adellgene

ADELLGENE SCAs

INSTRUCTIONS FOR USE

Kit for the determination by fluorescent fragment analysis of the presence of healthy and mutated alleles of the SCA-1, 2, 3, 6, 7, 8 genes

Rev. 02 - 09/11/2018

CE

Catalog No. AD-SCA-16

Storage at -18ºC to -30ºC

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1-Intended Use

Adellgene SCAs is a kit designed for use in clinical laboratories which detects the number of repetitions of CAG of the SCA 1, 2, 3, 6, 7 and CTA/CTG of SCA 8 (see Table 1), establishing healthy and disease alleles and aid clinical diagnosis associated with autosomal dominant spinocerebellar ataxias.

Large abnormal expansions described for SCA2, SCA7, and SCA8 with more than 200 repeats are not detected for this kit and it is necessary the use of other methodology to solve this kind of samples.

The use of this kit is the determination of both healthy and diseased individuals who have the corresponding range of repetitions [1, 2] (see section 2).

The technology is based on the polymerase chain reaction (PCR) of genomic DNA extracted and purified from peripheral blood followed by fluorescence analysis of the size of the PCR fragments obtained by genetic analyzer and conversion of that size in the number of CAG repeats.

Heterozygotes and possible homozygous with a size equal or less than 200 CAG repeats are detected.

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2-Summary and explanation

The autosomal-dominant spinocerebellar ataxias (ADCA) are a heterogeneous group of neurodegenerative disorders characterized by slowly progressive cerebellar dysfunction. Affected individuals have difficulty coordinating body movement, gait ataxia in particular, in addition to other associated findings [1, 2]. Over 17 distinct types of hereditary spinocerebellar ataxia (SCA) disorders have been described based on identification of different causative genes or chromosomal loci [1]. Because of variable expression and phenotypic overlap, the SCA disorders cannot be differentiated reliably on a clinical basis. Although algorithms may predict the likelihood of a specific type of SCA [3, 4] an accurate diagnosis depends on molecular testing that detects a mutation in a specific causative gene. With few exceptions, mutations that cause SCA disorders are an abnormally large numbers of nucleotide- repeat motifs.

Typical onset of SCA symptoms usually occurs between the ages of 30 and 40 years, and symptoms are slowly progressed. The expanded repeats are unstable between generations and this intergenerational instability gives rise to an unusual pattern of inheritance-anticipation, which means decreasing age at onset of symptoms and increasing disease severity in successive generations [5]. The phenomenon of genetic anticipation is based on the expansion of trinucleotide repeats with an unstable intergenerational transmission. However, the molecular mechanisms of genomic instabilities are still unknown. SCA subtypes among ADCA patients vary highly in different ethnic populations.

Table 1. TRINUCLEOTIDE REPEATS OF THE MOST COMMON SPINOCEREBELLAR ATAXIAS. Reprinted from Sequeiros J, Seneca S, Martindale J. Consensus and controversies in best practices for molecular genetic testing of spinocerebellar ataxias. Eur J Hum Genet 2010; 18, 1188–1195.

Ataxia	Gene affected	Repeat	Normal	Uncertain[b]	Reduced penetrance	Full penetrance	Refs.
SCA1	ATXN1	$(CAG)_n$ $(CAT)_n$ $(CAG)_n$ [c]	6-38; 39-44 CAT interrupted	--[d]	--	39-44 CAGs uninterrupted; 45-91	[9, 13, 16, 17]
SCA2	ATXN2	$[(CAG)_n$ CAA $(CAG)_n]_n$ [e]	14-31	32-34[f]	--	35-500	[10, 11, 18, 19, 20, 21, 22, 23, 24, 25, 54]
MJD/SCA3	ATXN3	$(CAG)_2$ CAA AAG CAG CAA $(CAG)_n$	11-44	45-59[g]	--	61-87	[26, 27, 28, 29, 30, 31, 55]
SCA6	CACNA1A	$(CAG)_n$	4-18	--	19	20-33	[13, 32, 33, 34, 35, 36, 37, 38]
SCA7	ATXN7	$(CAG)_n$	4-19	28-33	34-35	36-460	[39, 40, 41, 42, 43, 44, 45, 46, 47, 48]
SCA8	ATXN8/ ATXN8OS	(CTA/CTG)	14-42	--[h]	≥74-1000[h]	--[h]	[15, 49, 50, 51, 52, 53]

a Full references are available at: http://www.scabase.eu.

