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On-line nanoliter cycle sequencing reaction with capillary zone electrophoresis purification for DNA sequencing

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Abstract

An integrated system for DNA sequencing based on a nanoreactor for cycle-sequencing reaction coupled with on-line capillary zone electrophoresis (CZE) for purification and capillary gel electrophoresis (CGE) for separation is presented. Less than 100 nl of premixed reagent solution, which includes dye-labeled terminator pre-mix, bovine serum albumin and template, was hydrodynamically injected into a fused-silica capillary (75 μm I.D.) inside a laboratory-made microthermocycler for cycle sequencing reaction. In the same capillary, the reaction products were purified by CZE followed by on-line injection of the DNA fragments into another capillary for CGE. Over 540 base pairs (bp) of DNA can be separated and the bases called for single-standed DNA with 0.9% error rate. The total time was about 3.5 h, or a cycle time of 2 h with staggered operation. For double-stranded DNA, a longer reaction time was required and base calling up to 490 bp with 1.2% error rate was achieved. The whole system is readily adaptable to automated multiplex operation for DNA sequencing or polymerase chain reaction analysis.

Keywords: Sequencing methods; DNA

1. Introduction

DNA sequencing usually involves five steps: (1) preparation of template DNA; (2) Sanger reaction to produce sequencing fragments; (3) purification of Sanger reaction products; (4) separation of DNA fragments; (5) base calling. Driven by the Human Genome Project, lots of effort has been applied to developing DNA sequencing methods that are more rapid, accurate and cost-effective than traditional sequencing methodologies.

Recent advances came from the development of capillary gel electrophoresis (CGE) separation [1–4], and the laser-induced fluorescence (LIF) detection of dye labeled primers or dideoxy nucleotides [5–7]. As a direct result, the cost in the separation (electrophoresis) and identification (detection) tasks is substantially lowered with greatly improved throughput [8–10]. CGE and the more recent microchip electrophoresis [4] took advantage of the small volume of the separation column, which allowed the use of much higher electric fields. Nearly 3-fold improvement in resolution and 25-fold increase in the speed of the separation were achieved in CGE compared to traditional slab gel electrophoresis (SGE) [11]. Meanwhile, the amount of sample required for analysis dramatically dropped to the nanoliter regime, which could potentially offer significant savings in the use of costly reagents required for preparing the sequencing fragments.

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Unfortunately, the protocols for sample preparation and Sanger reaction did not keep pace with the low-cost high-speed high-throughput separation techniques. While capillary or microchip separations are scaled down to the nanoliter regime, the standard Sanger reaction and subsequent purification and injection are still performed in the microliter range because of difficulties in manipulating and transferring very small amounts of sample. Recently, miniaturization of polymerase chain reaction (PCR) or cycle-sequencing reaction in capillaries or chips has been demonstrated. Burns and colleagues developed an integrated nanoliter DNA analysis device which is capable of metering a small, accurate volume of fluid by means of a hydrophobic patch and injected air [12]. Soper et al. made use of the biotin/streptavidin/biotin linkage to tether biotinylated DNA sequencing templates to the wall of the capillary for solid-phase cycle sequencing [13–15]. A single-dye amplification was accomplished in a volume of 62 nl in an (aminoalkyl)silane-derivatized fused-silica capillary. The capillary reactor was connected to the gel column via a zero-dead-volume fused-silica connector for on-line analysis. However, that approach involved a time-consuming and labor-intensive capillary coating process which took more than 24 h. After a couple of runs, noticeable loss of anchored streptavidin was observed and capillary regeneration was required.

Compatible miniaturized thermal-cycling equipment is also important for microscale sample preparation. Traditional metallic thermal blocks require a fairly long temperature transition time because of their high thermal mass. Several novel heating methods have been demonstrated for fast temperature change in the PCR or cycle-sequencing reaction. A hot-air thermocycler was first commercialized by Idaho Technology, which achieved rapid cycle sequencing within 25 min with dye-labeled primers [16]. Infrared-mediated heating was also successfully applied to microchamber thermal cycling [17]. Resistive thin films have been used to heat up individual capillaries with very fast ramping rate although they may not be readily adapted for multiplex operation [18].

Finally, to fully profit from the high throughput of such miniaturized systems, the integration of all the different parts is required. Several reports have shown a variety of coupling schemes for fluid handling with capillaries and valves in order to produce, purify and inject the sequencing sample [19–22]. In our earlier work [19–21], freeze–thaw valves were used in combination with pressure-driven flow. These mechanical devices are bulky and add to the complexity of the system. Other reports showed total integration on microchips, performing PCR reactions, sample purification and separation [23], although the protocol requires operator intervention.

In this report, we describe an integrated system of capillary nanoreactor (reaction volume ~100 nl) for cycle sequencing reaction with capillary zone electrophoresis (CZE) purification coupled with CGE for DNA separation. No complicated accessories such as high-pressure pumps or valves were needed. The closed system is capable of continuous operation from the introduction of reagents to called bases. The advantages of this system are its simplicity, reliability, cost effectiveness, high speed and suitability for highly multiplexed operation.

