

## Flow-through polymerase chain reactions in chip thermocyclers

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### Abstract

The miniaturization of analytical devices by micromachining technology is destined to have a major impact on medical and bioanalytical fields. To meet the current demands for rapid DNA amplification, various instruments and innovative technologies have been introduced by several groups in recent years. The development of the devices was extended in different directions and adapted to corresponding applications. In this review the development of a variety of devices and components for performing DNA amplification as well as the comparison of batch-process thermocyclers with reaction chambers and flow-through devices for different purposes are discussed. The main attention is turned to a flow device concept for thermocycling using microfabricated elements for local heat flow management, for which simulations and considerations for further improvement regarding design, material choice and applied technology were performed. The present review article mainly discusses and compares thermocycling devices for rapid thermocycling made of silicon or of silicon and glass with a short excursion to the possibility of plastic chip devices. In order to perform polymerase chain reactions (PCRs) in the microreactors, special attention must be paid to the conditions of the internal surfaces. For microchips, surface effects are generally pronounced because the surface to volume ratio increases upon miniaturization. Solutions for solving this problem are presented. We propose an overview of layouts for batch-process thermocyclers with different parallelization of reaction chambers and also of different designs of continuous flow thermocycling chips, paying particular attention to the parameters which influence the efficiency of such chip devices. Finally we point out some recent issues for applications in the field of clinical diagnostics. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Flow-through reactor; Rapid polymerase chain reaction; Continuous flow PCR; Chip technology; Chip thermocycler; Lab-on-a-chip

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## 1. Introduction

### 1.1. Miniaturization of microfluidic chip devices: parallel vs. serial

Both a large number of samples for genotyping as well as for expression studies, and the huge number of different molecular species, call for methods of high-throughput in the specific amplification of nucleic acid molecules. There are two strategies for highly-efficient handling of larger groups of samples. Parallelization is nowadays the most popular strategy used in laboratories. By use of microtiter plates or nanotiter plates, hundreds to thousands of samples can be processed on one carrier in parallel. Serial processing of many samples in small time intervals is the alternative to parallel processing. Both strategies have specific advantages, but also characteristic disadvantages (Manz et al., 1998).

Parallel arrangements are well-suited for dispensing operations and for a parallel read-out of analytical information by imaging systems. A series of liquid handling steps can only be performed if the liquid surface can remain open. However, the evaporation of liquid is a problem resulting in concentration gradients, which has to be avoided by covering the top of the compartment. The covering of the arrays ensure the restriction of evaporation or contamination from the surroundings, but the liquid transfer into or from the single chambers or reactions sites is complicated. The problem of stability of liquid samples is particularly important in case of very small sample volumes and in the case of increased temperature. Both aspects are very important for the miniaturized polymerase chain reaction (PCR).

There are two strong arguments against a simple serial processing of samples in microfluid systems. One concerns the compatibility with the parallel sample processing devices usually used in laboratories, namely all devices around the microtiter plates (MTP). This argument is practically important, but neglects the fact that samples are frequently gained or produced serially. Moreover, a lot of MTP operations are not made in parallel, but step by step. Therefore, parallel han-

dling is advantageous for some operations, but is not the ultimate single principle for the handling of large numbers of samples. The second argument concerns the dispersion of concentration clouds in fluid channels and the cross-talk between samples in serially working devices. This argument is of fundamental importance in microchannels due to the extreme velocity gradients over the cross-section of streaming fluids. The huge amplification effect in PCR makes the situation still more serious in the case of homogeneous phase processes. However, the conditions are completely changed, if microheterogeneous phase systems are applied in microchannel devices instead of homogeneous phases. The existence of liquid/liquid interfaces reduces the dispersion drastically in cause of the interface tension. Additionally, an embedding liquid can insulate the reacting liquid volume fractions from the wall surfaces. Cross-talk as well as chemical gettering of templates and enzymes by reaction sites at the wall surfaces can be avoided. Therefore, serial sample processing can become a very interesting alternative to highly parallelized processing in DNA analyses.

The application results from the flow-through thermocycler devices in performing DNA amplification confirm the outlined arguments as demonstrated in this review.

### 1.2. Polymerase chain reaction and micro total analysis systems ( $\mu$ TAS)

During the progressive development of the PCR over the last 17 years, this method became one of the central techniques for nucleic acid analyses and is the most widely used method for amplification of defined DNA sequences today (Taylor and Robinson, 1998; Whitcombe et al., 1998; Mullis et al., 1994). The PCR is applied to a broad range of diagnostic questions like the sequencing of human genome, in the fields of validation and control in food production and environmental analysis, forensic medicine, molecular biotechnology and the pharmaceutical industry.

As a result of the keen demand for high-performance PCR devices with high sample-throughput, but low reagent consumption due to the continu-

ously increasing number of diagnostic samples, PCR became the focus of investigations for miniaturization of this biochemical process in order to enhance its efficiency.

The PCR is a temperature controlled and enzyme catalyzed process which consists of the periodical repetition of three reactions at different temperatures (Fig. 1). The initial reaction of denaturation at a temperature of approximately 94 °C serves to separate the template of double-stranded DNA into two single-stranded DNA for subsequent amplification. In the following reaction the temperature decreases to the temperature necessary for annealing of DNA primer, short nucleotides which serve as starter molecules for synthesis and flanks the DNA sequence to be amplified from both sides. The temperature for the primer annealing depends on their inherent nucleic acid sequences and is determined for each primer pair which is used. The primer extension is performed by increasing the temperature to approximately 72°C, the optimal temperature of the polymerase enzyme. Thus, in one cycle the number of DNA molecules is theoretically doubled. The cycles are repeated 20–30 times to yield a large amount of amplified molecules, i.e. a large amplification factor  $N$ . Because the PCR is not a

linear amplification process but an exponential method, already a small inhibition of individual samples can lead to drastic changes in product yield. From an initial number of template molecules before amplification ( $N_0$ ) the amount of PCR product ( $N$ ) is theoretically doubled in each cycle ( $n$ ); the function thereto is given in Eq. (1).

$$N = N_0 2^n \quad (1)$$

In practice, the amplification is better described by Eq. (2) by inclusion of the efficiency of amplification ( $E$ ), which demonstrates the fraction of the copies that are duplicated in each cycle. Ideally,  $E = 1.0$  for a fully optimized PCR and is constant throughout the exponential phase, but practically an efficiency of approximately 0.8–0.9 is reached.

$$N = N_0 (1 + E)^n \quad (2)$$

The amplification yield is an important parameter for the estimation of the productivity of miniaturized thermocycler devices on the basis of the application of materials for fabrication which are not always optimal for use in biological application. A maximal amount of amplified product in the range of approximately  $10^{13}$  molecules has been obtained, however, the amount can deviate from this to a much lower quantity in dependence of the adaptation and optimization of the chip devices to the PCR process.

Miniaturization has not only the potential of saving space and material, but offers also the opportunity for enhancing processes, such as optimized heat transfer due to the large surface to volume ratio. Also the mass transfer can be greatly improved by properly designed systems.