b 'Uncertain' range was defined whenever there was only one, or two or more contradictory reports.

c May be interrupted by 1 to 3, or exceptionally 4, CATs.

d One report of non-penetrance with a 44 repeat allele, but not described as pure or interrupted (Goldfarb et al, 1996).

e The CAG repeat may be pure or have 1 to 4 CAA interruptions.

f An interrupted 32 repeat allele found in a patient (Silveira et al, 2002); an uninterrupted 32 CAGs allele in a (young) asymptomatic person (Cancel et al, 1997); a 33 pure CAG repeat in one patient (Fernandez et al, 2000); a 34 interrupted repeat in one patient (Constanzi-Porrini et al, 2000); 32, 34 and 35 interrupted repeats found in patients with Parkinsonism.25

g A 45 CAG allele in one patient (Padiath et al, 2005); one family segregating a 51 CAG allele, apparently not associated with the disease (Maciel et al, 2001); a 51 allele in one patient from a MJD family (Gu et al, 2004); one family segregating 53 and 54 alleles associated with an 'abnormal phenotype' (van Alfen et al, 2001); a 54 CAGs allele in a patient from a MJD family (van Schaik et al, 1997); a 55 allele described in one patient (Egan et al, 2000); a 56 allele described in one patient (Takiyama et al, 1997).

h Pathogenic ranges and incomplete penetrance are very uncertain in SCA8; there is a large overlap of repeat sizes in patients and in persons with no symptoms and no family history of ataxia, but itmay depend on the family; however, expansions were also present in 0.4% of controls;31 Silveira et al29 had reported before different ranges, seeing no overlap in controls (15–91) and pathogenic (100–152 repeat) alleles; however, they found a very high instability in sperm (contractions and expansions), both for expanded and normal alleles; patients were found with schizophrenia or bipolar disorder, depression, or borderline personality disorder with 1140 and 1300 repeats (Vincent et al, 2000).

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The most common Autosomal dominant spinocerebellar ataxias are SCA1, SCA2, SCA3, SCA6, SCA7 which common motif is a CAG and SCA8 where a CTA/CTG motif is repeated (Table 1) [1, 6-8].

Expansions of the CAG triplets are at the beginning of the exons of the corresponding genes for SCA1 to SCA7 and in the 3´UTR region in the SCA 8 (Figure 1).

The loci and mutations associated with these subtypes of SCAs have been cloned: SCA1 (9) (MIM 164400), SCA2 [10, 11] (MIM 183090), SCA3 [12] (MIM 109150), SCA6 [13] (MIM 183086), SCA7 [14] (MIM 164500), and SCA8 [15] (MIM 603680).

Testing commonly employs radiolabeled primers to individually amplify the region containing the CAG or CTA/CTG-repeat motif from each of the six genes, polyacrylamide gel electrophoresis (PAGE) to resolve the amplicons by size, and autoradiography for visualization. To determine whether the amplicons have an abnormal number of repeats, their lengths are determined by co-migration with a DNA sequencing ladder. This method is labor intensive, requiring both the analysis of each locus individually and the precautions inherent in the use of radionucleotides.

