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Instructions for Use

imegen™ Friedreich

GAA expansion detection in FXN gene by PCR and TP-PCR

REF IMG-155

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Imegen is not liable for any damage, direct or indirect, resulting in economic loss or resulting from the use of this product by the purchaser or user.

All products sold by the Imegen are subjected to rigorous quality control. The **imegen-Friedreich kit** has passed all internal validation tests, ensuring the reliability and reproducibility of each assay.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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Amendments to the Instructions for Use (IFU)	
Versión 02	Amendment: Contents review

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Index

1. General Information

Friedreich's ataxia (FRDA, MIM # 229300) is the most frequent hereditary ataxia, affecting approximately 1 in 29,000 individuals. It presents a pattern of autosomal recessive inheritance and in most of patients (98 %) is due to tri - nucleotide GAA repetition homozygous expansion in the intron 1 of the FRATAXIN gene (FXN or X25 ; MIM * 606 829), located on the chromosomal region 9q13 .

The hallmark clinical features of FRDA include progressive afferent and cerebellar ataxia, dysarthria, impaired vibration sense and proprioception, absent tendon reflexes in lower limbs, pyramidal weakness, scoliosis, foot deformity and cardiomyopathy.

The diagnosis of FRDA is established by the identification of the number of GAA trinucleotide, being less than 33 the normal genotype. Table 1 shows the range of GAA-repeats in the normal and the mutated alleles.

Allele	(GAA)n* Repetitions number
Normal	< 33
Pre-mutated	34-65
Pathological	66-1300

Table 1. Information about the analysed expansions in the imegen-Friedreich kit

References

- Consensus clinical management guidelines for Friedreich ataxia. Louise A Corben, David Lynch, Massimo Pandolfo, Jörg B Schulz, Martin B Delatycki, On behalf of the Clinical Management Guidelines Writing Group Orphanet J Rare Dis. 2014; 9: 184. Published online 2014 Nov 30. doi: 10.1186/s13023-014-0184-7

2. Intended Use

imegen-Friedreich kit has been designed to identify the GAA expansion in the intron 1 of the FRATAXIN gene by PCR, TP-PCR and subsequently capillary electrophoresis. Besides, the kit offers a TP-PCR (triplet repeat primed PCR) system, for the samples bearing larger expansions, not detectable with conventional PCR.

PCR products will be separated by capillary electrophoresis and both the PCR as well as the TP-PCR will be detected by the 6-Carboxifluoresceina (6-FAM) labelling.

The triplet repeat primed PCR (TP PCR) assay uses a locus-specific primer flanking the repeat together with paired primers amplifying from multiple priming sites within the repeat, allowing thus by PCR and subsequently capillary electrophoresis, the detection of the expanded alleles undetectable by conventional PCR.

imegen-Friedreich kit has been designed for in vitro diagnostics and it is directed to professionals from the molecular biology sector.

3. Technical characteristics

This kit has been validated using reference EMQN samples, 2016, as well as using samples previously analysed in the Medical Genetic Unit of imegen. The kit provides robust and specific detection of the expansions for which has been developed.

The type of sample required for this analysis is genomic DNA extracted from peripheral blood, a total quantity of 50 ng will be necessary.

This product complies with the quality requirements established by ISO 9001, both in its validation and manufacturing process as well as in the materials used.

4. Warnings and precautionary statements

1. Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.

2. Do not pipette by mouth.

3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

4. You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.

5. Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.

6. In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.

7. The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to imegen.

8. This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.

9. Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.

10. This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.

11. The manufacturer is not responsible for the malfunction of the assay when one or more reagents included in the kit are replaced by other reagents not supplied by Imegen.

12. The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Imegen, considering them equivalent to those provided in the Kit.

5. Contents and Storage Conditions

The kit includes the following reagents, enough to analyse 12 samples:

- Friedreich Master Mix A: PCR Master Mix containing $MgCl_2$, nucleotides and Buffer solution, required for the PCR reaction.
- Friedreich Master Mix B: PCR Master Mix containing $MgCl_2$, nucleotides and Buffer solution, required for the TP-PCR reaction.
- PCR Master Mix and TP-PCR Master Mix: PCR Master Mix containing the oligonucleotides to perform the amplification of the target regions.
- Friedreich Taq: DNA polymerase required for the PCR reaction.
- General Master Mix IV: PCR Master Mix containing DNA polymerase and Buffer solution, required for the TP-PCR reaction.

