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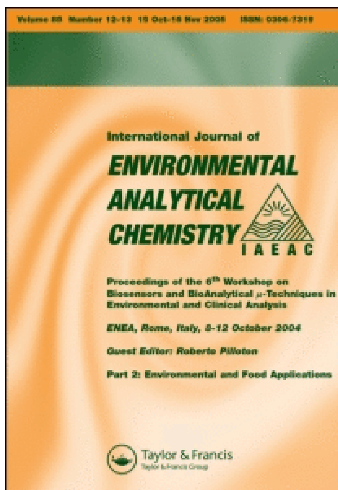

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DEVELOPMENT OF PCR CONDITIONS IN A SILICON MICROREACTOR DNA-AMPLIFICATION DEVICE

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A silicon microsystem was developed which functions as a miniaturised DNA-amplification device. The system represents a technology platform for performing a polymerase chain reaction (PCR) with reduced volumes of 7 μL. The silicon microreactor was fabricated using silicon bulk micromachining, and a platinum heater was fabricated on a Pyrex substrate. A miniaturised DNA-amplification system permitted rapid heating and cooling, and shorter reaction times of 30 min were achieved. In this work, biocompatibility issues are addressed; conditions for efficient PCR in a silicon-based microreactor are established for the amplification of 500 bp DNA from the *Escherichia coli* bacteriophage Lambda; and the conditions are verified by amplifying a 255 bp region from the *Mycobacterium tuberculosis rpoB* gene. This work describes the PCR volume *scale down* experiments that were conducted and concentrations of the reactants; Taq polymerase, oligonucleotides, $MgCl_2$ and template DNA were determined for DNA-amplification reactions with this novel device.

Keywords: DNA amplification; Miniaturisation; Silicon micromachining

INTRODUCTION

Since its development, the polymerase chain reaction (PCR) has become the most commonly used molecular biology tool [1]. The ability to achieve 10^{12}-fold amplification of individual molecules of nucleic acid strands in 2–3 h has facilitated enormous progress in many areas of biology, including genome sequencing, genetic engineering, forensic science, gene cloning, disease diagnostics and phylogenetic classification. This powerful enzymatic reaction is mediated by a thermostable Taq polymerase enzyme and is performed by repetitive cycling of reaction components at three different temperatures, for 25–30 cycles. At 95°C, the template DNA is denatured; at a temperature between 45°C and 65°C, the sequence-specific oligonucleotide primers hybridise to the single-stranded DNA template; and at 72°C, strand elongation occurs. The sequence specificity of

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DNA amplification and the generic nature of the reaction have facilitated the detection and identification of micro-organisms using this method.

The evolution of PCR technology began when heated water baths were used to achieve the necessary temperature transfer for PCR. Later applied robot arms moved the reaction vessels from one bath to the next. Today, the standard PCR process is performed in dedicated 'thermocycler machines'. These control the temperature in the reaction tube with the use of peltier elements, which are mounted on a metal block. Cycling is achieved by cooling and heating the massive metal block with a maximum speed of about 2–3°C/s. Conventional thermal cycling machines accommodate a number of parallel reactions with typical volumes of 25–100 μL. The sizeable thermal mass of these instruments means that long ramping times are required to archive the necessary temperatures. As a consequence, the time taken to perform a single three-temperature PCR cycle, is lengthened.

The development of low-cost, miniaturised PCR methods for clinical diagnostics and environmental analysis has been the focus of many groups. The application of MicroElectroMechanical Systems (MEMS) technology promises the creation of portable, inexpensive PCR devices where size, reaction volumes and power demands can be substantially reduced. This is due mainly to silicon processing being a mature manufacturing technology that lends itself to the production of high-volume, low-cost components, suitable for use as portable point-of-care diagnostic instruments. Much faster cycling times are afforded where smaller instruments and reduced reaction volumes are employed. The first reports of miniaturised PCR appeared in 1993 [2] with simple micromachined chemical reactors with integrated heaters for PCR. Research has continued for single microcavity-type devices [3,4], and flow-through devices are also being developed [5,6]. Different approaches, such as closed micro-chambers and flow-through thermocycling devices that allow reductions in PCR reaction rates from hours to minutes [7–10] have been reported by a number of groups.

