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Journal of Chromatography B, 714 (1998) 3–11

JOURNAL OF
CHROMATOGRAPHY B

On-line coupling of polymerase chain reaction and capillary electrophoresis for automatic DNA typing and HIV-1 diagnosis

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Abstract

We demonstrate an integrated on-line system with a fused-silica capillary as the microreactor for PCR and capillary gel electrophoresis with laser-induced fluorescence detection for DNA typing and disease diagnosis. Two applications have been investigated: the four short tandem repeat (STR) loci vWA, THO1, TPOX and CSF1PO (CTTv) for DNA typing, and DNA probe for human immunodeficiency virus (HIV-1) diagnosis. The CTTv are important loci in forensic and genetic linkage analysis. The PCR technique is a powerful tool in HIV research because it can detect the presence of the virus before any antibody response in the infected person. Thus it is important for early diagnosis. Multiplexed PCR in a fused-silica capillary, on-line injection, DNA denaturation and calibration based on a standard ladder have been successfully combined. Also, on-line liquid flow management, DNA separation and detection have been completely integrated. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Polymerase chain reaction; DNA; Human immunodeficiency virus

1. Introduction

Since the invention of polymerase chain reaction (PCR) in 1985 by Kary Mullis, the ultimate in sensitivity, together with increasing ease in implementation, have placed this technique in a central position in molecular biology research and in clinical diagnosis [1]. In the last decade, PCR has stimulated numerous investigations in genetic analysis, and is even being used to determine the genetic basis of complex diseases such as sickle cell anemia [2]. There is no need to reiterate the development of capillary electrophoresis (CE) as a powerful analytical tool in post-PCR analysis. A large amount of research has been done to explore the advantage of CE over traditional slab gel electrophoresis. These include high-speed, high-resolution restriction fragments analysis [3–8], high-speed, high-throughput DNA sequencing [9–15], rapid and precise DNA typing and sizing [16–22], single-base mutation analysis [23–28] and the analysis of disease-causing genes [29–33]. In particular, capillary array electrophoresis along with other microfabricated devices [34–36] are promising methods for the purpose of achieving high-throughput DNA analysis. Research laboratories have also begun to address issues in all areas of laboratory testing, from sample preparation and sample reaction to separation and detection, to match these high-throughput devices.

The ultimate goal is to devise an automatic system which can integrate all these steps, from a real biological sample to final readable results. A critical intermediate step is to integrate PCR with CE to allow on-line sample reaction, sample injection and DNA separation. The following basic requirements must be met to achieve this purpose: (1) faster PCR

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PII: S0378-4347(98)00105-4

to be compatible with the high separation speed of CE; (2) minimum PCR volume to reduce the cost of reagents and the amount of tissue or fluid sample, since CE only requires an extremely small amount of sample; and (3) automation in the sample handling process throughout, including sample injection and solution flow management. Based on these requirements, standard PCR protocols performed inside plastic vials with reaction volumes around 50~100 μl are not well matched to CE. On the other hand, a fused-silica capillary as a microreactor is compatible with an integrated on-line system for DNA sequencing, as was previously demonstrated by our group [37]. Here, we modified the system to allow one-step single strand (ss)- and double strand (ds)-DNA fragment analysis after PCR.

2. Experimental

2.1. Reagents, buffers and separation matrix

All chemicals for preparing buffer solutions were purchased from ICN Biochemicals (Irvine, CA, USA). Methanol, anhydrous sodium hydroxide and fuming hydrochloric acid were obtained from Fisher (Fairlawn, NJ, USA). Poly(ethylene oxide) (PEO) was received from Aldrich (Milwaukee, WI, USA). 1× TBE buffer is made of 89 m*M* Tris-(hydroxymethyl)aminomethane (THAM), 89 m*M* boric acid and 2 m*M* EDTA in deionized water (pH~8.3). The 1× TE buffer solution was prepared by dissolving 10 m*M* THAM and 2 m*M* EDTA in deionized water (pH~8.2).

