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Separation of dsDNA in the presence of electroosmotic flow under discontinuous conditions

Separations of φX-174/*Hae*III DNA restriction fragments have been performed in the presence of electroosmotic flow (EOF) using five different polymer solutions, including linear polyacrylamide (LPA), poly(ethylene oxide) (PEO), hydroxypropylcellulose (HPC), hydroxyethylcellulose (HEC), and agarose. During the separation, polymer solutions entered the capillary by EOF. When using LPA solutions, bulk EOF is small due to adsorption on the capillary wall. On the other hand, separation is faster and better for the large DNA fragments ($>$ 872 base pairs, bp) using derivative celluloses and PEO solutions. Several approaches to optimum resolution and speed by controlling EOF and/or altering electrophoretic mobility of DNA have been developed, including (i) stepwise changes of ethidium bromide (0.5–5 μg/mL), (ii) voltage programming (125–375 V/cm), (iii) use of mixed polymer solutions, and (iv) use of high concentrations of Tris-borate (TB) buffers. The DNA fragments ranging from 434 to 653 bp that were not separated using 2% PEO (8 000 000) under isocratic conditions have been completely resolved by either stepwise changes of ethidium bromide or voltage programming. Compared to PEO solutions, mixed polymer solutions prepared from PEO and HEC provide higher resolving power. Using a capillary filled with 600 mM TB buffers, pH 10.0, high-speed ($<$ 15 min) separation of DNA (pBR 322/*Hae*III digest, pBR 328/*Bgl*I digest and pBR 328/*Hin*fI digest) has been achieved in 1.5% PEO.

Keywords: Capillary electrophoresis / Polymer solutions / DNA / Laser-induced fluorescence
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1 Introduction

Capillary electrophoresis (CE) using polymer solutions has gained increased acceptance over the past ten years in DNA separation due to its speed, high resolving power, use of small amounts of samples, and ease of automation [1–3]. The polymer solutions are commonly prepared from linear polymers, including linear polyacrylamide (LPA) and its derivatives [4–6], cellulose and its derivatives [7–9], agarose [10], and poly(ethylene oxide) (PEO) [11, 12]. For separations of small DNA fragments, entangled polymer solutions are generally used. The entanglement threshold concentration (Φ^*) of a polymer solution is the approximate concentration above that interaction between polymer chains begins to affect bulk solution properties such as viscosity. The Φ^* is a unique property of a given polymer/buffer system, which is strongly dependent on the molecular mass of the polymer molecules. The length dependence of Φ^* may range from $N^{-1.2}$ for stiff and hydrophilic cellulose derivatives to $N^{-0.8}$ for extremely flexible polymers, where N is the number of monomers in the polymer chain [13, 14]. Furthermore, the viscosity and mesh size of polymer solutions are both dependent on N and concentration [15].

Variations of electroosmotic flow (EOF) and wall interactions with DNA are problematic using bare fused-silica capillaries, leading to poor resolution and irreproducibility. To improve reproducibility and accuracy, bare fused-silica capillaries rinsed with 0.1 M HCl between injections have been used for DNA sequencing in PEO solutions [16]. More commonly, DNA analysis is performed using coated capillaries, which can be prepared either through covalent bonding or dynamic coating [17–20]. Because the coated capillary is relatively expensive and has a short life, dynamic coating has recently gained more interest [21]. In the absence of EOF, filling capillaries with polymer solutions by any pressure means prior to DNA separations is needed, necessitating a large size of capillaries and low-viscosity polymer solutions.

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Abbreviations: EtB, ethidium bromide; **HEC**, hydroxyethylcellulose; **HPC,** hydroxypropylcellulose; **LPA**, linear polyacrylamide; **TB**, Tris-borate; **TBE**, Tris-borate-EDTA; **PEO**, poly(ethylene oxide)

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Recently, we have turned our interest toward DNA separation in the presence of EOF [22–24]. Polymer solutions entered capillaries filled with buffers such as Tris-borate (TB) by EOF after injecting DNA samples. As a result of DNA migration against EOF, large DNA fragments with small electrophoretic mobility (μ_{ep}) are detected earlier at the cathode end. The technique provides advantages over those in the absence of EOF, including no need to fill capillaries with polymer solutions prior to analysis, capability of performing DNA separations by gradient techniques, and optimization of resolution, concentration, and migration by regulation of EOF [25].

In the presence of EOF, choosing polymer solutions is important because resolution depends on sieving and the magnitude of EOF. To achieve optimum resolution, sieving matrices providing suitable mesh sizes are needed, which are related to polymer species and concentration. Because EOF decreased due to dynamic adsorption of polymers on the capillary wall, low-concentration and hydrophilic polymer solutions are superior for rapid separation. To suppress polymer adsorption, pH, ionic strength, and additives must also be well controlled [26, 27]. For instance, we have shown that high bulk EOF can be achieved in PEO solutions, pH 9.0, using a capillary filled with 400 mM TB buffers, pH 10.0 [25]. In an attempt to gain more insight into the effect of changes in bulk EOF on DNA separation, polymers prepared from PEO, LPA, agarose, hydroxyethylcellulose (HEC), or hydroxypropylcellulose (HPC) were tested. It is also our interest to explore DNA separations using mixed polymer solutions prepared from PEO with other polymers. In addition, we also demonstrated effects of buffer concentration, applied voltage, and ethidium bromide (EtB) concentration on resolution.