The advent of more sophisticated real-time PCR technology has dramatically reduced reaction times to less than 30 min. A number of reports demonstrate real-time PCR as a portable system based on this technology for the detection of infectious diseases [11–14]. An instrument weighing a mere 3.3 kg was fashioned for the amplification and detection of two types of *Bacillus* spores as well as characterisation of the single nucleotide polymorphism for the genetic hemochromatosis disorder [15]. An on-chip real-time PCR in a microarray format has been described; the reaction was performed in gel matrices and resulting amplicons were detected using a charge-coupled device (CCD) camera [16]. An integrated PCR and capillary electrophoresis chip was reported that permits on-chip concentration of the DNA sample prior to mobility sizing of the PCR products [17].

In this work, we are interested in demonstrating the suitability of using silicon as an appropriate material for the creation of microsystem devices that encompass DNA amplification. Conventional laboratory-based thermal cycler machines typically require 2–3 h for DNA amplification. The work presented here has focused on the characterisation of a DNA-amplification microreactor, fabricated using silicon micromachining. The biochemical parameters for the DNA-amplification reaction were established for this microreaction chamber. In this work, we show that it is possible to match the speed of real-time PCR technology with a low-cost, low-power, microsystem device.

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EXPERIMENTAL

PCR Chip Fabrication and Assembly

Microreactor Cavities

Microreactor cavities with a volume of ~7 µL were fabricated [18]. Briefly, silicon (100) wafers had 30 nm silicon dioxide and 100 nm silicon nitride deposited by low-pressure chemical vapour deposition. A wafer mask was designed to produce devices with a microreactor area with dimensions of 4.5 mm × 4.5 mm. In these areas, the silicon nitride and silicon dioxide were removed, using a plasma etch process, leaving an exposed silicon area for potassium hydroxide (KOH) etching. The silicon nitride acts as a hard mask during etching, protecting the wafer from the KOH. In the exposed areas, 29% KOH at 80°C was used to etch to a depth of 350 µm. The silicon nitride and silicon dioxide layers were removed, and a 100 nm layer of insulating thermal silicon oxide was grown on the silicon to improve the biocompatibility of the microreactor [19].

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Heating Element

A heating element was fabricated using a lift-off process to pattern the platinum onto the Pyrex wafers. Briefly, Pyrex wafers were cleaned, and a photoresist layer was patterned with the design of the platinum heater. The Pyrex was etched using the etching solution Silox Glass Etch 50 : 50 : 50 (Rockwood Electronic Materials, Alfreton, UK), containing a mixture of ammonium fluoride and acetic acid in water (13% NH_4F and 35% CH_3COOH) to a depth of 100 nm. The heater metals (5 nm titanium and 60 nm platinum) were then sputtered onto the wafer. Subsequently, the Pyrex wafers were placed in a photoresist etchant solution, which removed the resist and metal from the unpatterned areas, leaving the patterned heater on Pyrex. Finally, the heater wafers were cut to yield 8 mm × 8 mm heater chips [18,20], with a platinum heating element of 4 mm × 4 mm centred on the chip.

Microcavity Lid

A Pyrex lid, which matched the outer dimension of the silicon microreactor cavity, 6650 µm × 5700 µm, was fabricated. For the silicon microreactor cavities without inlet and outlet holes, an additional hole was drilled through the lid with diamond bits of 0.9 mm or 1 mm diameter.

