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Applications of Short-Chain Polydimethylacrylamide as Sieving Medium for the Electrophoretic Separation of DNA Fragments and Mutation Analysis in Uncoated Capillaries

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In capillary electrophoresis (CE), separation of DNA fragments is usually performed in covalently coated capillaries. Recent studies have demonstrated that certain polymers form a dynamic coating on the inner surface of the capillary, thereby suppressing the electroosmotic flow and DNA–capillary wall interactions. We developed a simple method for the synthesis of short-chain polydimethylacrylamide (PDMA) using isopropanol as a chain transfer agent. Capillary (<75 μm internal diameter) filling and replacement of this low-viscosity (14 cP at 4% PDMA) self-coating medium were easily carried out by commercial CE instruments. Using PDMA and uncoated capillaries, we first examined the separation of ϕX174 *Hae*III DNA digests and observed that the stability of the dynamic coating was markedly better at pH 7.8 than at pH 8.3. At this lower pH and nondenaturing conditions, high resolution of the ϕX174 *Hae*III DNA digests was obtained for more than 850 injections in the same capillary. We then exploited this sieving medium in CE using multiple approaches for mutation analysis of clinical DNA samples including separation of restriction enzyme cleavage products, analysis of single strand conformation polymorphisms, and simultaneous detection of several mutations using multiplex allele-specific PCR amplification. Our results demonstrate that CE in uncoated capillaries using PDMA as sieving medium is a simple, versatile, and reliable strategy for separation and mutation analysis of clinical DNA samples.

Capillary electrophoresis (CE)[2] has become an attractive alternative to slab gel electrophoresis for DNA analysis. CE can be automated and is characterized by short analysis time, small sample and reagents requirements, high separation efficiency, and, when coupled to laser-induced fluorescence (LIF) detector, unsurpassed sensitivity (1).

The early techniques for DNA separation by CE were based on filling the capillary with a polyacrylamide gel similar to that used in conventional slab gel electrophoresis (2–5). However, this approach was far from optimal. The capillary gel columns were difficult to prepare, the polymerization of the acrylamide inside the capillary often led to bubble formation and gel shrinkage, and the lifetime of the column was usually short (6, 7). A major step forward was the introduction in recent years of replaceable polymer solutions as separation or sieving media, including linear polyacrylamide (6–11), cellulose derivatives (8, 12, 13), and poly(ethylene oxide) (14, 15). These advances in matrix preparation have promoted the application of CE in DNA analysis, such as mutation detection (16–20), forensic genotyping (21, 22), DNA sequencing (5, 10, 15), and measurement of gene expression (23–25).

To perform DNA analysis in capillaries filled with a polymer solution, the inner surface first must be modified, usually by covalent bonding of hydrophilic polymers which thereby suppress the electroosmotic flow and the adsorption of DNA to the capillary surface (26). However, the coating procedure increases the cost and

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[2] Abbreviations used: CE, capillary electrophoresis; LIF, laser-induced fluorescence; i.d., internal diameter; o.d., outer diameter; PDMA, polydimethylacrylamide; CBS, cystathionine β-synthase; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; RSD, relative standard deviation.

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often causes problems related to capillary deterioration and coating inhomogeneity.

Certain polymer solutions adsorb to the capillary inner surface and form a stable dynamic coating which reduces the electroosmotic flow to a negligible level. This has been reported for poly(ethylene oxide) (15) and poly(vinylpyrrolidone) (27) which have been successfully used for DNA sequencing and genotyping (15, 27).

Madabhushi recently reported that using polydimethylacrylamide (PDMA) as a sieving medium in the uncoated capillary effectively suppresses both electroosmotic flow (EOF) and DNA–capillary wall interactions (28). The author reported that at least 100 successive sequencing runs could be performed in the same capillary.

