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Instructions for use

Imegen® SCAs

REF IMG-152

CE IVD

DISPOSITIVO MÉDICO ISO 13485 CERTIFICADO AEMPS [Nº 2019 09 0061 EN]

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Rev. 6 14/05/2021

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Imegen guarantees that all its products are free from defects, both in the materials used as well as in its manufacturing process. This guarantee extends through to the expiry date, so long as the storage conditions specified in this manual are observed.

Our products are designed for *in vitro* diagnostics. Imegen does not offer any other warranty, express or implied, that extends beyond the correct functioning of the components of this kit. Imegen's only obligation, with respect to the aforementioned guarantees, will be to replace the products, or to return the purchase price thereof, at the customer's discretion, provided that the existence of a defect in the materials or in the development of the products, is proven.

Imegen will not be responsible for any damage, direct or indirect, that results in economic losses or damages that may occur due to the use of this product by the buyer or user.

All products marketed by Imegen are subjected to rigorous quality control. **Imegen-SCAs** kit has passed all internal validation tests, which guarantee the reliability and reproducibility of each test.

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

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Amendments to the Instructions for Use (IFU)	
Version 03	Adaptation to the Regulation (EU) 2017/746 of the European parliament and of the Council on *in vitro* diagnostic medical devices
Version 04	Update of the reference ranges of the number of repetitions
Version 05 and 06	Content Review

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1 General Information

Spinocerebellar ataxia (SCA) comprises a large group of heterogeneous neurodegenerative disorders inherited in an autosomal dominant fashion. It is characterised by progressive cerebellar ataxia with oculomotor dysfunction, dysarthria, pyramidal signs, extrapyramidal signs, pigmentary retinopathy, peripheral neuropathy, cognitive impairment and other symptoms.

It is classified according to the clinical manifestations or genetic nosology. To date, 40 types of SCAs have been identified and are classified as SCA1 through SCA40.

The prevalence rate of SCAs in the general population is estimated to be 0.001-0.005%. The frequency of each SCA type varies in different populations, being the most frequent SCA1, SCA2, SCA3, SCA6, SCA7, SCA12 y SCA17.

Different genes associated with this pathology, whose mutation generally consists of the expansion of CAG repetition, have been described. Except in the case of SCA17, which is characterised by the expansion of the CAG and CAA trinucleotides. Therefore, the diagnosis of SCAs is carried out by identifying the number of repetitions of the trinucleotide that generates the expansion.

Ataxia	Gene	Chromosome location	Repeat N°			
			Normal	Uncetain	Intermediate penetrance	Complete penetrance, pathological
SCA1 [1] (MIM#164400)	ATXN1	6p22.3	6—38; 39—44 CAT interrupted		-	39—44 CAGs interrupted; 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 for oligonucleotide repeat sizes for the SCA loci analysed by Imegen-SCAs.

Additionally, **imegen-SCAs kit** includes the analysis of the Dentatorubral-pallidoluysian atrophy because it is a neurodegenerative disorder that shows similarities to the SCAs, such as the type of inheritance (autosomal dominant), mutation type (triplet CAG repeat expansion) and some symptoms (mostly cerebellar ataxia).

References

[1] SCA Base. Reference ranges for oligonucleotide repeat sizes at the main SCA loci. http://www.scabase.eu/table3.php

[2] 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

[3] 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.

2 Intended use

Imegen-SCAs kit has been designed to detect triplet CAG or CAG/CAA repeat expansion associated to spinocerebellar ataxia and Dentatorubral-pallidoluysian atrophy (DRLPA) by conventional PCR and capillary electrophoresis (fragment analysis). Furthermore, the kit offers a TP-PCR (triplet repeat primed PCR) system for the analysis of SCA2 and SCA7, for which longer expansions not detectable by conventional PCR have been described.

The triplet repeat primed PCR (TP PCR) assay uses a locus-specific primer flanking the repeat together with paired primers amplifying from multiple priming sites within the repeat, allowing thus by PCR and subsequently capillary electrophoresis, the detection of the expanded alleles undetectable by conventional PCR.

