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Large Pathogenic Expansions in the *SCA2* and *SCA7* Genes Can Be Detected by Fluorescent Repeat-Primed Polymerase Chain Reaction Assay

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Large expansions in the *SCA2* and *SCA7* genes (>100 CAG repeats) have been associated with juvenile and infantile forms of cerebellar ataxias that cannot be detected using standard polymerase chain reaction (PCR). Here, we describe a successful application of the fluorescent short tandem repeat-primed PCR method for accurate identification of these expanded repeats. The test is robust, reliable, and inexpensive and can be used to screen large series of patients, although it cannot give a precise evaluation of the size of the expansion. This test may be of practical value in prenatal diagnoses offered to affected or presymptomatic at-risk parents, in which a very large expansion inherited from one of the parents can be missed in the fetus by standard PCR. ***(J Mol Diagn 2006, 8:128–132; DOI: 10.2353/jmoldx.2006.050043)***

Large-repeat expansions in *SCA2* and *SCA7* genes have been associated with infantile- and juvenile-onset forms of spinocerebellar ataxia (SCA).[1] SCA2 neonatal- and infantile-onset cases with common (from 57 to 64 CAG repeats) and extreme triplet expansions (from 230 to 500 CAG repeats) have been reported.[2–4] However, the clinical manifestations of the latter are unusual and include one or more of the following symptoms: severe hypotonia, developmental delay, dysphagia, encephalopathy, chronic seizures, and retinopathy.[1,5] *SCA7* CAG repeats from 55 to 460 repeats can give a severe disease with onset in childhood and a rapid fatal course.[6–11]

Routine genetic tests for these genes can miss expansions >80 CAG repeats giving a false-negative result. Assays based on the separation of the polymerase chain reaction (PCR) products on agarose gels, blotting, and hybridization with a $(CAG)_n$ oligonucleotide have been applied to overcome this limit.[1,12,13]

Our aim was to develop a simple, robust, and rapid PCR-based test able to detect large *SCA2* and *SCA7* CAG expansions. Our approach is based on a fluorescent short tandem repeat (STR)-primed PCR, first described for myotonic dystrophy type 1 by Warner et al[14] and recently applied by our group to *SCA10*, *SCA12*, and *FRDA1* genes.[15]

Materials and Methods

Genomic DNA and Patients

Genomic DNA was extracted from blood samples using the QIAamp DNA mini blood kit (Qiagen, Hilden, Germany) or a standard phenol-chloroform extraction. From our DNA collection of SCA patients and healthy controls, we selected 34 samples with *SCA2* repeats in the normal range (22 to 34 CAG repeats) and 12 SCA2 patients carrying the most common repeats within the pathogenic range (37 to 53 CAG repeats). Two patients with very large *SCA2* expansions (>200 CAG repeats) were pro-

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Table 1. Comparison of STR-PCR with Standard PCR and PCR-Southern Blot in the Analysis of SCA2 Patients

Range of expansion	Genotypes (CAG)	N	Standard PCR	PCR-Southern	STR-PCR
Normal	22/22	25	+	n.d.	+
	22/23	4	+	n.d.	+
	22/25	1	+	n.d.	+
	22/27	1	+	n.d.	+
	22/29	1	+	n.d.	+
	22/32	1	+	n.d.	+*
	22/34	1	+	n.d.	+*
Common mutations	22/37	1	+	n.d.	+*
	22/38	1	+	n.d.	+*
	22/39	2	+	n.d.	+*
	22/40	1	+	n.d.	+*
	22/41	1	+	n.d.	+*
	22/45	1	+	n.d.	+
	22/47	1	+	n.d.	+
	22/49	2	+	n.d.	+
	22/52	1	±	n.d.	+
	22/53	1	±	n.d.	+
Rare large mutations	23/~200	1	−	+	+
	22/~350	1	−	+	+

*The fluorescent STR-primed PCR could not correctly classify the profile in the normal or expanded range.

PCR results on the three right columns refer to the larger allele. + and − indicate the ability of a technique to identify an expansion. When ± is present, the expansion was not always detected.

