



Adellgene®

SCAs

INSTRUCTIONS FOR USE

Kit for the determination of the number of triplets at *ATXN1* (SCA1), *ATXN2* (SCA2), *ATXN3* (SCA3), *CACNA1A* (SCA6), *ATXN7* (SCA7), *PPP2R2B* (SCA12), *TBP* (SCA17) and *ATN1* (DRPLA) genes by means of fragment analysis.

For In Vitro Diagnostic Use

Rev. 04 - 2026/01/15



Blackhills Diagnostic Resources S.L.U
Camino del Pílon 86, Casa 7, Local
50011 – Zaragoza (Spain)



www.bdrdiagnostics.com



Kit references:
AD-SCAs-16

UDI-DI:
8437016942437

Storage:
-30 °C to -18 °C
AD-RED-SCA vial at 4 °C after first use

Adellgene®

SCAs

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1- Information for safety

Please read these instructions for use carefully and follow them while using this IVD kit.

This IVD kit should be used by experts with extensive experience in DNA analysis and the interpretation of genetic results.

Consult the manufacturer if there are any doubts related to the description of the test method. Contact by phone +34 976 094 603 or at the email address customersupport@bdrdiagnostics.com.

This IVD kit has a limited shelf life. Make sure that the kit has not exceeded its expiry date before using it as the reagents could degrade, offering erroneous results. Dispose of expired reagents in accordance with the applicable regulations.

Contamination of the sample or reagent may result in incorrect results. Be careful during the DNA extraction process and the handling of reagents and samples.

This kit is susceptible to damage during transport or storage. Do not use the kit if it is suspected it was damaged during transport. Carefully follow the storage conditions described in the labelling and instructions for use.

The product box is sealed with a tamper-evident seal. Do not use the reagent if the seal is broken or missing.

Ensure that the waste is managed according to local regulations. Incorrect waste management can result in environmental contamination. Do not dump it into sewers or waterways. Dispose of the contents or container to an authorised waste manager.

The toxicological properties of the kit have not been studied in-depth, so it is recommended to avoid contact with skin and mucous membranes. Do not ingest. Wear gloves and protective clothing. Material Safety Data Sheets (MSDS) are available for the customers upon request and on the manufacturer's website. Do not handle the reagents until all safety precautions have been read and understood.

Make sure that the kit is suitable for the analysis requested by the clinician/physician.

2- Intended use

Adellgene® SCAs is a semi-automated *in vitro* diagnostic kit for the quantitative determination of the number of triplet repeats in genes *ATXN1* (SCA1), *ATXN2* (SCA2), *ATXN3* (SCA3), *CACNA1A* (SCA6), *ATXN7* (SCA7), *PPP2R2B* (SCA12), *TBP* (SCA17) and *ATN1* (DRPLA), as an aid in the clinical diagnosis of autosomal dominant spinocerebellar ataxias and dentatorubral-pallidoluysian atrophy (DRPLA).

The kit allows the quantification of the size of healthy, intermediate, uncertain and expanded alleles with a size equal to or less than 200 repeats. The expansions of more than 200 repeats described for SCA2 and SCA7 can be detected, but not quantified.

The procedure is based on the amplification of genomic DNA, extracted from whole blood and/or buccal swab, by polymerase chain reaction (PCR or TP-PCR) with fluorescent primers, subsequent analysis of the size of the amplified fluorescent fragments in a capillary sequencer and conversion of the fragment size into its corresponding number of repeats.

The patient referred by the corresponding health specialist (e.g., neurologist) may be subject to this determination, taking into account the compatibility of the symptoms presented (progressive cerebellar ataxia with motor abnormalities, dysarthria, ophthalmoplegia,

involuntary movements/tremors, peripheral neuropathy and cognitive impairment), and/or family history.

The intended user of this kit is technical personnel trained and qualified to perform the protocol described in the instructions for use and interpretation of its results.

3- Summary and explanation

Spinocerebellar ataxias (SCAs) are a heterogeneous group of inherited neurodegenerative disorders that mainly affect the cerebellum, causing a progressive loss of coordination and balance. These diseases are autosomal dominant and are characterised by great clinical and genetic variability.¹ More than 40 types of SCAs have been described, which are named as "SCA" followed by a number indicating the order in which the altered gene specific to each type was identified.²

At least twelve types of SCAs are caused by repeat expansions, among which six result from mutations caused by the expansion of the CAG trinucleotide, which encodes sections of polyglutamine in the proteins associated with these diseases: SCA1, SCA2, SCA3/Machado-Joseph disease, SCA6, SCA7 and SCA17. SCA12 is also caused by the expansion of the CAG trinucleotide, although the mutation is found in the 5' UTR region of the *PPP2R2B* gene, so its pathogenesis seems to be due to a modification of the expression of this gene. In addition, another disorder related to a translated CAG trinucleotide repeat, dentatorubral-pallidoluysian atrophy (DRPLA), is classified as an SCA due to its clinical characteristics.²⁻⁴

SCA1 is associated with an expansion of the CAG triplet in the *ATXN1* gene, causing progressive cerebellar ataxia and dysarthria. **SCA2** is caused by a similar expansion in the *ATXN2* gene and it is characterised by a combination of ataxia and peripheral neuropathy. **SCA3**, also known as Machado-Joseph disease, is the most common form of SCA and results from CAG expansion in the *ATXN3* gene, presenting with symptoms of ataxia, ophthalmoplegia and parkinsonism.^{5,6}

SCA6 is due to the expansion of CAG repeats in the *CACNA1A* gene and it mainly presents with pure cerebellar ataxia. **SCA7** is notable for its retinal involvement, which leads to progressive blindness in addition to ataxia, and is caused by CAG repeat expansion in the *ATXN7* gene. **SCA12** is less common and is characterised by a predominant tremor and ataxia, caused by an expansion of the same triplet in the *PPP2R2B* gene.^{5,6}

SCA17 is a rare form of SCA caused by an expansion of the CAG triplet in the *TBP* gene, which may present with a wide variety of neurological symptoms, including ataxia, dementia, and chorea. Finally, **DRPLA** is also due to a CAG expansion in the *ATN1* gene and it is characterised by a combination of ataxia, epilepsy, and dystonia.^{5,6}

4- Principles of the procedure

This test is based on capillary sequencing analysis of the size of the fluorescent fragments obtained by multiplex PCR (**R1** and **R2**) and TP-PCR (**R3** and **R4**) and fluorescent labelled primers. Each sample is analysed in 4 reactions: 2 multiplex PCR reactions (**R1** and **R2**) and 2 TP-PCR singleplex reactions (**R3** and **R4**).

To analyse the samples in **R1**, the following are used:

- A pair of primers for the amplification of a fragment of the *ATXN1* gene, a pair of primers for the amplification of a fragment of the *ATN1* gene and a pair of primers for the amplification of a fragment of the *TBP* gene. These amplified fragments include

the sequences where the triplet repeats of interest are found. One of the primers of the pairs that amplify the fragments of the *ATXN1*, *ATN1* and *TBP* genes is labelled at the 5' end with the fluorophore FAM, HEX and NED, respectively.

Reaction	Pathology	Gene	Expansion	Fluorophore
R1	SCA1	<i>ATXN1</i>	(CAG) _n (CAT) _n (CAG) _n	FAM
	DRLPA	<i>ATN1</i>	(CAG) _n	HEX
	SCA17	<i>TBP</i>	[(CAG) _n (CAA) _n (CAG) _n] _n	NED

To analyse the samples in R2, the following are used:

- A pair of primers for the amplification of a fragment of the *PPP2R2B* gene, a pair of primers for the amplification of a fragment of the *CACNA1A* gene and a pair of primers for the amplification of a fragment of the *ATXN3* gene. These amplified fragments include the sequences where the triplet repeats of interest are found. One of the primers of the pairs that amplify the fragments of the *PPP2R2B*, *CACNA1A* and *ATXN3* genes is labelled at the 5' end with the fluorophore FAM, HEX and NED, respectively.

Reaction	Pathology	Gene	Expansion	Fluorophore
R2	SCA12	<i>PPP2R2B</i>	(CAG) _n	FAM
	SCA6	<i>CACNA1A</i>	(CAG) _n	HEX
	SCA3	<i>ATXN3</i>	(CAG) _n	NED

The R1 and R2 amplifications of the Adellgene® SCAs kit are based on PCR multiplex, wherein the fragments of interest are amplified simultaneously and labelled with a fluorophore attached to the corresponding primer. The amplified and fluorescently labelled products obtained in the different reactions are separated according to their size and identified according to the fluorophore in a capillary electrophoresis (CE) instrument. The following image shows an electropherogram of R1 and R2.



