Electrophoretic separations of polymerase chain reaction-amplified DNA fragments in DNA typing using a capillary electrophoresis–laser induced fluorescence system

Kannan Srinivasan

Biotechnology Division, Chemical Sciences and Technology Laboratory, National Institute of Standards and Technology, Room A 353, Building 222, Gaithersburg, MD 20899 (USA)

James E. Girard

Department of Chemistry, The American University, Washington, DC 20016 (USA)

Patrick Williams, Rhonda K. Roby and Victor W. Weedn

Armed Forces DNA Identification Laboratory, Washington, DC 20306 (USA)

Sam C. Morris

Beckman Instruments, Columbia, MD 21045 (USA)

Margaret C. Kline and Dennis J. Reeder**

Biotechnology Division, Chemical Sciences and Technology Laboratory, National Institute of Standards and Technology, Room A 353, Building 222, Gaithersburg, MD 20899 (USA)

ABSTRACT

Analysis of polymerase chain reaction (PCR)-amplified DNA fragments for human identification requires high-resolution separation and efficient detection of amplified alleles. Capillary electrophoresis (CE) with its speed, automation, high resolution and efficiency shows promise for analyzing the amplified DNA fragments. CE with UV detection, however, suffers from lack of detector sensitivity owing to the limited detection path length of the capillary. By the use of intercalating dyes (TOTO and YOYO) a laser-induced fluorescence (LIF) detection system can provide much greater sensitivity for detecting DNA fragments. Femtogram quantities of dsDNA (ΦX174 *Hae*III restriction digest mixture) per nanoliter of injected volume have been detected. Application of CE–LIF to analysis of PCR-amplified DNA fragments from three different genetic loci (apolipoprotein B, VNTR locus D1S80, mitochondrial DNA) is shown here. Further, the resolving power of a polymer-network capillary separation system is compared to that of a capillary-gel separation system.

---

* Corresponding author.

INTRODUCTION

The ability of the polymerase chain reaction (PCR) to make multiple copies from minute quantities of DNA extracted from blood, hair or semen has enabled new forensic approaches in human identification [1,2]. Generally DNA identification is based on differences in inherited genetic markers. Analytical techniques are employed following the PCR reaction to distinguish individuals based on these genetic differences. Techniques such as slab gel electrophoresis, various hybridization strategies, dot blot assays and direct sequencing are used to analyze the amplified PCR products [3]. Capillary electrophoresis (CE), due to its automation, speed and ability to analyze small sample volumes shows high potential for PCR product analysis.

At present, DNA fragments are analyzed by two major CE approaches: a polymer-network capillary separation system [4–7] and a capillary-gel separation system [8–10]. The polymer-network system is based on buffers containing additives such as methylcellulose, hydroxypropylmethylcellulose, dextran or polyethylene glycol, which create a gel-like matrix inside the capillary. The migrating DNA fragments interact with the dynamic pores of this matrix resulting in separations based on size. The capillary-gel system on the other hand, uses an *in situ* polymerized acrylamide gel cross-linked to the walls of the capillary. Fixed pores in the gel retard migration of the DNA fragments in a manner similar to that in slab gels and, consequently, results in separations based on size. These approaches have their own limitations. The gel-filled capillaries are difficult to manufacture with sufficiently large pores, suffer from matrix contraction problems and often have problems associated with air bubbles, thus restricting their useful life. Nevertheless, gel-filled capillaries have proven useful in high-resolution separation of oligonucleotides or of smaller double-stranded DNA (dsDNA) fragments. Polymer-network capillaries owing to their wider range of pore sizes offer comparable or better separations to the gel-filled capillaries [11]. They are, however, restricted by the viscosity of the buffer–polymer mixtures, making it difficult to completely fill the capillaries. In addition to the buffer, capillary dimensions, applied field, injection, the amount of polymer as well as the chain length of the polymer affect the separations in both systems.

Since the validity of DNA typing increases with the number of marker systems used, we tested both CE approaches for their ability to effectively separate polymorphic PCR-amplified DNA fragments. We used three different genetic systems: (1) apolipoprotein system (apo B) [700–1000 base pairs (bp)] [12], (2) Variable number tandem repeat (VNTR) locus D1S80 (300–700 bp) [13] and (3) mitochondrial DNA system (mtDNA) (130–140 bp) [14]. The goal of this study was to find a separation system that would be useful in analyzing PCR-amplified DNA fragments from loci that differ in number of base repeats and in size.

