adellgene

Myotonic Dystrophy Screening

INSTRUCTIONS FOR USE

Rv. 04 / 2019-05-27

Kit for the determination by fluorescent fragment analysis of the presence of healthy, premutated and mutated alleles of less than 200 repeats of the DMPK gene of Myotonic Dystrophy type 1

CE

Catalog No. AD-MD-16

Store: Box 1 from -30ºC to -18ºC

Box 2 from 20ºC to 25ºC

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Intended Use

Adellgene® Myotonic Dystrophy Screening is a kit designed for use in clinical laboratories which detects the number of repetitions of CTG of 3´UTR region of the DMPK gene located in chromosome 19 resulting in Myotonic Dystrophy disease. It aims to aid clinical diagnosis associated with clinical findings in myotonic dystrophy type 1 (DM1) that span from mild to severe symptoms.

The use of this kit is the determination of healthy individuals who have between 35 to 49, patients with mild phenotype (50-150 repeats) and classic (100-1000) until 200 repeats. The technology is based on the polymerase 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 CTG repeats.

The Primer Mix Screening (PM1) together with the Polymerase Mix (POM) allows for the detection of heterozygotes and possible homozygous alleles with a size less than 200 CTG repeats, and its quantification.

In the cases of homozygous individuals which allele detected is less than 200 repeats or no allele is detected a subsequent study with Adellgene® Myotonic Dystrophy Confirmatory kit must be done to establish the definition of alleles of more than 200 repeats.

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Summary and explanation

Myotonic dystrophy type 1 or Steinert's disease is currently the most common form of muscular dystrophy in adults. It was first clinically recognized by Steinert (1) and Batten and Gibb (2) in 1909. Based on clinical ascertainment, worldwide prevalence is estimated to be 12.5/100000 (3), but it can be higher as many patients in older generation remain undiagnosed. Inheritance of this multisystem disease is autosomal dominant, and phenotypic expression is highly variable due to an unstable expansion CTG trinucleotide repeats in the 3´ untranslated region (3´UTR) of the dystrophia myotonica protein kinase gene (DMPK, MIM*605377) (4-6) located in the long arm of chromosome 19 (19q.21.3). The expanded DMPK gene produces a toxic RNA transcript that does not exit the nucleus (7).

Reference ranges for allele sizes were established by the Second International Myotonic Dystrophy Consortium (IDMC) in 1999 (8-11) for technical standards and guidelines for testing. Normal alleles: 5-34 CTG repeats; Mutable normal (premutation) alleles: 35-49 CTG repeats. Individuals with CTG expansions in the premutation range have not been reported to have symptoms, but their children are at increased risk of inheriting a larger repeat size and thus having symptoms (12). Full penetrance alleles >50 CTG repeats are associated with disease manifestations.

Clinical findings in myotonic dystrophy type 1 (DM1) span a continuum from mild to severe (13) and provide an excellent overview of all aspects of DM1. The clinical findings have been categorized into three somewhat overlapping phenotypes, mild, classic, and congenital, that generally correlate with CTG repeat size (Table 1) (8, 9, 14-17).

