

# Analysis of Single-Strand Conformation Polymorphism by Capillary Electrophoresis with Laser-Induced Fluorescence Detection Using Short-Chain Polyacrylamide as Sieving Medium

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**A rapid analysis of single-strand conformation polymorphism (SSCP) by capillary electrophoresis with laser-induced fluorescence (CE-LIF) detector was developed using a short-chain, linear polyacrylamide (PA) as sieving medium. Capillary filling of this low-viscosity medium and medium replacement were carried out by commercial capillary electrophoresis instruments. The approach was successfully applied to detect the C677T mutation of methylenetetrahydrofolate reductase gene. The influences of factors such as the concentration of polymers, voltage, temperature, and additives on the SSCP analysis were systematically investigated. Using 6% PA sieving medium and high electric field, four strands were resolved within 11 min in a DNA sample heterozygous for the C677T mutation, and a characteristic pattern was apparent for each of the three genotypes. When using multiple injection mode, the average analysis time per sample was reduced to about 4 min. In conclusion, our results indicate that CE-LIF may be an alternative to conventional SSCP analysis based on slab gel electrophoresis for the detection of genetic mutations. The technique is simple and rapid and is well suited to analysis of large numbers of clinical samples.** © 1997 Academic Press

Capillary electrophoresis (CE)<sup>2</sup> has been successfully used in the analysis of double-stranded DNA (dsDNA)

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<sup>2</sup>Abbreviations used: CE, capillary electrophoresis; dsDNA, double-stranded DNA; SSCP, single-strand conformation polymorphism; ssDNA, single-stranded DNA; PA, polyacrylamide; LIF, laser-induced fluorescence; TEMED, *N,N,N',N'*-tetramethylethylenediamine; APS, ammonium peroxydisulfate; PEO, polyethylene oxide; HPMC, hydroxypropylmethylcellulose; MTHFR, methylenetetrahydrofolate reductase; i.d., internal diameter.

fragments and polymerase chain reaction (PCR) products (1–3), and various CE techniques have been developed to detect genetic mutations. Ulfelder *et al.* (4) demonstrated a point mutation of ERBB2 oncogene based on restriction fragment-length polymorphism. Khrapko *et al.* (5) developed a constant denaturant CE technique to separate mutant from wild-type DNA sequences, and Gelfi *et al.* (6) utilized the Joule heating effect in the capillary to create a thermal gradient, which was then used to identify point mutations in the cystic fibrosis transmembrane conductance regulator gene. Cheng *et al.* (7) were able to detect multiple point mutations as well as gene deletion on the basis of heteroduplex DNA conformation polymorphism.

Single-strand conformation polymorphism (SSCP) analysis is based on the principle that single-stranded DNA (ssDNA) fragments with a base substitution often get an altered conformation and therefore migrate differently in a non-denaturing gel, and the method is frequently used in the detection of unknown genetic mutations (8, 9). SSCP analysis based on conventional gel electrophoresis is a simple and sensitive technique, but high resolution is of paramount importance (10). CE has now emerged as an alternative with the advantages of speed and automation, combined with high separation efficiency (11–14). Usually, the capillary is filled with a linear, long-chain polyacrylamide (PA) sieving medium which gives high resolution, but the viscosity of the high concentration of PA required in SSCP prevents the filling of capillaries with small internal diameters (i.d.), and in capillaries with large i.d., high electric field may cause marked Joule heating (12, 14, 15). The alternative of polymerizing the acrylamide *in situ* (i.e., inside the capillary) is hampered by the formation of bubbles during the polymerization step and needs of extensive preelectrophoresis and pre-

cludes replacement of sieving matrix. Thus, long-chain PA is unsuitable for rapid and automated analysis of SSCP by commercial CE instruments.

Recently, Grossman (16) synthesized a short-chain linear PA with low viscosity, which is easily filled into capillaries with small i.d. This sieving medium has been successfully used for CE separation of DNA (17) including DNA sequencing extension products (16). Notably, the resolution of dsDNA fragments using long- and short-chain PA is identical in 50- $\mu\text{m}$ -i.d. capillaries (17).

