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Capillary electrophoresis for the detection of known point mutations by single-nucleotide primer extension and laser-induced fluorescence detection

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Abstract

Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) was used to detect known point mutations using the method of single-nucleotide primer extension (SNuPE). Three different point mutations in human mitochondrial DNA associated with Leber's hereditary optic neuropathy (LHON) were detected by annealing a primer immediately 5′ to the mutation on the template and extending the primer by one fluorescently labeled dideoxy terminator complementary to the mutation. By using two or more differently labeled terminators, both the mutant and wild type could be simultaneously detected. The advantages of using CE–LIF for detecting SNuPE reactions include speed and ease of analysis, absence of radioactivity, and potential for automation. © 1997 Elsevier Science B.V.

Keywords: DNA; Point mutation; Single-nucleotide primer extension

1. Introduction

Capillary electrophoresis (CE) continues to be developed for the analysis of biological samples and to address biological problems. Separations of DNA have been performed in a CE format, usually in conjunction with laser-induced fluorescence (LIF) detection. Previous applications include ssDNA detection with fluorescently labeled primers or dideoxy terminators (e.g. DNA sequencing) or dsDNA with fluorescent intercalating dyes [1–3].

An important diagnostic marker of disease is the detection of point mutations in DNA. There are currently a variety of methods available for the determination of known point mutations, including: single-stranded conformation polymorphism, ligase chain reaction, denaturing gradient gel electrophoresis, allele-specific amplification, and direct sequencing [4,5]. Another effective method of point mutation detection involves annealing a primer immediately 5′ to the position of possible point mutation on the template and then extending the template by the complementary labeled base with polymerase (see Fig. 1). When the detection primer is labeled with biotin and is immobilized on beads or in wells coated with avidin, this method is known as minisequencing [6]. If, however, the extension reaction is run in solution, the procedure has been called single-nucleotide primer extension (SNuPE) [7,8]. Many applications of both the solid-phase [9–13] and solution-phase [14–19] primer extension by a single base have been reported, including extension with fluorescently labeled dideoxy terminators [20–22]. SNuPE is an effective technique

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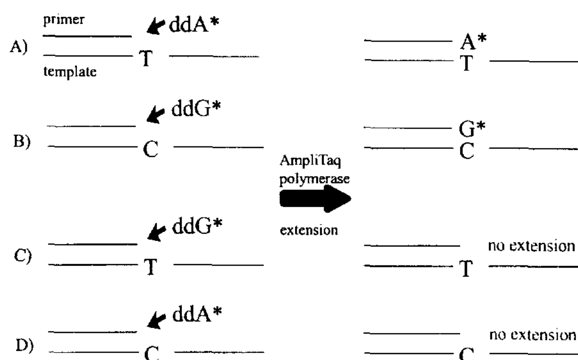

Fig. 1. Diagram of the principle of single-nucleotide primer extension (SNuPE). Addition of the complementary primer and fluorescently labeled dideoxy terminator results in extension of the primer by one nucleotide to yield a fluorescent product. (A) Mutant template+mutant terminator, (B) wild-type template+wild-type terminator, (C) mutant template+wild-type terminator, and (D) wild-type template+mutant terminator.

for the detection of known point mutations because it is a relatively simple single-step reaction.

Our laboratory has been interested in mutation detection by CE for some time [23,24]. Because of its speed and automation, CE should be a suitable method for the detection of fluorescently labeled single-nucleotide primer extension products. The goal of this work was to detect, using CE–LIF, three known point mutations in mitochondrial DNA (at positions 3460, 11 778 and 14 459) associated with Leber hereditary optic neuropathy (LHON) [25]. It will be demonstrated that CE–LIF is an effective and rapid means of analysis for the SNuPE procedure.

