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The impact of a plug of salts on the analysis of large volumes of dsDNA by capillary electrophoresis

A partially filling technique for the analysis of DNA markers and polymerase chain reaction (PCR) products by capillary electrophoresis in the presence of electroosmotic flow using polymer solutions is presented. Either after or prior to the sample injection, a plug of salts at high pH was hydrodynamically injected. During the separation, poly(ethylene oxide) (PEO) solution entered the capillary. We have found that the position, length, and composition of the plugs affect the sensitivity, resolution, and speed on the analysis of ΦX-174/*Hae*III DNA restriction fragments or a DNA mixture (pBR 322/*Hae*III digest, pBR 328/*Bgl*I digest and pBR 328/*Hinf*I digest) with different degrees. Through careful evaluation of the impact of anions and cations on the analysis of DNA, we have suggested that the optimal condition is applying a plug consisting of 32 mM NaCl and 0.01 M NaOH at 30 cm height for 60 s after sample injection. In the presence of such a plug, PEO adsorption reduces, and thus the separation is faster, as well as the sensitivity improves. Using this condition, the analysis of a DNA mixture (injected at 30 cm for 360 s) containing ten different PCR products amplified after 17 cycles was complete in 25 min. About a 2000-fold improvement in the sensitivity was achieved when compared to that by a conventional method (10 s injection) without applying a plug.

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

Owing to their ease of use and replacement, many hydrophilic polymer solutions like poly(ethylene oxide) (PEO) have been tested in capillary electrophoresis (CE) for the analysis of polymerase chain reaction (PCR) products and DNA sequencing for many years with varying degrees of success [1–11]. Despite the sieving ability of the polymer solution, the use of coated capillary to suppress electroosmotic flow (EOF) and to minimize DNA adsorption is generally required for high resolution and reproducibility [12–15]. Recently, we have demonstrated the analysis of DNA using PEO in the presence of EOF [16–21]. After injection of DNA, PEO enters the capillary filled with tris(hydroxymethyl)aminomethane (Tris) and borate (TB) buffer. Because DNA migrates against EOF, large DNA is detected earlier towards the cathodic end. Through a series of studies, we have learned that PEO adsorption on the capillary wall is easily removed by washing with 0.5 M NaOH at 1 kV for 10 min after each run. In order to control PEO adsorption and thus regulate EOF, the ionic strength and pH of the buffers used to fill the capillary and prepare polymer solutions are of considerable importance [17–20], especially, when on-line concentration and separation of DNA in the presence of EOF using PEO was performed [22].

In order to optimize DNA separation by CE, additives such as amines, metal ions, and urea have been added to background electrolytes [23–26]. This is because such additives could cause changes with different degrees in DNA conformation and charge density, the binding constant for intercalated DNA complex, viscosity, as well as the property of the capillary wall. For instance, it has been found that DNA separation is better in high-pH buffer containing NaCl than in conventional Tris-borate-EDTA (TBE) buffer [27], mainly due to a small diffusion coefficient of DNA. The increment in the mobility with increasing the concentration of alkaline metal ions and the improvement in the efficiency for the DNA separation in the presence of lithium ions have recently been reported [26]. However, the binding between DNA and ethidium bromide (EtBr) decreases with increasing the concentration of salts, leading to decreases in the sensitivity [28]. Although the separation of DNA using solutions containing suitable amounts of salts at high pH is quite promising, a particular attention must be paid to minimize Joule heating. To achieve this goal, our attempt was to develop a partially filling technique for DNA separation in the presence of EOF using PEO solutions. The effects of a plug of salts

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Abbreviations: EtBr, ethidium bromide; **PEO**, poly(ethylene oxide); **TB**, Tris-borate

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injected either before or after sample injection on the resolution and sensitivity in DNA separation were evaluated.