Packaging the PCR Chip

The totally packaged system will be referred to as the PCR chip in the following text. The Pyrex lid was attached to the top of the silicon microreactor cavity's outer margin (dimension of 0.6 mm) by using UV glue (Loctite 302) cured in a UV1250 Oven (Loctite, Welwyn Garden City, UK). The platinum on the Pyrex heater was assembled using UV glue on a small printed circuit board (PCB) dipstick 4 cm × 1 cm (ILFA Feinstleitertechnik GmbH, Hannover, Germany) for electrical access to the heater. The PCB contains a hole (6.5 mm × 6.5 mm) directly under the heater to facilitate more rapid cooling and heating times. The platinum contacts for the Pyrex heater

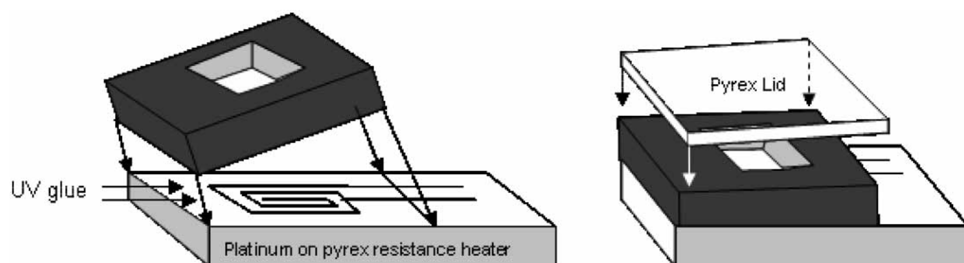

FIGURE 1 Assembly of the PCR chip.

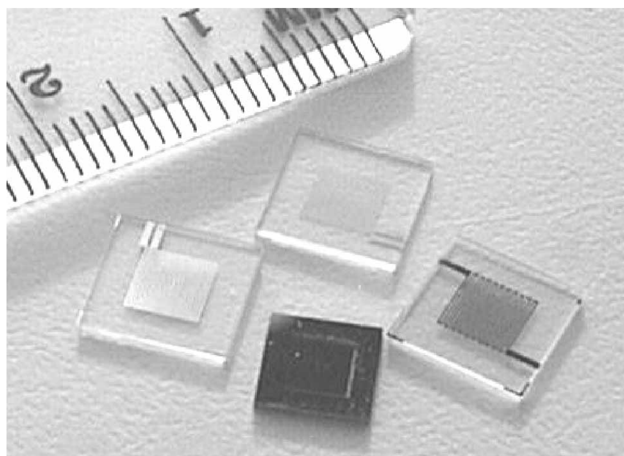

FIGURE 2 PCR microreactor and platinum Pyrex heater prior to packaging.

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were wire-bonded to the dipstick. The assembled microreactor cavity with lid was attached, using UV cure glue, to the platinum on Pyrex heater (Fig. 1). A Pt100 temperature sensor (Juchheim, Fulda, Germany) was packaged on top of the Pyrex lid, and a soldered contact connected the sensor to the dipstick. After sample delivery, the inlet/outlet hole was sealed with a transparent adhesive PCR sealing film (Eppendorf, Hamburg, Germany).

The final assembled micro-fabricated silicon thermal cycler (PCR chip) generates a small thermal cycler with a total mass of approximately 1.1 g. The microcavity comprises the following modules: temperature sensor Pt100, Pyrex lid, silicon microreactor cavity, platinum on Pyrex heater and PCB. The individual cavities and heaters are shown in Fig. 2, and the assembled system is shown in Fig. 3.

PCR Chip Thermal Cycling Control

The temperature profiles were achieved by applying a current to the platinum resistor heater. A programmable source meter (Keithley 2400 Source Meter) and a digital multimeter (Agilent 34401 6½ Digital Multimeter) were employed for temperature measurements from a calibrated Pt100 temperature sensor. The digital multimeter was connected to a PCI interface with a PC; a dedicated LabVIEW program was written for real-time data monitoring from the Pt100 [21].

