adellgene

Huntington Disease

INSTRUCTIONS FOR USE

Rv. 01 / 15-05-2017

Kit for the determination of the number of CAG triplets of the HD gene, by fluorescent fragment analysis

CE

Reference No. AD-HD-16

Store at -18ºC to -30ºC

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HUNTINGTON DISEASE

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Intended use

Adellgene Huntington Disease (HD) is an in vitro diagnostic kit designed for use in clinical laboratories. The kit detects the number of repetitions of the CAG (cytosine-adenine-guanine) triplet, located on exon 1 of the IT15 gene (HTT), which can result in the formation of Huntington's disease, also called Huntington's Chorea. It aims to aid the clinical diagnosis associated with Huntington's chorea, such as: subtle changes in coordination, involuntary minor movements, difficulty mentally planning, and often a depressed or irritable mood.

The use of this kit is for the determination of both healthy and unhealthy individuals, who have between 10 and 35 repetitions or more than 36 repetitions, respectively [1, 2]. There is a difference in penetrance between individuals with 36, and more than 42 repetitions [2] (see section 2).

The technology is based on the triplet repeat primed polymerase chain reaction (TP-PCR) of genomic DNA, extracted and purified from peripheral blood, followed by fluorescence analysis of the size of the PCR fragments obtained by the genetic analyzer and conversion of that size into the respective number of CAG repeats. Moreover, the observation of the pattern of peaks allows to identify expanded alleles and to confirm the samples with homozygous normal alleles.

Heterozygous and possible homozygous alleles with a size equal to or less than 121 CAG repeats are quantified. Possible alleles bigger than that size would be identified because of their pattern.

Summary and explanation

Huntington Disease (HD) is a progressive disorder of motor, cognitive, and psychiatric disturbances. The mean age of onset is 35 to 44 years; this is modified by the repeat length, epigenetic influences, and possibly environmental influences. The median survival time is 15 to 18 years after onset. Chorea, an involuntary movement disorder consisting of non-repetitive, non-periodic jerking of limb, face, or trunk, is the major sign of the disease. This disease occurs in 3-7 people per 100,000, in populations of western European descent. The disease is inherited in an autosomal dominant fashion, and is caused by the expansion of CAG trinucleotide repeats in exon 1 of a gene termed Huntingtin (HTT, originally known as IT-15) on chromosome 4p16.3 [3]. This trinucleotide codes for the amino acid glutamine. An amino-terminal fragment of mutant Huntingtin localizes to toxic neuronal intranuclear inclusions and dystrophic neuritis in the cortex and striatum of HD individuals [4]. Accumulation of Huntingtin within these structures is influenced by polyglutamine length.

Expression of the IT15 gene occurs mainly in neurons [5]. Huntingtin is involved in processes that counterbalance normal apoptotic pathways [6]. The disease is considered a disorder of inappropriate apoptosis, involving selective (localized) neural cell death, which may result in the neurodegeneration that is seen in HD [7]. It is as yet unknown how the polyglutamine stretches created by the repeat expansions facilitate selective neuron degeneration. Homozygotes for HD appear to have a similar age of onset to heterozygotes, but may exhibit an accelerated rate of disease progression.

A normal allele for gene IT15 contains 10-26 CAG repeats. Individuals with 27-35 repeats fall into the intermediate range, and their children are at risk of HD. The abnormal range varies between 36-121 alleles; individuals at the bottom of this range may or may not develop symptoms of HD. Expansions greater than 60 repeats result in juvenile onset, while expansions between 40 and 55 repeats induce adult onset [8].

Repeats between 27 and 35 can be meiotically unstable in paternal transmission. Descendants of men with repeats in this range have been known to inherit repeats of 40 or more, which are associated with the disease [9].

Repeats between 36 and 39 are also rare, and are associated with reduced penetrance; some individuals with repeats in this range develop HD and others do not [9]. Repeats of 40 or larger are associated with the expression of HD. Persons carrying repeats in this range will develop HD, assuming they do not die of other causes before onset.

