Methods and instruments for continuous-flow PCR on a chip

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INTRODUCTION

Since its inception in the early 1980ies by K. Mullis et al. [1,2], for which he was awarded the Nobel prize for Chemistry in 1993, the polymerase chain reaction (PCR) has developed into the standard method for the identification and replication of DNA strands and is nowadays a key element in modern techniques of genetic analysis which finds its application ranging from drug discovery to genetic fingerprinting in forensic laboratories [2,3]. Due to its high specificity, PCR is posed to play a major role in future individualized medicine, namely in point-of-care diagnostics (POC).

The principle of PCR is the in -vitro multiplication of defined strands of DNA with the use of enzymatic polymerase molecules. The duplication of a DNA strand is carried out in three principle steps (see Fig. 1):

In the first step, the original double stranded DNA is split in two single-stranded DNA-molecules. This "denaturation" (or "melting") of the DNA occurs at elevated temperatures of about 90-96°C. In a second step, carried out between 50 and 65°C, small complementary DNA-molecules (so-called primers) attach themselves to defined sites of the single -strand DNA molecules ("prime annealing"). In the third step, carried out around 70°C, starting from the prime location, the polymerase molecules attach themselves to the single-strand DNA and form the complementary DNA strand ("primer elongation"), leading finall to an identical copy of the original double-stranded DNA. These two molecules then undergo the same cycle again and again, which leads to an (ideally) exponential growth of the number of identical DNA-strands which finally exists in a sufficient amount to be used for further analysis or other purposes.

The equipment in which PCR is classically carried out is called a thermocycler. In a small plastic tube, the DNA sample together with the primer and polymerase molecules in a suitable carrier medium (buffer) is repeatedly temperature cycled through the phases described above, in typical applications between 15 and 25 times.

Due to its importance for genetic research, several concepts were developed in the 1990ies to perform PCR in miniaturized devices. The main reason for this development can be found in the fact that the thermal mass which has to be cycled through the various temperatures in a normal instrument is of the order of 0.5 gramms, while the amount of DNA which is really of interest is only 10^8 – 10^{-9} of this. In order to speed up the PCR process, it is therefore advisable to reduce the overall sample volume in order to reduce the thermal response time. As furthermore the reaction speed is determined by the time in which molecules find and bind to each other with the molecular transport being controlled by diffusion, the reduction of the diffusion length by reducing the size of the reaction vessel is advantageous. In a first miniaturization approach, PCR was
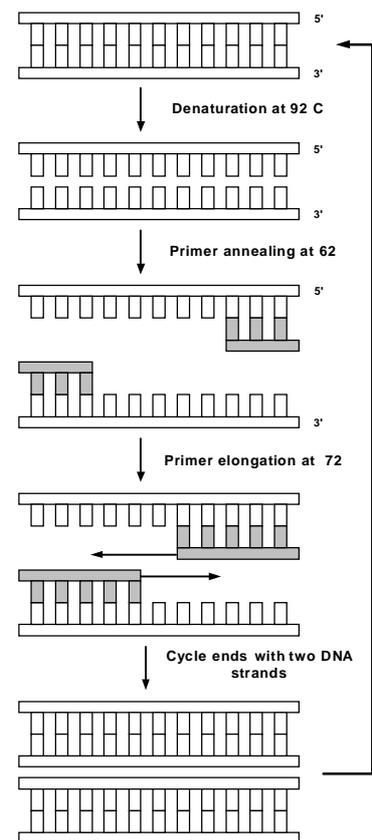

Fig. 1: Diagram of the PCR process

carried out in miniaturized reaction vessels, made with standard lithographic methods out of silicon and glass [4]. This allowed for reduced thermal mass as well as using several reaction vessels in parallel on a single chip due to the small footprint, thus increasing the amount of PCR products.

A more radical approach which makes use of the advantages of miniaturization by introducing a method not possible in the macroworld, was independently conceived by Köhler et al [5] and Kopp et al. [6] in the mid 1990ies. In this concept, the temperature cycling is achieved by transporting the sample in a microchannel through spatially fixed temperature zones. In this concept, only the moving liquid colum with a typical thermal mass of 0.1 mg (for a channel with dimensions of 500μm by 100 μm and an assumed sample plug length of 2 mm) has to undergo the temperatu e change between the PCR phases. Furthermore, this technique allows for a continuos flow of the process instead of the batch - processing of conventional thermocyclers. As a third advantage the possible integration of an analysis step in form of capillary electrophoresis on chip [7] can be achieved.