Electropherograms corresponding to R1 (left) and R2 (right)

The different peaks obtained are identified based on their fluorophore, and their size is subsequently converted to the number of repeats.

To analyse the samples in R3, the following are used:

- A pair of primers for the amplification of a fragment of the *ATXN2* gene where the triplet repeats to be determined are found. One of the primers of this pair is labelled at the 5' end with the FAM fluorophore.
- A third complementary primer with the sequence of repeated triplets.

Reaction	Pathology	Gene	Expansion	Fluorophore
R3	SCA2	<i>ATXN2</i>	[(CAG) <i>n</i> CAA (CAG) <i>n</i>] <i>n</i>	FAM

To analyse the samples in R4, the following are used:

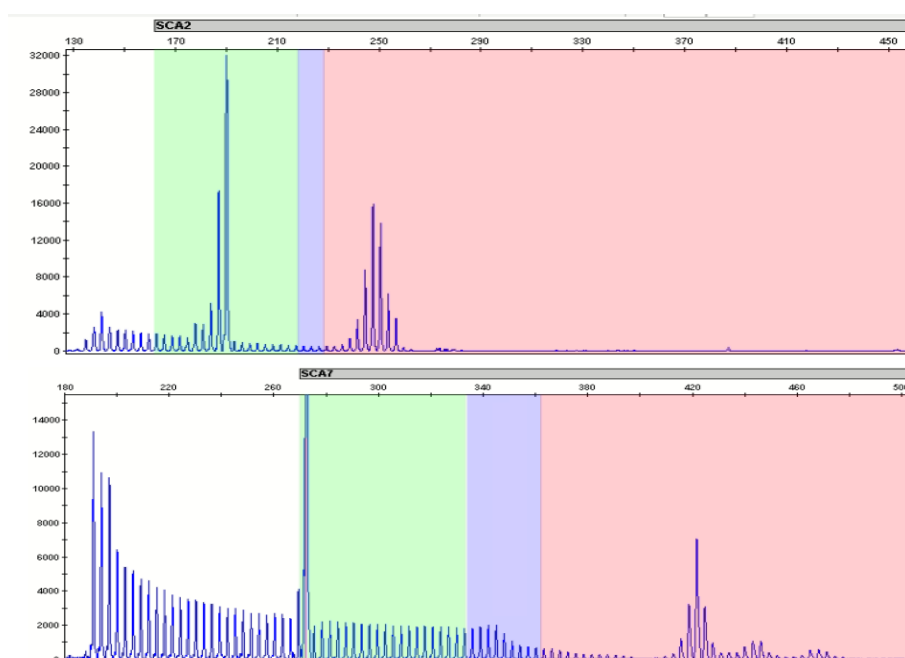
- A pair of primers for the amplification of a fragment of the *ATXN7* gene where the triplet repeats to be determined are found. One of the primers of this pair is labelled at the 5' end with the FAM fluorophore.
- A third complementary primer with the sequence of repeated triplets.

Reaction	Pathology	Gene	Expansion	Fluorophore
R4	SCA7	<i>ATXN7</i>	(CAG) <i>n</i>	FAM

The R3 and R4 reactions of the Adellgene® SCAs kit are based on Triplet-Repeat Primed PCR (TP-PCR), in which amplified fragments of different sizes labelled with the FAM fluorophore are generated depending on the existing repeats in the DNA being analysed. These fragments are then separated according to their size in a capillary electrophoresis (CE) instrument. The capillary electrophoresis analysis of the TP-PCR products generates:

- Allele-specific peaks to be identified and converted to the number of CAG repeats.
- CAG repeat primed peaks ("stutter" peak pattern) that correspond to individual PCR amplicons from each combination of the repeat primer with the reverse primer. These peaks are 3 base pairs apart.

The following image shows an electropherogram of R3 and R4.



Electropherograms corresponding to R3 (top) and R4 (bottom)

With this test the following is possible:

- Detecting and determining the size of alleles with less than 200 CAG repeats and
- Detecting alleles with more than 200 CAG repeats, but not determining their size. These alleles should be identified as >200 CAG repeats.

5- Contents of the kit

→ AD-SCA-16 (16 tests)

Reference	Vial	Colour	AD-SCA-16 (16 tests)
AD-SCA-PM1-A	Primer Mix A1	Blue cap	1 x 128 µl
AD-SCA-PM2-A	Primer Mix A2	Blue cap	1 x 128 µl
AD-SCA-PM3-B	Primer Mix B3	Blue cap	1 x 128 µl
AD-SCA-PM4-B	Primer Mix B4	Blue cap	1 x 128 µl
AD-SCA-MM-A	Master Mix A	Red cap	1 x 320 µl
AD-SCA-MM-B	Master Mix B	Red cap	1 x 320 µl
AD-SCA-C+	Positive Control	Green cap	1 x 40 µl
AD-RB	Reaction Blank (RB)	Transparent cap	1 x 100 µl
AD-RED-SCA	Red 1000 64 rxn	Brown vial	1 x 64 µl

6- Storage of the kit

All components of the kit should be stored between -30 °C and -18 °C after receipt, except the AD-RED-SCA component, which should be stored at 4 °C after first use. Under these conditions, the kit maintains its functionality until the expiry date indicated on the label.

Do not carry out more than 3 freeze/thaw cycles on the vials belonging to the kit as this may reduce the sensitivity of the test and affect the results. If the test is to be carried out on only a few samples, it is recommended to separate the components into aliquots to reduce the number of freeze/thaw cycles.

Due to the photosensitive nature of the reagent, avoid its continued exposure to light.

7- Materials required but not supplied

→ General

- Disposable gloves
- Lab coat

→ Consumables

- Tips with Filter (P1000, P20, P2)
- 1.5 mL autoclaved tubes
- PCR plates/tubes compatible with the equipment used with their corresponding caps/covers.

→ Reagents

- POP-7 Polymer (ref: 4363785, Applied Biosystems™), Spectrum Compact Polymer7 (ref: CE2507, Promega™) or equivalent
- Highly deionised (Hi-Di) formamide (ref.: 4311320, Applied Biosystems) or equivalent

→ Equipment

- Vortex mixer
- PCR plate/tube centrifuge
- Micropipettes (P1000, P20, P2)
- Nanodrop 1000 Spectrophotometer (Thermo Scientific) or equivalent
- Thermocyclers. The following equipment has been validated:
 - VeritiPro™ and VeritiFlex™ Thermal Cycler, 96-Well, Applied Biosystems™
 - Biometra Analytik Jena T Professional Thermocycler PCR W/ Module 96 070-91
- Capillary electrophoresis instruments. The following instruments have been validated:
 - For the POP-7 polymer:
 - 3130xl Genetic analyser, (*)
 - 3500xl Genetic Analyser, Applied Biosystems™ (*)
 - For the POP-1 polymer:
 - SeqStudio Genetic Analyser, Applied Biosystems™
 - For the Spectrum Compact Polymer7:
 - Spectrum Compact CE System, Promega™ (*)

(*) The equipment needs to be calibrated for the FAM, HEX, NED and ROX fluorophores (DS-30 Matrix Standard kit, Dye Set D, 4345827 ThermoFisher; or equivalent)

8- Sample collection and preparation

The samples must be collected in accordance with the instructions for use of the device used (not included in the kit) and the corresponding national and international guidelines.

This test should be performed with genomic DNA extracted from a buccal swab or whole blood collected in tubes with EDTA anticoagulant or citrate (see section 12).

The technique is compatible with several DNA extraction systems. Some potentially interfering substances that could interfere with the results have been tested (see section 12). Before issuing results for diagnostic purposes, a validation test must be carried out with the extraction method used.

CAUTION!



All biological and blood samples should be treated as potentially infectious. When handling them, the corresponding basic and universal precautions should be taken.

9- Procedure for use

→ Evaluation of the starting DNA

It is recommended to check the concentration (Absorbance at 260nm) and purity (Absorbance ratio 260/280nm and A260/230) of the input DNA using a spectrophotometer. The samples must meet A260/A280 >1.8 and A260/A230 >1.3 values. Samples with ratios values lower than the established values must be re-extracted.

For optimal performance, it is recommended the starting DNA concentration is adjusted to 10 ng/μl.

→ Preparation of the amplification reaction

Each sample is analysed in 4 different reactions (R1, R2, R3 and R4), which take place in 4 different tubes/wells.

CAUTION!



- Define pre- and post-PCR work areas, which should be separated to reduce the risk of contamination. Prepare the amplification reaction in the pre-PCR area.
- Wear a disposable lab coat and gloves.
- Work on ice or on a cold support. Minimise the time elapsed between the preparation of the reaction and the start of the test.
- It is recommended that the Reaction Blank (negative control) and Positive Control supplied with the kit be included in each reaction of each assay.