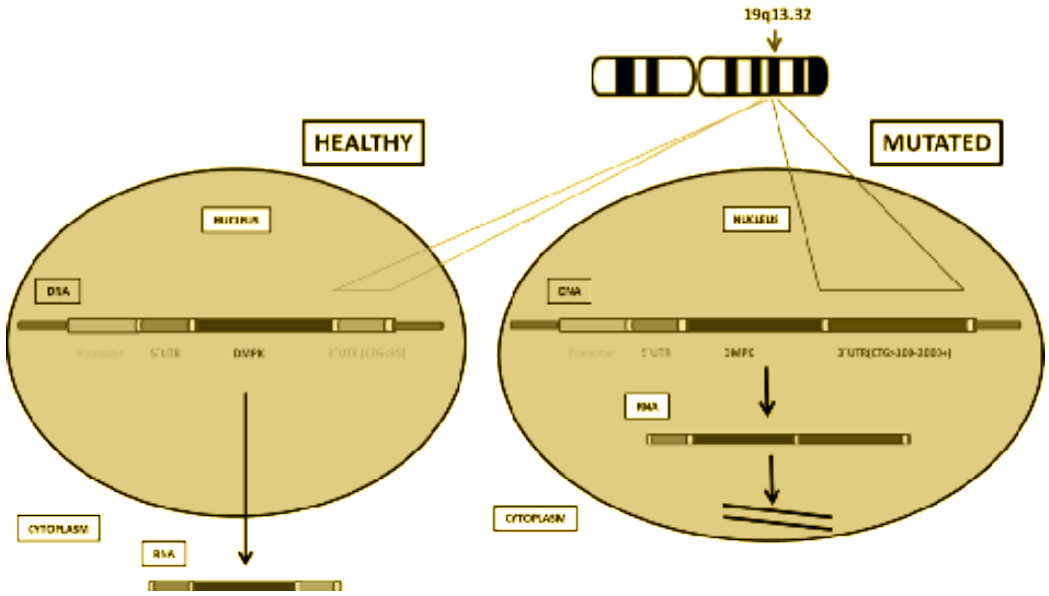

Figure 1. Representation of the DMPK gene types and their production depending on the degree of mutation

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Mild DM1 (50-150 CTG repeats) may have only cataract, mild myotonia, or diabetes mellitus. They may have fully active lives and a normal or minimally shortened life span (18).

Within Classic DM1 (100-1000 CTG repeats), only a rough correlation with severity of symptoms exists. Individuals with these CTG repeat sizes usually develop classic DM1 with muscle weakness and wasting, myotonia, cataracts, and often cardiac conduction abnormalities. While the age of onset for classic DM1 is typically in the 20s and 30s (and less commonly after age 40 years), classic DM1 may be evident in childhood, when subtle signs such as myotonic facies and myotonia are observed. In the case of Congenital DM1 (>1000 CTG repeats), a transmission ratio distortion at conception favors transmission of larger CTG repeats than those present in the parent (19). Presence of a large repeat may lead to earlier onset and more severe disease, known as congenital

Phenotype	Clinical Signs	CTG Repeat Size	Age of Onset
Mutable normal (premutation)	None	35 to 49	Non defined
Mild	Cataracts		
	Mild myotonia	50 to ~150	20 to 70 yrs
Classic	Weakness		
	Myotonia		
	Cataracts		
	Balding		
	Cardiac arrhythmia		
	Others	~100 to ~1000	10 to 30 yrs
Congenital	Infantile hypotonia		
	Respiratory deficits		
	Intellectual disability		
	Classic signs present in adults	>1000	Birth to 10 yrs

Table 1. Correlation of Phenotype and CTG Repeat Length in Myotonic Dystrophy Type 1

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DM1 (20, 21). Congenital DM1 often presents before birth as polyhydramnios and reduced fetal movement. After delivery, the main features are severe generalized weakness, hypotonia, and respiratory compromise. Mortality from respiratory failure is common. Surviving infants experience gradual improvement in motor function. Affected children are usually able to walk; however, a progressive myopathy occurs eventually, as in the classic form (15). These individuals may develop any of the typical features of DM1 including weakness, myotonia, cataracts, and cardiac problems. Intellectual disability is present in 50%-60% of individuals with congenital DM1. Autism spectrum disorder, common mood/anxiety disorders, impaired attention, and abnormal visual-spatial abilities have been reported (22, 23). Children with DM1 may have low visual acuity, hyperopia, or astigmatism (24).

The PM1 of this kit is optimized for the detection of about 200 CTG repeats so as to determine the heterozygous healthy individuals (35-49 repeats), heterozygous mild patients (50-150 repeats), and heterozygous classic patients (100-1000 but only until 200 repeats). It is based on the analysis of fragments generated with fluoresceinated primers and capillary electrophoresis in a genetic analyzer and fluorescence analysis with appropriate software for detection and interpretation.

Principles of the procedure

The detection method employed by the Adellgene® Myotonic Dystrophy Screening kit is based on the specific amplification of genomic DNA from purified fragment containing 3´UTR region of the DMPK gene, which containing the CTG repeats. PM1 includes 2 primers, one of which is labeled with a fluorophore for subsequent detection in a DNA fragment analyzer. The use of ROX1000™ size marker is recommended. The size of PCR products are converted into the number of CTG triplet repeats using conversion factors of mobility and size.

