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Autonomous microfluidic multi-channel chip for real-time PCR with integrated liquid handling

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Abstract We report on a novel, polymer-based, multi-channel device for polymerase chain reaction that combines, for the first time, rapid sample processing in less than 5 min with high throughput at low costs. This is achieved by sample shuttling, during which submicroliter sample plugs (~100 nl) are oscillated rapidly over three constant-temperature zones by pneumatic actuation with integrated system. The accuracy and the speed of the liquid handling have been significantly increased, while the design of the device can be kept very simple and allows for mass production using conventional low-cost polymer fabrication processes. Massive parallelization can lead to a throughput up to 100 samples in 10 min including the preparation time. The amplification can be optically monitored by means of online fluorescence detection. Successful real-time PCR and the determination of the threshold cycle, C_t, using the developed device were demonstrated with plasmid DNA in a fluorescent real-time format.

Keywords PCR · Microchip PCR · Sample shuttling · PDMS · Diffuser nozzle structures

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1 Introduction

There is a large interest in miniaturizing polymerase chain reaction (PCR) devices by developing autonomous, microfluidic-chip-based systems. The resulting small volumes and low thermal mass are the main benefits of using microtechnology as the systems react faster upon temperature changes, so that the heating and cooling speed can be drastically increased. The devices can, therefore, be operated close to the limit defined by the chemical reaction kinetics so that shortest possible analysis times can be obtained. Further advantages include the small sample volume that is required (microliter to nanoliter), and the possibility of manufacturing portable or hand-held devices, which are easy to use and can be applied as point-of-care devices. To this end, integrated systems combining different processes, such as sample pretreatment, the PCR process itself, a data post-processing stage, and, possibly, further analysis steps are desired.

Since 1994 a variety of miniaturized devices for rapid DNA amplification has been designed. Two comprehensive reviews of this research field were recently published by Auroux et al. (2004b) and Lee et al. (2003). PCR allows for the amplification of a specific deoxyribonucleic acid (DNA) sequence and was first introduced in 1985 by Kary B. Mullis. It comprises the repeated application of three different temperatures (95°C denaturation, 55–60°C annealing, and 72°C extension) to a mixture of the target DNA, primers that determine the region to be amplified, deoxynucleotides, a DNA polymerase, and cofactors such as Mg^{2+}.

There are potentially two different approaches for micro-PCR devices: chamber-type and continuous-flow-type. The chamber-type devices are generally a microfabricated

version of conventional thermocyclers (Giordano et al. 2001; Northrup et al. 1993; Wilding et al. 1994). The reaction mixture is kept stationary, while the temperature of the reaction chamber is cycled. In most cases, these devices are highly integrated featuring different metallizations for rapid heating, active cooling and temperature control, so that a rather complex and expensive fabrication procedure is required (Burns et al. 1998; Lagally et al. 2001). The alternative is a dynamic approach, a continuous-flow system, in which the reaction mixture is moved through three different temperature zones in a flow channel instead of cycling the whole device (Kopp et al. 1998; Schneegaß et al. 2001). Reducing the overall mass, the temperature of which has to be varied (essentially to the sample solution itself in a microchannel) decreases the time that is required for heating and cooling to the possible minimum. The relative residence time in each temperature zone, and the number of cycles are defined by the channel design so that there are some limitations in the process flexibility. However, Liu et al. (2002) circumvented this drawback by designing a rotary device, where a 12-nl-sample is pumped in a single circle over three integrated heaters and the cycling parameters could easily be adjusted.

Both of the presented basic approaches—chamber-type and continuous-flow-type—were optimized during the last years, with a main thrust being the integration of functional units into the same device so that a multitude of operations can be performed with a single system (e.g. cell lyses or sample pretreatment). PCR is exquisitely sensitive to cross-contamination so that all required analyses have to be performed in a sealed, disposable device. With respect to high throughput both methods are struggling with rivaling factors: The use of multi-chamber micro-thermocyclers (Yu et al. 2003; Zou et al. 2002) can increase the throughput significantly. But keeping the device simple and the fabrication costs low and, at the same time, ensuring an easy liquid handling without the risk of cross-contaminations is difficult. In continuous-flow systems multiple analyses can only be performed in a serial fashion, so that substantial efforts are necessary to avoid cross-contaminations in autonomous systems (for instance, two-phase flow, Schneegaß et al. 2001). For the rotary chip, so far, reliable and proven protocols suitable for parallelization have not been established yet.

