Anal. Chem. **2003,** *75,* 6029−6033

Cylindrical Compact Thermal-Cycling Device for Continuous-Flow Polymerase Chain Reaction

Nokyoung Park, Suhyeon Kim,† and Jong Hoon Hahn*

National Research Laboratory for Advanced Biotechnology and Biomedical Microinstrumentation, Department of Chemistry, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31 Hyoja-Dong, Pohang 790-784, South Korea

A compact, thermal-cycling device for high-throughput continuous-flow polymerase chain reaction (PCR) has been developed, which consists of a flow-through capillary and a cylindrical heating-block assembly. A 3.5-m-long fused-silica capillary coils helically, with 33 turns, up around the 30-mm-diameter assembly of three equally divided thermostating copper blocks for melting, annealing, and extension. An injected PCR mixture undergoes one cycle of PCR each turn. A continuous-flow PCR of one sample and also a segmented-flow PCR of four different samples have been successfully demonstrated. The present device can easily evolve into a parallel-processing, multistation compact device and be modified to have real-time PCR capability. This solid-based compact PCR device, therefore, has a potentiality to be the format of choice when developed for a portable system.

Polymerase chain reaction (PCR) has emerged as a powerful tool[1] in genetic analyses, medical diagnostics, and forensic applications, and increasing demands in these fields for analyzing a large number of samples have encouraged the development of high-throughput PCR techniques.[2−4] An early advancement employed rapid thermal cycling by using an air stream to heat and cool sealed glass capillaries containing PCR mixtures.[5,6] Recently, however, interests have been focused on developing microfabricated PCR devices, because they can provide enhanced speed, reduced reagent consumption, and automation. Such devices have been fabricated in various substrates: silicon,[7−9] glass,[10−12] silicon/glass hybride,[13−15] and plastic/glass hybride,[16] where PCR occurs in a confined space subjected to different temperatures in a repetitive sequence. Alternatively, DNA amplification can be achieved by letting a PCR mixture continuously flow through a single channel passing repetitively through three constant-temperature zones for melting, annealing, and extension.

This continuous-flow PCR has been demonstrated on a microchip by Manz and co-workers.[17] They have used glass as the substrate for the chip and linearly arranged three thermostated copper blocks beneath the chip in the order of temperature: 95 °C block for melting, 77 °C for extension, and then 60 °C for annealing. Such an arrangement is good to establish a rather smooth temperature gradient on the chip. But a melted sample, in this case, is subjected to the extension temperature before reaching the annealing zone. When passing the extension zone, the melted, single-stranded DNA fragments with a length defined by the distance between two primer-binding sites (mostly several hundred bp) likely form double strands by binding with the template strands or their complementary fragments, which could reduce the PCR yield significantly.[18,19] The annealing temperature in a thermal-cycling PCR increases with the size of primers, and therefore, at a typical extension temperature, single-stranded DNA fragments of several hundred bp long, in contrast to short primers (only 20−30 bp long), can bind to their complementary strands. Rearranging the order of temperature blocks to melting, annealing, and extension would require forced cooling, instead of heating, at the annealing stage, which would create more complications in building a system. Quake and co-workers have solved this problem by employing a circular arrangement of heating blocks

* To whom correspondence should be addressed. Phone: +82-54-279-2118. Fax: +82-54-279-3399. E-mail: hahn@postech.ac.kr.

† Present address: Digital Bio Laboratory, Samsung Advanced Institute of Technology, P.O. Box 111, Suwon 440-600, South Korea.