## 2. Requirements and advantages of chip thermocyclers over conventional thermocyclers

The PCR has advanced DNA amplification dramatically but in most cases requires long cycling times because of the large thermal mass of typical thermocycling systems in conventional devices

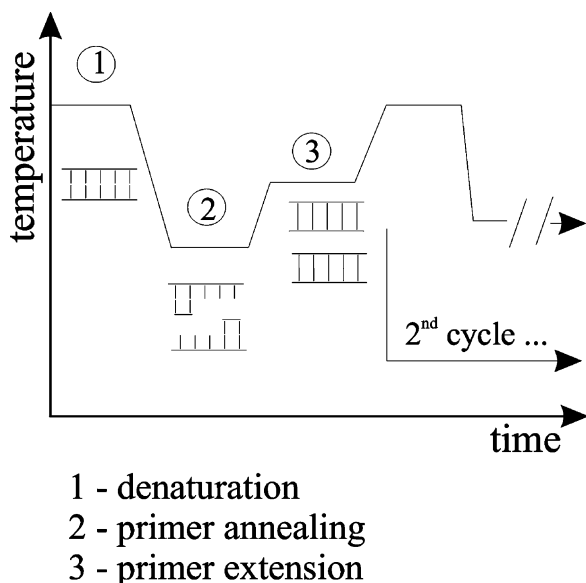


Fig. 1. Schematic diagram of the PCR process.

which accordingly have slow heating and cooling rates.

### 2.1. Properties of conventional thermocycler devices

Conventional thermocyclers are sizable devices accommodating multiple reaction vials. The sample heating and cooling, connected with the heating and cooling of the reaction vials and thermal blocks, is time consuming due to the large thermal mass of the individual components and results in cycling times of 1–5 min per cycle. Shorter cycling times require in contrast higher power devices for heating and chilling. The polymerase enzyme with a synthesis rate of approximately 100 nucleotides/s is not the time limiting factor for the amplification of products with a length of  $10^2$ – $10^3$  base pairs, but the thermal device itself. Therefore, the velocity of the process is dependent mainly on the development of devices for faster temperature changes and heat exchange between the device and the sample.

The problems of slow heating and cooling rates have been solved in part in the LightCycler of Boehringer Mannheim by placing samples in small glass capillaries and using heated air for tempering them, but the handling of the capillaries during insertion and removal of the samples can be complicated.

The relatively large dimension of the devices, correlated to the demand on high power, excludes the application of such devices outside of laboratories and also the construction of a portable, mobile and compact solution of a thermocycler device on the given basis.

### 2.2. Advantages of chip elements for rapid thermocycling

The problems of low process velocity and high power demands can be drastically reduced by decreasing the parasitic heat capacities of the thermocyclers. At present, most of the conventional thermocyclers achieve heating and cooling rates of approximately  $2$ – $10\text{ K s}^{-1}$  in a temperature range of  $40$ – $95^\circ\text{C}$ . Therefore, a consequent reduction of parasitic heat capacities is indispensable. The miniaturization of thermocyclers

offers a possibility to contribute to this point. The integration of functional elements for the generation of heat, for controlling the heat flow and for measuring the temperature is simply possible using the well-established technologies of thin-film deposition and microfabrication. From these technologies arise some important advantages of miniaturization of thermocyclers which should be noted. First, the heating and cooling elements possess small volumes and heat capacities, which yield at high heating and cooling rates such as  $15$ – $40\text{ K s}^{-1}$ . Secondly, heaters and sensors for local temperature measurement can be integrated as thin film resistors onto the reactor bottom in very close proximity to the liquid sample for fast temperature transition. Thirdly, the choice of geometries and materials in microsystems allows configurations of very well controlled local heat flow during all processes such as heating, cooling and temporal thermostabilization. Fourthly, with shrinking the reaction volumes in the microliter range, the consumption of expensive or dangerous reaction materials is also decreasing.

## 3. Fluidly stationary working microchamber chip-thermocyclers

Since the introduction of the thermostable *Taq* polymerase enzyme of the marine bacterium *Thermus aquaticus* into PCR technology in 1985 (Sakai et al., 1985), considerable effort has been expended in promoting the automation of the DNA amplification in thermocycler devices (Mastrangelo et al., 1998). The miniaturization of these devices offers the possibility to improve them further with the aim of shorter analysis times and higher sample throughput with minimal human intervention for less presence failure.

The fluidly stationary microchamber chip-thermocyclers work in the same manner as described for conventional macroscopic thermocyclers, where the amplification cocktail is placed in a reaction chamber which is processed with the appropriate thermal cycling program. After completion the samples are removed or detected on-line.

### 3.1. Rapid PCR in chamber thermocycling devices

A number of miniaturized devices have been developed to achieve faster thermal cycling by basically reducing the thermal mass present in conventional macroscopic systems.

The first of these devices was described by Northrup et al. (1993). This chip-thermocycler consists of a microwell cavity structure that serves as an appropriate reaction chamber formed by bulk micromachining using anisotropic wet etching. A 20-cycle amplification in a 50- $\mu$ l microwell was performed approximately four times faster than in a conventional device with a much lower power consumption.

The miniaturization of DNA amplification devices has also been investigated and promoted by several other groups over the last decade. Using a broad range of fabrication technologies and materials, different designs and application types such as single chamber thermocyclers (Cheng et al., 1996; Daniel et al., 1998; Woolley et al., 1996; Wilding et al., 1994; Baier et al., 1995; Poser et al., 1996) were prepared as well as array thermocyclers and flow-through devices (Kopp et al., 1998; Köhler et al., 1998; Poser et al., 1999; Litborn et al., 2000; Schneegaß et al., 2001).

### 3.2. Silicon chip thermocyclers with integrated heat flow barriers

In previous reports (Baier et al., 1995; Köhler et al., 1999; Poser et al., 1996, 1997a,b, 1999) our group has shown the development of chip-thermocyclers with different high integration rates of reaction chambers. Silicon-based thermocyclers with one, two and 10 chambers for volumes in the range of 4–8  $\mu$ l have been prepared (Fig. 2).

Because the dynamics of a cycler chip is mainly determined by the heat capacity and the thermal conductivity of the chip, we proved the possibility to optimize the chip-thermocycler for fast controlled temperature profiles by choosing materials and geometries well adapted to cycling processes for samples of a few microliters. FEM simulations were performed in order to evaluate the geometry of the cycler chips, particularly the local heat flows, the dynamics of the empty and the filled

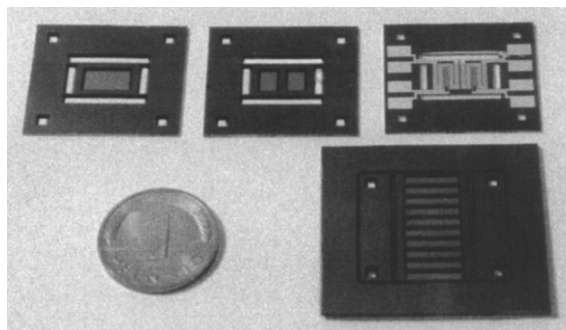


Fig. 2. A set of three different chip designs with various numbers of reaction chambers – view on the front side of a one- and a two-chamber chip as well as a view of their backside with appropriate heaters and circuits (upper part, from left to right) and a ten-chamber chip (lower part).

device and the temperature distribution in different phases of the PCR process (Poser et al., 1996, 1997b). A 32-cycle amplification was performed successfully in a total amplification time of approximately 16 min in the chip devices for an amplification product with a length of 850 bp. In comparison with conventional cyclers, a five-fold reduction of the time was achieved by reducing the amplification time to as low as 30 s per cycle. Similar results were obtained by Taylor et al. (1997) and Woolley et al. (1996). The final adaptation and construction showed a high spatial temperature uniformity in the chambers. We achieved experimental heating rates up to 80 K s<sup>-1</sup> with relatively low power consumption of 2.5 W per chamber. Under stationary conditions approximately 1.25 W per chamber were needed for a temperature of 94°C. By cooling down with a fan, cooling rates up to 40 K s<sup>-1</sup> were obtained. In connection with the programmable voltage source for giving temperature set points, the analog controller circuit works quickly and accurately with typical deviations of  $\pm 0.1$  K. The absolute accuracy of the monitored temperature was verified with test substances in the reaction chambers and by microscopic inspection of their melting points (Poser et al., 1997a).

