Instructions for use

Imegen® DM1

Ref. IMG-173

CE IVD

Manufactured by:
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Code: HIC-PT-KIT 03-F-03 V.02

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This product is designed for *in vitro* diagnostic use. Health in Code, S.L. makes no other express or implied guarantee, which extends beyond the proper operation of the components of this kit. The only obligation of Health in Code, S.L. in relation to the aforementioned guarantees will be to replace the products or refund the purchase price thereof, as requested by the customer, provided that the defect in the materials or the manufacture of its products is proven. Health in Code, S.L. shall not be liable for any direct or indirect damages resulting from economic losses or damages that may arise from the use of this product by the purchaser or user.

All the products marketed by Health in Code, S.L. undergo rigorous quality control. The Imegen® DM1 kit has passed all internal validation tests, which guarantee the reliability and reproducibility of each manufactured batch.

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

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Modifications to the instructions for use (IUF)		
Version 09	DEC 2023	Review and update of section "3. Technical characteristics".
Version 08	NOV 2023	Amendment of positive control (applied from batch 17323C015). Review of entire document. Update of table 1 in section 01 and results analysis in section 08.
Version 07	NOV 2022	Change of manufacturer's address: Health in Code, S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 06	SEP 2022	Change of manufacturer's identification: from Imegen to Health in Code, S.L.
Version 05	FEB 2020	Additional information in section 8.1, Positive control results.
Version 04	FEB 2020	Adjustment of the volume of the positive control included in the kit.
Version 03	SEP 2018	Updating the document for CE-IVD marking of the product.

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01 General information

Myotonic dystrophy type 1 (MIM #160900) is inherited in an autosomal dominant manner and is due, in 100% of cases, to the expanded CTG trinucleotide repeat of the 3' end of the *DMPK* gene (MIM *605377), located on chromosomal region 19q13.3.

The *DMPK* gene encodes myotonic dystrophy protein kinase, which plays a crucial role in the proper functioning of muscle, heart and brain cells. Myotonic dystrophy type 1 (MD1) results from an increase in the number of copies (expansion) of the CTG trinucleotide in the *DMPK* gene, generating a modified version of the messenger RNA that forms, sequesters and binds proteins, interfering in their correct functioning. MD1 presents with muscle weakness, atrophy of the facial muscles, cataracts, alopecia, gonadal atrophy and cardiomyopathies.

The following table shows the expansions analyzed with the Imegen® DM1 kit.

Allele	(CTG)n* Number of repeats	Stability	Clinical phenotype
Normal	5–34	Stable	No MD
Intermediate	35–49	May be unstable	No MD
Pre-mutated	50–150	Unstable	No MD, minimal, or classic MD
Pathological	> 150	Unstable	Classic, juvenile, or congenital DM

Table 1. Information on the expansions analyzed in the Imegen® DM1 kit. MD: Myotonic dystrophy. Last revision 11/11/2023

References

- *Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2. Erik-Jan K et al. Eur J Hum Genet. 202; 20(12): 1203-1208*
- *Incidence of amplification failure in DMPK allele due to allelic dropout event in a diagnostic laboratory. De Siena C et al. Clin. Chim. Acta. (2018) May 24: 484: 111-116*
- *Myotonic dystrophy type 1: role of CCG, CTC and CGG interruptions within DMPK alleles in the pathogenesis and molecular diagnosis. Santoro M et al. Clin Genet. (2017) Oct; 92(4): 335-364*

02 Intended use

Using the Imegen® DM1 kit, the CTG expansion of the 3' end of the *DMPK* gene can be analyzed by PCR and subsequent capillary electrophoresis. In addition, the kit offers a TP-PCR (triplet repeat primed PCR) system for cases with major expansions, which are undetectable by conventional PCR.

The TP-PCR assay uses a locus-specific tagged oligonucleotide that flanks the repeat, together with paired oligonucleotides that amplify from multiple sites of the expansion, allowing the detection of expanded alleles undetectable by conventional PCR by means of end-time PCR and subsequent capillary electrophoresis.

PCR products will be separated by capillary electrophoresis, and both PCR and TP-PCR will be detected by 6-Carboxyfluorescein (6-FAM) labeling.

Imegen® DM1 is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

This kit has been validated using samples analyzed by the EMQN (European Molecular Genetics Quality Network) interlaboratory, as well as reference materials from the Coriell Institute and samples previously analyzed by the medical genetics service of Health in Code, S.L. The kit specifically detects the expansions for which it has been developed.