2. Experimental

2.1. Instrumentation

The CE instrument with one photomultiplier tube (PMT) was assembled in-house from commercially available components. A 500-mW multiline argon ion laser (Laser Physics, Salt Lake City, UT, USA) was used in conjunction with a 514-nm narrow band pass filter (Oriel, Stratford, CT, USA) as the excitation source. The laser beam was directed onto a mirror (Newport, Fountain Valley, CA, USA) positioned 45° to both the incoming beam and the lens. This mirror directed the beam upwards through the lens to focus the light inside the capillary. The fluorescent emission was collected by a 40×0.65 numerical aperture microscope objective (Oriel) and focused through a 520-nm long band pass filter to block the laser excitation light, then through a 560-nm narrow band pass filter, and finally onto the PMT (Hamamatsu, Bridgewater, NJ, USA). The PMT was monitored with an Oriel Detection System Model 7070, and the signal was converted by a PE Nelson Model 970 Interface (Perkin-Elmer, Cupertino, CA, USA) and then transferred to an NEC PowerMate 386/33i PC equipped with TurboChrom 3.2 Chromatography Workstation (Perkin-Elmer). The high voltage power supply was a Model PS/MJ30P0400-11 from Glassman High Voltage (Whitehouse Station, NJ, USA). The photodiode array (PDA) system has been described elsewhere [26]. In addition, automated runs were performed on a Beckman P/ACE 5510 equipped with an argon ion laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA, USA). Excitation was at 488 nm, and detection was performed at 580 nm.

2.2. Reagents

Tris, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), EDTA, and urea (all analytical or electrophoresis grade) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. SeaKem Gold agarose was obtained from FMC (Rockland, ME, USA). The water used was deionized (18.2 MΩ) by an Alpha-Q water purification system (Millipore, Worcester, MA, USA). The AmpliTaq FS Cycle Sequencing Core Kit was purchased from Perkin-Elmer/Applied Biosystems (Foster City, CA, USA).

2.3. Capillary electrophoresis conditions

All capillaries were 365 μm O.D./100 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) and covalently coated with poly(vinyl alcohol) (PVA) [27]. For the PMT and PDA instruments, the capillaries had the following measurements: l=15 cm, and L=25 cm. On the P/ACE, the capillary was l=20 cm, L=27 cm. The buffer was 50 mM Tris, 50 mM TAPS, 2 mM EDTA and 3.5 M urea. A 10% (w/v) solution of 360 000 M_r polyvinylpyrrolidone

Table 1
Wild-type mitochondrial DNA sequence at LHON point mutations[a]

LHON mutation	Positions	Sequence	
3460	3435–3467	upper	CGG GCT ACT ACA ACC CTT CGC TGA CGC CAT AAA
		lower	GCC CGA TGA TGT TGG GAA GCG ACT GCG GTA TTT
11778	11 753–11 785	upper	TCA AAC TAC GAA CGC ACT CAC AGT CGC ATC ATA
		lower	AGT TTG ATG CTT GCG TGA GTG TCA GCG TAG TAT
14459	14 434–14 466	upper	CTC AGG ATA CTC CTC AAT AGC CAT CGC TGT AGT
		lower	GAG TCC TAT GAG GAG TTA TCG GTA GCG ACA TCA

[a]These LHON mutations are always **G→A** on the upper strand (and **C→T** on the lower strand). Primers were designed to probe the lower strand. All sequences were obtained from MITOMAP v3.0 (Genbank Accession #J01415).

(PVP, Sigma) prepared in this buffer served as the separation matrix, which was replaced after each run. The electric field strength was 300 V/cm, and samples were injected electrokinetically for 5 s at the running voltage. Both the in-house PMT and the PDA systems were operated without temperature control of the capillary, but the P/ACE-LIF was controlled at 25°C.

2.4. Amplification of templates

Mitochondrial DNA samples, each containing one specific point mutation at either position 3460, 11 778, or 14 459, were the generous gift of Dr. Michael Brown, Emory University (Atlanta, GA, USA). Table 1 presents the human mitochondrial DNA sequence at the locus of each mutation. As performed previously [24], three DNA fragments were amplified from the three mitochondrial DNA samples for use as templates in the SNuPE reactions. Each double-stranded template contained one specific point mutation. Oligo 5.0 software (National Biosciences, Plymouth, MN, USA) was used to optimize primer design. Each sample was amplified using an upper (U) and lower (L) primer to produce a double stranded template of the designated length (see Table 2). All primers were synthesized and purified by Midland Certified Reagent Company (Midland, TX, USA) and dissolved in water to a concentration of 20 pmol/μl. To produce the templates, 5 μl of Pfu buffer (200 m*M* Tris–HCl, pH 8.8, 100 m*M* KCl, 100 m*M* $(NH_4)_2SO_4$, 20 m*M* $MgSO_4$, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin) (Stratagene, La Jolla, CA, USA), 2 μl of each primer (40 pmol), 1 μl mitochondrial DNA, 34 μl of water and a wax bead (AmpliWax PCR Gem, Perkin Elmer, Norwalk, CT, USA) were heated to 94°C for 2 min and then cooled to room temperature. After the wax had solidified, 1.25 μl of each dNTP plus 1 μl of cloned Pfu DNA polymerase (2.5 U/μl) (Stratagene) was added, and the mixture was subjected to 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 2 min, followed by a 7-min extension at 72°C and soaking at 4°C. Each template batch was tested on a 1% SeaKem Gold agarose gel (FMC) in a MINNIE Submarine Agarose Gel Unit (Hoefer Scientific Instruments, San Francisco, CA, USA) with 1× TBE (89 m*M* Tris, 89 m*M* boric acid, 2.5 m*M* EDTA) running buffer. Ethidium bromide was added to both the buffer and gel at a dilution of 2×10^4 times (0.5 μg/ml). Equal 4-μl portions of the template reactions and a molecular