PCR products will be separated by capillary electrophoresis and both the PCR as well as the TP-PCR will be detected by the 6-Carboxifluoresceina (6-FAM) labelling.

The results will provide the clinician information on genotype -phenotype correlation that will aid to assess differential diagnosis, disease progression, symptoms monitoring and genetic counselling.

Imegen-SCAs kit has been designed for *in vitro* diagnostics and it is directed to professionals from the molecular biology sector.

3 Technical characteristics

This kit has been validated using reference EMQN samples (European Molecular Genetics Quality Network), reference DNA samples from Coriell Institute, as well as using samples previously analysed in the Medical Genetic Unit of Imegen. The kit provides robust and specific detection of the expansions for which has been developed.

The type of sample required for this analysis is genomic DNA extracted from peripheral blood, a total quantity of 50 ng for each system will be necessary.

Imegen is certified, in accordance with the standard **UNE-EN ISO 13485:2018 Medical devices – Quality management systems – Requirements for regulatory purposes**, by THE AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS for the Design, development and manufacturing of "in vitro" diagnostic medical devices:

- Kits for genetic testing
- Software for bioinformatic analysis of genetic data

4 Warnings and Precautionary statements

1. Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.

2. Do not pipette by mouth.

3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

4. You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.

5. Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.

6. In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.

7. The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to imegen.

8. This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.

9. Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.

10. This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.

11. The manufacturer is not responsible for the malfunction of the assay when one or more reagents included in the kit are replaced by other reagents not supplied by Imegen.

12. The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Imegen, considering them equivalent to those provided in the kit.

5 Contents and storage conditions

The kit includes the following reagents, enough to analyse 12 samples:

- Ataxia A Master Mix: PCR Master Mix containing $MgCl_2$, betaine and water reagents for the PCR reactions (A, B, C, D, E and F wells of SCAs Master Mix strip).
- Ataxia B Master Mix: PCR Master Mix containing Buffer, $MgCl_2$, dNTPs, betaine and water reagents for the PCR reactions (G and H wells of SCAs Master Mix strip) and TP-PCR.
- General Master Mix III: Conventional PCR enzyme for the PCR reactions (A, B, C, D, E and F wells of SCAs Master Mix strip).
- General Master Mix IV: Conventional PCR enzyme master mix for the PCR reactions (G and H wells of SCAs Master Mix strip) and TP-PCR.
- TP SCA2 y TP SCA7 Master Mix: Specific oligonucleotides for TP-PCR.
- Positive Control: Genomic DNA control with normal alleles of all the regions analysed (see genotype in Section 8 of this manual).
- SCAs Master Mix Strip: 8-well strip with specific oligonucleotides for conventional PCR of the target regions. See the distribution of each SCA-specific Master Mix in the Table 2.

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

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

Reagents	Colour	Volume	Storage conditions
Ataxia A Master Mix	White pad	720 µl	-20°C
Ataxia B Master Mix	Yellow pad	710 µl	-20°C
General Master Mix III	Orange cap	360 µl	-20°C
General Master Mix IV	Yellow cap	10 µl	-20°C
SCAs Master Mix Strip	-	8 x 60 µl	-20°C
TP SCA2 Master Mix	Green pad	60 µl	-20°C
TP SCA7 Master Mix	Purple pad	60 µl	-20°C
Positive Control	Blue cap	200 µl	-20°C

Table 3. imegen-SCAs kit contents

6 Equipment, reagents and material required but not supplied

Equipment:

- Conventional PCR Thermal Cycler
- Micropipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Multichannel pipette
- Vortex
- Centrifuge
- Sequencer

Reagents:

- GeneScan™ 500 LIZ® (Applied Biosystems cat. no. 4322682)
- Hi-Di™ formamide
- Nuclease free Water

Materials:

- Disposable micropipette filter tips (10 µL, 20 µL, 200 µL and 1000 µL)
- 1.5 mL sterile tubes
- 96-well plates or 0,2 mL tubes
- Films for 96-well plates
- Powder-free latex gloves

Note: This kit does not contain the reagents to perform capillary electrophoresis.

