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Integrated electroosmotically-driven on-line sample purification system for nanoliter DNA sequencing by capillary electrophoresis

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Abstract

An integrated on-line system is developed for DNA sequencing at the nanoliter scale. The technique involves the use of a nanoreactor for small-volume cycle-sequencing reaction, capillary zone electrophoresis (CZE) for purification of the sequencing fragments, and capillary gel electrophoresis (CGE) for separation of the purified DNA fragments. The nanoreactor and CZE are integrated into one capillary, where a 100-nl dye-labeled terminator cycle-sequencing reaction is carried out followed by CZE to separate excess dye-labeled terminators from the sequencing fragments. On-line electrokinetic injection of the purified DNA fragments into the CGE system is accomplished at a small-volume tee connector by which the CZE capillary is interfaced to the CGE system. The utility of the system is demonstrated in sequencing nanoliter volumes of single-stranded DNA (M13mp18) and double-stranded DNA (pGEM). The use of voltage to drive both CZE and CGE makes it feasible for automation and future adaptation of the whole system to a microchip.

Keywords: Sample handling; Instrumentation; DNA

1. Introduction

Miniaturization, integration and automation are all well-recognized trends in the development of various protocols for high-speed and low-cost DNA sequencing required by the Human Genome Project [1]. Initially, DNA sequencing based on slab gel electrophoresis (SGE) was performed with sample volumes at the scale of tens of microliters. Moreover, the individual steps of template preparation, cycle-sequencing reaction, product purification, gel separation and detection were manually carried out in isolated devices. Numerous efforts have been made to increase the speed and reduce the reagent and labor cost of DNA analysis by developing robotic workstations to perform the sequencing reaction, purification, denaturation and sample loading in SGE [2–4]. However, the inability to miniaturize the robotic workstation limits the miniaturization and integration of the individual parts of the whole sequencing process.

In the last decade, intensive research has been dedicated to developing miniaturized separation techniques, such as capillary gel electrophoresis (CGE) and microchip gel electrophoresis (MGE), as viable alternatives to SGE for DNA sequencing [5–10]. CGE requires extremely small sample volumes, typically in the low nanoliter regime, which provide a great opportunity to reduce the cost in the use of expensive DNA primers, template, enzyme and reagents. Multiplexed CGE [9,11,12] has been demonstrated to provide high efficiency, high speed and

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high throughput in DNA sequencing analysis. Reports have shown nearly a three-fold improvement in resolution and a 25-fold increase in speed in the separation by CGE compared to SGE, primarily due to the use of capillaries that can efficiently dissipate Joule heat generated at high electric field strengths [6]. Parallel to the development of CGE and MGE, miniaturization of cycle sequencing in a glass capillary or microchip channel has been demonstrated. Polymerase chain reactions (PCRs) have been successfully performed in 1.5 μl volumes, and even 60 nl volumes in a capillary format [13,14], and 4 μl to 240 nl volumes in microchip chambers [15,16]. Recently, the integration of nanoreactor and MGE in the microchip for PCR analysis at the nanoliter scale has also been explored by a number of research groups [16–18]. However, for DNA sequencing, the critical intermediate step of purification of the reaction products between cycle-sequencing reaction and gel separation has not been addressed.

Currently, purification of reaction products is predominantly accomplished by conventional methods such as acetate–ethanol precipitation [19], phenol–chloroform extraction [20], magnetic bead adsorption [21], and gel filtration in spin column or microtiter plate format [22–24]. These methods require large sample volumes and high-speed centrifugation, which do not facilitate miniaturization and automation. Recently, there appeared reports on integrated on-line systems which integrated the steps of DNA sequencing from cycling reaction, product purification to separation and detection [25,26]. The techniques involved the on-line coupling of capillary microreactor for sample amplification, size-exclusion chromatography (SEC) for product purification, and CGE for separation of the purified sequencing fragments. The whole process has been automated, and the analysis speed was greatly improved by the use of multiplexed CGE [27–29]. However, the volume of the reaction mixture was in the order of tens of microliters and therefore did not exploit the low (nl) sample requirement for CGE. The system is also quite complicated due to the use of pressure-driven fluidics, which is required by the SEC purification column. The SEC column also introduces substantial dilution prior to injection into the CGE capillary.

In this report, a simple, miniaturized and integrated on-line sample preparation system was developed for DNA sequencing at the scale of nanoliters. The whole system features the use of capillary zone electrophoresis (CZE) for purification of the cycle-sequencing products, the integration of nanoreactor and CZE in one capillary, and on-line coupling of CZE with CGE. Factors affecting the individual steps and their integration are examined and optimized. The performance of the system is demonstrated in the analysis of both single-stranded (ss) DNA (M13 mp18) and double-stranded (ds) DNA (pGEM). With further integration of mixing and injection at the nanoliter scale and the automation and multiplexing of the whole system, the protocol will ultimately provide an attractive approach for high-speed and low-cost DNA sequencing.

2. Experimental

2.1. Reagents, separation matrix and reaction mixture

Tris(hydroxymethyl)aminomethane (THAM), fuming hydrochloric acid (HCl) and anhydrous sodium hydroxide (NaOH) were purchased from Fisher (Fairlawn, NJ, USA). The 1× TBE premix powder was bought from Amresco (Solon, OH, USA). 1 *M* magnesium chloride solution was bought from Sigma (St. Louis, MO, USA). 2.5 mg/ml bovine serum albumin (BSA) solution was purchased from Idaho Technology (Idaho Falls, ID, USA). Poly(ethylene oxide) (PEO) and histidine were received from Aldrich (Milwaukee, WI, USA). M_r 1 000 000 poly(vinylpyrrolidone) (PVP) was from Polyscience (Warrington, PA, USA). Urea was from ICN Biomedicals (Aurora, OH, USA). The water used in all reaction and separation solutions was deionized with a Milli-Q water purification system (Millipore, Worcester, MA, USA). A Thermo-Sequenase dye-terminator cycle-sequencing reaction core kit and premix kit were purchased from Amersham Life Science (Cleveland, OH, USA). pGEM was bought from Promega (La Jolla, CA, USA).