2 Materials and methods

2.1 Equipment

The basic design of the separation system has been previously described [22]. Briefly, a high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) was used to drive electrophoresis. The entire detection system was enclosed in a black box with a high-voltage interlock. The high-voltage end of the separation system was put in a home-made plexiglass box for safety. A 4.0-mW He-Ne laser with 543.6-nm output from Uniphase (Mantence, CA, USA) was used for excitation. The light was collected with a 20 × objective (numerical aperture 0.25). One RG 610 cutoff filter was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The amplified currents were transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a PC. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm ID and 365 μm OD were used for DNA separations without any further coating process.

2.2 Chemicals

All chemicals for preparing buffer solutions and polymers were from Aldrich (Milwaukee, WI, USA). EtB was obtained from Molecular Probes (Eugene, OR, USA). Agarose (SeaPrep) was from FMC Bioproducts (Rockland, ME, USA). TB buffers were prepared from Tris adjusted with boric acid to pH 7.0, 8.2, 9.0, and 10.0, respectively. Unless otherwise noted, × mM TB buffers means × mM Tris solutions adjusted with a suitable amount of boric acid. Buffer solutions used to fill capillaries were 1 × TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) or 600 mM TB. Polymer solutions were prepared from different amounts of agarose, HEC (1.3 M), HPC (1 M), and PEO (4 M), and PEO (8 M), respectively, in 1 × TBE buffer or 100 mM TB buffer. In this study, HEC (1.3 M), HPC (1 M), PEO (4 M), and PEO (8 M) represent polymers with molecular weights 1 300 000, 1 000 000, 4 000 000, and 8 000 000, respectively. LPA solutions were prepared from polymerization of acrylamide using ammonium persulfate and *N*,*N*,*N*‘,*N*‘-tetramethylenediamine as initiators [28]. ΦX 174 RF DNA-*Hae*III digest was purchased from Pharmacia Biotech (Uppsala, Sweden). DNA markers V (pBR 322/*Hae*III digest) and VI (pBR 328/*Bgl*I digest and pBR 328/*Hin*fI digest) were purchased from Boehringer Mannheim (Mannheim, Germany).

2.3 Preparation of polymer solutions

Certain amounts of polymers were gradually added into the TB or TBE buffers in a beaker stirring in a water bath at 85–90°C. During the addition of polymers, a magnetic stirring rod was used at high speed to produce a homogeneous suspension. After addition was complete, the suspension was stirred for at least one more hour. Mixed polymer solutions of PEO and LPA were prepared by mixing the two polymer solutions. Finally, polymer solutions were degassed with a vacuum system in an ultrasonic tank. Polymer solutions stored in a refrigerator at 4°C were usable for at least a week.

2.4 Separation

Prior to analysis, capillaries were treated with 0.5 M NaOH overnight. After each run, capillaries were washed with 0.5 M NaOH at 25 V/cm for 10 min to remove polymer solutions and refresh the capillary wall, and subsequently filled with TB buffers. This treatment allowed reproducible

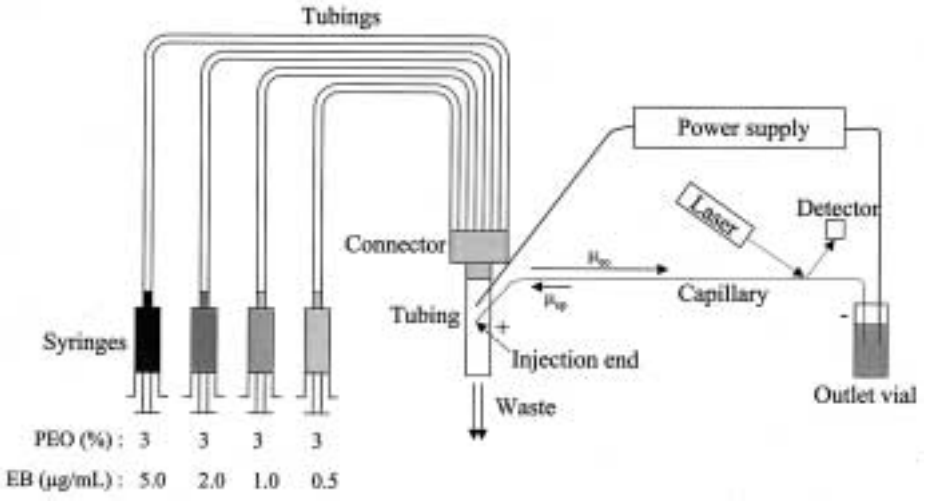

Figure 1. Schematic of a CE system for DNA separation by stepwise techniques using a number of polymer solutions containing different concentrations of EtB.

results RSD of EOF $<$ 3%) [23]. The injection of 1 or 25 μg/mL DNA samples was carried out by pressure at 30 cm height for 5 s. During analysis, polymer solutions entered the capillary by EOF and served as sieving matrices for DNA separations. For stepwise changes in EtB, the PEO solutions containing different amounts of EtB were injected by pressure to a Teflon connector, from wherein the PEO solutions entered the capillary by EOF (Fig. 1).