The material needed for this study is genomic DNA mainly from peripheral blood. The total quantity of DNA needed is 500 ng.

04 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not pipette by mouth.
- Do not smoke, eat, drink or apply cosmetics in the areas where kits and samples are handled.
- Any skin conditions, as well as cuts, abrasions and other skin lesions should be properly protected.
- Do not pour reagent residues into the drinking water system. It is recommended to use the waste containers set out by the legal regulations and to manage them via an authorized waste manager.
- In the case of accidental spillage of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with plenty of water.
- Material safety data sheets (MSDS) for all hazardous components contained in this kit are available upon request.
- This product requires the handling of samples and materials of human origin. It is recommended that all human-sourced materials be considered potentially infectious and handled in accordance with the OSHA Biosafety Level 2 standard for bloodborne pathogens or other relevant biosafety practices should be used for materials that contain or are suspected of containing infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive and do not cause biological environmental contamination.
- This kit has been validated with specific equipment and under specific conditions that may vary significantly in other laboratories. It is therefore recommended that each laboratory perform an internal validation when using the kit for the first time.
- The manufacturer is not responsible for the assay not working properly when the reagents included in the kit are replaced by other reagents not supplied by Health in Code, S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user includes reagents not validated by Health in Code, S.L., considering them equivalent to those supplied in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient reagents in order to make 12 determinations. The list of reagents included in the kit is as follows:

- ***MD1 Master Mix***: PCR Master Mix with the quantities of nucleotides, $MgCl_2$ and buffer necessary to carry out amplification reactions:
- ***PCR Master Mix***: Master Mix with the oligonucleotides necessary to carry out the PCR amplification of the kit's target region.
- ***TP-PCR Master Mix***: Master Mix with the oligonucleotides necessary to carry out the TP-PCR (triplet repeat primed PCR).
- ***Taq***: DNA polymerase necessary to carry out amplification reactions.
- ***Positive Control:*** genomic DNA from a reference sample with a normal allele with 12 repeats and a pre-mutated allele with approximately 75 repeats at an optimal amplification concentration.

Reagents	Color	Quantity	Storage
MD1 Master Mix	White pad	350 µl	-20°C
PCR Master Mix	Red pad	60 µL	-20°C
TP-PCR Master Mix	Blue pad	60 µL	-20°C
Taq	Orange cap	18 µL	-20°C
Positive control	Black cap	60 µl	-20°C

Table 2. Imegen® DM1 kit components

06 Equipment, reagents and materials not included in the kit

Equipment:

- Conventional thermal cycler
- 10 µL, 20 µL, 200 µL and 1000 µL micropipettes
- Vortex
- Centrifuge
- Capillary electrophoresis system

Reagents:

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

Materials:

- Pipette tips with filter (10 µL, 20 µL, 200 µL and 1000 µL)
- 1.5 mL sterile tubes.
- 0.2 mL 96-well tubes or plates
- Film for 96-well plates
- Latex gloves

NOTE: This kit does not include the reagents necessary to perform capillary electrophoresis.

Complementary kits

In order to analyze the expansions involved in other neurodegenerative diseases, Health in Code, S.L. also offers Imegen® SCAs **(Ref: IMG-152)**, Imegen® SBMA **(Ref: IMG-153)**, Imegen® Huntington **(Ref: IMG-154)**, and Imegen® Friedreich **(Ref: IMG-155)** kits. All of them, together with the Imegen® DM1 kit, have been designed using the same PCR program, so they can be analyzed together.

07 Assay protocol

07.1 | Preparation of amplification reactions

In order to estimate the quantity of reagents required, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend adding one more reaction or increasing the volume of each reagent by 10% when making the calculations.

Two different PCR mixtures will be prepared per sample, one for the PCR reaction and another for the TP-PCR. The recommended protocol for the preparation of amplification reactions is shown below:

01 Thaw all kit reagents and DNA from the samples. Vortex each of the reagents and keep cold.

02 Prepare the PCR mix in a 1.5 mL tube by adding the following reagents.

Reagents	Quantity per reaction
MD1 Master Mix	14.5 µL
Taq	0.5 µL
PCR Master Mix	5 µL

03 Prepare TP-PCR mix in a 1.5 mL tube by adding the following reagents.

Reagents	Quantity per reaction
MD1 Master Mix	14.5 µL
Taq	0.5 µL
TP-PCR Master Mix	5 µL

04 Vortex and spin the PCR and TP-PCR mixes. Dispense 20 µL into the corresponding 0.2 mL tubes.

05 Add 5 µL of the diluted samples at a concentration of 50 ng/µL. It is advised to include a negative PCR and TP-PCR control for each amplification batch to confirm the absence of contamination from reagents and also positive controls to verify allele size. Given the possibility of not having positive controls, the kit includes its own positive control.