(1) Brinkmann, B. *Methods Mol. Biol.* **1998**, *98*, 105−118.
(2) Manz, A.; Becker, H. *Microsystem Technology in Chemistry and Life Sciences*; Springer: Berlin, 1999.
(3) Verpoorte, E. *Electrophoresis* **2002**, *23*, 677−712.
(4) Reyes, D. R.; Lossifidis, D.; Auroux, P.; Manz, A. *Anal. Chem.* **2002**, *74*, 2637−2652.
(5) Wittwer, C. T.; Fillmore, G. C.; Hillyard, D. R. *Nucleic Acids Res.* **1989**, *17*, 4353−4357.
(6) Wittwer, C. T.; Fillmore, G. C.; Garling, D. J. *Anal. Biochem.* **1990**, *186*, 328−331.
(7) Wilding, P.; Kricka, L. J.; Cheng, J.; Hvichia, G.; Schoffner, M. A.; Fortina, P. *Anal. Biochem.* **1998**, *257*, 95−100.
(8) Daniel, J. H.; Iqbal, S.; Millington, R. B.; Moore, D. F.; Lowe, C. R.; Leslie, D. L.; Lee, M. A.; Pearce, M. J. *Sens. Actuators, A* **1998**, *71*, 81−88.
(9) Northrup, M. A.; Bennet, B.; Hadley, D.; Landre, P.; Lehew, S.; Richards, J.; Stratton, P. *Anal. Chem.* **1998**, *70*, 918−922.
(10) Taylor, T. B.; Harvey, S. E.; Albin, M.; Lebak, L.; Ning, Y.; Mowat, I.; Schuerein, T.; Principe, E. *Biomed. Microdevices* **1998**, *1*, 65−70.
(11) Waters, L. C.; Jacobson, S. C.; Kroutchinina, N.; Khandurina, J.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **1998**, *70*, 5172−5176.
(12) Lagally, E. T.; Simpson, P. C.; Mathies, R. A. *Sens. Actuators, B* **2000**, *63*, 138−146.
(13) Cheng, J.; Schoffner, M. A.; Hvichia, G.; Kricka, L. J.; Wilding, P. *Nucleic Acids Res.* **1996**, *24*, 380−385.
(14) Chaudhari, A. M.; Woudenberg, T. M.; Albin, M.; Goodson, K. E. *J. Microelectromech. Syst.* **1998**, *7*, 345−355.
(15) Nagai, H.; Murakami, Y.; Yokoyama, K.; Tamiya, E. *Biosens. Bioelectron.* **2001**, *16*, 1015−1019.
(16) Hong, J. W.; Fujii, T.; Seki, M.; Yamamoto, T.; Endo, I. *Electrophoresis* **2001**, *22*, 328−333.
(17) Kopp, M. U.; deMello, A. J.; Manz, A. *Science* **1998**, *280*, 1046−1048.
(18) Wittwer, C. T.; Garling, D. J. *Biotechniques* **1991**, *10*, 76−83.
(19) Innis, M. A.; Gelfand, D. H.; Sninsky, J. J. *PCR Applications*; Academic Press: San Diego, CA, 1999; pp 211−229.

10.1021/ac0346959 CCC: $25.00
Published on Web 09/26/2003

on a rotary microchip in the sequence of melting, annealing, and extension.[20]

Recently, Curcio and Roeraade have also used the circular arrangement in developing a continuous segmented-flow PCR device, where different PCR mixtures are loaded in the form of small segments one after another in a 15-m-long Teflon tube, coiled so as to be repeatedly exposed to the three temperature zones provided by thermostated water baths.[21] Water is an excellent medium for thermostating because of its high heat capacity. But it requires agitation for the homogeneity in temperature, and in an open bath, it suffers fast evaporation at high temperatures and the bath is restricted to a vertical configuration at a stationary place. Therefore, it would not be quite feasible to realize a compact, portable PCR system based on this liquid bath thermocycler.

We have developed, in this work, a compact, continuous-flow PCR device fabricated only from solid components and demonstrated the possibility of high-throughput PCR amplification in a segmented-flow mode. The device performs thermal-cycling PCR by having a PCR mixture flow through a long fused-silica capillary wound helically around a cylindrical thermal cycler with a fixed pitch per turn of the helix. Three thermally insulated metal heating blocks are arranged to form the cylindrical structure and run thermal cycles repetitively, in the sequence of melting, annealing, and extension. A similar method had been conceptually proposed previously by Reed,[22] but here we have realized a compact system proper for practical uses. This system is free of previously mentioned drawbacks of other continuous-flow PCR methods[17,21] and thus has greatly enhanced the practicability of continuous-flow PCR.