2. Experimental

2.1. Reagents, buffers and separation matrix

10× Bovine serum albumin (BSA) was obtained from Idaho Technology (Idaho Falls, ID, USA). 1× TBE with 7 *M* urea buffer was prepared by dissolving pre-mixed TBE buffer powder (Amresco, Solon, OH, USA) and urea (ICN Biomedicals, Aurora, OH, USA) in deionized water. The dynamic coating matrix was made by dissolving 2% (w/v) of M_r 1 300 000 poly(vinylpyrrolidone) (PVP) (Sigma, St. Louis, MO, USA) into deionized water. Tris, HCl, $MgCl_2$, Triton X-100, and poly(ethylene oxide) (PEO) was received from Aldrich (Milwaukee, WI, USA). 1× PCR buffer consisted of 10 m*M* Tris, 50 m*M* KCl, 1.5 m*M* Mg_2Cl and 0.1% Triton X-100. The CZE separation buffer was prepared by dissolving 10 m*M* Tris, 1.5 m*M* $MgCl_2$, 2 m*M* KCl and 0.3% (w/v) PVP in deionized water. The buffer was shaken for 30 s and left to stand for 1 h to remove bubbles. The CGE sieving matrix was an entangled polymer solution made by dissolving 1.5% (w/v) of M_r 8 000 000 PEO and 1.4% (w/v) of M_r 600 000

PEO in 1× TBE buffer with 7 *M* urea. The solution was stirred vigorously overnight until all material was dissolved and no bubbles can be observed. The bare fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). Fluoro carbon (FC)-coated capillary, poly(ethylene glycol) (PEG)-coated capillary, and DB-WAX-coated capillary were purchased from J&W Scientific (Folsom, CA, USA). Poly(vinyl alcohol) (PVA)-coated capillary was received from Beckman Instruments (Fullerton, CA, USA).

2.2. Sequencing reaction protocol

ThermoSequenase old-dye terminator pre-mix kit (Amersham Life Science, Cleveland, OH, USA) was used for on-line cycle sequencing. Modifications were made to fit the protocol to on-line capillary format since the original protocol was developed for the ABI Model 9600 or 2400 instrument.

The old-dye terminator premix included 125 m*M* Tris–HCl, 5 m*M* $MgCl_2$, 1.25 m*M* dITP, 0.25 m*M* dATP, 0.25 m*M* dCTP, 0.25 m*M* dTTP, 0.25 m*M* old-dye labeled ddNTP, ThermoSequenase, 3-[tris(hydroxy-methyl)-methyl]propanesulfonic acid (TAPS), Nonidet P40, Tween 20 and 6.25% glycerol. A typical cycle-sequencing reaction mixture consisted of 8 μl of such premix and 1 μl 5.0 μ*M* universal –17 M13 primer. 2 μl of 10× BSA was added to prevent adsorption of the enzymes on the surface of the capillary tubes and an additional 1.0 μl 25 m*M* $MgCl_2$ was added to compensate for the loss during the reaction due to diffusion. For single-stranded (ss) DNA sequencing, 2 μl of 0.2 μg/μl M13mp18 in 1× TE buffer (pH~7.5, Amersham) was used as the template. For double-stranded (ds) DNA, 4 μl of 0.2 μg/μl pGEM was used since only one strand of DNA served as template during reaction. The total reaction volume was made up to 20 μl in both cases with an appropriate aliquot of deionized water.

The temperature protocol for ssDNA cycle sequencing was adjusted to the following: the sample mixture was heated to 92°C and held for 2 min; 30 cycles were performed with denaturation at 94°C for 5 s, annealing at 50°C for 5 s and extension at 63°C for 2 min. When dsDNA was amplified, the sample mixture was first heated to 92°C and held for 2 min; 35 cycles were performed with denaturation at 96°C for 5 s, annealing at 50°C for 5 s and extension at 63°C for 4 min.

2.3. Instrumentation

Fig. 1 shows the schematic diagram for the setup. A single 75 μm I.D. capillary served as both the cycle-sequencing reaction vessel and the CZE purification column. The entire system consisted of a microthermocycler, a CZE and a CGE electrophoretic system. A microtee (Upchurch Scientific, Oak Harbor, WA, USA) was used to connect the CZE capillary, CGE capillary and a waste arm.

2.3.1. Microthermocycler

A laboratory-built microthermocycler was used for on-line reactions. Two major features were incorporated in the thermocycler design: rapid thermal cycling and good temperature accuracy and stability.

The reaction capillary (75 μm I.D.×360 μm O.D.) was sandwiched between two pieces of thin brass sheet (7.5 cm×2.5 cm×25 μm) (Small Parts, Miami Lakes, FL, USA) and a Kapton insulated flexible heater (7.5 cm×2.5 cm×0.25 mm, 10 W/in^2) (Omega, Stamford, CT, USA) as shown in Fig. 2. A thermal epoxy (Delta Bond 155, Wakefield Engineering, Wakefield, MA, USA) was used to bond the brass sheet and the flexible heater together. This microthermocycler has a very small thermal mass since the total thickness excluding the capillary reactor is ~300 μm so that it allows rapid temperature changes. The length of the heater allowed the future adaptation for 8 capillaries (9-mm spacing was used for the standard 8×12 tray format) or 16

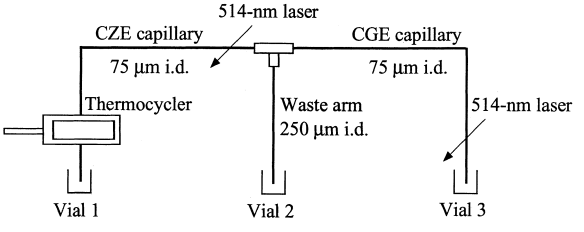

Fig. 1. Schematic diagram of on-line DNA sequencing system. On the left is the reaction/purification region and on the right is the separation/detection region.