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Kit contents

Reference AD-MD-16 (16 tests)

Box 1 of 2

- AD-MD-PM1: Primer Mix Screening (PM1). 1 vial x 79 µl
- AD-MD-POM: Polymerase Mix (POM). 1 vial x 344 µL

Box 2 of 2:

- AD-PUR-16: including all the reagents and plastics for purifying the amplicons of 16 samples:
    - Capture buffer: 9,8 mL
    - Wash buffer: 2.0 mL (see how to prepare in section Procedure / D)
    - Elution buffer: 300 µl
    - Micro - columns: 17 units
    - Collection tubes: 17 units

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-MD-POM) to reach room temperature before use. Shake gently all reagents except AD-MD-POM 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 the vial of Primer Mix (AD-MD-PM1), and Polymerase mix (AD-MD-POM) as this may reduce the sensitivity of the test and alter the results.It is recommendable to establish aliquots of these reagents, at first thawing cycle, depending of the use of the kit.
- Due to the photo-sensitive nature of the reagent AD-MD-PM1, avoid continued exposure to light.

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Materials required but not supplied

Isolation and Purification Reagents

- Reagents for DNA isolation and purification are not included. The DNA can be extracted by any method validated in the laboratory to ensure high quality and 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 y ROX (ABI, Ref.:4345827, 4345829 or equivalent)
- Size standard marker ROX ™ 1000 (ABI, Ref.: 401098; Eurogentec, Ref.: MW-0195-80ROX)

Supplies and equipment

- General equipment of a laboratory dedicated exclusively to performing the PCR.
- 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 Fragile-X syndrome or any cell line whose DNA corresponds with a validated sample

Sample collection and preparation

- All biological and blood samples should be treated as potentially infectious. When handling, 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.

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 50 ng (e.g.: 1 µl of DNA of 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+2 samples:

Reagent	Vol. for each sample (ul)
AD-MD-PM1	4,3
AD-MD-POM	18,7
Total volume for each reactions	**23**

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- 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-MD-PM1 could inhibit the PCR reaction

2. To pipette 23 µl of this mixture on an optical 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.

	Number of cycles	Temperature (ºC)	Time (mm:ss)
DENATURATION	1	98	05:00
CYCLES	30	97	00:35
		64	00:35
		68	04:00+ 00:20SEG/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

- Use the Box 2 of 2 included with the kit. The protocol is:

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 17 ul of PCR volume)

b. Mix thoroughly.

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

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

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

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

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

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

i. Add 500 ul Wash buffer to the micro-column.

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

k. Discard the collection tube and transfer the micro-column to a fresh DNase-free 1,5 ml microcentrifuge tube.

l. Add 10 ul Elution buffer to the center of the membrane in the assembled micro-column and sample collection tube.

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

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

o. Proceed to the general protocol.

2. Preparation of Samples for DNA analyzer

The following reaction mixture is performed with the PCR product. For amplicons of PM1, it is recommended to use 2ul the PCR product for the 3130/3130XL and 3730xl DNA analyzer or to check the optimal volume between 2 or 3 ul to charge in the DNA analyzer.

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

** These items are not supplied with the kit*

*** See recommended volume for the PCR product of PM1 depending on the analyzer.*

- 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 µAmps	5.0 µAmps
Pre-Run Voltage	15.0 KVolts	15.0 KVolts
Pre-Run Time	180 sec	180 sec
Injection voltage	3.0 KVolts	1.6 KVolts
Injection time	15 sec	30 sec
Voltage Number of steps	20 nK	20 nK
Voltage Step Interval	15 sec	15 sec
Data Delay Time	60 sec	60 sec
Run voltage	15.0 kVolts	15.0 kVolts
Run time	3000 sec	2200 sec

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

Results and interpretation

Adellgene® Myotonic Dystrophy Screening kit is a quantitative technique for identifying the number of CTG triplet repeats between 5 and at least 200 (see section 2).

The Adellgene® Myotonic Dystrophy Screening kit includes a series 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 Adellgene® Myotonic Dystrophy Confirmatory kit to assure or discard the homozygosis of the sample.

Using the reagents supplied in this kit, if the sample has a number of 5 CTG repeats, the size of the amplified fragment will be of 265 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 CTG triplet repeats. It is not necessary to use any passive reference.

In Figure 2 are expressed fragment analysis (see below) results of the kit for a homozygous healthy individual (sample 1), heterozygous healthy individual (sample 2), and unhealthy individuals (samples 3-5).

