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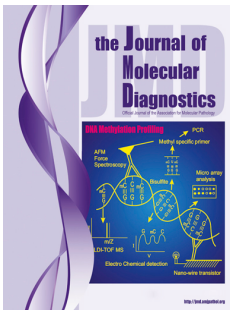

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SCA TETHERING-PCR: A RAPID GENETIC TEST FOR THE DIAGNOSIS OF SCA1-3, 6, AND 7 BY PCR AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

Spinocerebellar ataxias (SCA) type 1, 2, 3, 6, and 7, associated with a (CAG)n repeat expansion in coding sequences, are the most prevalent autosomal dominant ataxias worldwide (approximately 60% of the cases). In addition, the phenotype of SCA2 expansions has been now extended to Parkinson's disease and amyotrophic lateral sclerosis. Their diagnosis is presently based on a PCR to identify small expanded alleles, followed by a second-level test whenever the suspect of false normal homozygous, or a CAT interruption in SCA1 needs to be verified. Next-generation sequencing still does not allow efficient detection of these repeats. Here, we show the efficacy of a novel, rapid, and cost-effective method to identify and size pathogenic expansions in SCA1-3, 6, and 7 and recognize large alleles or interruptions without a second-level test. Twenty-five healthy controls and 33 expansion carriers were analyzed: alleles migrated consistently in different PCRs/capillary runs, and homozygous subjects were always distinguishable from heterozygous carriers of both common and large (>100 repeats) pathogenic CAG expansions. Repeat number could be calculated counting the number of peaks, except for the largest SCA2 and SCA7 alleles. Interruptions in SCA1 were always visible. Overall, our method allows a simpler, cost-effective, and sensibly faster SCA diagnostic protocol compared to the standard technique and to the still unadapted next-generation sequencing.

INTRODUCTION

Autosomal dominant cerebellar ataxias (SCA) are clinically and genetically heterogeneous neurodegenerative diseases characterized by the progressive loss of voluntary muscle control (ataxia) often associated with various neurological and extra-neurological signs, and cerebellar atrophy.
In two-thirds of all SCA patients, gait disorders are the prevalent symptoms at onset, and the first signs of disease appear in the third-fourth decade of life.[1]
The classification based on clinical signs and symptoms, originally proposed by Anita Harding[2] does not allow the discrimination of subtypes as: i) different subtypes share common phenotypic features; ii) clinical signs vary considerably between families with the same subtype; iii) extra-cerebellar clinical manifestations can be the predominant signs of the disease; iv) complicated forms can show extra-cerebellar signs only in the advanced stages of the disease.[3] Presently, a classification based on the gene/locus associated with the disease is preferred:[4] at the time of writing, more than 35 SCA subtypes have been identified, and the underlying genetic mutation is known for at least 29 of them (http://neuromuscular.wustl.edu/ataxia/domatax.html, last accessed November 17, 2017).

Mutations can be divided in expansions/insertions of a polynucleotide repeat (SCA1–3, SCA6–8, SCA10, SCA12, SCA17, SCA31, SCA36 and dentato-rubro-pallidoluysian atrophy/DRPLA), and conventional mutations.[5-7] It has been suggested that the most severe and complicated forms belong to the first group whereas the second includes mostly pure forms of ataxia.[6, 8]
SCA1 (MIM#164400), SCA2 (OMIM#183090), SCA3 (OMIM#109150), SCA6 (OMIM#183086), SCA7 (OMIM#164500), SCA17 (OMIM#607136), and DRPLA (OMIM#125370) belong to the group of $(CAG)_n$ repeat expansions in coding sequences. The mutation causes the polyglutamine-encoded tract to exceed a pathogenic threshold specific for each protein.[9]

In three genes, the CAG-tract can be interrupted: in SCA1, by CAT units, encoding for histidine; in SCA2 and SCA17, by CAA units, coding for the glutamine amino acid. The length and position of these interruptions is variable within the CAG-tract, and alleles carrying interruptions are less prone to meiotic

expansion. Furthermore, SCA1 expanded alleles with 39 to 44 repeats are not pathogenic in the presence of interruptions.[10] In SCA2, small interrupted repeat expansions and interrupted intermediate-size repeats have been associated with Parkinson disease and amyotrophic lateral sclerosis, respectively, in numerous studies.[11-15] A recent large survey, however, did not find any correlation between SCA2, 3, 6, and 17 expansions and Parkinson disease.[16]