To date, self-coating sieving media have been used for sequencing, performed under denaturing conditions and with purified DNA (10). However, many types of DNA analyses are based on nondenaturing conditions without the presence of urea. This includes analysis of double-strand and single-strand conformation polymorphisms, separation of restriction enzyme digests, detection of specific PCR products, and quantitation of gene expression (29). Moreover, unpurified PCR products, with or without subsequent enzyme digestion, contain protein and high salt concentration. In this article, we present a simple technique for the synthesis of PDMA, and demonstrate a number of applications in CE analyses of PCR-amplified DNA.

MATERIALS AND METHODS

Materials

Dimethylacrylamide was obtained from Aldrich-Chemie (Germany). *N*,*N*,*N*′,*N*′-Tetramethylenediamine (TEMED) and ammonium peroxydisulfate (APS) was purchased from Bio-Rad Laboratories (Hercules, CA). ϕX174 *Hae*III digests (11 DNA fragments) and *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) were obtained from Sigma Chemical Co. (St. Louis, MO). The pH of TAPS buffer was adjusted with 5 M sodium hydroxide. SYBR Green I (concentration not given) in dimethyl sulfoxide was purchased from Molecular Probes. Reaction tubes (thin-walled, Gene Amp) for PCRs were from Perkin–Elmer (Norwalk, CT). Fused silica capillaries with 50μm internal diameter (i.d.) and 192μm outer diameter (o.d.) were products of Polymicro Technologies Inc. (Phoenix, AZ) and fused silica capillaries with 75 μm i.d. and 365 μm o.d. were obtained from J & W Scientific (Folsom, CA). QIAquick PCR purification and QIAamp blood kits were products of QIAGEN Co. (Hilden, Germany). 5-Fluorescein-labeled primers and nonlabeled 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 PDMA

Short-chain linear PDMA was synthesized in water, using isopropanol as a chain transfer agent to control the molecular weight of PDMA. Briefly, 110 ml water and 3.8 ml isopropanol were added to the reaction vessel containing 12 ml dimethylacrylamide. The mixture was degassed with helium for 30 min and heated in a water bath at 50°C for 20 min. Then, 0.625 ml 10% (v/v) TEMED and 0.625 ml 10% (w/v) APS were added, and the polymerization took place for 1.5 h at 50°C.

The reaction product was extensively dialyzed against water for 2 days, using a 12,000 molecular weight cutoff dialysis membrane tubing (Thomas Scientific, Philadelphia, PA), lyophilized, and then weighed. The yield of the reaction was 88%. The sieving media were prepared by dissolving PDMA powder in the separation buffers.

Measurements of PDMA Molecular Weight and Medium Viscosity

The viscosity-average molecular weight (M_v) was measured from the intrinsic viscosity [η] of the polymer. The Kinematic viscosity was determined at 25°C using an Ubbelohde viscometer (SCHOTT, Germany). The density of polymer solutions was measured at 25°C using a density meter (PAAR DMA 601/602). The plot of η_{sp}/C (η_{sp}, specific viscosity; C, concentration of polymer) versus the concentration of polymer was extrapolated to zero concentration, producing [η] as the intercept. M_v was calculated according to the Mark–Houwink equation (28, 30). The M_v of PDMA was 36 kDa. The viscosity of 4% PDMA solution in 100 mM TAPS (pH 7.8) was 14 cP at 25°C.

DNA Extraction and PCR Reactions

Blood samples were from different subjects that had previously been genotyped with respect to variants in the CBS gene (31), the MTHFR gene (18, 32) and factor V gene (19).

DNA was extracted from whole blood using a QIAamp blood kit according to the instructions from the manufacturer and amplified by PCR, using a Perkin–Elmer 480 thermocycler. The PCR mixture contained 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100, 125 μM of each dNTP, 0.2 μM of each primer, 1 U *Taq* polymerase (Super Taq, HT Biotechnology Ltd., UK), and approximately 100 ng template DNA in a final volume of 100 μl. The sequences of primers, the size of PCR

