Instructions for use

Imegen® SCAs

Ref. IMG-152

CE IVD

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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	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 07	SEP 2022	Change of manufacturer's identification: from Imegen S.L. to Health in Code S.L.
Version 06	FEB 2022	Revision of the document's content.
Version 05	MAY 2021	Revision of the document's content.
Version 04	JUL 2019	Updating the reference ranges of the number of repeats.
Version 03	JAN 2019	Updating the document for CE-IVD marking of the product.

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01 General information

Spinocerebellar ataxias (SCAs) comprise a large, heterogeneous group of neurodegenerative disorders characterized mainly by progressive cerebellar ataxia with motor abnormalities, dysarthria, pyramidal and extrapyramidal symptoms, pigmentary retinopathy, peripheral neuropathy and cognitive dysfunction among other symptoms.

This group of diseases has an autosomal dominant pattern of inheritance. To date, 40 types of SCAs have been identified, classified from SCA1 to SCA40.

The prevalence of SCAs in the general population has been estimated at 0.001–0.005%. The frequency of each SCA varies in different populations, the most frequent being SCA1, SCA2, SCA3, SCA6, SCA7, SCA12 and SCA17.

Several genes associated with this pathology have been described, whose mutation generally consists in the expansion of the CAG repeat. An exception is SCA17, which is characterized by the expansion of CAG and CAA trinucleotides. Therefore, the diagnosis of SCAs is carried out by identifying the number of trinucleotide repeats that cause the expansion.

Ataxia	Gene	Chromosome location	No. of repeats			
			Normal allele	Uncertain allele	Intermediate penetrance	Full penetrance, pathological
SCA1[1] (MIM#164400)	*ATXN1*	6p22.3	6–38; 39–44 CAT interrupted		–	39–44 CAGs uninterrupted; 45–91
SCA2[1] (MIM#183090)	*ATXN2*	12q24.13	14–31	32–34	–	35–500
SCA3[1] (MIM#109150)	*ATXN3*	14q32.12	11–44	–	45–59	60–87
SCA6[1] (MIM#183086)	*CACNA1A*	19p13.13	4–18	–	19	20–33
SCA7[1] (MIM#164500)	*ATXN7*	3p14.1	4–19	28–33	34–35	36–460
SCA12[1] (MIM#604326)	*PPP2R2B*	5q32	4–32	40–45	–	51–78
SCA17[2] (MIM#607136)	*TBP*	6q27	25–40	–	41–48	> 49
DRPLA[3] (MIM#125370)	*ATN1*	12p13	6–35	36–47	–	≥ 48

Table 1. Reference ranges of the number of repeats of SCAs analyzed by Imegen® SCAs.

Additionally, the Imegen® SCAs kit includes the analysis of dentatorubral-pallidoluysian atrophy (DRPLA) because it is a neurodegenerative disorder that has similarities with SCAs,

such as the type of inheritance (autosomal dominant), the type of mutation (expansion of a CAG trinucleotide) and some symptoms (mainly cerebellar ataxia).

References

- *SCA Base. Reference ranges for oligonucleotide repeat sizes at the main SCA loci. http://www.scabase.eu/table3.php*
- *Toyoshima Y, Onodera O, Yamada M, Tsuji S, Takahashi H. Spinocerebellar Ataxia Type 17 [Internet]. GeneReviews®. University of Washington, Seattle; 1993. Available: http://www.ncbi.nlm.nih.gov/pubmed/20301611*
- *Veneziano L, Frontali M. DRPLA [Internet]. GeneReviews®. University of Washington, Seattle; 1993. Available: http://www.ncbi.nlm.nih.gov/pubmed/20301664*
- *Sun Y-M, Lu C, Wu Z-Y. Spinocerebellar ataxia: relationship between phenotype and genotype - a review. Clin Genet. 2016; 90: 305–314*

02 Intended use

The Imegen® SCAs kit analyzes the expansions of CAG or CAG/CAA triplets associated with cerebellar ataxias and dentatorubral-pallidoluysian atrophy (DRPLA) by conventional PCR and capillary electrophoresis. In addition, the kit offers a TP-PCR (triplet repeat primed PCR) system to detect major expansions of SCA2 and SCA7, 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.