1. Defrost the Primer Mix (PM), Master Mix (MM), Reaction Blank and Positive Control vials before starting the test. Vortex the Primer Mix and Positive Control vials and gently mix the Master Mix vials. Centrifuge briefly to collect the entire volume at the bottom of the vials.
2. Prepare the following reaction mixtures (one mixture for each of the reactions) for n+1 samples, using the amounts indicated in the following table:

Reagent	R1 mix	R2 mix	R3 mix	R4 mix	Vol. per sample (μl)
Master Mix	MM-A	MM-A	MM-B	MM-B	10
Primer Mix	PM1-A	PM2-A	PM3-B	PM4-B	8

Mix well and centrifuge briefly so that all the contents are deposited at the bottom of the tubes.

3. Add 18 μl of each of these mixtures to each tube/well on the PCR plate.
4. For each of the 4 reactions, add to each well 2 μl DNA (recommended concentration: 10 ng/μl), Reaction Blank or Positive Control properly homogenised. Each sample should be analysed in 4 different wells.
5. Close the tubes or seal the PCR plate using a suitable seal, mix and centrifuge briefly to deposit the entire volume at the bottom of the well.
6. Configure the amplification program as indicated in the following section.

→ Thermocycler configuration

1. Select the temperature ramp of the thermocycler at **3 °C/sg**.
2. Set the following amplification program:

No. cycles	Temperature (°C)	Time (mm:ss)
1	98	05:00
30	97	00:35
	60	00:35
	68	02:00
1	72	15:00
1	4	∞

3. Place the tubes/plate in the thermocycler and start the test.

After amplification, continue with the preparation of the sample for capillary electrophoresis. If not continuing directly with the sequencing, store the PCR products at 4 °C for a maximum period of 1 week or at -20 °C for longer periods.

→ Preparation of the samples for capillary electrophoresis



CAUTION!

Formamide should be handled with extreme caution, as it can cause skin, respiratory and ocular irritation. Avoid breathing its vapour by handling it in adequately ventilated hoods.

1. Defrost the formamide. Defrost the AD-RED-SCA if this is the first time it is being used. If it is not the first time, take this reagent out of the refrigerator. Vortex and centrifuge the tubes before use.
2. Prepare the following reaction mixture for n samples + 10% excess:

Reagent	Volume per sample (µL)
Hi-Di™ formamide	10
AD-RED-SCA	1
Total volume	11

Homogenise the mixture and centrifuge briefly so that the entire volume is deposited at the bottom of the tube.

3. Add 11 µL of the mixture to each well of the CE plate.
4. Add 2 µL of the amplified product to the plate and mix by pipette 5 times ensuring thorough mixing.
5. Seal the electrophoresis plate with a suitable cover and centrifuge briefly to ensure that the entire volume is deposited at the bottom of the well. Check that there are no bubbles.
6. Insert the plate into the thermocycler and configure the following denaturation program:

No. cycles	Temperature (°C)	Time (mm:ss)
1	95	02:00
1	4	5:00
1	4	∞

- Once the denaturation is completed, keep the plate at 4 °C protected from light until it is injected into the genetic analyser. Under these conditions, the preparation is stable up to 24 h.

➔ Configuration of the capillary sequencer

- Follow the instructions of the equipment manufacturer when configuring the data acquisition. The instrument must be calibrated for the detection of the FAM, HEX, ROX and NED fluorophores.
- Use the default *Fragment Analysis* working module for the specific polymer and capillary length of the instrument as the base protocol.
- Adjust both injection conditions and run time to the specific instruments where the assay will be carried out. The intensity of the peaks varies depending on, among other factors, the input gDNA concentration, the thermal cycler and temperature rate used during the amplification step, as well as the capillary electrophoresis instrument. In the following table, the settings used to reach the values included in the section 12 are shown:

Parameters	Capillary sequencer				
	3130xl	SeqStudio	3500xl	Spectrum Compact	
				R1 and R2	R3 and R4
Run voltage	15.0 kV	7000 V	15.0 kV	14.0 kV	14.0 kV
Run time	2700 s	2300 s	2600 s	2400 s	2400 s
Injection voltage	2.5 kV	1200 V	2.5 kV	2.5 kV	2.5 kV
Injection time	15 s	7 s	15 s	15 s	10 s
Oven temperature	60°C	-	60°C	60°C	60°C
Poly Fill Vol.	7300 steps	-	-	-	-
Current Stability	5.0 µA	-	-	-	-
Pre-Run Voltage	15.0 kV	-	15.0 kV	-	-
Pre-Run Time	180 s	-	180 s	-	-
Voltage Number of steps	20 nK	-	-	-	-
Voltage Step Interval	15 s	-	-	-	-
Data Delay Time	60 s	-	60 s	-	-

The previous settings are saturating conditions aimed to reach a sensitive detection of the mutant alleles with higher size. Under these conditions, the *wild-type* allele specific peaks with smaller size could appear saturated, which can prevent their accurate determination. When this happens, it is recommended to reduce the injection times between 50% and 75% until the wild-type allele peak intensities values are adjusted to the capillary electrophoresis instrument used.

→ Waste disposal

All the waste products must be disposed of in accordance with local regulations.

10- Results and interpretation

→ Presentation of results

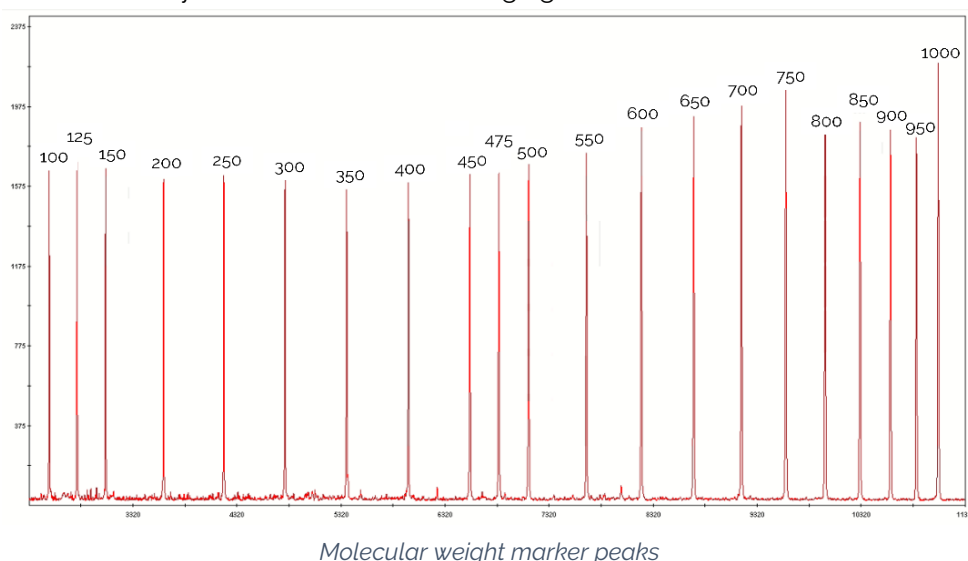
The results of the capillary electrophoresis run (*.fsa files) must be imported into analysis software such as GeneMapper® or equivalent. The results should be analysed in accordance with the instructions for use of the software manufacturer, and in accordance with the analysis method and configuration of the size marker set for the Adellgene® SCAs kit. The use of panels with repeat ranges established based on GeneReviews⁶⁻¹³ and on the values c_0 and m_0 obtained in our installations can make it easier to present the results. These files are available on request from the email address customersupport@bdrdiagnostics.com.

Presentation of the molecular weight marker

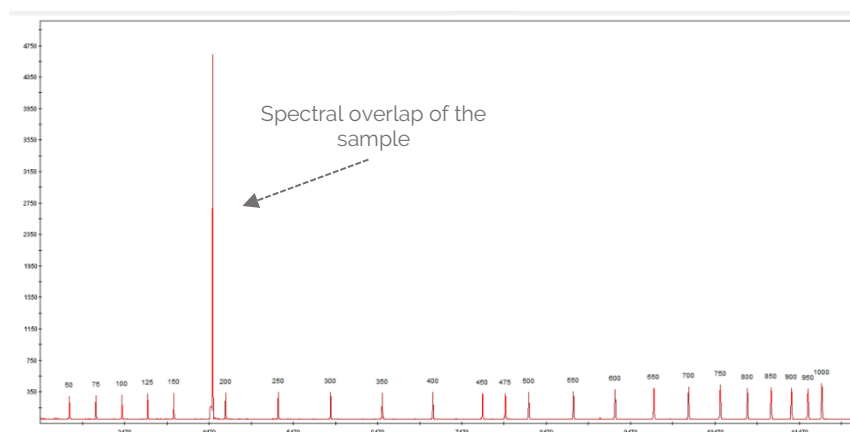
In order to carry out the correct determination of the size of the alleles, it is essential to add the molecular weight marker in all the wells that are going to be analysed in the capillary sequencer. Thus, the peaks corresponding to the molecular weight marker should be detected in all analysed samples (including Positive Control and Reaction Blank).

