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Polymerase Chain Reaction in Polymeric Microchips: DNA Amplification in Less Than 240 Seconds

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There is much interest in developing methods amenable to amplifying nucleic acids by the polymerase chain reaction (PCR) in small volumes in microfabricated devices. The use of infrared-mediated temperature control to accurately thermocycle microliter volumes in microchips fabricated from polyimide is demonstrated. Amplification of a 500-base-pair fragment of λ-phage DNA was achieved in a 1.7-μl chamber containing a thermocouple that allowed for accurate control of temperature. While previous work showed that *Taq* polymerase was inactivated when in direct contact with the thermocouple, this was circumvented with the polyimide chip by the addition of polyethylene glycol as a buffer additive. This, consequently, allowed for adequate amounts of PCR product to be observed after only 15 cycles, with a total time for amplification of 240 s.

Key Words: **polymerase chain reaction; thermocycling; PCR fragment; capillary electrophoresis; polyimide; polyethylene glycol; thermocouple; microchips.**

The use of electrophoretic separations on microfabricated devices has blossomed into a rapidly growing field geared toward the development of a stand-alone diagnostic platform. One of the overall goals is the exploitation of microchips for rapid detection of pathologies. In this laboratory (1–4) and others (5–7), the diagnostic capabilities of microchips have been demonstrated with the detection of B- and T-cell lymphomas (1), mutations in human DNA associated with breast cancer (2, 3), herpes simplex virus (4), Duchenne/Becker muscular dystrophy (5), C677T polymorphism (linked to neural tube defects), hyperhomocysteinemia and occlusive vascular disease (6), and assays to detect acetylcholinesterase inhibitors (7). Previous work has shown the rapid and reliable detection of these pathologies with microchip separations on the order of only a few hundred seconds. However, prior to microchip-based separation for diagnosis, samples had to undergo traditional DNA amplification via the polymerase chain reaction (PCR) (1–4, 6). Unfortunately, PCR and the pre-PCR processing (such as DNA extraction) are time-consuming steps, often requiring several hours. Moreover, the coupling of traditional sample preparation processes with microchip electrophoresis is wasteful. While 20–50 μl of PCR product is generated with conventional thermocycling, microchip analysis requires as little as 1 μl of sample with only a few picoliters actually consumed.

Fast DNA amplification coupled with fast separation and the ability to parallel process samples, as demonstrated by the 96-channel radial array electrophoretic microchips of Mathies and co-workers (6, 8), reduces the analysis time for multiple samples to less than a few seconds. The applicability of devices capable of high-throughput screening in both the clinical chemistry and genetic analysis laboratories would be enhanced dramatically if lengthy preelectrophoresis protocols could be reduced. Since only a small volume of sample is needed for the microchip separation, it is reasonable to reduce the volume of sample thermocycled for PCR amplification. A significant reduction in amplification time concurrent with reduced volumes should be observed based on previous reports on this topic (9–12). The sample transfer problems associated with small-volume PCR described previously in the

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literature (11) are not problematic if PCR is executed on the same device used for electrophoretic analysis.

A number of groups have demonstrated integrated PCR amplification with various microchip platforms (5, 13–23). Some of these have exploited conventional thermocycling instruments for heating and cooling (5, 13–17) while others have incorporated heating devices directly into the microchips (18–21, 23). Where traditional thermocyclers have been used, there was little, if any, improvement in the time required for PCR thermocycling (16, 17). The thermal mass of the metal block used in these thermocyclers is large compared to that of the reaction mixture, so heating and cooling rates (and thus the time for PCR amplification) are limited by the thermal properties and mass of the metal. Reports in the literature (9–11, 22) have described how increasing the rates for thermal transitioning of PCR mixtures results in more rapid temperature cycling without loss of PCR amplification efficiency. In these instances, faster heat transfer was achieved by increasing the surface area-to-volume ratio of the reaction chamber and lowering the volume of the reaction mixture, both of which allowed for faster thermocycling.