EXPERIMENTAL SECTION

Materials. Chemicals for PCR mixtures, including buffer, $MgCl_2$ solution, and Taq polymerase, were purchased from Promega (Madison, WI). All PCR mixtures have the same composition: 50 μL of PCR mixture contains 3 μL of 25 mM $MgCl_2$, 5 μL of 10× thermophilic DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100), 1 μL of 10 mM PCR nucleotide mix (10 mM each of dATP, dCTP, dGTP, and dTTP in water), 3.3 μL of 15 μM upstream primer, 3.3 μL of 15 μM downstream primer, 0.25 μL of 5 unit/μL Taq DNA polymerase, 1 μL (1 ng) of template DNA, and 33.15 μL of sterilized water. Two DNA templates and their primers were obtained from Promega (template, plasmid DNA from bacterial kanamycin resistance gene for amplifying a 323-bp fragment; upstream, 5′-GCC ATT CTC ACC GGA TTC AGT CGTC-3′; downstream, 5′-AGC CGC CGT CCC GTC AAG TCAG-3′) and Takara (template, λDNA for amplifying a 500-bp fragment; upstream, 5′-GAT GAG TTC GTG TCC GTA CAA CT-3′; downstream, 5′-GGT TAT CGA AAT CAG CCA CAG CGCC-3′). Two other DNA templates and their primers were supplied by a laboratory in the Department of Life Sciences, Pohang University of Science and Technology (template 1, PCS2HA/LM04 for amplifying a 497-bp fragment; upstream, 5′-GCC CTC GAG ATG GTG AAT CCG GGC AGC-3′; downstream, 5′-GCC CTC GAG TCA GCA GAC CTT CTG GTC-3′; template 2, Lhx3-LIM1 for amplifying a 267-bp fragment; upstream, 5′-G GAA TTC ATG CTG TTA GAA-3′; downstream, 5′-CGC GGA TCC CCG AAG CGC TTA AAG AAG TC-3′). Chlorotrimethylsilane (TMS), dimethylformamide (DMF), and imidazole were purchased from Sigma-Aldrich (St. Louis, MO).

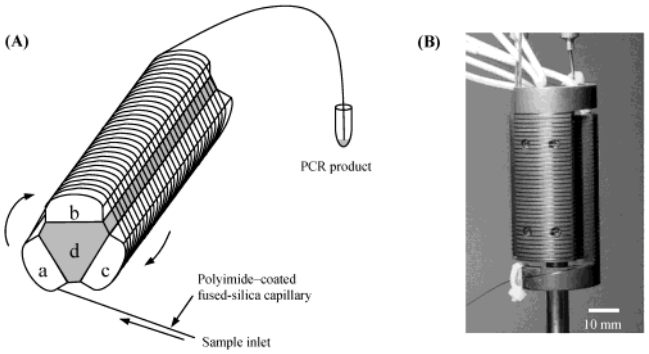

Figure 1. (A) Schematic and (B) photographic image of the cylindrical continuous-flow PCR device: (a) the copper heating block for melting (95 °C), (b) the annealing block (60 °C), (c) the extension block (72 °C), and (d) the thermally insulating plastic core.

Construction of Continuous-Flow PCR Device. Our continuous-flow PCR device (Figure 1A) consists of a cylindrical thermal-cycling assembly and a polyimide-coated fused-silica capillary wound up around the assembly. The thermal-cycling assembly has a plastic insulating core with an equilateral triangle-like cross section. Three copper blocks for melting, annealing, and extension are mounted on each side of the plastic core, respectively, forming the cylindrical appearance of the assembly (30 mm in diameter and 65 mm in height). The beginning of the melting block and the end of the extension block are elongated to help complete the initial melting and final extension steps, respectively (Figure 1B). Two adjacent copper blocks have an air gap of ∼5 mm between to secure thermal insulation. A helical groove of 250-μm width and 250-μm depth is formed on the surface of the cylinder, and a 3.5-m-long capillary (100-μm i.d. and 240-μm o.d.: Polymicro Technologies, Phoenix, AZ) is fitted into the groove to form a 33-turn helix with 1.5-mm pitch/turn of the helix. A pair of a heater cartridge (3.1 mm in diameter and 32 mm in length; Firerod, Watlow, St. Louis, MO) and a temperature sensor (1 mm in diameter and 27 mm in length; Watlow) are mounted into each copper block, as shown in Figure 1B, so that the temperature of each block can be controlled independently. The temperature distribution on the surface of the cycler is measured with a temperature sticker (Temperature Label D-50, Nichiyu Giken Kogyo, Japan).