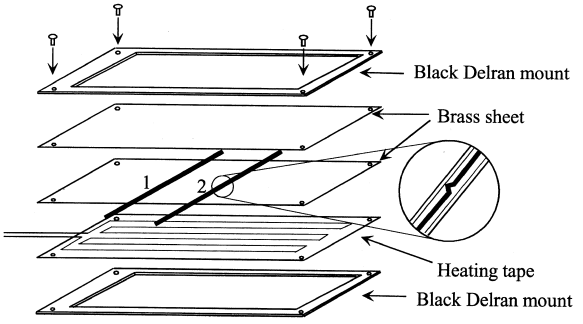

Fig. 2. Schematic diagram of microthermocycler. Thin brass plates provide heat transfer from the heating tape to the reaction capillary. The inset shows a thermocouple inside a similar O.D. capillary column for temperature feedback.

capillaries (4.5 mm spacing for the 384 tray format) for simultaneous reactions.

Thermal conductive silicone grease (Radio Shack) was applied onto the capillary surface and in between the brass sheets to ensure proper heat transfer. For 75 μm I.D. capillary, 23 mm reaction length corresponded to ~100 nl maximum reaction volume. One could change to different inner diameters to accommodate different reaction volumes.

In order to accurately measure and control the inner temperature of the reaction capillary, a miniature bare type K thermocouple (75 μm diameter, Omega) was inserted into a 250 μm I.D.×360 μm O.D. capillary which was filled with water and sealed with epoxy on both ends. The O.D. is identical to that of the reaction capillary so the total heat capacity should be similar. The thermocouple probe was placed in the middle of the thermocycler. Silicon thermal grease was applied to the surface of the probe capillary as well for better heat conduction.

A proportional-integral-derivative (PID) temperature controller (CN77300, Omega) was used to control the temperature profile for cycle sequencing. This method of control varies the magnitude of heat applied to the sample in proportion to the difference between the actual and the set temperature. The advantages of PID control over traditional ON/OFF control is that the system can be carefully tuned to compensate for temperature overshoot and ringing [24].

A 4-W aquarium air pump (Tetra Secondnature, Blacksburg, VA, USA) was used to blow room air onto the heater to cool it down quickly during the ramp from denature to annealing steps. A faster cooling rate can be obtained if desired by using a higher gas flow-rate offered by a more powerful pump or a compressed gas jet [25].

An laboratory-developed Labview program was used control the microthermocycler. Standard RS-232 serial port was used to communicate with the temperature controller. The digital TTL-level parallel port output was fed to a solid-state relay (Omega) to control the ON/OFF of the air pump. The control system can be easily adapted to any personal computer for a variety of applications such as on-line digestion [26] PCR, etc.

2.3.2. Microtee connection

A polyether ether ketone (PEEK) microtee with 9 nl dead volume was used to connect together the 35 cm long CZE capillary (75 μm I.D.), 63 cm long CGE capillary (75 μm I.D.) and a 10 cm long waste arm. A much larger I.D. capillary (250 μm I.D.) was chosen as the waste arm because of its small flow resistance for rinsing and gel filling. Tight connection was confirmed by the stable current during CE separation.

2.3.3. Detection system

Laser-induced fluorescence (LIF) detection was used both in CZE monitoring and DNA sequencing. A 15-mW Ar-ion laser (Uniphase, San Jose, CA, USA) was used for the excitation in both separation systems by splitting the laser beam 50:50. Both optical paths used an uncoated plano-convex lens with 12-mm focal length to focus the laser beams onto the capillaries. For CZE separation detection, a 10× microscope objective (Edmund Scientific, Barrington, NJ, USA) was used to collect the fluorescence into a photomultiplier tube (PMT) with the use of a 560-nm long-pass filter to cut off the laser scattering. For CGE, another 10× microscope objective was used. A 514-nm notch-plus filter (Kaiser Optical System, Ann Arbor, MI, USA) was used to block the laser scattering. Then a 80:20 beam splitter was used to split the fluorescence into two PMTs for simultaneous monitoring. One PMT (80% splitting ratio) had an additional RG630 long-pass filter in front of it. This arrangement allowed base calling for DNA sequencing [9] All PMTs (R928, Hamamats,

Bridgewater, NJ, USA) were terminated with 10-kΩ resistors before connecting to a 24-bit A/D interface (PC4350, National Instruments, Austin, TX, USA). A Pentium II 266 MHz computer (Dell, Austin, TX, USA) was used to control the system and for data acquisition.

2.4. Sample injection and on-column cycle sequencing

Before sample injection, the CZE capillary was filled with pH 8.2 separation buffer containing 10 m*M* Tris, 1.5 m*M* $MgCl_2$, 2 m*M* KCl and 0.3% PVP. Hydrodynamic injection of the cycle-sequencing mixture into the CZE capillary was performed by raising the microthermocycler by 11 cm. For the 75 μm I.D. capillary with total length of 35 cm, the reaction mixture was injected for 60 s and then the separation buffer was introduced for 60 s. Even though the sample vial contains a few microliters of reaction mixture, the injection process only takes up ~100 nl of sample. The premixed reaction mixture was reused more than 20 times without noticeable decrease in activity. This leads to a true saving in reagent costs. After injection, the microthermocycler was lowered to the original level and the capillary immersed into the vial containing separation buffer. Thirty to 35 cycles was then performed by the microthermocycler. No additional denaturing was needed before injection into the CGE capillary.