In this paper, we present a different approach by adding reliable and flexible fluid handling to a modified form of flow-through reactors. Shuttling a submicroliter sample of the PCR mixture back and forth over three heaters that feature the specific reaction temperatures combines the cycling flexibility of a chamber-type device with the fast temperature cycling possibility of continuous-flow devices (Auroux et al. 2004a). The time to reach a new temperature depends mainly on the time needed to transport the sample into the target temperature zone and to heat the liquid plug in the channel to the preset temperature. The parameters of the PCR, such as incubation time, temperatures and number of cycles can be fully controlled and adjusted to the specific template–primer combination.

In this paper we describe this novel approach, the design, the fabrication and the first amplification results of a disposable polymer-device, which features (a) simple and low-cost fabrication by conventional processes (e.g. casting or injection molding), (b) fast analysis times and the careful control of all cycling parameters and (c) high-throughput capabilities at a significantly reduced risk of cross-contamination. An externally actuated pneumatic pump and microfluidic components in the channels allow for a robust and autonomous sample manipulation. Moreover, the developed approach offers the possibility of arranging several channels side by side for massively parallel processing by means of simultaneous pump actuation.

2 Microfluidic device design

2.1 Principle

The miniaturization of a fluidic system requires a careful design since the governing laws and operating principles may significantly diverge from those of a macroscopic setup. Controlled fluid movement and the positioning of very small quantities of liquid at the microscale with sufficient precision turns out to be challenging. Moreover, only a few liquid-handling components and techniques for nanoliter-volumes are currently available. Owing to the

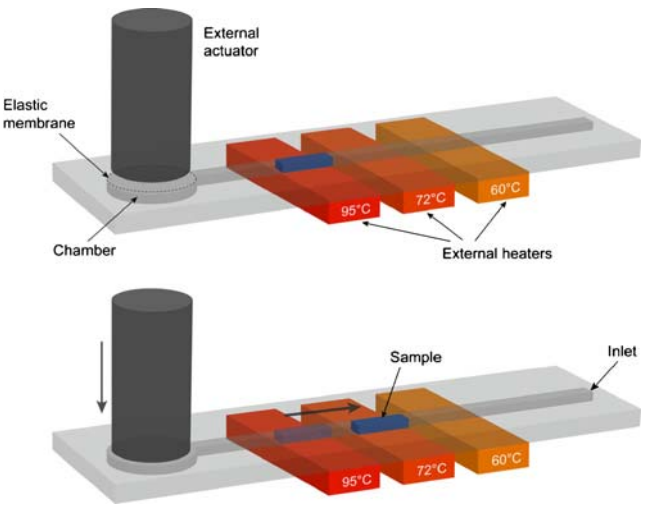

Fig. 1 Principle of sample shuttling: The PCR reaction is performed inside a straight channel ending in a chamber with a membrane which is deflected to move the liquid sample back and forth over three constantly heated regions. Actuation and heating is done externally so that the chip can be kept as simple as possible

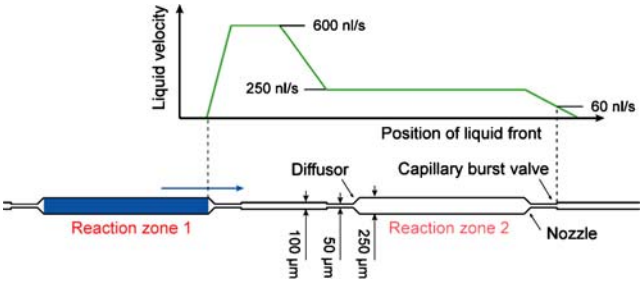

Fig. 2 The shuttling speed to change from one temperature zone to the next is depending on the functional design of the channel. Microfluidic components have been realized for an improved positioning of the sample: Once the liquid plug reaches the reaction zone, it is stopped by the nozzle and the capillary burst valve. After the reaction step, a large velocity gradient is applied so that the liquid passes the burst valve and moves to the next zone

large surface-to-volume ratios, surface-related effects such as interface forces, evaporation, absorption and friction become very significant.

