Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2018, 7(9): 120-126

Improved SSCP Analysis through Primers Addition to Screen Hotspot Exons in DNAH5 gene

Imed Mabrouk[1,2], Rahma Mani[1]*, Jihen Bouguila[3] , Lamia Boughamoura[4], Mongia Hachicha[5], Raoudha Boussoffara[6], Salma Ben Ameur[5], Samia Hammouda[3], Khadija Boussetta[4], and Zohra Soua[1]

[1] *Laboratory of Biochemistry, Faculty of Medicine, University of Sousse, Tunisia*

[2] *Department of Biology, Faculty of Science, Taif University, Kingdom of Saudi Arabia*

[3] *Children's Hospital Bashir Hamza, Tunis, Tunisia*

[4] *Farhat Hached Teaching Hospital, Sousse, Tunisia*

[5] *CHU Hedi Chaker, Sfax, Tunisia*

[6] *CHU Tahar Sfar, Mahdia, Tunisia*

**Corresponding e-mail: mani-rahma@hotmail.fr*

ABSTRACT

Background: *PCR-coupled single-strand conformation polymorphism (PCR-SSCP) is often used as a first line screening technique to facilitate mutation detection in large genes such as the DNAH5 gene. A modified protocol of PCR-SSCP by primers addition before denaturation may improve banding profiles.* ***Objectives:*** *The aim is to study the effect of primers addition and determine the optimal conditions to screen DNAH5 hotspot exons.* ***Materials and Methods:*** *Prior to SSCP screening, various concentrations of primers were added to PCR-products of DNAH5 hotspot exons. The resolution and intensity of the electrophoretic bands were then evaluated.* ***Results:*** *Herein we prove that specific primers addition at 4 µM concentration improves the resolution and efficiency of the method without altering the detection of the mutations. Moreover, an optimal result was obtained by adding both primers for some exons and only the reverse one for others.* ***Conclusion:*** *The modified PCR-SSCP protocol described in this present study could be used to screen the mutations in DNAH5 hotspot exons in patients with primary ciliary dyskinesia (PCD).*

Keywords: Primary ciliary dyskinesia, Mutations screening, Single-stranded DNA

INTRODUCTION

PCR-coupled single-strand conformation polymorphism (SSCP) has proved to be a powerful method for mutation screening. It is a widely applied method in recent research experiments [1,2]. In SSCP, single-stranded DNA (ssDNA) molecules fold in a sequence-specific manner. Normally, a wild-type homozygous profile should appear as two distinct electrophoretic bands corresponding to the complementary DNA strands. A mutation at a particular position in the primary sequence causes a change in the folded structure, which modifies the mobility of the conformer on a non-denaturing polyacrylamide gel. A homozygous mutation leads to a profile of two bands that migrate differently to the wild type. Heterozygous mutation creates a pattern of 4 distinct bands corresponding to the complementary strands of each allele [3,4]. In practice, the targeted sequence is first specifically amplified by PCR, and then amplicons are strongly denatured before being separated as single-stranded in non-denaturing polyacrylamide gel [5]. The three-dimensional conformation of simply stranded amplicons is unpredictable. Therefore, there is no standard protocol for SSCP screening method. To obtain stable conformers that give electrophoretic bands of different mobility, appropriate experimental conditions should be determined for each amplicon prior to its analysis [1].

SSCP screening method could be highly useful particularly to screen mutations in large cohorts of patients or hotspot

exons of large genes. DNAH5 is a large gene that comprises 79 exons plus an alternative first exon and spans 250 Kb [6]. It has been reported as the most frequently mutated in primary ciliary dyskinesia (PCD); a hereditary disease that alters the mucociliary clearance and thus results in upper and lower airway infections [7-9]. In literature, hotspot exons in DNAH5 gene (exons 33, 34, 36, 48, 49, 50, 63, 76 and 77) have been defined [10,11]. Therefore, SSCP screening of hotspot exons, prior to direct sequencing, would be of a great interest. However, SSCP screening methods have its weaknesses regarding the resolution and the intensity of the electrophoretic bands. In fact, it has been noted that re-annealing of ssDNA during electrophoresis decreases their proportion which declines their detection. Moreover, residual primers in PCR product and a part of ssDNA may re-pair creating new additional conformers which alter the significant electrophoretic profile [12-16]. In presetting steps, we noticed that purification of amplicons or addition of PCR primers to PCR product could troubleshoot the noise created by residual primers. Therefore, we aim in this work to deeply study the effect of primers addition to PCR product before denaturation in order to improve the effectiveness of SSCP screening of the hotspot exons of DNAH5 gene. This work would offer a reliable tool in the genetic diagnosis of PCD.