3 Results and discussion

3.1 Polymer solution

When DNA separation is performed in the presence of EOF, resolution is related to sieving and the magnitude of bulk EOF mobility (μ_{eo}). In this study, polymer solutions entered the capillary filled with TB buffers by EOF after DNA injection. With small mobility ($\mu_{eo}-\mu_{ep}$) towards the cathode end, DNA migrated into polymer solutions as soon as the separation started. Because of polymer adsorption on the capillary wall and increases in the fraction of the capillary filled with polymer solutions, EOF gradually decreased. As a result, resolution and speed should be dependent on the tendency of the polymer adsorption. To explore the effect of polymer solution on DNA separation in the presence of EOF, we separately tested solutions of agarose, HEC, HPC, LPA, PEO (4 M), and PEO (8 M). Table 1 shows the Φ* and the suitable concentration ranges of these polymer solutions for separating small DNA fragments in the presence of EOF. Because resolution was poor at lower concentrations, while separation time was long at high concentrations, the optimal concentrations, in terms of speed and resolution, for agarose, HEC, HPC, LPA, PEO (4 M), and PEO (8 M) were 1.6, 1.7, 3.4, 3.0, 3.0, and 2.0%, respectively.

Table 1. Suitable concentration range of polymer solutions for DNA separations in the presence of EOF

Polymer	Molecular mass (g/mol)	Φ*(w/w %)	Concentration[a)](%)
Agarose	120 000	NF[b)]	1.0–1.6
HEC	1 300 000	0.37	0.8–2.5
HPC	1 000 000	0.09	1.9–3.4
LPA	1 000 000[c)]	0.23	2.5–7.0
PEO	4 000 000	0.12	2.0–3.5
PEO	8 000 000	0.05	1.0–2.5

a) For complete separations of ΦX 174 RF DNA-*Hae*III digest in the presence of the EOF
b) Not found
c) M_r of LPA prepared in this study was not determined.

At the optimal concentrations, the separation time was comparatively shorter using HEC solutions as shown in Fig. 2. This is because EOF was high due to low viscosity and less adsorption when using 1.7% HEC. The fact that the peaks corresponding to the 72- and 118-bp fragments were small and not too narrow supports our suggestion. It has been suggested that the interaction of small DNA fragments with the capillary wall is more profound, especially when the capillary wall is not well covered with polymers [29, 30]. Compared to HEC solutions, relatively high concentrations of HPC solutions were needed for achieving comparable resolution. This is because stiffer and more extended HEC chains have larger radii of gyration in solution and occupy a greater coil volume on average than do HPC chains.

Figure 2 also shows that the migration times for the DNA fragments were comparatively longer and the peaks corresponding to 118- and 72-bp fragments were broader