Tris–HCl buffer (THM) used in the CZE separation was prepared by dissolving 50 m*M* Tris, 2.5 m*M* HCl and 0.1 m*M* $MgCl_2$ in deionized water (pH 9.0). The 1× TBE buffer solution was prepared by

dissolving 89 m*M* Tris, 89 m*M* boric acid and 2 m*M* EDTA sodium salt with 7 *M* urea in deionized water. The sieving matrix was made by dissolving 1.5% of M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO in 1× TBE buffer. The solution for coating the separation capillary was prepared by dissolving 2% of M_r 1 000 000 PVP in 1× TBE buffer.

Modifications to the original cycle-sequencing reaction mixture developed for ABI Model 9600 thermocycler were made to fit the small volume reaction in the capillary. A typical 20-μl reaction mixture was composed of 2 μl of 2.5 mg/ml BSA, 1 μl of 20 m*M* $MgCl_2$, 2 μl of 5 μ*M* 40M13 (5′-GTTTTCCCAGTCACGAC-3′) universal primer, 3 μl of 0.2 μg/μl ssDNA (M13mp18) or 5 μl of 0.2 μg/μl dsDNA (pGEM) in 1× TE buffer (10 mM Tris, 1 mM EDTA, pH~7.5), 8 μl of sequencing reagent premix, and 4 μl of deionized water. The reagent premix consists of 125 m*M* Tris–HCl, pH 9.5, 5 m*M* $MgCl_2$, 1.25 m*M* dITP, 0.25 m*M* each dATP, dCTP, dTTP, ddATP (dye-labeled), ddCTP (dye-labeled), ddGTP (dye-labeled) and ddTTP (dye-labeled), Thermo-Sequenase DNA polymerase, Thermoplasma acidophilum thermostable inorganic pyrophosphatase (TAP), Nonidet P40, Tween 20 and 6.25% glycerol.

2.2. Instrumentation and operation

Fig. 1 is a schematic diagram of the entire instrumental set-up.

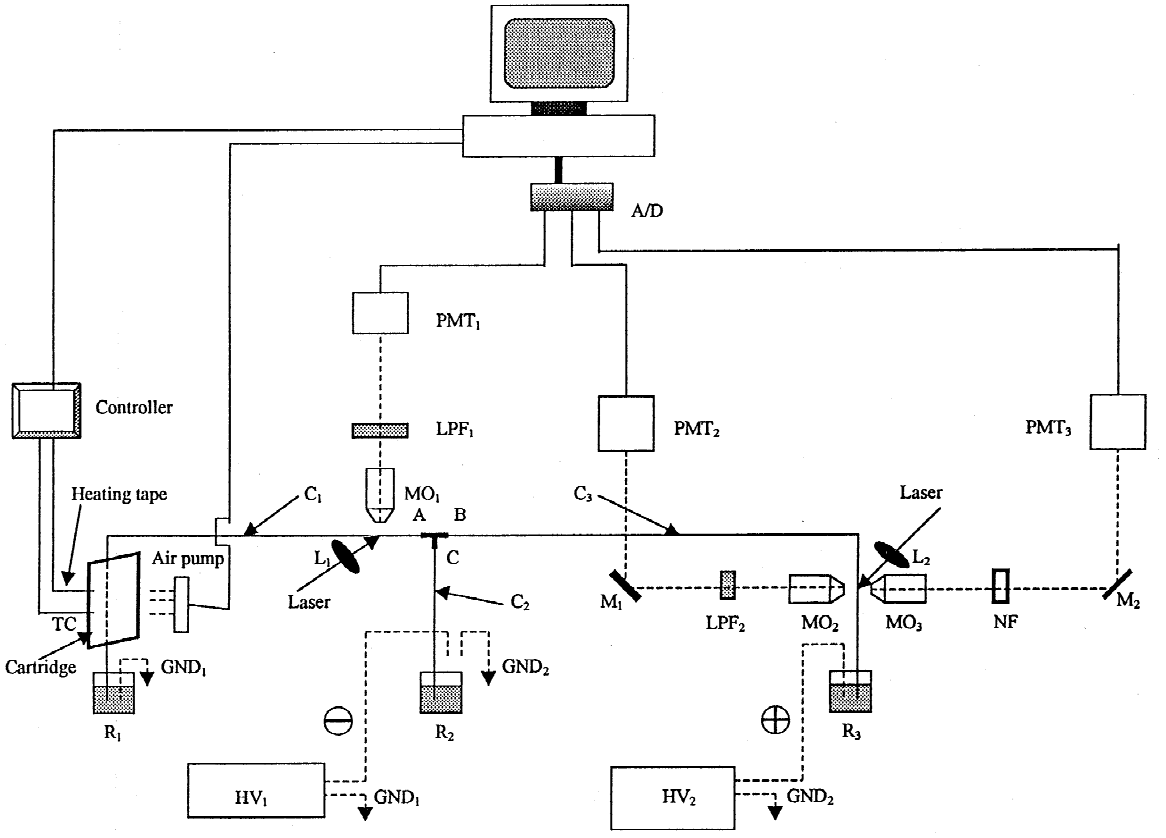

Fig. 1. Schematic of the integrated on-line cycle-sequencing–CZE–CGE system. TC, Thermocouple; C_1 and C_2, CZE capillaries; C_3 CGE capillary; R_1, R_2 and R_3, buffer reservoirs; HV_1, negative-voltage power supply; HV_2, positive-voltage power supply; GND_1 and GND_2, grounded lines for HV_1 and HV_2; L_1 and L_2, lenses; MO_1, MO_2 and MO_3, microscope objectives; LPF_1, 550-nm long-pass filter; LPF_2, 610-nm long-pass filter; NF, 543-nm notch filter; M_1 and M_2, mirrors; PMT_1, PMT_2 and PMT_3, photomultiplier tubes; A/D, data acquisition board.

2.2.1. Nanoliter DNA sequencing reaction in the μ-thermocycler

The μ-thermocycler cartridge was constructed by holding two frames (7.0 cm×3.5 cm×0.3 cm) together with screws around the four corners (Fig. 1). The frame was made of thermally stable polypropylene. The base frame was affixed to one side of the heating tape (Omega, Stamford, CT, USA), and a thin (0.025 mm) brass sheet (Small Parts, Miami Lakes, FL, USA) was glued to the other side with thermally conductive epoxy. The cover frame was affixed to the second brass sheet also by thermally conductive epoxy. The μ-thermocycler was heated by the heating tape (Omega), with 2 W/cm^2, and cooled down by air blown from an air pump. The temperature was monitored using a small K-type thermocouple (Omega) inside a capillary (7 cm×100 μm) which was positioned parallel to the CZE capillary within the μ-thermocycler. The thermocouple (TC), heating tape, air pump and temperature controller formed an active feedback loop controlled by Labview (National Instruments, Austin, TX, USA) and a desktop computer. The heating and cooling rates of the thermocycler were, respectively 70 and 100°C/min.