Silanization of Capillary Inner Surface. To discourage the adsorption of biomolecues on the inner surface of a fused-silica capillary, the surface is silanized using the process previously developed.[23] The capillary is flushed with methanol for 30 min, dried at 40 °C for 12 h, and kept filled with a DMF solution containing 0.02 M TMS and 0.04 M imidazole at room temperature for 1 day. When the silanization reaction is completed, the capillary is rinsed with methanol and then with sterilized water.

PCR at Various Flow Rates. PCR of the 323-bp fragment is performed at different flow rates of the corresponding PCR mixture through the thermal-cycling capillary. The melting, annealing, and extension blocks are thermostated at 95, 60, and

(20) Liu, J.; Enzelberger, M.; Quake, S. *Electrophoresis* **2002**, *23*, 1531−1536.
(21) Curcio, M.; Roeraade, J. *Anal. Chem.* **2003**, *75*, 1−7.
(22) Reed, K. C. *Australas. Biotechnol.* **1996**, *6*, 280−281.
(23) Chen, Y.; Gerhardt, G.; Cassidy, R. *Anal. Chem.* **2000**, *72*, 610−615.

72 °C, respectively. The flow rate controlled by a syringe pump (22 Multiple Syringe Pump, Harvard Apparatus, Holliston, MA) ranges from 0.3 to 5.0 μL/min. At each flow rate, the PCR mixture is introduced continuously into the lower end of the capillary (at the beginning of the melting block), the PCR product is collected from the upper end (at the ending of the extension block), and 10 μL of the product is analyzed by 2% agarose gel electrophoresis in TBA buffer. PCR for a positive control is also performed in a commercial machine (MBS 0.2G, Hybaid, U.K.) using the same PCR mixture. The temperature program for this PCR is initiated at 95 °C for 2 min, and the subsequent temperature steps are 95 °C for 0.5 min, 60 °C for 1 min, and 72 °C for 2 min. These steps are repeated 33 times and then the temperature is held at 72 °C for 5 min to complete chain extension. The program is concluded by cooling the PCR product to 4 °C.

Carryover Checking. The PCR mixture for 323-bp amplification and a mixture for a negative control (containing all the reagents in the PCR mixture but the DNA template) are sequentially injected by 2 μL at regular intervals, and sample segments flow through the thermal-cycling capillary at the flow rate of 0.3 μL/min. Between each mixture injected, we interpose bromophenol blue buffer (30% glycerol, 30 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol; Takara) and small air gaps (<1 cm) on both sides of the buffer segment. This sequence of injections is repeated so that 10 μL of each product can be collected separately for analysis by agarose gel electrophoresis. The whole procedure is repeated for different volumes (0, 0.6, 0.9, and 2 μL) of the intervening blue buffer. A tiny air bubble responsible for the small air gap is inevitably introduced into the capillary during every manual change in injection from a sample to the blue buffer or vice versa.

Segmented-Flow PCR of Different DNA Samples. PCR mixtures containing four different DNA templates for amplifying 500-, 323-, 497-, and 267-bp fragments are sequentially injected 2 μL each at regular intervals, and sample segments flow through the thermal-cycling capillary at the flow rate of 0.3 μL/min. Between each sample injected, we interpose an air gap, 2 μL of bromophenol blue buffer, and then an air gap. This sequence of injections is repeated so that 10 μL of each product can be collected separately for the analysis by agarose gel electrophoresis.