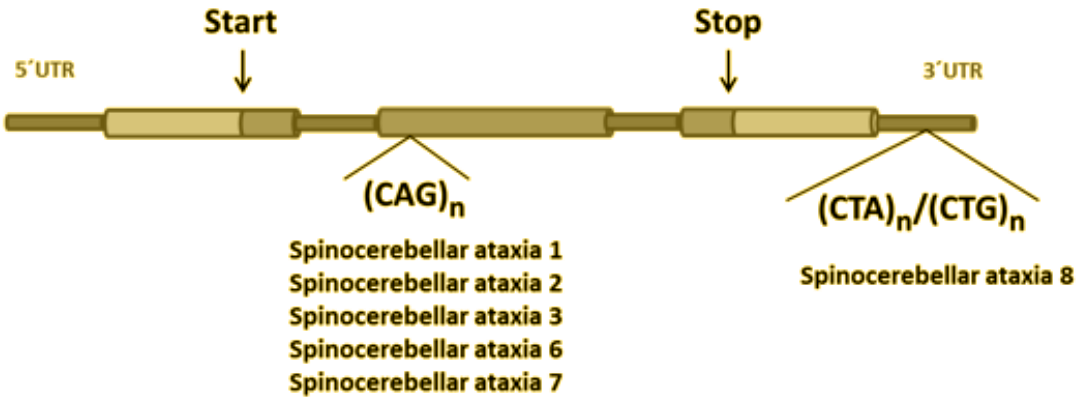

Figure 1. Expansions of the Spinocerebellar ataxias

The Adellgene SCAs kit is a rapid and precise method to assay SCA1, SCA2, MJD (SCA3), CACNA1A (SCA6), SCA7, and SCA 8 by multiplex amplification and capillary electrophoresis (CE).

This kit Adellgene SCAs is optimized for carrying out a rapid screening for detection of approximately 10-200 of the corresponding CAG or CTA/CTG triplet repeats so as to directly discard the people with normal or mutated (until 200) number of repeats (see Table 1). It is based on the analysis of fragments generated with a multiplex PCR with fluorescence primers and capillary electrophoresis in a genetic analyzer of fluorescence with the appropriate software for detection and interpretation. Those cases of people with only one peak, mainly, corresponding to a normal SCA2, 7, or 8 number of repeats have to go to a later study by an appropriate methodology.

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3-Principles of the procedure

The detection method employed by the Adellgene SCAs is based on two multiplex PCR with a specific amplification, from purified genomic DNA, of the fragment containing the gene region which presents the corresponding SCA-1, 2, 3, 6, 7, 8 CAG or CTA/CTG repeats, one of which is labeled with a different fluorophore in each multiplex PCR for subsequent detection in a DNA fragment analyzer. The kit includes primers, polymerase mix and a specific buffer for amplification of the corresponding regions. The use of LIZ 500™ tagged fragments is recommended. The size of PCR products are converted into the number of CAG or CTA/CTG triplet repeats using conversion factors of mobility and size.

4-Kit contents

Reference AD-SCA-16

REACTION 1

For determination of the CAG triplets of SCA-1, 3, 7

- AD-SCA-PM1: Primer Mix 1 (PM1). 1 vial x 34 µL

REACTION 2

For determination of the CAG of SCA-2 and SCA-6 and CTA/CTG of SCA8 triplets.

- AD-SCA-PM2: Primer Mix 2 (PM2). 1 vial x 34 µL

AMPLIFICATION MIX

For amplification of all regions included in PM1 and PM2.

- AD-SCA-POM: Polymerase Mix (POM). 2 vials x 110 µL
- AD-SCA-BUF: PCR Buffer (BUF). 2 vials x 52 µL

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5-Kit storage

- All kit reagents should be stored at - 18°C to -30°C and are stable at this temperature until their expiration date on the package.
- Allow the reagents (except AD-SCA-POM) to reach room temperature before use. Shake gently all reagents except AD-SCA-POM after thawing.
- Before to open the reagents tubes, perform a gentle centrifugation of each component so that the reagents are deposited on the bottom of the tube and no loose by the walls thereof.
- The test should be done keeping reagents on ice or cold block.
- Do not make more than 3 cycles of freeze / thawing the vials of Primer Mix (AD-SCA-PM1/2) and Polymerase Mix (AD-SCA-POM), as this may reduce the sensitivity of the test and alter the results.
- Due to the photo-sensitive nature of the reagents AD-SCA-PM1 and AD-SCA-PM2 avoid continued exposure to light.