06 Place the tubes in the thermal cycler and run the following amplification program:

Fields	Stage 1 Enzymatic activation	Stage 2 PCR or TP-PCR			Stage 3	
No. of cycles	1 initial cycle	30 cycles			1 cycle	
		Denaturation	Primer binding	Extension	End of PCR 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 and TP-PCR program, optimal for Biometra T3 equipment, SimpliAmp Thermal Cycler and GENEAMP® PCR System 2720 (Applied Biosystems).

↘ It is possible to stop the protocol at this point. PCR products can be stored at 4°C if the protocol is to be continued within the next 24 hours or at -20°C for longer periods of time.

07.2 | Preparation of amplified fragments

Prepare the plate for fragment analysis from the PCR and TP-PCR products as follows:

01 Add the following reagents to a 1.5 mL tube:

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

We recommend adding one more reaction or increasing the volume of each reagent by 10% when making the calculations.

NOTE: The volume of the size marker can be increased or decreased to adjust the intensity of the peaks observed in the electropherogram.

02 Dispense 18.5 µL of the above mixture into each well.

03 Add 1 µL of the DNA obtained from the PCR and TP-PCR reactions.

NOTE: The sample volume can be increased or decreased (by diluting the samples) to adjust the intensity of the peaks.

04 Cover the plate, spin and denature in a thermal cycler for 5 minutes at 98°C.

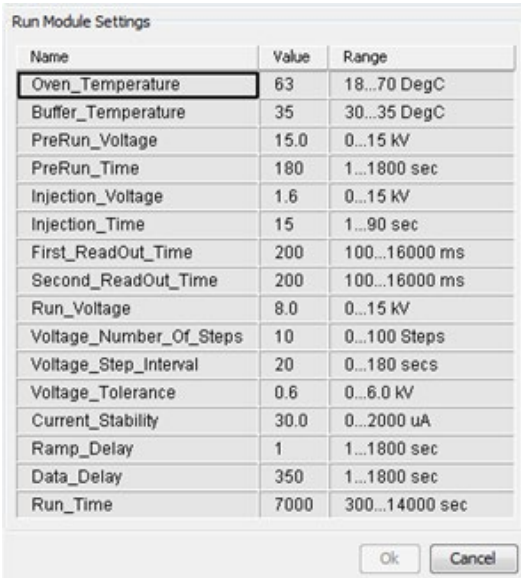
05 Store the plate at 4°C until it is put into the sequencer.

07.3 | Capillary electrophoresis

Once the fragment plate has been prepared, the reactions should undergo capillary electrophoresis. Depending on the sequencer model used, the electrophoresis conditions recommended by the manufacturer will be used.

In order to program the capillary electrophoresis conditions, it should be considered that the amplification range varies approximately between 100 and 500 bp, that 6-FAM-labeled primers are used and that the molecular weight standard is labeled with GeneScan™ 500 LIZ.

The following image shows the optimized conditions for the 3730xl DNA Analyzer sequencer (Thermo Fisher Scientific), using the POP-7™ polymer.


Run Module Settings

Name	Value	Range
Oven_Temperature	63	18...70 DegC
Buffer_Temperature	35	30...35 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.6	0...15 kV
Injection_Time	15	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	8.0	0...15 kV
Voltage_Number_Of_Steps	10	0...100 Steps
Voltage_Step_Interval	20	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	1	1...1800 sec
Data_Delay	350	1...1800 sec
Run_Time	7000	300...14000 sec

Ok Cancel

Figure 1. Optimized parameters for the 3730xl DNA sequencer.

Detection intensity may vary between different equipment, depending on the model, the state of the optical system of the equipment, and the injection time and voltage. Therefore, it may be necessary to increase or decrease the quantity of size marker or PCR product required to perform capillary electrophoresis.

08 Analysis of results

It is recommended to follow the indications below for the results to be analyzed properly:

- To analyze the samples, it is necessary to use specific software and the .fsa file obtained as a result of capillary electrophoresis.
- Check that the negative PCR control does not contain any peaks larger than 150 base pairs in the electropherogram. If amplification is detected, it is recommended to repeat the assay to rule out accidental contamination.
- Sample analysis.