TABLE 1

Sequence of Primers, PCR Conditions, and Size of PCR Products

Gene	Mutation	Sequence of primer (5′–3′)	PCR condition	Size of PCR product (bp)
MTHFR	A1298C	CTTTGGGGAGCTGAAGGACTACTAC CACTTTGTGACCATTCCGGTTTG	94°C 30 s, 62°C 30 s, 72°C 20 s, 36 cycles.	163
Factor V	G1691A	GGCAGGAACAACACCATGAT TCAAGGACAAAATACCTGTATTC	94°C 30 s, 55°C 30 s, 72°C 30 s, 38 cycles.	232
CBS	C785T (exon 7)	CCAGGCAGGGACCCAAGAAT CCACTCCGCACTGTCCCTCT	94°C 30 s, 58°C 30 s, 72°C 30 s, 36 cycles.	170

products and the PCR thermocycles are given in Table 1.

Restriction Enzyme Digestion

The A1298C polymorphism of the MTHFR gene abolishes a *Mbo*II restriction site (33). A DNA fragment (163 bp) was obtained by PCR, and the PCR product (25 μl) was treated with *Mbo*II in a final volume of 50 μl, according to the instructions from the manufacturer. The digests were diluted 10 times in water prior to CE analysis.

SSCP Analysis

The fluorescent-labeled PCR products encompassing the A1298C MTHFR, G1691A factor V, and C785T CBS mutations were obtained by PCR using the fluorescein-labeled primers. The PCR products were purified with the QIAquick PCR purification kit according to the instructions from the manufacturer. This purification step was included to remove fluorescent materials and fluorescent primers which can interfere with the SSCP analysis. The final volume of the purified DNA sample was 50 μl. The samples were stored at −20°C until analysis. Immediately before CE, the PCR products were diluted 1:10 in water, heated to 95°C for 5 min, cooled in ice water for 10 min, and subjected to CE analysis.

Capillary Electrophoresis Instruments and Procedures

Two commercial CE instruments were used in the study.

A Beckman P/ACE System 2100 was equipped with a LIF detector (Beckman Instruments, Palo, CA) in which an argon ion laser source provided an excitation light at 488 nm. A 520-nm bandpass filter was used as an emission cutoff filter. Beckman System Gold software (version 8.10) was used in system control, data collection, and processing. The P/ACE system was only used in the reproducibility determinations.

The other commercial CE instrument was from Prince Technologies (Emmen, The Netherlands). It was coupled to an in-house built LIF detector equipped with a sheath-flow cuvette constructed essentially as described by Dovichi *et al.* (34). An argon ion laser (Uniphase Ltd., Hertfordshire, UK) with 488 nm emission (20 mW) was focused on the sheath-flow cuvette 30

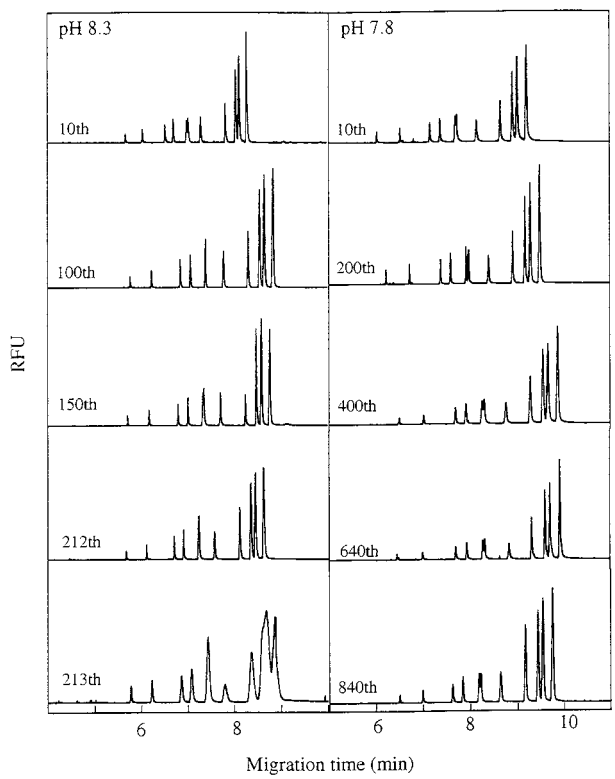

FIG. 1. Separation of DNA fragments in a PDMA matrix at pH 8.3 and 7.8. A *Hae*III digest of ϕX 174 (50 ng/ml), containing DNA fragments of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353 bp, was separated in an uncoated capillary (40 cm length and 50 μm i.d) filled with 4% PDMA in 60 mM TAPS, pH 8.3, or 100 mM TAPS, pH 7.8. Electrokinetic injection at −2 kV for 6 s was used, the temperature was 22–24°C and the applied voltage was −20 kV. RFU, relative fluorescence units. The numbers in the lower left corners refer to the injection number.