6.1 Related Kits

In order to analyze expansions involved in other neurodegenerative diseases Imegen offers the kits imegen-DM1 (Ref: IMG-173), imegen-SBMA (Ref: IMG-153), imegen-Huntington (Ref: IMG-154) and imegen-Friedreich (Ref: IMG-155). All these kits, including imegen-SCAs, has been designed to amplify with the same PCR program, for a simultaneous analysis.

7 Assay protocol

7.1 PCR reactions preparation

Imegen-SCAs Kit is designed to perform 10 reactions per sample (8 PCRs and 2 TP-PCRs), for a total of 12 samples.

It is recommended to include a negative control in each assay, to rule out PCR contamination, and a positive control, to verify the correct amplification of each system and normalise the results according to the fragment size obtained for each assay.

In order to estimate the amount of necessary reagents, we recommend scaling up the volume of reagents to take into account the total number of samples and controls to be simultaneously analysed. In addition, it is recommended to include reagents for one more reaction or an additional 10% of each reagent. .

Using **imegen-SCAs kit**, two master mixes should be prepared: one for PCR reactions of SCA1, SCA3, SCA6, SCA12, SCA17 and DRPLA (Mix A), and another mix for the PCR reactions of SCA2, SCA7 and TP-PCR of SCA2 and SCA7 (Mix B).

1. Thaw all reagents contained in the kit and DNA samples. Vortex each reagent and keep cold.
2. In order to prepare the PCR master mix A, add into a fresh 1.5 mL tube the following reagents. Vortex and spin.

Reagents	Amount per reaction
Ataxia A Master Mix	60 µL
General Master Mix III	30 µL

3. In order to prepare the PCR master mix B and TP-PCR mix, add into a 1.5 mL tube the following reagents. Vortex and spin.

Reagents	Amount per reaction
Ataxia B Master Mix	59.2 µL

General Master Mix IV 0.8 µL

4. In a 96 well plate dispense 15 µL of the PCR Master Mix A in corresponding wells of A, B, C, D, E and F rows of the plate. The number of columns utilised per plate will depend on the number of samples or controls simultaneously analysed.
5. Likewise, dispense 15 µL of the PCR Master Mix B in corresponding wells of G and H rows of the plate for SCA2 and SCA7 reactions. Similarly, the number of columns utilised per plate will depend on the number of samples or controls simultaneously analysed.
6. Then, dispense 15 µL of the PCR Master Mix B in the wells where TP-PCR reactions of SCA2 and SCA7 will be performed.
7. Add 5 µL of the SCA-specific master mixes included in the SCA Master Mix 8 well-strip with a multichannel pipette to each column depending on the samples or controls.
8. Add 5 µL of the SCA-specific master mixes of each TP-PCR in the wells where TP-PCR has to be performed.
9. Add 5 µL of sample DNA at 10 ng/µL into each reaction well, 5 µL of water in the negative control and 5 µL of the given positive control.
10. Place the samples in a thermal cycler and perform the following PCR programme:

Fields	Step 1 Enzymatic Activation	Step 2 PCR or TP-PCR			Step 3	
Cycle Number	1 Initial Cycle	30 cycles			1 cycle	
		Denaturation	Primers binding	Extension	Final step 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 optimal programme. This program has been validated on Biometra T3 equipment, SimpliAmp Thermal Cycler and GeneAmp® PCR System 2720 (Applied Biosystems)

After the amplification is completed, the protocol can be stopped. If the second PCR will be carried out within the next 24 hours, keep the PCR product at 4ºC. Otherwise, store at -20ºC until it is needed.

7.2 Fragment analysis preparation

In order to perform the analysis of expansions, fragment analysis is required. Thus, a PCR plate containing the PCR and TP-PCR products has to be prepared. The recommended protocol for its preparation is showed below:

1. Add the following reagents into a fresh 1.5 mL tube to prepare the Fragment master mix:

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

   To estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples to be simultaneously analysed, and then considering one more reaction, or increase a 10% the volume of each reagent.