PATIENTS AND METHODS

DNA Extraction

Blood samples were obtained from clinically-diagnosed PCD patients and a healthy subject. Written informed consent was obtained from all the studied subjects or their parents. Genomic DNAs were isolated from 5 ml of blood using the salting-out method modified in our laboratory [17]. Briefly, buffy coats of nucleated cells are purified twice by 10 ml red-cells lysis buffer (1mM Tris, 0.5mM MgCl2, 1mM NaCl, pH 7.5). The white-cells pellet is washed with 10 ml PBS1X and then digested overnight at 45°C with 1.8 ml white-cells lysis buffer (10mM Tris-HCl, 400 mM NaCl and 2 mM Na2 EDTA, pH8.2), 0.5% SDS and 50 µg/ml protease K. Protein precipitation is performed by adding 0.5 ml of a saturated NaCl solution (6 M). Two volumes of pure ethanol are added to the recovered supernatant to precipitate DNA. DNA quantity and quality checking were performed by nanodrop ND-1000 measurement.

Polymerase Chain Reaction PCR

Amplification was performed in a 50 µl reaction containing 50 mg of the genomic DNA, 0.1 µM of each primer, 0.2 mM of dNTPs, 2 mM of $MgCl_2$ and using the G2 hot start Taq DNA polymerase (Promega MP704) according to the manufacturer instructions. For all exons, the PCR program consisted of 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C, with a final extension of 10 min at 72°C. Primers used for PCR were either taken from a published paper or was newly designed to encompass DNAH5 hot spot regions (Table 1) [11]. PCR was followed by electrophoresis in 8% polyacrylamide gels (29:1) using 0.5X TBE buffer. Gels were colored with SYBER® gold nucleic acid gel stain (Invitrogen MP 11494) following the manufacturer instructions.

Table 1 List of PCR primers

Exons	Primer	Sequence (5'"3')	Length
33	DNAH5-33F1[a]	TCTTTGGGAAGGACAAGTCAA	21
	DNAH5-33R2[a]	GCTTACAATAGGTAATGTCTGA	22
34	DNAH5-34F1	CAGCTGTTGATCACATTGTATTTCT	25
	DNAH5-34R2	TTCTACTGGGTAAATGCAGATAGTGT	26
36	DNAH5-36F1	GGACAATGGCCTTTTGTTTT	20
	DNAH5-36R2	AATGTGGCCAATTGTTGAAA	20
48	DNAH5-48F1	GGGGGAAATTTGGCTTTTTA	20
	DNAH5-48R2	TTGAGTGTTTCCAAAGAAAGAAC	23
49	DNAH5-49F1	GAGTTAGGCTCCGGGGATAG	20
	DNAH5-49R2	AAAAAGGAACCCAAGCAGGT	20
50	DNAH5-50F1	CATTTTTGCAAACCCTATGC	20
	DNAH5-50R2	CCATGCTGAAACATTCCAAA	20

63	DNAH5-63F1	TTTTTAATATGCTGTACAATCACACG	26
	DNAH5-63R2	GGATTTTTGTTTATCTGAGGCAAT	24
76	DNAH5-76F1	CCAGGGCACCAGGTCTATAA	20
	DNAH5-76R2	TGCTTTCCACTTGCCAATTA	20
77	DNAH5-77F1*	CATAGCTGGTTTACACTGCAG	21
	DNAH5-77R2*	GATGGAAAATCCTGTGGAGG	20
Mutagenic Primers			
63	DNAH5-63 F2	AACATCTCACTG[G]GATTTAGGTTCTT	26
	DNAH5-63 R1	AAGAACCTAAATC[C]CAGTGAGATGTT	26
77	DNAH5-77 F2	CCATTGAAA[G]CCCACGAGGTA	21
	DNAH5-77 R1	TACCTCGTGGG[C]TTTCAATGG	21