Table 2
Primers for LHON template synthesis

LHON mutation	Product length (bp)	Positions	Primers
3460	199	3367–3545	U: ATG CTT ACC GAA CGA AAA L: TTC ATA GTA GAA GAG CGA TGG
11778	300	11 532–11 811	U: CCT ACC CCT TCC TTG TA L: AAA AAG CTA TTA GTG GGA GTA
14459	406	14 156–14 5 41	U: CCC CGA GCA ATC TCA AT L: TCG GGT GTG TTA TTA TTC TGA

weight standard, DNA Mass Ladder (Gibco-BRL, Gaithersburg, MD, USA) were each mixed with 0.5 μl of loading buffer. The gel was run at 10 V/cm for approximately 1 h and visualized on a UV trans-illuminator (Hoefer Scientific Instruments). The amount of template produced for each of the mutations was 25–50 ng/μl, based on the intensity of the ladder standard. Then each template reaction was digested with 8 μl of shrimp alkaline phosphatase (USB, Cleveland, OH, USA) and exonuclease I (USB) at 37°C for 1 h and deactivated at 65°C for 20 min to digest the primer and deactivate the nucleotides. Finally, enzyme and salts were removed by treatment with Ultrafree-MC Probind filtration (Millipore, Bedford, MA, USA) and CentriSpin 10 columns (Princeton Separations, Adelphia, NJ, USA).

2.5. Single terminator extension

All single-nucleotide extension reactions were run using components from the AmpliTaq FS Cycle Sequencing Core Kit (Perkin-Elmer/Applied Biosystems). The single SNuPE reactions to detect the mutated base on the lower strand (see Table 1) used one R6G-labeled dye terminator, ddA*. All primers (see Table 3) were purchased from Midland Scientific and dissolved in water to a concentration of 20 pmol/μl. For each SNuPE reaction, 3 μl (~100 ng) of template was combined with 4 μl of 5× AmpliTaq FS buffer, approximately 10 pmol of primer (either a single primer or three primers for positive control), 1 μl of ddA*, 1 μl of AmpliTaq FS (8 U/μl), and diluted with water to a total volume of 20 μl. The reactions were then subjected to the 'touchdown' program ((1) 94°C for 1 min; (2) 92°C for 30 s; (3) 70°C for 40 s with −0.5°C/cycle; (4) go to step (2) 19×; (5) 92°C for 30 s; (6) 60°C for 40 s with +1 s/cycle; (7) go to step (5) 19×) on a PTC100 thermocycler (MJ Research, Watertown, MA, USA). This program was used to rapidly cover a range of annealing temperatures without requiring the optimization of each annealing temperature.

3. Results and discussion

3.1. Template preparation

The experiments were designed to test the detection of both single and multiple dye terminator extensions of three well-characterized point mutations in mitochondrial DNA (3460, 11 778 and 14 459) associated with Leber hereditary optical neuropathy (LHON). Instead of using the entire mitochondrial DNA as a template for the SNuPE reactions, three separate, smaller LHON templates were prepared by PCR amplification, each containing a separate point mutation. These templates, LHON 3460, LHON 11 778, and LHON 14 459, were 199, 300 and 406 bp in length, respectively [24]. As previously mentioned, the estimated concentration of

Table 3
Primers for single nucleotide primer extension of LHON mutations[a]