Besides these chip devices a chamber array of 90 chambers with a volume of 1  $\mu$ l per well and with outer dimensions of 26  $\times$  30 mm for high-throughput of samples was designed, constructed

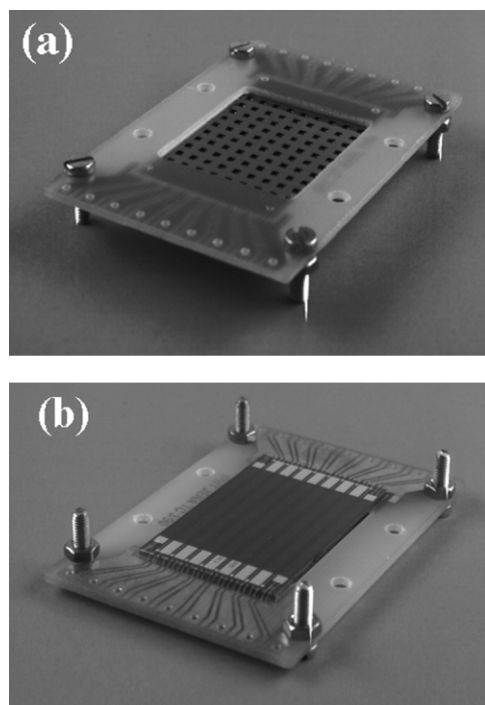


Fig. 3. The front view (a) of a 90-well array for thermocycling with uncovered reaction vessels and the back view (b) with the heaters and wire bonding pads.

and tested (Fig. 3). The parallel arrangement of nine thin-film transducers and especially the short distances between the reaction vessels ( $200\ \mu\text{m}$ ) assure a high thermal uniformity over the whole

reaction area with a heating rate of  $18\ \text{K s}^{-1}$  and a cooling rate of  $9\ \text{K s}^{-1}$ .

We could demonstrate the ability of these devices for fast thermocycling on different biological test systems (Poser et al., 1996, 1997b).

The integrated optical windows in the lid of the thermocycler chambers allow the on-line detection of the amplification products using fluorescence labeling. A high amplification rate could be obtained using the devices. The results shown in Fig. 4 demonstrate the fourth cycle to be the threshold cycle, where the amplification already reaches detectable quantities of nucleic acids.

## 4. Flow-through thermocyclers

### 4.1. Microsystem technology and microfluidics

In recent years, the combination of microsystem technology and microfluidics has been promoted for various applications, such as filtering (Qin et al., 1998), cell sorting (Sikorski and Peters, 1999), electrophoresis (Colyer et al., 1997; Lagally et al., 2000; Manz et al., 1992), DNA sequencing (Carrilho, 2000; Woolley and Mathies, 1995), immunoassays (Chiem and Harrison, 1997), cell lysis (Waters et al., 1998) and several other application fields (Ehrlich and Matsudaira, 1999; Ehrfeld et al., 1998). Pioneer research groups

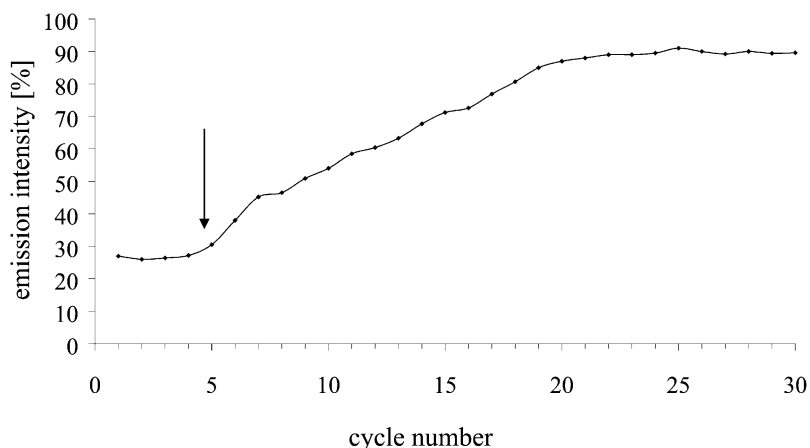


Fig. 4. Diagram of the fluorescence signal of amplified DNA as a function of the number of cycles. At the fifth cycle detectable amounts of nucleic acids are obtained.

such as G.M. Withesides, R.A. Mathies, A. Manz and J.M. Ramsey, D.J. Harrison, A. de Mello and co-workers supported, with their investigations, the development of microfluidic chip devices for the most manifold applications (Freemantle, 1999). The combination of microfluidic components with different elements for temperature and flow measurement offers the possibility to establish complex integrated microfluidic systems.

The future trend is directed toward the realization of miniaturized total analysis systems ( $\mu$ TAS), where all sample-handling steps are performed in an integrated fashion. Unit operations form the basis of  $\mu$ TAS. Basic  $\mu$ TAS systems that combine analysis with a single reaction have been reported (Lagally et al., 2000). Many protocols for genetic analysis require the execution of a complex series of preparative steps. The ultimate manifestation of  $\mu$ TAS is a fully integrated device that receives a sample, performs a multi-step process, and analyzes the result. Such devices will find their application mainly in analytical chemistry with an expansion into other fields such as biology, medicine, materials science, physics, microtechnology and environmental and nutritional sciences (Jakeway et al., 2000; Qin et al., 1998). Micromachining technologies incorporating advanced microfluidic techniques allow faster and fully automated analyses, improved sensitivity, and enhanced precision.

A  $\mu$ TAS device for DNA amplification, where a PCR chamber was connected with a microfluidic system and several vents for sample loading and removing was presented by Burns and co-workers (Burns et al., 1998). In their device the fluid is moved as discrete drops by applying pressure and is positioned by hydrophobic patches (Handique et al., 1997). No information regarding possible cross-contaminations of a consecutive sample application, which might be observed by the used stop-flow mode, was given.

#### 4.2. Surface to volume ratio and heat transfer-microcentrifuge tube vs. capillary tube

The microcentrifuge tube is the standard vessel in molecular biology. However, it is not suitable for rapid thermocycling because of its wall thick-

ness and because of the conical geometry of the tubes, resulting in limited conditions for heat transfer. Also the thermal distribution within the sample is more limited in microcentrifuge tubes than in capillary tubes. There the cross-sections are smaller with, respectively, shorter ways for thermal convection (Wittwer et al., 1994), and the thermal distribution is enhanced according to Fick's law, because the time needed for heat dissipation is directly proportional to the second power of the channel cross-section. On that basis, the time to reach a temperature equilibrium in each temperature zone determines the rate for reduction of the process time. For a channel, which is 40  $\mu$ m deep and 90  $\mu$ m wide, a time for achieving the temperature equilibrium of less than 100 ms has been calculated (Kopp et al., 1998).

The compatibility of wall surfaces is a major problem in the application of chip-thermocyclers. There is a contact area of approximately 40 mm<sup>2</sup> with wall surfaces in the case of a reaction volume of approximately 20  $\mu$ l in a plastic cup or in a well of a microtiter plate. That means, we find a surface to volume ratio, which is in the order of approximately 2 mm<sup>-1</sup> in this classical case for PCR. This ratio increases up to approximately 10 mm<sup>-1</sup>, if stationary chip-thermocyclers with chamber volumes of a few microliters and a capillary slit chamber with a depth of 0.4 mm, nanotiter plates with chamber volumes in the sub-microliter range or flow-through chip-thermocyclers with larger capillary diameter are applied. The ratio increases further up to 40 mm<sup>-1</sup>, if capillaries with a diameter in the range of 100  $\mu$ m are used.