In the present work, we demonstrate the use of short-chain PA as sieving medium in an automated SSCP analysis by CE-LIF to detect a point mutation of the methylenetetrahydrofolate reductase gene (18–20). Use of high electric field during separation and a multiple injection technique shortened the analysis time to <5 min per sample.

## MATERIALS AND METHODS

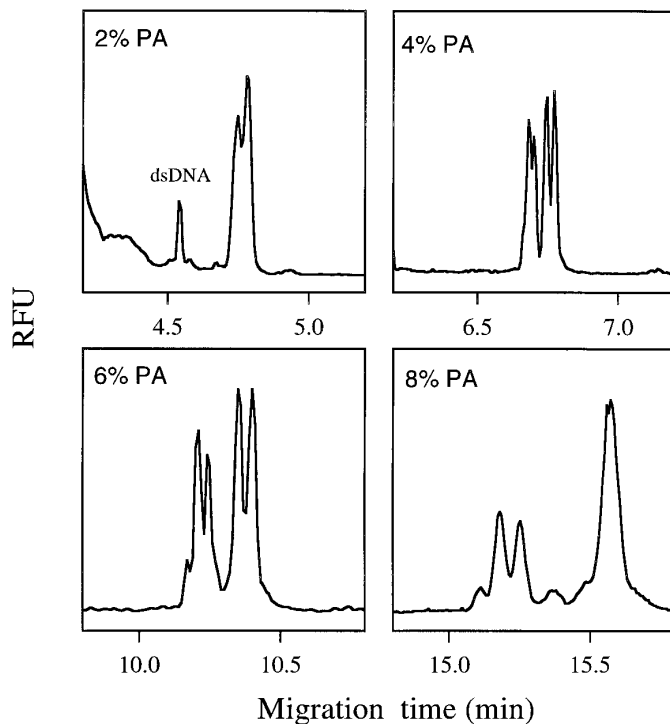
### Materials

Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), and ammonium peroxydisulfate (APS) were purchased from Bio-Rad Laboratories (Hercules, CA). Different molecular weight polyethylene oxides (PEO, with an average  $M_r$  200,000–8,000,000) were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). 3-Methacryloxypropyltrimethoxysilan was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Hydroxypropylmethylcellulose (HPMC, 4000 cp, 2% aqueous solution, 25°C) and other chemicals (molecular biology grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Reaction tubes (thin-walled, Gene Amp) for PCR were from Perkin-Elmer (Norwalk, CT). Fused capillaries (50  $\mu\text{m}$  i.d., 192  $\mu\text{m}$  outer diameter) were products of Polymicro Technologies Inc. (Phoenix, AZ). 5-Fluorescein labeled primers were synthesized by Eurogentec (Seraing, Belgium). Water, double-distilled and purified on a Milli-Q Plus water purification system (Millipore, Bedford, MA), was used for preparation of all aqueous solutions.

### Synthesis of Short-Chain Linear Polyacrylamide

Solutions of short-chain linear PA, used as the sieving medium in SSCP, were synthesized according to the procedure described by Grossman (16). Briefly, 111 mL water and 3.75 mL isopropanol were added to the reaction vessel containing 12.5 g acrylamide. The solution was degassed with helium for 1 h and heated to 60°C in a water bath. Then, 0.625 mL 10% (v/v) TEMED and 0.625 mL 10% (w/v) APS were added, and the polymerization took place for 2 h.

The product was extensively dialyzed against water for 2 days, using a 12,000 molecular weight cut-off dialysis membrane (Thomas Scientific, Philadelphia, PA),



**FIG. 1.** Separation of ssDNA components of a heterozygous sample using various concentrations of polyacrylamide (PA) as sieving matrix. Electrophoretic conditions: Buffer, 1 $\times$  TBE; temperature, 25°C; applied voltage, -20 kV. RFU, relative fluorescence units.

lyophilized, and then dissolved in separation buffer (TBE; 89 mM Tris-borate, containing 1 mM EDTA, pH 8.33) at different concentrations. These PA solutions were stored at 4°C. Filling of the capillary (39.5 cm length, 50  $\mu\text{m}$  i.d.) with 6% PA solution was accomplished by applying a pressure of 2 bar for 2 min.