2.5. CZE purification

The same 75 μm I.D. reaction capillary with effective length of 31 cm was used for CZE purification to get rid of the excess dye terminators and salt. 10 kV was applied to vial 1 and vial 2 (Fig. 1) which contained the separation buffer. Purification was completed in 10 min. When the DNA fragments were detected at the detection window, an appropriate delay was estimated to allow for the fragments to move to the microtee. Electrokinetic injection of the DNA into the CGE capillary was initiated by switching the positive high-voltage electrode from vial 2 to vial 3. After injection at 20 kV for 2 min, the CZE capillary was flushed with 1×TBE buffer with 7 *M* urea to remove the uneluted excess dye terminators and salts. Vial 2 was replaced by 1×TBE buffer with 7 *M* urea for sequencing separation.

2.6. CGE Sequencing

The CGE capillary (63 cm long, 50 cm effective length) was flushed with deionized water and 2% PVP, and filled with PEO gel (1.5% M_r 8 000 000 and 1.4% M_r 600 000) before the CZE capillary was flushed with the separation buffer and loaded with the reaction mixture. 11 kV was applied to vial 2 and vial 3 to perform DNA sequencing. Two-channel detection was used for base calling [9].

2.7. Base calling

Two-channel electropherograms were smoothed and baseline corrected by commercial Grams32 software (Galatic Industries) before peak picking. The software could export the peak table to an Excel worksheet. The peak-height ratios of corresponding peaks and absolute peak heights in the red channel were set as criteria for base calling in Excel [9]. Peaks were inserted as a G base when a gap was detected by calculating the distance between neighboring peaks.

3. Results and discussion

3.1. Microthermocycler performance

Fig. 3 documents the reactor temperature as a function of time for the cycle-sequencing amplification of M13mp18. The lower panel shows the temperature profile of several cycles in greater detail. Because of the low thermal mass, very rapid temperature changes were achieved. Cooling from the denaturation temperature (94°C) to the annealing temperature (50°C) takes ~10 s, corresponding to a cooling rate of 4.4°C/s. The heating rates are even higher, ~10°C/s. These fast heating and cooling rates are essential for speeding up sample preparation, increasing the yield and suppressing nonspecific binding and false priming problems that are normally associated with PCR amplification. The temperature profile also exhibited very good stability. An overshoot of about 2°C is evident in Fig. 3, but the inner

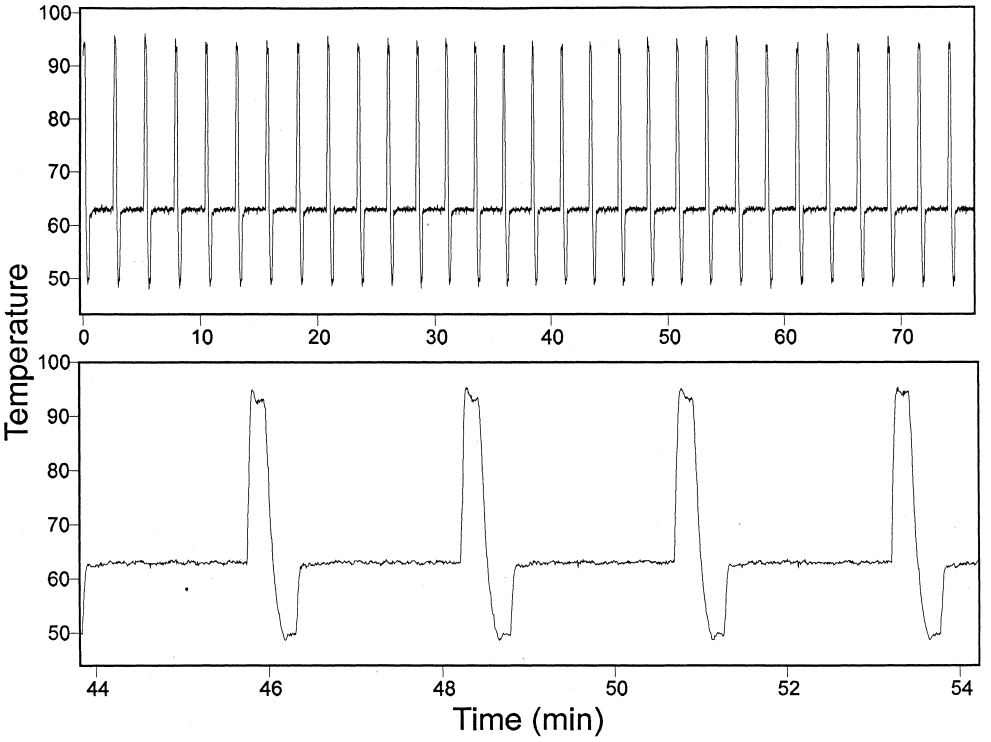

Fig. 3. Temperature profile of cycle-sequencing amplification of M13mp18.

capillary temperature profile was reproduced to within ±0.5°C in each cycle. We also moved the reaction capillary around and performed the sequencing reaction at different locations of the thermocycler. All experiments showed very similar yields. This level of homogeneity makes the current design suitable for adaptation to multiplexed capillary cycle-sequencing reactions. The microthermocycler allows the use of very short reaction capillaries (and thus smaller reaction volumes) compared to that needed in an air-thermocycler. It is also much less expensive to implement.