The ROX channel electropherogram of all tested wells should show 23 peaks (from 50 to 1000 bp) from the molecular weight marker. The height of the peaks is as follows: 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 and 1000 base pairs.

It is necessary to check the molecular weight marker behaves as expected in all the analysed samples before proceeding with the identification of peaks. The 23 peaks must be detected and labelled correctly, as shown in the following figure.



In some cases, spectral overlap of the PCR product can be observed in the ROX channel, which appears between the peaks of the size marker. The existence of this peak should not interfere with the size calibration algorithm of the analysis software. If it interferes, deselect this peak and manually correct the assignment of marker peaks.



Peaks of the molecular weight marker showing a signal from the spectral overlap of the PCR product

Wells having less than 23 size marker peaks should be excluded from the analysis and retested, increasing the run time or modifying the analysis method if necessary.

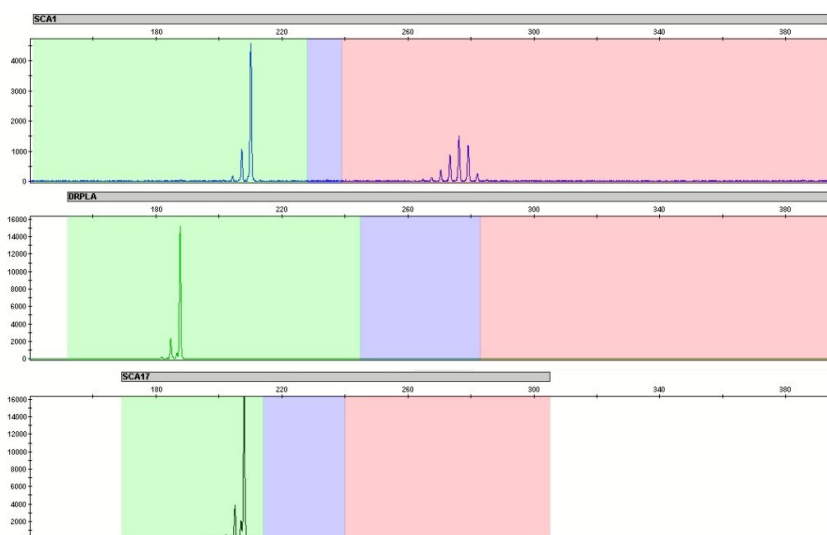
Once the correct behaviour of the molecular weight marker of each sample has been verified, proceed with the display and selection of peaks in the electropherograms, as indicated below.

Visualization of multiplex PCR reactions (R1 and R2)

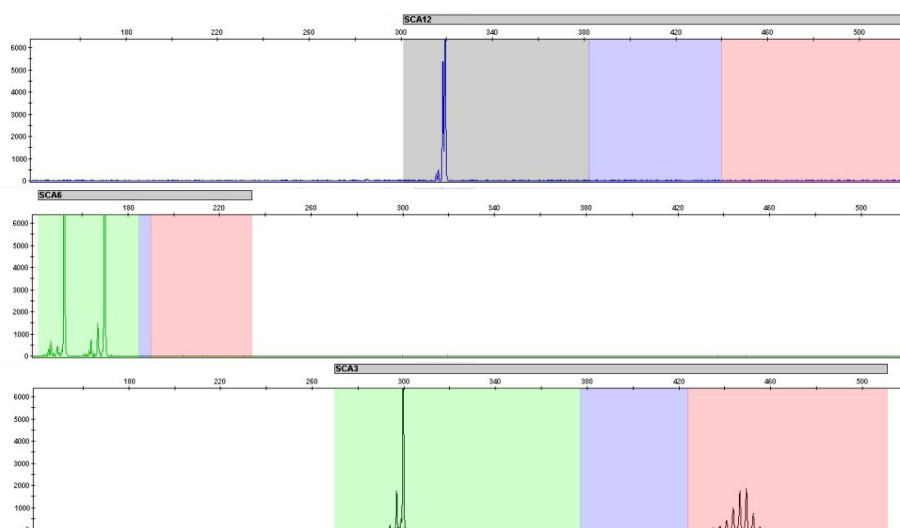
After the CE, signals from the amplified fragments generated in multiplex PCR reactions should be observed in FAM, HEX and NED channels. Allele-specific peaks should exceed the intensity values indicated in the following table.

Capillary analyser	Cut-off point (RFU)
3130xl	50
SeqStudio	175
3500xl	175
Spectrum Compact	175

The identity of the gene is determined from the fluorescence channel and the size of the fragment from the base pairs. The fluorescence of FAM, HEX and NED channels is represented with blue, green and black peaks, respectively. Typical electrophoretic patterns of multiplex PCR reactions are shown in the following images.



Electropherogram corresponding to the R1 reaction: heterozygous sample with expanded allele for SCA1 (FAM), healthy homozygous samples for DRPLA (HEX) and SCA17 (NED)



Electropherogram corresponding to R2: healthy homozygous sample for SCA12 (FAM), healthy heterozygous for SCA6 (HEX) and heterozygous with a mutated allele in SCA3 (NED)

Visualization of TP-PCR reactions (R3 and R4)

After the CE, signals from the amplified fragments generated in the TP-PCR reaction should be observed in the FAM channel:

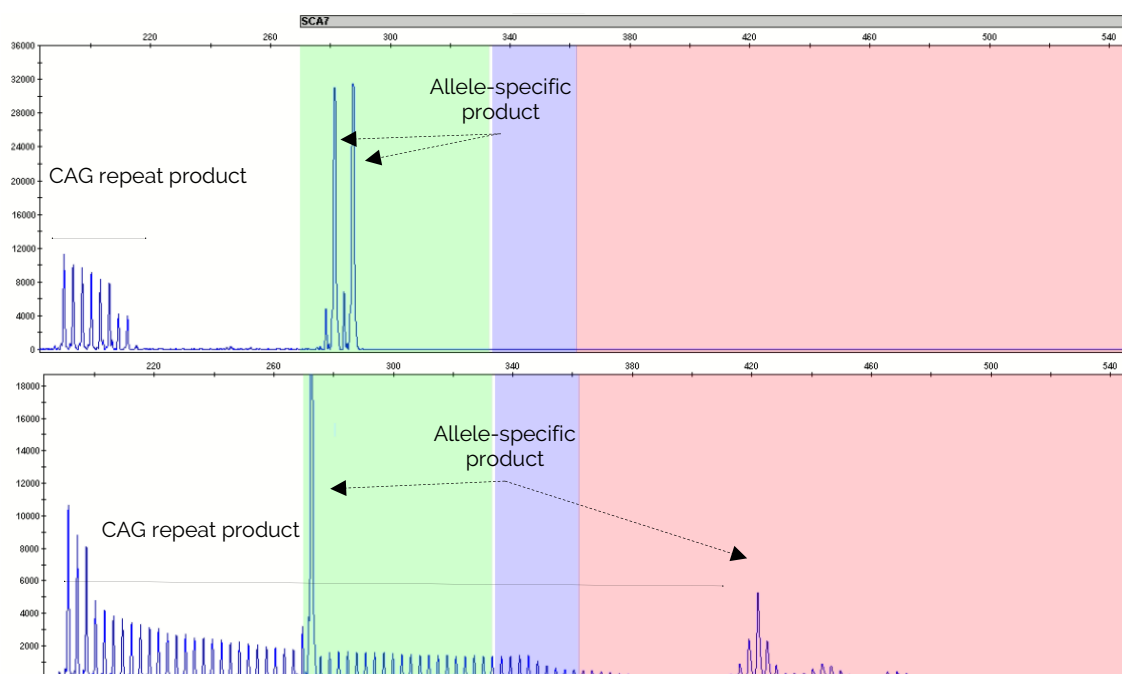
- Allele-specific peaks should exceed the intensity values indicated in the following table.

Capillary analyser	Cut-off value (RFU)
3130xl	50
SeqStudio	175
3500xl	175
Spectrum Compact	175

- The "stutter" peak pattern indicates the presence of CAG repeats. The fluorescence intensity of the "stutter" peak pattern can be below the intensities established for allele-specific peaks, as detailed in the following table:

Capillary analyser	Cut-off value (RFU)
3130xl	10
SeqStudio	50
3500xl	50
Spectrum Compact	50

Typical electrophoretic patterns for the TP-PCR reaction are shown in the following images.