Taken together, SCA1-3, 6, and 7 are the most prevalent autosomal dominant ataxias worldwide, accounting for ~60% of the cases.[5, 17-28] The diagnosis of these SCAs is presently based on a standard PCR able to identify small-expanded alleles, followed by a second-level test whenever an interruption in SCA1 is to be verified or the PCR shows a single allele in a symptomatic subject (false homozygous), raising the question if an expanded allele failed to be amplified. [29]

Here, we show the efficacy of a novel, rapid, and cost-effective method based on PCR and capillary electrophoresis, named tethering PCR (tPCR), to identify and size pathogenic expansions in SCA1-3, 6 and 7 and recognize large alleles or interruptions without a second-level test. This method advances our previous technique to detect large SCA2 and SCA7 expansions[30] and is required for efficient and reliable diagnosis as next-generation sequencing (NGS) techniques still fail to detect these repeats.

MATERIALS AND METHODS

Twenty-five healthy controls, tested negative for SCA1-3, 6, and 7 using a standard diagnostic technique (based on PCR amplification and CAG-repeat assessment using the amplicon size as a reference), and 33 expansion carriers (SCA1, n=9; SCA2, n=8; SCA3, n=4; SCA6 n=6; SCA7, n=6) were collected at the "Città della Salute e della Scienza" University Hospital of Torino, Fondazione IRCCS Istituto Neurologico "Carlo Besta", the Pitié-Salpêtrière University Hospital of Paris according to local ethical rules (Department of Medical Sciences, Istituto Neurologico "Carlo Besta" and Paris-Necker Ethics Committees) (Table 1). Blood samples were obtained with the written consent of the subjects, and DNA was extracted using standard procedures.

Expansion carriers were diagnosed using standard PCR, a combination of standard PCR and Southern blot for the larger alleles, or a combination of PCR and enzymatic digestion to assess the presence of CAT interruption in SCA1 expanded alleles (Table 2).[31]

To precisely assess the number of CAG repeats, seven subjects to have two homozygous controls, carrying the more common allele according to literature data, for SCA1, 3, 6, and 7, and a homozygous (allele size = 22 CAG) and a heterozygous (allele size = 22/23 CAG) subject for SCA2 (Table 1) were Sanger-sequenced. Three of these subjects (CTR1, CTR5, and CTR15; Table 1) were also used to verify the reproducibility of amplicon size. Each sample used for the set-up was PCR-amplified five times, the first PCR was run five times (capillary replicates), and PCRs 2^{nd}-5^{th} were run once (PCR replicates).

A locus-specific fluorescently labeled forward primer was designed for each SCA upstream of the unstable repeat. The reverse primer contained a locus-specific sequence followed by five CTG units complementary to the $(CAG)_n$ repeats (Table 3, Figure 1). This design allows: i) the amplification of the entire repeat, obtaining amplicons of different sizes for each SCA (SCA1 = PET, 104-200 bp; SCA2 = ATTO, 255-327 bp; SCA3 = FAM, 170-272 bp; SCA6 = VIC, 70-108 bp; SCA7 = FAM, 115-157 bp; sizes are referred to alleles in the normal range); ii) the random annealing inside the unstable sequence, generating a stutter of bands starting from five/nine CAG repeats and differing from each other by one repeat (Figure 1). This is useful for manually counting the repeats number and can allow automatic binning.

Amplifications were performed using different combinations of $MgCl_2$ (Promega, Madison, WI), Betaine (Sigma-Aldrich, Milan, Italy), DMSO 100% (Sigma-Aldrich), and GoTaq G2 Hot-start polymerase with Flexi Buffer (Promega) as reported in Table 4, on a Biometra T Professional or a Biometra T Gradient thermal cycler (Analytik Jena, Jena, Germany). PCR products were subsequently pooled in two groups (SCA1, 3, 6 and SCA2, 7) and loaded on an ABI Prism 3730XL automatic sequencer (Thermo Fisher Scientific, Foster City, CA) on a 36 cm capillary with the POP7 polymer and the GS-500 Liz size-standard. Oven temperature was set to 60 °C, injection voltage to 2.5 kV, and injection time to 20 sec.