3.2. Sample introduction

According to the Poiseuille equation, the linear flow-rate (V) of an analyte in the column can be calculated from

$$V = \rho g r^2 \Delta h / 8\eta L$$

where ρ the solution density, g is the gravitational force constant, r is the internal radius of the capillary, Δh is the difference in height between the two ends of the capillary, η is the solution viscosity, and L is the capillary length. The reaction mixture was introduced for 10 s hydrodynamically. For a 75 μm I.D. capillary, when vial 1 was raised by 11 cm relative to vial 2, the flow-rate (V) was around 2.3 cm/min. So, 1 min hydrodynamic injection introduced a 2.3-cm reaction mixture plug, which corresponded to ~100 nl, into the capillary. Then, separation buffer was hydrodynamically injected for 1 min to push the reaction mixture to the part of the capillary which was sandwiched in the microthermocycler. The sequencing results demonstrated that this process was highly reproducible.

3.3. On-line thermal cycling

The most convenient way to perform automated DNA sequencing today is by the dye-terminator chemistry since only one-tube sequencing reaction and single-capillary separation is needed for each DNA sample. Unfortunately, incorporating the dye

terminators involves fairly slow kinetics. Usually, 4-min extension time is required. With our microthermocycler, only 2-min extension time was needed to produce enough DNA fragments to sequence M13mp18. Fig. 4 shows the relative signal of DNA fragments in CZE separation with different extension times. The dips before injection and after the analysis are artifacts due to the interruption of data acquisition while manually switching the electrodes in this experiment. Only 15% signal loss was observed when the extension time was reduced from 3 min to 2 min. Further reduction of the extension time to 1 min would reduce the DNA signal by more than 35%, which was not sufficient for sequencing of the DNA fragments longer than 250 base pairs (bp). So 2-min extension time was used for ssDNA amplification and 30 cycles were completed within 80 min.

However, amplification of dsDNA such as pGEM is more difficult than ssDNA because the complementary strand of DNA may compete with the primer for annealing. Higher denaturing temperature (96°C) and longer extension time (4 min) were needed to achieve comparable efficiencies. Also, increasing the BSA concentration to 0.5 μg/μl proved to be helpful for the cycle sequencing of pGEM.

No special pretreatment of the fused-silica capillary was needed. The reaction capillary was completely regenerated simply by rinsing the capillary with separation buffer. For over 1 month of experiments, the same capillary was used for over 40 reactions without noticeable loss of reaction efficiency.

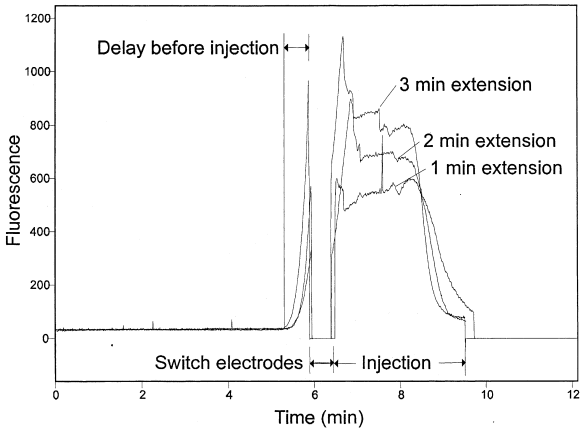

Fig. 4. Purified M13mp18 DNA fragments from cycle-sequencing amplification with different extension times.

3.4. CZE purification with suppressed EOF

Since the excess old-dye terminators comigrate with ~60 bp and ~110 bp DNA fragments in CGE, they will mask several base pairs around those regions in the sequencing separation and introduce errors in base calling. Also the high salt content of the reaction mixture makes electrokinetic injection to the CGE capillary extremely difficult. Proper purification of the cycle-sequencing product to get rid of these interferences must be incorporated into the on-line system. In our previous work, several separation schemes have been devised such as size-exclusion chromatography in a 250 μm I.D. capillary [27] and CZE separation [28]. CZE separation is especially attractive because it does not require additional instrumental components such as a high-pressure pump. However, in bare fused-silica capillaries, since electroosmotic flow (EOF) dominates the direction of migration, the dye terminators, which have smaller electrophoretic mobilities, migrate faster than the DNA fragments. The strong tailing of the terminator peaks, which is probably due to their strong hydrophobicity, makes the separation very difficult. Furthermore, the uncertainty in EOF due to variable surface conditions of the capillary inner wall makes the migration times in a capillary array very irreproducible [22]. This makes multiplexed CZE purification, where very precise timing and very short injection time to the CGE capillary is required, almost impossible.