Shuttling submicroliter samples back and forth cannot be compared to flow-through devices, where a constant pressure difference is applied to control the flow rate. The sample has to be accelerated and has to be very precisely stopped at the location of the heaters. The plug movement is very sensitive to minute disturbances and is not only depending on the applied pressure difference, but also on fluid inertia and, therefore, on the pressure gradient.

The device presented in this paper is designed as a disposable polymer unit consisting of a straight channel that is pneumatically connected to a chamber, which is used as an integrated pump (see Fig. 1). Deforming the elastic chamber wall or membrane results in a volume change of the chamber and, hence, displaces the liquid plug in the channel by the same volume (neglecting the compressibility of air at such small volumes). The integration of the pump allows for a precise and fast manipulation of the sample at a sub-second time scale and reduces the gas volume of the pneumatic pump to a minimum. This entails a negligible hysteresis (as a consequence of the compressibility of the gas) and better response characteristics of the sample movement upon membrane actuation. Furthermore, this approach enables the deformation of the membrane by an external actuator so that the chip can be kept very simple.

2.2 Fluidic handling

To ensure robust fluid handling and an accurate placement of the liquid plug, the channel geometry has been optimized by applying capillary burst valves and by designing reaction chambers with diffusers and nozzles (Fig. 2). The nozzle at the end of each reaction chamber increases the flow resistance, while the capillary burst valve stops the overflowing liquid by forming a capillary-pressure barrier that develops when the channel cross-section abruptly increases. This type of passive flow element provides a precise control of the sample movement in microfluidic devices. The liquid that reaches the valve is stopped by a pressure drop

$$\Delta P = \frac{\sigma \cdot \cos\theta \cdot L_{\text{wetted}}}{A}$$

where σ denotes the surface tension of the liquid, θ the contact angle between the liquid and the channel wall, L_{wetted} the channel perimeter and A the cross-section of the channel. The precise placement of the sample plug is assured for movements in both flow directions due to an adapted and well-controlled velocity profile (Fig. 2): At a low flow-rate of 60 nl/s the flow resistance increases by 20%, whereas at a high flow-rate of 600 nl/s it increases by only 2% so that the valve can be passed easily by the liquid. The chosen microfluidic architecture allows for a reproducible and continuous cycling with liquid velocities up to 100 cm/s and for an accurate plug positioning at less than 1% misplacements during 20 cycles.

2.3 Chip description

A schematic view of the device (chip size: ~1×6 cm^2) is shown in Fig. 3. The channel height is 100 µm over the total length, while the channel width is varied in order to realize a channel design as explained before: 250 µm channel width in the reaction chamber zones continuously decreasing to 50 µm towards the chamber ends (nozzle, diffuser) and, after that, an abrupt increase of the channel width to 100 µm (capillary burst valve).

The pumping chamber for pneumatic actuation has a diameter of 5 mm and is 800 µm high. The ceiling

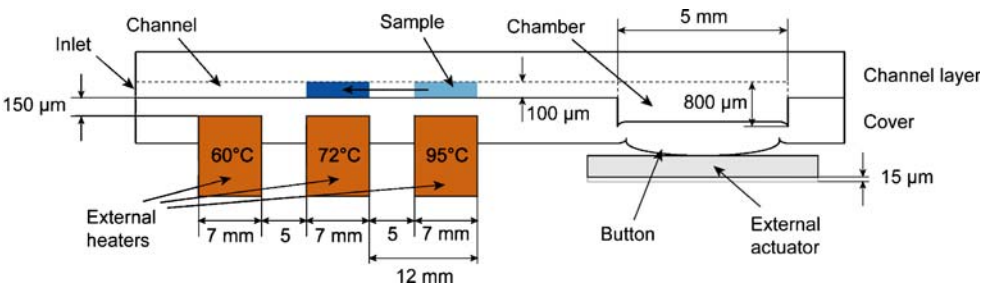

Fig. 3 Schematic cross section through the chip showing the geometric dimensions and illustrating the actuation principle