N, number of tested subjects. n.d., not done.

Table 2. Comparison of STR-PCR with Standard PCR and PCR-Southern Blot in the Analysis of SCA7 Patients

Range of expansion	Genotypes (CAG)	N	Standard PCR	PCR-Southern	STR-PCR
Normal	9/9	5	+	n.d.	+
	9/10	1	+	n.d.	+
	10/10	16	+	n.d.	+
	10/11	3	+	n.d.	+
	10/12	4	+	n.d.	+
	10/13	2	+	n.d.	+
	10/14	1	+	n.d.	+
	10/15	1	+	n.d.	+
	10/16	1	+	n.d.	+
	11/12	1	+	n.d.	+
	11/14	1	+	n.d.	+
	12/12	1	+	n.d.	+
	13/13	1	+	n.d.	+
	12/34	1	+	n.d.	+*
Common mutations	10/36	1	+	n.d.	+*
	11/39	1	+	n.d.	+*
	10/41	1	+	n.d.	+
	7/42	1	+	n.d.	+
	10/42	5	+	n.d.	+
	12/42	1	+	n.d.	+
	10/43	2	+	n.d.	+
	12/43	1	+	n.d.	+
	10/44	1	+	n.d.	+
	10/47	2	+	n.d.	+
	10/48	1	+	n.d.	+
	10/49	1	+	n.d.	+
	10/54	2	+	+	+
Rare large mutations	10/58	1	+	+	+
	12/61	1	+	+	+
	10/65	1	+	+	+
	13/66	1	+	+	+
	10/77	1	+	+	+
	10/90	1	±	+	+
	13/106	1	±	+	+
	10/306	1	−	+	+

*The fluorescent STR-primed PCR could not correctly classify the profile in the normal or expanded range.

PCR results on the three right columns refer to the larger allele. + and − indicate the ability of a technique to identify an expansion. When ± is present, the expansion was not always detected.

N, number of tested subjects. n.d., not done.

vided by the Molecular Genetics Laboratory of the Mayo Clinic (Rochester, MN) (Table 1). The approximate expansion size of the latter had been determined by PCR-blot assay.[1]

For *SCA7* analysis, we selected 39 normal subjects with alleles ranging from 9 to 34 CAG repeats and 20 SCA7 patients carrying a 36- to 54-CAG repeat allele. Eight additional patients carried large uncommon alleles of 58 to 306 CAG repeats (Table 2). All normal and expanded alleles had been confirmed by Southern blot-PCR for *SCA7*.[8,12]

Standard PCR and Repeat-Primed PCR

Standard PCR analyses of the *SCA2* and *SCA7* genes were performed following previous reports.[12,16,17]The fluorescent STR-primed PCR was based on a previously described method[14] that we successfully applied to analyze the *FRDA1*, *SCA10*, and *SCA12* genes.[15] Briefly, a fluorescent-labeled primer was designed in a locus-specific region upstream of the unstable repeat of interest. The companion reverse primer (on the complementary strand) consisted of five CTG units and a 5′ tail that was used as an anchor for a second reverse primer, which prevents progressive shortening of the PCR products during subsequent cycles.

PCR reactions were performed on 200 to 1000 ng of genomic DNA with 144 μmol/L dNTPs, 1.3 to1.5 mmol/L $MgCl_2$ (for the *SCA7* and *SCA2* genes, respectively), 1 mol/L betaine (B0300; Sigma-Aldrich, St. Louis, MO), 1 U of TaqGold in 1× TaqGold buffer (Applied Biosystems, Foster City, CA) in the presence of 0.8 μmol/L of the locus-specific primer (SCA2F, 5′-HEX-ggg ccc ctc acc atg tcg, or SCA7F, 5′-HEX-gcg gtc cca aaa ggg tca gtt gtt aca ttg tag gag cgg aa), 0.08 μmol/L of the repeat-specific oligonucleotide (R1, 5′-tac gca tcc cag ttt gag acg ctg ctg ctg ctg ctg) and 0.8 μmol/L of the "common" flag primer" (R2, 5′-tac gca tcc cag ttt gag acg).