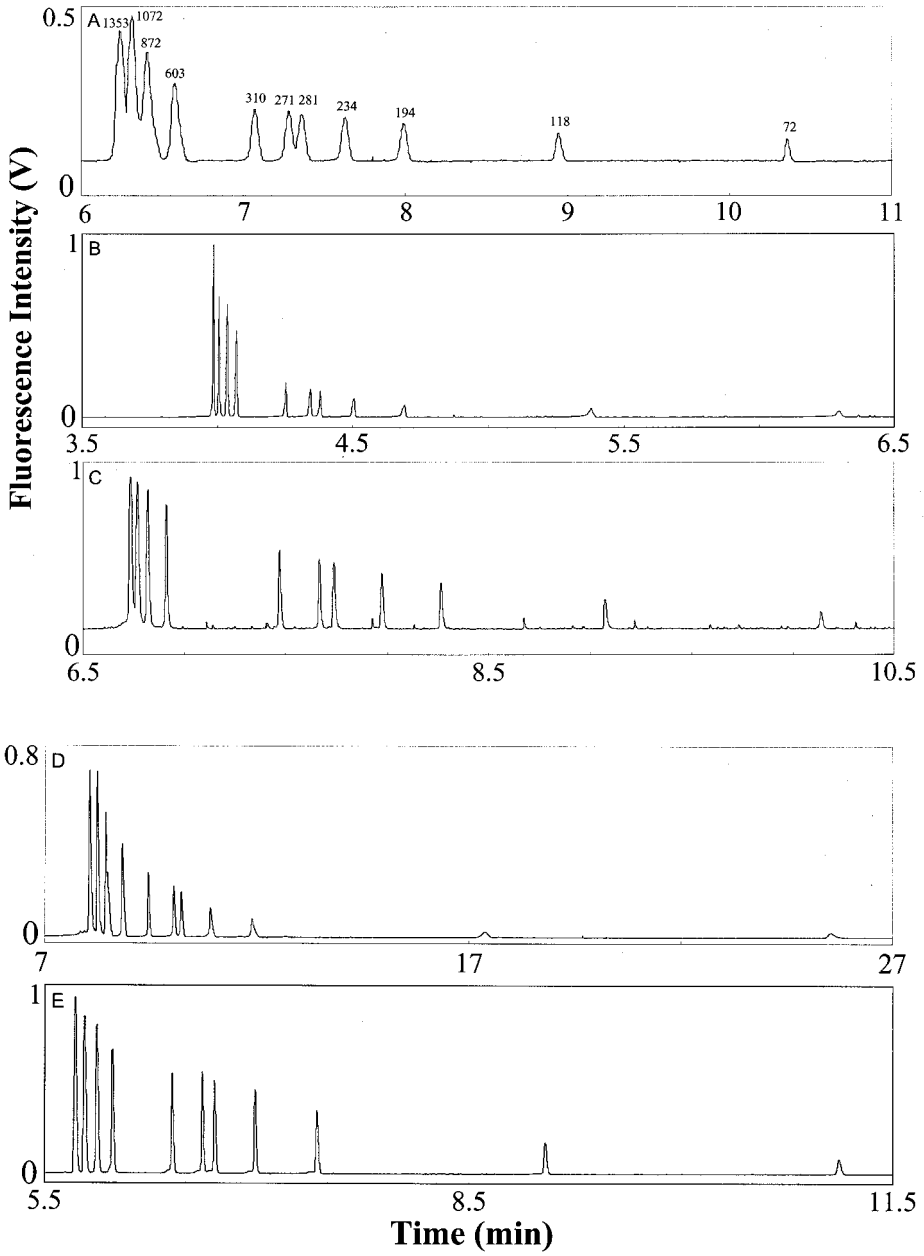

Figure 2. Separations of 25 μg/mL ΦX 174 RF DNA-*Hae*III digest in the presence of EOF at 375 V/cm using five polymer solutions containing 5 μg/mL EtB prepared in 1 × TBE, pH 8.2, respectively. (A) 1.6% Agarose, (B) 1.7% HEC, (C) 3.4% HPC, (D) 3.0% LPA, (E) 2.0% PEO (8 M). Capillary: 40 cm in total length and 30 cm in effective length, filled with 1 × TBE buffers, pH 8.2.

(bandwidth at the base is 18 s, while only 6 s when using HEC or PEO) when using LPA solutions. As a result of a long migration time, diffusion played a significant role in the band broadening. Since the viscosity of 3% LPA is only slightly higher than that of 1.7% HEC, the results implied that LPA adsorption was marked. Another fact to support our suggestion is that after using LPA, the capillary had to be washed with 0.5 M NaOH at 25 V/cm for 20 min to obtain reproducibility (RSD $<$ 3%) comparable to that of using other polymer solutions. It is interesting to note that the bandwidths for the large DNA fragments ($>$ 310 bp) were much wider (bandwidth at the base is 9 s for the 603-bp fragment, while only 3 s when using HEC or PEO) when using agarose solutions. This is probably because (i) the capillary wall was not fully covered with agarose molecules (many charged groups present on the polysaccharide, mostly pyruvate and sulfate), (ii) the morphology of agarose matrices (low gelling point) was changed due to Joule heating, (iii) agarose migrated against EOF (it took a longer time for DNA migrating from the sample zone to the agarose solutions). To clearly evaluate effects of polymer adsorption on EOF and separation, some data obtained from Fig. 2 were tabulated in Table 2.

Because EOF mobility was higher than the electrophoretic mobility of the DNA fragments that migrated against EOF, the large DNA fragments were detected earlier at the cathode end. As a result, the migration times for the DNA fragments were generally shorter at a high EOF. Please note that EOF gradually decreased. Thus, a long migration time for the last peak (or a wide separation window) generally indicated that strong adsorption took place on the capillary surface if the viscosities among the five polymer solutions are similar. Besides, viscosity and sieving dependence had to be considered. We concluded that the interaction of the polymer molecules with the capillary wall increased in the following order: agarose $<$ HEC $<$ HPC $<$ PEO $<<$ LPA. This increased order was in agreement with the hydrophobicity of the polymers, which suggested that hydrophobic interactions play a crucial role in adsorption. The RSD values shown in Table 2 all were $<$ 3.0%, suggesting that the capillary was refreshed after treated with 0.5 M NaOH at 25 V/cm for 20 min when using LPA or for 10 min when using other polymer solutions. The result implied that separation results were not greatly dependent on the history of the system.

The resolutions shown in Table 3 support the fact that LPA is better than agarose for the small DNA fragments. At higher concentrations, resolution values between the small DNA fragments were much higher in this study than those obtained by conventional methods (in the absence of EOF). This is because the differential migration mobility became greater and the sum of the average mobility of the two analytes (μ_{av}) and the EOF mobility (μ_{eo}) decreased when DNA migrated against a slow bulk EOF, according to Eq. (1) [31]:

$$R = 0.177\ (\mu_{ep1} - \mu_{ep2})\ [V / D\ (\mu_{av} + \mu_{eo})]^{1/2} \quad (1)$$

where V is the applied voltage, D is the diffusion coefficient, and μ_{ep1}, μ_{ep2} are the effective mobilities of two adjacent DNA fragments, respectively. The suggestion was further supported by the fact that the large DNA fragments were not separated in 2% LPA solutions in comparison to small ones (not shown). Although band broadening due to diffusion and interactions with the capillary wall might take place, they did not cause dramatic loss of resolution between any two large adjacent DNA fragments ($>$ 194 bp). It is, however, a matter when the migration time was long and the capillary wall was not covered effectively with polymers. For example, loss of resolution between any two small DNA fragments ($<$ 194 bp) was marked most likely due to diffusion (long migration times) when using high concentrations of LPA.

3.2 Separation using mixed polymer solutions

As addressed above, it is possible to optimize resolution and speed by controlling EOF and sieving matrices. To achieve high speed, one would like to use hydrophilic polymer solutions. On the other hand, for optimum resolution between small DNA fragments, use of hydrophobic polymer solutions with small mesh sizes (higher concentrations) is appropriate. From these views, use of polymer solutions prepared from hydrophilic and hydrophobic polymers may be useful. To test this hypothesis, we performed DNA separations using mixed polymer solutions prepared from PEO and LPA. For comparison, separations were also carried out using mixed polymer solutions of PEO and HEC. Table 4 shows that resolution values obtained were higher when using mixed polymer solutions of LPA and PEO, which were mainly due to greater differential mobilities. Because the viscosity of 1.5% PEO + 0.5% LPA is close to that of 2% PEO solutions, a smaller bulk EOF (longer t_0) using mixed polymer solutions was mostly due to LPA adsorption. Table 4 also shows that, compared to 2% PEO solutions, mixed polymer solutions of 1.85% PEO and 0.15% HEC (1.3 M) provided better resolution in comparable times. A similar result has been found using PEO solutions prepared from a number of PEO polymers with different sizes [32]. It is interesting to note that, at the same concentration, separation times were longer using mixed polymer solutions containing HEC (250 k) than those containing HEC (1.3 M). Most likely, this is because the capillary wall was not effectively covered with large-size HEC [20, 29, 30]. Of course, different morphologies among mixed polymers might attribute to different separation results.