The front end (ca. 6 cm) of capillary C_1 (45 cm×75 μm I.D.) passed through the μ-thermocycler cartridge (Fig. 1) with the inlet tip of the capillary about 2 cm away from the edge of the cartridge. The inlet tip of the capillary C_1 was immersed in the THM buffer in R_1. The back end of the capillary C_1 was connected to the A port of a μ-tee (Upchurch Scientific, Oak Harbor, WA, USA). The μ-tee had a 29-nl volume. The inlet tip of the CGE capillary (C_3) filled with PEO gel (70 cm×75 μm I.D.) was connected to the B port of the μ-tee. A short capillary C_2 (13 cm×75 μm) used for the both CZE and CGE separations was connected to the C port. The outlet tip of the short capillary was immersed in the buffer solution R_2.

The capillaries C_1 and C_2 were conditioned by 1 *M* NaOH, deionized water and THM buffer. Before loading cycle-sequencing reaction mixture, the liquid levels in R_1 and R_2 were equilibrated for 20 min by using a wide-bore plastic tube (30 cm×3 mm I.D.) filled with THM buffer to connect R_1 and R_2. R_1 and R_2 remain connected during cycle sequencing. In addition, the liquid level in R_3 was also manually adjusted to the same level as that in R_2. This was to ensure that hydrodynamic flow in the three capillaries was nearly zero, and the small-volume reaction mixture stayed in the same location in the capillary during the entire period (ca. 2.5 h) of reaction.

A small volume of reaction mixture was introduced into the reaction region in the thermocycler by hydrodynamic injection followed by a plug of THM buffer. The distance between the inlet tip and the reaction region was ca. 3 cm. The reaction volume was in the range of 90–120 nl with corresponding length in the range of 2–3 cm. The temperature protocol for the on-column cycle-sequencing reaction was adjusted to the following: the sample mixture was heated to 96°C and held for 1 min; 35 cycles were performed with denaturation at 96°C for 10 s, annealing at 45°C for 5 s, and extension at 60°C for 3 min; then the temperature was ramped to 96°C and held for 2 min. For off-line experiments, reaction was carried out in either a plastic vial using an aluminum block thermocycler (Perkin-Elmer, Foster City, CA, USA), or in a long capillary using a hot-air thermocycler (Idaho Technology).

2.2.2. Purification and separation of DNA sequencing products

After the cycle sequencing reaction, the wide-bore plastic tube was removed from R_1 and R_2. A negative high-voltage power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) was used to drive electrophoresis for purification from the anode (R_1) to the cathode (R_2). Note that the anode in R_1 should be grounded in order to avoid electrical arcing in the μ-thermocycler when the high voltage is applied. Also, the electrode in R_3 needed to be removed from the buffer during the CZE separation. Otherwise, the current in the CZE capillary will be seriously affected even if HV_2 was not applied. During CZE separation, dye-terminators passed the detection window first. Once the DNA fragments were detected by PMT_1, timing was initiated. When the peak of the DNA fragments was expected to go past the tee and enter capillary C_2 (based on observed time to the detection window extrapolated to include the extra distance), HV_1 was turned off. The electrode at the cathode from HV_1 was removed from the buffer in R_2. The ground electrode from a positive high-voltage power supply (HV_2) was

immersed in the buffer in R_2, and the anode was placed in the 1× TBE buffer in R_3. Then, a positive voltage (9 kV) was applied between R_2 and R_3 for 40–50 s to inject negatively charged DNA fragments into the CGE capillary. A steel wire with its two ends immersed in the buffer in R_1 and R_2 should be used to equalize the potential of R_1 and R_2 during injection and subsequent CGE separation. After injection of the DNA ladder into C_3, HV_2 was turned off. The THM buffer solutions in capillaries C_1 and C_2, and in R_1 and R_2 were replaced by 1× TBE buffer. Then, HV_2 was turned on to apply 12 kV between R_2 and R_3 to start the separation of the DNA ladder in CGE.

2.2.3. Detection and base calling

An air-cooled Ar^+ laser (Uniphase, San Jose, CA, USA, Model 2213-150 ML) with multi-line emission was used as the light source for fluorescence excitation. The 514-nm line was separated out with an uncoated 60° glass prism (Edmund Scientific, Barrington, NJ, USA). The laser was further divided into several beams by the prism. The strongest beam (6 mW) was used for CGE detection, while the weaker one (ca. 0.6 mW) was used for CZE detection. Uncoated plano-convex lens L_1 and L_2 (Edmund Scientific) with 12 mm focal length were used to focus the laser beams to the capillary windows. 10× microscope objectives MO1, MO2 and MO3 (Edmund Scientific) were used to collect the fluorescence perpendicular to the excitation laser. A 540-nm long-pass filter (LPF_1) was employed to block the scattered light from entering PMT_1 (R928, Hamamatsu, Bridgewater, NJ, USA). A 610-nm long-pass filter (LP_2) was used to reduce the stray light entering PMT_2 which was used for red-channel detection. A notch filter (NF) was deployed to prevent the stray light from going into PMT_3 which was used for blue-channel detection. All PMTs were operated at 1000 V. The signal from PMT_1 was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface operated at 5 Hz (Lawson Lab., NJ, USA) in the computer. The signals from PMT_2 and PMT_3 were monitored simultaneously and processed by ChromPerfect software (Justice Innovations, San Jose, CA, USA) in the computer. Base calling was manually conducted based on the two-color ratio in the blue and red channels [30].

2.2.4. Regeneration of the CZE and CGE capillaries

Recycling the capillaries is an important issue in on-line DNA sequencing. In the manual mode in this work, regeneration of whole system was performed after CGE separation was complete. The CZE capillary, including the reaction section and the μ-tee, was regenerated by flushing with 50 μl of deionized water, 60 μl of 1 *M* NaOH, 50 μl of deionized water, and 60 μl of Tris–HCl buffer, sequentially. The CGE capillary was simply regenerated by flushing with a large amount (ca. 300 μl) of deionized water, 30 μl of 2% PVP and 12 μl of PEO gel.