LHON mutation	Primer	Sequence
3460	K1	TAC TAC AAC CCT TCG CTG AC
Internal standard	K1A	TTC ACC AAA GAG CCC CT
Internal standard	K1B	ACA TCT ACC ATC ACC CTC TAC ATC
11778	K2	CTA CGA ACG CAC TCA CAG TC
Internal standard	K2A	ATC CAA ACC CCC TGA
Internal standard	K2B	TTA CAT CCT CAT TAC TAT TCT GCC T
14459	K3	GAT ACT CCT CAA TAG CCA TC
Internal standard	K3A	CCC CGC ACC AAT AGG
Internal standard	K3B	TAA CTA CTA CTA ATC AAC GCC CAT A

[a]K1–K3 are the primers used for detection of the LHON 3460, 11 778 and 14 459 mutations, respectively. The A and B primers (K1A, K1B, etc.) are the shorter and longer primers, respectively, used for internal standards bracketing the SNuPE product which were simultaneously annealed to their respective templates upstream of a thymidine at different locations on the LHON templates. All primer sequences are listed 5′→3′.

these templates prior to purification was 25–50 ng/μl, based on a comparison to a DNA standard ladder.

3.2. Single dye terminator reaction

The initial experiment involved adding ddA* to the reaction mixture of template, primer and enzyme. The primers for the SNuPE reactions were designed to be 20 bases in length with 3′ ends that would anneal immediately upstream of the mutation site. Referring to Fig. 1, if the mutation were present, the ddA* would be incorporated and the fluorescent 21-mer detected. Alternatively, if no mutation were present, the ddA* would not be incorporated, and no fluorescent product would be observed.

A 'touchdown' program on the thermocycler was utilized to circumvent the problem of finding the optimum annealing temperature for each reaction, and this approach yielded a significant amount of fluorescently labeled 21-mer for each mutation. This program was a two-step procedure (denature; anneal/extend) where a range of annealing temperatures was covered in one protocol by decreasing the anneal/extend temperature by 0.5°C/cycle during the first half of the program (19 cycles). The amplification was linear during the 40 cycles, and the total cycling time was about 1.5–2 h. Ultimately, exact temperature protocols can be used for screening purposes and the cycling time thus reduced.

At the conclusion of each SNuPE reaction, primer extension product, free dye terminator, enzyme, and a considerable amount of salt remained. Fig. 2 shows the results of samples before and after desalting with a CentriSpin 10 column. All of these runs were made on a manual CE system with a PMT detector. In the first three electropherograms (Fig. 2A–C), the signal for the extension product can be seen at a migration time of approximately 6 min, while the remaining free ddA* peaks (confirmed by injection of free ddA*) can be detected between 12 and 20 min. Upon desalting, the signal for each primer extension product was dramatically increased, and the free ddA* peaks virtually disappeared. It is well-known that salt and other contaminants with high electrophoretic mobility compete when samples are electrokinetically injected in CE [28]. The small peak which migrated before the large SNuPE peak in Fig. 2 is an

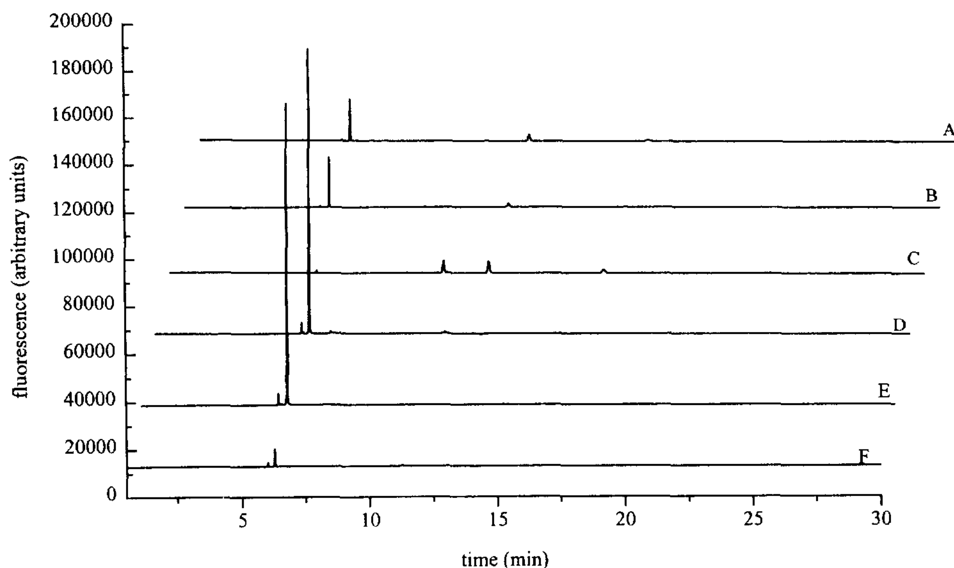

Fig. 2. CE–LIF of non-desalted and desalted SNuPE reactions prior to injection. (A–C) Not desalted using templates with LHON mutations 3460, 11 778, and 14 459, respectively. (D–F) Desalted prior to CE injection, again using LHON 3460, 11 778 and 14 459, respectively. The small signal for F (14 459 desalted) is discussed in the text. The electropherograms have been displaced along the x-axis for clarity. See Section 2.3 for CE conditions.