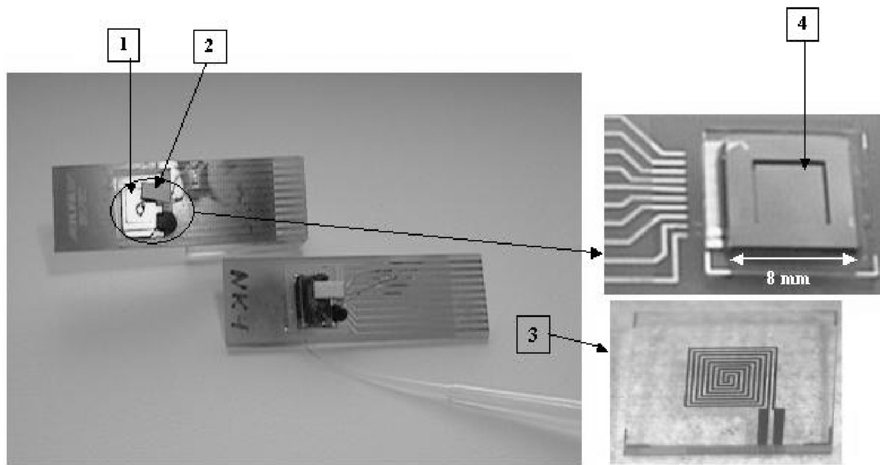

FIGURE 3 Assembled PCR chip on the PCB (with components indicated): Pyrex lid (1), Pt100 temperature sensor, (2) platinum Pyrex heater (3), which is positioned underneath the silicon microreactor cavity (4).

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DNA Amplification in Standard Thermal Cycler

A 500 bp fragment from the lysin gene of *Escherichia coli* bacteriophage Lambda (GeneBank accession number J02459.1) was amplified using the oligonucleotide primer I: 5′-GATGAGTTCGTGTCCGTACAACTGG-3′, and primer II: 5′-GGTTATCGAAATCAGCCACAGCGCC-3′ (Proligo, Paris). Standard thin-walled polypropylene tubes (200 μL volume) were used for the amplification reactions, which had final volumes of 25 μL. The PCR mixture comprised: 2 mM $MgCl_2$, 0.2 mM dNTPs (deoxynucleoside triphosphates), 10 nM of each primer, 10 pg/μL of template lambda DNA (Sigma Aldrich, Dublin), AmpliTaq Gold and corresponding buffer (Applied Biosystems, Nieuwerk, The Netherlands). A Mastercycler Gradient (Eppendorf) was used to perform the thermal cycling with the following temperature profile: an initial denaturation step of 95°C for 4 min, followed by 25 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 30 s. A final elongation step of 72°C for 10 min was performed, and the reaction was completed in 120 min.

DNA Amplification Reaction Transfer to the PCR Chip

The underside of the PCR chip with its lid were attached to a sheet of Thermofoil (Eppendorf) material and placed into the Mastercycler machine. A series of DNA-amplification reactions were performed, and the concentrations of the following reaction constituents were varied; $MgCl_2$, Taq polymerase, oligonucleotides and template DNA. In addition, known PCR enhancing agents, Surfasil (Pierce Biotechnology, Rockford, IL), dimethylsulfoxide (DMSO) and bovine serum albumin (BSA), were included (Sigma-Aldrich, Dublin).

DNA Amplification in PCR Chip

The PCR chips were cleaned by washing with sterile distilled water, and then the chips were autoclaved. The PCR mixtures were prepared (as above) in 25 μL aliquots, and

7 μL of this was used to fill the microcavity in the PCR chip. Liquid access to the PCR chip was achieved using GELoader Tips (Eppendorf).

Gel Electrophoresis

Resultant amplicons from all PCR experiments were analysed by gel electrophoresis. Briefly, 2% agarose gels were prepared and stained with ethidium bromide [22]. DNA molecular-weight markers (50 bp and 100 bp ladders) were supplied by Promega (Madison, WI).

Verification of Method for Amplification of *Mycobacterium tuberculosis* Gene

A 255 bp fragment from the *rpoB* gene *Mycobacterium tuberculosis* was amplified using the oligonucleotide primers RP4T: 5′-GAGGCGATCACACCGCAGACGT-3′, and RP8T: 5′-GATGTTGGGCCCCTCAGGGGTT-3′ (Proligo, Paris) [23]. A Mastercycler Gradient (Eppendorf) was used to perform the thermal cycling, and the reaction was completed in 120 min. The PCR mixture comprised: 2 mM $MgCl_2$, 0.2 mM dNTPs (deoxynucleoside triphosphates), 1 μM of each primer (Sigma Aldrich, Dublin), AmpliTaq Gold and corresponding buffer (Applied Biosystems, Nieuwerk, The Netherlands).