   Note: The size-marker volume can be increased or decreased to adjust peaks intensity.

2. Dispense 18.5 µL of the Fragment Master Mix in each well.
3. Add 1 µL of the DNA obtained from the PCR and TP-PCR reactions.
   Note: The volume of the sample can be adjusted by diluting DNA sample in order to optimise the fluorescent peak intensity.
4. Seal the plate, spin down and denature in a thermal cycler for 5 minutes at 98°C.
5. Store the plate at 4°C until the sequencer is ready to perform the capillary electrophoresis.

7.3 Capillary electrophoresis

Once prepared fragments plate, reactions should be subjected to capillary electrophoresis. Depending on the type of sequencer used, electrophoresis conditions recommended by the manufacturer shall be used.

To set these conditions, it should be taken into account that amplification range varies approximately between 100-500 bp, that are employed FAM-labelled primers and that molecular weight standard is labelled with GeneScan™ 500 LIZ.

The following image shows the optimized parameters for the 3730xl DNA Analyzer sequencer, with POP-7™ polymer.

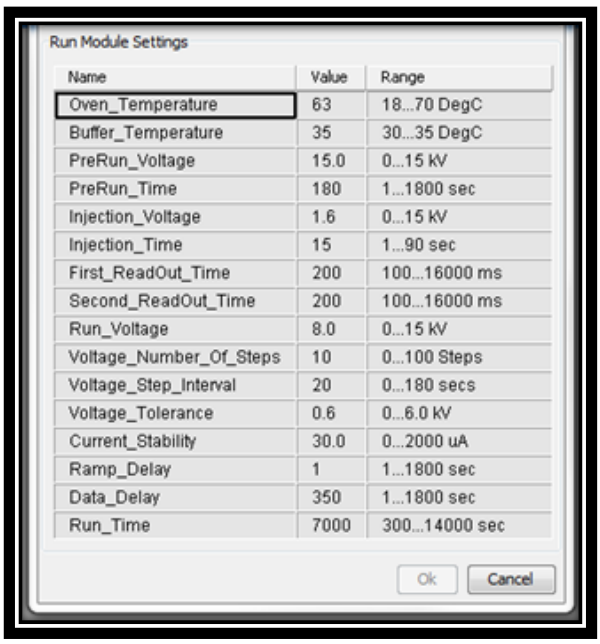

Figure 1. Optimized parameters for the 3730xl DNA Analyzer sequencer

Detection intensity may vary between different equipment, depending on the model and the conditions of the equipment optical system. Therefore in some cases it may be necessary to dilute the samples before performing capillary electrophoresis.

8 Results analysis

For a correct analysis of the results it is recommended to follow these indications:

- To analyze the samples, specific software and the .fsa file obtained as a result of capillary electrophoresis must be used.
- Check that in the negative PCR control there is no presence of peaks of 130 - 300 base pairs in the electropherogram. If amplification is detected, it is recommended to repeat the test to rule out that accidental contamination has occurred.
- Sample analysis:

  The following formula can be used to calculate the number of repetitions:

$$Number\ of\ Repetitions = \frac{Size_{Allele\ x} - X}{3}$$

  where **X** varies depending on the SCA of study.

SCAs	8.1.1.1.1.1 X bp
SCA1	139
SCA2	97
SCA3	160
SCA6	96
SCA7	248
SCA12	123
SCA17	127
DRPLA	112

Table 5. X value to apply to each PCR system

Note: the "X" value is intrinsic to the amplification system used for each *SCA* gene, and it has been validated *in silico* as well as empirically.

A variation of one repetition might be detected between laboratories, caused by factors such as the reagents or the capillary electrophoresis equipment used for fragment analysis. Therefore, we recommend using a sample with a known repetition size (for example: 8 repetitions):

$$Number\ of\ Repetitions\ Number = \frac{\mathrm{Size}_{\mathrm{Allele\ x}} - \mathrm{Size}_{\mathrm{Allele\ 8\ rep.}}}{3} + 8$$

The following images show examples of expected fragment analysis results.