Normally, the heat source, the microchip itself, and the reaction mixture all contribute to the thermal mass of the PCR system, and heat must be transferred from the heat source through the microchip to the solution, limiting the rates for heating and cooling. The approach of Manz and co-workers (19) focused on eliminating this problem by utilizing localized resistance heaters to keep temperatures constant on various regions of a glass microchip. By flowing the PCR mixture through each of the temperature zones on the microchip in a repeated manner, amplification was achieved as judged by off-chip electrophoretic analysis. Since the PCR mixture flow path and number of cycles were determined by the microchip design, and therefore fixed, limited control of the reaction conditions was possible only through changes in chip geometry or variations in flow rate. Mathies and colleagues (20) have used localized resistance heaters in a similar fashion in heating nanoliter-volume microchambers integrated with separation channels. They achieved amplification with as few as five starting copies in the microchamber. However, as with other examples, the microchip remains the primary contributor to thermal mass. Ramsey and co-workers (23) have recently demonstrated an integrated system for PCR analysis, which utilizes localized Peltier thermoelectric elements for DNA amplification coupled to a microchip-based separation. They have presented an integrated device with a total analysis time of approximately 20 min per sample (23).

The work described above represents some of the most promising work in the development of a fully integrated diagnostic device. However, in each case the substrate used is glass. An alternative to glass in the manufacture of microfluidic devices is the use of polymers, such as polymethylmethacrylate (PMMA)[4] (24, 25), poly(dimethylsiloxane) (26), parylene (27), and Vivak (28). Each of these polymers has been used as a device substrate for DNA separations, with a PMMA chip used in a diagnostically significant fashion in the detection of hepatitis C PCR products (25). Some of the advantages afforded by the use of polymeric materials include improved compatibility for a broad range of bioanalyses, ease of stable chemical modification of many polymeric materials, and the ability for mass production by a variety of microfabrication techniques. However, many questions remain in this nascent stage of microchip research, and the understanding of the strengths and limitations of polymers as substrates is still very much in its infancy. Clearly, there are many challenges associated with matching the requirements for use in bioanalysis (i.e., biofouling resistance, chemical stability, mechanical stability, compatibility with detection needs, etc.) with both fabrication and assembly requirements.

The thermal mass problem in thermocycling has been eliminated by a novel method using a tungsten lamp to specifically heat the PCR solution (29, 30). Direct heating of the solution takes place by excitation of the vibrational bands of water by the IR radiation produced by the lamp. The thermal mass is reduced by eliminating heating of the reaction chamber and can be reduced further by decreasing the volume of the sample being thermocycled. Cooling rates are increased because cooling of the PCR mixture takes place independent from cooling of the heat source. This method has been shown to be effective for accurate control and ultrafast temperature cycling of small volumes (29, 30).

In this report, a novel polymeric microchip used in conjunction with IR-mediated thermocycling is described. The use of the small volumes expected to be needed for PCR amplification of DNA on integrated devices is demonstrated in these polyimide microchips. This material represents a possible substrate for use in microchips with a more complex architecture. Some of the properties that make it desirable as a material for integrated microchips for DNA analyses include high breakdown voltage (>4 kV/mil or 1500 kV/cm), optical transmission in the useful IR range in which the IR-mediated thermocycling is done, and a high glass transition temperature (350°C).

EXPERIMENTIAL METHODS

Noncontact thermocycler. The IR-mediated thermocycling system was constructed in-house and is

[4] Abbreviations used: PMMA, polymethylmethacrylate; PEG, polyethylene glycol.