The use of glass and silicon for the construction of chip-thermocyclers is mainly due to their thermal properties, besides their ability for common use in micromachining technologies. Silicon possesses a very high heat conductance, whereas glass is a comparatively bad thermal conductor. Therefore, the heat conditions for heat flow in microdevices can be controlled easily by choosing both these materials in the correct geometries. Silicon can be used as wall material in order to construct regions with a very good local equilibrium of temperature. It is also used in order to couple Joule heat originating from thin film resis-



thin film of sample behind the backside of the sample volume. This film can cross-contaminate the next sample, which will pick up this small volume of the film. The thickness of the remaining liquid film behind the back-meniscus of the sample is related to the internal friction within the liquid, the contact angle of the surface and the flow rate used for sample movement (Litborn et al., 2000). Transporting liquid through a capillary tube with a surface of low wettability, no cross-contamination between different sample plugs appears. However, this effect can be suspended by micro roughness of the surface, contact angle hysteresis as well as chemical affinities. One of the dynamic effects that can lead to hysteresis is slow adsorption and desorption of solute molecules dissolved in the liquid (Israelachvili, 1994). In respect of this fact, a careful choice of the channel material with low affinity to the compounds of the PCR mixture or an appropriate chemical modification is necessary for continuous sample flow.

#### *4.5. Two-phase system for flow segmentation in the serial flow mode*

A concept with the best potential for generating serial sample sequence reducing surface contacts, is the use of a system with two immiscible fluids, a liquid/liquid two-phase system. For this purpose we checked a number of compounds immiscible in water for the usage in PCR. For example, dodecane as carrier flow medium did interfere with the PCR and resulted in low product amounts (no remarkable product yields were obtained). Because of its volatility, an additional adaptation to maintain the flow on a constant rate was essential. Finally the use of non-volatile mineral oil, which was commonly utilized in the past to prevent sample evaporation in thermocyclers without heated lid, led to the ideal sample transport and showed the expected amplification results. In contrast, Litborn and co-workers recently reported a successful DNA amplification using the hydrocarbon octane for generating a carrier flow (Litborn et al., 2000).

Using mineral oil for generation of a two-phase system for sample transfer, we could demonstrate,

that an aqueous sample droplet in a continuous carrier flow of the oil in a hydrophobized channel moves well separated from the subsequent sample droplet as confirmed by the findings of Katsura et al. (2001) who used a water-in-oil emulsion for the formation of droplets as ‘microreactors’. The aqueous sample droplet detaches from the channel surface because the oil forms a thin layer on the channel surface and reduces the surface contact of the sample. The sample easily repels from the surface due to the smaller contact angle, which leads to a lower risk in cross-contamination of samples. In addition, the mineral oil does not interfere with the analysis method of gel slab electrophoresis we used for visualization of amplification results.

#### *4.6. Sample handling and instrumentation arrangement for serial sample sequence*

The sequential injection of samples into a continuous flow, which was developed mainly for chromatographic separation, was recently demonstrated also for the rapid sample loading in miniaturized analysis devices, e.g. the bead injection for detection of enzymatic ligand reaction (Ruzicka, 2000).

For the creation of a serial flow mode we periodically injected small sample volumes into the continuous carrier flow, which was generated by a syringe pump (P-200 module, World Precision Instruments). The sample injection was performed automatically by a precision syringe drive module (PSD/2, Hamilton) with integrated valve for sample uptake and a connected six position valve with a sample loop (PR-750, Rheodyne). The detailed module arrangement has been described previously (Schneegaß et al., 2001). This module arrangement allowed a sample injection free of air bubbles, but the valve composition possesses a comparatively large dead volume of a few microliters preventing ultra-high sample sequence. For a higher sample throughput the use of an adapted ‘T’-shaped injector channel is conceivable (Weigl and Yager, 1999; Li and Harrison, 1997) or of a ‘wedge’-shaped injector channel as reported for capillary electrophoresis (Bousse et al., 2000). The fast sample injection for

capillary electrophoresis attained by an electrokinetic flow was recently reported by several authors (Alarie et al., 2001; Kwok and Manz, 2001; Smith et al., 2001; Attiya et al., 2001).

#### 4.7. Surface passivation, PCR mixture composition and effect of additives

Achieving a fluid flow without significant bubble formation has been a notable challenge requiring careful attention to surface chemistry (Woolley et al., 1996). As already elucidated above, special attention must be paid to the surface conditions of the fluid channel. Unfortunately, glass as a microchip substrate is less ideal for amplifying DNA because of the proclivity of protein (e.g. *Taq*-polymerase) to be deactivated by adsorption to the surface. Adsorption of molecules in solution to surfaces can be influenced by regulating the kind and number of binding groups on the surface. This feature can often be changed by chemically modifying surfaces or by addition of certain solute molecules into the medium that adsorb on the surface. For example, addition of surface active molecules such as detergents to water can cause a wide spectrum of contact angles (Israelachvili, 1994). Beside hydrophobizing procedures of the channels by different silanizing reagents like hexamethyldisilazane, trimethylchlorosilane or octadecyltrichlorosilane, we supplemented the PCR mixture with several additives with different effects. Tween 20, a non-ionic surfactant, was added to the PCR mixture to reduce the surface tension, thus stabilizing the sample droplets in the oil flow and simultaneously the polymerase during PCR (Katsura et al., 2001; Chiem and Harrison, 1997). The stabilization of the annealing of primers and the polymerase to the DNA template was supported by glycerol and bovine serum albumin (BSA), which also serves in preventing denaturation of the polymerase on the channel surface by deactivating of possibly available reactive groups on the surface (Taylor et al., 1997). The addition of polyethylene glycol (PEG) for preventing protein adsorption is also described by several authors (Giordano et al., 2001). On the basis of the application of the described methods for modification of surface effects, we

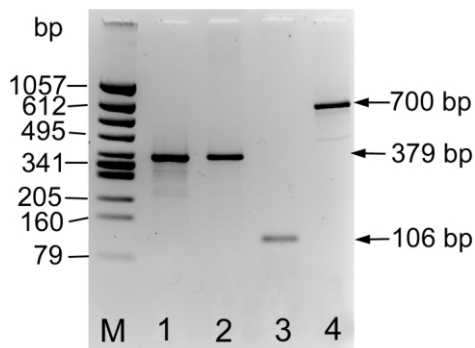


Fig. 6. PCR products of three different PCR systems amplified in the flow-through PCR chip (lanes 2–4). Product detection was performed by gel electrophoresis in agarose gels (3%); In lanes 1 and 2, the product yield and specificity of the flow cyclers is compared with that of a conventional thermocycler and is represented by the example of the GAPDH system. M, marker; lane 1 and 2, product of the gene encoding for the glutaraldehyde 3-phosphate dehydrogenase (GAPDH); lane 3, product of the CATCH system (a completely synthetic system [Cooperative Amplification of Templates by Cross-Hybridization] for investigations of evolution in cooperatively coupled amplification systems); lane 4, the Miz1-KO system (system that encodes for a zinc finger protein relevant in human pathogenesis).

could demonstrate amplification results in our miniaturized devices with an adequate product yield and specificity comparable to the results achieved in a conventional thermocycler (Mastercycler gradient, Eppendorf) (Fig. 6). This was proved for several biological systems (Poser et al., 1999; Schneegeß et al., 2001).

Combined with the progress in developing devices for genetic diagnostics, several groups have developed individual methods and protocols of surface passivation for preventing the molecule adsorption and have shown successful DNA amplification in their devices (Giordano et al., 2001; Shoffner et al., 1996; Wittwer et al., 1994; Li and Harrison, 1997; Lao et al., 2000).