Initial efforts to analyze PCR-amplified products failed when we used UV detection at 260 nm both in capillary gels and in polymer-network capillaries [15]. Nonetheless, we were able to analyze the same products using slab-gel electrophoresis with silver staining (data not shown). We have observed that some PCR-amplified samples are not amenable for analysis by UV, even when samples are desalted or dialyzed [16]. Failure to observe PCR products could be a result of amplification problems associated with the PCR or lack of sensitivity of UV detection. To compete with the sensitivity of silver staining, we employed a laser-induced fluorescence (LIF) detection system.

The utility of a CE–LIF system for analyzing PCR products was first shown by Schwartz and Ulfelder [17] who used thiazole orange dye in the CE run buffer. They showed roughly 400 times better sensitivity than that achieved with UV. We investigated asymmetric cyanine dyes TOTO (TOTO-1) and YOYO (YOYO-1) (Molecular Probes, Eugene, OR, USA)[a] based on a report of enhanced detection of DNA fragments

___

[a] Certain commercial equipment, instruments and materials are identified in this paper in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument or equipment identified is necessarily the best available for the purpose.

by Rye et al. [18]. TOTO and YOYO are non-fluorescent dyes when free but fluoresce upon binding to dsDNA; this property makes them useful in applications that require high sensitivity [19,20]. Our studies showed suitability of TOTO and YOYO in detecting PCR-amplified DNA fragments [21]. We have also shown the analysis of PCR-amplified DNA fragments without any sample pretreatment step (dialysis or filtration) in an earlier publication [22]. Our approach included prestaining the samples in addition to incorporating a small amount of the dye to the run buffer. In this study we compare the polymer-network capillaries to gel-filled capillaries for their usefulness in DNA typing by using a CE–LIF system with TOTO.

EXPERIMENTAL

Instrumentation

A P/ACE system 2100 CE apparatus coupled with an Ar ion laser source (Beckman Instruments, Fullerton, CA, USA) was used with negative source polarity. The laser source provides 4 mW excitation at 488 nm. A 530-nm band pass filter was used as an emission cut-off filter. Data were collected at 5 Hz and analyzed using System Gold software. The temperature was set at 25°C and electrophoretic runs were performed at the conditions specified in the figure captions. The coated capillary was flushed with buffer for 3 min between runs.

Capillaries

The polymer network separations were performed in a DB-17 (50% phenyl, 50% methyl silicone)-coated capillary (J&W Scientific, Sunnyvale, CA, USA). The gel separations were performed in 3%T, 3%C^a urea and non-urea based gel-filled capillaries (J&W Scientific). The dimensions of the capillaries are indicated in the figure captions.

Dyes

TOTO was supplied as a 1 mM solution. [TOTO-1 is 1,1'-(4,4,7,7-tetramethyl-4,7-dia-

zundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide].

Buffers

CE run buffer (1 ×) for all the polymer-network separations consisted of 89 mM Tris, 89 mM boric acid, 2 mM EDTA at pH 8.5. To this buffer was added 0.5% (w/w) methylcellulose (Sigma, St. Louis, MO, USA). Just prior to the CE analysis, 1 µl of a 1 mM dye solution of TOTO was added to 15 ml of the run buffer. Gel separations were performed on (urea and non-urea based) µPage buffers (J & W Scientific) and supplemented by the addition of 1 µl of 1 mM TOTO to 15 ml of the buffer.

Polymerase chain reaction protocol

DNA was extracted using a modified Chelex-100 procedure [23] by incubating three human hair roots in a 5% Chelex-100 suspension overnight at 56°C. After a 5-min heating in a boiling-water bath, the samples were ready for PCR amplification on a Perkin-Elmer Cetus 9600 thermal cycler. The region coding for the genetic system D1S80 [13] and apo B [12] were amplified using prescribed primers at a concentration of $2.5 \cdot 10^{-5}$ µM MgCl$_2$ was added to achieve a 2.0 mM concentration in the PCR reaction tube. Each cycle was programmed to denature at 94°C for 10 s, anneal for 20 s at 68°C and extend at 72°C for 30 s. The samples were analyzed after 30 amplification cycles. The mtDNA PCR samples were amplified using DNA extracted from whole blood with 100 ng of prescribed primers [14] in a total volume of 50 µl. The conditions of PCR include 1 cycle at 94°C for 30 s followed by 30 cycles programmed to denature at 94°C for 10 s, anneal at 50°C for 10 s and extend at 72°C for 1 s.

Samples and standards

Samples of ΦX174 *Hae*III DNA (BRL Labs., Gaithersburg, MD, USA) restriction fragments were diluted to the required concentration using deionized water. The PCR samples were pretreated on a 0.025 µm pore size, 25 mm diameter MF-Millipore membrane filter (Millipore, Milford, MA, USA) for 20 min [16]. For prestaining, the DNA samples were always added to

---

^{a} T = [2.91 g acrylamide monomer + 0.09 g N,N'-methylene-bisacrylamide (Bis)]/100 ml solution; C = g Bis/%T.

the dye as recommended by Molecular Probes, at the desired DNA base pair-to-dye molar ratio of 5:1. The samples were incubated at room temperature for at least 20 min before analysis. The PCR samples were prestained by diluting 1 μl of the 1 mM dye to 1 ml and adding 1–20 μl of the sample.