The age of disease onset is inversely correlated with repeat length [10, 11]. Together, these two factors explain the phenomenon of anticipation [12], in which the age of onset tends to decrease in successive generations; on average, affected children will develop the disease 8 years earlier than their affected father. The clinical manifestation of anticipation can be dramatic; at times the children of HD-gene carriers will develop the disease before their parents.

The correlation is stronger for high repeat numbers (and low ages at onset) and is much weaker for low repeat numbers (and older ages at onset). This implies that, although CAG repeat length is a major determinant of age of onset in juvenile-onset patients, factors other than CAG repeat length contribute significantly to the onset of HD in the elderly [13, 14, 15].

The "Adellgene Huntington Disease kit" is optimized for the detection and quantification of approximately of approximately 121 CAG repeats, in order to determine the healthy individuals (10-35 repeats) and patients (36-121 repeats). It is based on the analysis of fragments generated with fluorescent primers, capillary electrophoresis in a genetic analyzer, and subsequent detection of the fluorescence with appropriate software for detection and interpretation.

In the cases where only one peak appears, and any errors inherent to the process (low amount of DNA, DNA extracted poorly, failed PCR reaction etc.) have been discarded, t is necessary to observe the pattern of the peaks in order to confirm the homozygosity of the sample. This kit resolves the cases of suspected homozygous individuals based on the resulting electropherogram pattern of the full length PCR products generated from the CAG repeat primed peaks. These peaks are separated by 3 bp, as expected and set up as a decreasing peak´s saw. The profile of peaks provides confirmatory information about a homozygous sample or , to the contrary, the presence of an expanded allele.

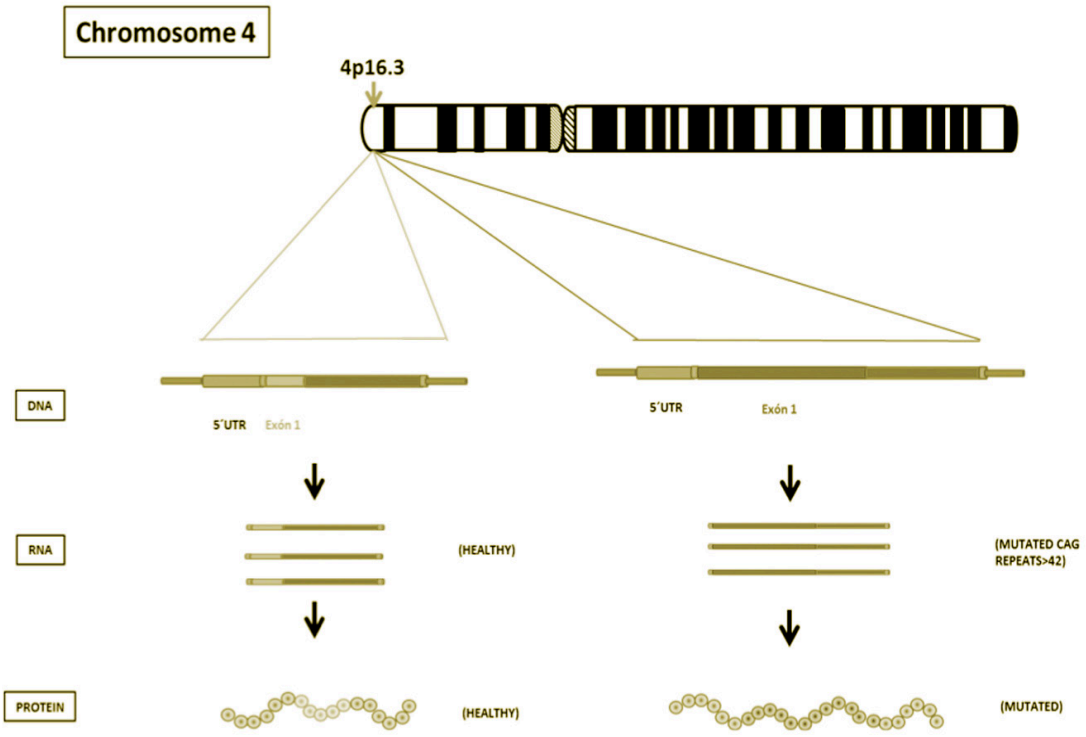

Figure 1. Representation of the different types of HD gene and their products depending on the degree of mutation