3. Results and discussion

3.1. Purification of sequencing reaction products by CZE

In the analysis of DNA fragments produced from the dye-labeled terminator-sequencing reaction, the major interference comes from the unincorporated dye-labeled terminators (d-ddNTPs) and the high concentration of salt. The d-ddNTPs will interfere with the detection of the dye-labeled DNA ladder (d-DNAs) in CGE, and seriously reduce the accuracy of base calling. The high concentration of salt will significantly reduce the amount of DNA injected electrokinetically into the CGE capillary (lower effective potential), and will also degrade the separation of the DNA ladder (perturbation of the separation buffer).

3.1.1. Separation of d-ddNTPs and d-DNAs

In previous sample clean-up methods, the separation of d-ddNTPs and d-DNAs was based on their solubility differences in organic solvent (e.g., ethanol precipitation) or size differences (e.g., SEC). In the new approach investigated here, the separation of d-ddNTPs and d-DNAs is attained on the basis of their mobility differences in CZE. In CZE, the electrophoretic mobility of a molecule is primarily determined by its charge-to-mass ratio (z/m). It is well known that DNA fragments of different sizes (>ca. 20 base pairs, bp) have almost equal electrophoretic mobilities (μ_{ele}) in free solution electrophoresis due to their very similar z/m ratios [31–33].

When a dye is attached to the DNA fragments, μ_{ele} should decrease due to the fact that the dye has much smaller z/m ratio than the DNA fragments. However, the decrease of μ_{ele} should be very small for DNA fragments with more than 20 bp, i.e., all fragments larger than the primer, as the z/m ratio of the whole fragment is primarily determined by the nucleotides. So, various d-DNAs are expected to migrate close to each other in free solution CZE. On the other hand, the attachment of rhodamine dye to the ddTTP makes its z/m reduced by about half from ca. $-3/500$ to $-3/1000$. Note that both ddTTP and d-ddTTP have net -3 charge in weakly basic solutions, and ddTTP and rhodamine dye have similar molecular masses (around 500). Additionally, the d-ddTTP will form a stable 1:1 complex with Mg^{2+} present in the reaction mixture [34,35], reducing its net charge from -3 to -1. Hence, the z/m for ddTTP–Mg^{2+} complex is only about $-1/1000$. On the other hand, the z/m ratio of d-DNA_{20} is only slightly reduced by the attachment of dye and the presence of Mg^{2+}, and is thus slightly lower than $-1/500$. Therefore, it can be expected that the μ_{ele} of d-DNAs should be higher than that of the ddNTPs in basic solution, which forms the basis of their separation by CZE.

To study the separation of d-DNAs and d-ddNTPs, off-line cycle-sequencing reaction was performed and the reaction products were directly injected into the CZE system for analysis. Fig. 2 shows the separation of d-DNAs and d-ddNTPs by CZE in weakly basic THE buffer (pH 7.8). The first cluster of peaks are the four d-ddNTPs while the second peak is the dye-DNA nested set. The migration order of d-ddNTP and d-DNAs in CZE was just opposite to that in SEC. This is because the bulk solution in CZE is driven by electroosmotic flow that is in the opposite direction to electrophoretic movement of the negatively charged d-ddNTPs and DNA ladder. As can be seen, different fragments of d-DNAs migrate within one narrow peak with peak width at half height of only 8 s, corresponding to a peak volume of 160 nl. In addition, baseline separation of d-DNAs and d-ddNTPs was easily achieved in less than 6 min in the THE buffer. However, the THE buffer posed several problems when it was used together with the on-column cycle-sequencing reaction, as discussed below.

The reaction buffer used for the sequencing reaction consisted of ca. 55 m*M* Tris–HCl (pH 9.0) and 3 m*M* $MgCl_2$. The separation buffer used in CGE was 1× TBE. It is expected that a CZE buffer with similar composition and pH value to those of the reaction buffer, and lower ionic strength than that of the CGE separation buffer should be favorable for both on-column reaction and on-column sample-stacking injection. Fig. 3 illustrates the CZE separation of d-ddNTPs and d-DNAs in 50 m*M* Tris–HCl buffer (pH 9.0) with increasing concentrations of Mg^{2+}. Without $MgCl_2$ (Fig. 3a), the migration

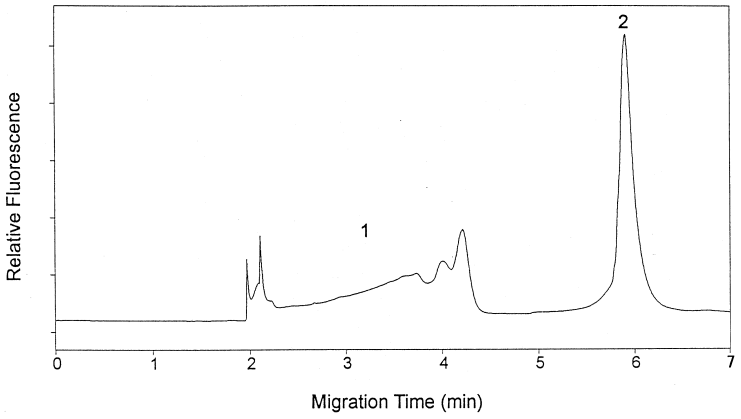

Fig. 2. CZE separation of d-ddNTPs and d-DNAs using THE buffer. Conditions: THE buffer, 2 m*M* Tris–6 m*M* histidine–1 m*M* EDTA, pH 7.8; bare fused-silica capillary, 50 cm (effective length 35 cm)×75 μm I.D.; applied voltage, 15 kV; hydrodynamic injection 12 cm×15 s; sample, untreated crude sample from off-line cycle-sequencing reaction with M13mp18 as template. Peaks: 1=clustered peaks of d-ddNTPs, 2=d-DNAs.

