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Technical Advance

Design and Validation of a Conformation Sensitive Capillary Electrophoresis-Based Mutation Scanning System and Automated Data Analysis of the More than 15 kbp-Spanning Coding Sequence of the *SACS* Gene

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In this study, we developed and analytically validated a fully automated, robust confirmation sensitive capillary electrophoresis (CSCE) method to perform mutation scanning of the large *SACS* gene. This method facilitates a rapid and cost-effective molecular diagnosis of autosomal recessive spastic ataxia of Charlevoix-Saguenay. Critical issues addressed during the development of the CSCE system included the position of a DNA variant relative to the primers and the CG-content of the amplicons. The validation was performed in two phases; a retrospective analysis of 32 samples containing 41 different known DNA variants and a prospective analysis of 20 samples of patients clinically suspected of having autosomal recessive spastic ataxia of Charlevoix-Saguenay. These 20 samples appeared to contain 73 DNA variants. In total, in 32 out of the 45 amplicons, a DNA variant was present, which allowed verification of the detection capacity during the validation process. After optimization of the original design, the overall analytical sensitivity of CSCE for the *SACS* gene was 100%, and the analytical specificity of CSCE was 99.8%. In conclusion, CSCE is a robust technique with a high analytical sensitivity and specificity, and it can readily be used for mutation scanning of the large *SACS* gene. Furthermore this technique is less time-consuming and less expensive, as compared with standard automated sequencing. ***(J Mol Diagn 2009, 11:514–523; DOI: 10.2353/jmoldx.2009.090059)***

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) belongs to a heterogeneous group of neurodegenerative disorders characterized by ataxia, mostly due to progressive degeneration of the cerebellum and associated structures. The clinical phenotype of these disorders is broad and quite variable. Clinically it can sometimes be hard to distinguish the different types of cerebellar ataxia. Molecular diagnosis can be extremely helpful to differentiate between the different forms of ataxia.

Using DNA analysis to confirm the clinical diagnosis of ARSACS (MIM 270550) has been hampered in the majority of patients worldwide due to the lack of an efficient and cost-effective mutation scanning system for the *SACS* gene. The large size of the total coding sequence of this gene has precluded sequencing of the total coding sequence of the *SACS* gene as a routine diagnostic service. Since automated sequencing of this large gene is labor-intensive, costly, and time-consuming, we developed mutation screening by designing a heteroduplex analysis system using conformation-sensitive capillary electrophoresis (CSCE), which is a simple, rapid, reliable, and sensitive technique enabling a high sample throughput[1] and automated DNA variant identification. We have chosen this method because it can be used for the analysis

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of larger fragment sizes (up to 500 bp), it has a higher sensitivity, and it can easily be implemented in existing automated sequencer infrastructure. DNA analysis therefore becomes more reliable, more robust, less expensive, and has a higher throughput, in comparison with established techniques such as single-strand confirmation polymorphism, denaturing gradient gel electrophoresis, and denaturing high performance liquid chromatography.[2]

The *SACS* gene involved in ARSACS is located on chromosome 13q12.12 and encodes the large protein sacsin.[3] The gene consists of at least 11 exons, of which 9 contain coding sequences, spanning 13,737 bp and 4579 amino acids (NM_014363.4). To date, apart from the two mutations originally identified in the ARSACS patients from Québec, 30 different additional mutations, including nonsense, missense, small deletions and insertions, have been identified in ARSACS patients from different countries. These mutations occur throughout the entire gene.

Not all types of mutations will be detected by sequencing or alternative subtle mutation scanning techniques such as CSCE. For instance, recently a Belgian ARSACS patient has been described with a 1.5-Mb deletion on chromosome 13q12.12 encompassing the *SACS* gene and a missense mutation on the other allele.[4] This underlines that additional mutation detection techniques will still be needed.

Here, we report the development of a fully automated, robust CSCE method to perform mutation scanning of the large *SACS* gene, facilitating a rapid and cost-effective molecular diagnosis of ARSACS patients. Since the cohort of Dutch ARSACS patients had been thoroughly analyzed for *SACS* mutations by automated direct sequencing, a good starting point for the analytical validation of the CSCE mutation scanning including automated heteroduplex pattern analysis had already been created. A technical validation of the CSCE technique in general has been performed as an interlaboratory collaborative project previously[5]; details will be published elsewhere. The analytical validation of CSCE specifically for the *SACS* gene presented here has been performed in two phases: a retrospective analysis of samples containing known DNA variants in the *SACS* gene and a prospective analysis of samples of patients for whom *SACS* mutation analysis was requested by a physician. Issues addressed during the development of a CSCE system for mutation scanning are discussed and illustrated with examples. These include the position of a DNA variant relative to the primers, and the CG content of the amplicons. Finally an overview of all novel SACS mutations and single nucleotide polymorphisms (SNPs) identified in a cohort of 76 patients for whom *SACS* mutation analysis was requested is presented (Tables 1[5] and 2).

Materials and Methods

Patients

Peripheral blood was collected using EDTA as an anticoagulant from index patients with recessive cerebellar ataxia. Automated genomic DNA extraction was performed using a Tecan Freedom EVO 150 workstation combined with a Chemagic Magnetic Separation Module 1 from Chemagen. The concentration of DNA samples were standardized at 100 ng/μl. For the validation, in total 52 DNA samples of patients were used who either have ARSACS confirmed at the DNA level or known variants in the *SACS* gene (retrospective phase), or who were suspected of having ARSACS (prospective phase). The remaining 24 patients for whom mutation data were available have not been included in the validation study to avoid redundancy, ie, no additional fragments would have been validated by their inclusion. To facilitate the detection of homozygous mutations, before CSCE, the PCR product of a patient was mixed with the PCR product of a mutation-negative reference sample to allow the formation of heteroduplex bands. For this purpose, we used two different reference samples (V3 and V4). These samples were sequenced in advance to identify the DNA variants present in the different amplicons of the *SACS* gene. Informed consent was obtained from all patients included in this study.