The bioinformatics analysis was performed using the GeneMapper ver4.0 (Thermo Fisher Scientific) and GeneMarker ver.1.95 (Softgenetics, State College, PA) software by three independent operators.

RESULTS

The rationale to set up a rapid technique for the diagnosis of the main SCAs associated with polyglutamine expansions (SCA1-3, 6, and 7), was to minimize the number of PCR reactions, thermal cycling conditions, and capillary electrophoresis runs. Different combinations of commercially available reagents, and co-solvents commonly used to amplify CG-rich regions (eg, Betaine and DMSO) were tested. The optimal conditions and mix of reagents for each SCA are reported in Table 4 and 5 and in the *Materials and Methods*. As a final procedure, three different chemical protocols (SCA2 and SCA7 required specific concentrations of DMSO and $MgCl_2$ compared to SCA1, 3, and 6), and a single cycling condition were used to amplify all five SCAs.

To determine the exact number of repeats for each tested SCA, and have the standard profile of a homozygous subject, one homozygous genotype was selected for each SCA allele in the normal range. These SCA alleles were Sanger-sequenced to be used as calibrators in subsequent reactions. The size of the alleles was deduced from the number of peaks in the arrays as specified below. In both capillary electrophoresis and PCR replicates, the same allele migrated consistently, with a standard deviation ≤ 0.41 base pair (Supplemental Table S1).

Then, 25 healthy controls and 33 expansion carriers were tested using this method. Electropherograms showed a series of discrete peaks: using the three calibrators, the first peak corresponded to five CAG repeats for SCA1, 2, 6, and 7, and to nine CAG repeats for SCA3 (Figure 2, 3). Each subsequent peak corresponded to an increment of one CAG repeat. The average distance between two consecutive peaks was ~2.9 bp, slightly smaller than the expected 3 bp (one CAG-repeat).

Peaks with the highest intensity corresponded to the size of the genotyped allele(s).

A homozygous subject was always distinguishable from a heterozygous carrier of an expansion (Figures 2, 3). If the expansion was <~100 repeats, the second allele was detectable as a bell-shaped array of peaks for SCA1, 2, 3, and 7: the tallest peak within the bell was considered for allele-calling (Figure 3). In SCA6, the small size of the pathological expansion prevented the formation of the bell-shaped curve (Figure 3). The hallmark for large SCA2 and SCA7 expansions (>100 CAGs) was a slowly degrading array of peaks (Figure 3): the size of the larger allele could not be determined by our technique.

Sizes detected by our method and the standard PCR technique in controls and expansion carriers were compared (Table 1 and 2). A systematic difference in SCA1 was found (-1 to -3 repeats). In these cases, the presence of CAT interruptions could be clearly determined as a gap in the array of peaks (Figure 2). In SCA2, the measure was concordant with the standard PCR (Table 1 and 2). A very large SCA2 expansion carrier (EXP11, ~200 CAG as estimated by Southern blot[32]) was recognizable by the descending array of peaks starting from the normal allele (Figure 3). The presence of a CAA-interrupted allele was always clearly detectable (Figure 2 and 3).

In SCA3, the standard PCR overestimated the CAG-repeat size of the smaller allele by one unit in 11 controls (Table 1). In the four expansion carriers, both the normal and the expanded allele were over- or underestimated by the standard PCR by 1-4 CAG (Table 2). Using the standard diagnostic technique, one out of four expanded alleles (EXP20, Table 2) fell out of the ± 3 tolerance recommended by the European Molecular Genetics Quality Network (EMQN) guidelines.

In SCA6, the standard PCR overestimated the CAG-repeat size by one unit in four out of 25 controls (Table 1). The sizing of expanded alleles using our technique was precise, as recommended by the EMQN guidelines (± 1 CAG).

In SCA7, the standard PCR overestimated one control and one expanded allele by one CAG unit. (Table 2). The three large expansions (EXP31, 32, and 33) were estimated by Southern blot analysis [33, 34], and were always recognizable with our technique because of the descending array of peaks starting from the normal allele (Figure 3).