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6-Materials required but not supplied

Isolation and Purification Reagents

- Reagents for DNA isolation and purification are not included. The DNA can be extracted by any method validated in the laboratory to ensure high quality and that the DNA is intact.

Capillary Electrophoresis Reagents (recommended)

- Genetic Analyzer for polymer POP-7 (ABI: 3130, 3730 or 3500).
- POP-7 polymer (ABI, Ref: 4363785 or equivalent)
- High purity formamide (Hi-Di Formamide; ABI, Ref.: 4311320 or equivalent)
- Calibrators for fluorophores FAM, HEX, ROX and LIZ (ABI, Ref.: 4345833 or equivalent)
- Size standard marker LIZ 500 (ABI, Ref.: 4322682)

Supplies and equipment

- General equipment of a laboratory dedicated exclusively to performing the PCR.
- Thermo cycler (ABI, 9700, Veriti or equivalent)
- Centrifuge for 96-well plates
- Shaker (Vortex)
- Micro-centrifuge
- Pipettes (P1000, P200, P20 and P2) and filter tips specific pipette.
- PCR plates and sealers for PCR
- Plates for capillary electrophoresis

Positive Control

- The recommended WHO standard for the different SCAs tested or any cell line who DNA corresponds with validated sample.

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7-Sample collection and preparation

- All biological and blood samples should be treated as potentially infectious. When handling, basic or 'universal' precautions must be observed. Any sample handling should be done with appropriate personal protection such as goggles, gloves and appropriate clothing.
- This test should only be performed with whole blood samples treated with EDTA or ACD anticoagulants. Heparin can interfere with PCR and should not be used in this procedure. No hyperlipemic, hemolyzed, jaundiced or proteinemia blood samples should be used
- DNAses contamination can produce DNA degradation, so should be used with filter pipette tips and DNAse-free tubes. To take care with handling, pipetting and working systems to avoid PCR failure.
- Before giving results with in vitro diagnostics purpose, perform a validation assay.

Caution

Kit toxicological properties have not been studied in depth so it is recommended to avoid contact with skin and mucous membranes. Do not ingest. Manual Safety Data Sheets (MSDS) are available to the customer.

8-Procedure

A. DNA extraction

Extraction of genomic DNA by standard methods from whole blood collected with EDTA is compatible with the use of this kit. It is recommended to perform the evaluation of the quality and quantity of DNA obtained by measurements of absorbance at 260nm (OD260; concentration) and the ratio of the measurements at 260 and 280 nm (OD; purity). The DNA obtained can be stored at -20°C until use. The appropriate amount of DNA in each PCR reaction is about 25-30 ng (p.e.: 1 µl of DNA at 30 ng/µl).

B. Preparation and conditions of the PCR reaction

Precautions

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

1. Mix preparation of the Primer Mix, Polymerase Mix and PCR Buffer for n+1 samples:

Reagent	Vol. for each sample (ul)
AD-SCA-PM1	1.8
AD-SCA-POM	5.9
AD-SCA-BUF	2.8
Total volume for each reactions	**10.5**

Reagent	Vol. for each sample (ul)
AD-SCA-PM2	1.8
AD-SCA-POM	5.9
AD-SCA-BUF	2.8
Total volume for each reactions	**10.5**

- To shake the sample gently 3-5 times before distribution in PCR tubes ensuring of the complete homogenization
- To perform a pulse centrifugation to ensure that there is no sample in the tube walls or bubbles.