Interpretation using a control sample or positive control from the kit

To calculate the number of repeats, a control sample or positive control from the kit may be used, as it has a known number of repeats, a normal allele with 12 repeats and an allele with approximately 75 repeats (see Figure 3). This form of interpretation is recommended by Health in Code, S.L., as it minimizes possible variations between assays and laboratories.

$$No.of\ repeats = \frac{Allele_{X\ size} - Control_{Allele\ size}}{3} + No.rep\ control\ allele$$

Where:

- **Size of X allele**: Size (bp) of the peak obtained from the sample analyzed.
- **Size of control allele**: Size (bp) of the peak obtained from the positive control.
- **No. control allele rep.**: The number of repeats with the allele used for the positive control.

For instance, the results obtained for the sample in Figure 2 when applying the above formula are shown in Table 4. It is recommended to use the values of a normal, stable allele with 12 repeats and high fluorescent intensity, which will facilitate interpretation.

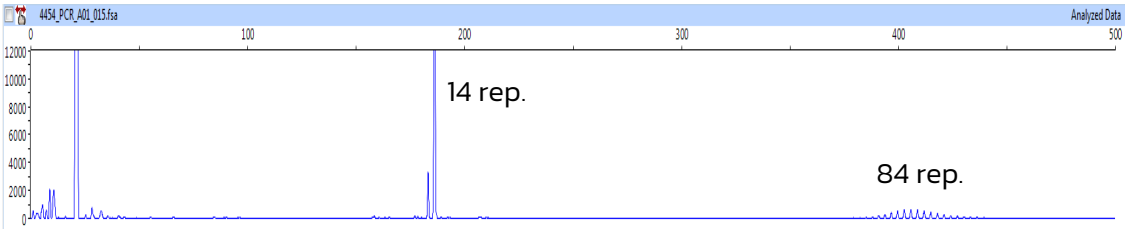


Figure 2. Sample with one normal allele and one expanded allele for the DM1 system.

Size of X Alleles		Size of Control Allele	No. sample rep.	
			Allele 1	Allele 2
189	399	183	14	84

Table 4. Number of repeats of a sample with a normal allele and another expanded one, bearing in mind the size and number of repeats of the normal positive control allele.

$$Number\ of\ repeats\ Allele\ 1 = \frac{189 - 183}{3} + 12 = 14$$

$$Number\ of\ repeats\ Allele\ 1 = \frac{399 - 183}{3} + 12 = 84$$

The image below is an example of the result obtained for Control-MD1, alongside a table with the correlations between the number of repeats and the base pair size of this control.

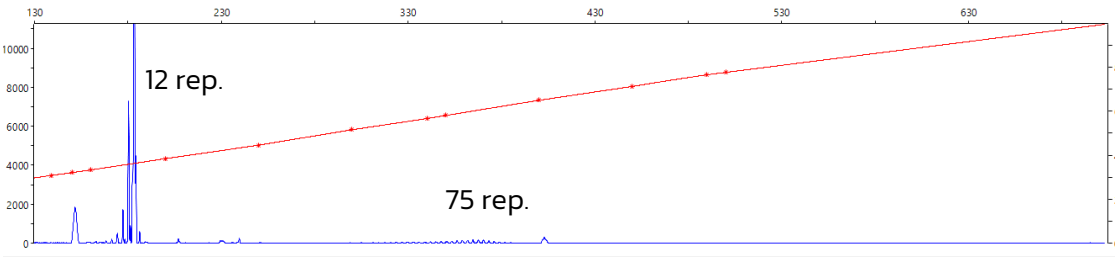


Figure 3. Results of the Positive Control in PCR DM1.

Positive control alleles	No. of repeats	Size (bp)
Normal allele	12	183
Expanded allele	75	372

Table 5. Positive control repeats and size.

Interpretation in the absence of a control sample or positive control from the kit

Another way to calculate the number of repeats, in the absence of a control sample, is to take into account the size of the PCR product obtained with the amplification system, by applying the following formula:

$$No.of\ repeats = \frac{Allele_{X\ size} - 147}{3}$$

Where:

- **The value "147"**: The amplicon size of the reference allele, confirmed both *in silico* and at our laboratories during the kit validation.
- **Size of X allele**: Size (bp) of the peak obtained from the sample analyzed.