DISCUSSION

Here, we describe a new method to determine the presence of pathogenic expanded SCA1-3, 6, and 7 alleles, named tethering-PCR. To go beyond the standard PCR method and the STR-primed PCR technique developed in our laboratory for several triplet expansion disorders,[30, 35, 36] a SCA testing protocol was implemented, following the procedure described for Huntington's disease.[37]
In this protocol, a forward fluorescent-labeled primer was coupled with a reverse primer containing the last five CTG units of the polyglutamine encoding repeat followed by 15 to 16 bp of the gene-specific region. After the capillary run, an array of peaks was obtained and interpreted.

The test overcomes several limits of the standard diagnostic technique, which is performed amplifying the region containing the repeat with primers external to the CAG-tract and measuring the amplimer after capillary electrophoresis. Moreover, in the STR-primed PCR the reverse primer anneals in the repeat only, and a third primer, annealing on the reverse's tail, is required for the final amplification. Although this allows the formation of an array of stutter band, the normal alleles are not clearly distinguishable, a precise CAG-size counting is not possible, and interruptions in SCA1 and SCA2 are not visible.

The size of the alleles measured using the formula

[(amplicon size – "constant region")/3]

is incorrect. Allele migration is influenced by the fluorochrome in the labeled primer, the type of capillary, the polymer, and the presence of the repeat itself. Thus, the "constant region" size needs to be corrected experimentally, running a known control sample validated by Sanger sequencing. Moreover, the sizing error increases with the number of repeats, as also shown for other triplet repeat disorders.[36] In this work, an average distance between two CAG-consecutive peaks of ~2.9 bp was calculated, instead of the expected 3 bp, causing an error in the measure of CAG-alleles that increases with their size. With this

method, allele sizing is performed counting the number of peaks, each corresponding to a CAG unit, starting from the first visible one. However, since SCA1 and SCA2 interrupted alleles contain traits in which the peaks are not visible, it would still be advisable to run a standard sample whose CAG-repeat number has been ascertained by Sanger sequencing, and/or to generate a sizing bin based on the carrier of an uninterrupted expansion allele, in which all peaks are visible. The first peak corresponded to five CAGs for SCA1, 2, 6, and 7, as was expected because the reverse primer contains five CTG units. For SCA3, the first peak corresponded to nine CAGs: this is because SCA3 polyglutamine trait starts with an "irregular" and interrupted CAGCAGCAAAAG sequence, encoding for the amino acids Glu-Glu-Glu-Lys and included in the count of CAG repeats [38]. The reverse primer is most likely unable to anneal because of the mismatches, skips these four units, and then anneals from the fifth to the ninth CAG, producing a first peak corresponding to nine CAG units (Supplemental Figure S1).

The standard technique cannot discriminate between homozygous alleles and an expansion carrier with a dropped-out allele. Indeed, standard PCR in triplet repeat disorders is unable to amplify large alleles due to technical limitations.[36, 39-41] Large alleles have been described in SCA2 neonatal and infantile-onset cases (230-500 CAG repeats),[42-44] and in SCA7 severe forms with onset in childhood and a rapid fatal course (55-460 CAG repeats).[33, 45-49] It is therefore mandatory to perform a second-level test (eg, Southern blot) when a homozygous genotype is found in infantile cases or in prenatal tests where one of the parents is a carrier. With this method, carriers of an expanded allele, the size of which falls out of the PCR sensitivity range, are clearly discernible from normal homozygous subjects because of the presence of a slowly descending array of peaks starting from the main one. This array is never found on a normal subject (Figures 2 and 3). In carriers of smaller expansions, the expanded allele is visible as a bell-shaped array of peaks for SCA1-3, and 7. In SCA6, such an array does not form due to the small number of repeats even in pathologically expanded alleles and it is sufficient to count the number of peaks.

This method is able to clearly detect both SCA1 and SCA2 interruptions (Figure 2 and 3). In routine diagnostics, when a SCA1 allele between 36 to 44 CAG repeats is found, a second level test to determine

the presence of CAT interruptions is used to discriminate if the allele is not associated with the disease (interrupted), is a mutable normal allele (36 to 38 non-interrupted CAGs), or is a full-penetrance allele (39 to 44 non-interrupted CAGs). Moreover, recent findings strongly suggest the association of SCA2 interrupted intermediate alleles with amyotrophic lateral sclerosis.