### Capillary Coating

The inside wall of the capillary was coated with linear polyacrylamide as described by Hjertén (21). A new capillary was rinsed with 0.1 N HCl and 0.04 N NaOH. The capillary was filled and left standing, first for 1 h with 50% acetic acid solution containing 2% 3-methacryloxypropyltrimethoxysilan and then for another hour with 3% acrylamide solution (5 mL 3% acrylamide solution containing 2  $\mu\text{L}$  TEMED and 20  $\mu\text{L}$  10% APS).

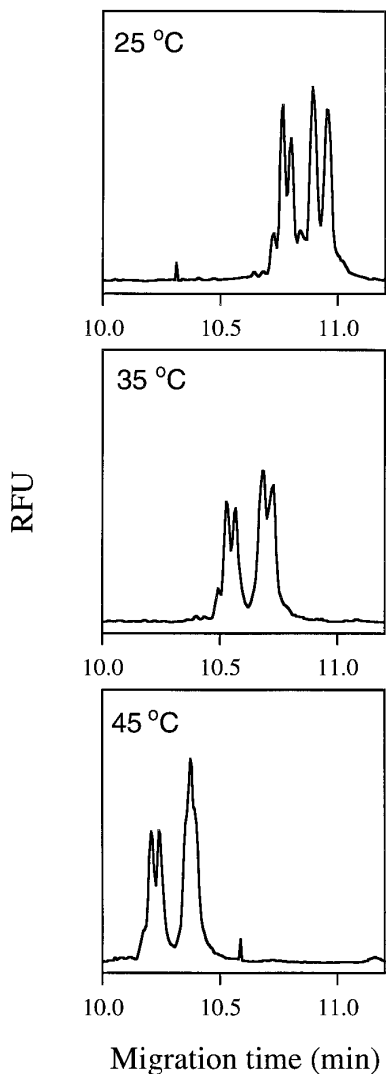
### DNA Extraction and Genotyping

DNA was extracted from whole blood using the DNA Direct kit (Dynal, Oslo, Norway). The C677T mutation in the MTHFR gene creates a *HinfI* restriction site, and the MTHFR genotype was determined by PCR amplification and *HinfI* digestion. The digestion fragments were detected either by conventional gel electro-

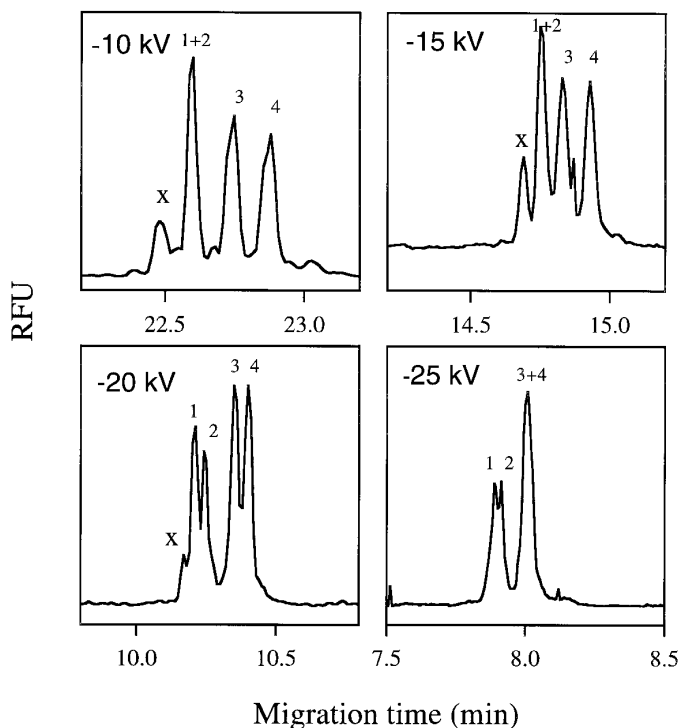
phoresis as described by Frosst *et al.* (20) or by capillary electrophoresis as described by Ulvik *et al.* (22). DNA from subjects of the normal homozygous (CC), the heterozygous (CT), and the mutant homozygous (TT) genotype was used as template DNA in the SSCP analyses.