TABLE 2

Reproducibility of Migration Time

	Within-day (n = 10)		Between-day (N = 5[a])		Between-capillaries (N = 4[a])	
DNA (bp)	MT (min)	RSD (%)	MT (min)	RSD (%)	MT (min)	RSD (%)
72	9.22	0.84	9.64	3.5	9.47	1.1
118	10.18	0.87	10.47	3.9	10.45	0.93
194	11.36	0.94	11.69	4.0	11.70	0.45
234	11.92	0.97	12.31	4.4	12.25	0.80
271	12.46	1.1	12.83	4.1	12.80	0.60
281	12.57	0.99	12.99	4.5	12.92	0.72
310	13.51	1.4	13.85	3.8	13.8	0.88
603	14.55	1.2	14.92	4.7	15.05	1.4
872	15.11	1.2	15.52	4.9	15.66	1.3
1078	15.28	1.3	15.70	4.8	15.83	1.2
1353	15.68	1.4	16.11	4.6	16.20	1.2

Note. The reproducibility experiments were performed with a Beckman P/ACE 2210 system using an uncoated capillary (37 cm total length, 30 cm effective length and 75 μm i.d) filled with 4% PDMA in 100 mM TAPS, pH 7.8. The concentration of ϕX174 *Hae*III digests was 0.5 μg/mL. Electrokinetic injection was −6 kV for 12 s. The applied voltage, and separation temperature was −12 kV and 20°C, respectively. MT, average migration time; RSD, relative standard deviation.

[a] On 5 consecutive days or with 4 capillaries, 10 parallel injections were carried out.

μm below the capillary outlet. The fluorescence emission signal was collected at 90°C with a microscope objective, amplified by a photo-multiplier (Hamamatsu, Japan), and transferred to a computer. Prince software (version 1.14) and Caesar software were used in system control, data collection, and processing.

A new uncoated capillary was first rinsed with 1 M HCl solution for 30 min, then with water for 5 min and finally filled with PDMA sieving medium. For double-strand DNA analysis, SYBR Green I was added to PDMA medium at a dilution of 1:10,000.

Samples were introduced by electrokinetic injection, and the electrophoresis was performed at reverse polarity mode. The compositions of the sieving medium and electrophoresis buffer are specified in the figure legends. Between each run, the capillary was rinsed with water for 2–2.5 min and then filled with the sieving medium. The capillary was filled with PDMA sieving medium before storage.

RESULTS AND DISCUSSION

pH of the PDMA Sieving Medium

Figure 1, left row of panels, shows the separation of ϕX174 *Hae*III DNA fragments at pH 8.3, which is commonly used in DNA separation based on slab gel electrophoresis or CE. The system was characterized by high separation efficiency, and the ϕX174 DNA fragments were baseline resolved under the conditions. However, the separation efficiency decreased dramatically after about 200 injections (Fig. 1). This phenomenon was consistently observed with 4 capillaries, and suggests deterioration of the dynamic capillary coating at pH 8.3, possibly related to adsorption of DNA or protein on the capillary surface. The capillary can be regenerated by rinsing with HCl and water, but separation efficiency was again lost after only 130 injections (data not shown).

Figure 1, right row of panels, shows the separation of ϕX174 *Hae*III DNA fragments at pH 7.8. At this pH, the high separation efficiency was maintained for more than 800 injections, carried out over 20 days.