2. For each samples two wells must be keep. To pipette 10.5 µl of Reaction 1 and 10.5 µl of Reaction 2 mixtures on two different wells of a PCR plate or sterile tubes and add DNA volume subsequently needed to get 25-30 ng (i.e.: 1 µl of DNA at 30 ng/µl) or negative control in the case of contamination control well.

3. To seal the plate or the tubes and perform a pulse centrifugation to ensure mixing and there are no bubbles.

4. To place the plate or tubes in the thermal cycler and perform the PCR reaction.

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Thermocycler conditions

	Number of cycles	Temperature (ºC)	Time (mm:ss)
DENATURATION	1	98	05:00
CYCLES	30	97	00:35
		60	00:35
		68	06:00
FINAL EXTENSION	1	72	30:00
COOLING	1	4	∞

C. Confirmation of the amplification products

Confirmation of the amplification products could be made through an appropriate system as the horizontal electrophoresis in agarose gels. To prepare an agarose gel at a concentration of 1-1.2% w / v validated according to the lab protocol and analyze 2ul of each amplified to certify that the PCR was successful.

D. Preparation of the samples for capillary electrophoresis

1. Preparation of Samples for DNA analyzer

- The following reaction mixture is performed with the PCR product. It is recommended to add 1 or 2 µl of the PCR product for the 3130/3130XL and 0.5 or 1 µl for the 3730xl DNA analyzers to obtain better results or to check the optimal concentration to charge in the DNA analyzer.

Reagent	Vol. for each sample (µl)
PCR product*	1 *
Hi-Di-Formamide**	10
LIZ 500™ Marker**	0.5
Total volume for each reactions	**11.5**

** See recommended volume of PCR (see above) - ** These items are not supplied with the kit*

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- To add the appropriate amount of PCR product in each well of the plate for capillary electrophoresis.
- To mix Formamide and Marker in a tube multiplying for the number of samples that are going to be injected. To perform a pulse of centrifugation (3-5 times) to ensure mixing of the reagents and transfer 10,5 µL of the mixture to the corresponding well of the plate for capillary electrophoresis
- To seal the plate, shake and centrifuge to remove bubbles and transfer to the thermal cycler.
- To denature 2 minutes at 95°C and transfer to ice protecting the sample from light until injection into the genetic analyzer. It is recommended to use a positive control with a control sample which the number of repetitions is known (see section 6).

2. Module of DNA analyzer

The following module of work is recommended for the corresponding DNA analyzer:

	DNA ANALYZERS	
PARAMETERS	3130/3130xl*	3730xl
Oven Temperature	60 ºC	63 ºC
Poly Fill Vol	7300 steps	6500 steps
Current Stability	5.0 uAmps	5.0 uAmps
Pre Run Voltage	15.0 KVolts	15.0 KVolts
Pre Run Time	180 sec	180 sec
Injection voltage	3.0 KVolts	1.6 KVolts
Injection time	15 sec	30 sec
Voltage Number of steps	20 nK	20 nK
Voltage Step Interval	15 sec	15 sec
Data Delay Time	60 sec	60 sec
Run voltage	15.0 kVolts	15.0 kVolts
Run time	3000 sec	2200 sec

* *In both cases is recommended the use of polymer POP7*

9-Results and interpretation

Adellgene SCAs kit is a quantitative technique for identifying the corresponding number of CAG or CTA/CTG triplet repeats between 4 and 200 (see Table 1) to directly discard the people with normal or mutated (until 200) number of repeats. It is based on the analysis of fragments generated with a multiplex PCR with fluorescence primers and capillary electrophoresis in a genetic analyzer of fluorescence with the appropriate software for detection and interpretation. Those cases of people with only one peak, mainly, corresponding to a normal SCA2, 7, or 8 number of repeats have to go to a later study by the appropriate technique.