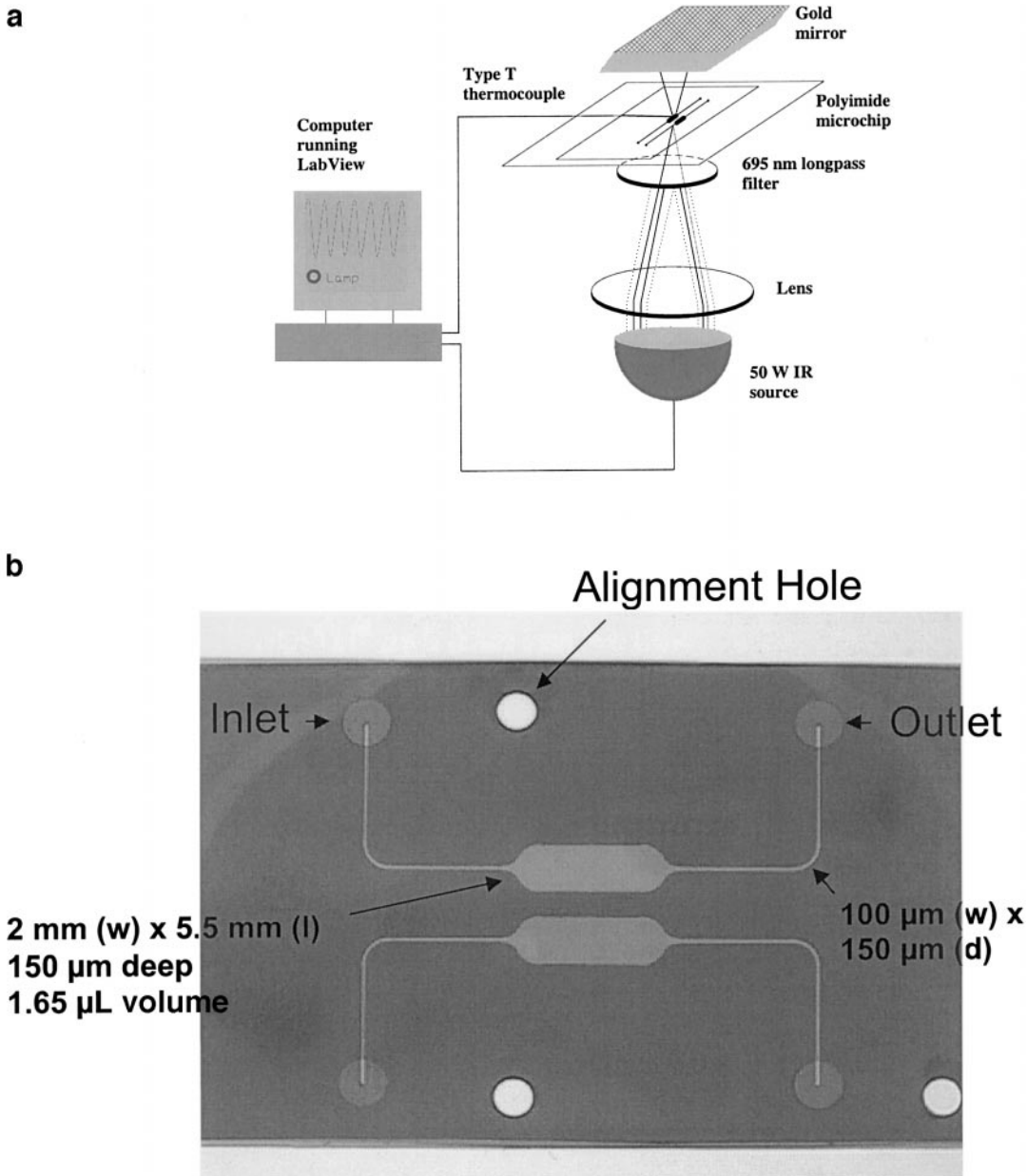

FIG. 1. (a) The setup for noncontact heating of the PCR chamber. (b) The polyimide microchip.

shown in Fig. 1a. The device utilized a PC outfitted with an analog-to-digital (A/D) converter board (ComputerBoards Inc., Mansfield, MA) and a tungsten lamp (CXR, 8 V, 50 W, General Electric, Cleveland, OH) as the IR source. The lamp was focused on the PCR chamber using a plano-convex lens (Edmond Scientific, Barrington, NJ) and filtered through an IR-transmitting black glass filter (Melles Griot). A gold-coated mirror placed above the microchip reflected IR light back onto the PCR chamber to enhance the rate of heating. Temperature cycling was controlled via a LabVIEW application, which monitored the temperature in the PCR chamber using a small-diameter (0.002") type-T thermocouple (Physitemp Instruments) and appropriately modulated the state of the lamp—on or off—using the A/D board.

Polyimide microchips. Research prototype polyimide microchips were supplied by Agilent Technologies. The PCR chambers were formed by laser ablation of a single layer (150 μm thick) of polyimide. This layer is sandwiched between two additional layers of polyimide (125 μm thick), one of which contains two laser-ablated ports, producing a 150-μm-deep chamber. Total thickness of the microchip is 400 μm with a chamber volume

of 1.7 μl. The microchip is pictured in Fig. 1b. The thermocouple is inserted into the center of the chamber through one of the access channels.

