Chapter 13
Nucleic Acid Amplification in Microsystems

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1. General Elements of Amplification

One of the most basic sources of information in biology is nucleic acid. While the nature of DNA (desoxyribonucleic acid) is comparable to a library where all details are stored, RNA (ribonucleic acid) is more involved in information transfer. In the century of information technology, it is only natural to analyze both kinds of nucleic acids for identifying predispositions (reading DNA) as well as the actual status of a biological system (reading RNA). However, the usually very low number of nucleic acid molecules, e.g., only two DNA strands of a single gene within a mammalian cell, lies beyond detection limits as long as detection techniques do not reach single molecule sensitivity. Moreover, the effort needed for electrical, optical, or chemical signal amplification is so high that such a technique cannot be applied to numerous applications, especially in the field of point of care testing. Hence, the solution is not improving detection limits, but performing target amplification.

For the nucleic acid amplification to occur, some type of copying machine is required, because the sequence of a gene is a priori not known and currently there exists no sufficiently cheap method to read the information that would enable a subsequent synthesization in a more or less chemical approach. At this point, biology helps by providing different kinds of biological copying mechanisms. As a consequence, most amplification strategies imitate microbiological processes, e.g., TMA (transcription mediated

amplification) has its equivalent in reverse transcription, HDA (helicase dependent amplification) in DNA unwinding, SDA (strand displacement amplification) in excision DNA repair, Q-beta-replicase amplification in bacteriophage replication, etc. [1].

In this sense, NASBA (nucleic acid sequence based amplification) is the in vitro version of the natural replication of retroviral RNA, formerly known as 3SR (self-sustaining sequence replication) [2, 3]. The reaction takes place at 42°C and is initialized by binding a primer, a short oligonucleotide strand that serves as a starting point, to the RNA-template. The enzyme reverse transcriptase translates the RNA into a complementary DNA (cDNA) and the RNA of the hybrid is digested by RNAse H. Afterwards, a second primer binds to the previously generated single-stranded cDNA and is extended by the polymerase activity of the reverse transcriptase. In this step, a new promoter is also generated at which the T7 polymerase binds and synthesizes about 40–100 new RNA strands per double-stranded DNA. From this point on, the above steps continue in a cyclic manner leading to an amplification of up to 12 orders of magnitude within 90 min. Since the product of one cycle provides the starting templates for the following cycle, the amplification is in principle exponentially. However, because of depletion effects and generation of side products, this is only true in the beginning. Later, a linear behavior is found that finally saturates [4].

In contrast to isothermal protocols like NASBA, there exists another group of thermal cycling methods with its most prominent representative named PCR (polymerase chain reaction). During PCR, specific DNA sequences are amplified. In the first step, the DNA double helix is denatured at about 95°C, meaning that the DNA is separated into two single strands. In the second step, the temperature is lowered to 40–65°C and two primers, about 17–28 bp long, specifically bind to the single-stranded DNA. During the last step of the cycle, these primers are extended by the enzyme Taq polymerase, cf. Fig. 1. The Taq polymerase is a thermostable polymerase first isolated from *Thermus aquaticus*, a bacterium that lives in hot springs, and is able to generate a double strand from initially one single strand. Hence, the number of DNA strands increases exponentially with the number of cycles. As for NASBA, the exponential behavior finally saturates leading to an amplification of up to 12 orders of magnitude.