Electropherograms corresponding to a sample with two wild-type alleles (top) and a sample with one wild-type allele and another mutated allele (bottom) obtained with the Adellgene® SCAs kit.

➔ Identification of peaks

The shape of the allele-specific peaks depends on their height, i.e. on their position in the electropherogram.



WARNING!

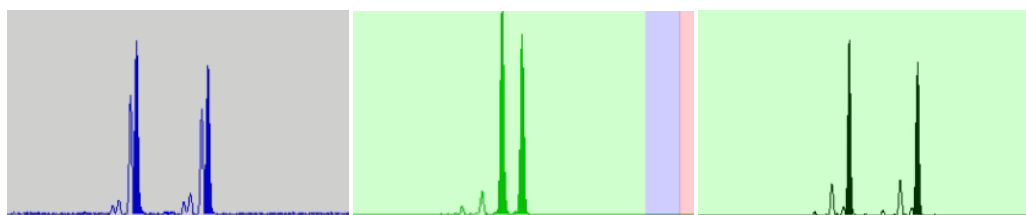
This test is optimised to detect mutated alleles, larger in size than the wild-type alleles, from 20 ng of total DNA (2 µl to 10 ng/µl).

On occasions, it is possible to observe peaks with saturated fluorescence that may generate signals in another fluorescence channel and/or problems in correctly assigning the height of the saturated peak. In these cases, it may be necessary to reanalyse the amplified product after a 1:10 (R1, R2) or 1:100 (R3, R4) dilution in water using the above-described conditions or to reanalyse the initially prepared sequencing mixture by decreasing the injection time between 50 and 75%.

If a fluorescence signal is obtained close to the cut-off point, it is possible to increase the injection time by 1/3 to increase the signal of the larger alleles.

Selection of peaks in multiplex PCR reactions (R1 and R2)

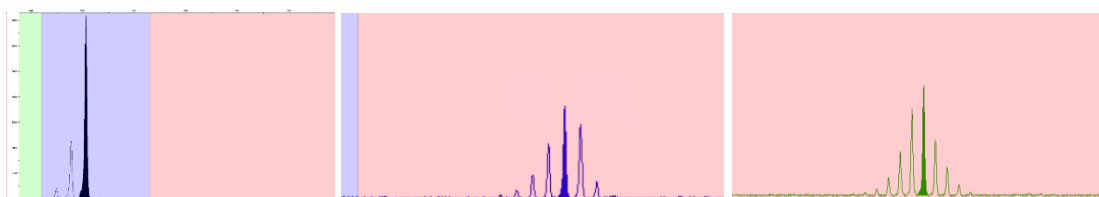
The allele-specific peaks with few repeats appear on the left side of the electropherogram as high fluorescence intensity peaks preceded by lower intensity peaks. Select the highest peak as the allele-specific peak, which is usually the one located furthest to the right. Samples with alleles of similar sizes have two nearby peaks. In these cases, select the two highest peaks as specific peaks.



Electropherograms of wild-type samples for R2. Heterozygous sample in SCA12 (left), heterozygous for SCA6 with two alleles that differ by 1 repeat (centre) and heterozygous for SCA3 (right)

As the number of repeats and the size of the alleles increase, the shape of the peaks changes from a peak of high fluorescence intensity to a cluster of mountain-shaped peaks of lower intensity. Select the highest peak in the cluster as the allele-specific peak.

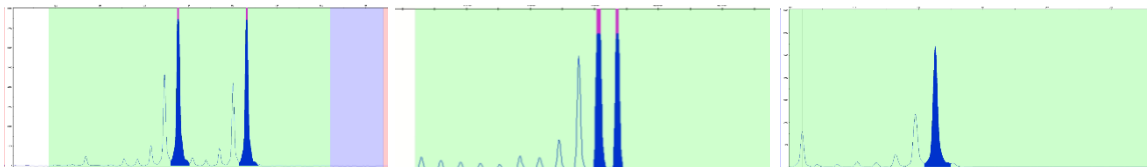
The base pairs of the allele-specific peaks should be converted to number of repeats, as indicated in the next section.



Electropherograms of reduced penetrance and mutated alleles for R1. Allele with reduced penetrance for SCA17 (left), mutated alleles in SCA1 (centre) and DRLPA (right).

Selection of peaks in TP-PCR reactions (R3 and R4)

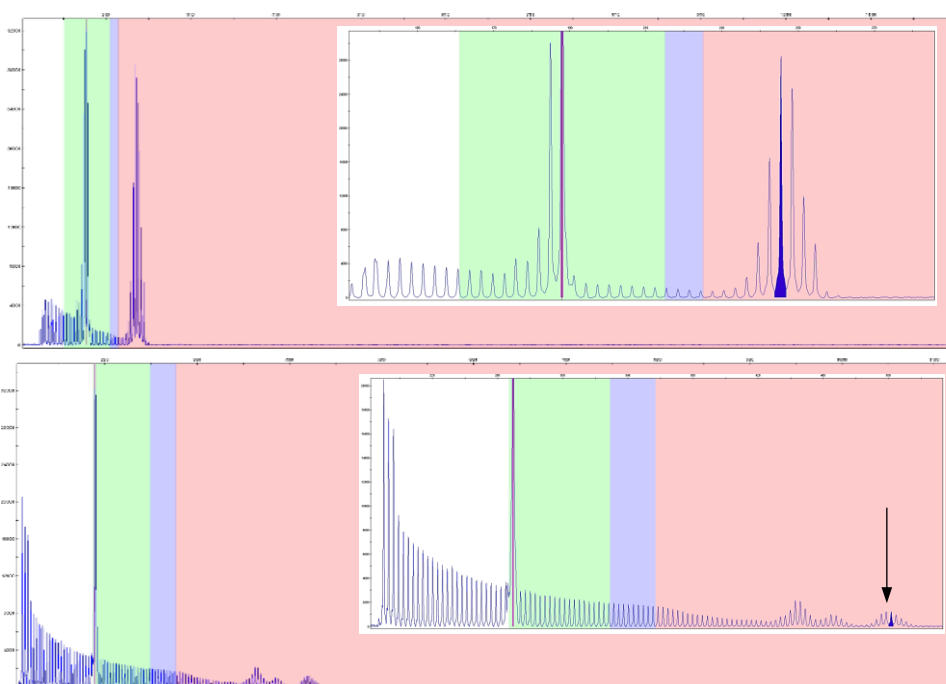
Alleles with a low number of repeats appear on the left side of the electropherogram, and usually have a high intensity peak preceded by one, two or a series of peaks of lower intensity. Select the highest peak height as the allele-specific peak. Samples with alleles of similar sizes have two nearby peaks. In these cases, select the two highest peaks as specific peaks.



Electropherogram of wild-type heterozygous sample (left), wild-type heterozygous sample with alleles differing by 1 repeat (centre) and wild-type homozygous sample (right).

In some cases, it may be necessary to reduce the injection time for the correct determination of the size of the wild-type alleles.

As the size of the alleles and the number of repeats increase, the shape of the peaks becomes a cluster or clusters of peaks. In addition, a "stutter" peak pattern appears from the beginning of the electropherogram to the last cluster of peaks. Select the highest peak of the last cluster of peaks as the allele-specific peak.



Electropherograms of two heterozygous samples with one wild-type and one mutated allele (<200 CAG repeats). A high fluorescence intensity allele-specific peak is observed at the start of the electropherogram, along with a sawtooth fluorescence signal ending in a cluster (top) or clusters of peaks (bottom).

Alleles with a high number of repeats (>200 CAG) appear as a "stutter" peak pattern from the beginning of the electropherogram that weakens as the size of the fragments increases. At the end of the electropherogram the specific product of the allele will appear as a mountain of stacked peaks.

The base pairs of the allele-specific peaks should be converted to the number of repeats, as indicated in the next section.



WARNING!

The peaks corresponding to a number of CAG repeats greater than 200 should be categorised as >200 CAG repeats, regardless of the number of repeats obtained in the calculation.

→ Calculation of the number of triplet repeats

The base pairs of the peak of interest are obtained by comparison with the size of the molecular weight marker fragments. The fragments with CAG repeats and the fragments of the molecular weight marker have different mobilities so, to correctly convert the base pairs to the number of repeats, it is necessary to use the size correction factors (c_0) and the mobility correction factor (m_0). The base pairs of the peaks should be converted to the number of CAG repeats using the following formula:

$$\text{No. of triplet repeats} = \frac{(\text{Base pairs of the peak} - C_0)}{m_0}$$

The following table shows the m_0 values calculated in our facilities:



WARNING!