PCR cycling parameters were as follows. SCA2: initial denaturation of 7 minutes at 95°C, 14 cycles consisting of 30 seconds at 95°C, 30 seconds at 63°C −0.5°C/cycle, 1 minute at 72°C, 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 56°C, 1 minute at 72°C + 10 seconds/cycle, and 10 minutes of final extension at 72°C; SCA7: initial denaturation of 7 minutes at 95°C, 14 cycles consisting of 1 minute at 95°C, 1 minute at 63°C −0.5°C/cycle, 1 minute at 72°C, 35 cycles consisting of 1 minute at 95°C, 1 minute at 56°C, 1 minute at 72°C + 20 seconds/cycle, and 10 minutes of final extension at 72°C.

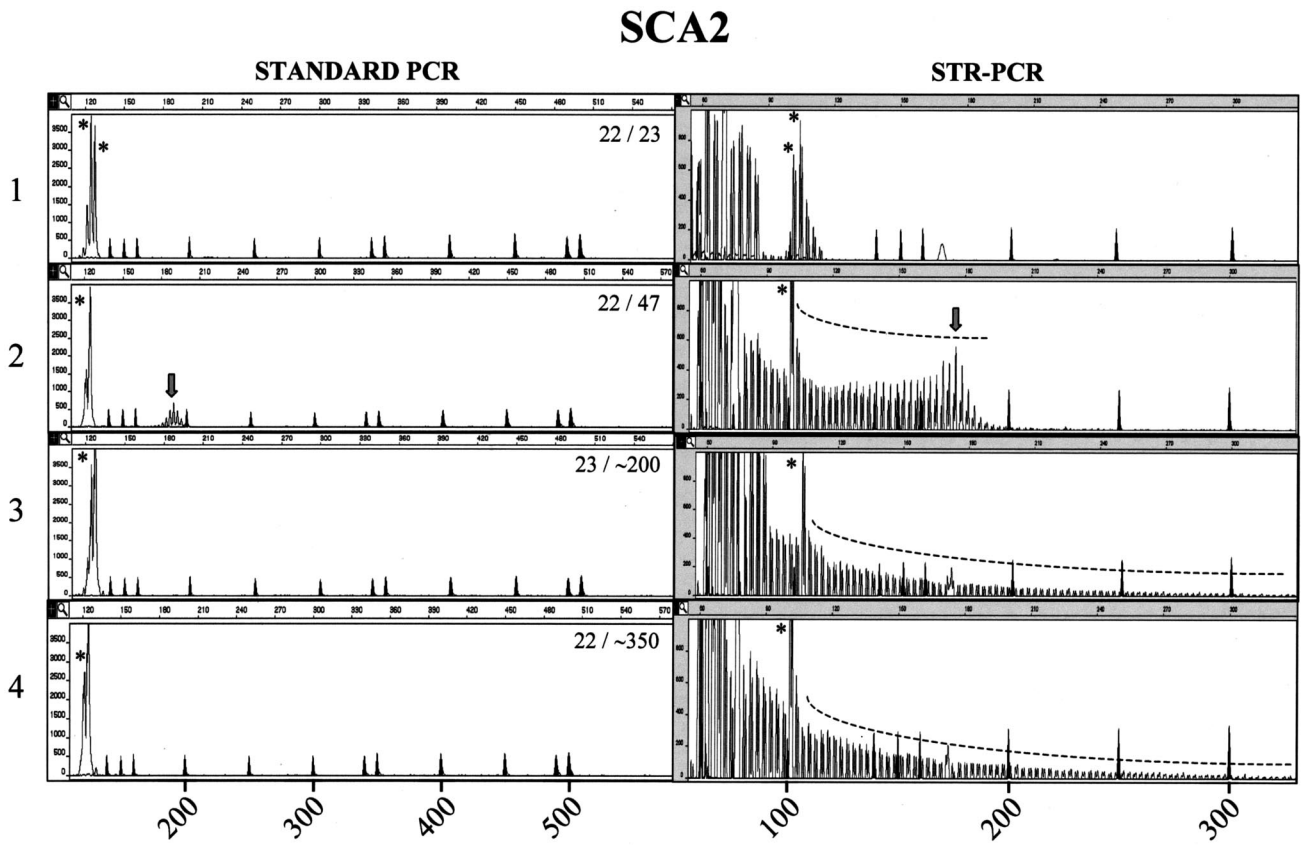

Figure 1. Comparison between standard and STR-primed PCR analysis in the *SCA2* locus. Left and right panels represent the analysis of *SCA2* CAG repeat in the same subject using standard PCR and STR-primed PCR, respectively. The STR-primed PCR peak profile of a healthy control with two alleles in the normal size range (**panel 1**) is distinct from the profiles found in a subject carrying a pathogenic allele (**panel 2**). In the case of an extreme expansion, standard PCR is unable to detect the pathogenic allele, whereas the STR-primed PCR profile clearly shows a gradually descending array of peaks, revealing the presence of a large CAG tract (**panels 3** and **4**). The genotype of *SCA2* CAG triplets is shown in the left panels. Marker peaks are shadowed. On the left panels, their sizes are: 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp. Marker sizes on the right panels are: 75, 100, 139, 150, 160, 200, 250, and 300 bp. Size in base pairs is also reported on the abscissa, whereas the ordinate shows fluorescence intensity as arbitrary unit. An **asterisk** indicates the peaks corresponding to normal alleles. The **arrow** points to the central peak of small expansions. *SCA2* profiles of normal alleles typically contained one or two major peaks of 101/104 bp corresponding to the most frequent 22- and 23-CAG alleles.

Analysis of Fluorescent PCR Fragments and Interpretation of Data