In conclusion, the current diagnostic procedure for polyglutamine expanded SCAs is time-consuming and requires distinct amplification protocols, PCR conditions, and capillary runs. Having the same thermal cycling protocol, our new method allows the simultaneous amplification of five SCAs on a single thermal cycler. Primer design allows two pooled runs for the capillary electrophoresis. These features lead to a simpler, cost-effective, and significantly faster SCA diagnostic protocol compared to the standard technique.

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FIGURE LEGENDS

Figure 1. Schematic of the proposed PCR procedure. An ideal heterozygous subject, carrying 11 and 12 CAG repeats (small rectangular bars), is represented. The forward primer (F), marked with a fluorochrome (green), is designed to anneal upstream of the repeat. The reverse primer (R) anneals partly on the repeat, and partly downstream. Given this particular design, it can amplify the whole repeat (upper part) generating two main peaks (heterozygous subject), and, at the same time, it can anneal inside the repeat giving rise to a discrete number of stutter bands with a 3 bp interval (lower part). The electropherogram shows two main peaks (11 and 12 CAG repeats from left to right) preceded by an array of smaller peaks starting from five CAG repeats.

Figure 2. The electropherograms of one homozygous and one heterozygous normal subject for each SCA tested are reported. Homozygous subjects' alleles were also sized by Sanger sequencing. Each panel contains the subject code (see also Tables 1 and 2), the allele size (expressed as number of CAG repeats), and the main peak(s) size. SCA1: peaks start from 104 ±1 bp, corresponding to 5-CAG repeats; SCA2: peaks start from 255 ±1 bp, corresponding to 5-CAG repeats; SCA3: peaks start from 170 ± 1 bp, corresponding to 9-CAG repeats; SCA6: peaks start from 70 ±1 bp, corresponding to 5-CAG repeats; SCA7: peaks start from 115 ±1 bp, corresponding to 5-CAG repeats. Main peaks are marked by a black arrowhead. The size of the first peak is reported only in left panels. The order reflects the two different pools prepared before capillary electrophoresis (SCA1, 3, 6 and SCA2, 7). For SCA1 and SCA2 homozygous subjects, a zoomed panel shows the tract in which peaks are absent because of the presence of CAT and CAA interruption, respectively.

Figure 3. On the left, electropherograms of one expansion carrier for each SCA tested are reported. A black arrowhead indicates the main peak and the peak corresponding to the expanded allele. On the right, two subjects carrying large SCA2 and SCA7 expansions are reported. A black arrowhead indicates the main peak whereas the expanded allele is not visible. A zoomed panel shows a slowly descending array of peaks, the hallmark of the presence of an expanded allele. Each panel contains the subject code (see also Tables 1 and 2), the alleles size (expressed as number of CAG repeats), and peak size. The order reflects the two different pools prepared before capillary electrophoresis (SCA1, 3, 6 and SCA2, 7).

Table 1. Controls (CTR) analyzed for each SCA.