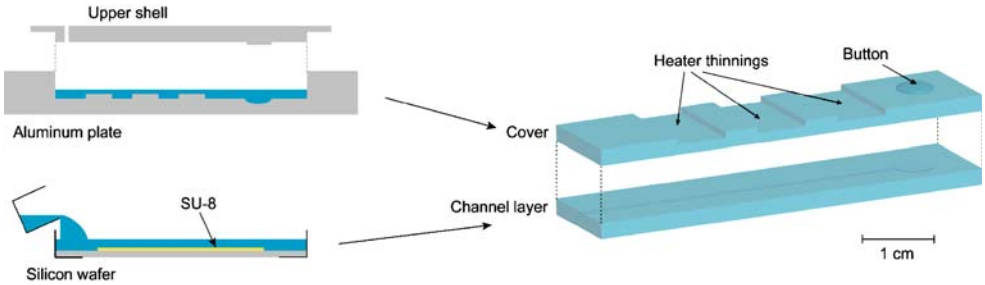

Fig. 4 Fabrication of the two layers of the PCR-device prototype: PDMS casting using a 3-dimensional precision-milled aluminum mold for the cover and a SU-8 patterned silicon wafer for the channel structure. The simple design allows for the mass production of a cost-effective, disposable device fabricated by conventional polymer processing (e.g. injection molding)

membrane (thickness: ~0.7 mm) is deformed by a high-precision, electrical actuator to change the chamber volume. Integrating a protruding button on the membrane to realize a defined contact point is indispensable for a reliable and parallel operation of multiple channels and chambers. The transport of the sample plug from one heater to the next (12 mm distance) takes less than 0.5 s and requires a membrane deflection of 15 μm. This value is very close to the calculated value under conditions of neglected air compressibility. Three thermocontrolled copper blocks of 7 mm width constitute the external heaters. Resistive heaters driven by a defined constant current in a steady state are used to control the temperature (between 220 and 520 mA). The heat transfer to the sample is optimized by a localized thinning of the PDMS walls of the microfluidic unit to 150 μm. These cut-outs also allow for a simple and accurate positioning of the microfluidic unit on the heaters and with regard to the actuator.

2.4 Chip fabrication

The external pumping via the membrane on the polymer chip and the use of an external heating source allow for a cost-effective fabrication of the device using conventional polymer processing (e.g., casting or injection molding). The chip can be designed as a simple, low-integration, cost-effective disposable device, to be inserted in a programmable desktop unit that provides the external heating and pumping. The prototype chips consist of two layers of poly (dimethylsiloxane) (PDMS) fabricated by rapid prototyping: For the channel layer a silicon wafer patterned with SU-8 50 is used as master (lower part in Fig. 4). The PDMS (Sylgard 184, Dow Corning, USA) was poured on the channel structure and cured for 4 h at 65°C. The cover is fabricated in a similar way by casting from a precision-milled aluminum plate containing the heater cut-outs and the actuation button (pump). An optional upper shell was clamped onto the mold after pouring of the PDMS to assure a defined thickness (down to 150 μm at the cut-outs) and to provide a larger chamber height for an increased total chamber volume. For bonding, the surfaces were cleaned using acetone and isopropanol and subsequently activated by an oxygen-plasma for 1 min at 100 W.

Due to the hydrophobic nature of PDMS, the channel walls were first exposed to an oxygen-plasma (80 min at 70 W) to form a negative charge on the walls, followed by coating the channels with a monolayer of poly(L-lysine) grafted with poly(ethylene glycol) (PLL-g-PEG). PLL-g-PEG is a polycationic copolymer that is positively charged at neutral pH and adsorbs spontaneously from aqueous solution onto the negatively charged surface. This treatment yielded stable polymeric monolayers and rendered the surfaces highly resistant to nonspecific adsorption (Pasche et al. 2003). As a result a hydrophilic, protein-resistant surface for improved liquid handling and good PCR performance was generated.