3.3 Separation under discontinuous conditions

There have been several techniques developed for optimum resolution under discontinuous conditions, most of which are by voltage or temperature programming in the absence of EOF [33–36]. Recently, we have performed stepwise changes of polymer solutions and EtB concentrations for optimum resolution and speed in the presence of EOF [24, 37]. In this study, we further tested the effect of capillary length and molecular weight of PEO on DNA separations under discontinuous conditions. Figures 3A-C show the separation of DNA markers V and VI in the presence of EOF under isocratic conditions, by stepwise changes of EtB and by voltage programming, respectively. Figure 3A shows that eight peaks corresponding to DNA fragments ranging from 434 to 653 bp were resolved using 3% PEO (4 M), which were not completely resolved using 2% PEO (8 M) under the same conditions [37]. Figure 3B demonstrates that the two 234-bp fragments and the 123- and 124-bp fragments were further

Table 2. Reproducibility for DNA separations at 375 V/cm in the presence of EOF using different polymer solutions

Solution	Concentration (%)	t_0[a] (min)	μ_{eo}[b] ($\times 10^{-4}cm^2V^{-1}s^{-1}$)	First peak (min)	μ_{app}[c] ($\times 10^{-4}cm^2V^{-1}s^{-1}$)	Last peak (min)	μ_{app}[c] ($\times 10^{-4}cm^2V^{-1}s^{-1}$)	W[d] (min)
Agarose	1.0	3.20 (1.40%)[e]	4.17	5.95 (3.26%)	2.24	9.21 (3.10%)	1.45	3.26
	1.2	3.33 (1.12%)	4.00	6.08 (3.20%)	2.19	9.58 (2.90%)	1.39	3.50
	1.6	3.71 (1.19%)	3.59	6.24 (3.08%)	2.14	10.34 (2.89%)	1.29	4.10
HEC	0.8	2.40 (1.69%)	5.56	3.64 (2.17%)	3.66	6.29 (2.83%)	2.12	2.65
	1.0	2.99 (2.02%)	4.46	4.23 (2.44%)	3.15	7.18 (3.37%)	1.86	2.95
	1.7	2.99 (1.81%)	4.46	3.99 (2.06%)	3.34	6.47 (3.77%)	2.06	2.48
HPC	1.9	3.08 (1.26%)	4.33	4.28 (2.04%)	3.11	6.32 (3.11%)	2.11	2.04
	2.6	3.91 (1.49%)	3.41	5.15 (1.35%)	2.59	7.54 (2.45%)	1.77	2.39
	3.4	5.56 (1.43%)	2.40	6.74 (1.79%)	1.98	10.14 (2.16%)	1.31	3.40
LPA	3.0	4.04 (2.09%)	3.30	8.06 (1.79%)	1.65	25.55 (3.53%)	0.52	17.49
	4.0	4.15 (2.16%)	3.21	10.83 (1.14%)	1.23	65.05 (3.33%)	0.21	54.22
	6.0	5.31 (2.52%)	2.51	17.84 (1.13%)	0.75	52.71 (2.91%)[f]	0.25	–
PEO (8 M)	1.5	3.41 (1.45%)	3.91	5.77 (0.89%)	2.31	12.36 (0.26%)	1.08	6.59
PEO (8 M)	2.0	3.56 (0.89%)	3.74	5.71 (0.72%)	2.34	11.19 (1.93%)	1.19	5.48
PEO (4 M)	2.5	4.09 (2.77%)	3.26	6.71 (1.62%)	1.99	14.24 (0.89%)	0.94	7.53

a) Time when the baseline shifted due to detection of polymer solutions
b) Bulk EOF mobility
c) Apparent electrophoretic mobility
d) Time scale between the first and the last detected peak
e) % RSD, $n = 3$
f) 310-bp DNA fragment