The data presented in Fig. 1 suggest that dynamic coating of the capillary surface is related to the pH of the sieving medium. This could be explained by the involvement of hydrogen bonds in the interaction between silica surface and PDMA (N(CH3)2-R):

$$-\text{Si}-\text{Si}-\text{Si}-\text{O}^- \underset{\text{OH}^-}{\overset{\text{H}^+}{\rightleftharpoons}} -\text{Si}-\text{Si}-\text{Si}-\text{OH} \qquad [1]$$

$$-\text{Si}-\text{Si}-\text{Si}-\text{OH} + \text{N(CH3)2-R} \rightleftharpoons -\text{Si}-\text{Si}-\text{Si}-\text{O}\cdots\text{H}\cdots\text{N(CH3)2-R} \qquad [2]$$

The ionization of the silanol groups is decreased at low pH, thereby shifting the equilibrium toward the silanol (Si–Si–Si–OH) form which forms hydrogen bonds with the nitrogen of the PDMA backbone. The electron cloud density around the nitrogen atoms is high due to the electron repulsion of the methyl groups. This may lead to more efficient dynamic coating of the capillary by PDMA compared to polyacrylamide which is not methylated.

Reproducibility

We tested the within-day, between-day, and between-capillary reproducibility for the separation of the ϕX174 *Hae*III DNA restriction fragments in PDMA at pH 7.8. In these experiments, we used a Beckman P/ACE with liquid cooling control to minimize variation in capillary temperature. The results obtained (Table 2) demonstrate a high reproducibility (within-day RSD, <1.5%; between-day RSD, <5%). Notably, the RSD between capillaries was <1.5%.

Applications

The results reported above demonstrate efficient and reproducible separation of the ϕX174 DNA fragments in an uncoated capillary using PDMA as sieving medium. We also wanted to investigate the suitability of PDMA for the analysis of samples containing various buffer, salts (from PCR), or biological materials (from unpurified blood). We investigated three applications requiring separation of double- or single-stranded DNA fragments.

1. Mutation analysis by restriction enzyme digestion. The A1298C polymorphism in the MTHFR gene abolishes a *Mbo*II restriction site (33). Figure 2 shows the electropherograms of 163-bp PCR product before digestion, and the profiles obtained after *Mbo*II digestion of amplified fragments from the three different genotypes, i.e., homozygous normal (− −), homozygous mutant (+ +), and heterozygous (+ −). The profile of heterozygous (+ −) sample (lower panel) shows the efficient separation of fragments of 81, 56, and 18 bp and the triplet of 28, 30, and 31 bp. These results demonstrate the application of the PDMA sieving medium for the analysis of nonpurified restriction digests of PCR products.

2. SSCP analysis. Figure 3 shows SSCP profiles of homozygous normal (− −), homozygous mutant (+ +) and heterozygous (+ −) specimens of three genes, i.e., the A1298C polymorphism of MTHFR, the G1691A mutation of factor V, and the C785T mutation of CBS. Three different genotypes for each gene were clearly differentiated. The genotypes were identified by comparison with migration profiles for samples analyzed by reference techniques, and by identification of peaks according to their migration times. In the heterozygous samples, components derived from each allele were identified (Fig. 3). The DNA samples shown in Fig. 3 were purified using the QIAquick PCR purification kit to remove interfering fluorescent materials occasionally present in the primer preparation. However, the same SSCP profiles were obtained with nonpurified samples (data not shown). Our data show that single-strand DNA conformations are efficiently separated by CE using PDMA as sieving medium.