## 5. Design considerations for chip-thermocyclers and their construction

### 5.1. Various designs of flow-through thermocyclers — their assets and drawbacks

We have utilized the principle of a continuous

flow thermocycler in designing various flow-through thermocyclers, where pre-mixed samples are transported through a single channel, while they are subjected to thermal cycling.

To design a flow-through thermocycler the following aspects, besides the choice of the material, must be taken into consideration: the number of cycles to be processed, the channel cross-section or variation on it, the length of the channel of each of the three thermal zones, the kind of fluid delivery, the geometry of the heaters, the local position of the thermal sensors, and the kind of thermal insulation of the three zones. The arising design influences the chip periphery which has to be provided, such as the chip mounting and packaging, a detection unit as well as the electric and fluidic control.

According to the requirements of the PCR process, the first design we created, TC1997, was a chip consisting of three sections representing the individual thermal zones (Köhler et al., 1998). In this first layout of a flow-through thermocycler the flow channel was worked out in Si(100) and covered by glass (Fig. 7). There the thermal zones for 16 cycles were arranged in the order of the denaturation zone, followed by the annealing zone and the extension zone. The layout resulted in a very narrow area, where the temperature reaches the temperature set point due to high temperature gradients within each thermal zone. Also the residence time of the sample was reduced in comparison to the time which was previously estimated according to design. We obviated this problem by the exchange of the annealing zone with the extension zone whereby the temperature profile of the temperature transitions was improved. The insulation of the thermal zones was performed by inserting glass chips using the lower thermal conductivity of the material. The channel part between the outer zone of extension to the opposite denaturation zone was realized by a 'connecting channel part' with a 16-fold decrease of cross-section to accelerate the flow in this pass-over area (Fig. 8). Thus, a prolonged stay of the sample in the annealing zone after the primer extension could be avoided. The drawback of this construction is shown in the flow behavior. Because the channel was fabricated in silicon, the

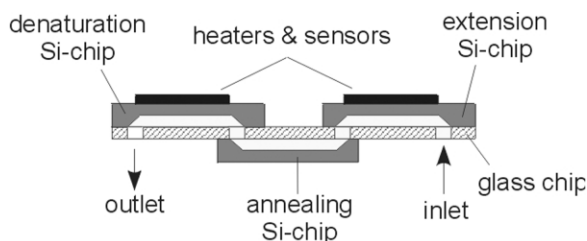


Fig. 7. Schematic sketch of the cross-section and the construction principle of the flow thermocycler design TC1997 consisting of three Si-chips, which represent the individual thermal zones. The annealing zone is coupled to a cooling block. Thermal insulation is achieved by incorporating a glass chip with drilled holes and additional thermal gaps.

channel loop turns featured angular structures due to the anisotropic etching of silicon. Furthermore, the discontinuous cross-section of the channel resulted in formation of air bubbles at the border of cross-section changes and also at the sharp-edged channel turns. Thereby, a continuous flow rate of the sample could be generated only with difficulty due to flow disturbances by bubble formation. In the case of the serial flow mode, turbulence and mixing of the carrier flow and samples was observed. Therefore, this design could be used for a continuous sample flow with a few limitations, but its application to a serial flow mode with a consecutive sample sequence was excluded.

In the next chip, TC1999, which was designed with channel loops for 25 amplification cycles, the

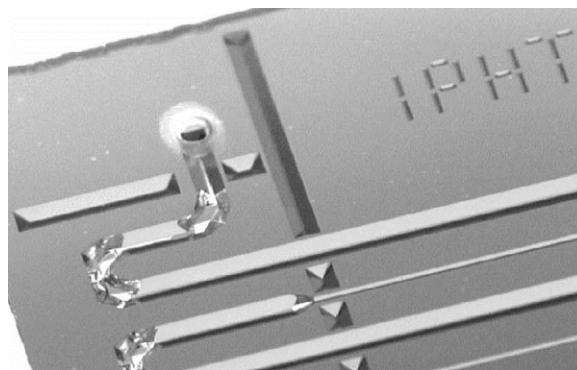


Fig. 8. Photograph of a section of the thermocycler chip (TC1997), where the variation of the channel cross-section for accelerating the flow in the intermediate zone and several thermal gaps for isolation of the zones are shown.

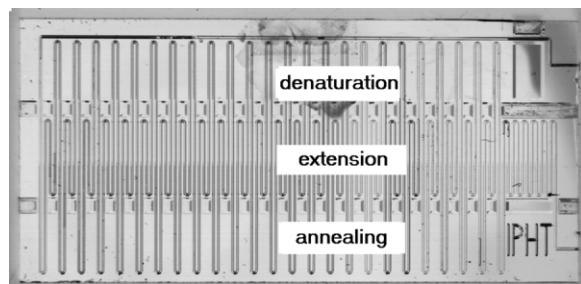


Fig. 9. Top-view of the flow-through thermocycler (TC1999) with the fluid channel layout for 25 PCR-cycles and the individual temperature zones insulated by thermal gaps.

extension zone was arranged in the chip center, the denaturation and the annealing zone at the opposite sides. The outer dimensions of the chip-thermocycler were 26 mm × 50 mm. The cross-section of the channel was constant and without any variation. In this case the channel was made of glass and possessed smooth edges due to the fabrication technology. The channel of the extension zone, positioned in the middle of the chip, was double-looped to enlarge the channel and thus the residence time of the sample. An additional part of a loop for primer extension was integrated at the outlet side of the chip layout to ensure a completed product synthesis. At the inlet side an additional channel over the whole length of the denaturation zone is integrated in order to realize a complete initial denaturation of the template DNA prior to the first PCR cycle (Fig. 9). The application of this chip design to the PCR process demonstrated a very good conformity of the results of the conventional and the chip device (Poser et al., 1999). A further im-

provement of the chip design was made by re-designing the loops of the primer extension zone from two longitudinal loops to nine smaller transversal loops (Fig. 10). Thus, the thermal behavior was improved by avoiding the sample to move from the border of the primer annealing zone to the border of the denaturation and back to the border of the denaturation zone and thereby generating a relatively high, wavelike temperature gradient within the extension zone in which the DNA molecules, moving toward the annealing border after the turn to the denaturation border, might be already partially denatured resulting in less amplified molecules. The new layout of the channels in the extension zone realized a continuous thermal gradient, which is favored for DNA synthesis in the extension zone. Although initially conceived as the better design, this could be confirmed mainly for the thermal behavior, but not in every respect for the flow behavior. In this design, the number of channel turns, which are involved in a higher affinity for molecule adsorption because of the flow behavior, was increased. On this basis, the previous chip design was more advantageous according to the flow behavior of small sample droplets in the serial flow mode. A distortion of the sample plugs at the channel turns could be reduced, resulting in a more uniform sample flow correlated to a high reproducibility of amplification yields. The thermal isolation of the individual zones was realized by thermal gaps in the silicon, respectively, to the previous design.

The recently new-designed flow-through thermocycling chip, TC2000, consists of a minimal

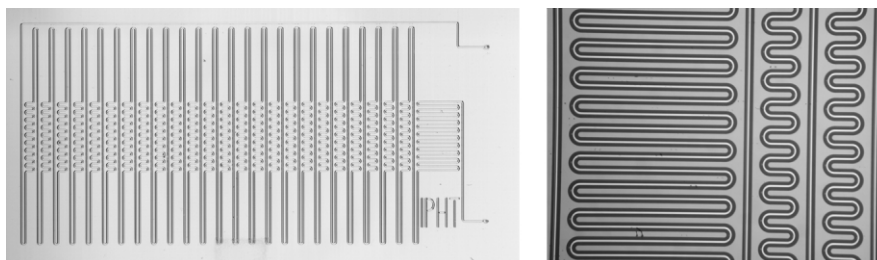


Fig. 10. Top-view of the flow-through thermocycler with a fluid channel layout for 25 PCR-cycles and the individual temperature zones insulated by thermal gaps and aside an enlarged detail of the looped channel of two extension zones as well as an additional extension zone. The layout of the channel in the extension zone is redesigned in comparison to the flow cycler design TC1999.