2.5 Instrumental set-up

The PDMS chip is placed onto a custom-made PMMA device holder comprising the heaters that can be precisely positioned with regard to the external bolt actuator by

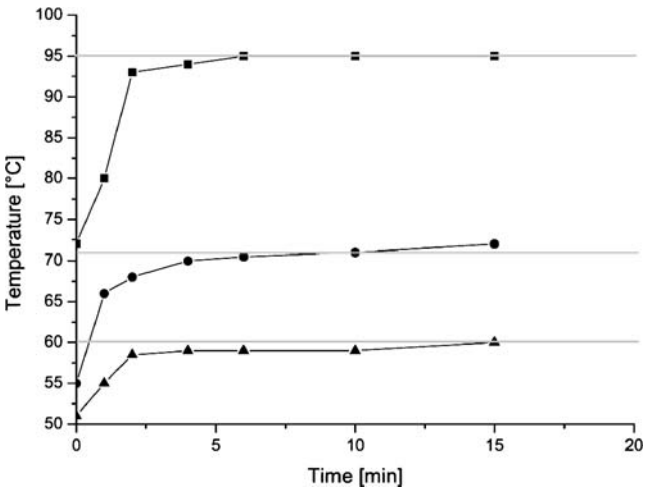

Fig. 5 Measurement of the temperature in the three reaction zones during the calibration of the heaters

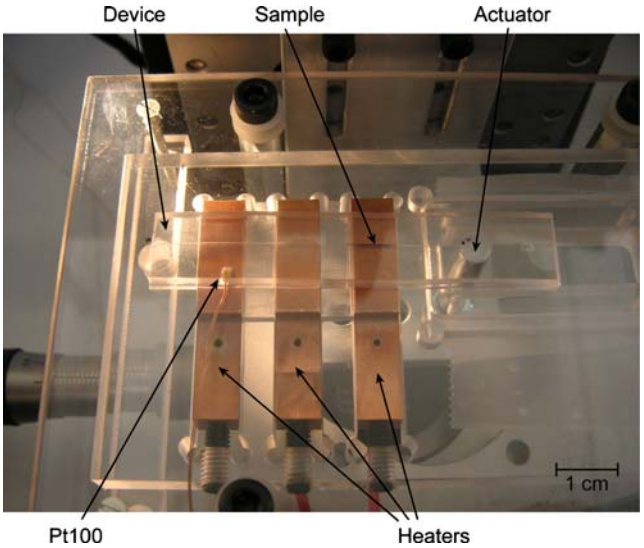

Fig. 6 Instrumental set-up: The chip can be easily placed on the heaters in the chip holder and positioned with regard to the pump actuator by means of a *xy*-table. Pt100 elements are used to control the temperature of the heated regions

means of a *xy*-table. Since pump actuation and heating is done externally the temperature and procedure protocols can be adapted any time by an adjustment of the heating current or the pump actuation program. The heaters consist of conventionally milled copper blocks with a resistive thermofoil heater (Model-Nr. 5568, R=13.1 Ω, Minco Products Inc., Minneapolis, USA) affixed to the blocks by an epoxy glue (EPO-TEK H72, Epoxy Technology Inc., Billerica, USA). They were previously calibrated by introducing a miniature thermocouple into the channel of the PDMS chip, once placed in the holder. Figure 5 shows a graph of the temperature of the reaction region over the heaters in each zone. The temperature is reached in less than 5 min after placing and adjusting of the chip into the holder and can be controlled by varying the current within 1–2°. The pumping actuation is performed by a computer-controlled, high-precision, linear motor (linear DC-Motor M-125.10, Physik Instrumente (PI), Germany). Figure 6 shows the set-up.

A continuous monitoring of the DNA amplification after every cycle could be easily performed owing to the facts that (a) the chip and the device holder are optically transparent, and (b) the liquid plug is always returning to the same positions during every step of the cycle. For monitoring, the set-up was mounted on an inverted fluorescence microscope (Zeiss Sedival) that was focused on the channel in the second reaction chamber, where the extension was performed. The read-out was done by a photomultiplier tube (PMT, Modul H5784, Hamamatsu Photonics, Switzerland) that detects the fluorescence intensity at 514 nm, which was recorded from the voltage output of the PMT by means of a personal computer running a Labview program. Fluorescence excitation was performed using a 100 W halogen lamp with a 475 nm interference filter.