The different PCR products are labeled with different fluorophores, which makes possible the subsequent analysis in the appropriate fragment analysis software:

SCA gene	Fluorophore(color)
SCA1	HEX (green)
SCA2	ROX (red)
SCA3	ROX (red)
SCA6	HEX (green)
SCA7	FAM (blue)
SCA8	FAM (blue)

The size of the target amplicon is derived from comparison to a co-injected size standard. This kit incorporates two correction factors for conversion of size in base pairs to the number of CAG or CTA/CTG repeats for each allele to adjust different mobility on capillary electrophoresis between the size ladders and the GC-rich amplicons. The size of product peaks can be converted to repeat length by the following equation:

$$\text{Nº repeats} = \frac{\text{Peak}_i - c_0}{m_0}$$

Peak_i - size in base pairs of a given product peak

c_0 - size correction factor,

m_0 - mobility correction factor

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Correction factors for the recommended CE instrument may have the values listed in the following the Table.

Instrument	m_0
3130, 3130xl 36cm	2.970
3130, 3130xl 50cm	2.963
3730, 3730xl 50cm	2.995

SCA genes	c_0
SCA1	123.8
SCA2	122.8
SCA3	227.8
SCA6	236.8
SCA7	244.8
SCA8	232.0

Correction factors can be changed by CE instruments, capillary length, polymer type or run conditions. The calculation of correction factors is suggested using positive controls when the kit is used first time.

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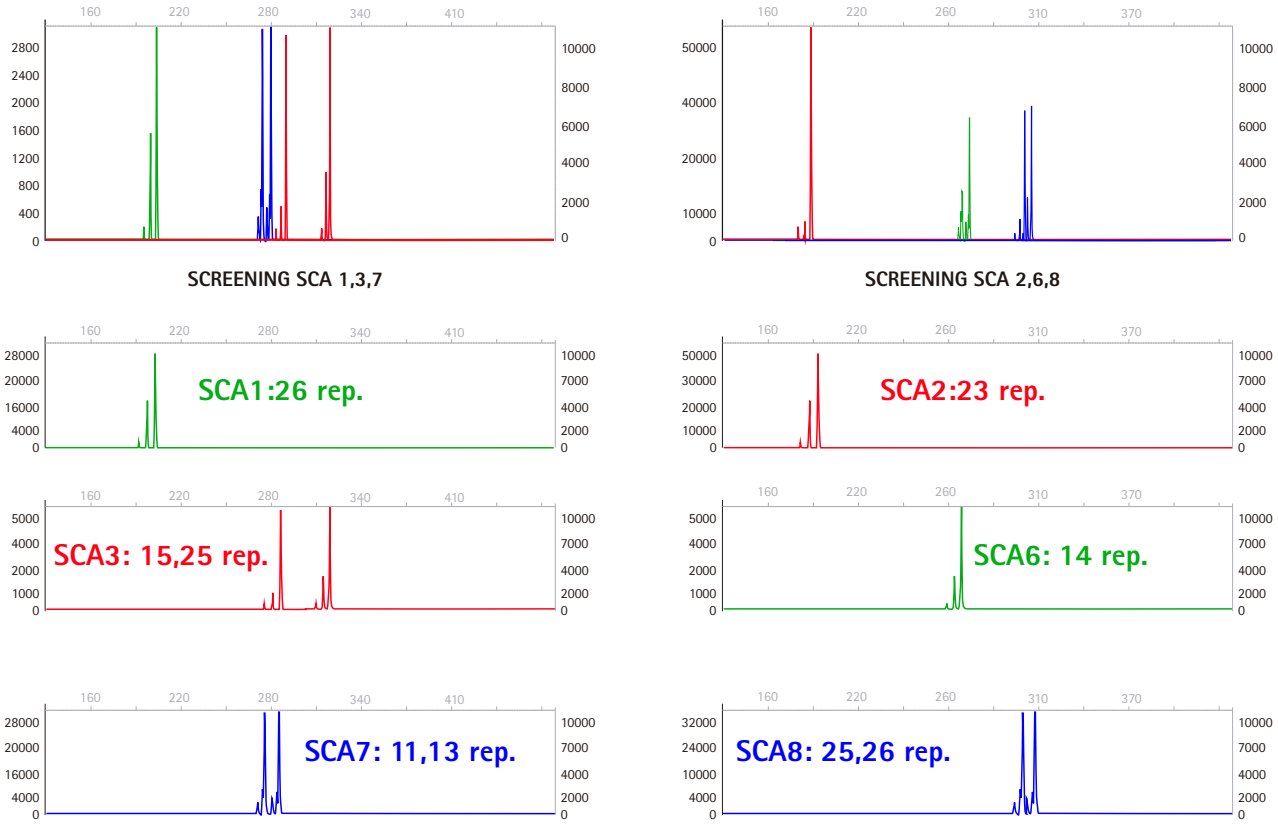