2 Materials and methods

2.1 Equipment

The basic design of the separation system has been previously described [16]. Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., 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 laboratory-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 10 × objective (numerical aperture = 0.25). One RG 590 cutoff filter was used to block scattered light before the emitted light reached the photomultiplier tube (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 personal computer. 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). EtBr was obtained from Molecular Probes (Eugene, OR, USA). Tris and boric acid were used to prepare TB buffers, pH 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 400 mM TB, pH 10.0. Certain amounts of PEO (8 000 000) were used to prepare polymer solutions in 200 mM TB buffer, pH 9.0, containing 5 μg/mL EtBr. ΦX-174 RF DNA-*Hae*III digest was purchased from Pharmacia Biotech (Uppsala, Sweden). The DNA sample was prepared by mixing equal volumes of DNA markers V (pBR 322/*Hae*III digest) and VI (pBR 328/*Bgl*I digest and pBR 328/*Hin*fI digest) that were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

2.3 DNA extraction and PCR products

The blood sample was from a normal male. Human genomic DNA from buffy coat was extracted using the QIAamp DNA blood mini kit in accordance with manufacturer's instructions. Amplification of the DNA sample was conducted as suggested by the manufacturers. Briefly, 22.5 μL PCR master mix component was prepared by mixing 17.45 μL sterile water, 2.50 μL STR 10 × buffer, 2.50 μL 10 × primer pairs, and 0.05 μL *Taq* polymerase (5 u/μL). To the PCR master mix, 2.5 μL of human genomic DNA (25 ng) was added. PCR was conducted as: initial incubation at 96°C for 2 min; cycling for the first 10 cycles at 94°C for 1 min, at 64°C for 1 min, and at 70°C for 1.5 min; and cycling for the last 7 or 22 cycles, at 90°C for 1 min, at 64°C for 1 min, and at 70°C for 1.5 min.

2.4 Separation

Prior to analysis, capillaries were treated with 0.5 M NaOH overnight. After each run, the capillary was washed with 0.5 M NaOH at 1 kV for 10 min to remove polymer solutions and refresh the capillary wall, and subsequently filled with TB buffers. This treatment allowed reproducible results (relative standard deviation (RSD) of EOF $< 3\%$) [19]. Injection of the DNA samples was carried out by electrophoresis for 1 kV 10 s or 15 kV 360 s. Plugs consisting of salts and 0.001, 0.01, or 0.1 M NaOH were hydrodynamically injected either before or after the sample injection. During the analysis, 2% PEO in the anodic reservoir entered the capillary by EOF and served as sieving matrices for DNA separations. For the analysis of a DNA mixture containing ten different PCR products, the plug consisting of 32 mM NaCl and 0.01 M NaOH, was injected after the sample injection at 30 cm for 60 s.

3 Results and discussion

3.1 Separation in the absence or presence of a plug

It is difficult to detect small DNA fragments when conducting DNA separations in the presence of EOF using polymer solutions because they migrate with greater electrophoretic mobilities against decreased EOF and intercalate less amounts of EtBr (low fluorescence intensities) when compared to large ones. A typical example is given in Fig. 1A that shows only the DNA fragments greater than 51 bp were detected in 18 min, despite with a single-base resolution for 123 and 124 bp fragments. To overcome this shortage, a method providing a high EOF and better sensitivity is required. On the basis of others' and our results [21, 27, 29], we started exploring the impact of pH and salts on DNA separations from applying a plug of 0.01 M NaOH and 32 mM NaCl. As expected, Fig. 1B shows that the DNA fragments greater than 18 bp were resolved in 20 min, with a 4-fold improvement in the