Table 3. Effect of polymer solutions on resolution

Polymer solution	Concentration (%)	Resolution									
		1353/1078	1078/872	872/603	603/310	310/271	271/281	281/234	234/194	194/118	118/72
Agarose	1.0	1.39	1.56	3.41	15.17	4.55	1.48	4.79	6.49	22.50	27.24
	1.2	0.77	1.13	2.09	8.16	3.07	1.06	4.13	5.51	15.58	18.64
	1.6	1.48	1.53	2.26	9.44	3.60	1.94	5.31	4.87	15.19	20.16
HEC	0.8	3.25	3.54	3.54	10.62	2.87	0.96	3.69	4.85	13.52	13.08
	1.0	3.54	4.72	5.01	13.72	4.62	2.24	7.79	10.32	38.05	36.73
	1.7	3.54	4.81	7.08	15.69	17.11	3.54	11.21	16.81	63.42	60.18
HPC	1.9	0.91	1.33	1.67	6.97	3.54	1.36	3.75	5.36	17.97	25.13
	2.6	1.25	2.19	3.44	18.68	8.36	3.24	9.05	12.10	29.92	35.47
	3.4	1.77	2.95	5.31	33.04	11.50	4.33	11.80	14.75	40.88	47.13
LPA	2.0	0.00	0.00	0.00	6.80	5.25	1.03	3.53	4.96	18.12	6.25
	4.0	2.87	2.43	4.89	10.84	18.52	6.28	18.26	23.11	87.94	29.65
	6.0	2.59	3.80	9.44	29.77	17.50	5.26	31.97	50.98	ND[a]	ND[a]
PEO	1.5[b]	1.11	1.57	2.20	11.76	6.72	2.77	9.35	13.73	41.65	45.83
	2.0[b]	2.46	3.09	4.05	17.56	9.25	3.65	11.69	16.50	52.63	50.67
	2.5[b]	1.95	2.75	4.25	15.51	7.47	3.06	9.21	12.41	37.36	25.62

a) Not detected
b) 8 M
c) 4 M

Table 4. Comparison of resolution and separation time when using sole polymer solutions and mixed polymer solutions

	Resolution						Time (min)	
	1353/1078	872/603	271/281	234/194	118/72	t_0[a)]	First peak	Last peak
1.5% PEO (8 M)	1.1	2.2	2.8	13.7	45.8	3.41	5.77	12.36
1.5% PEO + 0.5% HEC (0.25 M)	1.8	3.5	4.3	16.7	17.8	3.49	5.64	12.49
+ 0.5% HEC (1.3 M)	1.9	3.1	3.3	14.4	50.5	3.21	5.17	10.41
+ 0.5% LPA	4.9	10.4	6.0	24.7	65.5	3.60	8.35	24.95
1.85% PEO + 0.15% HEC (0.25 M)	1.5	2.3	1.6	5.9	48.6	3.49	5.94	12.65
+ 0.15% HEC (1.3 M)	3.3	5.5	4.4	19.2	57.1	3.44	5.73	12.25
2.0% PEO	2.5	4.1	3.1	16.5	50.7	3.56	5.71	11.19

a) As in Table 2

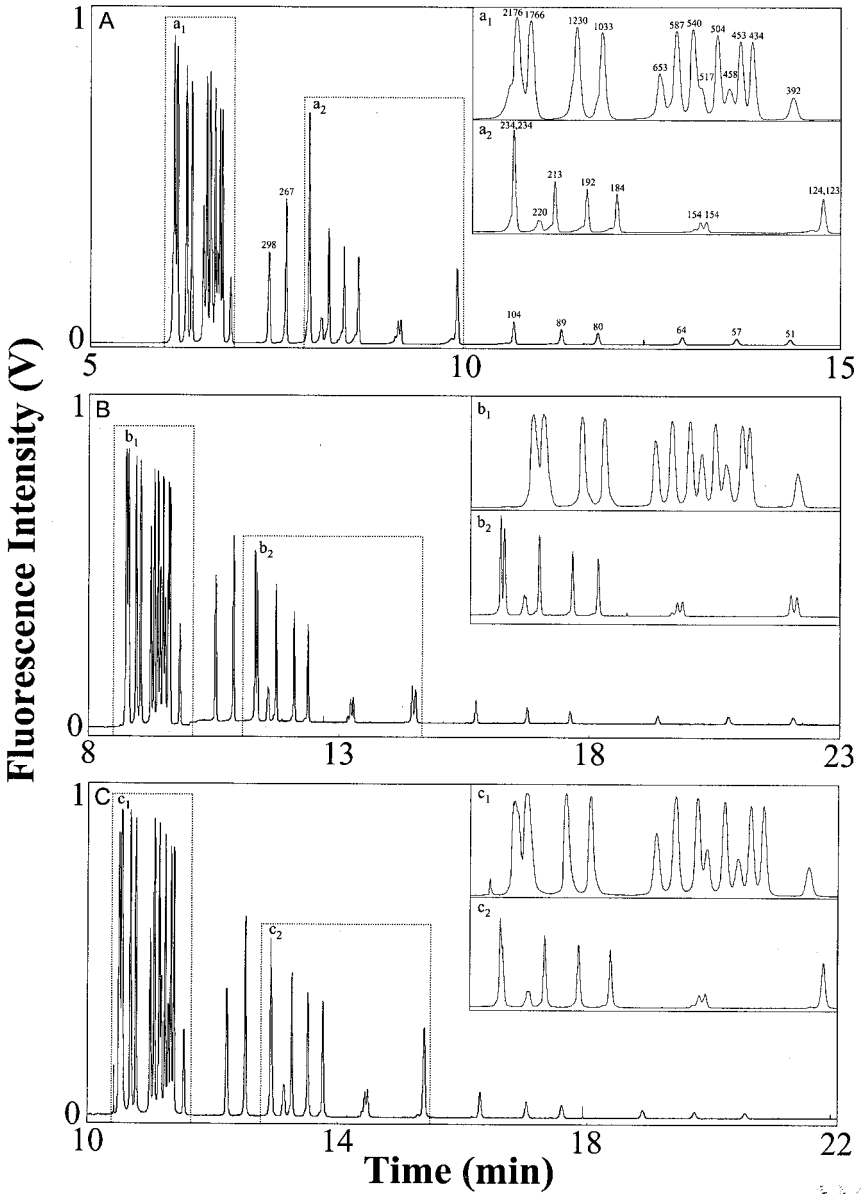

Figure 3. Separation of DNA markers V and VI in the presence of EOF under isocratic conditions, stepwise changes of EtB and voltage programming, respectively. (A) 3% PEO (4 M) containing 0.5 μg/mL EtB; (B) 3% PEO (4 M), stepwise form was 0.5 μg/mL EtB for 210 s, 1.0 μg/mL EtB for 90 s, 2.0 μg/mL EtB for 90 s, and 5.0 μg/mL EtB for the rest; (C) 3% PEO (4 M) containing 0.5 μg/mL EtB, 125 V/cm for 5 min and subsequently 375 V/cm for the rest. Other conditions were as in Fig 2.