The results will provide the clinician with a phenotype-genotype correlation, which will facilitate differential diagnosis, disease course prediction, symptom monitoring and genetic counseling.

Imegen® SCAs 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 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 50 ng for each amplification system.

Health in Code S.L. is certified according to the standard UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems – Requirements for regulatory purposes by AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS (AEMPS, Spanish Agency for Medicinal Products and Medical Devices) for the design, development and production of *in vitro* diagnostic medical devices:

- Genetic analysis kits
- Software for the bioinformatics analysis of genetic data

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:

- *Ataxia A Master Mix*: PCR Master Mix with the quantities of nucleotides, betaine, $MgCl_2$ and buffer necessary to carry out reactions: A, B, C, D, E and F wells of the SCAs Master Mix strip.
- *Ataxia B Master Mix*: PCR Master Mix with the quantities of nucleotides, betaine, $MgCl_2$ and buffer necessary to carry out PCR reactions: G and H wells of the SCAs Master Mix strip and TP-PCR.
- *General Master Mix III*: DNA polymerase necessary to carry out PCR reactions: A, B, C, D, E and F wells of the SCAs Master Mix strip.
- *General Master Mix IV*: DNA polymerase necessary to carry out PCR reactions: G and H wells of the SCAs Master Mix strip and TP-PCR.
- *TP SCA2 and TP SCA7 Master Mix*: It contains the oligonucleotides necessary to perform the TP-PCRs included in the kit.
- *Positive Control*: Genomic DNA at the optimal amplification concentration at which normal alleles are present for all analyses performed with the kit (see genotyping in section 8 of this document).
- *SCAs Master Mix strip*: The 8-well strip contains the oligonucleotides necessary to perform conventional PCR amplification of the kit's target regions. See the distribution of each specific *Master Mix* in table 2.

Well	SCA Master Mix
A	SCA1
B	SCA3
C	SCA6
D	SCA12
E	SCA17
F	DRPLA
G	SCA2
H	SCA7

Table 2. Distribution of each specific Master Mix on the 8-well strip

Reagents	Color	Quantity	Storage
Ataxia A Master Mix	White pad	720 µL	-20ºC
Ataxia B Master Mix	Yellow pad	710 µL	-20ºC
General Master Mix III	White cap	360 µL	-20ºC
General Master Mix IV	Yellow cap	10 µL	-20ºC
SCAs Master Mix strip	8-well strip	8 x 60 µL	-20ºC
TP SCA2 Master Mix	Green pad	60 µL	-20ºC
TP SCA7 Master Mix	Purple pad	60 µL	-20ºC
Positive Control	Blue cap	200	-20ºC

Table 3. Imegen® SCAs kit components

06 Equipment, reagents and materials not included in the kit

Equipment:

- Conventional thermal cycler
- Micropipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Multichannel pipette (0.5-10 µL)
- 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.

Complementary kits

In order to analyze the expansions involved in other neurodegenerative diseases, Health in Code S.L. also offers Imegen® DM1 (Ref: IMG-173), Imegen® SBMA (Ref: IMG-153), Imegen® Huntington (Ref: IMG-154), and Imegen® Friedreich (Ref: IMG-155) kits. All of them, together with the Imegen® SCAs kit, have been designed using the same PCR program, so they can be analyzed together.

07 Assay protocol

07.1 | Preparation of amplification reactions

The Imegen® SCAs kit has been designed to perform 10 reactions for each sample to be analyzed (8 PCR and 2 TP-PCR) for a total of 12 determinations.

It is recommended to add a negative PCR control for each assay to check that the reagents used are not contaminated with DNA and a positive control to check the operation of all the amplification systems and to normalize the result obtained based on the size of the fragment obtained for each type of analysis.

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 mixes will be prepared, one for the PCR reactions of SCA1, SCA3, SCA6, SCA12, SCA17 and DRPLA (Mix A), and one for the PCR reactions of SCA2, SCA7 and TP-PCR of SCA2 and SCA7 (Mix B).

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 PCR Mix A in a 1.5 mL tube by adding the following reagents. Vortex and spin.