For short tandem repeat (STR) loci analysis, the sieving liquid polymer was 2.0% M_r 8 000 000 PEO and 1.4% M_r 600 000 PEO in 1× TBE buffer with 3.5 *M* urea. For the human immunodeficiency virus (HIV) PCR fragment test, the sieving liquid polymer was 1.5% M_r 8 000 000 PEO in 1× TBE buffer without urea. Ethidium bromide (EtBr) was used as the intercalating dye in this case. The concentration of EtBr was 1 μg/ml in the electrophoresis buffer.

2.2. Polymerase chain reaction

2.2.1. Multiplexed PCR for the STR loci

The GenePrint Fluorescent STR system was purchased from Promega (Madison, WI, USA). This kit included four loci: vWA, THO1, TPOX and CSF1PO. Table 1 shows some information about the allelic ladders for the four loci. One of each pair of primer was labeled with fluorescein at the 5′ end. The original PCR protocol was designed for the Perkin-Elmer DNA thermal cycler Model 480 or 9600. The total reaction volume in the protocol was 22.5 μl and the reaction time needed is around 2 h. Modifications were made both in the amounts of PCR reagents and the temperature and time protocol to fit a hot-air thermal cycler (Rapid-cycler from Idaho Technology, Idaho Falls, ID, USA), which uses a soft-glass tubing as a PCR reactor.

The Taq DNA polymerase in storage buffer A was obtained from Promega. The original concentration was 5 U/μl. This enzyme solution was diluted five-times by enzyme diluent which contains 2.5 mg/ml bovine serum albumin (BSA) (Idaho Technology) as the working solution. The PCR was multiplexed by having the four loci amplified together in one tubing. The total PCR volume was 10.0 μl, containing: 1.0 μl of CTTv primers (5 μ*M*), 1.0 μl of Taq polymerase working solution, 1.5 μl 40 m*M* $MgCl_2$ solution with 2.5 mg/ml BSA, 1 μl 2.5 mM dNTPs (ABI, Foster City, CA, USA), and 5.5 μl genomic DNA (~50 ng) stored in 1× TE buffer.

For off-line experiments, 10.0 μl of the reaction

Table 1
Multiplexed STR loci for genetic sequencing

Locus	Repeat sequence	Range of alleles	Locus size range
vWF	AGAT	13–20	139–167
THO1	AAGT	5–11	179–203
TPOX	AAGT	6–13	224–252
CSF1PO	AGAT	7–15	295–327

Table 2
PCR temperature and time profile for multiplexed probing

Cycle No.	Denaturation	Annealing	Extension
2	95°C, 1 min	65°C, 30 s	70°C, 30 s
30	95°C, 1 s	65°C, 30 s	70°C, 30 s
1	70°C, 2 min		

mixture was sealed in the 1.02 mm O.D.×0.56 mm I.D. soft-glass tubing by using a micro-flame torch to melt both ends of the capillary. The PCR was performed according to the following profile, which was tested to be optimum: denaturation at 95°C, 0 s; annealing at 60°C, 0 s; extension at 70°C, 10 s; and cycle number, 30. Initially, we tried to use the same protocol when the PCR was adapted to a 360 μm O.D.×250 μm I.D. fused-silica capillary. However, it failed completely. It is known that PCR is sensitive to surface effects. We therefore tested many different protocols. The one in Table 2 gave the best signal for all loci. The possible reasons why different protocols are needed in the two cases are discussed later.

2.2.2. PCR for HIV diagnosis

The HIV test kit (Perkin-Elmer, Foster City, CA, USA, part No. N808-0015) included: positive control DNA which includes all parts of the HIV-1 genome; negative control DNA; and HIV primers for amplifying the HIV-1 *gag* region. The optimized PCR reagent mixture is described in Table 3. The protocol for the thermal cycler begins with 2 cycles of denaturation at 95°C for 15 s, and annealing and extension at 62°C for 1 min. The next 25 cycles involve denaturation at 95°C for 0 s, and annealing and extension at 62°C for 1 min.