IR-mediated PCR using λ-phage DNA template. The ability to use IR-mediated thermocycling in a polyimide chip was tested using the control λ-phage DNA reaction supplied with the GeneAmp PCR reagent kit with Ampli*Taq* DNA polymerase (Perkin–Elmer); a specific DNA sequence of 500 bp in length is amplified using the supplied primers. The reaction mixture was prepared in a 50-μl stock solution according to the following mixture: 10 mM Tris–HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM of each dNTP, 0.2 μM of the appropriate primers, 0.1 ng of λ-phage DNA, 10 units of Ampli*Taq* DNA polymerase, and 0.75% (w/v) polyethylene glycol 8000 (EM Science). With the exception of an elevation in the initial amount of Ampli*Taq* DNA polymerase and the presence of polyethylene glycol, the reaction mixture is as recommended by the manufacturer. The stock solution was divided into two parts, one used in the microchip and another in a conventional thermocycler. The PCR chamber in the polyimide microchip was filled with approximately 1.7 μl of the stock solution (total starting copies of template DNA $\approx$ 69,000); several flushes of the mixture were used to remove any air bubbles that may have been trapped when filling the chamber. The chamber was then sealed using Teflon septa to cover the reservoirs. Thermocycling conditions were as follows: 10 s at 94°C for initial denaturation of the DNA, 15 cycles of 2 s at each of the following temperatures: 68°C (annealing), 72°C (extending), 94°C (denaturing), followed by 10 s at 72°C for a final extension. No evaporation of the PCR mixture was observed after amplification.

The remainder of the PCR mixture was utilized in a control experiment using a conventional thermocycler (GeneAmp 2400, Perkin–Elmer). A polypropylene insert containing 5 μl of the stock solution was placed in the conventional thermocycler and cycled using the same dwell times and temperatures and number of cycles as in the microchip experiments. The volume used in the conventional thermocycler is the minimum volume allowed by the controlling software.

PCR chambers were cleaned with several volumes of ethanol and thoroughly flushed with sterile water between runs. To ensure that cross-contamination was not an issue, several runs were performed after cleansing the chamber. PCR mixture without template DNA was thermocycled using the above profile and no product was observed.

Analysis of PCR product by capillary electrophoresis. PCR product from the microchip was removed with 4-vol washes of the chamber with nanowater. Amplified DNA was detected on a Beckman 2100 P/ACE station equipped with laser-induced fluorescence detection. Electrophoresis was carried out in a 50-μm by 27-cm FC-coated capillary using reverse polarity at 4 kV total field strength. Separation buffer was 89 mM Tris–borate at pH 8.5, with 3 mM EDTA, 1.5% (w/v) hydroxyethylcellulose (Aldrich), and 1 mM 1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethyl-ammonium propanone diodide (YO-PRO-1) (Molecular Probes). The capillary was thermostated at 20°C and samples were electrokinetically injected for 10 s at 10 kV. For samples thermocycled using the conventional thermocycler, aliquots were diluted 1 in 5 before electrophoresis.

RESULTS AND DISCUSSION

Polyimide as a material for microchip technology. The integration of PCR-based DNA amplification into polymeric microchips requires the careful selection of a suitable material that combines favorable surface and thermal properties, both of which must be conducive to fast and efficient thermocycling. Polyimide has two desirable properties that make it a good candidate for consideration as a material for a PCR compartment in integrated DNA analysis microchips. First, it has a glass transition temperature of 350°C and therefore holds its structural integrity over the temperature ranges used during thermal cycling. Second, it is transparent in the 600- to 3000-nm range, so that the radiation heats the solution alone and not the substrate. This property of polyimide preserves the major advantage of the IR-mediated thermocycling approach (29, 30) and makes this microchip unique from the selection of polymeric materials considered for the fabrication of PCR microreactors.

Thermocycling in a polyimide microchip. We have previously described that the major advantage of IR-mediated heating is the ability to selectively heat aqueous solutions before heating the vessel in which it is contained (29, 30). Water absorbs IR light in the wavelength range of 900–4000 nm, where transitions between vibrational states result in bulk heating of the solution. As indicated in prior work (30), effective IR-mediated heating can occur if the vessel containing the solution does not significantly absorb the transmitted light. Over 60% of the electromagnetic radiation is transmitted through the microchip substrate at wavelengths between 500 and 2700 nm. This results in a large percentage of near-IR light in the important wavelength range reaching the PCR mixture and, thus, should afford rapid heating of small volumes of aqueous mixtures. Heating rates of roughly 10°C/s were found with the lamp at full intensity, and cooling rates of 10°C/s were observed with no forced cooling in place. To test the ability to IR temperature cycle in a polyimide microchamber, PCR was carried out using the