Here, we use CZE with suppressed EOF to separate the dye terminators from the DNA fragments. Several separation systems were studied to optimize the on-line system. The most obvious idea would be to use the 1×PCR buffer as the separation buffer (pH 8.9), which is completely compatible with the cycle-sequencing reaction. Because of its high ion strength, the cations would shield the deprotonated silanol groups on the inner wall of the capillary to suppress EOF. Very good separation was achieved (Fig. 5a). The DNA fragments migrated as a narrow band because of their similar mass-to-

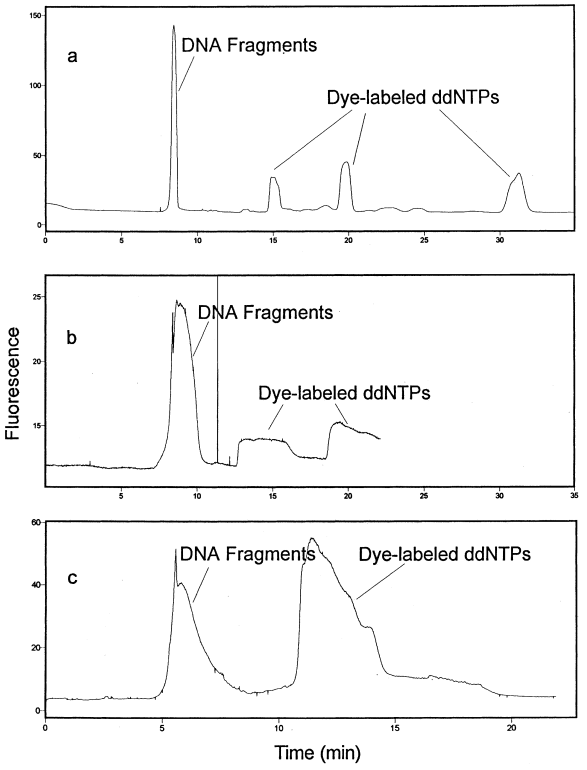

Fig. 5. CZE purification of cycle-sequencing product. (a) Bare fused-silica capillary, 1×PCR buffer with 50 m*M* KCl. (b) PVA-coated capillary, 1×PCR buffer with 4 m*M* KCl. (c) Bare fused-silica capillary, 1×PCR buffer with 2 m*M* KCl, 0.3% M_r 1 000 000 PVP.

charge ratios. However, when high-voltage is applied across the CZE capillary and the CGE capillary during electrokinetic injection, most of the voltage will drop along the CGE capillary. Thus, the field strength in the CZE capillary will be much smaller than that in the CGE capillary, which is just the opposite scenario as in electro-stacking. Therefore, electrokinetic injection turned out to be very inefficient.

One of the other choices would be using coated capillaries. Different coated capillaries, such as FC, PEG, PVA, DB-WAX coated capillaries, were tested as the integrated reaction vessel and separation column. 1×PCR buffer with low concentrations of KCl (pH 8.9) was tested as the separation medium. PVA-coated capillary proved to be the best choice in terms of suppressing EOF. Good separation from the dye terminators was achieved (Fig. 5b), although the DNA fragments appeared as a broader band. Different concentrations of KCl were tested for the CZE separation and electrokinetic injection efficiency (Table 1). 2~4 m*M* KCl proved to be best separation condition as a tradeoff between DNA bandwidth and on-line injection efficiency, which was confirmed by good DNA signals and sequencing separation with base calling up to 500 bases. No dye terminator interference was observed. However, there are two inevitable problems inherent to coated capillaries. One is the cost in a multiplexed system and the other is degradation of the coating. After about 20 reactions and CZE separations, noticeable decrease in the reaction efficiency was observed. Also, EOF gradually increased. Attempts to regenerate the capillary by washing it with methanol and deionized water were not successful.

In the previous studies, we demonstrated that EOF

Table 1
Effect of KCl concentration on CZE separation and electrokinetic injection

KCl conc. (m*M*)	Current in CZE (μA)[a]	Injection current (μA)[b]	Field strength during injection[b]		FWHM (min)
			CZE capillary (V/cm)	CGE capillary (V/cm)	
0	6.8	6.8	300	146	2.9
2	9.5	8.0	220	170	2.4
4	12.2	8.8	190	190	1.5
6	15.5	9.5	160	200	1.3

[a] Voltage applied across the CZE (L_1 = 35 cm) and waste arm (L_2 = 10 cm) capillaries was 11 kV, corresponding to ~300 V/cm field strength in CZE capillary.

[b] Voltage applied across the CZE (L_1 = 35 cm) and CGE (L_3 = 64 cm) capillaries was 21 kV. The CGE capillary was filled with PEO gel while the CZE capillary was filled with 1×PCR buffer. When 150 V/cm was applied across the CGE capillary during sequencing separation, the current was ~7.0 μA.

of bare fused-silica capillary could be substantially suppressed by the dynamic coating of PVP solution due to the strong hydrogen bonding between the hydrophilic carbonyl group of PVP and the residual hydroxyl group on the capillary wall [29]. Compatibility of the PVP coating with the cycle-sequencing reaction was therefore studied by flushing the long capillary loop with 2% PVP followed by placing the capillary in the commercial air thermocycler (Idaho Technology) for offline cycle-sequencing reaction. After reaction, about ~2 μl of reaction product was collected and purified by spin column. CGE separation of the purified DNA product showed similar signal strength as that without PVP coating, which confirmed that PVP did no harm to the reaction.