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RESULTS

Establishing Conditions for Reduced Volume PCR in Polypropylene Tubes

In order to scale down the PCR reaction to suit the miniaturised PCR chip, the reaction conditions were first established in the standard polypropylene tubes (Eppendorf) using a 7 μL reaction volume.

Annealing Temperature

The PCR reaction annealing temperature was established using the Gradient function of the MasterCycler and temperature values between 46 and 55°C. Equal amounts of PCR product were observed for each of the annealing temperatures tested (Fig. 4). On that basis, the intermediate temperature of 50°C was employed in order to ensure DNA hybridisation.

Taq Polymerase

Enzyme concentrations of 0.35, 0.75, 1.25, 2.75 and 3.25 unit per reaction (U/reaction) were chosen for a 7 μL PCR reaction, where 1.25 U/reaction is the amount recommended by the supplier. All of the amounts tested were equally sufficient to perform the PCR reaction (results not shown).

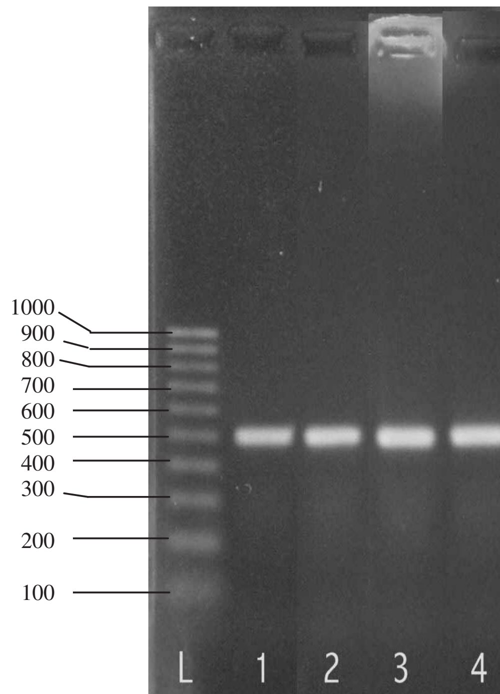

FIGURE 4 Influence of different annealing temperatures on the PCR reaction. Annealing temperatures: lane 1, 46.6°C; lane 2, 49.0°C; lane 3, 51.8°C; lane 4, 54.1°C. L: 100 bp molecular-weight ladder.

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Oligonucleotide Primers

Concentrations of 0.001, 0.01, 0.1, 1 and 10 μM were examined for each primer. The resulting agarose gels suggest that the most appropriate concentration for sufficient amplicon yield is 0.1 μM (results not shown).

$MgCl_2$

The establishment of the $MgCl_2$ concentration was performed for concentrations including 2, 3, 4, 5 and 6 mM. The most successful PCR reaction was that in which the $MgCl_2$ concentration was 2 mM (results not shown), whereas higher concentrations appeared to be successively inhibitory to yield of PCR product and contribute to the appearance of non-specific smearing at >500 pb.

DMSO

The PCR-enhancing agent DMSO was added to the PCR reaction at concentrations of 1, 2, 3, 4 and 5%, and every resultant PCR product was similar in yield to the positive control (results not shown). We did not pursue the role of PCR-enhancing agents any further.

Establishing PCR Conditions for the Reactions in the PCR Chip

The conditions for PCR in the silicon-based microreactor were established by placing the PCR reaction mixture into the silicon cavities, attaching the underside of the cavities to the thermofoil and placing this on top of the thermoblock of the Mastercycler. Initially, the PCR conditions chosen were those established parameters elucidated above (Table I). However, no amplicons could be obtained. Further research was performed to establish the conditions for PCR using these microcavities.