PCR Amplification

We designed 45 overlapping PCR primer pairs for the nine coding exons (Table 3), including the gigantic exon 9 described previously[3] and the flanking intronic sequences of the *SACS* gene (GenBank reference sequence accession number NM_014363.4, NCBI). All forward primers (FPs) and reverse primers (RPs) contain next to the specific primer an universal primer tag (M13), (FP) 5′-TGTAAAACGACGGCCAGT-3′ and (RP) 5′-CAGGAAACAGCTATGACC-3′. The primer sequences, positions and size of each amplicon are listed in Table 3. The DNA-amplification mix included (per amplicon per sample) 1.0 μl genomic DNA, 0.36 μl (8.33 mmol/L) dNTPs, 1.44 μl $MgCl_2$ (50 mmol/L, AB), 1.80 μl 10× PCR Gold Buffer (AB), 0.14 TaqDNA polymerase Gold (5 U/μl), 6.26 μl distilled H_2O (mQ), and 7 μl FP and RP (10 pmol/μl) from a mix of 0.16 μl FP and RP per 1 μl mQ. The reaction volume per sample was 18 μl. The samples were PCR amplified using the following PCR program on a Perkin-Elmer (ABI) Geneamp 9700: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 1 minute, with a final step at 72°C for 7 minutes, after which the samples are cooled down to 10°C. Before CSCE, 5 μl of each PCR-product with 7 μl Loading Orange G was run on a 1% agarose gel with a 100-bp marker for comparison. Next, all PCR-products (13 μl) were purified using a Multiscreen $PCR_{\mu 96}$ filter plate (Millipore, Tullagreen, Carrigtwohill, County Cork, UK).

For fragment 2 (exon 2) a different PCR program and amplification mix is used; 0.5 μl (8.33 mmol/L) dNTPs, 3.0 μl $MgCl_2$, 3.0 μl PCR Gold Buffer, 7.5 μl Betaine, 0.2 μl Tag DNA polymerase Gold, 13.8 mQ, 1 μl FP and RP (10 pmol/μl) with 1.0 μl genomic DNA in a total reaction volume of 30 μl per sample. The PCR program is as follows: initial denaturation at 94°C for 5 minutes,

Table 1. Known and Novel Mutations Detected by CSCE and Sequencing

Exon	Amplicon	Variant	Predicted protein change	Predicted consequence	Mutation type	Position	Number of occurrences
6	6	c. 502G>T	p. Asp168Tyr	Amino acid substitution	Missense	174/390	2
7	8	c. 961C>T	p.Arg321X	Premature termination codon	Nonsense	226/466	2
	9//10	c. 1475G>A	p.Trp492X	Premature termination codon	Nonsense	448/484//84/449	1
8	12	c. 2094-2A>G		Aberrant splicing	Splice site	92/2 99	1
		c. 2182C>T	p.Arg728X	Premature termination codon	Nonsense	182/299	2
		c. 2185+1del		Aberrant splicing	Splice site	186/299	2
9	18*	c. 4108C>T	p.Gln1370X	Premature termination codon	Nonsense	365/464	
	21	c. 4957G>T	p.Glu1653X	Premature termination codon	Nonsense	100/484	2
		c. 5125C>T	p.Gln1709X	Premature termination codon	Nonsense	268/484	1
		c. 5143A>T	p.Lys1715X	Premature termination codon	Nonsense	286/484	1
	23/24	c. 6000_6004del	p.Arg2002fs	Premature termination codon	Deletion	468–472/492//100–104/466	1
	26*	c. 6835dupA	p.Glu2280fs	Premature termination codon	Insertion	241–242/463	
	27*	c. 7250_7254del	p.Thr2417fs	Premature termination codon	Deletion	318–322/486	
	30	c. 8401-8403del	p.Gln2801del	Single amino acid deletion	In frame deletion	319–321/489	2
	34/35	c. 9911_9912del	p.Leu3304fs	Premature termination codon	Deletion	413–414/481//49–50/474	1
	36	c. 10442T>C	p.Leu3481Pro	Amino acid substitution	Missense	260/496	1
	37	c. 10906C>T	p.Arg3636X	Premature termination codon	Nonsense	393/464	1
	41	c. 12160C>T	p.Gln4054X	Premature termination codon	Nonsense	195/485	11
	43	c. 12992G>A	p.Arg4331Gln	Amino acid substitution	Missense	351/486	1

*Known Italian mutations (Grieco et al[5]).

then 38 cycles of denaturation at 98°C for 5 seconds, annealing at 68°C for 30 seconds, and elongation at 72°C for 50 seconds, with the final step at 72°C for 7 minutes, after which the samples are cooled down to 10°C.

CSCE

During CSCE, the target gene is PCR-amplified and PCR products are denatured and re-annealed slowly, so that four different double stranded (ds) DNA molecules, ie, two homoduplexes and two heteroduplexes, can be formed if a heterozygous mutation is present. To identify conformational changes resulting in a mobility shift, the re-annealed PCR products can be analyzed using any capillary DNA sequencer, and by using non-denaturing conditions, ie, an ABI 3730 sequencer with conformation analysis polymer-containing capillaries. The re-annealed PCR products can be detected by using a four-dye filter set, and fluorescently labeled primers. The presence of many SNPs in the gene of interest will lead to the identification of these as DNA variants in several samples, depending on their allele frequency. These DNA variants will all have to be subsequently analyzed by automated sequencing. CSCE therefore is most suited for genes containing just a limited number of (common) SNPs. The peak patterns are analyzed automatically by specially designed software (BioNumerics, Applied Maths, St-Martens-Latem, Belgium).

CSCE Conditions

Before CSCE, 5 μl purified PCR-product was mixed with 2 μl PCR-product of a mutation-negative reference sample (V3). For the amplicons 7 and 10 of the *SACS* gene a different reference sample (V4) was used since the V3 sample was heterozygous for common SNPs in these amplicons. The labeling-PCR mix contained 0.2 μl 6-carboxyfluorescein-labeled FP, 0.2 μl reverse M13 primer, 0.5 μl dNTPs (8.33 mmol/L), 1.5 μl $MgCl_2$ (50 mmol/L, AB), 2.5 μl 10× PCR Gold Buffer (Applied Biosystems, Inc, Foster City, CA), 0.2 μl TaqDNA polymerase Gold (5 U/μl), and 17.9 μl mQ. Of this mix 18 μl was used per sample for the labeling-PCR. The following PCR program was used for the labeling-PCR: an initial denaturation at 94°C for 5 minutes, followed by 10 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation for 1 minute at 72°C, with a final