RESULTS AND DISCUSSION

The purpose of this work was to identify a single separation method for analysis of PCR-amplified DNA fragments of forensic origin (irrespective of the marker system under consideration). High-resolution separation and sensitive detection of the amplified products are primary requirements for typing. Our earlier work has shown the suitability of methylcellulose polymer-based separation systems in analyzing VNTR locus D1S80 alleles with TOTO as an additive. Using the same system we show here

the analysis of both apo B DNA (14 bp repeat) and mtDNA (2 bp repeat).

Fig. 1 (inset) demonstrates the sensitivity of the CE–LIF system with an analysis of a standard DNA ladder (0.5 pg/μl) consisting of 11 fragments. Femtogram amounts are detected per nanoliter of injected volume. The peak marked 118 contains roughly 2% of the total amount of DNA injected (assuming that equal amounts of the different fragments are injected). The migration times at two different concentrations 150 pg/μl and 0.5 pg/μl showed very little difference, indicating that the effect of DNA concentration on migration was minimal in the presence of TOTO under the conditions shown here. At low DNA concentrations, however, we have observed that some smaller-sized fragments showed higher relative fluorescence than the larger ones. This is possibly associated with differences in dye binding kinetics. Reproducibility studies with a ΦX174 HaeIII digest using an internal standard (PCR-LIF kit, Beckman In-
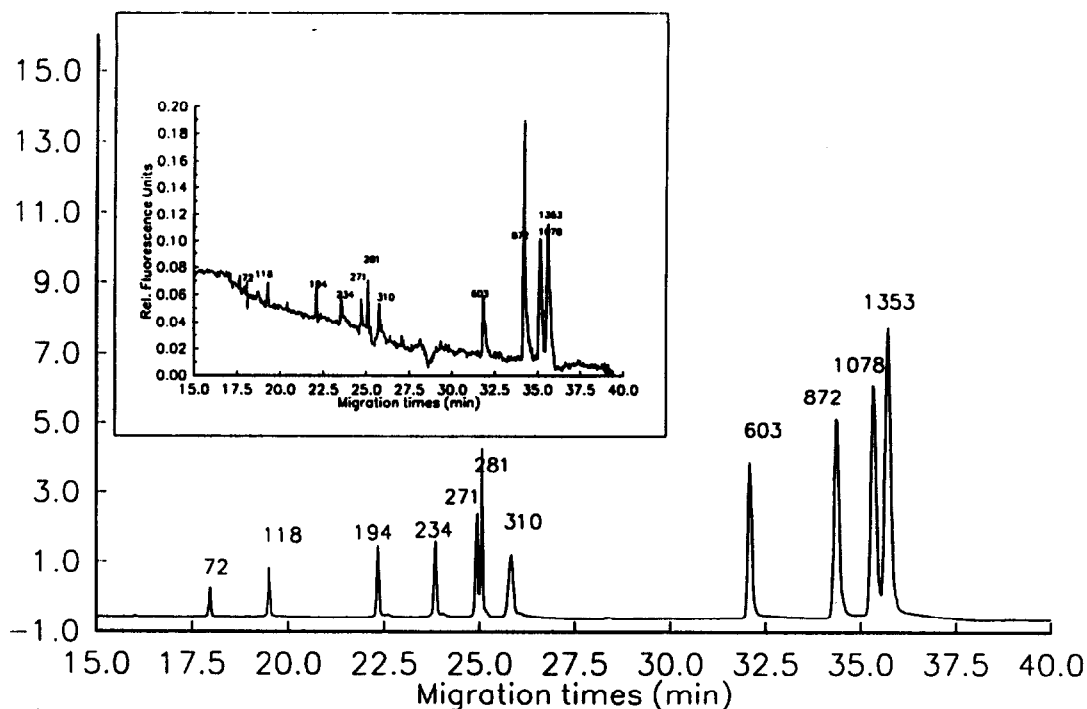

Fig. 1. Analysis of a ΦX174 DNA restriction fragment mixture with sizes as indicated. Conditions: A DB-17 capillary from J & W Scientific (40 cm × 100 μm); electrophoretic run at constant voltage of 10 kV; injection at 10 kV for 5 s; Sample 0.15 ng/μl ΦX174 DNA HaeIII digest. Inset shows the analysis of the same digest at a concentration of 0.5 pg/μl under the same conditions.

struments) showed a relative standard deviation (R.S.D.) of less than 1.5% in migration times ($n = 10$).