Table 5. Comparison of resolution and separation time obtained under isocratic and discontinous conditions

	Resolution										Time (min)		
	653/587	587/540	540/517	517/504	504/458	458/453	453/434	234/234	154/154	124/123	t_0	First peak	Last peak
PEO (4 M)													
Isocratic[a)b)]	1.59	1.59	0.54	1.43	1.21	1.18	1.31	0	1.08	0	3.73	6.12	14.32
Stepwise EtB[a)c)]	1.73	1.81	1.32	1.62	1.10	1.64	0.81	1.18	1.34	1.21	5.39	8.75	22.06
Voltage[a)d)]	2.02	2.26	1.13	2.18	1.50	1.45	1.65	0.43	0.94	0	7.99	10.51	20.51
PEO (8 M)													
Isocratic[a)e)]	1.84	1.67	1.48	1.43	0	2.10	0	0	0.52	0	3.34	5.55	12.95
PEO (8 M)													
Isocratic[f)]	1.56	2.84	1.38	2.51	1.77	1.67	2.89	0.55	1.24	0	5.75	10.28	36.89
Stepwise[f)g)]	2.00	2.71	1.40	2.04	1.33	2.25	1.70	0.32	1.28	2.08	5.79	9.96	36.04

a) 40-cm capillary
b) 3% PEO (4 M) solutions containing 0.5 μg/mL EtB; running voltage, 375 V/cm
c) Stepwise change of EtB using a similar setup shown in Fig. 1: 0.5 μg/mL EtB for 210 s, 1 μg/mL EtB for 90 s, 2.0 μg/mL EtB for 90 s, and 5 μg/mL EtB for the rest
d) Voltage programming: 125 V/cm for 5 min, and 375 V/cm for the rest, other conditions as b)
e) 2% PEO (8 M) solutions containing 0.5 μg/mL EtB and the running voltage was 375 V/cm
f) 50-cm capillary
g) Stepwise change of EtB: 0.1 μg/mL EtB for 90 s, 0.5 μg/mL EtB for 60 s, 1.0 μg/mL EtB for 60 s, and 2 μg/mL EtB for the rest

resolved under stepwise changes of EtB. Resolution was optimized because changes in DNA conformations (becomes stiffer) when intercalating with EtB at high concentrations (5 μg/mL).

To minimize the field dependence of the DNA fragments (especially the large ones) [15, 38, 39], we performed DNA separation under stepwise changes of voltages from 125 (5 min) to 375 V/cm. Resolution between any two adjacent peaks corresponding to 1230 to 434-bp fragments was slightly improved (Fig. 3C). The reason not to start from lower applied field strengths was because separation time was long, some small DNA fragments were not detected, or resolution between any two large DNA fragments ($>$ 434 bp) was not improved markedly. Table 5 compares the results of separating DNA markers V and VI obtained under different conditions. It clearly shows that resolution between DNA fragments 504/458, 453/434, and two 154/154 of DNA markers V and VI, was improved using PEO (4 M) solutions instead of using PEO (8 M) solutions. Regardless of the separations that were performed under isocratic or stepwise conditions, it is apparent that resolution was further improved using a longer capillary. As the capillary length was increased, the migration times for DNA were longer because of a greater migration length and small bulk EOF mobility. Please note that the bulk EOF decreased gradually as a result of increase in the fraction of polymer solutions inside the capillary and adsorption of polymers during separation. Although improvement of resolution between any two large DNA fragments ($>$ 392 bp) was greater when separating at electric field strengths $<$ 125 V/cm than that using a 50-cm capillary, the peaks corresponding to the small DNA fragments became very broad or not detectable. In comparison, changes in the capillary length seem to be more straightforward than voltage programming in this study.

3.4 Separation at high TB buffer concentrations

High-speed DNA separation can be achieved if PEO adsorption can be further minimized. Recently, we have found that PEO adsorption diminished using a capillary filled with high concentrations of TB buffers, pH 10.0 (manuscript in preparation). This is because hydrogen bonding between PEO and the capillary wall is weakened, presumably because there are a lot of charged species adsorbed on the capillary surface at high pH and in the presence of high salts. For this reason, we performed the separation of DNA marker V and VI in 1.5% PEO (8 M) solutions, prepared in 100 mM TB, pH 9.0, using a capillary filled with 600 mM TB, pH 10.0. Several important results from Fig. 4 must be emphasized, including (i) separation was accomplished in 15 min, (ii) two 234-bp fragments, and 123- and 124-bp fragments were separated, and (iii) the smallest DNA fragment detected was the 11-bp one. Separation was short because PEO adsorption had a less effect on decreases in bulk EOF