Table 2. Polymorphisms Detected by CSCE and Sequencing

Exon	Amplicon	Variant	Predicted protein change	Predicted consequence	Mutation type	Position	Number of occurrences
1	1	c.1-219 A>G				145/585	10
		c.1-13A>G				351/585	18
2	2	c.171+6C>T					3
		c.171+24G>A					1
3	3	c.172-129A>G				1/343	1
		c.259+28A>G				245/343	1
5	5	c.346-143G>A				79/391	4
7	7	c.696T>A	p.Asn23 2Lys	Amino acid substitution	Missense	189/327	13
	8	c.909A>G	p.Ala3 03Ala	Amino acid substitution	Missense	174/466	9
		c.973G>A	p.Gly325Arg	Amino acid substitution	Missense	238/466	1
	10	c.1656A>G	p.Leu552Leu	Amino acid substitution	Missense	265/449	14
	11	c.1846G>C	p.Ala6 16Pro	Amino acid substitution	Missense	385/488	1
		c.2080G>A	p.Ala6 94Thr	Amino acid substitution	Missense	151/488	7
9	13/14	c.2472G>A	p.Ser 824Ser	Amino acid substitution	Missense	374/459//76/475	1
	15	c.2983G>T	p.Val995Phe	Amino acid substitution	Missense	252/494	1
		c.2988A>G	p.Leu996Leu	No change	Missense	257/494	2
	19	c.4188C>T	p.His1396His	No change	Missense	86/498	2
		c.4466A>G	p.Asn14 89Ser	Amino acid substitution	Missense	364/498	3
	24	c.6195T>C	p.Ile 2065Ile	No change	Missense	294/466	40
		c.6267G>A	p.Ser 2089Ser	No change	Missense	366/466	2
	26	c.6781C>A	p.Leu2261Ile	Amino acid substitution	Missense	187/463	1
		c.6946A>G*	p.Thr 2316Ala	Amino acid substitution	Missense		1
	27	c.7140T>A	p.Asn24 80Lys	Amino acid substitution	Missense	208/486	1
		c.7149C>T	p.Arg2383Arg	No change	Missense	217/486	1
	28	c.7539C>T	p.Val2513Val	No change	Missense	243/498	1
	31/32	c.8853T>C	p.Val2951Val	No change	Missense	404/491//52/494	44
	34	c.9801A>G*	p.Thr 3267Thr	No change	Missense		1
		c.9846A>G	p.Pro3282Pro	No change	Missense	348/481	1
	35	c.9981T>C	p.Ala3327Ala	No change	Missense	119/473	87
		c.10106T>C	p.Val3369Ala	Amino acid substitution	Missense	244/473	43
	36	c.10338G>A	p.Gln344 6Gln	No change	Missense	156/494	4
	38	c.11032C>G	p.Pro36 78Ala	Amino acid substitution	Missense	153/499	5
	41	c.12304T>C	p.Leu4102Leu	No change	Missense	339/485	27
	43	c.12813T>C	p.Pro42 71Pro	No change	Missense	121/486	1
	45	c.13522A>C	p.Lys4508Gln	Amino acid substitution	Missense	183/483	1
		c.13717A>C	p.Asn45 73His	Amino acid substitution	Missense	377/483	1

*SNP not validated.

step at 72°C for 7 minutes after which the samples are cooled down to 10°C. After the labeling PCR, the products (25 μl) are again purified as described before. Per sample 1 μl pooled PCR product, was mixed with 9 μl standard LIZ-500 (Applied Biosystems)/mQ-mix (in proportion 0.2 μl Liz to 8.8 μl mQ), before running the actual CSCE. The analysis was performed using an ABI 3730 automated sequencer (Applied Biosystems) using capillaries containing conformation analysis polymer. Results were analyzed using BioNumerics 5.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) software. We choose to run four patient samples in a single CSCE run.

Automated CSCE Pattern Analysis

To automatically analyze the CSCE electropherograms of the different amplicons of the *SACS* gene by the data analysis software program Bionumerics an optimized set of parameters (quantified characteristics of the shapes of the electropherogram) has been used. The parameters include (1) the relative position of the peak within the electropherogram, and (2) the signal-width as a percentage of the run length, and (3) the left and right control range, ie, the area flanking the main peak signal in which the algorithm will search for additional peaks. To further characterize the shape of the peaks (curves), the algorithm calculates five different pattern-matching parameters, ie, SRMS, MAX, SECPK, DFH3, and DFH4. SRMS stands for single-sided root mean square value, and is calculated from the highest 50% of square differences between the curves compared. MAX determines the highest difference between any corresponding values on the two curves. SECPK calculates the proportion of a secondary peak relative to the primary peak of the curve. DFH3 is a parameter for the difference in H3 parameters between the signal and the reference (H3 is a measure of the deviation from symmetry, compared with a Gaussian curve derived from reference samples without DNA variants), whereas DFH4 determines the deviation from a theoretical Gaussian curve. The parameters SRMS and MAX take into account any difference between the curves, the parameters SECPK, DFH3, and DFH4 each apply to a specific component of the curve. For a correct match interpretation a peak height has to be above 1000 and below 32000 relative fluorescence units. Peak heights outside this range will be regarded as failures by the BioNumerics program.