Resolution and DNA typing

In DNA typing applications it is important to differentiate heterozygous from homozygous individuals. Lack of resolution of the PCR-amplified fragments would result in inaccurate typing results. To optimize resolution of apo-B PCR-amplified products, we varied the applied voltage. Reducing the field strength improved separations of the larger fragments at the expense of time as shown in Fig. 2. In addition to voltage effects, increased resolution may also be attributed to increased interactions with the sieving matrix. Fig. 3 shows the effect of sample load on separation; increased sample loads decreased both resolution and efficiency of separa-

tions. A 10 kV s injection was found to be optimal for analyzing PCR-amplified DNA fragments.

Analysis of the apo B PCR-amplified DNA fragments from four different individuals is shown in Figs. 4 and 5. Using the polymer-network capillary separation system we could distinguish all four. Average efficiencies of greater than $1.5 \cdot 10^6$ plates/m were achieved. A 3%T, 3%C urea-based gel-filled capillary, when used for analyzing one of the apo B samples, resulted in multiple peaks with excessive band broadening (Fig. 6). Additionally, the same PCR-amplified products when analyzed on a 3%T, 3%C non-urea gel were not optimally resolved and showed broad peaks as shown in Figs. 7 and 8.

We hypothesize that the 3%T, 3%C urea-based gel-filled capillaries are not as effective in
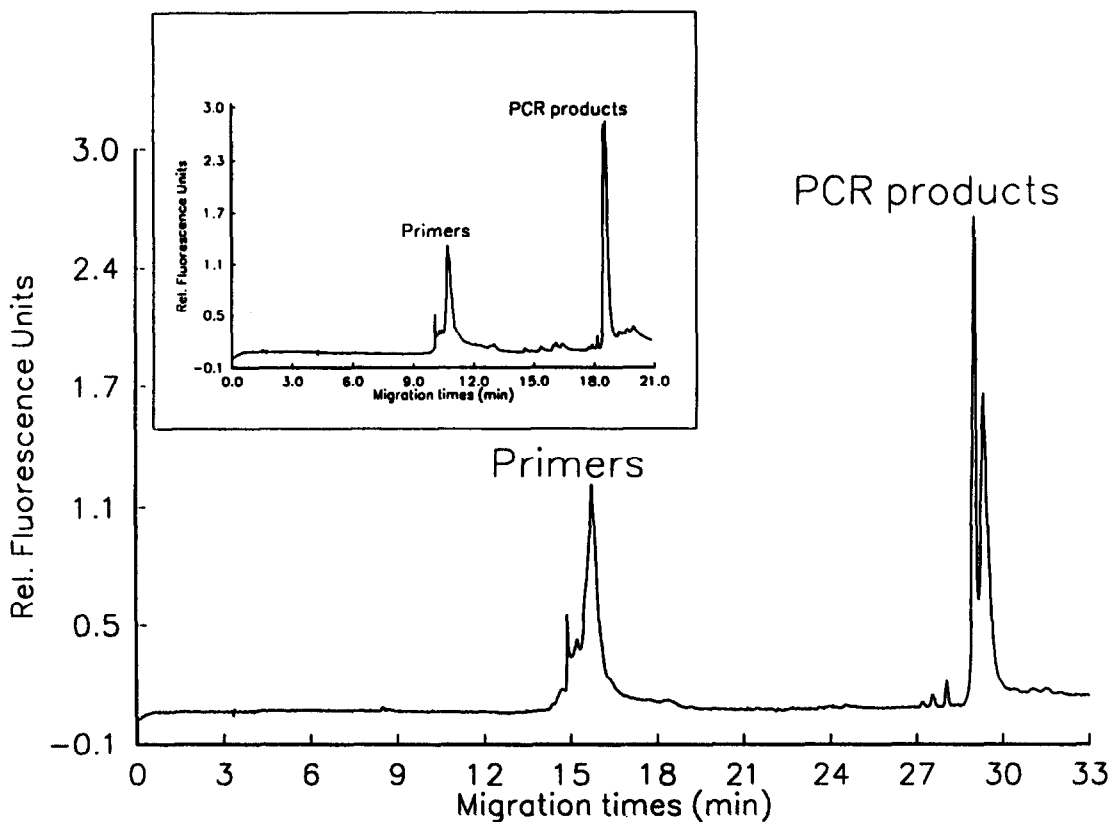