PCR-Enhancing Agents

This work commenced by investigating the effect of PCR enhancers on PCR in the microcavities. First, coatings of the silicon surface were investigated. Both Shoffner *et al*. [19] and Taylor *et al*. [24] recommend coating the silicon surface with BSA or with different polymers, e.g. Surfasil. BSA concentrations of 0.1–0.4% were used to coat the silicon microreactor, no obvious benefit to the outcome of a PCR reaction was observed. Similarly, when identical concentrations of BSA were added to the PCR mixture, no effect on the reaction outcome was observed. Also, the use of Surfasil as the surface coating did not have any effect on the PCR product.

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Taq Polymerase

Concentrations of 3.25, 2.75, 1.25 and 0.75 U/reaction were used for this experiment. It appears that quite a high concentration of 3.25 U/reaction is necessary for adequate amplification. This compares to the supplier's recommended amount of 1.25 U/reaction when using a standard plastic type tube. Lower Taq polymerase concentrations did not amplify the desired fragment (Fig. 5A).

$MgCl_2$

Appropriate $MgCl_2$ concentrations were established by using concentrations of 2, 3, 4, 5 and 6 mM. As for the reduced volume PCR, here the most successful amplification reaction was achieved with 2 mM (Fig. 5B).

Oligonucleotide Primers

Concentrations of 0.001, 0.01, 0.1, 1 and 10 μM for each primer were used to perform the experiments. A primer concentration of 10 μM is necessary for amplification (Fig. 5C), compared with 0.1 μM for the reduced volume PCR.

DNA Template

Lambda phage genomic DNA was utilised for DNA amplification at concentrations of 0.01, 0.1, 1 and 10 ng/μL. Concentrations of 0.01 ng/μL did not yield a PCR product, while 0.1, 1 and 10 ng/μL were sufficient for DNA amplification (Fig. 5D). However, a template concentration of 10 ng/μL was clearly excessive and detrimental to the outcome of the reaction.

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TABLE I Conditions chosen for establishing the concentrations of the components of the PCR mixture for DNA amplification from Lambda genomic PCR and verification of these conditions using *M. tuberculosis* PCR reaction; standard reaction in polypropylene tubes of 25 μL (a), reduced volume reactions of 7 μL with the silicon microcavity cycled using the conventional thermal cycler (b) amplification using the miniaturised PCR system (c) and amplification using the miniaturised PCR system for the amplification of *M. tuberculosis rpoB* gene (d)

PCR component	*Concentration range*	*Established concentrations*			
		a	*b*	*c*	*d*
$MgCl_2$	2, 3, 4, 5, 6 mM	2 mM	2 mM	2 mM	2 mM
Primer	0.001, 0.01, 0.1, 1, 10 μM	10 nM	0.1 μM	1 μM	1 μM
Template	0.01, 0.1, 1, 10 ng/μL	0.01 ng/μL	0.01 ng/μL	5 ng/μL (also yields a detectable product with 0.1 ng/μL and 1 ng/μL)	1 ng/μL
Taq polymerase	0.35, 0.75, 1.25, 2.75, 3.25 U/reaction	0.35–1.25 U/reaction; producer recommends 1.25 U/reaction	0.35–1.25 U/reaction; producer recommends 1.25 U/reaction	2.75–3.25 U/reaction; best result with: 3.25 U/reaction	2.75–3.25 U/reaction; best result with: 3.25 U/reaction
Temperature profile		95°C for 4 min, followed by 25 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 30 s, a final step of 72°C for 10 min	95°C for 4 min, followed by 25 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 30 s, a final step of 72°C for 10 min	95°C for 3 min, followed by 24 cycles of 50°C for 16 s, 72°C for 14 s 94°C for 12 s, a final step of 72°C for 5 min	95°C for 3 min, followed by 24 cycles of 65°C for 16 s, 72°C for 14 s 94°C for 12 s, a final step of 72°C for 5 min
Total reaction time		120 min	120 min	30 min	20 min