Table 3. PCR Primers and Amplicon Size

Exon	Amplicon	Primer sequence		Amplicon size (bp)	GC %
1	1	F: 5′-ACGTTTTCCCCAAGAACCTT-3′	R: 5′-TTTAACCCTTGGCTTGAAAAA-3′	585	38
2*	2	F: 5′-GGCTTCCTCTAGCGTTTCCTCCT-3′	R: 5′-ACGGAAAAGGCAAGTGATGA-3′	577	74
3	3	F: 5′-TCCGTGGAATATTCACTTCTCC-3′	R: 5′-AGGGCGAGACTCAGTTTCAA-3′	343	33
4	4	F: 5′-TTGAATCTGCTTTACTTTCTGCTG-3′	R: 5′-GCCCCAGAAAACCTCACATA-3′	475	36
5	5	F: 5′-TGTTGCAAATAGTGGGTTTCC-3′	R: 5′-CCCACCAAAAGCAGAGAAAA-3′	391	30
6	6	F: 5′-TTCAGTCACTCTATTCACTCCTCTC-3′	R: 5′-GAATTTGGGTAAGATGGCTTTT-3′	391	39
7	7	F: 5′-TTTAATGGCTCATGCTTTTTCA-3′	R: 5′-TTGTAGGCGAAGAGGGAAAC-3′	328	36
	8	F: 5′-CAGTTTGCACCATTTGTTGG-3′	R: 5′-GCCCACCCACACTGTTACAC-3′	466	41
	9	F: 5′-ATGAGCGGCCGAATTCTAT-3′	R: 5′-GCTTTGGGGACAACATTCAT-3′	484	44
	10	F: 5′-CAGTGGGTTCTTTGGCCTTA-3′	R: 5′-CTGAACAGCAGCATCCACAT-3′	449	47
	11	F: 5′-AGCTGTGACTGGGTCAGGTT-3′	R: 5′-CCATTGAATTTCACAACAAAGG-3′	488	50
8	12	F: 5′-TCGGCTTAACTGACTTGAAAA-3′	R: 5′-GCTTGAGCCATAAGAAATTGTTG-3′	299	32
9	13	F: 5′-CCTTCCAGTACTGTGTTATTTGTGA-3′	R: 5′-AAGGACAAACCCTCCAAGTTT-3′	459	37
	14	F: 5′-GATGCCACTTATCCCCAGAA-3′	R: 5′-TGGGAGTTTGGCAGTATGGT-3′	475	37
	15	F: 5′-CCGATAGCAGTGAGAAAGAGAAA-3′	R: 5′-GGGTGGGAAATAGGTTCCTT-3′	494	36
	16	F: 5′-TCCAAATGTGCTTGAGTGGT-3′	R: 5′-ATTGCATGGCCTACATCACA-3′	498	40
	17	F: 5′-GAAAGGCCTCCAAATTATCCA-3′	R: 5′-CTGGGCAAGTGGACAAAACT-3′	384	39
	18	F: 5′-CCAGCATATTTTGCTTGAGATTT-3′	R: 5′-TAATGTCACAATAACAGCATTC-3′	465	37
	19	F: 5′-CAGTGAACAAGAAAGCAAACAAA-3′	R: 5′-TCCCTGGGTCTAGGAGATTC-3′	498	38
	20	F: 5′-TTCTGGAAGAATACCCTTCAGTG-3′	R: 5′-CTGTTGAGTTCTAAAGGACAGTCG-3′	500	36
	21	F: 5′-CCATTTATAGATGTATTTGGCTGTCA-3′	R: 5′-GTGGCGACTGTAAATCAGCA-3′	484	38
	22	F: 5′-AGGCTGCTAAGCTCATGAAGA-3′	R: 5′-TAACAGCAAAGCACCCATTG-3′	477	45
	23	F: 5′-CATGTGGGGCAGTAGGAGTT-3′	R: 5′-AGGCTGCTGAACCAACATCT-3′	492	41
	24	F: 5′-GCTCATGGAAAAGGGAAAGA-3′	R: 5′-ACTTTGCAACTCGTCCTTCG-3′	466	38
	25	F: 5′-GGAGTTCTTCGTGTTACTCC-3′	R: 5′-GTCAGTTGCTGCAAACATGG-3′	466	39
	26	F: 5′-AAGGGATCCTAGAGCAAAGGA-3′	R: 5′-TGCATTCTCAACTAGAATGAAGC-3′	463	37
	27	F: 5′-CCAGGAGAATATCACCAATGC-3′	R: 5′-CAAGGGCAATCATTGTAGCA-3′	486	35
	28	F: 5′-TGGAGTCTCATTAGAGAAAAGAAAC-3′	R: 5′-TTCTGTAAATGGCTGGTTGTTG-3′	498	39
	29	F: 5′-TTCTGTAAATGGCTGGTTGTTGA-3′	R: 5′-GCGCAGTTTGTCCAAAAGAT-3′	496	42
	30	F: 5′-TGCACAATGTTCAGATTTCCTC-3′	R: 5′-AATGCAGGCAGCTACTCCAC-3′	489	38
	31	F: 5′-CGTGGCTAATTTGTAATAGATCAGG-3′	R: 5′-TGCTTTCACTAGACAATATAAATCTGG-3′	491	40
	32	F: 5′-CCCTGGTTCTGATCCAACAT-3′	R: 5′-TGATATCAGCAGGGGTCACA-3′	494	36
	33	F: 5′-CACGCAAAACAGTAGCAGAGA-3′	R: 5′-TGGAAATGTCAAACACTTTTGC-3′	498	34
	34	F: 5′-GATGCAAAACGACCCAAGTT-3′	R: 5′-GCTTTCATTAGAGCATGAAAAAC-3′	481	36
	35	F: 5′-CTGTTTCAGCCAACCAGCTT-3′	R: 5′-TGTCCATTTCTCCACTTCAGC-3′	473	39
	36	F: 5′-TTGAATCATTTGATGTCCCAAG-3′	R: 5′-GGAATAAACAATTTTTCAGGAAGC-3′	494	34
	37	F: 5′-CAAGTGCTGAGGAATTATCAGAGA-3′	R: 5′-GGCCCGCTCAGGACATAA-3′	464	33
	38	F: 5′-TCCTTCTGCATCATATATTCCAA-3′	R: 5′-CCTCGCAACTGAAAACGAA-3′	499	38
	39	F: 5′-ATGCAACATAACGACGTTGG-3′	R: 5′-AAGCGCAAGGTCTCGTACAT-3′	457	38
	40	F: 5′-TGAGGGCAAACAATTAGATCC-3′	R: 5′-TGGCCAGAAAAGCATTATCA-3′	497	40
	41	F: 5′-TTTGTCAGTTTGGAGCGTTG-3′	R: 5′-TTTTGATGGCTCCGAAGAGT-3′	485	36
	42	F: 5′-GGCAATGACTCTTAAATCAGCA-3′	R: 5′-GGCTGGTTGGTGTAGAAGGA-3′	485	38
	43	F: 5′-TGCTGACAATTCTAGTTTTCTAGG-3′	R: 5′-GAGGCCCTGTCTGCATTTT-3′	486	40
	44	F: 5′-TGAAATGGCATCCTGACAAA-3′	R: 5′-TCAGACTTTCCCCTCACAGC-3′	490	42
	45	F: 5′-CACGCAGATGGCTAAGACAA-3′	R: 5′-TGGCAATGAAGCTTAATGAAGTA-3′	483	39

*Amplicon that has not been validated.