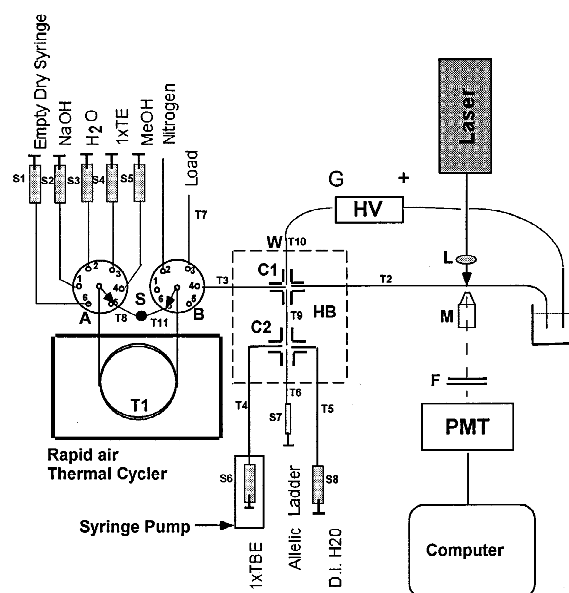

Fig. 1. Schematic of instrumental set-up for integrated on-line PCR–CE. A and B: Six position selection valves; S: septum for sealing the two capillary ends; T1: reactor for PCR, 40 cm×248 μm I.D.×360 μm O.D. fused-silica capillary; T3: capillary for transferring PCR products from B to C1 for injection; C1: quartz cross; C2: stainless steel cross; HB: heating block; T2: separation capillary, 75 μm I.D.×365 μm O.D.; W: waste line with a small diameter stainless steel wire threaded into the capillary; HV: high-voltage power supply; L: lens; M: 10× microscope objective; F: spectral filters for collecting fluorescence; PMT: photomultiplier tube; S1: 200-μl microsyringe; S7: 100-μl microsyringe; S2–S6 and S8: 5-ml syringes.

2.3. Instrumentation and operation

Fig. 1 is a schematic diagram of the entire instrumental set-up. There were 10 capillary tubes

Table 3
Reagent mixture for multiplexed PCR

Component	Concentration	Volume (μl)
HIV-1 primer 1	25 μ*M*	1.0
HIV-1 primer 2	25 μ*M*	1.0
Taq DNA polymerase working solution	0.8 U/μl	1.0
HIV positive control DNA or negative control DNA	10^3 copies/μl	1.0
$MgCl_2$/BSA	40 m*M*/2.5 mg/ml	1.5
1×TE		4.5

and eight syringes. T1, T3, T6, T7, T8, T9, T10 and T11 were fused-silica capillaries of 360 μm O.D.× 250 μm I.D. T2 was the separation capillary filled with gel, with an I.D. of 75 μm and an O.D. of 370 μm. T4, T5 and all tubes connecting syringes with the two six-position valves (Alltech) were 0.38 mm I.D.×1.6 mm O.D. PTFE tubings (Valco Instruments, Houston, TX, USA). From left to right, the whole set-up could be divided into three parts: PCR, flow control, and separation and detection.

2.3.1. PCR

T1 is the 40 cm long capillary for PCR. The capillary was placed inside the thermocycler compartments by passing two ends of capillary through the holes on the capillary holders of the thermocycler. The two ends of T1 outside the capillary holder were connected into the center ports of two six-position valves. The fitting between the capillary and the valves was described by Tan and Yeung [37]. By switching valve A to A6, B to B3, the PCR mixture was loaded from port B3 to capillary T1 by the vacuum produced in the empty syringe S1 at port A6. When PCR was in progress, valves A and B must be switched to positions A5 and B6 so that the capillaries T8 and T11 were sealed by a septum. The purpose is to seal the air inside both ends of the capillaries so that the pressure prevents the segmentation of the PCR mixture inside capillary T1 due to the rapid change of temperature during PCR cycling. After completion, the PCR products were pushed gently by syringe 1 to the cross junction C1 for injection, with A at position A6 and B at position B4. The solutions in syringes S2–S5 were used for washing the reactor and loading capillary T7. Nitrogen gas was used to dry the capillary T1 and T3 after washing.