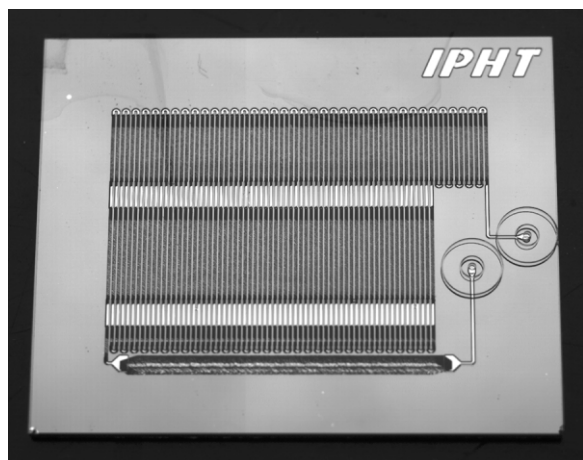


Fig. 11. Top-view of the flow-through thermocycler chip (TC2000) with a fluid channel layout for 32 PCR-cycles. The fluid channel alternately passes the upper glass-chip and the bottom chip made of Si (110). The part, which is located at the glass chip serves for thermal insulation in addition to incorporated thermal gaps.

looped fluid channel, which is made of glass as well as of Si(110) (Fig. 11). The advantage of the use of Si(110) is found in the possibility to manufacture channels with two rectangular side walls and two opposite sites, which are sloped at a wide angle. This construction supports a smooth flow transition from a silicon channel into a glass channel. The insulation of the thermal zones is performed by leading the channel through a glass substrate, additional to the thermal gaps as prepared in the previous chip layout. This layout combines all important advantages of the previous designs and improves the flow behavior and

fluid control by avoiding the discovered drawbacks.

To check the efficiency of the created layouts, the amplification tests were conducted using various biological systems with different lengths of templates and products as well as diverse cycling parameters as described previously. To compare the amplification yield and specificity, the PCR in the chip device was always accompanied by a control sample in a conventional thermocycling device.

In conclusion to our experiences in designing a flow-through PCR chip, special attention must be paid not only to parameters concerning the flow behavior but also to the heat distribution and dissipation to obtain a chip device which is suitable for continuous sample application as well as for serial sample flow mode.

Comparing few characteristic parameters of the various outlined designs of flow-through chips in correlation to the level of miniaturization, the last design proved to be the best. In Table 1 defined parameters like the number of cycles or channels respectively ( $N$ ), the area occupied by them ( $A$ ), the lateral distance between the loop of two cycles, the cross-section area of the channel (XSA), and the relative thermal resistance ( $R'$ ), i.e. the thermal transmission from one to the other thermal zone calculated in consideration of the remaining substrate beams, is put into correlation. The characteristic parameter as a function of selected features specify the efficiency of miniaturization by keeping the rules for PCR. The higher the value of the specific parameter the

Table 1

Comparison of characteristic parameters of three different designs of flow-through devices correlated to the level of miniaturization

			TC1997	TC1999	TC2000
Number of cycles	$N$		16	25	32
Volume per cycle	$V$	( $\mu\text{l}$ )	3.4	1.5	2.0
Total volume	$V'$	( $\mu\text{l}$ )	54.4	37.5	64.0
Lateral extension per cycle	$l$	(mm)	2.3	1.8	0.56
Cross-section area of channels	XSA	( $\text{mm}^2$ )	0.113	0.021	0.1
Occupied active area	$A$	( $\text{mm}^2$ )	1196.0	1404.5	260.6
Relative thermal resistance between the zones	$R'$	( $\text{mm}^{-1}$ )	9.419	4.363	17.857
Characteristic parameter	$N \frac{V'R'}{A}$		0.428	0.117	4.385

better the achieved miniaturization (Zieren, M., 2000, private communication).

### 5.2. Material, geometry and power parameter of thermal transducers

The thermal sensor elements manufactured by thin-film deposition normally possess a resistance value, which can slightly differ from the theoretically calculated value. This is caused by inhomogeneity of the layer structure, minor changes in structure transfer from the lithographic mask or slight variations of the layer thickness. Therefore, for temperature measurements the sensor elements have to be calibrated prior to use, in order to refer different resistance values to their correct temperature. We favored the use of platinum as transducer material, because the characteristic of the integrated platinum resistance sensor is linear in an operating range from room temperature up to 100°C with a high temperature coefficient of the resistance (Poser et al., 1997b). In this manner, with knowledge of slope and offset, a simple calculation and monitoring of temperature can be realized. The nickel–chromium in contrast, has a much lower temperature coefficient and was therefore excluded from the application.

The thin-film platinum heating resistors of the first flow-through layout, TC1997, consisting of three individual Si-chips, were designed, according to our knowledge, from the chamber thermocycler (Fig. 12), using meandered geometries of the heater channels. They were designed for 14.6 and 16.6 W maximum power for the zone of primer extension and denaturation, corresponding to resistances of 220 and 250  $\Omega$  in the case of 16 cycles. The primer annealing zone was connected to a cooling block to avoid overheating from the adjacent zones. Hence, for this chip no active heating element was necessary.

A serpentine geometry was selected to save space, to cover a broad area of the chip with heaters and to realize resistive sensors with an increased resistive length on a small area. The transducers convert electrical energy into heat

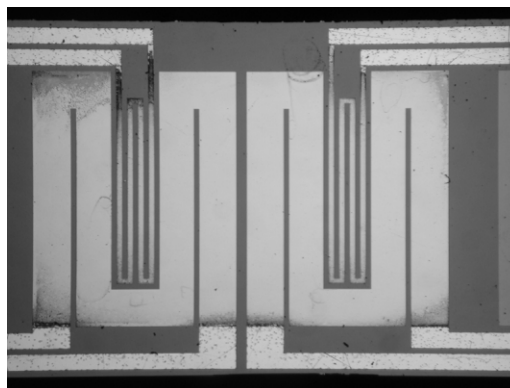


Fig. 12. Thin-film platinum transducers with meandered geometries. Two symmetric structures consisting of wide heaters and fine sensors are shown. They were mainly applied to the miniaturized chamber thermocycler chip.

utilizing Ohm's resistance. For Ohm's resistance ( $R$ ) the following equation is valid:

$$R = \frac{\rho l}{A} \quad (3)$$

where  $\rho$  is the specific resistance,  $l$  the length of the transducer element and  $A$  the cross-section. Assuming the resistive cell to have a quadratic form ( $l = b$ , where  $b$  is the width of the transducer element) and  $A = hb$  ( $h$  is the layer thickness); then the quadratic resistance  $R_Q$  results in:

$$R_Q = \frac{\rho}{h} \quad (4)$$

The value of the quadratic resistance calculated with the specific resistance of compact platinum using Eq. (4) is  $R_Q = 480 \text{ m}\Omega$ . Including the number of quadratic resistive cells  $n$ , the overall resistance  $R$  can be calculated

$$R = nR_Q \quad (5)$$

The maximal current density  $S$  is determined by the current density of the migration, which is determined for platinum with  $S_{\text{Pt}} \sim 10^9 \text{ A m}^{-2}$  (Dagge et al., 1996). The maximal achievable