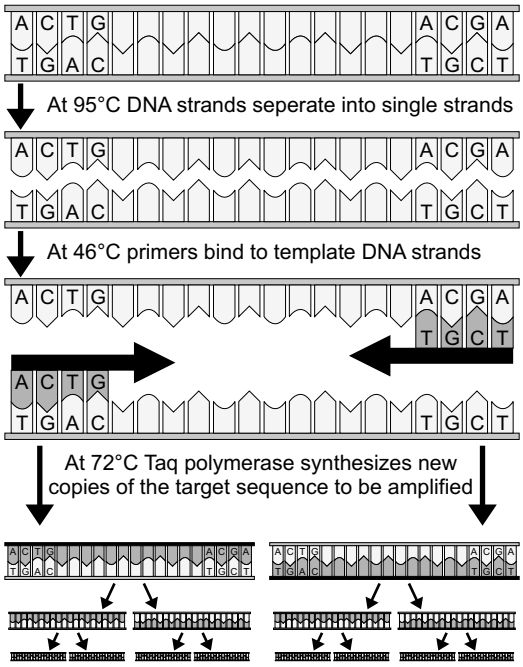

Fig. 1. Principle of the polymerase chain reaction

A possible classification is described by Zhao who identified three different types of amplification processes [1]. The first one is target based, meaning that the target gene of interest is amplified. The second one is probe based, relying on the amplification of a probe analog to a gene sequence. The third type is based on signal amplification where the specificity is not achieved during amplification but prior to it, e.g., by antibody-antigen interaction as a specific binding event known from immuno-PCR. A fourth set of amplification methods, which was not mentioned by Zhao, are unspecific amplifications like the whole genome amplification. Zhao decided not to include this class because unspecific amplification is mainly used as a preparative and not as a diagnostic step unless it is followed by specific assay steps.

Some of these different amplification methods are described in more detail later in this chapter. Nevertheless, it should be noted that the most widely used chemical procedures are too expensive for most amplification methods to be considered economically responsible, except for the fact that they may offer very sensitive analytical assays.

For the detection of amplified nucleic acids, principally most of the microtechnological detection methods can be applied, e.g., optical (fluorescence), electrochemical, gravimetrical (mechanical), or magnetic. These detection methods can be divided into two classes. The first class is based on labels, e.g., fluorescence or magnetic detection. The second class works label-free, e.g., electrochemistry and gravimetry. However, even though detection without labels is possible, labels are primarily used as they provide an increased specificity. One of the best examples is real-time amplification using fluorescent dyes, which indicate the total amount of DNA. The counterpart to this class of detection methods is the endpoint detection. The main representatives here are electrophoresis, including slab gel and capillary electrophoresis, and microarrays. During electrophoresis, the end-point product is separated by size followed by a detection using one of the above-mentioned sensor methods. In the case of microarrays, immobilized oligonucleotides are prepared while the amplified single-stranded DNA is brought into contact for hybridization and final detection. However, in the case of unspecific amplifications, a specific response reaction, e.g., hybridization on an array, is carried out subsequently.

2. Micro–Macro Comparison

By definition, the only element characterizing micro- or nanosystems is their typical length scale. Therefore, direct effects due to downscaling are discussed at first, which, compared to macroscopic systems, lead to generic differences. Further but indirect effects also occur in microfluidic designs and have to be considered, e.g., enhancement of unspecific binding. The third type of differences represents new effects unknown in macroscale, e.g., quantum effects. Since only a macroscopically established process is downscaled, these effects are irrelevant here. Below, typical length scales are identified and followed by a discussion relating to volumetric, two-dimensional, and one-dimensional effects.

2.1. Typical Length Scales

For the direct comparison, a 96-well microtiter plate is chosen as a macroscopic reference system. Here the well-to-well distance is 9 mm. Since the walls have a finite thickness and there is usually additional spacing between neighboring walls, a typical length of 4 mm is defined. This leads to a typical volume of 64 μL. To keep the discussion mathematically simple, for the microsystem a typical length scale of 400 μm is set, which corresponds to a volume of 64 nL. If not mentioned otherwise, this

comparison between the 4 mm and the 400 µm is taken as the standard example for the dissections below. The reader should notice that 400 µm is not the lower limit for miniaturized biosystems at all and might find microfluidic systems with typical length scales that are one or two orders of magnitude smaller, i.e., 40 µm or 4 µm resulting in 64 pL or 64 fL. However, the limit is given by the biology itself, e.g., cell sizes are in the order of 2–10 µm, and because of the small size of enzymes and DNA, approximately a few nanometers, nucleic acid amplification processes also allow nanometer-sized systems.