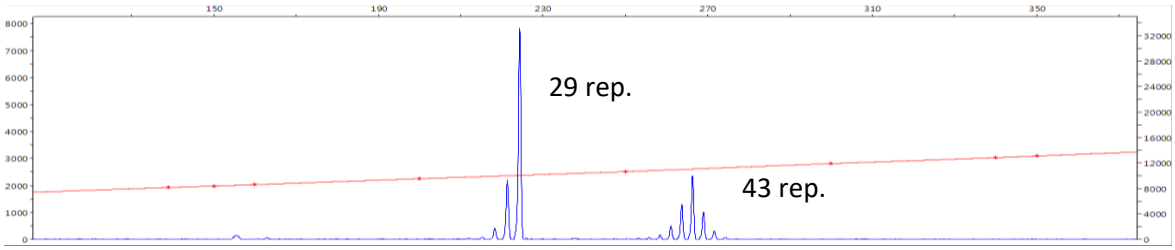

Figure 2. Examples of results, wild type allele and a SCA1 expanded allele.

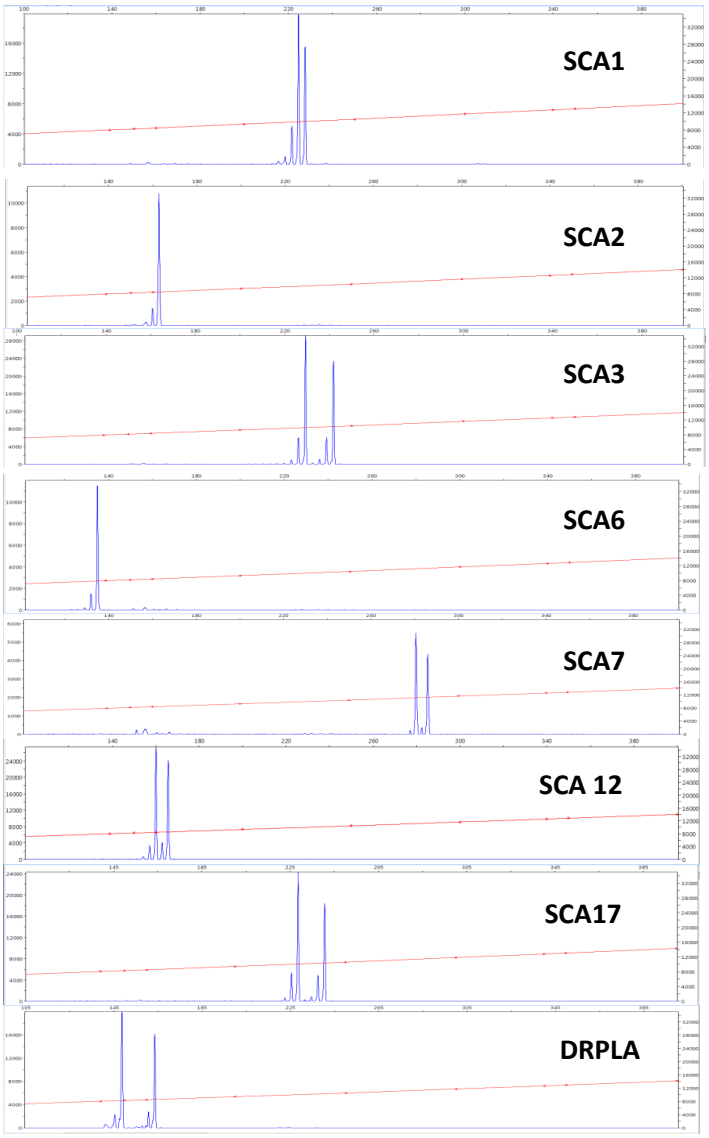

Figure 3. Expected results for the SCAs Positive Control for each PCR system included in the kit

SCA System	Base pairs number		Repetition number	
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

Tabla 6. Repetitions and size (bp) of SCA Positive control alleles in each PCR system

Excess fluorescently labelled oligonucleotides and primer dimers produced during the PCR and TP-PCR might be detected as unspecific peaks. However, if present, such unspecific peaks will be detected out of the detection range thus not affecting the interpretation of the results. Moreover, the structure of such unspecific peaks will never display the characteristics of triplet tandem repeats, such as stutter peaks.