CTR	SCA1		SCA2		SCA3		SCA6		SCA7	
	S	T	S	T	S	T	S	T	S	T
1	28/29	29/30	22/22	=	18/26	=	**13/13**	**13/13**	**10/10**	**10/10**
2	28/29	29/30	22/22	=	21/21	=	12/13	=	10/10	=
3	**29/29***	**30/30***	22/22	=	18/19	=	**11/11**	**11/11**	10/10	=
4	28/30	29/31	**22/22**	**22/22**	13/21	12/21	13/13	=	10/10	=
5	**28/28***	**29/29***	22/22	=	**21/21**	**21/21**	11/12	=	10/10	=
6	28/32	29/33	22/22	=	13/25	12/25	4/11	=	10/10	=
7	28/30	29/31	22/22	=	17/20	=	713	=	10/10	=
8	29/32	30/33	22/22	=	13/22	12/21	7/12	=	9/12	=
9	28/30	29/31	22/22	=	13/18	12/18	11/13	=	11/12	10/12
10	28/31	29/32	22/23	=	19/25	=	7/11	=	10/14	=
11	29/29	30/30	22/22	=	**13/13***	**12/12***	11/13	=	10/10	=
12	28/31	29/32	22/22	=	13/21	12/21	12/13	=	10/13	=
13	29/29	30/30	22/22	=	19/21	=	7/13	=	10/12	=
14	28/29	29/30	22/23	=	19/22	=	7/11	=	10/10	=
15	28/29	29/30	**22/23**	**22/23**	13/20	12/20	11/12	=	10/12	=
16	28/28	29/29	22/22	=	13/21	12/21	9/10	10/11	10/13	=
17	28/29	29/30	22/22	=	21/25	=	13/13	=	10/12	=
18	28/32	29/33	22/22	=	18/21	=	7/11	=	10/10	=
19	29/31	30/32	22/22	=	13/22	12/22	10/12	11/13	10/13	=
20	30/31	31/32	22/22	=	15/21	14/21	10/11	11/12	10/12	=
21	28/31	29/32	22/22	=	21/24	=	4/13	=	**12/12**	**12/12**
22	26/29	27/30	22/22	=	19/21	=	12/12	=	10/12	=
23	28/31	29/32	22/22	=	13/19	12/19	10/12	11/12	10/10	=
24	28/29	29/30	22/22	=	21/25	=	12/13	=	10/10	=
25	28/29	29/30	22/22	=	13/13	12/12	7/13	=	10/10	=

Allele sizes are expressed as CAG-repeat number. Subjects used to set-up the technique are underlined. Alleles sequenced for SCA2, SCA3, SCA6, and SCA7 for which sPCR and tPCR were concordant are in bold.

*sequenced alleles for which sPCR and tPCR were discordant (SCA1, CTR3 and CTR5, and SCA3, CTR11): in all three cases sPCR under- or over-estimated the number of repeats measured by Sanger sequencing.

S = sPCR (standard PCR); T = tPCR (tether PCR).

Values in "T" columns are indicated with an equal sign (=) when the measures obtained by sPCR and tPCR were identical.

Table 2. Expansion carriers (EXP) analyzed for each SCA.

	EXP	Standard PCR	Tether PCR
SCA1	1	28/56	29/58
	2	29/42	30/44
	3	29/48	30/50
	4	35/53	36/55
	5	31/36	=
	6	28/34	29/37
	7	31/56	30/57
	8	31/51	30/51
	9	29/44	28/44
SCA2	10	22/36	=
	11	23/~200 *	=
	12	-	22/41
	13	-	22/36
	14	22/45	=
	15	22/36	=
	16	22/33	=
	17	22/36	=
SCA3	18	22/67	23/70
	19	25/70	24/71
	20	24/74	20/70
	21	29/65	28/66
SCA6	22	11/23	=
	23	11/23	=
	24	11/23	=
	25	13/23	=
	26	13/22	=
	27	13/24	=
SCA7	28	-	12/34
	29	10/44	=
	30	10/41	10/40
	31	10/~86*	10/90
	32	12/~140*	12/>100
	33	12/~81*	12/85

* Estimated by Southern blot analysis.
Allele sizes are expressed as CAG-repeat number.
Equal sign (=) indicates when the measures obtained by standard and tether were identical. A dash (-) indicates that the subject was not analyzed by standard PCR.

Table 3. Primer sequences.

GENE	FORWARD	REVERSE
SCA1	5‘-PET-TTTGCTGGAGGCCTATTCCACTCT-3’	5‘-GAGCCCTGCTGAGGTGCTGCTGCTGCTGCTG-3’
SCA2	5‘-ATTO-TTTCGGCGGCTCCTTGGTCTC-3’	5‘-AGCCGCGGGCGGCGGCTGCTGCTGCTGCTG-3’
SCA3	5‘-FAM-AGTCCAGTGACTACTTTGATTCG-3’	5‘-GTCCTGATAGGTCCCCCTGCTGCTGCTGCTG-3’
SCA6	5‘-VIC-TTTTTCCCCTGTGATCCGTAAGG-3’	5‘-CGGCCTGGCCACCGCCTGCTGCTGCTGCTG-3’
SCA7	5‘-FAM-TTTGAAAGAATGTCGGAGCGGG-3’	5‘-CTGCGGAGGCGGCGGCTGCTGCTGCTGCTG-3’

Table 4. PCR Reagents.