Fig. 2. Analysis of PCR-amplified DNA fragments coding for the apo B gene at two different voltages. Conditions: A DB-17 capillary from J & W Scientific (50 cm × 50 μm); electrophoretic run at constant voltage of 10 kV; injection at 10 kV for 5 s. Inset shows separation of the same product at constant voltage of 15 kV; injection at 10 kV for 5 s.
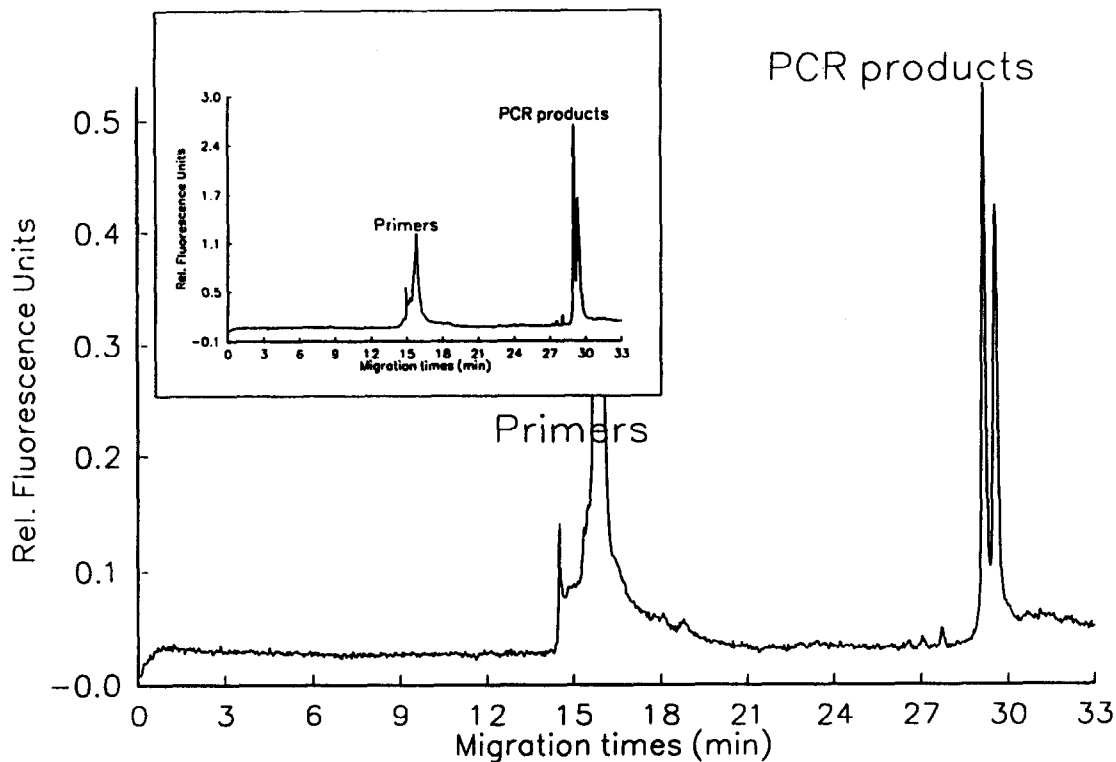
Fig. 3. Analysis of PCR-amplified DNA fragments coding for the apo B gene at two different injections. Conditions: A DB-17 capillary from J & W Scientific (50 cm × 50 μm); electrophoretic run at constant voltage of 10 kV; injection at 10 kV for 1 s. Inset shows separation of the same product at 10 kV; injection at 10 kV for 5 s.
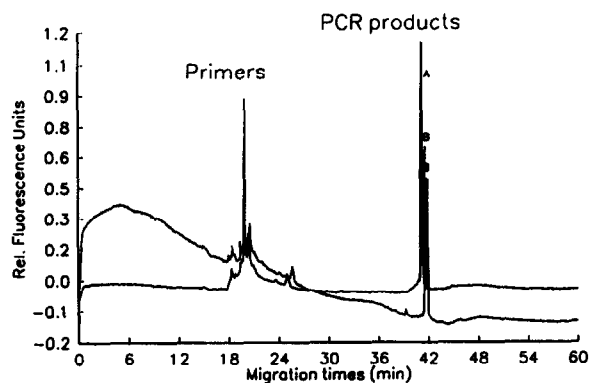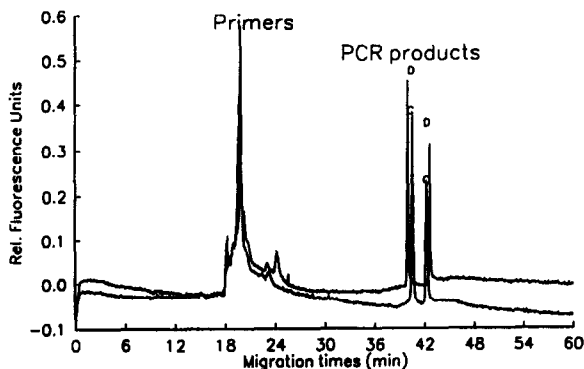

Fig. 4. Analysis of PCR-amplified DNA fragments coding for the apo B gene of a homozygous and a heterozygous individual A and B. Conditions: A DB-17 capillary from J & W Scientific (50 cm × 50 μm); electrophoretic run at constant voltage of 8 kV; injection at 10 kV for 1 s.