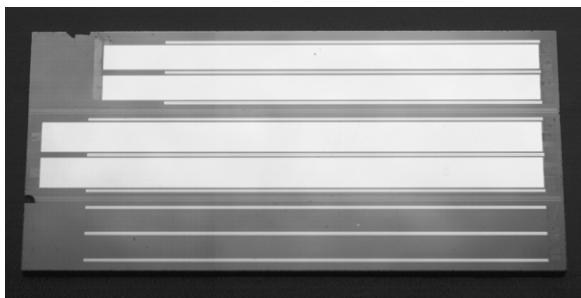


Fig. 13. Back view of the silicon chip of the flow-through thermocycler with integrated Pt thin-film transducers. Heaters are displayed by wide structures and sensing elements by fine structures.

power  $P_{\max}$  for the thin-film heater is calculated by:

$$P_{\max} = R(S_{\text{Pt}}bh)^2 \quad (6)$$

Further designs were equipped with parallel heating channels with a thickness of  $0.1 \mu\text{m}$  to create the heating areas on which a set of fluidic channels was arranged (Fig. 13). For the given resistance of  $20 \Omega$  of the platinum heating element per zone where a maximum operating voltage of  $17 \text{ V}$  is applied, a maximum power of  $14.45 \text{ W}$  results for the heater of each thermal zone.

The resistive transducers were positioned on the silicon part of the chip devices, because of the high temperature conductivity of the material, whereby the wafer thickness of less than  $400 \mu\text{m}$  used was negligible for thermal transition. For accurate temperature control, each heating element is flanked by two sensing elements to observe the whole area for thermal homogeneity. The temperature distribution over the whole chip was checked by an IR-thermocamera.

## 6. Fabrication of chip devices

The combination of silicon–glass technology and thin-film elements enables the fabrication of thermocycler chips with a high thermal stability and sufficient homogeneity of the temperature over the whole area. It allows the performance of a single thermal cycle within the range of a few

seconds. We fabricated the devices by using standard microphotolithographic techniques (Chen and Pépin, 2001).

### 6.1. Channels in glass

Etched channels into glass are often preferred to channels in Si(100), because they show smooth and rounded edges and curves as well as constant cross-sections. A rounded shape of channel loops should support not only a continuous flow PCR, but also a homogeneous flow behavior of a serial flow regime. For preparation of channels in glass, borosilicate glasses like Pyrex (Corning #7740) and Borofloat® (Schott) were used; the glass wafers were  $400 \mu\text{m}$  thick and polished. A solution of  $\text{HF}/\text{HNO}_3$  was used to etch the glass to a depth of  $100 \mu\text{m}$  through a Ni/Cr metal mask. According to isotropic etching, a  $100\text{-}\mu\text{m}$ -deep channel was approximately  $240 \mu\text{m}$  wide at the surface.

### 6.2. Channels in silicon

Fluidic channels in silicon were fabricated by using a  $\text{Si}_3\text{N}_4$  layer for masking. Thermal gaps were etched  $470 \mu\text{m}$  into the front which was faced to the channels after bonding. The remaining membrane of  $30 \mu\text{m}$  was mechanically opened after chip completion. A very narrow silicon beam between the thermal gaps served in covering the glass channel. At the reverse of the silicon wafer a metal layer of platinum for fabricating the thermal transducers and an aluminum layer for generating bonding pads, both  $0.1 \mu\text{m}$  thick, were deposited by sputtering.

### 6.3. Chip assembly

The prepared chips were assembled by anodic bonding. Fluid connections were prefabricated mechanically and attached by glue bonding on top of the glass chip, where holes were drilled by ultrasonic drilling. The careful alignment was of prime importance, especially in the case of the chip designs, where the fluid channel crosses either both substrates or different silicon chips and the correct alignment was essential for a consistent fluid stream (see design TC1997 and TC2000).

## 7. Plastics as material of choice for fabrication of chip devices

### 7.1. Fabrication of microfluidic devices and chamber thermocycler chips in plastics

Silicon and glass has been the most popular material for fabricating chip devices but increasingly, plastic materials appear to be more and more attractive for fabrication of devices for biomedical diagnostics because the materials are cheap, they enable the manufacturing of single use, disposable devices by technologies, which are not limited to formation of only planar structures and do not require routine access to cleanrooms as in the case of silicon and glass (Quake and Scherer, 2000; Chou et al., 1999; Unger et al., 2000; Becker and Heim, 2000). Whitesides and co-worker have written several recent reviews on the alternative microfabrication strategy of soft lithography and gave an overview of the most important technologies covering microcontact printing, micromolding, microtransfer molding, replica molding, solvent assisted micromolding, and near-field contact-mode lithography (McDonald et al., 2000; Qin et al., 1998).

With progress in the development of chip-thermocycler for the batch mode, several groups introduced the use of the alternative polymer material for preventing the described problems of surface effects and biological incompatibilities, since polyethylene for example is used as tube material in conventional thermocyclers (Taylor et al., 1998; Woudenberg et al., 1996). Special efforts were made on the field of combination PCR with capillary electrophoresis, where both modules were fabricated mainly in poly(dimethylsiloxane)

(PDMS) (Effenhauser et al., 1997; Gawron et al., 2001), but also in other plastics such as polycarbonate or poly(methylmethacrylate) (PMMA) (Lee et al., 2001). The combination of PDMS and glass in a hybrid chip device was also investigated (Hong et al., 2001).

### 7.2. Fabrication of a flow-through thermocycler chip in PDMS

We utilized the transparent elastomer PDMS (Sylgard 184, Dow Corning) for micromolding a flow-through thermocycler chip. PDMS is known for its low interfacial free energy, low reactivity and the possibility of conformal contacting and easy releasing (Chen and Pépin, 2001). As a pre-master we used the microfabricated glass chip with the fluid channel of our designed device, which was chemically modified by a silanizing reagent to support easy repelling of the polymer. The resulting mold was silanized and used as a final master. Thus the chip part holding the fluidic channel could be precisely molded, as demonstrated in comparative pictures of a fluid channel cross-section in silicon and the analog in PDMS molded channel (Fig. 14). A second chip was prepared for covering the channel and facing the heater platform by using the spin-on technology to create a comparably thin layer of PDMS (less than 80  $\mu\text{m}$ ) on a silanized glass chip, which was removed after bonding. To bond both PDMS parts to a complete chip device, a technique for activation of the PDMS surface by treatment in a  $\text{O}_2$ -plasma was used (Duffy et al., 1998; Patel et al., 1999). The treatment with plasma and the subsequent assembly of the parts led to their irreversible binding due to the formation of covalent

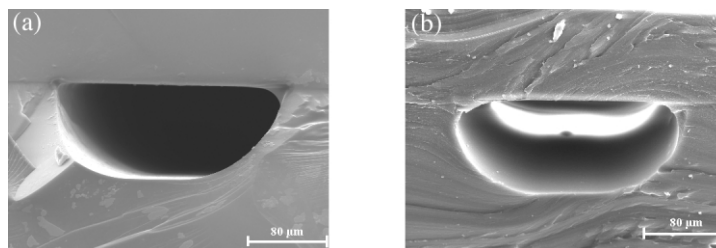


Fig. 14. Scanning electron microscopy photographs of the channel geometry in silicon/glass (a) and in PDMS (b).