Sequence Analysis

In this validation study, all samples have been analyzed in parallel by CSCE and by automated sequencing. Sequence reactions were run on an ABI 3730 automated sequencer and analyzed with a combined software program: Phred, Phrap, and Polyphred (University of Washington). In affected siblings or relatives of patients with one homozygous or two compound heterozygous mutations in the *SACS* gene, the presence of the mutations was identified or excluded using direct sequencing. Due to the occurrence of two relatively frequent polymorphisms in amplicon 35, which are heterozygous in both reference samples V3 and V4, direct sequencing is preferred over CSCE for this amplicon, since aberrant heteroduplex patterns will occur in the majority of cases. As the PCR-mix and program conditions for exon 2 of the *SACS* gene differ substantially from standard conditions, CSCE conditions for this specific exon have not been developed.

Results

Design

Primers were designed (a) to allow for an identical annealing temperature for all 45 amplicons, except for amplicon 2 (exon 2) for which a different annealing temperature is used because of the high CG-content of this fragment, (b) to obtain amplicon sizes ranging from 299 to 585 bp in length, (c) to allow for a distance of at least 30 bp between the 5′-end of a particular primer and the nearest exon-intron boundary within the amplicon, and

(d) to have at least 100 bp overlapping sequence for neighboring amplicons of the same exon. An overlap will ensure the identification of a heterozygous deletion encompassing a single or multiple amplicons that could otherwise escape detection. Furthermore a minimum of 30 bp between the beginning of a primer and an exon-intron boundary are present in each amplicon, where applicable. To enable a rapid confirmation by automated sequencing of sequence variants identified by CSCE, the amplicons to be analyzed by CSCE were identical to the amplicons to be analyzed by sequencing. This approach also reduces the consumable costs, since identical primer pairs for both CSCE as well as sequencing can be used. This method is feasible, since there is no need to split amplicons into smaller fragments to comply with the presence of multiple melting domains, like in denaturing gradient gel electrophoresis- or denaturing high performance liquid chromatography-based approaches.

To automatically analyze the CSCE electropherograms of the different amplicons of the *SACS* gene by the data analysis software program Bionumerics an optimized set of parameters (quantified characteristics of the shapes of the electropherogram) has been used that was developed during a separate technical validation of CSCE. Essentially, the position and shape of the peak of a normal (no DNA variant-containing) reference electropherogram is compared with the peak positions and shapes of the electropherogram to be analyzed. The parameters used are described in the *Materials and Methods.*

Validation

A generic technical validation of the experimental conditions of CSCE and the optimal general settings of the software program BioNumerics has been performed previously. In this collaborative study (Interlaboratory Diagnostic Validation of Confirmation Sensitive Capillary Electrophoresis, to be published in detail elsewhere) parameters including (1) CG-content of fragments, (2) position of the DNA-variant relative to the primers, (3) fragment length, and (4) temperature during electrophoresis have been analyzed. The main results are that (1) CG-content of 30% to 60% gives optimal results, whereas for amplicons with a CG-content up to 80% different conditions may be necessary (2), SNPs close to a primer may escape detection, an issue that can be circumvented by the design of the analytical strategy, eg, by taking sufficient overlap of amplicons into account, (3) length of amplicons up to 500 bp can still be analyzed reliably, and (4) cooling of the oven temperature below 15°C, which improves separation of heteroduplex bands.[6]

As a result of the design and analytical validation of the CSCE system for mutation scanning of the *SACS* gene presented in this paper, the five pattern matching parameters were set at specific lower and upper threshold values (Table 4). Patterns with parameter values above one or more of these thresholds are considered to be divergent (indicated by red) and patterns with one or more parameter values below a certain threshold are considered to be normal (indicated by green). Patterns with one or more parameter values between these two thresholds are slightly divergent (indicated by orange). These patterns always need to be visually inspected.

Table 4. Peak Matching Parameters

Parameter	Threshold			
	Green (II)	Green (I)	Orange	Red
SRMS	<3.50	<7.00	↔	>20.00
MAX	<3.50	<6.00	↔	>20.00
SECPK	<200.00	<200.00	↔	>400.00
DFH3	<1.00	<1.00	↔	>1.70
DFH4	<0.70	<0.70	↔	>1.20

The parameters and settings are described in the *Materials and Methods*. Peaks indicated in green should be considered as normal and do not require subsequent sequencing. Green II indicates the adapted parameters. Peaks indicated in orange need further visual inspection and peaks indicated in red always require sequencing.

For analytical validation of the use of CSCE to the detection of DNA variants specifically in the *SACS* gene in total 52 DNA samples containing a total of 51 different variants occurring in 32 different amplicons of the *SACS* gene have been analyzed. The PCR products ranged in length from 299 to 585 bp. The validation process consisted of a retrospective phase and a prospective phase. In the first phase, 32 DNA samples of index patients with known variants, previously identified by sequencing of the *SACS* gene were used retrospectively to determine the analytical sensitivity of the CSCE approach. In the second phase, to further assess the analytical sensitivity and to determine the analytical specificity, we performed CSCE and subsequent sequencing of the *SACS* gene prospectively in a cohort of 20 DNA samples from patients for whom mutation analysis of the *SACS* gene was requested for clinical purposes.

Phase One (Retrospective Analysis)

To test the analytical sensitivity of the CSCE 32, DNA samples containing 41 different known variants were retrospectively used. These 41 known variants occur in 25 different amplicons (4/29 amplicons are redundant due to overlaps with flanking amplicons) of the *SACS* gene. Of these 41 variants 24 are nonpathogenic SNPs (Table 2) and 17 of these are pathogenic mutations (Table 1). Fourteen out of these 17 have recently been described as a novel pathogenic mutation.[7] Two DNA samples, with three different known mutations in the *SACS* gene, were kindly made available by Dr. F.M. Santorelli from Italy.[5]

Samples carrying a homozygous variation were mixed with a mutation-negative reference sample. All 41 variations could be detected by CSCE, except for two SNPs in amplicons 3 and 11, respectively. The first SNP c.172-129A>G, which is located just one bp next to the forward primer of amplicon 3 (exon 3), displayed a completely normal pattern and could not be distinguished from the reference pattern (Figure 1). All pattern parameters had values below the initial threshold settings (Table 4). The pattern of the second SNP c.2080G>A at position 151 of the 488 bp long amplicon 11 (exon 7) did not show a clear heteroduplex band. The pattern differed just slightly