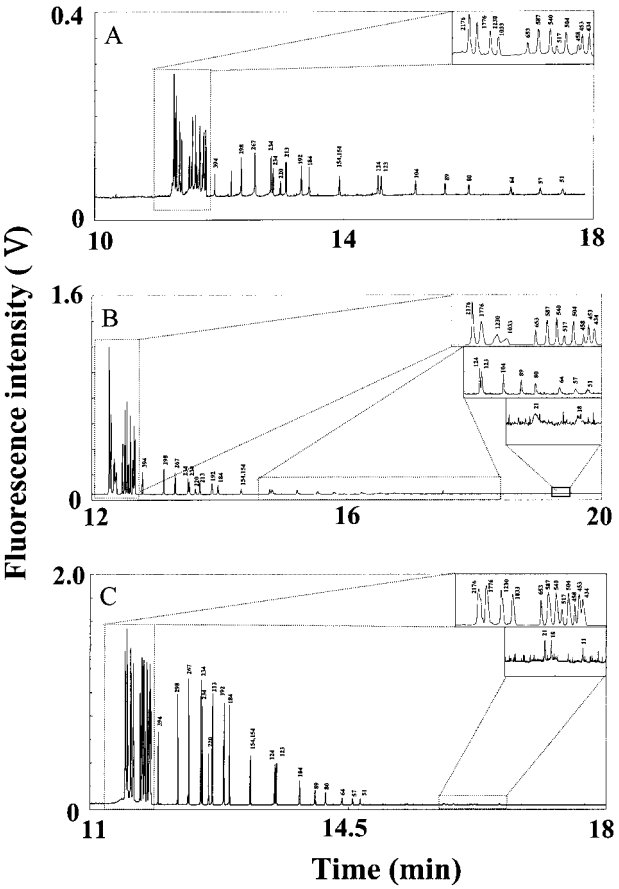

Figure 1. Separations of 5 μg/mL DNA markers V and VI in the presence of EOF at 15 kV using 2.0% PEO containing 5 μg/mL EtBr prepared in 200 mM TB, pH 9.0. (A) Without a plug; (B), (C) plugs consisting of 32 mM NaCl and 0.01 M NaOH were hydrodynamically injected at 30 cm height for 30 s prior to and after the sample injection, respectively. Capillary, 40 cm in total length, 30 cm in effective length, filled with 400 mM TB buffer, pH 10.0.

sensitivity, when injecting such a plug prior to the sample injection (cathodic side). Because of migrating against EOF, the DNA fragments possibly interacted with hydroxide and chloride ions from the plug. Upon exposing at salts, the ion atmosphere is dispersed in front of the migrating macroions and rebuilt on its back, leading to higher electrophoretic mobilities. This was supported by a longer migration times for the large DNA fragments. The increased fluorescence intensity also supported that DNA migrated in higher pH environments [28]. Together with weakened interactions of DNA and PEO with the capillary wall at high pH, a greater EOF and thus the detection of 18 bp fragment were achieved [21, 30]. Figure 1C shows that the 11 bp fragment was detected as a result of an improvement in the sensitivity when a plug was injected after the sample injection (anodic side). With such a plug, the migration time for the 51 bp fragment shortened from 17.5 (Fig. 1A) to 14.7 min. In comparison to that shown in Fig. 1B, the separation was also faster and the sensitivity improvement was greater. The electropherogram also shows that the peak profiles are much sharper and suggests that the EOF mobility is greater, indicating reduced interactions of DNA and PEO with the capillary wall. Unlike the role of anions playing in improving the sensitivity and changes in resolution (Fig. 1B), sodium ions should be one of the important contributors, which is in good agreement with a reported result [26].

Several groups have investigated the effect of cations as well as anions on DNA conformation and the charge density [27, 29, 31, 32]. It has also been found that the ionic strength of background electrolytes plays a significant role in determining resolution and has various impacts on DNA with different structures [33]. To further explore the effect of other cations on DNA separation, salts such as $CaCl_2$ and KCl at a concentration of 32 mM in 0.01 M NaOH were separately injected after sample injection. The results show that the plug of KCl has a similar effect as that of NaCl, while the sensitivity improvement is slightly less when applying $CaCl_2$ plug. This is mainly due to reduced interactions with the capillary wall, stacking effects, and different effects of metal ions on DNA conformation, supported by narrower peak profiles in the presence of the plugs. On the other hand, to compare the effect of anions on DNA separation, salts such as NaI and NaF at a concentration of 32 mM in 0.01 M NaOH were separately injected prior to sample injection. The results were similar to that with the plug of NaCl, indicating that pH should play a more important role in improving the sensitivity. Compared to the result obtained by a fluorometer at the same pH, salts cause greater improvements in the sensitivity for DNA analysis by CE, suggesting that stacking and/or reduced adsorption of DNA took place.