2.6 PCR reagents

The PCR system used to assess the performance of the device has been provided by Promega Corporation, USA. This PCR system (PCR Core System II) is designed for exponential amplification of the specific regions of a template using Taq DNA Polymerase. The system also includes positive control primers and a control template. This mixture was used for all PCR reactions performed in this work. Table 1 lists all the ingredients of the PCR master mix. The reaction is initiated by adding 1 µl of the positive control plasmid DNA, which corresponds to 1 ng and is equivalent to 2.2×10^8 molecules.

The detection of the amplified DNA is done by using a fluorescent SYBR Green I staining. The SYBR Green I dye exhibits a remarkable affinity for double-stranded DNA and a large fluorescence enhancement upon intercalating into the DNA. SYBR Green I stain has its excitation maximum at 497 nm and emits fluorescence at 520 nm.

3 Results and discussion

3.1 Fluidic handling

Figure 7 shows the fluorescence intensity that was recorded by the photomultiplier tube (sampling rate 10 Hz) while a fluorescent sample plug was shuttled back and forth. The sample consisted of fluorescently labeled BSA (BSA-FITC:

Table 1 Components of the PCR master mix[1]

Component	Volume (µl)
$MgCl_2$, 25 mM solution	3.0
Thermophilic DNA polymerase 10× buffer	5.0
PCR nucleotide mix, 10 mM	1.0
Upstream control primer, 15 µM	3.3
Downstream control primer, 15 µM	3.3
Taq DNA polymerase, 5u/µl	0.25
SYBR green mix	5.0
Nuclease-free water	28.0
Total	49.0

[1]The polymerase 10X buffer consists of 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100 and 15 mM $MgCl_2$. The nucleotide mix is composed of 10 mM of dATP, dCTP, dGTP and dTTP in water. The upstream control primer (5′-GCCATTCTCACCGGATTCAGTCGTC-3′) and the downstream control primer (5′-AGCCGCCGTCCCGTCAAGT CAG-3′), 15 µM each, are provided in nuclease-free water (Technical Bulletin No. 254, Instructions of the Use of PCR Core System M7665, Promega Corporation, Madison, USA).

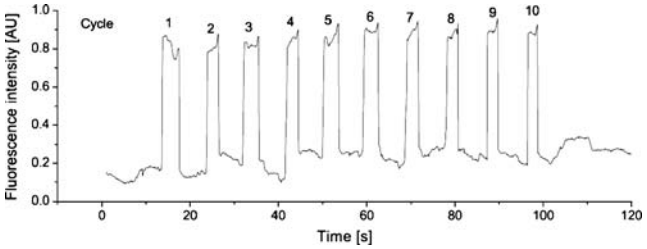

Fig. 7 Fluorescence intensity changes in the reaction zone 3 (95°C) due to shuttling a plug of BSA-FITC solution according to a protocol similar to that of a PCR

10 mg in 4 ml 50 mM Tris Buffer at pH 7.4). The detection area of the microscope included the whole reaction chamber at the third heater (95°C). The graph shows the periodic fluorescence emissions as a consequence of the sample shuttling during 10 cycles with less than 0.1 s needed to move from one reaction zone to the next. Due to some evaporation at the dead-end side of the channel (chamber side), which entailed an increase of the pneumatic air volume, the sample position constantly drifted towards the outlet of the channel. However the pump actuator can be programmed to compensate for this drift: The pumping volume was increased according to the drift per cycle so that the sample could be moved precisely to the intended position.

The sample introduction of 125-nl volumes can be done in a very simple way: A droplet of the PCR mix is placed at the inlet of the channel, where a small slice of the PDMS chip has been previously cut off to reestablish the hydrophobic surface conditions of the natural, untreated PDMS polymer. Owing to this hydrophobicity, the droplet will keep its convex shape and stay at the vertical channel end. After dragging the desired volume into the channel using the pneumatic actuator, the excess solution is removed, and the liquid plug in the chip can undergo the shuttling program. An operation of the chip in a vertical arrangement of the set-up would moreover allow the sample to be applied horizontally on the topside of the chip and assure sample introduction even for hydrophilic surfaces. Gravitation forces will not influence the shuttling of the sample, due to the small sample mass and the vertical, opposite actuation of the pneumatic pressure of the pump.

