Instructions for use

Imegen® Huntington

Ref. IMG-154

CE IVD

Manufactured by:
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Our products are 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® Huntington 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 (IFU)		
Version 08	DEC 2023	Review and update of section "3. Technical characteristics".
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 S.L. to Health in Code S.L.
Version 05	JUL 2020	Modification of section 2, Intended use. Addition of section 3, Technical characteristics. Positive control is added to section 5 Kit contents and conditions
Version 04	JUL 2020	Updating of section 7 Assay protocol and section 8 Analysis of results.

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01 General information

Huntington's disease (HD, OMIM #143100) is an inherited disease that causes progressive degeneration of neurons in the brain. Huntington's disease has a wide impact on a person's functional abilities and usually results in movement, thinking (cognitive) and psychiatric disorders.

Most people with Huntington's disease develop signs and symptoms between the ages of 35 and 50. However, the disease may appear earlier or later in life. When the disease occurs before the age of 20, it is called "juvenile Huntington's disease". Earlier onset usually results in a somewhat different set of symptoms and more rapid disease progression.

Huntington's disease is inherited in an autosomal dominant manner and is due, in 100% of cases, to the expanded CAG trinucleotide repeat of the 5' end of the first exon of the huntingtin gene (HTT; MIM 613004), located on chromosomal region 4p16.3.

The diagnosis of Huntington's disease is based on the number of CAG triplet repeats, with a value under 26 repeats being considered the normal genotype. In addition, an inversely proportional relationship has been established between the number of repeats and age at symptom onset. This relationship is particularly strong for high repeats. Table 1 shows the range of CAG repeats in the normal and mutant alleles.

Allele	Repeats
Normal	6-26 CAG repeats
Intermediate	27-35 CAG repeats
Pathological	> 36 CAG repeats

Table 1. Information on the expansions analyzed in the Imegen® Huntington kit.

References

- *Technical standards and guidelines for Huntington disease testing. Potter NT, Spector EB, Prior TW. Genet Med. 2004 Jan-Feb; 6(1): 61-5.*
- *Losekoot M1, van Belzen MJ, Seneca S, Bauer P, Stenhouse SA, Barton DE (2013) EMQN/CMGS best practice guidelines for the molecular genetic testing of Huntington disease. European Molecular Genetic Quality Network (EMQN). Eur J Hum Genet. 21(5): 480-486*

02 Intended use

Imegen® Huntington analyzes the CAG expansion of the 5' end of the first exon of the *HTT* gene by two PCRs (short and long PCR). Short PCR (HTT-S) only amplifies the disease-causing poly-CAG fragment, whereas long PCR (HTT-L) amplifies the poly-CAG fragment and an adjacent polymorphic poly-CCG fragment.

It is necessary to evaluate the results of HTT-L PCR in those samples where only one allele has been detected by short PCR (HTT-S), which could be caused by allelic loss due to the presence of polymorphism in the binding site of one of the short PCR (HTT-S) primers. Long PCR (HTT-L) simultaneously amplifies the poly-CAG fragment and the CCG polymorphism, which can vary between 7 and 11 repeats.

PCR products labeled with 6-Carboxyfluorescein (6-FAM) and NED™ are separated by capillary electrophoresis to analyze the size of the amplicons.

Imegen® Huntington 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 samples previously analyzed by the medical genetics service of Health in Code, S.L. The kit enables the specific detection of 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 25 ng for each amplification system.

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:

- ***Huntington Master Mix***: amplification buffer with dNTPs, $MgCl_2$ and buffer necessary to carry out amplification reactions.
- ***HTT-L Master Mix***: specific oligonucleotides labeled with the NED™ fluorophore to perform the long PCR amplification reaction. Includes the poly-CAG fragment and the adjacent polymorphic poly-CCG fragment.
- ***HTT-S Master Mix***: specific oligonucleotides labeled with the 6-FAM fluorophore to perform the short PCR amplification reaction. Includes the poly-CAG fragment.
- ***General Master Mix IV***: DNA polymerase necessary to carry out amplification reactions.
- ***Positive Control***: Synthetic DNA containing an allele with 26 repeats.