8.2 TP-PCR results

In SCA2 and SCA7, PCR might result in a single amplification peak resulting from a homozygous sample for the given allele or due to the sample being heterozygous with a wild-type allele and an expanded allele undetectable by conventional PCR. In order to differentiate between homozygous samples for the normal allele and samples with a normal allele and an undetected expanded allele, the TP-PCR assay (Triplet Repeat primed polymerase chain reaction) of **imegen-SCAs kit** has been developed.

TP-PCR uses a fluorescently labelled locus-specific primer and paired primers capable to amplify the repeat from multiple priming sites. This enables the detection of expansions at all sizes although generally it does not enable to quantify the number of repetitions.

The images below show the expected results from a sample with and without

expanded alleles in SCA2 and SCA7 genes analysed by TP-PCR.

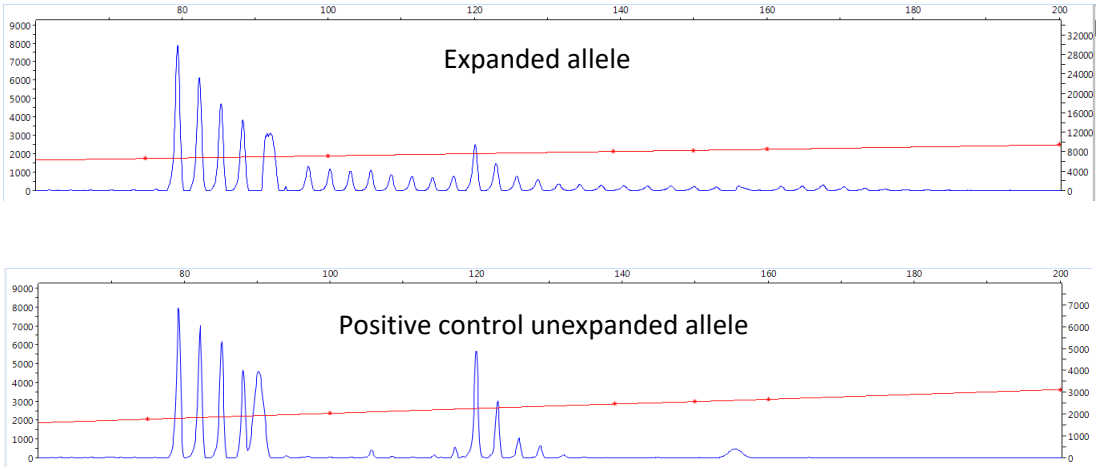

Figure 4. Expanded and unexpanded alleles for SCA2 TP-PCR system.