Most of the above mentioned publications describe continuous-flow PCR chips manufactured in silicon or glass. Fo practical purposes however it is necessary to conceive a system which is capable of delivering the se results with chips that can be used as disposables and hence must be affordable in microfabrication. Polymer microfabrication methods [8], especially replication methods allow the high volume manufacturing of such chips. Some publications already have proven the suitability of polymer microfluidic chips for PCR applications [9 -12]. They however mainly concentrate on the chip itself. In this paper we describe a complete continous flow-PCR system comprising the polymer -based PCR chip, the operating instrument to perform PCR as well as the necessary kits to generate PCR results comparable in yield with existing macroscopic systems but at signicficantly higher speed.

EXPERIMENTAL SETU

The chips used for continous flow PCR can be seen schematically in Fig. 2 and as a fotograph in Fig. 3. They are made out of polycarbonate (PC, Makrolon CP 2005 from Bayer Material Science, glass transition temperature of 148°C) and wer manufactured by injection molding. The mold insert was microstructured using standard photolithography with a positive photoresist SU-8 and the subsequent electroplating of the developed resist structure with nickel. The channel dimensions are 500 μm width, 100 μm depth with an overall channel length of 818 mm. After the inlet port (upper right corner of the chip in Fig. 2), the liquid passes through a longer section of the microchannel at the denaturation temperature. The chip then contains 15 temperature cycles, represented by a 15 times repetition of a meandring pattern through all three temperature zones before exiting the chip afte a post -elongation period. The channel part in the denaturing zone measures 8.6 mm, 10.8 in the annealing zone and 18.3 mm in the elongation zone (a ratio of 4 to 5 to 8.5, the phase ratio for a typical PCR protocol), leading to a total length per PCR cycle of 47.35 mm (representing a maximum sample volume which undergoes the thermal cycling of 1.885 μl as compared to a liquid volume of 200 μl in a typical PCR cup). The outer dimensions of the chip are 75.5 mm by 25.5 mm, the size of a microscopy slide for easy interface with standard laboratory equipment. The thickness of the
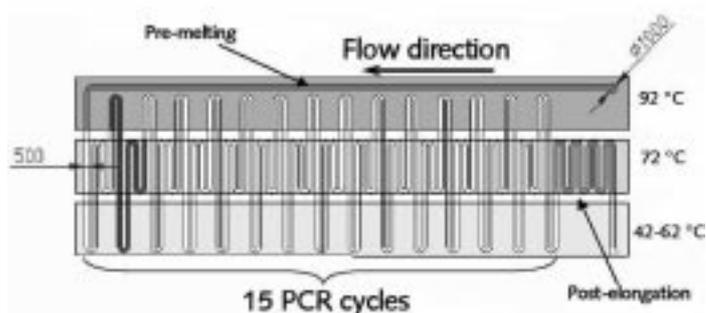

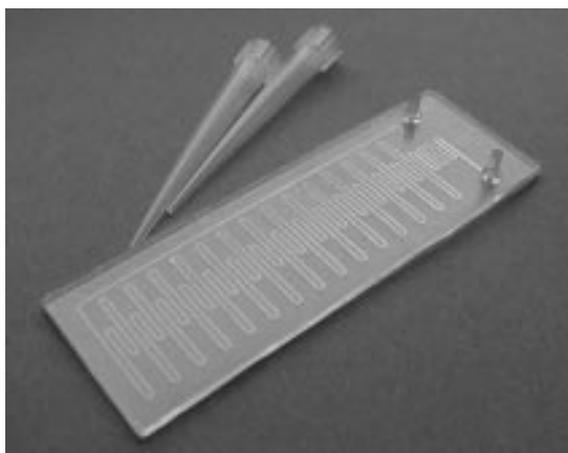**Fig. 2**: Schematic drawing of the PCR chip.

Fig. 3: PCR Chip

structures part of the chip is 1.5 mm, the microchannels are covered by a 250 µm thick polycarbonate film to allow a rapid heat transfer while having a small lateral heat dissipation (see Fig. 7 below). At the in - and output of the channel, olives with an inner diameter of 0.8 – 1 mm for the connection of tubing are integrated in the chip [13], allowing for an easy interfacing of the chip with a variety of pumps.