During CZE separation, PVP was added to the separation buffer to achieve more even coating. Different concentrations of PVP were investigated for efficiency for suppressing EOF and for separation. At pH 8.9, 1.0% PVP was required to consistently suppress EOF. However, since the entanglement limit of M_r 1 000 000 PVP is about 0.7%, at such a high PVP concentration the DNA fragment peak was substantially broadened by the sieving effect. An alternative approach was to lower the pH of the separation buffer. It was found that at pH 8.2, only 0.3% PVP was needed to suppress EOF. Different sized DNA fragments comigrated as a narrow band with half peak width of ~1.5 min (Fig. 5c). More important was that there was no compromise in the reaction efficiency in such a low pH buffer. The surfactant, Triton X-100, was taken out from the buffer to avoid bubble formation around the microtee connection during CZE separation.

Very reproducible separation was achieved with PVP dynamic coating. The bare fused-capillary could be reused again and again by simply flushing the capillary with the separation buffer between runs. For over 1 month of experiments, more than 40 reactions and separations were performed on the same capillary. No decay in the on-line reaction efficiency was observed and the DNA fragments showed very reproducible migration times.

3.4.1. DNA sequencing separation

Both ssDNA and dsDNA were successfully amplified on-line, purified and injected into the CGE capillary for two-channel detection. Fig. 6 shows the two-channel electropherograms of ssDNA M13mp18 [9]. No dye-labeled ddNTP peak was detected. As summarized in Table 2, there were no errors in base calling up to 400 bp. The error rate was below 1% up to 540 bp.

Fig. 7 is the two-channel sequencing data for dsDNA pGEM [9]. A shorter read length compared to that of M13mp18 was achieved. This may be partially due to the lower efficiency in the amplification of dsDNA. Other reasons might be the reannealing of the Sanger fragments with the complementary strand of the template, since the pGEM has a partial sequence which has a high content of G and C nucleotides. However, no error occurred till 460 bp and the error rate was kept below 1% up to 480 bp. If some heating element is placed before the tee or the tee itself is heated before injection into the CGE capillary, even better base calling result should be attainable because of additional denaturing [20]

4. Conclusions

Here we demonstrated a simple but reliable microthermocycler which is capable of cycle-sequencing amplification of both ss and dsDNA. Fast nanoliter-scale reaction was accomplished in the same bare fused-silica capillary used for subsequent purification. If this is combined with nl reagent dispensing [25], true savings in material cost will be realized for DNA sequencing. PVP proved to be a reliable and efficient dynamic coating material in the CZE purification of Sanger fragments from the cycle-sequencing reaction. Purified DNA was successfully injected into the CGE capillary for sequencing separation by simply switching the high-voltage electrode. This closed system avoids external sample transfer and consumes only nanoliter volumes of expensive cycle-sequencing reagents in each run. It greatly reduces contamination and allows for faster execution of analysis at a substantially lower cost. The whole system is ready for conversion into a multi-capillary on-line reaction, purification and gel separation instrument for high throughput operation. Adaptation to a microchip format should also be possible, since no high-pressure fluidics is involved.