Reagents	Color	Quantity	Storage
Huntington Master Mix	Yellow pad	460 µl	-20°C
HTT-L Master Mix	Green pad	66 µL	-20°C
HTT-S Master Mix	Purple pad	66 µl	-20°C
General Master Mix IV	Yellow cap	10 µl	-20°C
Positive control	Black cap	40 µL	-20°C

Table 2. Imegen® Huntington 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 equipment

Reagents:

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

Materials:

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

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

07 Assay protocol

07.1 | Preparation of amplification reactions

The Imegen® Huntington kit is designed to perform two PCR reactions for each sample to be tested.

In order to estimate the quantity of reagents needed, the total number of samples and controls to be analyzed simultaneously should be taken into account and the volume of each reagent should be increased by an additional 10%.

The recommended protocol for the preparation of amplification reactions is shown below:

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

02 Prepare the short PCR (HTT-S) mix in a 1.5 mL tube.

Reagents	Quantity per reaction
Huntington Master Mix	17.3 µL
General Master Mix IV	0.2 µL
HTT-S Master Mix	5 µL

03 Prepare the long PCR mix (HTT-L) in a 1.5 mL tube:

Reagents	Quantity per reaction
Huntington Master Mix	17.3 µL
General Master Mix IV	0.2 µL
HTT-L Master Mix	5 µL

04 Vortex and spin the HTT-S and HTT-L mixes.

05 Dispense 22.5 µL to the corresponding 0.2 mL tubes and add 2.5 µL of the diluted samples at a concentration of 10 ng/µL.

NOTE: It is recommended to include a negative PCR control for each amplification batch to confirm the absence of contamination and also positive controls to verify allele size. If there are no positive controls, the kit includes a positive control with 26 repeats at the concentration of use.

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

Fields	Stage 1 Enzymatic activation	Stage 2 HTT-S and HTT-L 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. Optimal PCR program 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 HTT-S and HTT-L 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.

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

03 Add 1 µL of the DNA obtained from each PCR reaction.

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.

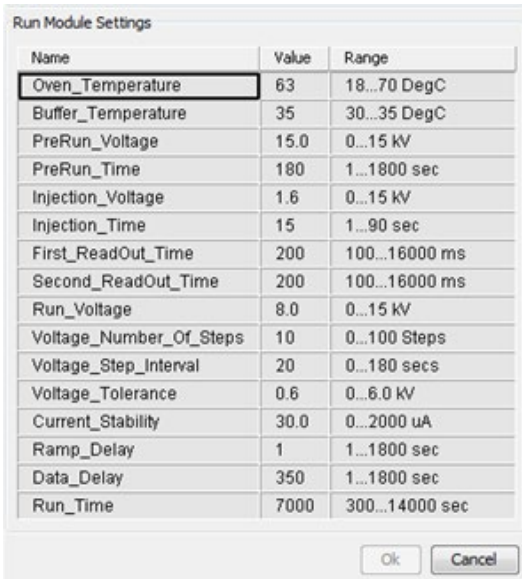
05 Store the plate at 4°C until it is put into the sequencer.

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 and NED™-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:

- In order to analyze the samples it is necessary to use specific software and the .fsa file obtained as a result of capillary electrophoresis.
- Check the absence of peaks in the negative PCR control. If amplification is detected, it is recommended to repeat the assay to rule out accidental contamination.
- Sample analysis:

  Short PCR (HTT-S) allows the user to detect > 100 CAG repeats. However, since a non-pathological CCG insertion frequently occurs in the DNA region complementary to the HTT-S binding oligonucleotides, it is necessary to evaluate the results obtained by long PCR (HTT-L), especially samples where only one allele is detected with HTT-S. Long PCR (HTT-L) allows the user to amplify a larger DNA region, containing both the number of CAG repeats and the non-pathological CCG polymorphism repeats, if any. This highly repetitive GCC polymorphism, amplified by long PCR (HTT-L), can vary between 7 and 11 repeats:

HTT-S Master Mix

The following formula can be used to calculate the exact number of CAG repeats in a given sample.

$$No.of\ repeats\ (CAG) = \frac{Size_{Allele\ x} - 68\ pb}{3}$$

NOTE: Variations may occur ± 1 CAG repeat. This is due to variations in the reagents used or the capillary electrophoresis equipment used for fragment analysis.