Reagents	Quantity per sample
Ataxia A Master Mix	60 µL
General Master Mix III	30 µL

03 Prepare PCR Mix B and TP-PCR in a 1.5 mL tube by adding the following reagents. Vortex and spin.

Reagents	Quantity per sample
Ataxia B Master Mix	59.2 µL
General Master Mix IV	0.8 µL

04 In a 96-well plate, dispense 15 µL of Mix A into the wells of rows A, B, C, D, E and F of the plate. As many columns should be filled as the number of samples or controls to be analyzed simultaneously.

05 Similarly, dispense 15 µL of Mix B into the wells of rows G and H of the plate for the SCA2 and SCA7 PCR reactions. As many columns should be filled as the number of samples or controls to be analyzed simultaneously.

06 Once the mixes for the PCR reactions have been dispensed, dispense 15 µL of Mix B into the wells where the TP-PCR reactions of SCA2 and SCA7 are to be performed.

07 Using a multichannel pipette, add 5 µL of the SCA-specific Master Mixes included in the 8-well strip of the kit to each column according to the number of samples or controls.

08 Add 5 µL of the specific Master Mixes for each TP-PCR (*TP SCA2 Master Mix* and *TP SCA7 Master Mix*) to the wells where these reactions are to be performed.

09 Add 5 µL of the diluted samples at a concentration of 10 ng/µL to each well or 5 µL of water if it is a negative PCR control or 5 µL of the positive control, which is already at the optimal amplification concentration.

10 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 4. 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 | Real-time PCR program setup

In order to perform capillary electrophoresis, PCR and TP-PCR products (fragments) must be prepared in a 96-well plate compatible with the capillary electrophoresis equipment 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 with nuclease-free water) to adjust the intensity of the peaks observed in the electropherogram.

04 Seal the plate with a film, 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 (ThermoFisher 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 that the negative PCR control does not contain any peaks of 130-300 base pairs in the electropherogram. If amplification is detected, it is recommended to repeat the assay to rule out accidental contamination.
- Sample analysis:

  In order to calculate the number of repeats of an unknown allele, the following formula can be used:

$$No.of\ repeats = \frac{Size_{Allele\ X} - Size_{Allele\ 8\ rep}}{3} + 8$$

X being different for each of the systems.

SCAs	X bp
SCA1	SCA1
SCA2	SCA3
SCA3	SCA6
SCA6	SCA12
SCA7	SCA17
SCA12	DRPLA
SCA17	SCA2
DRPLA	SCA7

Table 5. Value of X to be applied in each PCR system

NOTE: The value of X 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.

Due to the described variations of a repeat between different laboratories, Health in Code S.L. recommends the use of a sample with a known repeat size, using the following formula (e.g.: 8 repeats).

$$No.of\ repeats = \frac{Size_{Allele\ X} - Size_{Allele\ 8\ rep}}{3} + 8$$

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

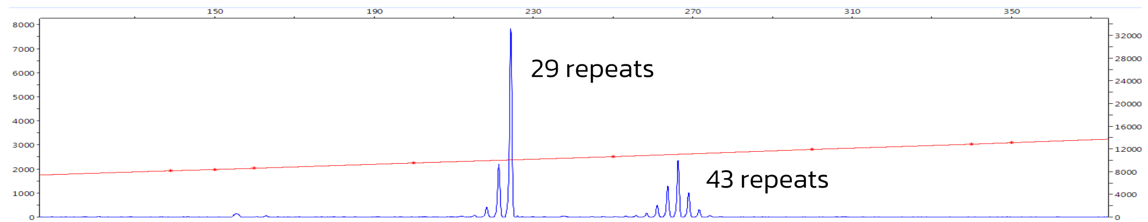

Figure 2. Sample with one normal allele and one expanded allele for the SCA1 system.