Fig. 5. Analysis of PCR-amplified DNA fragments coding for the apo B gene of two different heterozygous individuals C and D. Conditions as in Fig. 4.
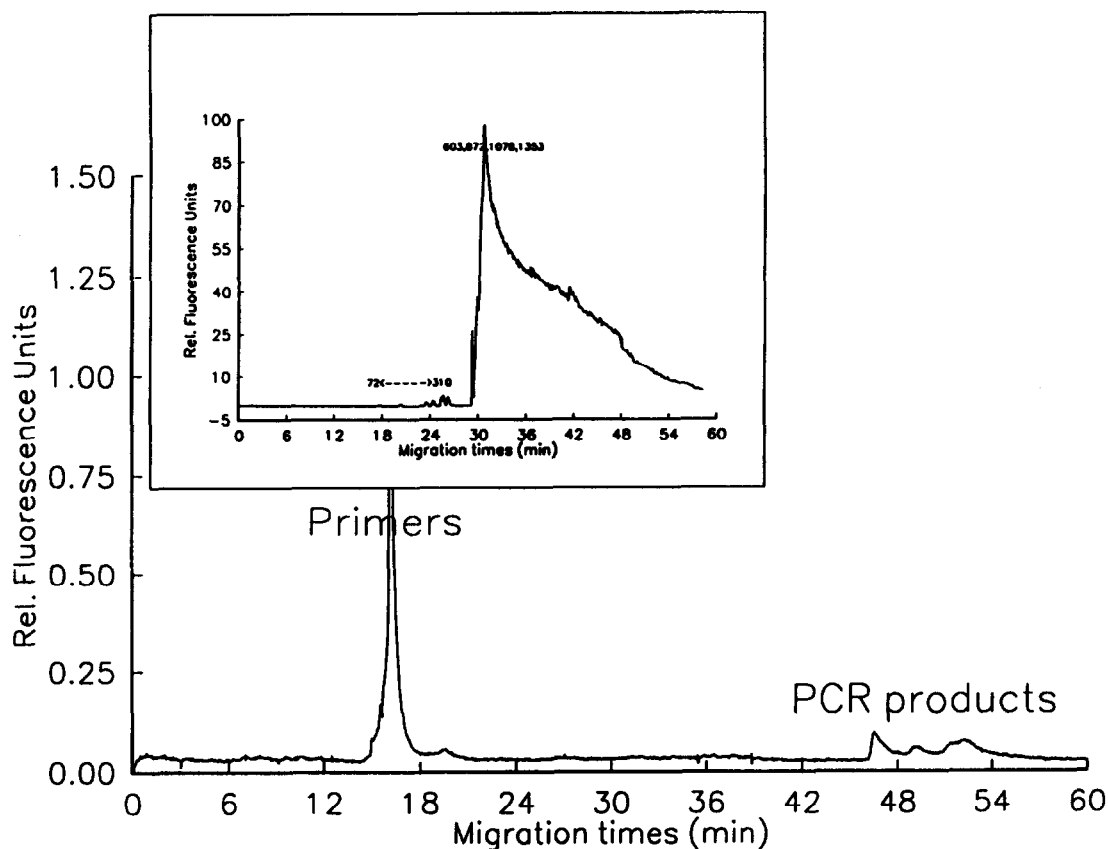
Fig. 6. Analysis of PCR-amplified DNA fragments coding for the mt locus of an individual. Conditions: A 3%C, 3%T urea-gel capillary from J & W Scientific (40 cm × 50 μm); electrophoretic run at constant voltage of 10 kV; injection at 5 kV for 5 s. Multiple peaks with band broadening are observed for this heterozygous individual. Inset shows the analysis of a ΦX174 DNA restriction fragment mixture showing the same behavior for the bigger fragments.

resolving larger DNA fragments due to increased non-specific interactions with the intercalating dye. Urea is known to denature DNA fragments, even at low urea concentrations. TOTO has a high binding affinity for DNA and, in a denaturing environment, binds to single strands of DNA to induce further conformational changes. In addition, we speculate that the TOTO in the run buffer binds to the limited number of free capillary surface silanols that were not masked by the gel-coating process resulting in cationic sites being available along the capillary walls for interaction (TOTO has 4 cationic charges available). These charges may interact with DNA fragments resulting in band broadening. These effects are more obvious for the larger fragments possibly due to their size and longer residency in

the capillary column. Use of a non-urea-based gel provided us with separations of some of the higher-molecular-mass species showing that urea has some deleterious effect. In this case, resolution was not optimal possibly due to the rigid pore structure of these gels.