3.2 Effect of the composition and length of the plug

To further investigate the effect of NaCl on the separation of DNA, the separations were separately conducted in the presence of a plug of NaCl, at concentrations of 1, 10, 32, 64, 100, 200, and 300 mM. When the plugs were in the cathodic side of the sample, the sensitivity improved with increasing the concentration of NaCl for the large fragments ($>$ 281 bp), while slightly decreased for the small ones. The maximum sensitivity improvement was 12-fold (RSD 5.1%) for the 281 bp fragment at 300 mM NaCl. However, the migration time for the 72 bp fragments increased from 11.23 to 19.06 min when the concentration of NaCl was increased from 1 mM to 300 mM.

Table 1. The impact of plugs on migration time and peak height enhancement (PHE)

Plug position[a)]	Time (s)[b)]	Migration time (min) Mean (RSD%)			PHE[c)] Mean (RSD%)		
		t_0[d)]	1353 bp	72 bp	603 bp	281 bp	72 bp
None		4.33 (0.6)	6.05 (0.9)	11.13 (1.5)	1	1	1
Cathodic side	30	4.82 (0.8)	6.69 (1.1)	11.58 (0.9)	5.3 (1.9)	4.1 (2.4)	1.6 (2.9)
	60	5.07 (1.0)	7.5 (1.3)	12.08 (1.7)	5.9 (2.1)	5.4 (2.4)	1.5 (3.6)
	90	5.56 (1.5)	8.02 (0.5)	14.81 (2.8)	6.7 (2.7)	7.6 (3.5)	1.4 (3.7)
	120	7.6 (1.1)	10.18 (1.3)	18.28 (2.9)	6.8 (3.0)	8.9 (4.3)	1.1 (5.5)
Anodic side	30	6.77 (1.0)	8.6 (0.7)	11.99 (1.0)	5.4 (1.8)	7.8 (2.2)	3.2 (2.6)
	60	7.82 (1.1)	9.58 (1.1)	11.65 (1.3)	5.4 (2.1)	8.3 (2.4)	3.2 (2.7)
	90	8.07 (0.7)	9.78 (0.8)	10.75 (0.8)	5.5 (2.6)	9.9 (3.1)	5.6 (3.3)

a) The plug contained 32 mM NaCl and 0.01 M NaOH and the position was related to the sample zone. The separation was conducted in 2% PEO prepared in 200 mM TB, pH 9.0, using a capillary filled with 400 mM TB, pH 10.0.
b) Injection of the plug at 30 cm height.
c) The ratio of the peak height obtained in the presence of the plug to that obtained without a plug.
d) Time when the baseline shifted due to detection of PEO.

While the sensitivity for all of the DNA fragments increased with increasing the concentration of NaCl when the plug was in the anodic side of the sample zone. The maximum improvement was 9-fold (RSD 4.7%) for the 281 bp fragment at 100 mM NaCl. A different effect on small DNA fragments at higher concentrations of NaCl in the two cases is due to the impact of chloride ions on peak profiles when the plug is in the cathodic side. Although the sensitivity improved in both cases, resolution decreased with increasing the concentration of NaCl, especially for the large ones, whether the plugs were in the anodic or cathodic side. For example, the resolution values between 1352 and 1032 bp fragments were 3.0, 1.4, and 0.5, at 32, 100, and 300 mM in the anodic side, respectively. The loss of resolution is mainly due to small differential mobilities when the separation is faster and nonuniform distribution of the electric field inside the capillary in the presence of a plug of salts. Overall, the deterioration of resolution was slightly greater when the plugs were in the anodic side, mainly due to smaller differences in migration times (DNA did not enter PEO zones immediately). In the end, we suggested that a plug consisting of 32 mM NaCl and 0.01 M NaOH in the anodic side was more appropriate with respect to resolution, speed, and sensitivity.