impurity associated with the template. Different template batches showed small impurity peaks before or after the main SNuPE peak.

The LHON 14 459 reaction (Fig. 2C and F) showed a small amount of primer extension product relative to the other two reactions. A higher template concentration (relative to LHON 3460 and 11 778) was suspected to adversely shift the competition between template and primer and reduce the amount of primer that annealed. This effect was observed by analysis of the template by CE with ultraviolet (UV) detection and dilution of a LHON template prior to SNuPE. It can be seen in Fig. 3 that when concentrated LHON 3460 was used in the SNuPE reaction (Fig. 3A), a zone of multiple peaks around 8 min was detected rather than the expected peak at approximately 6 min. When the same template was diluted 10× in water and used in an extension reaction, the expected product peak was clearly visible at 6 min (Fig. 3B), and a further dilution of template to 1/50th of the original concentration yielded an even higher signal (Fig. 3C). Because of the high sensitivity of CE–LIF, dilute template concentrations can be easily used while still detecting the primer extension product.

3.3. Controls

The preliminary reactions yielded a single peak when a mutation was present. Recognizing the separation power of CE, two additional primers could be added to the SNuPE reaction to function not only as internal standards, but also as sizing standards to bracket the mutation product. These control primers were also immediately 5′ to a thymidine on other regions of the LHON templates, with the next nucleotide to be incorporated a deoxyadenosine, similar to the 20-mer primers K1–K3 in Table 2. Moreover, by selecting one primer shorter and another one longer than K1–K3, the additional two peaks should bracket the 21-mer extension product and function as size markers (see Table 3). The control primers were designed to have similar annealing temperatures as K1–K3 so that the reaction could be run at a single annealing temperature if optimized. Fig. 4 shows the electropherograms of the internal standard reactions for LHON 14 459. The internal standards showed that additional primers could anneal at various locations along the template and incorporate ddA*, demonstrating the potential for multiplexing.

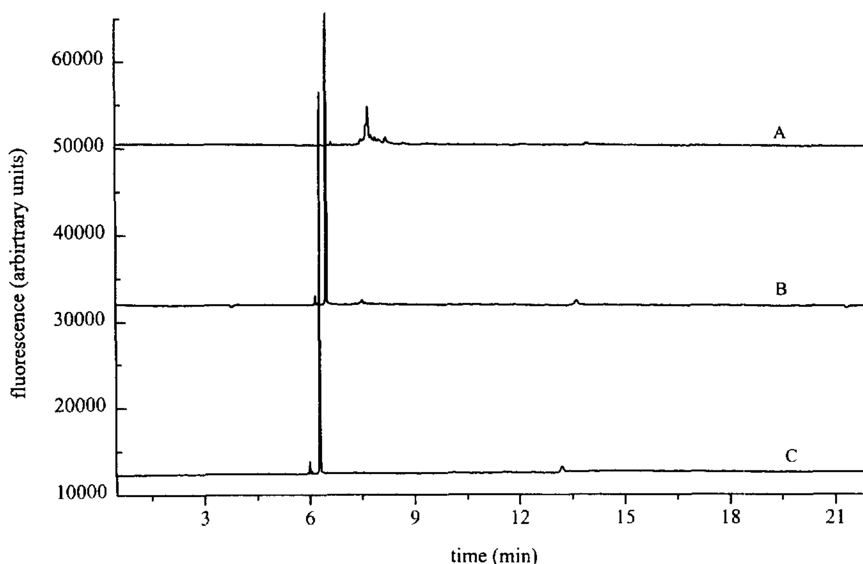

Fig. 3. Effect of template concentration on SNuPE of LHON 3460 by CE–LIF. (A) Undiluted LHON 3460 template, (B) 10× diluted LHON 3460 template and (C) 50× diluted LHON 3460 template. See Section 2.3 for CE conditions.