RESULTS AND DISCUSSION

The thermal cycling for a continuous-flow PCR is realized in this study by winding up a long fused-silica capillary helically around a cylindrical thermal cycler with a fixed pitch per turn of the helix. Since three separate heating regions (for melting, annealing, and extension) are placed around the surface of the cylinder, a single amplification cycle in PCR is completed whenever a DNA mixture solution flows one turn through the helical capillary coil. Three identical copper heating blocks are arranged symmetrically around the thermally insulating plastic core structure (Figure 1A). Deviation in temperature over the surface of a heating block has not been observed, indicating that employing both a highly heat-conducting material, copper in this case, and a long heater cartridge and efficient thermal insulation provided by the plastic core and the 5-mm air gaps between blocks help to establish a homogeneous temperature over a block. Secure fixing of the capillary on the cylindrical thermal-cycling assembly and also efficient heat transfer from the heating blocks to the capillary are provided by fitting the 240-μm-o.d. capillary into the U-shaped groove of 250-μm width and 250-μm depth. The present dimensions of our device are very small compared with commercially available ones, but we have much room to reduce them. Since a 100-μm-i.d. capillary is used in this work and the passing length of the capillary on each block is 22 mm, the dwell time of a PCR mixture on each temperature region is 34 s at a flow rate of 0.3 μL/min. But it has been reported that short annealing and melting times (<1 s for both) are strongly recommended for high-yield specific amplification.[19] Conservatively, we could reduce the width of the annealing and melting blocks to ~3 mm, which results in a diameter half of the present one. The height can be also easily decreased to half because there is much room in reducing the pitch.

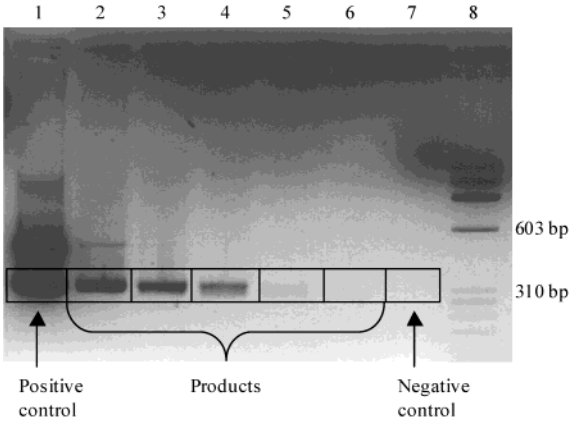

Figure 2. Agarose gel electrophoresis of PCR products of the 323-bp fragment. Lane 1: positive-control PCR product obtained from a conventional PCR machine. Lanes 2−6: continuous-flow PCR products at various flow rates, 0.3, 0.5, 1.0, 3.0, and 5.0 μL/min, respectively. Lane 7: negative control, unamplified PCR mixture. Lane 8: DNA size markers (φX 174-*Hae*III digest). Boxes represent areas over which the amounts of the PCR products have been determined using an image integration software (ImageGauge, Version 3.12, Fuji Film).

The flow-through PCR system is constructed to have 33 identical cycles. The time for each cycle is evenly divided for melting, annealing, and extension and determined by the inner diameter of the capillary tubing and the flow rate of a PCR mixture through the capillary. It is expected that a greater amount of the PCR product will be obtained with a decrease in the flow rate because a longer time for extension, especially, will make polymerization more complete. Figure 2 shows the results from gel electrophoresis of PCR products (323-bp fragment) obtained at various flow rates ranging from 5.0 to 0.3 μL/min. The corresponding time for flowing through the 3.5-m-long capillary ranges from 5.5 to 92 min. The results clearly show that the amount of the PCR product decreases as the flow rate increases. At a flow rate of 0.3 μL/min (Figure 2, lane 2), the amount of PCR product is only ~2-fold less than that from the positive control performed in a commercial thermal cycler (Figure 2, lane 1), indicating a continuous-flow PCR mixture is amplified well in our PCR device.