As the distribution of the electric field strength inside the capillary depends on the local environment, the plug length plays a crucial role in determining DNA migration and thus resolution. From a dynamic point of view, the interaction between DNA and the component in the plug is also length-dependent. When the plug is in the anodic side, the time that DNA migrating in the PEO zone also depends on the plug length. With increasing the length of the plug consisting of 32 mM NaCl and 0.01 M NaOH in the cathodic side, the peak widths for the small DNA fragments increased dramatically. For example, the peak widths at the baseline for the 72 bp fragment were 2.4 and 18.1 s when the plugs were applied for 30 and 120 s, respectively. On the other hand, the peak widths for the 72 bp fragment were almost the same ($<$ 1.4 s) when the plugs in the anodic end were applied for 30 to 90 s. This again shows the role of chloride ions played in affecting peak profiles. Table 1 shows that the optimal plugs in the anodic and cathodic sides were conducted at 30 cm height for 60 and 90 s, respectively, on the basis of the sensitivity improvement for all DNA fragments. However, the loss of resolution increased with increasing the plug length in both cases, while 11 fragments were still well resolved. It was more profound when the plug was in the anodic side, simply because DNA did spend more time in free solution before entering PEO zone. This is supported by decreases in the migration time for the 72 bp fragment.

Next, we separately compared the effect of the plug with and without 32 mM NaCl in 0.001, 0.01, or 0.1 M NaOH. Figure 2 shows that, in the absence of 32 mM NaCl, the sensitivity enhancement was more marked in the presence of a plug of 0.01 M NaOH (cathodic side), which agreed with a reported result [28]. In addition, pH junction should contribute to the sensitivity improvement, on the basis of our previous result [21]. In the presence of a plug of 0.1 M NaOH, the sensitivity decreased slightly, except that for small ones ($<$ 118 bp), most likely due to partial denaturation of DNA, supported by increases in the migration times for all DNA fragments. It has been shown

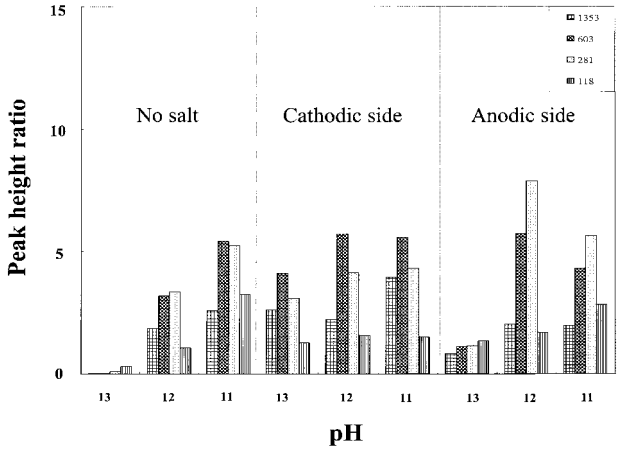

Figure 2. Effects of pH and salt on the separation of ΦX-174 RF DNA-*Hae*III digest. A plug was injected to the capillary prior to or after the sample injection. Peak height ratios are the peak heights obtained in the presence of plugs to that without plugs. Other conditions were as in Fig. 1.

that the electrophoretic mobility of DNA increased due to an increased effective charge and denaturation at high pH [28]. On the other hand, in the presence of NaCl, the sensitivity enhanced in the plug of 0.1 M NaOH, presumably because DNA were compact and the interaction with the capillary wall reduced [21, 27]. When compared the difference obtained in the absence and presence of NaCl, a greater improvement in the sensitivity in 0.1 M NaOH than in 0.01 M NaOH suggests that DNA is stable in the presence of salts under alkaline conditions. Overall, Fig. 2 shows that the best condition for the separation of DNA was in the presence of a plug consisting of 32 mM NaCl and 0.01 M NaOH in the anodic side.