2.2. Volumetric Effects

Volumetric effects show cubic dependence on system dimensions. Supposing that the concentrations of the used reagents could be kept constant, which is usually not possible, the amount of reagents for a single assay has to be reduced by a factor of 1,000 with respect to the above example. However, using an optical real-time assay, the fluorescence signal intensity is also reduced by three orders of magnitude. But microsystems are usually integrated solutions in contrast to our macroscopic example. This leads to none generic differences that are introduced by special microsystem designs. Components reducing the loss of fluorescent light can be easily integrated and provide a signal enhancement of about one order of magnitude. On the other hand, only by an improved focusing of the excitation light source, the amount of excitation light emitted from fluorescence labels can be additionally increased by a factor of 100. This means that, although the signal intensity is probably reduced in microsystems, integrated components and modified setups can help to reduce the negative light intensity effect.

Assuming that the ratio between active fluidic volume and thermally controlled surrounding material is kept fixed, the volumetric downscaling would result in a reduction of power consumption by the same factor of 1,000. This kind of difference is again not generic, however. As a result of manufacturability and structural stability requirements, the wall thicknesses do not decrease that much during miniaturization. Consequently, the above argumentation cannot be followed. It is obvious that the power consumption will decrease by miniaturization, even if it is in a linear or quadratic rather than in a cubic way. Furthermore, some microsystems have taken the approach of stationary temperature fields, where the fluid itself is transported to individual temperature zones. Because of such a different technological approach, the scaling cannot be applied for estimating power consumption or material changes during the course of miniaturization.

We have assumed above that the concentrations of reagents stay constant, but in many applications the total number of molecules becomes relevant, e.g., in single cell analysis, only two DNA copies for each gene are present. In return, the concentration of such reagents increases by a factor of 1,000 during miniaturization. With regard to the above mentioned example of a single cell, the calculated concentration of the target-DNA, or any other two molecules, is 0.1 nM when choosing a well of 4 μm in size. Since detection limits are often said to be in femto- or ato-Molar range, it could be assumed that detection does not seem to be a critical factor in miniaturized systems. But in this case, not the reagent concentration itself but the total number of molecules defines the detection limits. So, the consideration of the total number of molecules helps to avoid false routes of developments, which may occur when only reagent concentrations are taken into account.

2.3. Surface Effects

Surface effects scale quadratically with the characteristic system size and the most obvious example of this is that the number of spots on an array decreases by a factor of 100 if the spot pitch is kept the same and only the total surface area of the array is reduced. Another example is the signal intensity of a "surface sensor," e.g., an electrochemical sensor, which decreases by two orders of magnitude once its size is reduced by only one order of magnitude. However, the signal intensity loss of an electrochemical microsensor due to downscaling is much less if the corresponding current is not only dependent on the total surface area of the sensor, but also on the thickness of the diffusion layer. In those cases, the above scaling argument becomes invalid because the signal intensity is no longer dependent on the entire area. The two initial examples have been chosen because in most cases the amplification is followed by detection. Again, picking up the point of reagent consumption, the miniaturization allows a saving of a factor of 100 relating to the surface chemistry compared to a factor of 1,000 relating to the volume chemistry. As a consequence, the problem of unspecific binding also becomes more important. Since this is one of the biggest challenges for miniaturized amplification assays, it is scrutinized here in more detail.

Let us assume a molecule of 40 nm in size that finds a binding site every 400 nm on average. A further assumption shall be that equilibrium is completely on the binding site, thus all molecules remain bound once they have reached one. In the macroscopic example, 6×10^8 molecules can bind to the surface that leads to a concentration of 16 pM. In the microsystem example, there exists only 6×10^6 binding sites, but the corresponding concentration

would be 160 pM. One may argue that a change by one order of magnitude is not all that dramatic. But this one order of magnitude is due to scaling only. In terms of the macroscopic example, the reagents are usually transported by pipetting robots and, therefore, all long distances are covered by mechanical and not by fluidic movement. Also, the amplification is carried out without any fluid movement. Hence, the surface that is exposed to the reagents for unspecific binding remains virtually constant, within a factor of 2 or 3 relating to the total surface area of a single well. In contrast, in nearly all microamplification systems, the reagents are mixed and brought to the temperature control zones via fluidic movement [5–8]. Thus, the surface presented to the reagents is increased by two orders of magnitude only by transport processes. In this case, the amount of absorbed molecules is the same as in macroscopic devices, which leads to a concentration of 16 nM compared to 16 pM as in the case of the macroscopic example. For even smaller systems (4 μm), the concentration can further increase reaching a concentration of 16 mM.