(a) Primers taken from publication (Djakow, et al., 2012); The introduced substitutions are written between brackets in the mutagenic primers

Mutagenesis by PCR-Driven Overlaps Extension

This technique involves three separate PCRs [18]. In the first two PCRs, two amplicons (A1 and A2) were produced separately with the same conditions mentioned above. The PCR program consisted of 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 45 sec at 55°C and 30 sec at 72°C, with a final extension of 10 min at 72°C. The A1 amplicon was generated using the primer F1 and the mutagenic primer R1. Likewise, a mutagenic primer F2 and a primer R2 were used to produce the A2 amplicon. All the primers are listed in (Table 1). Mutagenic primers are complementary and were designed in order to introduce a T→C substitution at about the middle of the amplicons of 63 and 77 of DNAH5 gene. The amplicons (A1 and A2) were mixed and puriied on low melting point agarose gels according to standard protocol and were used as a template for the third PCR. The third PCR was also performed with the same conditions mentioned above using 5 µL of the puriied mixture of amplicons (A1 and A2) and flanking master primers (F1 and R2). The PCR program consisted of 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C, with a inal extension of 10 min at 72°C.

SSCP Analysis

A volume of 3 µl of PCR products (≈50ng) is diluted 3.5 folds in 40% formamide, 1 X gel loading buffer and 8 µM forward and/or reverses PCR primers (Table 1). The mixture is heated for 15 min at 95°C on thermocycler heat block and then immediately snap-cooled into ice for 2 min. The samples were rapidly loaded on 8% non-denaturing polyacrylamide (29:1) gels supplemented with 5% glycerol. Electrophoresis was performed at 80 V for 18 hours at 4°C in 0.5X TBE buffer. Gels were stained with SYBR gold stain according to the manufacturer instructions.

Sequencing

Purified PCR products were directly sequenced using the PCR forward or both primers in ABI 3730xl DNA analyzer. Sequence alignments were carried out using the NCBI-BLAST algorithm.

RESULTS AND DISCUSSION

Primers Addition Effect

As a first essay, we carried-out PCR-SSCP analysis of all the targeted DNAH5 hotspot exons (33, 34, 36, 48, 49, 50, 63, 76 and 77) according to the conventional protocol-without primers addition [5]. The wild-type controls were prepared from the healthy subject by PCR amplification of all the exons and verified by sequencing. In Figure 1, the results of SSCP analysis of the exon 76 are illustrated. For all samples including patients and control, non-informative profiles of 3 bands were revealed with low and variable intensities. The same result was obtained for all the other exons. In fact, during folding, residual PCR primers may pair to their complementary sequence in some of the single-stranded molecules creating new additional conformers. These new conformers migrate differently and lead to ambiguous profile with 3 bands. To overcome this ambiguity, PCR product could be purified or a saturating

amount of primers could be added to pair with all single strands. To test the second alternative, we carried-out SSCP screening according to the modified protocol in which 4 µM of primers were added to the PCR product before denaturation. A homozygous profile with two very intense single-stranded bands in all samples is observed (Figure 1). In fact, primers addition has a double effect. Firstly, all simple strands pair with primers and fold in the same manner. Secondly, primers' pairing prevents single strands re-annealing, increases their amount and enhances their staining. This confirms that primers addition in PCR product eliminates the ambiguity brought by the emergence of a third band and strongly improves the yield of single-stranded folding indicated by the intensity decrease of the double-stranded band. In literature, only three reports have briefly described the effect of adding PCR primers in SSCP analysis of other PCR fragments [12,14,16]. Herein, we demonstrate and further describe the improvement of SSCP analysis through primers addition to PCR product.