Comparisons of the separation times of the amplified mtDNA fragments on both systems showed very little difference (Figs. 9 and 10). However, the gel-filled capillary separations showed broad peaks with much lower efficiencies and resolution. The polymer-network capillaries show better separations and a broader range of applicability than the gel-filled capillaries used in this study. Fig. 11 shows the separation of the larger D1S80 alleles of a single individual using the polymer-network capillary system. The poly-
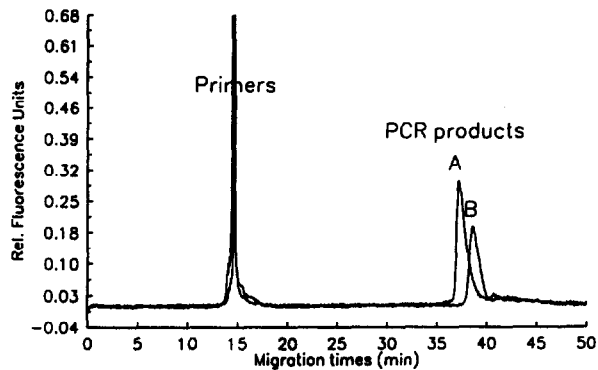
Fig. 7. Analysis of PCR-amplified DNA fragments coding for the apo B gene of a homozygous and a heterozygous individual A and B. Conditions: A 3%C, 3%T non-urea gel capillary from J & W Scientific (40 cm × 50 μm); electrophoretic run at constant voltage of 10 kV; injection at 5 kV for 5 s. The heterozygous individual B appears homozygous in this separation.
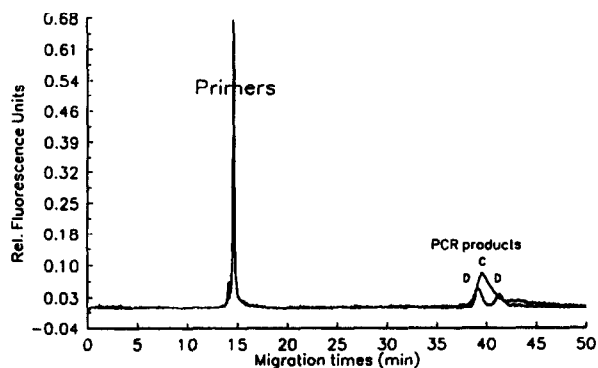

Fig. 8. Analysis of PCR-amplified DNA fragments coding for the apo B gene of two different heterozygous individuals C and D. Conditions as in Fig. 7. The heterozygous individual C appears as homozygous in this case.
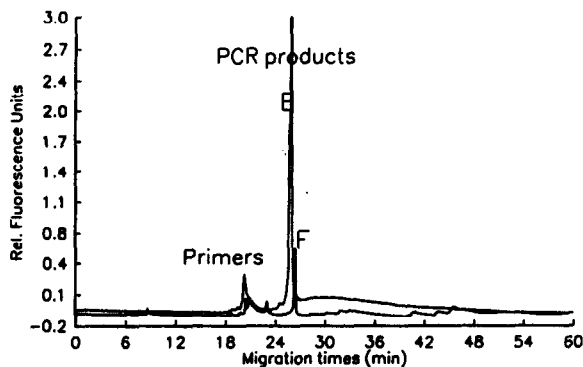

Fig. 9. Analysis of PCR-amplified mtDNA fragments of two different individuals E and F using a polymer-network capillary. Conditions as in Fig. 4.
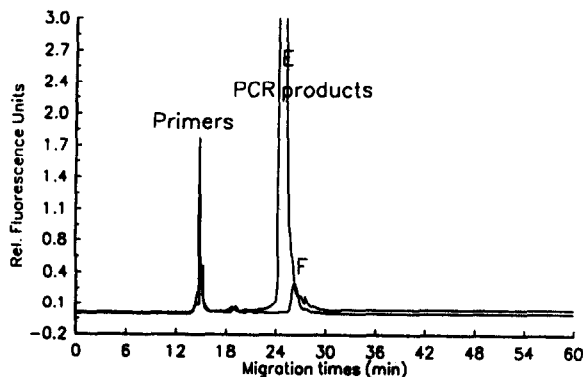