3.3 PCR analysis

The analysis of PCR products by CE is of considerable importance and interest in many realms like forensics [34, 35]. As a request for analyzing a vast number of DNA samples in a day, several approaches, based on the array techniques, have been proposed [36–39]. These techniques provide advantages of high throughput (rapidity) and low cost (small amounts of sample and chemicals), and easy automation over the traditional slab gel electrophoresis. In an attempt to reduce the cost for the analysis of PCR products used in forensics, a plug consisting of 32 mM NaCl and 0.01 M NaOH in the anodic side was applied. Figure 3A shows the analysis of a sample containing ten PCR products after 32 cycles, with faster and greater efficiency when compared to that without such a plug. It is noted that the separation was not quite successful (some peaks were not resolved) without applying such a plug using the same conditions. Figure 3B shows the analysis of injecting a sample containing ten PCR products after 17 cycles at 30 cm height for 360 s. Narrow peak profiles and resolved peaks shown in the electropherogram indicates that DNA stacked as a result of decreases in the electrophoreteic mobility due to sieving and increase in viscosity when migrating from the sample zone to PEO solution [22]. In comparison to that by a conventional method (10 s injection) without applying a plug of salts, the sensitivity improvement was at least 2000-fold. Moreover, this method provides a greater sensitivity improvement, higher resolving power, and faster analysis time than our previous results [22], showing a merit of applying such a salt plug. Owing to only 17-cycle reactions conducted, the total analysis time (PCR amplification, injection, and separation) was reduced from 139 to

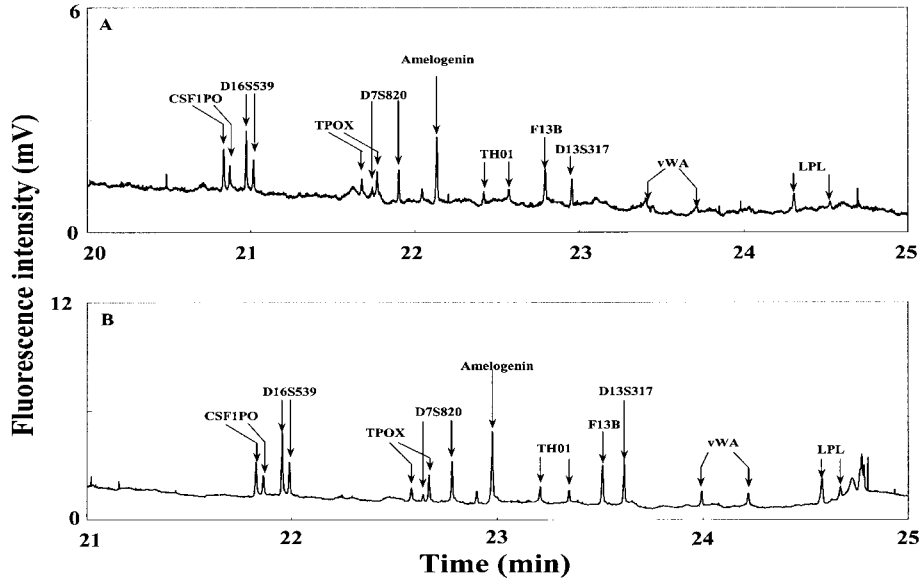

Figure 3. Separations of DNA mixtures containing ten different PCR products at 20 kV. (A), (B) The samples amplified after 32 and 17 cycles were hydrodynamically injected at 30 cm height for 10 and 360 s, respectively. A plug consisting of 32 mM NaCl and 0.01 M NaOH was conducted at 30 cm height for 60 s after sample injection. Capillary, 60 cm in total length, 50 cm in effective length. Other conditions were as in Fig. 1.

93 min. In addition, the cost of the analysis of PCR products should be dramatically reduced when using small amounts of primers and reagents to perform PCR for 32 cycles.

4 Concluding remarks

Improvement in sensitivity will result in great benefits for DNA analysis in CE, especially for trace amounts of samples. This paper describes the progress in the analyses of DNA markers and PCR products by CE in the presence of EOF using a partially filling technique. We have investigated the impact of the plugs consisting of salts at high pH on the analysis of DNA and the results show promising in terms of sensitivity and speed. By applying such a plug, problems associated with Joule heating and instability of PEO at high pH are prevented. It has been found that a plug consisting of 0.01 M NaOH and 32 mM NaCl applied after sample injection was appropriate. Owing to improvements in the sensitivity and reductions of the interaction with the capillary wall, this method allows detection of 11 bp DNA fragment, which was not detected by using our previous method [22]. Together with on-line concentration techniques, this method allows the analysis of a sample consisting of ten different PCR products amplified after 17 cycles. In the presence of a plug of salts, the sensitivity improvement is greater and the separation time is shorter. Overall, the results have shown a high potential for forensics by using such a sensitive, simple, and cost-effective method.

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