The thermocycler unit (see Fig 4) allows for two PCR chips per module to be used simultaneously. Each chip sits on three aluminum blocks which can be independently temperature controlled to a measured tolerance of 1°C, having a size of 6 by 69 mm (width × length) with a height of 9 mm, each aluminum block is separated by 1.2 mm from the other. The fixation of the chips onto the thermal blocks is realized using a commercial plastic microscopy slide holder The chip chamber can be closed with a lid to minimze heat loss.

A variety of pump types have been evaluated with the chip, all following experiments were carrid out using a multi - channel syringe pumpe systems neMESYS by Cetoni GmbH. The pumps allows syringes with an inner diameter of 6-30 mm and a drive speed of 0.012 – 6 mm/s, resulting in a minimum volume flow of 0.34 µl/s. The tubing used was standard PE-tubing with an ID of 0.5 mm, the connection of the various system components with the PE tubing was realized using short pieces of silicone tubing with the same ID.

Fig. 4: Chip thermocycler unit which can be equipped with two PCR chips simultaenously.

The PCR products were evaluated using a standard CE agarose gel, the fluorescence signal of the nucleic acid molecules was generated using a transluminator at 265 nm wavelength and the gel images were recorded with a digital camera and evaluated using the software ImageJ 1.34s from Wayne Rasband, National Institutes of Health.

As a DNA sample, a 4 kbp plasmid pCRII (Invitrogen) was used, the polymerase was Thermoprime Plus DNA from Abgene.

EXPERIMENTAL RESULTS: SYSTEM SETU

For a continuous-flow PCR system, a stable and well controlled flow behaviour is of the essence, as all subsequent biochemical results depend strongly on the residence time of the sample at the various temperature zones which are in this concept given purely by the chip geometry and the flow speed. Figure 5 shows the dependence of the time of a single PCR cycle on the flow speed of the liquid for the given chip design.

The flow speeds as a result of the selected volume flows at the pump are given in Fig 6. For the pumps used it is important to show a linear behaviour over the selected flow range.
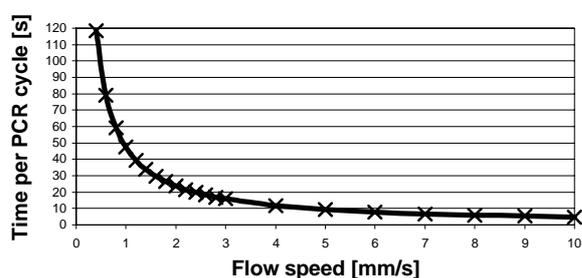

Fig. 5: Cycle time vs. flow speed in a microchannel of dimensions 500 µm by 100 µm.
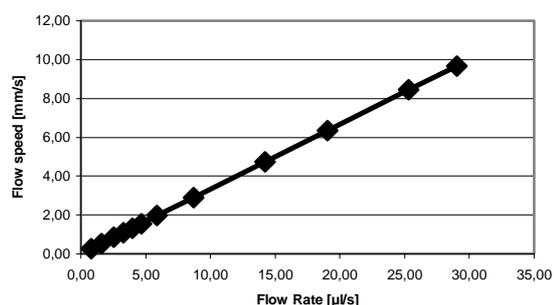

Fig. 6: Flow speed vs. flow rate for the pump/chip system used.

For good PCR yields it is important that the transition from one temperature zone to the other is a well defined as possible. To evaluate the temperature profiles in the system, thermal images were taken using a infrared camera system (THERMOCAM PM 280, Inframetrics). Figure 7 shows the thermal distribution in three different configurations: a) the temperature distribution of the empty aluminum heating blocks, b) the distribution after the 250 µm PC cover foil (this temperature distribution is actually seen by the sample molecules in the microchannel) and c) the distribution on the top side of the 1.5 mm thick PCR chip.
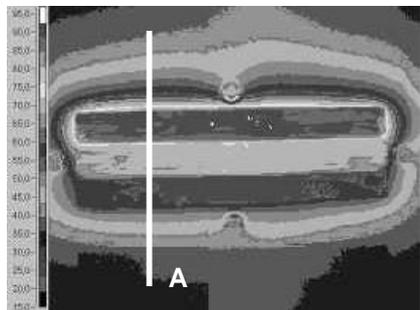

Fig. 7a:Thermal distribution of the aluminum heating blocks.