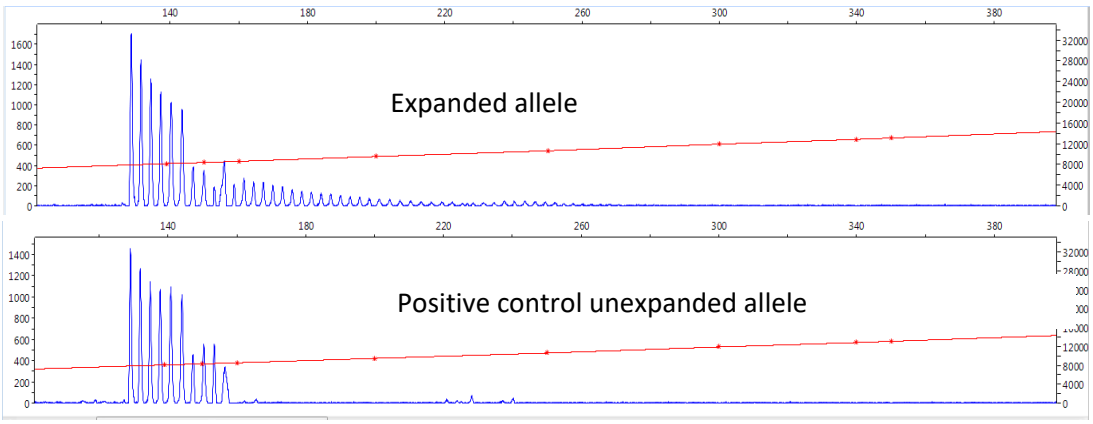

Figure 5. Expanded and unexpanded alleles for SCA7 TP-PCR system

9 Troubleshooting

The following table resume problems or difficulties that can occur in the results of the samples, Positive control, the size marker and the negative control. In the eventuality of an unexpected result check for the probable cause in the table below.

Symptoms	Analysed sample	Positive control	Size Marker	Negative control	Possible causes
Weak fluorescent signal for allele peaks				√	Expected result
	√			√	Insufficient/poor quality DNA template. [1] Impure DNA template.[2]
	√	√	√	√	Poor capillary electrophoresis injection.[3] Samples were not properly denatured before loading.[4]
	√	√		√	Thermal cycler or tube problems.[5]
Excessive fluorescent signal for allele peaks	√				Too much template DNA.[6]
	√	√			
Presence of more peaks than expected	√	√		√	Contamination.[7]
	√				Contamination.[7] Mosaicism.[9]
	√	√			Artifacts typical of expansions.[8]

Table 7. Problems or difficulties in the results of the imegen-SCAs Kit

[1] Insufficient/poor quality DNA template: Make sure DNA has been accurately quantitated and use the recommended amount of template DNA. In the case of the DNA was correctly quantified, check for DNA integrity and eventually repeat DNA preparation.

[2] Impure DNA template: High salt concentration or altered pH can inhibit PCR amplification. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover

from DNA sample of K+, Na+, Mg2+ or EDTA can negatively affect PCR. Changes in pH also may affect PCR. If it's so, clean the DNA sample or repeat DNA preparation.

[3] Poor capillary electrophoresis injection: Check if the instrument parameters are the specified ones and re-inject sample.

[4] Samples were not properly denatured before loading: Heat-denature samples for the recommended time (section 7 of this manual) and cool on crushed ice or in an ice-water bath immediately prior to loading.

[5] Thermal cycler: Check if PCR program is the specified one.

[6] Too much template DNA: Make sure DNA was accurately quantitated. If it is so, dilute the PCR product in sterile deionized water and repeat sample denaturation and loading.

[7] Contamination: It may be caused by another template DNA or a previously amplified DNA. Cross-contamination can lead to false positives or negative results, and consequently to problems in results interpretation. Use aerosol-resistant pipette tips, and change gloves regularly

[8] Artefacts typical of expansions: Amplification of expansions generates artifacts that appear as smaller peaks 3bp above or below the prominent repeat allele.

[9] Mosaicism: In some SCAs, such as SCA2 and SCA6, mosaic patients have been described, therefore it is possible to find more than one genotype in the same sample. In this case we recommend to repeat the PCR and, if the result is the same, use a patient's different sample, possibly from a different tissue (ex. oral swab).

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 brand or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical service for any query or clarification.

Imegen-SCAs has been validated using the following high-throughput sequencing platform:

- 3730xl DNA Analyzer (ThermoFisher Scientific)

This kit is valid only with the polymers compatible with 6-Carboxifluoresceina (6-FAM) labeling. If you use another brand or model of sequencer, follow the instructions and protocol recommendations of that instrument.

10.2 Reagents

Imegen-SCAs has been validated using the reagents included in the kit and the ones recommended in the section 6 of this manual [Equipment and materials required but not supplied].

For the capillary electrophoresis it is advised to use the reagents recommended by the supplier of the sequencer: ThermoFisher Scientific.

Please contact our technical service for any query or clarification.

10.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied from the reception of the kit until the expiry date assigned to each production batch.