2.4. Linear, Timescale and Other Effects

In contrast to amplification, transport and separation processes show a linear dependence on the characteristic system size. Furthermore, during miniaturization, dissipation processes usually increase quadratically divided by a typical length that, on its part, decreases linearly. Thereby, dissipation processes, e.g., diffusion of mass, diffusion of momentum, better known as liquid friction, and heat conduction, become more important with decreasing length and finally dominate. Good examples are the drastically reduced power consumption for heating and the existence of laminar flow instead of turbulent flow. In contrast, inertia effects are volume- dependent and become less important during miniaturization.

Looking at the increase of dissipative processes, an example of timescale reduction due to miniaturization is the fast heat exchange. For the scaling case specified above, a speedup of a factor of 100 is reached if the heat transfer is conduction limited. In the case of power limitation, a reduced heating time by a factor of 1,000 is achieved. However, the first case is more realistic. Using these properties, Kopp et al. presented an ultrafast amplification and even reached the point where the reaction rate of the polymerase became the limiting factor [5]. A second example for this accelerated dissipation is the increased diffusion of molecules from the centre of the fluid toward the walls and vice-versa. In the case of thermal transport, the process is accelerated by a factor of 100 relating to the initial scaling example. A typical diffusion constant for a midsize molecule in water is 1×10^{-10} m^2 s^{-1}, which leads to a typical diffusion time of about 11 h in the macroscopic

system. This means that in the case of an absorbing wall, unspecific binding can be neglected because amplification is generally finished within 1h. Compared to the microsystem, the diffusion time is already reduced to approximately 7 min. Thus, even in fast micro-PCR systems, molecules will certainly meet the walls. In addition, this process is accelerated by the convective exchange close to the wall. In smaller systems, like cavities of 40 μm, which are interesting for single cell analysis, the timescale for the diffusion is further reduced to 4 s. Accordingly, supported by higher surface-to-volume ratios and decreased diffusion times, unspecific binding remains a big challenge in microscopic approaches.

At the end of this micro–macro comparison it is noteworthy that the amplification reactions themselves, and in particular their rates, remain unchanged via miniaturization as long as the concentration and the quality of temperature control stay constant. As a consequence, all isothermal amplification processes that do not rely on surface reactions cannot be accelerated. For example, a NASBA microchip is as fast as its macroscopic counterpart. This means that, very likely, only PCR can reach a strong acceleration via miniaturization toward ultrafast amplification.

The comparison between microscopic and macroscopic approaches can be summarized in four points:

1. Microsystems have the potential for faster operations if the macroscopic amplification is transport limited.
2. Unspecific binding is the biggest additional challenge introduced by miniaturization.
3. The signal intensity in real time approaches is reduced.
4. The amount of reagents needed is also reduced.

3. Microfluidic Realization Methods

The most commonly used and automated PCR systems have been realized in macrosystems and there are several attempts to fulfill the demands of PCR protocols. Generally, those so-called PCR thermal cyclers consist of a specific number of well adapters, a heating block designed for rapid and accurate temperature change, and electronic controllers. The wells can either be tubes or well plates, which are commonly comprised of 96 or 384 wells. In recent years, 1,536-well plates have also been increasingly used. These were already introduced by Sasaki and co-workers in 1997, and each well maintained a reaction volume below 1 μL [9]. To heat and cool the heating block Peltier cells, which can reach heating rates up to

6°K s^{-1} and lower cooling rates of approximately 1.5–4.0°K s^{-1}, are generally used. Using tempered air circulating around capillaries, in which the samples are placed, those heating and cooling rates can also be increased up to 20°K s^{-1} (LightCycler®, Roche Diagnostics). The main advantage of macro-PCR thermal cyclers is the parallelization – meaning the number of amplifications performed at the same time. But they are time-consuming and less applicable for point of care diagnostics or integrated systems.

Because of the price of enzymes, the costs associated with each reaction are still quite high. Accordingly, one main intention to realize microfluidic PCR systems is the reduction of sample amounts. Furthermore, because of their smaller mass compared to macroscopic systems, miniaturized systems show less thermal inertia. As discussed before, this property allows increased heating and cooling rates, which lead to drastically reduced total amplification times.