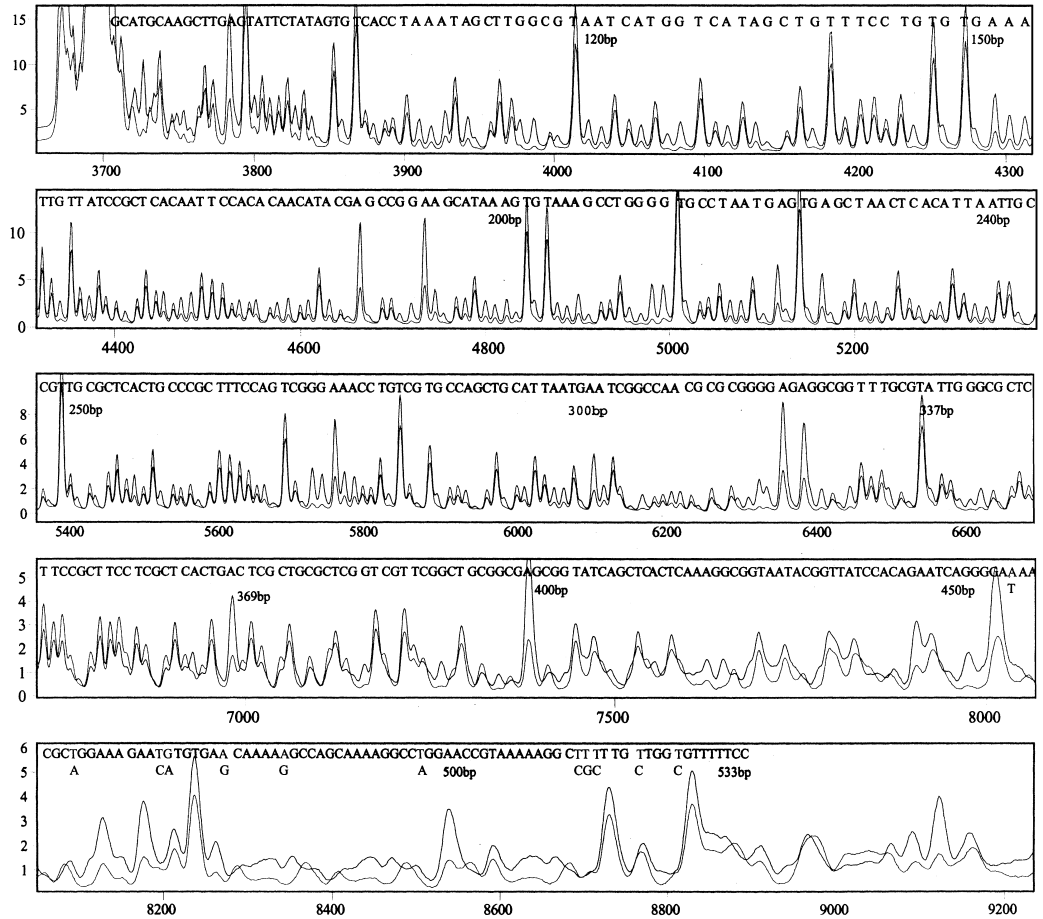

Fig. 6. Online cycle sequencing of ssDNA M13mp18 with 2-color base calling. Errors are marked underneath the line of corrected sequences.

Without much modification, the whole system could also be adapted to many other applications such as genotyping [21], peptide mapping [30], and combinatorial screening [31].

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Table 2
Base-calling results

ssDNA M13mp18			dsDNA pGEM		
bp number	Number of errors	Error rate (%)	bp number	Number of errors	Error rate (%)
400	0	0	450	0	0
500	2	0.4	470	3	0.6
540	5	0.9	480	4	0.8
590	15	2.5	490	6	1.2

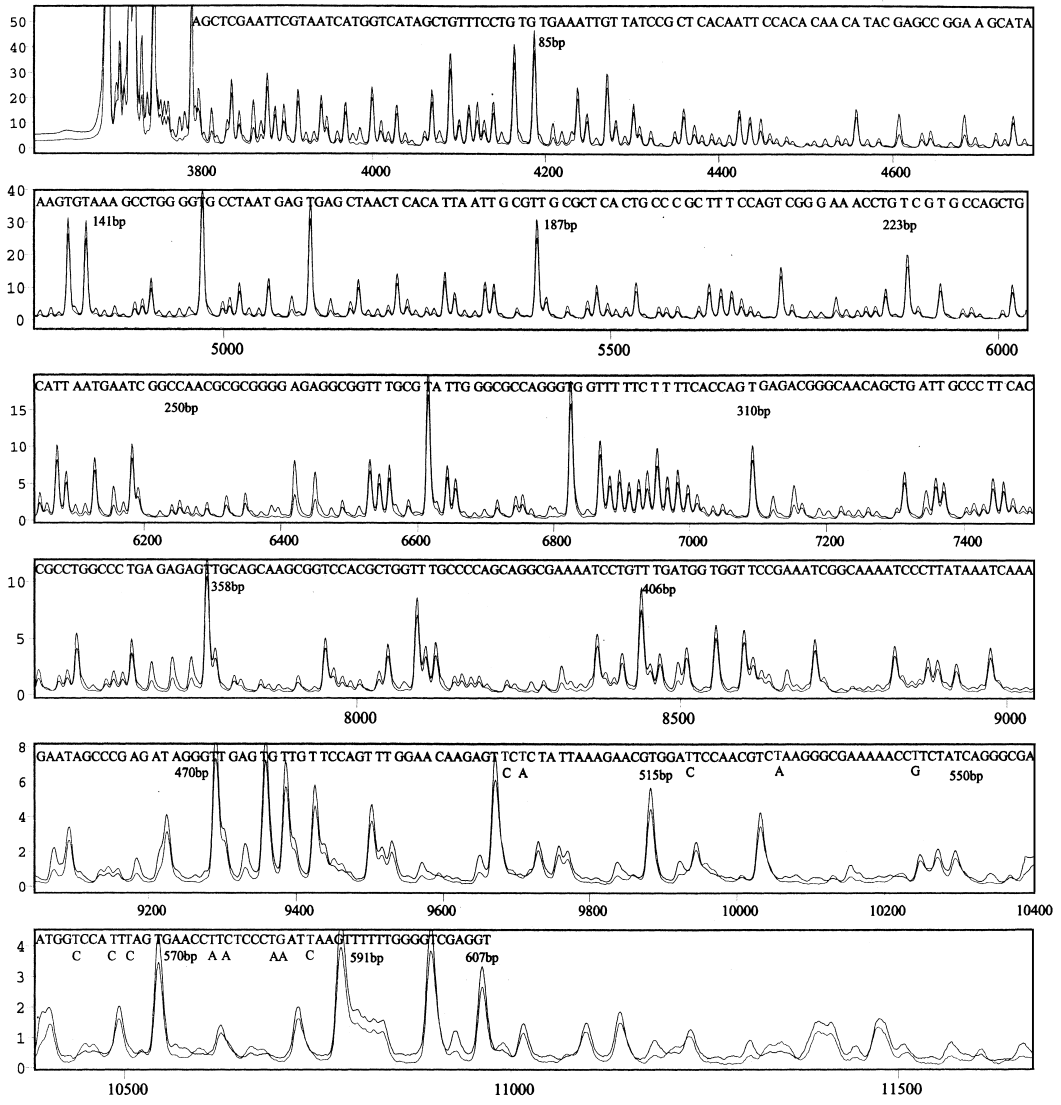

Fig. 7. Online cycle sequencing of dsDNA pGEM with 2-color base calling. Errors are marked underneath the line of corrected sequences.

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