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Procedure principles

The detection method employed by the "Adellgene Huntington disease kit" is based on the specific amplification of genomic DNA from purified fragments containing exon 1 of the IT15 gene (HTT), which contains the CAG repeats. The kit includes several primers, one of which is labeled with a fluorophore for subsequent detection in a DNA fragment analyzer. The use of LIZ500™ size marker is recommended. The size of PCR products are converted into the number of CAG triplet repeats using conversion factors of mobility and size.

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Kit contents

Reference AD-HD-16 (16 tests)

- AD-HD-POM2: Polymerase Mix (POM) 1 vial x 390 µL
- AD-HD-PM2: Primer mix (PM) 1 vial x 46 µL

Kit storage

- All kit reagents should be stored at -18 ° C to -30 ° C and are stable at this temperature until the expiration date as indicated on the package.
- Allow the reagents (except AD-HD-POM2) to reach room temperature before use. Gently shake all reagents except AD-HD-POM2 after thawing.
- Before opening the reagent tubes, perform a gentle centrifugation of each component so that the reagents are deposited at the bottom of the tube and not loose on the walls.
- The test should be carried out whilst keeping the reagents on ice or on a cold block.
- Do not make more than 3 cycles of freeze / thawing of the Primer Mix (AD-HD-PM2) and Polymerase mix (AD-HD-POM2) vials as this may reduce the sensitivity of the test and alter the results.
- Due to the photo-sensitive nature of the reagent AD-HD-PM2, avoid continued exposure to light.

Materials required but not supplied

DNA isolation 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

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 LIZ (ABI, Ref.: 4345833 or equivalent)
- Size standard marker LIZ 500™ (ABI, Ref.: 4322682)

Supplies and equipment

- General equipment of a laboratory dedicated exclusively to performing the PCR
- Thermocycler (ABI, 9700, Veriti or equivalent)
- Centrifuge for 96-well plates
- Shaker (Vortex)
- Micro-centrifuge
- Pipettes (P1000, P200, P20 and P2) and filter tips for specific pipette
- Multichannel pipette capable of dispensing of 1-10µl
- PCR plates of 96 wells (optional)
- PCR Plate Sealer (optional)

Positive Control

- The recommended WHO standard for Huntington Disease or any cell line whose corresponding DNA has been validated

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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 carried out with appropriate personal protection such as goggles, gloves and appropriate clothing.
- The 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. Furthermore, no hyperlipemic, hemolyzed, jaundiced or proteinemia blood samples should be used.
- Contamination of DNAses can cause DNA degradation, so filter pipette tips and DNAse-free tubes should be used. Take care with handling, pipetting and using devices to avoid PCR failure.
- Do not use components from different lots. Do not use reagents beyond the expiration date.
- Before using the kit, ensure that the equipment (thermal cycler, genetic analyzer etc.) 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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Procedures

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 100ng (i.e. 1 µl of DNA to 100ng/µl or 2 µl of DNA to 50ng/µl).

B. Preparation and conditions of the PCR reaction

Precautions

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

1. Mix preparation of the Primer and Taq polymerase for n+2 samples:

Reagent	Vol. for each sample (ul)
AD-HD-PM2	2.5
AD-HD-POM2	21.2
Total volume for each reactions	**23.7**

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- Shake the sample gently 3-5 times before distribution into the PCR tubes, to ensure complete homogenization
- Perform a pulse centrifugation to ensure that there are no bubbles or sample on the tube walls.