3.1. Substrates

In the first half of the 1990s, miniaturized biomedical and microfluidic systems, besides intelligent sensors for automotive applications, developed as one of the most concrete and vivid application fields of microsystem technologies. At this time, most microsystem solutions consisted of silicon and the manufacturing was adopted from established processes of the microelectronics industry. This is the reason that the first classification of miniaturized PCR systems was made according to the chosen material, silicon or glass [5, 10]. Later, polymers like polymethylmethacrylate (PMMA), polydimethylsiloxane (PDMS), and polycarbonate (PC) as alternative substrates continued to increase in popularity. Today, there is still an ongoing search for further materials, mainly polymers, which can be used for PCR microsystems. An overview of typically used materials is given in Table 1.

In most silicon-based PCR systems, the temperature control system of a chamber consists of integrated thin film heaters and temperature sensors, which are controlled by a personal computer or an electronic control unit [11]. The integrated setup implies that after every use these chambers have to be cleaned to avoid crosscontamination.

Table 1. Material used for miniaturized PCR systems

Material	References
Silicon	11–17
Glass	5, 18–32
Silicon + glass	8, 10, 13, 32–54
Polyimide	55
PTFE (polytetrafluoroethylene, Teflon)	56–60
PC (polycarbonate)	61–68
PMMA (polymethylmethacrylate)	7, 69–71
PDMS (polydimethylsiloxane)	72–82
COC (cyclic-olefin-copolymer)	7, 83, 84
PET (polyethylene-terephthalate)	85
PP (polypropylene)	86, 87
SU-8	88, 89
Fiber optic face plates	90

Because of high fabrication costs of silicon, a general trend toward low-cost polymeric disposables for micrototal analysis systems (μTASs) is observed, and therefore toward setups where the temperature control system and the PCR chamber itself are not monolithically integrated. The advantages over silicon or glass are lower material costs, flexibility, transparence to visible and UV light, easy molding, and improved biocompatibility due to possible surface modifications. Rapid prototyping can be performed by micromilling (down to 50 μm) and laser ablation (down to 5 μm), which permit frequent design changes. After a design freeze, microinjection molding and hot embossing (for thermoplastics) or casting (for elastomers like PDMS) are good choices for inexpensive mass fabrication. On the other hand, the use of polymers also implies some disadvantages, such as low thermal and electrical conductivity.

3.2. Types of Setup

The first realizations of chip-based PCR systems were presented by Northrup et al. in 1993 and Wilding et al. in 1994 [10, 39]. In the sense of current integrated systems, these microfabricated and silicon-based reaction wells were not fully developed microfluidic systems, but already combined the advantages of miniaturized devices.

The different setup types can be divided into three main groups: the amplification in wells where a sample is injected into a chamber and remains at this position after the amplification process is completed; the amplification by continuous flow-through devices where one or multiple samples are

transported through different temperature zones and collected at the channel outlet; and special realization methods that utilize, for example, buoyancy forces or electrowetting for sample transport.

3.2.1. Amplification in Wells

The basic microstructure of PCR wells consists of a heating element, a well in which the sample is injected, and a cover plate, cf. Fig. 2. To fabricate wells with integrated heaters and sensors, silicon and the long experience in using this material offer great possibilities and has been first utilized by Northrup et. al. [10]. Their device consisted of a reaction chamber, which was embedded in a silicon chip and a silicon nitride membrane serving as the chamber bottom, onto which a thin polysilicon heater was deposited. Finally, the complete chamber was covered by a glass slide and the sample was injected through polyethylene tubing. Up to the present, this basic structure and principle have been replicated and modified by numerous groups [11, 13, 35, 38].