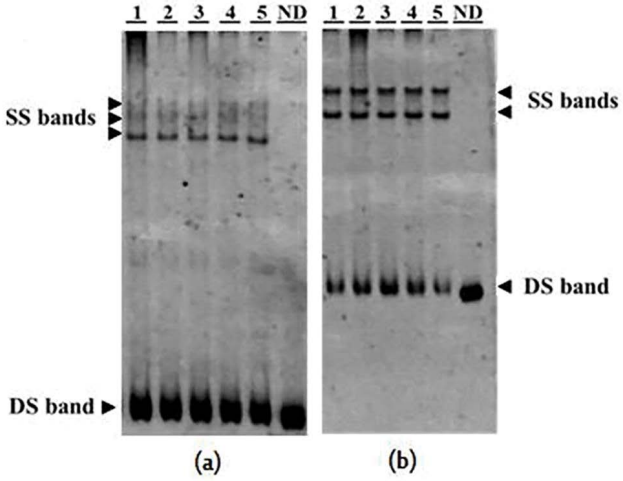

Figure 1 PCR-SSCP analysis of exon 76 of DNAH5 gene with (b) and without (a) primers' addition. The figured gel in (a) shows a non-informative profile with three single-stranded bands. In (b), two intense single-stranded bands are revealed indicating a homozygous wild-type profile in samples (1-4) and control (5). Electrophoresis was performed in two different time durations in (a) and (b). (ND: non-denatured sample, SS: Single-Stranded bands, DS: Double-Stranded band)

Primers Amount and Specificity Effects

To determine the perfect amount of added primers, the two specific forward and reverse primers were simultaneously added at different concentrations to PCR product of all exons amplified on control DNA. SSCP analysis shows that the ambiguity of the banding profile is eliminated by primers addition in a dose-dependent manner (Figure 2). Indeed, a third band is gradually eliminated when primers' concentration increases (lanes 2-8). A saturating effect was reached from 4 µM of each primer leading to a significant profile of two intense bands. The same saturating effect has been reached with fewer amounts of primers in a previous study. Zhu, et al., have reported a saturating amount of only 3 µM of primers [16]. This variability may be explained by differences in PCR yield and differences in the amounts of residual primers in PCR products. Thus, an optimization of the added amount of primers is recommended prior to SSCP analysis of new PCR fragments. Remarkably, when forward or reverse primer was added separately at saturating amount (4 µM), only one specific band shifted and showed the stronger intensity (lanes 9 and 10). This highlights the effect of residual PCR primers' pairing to their complementary sequences in single strands. This result was proven by the addition of a non-specific pair of primers at 4 µM which showed no effect (lanes 11 and 12). Despite its informative profile, the analysis of the purified fragment shows bands with unsatisfactory intensity (lane 1). All taken together, it is clear that the non-significant profile previously obtained is doubtless due to residual primers in PCR product and it is improved by optimized primers addition (Figure 2).

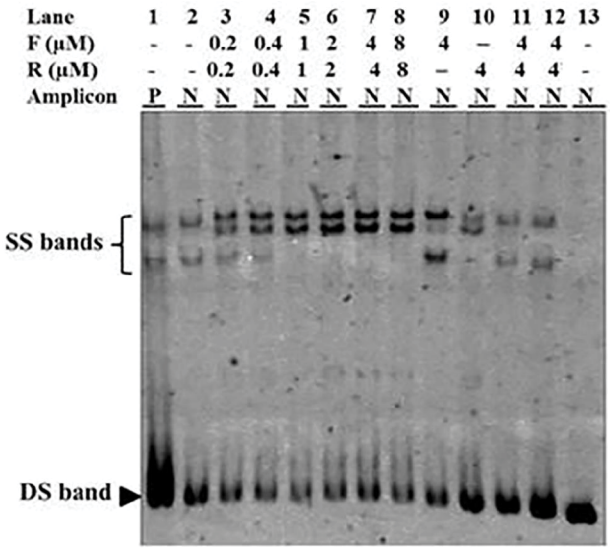

Figure 2 PCR-SSCP analysis of exon 49 of the DNAH5 gene. Purified (P) PCR product was loaded in lane (1) without adding primers. Non-purified (N) PCR products were loaded in lanes (2-8) with specific forward (F) and reverse (R) primers' addition in a dose increasing manner. A third band is gradually eliminated when primers concentration increases. In lanes (9 and 10) with respectively only specific forward and reverse primer addition, only one specific band shifted. In lanes (11and 12) with non-specific primers' addition, no effect is shown compared to lane (2). Non-denatured PCR product was loaded in lane (13). Amounts of the added primers are indicated on top. (SS: Single-Stranded bands, DS: Double-Stranded band)

Given that single-stranded bands shift with unpredictable amplitudes following the addition of primers, the resolution decreases for some exons (Figure 3). In fact, adding both forward and reverse primers may bring the bands closer but adding only one primer may improve the resolution. Therefore, we aimed to test the effect of the addition of the specific forward and/or reverse primers for each of the studied exons prior to screening. Interestingly, the optimal resolution was obtained by adding only the reverse primer for exons 36 and 77 and both primers for the other exons (Table 2).