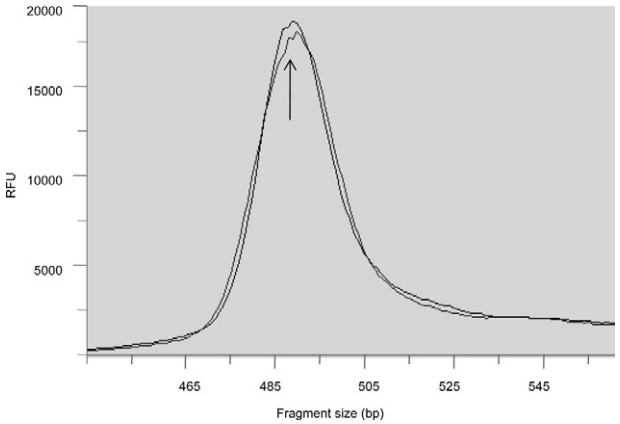

Figure 1. Example of initially false negative capillary heteroduplex electropherogram. This electropherogram shows the analysis of amplicon 11 containing the heterozygous SACS c.2080G>A variation, indicated by the **arrow**. The pattern of the sample containing the variation and the pattern of the normal genotype sample are virtually identical. This variation is located at nucleotide 151 of the 488 nucleotides in total. This variation could be detected after optimization of the threshold values of the data analysis software (Bionumerics). This underlines that optimization of the data analysis program is crucial.

from the reference peak. All pattern parameter values were below the initial threshold settings, except for the MAX value (6.1%), which was just above the threshold. The CG-content of this amplicon was 50%, which is the highest of all (mean, 39%). All other variants could clearly be detected regardless of their position in the amplicon or the CG-content of the amplicon. On duplication, identical results were obtained.

Phase Two (Prospective Analysis)

To further assess the analytical sensitivity and to determine the analytical specificity of CSCE for the *SACS* gene, CSCE and sequencing was performed in parallel for all 44 amplicons (except amplicon 2) of the *SACS* gene in a cohort of 20 individuals for whom mutation analysis of the *SACS* gene was requested for clinical purposes. In this cohort an additional 10 novel variants, including two pathogenic mutations (c.9911_9912del/p.Leu3304fs and c.10442T>C/p.Leu3481Pro) were detected. Four of these additional variants occurred in three additional amplicons that could not be assessed in the first phase, since no samples containing variants in these amplicons were available at that stage. All 73 variants present in this cohort, including eleven known variants, occurring in 18 different amplicons (including two overlapping amplicons) could be detected by CSCE, except again for the SNP in fragment 11 (exon 7), which gave a false negative score. This results in an analytical sensitivity of 98.6% before further optimization of the design, and of 100% after optimization. This optimization includes an optimized primer design for amplicon 3, resulting in successful detection of SNP c.172-129A>G, and of relaxation of parameter settings of the data analysis program (Table 4 II), resulting in the successful detection of SNP c.2080G>A in amplicon 11 (exon 7).

Two true false-positive results were observed in two different amplicons 38 (exon 9) (Figure 2) and 39 (exon 9)

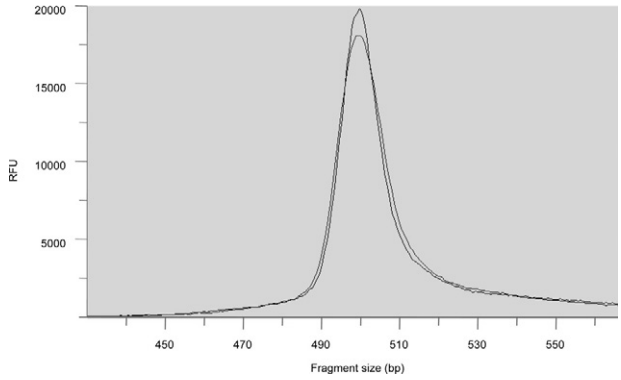

Figure 2. Example of false positive capillary heteroduplex electropherogram. This electropherogram shows the true false-positive result in amplicon 38 (exon 9).

respectively, giving a false-positive variant detection rate of 0.25% with an analytical specificity of 99.8%.

As all samples are mixed with PCR product of a reference sample negative for pathogenic *SACS* mutations, it cannot be determined from the raw data whether a DNA variant that has been detected is homozygous or heterozygous in the test sample. Out of 73 alterations detected, nine turned out to be homozygous variations after sequencing.

Due to overlap in the amplicons, five variations have been detected in two consecutive amplicons (Tables 1 and 2; one example is shown in Figure 3). In three samples, two variations per amplicon (15, 35, and 41 [exon9]) were present, resulting in a pattern of multiple conformational changes (cf. example in Figure 4A and B). The separate patterns when occurring as a single variation are displayed beneath for comparison.

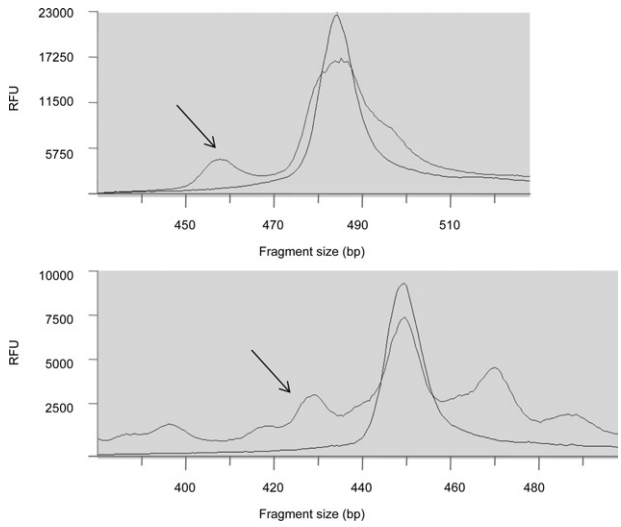

Figure 3. Example of a variation in two overlapping fragments. Due to overlap in the amplicons, five variations have been detected in two consecutive amplicons (Tables 2 and 3). Figure 3 shows the mutation (indicated by the **arrow**) c.1475G>A in amplicon 9 (located at nucleotide 448 of the 484 nucleotides in total) and 10 (at nucleotide 84 of the 449 in total).