Reagents	Colour	Amount	Storage
Friedreich Master Mix A	White pad	225 µl	-20°C
Friedreich Master Mix B	Yellow pad	225 µl	-20°C
PCR Master Mix	Red pad	66 µl	-20°C
TP-PCR Master Mix	Blue pad	66 µl	-20°C
Friedreich Taq	Orange cap	10 µl	-20°C
General Master Mix IV	Yellow cap	5 µl	-20°C

Table 2. imegen-Friedreich kit contents

6. Equipment, reagents and materials required but not supplied

Equipments:

- Thermal Cycler
- Micropipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Vortex
- Centrifuge
- Sequencer

Reagents:

- GeneScan™ 500 LIZ® (Applied Biosystems cat. no. 4322682)
- Hi-Di™ formamide

Materials:

- Disposable micropipette filter tips (10 µL, 20 µL and 200 µL)
- 1.5 mL sterile tubes
- 96-well plates or 0,2 mL tubes
- Films for 96-well plates
- Powder-free latex gloves

Note: This kit does not contain the reagents to perform capillary electrophoresis.

6.1 Related Kits

In order to analyse expansions involved in other neurodegenerative diseases imegen offers the kits imegen-SCAs (ref IMG-152), imegen-DM1 (ref IMG-173), imegen-Huntington (ref IMG-154) and imegen-SBMA (ref IMG-153).

All these kits, including imegen-Friedreich, has been designed to amplify with the same PCR program, for a simultaneous analysis.

7. Assay Protocol

7.1 PCR reactions preparation

imegen-Friedreich Kit is designed to perform 2 reactions per sample (1 PCR and 1 TP-PCR).

In order to estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples to be simultaneously analysed, and then considering one more reaction, or increase a 10% the volume of each reagent.

Recommended protocol for preparation of amplification reactions is showed below:

1. Thaw all reagents contained in the kit and samples DNA.
2. Vortex each reagent and keep cold.
3. In order to prepare the PCR mix, add into a 1.5 mL tube, the following reagents:

Reagents	Amount per reaction
Master Mix Friedreich A	17 µL
Taq Friedreich	0.5 µL
PCR Master Mix	5 µL

4. In order to prepare the TP-PCR mix, add into a 1.5 mL tube, the following reagents:

Reagents	Amount per reaction
Master Mix Friedreich B	17.3 µL
General master Mix IV	0.5 µL
TP-PCR Master Mix	5 µL

5. Vortex and spin PCR and TP-PCR mix tubes and dispense 22.5 µL into corresponding 0.2 mL tubes.
6. Add 2.5 µL of sample DNA at 10 ng/µL into corresponding wells. It is recommended to add a PCR negative control in order to rule out PCR contamination, and also positive controls to verify the allele's sizes.
7. Place the samples in a thermal cycler and perform the following PCR programme:

- Optimal Programme:

Fields	Step 1 Enzymatic activation	Step 2 PCR or TP-PCR			Step 3	
Cycle Number	1st Cycle	30 cycles			1 cycle	
		Denaturation	Annealing	Extension	Final step and storage	
Temperature	94°C	94°C	60°C	72°C	72°C	4°C
Time	5 minutes	1 minute	1 minute	2 minutes	10 minutes	∞

Table 3. PCR optimal program.

Note: This program has been validated on Biometra T3 equipment and GeneAmp® PCR System 2720 (Applied Biosystems).

At this point of the protocol can be stopped. If the second PCR will be carried out within the next 24 hours, keep the PCR product at 4°C. Otherwise, store at -20°C until it is time for their use.

7.2 Fragments analysis preparation

In order to perform the expansions analysis, a fragment analysis is required. Thus, a fragments plate with PCR and TP-PCR products has to be prepared. Recommended protocol for its preparation is showed below:

1. Add into a 1.5 mL tube, the following reagents:

Reagents	Amount per reaction
Formamide	18 µL
GeneScan™ 500 LIZ marker	0.5 µL

To estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples to be simultaneously analysed, and then considering one more reaction, or increase a 10% the volume of each reagent.
Note: The size-marker volume can be increased or decreased to adjust peaks intensity.

2. Dispense 18.5 µL of the Fragment plate Master Mix in each well.

3. Add 1 µL of the DNA obtained in PCR and TP-PCR reactions.
   Note: The volume of the sample can be increased or decreased (by diluting DNA sample) to adjust peaks intensity.
4. Cover and spin the plate and denature in a thermal cycler for 5 minutes at 98°C.
5. Store the plate at 4°C until the moment of the capillary electrophoresis.

7.3 Capillary electrophoresis

Once prepared fragments plate, reactions should be subjected to capillary electrophoresis. Depending on the type of sequencer used, electrophoresis conditions recommended by the manufacturer shall be used.

To set these conditions, it should be taken into account that amplification range varies approximately between 150 - 650 bp, that are employed FAM-labelled primers and that molecular weight standard is labelled with GeneScan™ 500 LIZ.

The following image shows the optimized parameters for the 3730xl DNA Analyzer sequencer, with POP-7™ polymer.