The values of c_0 and m_0 vary depending on factors including the capillary sequencer, the length of the capillary, the type of polymer and the run conditions. To ensure accurate estimations of the number of repeats it is recommended, before using the kit, that specific correction factors c_0 and m_0 are calculated by using samples with a known number of repeats.

	3130xl	SeqStudio	3500	Spectrum Compact
m_0	2.9437	2.9687	2.948	2.9232

The following table specifies the values of c_0 obtained in our facilities to calculate the number of repeats in each of the analysed genes:

Reaction	SCAs	C_0			
		3130xl	SeqStudio	3500	Spectrum Compact
R1	SCA1	122.63	123.35	123.67	124.84
	DRPLA	139.34	140.87	139.49	139.81
	SCA17	94.921	95.038	94.864	95.953
R2	SCA12	288.98	289.33	288.79	289.52
	SCA6	129.77	130.69	129.82	130.04
	SCA3	228.21	227.94	228.00	229.03
R3	SCA2	123.24	124.98	122.76	124.76
R4	SCA7	246.59	252.96	248.75	252.21

The alleles of the peaks for which a number greater than 200 repeats is obtained must be categorically typed as >200 CAG repeats, regardless of their position on the electropherogram.

→ Interpretation of results

Depending on the number of repeats obtained, and the ranges proposed in GeneReviews⁶⁻¹³, the alleles can be classified as indicated in the following table:

Reaction	Pathology	Expansion	Repeat ranges			
			Wild-type	Uncertain	Reduced penetrance	Complete penetrance (Mutated)
R1	SCA1	(CAG) _n (CAT) _n (CAG) _n	6-35	36-38	-	39-44 CAGs (uninterrupted)
			39-44 CAT (interrupted)	-	44	46-70 (interrupted)
	DRPLA	(CAG) _n	6-35	-	35-47	48-93
	SCA17	[(CAG) _n (CAA) _n (CAG) _n] _n	25-40	-	41-48	49-66
R2	SCA12	(CAG) _n	7-31	32-50	-	51-78
	SCA6	(CAG) _n	4-18	19	-	20-33
	SCA3 (*)	(CAG) _n	12-44	45-59	-	60-87
R3	SCA2	[(CAG) _n CAA (CAG) _n] _n	14-31	31-32	33-34	37->200
R4	SCA7	(CAG) _n	7-27	28-33	34-36	37-460

(*) the number of CAG repeats calculated for SCA3 **DOES** include the fixed-length motif (CAG)₂ CAA AAG CAG CAA

11- Quality control

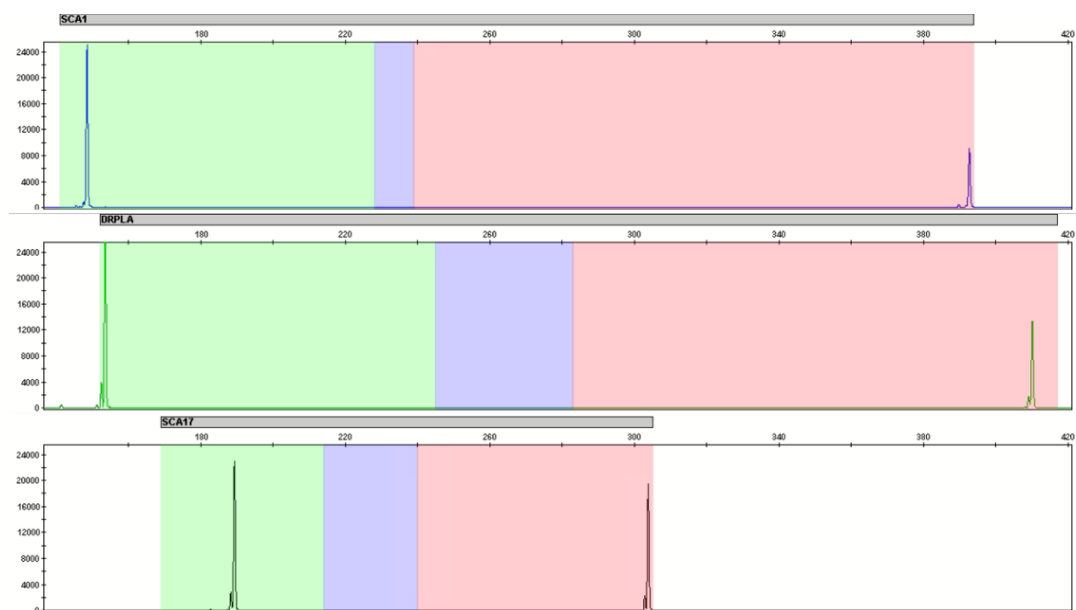
The kit includes a Reaction Blank and a Positive Control that must be included in each test. If the controls behave as expected, this is a guarantee for the proper functioning of each reaction.

For the Reaction Blank wells, only 23 peaks should appear in the ROX channel for the molecular weight marker.

For each of the 8 genes analysed, the electropherogram of the Positive Control should present a peak at the beginning and another at the end of the electropherogram. The following table shows the equivalent number of repeats for the positive control.

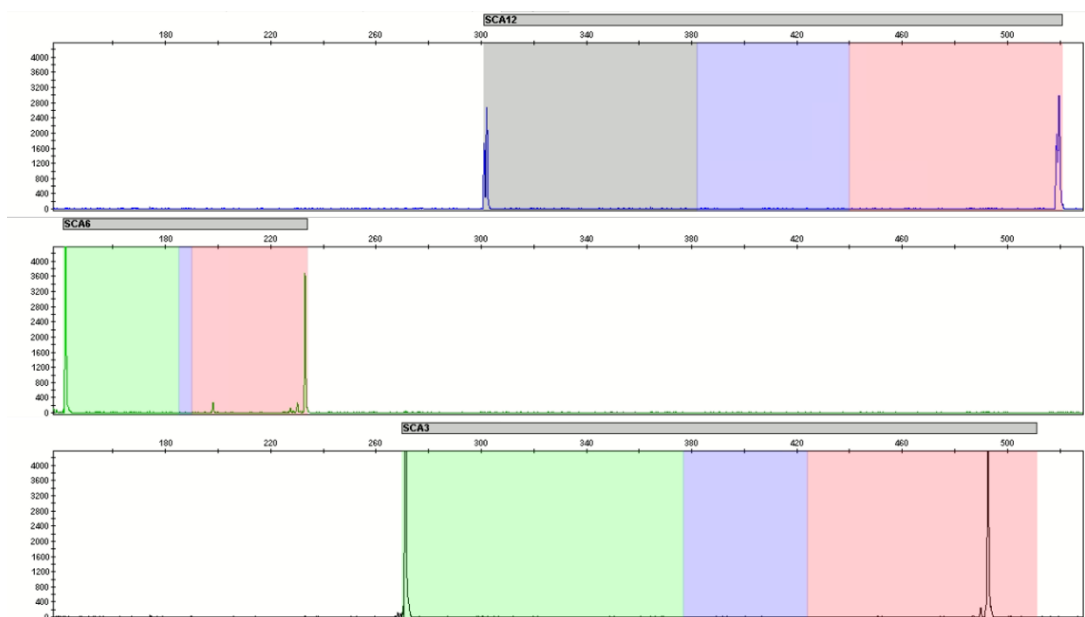
Reaction	SCAs	Equivalent number of repeats	
		Peak 1	Peak 2
R1	SCA1	8 ± 1	91 ± 3
	DRPLA	4 ± 1	91 ± 3
	SCA17	32 ± 1	70 ± 3
R2	SCA12	4 ± 1	78 ± 3
	SCA6	4 ± 1	34 ± 1
	SCA3	14 ± 1	89 ± 3
R3	SCA2	13 ± 1	>200
R4	SCA7	7 ± 1	>200

Positive Control R1



Electropherogram corresponding to the positive control for reaction R1. SCA1 (FAM) in the top panel. DRPLA (HEX) in the middle panel. SCA17 (NED) in the bottom panel.

Positive Control R2



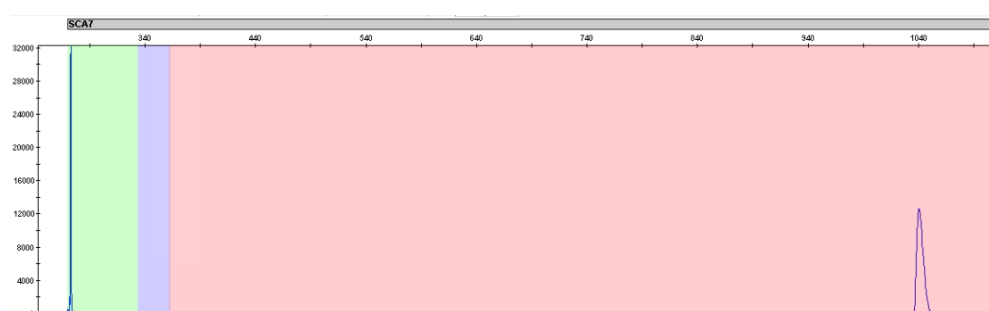
Electropherogram corresponding to the positive control for reaction R2. SCA12 (FAM) in the top panel. SCA6 (HEX) in the middle panel. SCA3 (NED) in the bottom panel.