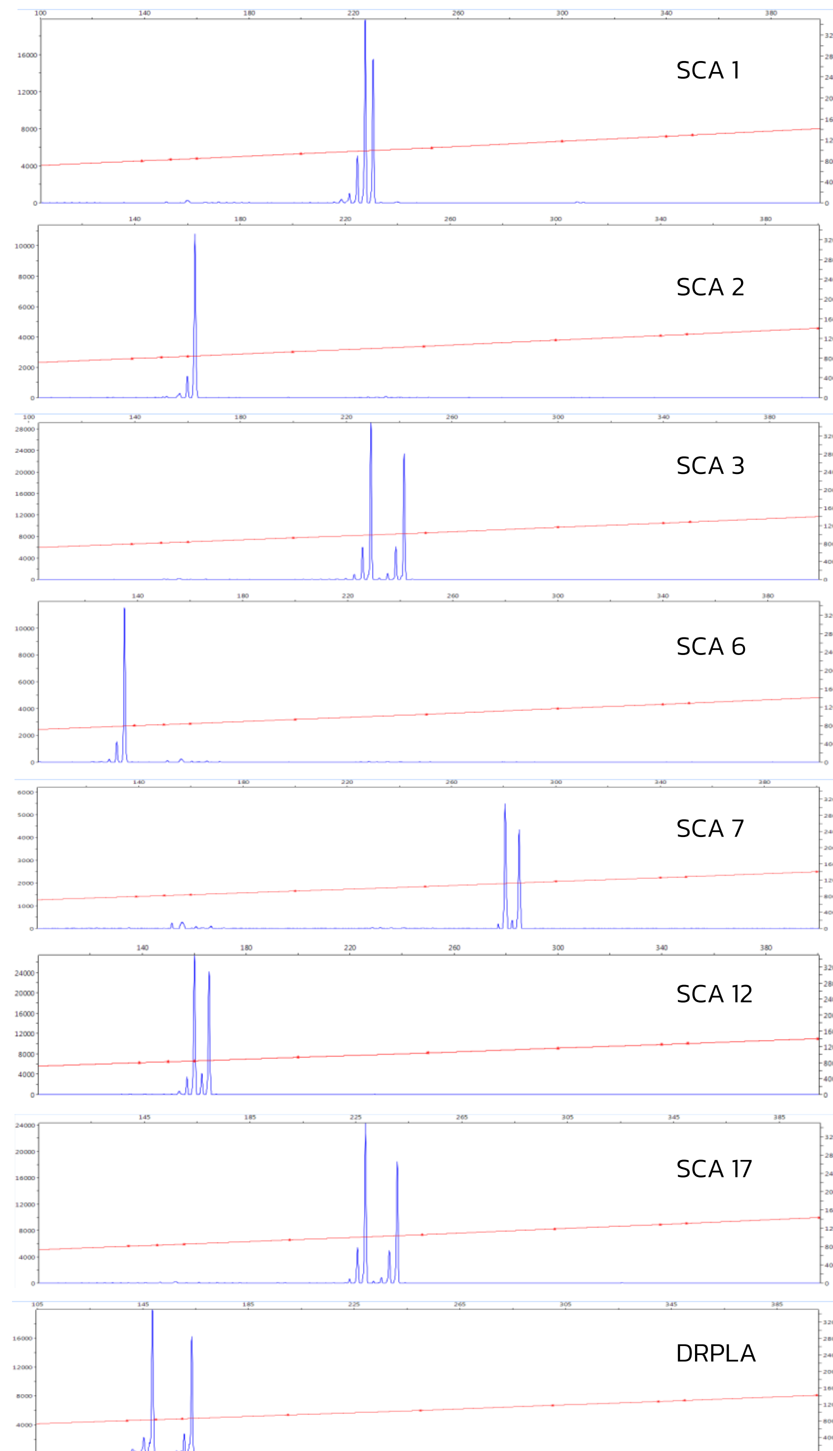

Figure 3. Expected results with the positive control for each of the PCR systems included in the kit.

System	No. of base pairs		No. of repeats	
SCA1	226	229	29	30
SCA2	163	163	22	22
SCA3	229	241	23	27
SCA6	135	135	13	13
SCA7	281	287	11	13
SCA12	159	165	12	14
SCA17	229	241	34	38
DRPLA	148	163	12	17

Table 6. Repeats and size of the positive control alleles in each system.

Due to the presence of unconsumed traces of labeled primers during PCR and TP-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 present 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.