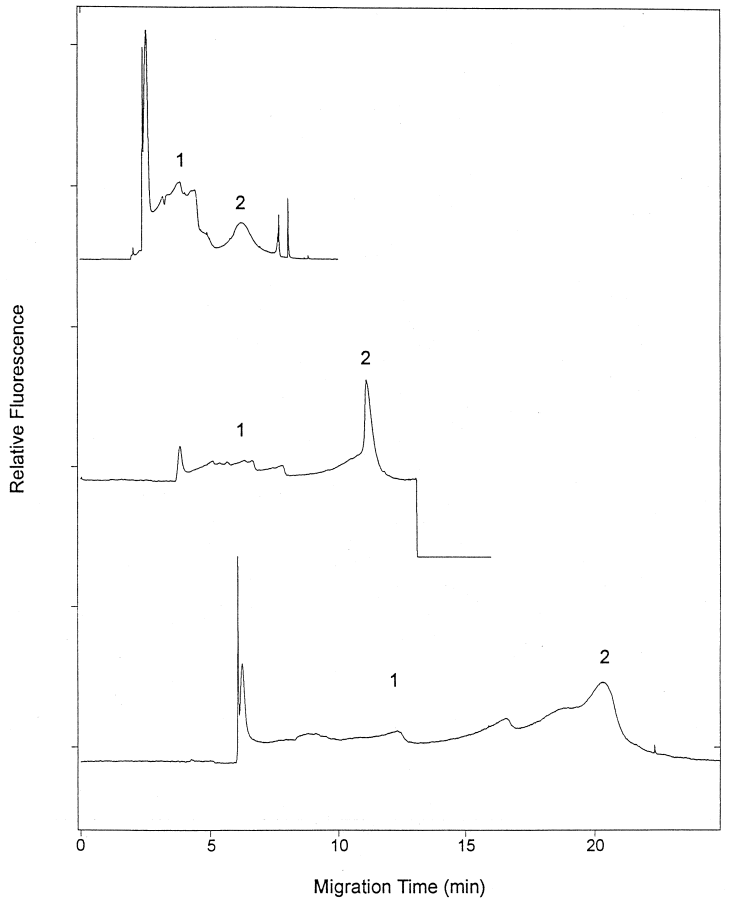

Fig. 3. CZE separation of d-ddNTPs and d-DNAs using 50 m*M* Tris–HCl (pH 9.0) buffer with different concentrations of $MgCl_2$. Conditions: bare fused-silica capillary, 57 cm (effective length 40 cm)×75 μm I.D.; separation buffer: top trace, no $MgCl_2$; middle trace, 0.1 m*M* $MgCl_2$; bottom trace 0.5 m*M* $MgCl_2$. Other conditions as in Fig. 2.

times were short, and the separation was poor. Moreover, the peak of d-DNAs was broad.

The addition of 0.1 m*M* $MgCl_2$ (Fig. 3b) brought about a significant increase in the migration times, much improvement in the separation, and interestingly, a decrease in the peak width of d-DNAs. The increase in migration time is due to the decrease in electroosmotic flow caused by the adsorption of divalent Mg^{2+} on the fused-silica surface, lowering the zeta potential of the capillary wall. A similar effect of Mg^{2+} had been reported by a number of published works in the separation of amino acids, anionic surfactants and oligonucleotides [36,37]. As the concentration of Mg^{2+} was raised to 0.5 m*M*, the migration time for d-DNAs nearly doubled to about 20 min (Fig. 3c). In addition, the peak was significantly broadened, probably due to adsorption of the solutes on the positive (metal) sites at the surface of fused-silica. Therefore 0.1 m*M* $MgCl_2$ provided the best results in terms of separation resolution, migration time and peak profile.

3.1.2. Reduction of salt concentration by matrix switching

In addition to the removal of unincorporated d-ddNTPs, the high salt concentration in the reaction mixture should also be eliminated or reduced in order that the purified DNA ladder can be effectively injected into the CGE system. Experiments were performed to compare the conductivities of the

reaction mixture, the CZE buffer (THM), and the CGE buffer (1× TBE). The results showed that the conductivity of the THM buffer was about 1/7 of that of the reaction mixture, and 1/3 of that of the 1× TBE buffer. Although CZE, unlike spin column, did not remove the salt completely, it transferred the d-DNAs from the reaction condition with high ionic strength to the CZE separation condition with much lower ionic strength, and thus increased the amount of DNA ladder injected into the CGE system. For comparison, standard dry M13mp18 samples were dissolved either in the reaction buffer or the THM buffer, and then injected into the CGE system. Under identical injection conditions, the signal intensity of d-DNAs for the sample dissolved in the THM buffer was about five-times higher than that in the reaction buffer.

3.2. On-column nanoreactor

In previous work on off-line or on-line sequencing reactions in capillaries, both ends of the reaction capillary were sealed by flame, mechanical or freeze–thaw valves, or flanked by long capillaries to prevent evaporation of the reaction mixture during long periods at high temperature [25–27,38]. The cycle-sequencing reaction and sample purification were carried out in two different columns. These complicated the system controls and did not facilitate further miniaturization and integration. The system here is much simpler because on-column sequencing reaction was carried out within a short section (2–3 cm) in a capillary (58 cm×75 μm) with the two ends of the reaction zone surrounded by the CZE separation buffer. However, some problems needed to be addressed in order that adequate reaction efficiency was attained.

3.2.1. Effect of sample plug length

When the cycle-sequencing reaction is performed in a long capillary with the two ends sealed, the reactants are always evenly distributed along the capillary. However, the influence of diffusion of the reactant (especially those with small molecular mass) on the reaction will become large when the length of the reaction mixture is reduced. Diffusion will cause mixing of the reaction mixture and the CZE buffer and decrease the concentration of the reactants in the reaction mixture.

To study the influence of the sample plug length on the reaction efficiency, off-line reactions were carried out in capillaries using the hot-air thermocycler. Five capillaries (24 cm×75 μm I.D.) were flushed with 1 *M* NaOH, water and THM buffer, respectively. Then, THM buffer in the capillary was completely or partially displaced by the reaction mixture, resulting in sample plugs of different lengths in the capillaries. After reaction, the reaction mixture together with the THM buffer was pushed into small plastic vials and then injected into the CZE system. The peak area ratio of d-DNAs to d-ddNTPs was used to evaluate the reaction efficiency. As can be seen in Fig. 4, the reaction efficiency remained almost constant when the length of sample plug decreased from 20 cm to 5 cm, then became lower with further decrease in the length of the sample plug. As compared to that for a long sample plug (20 cm), 75% of the reaction efficiency was preserved for a 2.5 cm sample plug. It is therefore possible to perform DNA sequencing in a volume of 110 nl.