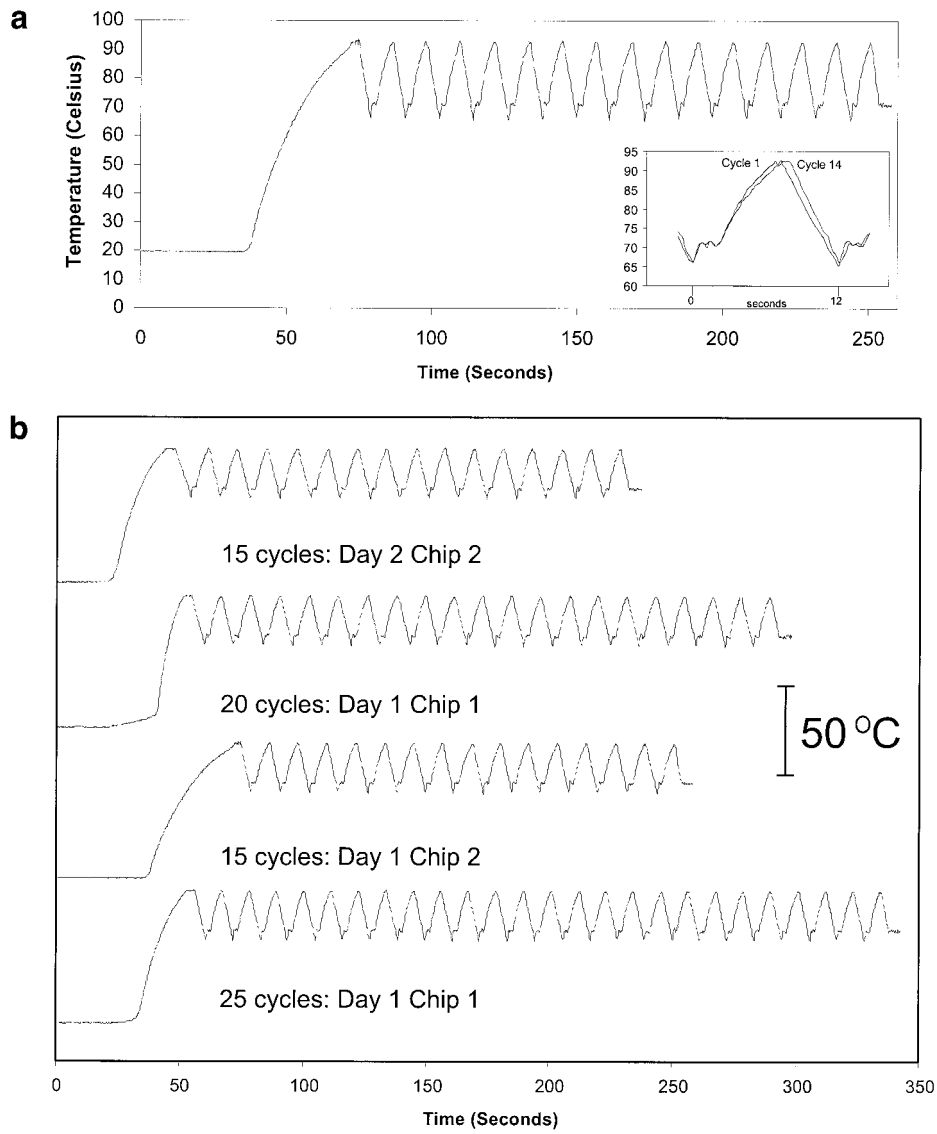

FIG. 2. Temperature cycling in the polyimide microchip. (a) From 0 to 40 s, the microchip is not positioned in the path of the IR source. The average cycle time is 12 s. (Inset) Comparison of the 1st cycle with the 14th cycle. (b) Comparison of thermocycling performance, including varieties of microchip utilized, day that thermocycling was performed, and number of cycles performed. Dwell times are maintained throughout as described in the text.

dwell times and temperatures described under Experimental Methods. Figure 2a presents the thermocycling profile for a microchip that underwent 15 cycles, with an initial dwell of 10 s at 94°C as a denaturation step and a final dwell at 72°C as a final extension step. The first 40 s of the profile represents the time during which the chamber is not accurately positioned at the focal point of the tungsten lamp. As the temperature increases from room temperature to 94°C for the initial 10-s denaturation, the microchamber is optimally positioned. With the 10°C/s rates for heating and cooling and the 2-s dwell times at each temperature, each cycle takes approximately 12 s. Taking into account the initial denaturation at 94°C (10 s) followed by 15 cycles between 68 and 94°C and a final 10-s extension, the total time for the PCR amplification was approximately 200 s. The reproducibility of the IR temperature cycling within a given thermocycling run is illustrated by the inset in Fig. 2a, which shows a comparison of cycles 1 and 14. It is clear from this figure that not only is the rate of temperature transitioning consistent throughout the cycling program, but also temperatures reached from cycle to cycle are reproducible. In contrast, the conventional thermocycler used in these experiments is capable of optimum rates of heating of 0.9°C/s and cooling of 3.9°C/s, resulting in an overall temperature cycle (denaturation, annealing, and extension as described under Experimental Meth-