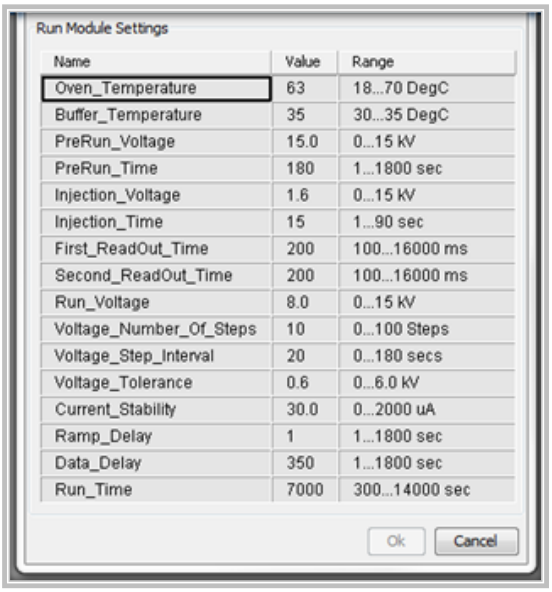

Figure 1. Optimised parameters for the 3730xl DNA Analyzer sequencer

Detection intensity may vary between different equipment, depending on the model and the conditions of the equipment optical system. Therefore, in some cases it may be necessary to dilute the samples before performing capillary electrophoresis.

8. Results analysis

Once the analysis results are displayed in the analysis software, in order to calculate the exact number of repetitions of an unknown allele, the following formula can be used:

$$Repetitions\ Number = \frac{Size_{Allele\ x}(bp) - 244}{3}$$

Note: the value "244" in the formula comes from a size conversion of the amplicon obtained with the oligonucleotides designed for the kit, and it has been validated in silico as well as in the laboratory.

It has been described variation in one repetition between different laboratories, depending on reagents and capillary electrophoresis equipment used for fragment analysis. Therefore, we recommend to use a sample with a known repetition size (for example: 36 repetitions):

$$Repetitions\ Number = \frac{Size_{Allele\ x}\ (pb) - Size_{Allele\ 8\ rep.}}{3} + 8$$

In the following image samples with different GAA expansions are shown:

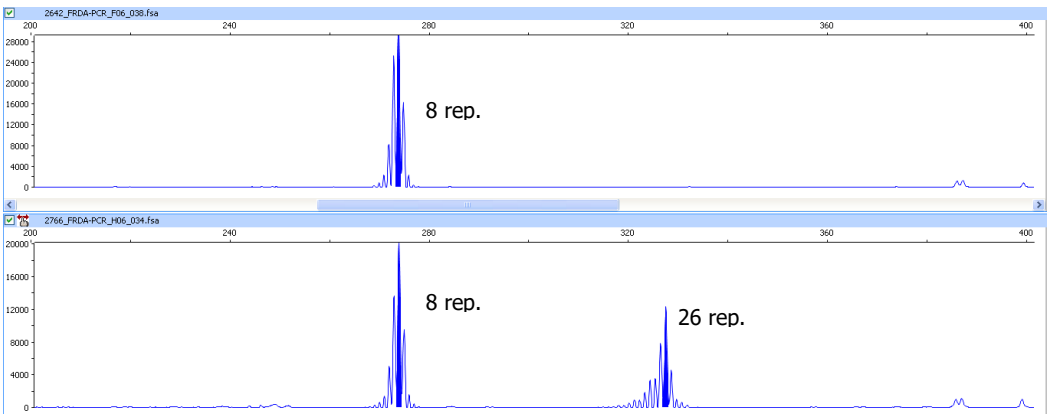

Figure 2. Examples of the obtained results with the PCR mix of the imegen-Friedreich kit

8.1 TP-PCR results

In those samples where only one allele is detected by using the PCR assay, this result could be due to the sample being homozygous for this allele or the sample being heterozygous with a wild type allele and an expanded allele undetectable by conventional PCR.

In order to differentiate between samples which are homozygous for the normal alleles and samples with a normal allele and an undetected expanded allele, the TP-PCR assay (Triplet Repeat primed polymerase chain reaction) of **imegen-Friedreich kit** has been developed.

As this technique uses a fluorescently labelled locus-specific primer and paired primers amplifying from multiple priming sites within the repeat, it allows the detection of expansions of any size, although smaller allele identification could be complicated and generally does not allow determining the number of repetitions.