08.1 | TP-PCR results

It is necessary to evaluate the TP-PCR (triplet repeat primed polymerase chain reaction) results for those samples where SCA2 and SCA7 are being analyzed, and where only one allele (one peak) has been observed in the electropherogram resulting from the SCA2 and SCA7 PCR. Therefore, TP-PCR allows samples homozygous for normal alleles to be differentiated from samples with expanded alleles that cannot be detected by conventional PCR due to their large size.

This technique allows expansions of any size to be detected, although the identification of the largest allele generally does not allow the number of repeats to be determined.

A series of images are given below as an example of the result obtained for a sample with an expanded allele and a sample without expansion for the two systems analyzed by TP-PCR.

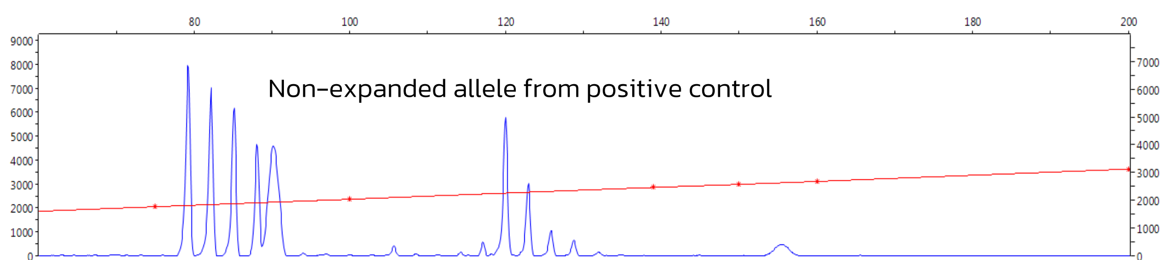

Figure 4. Expanded and non-expanded alleles for the SCA2 TP-PCR system.

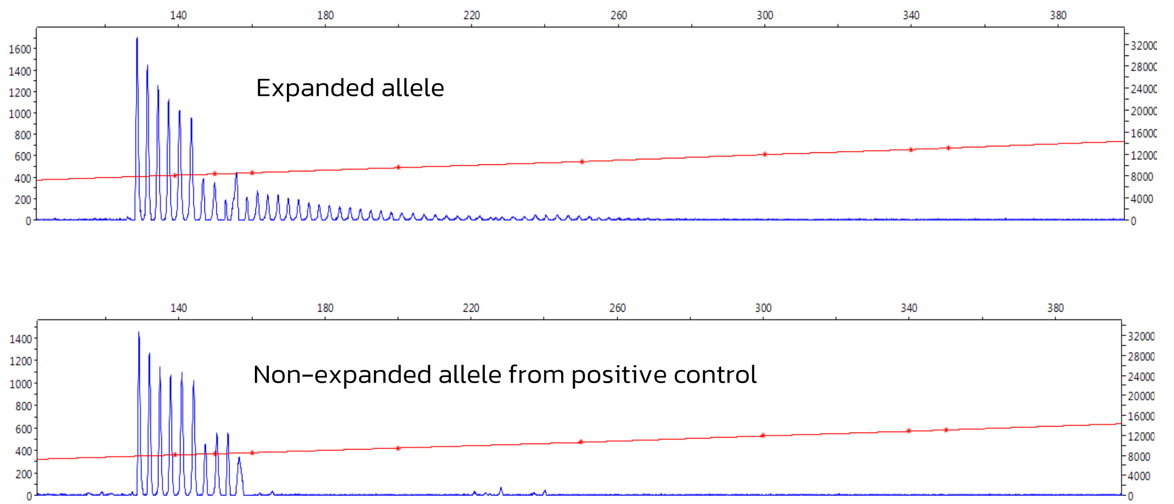

Figure 5. Expanded and non-expanded alleles for the SCA7 TP-PCR system.

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 7. Interpretation of possible results with the Imegen® SCAs 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. 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.

(9) Mosaicism: In some SCAs, such as SCA2 and SCA6, cases of mosaicism have been described. Therefore, it is possible, although unlikely, 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® SCAs has been validated using the following PCR thermal cyclers:

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

If you use another 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® SCAs has been validated using the following sequencing platform:

- *3730xl DNA Analyzer* (ThermoFisher 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® SCAs 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: ThermoFisher 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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Find out about all our **diagnostic kits**