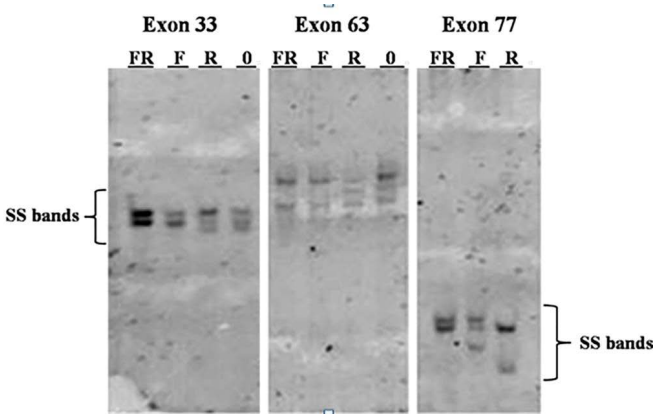

Figure 3 PCR-SSCP analysis of exons 33, 63 and 77 of DNAH5 gene with the addition of both forward and reverse primers (lanes FR), only forward primer (lanes F) and only reverse primer (lanes R). PCR products were loaded without primers' addition in lanes (0). A high resolution is obtained with both primers addition in exons 33 and 63 and with the addition of only the reverse primer in exon 77. (SS: Single-Stranded bands)

Table 2 Optimal concentrations of added primers in SSCP screening of DNAH5 hot spot exons

Exon	33	34	36	48	49	50	63	76	77
Forward (µM)	4	4	0	4	4	4	4	4	0
Reverse (µM)	4	4	4	4	4	4	4	4	4

The sensitivity of PCR-SSCP with primers addition

Mutagenesis by PCR-driven overlaps extension is performed to test the sensitivity of SSCP analysis when primers were added [18]. Therefore, internal mutagenic primers were designed to introduce a T→C substitution in the amplicons of exons 63 and 77 of DNAH5 gene (Table 1). Homozygous mutations were obtained through mutagenesis and verified by sequencing. These mutations were generated in the heterozygous state by mixing equal amounts of mutated and wild type PCR products. All these mutations were analyzed by SSCP with primers addition. Comparing with the wild-type, homozygous and heterozygous mutations are clearly highlighted (Figure 4). It is obvious that the primers' addition does not alter the detection of mutations. Thus, we believe that PCR-SSCP analysis with primers addition is a reliable screening technique.

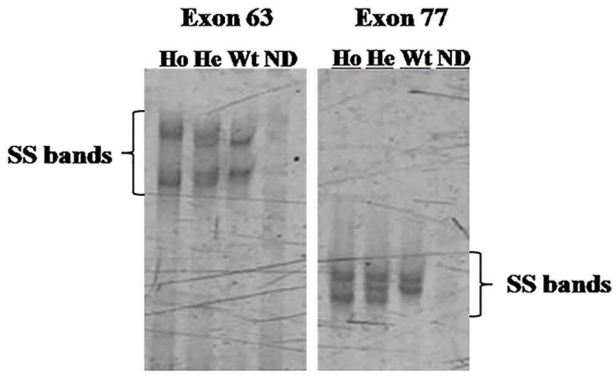

Figure 4 PCR-SSCP analysis with primers' addition of exons 63 and 77 of DNAH5 gene with introduced mutations. The homozygous (Ho) and heterozygous (He) genotypes of mutations were compared with the wild type (Wt). It is clear that primers addition in the PCR-SSCP analysis do not alter the detection of sequence variations (ND: non-denatured sample, SS: Single-Stranded bands)

To our knowledge, this is the first study that highlights the usefulness of primers addition in the PCR-SSCP screening of DNAH5 hotspot exons. If this protocol would be applied to screen other sequences, it is recommended to experimentally determine the optimum conditions of primers addition prior to SSCP analysis.