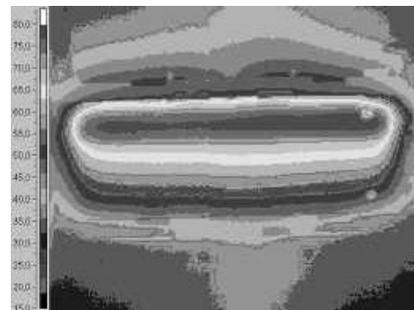**Fig. 7b**:Thermal distribution after a 250 µm cover foil.

Fig. 7c:Thermal distribution on top of the 1.5 mm thick PCR chip.

A measured temperature profile across these images along the indicated line "A" is shown in Fig. 8. As can be seen, the temperature profile after the c ver foil (and hence in the liquid sample) follows closely the temperature profile of the heating elements, creating sharp temperature zones with little lateral thermal diffusion due to the lo thermal conductivity of the polymer. The thermal diffusion in the bulk of the chip leads to a fuzz temperature profile at the top of the chip with no differentiatable zone structure anymore, with heat losses to the ambient air leading to temperatures which are generally 5-10°C lower than the selected temperatures of the heating elements.
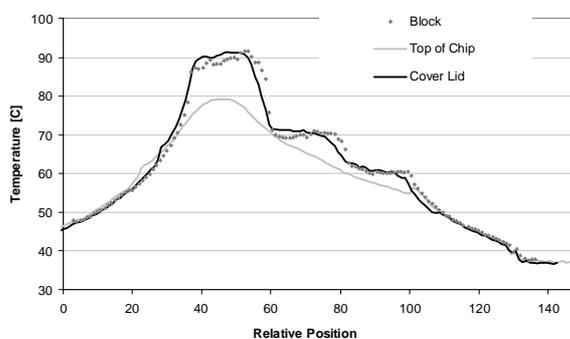

Fig. 8: Temperature profiles along line "A" in Fig. 7 a-c.

EXPERIMENTAL RESULTS: PCR EXPERIMENTS

In a first experiment, the sensitivity of the system has been evaluated using 9 different DNA concentrations between 0.001 ng/µl and 0.2 ng/µl in a 10µl sample volume (equivalent to 10^6 to 2×10^7 molecular copies). The volume flow was set to 3.1 µl/min. The resulting gel electrophoresis image for the 120 bp DNA fragment is shown in Fig. 9. Lane 1 shows a 1 kb ladder as a reference marker, lane 2 a conventional thermocycler result with a 0.1 ng/µl DNA concentration for comparison. Remarkably, already with the lowest concentration (lane 3), a weak fluorescence signal can be detected, with increasing product intensities as the DNA concentration increases. The fluorescence signal vs. concentration is shown in Fig. 10. The typical logarithmic increase of the fluorescence signal with increasing concentration can be seen, with a saturation of the signal above a concentration of 0.1 ng/µl, indicating that all DNA molecules or polymerase molecules available for the reaction have been consumed. Therefore, no further increase in the amount of PCR products can be achieved under the given conditions.
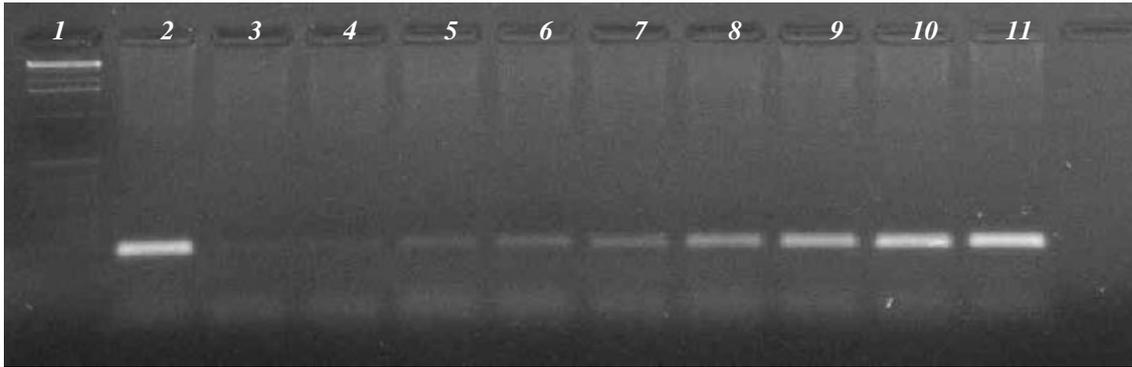
Fig 9: Lane 1: 6 µl Marker (GIBCO 1kb ladder), Lane 2: desktop cycler with 0,1 ng/µl DNA, Lane 3: sample with 0,001 ng/µl DNA, Lane 4: sample with 0,0025 ng/µl DNA, Lane 5: sample with 0,005 ng/µl DNA, Lane 6: sample with 0,0075 ng/µl DNA, Lane 7: sample with 0,01 ng/µl DNA, Lane 8: sample with 0,025 ng/µl DNA, Lane 9: sample with 0,05 ng/µl DNA, Lane 10: sample with 0,1 ng/µl DNA, Lane 11: sample with 0,2 ng/µl DNA**.**
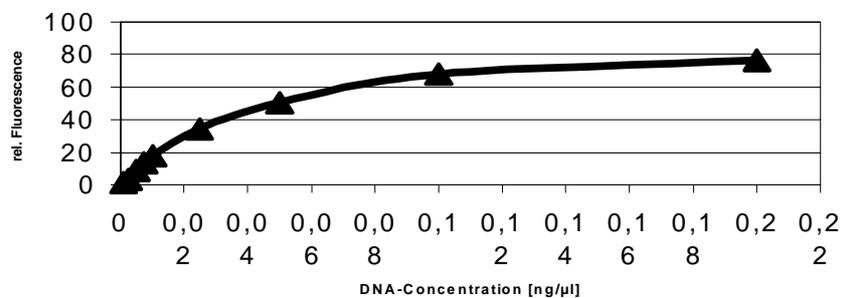