HTT-L (Long) Master Mix

The following formula can be used to confirm the exact number of CAG repeats in a given sample and calculate the number of GCC repeats.

$$No.of\ repeats\ (CAG + CCG) = \frac{Size_{Allele\ x} - 86\ pb}{3}$$

NOTE: The value of 68 bp (HTT-S) and 86 bp (HTT-L) comes from a conversion of the amplicon size obtained with the oligonucleotides designed for this kit, both confirmed *in silico* and at our laboratories during kit validation.

Given that the number of CAG repeats is obtained from the results of the formula for HTT-S:

$$No.of\ repeats\ (CCG) = No.of\ repeats\ (CAG + CCG) - No.of\ repeats\ (CAG)$$

Similarly, the repeats can be calculated using the amplicon sizes generated from a sample with a known number of size repeats (e.g. 21 CAG repeats and 7 CCG repeats):

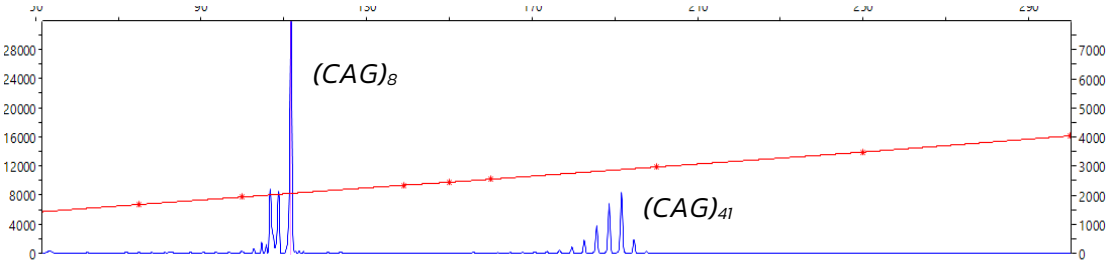
$$No.of\ repeats\ (CAG) = \frac{Size_{Allele\ x} - Size_{Allele\ 21\ rep}}{3} + 21$$

$$No.of\ repeats\ (CAG + CCG) = \frac{Size_{Allele\ x} - Size_{Allele\ 21+7\ rep}}{3} + (21 + 7)$$

Some images are shown below as an example of the possible results.

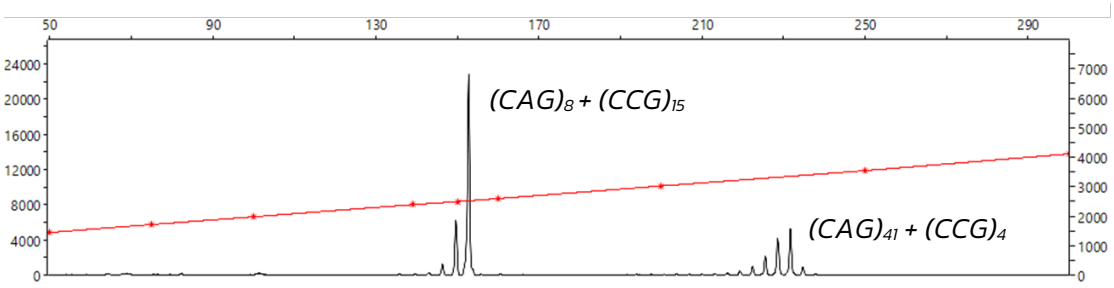
↘ HTT-S results (6-FAM)

- ◇ **Allele 1**: 8 CAG repeats (92 bp fragment)
- ◇ **Allele 2**: 41 CAG repeats (191 bp fragment)



↘ HTT-L results (NEDTM)

- ◇ **Allele 1**: 8 CAG repeats + 15 CCG repeats (155 bp fragment)
- ◇ **Allele 2**: 41 CAG repeats + 4 CCG repeats (221 bp fragment)



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]
	✔	✔			Artifacts characteristic of expansions [8]

Table 4. Interpretation of possible results with the Imegen® Huntington 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 that appear as smaller peaks, 3 base pairs away from the predominant peak.

10 Limitations

10.1 | Equipment

Imegen® Huntington 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® Huntington 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) and NED™ labeling. In the case of using equipment different from that mentioned above, follow the protocol specifications for those platforms.

10.2 | Reagents

Imegen® Huntington 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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