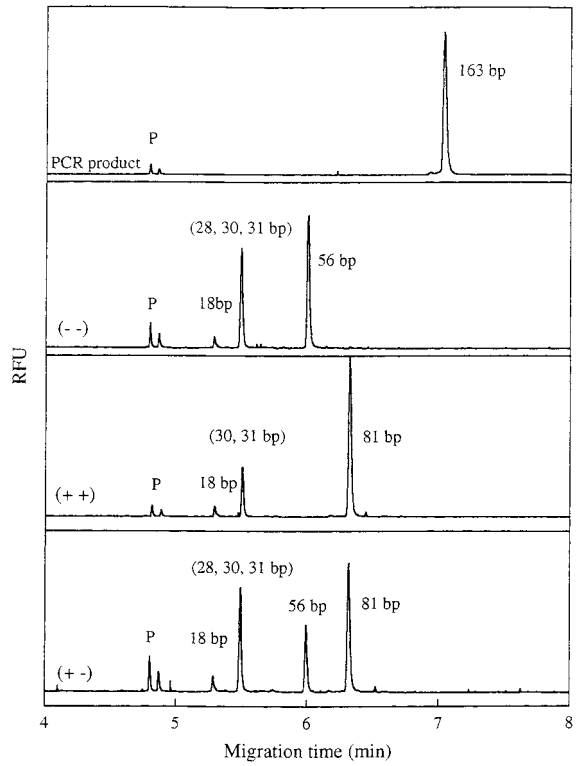

FIG. 2. Mutation analysis by restriction enzyme digestion. A PCR product of 163 bp encompassing position 1298 of the MTHFR gene was amplified, using the primers listed in Table 1, and then subjected to *Mbo*II digestion. Three previously genotyped samples, including a homozygous normal (− −), a homozygous mutant (+ +), and a heterozygous (+ −), were analyzed by CE, using 4% PDMA in 100 mM TAPS, pH 7.8 as separation matrix. The peaks denoted P derived from primers. Electrokinetic injection at −5 kV for 6 s was used; other conditions were as described in the legend to Fig. 1. RFU, relative fluorescence units.

3. Mutation analysis by multiplex mutagenically separated PCR. We used a multiplex mutagenically separated PCR method for the simultaneous analyses of 6 possible alleles in two different genes**.** The method is a modification of a method originally described by Ulvik *et al.* (19). Unpurified whole blood was used as template DNA for the PCR which was run in a single tube with 9 primers. Before CE analysis, the PCR products were diluted 1:30 with water and introduced into the capillary by electrokinetic injection. The mutations/polymorphisms analyzed were the A1298C of MTHFR, the G1691A of the factor V, and the C677T of the MTHFR genes. The allele-specific PCR products obtained were 92 bp (1298C allele of MTHFR), 101 bp (1298 A allele of MTHFR), 117 bp (1691G allele of factor V), 125 bp (1691A allele of factor V), 197 bp (677 T allele of MTHFR), and 207 bp (677C allele of MTHFR). Six different samples comprising 6 different genotypes and all 6 base substitutions were analyzed.

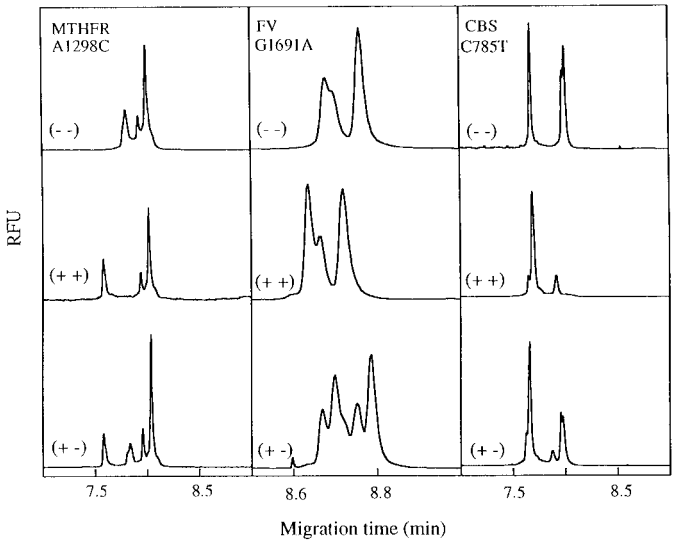

FIG. 3. SSCP analysis. The A1298C polymorphism of the MTHFR, the G1691A mutation of factor V, and the C785T mutation of CBS genes were determined by SSCP. The homozygous normal (− −), homozygous mutant (+ +), and heterozygous genotypes are indicated in the lower left corners of the panels. The single-stranded DNA fragments were separated in 6% PDMA in 100 mM TAPS, pH 7.8, containing no SYBR Green I. The samples were electrokinetically injected at −3 kV for 6 s; other conditions were as described in the legend to Fig. 1. RFU, relative fluorescence units.