Figure 2. SCA 1, 2, 3, 6, 7, 8 screening in healthy individual

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10-Quality control

Due to the quantitative nature of this test, it is necessary to perform calibration of the FAM, ROX, HEX and LIZ fluorophores in the genetic analyzer.

Furthermore, as described in the procedure, the addition of the marker fragment analysis LIZ™ 500 for determining the size of the DNA fragments is required.

A contamination control should carry out by replacing the DNA for a negative control and a positive control of a known size (see section 6).

The user should consider all precautions named in section 7 and the limitations in section 12 of this procedure.

The test shall be carried out as recommended by this protocol, as well as with other quality control procedures that comply with local, state, federal and / or certifying agencies.

11-Specific data of performance

1. Analytical specificity and sensitivity

The primers of this kit are specific for the corresponding genes of the Spinocerebellar ataxias 1, 2, 3, 6, 7, and 8 (see Table 1). The specific amplification of these regions of the corresponding genes were verified by DNA sequencing and by assaying samples of healthy individuals and patients well characterized. There is no reported case of cross-reactivity with another gene from genomic DNA.

2. Diagnostic specificity

This kit Adellgene SCAs is optimized for carrying out a rapid screening for detection of approximately 10-200 of the corresponding CAG or CTA/CTG triplet repeats so as to directly discard the people with normal or mutated (until 200) number of repeats (see Table 1).

Mutations (point mutations, insertions, deletions) at amplification primer sites are possible and may result of the lack of allele definition. Other technologies could be necessaries to resolve the typing. Homozygous results must be confirmed by alternative procedures. It is recommended to have a generic typing (two digits) of the simple before to do the sequencing process.

3. Range of

- DNA concentration

  For the best range of amount of DNA in each assay tests were performed between 20 ng to 100 ng with a sample. The assignment of peaks obtained size was independent of the amount of DNA used. The recommended working range is between 25-30 ng of DNA by PCR.

- Results of the kit

  The method of this kit can assign a number of CAG or CTA/CTG triplet repeats of between 4 and 200 alleles which is healthy or mutated, respectively. Those cases of people with only one peak, mainly, corresponding to a normal SCA2, 7, or 8 number of repeats have to go to a later study by the appropriate methodology.

4. Accuracy

- Assignment of fragment size

The assignment of the peaks is given by the size of the amplicon obtained and peak morphology. If there are several peaks, it will be considered the highest and the central peak. The accuracy of the number of the repeats was determined comparing sequenced samples with the sizes obtained with the present kit comprising a variability of No. of repeats of ±1 for alleles <50, and ±3 repeats in the case of alleles between 50 and 200 repeats.