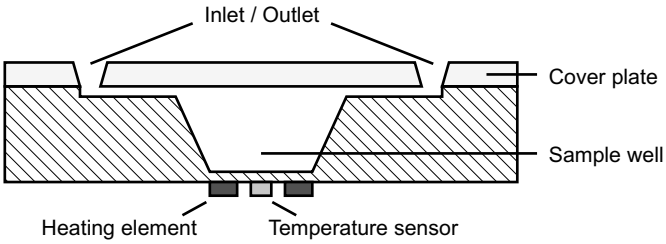

Fig. 2. DNA amplification in wells. First, the sample is injected though the inlet port and positioned inside the well. After the ports are sealed, the chip is heated and cooled according to the PCR protocol. The temperature is monitored by an integrated temperature sensor

These systems differ in the used material and layout. The total volume of realized chambers lies between 40 pL [90] and 50 µL [10] but most designs use wells between 1 and 10 µL [13, 34, 39, 55, 72], volumes that can still be handled with conventional laboratory equipment. In addition, the heat transfer and the temperature distribution of the devices during PCR process have been analyzed by FEM (finite element method) simulations in order to reach higher yields by optimizing the reaction conditions or to reduce the total time needed for amplifications [38, 43, 47, 69, 72, 88]. A fast amplification performed in a well has been presented by Yoon et al. [38]. By using additional cooling fins and a fan, they reached heating and cooling rates of about 36° K s^{-1} and 22° K s^{-1} and achieved 30 cycles within 3 min. Poser et al.

even reached heating and cooling rates of 80° K s^{-1} and 40° K s^{-1}, respectively [13]. By such an acceleration of temperature changes, different groups reached a successful DNA amplification within 10 min or less [11, 69, 73]. It should be pointed out that nearly all presented microdevices require less than 60 min and that the typical temperature precision stated is below ± 0.2°C [13, 38, 44, 46, 49, 77].

Apart from bulky copper heating blocks, most microchambers are equipped with thin-film heaters and temperature sensors made of platinum [34], indium-tin-oxide [25], polysilicon [10, 12, 87], or tungsten [74]. The structures are in direct contact to the chamber and heat can be easily transferred due to thermal conductions. As a result of the low power consumption, a few systems can be operated using commercially available batteries [49, 73]. Additionally, systems based on noncontacting heat transfer, e.g., infrared [26, 55, 91] and induction heating [49], have been realized. In case of an infrared-mediated temperature control, Giordano et al. used a 50 W tungsten lamp, which was focused on the microchamber through a convex lens. A small T-type thermocouple was placed inside the chamber to measure the temperature directly. The total reagent volume was 1.7 µL and 15 cycles were completed within 240 s [55]. Fermér and co-workers performed PCR in tubes using microwaves demonstrating a further possible noncontacting heating source for miniaturized PCR systems [92].

Decreasing the reaction volume required for amplifications and increasing the number of simultaneously performed amplifications are the main efforts to maximize time and cost efficiencies. Zou et al. designed a 16-well system [85]. The reaction chip was made of low-cost polymer and mounted on a thermally nonconductive substrate for thermal insulation between the wells. The substrate itself accommodated independent silicon heating blocks that allowed thermal multiplexing, i.e., different temperature settings for each well at the same time, showing a thermal cross talk as low as 0.2%. Nagai et al. presented parallel amplifications of high density [14–16]. A single test fragment was amplified in 10,000 reaction wells, which were 86 pL in volume and etched in a single silicon wafer. One of the most impressive combinations of sample reduction and massive parallelization has been achieved by Leamon et al. [90]. Their well plate permitted a simultaneous amplification of 300,000 discrete PCR reactions in wells of 40 pL in volume. To provide the PCR product for subsequent processing, it was immobilized to a DNA capture bead. In the case of a uniform temperature protocol for all wells, the achieved temperature uniformity of microarrays can be measured by liquid crystals thermometry, also applicable for characterizing any other heated microfluidic devices [93].

Because PCR is a temperature-controlled and enzyme-catalyzed process, which requires temperatures up to 95°C, the chamber sealing is essential to

avoid evaporation of reagents. For well-based systems, the chambers are often sealed by tape [34, 36, 72], rubber [38], or oil droplets [11, 26, 31, 49, 69, 94]. An advanced yet feasible system was presented by Oh and coworkers [43]. It included four PCR chambers and a world-to-chip microfluidic interface with built-in valves. The sample was injected by a pipette supported by pipette tip guides. Once the samples were loaded on to the microchambers, plastic fittings with rubber sheets were slid to the "sealing mode" position, closing the inlets without dead volume. Phase change valves, another technique for PCR chamber sealing, have been presented by Chen et al. [95]. For this purpose, the used liquid plug can be thermally actuated and either be frozen or melted.