Our PCR method using a continuous flow of a PCR mixture can be modified to perform sequential DNA amplifications using a continuous segmented flow of different PCR mixtures. For the success of this PCR mode, the carryover from one segment to

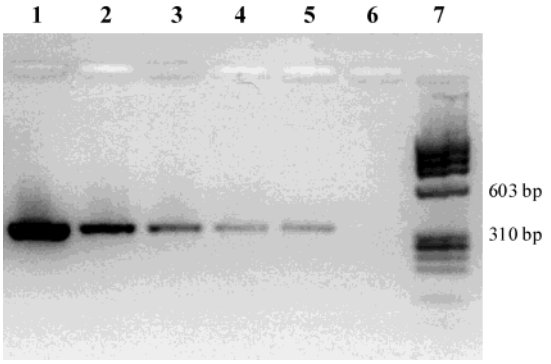

Figure 3. Agarose gel electrophoresis of separately collected PCR products for carryover checking. Lane 1: positive-control PCR product obtained from a conventional PCR machine. Lane 2: amplified product of 323-bp fragment. Lanes 3−6: negative-control PCR products obtained from PCR mixtures without DNA template at different volumes of the bromophenol blue buffer (0, 0.6, 0.9, and 2 μL, respectively) intervening between each sample segment. Lane 7: DNA size markers (ϕX 174-*Hae*III digest).

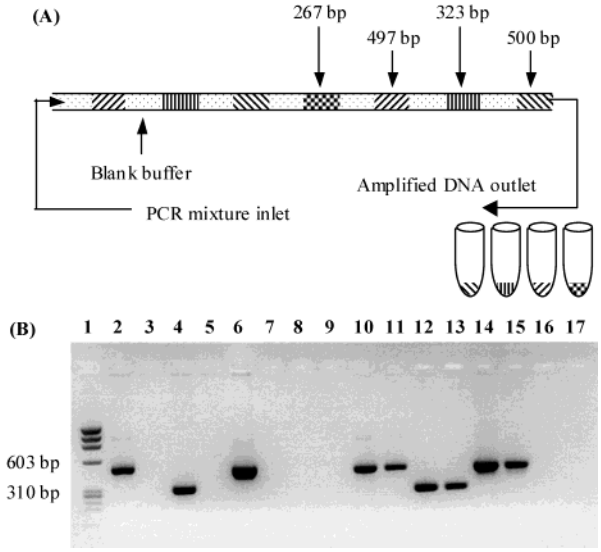

Figure 4. (A) Segmented-flow mode for PCR amplification of four different DNA fragments: 500, 323, 497, and 267 bp. The small air gaps are not shown here. (B) Agarose gel electrophoresis of separately collected PCR products. Lane 1: DNA size markers (ϕX 174-*Hae*III digest). Lanes 2 (10), 4 (12), 6 (14), and 8 (16): positive-control PCR products, obtained from a conventional PCR machine, of 500-, 323-, 497-, and 267-bp DNA fragments, respectively. Lanes 3, 5, 7, and 9: continuous-flow PCR results of 500-, 323-, 497-, and 267-bp DNA fragments, respectively, using a bare fused-silica capillary. Lanes 11, 13, 15, and 17: continuous-flow PCR results of 500-, 323-, 497-, and 267-bp DNA fragments, respectively, using a fused-silica capillary whose inner surface is silanized with TMS. Lanes 8, 16, and 17: no bands from PCR of 267-bp fragment (as in a commercial PCR instrument, the fragment could not be amplified in our system.).

the following one should be discouraged. In this work, to cleanse the capillary surface contaminated by the preceding segment, we interpose bromophenol blue buffer between each sample segment. Since the buffer is widely used in loading DNA samples on electrophoresis gels, DNA molecules would dissolve into it very well. Moreover, it is blue, and therefore, each sample segment is easily located. There is a small air gap (<1 cm) between each segment (sample/air or air/sample), which avoids direct mixing between them. To determine the volume for sufficient cleansing, a PCR mixture for 323-bp amplification and a mixture without the DNA template, for a negative control are sequentially injected, with varying volumes of the intervening buffer. We have found that carryover is very significant in the absence of the cleansing buffer (Figure 3, lane 3), that the increase of the buffer volume reduces carryover (Figure 3, lanes 3−6), and that carryover is almost completely discouraged with 2 μL of the cleansing buffer (Figure 3, lane 6). Note that the silanization of the capillary inner surface, which minimizes the adsorption of biomolecules on the surface, also contributes to reduce carryover and that instead of using air to make gaps between segments, the use of an immiscible, uncompressible liquid like perfluorodecalin[21] will be highly recommended.