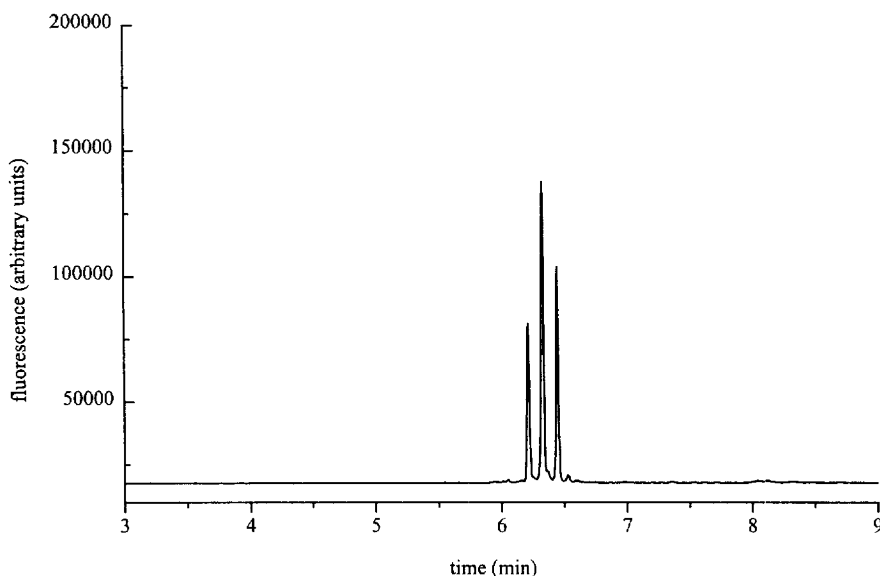

Fig. 4. Positive controls with shorter (K3A) and longer (K3B) primers for simultaneous extension at other regions of the LHON 14 459 template adjacent (5′) to a thymidine. The additional primers were chosen so that all three primers would have similar annealing temperatures. See Table 3 for primer sequences. See Section 2.3 for CE conditions.

In order to ensure that extension by a single dye terminator would take place only under the conditions of the analysis, a set of negative controls for LHON 14 459 was prepared with the following: (a) LHON 11 778 template, (b) K2 primer, (c) ddT* terminator, (d) no template, (e) no primer, (f) no terminator and (g) no polymerase. For all of these reactions, the buffer concentration and final volume was maintained constant. A large primer extension peak was only detected when the proper template, primer and terminator were added; no other solution produced the product peak. These results demonstrated that the SNuPE reaction was valid to detect the mutation.

3.4. Simultaneous extension with different dye terminators

Because the original mitochondrial DNA sample was heteroplasmic, it was expected that both the mutant and wild-type forms would be present in the resulting template for each mutation. Both the mutant and wild-type strands could be detected concurrently if ddA* and ddG* dye terminators were added to the same SNuPE reaction. Because each of the four dideoxy terminators is labeled with a different fluorophore, it was possible to simultaneously detect primers extended by A, C, T and G. Reactions were run for all three LHON templates. Fig. 5 shows the SNuPE reaction for LHON 14 459 when both ddA* and ddG* were added to detect both the mutant and wild type simultaneously on the CE-photodiode array instrument. The solid trace is for the ddA* (mutant) channel and the dotted for ddG* (wild type). It is noted that, although the extension products are the same length, they migrate at different rates because of the different mobilities of the two dye terminators.

To demonstrate the generality of the approach, all three SNuPE reactions were performed, desalted and then run on the automated commercial Beckman P/ACE. Fig. 6 shows the extension products from LHON 3460, LHON 11 778, and LHON 14 459, respectively. When three injections were made of each sample, replacing the PVP matrix each time, the migration time variations were 0.1% for LHON 3460, 0.6% for LHON 11 778, and 1.3% for LHON 14 459, respectively. The automated CE–LIF method with the P/ACE provides good sensitivity for detect-