CONCLUSION

In the present study, we showed that the addition of saturating amounts of specific forward and/or reverse primers to PCR products before SSCP analysis improves the resolution and intensity of the single-stranded bands and offers a better interpretation of results. Indeed, the optimized primers' addition, presented here, is being used to screen DNAH5 hotspot exons in a cohort of clinically diagnosed PCD patients.

DECLARATIONS

Acknowledgment

This work was supported by grants from Ministry of higher education and scientific research of Tunisia.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

[1] Konstantinos, Kakavas V., et al. "PCR-SSCP: A method for the molecular analysis of genetic diseases." *Molecular Biotechnology,* Vol. 38, No. 2, 2008, pp. 155-63.

[2] Zheng, Xuelian, et al. "Effective screen of CRISPR/Cas9-induced mutants in rice by single-strand conformation polymorphism." *Plant Cell Reports,* Vol. 35, No. 7, 2016, pp. 1545-54.

[3] Orita, Masato, et al. "Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms." *Proceedings of the National Academy of Sciences,* Vol. 86, No. 8, 1989, pp. 2766-70.

[4] Nataraj, Arun J., et al. "Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection." *Electrophoresis: An International Journal,* Vol. 20, No. 6, 1999, pp. 1177-85.

[5] Gasser, Robin B., et al. "Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation." *Nature Protocols,* Vol. 1, No. 6, 2006, p. 3121.

[6] Chapelin, Catherine, et al. "Isolation of several human axonemal dynein heavy chain genes: genomic structure of the catalytic site, phylogenetic analysis and chromosomal assignment." *FEBS Letters,* Vol. 412, No. 2, 1997, pp. 325-30.

[7] Damseh, Nadirah, et al. "Primary ciliary dyskinesia: mechanisms and management." *The Application of Clinical Genetics,* Vol. 10, 2017, p. 67.

[8] Lucas, Jane S., et al. "European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia." *European Respiratory Journal,* 2016.

[9] Vanaken, Gert Jan, et al. "Infertility in an adult cohort with primary ciliary dyskinesia: phenotype–gene association." *European Respiratory Journal,* Vol. 50, No. 5, 2017, p. 1700314.

[10] Hornef, Nada, et al. "DNAH5 mutations are a common cause of primary ciliary dyskinesia with outer dynein arm defects." *American Journal of Respiratory and Critical Care Medicine,* Vol. 174, No. 2, 2006, pp. 120-26.

[11] Djakow, Jana, et al. "Effectiveness of sequencing selected exons of DNAH5 and DNAI1 in the diagnosis of primary ciliary dyskinesia." *Pediatric Pulmonology,* Vol. 47, No. 9, 2012, pp. 864-75.

[12] Almeida, Teresa A., Vicente M. Cabrera, and Jose G. Miranda. "Improved detection and characterization of mutations by primer addition in nonradioisotopic SSCP and direct PCR sequencing." *BioTechniques,* Vol. 24, No. 2, 1998, pp. 220-21.

[13] Hennessy, Lori K., John Teare, and Christopher Ko. "PCR conditions and DNA denaturants affect reproducibility of single-strand conformation polymorphism patterns for BRCA1 mutations." *Clinical Chemistry,* Vol. 44, No. 4, 1998, pp. 879-82.

[14] Cai, Qiu-Qiong, and Isabelle Touitou. "Excess PCR primers may dramatically affect SSCP efficiency." *Nucleic Acids Research,* Vol. 21, No. 16, 1993, p. 3909.

[15] Selvakumar, Nagamiah, B. Ding, and Stuart M. Wilson. "Improved resolution of asymmetric-PCR SSCP products." *BioTechniques,* Vol. 22, 1997, pp. 606-08.

[16] Zhu, Xilin, et al. "Improvement of the sensitivity and resolution of PCR-SSCP analysis with optimized primer concentrations in PCR products." *Journal of Genetics,* Vol. 85, No. 3, 2006, pp. 233-35.

[17] Miller, S. A., D. D. Dykes, and H. F. R. N. Polesky. "A simple salting out procedure for extracting DNA from human nucleated cells." *Nucleic Acids Research,* Vol. 16, No. 3, 1988, p. 1215.

[18] Heckman, Karin L., and Larry R. Pease. "Gene splicing and mutagenesis by PCR-driven overlap extension." *Nature Protocols,* Vol. 2, No. 4, 2007, p. 924.