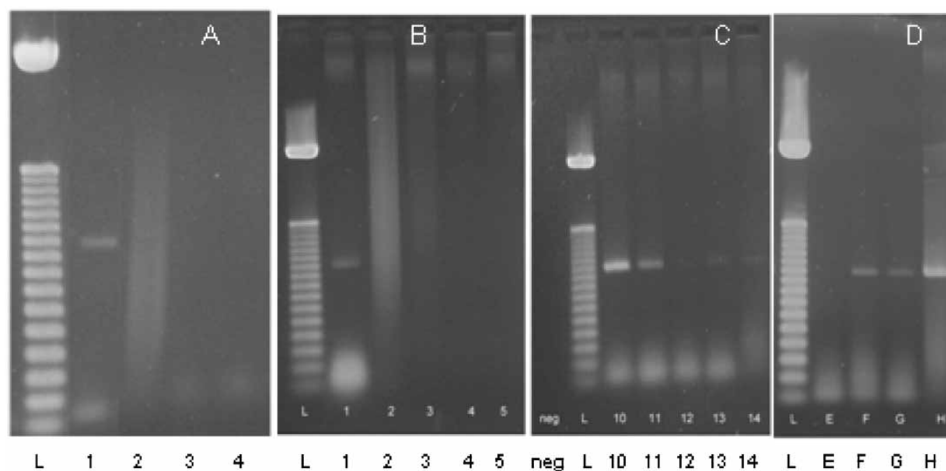

FIGURE 5 Results of the experiments to establish PCR conditions in the silicon microreactor with Lambda genomic PCR. (A) Influence of Taq polymerase concentration: lane 1, 3.25 U/reaction; lane 2, 2.75 U/reaction; lane 3, 1.25 U/reaction; lane 4, 0.75 U/reaction. (B) Influence of different primer concentration (4 mM $MgCl_2$): lane 1, 10 μM/μL; lane 2, 1 μM/μL; lane 3, 0.1 μM/μL; lane 4, 0.01 μM/μL; lane 5, 0.001 μM/μL. (C) Influence of different $MgCl_2$ concentrations (primer 1 μM/μL): neg, negative control without Taq polymerase; lane 10, 2 mM; lane 11, 3 mM; lane 12, 4 mM; lane 13, 5 mM; lane 14, 6 mM. (D) Influence of template concentration: lane E, 0.01 ng/μL; lane F, 0.1 ng/μL; lane G, 1 ng/μL; lane H, 10 ng/μL. In all cases, L is the 50 bp ladder.

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The established concentrations for the PCR mixture for genomic Lambda PCR for standard and reduced volume PCR in polypropylene tubes and the PCR chip are summarised in Table I. The conditions for the verification reaction where the *rpoB* gene from *M. tuberculosis* was amplified are also given in Table I.

PCR in Microfabricated Silicon Thermal Cycler

This established PCR mixture, determined by previous experimentation (Table I), was used in a DNA-amplification protocol in the PCR chip, where the thermal cycling was peformed using the integrated platinum resistance heating element. The reactants were cycled for the following temperature profile: 95°C for 3 min, 24 cycles of 50°C for 16 s, 72°C for 14 s, 94°C for 12 s and a final elongation step of 72°C for 5 min. The DNA-amplification time required was 30 min. The resultant amplicon was detected by gel electrophoresis (Fig. 6).

Verifying the Established PCR Chip Conditions in the Silicon

The concentrations of the PCR reactants were also shown to be appropriate for the amplification of a DNA fragment of 255 bp in size from the *rpoB* gene of the microorganism *M. tuberculosis* (Fig. 7). While the same PCR temperature profile was employed, the time taken for the reaction to reach completion was 20 min, 10 min shorter than that for the Lambda PCR product. This time-saving is due to a higher annealing temperature (65°C for *M. tuberculosis* PCR), as the device is passively cooled.