- General testing

Gene	Number of samples tested	Number of healthy individuals	Number of mutated individuals*
SCA1	7	7	0
SCA2	5	4	1
SCA3	6	5	1
SCA6	8	7	1
SCA7	9	7	2
SCA8	17	12	5

**The allele >200 repeats was tested by other technologies (RT-PCR)*

- Interferences

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

12-Procedure limitations

- The method detects all alleles with a number or repeats of less than 200 triplets (see sention 9).
- The conditions described for the PCR should be accurately controlled. Deviations from these parameters can lead to poor outcome.
- All work from Adellgene SCAs kit must be made in accordance with good laboratory practices and in compliance with local regulations, such as the international standard.
- Before to use the kit, to ensure that the equipment (thermal cycler, genetic analyzer,..) has been calibrated according to the manufacturers´ instructions.
- The DNA analyzer must be calibrated according to the manufacturer's recommendations for the fluorophores used in compliance with the limits specified by them.
- Do not mix components from other kits and lots.
- Do not use the kit after the expiry date exceeded.
- Do not use the kit in case of suspected loss of reactivity, contamination, deterioration of the container or any other incident that may affect performance.
- The interpretation of the data and typing results should be reviewed by qualified personnel.
- Remove expired reagents following applicable regulations.

13-Troubleshooting guide

PCR negative control is positive

- **Primer mix contamination**
  - Repeat the experiment with new aliquots of Primer mix / Negative Control
  - Handle kit components provided as commonly accepted practices to avoid contamination.
  - Check storage and handling conditions.
  - Discard contaminated reagents
- **Polymerase Mix contamination**
  - Repeat the experiment with new aliquots of Polymerase / negative control water.
  - Handle kit components provided as commonly accepted practices to avoid contamination.
  - Check storage and handling conditions.
  - Discard contaminated reagents.
- **Contamination in the Pre-PCR area**
  - Confirm that the necessary precautions have been followed in the PCR area.
  - Check possible contamination problems in other PCR techniques.
  - Confirm the suitability of the consumables used (tubes, pipette tips).
  - Confirm that the Taq is not contaminated.
- **Pipetting error**
  - Verify that the sample added in a given position always corresponds to the assigned worksheet.

Weak or absent signal in the PCR product

- **Poor quality of DNA samples**
  - Repeat the extraction of DNA
- **Samples with very low concentration of DNA**
  - Check the DNA concentration
- **DNA samples with high concentration**
  - Make a preliminary assessment of the extraction system by testing sample dilutions

- **Presence of PCR inhibitors in genomic DNA**
  - Avoid the use of whole blood containing heparin. Re-extract DNA an drepeat PCR wher possible.
- **Polymerase Mix not added to mix or insufficient mixing of PCR mix**
  - Repeat PCR ensuring all components are added and mixed sufficiently
- **Thermal cycling problems**
  - Check the thermal cycling run parameters and ensure thermal cycler is operating according to manufacturer´s specifications and maintenance.
- **No ethidium bromide (or other alternative DNA colorants) is added**
  - Ensure ethidium bromide is added to gel and electrophoresis buffer.

Incorrect band size or number of bands

- **Incorrect kit used**
  - Check the appropriate kit is used
- **Incorrect thermal cycling program used**
  - Check thermal cycler parameters
- **PCR contamination**
  - Check the negative control. Proceed with descontamination protocols and repeat PCR to identify the origin of contamination

Weak signal of electropherograms

- **Degradation of the kit**
  - Confirm proper storage of the kit
  - Avoid over 3 cycles of freeze / thaw of reagents
  - Perform aliquot fractions reagents as necessary.
  - Repeat with fresh reagents batch
- **Weak PCR product**
  - Check gel image and proceed accordingly
- **Insufficient reaction products applied to DNA analyzer**
  - Check analyzer parameters

Negative control simple yields a positive result

- Cross-contamination
  - Handle always the kit components according to commonly accepted practice to avoid contamination.
- Pipetting error
  - Verify that the sample added in a given position always corresponds to the assigned worksheet

High fluorescence intensity

- Too much PCR product
  - Check gel image. Dilute PCR product
- Too much product applied to the DNA analyzer
  - Check instrument parameters
- Pipetting error
  - Verify that the volume added in each well is correct

High background (noisy baseline)

- PCR product contaminated
  - See above
- Poor PCR purification
  - Ensure purification process is made according manufacturers' instructions.

14-References

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