Positive Control R3



Electropherogram corresponding to the positive control for reaction R3 (SCA2)

Positive Control R4



Electropherogram corresponding to the positive control for reaction R4 (SCA7)

If the controls of a test behave as expected, proceed with the interpretation of the results as indicated in the preceding section of this document.

The results are considered invalid and should be repeated if:

- Fluorescence signal is observed in FAM, HEX or NED in the Reaction Blank wells.
- The expected signals/expected sizes are not observed in the Positive Control wells.
- Improper behaviour of the molecular weight marker. These samples should be considered doubtful and must be re-analysed

12- Specific operation data

→ Analytical specificity

As part of the activities to determine analytical specificity, an in-house study was carried out with different genomic DNA samples whose number of repeats had previously been determined with a system other than Adellgene®. The results obtained with the Adellgene® SCA kit and the typing results obtained with an alternative method are in complete agreement as shown in the following table:

Gene	wild-type	No. alleles tested premutation/ intermediate	Mutant	Agreement of the result*
SCA1	182	1	3	Yes
DRLPA	190	0	3	Yes
SCA17	184	0	3	Yes
SCA12	149	0	0	Yes
SCA6	177	1	1	Yes
SCA3	182	0	5	Yes
SCA2	135	0	2	Yes
SCA7	161	0	3	Yes

* According to the tolerance criteria established for this kit (±1 repeats for wild-type alleles ±3 repeats mutated alleles in SCA1, DRLPA, SCA17, SCA12 and SCA3. ±1 repeats for SCA6. ±1 repeats for ≤57 repeats; ±3 repeats between 58-120 repeats; ±5 % 121-200 repeats; NA >200 repeats in SCA2 and SCA7)

In addition, the alignment of the different primers was analysed *in silico* against a genomic DNA database. No non-specific alignments are detected and no cross-reactivity phenomenon with genomic DNA has been reported.

The presence of variants at the binding sites of primers may affect the results obtained. Synthetic plasmids containing the following variants in the binding sites of primers were analysed with this Adellgene kit® in triplicate: rs761867883, rs747033709 and rs1388055144 in *TBP*, rs782402665 in *ATN1*, rs35216501, rs1236957623 and rs968565273 in *ATXN1*, rs372907618 and rs761293315 in *ATXN3*, rs1600075611 rs1351010453 and rs1050540062 in *CACNA1A*, rs964520058, rs1293527019 and rs1366921504 in *PPP2R2B*, rs749012233, rs774241164 and rs1259430760 in *ATXN2* and rs1575882626 and rs370299464 in *ATXN7*. The presence of the variants does not affect the size (base pairs) of the allele-specific peak in the plasmids used. Alleles with variants rs372907618, rs1351010453, rs1050540062 and rs964520058 do not produce amplification of the gene where they are located. The presence of variants rs761867883, rs1236957623, rs1600075611, rs1293527019, rs1259430760 and rs1575882626 is associated with a significant decrease in the fluorescence of the allele-specific peak in the plasmids used. The presence of the variants rs35216501, rs968565273, rs761293315 and rs370299464 is associated with a moderate decrease in the fluorescence of the allele-specific peak in the plasmids used.

Additionally, the presence of the rs547615608 variant in *ATX7* gene was analysed in samples and, although the size of the allele-specific peaks was not affected, the fluorescence was moderately affected.

The effect of some potentially interfering substances on the results obtained with this Adellgene® kit was also analysed. These substances were added to the genomic DNA that was subsequently analysed with the kit. Expanded allele loss and/or "stutter" signal loss and/or wild-type allele loss is observed in the presence of some interfering substances at concentrations higher than the IC₅₀. The following table details the IC₅₀ values set for this kit.

Interfering substance	IC ₅₀
Haemoglobin	6.21 g/L
IgG	14.39 g/L
FeCl ₃	1.01 mM
K ₂ -EDTA	6.66 mM
Citrate	2.66 mM
Ethanol	10.59 %

The presence of potentially interfering substances must be eliminated during the DNA extraction and purification steps. A validation of the extraction and purification method should be carried out before results are issued for diagnostic purposes.

➔ Analytical sensitivity

The limit of detection was determined as the amount of mutant allele that can be detected in a *wild-type* allele background. For this purpose, for each reaction, different mixtures were prepared from a mutant and a *wild-type* heterozygous sample with the following content 50%, 40%, 25%, 20%, 10%, 5%, 2.5% and 1% in mutant allele and keeping the amount of total DNA at 20 ng. Fluorescent "stutter" patterns and allele-specific peaks were observed above the established cut-off values in the presence of the 20% mutant allele, which is equivalent to the detection of 4 ng of mutant allele in presence of 16 ng of *wild-type* allele. The limit of detection that ensures a robust and reproducible detection of the mutant allele is established for this Adellgene® kit at 25% mutant allele in presence of 75% *wild-type* allele.

Samples with a known number of repeats were tested covering, as far as possible, the range of described repeats. The following results were obtained in terms of linear range and measurement range:

Gene	No. repeats	
	Linear range	Measurement range
SCA1	6-91	6-91
DRLPA	6-93	6-93
SCA17	25-66	25-66
SCA12	4-78	4-78
SCA6	4-33	4-33
SCA3	11-87	11-87
SCA2	14-200	14-500
SCA7	4-200	4-460

A test was performed with serial dilutions (between 25 and 0.5 ng of input DNA (**)) with 3 DNA samples (heterozygous with wild-type and mutant alleles). Each dilution level was tested in triplicate. The minimum amount of DNA that produces accurate results was set at 15 ng.

Samples with alleles of different sizes were analysed (heterozygous with wild-type and mutant alleles) in serial dilution, covering concentrations between 800 and 2.5 ng (**). The upper limit was set at 50 ng DNA. At higher input concentrations of DNA, to determine the base pairs of the wild-type peaks, it may be necessary to reanalyse the mixture in the

capillary sequencer using a shorter injection time. The precision regarding the number of repeats was not affected by the amount of starting DNA.

For optimal reaction performance, it is recommended to add 20 ng DNA to the mixture (2 µl DNA at 10 ng/µl). It is recommended to work with input DNA amounts between 20 and 40 ng.

(**) The DNA concentration was measured with a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

→ Accuracy

The repeatability study consists of the measurement of intra-test variability by analysing the possible genotypes that the kit can detect (wild-type, pre-mutant and mutant alleles). Each sample was analysed in duplicate.

The Adellgene® SCAs kit showed 100% repeatability.

The reproducibility study allows the variability between experiments, batches and instruments to be assessed. Samples with the different genotypes that the kit can detect were included in this study (wild-type, pre-mutant and mutant alleles). Three batches of reagent were tested on three different equipment units on different days.

The Adellgene® SCAs kit showed 100% reproducibility.

The proximity in the number of repeats calculated with this kit to those calculated using the reference Sanger method has been evaluated in samples with repeats comprised between 4 and 41 repeats. The results obtained with this kit remain stable between batches and coincide with those obtained by Sanger sequencing.

The accuracy in the number of repeats calculated is dependent on the size and morphology of the peaks. All repeat numbers were determined using the highest peak of the cluster of peaks. The accuracy in terms of the number of repeats was established against Sanger in the range between 4 and 41 repeats. Between 42 and 200, the precision was referenced against the number of individual CAG peaks in the "stutter" peak pattern. Above 200 repeats alleles should be categorically identified as >200.

R1 (SCA1, DRLPA, SCA17) and R2 (SCA3, SCA6 and SCA12)	
Alleles	Tolerance in the number of CAG repeats
Wild-type	± 1
Mutant	± 3 (*)

(*) the tolerance for SCA6 is ± 1, regardless of whether the allele is wild-type or mutated. Considering mutated for SCA1 ≥ 39 repeats, for SCA3 ≥ 60, SCA12 ≥ 51, SCA17 ≥ 49 and for DRPLA ≥ 48 CAG.