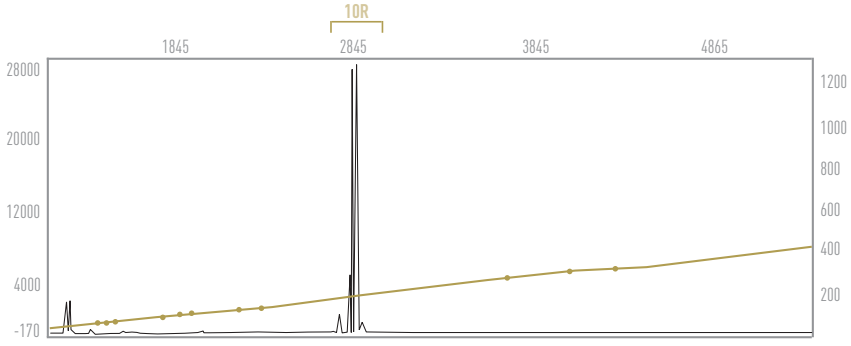

Figure 1. Homozygous 10,10 repeats (10R)

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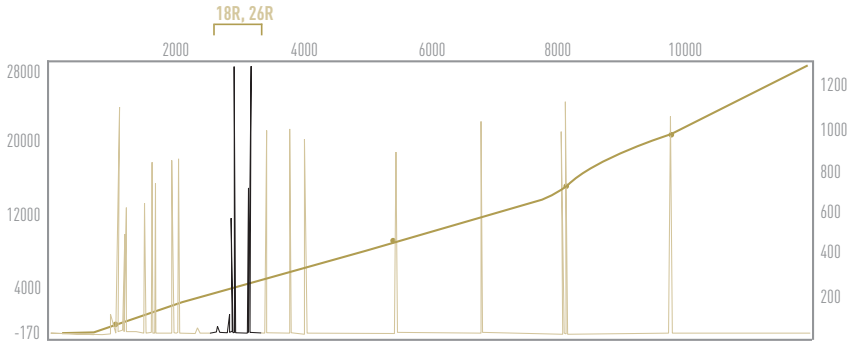
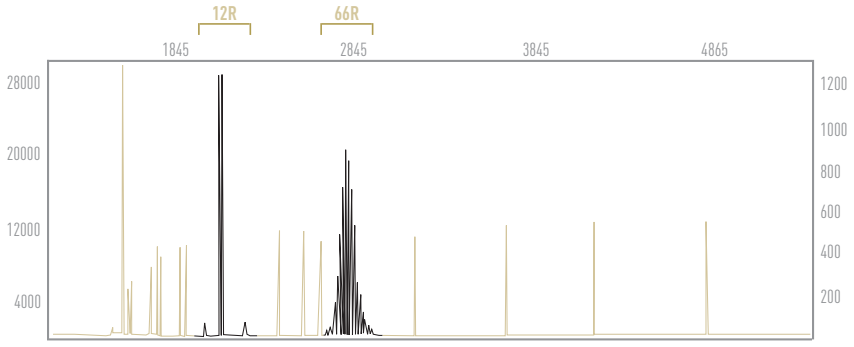
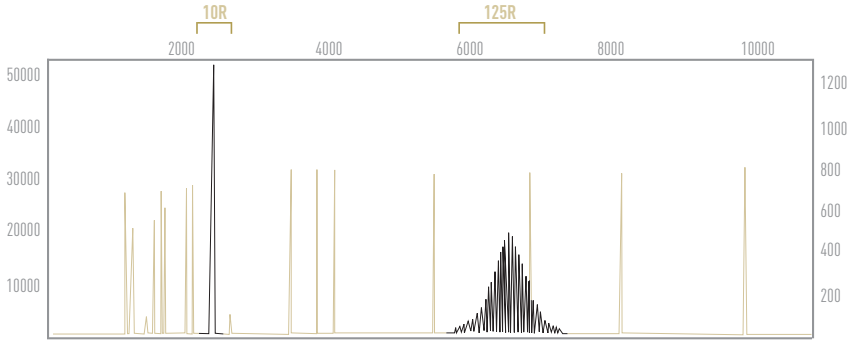