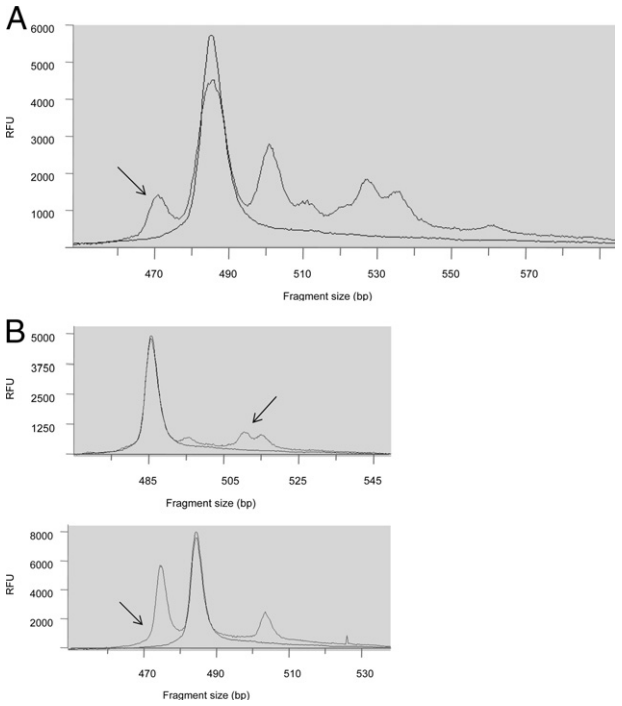

Figure 4. Example of two variations in a single amplicon. In three samples, two variations per amplicon (15, 35, and 41 [exon 9]) were present, resulting in a pattern of multiple conformational changes. For example, **panel A** (amplicon 41 [c.12160C>T and c.12304T>C]) shows multiple conformational changes as the variants are located on different alleles. The separate patterns when occurring as a single variation (**B**) are displayed beneath for comparison. The **arrows** indicate the variations.

Discussion

DNA diagnostic service laboratories are receiving a steadily increasing number of requests for confirmation or exclusion of the clinical diagnosis of a particular genetic disease by DNA techniques. This requires efficient mutation scanning of disease-causing genes by techniques such as automated sequencing, denaturing high performance liquid chromatography, denaturing gradient gel electrophoresis, melting curve analysis, or CSCE. For very large genes, such as the *SACS* gene, mutation scanning is labor intensive and costly with regards to consumable use. Therefore it is desirable to develop alternative scanning methods that are reliable, rapid and inexpensive. CSCE is such a method, and here we describe in detail the design and analytical validation of CSCE for the *SACS* gene, including the pitfalls and difficulties encountered.

Development and Design

To design and test the use of CSCE as a fully automated scanning method for the large *SACS* gene, 52 DNA samples were used for development of optimal running conditions for the different amplicons, for development of optimal automated data analysis parameters, and for subsequent analytical validation. A first important conclusion we can draw from the process of developing a CSCE system is that the developmental and validation stages of the process have to be performed consecutively in clearly distinct phases. The distinction has to be made to prevent that adaptations made to the CSCE system or the analysis software criteria that may be necessary to make to comply with the performance criteria, result in a disturbed validation process. This requires the definition of clear and unambiguous performance criteria for the CSCE system and the automated data analysis software before the development process has started. These *a priori* performance criteria are a high analytical sensitivity of at least 99% and an analytical specificity of at least 96% for the CSCE system. In addition, the robustness of the technique has to be very good (less than 5% failure rate) and the costs compared with automated sequencing of the complete coding region of the gene under study should be less than 50%.

To detect a homozygous mutation by CSCE in an autosomal recessive condition such as ARSACS, DNA of the parents could in principle be used for analysis. However, since DNA of parents is not always available we have chosen to mix PCR product of a patient (test-DNA) with PCR product of a mutation-negative reference sample (reference-DNA). In this procedure, it is very important to monitor the amount of the PCR product of interest on an agarose gel before CSCE analysis by estimating the ethidium bromide staining intensity to avoid aberrant results, eg, by erroneously analyzing just reference-DNA, or reversely unmixed test-DNA.

For amplicon 2 (exon 2) a different PCR mix and PCR program is used because of its high CG-content (75%). With CSCE no reliable results could be obtained for this amplicon, and therefore this amplicon is excluded from the CSCE analytical strategy, and directly sequenced. It has been documented previously that a very high or a very low CG-content reduces the sensitivity of heteroduplex methods.[8]

After identifying the false-negative SNP c.2080G>A in amplicon 11 (exon 5) the lower limits of the peak matching parameters were changed into more relaxed parameters (Table 4). With these newly lower limit parameters the false-negative SNP in amplicon 11 could easily be detected. The other false negative SNP c.172-129A>G in amplicon 3 (exon 3) underlines the importance of designing primers not to close to the intron-exon boundary, as a variant that is located close to, and in this instructive example only one bp apart from the primer, will not be detected by CSCE. In our cohort the position of the variants detected ranged from +1 bp distance to the forward primer to 10 bp distance to the reverse primer. In conclusion, after adaptation of the parameters of the data analysis program, and redesign of the amplicons also these two false-negative SNPs could be detected by CSCE. This results in an overall final analytical sensitivity of 100%.

Four primers (18RP, 19FP, 20FP, 20RP) had to be redesigned, due to four different variants that occurred underneath the primers. Two of these are pathogenic mutations (c.4108C>T/p.Gln1370X and c.4957G>T/p.Glu1653X) and the other two are SNPs (c.4188C>T, c.4466A>G). These redesigned primers have also been tested and validated, and showed normal results.

In Figure 4, the capacity of the CSCE technique to detect multiple variants located on different alleles in a single amplicon is shown. This could be used to design a strategy to discriminate a mutation from known SNPs with relatively high occurrence in the population by admixture of a reference fragment containing the particular SNP with the corresponding fragment of a patient before heteroduplex formation. This strategy could be extended to all amplicons containing known frequently occurring SNPs. By this strategy the number of additionally required subsequent sequencing reactions can be reduced.