Since the chip is designed as a disposable device for quantitative PCR reactions, there is no need to collect the submicroliter sample again after processing. In fact, this would be quite difficult due to the small sample volume, so that further processing should be done directly on the same chip if necessary. The monitoring of the DNA amplification is done online by measuring the fluorescence intensity in the channel. Thereafter the device is disposed of.

3.2 DNA amplification

Once the heaters reached their programmed temperatures, the chip was placed and positioned in the device holder. The reaction areas are equilibrated to the preset temperatures within 4 min and controlled by using miniature Pt100 sensors. The walls of the channels were chemically saturated in an initial step using a template-free PCR mix to

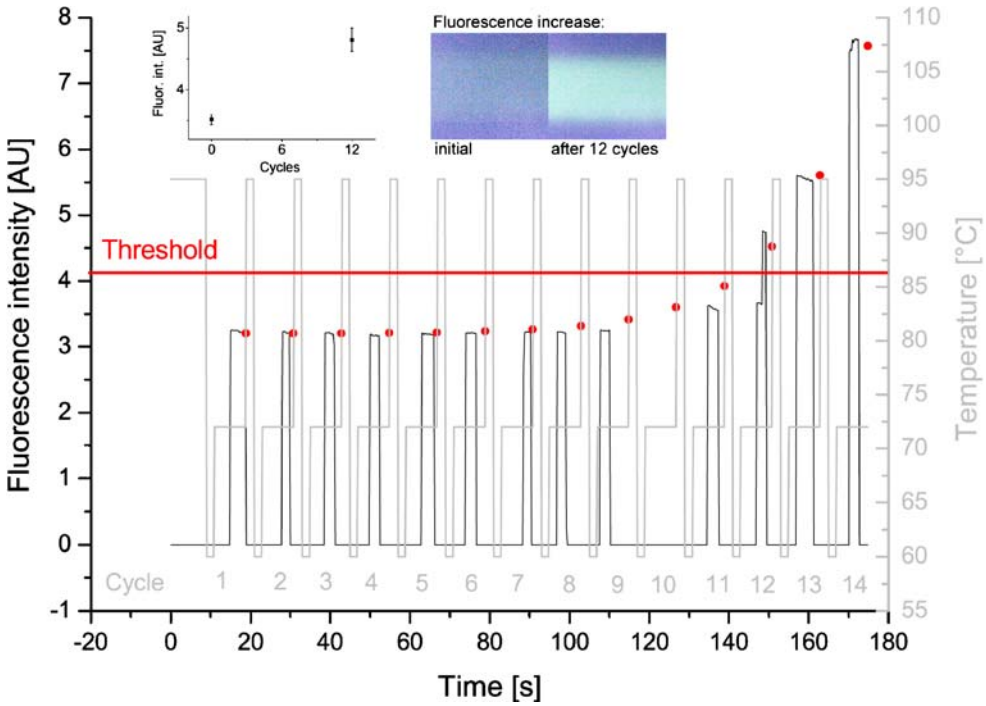

Fig. 8 The exponential increase of the fluorescence intensity appears already after the 12th cycle of amplification (inserts are measurements without online monitoring, n=4). The real-time read-out was always done at the end of the extension time for 1–2 s (*black line*). The *gray line* displays the cycling protocol (times and temperatures), the *red dots* represent a calculated exponential amplification curve with an efficiency of 82%. Cycle 10 could not be recorded due to a timing issue with the photomultiplier. The protocol was 2 s denaturation, 2 s annealing and 10 s extension