Fig. 10: Fluorescence signal extracted from Fig.9 as a function of the DNA concentration in the sample.

One critical parameter for a continuous-flow PCR is the flow speed of the sample which determines the individual cycle time and thus the overall process time. We therefore measured the PCR yields of a 5 µl sample with a DNA concentration of 0.1 ng/µl with respect to a variation in flow speed in a range between 0.74 mm/s and 7.4 mm/s, equivalent to a overall process time of the 15 PCR cycles including sample transport to and from the connector ports between 24 and 2.3 minutes. The flow speeds used and the resulting cycle times as well as the overall process times, as observed my measuring the flow front of the sample are given in Table 1.

Table 1: Flow speeds and resulting single cycle and complete process times

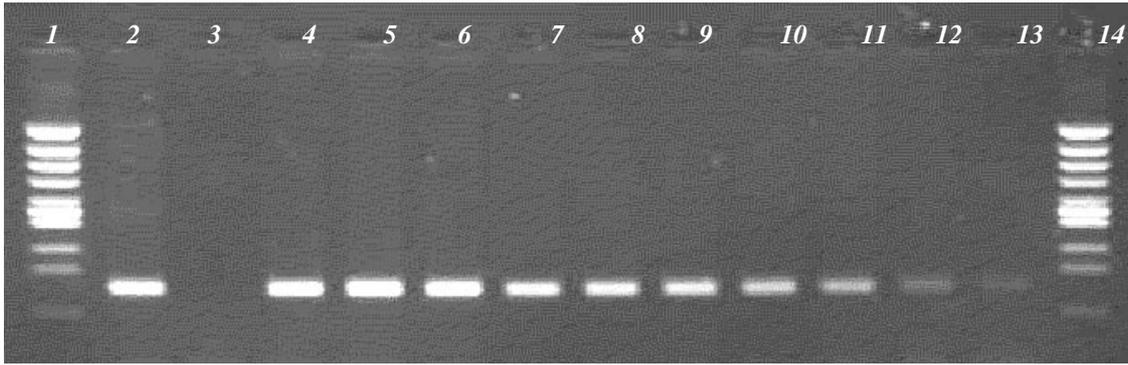| Sample No.	Flow speed [mm/s]	Time for single cycle [s]	Time for complete PCR process [min]
1	0,74	64,0	24,0
2	1,11	42,7	16,0
3	1,48	32,0	12,5
4	1,85	25,6	10,0
5	2,22	21,3	8,0
6	2,59	18,3	7,0
7	2,96	16,0	6,0
8	3,70	12,8	5,0
9	5,55	8,5	3,0
10	7,40	6,4	2,3