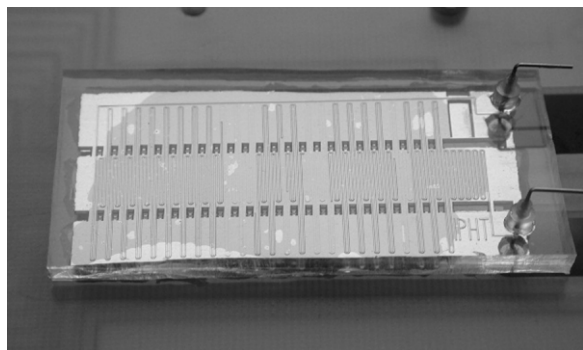


Fig. 15. Flow-through PCR chip in PDMS which is mounted on the platform for heating management and control.

lent siloxane bonds. The resulting chip is pliable, yet extremely durable, biocompatible and rapidly produced. In Fig. 15 the completed flow-through thermocycling chip made of PDMS is shown, which is mounted to the silicon part of the silicon-glass thermocycler with integrated thin-film transducers at the reverse serving as a basis for thermocycling. The used heating platform enables a successful DNA amplification in this disposable device. The property of PDMS easily adheres to all smooth surfaces forming a very close connection supporting heat dissipation.

### 8. *In cell* PCR – a specific application of the flow-through thermocycler at the single cell level

The application of single cells for intracellular amplification of mRNA in our flow-through thermocycler device demonstrated the high potential and relevance of the device in *in situ* PCR, which deals with expression studies of selected genes.

We could show the successful amplification and detection of intracellular mRNA of the gene MDR1 encoding glycoprotein P-170 which is mostly the molecular base of multidrug resistance (MDR) providing a channel for an active efflux of cytostatics out of the cells and is often induced by applied chemotherapeutics within leukocytes of leukemic patients (Dassow et al., 1998; Lassner et al., 1997).

The DNA of the leukocytes was stained by propidium iodide. To distinguish between ampli-

fied and initial non-amplified DNA, specific priming for the MDR1 gene with FITC-labeled primers was performed. Thus according to the detected fluorescence, it could be distinguished on the cellular level between affected cells stained in yellow-green and the ‘healthy’ leukocytes in red (Fig. 16).

The gene sequences were amplified after loading PCR master mix containing fixed and permeabilized leukocytes into the fluid channel. After PCR completion, the intracellular amplification, i.e. the amplification products remain inside the cell due to their size, was analyzed by a fluorescence microscope.

The described example for *in cell* application offers for instance a tool for fast determination of MDR1-mRNA positive cells in the whole blood of leukemic patients for estimation of successful therapies.

A similar basic approach was made by Wilding et al. (1998), when he performed a PCR with white blood cells in a silicon chamber device after the cells were isolated from the blood on a integrated microfilter. However, the chip device was not constructed with the aim of single cell analysis and is therefore not suited for it.

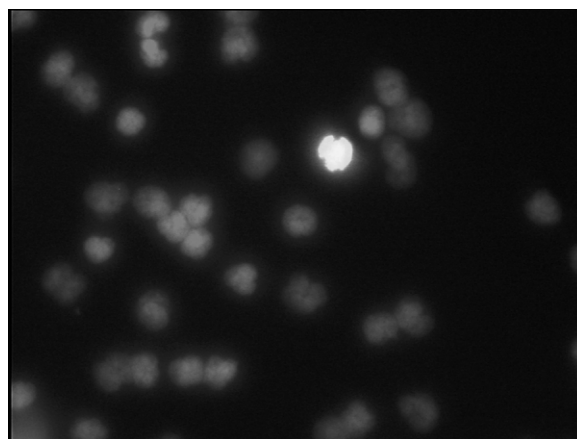


Fig. 16. Photograph of a cell population of leukocytes, which was subjected to *in cell* PCR, where a specific primed sequence is amplified inside the cell. Thereby, FITC-labeled primers (bright spot) were incorporated into amplified products. The background consists of cells with propidium iodide stained DNA (grey spots) where no amplification occurred.

## 9. DNA detection by integrated analytic modules

From most of the miniaturized PCR devices the sample must be manually transferred to an analysis device, increasing the time needed for the assay and the possibility of introducing external contamination.

Integrated systems combining rapid thermocycling with analysis modules such as for capillary electrophoresis address the outlined problem (Jakeway et al., 2000). Toward this goal, several research groups have investigated the integration of detection modules into the PCR device (Mastrangelo et al., 1998). Therefore, the DNA detection on the chip by an implemented optical fiber was mentioned (Woolley et al., 1998). The integration of capillary electrophoresis (CE) has made considerable progress, since the CE belongs to one of the first miniaturized biochemical processes (Woolley et al., 1996; Harrison et al., 1993; Waters et al., 1998) and an analyzer is now available commercially ([www.agilent.com](http://www.agilent.com)). Various detection methods are used for on-chip detection. Laser-induced fluorescence is the preferred detection method of DNA in microchannels. Beside this technique, also other methods such as chemiluminescence (Hsueh et al., 1996) and electrochemical detection (Woolley et al., 1998) are reported.

Currently we are investigating the integration of a detection system with an additional module for chip based separation in our chip design. To our knowledge no integrated module for DNA detection on a flow-through thermocycler-chip is reported to date.

During the last years considerable effort has been expended in developing  $\mu$ TAS devices on the basis of microchamber thermocycling devices with the aim of initiating the construction of lab-on-a-chip devices (Colyer et al., 1997; Lagally et al., 2000).

## 10. Conclusions and prospects

As we have shown, the field of continuous analysis in miniaturized devices offers an opportunity to discover and explore fundamental new

principles for biochemical processing, with the potential to greatly improve the performance, sensitivity, and cost of diagnostic procedures.

It could be demonstrated, that chip thermocycler devices, which are fabricated in glass and silicon and equipped with thin-film transducers for temperature control are powerful tools for rapid DNA amplifications due to their low thermal mass.

Comparing continuous flow chip devices to batch-process chip devices we notice advantages as well as disadvantages on both sides. Therefore, it would be greatly helpful for biomedical and bioanalytical applications, e.g. diagnostics and drug screening, and research on genomics and proteomics to combine continuous flow and batch-process devices.

Concerning PCR in chip devices, special attention must be paid to the conditions of the internal surfaces. For miniaturized chip devices, surface effects are generally pronounced due to the increasing surface to volume ratio. On the basis of an optimal thermal and fluidic design, surface treatments and PCR processing, PCR results, which are equivalent in yield and specificity to a conventional thermocycler, can be achieved for the flow-through thermocycler chip.

Miniaturized continuous flow and serial flow DNA amplification are both very promising techniques for nucleic acid analysis. The application of the serial flow mode with a liquid/liquid two-phase system enables a high-throughput of small samples without cross-contaminations and establishes the feasibility of performing regular screening assays for clinically relevant sequence. The continuous flow amplification was demonstrated to be especially effective using larger sample volumes of a few microliters. However, it could not be proved to be consistently free of cross-contaminations, a problem which was solved by the introduction of the two-phase system.

The reliable operation of the flow-through chip device in amplifying DNA was verified by various amplification systems, and it was proven that flow-through thermocycler chips are suited for rapid  $\mu$ -PCR and are applicable to very different templates. The amplification is efficient and highly selective for all templates; even higher product

selectivity of the chip thermocycler was found for distinct amplification systems in comparison to commercial thermal block devices. The time consumption for amplification is low. Also power consumption of the microchip is small, and the device is capable of being integrated in battery supplied portable compact analysis or diagnostic systems.

A disposable microchip in plastics for continuous flow was fabricated with relatively simple and inexpensive methods by molding of PDMS using a microfabricated master. Its application indicates comparable results to the silicon–glass chip device.

In conclusion, it seems possible to integrate several modules with complementary functions on a single amplification chip and to equip the device with all essential functions for automatic management from the sample load to the final result in order to introduce it into the clinical environment.

In our opinion, flow-through devices and systems will establish themselves as valuable tools in the diagnostic laboratory and, because of their potential for integrated sample handling and multiple sample processing, will greatly expand the repertoire of nucleic acid analysis.

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