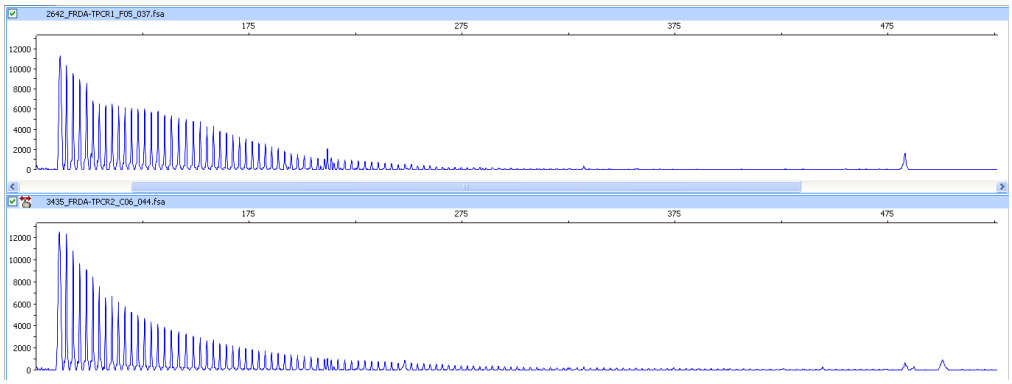

Figure 3. TP-PCR results corresponding to *FRATAXIN* expanded alleles.

9. Troubleshooting

The following table resume problems or difficulties which can occur in the results of the samples, Positive control, the size marker and the negative control. In the eventuality of an unexpected result check for the probable cause in the table below.

Symptoms	Analysed sample	Size Marker	Negative control	Possible causes
Weak fluorescent signal for allele peaks			√	Expected result
	√		√	Insufficient/poor quality DNA template. [1] Impure DNA template.[2]
	√	√	√	Poor capillary electrophoresis injection.[3] Samples were not properly denatured before loading.[4]
	√		√	Thermal cycler or tube problems.[5]
Excessive fluorescent signal for allele peaks	√			Too much template DNA.[6]
	√			
Presence of more peaks than expected	√		√	Contamination.[7]
	√			Contamination.[7] Mosaicism.[9]
	√			Artefacts typical of expansions.[8]

Table 7. Problems or difficulties in the results of the imegen-Friedreich Kit

[1] **Insufficient/poor quality DNA template:** Make sure DNA has been accurately quantitated and use the recommended amount of template DNA. In the case of the DNA was correctly quantified, check for DNA integrity and eventually repeat DNA preparation.

[2] **Impure DNA template:** High salt concentration or altered pH can inhibit PCR amplification. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover from DNA sample of K+, Na+, Mg2+ or EDTA can negatively affect PCR. Changes in pH also may affect PCR. If it's so, clean the DNA sample or repeat DNA preparation.

[3] **Poor capillary electrophoresis injection:** Check if the instrument parameters are the specified ones and re-inject sample.

[4] **Samples were not properly denatured before loading:** Heat-denature samples for the recommended time (section 7 of this manual) and cool on crushed ice or in an ice-water bath immediately prior to loading.

[5] **Thermal cycler or tube problems:** Check if PCR program is the specified one.

[6] **Too much template DNA:** Make sure DNA was accurately quantitated. If it is so, dilute the PCR product in sterile deionized water and repeat sample denaturation and loading.

[7] **Contamination:** It may be caused by another template DNA or a previously amplified DNA. Cross-contamination can lead to false positives or negative results, and consequently to problems in results interpretation. Use aerosol-resistant pipette tips, and change gloves regularly

[8] **Artifacts typical of expansions:** Amplification of expansions generates artifacts that appear as smaller peaks 3bp above or below the prominent mononucleotide repeat allele

[9] **Mosaicism:** Mosaic patients have been described in FRIEDREICH, therefore it is possible to find more than one genotype in the same sample. In this case we recommend to repeat the PCR and, if the result is the same, use a patient's different sample, possibly from a different tissue (ex. oral swab).

10. Limitations

10.1 Equipment

imegen-Friedreich kit has been validated using the following PCR Thermal Cyclers:

- SimpliAmp Thermal Cycler (ThermoFisher Scientific)
- GeneAmp PCR System 2720 (ThermoFisher Scientific)
- T3000 Thermocycler 48 (Biometra)

If you use another brand or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical service for any query or clarification.

imegen-Friedreich kit has been validated using the following high-throughput sequencing platform:

- 3730xl DNA Analyzer (ThermoFisher Scientific)

This kit is valid only with the polymers compatible with 6-Carboxifluoresceina (6-FAM) labeling. If you use another brand or model of sequencer, follow the instructions and protocol recommendations of that instrument.

10.2 Reagents

imegen-Friedreich kit has been validated using the reagents included in the kit and the ones recommended in the section 6 of this manual [Equipment and materials required but not supplied].
For the capillary electrophoresis it is advised to use the reagents recommended by the supplier of the sequencer: ThermoFisher Scientific.
Please contact our technical service for any query or clarification.

10.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied from the reception of the kit until the expiry date assigned to each production batch.