Fig. 11: Lane 1, 14: 2μl marker each, Lane 2: desktop cycler, Lane 4: 5 sample with 0,7 mm/s, La ne 5: sample with 1,11 mm/s, Lane 6: sample with 1,48 mm/s, Lane 7: sample with 1,85 mm/s, Lane 8: sample with 2,22 mm/s, Lane 9: sample with 2,59 mm/s, Lane 10: sample with 2,96 mm/s, Lane 11: sample with 3,7 mm/s, Lane 12: sample with 5,55 mm/s, Lane 13: sample with 7,4 mm/s

The results are shown in Fig. 11. Lanes 1 and 14 again show the marker ladder, lane 3 the result of the equivalent sample in a conventional thermocycler. Lanes 4-6 (flow speeds 0.7 mm/s, 1.11 mm/s and 1.48 mm/s) show intensities comparable to the conventional system, indicating similar PCR yields. The fluorescence intensit vs. flow speed is shown in Fig. 12, indicating saturation in the PCR yield at low flow speeds (up to 1.5 mm/s) while for higher flow speeds the intensity decreases, indicating the operating range of the system in the competing parameter field of speed (inversl proportional to the sample residence time in the various temperature zones) vs. reaction yield. Useful amplification could be generated even with the highest flow speed, i.e. detectable amounts of PCR products could be generated within 2.3 minutes as compared to a conventional thermocycler which used 59 minutes for a similar protocol.
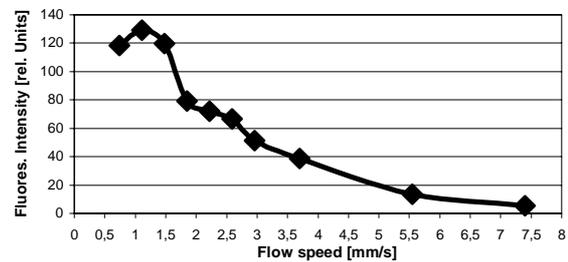

Fig. 12: Fluorescence intensity as a function of the selected flow speed.

Finally tests for the comparison of the chip PCR system with a conventional desktop thermocycler were performed using a variety of commercially available PCR kits. Fo the PCR chip, the parameters determined in the previous experiments, namely a DNA concentration of 0.1 ng/μl and a flow speed of 1 mm/s (resulting in an overall process time of 14 minutes) were used, as they produced PCR yields comparable to the desktop PCR system. The desktop system was set according to a standard PC protocol to 30 seconds denaturation at 95 °C, 30 seconds annealing at 72 °C and 60 seconds elongation at 62 °C wit the addition of predenaturation of 2 minutes and a post-elongation period of 2 minutes. The overall process time for the amplification with 15 cycles was 59 minutes. The resulting fluorescence intensities are shown in Fig. 13, with the left column showing the results of the chip-PCR system and the right column the desktop system. It is important to stress the fact that while the PCR yields show similar results (comparable fluorescence intensities), the chip-PCR system generates these results in a quarter of the time needed fo the desktop system.
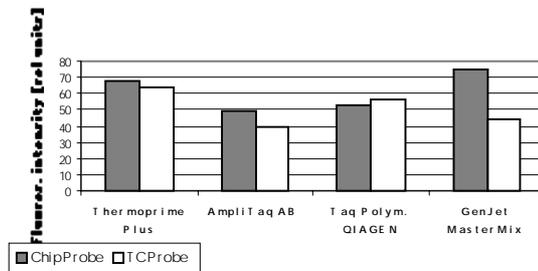

Fig. 13: Comparison of the results obtained with the chip-based PCR (dark columns) and with a conventional desktop cycler

CONCLUSION AND OUTLOOK

We have presented in this paper a complete chip-based system for continuous flow PCR comprising a polycarbonate microfluidic chip, the thermocycling unit for temperature control as well as PC protocols to perform the PCR. We could demonstrate that the described experimental setup is well suited to perform fast reactions with yields comparable to existing macroscopic systems in significantly less time. In a next step, the system will be extended with chips having different number of PCR cycles. A second concept are chips with a freely selectable number of PC cycles (see Fig. 14), where the sample is pumped back and forth across the chip exactly through as many temperature cycles as desired. Furthermore, additional functional intergrations are planned, with chips performing additional on-chip cell lysis fo direct cell DNA amplification as well as on -chip capillary electrophoresis. We are convinced that chip-based PCR methods will have a good chance of becoming part of the daily laborartory routine, particularly if a complete instrument, chip and reagent kit package can be presented.

Fig. 14: PCR chip with freely programmable number of cycles.

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