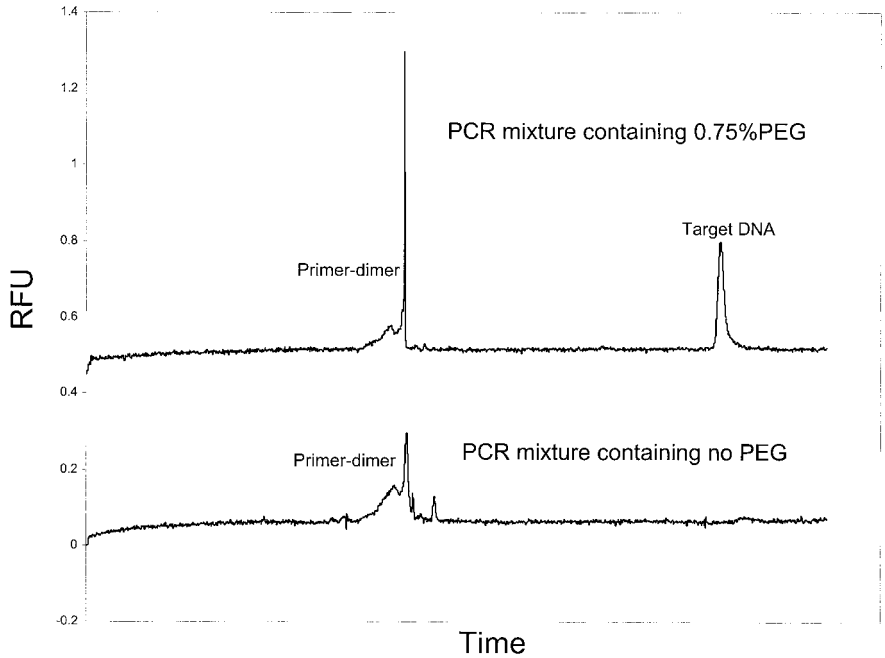

FIG. 3. Capillary electrophoretic analysis, as described under Experimental Methods, of PCR product formed in the presence and absence of 0.75% (w/v) polyethylene glycol 8000.

ods) taking approximately 40 s. In practice, the time necessary to complete 15 cycles was approximately 1300 s (85 s per cycle). The rapid rates of heating and cooling with the polyimide microchip illustrate that while the microchip makes up the majority of the thermal mass of the system, it contributes minimally to the thermal load. Simply put, the substrate does not absorb significant amounts of energy in the heating process, nor does it radiate dissipated heat back to the PCR solution during the cooling process. The reduced amount of heat input that is required for successful temperature cycling should be a major benefit in the design of integrated DNA analysis microchips. The capability of IR thermocycling to selectively heat the solution will place fewer restrictions on the distance between adjacent PCR compartments on the chip. Highly multiplexed microchips for DNA analysis that incorporates multiple PCR compartments should be easier to design and implement.

To determine the reproducibility of polyimide microchip thermocycling, cycle-to-cycle, intrarun, intraday, and intrachip performance was tested. This is illustrated in Fig. 2b, which shows thermocycling in two polyimide microchips on the same day with different numbers of cycles, as well as cycling carried out on different days. While the cycling profiles have slight differences with respect to temperature ramping speeds, they compare favorably; the average time for a single cycle remains at approximately 12 s regardless of the number of cycles or the microchip used. Minor variations can be attributed to slight variations in the alignment of the microchip in the path of the IR source. In all cases, successful PCR amplification, as described below, was achieved, illustrating the reproducibility of IR-mediated thermocycling in the polyimide microchips.

The ability to carry out simultaneous PCR amplification of multiple samples represents the first step toward multiplexed PCR on a microchip and an area where reproducibility is a concern. Two chambers in close proximity to one another (see Fig. 1b) were utilized with the assumption that they would experience the same exposure to IR heat. A 15-cycle amplification was carried out on one chip and the product collected from both chambers. Within experimental error, the amount of 500-bp fragment recovered from each chamber was equivalent (data not shown). The size of the focused IR beam used in these experiments prevented simultaneous amplification of more than two chambers.