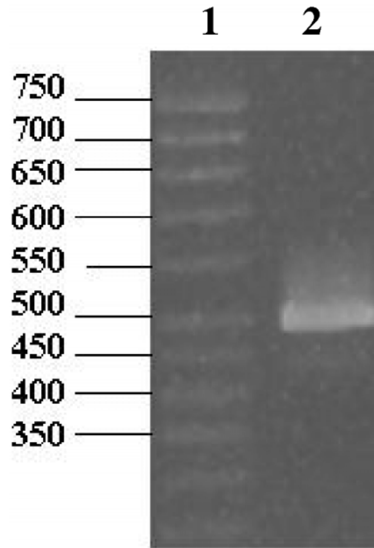

FIGURE 6 PCR product from DNA amplification reaction in the PCR Chip using the platinum resistance heating element for thermal cycling. 1: 50 bp ladder; 2: PCR product (500 bp).

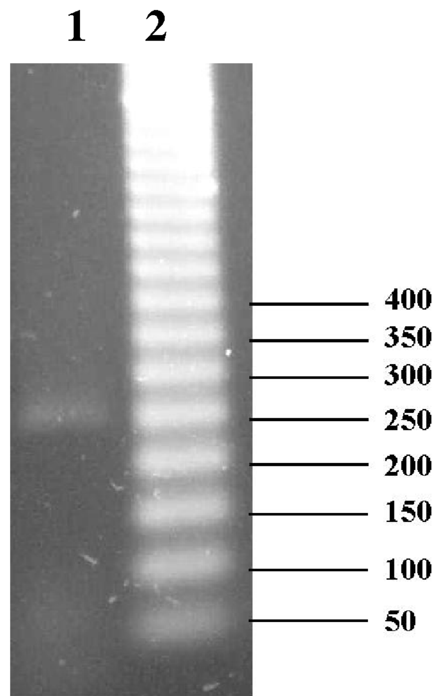

FIGURE 7 Gel electrophoresis showing the results of the verification reaction which employed the conditions established for the silicon microreactor PCR system and employed a reaction to amplify a 255 bp region of DNA from the *rpoB* gene of the organism *M. tuberculosis*. 1: PCR product (255 bp); 2: 50 bp ladder.

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DISCUSSION

The work described here illustrates that a silicon microfabricated reaction device with a miniaturised resistor-based heater can be used as a miniaturised PCR thermocycling device. We have shown that successful DNA amplification is possible in silicon-fabricated microcavities without the addition of enhancer additives or post-fabrication surface passivation. The results also demonstrated that PCR-enhancing agents did not improve the outcome of the reaction; interestingly, these agents were described by other authors as significant for PCR using different materials for miniaturisation [19,24–26]. Additionally, sufficient DNA amplification using the microreactor from 24 PCR cycles was achieved in 30 min.

Results on the established reaction components and their concentrations are presented in Table I and show that higher concentrations of oligonucleotide primers, genomic Lambda template DNA and Taq polymerase were all necessary for successful PCR in the microcavity. These reactions conditions were verified as appropriate for the amplification of a DNA fragment from *M. tuberculosis*. It is possible to speculate that a number of factors may contribute to this situation, including the high surface-to-volume ratio of the PCR chip. It may also be that the reaction components themselves are passivating the surface of the microreactor and are thus unavailable for the reaction.

The system we described comprises a PCR chip on a PCB of the size of 6×1 cm. This chip can be easily mounted and exchanged in a small box with a fan of the total size $4.7 \times 9.8 \times 3.9$ cm. It is conceivable that the additional instruments (digital multimeter and power source) may be miniaturised on a PCB, giving rise to a truly portable DNA-amplification system suitable for DNA detection.

The use of microfabricated structures to perform PCR has numerous advantages over conventional thermal cycling due to large decreases in thermal mass, which results in faster cycling times, low power consumption, a reduction in erroneous sequence amplification and suitability for point-of-care devices. Future generations of this thermocycler system could also include integrated optical PCR product detection methods to allow real-time PCR. Such a highly integrated silicon microreactor would have uses in all areas of research and diagnosis where DNA amplification is proving very valuable.

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