For instance, the results in Table 6 are obtained for the sample in Figure 2 with a normal allele and an expanded allele by applying the above formula.

Size of X Alleles		No. sample rep.	
		Allele 1	Allele 2
189	399	14	84

Table 6. Number of repeats of a sample with a normal allele and another expanded one, without taking into consideration the size and number of repeats of the normal positive control allele.

$$Nº\ repeticiones\ Alelo\ 1 = \frac{189 - 147}{3} = 14$$

$$Nº\ repeticiones\ Alelo\ 2 = \frac{399 - 147}{3} = 84$$

However, the most suitable way to calculate the number of repeats is by using a control sample or the positive control from the kit.

Due to the presence of unconsumed traces of labeled primers during PCR and primer dimers, unspecific peaks can be detected, but always outside the analysis range. Therefore, they do not interfere with the good interpretation of the results. Moreover, these peaks will never have the characteristics of peaks due to trinucleotide tandem repeats: The presence of a real peak of larger size, and one or several stutter peaks, always smaller than the real one and separated from it by three base pairs.

Images of different CTG expansion genetic profiles are given below:

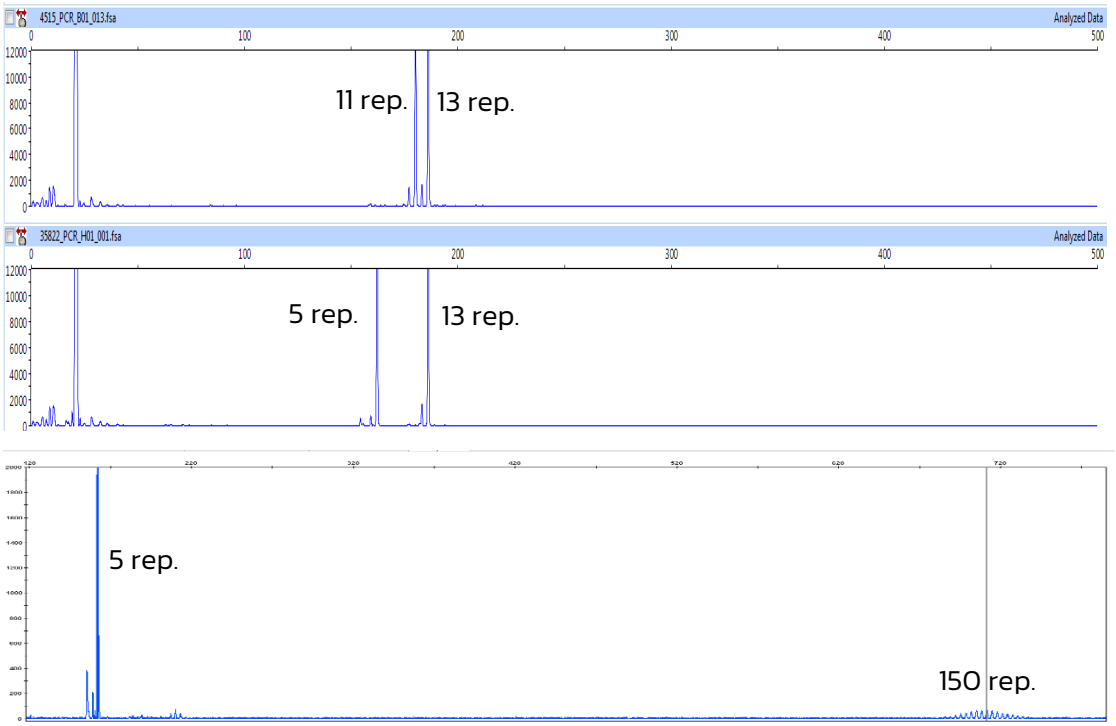


Figure 4. Examples of results obtained with the PCR mix of the Imegen® DM1 kit.

NOTE: To correctly analyze expansions larger than 150 repeats with the PCR system, Health in Code, S.L. advises to use a size marker with a range greater than 600 base pairs.

08.1 | TP-PCR results

In samples where only one allele has been detected using the PCR system, the genotype of the sample could be homozygous for that allele or heterozygous with one normal allele and one expanded allele, which is undetectable by conventional PCR.

The TP-PCR system (triplet repeat primed polymerase chain reaction) of the Imegen® DM1 kit has been designed to differentiate between homozygous and heterozygous samples with an expanded allele not detectable by conventional PCR.