Analysis of the fluorescent PCR products was performed using an ABI-Prism 3100 Avant automatic sequencer on a 36-cm capillary array with the POP4 polymer and a ROX-GS500 internal standard marker (Applied Biosystems). Data were examined using the Genescan 3.1 software. Three independent examiners blindly evaluated STR-primed PCR profiles.

Results and Discussion

Fluorescent STR-primed PCR is a robust technique used to detect large-repeat expansions that cannot be amplified using standard PCR.[14,15,18,19] Here, we present an application of this technique to *SCA2* and *SCA7* genes. Their pathogenic CAG expansions are mostly within the PCR detection range, but large expansions have been described in both genes and need Southern-based approaches to be detected.[1]

Fluorescent STR-primed PCR showed a short series of discrete peaks with 3-bp periodicity when subjects within the normal range were tested; a few peaks, probably generated by the alternative annealing sites of the reverse primer, always preceded the main allele (Figures 1 and 2, panel 1). To evaluate the consistency of STR-primed PCR profiles on expanded alleles, we tested 12 SCA2 and 20 SCA7 patients within the most common mutation ranges of 37 to 53 and 36 to 54 CAG repeats, respectively (Tables 1 and 2). Both *SCA2* and *SCA7* showed a proportionally larger array of peaks compared with controls. For the smallest expansions, the profile assumed a bell shape on the right end of the panel (Figures 1, panel 2, and 2, panel 2), with the highest peak in the bell-shaped array corresponding to the size of the larger allele with a maximum error of ± 2 repeats (Figures 1, panel 2 [right], and 2, panel 2 [right]). Large expansions in *SCA2* (two patients; ~200 and ~350 CAG repeats) and *SCA7* (eight patients; range, 58 to 306 CAG repeats) yielded clearly distinguishable profiles, with multiple peaks of progressively lower intensity down to a fluorescence close to zero (Figures 1, panel 3, and 2, panels 3–6). It is notable that *SCA7* expansions above 90 repeats were not constantly amplified in our laboratories and that *SCA2* mutations >200 CAG repeats and SCA7