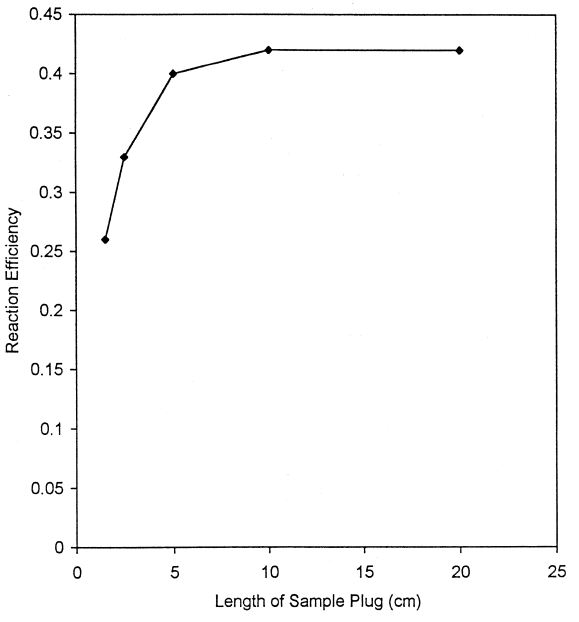

Fig. 4. Influence of sample plug length on the on-column reaction. The peak area ratio of d-DNAs to the sum of d-DNAs and d-ddNTPs is used to evaluate the reaction efficiency.

3.2.2. Effect of magnesium

The reaction mixture is a complex sample that includes both small molecules and macromolecules. With the use of a short sample plug (e.g., 2.5 cm), the influence of diffusion on the concentration of macromolecules (e.g., BSA and enzyme with M_r over 600 000) in the reaction region should still be negligible but the opposite is true for small molecules such as Mg^{2+}. Since Mg^{2+} plays a critical role in the DNA sequencing reaction [38], Mg^{2+} with higher than the standard concentration was added in the reaction mixture to compensate for the gradual decrease in the concentration of Mg^{2+} during the reaction period. We found that the reaction efficiency was improved by about 30% with the addition of 4× the Mg^{2+} in the reaction mixture. However, further increase of Mg^{2+} concentration seemed to have no enhancement effect on the reaction, but caused more peak broadening in the subsequent CZE separation.

3.3. On-line injection

Optimized on-line injection is essential for the complete removal of d-ddNTPs as well as the efficient separation and sensitive detection of d-DNAs in CGE. During injection, the potentials of the ends of the C_1 and C_2 needed to be equalized by immersing two ends of a wire in the two buffer vials. The presence of the unstable electroosmotic flow from C_1 to C_2 was observed if one end of C_1 was left in the floating state, which caused poor reproducibility and low efficiency in the injection.

In previous work on on-line injection of d-DNAs from SEC to CGE, injection was initiated when the top of the d-DNAs peak reached the center of the cross junction [25]. In the present system, however, it was observed that small amounts of d-ddNTPs would be injected together with the d-DNAs if the same protocol was utilized. Moreover, the reproducibility of the signal intensity in CGE was poor. This confirms that electroosmotic flow is less reliable than pressure-driven flow. To circumvent this problem, voltage switching was performed after the d-DNAs peak entered C_2 and reached a point about 1.5 cm beyond the center of the tee junction. This was to ensure that the d-DNAs were totally inside C_2 despite the run-to-run variations in migration time. Since the electroosmotic flow from C_3 to C_2 was largely suppressed by the PEO gel in C_3, the d-DNAs moved faster than d-ddNTPs during injection. Therefore, it became easier to control the injection of d-DNAs while avoiding the injection of d-ddNTPs.

Proper selection of injection time and voltage is critical for selective and efficient on-line injection of d-DNAs. For accurate control, the combination of lower injection voltage and longer injection time is preferred to that of higher injection voltage and shorter injection time. With the injection voltage fixed at 9 kV (electric field strength 220 V/cm), injection time was tested in the range of 20–120 s. It was found that the highest signal was obtained in the range of 30–50 s. Further increase of injection time brought about little increase in the signal intensity of d-DNAs, but introduced increased interference of d-ddNTPs peaks and poorer resolution of the large d-DNA fragments in CGE. In addition, careful flushing of the CZE capillary after injection is also essential for preventing d-ddNTPs from entering the CGE capillary. The capillary should be flushed from C_1 to C_2 with about 10 μl 1× TBE buffer to completely remove the d-ddNTPs left in C_2.

3.4. Integration of reaction, purification, separation and detection

In the search for an appropriate buffer for the CZE separation, THE buffer was found to provide better separation than THM buffer. However, when on-column reaction was carried out with the CZE capillary filled with THE and THM, respectively, it was discovered that the reaction efficiency was about 35% lower in the former case. The reason for this may be due to the diffusion of EDTA and H^+ from THE (pH 7.8) into the reaction mixture (pH 9.0), which caused considerable reduction in the concentration of free Mg^{2+} and lowered the pH value in the reaction region. Also, THE buffer has a low capacity. The migration times changed in a wide range, e.g., 5 to 10 min for d-DNAs. The poor reproducibility in migration time made it difficult to perform effective on-line injection.

On the other hand, THM was similar to the reaction buffer in composition, pH value, and buffer capacity. THM thus formed a favorable environment surrounding the short sample plug for on-column

cycle-sequencing reaction. Additionally, THM maintained good run-to-run reproducibility in migration time (RSD of 3.5% for d-DNAs).

As shown in Fig. 4, reaction efficiency was high when the sample plug was 5 cm, but the separation of d-ddNTPs from d-DNAs greatly deteriorated. On