2.3.2. Flow control

C1 was a quartz cross with four arms about 1 cm long (Phoenix, AZ, USA). The inner diameter of the four arms Polymicro Technologies were around 370 μm, which could fit the capillaries directly. T2, T3, T9 and T10 were inserted into the center of C1, with their tips close to each other but without touching. C1 served as the sample injection cross and the ground end for the electrophoresis. T10 was a 365 μm O.D.×250 μm I.D. capillary. A 100-μm diameter stainless steel wire (which was connected to the high-voltage power supply) was passed through T10 to serve as the ground electrode. T10 also served as the waste line for the solutions from sample injection and from the buffer solution being circulated by the syringe pump. T3 was used to transfer the PCR products to C1 for injection. T2, which was a capillary filled with sieving liquid polymer for separation, was connected on the opposite side of T3.

C2 was a stainless steel cross (Upchurch Scientific, Oak Harbor, WA, USA) with 0.020 in. holes (1 in.=2.54 cm). C2 controlled the flow of the 1× TBE buffer for electrophoresis, standard ladder for calibration, and deionized (d.i.) water for cleaning. First, the standard ladder was introduced into C1 for injection by pushing syringe S7. After injection, d.i. water was sent to C1 by pushing S8 to wash away the excess ladder. Then, the PCR products were transferred to C1 for injection. Each injection lasted for 5–10 s. Finally, the 1× TBE buffer in S6 was pumped to C1 with a flow-rate of 100 μl/min for 1 min without applying the high voltage. This step was necessary to clear out the extra PCR products at C1. High voltage was then applied to begin electrophoresis separation.

2.3.3. CE separation and laser-induced fluorescence detection

The experimental set-up was similar to that described before [18,20]. An air-cooled Ar^+ laser (Uniphase, San Jose, CA, USA, Model 2213-150 ML) with multi-line emission was used. The 488-nm line was isolated with a glass prism for excitation in the case of STR analysis. A 520-nm edge filter (Ealing Electro-optics, Holliston, MA, USA) and a 540-nm long-pass filter (Edmund Scientific, Barrington, NJ, USA) were combined to reject the scattered laser light. The total length of the capillary was 60 cm (effective length 50 cm). The running voltage was 12 kV. When analyzing the HIV DNA fragment, the 514-nm laser line was isolated for excitation. Two RG610 long-pass filters were used in front of the PMT. The separation capillary was 50 cm (effective length 40 cm). The running voltage was 9 kV. The fluorescence signal was transferred directly through a 10-kΩ resister to a 24-bit A/D interface at 5 Hz and stored in a computer.

3. Results and discussion

3.1. Effect of reactor surface and surface to volume ratio (SVR) on PCR

Some research done in the investigation of PCR in microfabricated chips showed that native silicon was an inhibitor of PCR. Amplification in an untreated chip had a high failure rate [38,39]. Complicated surface treatments are necessary such as silanization followed by coating of a selected protein or polymer and the deposition of a nitride or oxide layer onto the silicon surface. However, in a previous study of DNA sequencing reaction [37] and in this study, we found that high concentrations of BSA in the PCR mixture can prevent surface inhibition. No surface modification was needed for fused-silica capillary as a reactor if BSA was present in PCR mixture.

It was found that SVR affects PCR dramatically. In a 1.02 mm O.D.×0.56 mm I.D. soft-glass capillary, which had a SVR of 72.1/cm, PCR could be finished in less than 10 min. The fast reaction could be explained by the fact that the high SVR provided more efficient thermal conduction and dissipation so that shorter cycle times could be used compared to conventional plastic vials (with SVR 15.4/cm) [40]. However, PCR in a 360 μm O.D.×250 μm I.D. fused-silica capillary (with SVR 160/cm) failed to produce satisfactory amplification when the same protocol was used. Much longer reaction times were needed, resulting in a total time of around 40 min. This is probably due to the presence of surface chemistry and the limited dynamic diffusion of molecules in the PCR mixture in such a narrow tube.