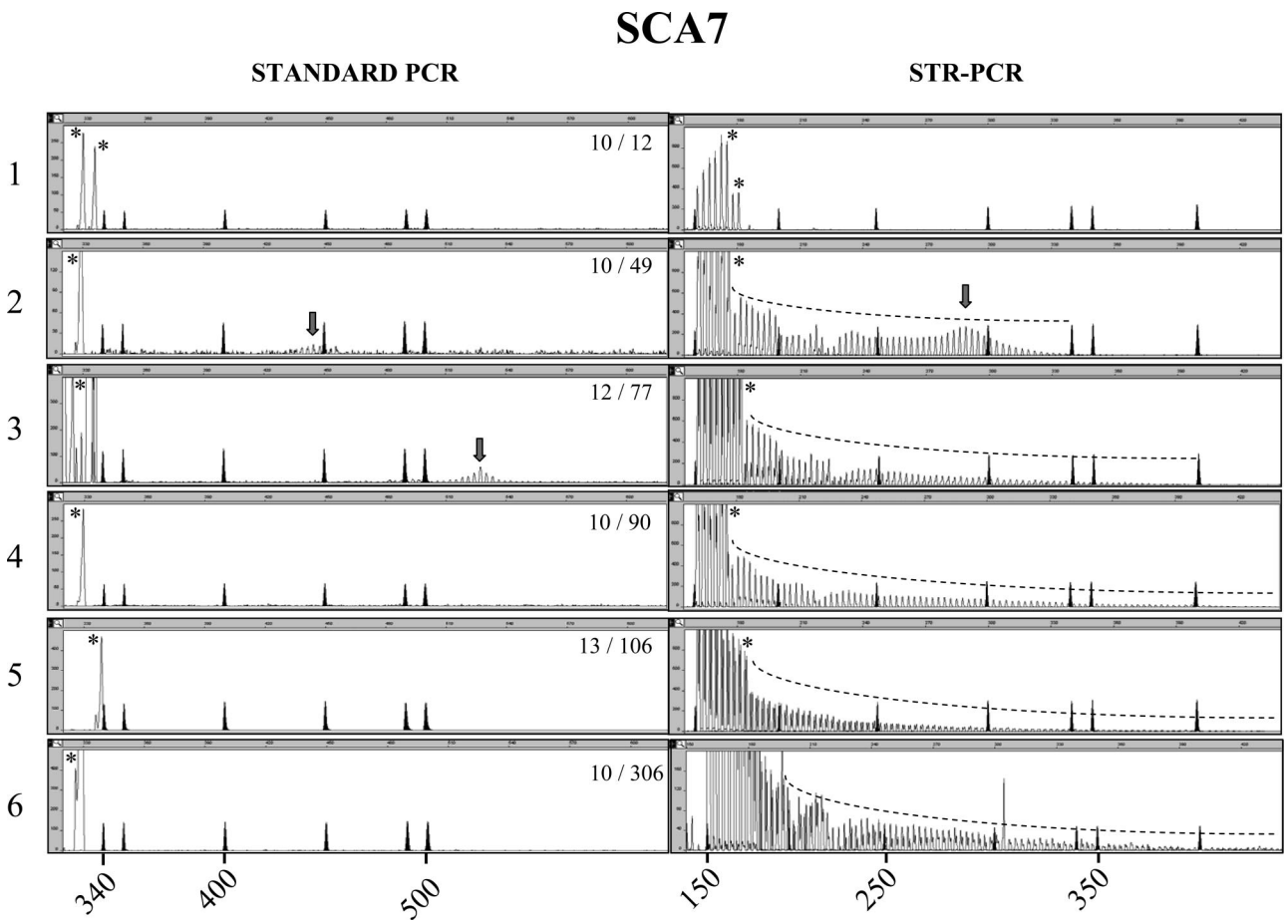

Figure 2. Comparison between standard PCR and STR-primed PCR analysis in the *SCA7* locus. Left and right panels represent the analysis of *SCA7* CAG repeat in the same subject using standard PCR and STR-primed PCR, respectively. The genotype of the *SCA7* CAG triplets is shown in the left panels. The STR-primed PCR peak profile of a healthy control with two alleles in the normal size range (**panel 1**) is distinct from the profiles in a subject carrying pathogenic 49 and 77 CAG repeats (**panels 2** and **3**). In patients carrying >80 CAG repeats, standard PCR often failed to amplify the repeat, whereas STR-primed PCR always detected the pathogenic allele (**panels 4–6**). Marker peaks are shadowed. Their sizes on the left panels are 340, 350, 400, 450, 490, and 500 bp. Marker sizes on the right panels are 160, 200, 250, 300, 340, 350, and 400 bp. Size in base pairs is also indicated on the abscissa, whereas ordinate shows fluorescence intensity in arbitrary units. An **asterisk** indicates the peaks corresponding to the normal alleles. The **arrow** points to the central peak of visible expansions.

mutations >106 were never detected by standard PCR (Figures 1, panels 2 and 3 [left], and 2, panels 4–6 [left]).

These results suggest that fluorescent STR-PCR is reliable for large CAG expansions in the *SCA2* and *SCA7* genes, although their detection is proved only up to 350 triplets in *SCA2* and up to 306 triplets in *SCA7*. However, it must be considered that this technique was used to detect CAG expansions up to 5 kb in the myotonic dystrophy type 1 gene,[14] >1400 GAA in the *FRDA1* gene, and >3500 ATTCT in the *SCA10* gene.[15] Also, considering its rationale, which does not rely on the amplification of the repeat region, we hypothesize that the upper detection limit is virtually absent.

The golden choice for large-repeat expansion detection has been the genomic- or PCR-Southern blot. We believe that STR-PCR has several advantages on these techniques: 1) it uses a small amount of DNA versus standard Southern blot (100 ng versus 10 μg); 2) it is easy to set up, quick to complete (1 to 2 versus 7 to 15 days), and does not need the use of radiochemicals; 3) it is easier to use on screening; 4) in our experience, PCR-Southern blot can give false-positive results on DNA samples extracted from chorionic villi, because of minor maternal contamination and its high sensitivity. On the other hand, STR-PCR requires automated genotyping equipment, which is not available in every laboratory.