Fig. 10. Analysis of PCR-amplified mtDNA fragments of two different individuals E and F using a non-urea gel-filled capillary. Conditions as in Fig. 7.
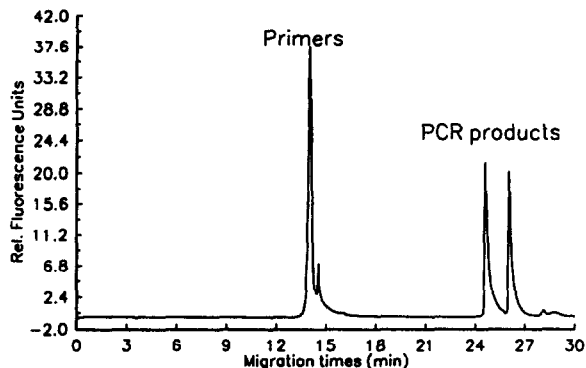

Fig. 11. Analysis of PCR-amplified DNA fragments coding for the VNTR locus D1S80 of an individual using a polymer-network capillary. Conditions of this run as in Fig. 1.

mer-network capillaries may have a broader effective size range due to their flexible and larger effective pore structure.

CONCLUSIONS

CE–LIF detection results in a powerful approach to analyze PCR-amplified DNA fragments. The polymer-network capillaries offer higher separation efficiencies and resolution when compared to gel-filled capillaries used in this study. The polymer-network capillaries also have a broader DNA size range of effectiveness

as demonstrated by the separations of various PCR-amplified fragments from different VNTR loci. The gel-filled capillaries did not provide us with efficient separations possibly due to interactions of the dye with the capillary and with the separation matrix. Further studies in terms of interactions of the dyes TOTO and YOYO with dsDNA [24] and the separation matrix are being pursued.

ACKNOWLEDGEMENTS

REFERENCES

1 K.B. Mullis and F. Faloona, *Methods Enzymol.*, 155 (1987) 335.
2 R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn and H.A. Erlich, *Science*, 239 (1988) 487.
3 R. Reynolds, G. Sensabaugh and E. Blake, *Anal. Chem.*, 63 (1991) 2.
4 M. Zhu, D.L. Hansen, S. Burd and F. Gannon, *J. Chromatogr.*, 480 (1989) 311.
5 M. Strege and A. Lagu, *Anal. Chem.*, 63 (1991) 1233.
6 W.A. MacCrehan, H.T. Rasmussen and D.M. Northrop, *J. Liq. Chromatogr.*, 15 (1992) 1063.
7 P.D. Grossman and D.S. Soane, *J. Chromatogr.*, 559 (1991) 257.
8 D.K. Heiger, A.S. Cohen, B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.
9 A. Guttman and N. Cooke, *Anal. Chem.*, 63 (1991) 2038.
10 B. L Karger, A.S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585.
11 H.E. Schwartz, K. Ulfelder, F.J. Sunzeri, M.P. Busch and R.G. Brownlee, *J. Chromatogr.*, 559 (1991) 267.
12 E. Boerwinkle, S.S. Lee, R. Butler, V.N. Schumaker and L. Chan, *Atherosclerosis*, 81 (1990) 225.
13 K. Kasai, Y. Nakumara, and R. White, *J. Forensic Sci.*, 35 (1990) 1196.
14 A. Bodenteich, L.G. Mitchell, M.H. Polymeropoulos and C.R. Merril, *Human Molecular Genetics*, 1 (1992) 40.
15 K. Srinivasan, J.E. Girard, D.J. Reeder, R.K. Roby, P. Williams and V.W. Weedn, presented at the *5th International symposium on High Performance Capillary Electrophoresis, Orlando, FL, Jan. 1992*, Poster T205.
16 P. Williams, in *CE Application Note No. 2*, J & W Scientific, Sunnyvale, CA, August 1992.
17 H.E. Schwartz and K.J. Ulfelder, *Anal. Chem.*, 64 (1992) 1737.
18 H.S. Rye, S. Yue, D.E. Wemmer, M.A. Quesada, R.P. Haugland, R.A. Mathies and A.N. Glazer, *Nucleic Acids Res.*, 20 (1992) 2803.
19 I.D. Johnson, E.M. Marcus, S. Yue and R.P. Haugland, *Biophys. J.*, 61 (1992) A314.
20 A.N. Glazer and H.S. Rye, *Nature*, 359 (1992) 859.
21 K. Srinivasan, J.E. Girard, S.C. Morris and D.J. Reeder, presented at the *3rd Annual Frederick Conference on Capillary Electrophoresis, Frederick, MD, Oct. 1992*, Poster p28.
22 K. Srinivasan, S.C. Morris, J.E. Girard, M.C. Kline and D.J. Reeder, *Appl. Theor. Electrophoresis*, in press.
23 P.S. Walsh, D.A. Metzger and R. Higuchi, *BioTechniques*, 10 (1991) 506.
24 K. Srinivasan, S.C. Morris, J.E. Girard and D.J. Reeder, in preparation.