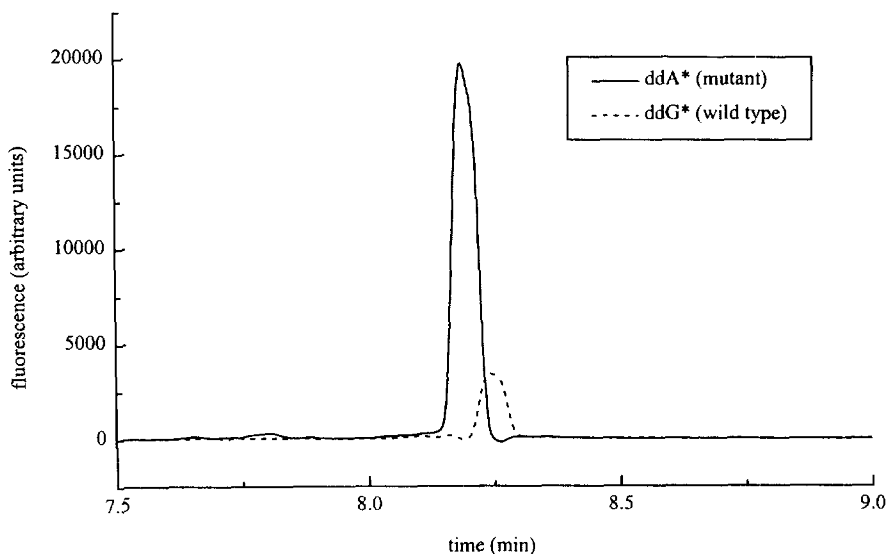

Fig. 5. Simultaneous detection of LHON 14 459 mutant and wild type with ddA* and ddG* dye terminators. By using terminators with different fluorescent dyes and detecting with a photodiode array, both the mutant and wild type could be identified in the same experiment. See Section 2.3 for CE conditions.

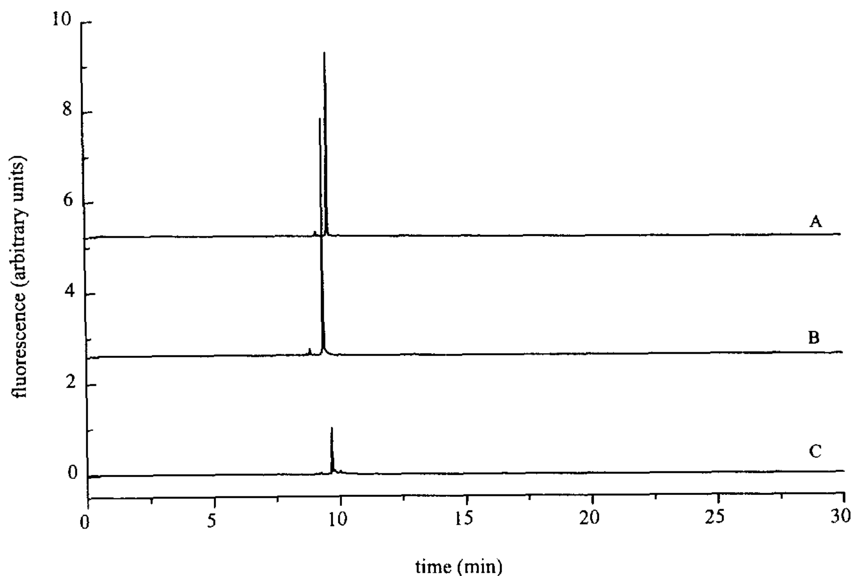

Fig. 6. Desalted SNuPE reactions on an automated CE instrument (Beckman P/ACE). (A) LHON mutation 3460, (B) LHON mutation 11 778 and (C) LHON mutation 14 459. See Section 2.3 for CE conditions.

ing these reactions as well as increased reproducibility in migration times because of capillary thermostatting.

4. Conclusion

This preliminary study has demonstrated that CE–LIF has the potential to be an effective tool in detecting the products of single-nucleotide primer extension with fluorescent dye terminators. Advantages of this technique include the speed and ease of analysis, the absence of radioactivity, and the potential for automation. Similar methods of point mutation detection can be analogously performed by CE–LIF, including variable termination, competitive priming with end mismatch, etc. There are several ways to increase the power of this method. By using multiplex detection, several different point mutations could be detected in the same reaction tube by different length primers. An alternative approach would be to run the reactions separately and pool them to analyze by CE–LIF. This would allow individual control of the annealing conditions of each primer, if necessary. With the use of four or more fluorescently labeled dye terminators, one can easily detect primers extended by different bases. In addition, with increased electric field and shorter capillaries, very rapid analysis of SNuPE products would be possible. Finally, the use of capillary array electrophoresis (CAE) would increase throughput by enabling the simultaneous analysis of multiple reactions.

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