R3 (SCA2) and R4 (SCA7)	
No. CAG repeats	Tolerance in the number of CAG repeats
≤ 57	± 1
58 - 120	± 3
121 - 200	± 5%
> 200	NA

→ Diagnostic specificity and sensitivity

DNA samples were analysed with this kit in different external laboratories. The numbers of repeats had been determined with a methodology other than Adellgene®. The following results were obtained:

Gene	No. <i>wild-type</i> alleles	No. alleles reduced/complete penetrance	Agreement of the result*
SCA17	11	1	Yes
SCA1	28	0	Yes
DRLPA	10	0	Yes
SCA3	28	0	Yes
SCA6	27	0	Yes
SCA12	10	0	Yes
SCA2	28	1	Yes
SCA7	27	0	Yes

* According to the tolerance criteria established for this kit (±1 repeats for wild-type alleles ±3 repeats mutated alleles in SCA1, DRLPA, SCA17, SCA12 and SCA3. ±1 repeats for SCA6. ±1 repeats for ≤57 repeats; ±3 repeats between 58-120 repeats; ±5 % 121-200 repeats; NA >200 repeats in SCA2 and SCA7)

There is a 100 % agreement between the results obtained with the Adellgene® SCAs kit and the genotypes obtained with another system for determining the number of CAG triplet repeats in SCAs.

13- Limitations of the procedure

- The kit can detect alleles with a number of repeats from 5 and quantify up to 200.
- The presence of variants (point mutations, insertions, deletions) in the regions complementary to the sequence of primers is possible and can lead to an allele not being defined. Other technologies may be necessary for the resolution of these alleles. More details can be found in the analytical specificity section of this protocol.
- The presence of insertion or deletion variants in regions adjacent to the primer sequence are possible and may lead to an incorrect estimation of the number of calculated repeats. Other technologies may be necessary for the resolution of such alleles.
- The number of CAG repeats obtained in SCA3 does include the fixed-length motif CAGCAGCAAAAGCAGCAA.
- The presence of interruptions in the sequence of CAG triplets can produce aberrant patterns or failure in the detection of expansions. To exclude false positives, it is recommended additional tests (bidirectional TP-PCR or Southern Blot) to be performed.
- The recommendations contained in this document must be followed precisely. Any deviation from these recommendations may lead to errors in the results.
- Do not use the kit if there is any suspicion of possible loss of reactivity, contamination, deterioration of the outer box or any other incident that could affect the performance of the kit.
- All handling of Adellgene® reagents must be carried out in accordance with general good laboratory practices, which should be adapted to local regulations.
- Do not mix components from other kits and/or batches.

- Do not use the kit after its expiry date. Discard expired reagents in accordance with current local regulations.
- All the laboratory equipment used to perform this test must be calibrated and/or maintained in accordance with the manufacturer's recommendations, and must be used following its instructions for use.
- The interpretation of the results obtained must always be supervised by qualified personnel.
- The kit does not allow the quantification of the number of alleles, therefore, in the presence of a single allele specific peak, genotyping should be stated as presumable/most likely/consistent with homozygote, as the presence of allelic dropout could not be excluded.
- This product is an auxiliary tool for the diagnosis of patients with suspected spinocerebellar ataxia or DRPLA. Use these results together with the clinical data and the results of other tests performed on the patient.

14- Troubleshooting guide

→ Signal is detected in the Reaction Blank

- Dispensing error (pipetting).
 - Change the tip of the pipette every time you add DNA to a well.
 - Check that the sample added to the well corresponds to that recorded on the worksheet.
- Contamination of the Primer Mix/Master Mix/Reaction Blank vial.
 - Repeat the test with new aliquots.
- The PCR preparation site is contaminated.
 - Clean surfaces, instruments, lab coats and change consumables and reagents. Repeat the test.

→ No signal is detected in any tested sample or the intensity is very low

- The injection parameters of the capillary sequencer and/or the PCR protocol are not suitable:
 - Check the injection and amplification protocols of the sample. Check the matrix used.
- The indicated amounts of each of the reagents have not been added to the reaction mixture/Poor mixing of the components of the reactions:
 - Repeat the PCR/capillary electrophoresis reaction making sure that all the components have been added and mixed in the appropriate amount.
- The reagent is not working properly
 - Make sure that the kit is stored at an appropriate temperature (between -30 °C and -18 °C) and protected from light. Avoid unnecessary freeze/thaw cycles. Do not use beyond the expiry date.

→ Weak or non-existent signal in any sample tested

- Poor quality of the DNA sample used:
 - Check the value of Absorbance 260/230 and Absorbance 260/280 and discard samples of low quality. Avoid the presence of inhibitors. Repeat the sampling and DNA extraction.
- Unsuitable DNA concentration.

- Adjust the DNA concentration to the recommended concentration range.
- Check that the DNA concentration is above the detection limit.
- Insufficient amount of PCR product injected into the DNA analyser:
 - Adjust the amount of PCR product.
 - Reanalyse the sample by increasing the injection time to the capillary sequencer.
- No sample has been added:
 - Repeat the test by adding a sample.
- Presence of polymorphisms or mutations at the primer binding sites:
 - Contact our Technical Support department via the email address customersupport@bdrdiagnostics.com.
- Presence of interfering substances:
 - Avoid the presence of inhibitors. Repeat the test with a new DNA sampling and extraction.

→ High fluorescence intensity

- Pipetting error:
 - Verify that the volume added in each well is correct.
- Too much PCR product:
 - Dilute the PCR product.
 - Reanalyse the sample by decreasing the injection time to the capillary sequencer.
- Incorrect parameters in the capillary sequencer:
 - Check the injection parameters of the capillary sequencer and adjust if necessary.

→ Problems in the size marker

- The 23 peaks of the size marker are not well assigned:
 - Manually correct and assign the correct sizes to the peaks in accordance with the scheme described in point 10 of this guide.
- There are more than 23 peaks in the size marker:
 - Some of those peaks do not belong to the marker. Assign the correct size to the marker peaks in accordance with the scheme described in point 10 of this guide.
- There are less than 23 peaks in the size marker:
 - Increase the run time so that all the peaks of the marker are detected.
 - Repeat the sequencing of the sample from the PCR product. Make sure that the formamide is thawed before preparing the sequencing mixture/use of correct volume of the size marker/it is properly mixed/ there are no bubbles in the well.
- The molecular weight marker does not work properly:
 - Make sure that the molecular weight marker is stored at the appropriate temperature (at 4°C after first use) and protected from light. Do not use it beyond the expiry date.

→ Presence of more peaks than expected

- Use of the wrong kit
 - Check that the correct kit has been used
- The size marker does not behave as expected during capillary electrophoresis/misallocation of peaks.
 - Review size marker peaks
- Contaminated PCR product:

- Check the electropherogram of the Reaction Blank.
- Unsuitable parameters in capillary electrophoresis
 - Check the parameters and the spectral matrix used.
- Contaminated starting DNA:
 - Repeat the test from a new sampling.

15- Bibliography

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16- Notice to purchaser

- This product has been developed for *in vitro* diagnostic purposes.
- When this kit is used in any Member State of the European Union, if there is any incident related to the use thereof, the user must report it to the manufacturer (regulatory@bdrdiagnostics.com) and to the Competent Authority of their country and/or the patient's country. The reporting of serious incidents for all other countries should be carried out in accordance with the local requirements of each country.
- The *Summary of Safety and Performance* (SSP) is uploaded to the EUDAMED database and is accessible to all users of the kit (<https://ec.europa.eu/tools/eudamed/#/screen/home>).
- Blackhills Diagnostic Resources S.L.U. products must not be resold, modified for resale or used to manufacture other commercial products without written authorisation from Blackhills Diagnostic Resources S.L.U.

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- GeneMapper® is a registered trademark of Applied Biosystems.
- Adellgene® is a trademark of Blackhills Diagnostic Resources S.L.U.

17- Changes control

Version	Description of the modification
Rev. 03	For research use only (RUO). Changes in the genes analysed. Change in the format and presentation of the kit. Inclusion of Positive Control, Reaction Blank and Red 1000 Size Ladder. Change in protocol and reaction volumes. Change in the interpretation of results. Change in the molecular weight marker. Change in the operating data. The number of CAG repeats calculated in SCA3 does include the fixed-length motif CAGCAG CAAAAGCAGCAA.
Rev. 04	CE marked under IVDR. New reference for the Spectrum Compact Polymer7 reagent (Promega™). Update of the capillary sequencer "Spectrum Compact" values in Section 9. The C ₀ values for the "Spectrum Compact" have been updated in Section 10. Section 13: the second limitation has been added. Section 14: the latest troubleshooting guidance has been included in case the molecular weight marker does not function properly.

18- Symbols used in labelling

	<i>In vitro</i> diagnostic medical device		Use-by date
	Catalogue number		Contains sufficient for <n> tests
	Batch code		Manufacturer
	Temperature limit		Keep away from light sources
	Positive Control		Consult electronic Instructions for Use
	This product fulfils the requirements of Regulation (EU) 2017/746 on <i>in vitro</i> diagnostic medical device		