Another approach to perform PCR in microscale is the use of virtual reaction chambers as reported by Neuzil et al. [18]. Here, a 1 μL sample was placed on a glass plate and encapsulated with mineral oil to prevent evaporation of water, cf. Fig. 3. The heating and cooling rates were about 40 K s^{-1} and 20 K s^{-1}, respectively. The surface of such a system has to be hydrophobic as well as oleophobic. Thereby, a small amount of liquid, aqueous or oily, will form a droplet that acts like a closed compartment. For additional transport of those droplets, Guttenberg et al. used surface acoustic waves generated by piezoelectric materials [96]. They first placed the PCR solution and the oil droplet separately on the chip and brought them into contact by moving them toward the same heater element. After amplification, the oil-covered sample droplet was joined with another additional oil droplet encapsulating a sample of concentrated hybridization buffer. As soon as the two aqueous solutions made contact inside the oil, a heater was set on temperature and the hybridization process started.

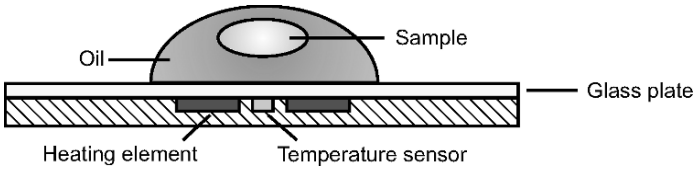

Fig. 3. Virtual reaction chamber. The PCR sample is covered with mineral oil and positioned above chip heater and temperature sensor. The glass plate [18] can also be replaced by other materials or setups [44]

3.2.2. Amplification by Continuous Flow-Through Devices

The second main group of miniaturized PCR systems relies on a continuous flow of reagents through three thermostatic zones representing the three steps of a PCR: denaturation, annealing, and extension. A simple system consisting of a 5 m long, thin capillary tube and a pump was already presented by Nakano et al. in 1994 establishing a basis for chip-based devices [59]. A 50 μL sample was introduced into the capillary and pumped through three different tempered oil baths in a loop. The duration of the three temperature steps was determined by the length of the capillary in each bath, the cross-section, and the flow rate, respectively. Additionally, a constant continuous flow was essential for the reproducibility of the residence time in each cycle. A similar system using a Teflon tube and immiscible organic liquid to separate the sample plugs was also performed by Curcio et al. [58]. Instead of using temperature baths, Park et al. presented a cylindrical assembly consisting of three equally divided thermostating copper blocks and a fused silica capillary, which wound around the block 33 times [24].

The first continuous flow-through PCR integrated on a chip was introduced by Kopp and his co-workers [5]. It consisted of a glass plate comprising etched microchannels in the form of 20 meanders representing the number of cycles. The chip was mounted on three copper heating blocks which were kept at constant temperature, cf. Fig. 4. Compared to amplifications in microwells, the heat inertia is reduced to the thermal mass of the sample itself, thus allowing high heating and cooling rates which reduce the time of amplification. The calculated time for achieving temperature equilibrium inside a channel of a cross section of 40 × 90 μm is less than

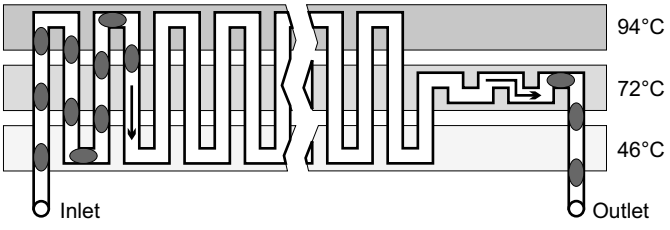

Fig. 4. Basic principle of DNA amplification within a continuous flow device on chip. The samples, denoted by dots, are introduced at the inlet and separated by, e.g., mineral oil for serial flow mode. Three individual heaters are maintained at constant temperatures providing adequate thermocycling conditions. The number of cycles is given by the number of windings and the final cycle is followed by an additional extension step

100 ms [5]. The total amplification time of different devices varies from 30 min [33, 41] down to a few minutes [5, 19, 63] depending on the number of cycles and flow rates.