Note

Excess AD-HD-PM could inhibit the PCR reaction.

2. Pipette 23,7µl of this mixture into an sterile plate or tube and add the volume of DNA, or negative control in the case of contamination control well, required to reach 100ng (i.e. 1 µl of DNA to 100 ng/µl). In the case of low DNA concentration, a higher volume of DNA could be added independently of the increase in total volume of the PCR reaction. However, please be aware that LOWER OR HIGHER DNA CONCENTRATIONS greatly DIMINISH THE QUALITY OF THE RESULTS.

3. Seal the plate or tube with appropriate coverslips and perform a pulse centrifugation to ensure throrough mixing and the removal of bubbles.

4. Place the plate in the thermocycler and perform the PCR reaction under the following conditions:

	Number of cycles	Temperature (ºC)	Time (mm:ss)
DENATURATION	1	98	05:00
CYCLES	10	97	00:35
		62	00:35
		68	04:00
CYCLES	25	97	00:35
		64	00:35
		68	06:00
FINAL EXTENSION	1	72	10:00
COOLING	1	4	∞

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C. Confirmation of the amplification products

Confirmation of the amplified products can be made through an appropriate system, such as horizontal electrophoresis in agarose gels. To carry out this method prepare an agarose gel at a concentration of 1-1.2% w/v, validated according to the laboratory protocol and analyze 2µl of each amplified product to certify that the PCR was successful.

D. Preparation of the samples for capillary electrophoresis

1. Preparation of Samples for DNA analyzer

- The following reaction mixture should be added to the PCR product. It is recommended to dilute the PCR product to ½ or ¼ for the 3730xl DNA analyzers with distilled water, to improve results or to check the optimal dilution to charge in the DNA analyzer.

Reagent	Vol. for each sample (µl)
PCR product *	1
Hi-Di-Formamide **	10
LIZ500™ Maker **	0.3
Total volume for each reactions	**11.3**

** Not diluted for the 3130/3130xl. 1/2 or 1/4 diluted for the 3730xl.*

*** These items are not supplied with the kit*

- Perform a pulse of centrifugation (3-5 times) to ensure thorough mixing of the reagents and transfer 11 µl of this mixture to the corresponding plate for capillary electrophoresis.
- Seal the plate, shake and centrifuge to remove bubbles, and transfer to the thermal cycler.
- Heat the mixture at 95°C for 2 minutes to denature the DNA and then transfer to ice whilst also protecting the sample from light, until injection into the genetic analyzer.
- It is recommended to use a positive control in which the number of repetitions is known (see section 'Materials required but not supplied').

2. Module of DNA Analyzer

The following module of work is recommended for the corresponding DNA analyzer, in a 36cm capillary array.

	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 the use of polymer POP7 is recommended.

Results and interpretation

Adellgene Huntington Disease kit is a technique with a quantitative component and a qualitative one. The quantitative component makes reference to the ability to quantify the number of CAG triplet repeats, between 10 and at least 121 (see Summary and Explanation section). The qualitative component refers to the necessity of observe the pattern of the saw of peaks that is obtained. The shape of these group of peaks determine the presence of short or expanded alleles.

Using the reagents supplied in this kit, if the sample has a number of 30 CGG repeats, the size of the amplified fragment will be 150 bases (Table 1). All other numbers of repetitions obtained with this component of the kit may be tabulated based on this size. The introduction of a fragment marker allows the genetic analyzer software to provide the size of the amplified product directly and therefore the number of CAG triplet repeats. It is not necessary to use any passive reference. Figures 2, 3 and 4 are fragment analysis graphs for the results of healthy and unhealthy individuals, tested using this kit.