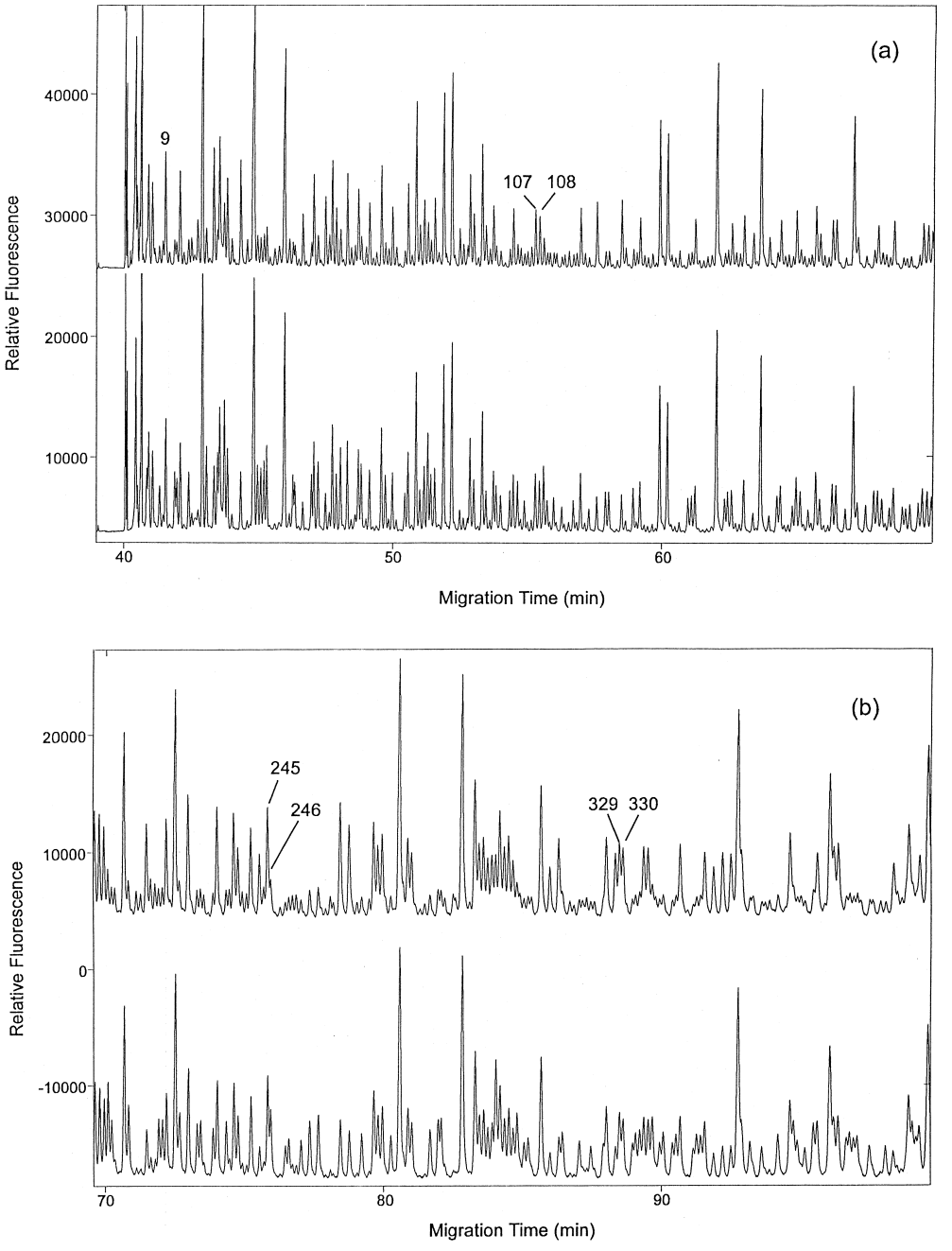

Fig. 5. Sequence analysis of M13mp18 by integrated on-line system with two channel detection: blue channel, top; red channel, bottom; volume of reaction mixture: 110 nl; on-line injection, 9 kV×40 s; electric field in CGE, 170 V/cm.

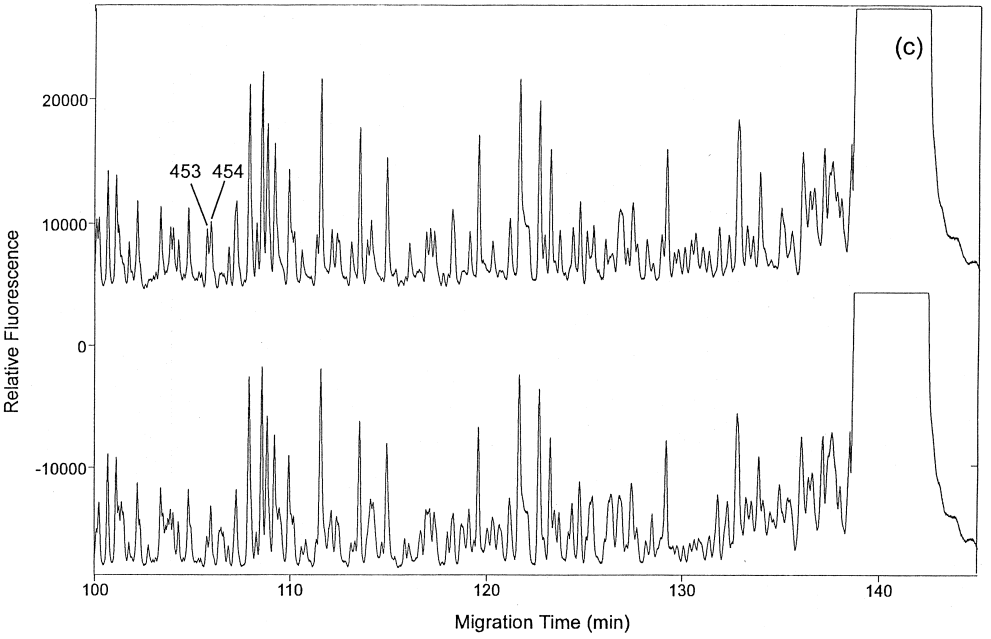

Fig. 5. (*continued*).

the other hand, a 1.5 cm sample plug showed good separation in CZE but inadequate S/N ratio of d-DNAs in CGE. It was found that a 2.5 cm sample plug (110 nl) provided a compromise between the reaction efficiency and separation resolution. This volume is only 1/10 to 1/100 of standard reaction volumes and provides substantial savings in reagent cost for DNA sequencing.

On-line injection was also re-examined when the whole system was integrated. The separation was degraded from that in Fig. 3b to some extent due to the diffusion of d-ddNTPs over the long period of reaction. Hence, the time for voltage switching and injection needed to be controlled more accurately. With an applied voltage at 9 kV, 40 s of injection was found to give the best signal intensity without compromising the resolution of d-DNAs from d-ddNTPs in CGE.