3.2. Multiplexed PCR for the four STR loci

Simultaneous amplification of more than one DNA region of interest in one reaction mixture reduces labor, time, cost and cross contamination, since sample handling is minimal. For multiplexed amplification, there is competition among the four pairs of primer. There are differences in the optimum conditions for the individual loci. Therefore, a multiplex system has much narrower tolerance limits than single PCR systems. Many factors may affect the amplification results, such as template DNA concentration, polymerase concentration, concentrations of primers and dNTPs, number of amplification cycles, denaturation and annealing temperatures, ionic strength and pH, etc., [40]. In this study, we found that the annealing temperature and the duration of each cycle were the key factors for obtaining strong signals for all four loci. Also, the temperature and time for both denaturation and extension were important factors.

3.2.1. Annealing temperature

Fig. 2A–C show the dramatic PCR results as a function of annealing temperature. Two loci were tested: THO1 and TPOX. There was no specific

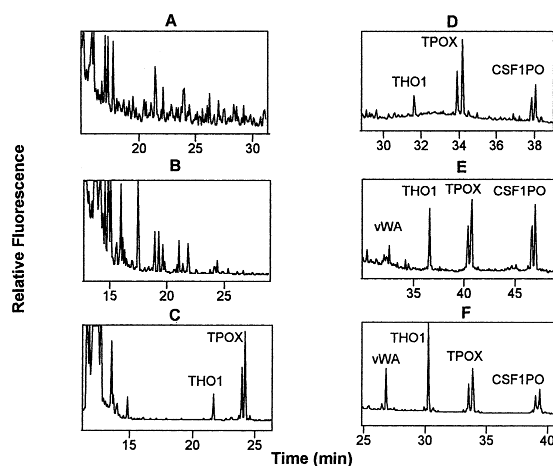

Fig. 2. The effect of temperature and time on multiplexed PCR for CTTv analysis. (A) Simultaneous amplification for loci THO1 and TPOX. Two cycles of denaturation at 92°C for 1 min, annealing at 42°C for 5 s and extension at 72°C for 72 s, followed by denaturation at 92°C for 1 s, annealing at 42°C for 5 s and extension at 72°C for 72 s; (B) same as (A) except the annealing temperature was 60°C; (C) same as (A) except the annealing temperature was 65°C; (D) simultaneous amplification of all four loci CTTv. Two cycles of denaturation at 92°C for 1 min, annealing at 65°C for 7 s, extension at 72°C for 72 s, followed by denaturation at 92°C for 1 s, annealing at 65°C for 7 s and extension at 72°C for 72 s; (E) simultaneous amplification of all four loci CTTv. Two cycles of denaturation at 92°C for 1 min, annealing at 65°C for 30 s, extension at 70°C for 70 s, followed by denaturation at 92°C for 1 s, annealing at 65°C for 30 s and extension at 72°C for 70 s; and (F) simultaneous amplification of all four loci CTTv. Two cycles of denaturation at 95°C for 1 min, annealing at 65°C for 30 s, extension at 70°C for 30 s, followed by denaturation at 95°C for 1 s, annealing at 65°C for 30 s and extension at 72°C for 30 s. The multitude peaks in (A) and (B) are non-specific PCR products. The peaks in (C–F) are labeled by the name of each STR locus.

primer annealing at 42°C at all. At 60°C, the amplified alleles were observed, but there were still a lot of non-specific peaks. The PCR products became unique as the temperature was raised to 65°C. The signals were intense and the homozygous DNA type at THO1 and the heterozygous DNA type at TPOX were clearly depicted.

3.2.2. Annealing time

When four-loci co-amplification was tested by using the same conditions as in Fig. 2C, only TPOX and CSF1PO loci were amplified. There were no signals for either vWA or THO1. We tried to change other conditions, but no improvements were found until the annealing time was extended from 5 s to 7 s. Signal corresponding to the THO1 locus was then obtained. This led us to further increase the annealing time to 30 s, when the PCR products of all four loci were obtained. The remaining problem is that there were ''stutter'' products in the vWA locus. This phenomena was commonly found in STR amplification, especially in the vWA system. The extra products result from a process known as ''slippage'' due to mispairing of the template and the newly synthesized strand during extension [41,42]. However, the amplification of vWA locus was improved (Fig. 2D–F) when a higher denaturation temperature (95°C) was used together with two cycles of long denaturation time (1 min) at the beginning. Also, the optimum extension temperature was found to be 70°C.