Reaction mixture additives for optimized polyimide microdevice PCR. Previous work with small-volume PCR (30) had found that inclusion of a type-T thermocouple in the PCR chamber inhibited the amplification. This problem was circumvented with the use of a "reference" chamber filled with the same solution and positioned adjacent to the sample amplification chamber; monitoring and controlling the temperature in the reference chamber allowed for amplification of DNA in the sample chamber (30). In working with the polyimide devices, it was observed that PCR product was

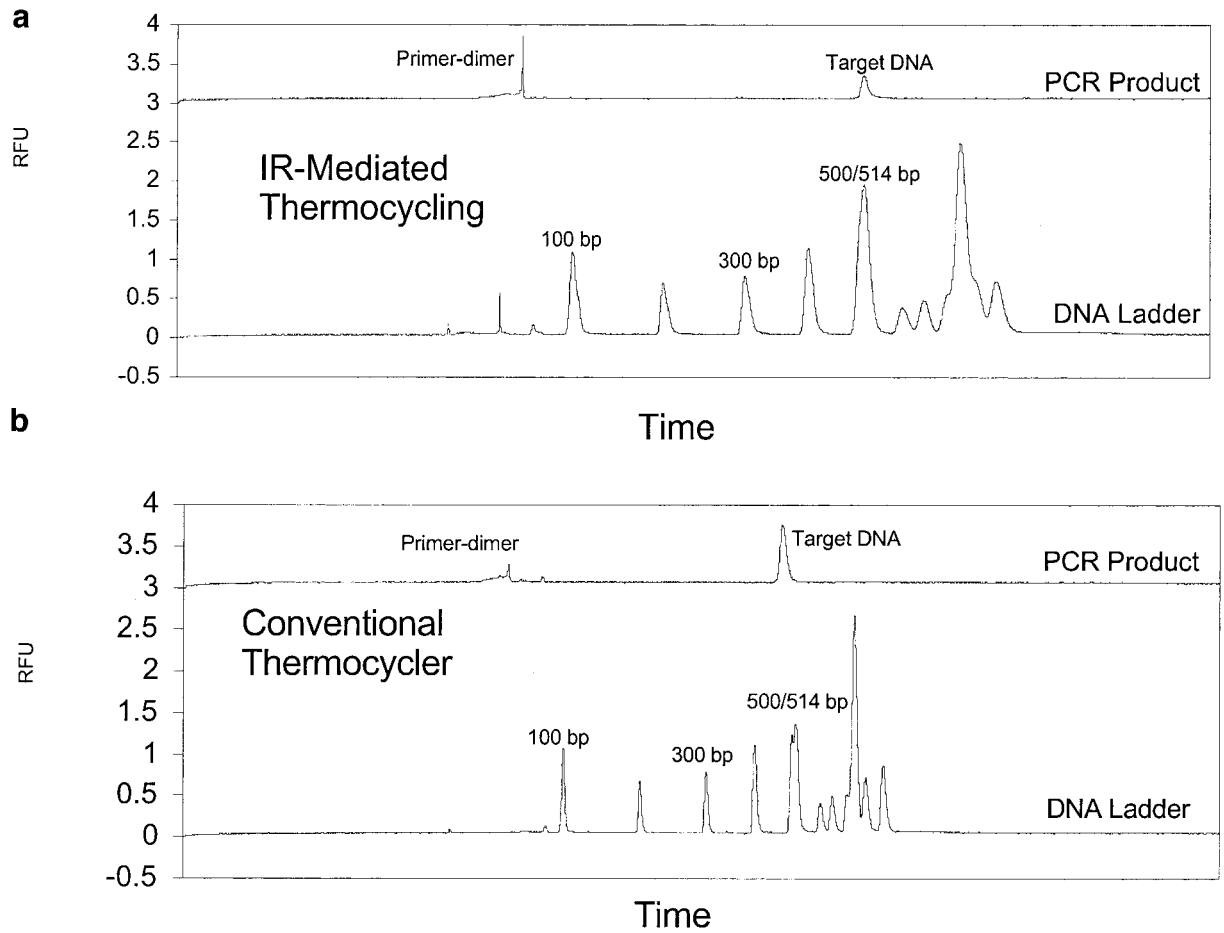

FIG. 4. Capillary electrophoretic analysis, as described under Experimental Methods, of PCR product from the IR-mediated thermocycling in a polyimide microchip and the conventional thermocycler in a polypropylene tube. (a) Electropherograms of PCR product (diluted 1 in 5) from approximately 69,000 starting copies achieved on chip using IR-mediated thermocycling and a 100-bp DNA ladder. (b) Electropherograms of PCR product (diluted 1 in 5) from approximately 160,000 starting copies using a conventional thermocycling and a 100-bp ladder.