Since it is a PCR that uses a locus-specific labeled oligonucleotide and paired oligonucleotides that amplify at any point in the expansion, this technique can detect expansions of any size, although it cannot determine the number of repeats.

Below is a series of images showing the results obtained from the TP-PCR.

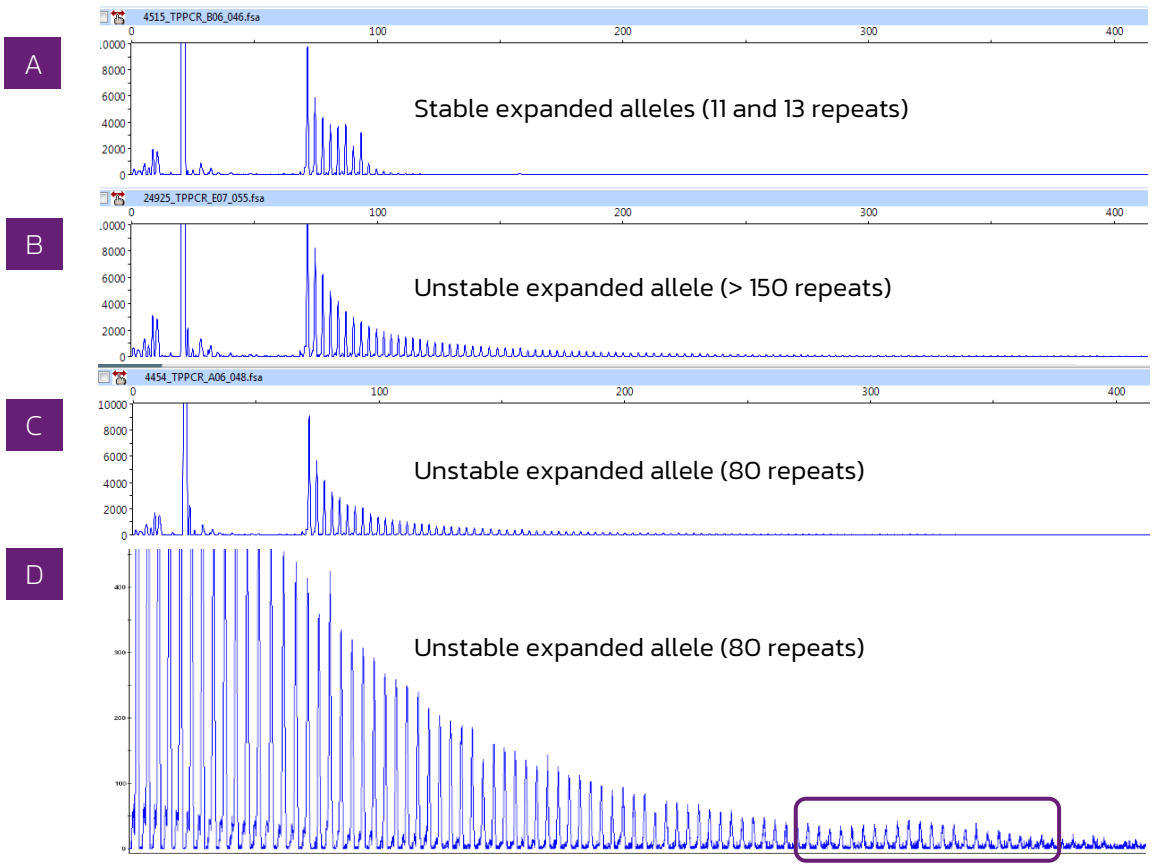


*Figure 5. TP-PCR results. Profile **A** compatible with the absence of an expanded allele in the sample. Profiles **B** and **C** compatible with expanded alleles in the sample. Profile **D**: Small increase in fluorescence intensity, compatible with expanded alleles of 80 repeats.*

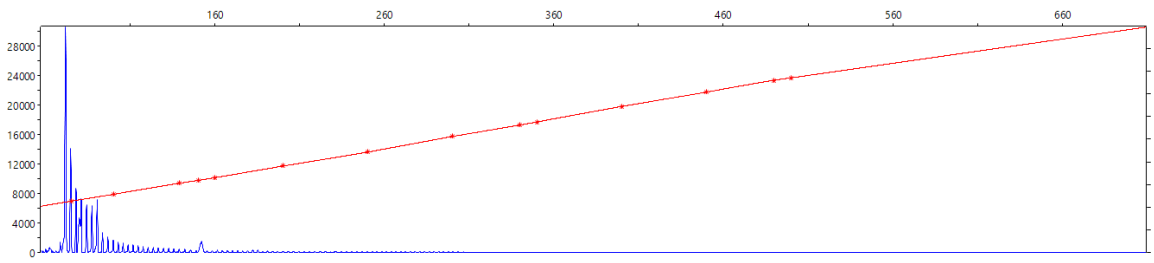


Figure 6. TP-PCR results from the positive control included in the kit.