In our continuous segmented-flow PCR, four different PCR mixtures for amplifying different DNA fragments (500, 323, 497, and 267 bp) are injected into the thermal-cycling capillary in series (Figure 4A). Figure 4B demonstrates that different DNA fragments can be amplified sequentially in our continuous-flow PCR, almost as well as in standard thermal-cycling methods. PCR of the fourth mixture for amplifying the 267-bp fragment failed in our system as in a commercial one. It is noted that the silanization of the inner surface of the capillary is critical to the success of PCR. Since the volume of each sample or intervening buffer segment is 2 μL, an amplified DNA fragment is obtained every 13 min at a flow rate of 0.3 μL/min. Recently, Hahn and co-workers developed a pumping technique for microfluidic devices and demonstrated 50-pL segmentation.[24] They also developed an easy interfacing technique between a fused-silica capillary and a microchannel in a microfluidic device.[25] When using these techniques, we might introduce segmented PCR mixtures of 50 pL each and obtain each amplified DNA fragment every 20 ms. Considering this rather conservatively, a continuous PCR with a high throughput of one PCR product per second would be quite feasible. In this situation with extremely small segments, a more efficient way to discourage carryover might be required.

CONCLUSIONS

The cylindrical PCR device developed in this work consists of only solid-state components. When a fluid is used as a thermostating medium for thermal cycling,[5,6,21] however, mechanisms for blowing gases or agitating liquid in baths are needed, which also demand space. In contrast, our solid-based device is very compact and free in its spatial configuration because even PCR mixture solution is confined in a closed container, the coiled capillary for continuous-flow PCR. These features are very advantageous for developing commercial products, especially ones mounted on mobile vehicles, and portable PCR systems. The flow-through capillary can be easily interfaced with sample-processing and product-analysis microfluidic devices. When combined with a picoliter segmentation technology,[24] our device might achieve an unprecedented high throughput in PCR (more than 1 sample/s).

(24) Lim, K.; Kim, S.; Hahn, J. H. *Sens. Actuators, B* **2003**, *92*, 208−214.

(25) Kim, H.; Ro, K. W.; Lim, K.; Park, N.; Kim, M.; Hahn, J. H. *Micro Total Analysis Systems 2002: Proceedings of the μTAS 2002 Symposium*; Baba, Y., Shoji, S., van den Berg, A., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2002; pp 401−403.

Moreover, since the single cylindrical device is so compact, a multistation device can be easily built in small dimensions. Such a parallel-processing thermal cycler will provide independent temperature settings for different stations and, thus, can accomplish optimization-free PCR using a single unit. This multistation device also can be used to rapidly determine the optimum PCR condition of a sample, using a minimal amount of the sample, before a large-scale amplification.

In addition, real-time PCR would be easily realized in our continuous-flow technique. Since there is an air gap between the extension and melting blocks, the parallel, bridging parts of the capillary can be severed as windows of laser-induced fluorescence (LIF) detection for quantitating a DNA fragment during the course of amplification. In this case, it would be favorable to use a transparent capillary coated with PTFE,[26] instead of making parallel windows on a coiled polyimide-coated capillary, where high-temperature burning of the polyimide coating is often involved, the transparent, bare-capillary parts thus formed are extremely fragile, and the high-temperature treatment possibly damages the antiadsorption coating on the inner surface of the capillary. Real-time PCR could be also performed in the segmented-flow mode, where a periodic scanning of the LIF detector along the length of the cylinder will be required. When these features and capabilities of our cylindrical compact thermal cycler, mentioned in this section, are fully developed, this type of continuous-flow PCR will revolutionize the advancement in bioscience and biotechnology.

(26) Kim, S.; Yoo, H. J.; Hahn, J. H. *Anal. Chem*. **1996**, *68*, 936−939.

ACKNOWLEDGMENT

This work has been financially supported by the National Research Laboratory Program (2000-N-NL-01-C-132) of the Ministry of Science and Technology, South Korea. Dr. E. Park has kindly supported the authors through supplying DNA samples and running electrophoresis of PCR products.

Received for review June 26, 2003. Accepted August 27, 2003.

AC0346959