The performance of the optimized integrated on-line system was investigated in actual DNA sequencing. Fig. 5 shows the electropherograms of M13mp18 recorded by using one-wavelength excitation and dual-wavelength detection. Data in both channels show high S/N ratios, and adequate resolution for base calling from 5 to 460 bp with an accuracy of 97%. The majority of miscalled bases were from small G peaks following high T peaks. Fig. 5 implies that the on-column reaction generated more DNA fragments terminated by ddTTP as compared to off-line reaction, e.g., in Ref. [30]. Note that a minimal amount of dye was present around 20 bp which however did not interfere with base calling. Related experiments indicated that the small amount of dye present in this region was not introduced during injection, but might be caused by diffusion of the concentrated dye labels as it initially passed the center of the μ-tee.

Experiments with pGEM showed that the efficiency of on-column reaction for pGEM was about 1/2 that of M13mp18. The reason for the decreased reaction efficiency for ds-DNA was not clear. Nevertheless, correct base calling of pGEM could be manually performed from 10 to 375 bp with 96% accuracy. Longer reads can be expected with further optimization of the efficiency of the on-column reaction in the future.

4. Conclusions

An integrated on-line system for nanoliter DNA sequencing analysis from cycle-sequencing reaction, product purification to separation and detection has been demonstrated. With the use of CZE, the purification of DNA sequencing products was achieved at the nanoliter scale for the first time. Although the on-line mixing of nanoliter-sized reagents and template has yet to be integrated into the present system, the elimination of a high-pressure pump and a large-volume SEC column from previous set-ups [27–29] should be important steps towards greatly simplifying the operation and reducing the cost of DNA sequencing. Moreover, the use of CZE instead of SEC for sample purification will facilitate adaptation of the whole system to the microchip format in the future.

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References

[1] R.F. Service, Science 282 (1998) 399.
[2] E.R. Mardis, B.A. Roe, BioTechniques 7 (1989) 840.
[3] R.K. Wilson, A.S. Yuan, S.M. Clark, C. Spence, P. Arakelian, L.E. Hood, BioTechniques 6 (1988) 776.
[4] M.M. DeAngelis, D.G. Wang, T.L. Hawkins, Nucleic Acids Res. 23 (1995) 4742.
[5] A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith, B.L. Karger, Proc. Natl. Acad. Sci. USA 85 (1987) 9660.
[6] E.N. Fung, E.S. Yeung, Anal. Chem. 67 (1995) 1913.
[7] H. Swerdlow, R.F. Gesteland, Nucleic Acids Res. 18 (1989) 1415.
[8] H. Drossman, J.A. Luckey, A.J. Kostichka, J. D'Cunha, L.M. Smith, Anal. Chem. 62 (1990) 900.
[9] X.C. Huang, M.A. Quesada, R.A. Mathies, Anal. Chem. 64 (1992) 967.
[10] S.C. Jacobson, R. Hergenroder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 1114.
[11] K. Ueno, E.S. Yeung, Anal. Chem. 66 (1994) 1424.
[12] N.J. Dovichi, H. Swerdlow, J.Z. Zhang, D.Y. Chen, H.R. Harke, Anal. Chem. 63 (1991) 2835.
[13] D.R. Meldrum, R.C. Seubert, R.H. Kraft, P.J. Wiktor, N. Friedman, in: Third International Conference on Automation in Mapping and DNA Sequencing, Berkeley, CA, 1995, p. 37.
[14] S.A. Soper, D.C. Williams, Y. Xu, S.J. Lassiter, Y. Zhang, S.M. Ford, R.C. Bruch, Anal. Chem. 70 (1998) 4036.
[15] P. Wilding, M.A. Shoffiner, L. Kricka, J. Clin. Chem. 40 (1994) 1815.
[16] M.A. Burns, B.N. Johnson, S.N. Brahmasandra, K. Handique, J. Webster, M. Krishnan, T.S. Sammarco, P.M. Man, D. Jones, D. Heldsinger, C.H. Mastrangelo, D.T. Burke, Science 282 (1998) 484.
[17] A.T. Woolley, D. Hadley, P. Landre, A.J. deMello, R.A. Mathies, M.A. Northrup, Anal. Chem. 68 (1996) 4081.
[18] R. Sikorski, R. Peters, Science 282 (1998) 433.
[19] J. Sambrook, E.F. Fritsch, T. Maniaties, in: Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, Section 9.49.
[20] D. Figeys, H. Ahmadzedeh, E. Arriaga, N.J. Dovichi, J. Chromatogr. A 744 (1996) 325.
[21] D. Hilderman, D. Muller, BioTechniques 22 (1997) 878.
[22] X. Tong, L.M. Smith, Anal. Chem. 64 (1992) 2672.
[23] J. Devaney, M. Marino, P. Williams, K. Weaver, K. Truner, P. Belgrader, Appl. Theor. Electrophoresis 6 (1996) 11.
[24] K. Wang, L. Ban, C. Boysen, L. Hood, Anal. Biochem. 226 (1995) 85.
[25] H. Tan, E.S. Yeung, Anal. Chem. 69 (1997) 664.
[26] H. Swerdlow, B.J. Jones, C.T. Wittwer, Anal. Chem. 69 (1997) 848.
[27] H. Tan, E.S. Yeung, Anal. Chem. 70 (1998) 4044.
[28] N. Zhang, H. Tan, E.S. Yeung, Anal. Chem. 71 (1999) 1138.
[29] Y. Zhang, H. Tan, E.S. Yeung, Anal. Chem. 71 (1999) 5018.
[30] Q. Li, E.S. Yeung, Appl. Spectrosc. 49 (1995) 1528.
[31] D.M. Goodall, S.J. Williams, D.K. Loyd, Trends Anal. Chem. 10 (1991) 272.
[32] D. Rickwood, B.D. Hames (Eds.), Gel Electrophoresis of Nucleic Acids – A Practical Approach, IRL Press, Washington, DC, 1983.
[33] G.L. Linda, R.C. Charles, L.W. Sam, D.C. Richard, F.M. Bernard, W.F. Carl, D.H. Nicolette, K.W. Richard, Nucl. Acids Res. 20 (1992) 2471.
[34] H. Sigel (Ed.), Metal Ions in Biological Systems, Marcel Dekker, New York, 1979.
[35] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. USA 74 (1977) 5463.
[36] A.S. Cohen, S. Terabe, J.A. Smith, B.L. Karger, Anal. Chem. 59 (1987) 1021.
[37] D.J. Dpietrzyk, S. Chen, B. Chanthanwat, J. Chromatogr. A 775 (1997) 327.
[38] C.T. Wittwer, D.J. Garling, BioTechniques 10 (1991) 76.