#### PCR and SSCP

The PCR mixture contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 0.1% Triton X-100, 125 μM each dNTP, 0.2 μM each primer, 0.2 U *Taq* polymerase (Super Taq, HT Biotechnology Ltd., UK), and approximately 100 ng template DNA in a final volume of 100 μL. The PCR was per-



**FIG. 2.** Separation of ssDNA components of a heterozygous sample at various temperatures. Electrophoretic conditions: Buffer, 1× TBE; sieving medium, 6% PA; applied voltage, -20 kV. RFU, relative fluorescence units.



**FIG. 3.** Separation of ssDNA components of a heterozygous sample at various field strengths. The peaks denoted x, 1, and 4 are derived from the CC genotype and 2 and 3 from the TT genotype. Electrophoretic conditions: Buffer, 1× TBE; sieving medium, 6% PA; temperature, 25°C; applied voltage, -10 to -25 kV. RFU, relative fluorescence units.

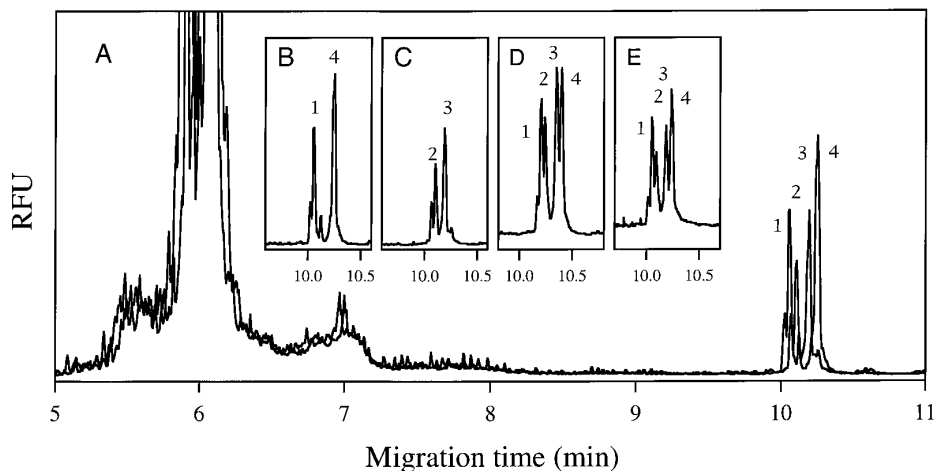
formed on a Perkin-Elmer 480 thermocycler, using a two-step thermocycling profile with 32 cycles of 94°C for 15 s and 60°C for 30 s, preceded by 94°C for 2 min and ended with 72°C for 5 min.

The primers used were 5'-fluorescein-GGAGCTTTG-AGGCTGACCTGAA-3' (forward) and 5'-fluorescein-GACGATGGGGCAAGTGAT-3' (reverse). The primers define the PCR product of 146 bp in length from position 616 to 762, which includes position 677 (C677T mutation).

To remove salt and thereby increase resolution of ssDNA (1), the PCR products were precipitated with 70% ethanol (23), and then dissolved in 50 μL dilute buffer (0.1× TBE). The samples were stored at -20°C until analysis. Immediately before CE, the PCR products were diluted 1:10 in water, heated to 94°C for 5 min, and then cooled in ice water for 10 min.

#### Capillary Electrophoresis Instrumentation

CE was performed on a commercial CE instrument (Prince Technologies, Emmen, The Netherlands). The LIF detector is an in-house-built unit with a sheath flow cuvette constructed essentially as described by



**FIG. 4.** Electropherograms of homozygous (CC), heterozygous (CT), and mutant homozygous (TT) samples. (A) Superimposed electropherograms of a CC and TT sample; (B–D) electropherograms of DNA samples of types CC, TT, and CT, respectively; (E) a CC and TT sample mixed prior to injection. Electrophoretic conditions: Buffer,  $1\times$  TBE; sieving medium, 6% PA; temperature,  $25^{\circ}\text{C}$ ; applied voltage,  $-20$  kV. RFU, relative fluorescence units.

Zarrin and Dovichi (24). An argon ion laser (Uniphase Ltd., Herts, UK) with 488 nm emission (20 mW) was focused on the sheath flow cuvette  $30\ \mu\text{m}$  below the capillary outlet. A fluorescence emission signal was collected at  $90^{\circ}$  with a microscope objective, amplified by a photomultiplier (Hamamatsu, Japan), and the signal transferred to a computer. For the instrument control we used Prince software (version 1.14) and for data collection Caesar software (version 4.0), both from Prince Technologies.