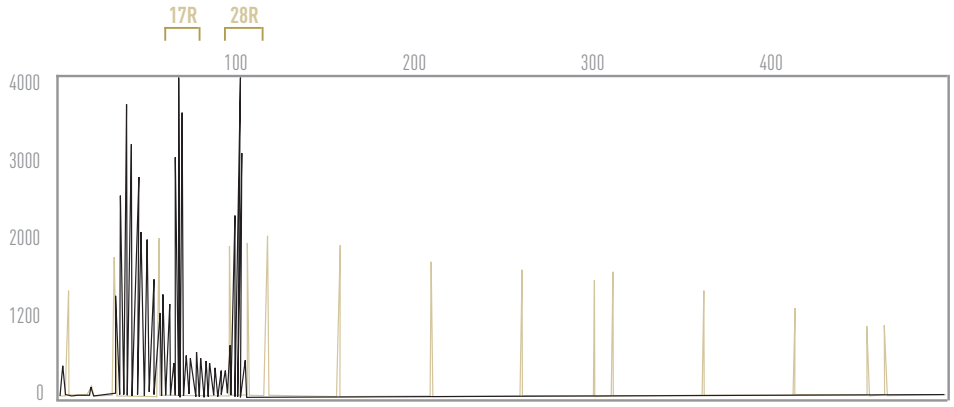

Figure 2. Healthy heterozygous individual (17/28 CAG repeats).

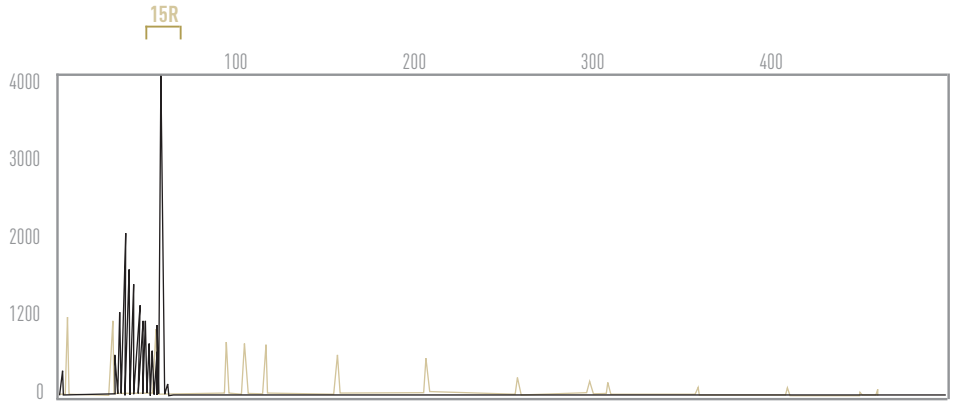

Figure 3. Healthy homozygous individual (15/15 CAG repeats).

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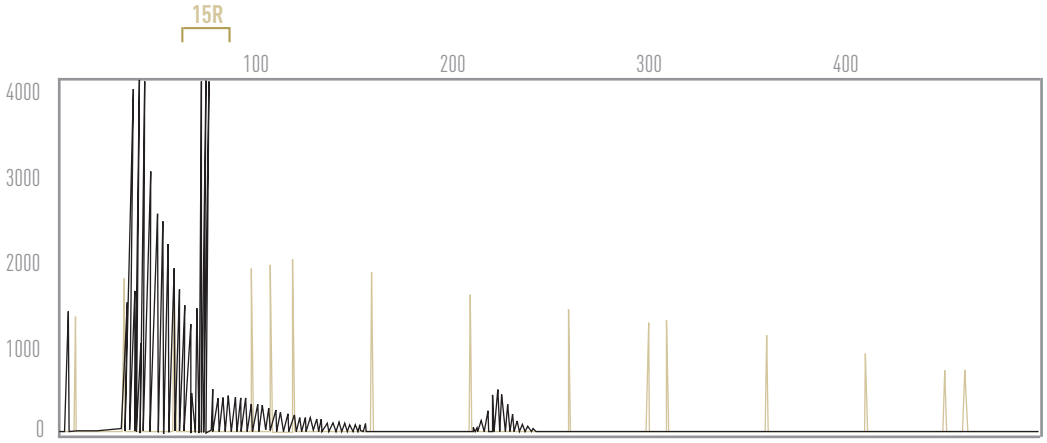

"Figure 4. Unhealthy individual (19/65 CAG repeats)."