09 Troubleshooting

The following table shows the results that could be obtained for the analyzed samples, the positive control, the size marker and the negative control. In the case of an unexpected result, the interpretation and the most probable reason for such a result are given in the following table:

Problem	Samples analyzed	Positive control	Size marker	Negative control	Results/interpretation
Weak or no fluorescence signal				✔	Expected result
	✔			✔	Insufficient quantity and/or quality of template DNA [1] Impure template DNA [2]
	✔	✔	✔	✔	Failed capillary electrophoresis [3] Failed denaturation[4]
	✔	✔		✔	Failed PCR [5]
Excessive fluorescence signal	✔				Excessive DNA quantity [6]
	✔	✔			
Presence of more peaks than expected	✔	✔		✔	Contamination [7]
	✔				Contamination [7]; Mosaicism [9]
	✔	✔			Artifacts characteristic of expansions [8]

Table 5. Interpretation of possible results with the Imegen® DM1 kit

(1) Insufficient quantity and/or quality of template DNA: Check that the DNA has been correctly quantified and use the indicated quantity of template DNA. If the DNA has been correctly quantified, check its integrity and perform a new extraction if necessary.

(2) Impure template DNA: High salt concentrations or altered pH can inhibit PCR. If you are using template DNA dissolved in an elution buffer with a pH other than 8 or at high EDTA concentrations, the volume of DNA should not exceed 20% of the total reaction volume. Traces of the reagents used during extraction can also affect the PCR reaction. If so, clean the DNA or prepare a new extraction.

(3) Failed capillary electrophoresis: Check if the equipment parameters are as specified and reinject the samples.

(4) Failed denaturation: For a correct denaturation, the samples must be heated for the time indicated in section 7 of this document, and then kept cold until loading into the sequencer.

(5) Failed PCR: Check that the PCR program is the indicated one.

(6) Excessive DNA quantity: Make sure you are using the right quantity of DNA. If so, dilute the PCR product in sterile deionized water and prepare again for denaturation and loading into the sequencer.

(7) Contamination: This can be caused by another template DNA or by a previously amplified DNA. Cross-contamination can lead to false positives and negatives, resulting in problems in the interpretation of results. Use pipette tips with filters and change gloves regularly.

(8) Artifacts characteristic of expansion: The amplification of expansions generates artifacts (peaks in the electropherogram) that appear as less intense, smaller peaks (3 base pairs smaller) than the predominant peak.

(9) Mosaicism: Cases of mosaicism have been described in MD1, therefore, it is possible to find more than one genotype in a sample. In this case we recommend repeating the PCR, and if the same pattern is obtained, use a new sample from the patient, preferably from another tissue (e.g. buccal mucosa).

10 Limitations

10.1 | Equipment

Imegen® DM1 has been validated using the following PCR thermal cyclers:

- *SimpliAmp Thermal Cycler* (Thermo Fisher Scientific)
- *GeneAmp PCR System 2720* (Thermo Fisher Scientific)
- *T3000 Thermocycler 48* (Biometra)

If you use another make or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical support for any questions or clarifications.

Imegen® DM1 has been validated using the following sequencing platform:

- *3730xl DNA Analyzer* (Thermo Fisher Scientific)

This kit is valid for polymers compatible with 6-Carboxyfluorescein (6-FAM) labeling. In the case of using equipment different from that mentioned above, follow the protocol specifications for those platforms.

10.2 | Reagents

Imegen® DM1 has been validated using the reagents included in the kit and those recommended in section 6 of this document (Equipment, reagents and materials not included in the kit).

It is recommended to use the reagents recommended by the sequencer supplier for capillary electrophoresis: **Thermo Fisher Scientific**.

In the case of doubt, please contact our technical service.

10.3 | Product stability

The optimum performance of this product is confirmed provided that the recommended storage conditions according to the optimum product date for each production batch are followed.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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