#### Capillary Electrophoresis Procedure

The PA-coated capillary (39.5 cm in length) was rinsed with TBE for 5 min and then filled with sieving medium. TBE was used as the separation buffer. Samples were introduced by electrokinetic injection at  $-2$  kV for 6 s. Electrophoresis was performed at reverse polarity under the conditions specified in the figure legends. The sieving medium in the capillary was replaced between each run.

## RESULTS AND DISCUSSION

### Separation Matrix

Several water-soluble polymers, including cellulose derivatives, non-cross-linked linear PA, and PEO, have successfully been applied in the separation of dsDNA by CE (2, 25–27). We first compared various sieving media and found that HPMC and large-molecular-weight PEO were unsuitable for SSCP analysis, due to inadequate resolution or high viscosity at the concentrations required for adequate resolution (data not shown).

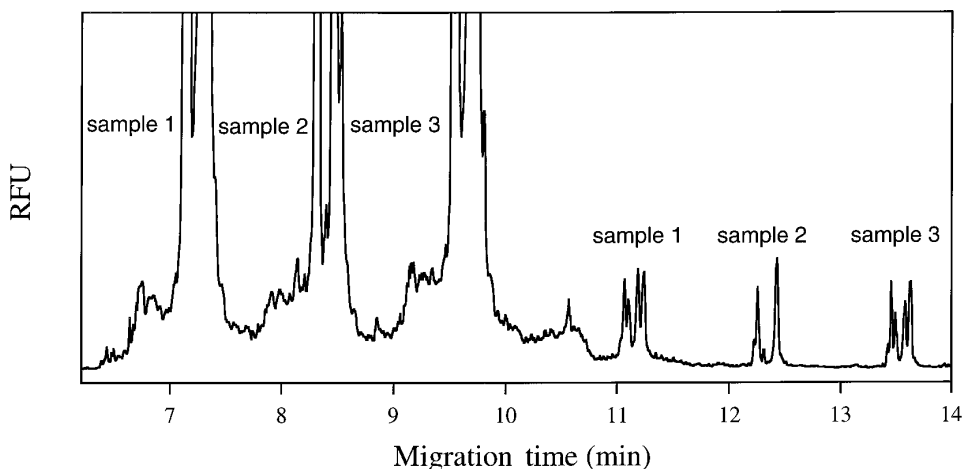
Figure 1 shows SSCP analysis using various concen-

trations of PA. A maximum of four peaks were detected after denaturation of dsDNA in a heterozygous (CT) sample. The best resolution of the single strands was obtained with short-chain PA. At 2% PA, only two peaks were resolved, whereas at 6% PA we obtained separation of four single strands. Further increase in PA concentration was associated with a decline in resolution with coelution of the last two peaks. Thus, the resolution was markedly dependent on the PA concentration, which is probably related to the change in pore size of the sieving medium with increasing concentration of the polymer (27). A PA concentration of 6% was optimal for the SSCP analysis of the C677T mutation, and this concentration was therefore used in the experiments described below.

Some reports indicate that glycerol in a cross-linked PA sieving medium improves resolution of SSCP bands (9, 14). However, we found that glycerol decreased the resolution of ssDNA species and primers (data not shown). Conceivably, glycerol may have different effects on the migration of ssDNA in short-chain PA compared to cross-linked PA. The former creates a dynamic network structure with pores that change in size probably depending on the concentration of additives such as glycerol, whereas the latter forms rigid and constant-sized sieving pores (25).

### Temperature

Figure 2 shows that increasing temperature decreased the resolution of the four single DNA strands in a heterozygous (CT) sample. This finding concurs with previous reports (12, 14) and may be explained by conformational change in ssDNA (12, 14) and possibly altered network structure of the sieving medium.



**FIG. 5.** Multiinjection electropherograms of three samples. Samples 1 and 2 were of the CT and CC genotypes, respectively, and sample 3 was a mixture of types CC and TT. The time interval between each of the three injections was 1.2 min. Electrophoretic conditions were as described in the legend to Fig. 4. RFU, relative fluorescence units.