The genotypes were differentiated on the basis of the number and the migration times of peaks in the electropherogram, and by comparison with migration profile for samples of known genotypes.

The data presented in Fig. 4 demonstrate efficient separation of six PCR fragments, interfering materials, and the primers, and each genotype was unambiguously identified. Notably, these samples were not purified to remove salts, enzyme derived from the PCR step and blood constituents prior to CE analysis. Thus, complex samples obtained by multiplex mutagenically separated PCR were analyzed without intervening purification steps by electrophoresis in uncoated capillary using PDMA as the matrix. The analysis was characterized by robustness and high reproducibility.

CONCLUSION

PDMA forms the dynamic coating of the inner surface of the silica capillary, thereby suppressing the electroosmotic flow and inhibiting solute adsorption to the surface. The stability of the dynamic coating is highly dependent on the pH value of the sieving medium and was markedly improved by reducing the pH from 8.3 to 7.8. We demonstrated the successful application of uncoated capillary filled with PDMA, pH 7.8, for the efficient and reproducible separation of complex DNA samples, including restriction enzyme digests, fluorescent-labeled single-strand DNA fragments in SSCP analysis, and multiplex PCR products. Thus, PDMA has a wide application in CE beyond its use in separation of DNA-sequencing extension products. Since low-viscosity PDMA facilitates easy capillary filling, and laborious capillary coating is not required, this sieving medium may become an attractive alternative

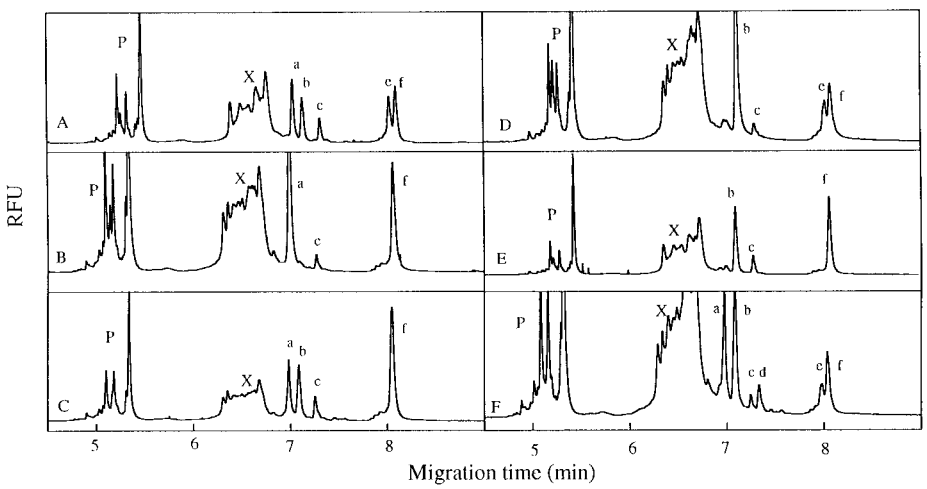

FIG. 4. Separation of six mutagenically separated PCR products derived from two genes. The mutations/polymorphisms A1298C of MTHFR, the G1691A of factor V, and the C677T of the MTHFR genes were analyzed by multiplex mutagenically separated PCR in six blood samples. The peaks corresponding to each allele are marked a (1298C allele of MTHFR), b (1298A allele of MTHFR), c (1691G allele of factor V), d (1691A allele of factor V), e (677T allele of MTHFR), and f (677C allele of MTHFR). The peaks denoted P and X derived from primers and primer dimers, respectively. The samples were electrokinetically injected at −5 kV for 6 s; other conditions were as described in the legend to Fig. 1. RFU, relative fluorescence units.

to other non-crossed-linked polymers commonly used in CE.

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