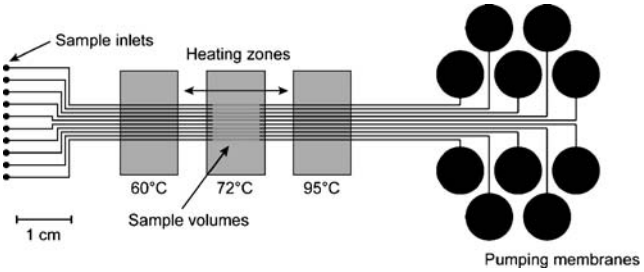

Fig. 9 Concept of a parallel arrangement of ten channels, where various samples can be simultaneously processed by synchronous actuation of the pumping membranes. If interfacing to the outside world can be established, the parallelization can be in theory scaled up to hundreds of channels side by side

reduce the adhesion of the DNA to the PDMS in the channels. Then, the complete PCR mix was introduced and was first denaturized for 20 s before the following cycling protocol was started: 2 s at 95°C for denaturation of the double strands, 2 s at 60°C for annealing of the primers to the single-stranded DNA and 10 s at 72°C for the extension of the primer DNA (synthesis of DNA). This protocol has been plotted as a grey line in Fig. 8. The inserts show a graph (n=4) and photographs of the fluorescence increase after 12 cycles. For the online monitoring, at the end of every extension step the light from the channel was directed towards the photomultiplier tube (PMT) for 1–2 s, and the fluorescence intensity was recorded (black line). The cycling was additionally observed through the microscope to ensure that the sample plug was correctly positioned. Again, a significant increase of fluorescence intensity was measured already after cycle 12, which determines the threshold cycle C_t. The threshold cycle C_t is defined as the point of the curve, at which the intensity of the fluorescence begins to rapidly increase. This is usually the case when the fluorescence rises above the baseline by more than 3–5 times the standard deviation.

Even though no real optimization of the cycling protocol was performed, the measurements are in good agreement with the early exponential phase as described for a typical DNA amplification process (dots in Fig. 8). By curve fitting of the experimental data, the efficiency of the PCR experiment can be determined here by 82%. This demonstrates that efficient PCR can be performed in this straightforward, but highly miniaturized format.

For control purposes, the same reaction was done using (a) a PCR mix without template and (b) a PCR mix without primers. In both cases no increase in fluorescence could be observed, even after 20 cycles. Especially the second control experiment strongly indicates that the fluorescence intensity increase observed with the complete PCR mixture is due to newly synthesized DNA and the resulting intercalation of the SYBR Green dye and is not a consequence of the renaturation of DNA single strands.

3.3 Parallelization

The PCR approach based on sample shuttling proposed here also offers the possibility of using multiple channels in parallel to simultaneously perform a multitude of PCR reactions without any risk of cross contamination. The pump actuation can be performed by a single actuator deflecting all pumping membranes via the buttons at the same time (Fig. 9). This parallel operating scheme combined with the high processing speed of the device allows for high throughput using a single or several devices in parallel. Exactly the same polymer fabrication techniques as used for the single channel device also work for the multichannel version with very little extra efforts and cost.

4 Conclusions and outlook

In general, miniaturization significantly reduces the amount of required sample liquid and, consequently, shortens the overall reaction and processing time. The idea of shuttling a small amount of liquid between different temperature zones by means of a pneumatic pump offers decisive advantages over methods published so far: (a) a precise and fast sample manipulation for rapid cycling; (b) the use of external pumping actuation and external heating reduces the fabrication efforts and costs so that the mass production of disposable devices by means of conventional polymer processes is feasible, (c) no cross-contamination, and (d) the possibility to arrange multiple channels in parallel to increase the throughput without changing the overall processing time.

Moreover it has been shown that the device can be successfully used for real-time PCR and that it is possible to use PDMS for rapid prototyping of PCR devices. However, it should be pointed out that PDMS is clearly not ideal for this type of systems, both due to the difficult fluid handling (hydrophobicity) and due to the high absorption of biomolecules. As a consequence, other polymers, possibly hybrid laminates with eventual selective surface treatments, will be evaluated and are suggested for the production phase.

Furthermore, optimizations of the PCR protocol, especially the adaptation of reaction times and temperatures with respect to template primer combinations, will be necessary to make the operation of the device more robust and reliable and to improve amplification efficiency. For an advanced integrated liquid handling the optical read-out could be also used as a feedback control for sample placement. Finally, it would be possible to integrate sample pretreatment units on the device, which allows using the system at the point of care without external pretreatment procedures.

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