### Electric Field Strength

In most previous studies, capillaries with large i.d. (100  $\mu\text{m}$ ) filled with high-viscosity sieving media have been used (11, 12, 14), and high field strength in such capillaries may cause Joule heating. This increases the temperature in the center of the capillary which may alter the conformation of ssDNA, increase diffusion, and thereby cause peak broadening and insufficient resolution (28). The low-viscosity short-chain PA allowed small i.d. capillaries (50  $\mu\text{m}$ ) to be used. Such capillaries efficiently dissipate heat, and a high electric field (–20 kV) produced a low current (12  $\mu\text{A}$ ). This allowed a systematic investigation of the influence of electric field strength on SSCP (Fig. 3).

At –25 kV, three peaks (denoted by 1, 2, and 3 + 4) were detected in a heterozygous DNA sample. At –20 kV, the last peak was split into two, and four major peaks corresponding to the two mutated strands and the two normal strands were detected at this voltage. At the lower voltages of –15 and –10 kV, the two peaks 1 and 2 coeluted and an additional peak (denoted x) deriving from the CC genotype separated from the other peaks. Apparently, the field strength influenced the resolution of the single strands.

We obtained optimal resolution at an electric field strength of –500 V/cm (–20 kV), in line with the results reported by Chang *et al.* on CE separation of dsDNA in a 50- $\mu\text{m}$ -i.d. capillary (27). Under the optimal conditions (6% PA, –20 kV, 25°C), we obtained about  $3 \times 10^6$  plates/m for some single strands, which is comparable to the plate number reported for capillary gel electrophoresis by others (29).

### Identification of Genotypes

Figures 4A–4E demonstrate the SSCP profiles of homozygous (CC), heterozygous (CT), and homozygous

mutant (TT) DNA samples. The high peaks at about 6 min derived from the primers. The three different genotypes had clearly distinguishable electropherograms. Moreover, the patterns of a heterozygous CT sample (Fig. 4D), a sample containing a mixture of CC and TT specimens (Fig. 4E), and the superimposed traces of a CC and a TT sample (Fig. 4A) were essentially equal. The migration times of the ssDNA fragments were 10–11 min with a RSD (%) < 0.23 between runs ( $n = 10$ ), < 1.8 between days ( $n = 5$ ), and < 1.4 between capillaries ( $n = 3$ ). Sufficiently high resolutions to separate the four main peaks were maintained through about 100 injections on one capillary.

The homozygous genotypes CC and TT were represented by two peaks denoted 1 and 4 (defining genotype CC) and 2 and 3 (defining genotype TT). Notably, the time interval between peaks 1 and 4 (0.185 min) was significantly ( $P < 0.0001$ ) different from the interval between peaks 2 and 3 (0.094 min). Thus, the relative migration of the major peaks 1, 2, 3, and 4 clearly distinguished between the CC and TT genotypes. The heterozygous CT genotype was characterized by the pattern comprising four major peaks. Our data demonstrate that SSCP analysis on CE allows the genotyping of the C677T mutation and may represent an alternative to the conventional PCR-restriction enzyme digestion technique based on gel electrophoresis (19, 20)

### Multiple Injection Mode

To reduce analysis time per sample, we applied a multiple injection mode similar to the one described by Ulvik *et al.* (22). The principle is that injection of new sample(s) takes place before previous sample(s) have eluted from the capillary. The SSCP analysis carried

out by the multiple injection mode is shown in Fig. 5. We performed three injections with 1.2-min intervals in one run. Analysis of three samples was completed in 14 min, i.e., a mean analysis time per sample of <5 min. This injection procedure did not affect the resolution of the ssDNA fragments, and the migration patterns characterizing each genotype were maintained.

## CONCLUSION

A low-viscosity short-chain PA, which affords high resolution of ssDNA, was well suited to SSCP analysis by a commercial CE instrument. Using a capillary with small i.d., high electric field improved the resolution and shortened analysis time. Further reduction in average analysis time per sample was obtained using a multiple injection mode. Our results indicate that SSCP analysis by CE-LIF can be used for rapid detection of the C677T mutation in the MTHFR gene in large numbers of clinical samples. The principles described can be transferred and applied in other SSCP-based analyses.

## ACKNOWLEDGMENTS

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