Reagent	Quantity (µL)		
	SCA1, 3, 6	SCA2	SCA7
dNTPs 2.5mM	1.8	1.8	1.8
$MgCl_2$ 25mM	1.8	1.8	1.1
Buffer 5X	5.0	5.0	5.0
Betaine 5M	7.5	7.5	7.5
GoTaq 5u/µl	0.1	0.1	0.1
Primer F 10uM	1.0	1.0	1.0
Primer R 10uM	1.0	1.0	1.0
DMSO 100%	-	1.3	1.3
DNA	50-200 ng	50-200 ng	50-200 ng
H_2O	up to 25µL	up to 25µL	up to 25µL

Table 5. PCR thermal cycling conditions

t (°C)	Time (sec)	Cycles
95°C	7’	
95°C	1’	
63°C – 0.5°C/cycle	1’	14
72°C	1’	
95°C	1’	
56°C	1’	
72°C	1’ + 20’’/cycle	35
72°C	10’	

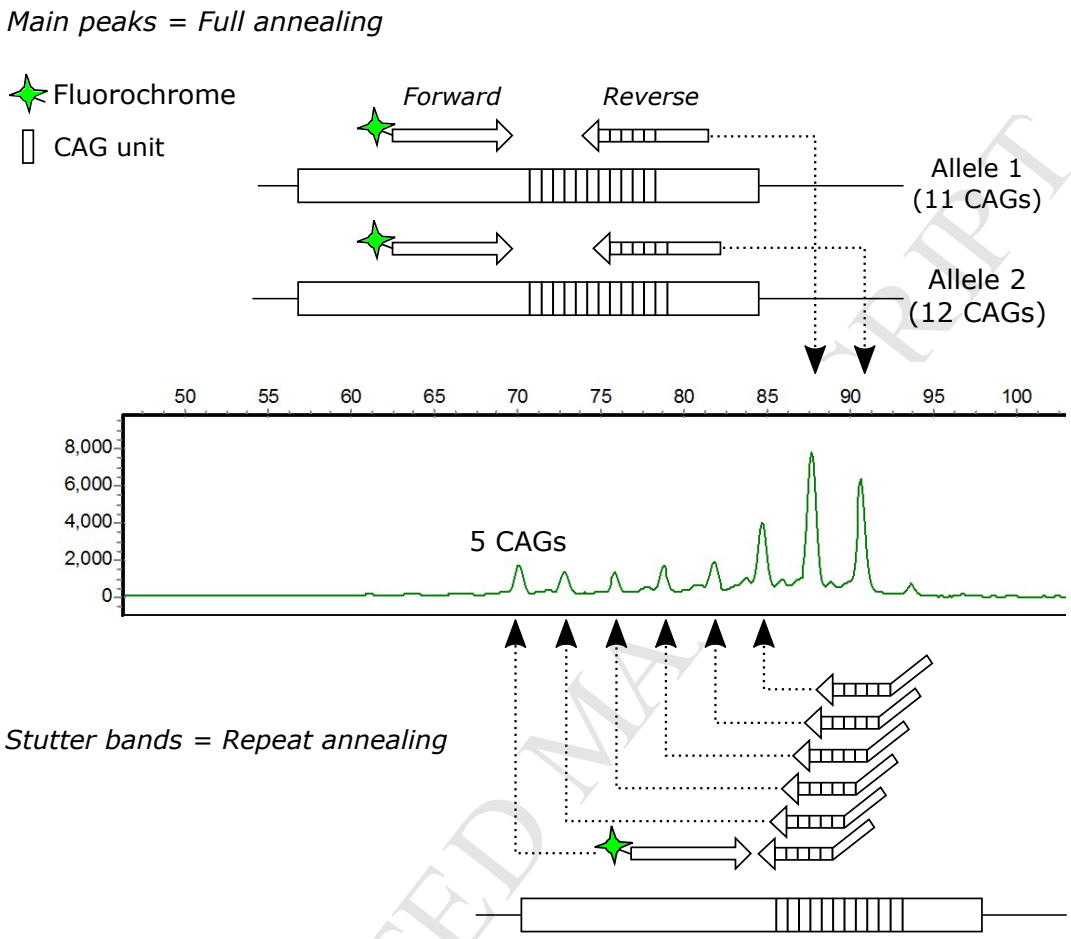

Figure 2

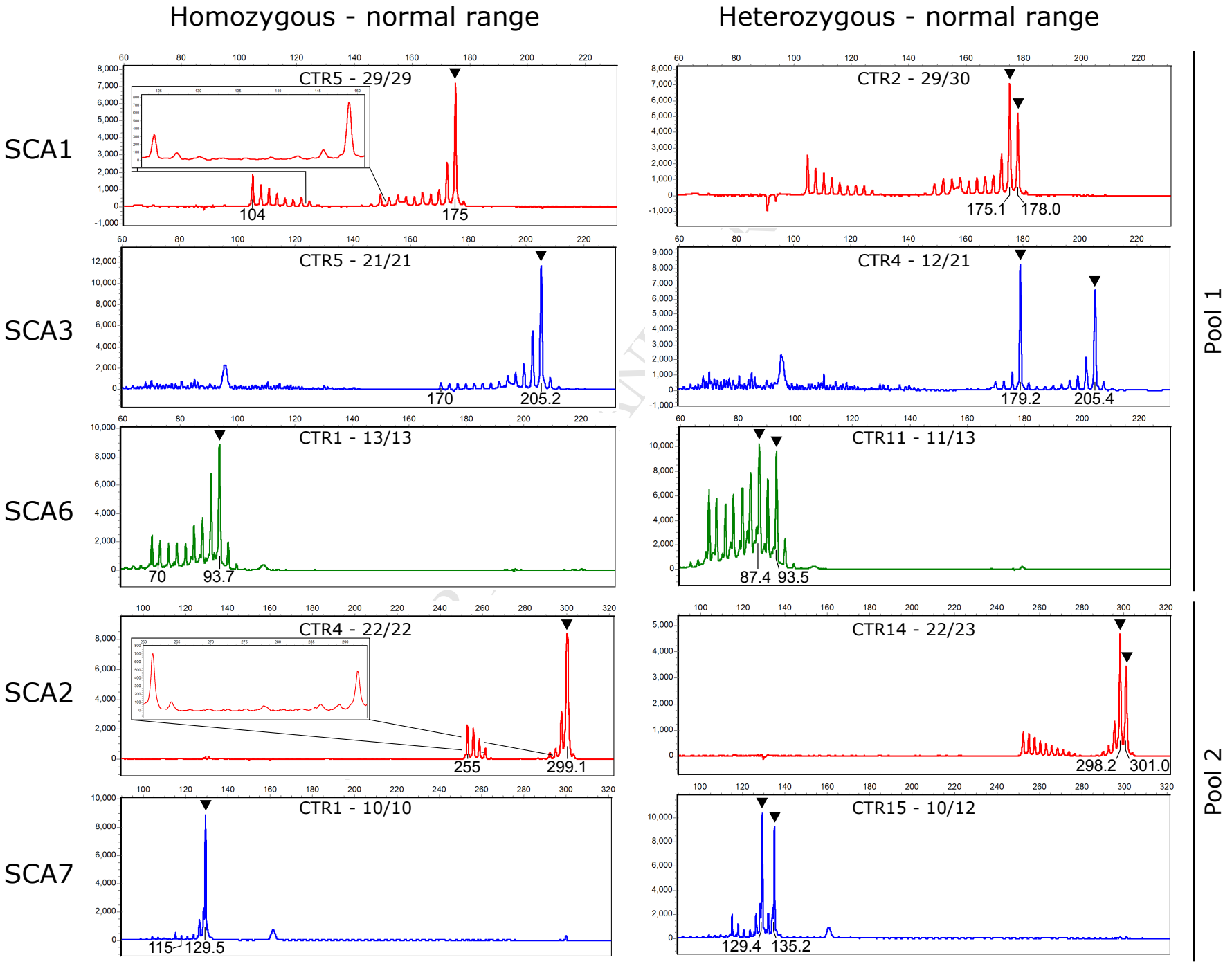

Figure 3