A misinterpretation of the expanded profile is possible because of technical reasons: 1) the presence of a very low fluorescent signal (<100 fluorescence units) that, however, must induce to repeat the test trying different DNA dilutions; 2) the presence of a possible somatic mosaicism in which two cell populations co-exist, one with two normal alleles and one heterozygous for an expansion; this event is, however, probably extremely rare.

The lower detection limit of fluorescent STR-PCR was estimated in controls and patients with small CAG expansions. The profiles of normal SCA2 subjects with 32 to 34 CAG repeats could not be precisely distinguished from those of affected patients with small expansions of 37 to 41 repeats (data not shown). In SCA7, a control subject carrying 34 CAG repeats was not distinguished from patients with 36 to 39 repeats (data not shown). A gray zone in which subjects could not clearly be classified as

carriers of a mutation exists approximately between 34 and 41 repeats. However, above this range, patients were clearly assigned to the mutated range.

Further data may prove that STR-primed PCR can be routinely used to detect expansions. At present, we propose that routine PCR should precede STR-PCR, which can be applied in case of apparent homozygosity. At this regard, it is noteworthy that, in our experience, common expansions >50 repeats might not be detected by standard PCR. Technical artifacts might affect the results obtained by standard PCR (eg, the phenol used in DNA extractions), disfavoring the amplification of pathological alleles. In such cases, a normal homozygous genotype can be wrongly attributed (Figure 2, panel 2). This was not the case for either gene using the fluorescent STR-PCR technique, providing an alternative method, or a second confirmatory technique and is particularly important in SCA patients with infantile or juvenile onset and an apparently homozygous normal allele when tested with standard techniques.

We also suggest using fluorescent STR-PCR in case a single normal allele is detected in prenatal diagnosis of affected or pre-symptomatic at-risk parents, because in such a case, a very large expansion inherited from one of the parents can be missed in the fetus by standard PCR. Finally, it is also possible to apply our test to routine screening for infantile- or juvenile-onset diseases that suggest the involvement of large expansions in these genes.

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