detected when 0.75% polyethylene glycol (PEG) was included in the reaction mixture (temperature mediated through the dummy chamber), while no product formed in control experiments without PEG. In addition, the inhibition of DNA amplification previously observed when the thermocouple was in direct contact with the PCR mixture (29, 30) was not observed in the presence of 0.75% PEG (Fig. 3). This indicates that accurate control of the temperature of the PCR mixture undergoing enzymatic amplification (not the reference chamber) can be accomplished. This not only enhances the possibility of executing the thermocycling and amplification efficiently, but also simplifies the PCR microchip design. As was the case with the chamber without the thermocouple, cross-contamination between amplification was not observed after extensive cleaning.

However, temperature control in this approach is still challenging. It remains to be shown that the incorporation of a temperature control element, e.g., thermocouple, is less problematic in polyimide devices than in glass devices where established thin-film deposition techniques could be applied to produce an integrated thermocouple.

Amplification of λ-phage DNA. The thermocycling profile shown in Fig. 2a represents a cycle time of 12 s and a total amplification time of 200 s for 15 cycles. The corresponding results from capillary electrophoretic analysis of the amplified products, obtained by IR-mediated thermocycling in a polyimide microchip and conventional thermocycling in a polypropylene tube, are given in Fig. 4. For DNA fragment analysis, a 100-base-pair DNA ladder was separated prior to and following analysis of the neat PCR products and is included in the figure. The PCR product, expected to be 500 bp in size, obtained in both the polyimide chip (Fig. 4a) and the polypropylene tubes (Fig. 4b) has a migration time consistent with the 500-/514-bp unresolved peaks in this ladder. It is important to note that, in

both cases, the neat PCR product has been diluted prior to separation by capillary electrophoresis. Four chamber volumes (4×2 μl) were used to flush the PCR mixture out of the chamber in the polyimide microchip. This was necessary for accurate electrokinetic injection and for removal of the PCR solution from the microchip. To make a direct comparison of the IR-mediated and conventional thermocycling products, the conventional product was diluted 1 in 5, as well.

Previous work in this laboratory had shown that for nanoliter volumes, utilizing IR-mediated thermocycling results in more specific and more rapid amplification, but more importantly, more PCR product (30). In the 1.7-μl volume chambers on the microchips, efficiency comparable to that found using the same cycling profiles in the conventional thermocycler was observed. The high level of efficiency observed in previous work (30) is due to the much smaller volume utilized (160 nl), coupled with the higher rates of temperature change in the thermocycling profile. The rate of heating was approximately 60°C/s with cooling at a rate of 20°C/s (forced-air cooling). These rapid temperature transitions (due to the small thermal load of the PCR mixture) limit the nonspecific binding of primers to the amplicon. In this regard, the results presented in this paper are not surprising. By utilizing microchips with volumes on the order of a few hundred nanoliters, efficiency and temperature transition rates will increase.

CONCLUSIONS

The accurate, rapid, and reproducible thermocycling of 1.7-μl volumes of PCR solution in a polyimide microchip has been demonstrated using IR-mediated thermocycling. Heating and cooling rates of 10°C/s were achieved in this system using a tungsten lamp and natural convection cooling. These rates are approximately 10 times and 3 times faster, respectively, than can be achieved using a typical conventional thermocycler. Cycle times as short as 12 s were found using dwell times of 2 s each at the denaturing, annealing, and extension temperatures. Detectable amounts of PCR product were produced in 15 cycles, giving a total reaction time of just over 200 s. Problems with the inhibition of DNA amplification in the polyimide chamber and by the thermocouple were mediated by inclusion of 0.75% (w/v) polyethylene glycol into the PCR mixture. The efficient amplification of DNA demonstrates that the desirable thermal and surface material properties of polyimide, in combination with remote heating using an IR source, form a unique approach for the application of PCR in polymeric microchips.

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