CAG repeats	Fragment size	CAG repeats	Fragment size
10	90	65	255
15	105	70	270
20	120	75	285
25	135	80	300
30	150	85	315
35	165	90	330
40	180	95	345
45	195	100	360
50	210	110	375
55	225	115	390
60	240	120	405

Table 1. Correlation between the fragment size and the number of CAG repeats.

Quality control

Due to the quantitative nature of this test, it is necessary to perform a 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 is required to determine the size of the DNA fragments.

A contamination control should be carried 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 should 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.

The kit has been tested in 3130/3130xl and 3730xl DNA analyzers.

Specific operation data

Analytical specificity and sensitivity

The primers of this kit are specific for the human Huntington Disease gene (HTT, originally known as IT-15) (NC_000004.11) and comprise the CAG repeat region within exon 1 of the gene (NT_006051.18). The specific amplification of this region was verified by DNA sequencing, and by assaying samples of healthy individuals and well characterized patients. There is no reported case of cross-reactivity with another gene from genomic DNA.

Diagnostic specificity

ADELLGENE HUNTINGTON DISEASE kit provides a specific assay of the exon 1 region of the HTT gene, which detects the number of CAG repeats. Also determines the presence of an expanded allele through the shape of the pattern of peaks.

Mutations (point mutations, insertions, deletions) at amplification primer sites are possible and may result in the lack of allele definition. Other technologies could be necessary to resolve the problem. Homozygous results must be confirmed by alternative procedures.

Range to

- DNA concentration. For the best range of volume of DNA required in each assay, tests were performed between 10ng to 200ng with a heterozygous female sample. The recommended working range was determined to be about 100 ng of DNA by PCR.
- Results of the kit. This kit can assign a number of CAG triplet repeats between 10 and 121. The use of this kit is for the determination of both healthy individuals, with 10 to 35 repetitions, and unhealthy individuals with 36 repetitions or more, with a difference in penetrance between 36 and more than 42 repetitions (see section 1).

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 most central peak. The accuracy of the number of the repeats was determined by comparing sequenced samples with the sizes obtained with the present kit. A variability of number of repeats with an acceptable error limit of ±1 repeat, for alleles less than or equal to 42, and ±3 repeats for alleles more than 42 was determined.

- General testing.

Region Exon 1/HTT	Samples Tested
Healthy heterozygous samples	25
Healthy homozygous samples	3
Samples with an expanded allele	34
Number of samples tested	**12**

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

Procedure limitations

- The method detects all alleles between 10 and at least 121 CAG triplet repeats (see section 9).
- The conditions described for the PCR should be accurately controlled. Deviations from these parameters can lead to a poor outcome.
- All work from ADELLGENE HUNTINGTON DISEASE KIT must be made in accordance with correct laboratory practices and in compliance with local regulations, such as the international standard.
- The thermocycler must be calibrated according to manufacturers' recommendations and used within the limits specified by them.
- The DNA analyzer must be calibrated according to the manufacturers' recommendations for the fluorophores and 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 has 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 with 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

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- **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 the 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 decontamination 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 freezing / thawing of the reagents
  - Perform aliquot of fraction reagents as necessary
  - Repeat with fresh batch of reagents
- **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 sample yields a positive result

- Cross-contamination
  - Always handle the kit components according to commonly accepted practices, to avoid contamination
- Pipetting error
  - Verify that the sample added 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 and possible dilutions of the PCR product.
- Pipetting error
  - Verify that the volume added in each well is correct

High backgroud (noisy baseline)

- PCR product contaminated
  - See above

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