the size of all alleles of between 5 and 200 GAA triplet repeats, and it is capable to detect the presence of alleles over this size (see section 9).
- The conditions described for the PCR should be accurately controlled. Deviations from these parameters can lead to poor outcome.
- All work from Adellgene Friedreich's Ataxia kit must be made in accordance with good laboratory practices and in compliance with local regulations, such as the international standard.
- The thermo cycler 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 exceeded.
- Do not use the kit in case of suspected loss of reactivity, contamination, deterioration of the container or any other incident that may affect performance.
- The interpretation of the data and typing results should be reviewed by qualified personnel.
- Remove expired reagents following applicable regulations.

13-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 as 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).
  - Confirm that the Taq is not contaminated.
- **Pipetting error**
  - Verify that the sample added in a given position 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 an drepeat PCR wher possible.
- **DNA polymerase not added to master mix or insufficient mixing of PCR mix**
  - Repeat PCR ensuring all components are added and mixed sufficiently

- 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 colorants) is added
  - Ensure ethidium bromide is added to 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 descontamination 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 freeze / thaw of reagents
  - Perform aliquot fractions reagents as necessary.
  - Repeat with fresh reagents batch
- Taq polymerase activity lost
  - Confirm the activity of Polymerase Mix
  - Repeat with a new vial of Polymerase Mix.
- 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

Negative control simple yields a positive result

- Cross-contamination
  - Handle always the kit components according to commonly accepted practice to avoid contamination.
- Pipetting error
  - Verify that the sample added in a given position always corresponds to the assigned worksheet

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 background (noisy baseline)

- PCR product contaminated
  - See above
- Poor PCR purification
  - Ensure purification process is made according manufacturers' instructions.

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