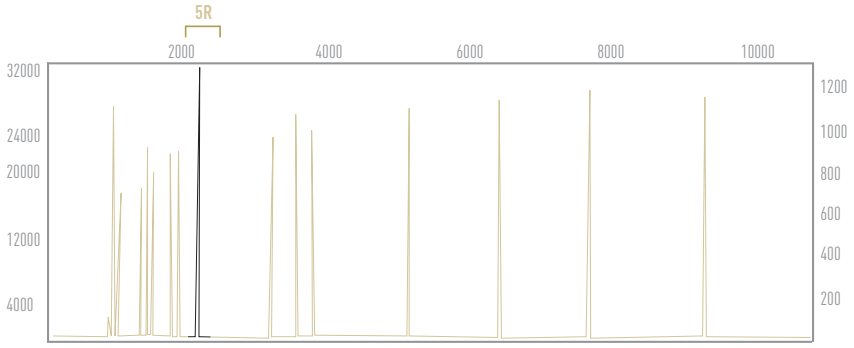
Sample 2. Heterozygous 18,26 repeats (18R,26R)



Sample 3. Heterozygous 12,66 repeats (12R,66R)



Sample 4. Heterozygous 5,125 repeats (5R,125R)



Sample 5. Heterozygous 5,>125 repeats (5R,>200R)

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CGG repeats	Fragment size (bp)	CGG repeats	Fragment size (bp)
5	265	105	565
10	280	110	580
15	295	115	595
20	310	120	610
25	325	125	625
30	340	130	640
35	355	135	655
40	370	140	670
45	385	145	685
50	400	150	700
55	415	155	715
60	430	160	730
65	445	165	745
70	460	170	760
75	475	175	775
80	490	180	790
85	505	185	805
90	520	190	820
95	535	195	835
100	550	200	850

Black numbers indicate healthy alleles. Gold numbers indicated premutated or mutated alleles.

Important note

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

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Quality control

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

Furthermore, as described in the procedure, the addition of the marker fragment analysis LIZ 500™ 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 Materials required but not supplied).

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

The test 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.

Specific data of performance

1. Analytical specificity and sensitivity

The primers of this kit are specific of the human Myotonic Dystrophy gene DMPK and comprise the CTG repeat region within of 3´UTR of the gene. The specific amplification of this region 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® Myotonic Dystrophy Screening PM1component is 3´UTR region DMPK specific assay to detect the number of CTG repeats. If only one peak is found, the use of Adellgene® Myotonic Dystrophy Confirmatory kit or other alternative technique is necessary to confirm the presence or not of unhealthy alleles (>200 CTG 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. Homozygous results must be confirmed by alternative procedures.

3. Range of

- DNA concentration

  For the best range of amount of DNA in each assay tests were performed between 10 ng to 200 ng with a sample heterozygous female. The assignment of peaks obtained size was independent of the amount of DNA used. The recommended working range is between 45-50 ng of DNA by PCR.

- Results of the kit

  The method of this kit can assign a number of CTG triplet repeats of between 5 and 200 repeat alleles. The use of this kit is the determination of both healthy individuals (5 to 49) repetitions and patients affected with 50 repetitions or more with different penetrance (see section 2).

4. Accuracy

- Assignment of fragment size

  The assignment of the peaks is given by the size of the amplicon obtained and peak morphology. If there are several peaks, it will be considered the highest and the 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 for healthy alleles and ±3 for 50-200 repetitions alleles.

- General testing

Region 3'UTR/DMPK	Samples Tested
Analyzed samples with 2 normal alleles (5-34 rep.)	17
Analyzed samples with 1 premutated allele (35-49 rep.)	1
Analyzed samples with 1 mutated allele (>50 rep.)	38
Total number of analyzed samples	**56**

- 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.

Procedure limitations

- The method detects all alleles of between 5 and 200 CGG triplet repeats (see Results and interpretation).
- The conditions described for the PCR should be accurately controlled. Deviations from these parameters can lead to poor outcome.
- All work from Adellegene Myotonic Dystrophy Screening 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.

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 always corresponds to the assigned worksheet

Weak or absent signal in the PCR product

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

- **Thermal cycling problems**
  - Check the thermal cycling run parameters and ensure thermal cycler is operating according to manufacturer´s specifications and maintenance
- **No Ethidium bromide (or other alternative DNA colorant) is added**
  - Ensure Ethidium bromide is added to 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 Taq polymerase
  - Repeat with a new Taq polymerase
- **Weak PCR product**
  - Check gel image and proceed accordingly
- **Insufficient reaction products applied to DNA analyzer**
  - Check analyzer parameters
- **Wrong purification process**
  - Take extreme care in purification process

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 backgroud (noisy baseline)

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

References

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