3.3. HIV-1 gag amplification

HIV-1 primers and template were added to the optimized PCR mixture (PCR buffer, Mg^{2+} concentration, BSA concentration, pH, enzyme concentration, ionic strength). Then various protocols for the thermocycler were tested. It was found that the optimum extension temperature was very low, similar to that suggested by the test-kit manufacturer. So the annealing and extension steps became a single step. The PCR mixture was found to be universally applicable to different primers and DNA templates.

3.4. On-line injection, denaturation and separation

Almost all DNA typing and DNA diagnosis need appropriate size standards to help interpret the results. It is well known that migration times alone cannot be used for identifying DNA fragments in CE, since one capillary can be very different from another [34]. Calibration based on size standards is not entirely satisfactory because base composition can influence mobility. Therefore, calibration for DNA allele typing here is based on the principle of co-injection of an allelic ladder as the absolute standard [18]. However, the 115 base pair (bp) HIV *gag* fragment was recognized by co-injection with 100 bp DNA standard ladder (Boehringer Mannheim, Indianapolis, IN, USA), since an appropriate standard was not available. The ladder consists of fragments 50, 100, 200, 300, 400, 500, 700 and 1000 bps. No peak broadening due to the consecutive injections or the washing step between the two injections was found. The d.i. water had two functions. First it reduced the ionic strength at C1 so that the DNA sample could be efficiently injected. Its second function was to clean C1 after the standard ladder was injected so that the PCR products could be injected at C1 again without contamination. Adsorption of DNA at valve B and C1 was not found. There was also no need to use reagents such as BSA to coat C1 before injection.

In the case of STR analysis, it was found that this sample was very difficult to keep denatured. Extra peaks due to rehybridization could often be seen [20]. Here, we placed both crosses C1 and C2 on a heating block with the temperature set at 90°C. Then the syringe was pushed gently to load the samples to the center of C1. After waiting for 30 s, the sample was injected. Initially, the heating block was removed after both the allelic ladder and the PCR products were injected, and after C1 had been washed by 1× TBE for 1 min. High-voltage for CE was immediately applied to begin separation. However, rehybridization of the fragments was still observed. Finally, we found that keeping C1 and C2 on the heating block all the time (during injection and separation) and keeping the beginning 8 cm of the separation capillary at 90°C resolved the rehybridization problem. The final results are shown in Fig. 3. Because of the presence of an absolute standard, the sample fragments show up as an increase in intensity for the specific allelic peaks.

No sample denaturation was necessary when testing for the HIV-1 *gag* fragment because ds-DNA was analyzed. Because a relatively low resolution is

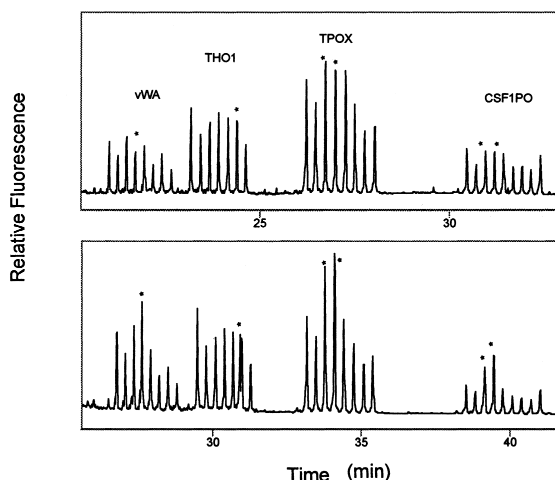

Fig. 3. On-line PCR–CE for CTTv typing. The upper electropherogram shows the on-line injection and separation of CTTv standard allelic ladder only. The lower one shows the result of on-line PCR, co-injection of standard allelic ladder and PCR products and CE separation. Notice the change of peak intensities in the peaks marked with an asterisk (*). The genotype is named by the number of repeat sequence contained in the allele (see Table 1). The results are: vWA=16,16; THO1=9,3; TPOX=8,9; and CSF1PO=9,10.