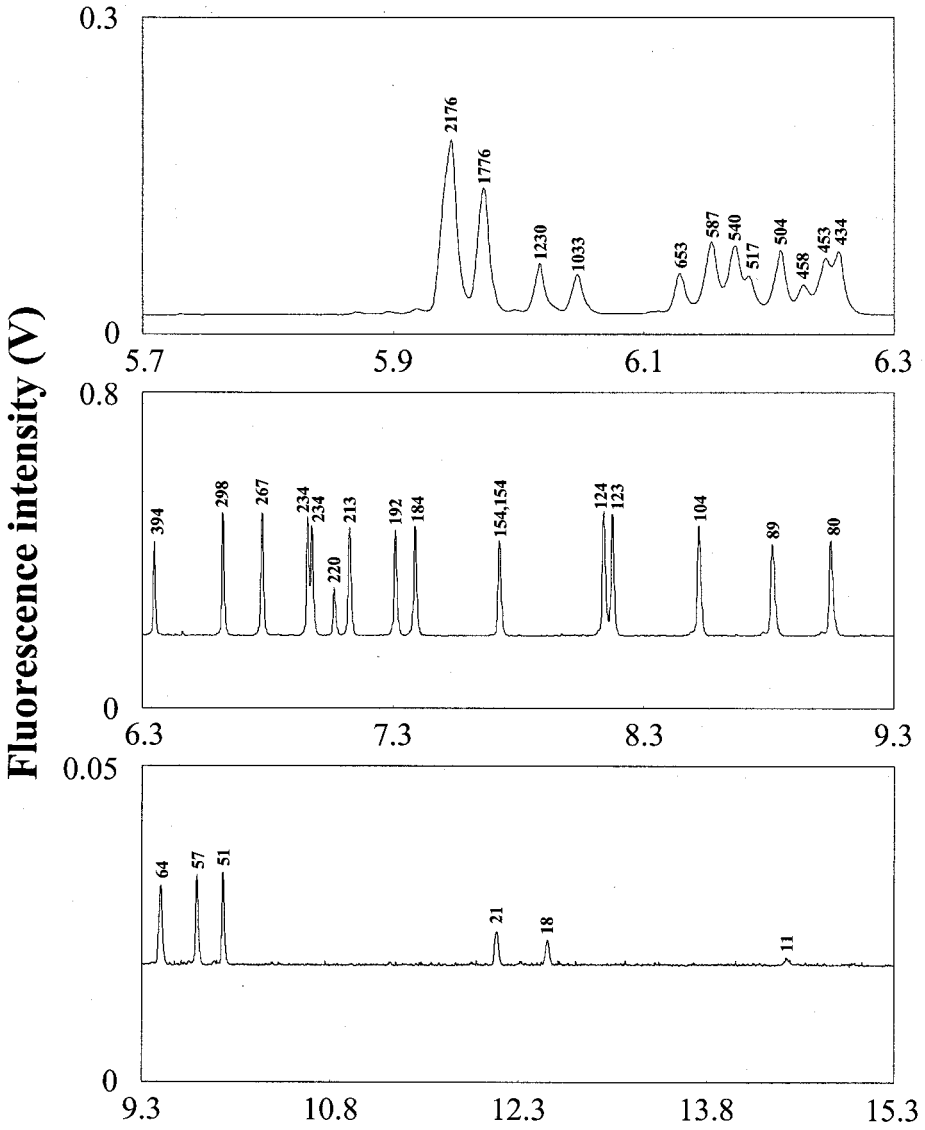

Figure 4. Separation of DNA markers V and VI using 1.5% PEO (8 M) solutions containing 5 μg/mL EtB prepared in 100 mM TB, pH 9.0, using a capillary filled with 600 mM free TB buffer, pH 10.0. Capillary: 40 cm in total length and 30 cm in effective length.

(RSD 1.4%) compared to that (RSD $>2\%$) using $1\times$ TBE, pH 8.2. Higher μ_{ep} and stiffer DNA structures at high concentrations of TB should also contribute to optimum resolution. The 11-bp fragment was detected because interaction with the capillary was weakened at high TB concentrations. In addition, the pH junction between PEO solutions and TB buffers should also play a crucial role in determining resolution, presumably because DNA became stiffer and its diffusion coefficient became small at high pH [40].

4 Concluding remarks

To obtain rapid DNA separation in the presence of EOF, a high bulk EOF is needed. To achieve this goal, the capillary surface must be washed with 0.5 M NaOH between runs, hydrophilic polymer solutions (HEC or PEO) are used, and the capillary filled with high concentration of TB buffers is suggested. We have also shown that resolution is optimized because DNA migrated against EOF. This is especially true for small DNA fragments with high μ_{ep} against small bulk EOF. Although we are able to improve reproducibility (RSD values of EOF $<$ 3%) by washing the capillary with NaOH, one major shortcoming of this technique is that EOF does not keep constant during the separation, which makes the calculation of the real μ_{ep} impossible. As results have shown in DNA sequencing using PEO or HEC polymer solutions [41, 42], we believed that using mixed polymer solutions prepared from PEO and HEC should be suitable because they provide better resolving power than that of HEC or PEO solution. As several stepwise techniques (EtB, voltage, PEO) have been

performed to optimize resolution and speed in the presence of EOF [37], we feel confident that this technique associated with PCR has the potential for analysis of single point mutation in DNA. For example, we will focus on analysis of PCR products by stepwise changes of urea that are not easily performed in the absence of EOF (conventional method).

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