Validation

The use of CSCE as a fully automated mutation scanning method for the large *SACS* gene has been validated. A recurrent issue in the discussion on how to perform a reliable validation of a DNA-variant (mutation) scanning technique is whether it is necessary to verify the capacity to identify the presence of a DNA-variant in each amplicon of the scanning system. For a rare disease such as ARSACS, not in all amplicons of the *SACS* gene a DNA-variant is known. Furthermore, it is virtually impossible to obtain samples containing known variants from other laboratories on request. To get an impression on the response rate, requests for samples containing known DNA variants from patients known from literature were sent out to six different laboratories, to be able to test the detection capacity of an additional six amplicons. From just one laboratory (Dr. F.M. Santorelli) two samples containing three mutations in three different amplicons were obtained. Therefore 12 of the 44 amplicons could not be directly tested for their capacity to detect a DNA variant in our validation process. Since in the technical validation of CSCE performed in collaboration with the groups of Matthijs (Leuven, Belgium), Mattocks (Salisbury, UK) and Applied Biosystems (Interlaboratory Diagnostic Validation of Confirmation Sensitive Capillary Electrophoresis), to be published in detail elsewhere, it could be shown that all DNA variants could be detected by CSCE when taking a number of running conditions into account. We concluded that an analytical validation of the CSCE technique for the application of the *SACS* mutation scanning would suffice when using identical running conditions, and that the analysis of eg, PCR products with artificially created SNPs for amplicons for which no known SNPs were available would be superfluous.[6] These running conditions and the recipe of the conformation analysis polymer used have been summarized in Table 5 and 6, respectively. We could conclude that an oven temperature of 12°C results in an optimal separation of heteroduplex bands here fore a specific adjustment to the ABI 3730 automated sequencer had to be made to set this oven temperature (Table 6).

To assess the analytical sensitivity and the analytical specificity of CSCE for the *SACS* gene, we compared it to DNA sequencing. In the analytical validation 32 of the 44 amplicons of the *SACS* gene could be assessed directly. Initially, two SNPs could not be detected. After further optimization, an overall analytical sensitivity of 100% was determined since all 114 DNA variants identified by DNA sequencing could also be identified by CSCE. The overall analytical specificity was 99.8% as a result of two false-positive results. Settings described in this study demonstrate the experimental conditions resulting in an optimal balance between sensitivity and specificity. However, to be safer with regard to the analytical sensitivity and thus taking less risk in obtaining false negative results, the stringency of the settings could be relaxed, accepting the possibility of a slightly lower specificity.

Mismatches in heteroduplex molecules can be detected best in smaller fragments. We have observed that to achieve optimal sensitivity amplicons should have a maximum length of 500 bp.[6] However in this study we noticed that amplicons even longer than 500 bp can easily be analyzed by CSCE.

Table 5. Running Conditions CSCE

Run condition	Value	Range
Oven temperature	12°C	12–70
Buffer temperature	35°C	30–35
Pre-run voltage	15 kV	0–15
Injection time	20 seconds	1–90
Pre-run time	180 seconds	1–1800
Injection voltage	3 kV	0–15
First read out time	180 ms	100–16,000
Exposure time	0 ms	0–5000
Run voltage	13 kV	15
Voltage number of steps	10 kV	0–100
Voltage step interval	20 seconds	0–180
Voltage tolerance	0.6 kV	0–6
Current stability	500 mA	0–2000
Ramp delay	200 seconds	1–800
Data delay	750 seconds	1–1800
Run time	5200 seconds	300–14.000

Buffer to run consists of 1× TTE, 10% glycerol (national diagnostic, EC-871).

Robustness

Initially, capillary overloading resulting in high peaks that cannot be interpreted by the analysis software was observed in a number of samples. This indicates that the DNA concentration of the samples needs to be monitored in process, and will be better controllable by automation of liquid handling during the amplification and sequencing steps. Introduction of DNA concentration monitoring and automated liquid handling resulted in a reduction of the failure rate to below 5%.

Table 6. Recipe CAP+

CAP (Applied Biosystems, 434037)	12.67 g
Ureum (1 kg, pro analyse, Merck)	5.27 g
Sucrose (250 g, for biochem, Merck)	2.03 g
20× TTE (national diagnostic, EC-871)	1.14 g
MQ	1.72 g

After a generic technical intern validation of the experimental conditions of CSCE an oven temperature of 12°C resulted in optimal separation of heteroduplex bands. A specific adjustment to the ABI 3730 automated sequencer had to be made to set this oven temperature.

To improve *in process* quality control the use of a Liz 500 size standard containing a DNA fragment in which a heterozygous DNA variant has been created has been introduced, which allows monitoring of the capillary separation capacity of the conformation analysis polymer.

Costs

To improve cost-effectiveness CSCE with two differently labeled primers (5-carboxyfluorescein and VIC) can be considered. However, in our experience the use of a single labeled primer (5-carboxyfluorescein) is preferable, due to translucency and interference between the differently labeled peaks. Furthermore, to avoid substantial differences in signal intensity between the differently labeled peaks, an additional effort in optimization would be required.

Roughly CSCE is, by a factor of six, cheaper than sequencing. As only those amplicons that show a divergent CSCE pattern need to be sequenced, instead of the entire coding sequence of the gene, a substantial cost reduction is achieved. If two differently labeled primers would be used the difference in costs compared with automated sequencing would even be a factor of 12.

Since one of the mutations (c.12160C>T/p.Gln4054X) has a high relative frequency in ARSACS patients of Dutch descent (identified either homozygously or heterozygously in seven of the so far 16 families identified) we are considering to perform a pre-screening by direct sequencing of this amplicon (41, exon 9). For reasons of efficiency, this will then be combined with the sequencing of amplicon 35 (exon 9) containing two common SNPs, with a high relative frequency, eg, for one of these SNPs (c.9981C>T) both reference samples V3 and V4 are heterozygous.

Mutation Spectrum

So far we have performed mutation analysis of the *SACS* gene in 76 index patients. In total, 16 novel pathogenic mutations were identified in 16 different families consisting of 23 patients. In the remaining 60 index patients no mutations were identified, which most likely reflects the fact that they have a different genetic condition. Most mutations (13) identified are truncating mutations, and three are missense mutations. Furthermore 36 different SNPs were identified of which one is a very common SNP (c.9981C>T) with an estimated minor allele frequency of 0.37. Large deletions of the *SACS* gene as identified in a Belgian ARSACS patient[4] will be missed by both CSCE and sequencing. Therefore in the near future an MLPA-test for the *SACS* gene will be set up.

Conclusion

Based on the various locations of the 52 variations in 32 different amplicons with different fragment lengths, and different CG-content our results show a high sensitivity (~ 100%) and specificity (99.8%) for the use of CSCE as a mutation scanning method for DNA variant identification of the *SACS* gene.

Our recommendation would be to perform sequence analysis of all amplicons with divergent peaks (orange or red) after CSCE and to perform direct sequence analysis of all amplicons with common SNPs with a high minor allele frequency (eg, over 0.25) in the population.

We can conclude that CSCE is a robust technique with a high sensitivity and specificity, and is highly suitable for application in mutation scanning for large genes such as the *SACS* gene.

Acknowledgment

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