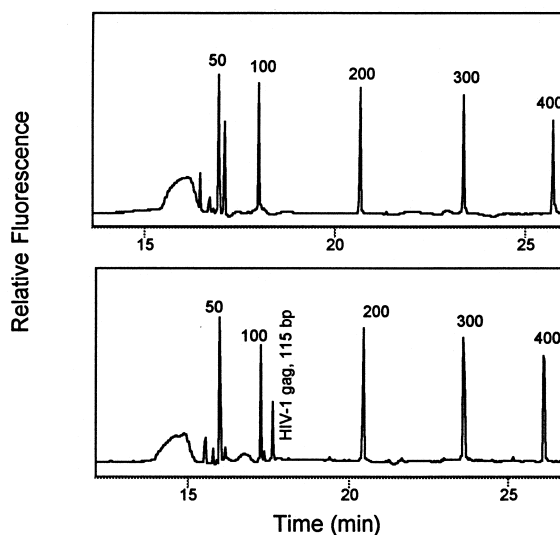

Fig. 4. On-line PCR–CE for HIV diagnosis. The upper electropherogram shows the negative control DNA after amplification. The lower one shows the positive HIV control DNA after amplification. The 115 bp fragment of HIV-1 gag is clearly identified as the peak immediately after the 100 bp fragment in the standard size ladder.

needed in the separation, lower viscosity sieving liquid polymer (1.5% M_r 8 000 000 PEO) and a shorter capillary (50 cm, 40 cm effective length) were used to provide faster separation. Before any injection, the separation column must be equilibrated at the running voltage so that ethidium bromide can be distributed in the gel before sample injection. The results in Fig. 4 show on-line analysis of the amplified positive control DNA and the amplified negative control DNA.

3.5. Regeneration of PCR capillary reactor

Because there were numerous species in the PCR mixture, careful washing was necessary to clean the capillary, especially its surface. Washing the capillary consecutively with NaOH, 1× TE, MeOH and d.i. water at room temperature was not sufficient, as indicated by a low-intensity PCR signal found after the capillary was treated in this way. We found that it was important to wash the capillary with 0.1 *M* NaOH at high temperature (95°C) for 3 min, followed by washing with 5 ml MeOH and d.i. water at room temperature. Five consecutive reactions were processed in this manner in one capillary. No obvious degradation or cross-contamination was observed.

4. Conclusion

The on-line coupling of PCR and CE for separation and detection has been successfully demonstrated here. No off-line manipulation was employed starting from extracted DNA to PCR and final CE separation and detection. The whole set-up is conceptually simple and easy to operate. Both ss-DNA and ds-DNA analysis can be performed. Also, the system is flexible and allows modification of the conditions to fit different analytical applications. This system shows the possibility of future adaptation of on-line sample preparation and analysis in a capillary array format for DNA typing and disease diagnosis. No purification of the PCR products was needed in almost all cases because the interference from dye-labeled primers or signals caused by intercalating dyes do not overlap with the DNA region of interest. Also, PCR products prepared as

described can be directly injected without sample desalting.

An on-line system for PCR analysis and for cycle sequencing was also reported recently [43]. However, here we have a much more difficult situation in the simultaneous amplification of four loci, particularly ones that readily show slippage or rehybridization, and the incorporation of an absolute DNA standard (allelic ladder) for calibration during each CE run. The elimination of a clean-up step also allows the use of low-pressure syringe pumps rather than a liquid chromatography pump. Also, actual applications to genetic typing and to HIV DNA analysis are shown here.

It is well known that DNA extraction from real samples (biological fluids) is always a time-consuming procedure. This procedure usually takes at least half of the total analytical time. It will be important to extend the present system to include DNA extraction and purification from real samples in an on-line instrument to save time, reduce labor and minimize the exposure of workers to infectious samples. Demonstration of the present scheme for simultaneous analysis in an array system will also be valuable. Computer-controlled valves and flow distribution networks will allow parallel systems to be developed based on Fig. 1. These will be some of the future efforts in our group.

Acknowledgements

The Ames Laboratory is operated for the US Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Health and Environmental Research. We also thank Hongdong Tan for valuable discussions.

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