In contrast to continuous flow, the serial flow mode offers the possibility to enhance the number of samples drastically. To create a serial flow, the samples can be separated by either gas or liquid. Gas–liquid systems pose more challenges concerning the realization of reproducible flow conditions [6]. Segmenting the samples by liquid, the interactions between the liquids themselves and between liquid and wall have to be considered. Once a sample passes a wettable surface, a thin film of it will be left at the wall. The remainder could crosscontaminate the next sample segment after reaching this area. Using a channel wall material with low wettability, the problem of crosscontamination can be reduced or almost eliminated.

The best way to prevent surface contacts of the sample is the use of a two-phase system consisting of immiscible liquids. Since the PCR mixture is mainly based on water, nonvolatile mineral oil, as mentioned above, or other types of organic liquids [97] have been used as a carrier liquid for sample droplets. When using a hydrophobized channel wall surface, the oil will form a thin layer. Thus the direct sample–wall contact is omitted and crosscontamination between individual sample droplets can be virtually excluded. A detailed examination concerning the problem of cross-contamination of droplet microreactors and plug-shape reactors has been presented by Barrett et al. [98]. Creating a two-phase and air bubble-free flow, a typical setup consists of two syringe pumps, one of which constantly provides the carrier liquid and one for the actual sample injection [33]. The final amplification products can either be analyzed by, for example, laser detection [58] or slab gel electrophoresis [5, 20, 33].

Further improvements of continuous flow-through PCR have been presented by Li and co-workers [23]. They developed a microchip including a local velocity control and a heating configuration for a more efficient DNA amplification. By varying the widths of the channel, the flow velocities of the samples can be regulated. Because of the modified channel shape, the transport time of a sample between two temperature zones as well as the time needed for temperature transition can be reduced. A detailed temperature analysis has been performed by Zhang et al. [32]. They compared a glass–glass bonding chip with a silicon–glass bonding chip using FEM simulations. Obeid et al. designed a modified chip adapted for both DNA amplification and reverse transcriptase-PCR analyzing RNA templates [19]. Besides silicon and glass, Schneegaß et al. also introduced a flow-through system made of the transparent elastomer PDMS with the benefit of a disposable unit [33]. To stabilize the flow and to prevent air bubble generation during the initial injection of the sample volume into the microchannel,

Nakayama et al. placed a fluorinated oil plug in front of the sample volume [81]. It helped to increase the internal pressure of the latter solution and led to stabilized flow conditions. Circumventing the restriction on the number of cycles, Liu et al. developed a rotary device, also made from PDMS [74]. A 12 nL sample was introduced into a loop and can be circulated by an integrated pump at a rate of 2–3 revolutions/min. Reaction temperatures were provided by three integrated heaters. A numeric simulation with regard to fluid transport mechanism was presented by Gui and Ren [99]. Instead of using a pressure-driven flow, they presented an alternative approach based on electrokinetic flow.

3.2.3. Special Realization Methods

The majority of microfluidic PCR systems is based either on wells or on continuous flow. But new approaches for DNA amplifications have also proven functional efficiency. Instead of pumping continuously only in one direction, a sample in the shape of a plug can be transported back and forth in an oscillating manner while the microchannel is mounted on three separately controlled temperature zones, cf. Fig. 5. The setting of different PCR protocols regarding plug transport, number of cycles, temperature ramps, residence times in each temperature zone, and, of course, the temperature of each zone itself can be easily performed by electronic control units and is not fixed by the design structure itself. Similar to continuous flow systems, the setup provides a rapid heat transfer from the channel walls to the plug due to the three independent thermostatic zones. Computer fluid dynamics (CFD) simulations of the heating and cooling process indicate a temperature equalization of the plug only within 80 ms [7]. This is not only caused by the short thermal diffusion path but also by the recirculating flow conditions within the plug, which speeds up the heat transfer by convection.

The oscillating plug flow system presented by Münchow and co-workers consisted of a structured polymeric chip that was placed on three heating blocks [7]. To avoid crosscontamination, it is used as a disposable. The sample plug was driven by a ferrofluidic actuator pneumatically connected to the PCR chip. It provided a precise, pulsation-free, and reliable micropump that is essential for an accurate positioning of the plug during cycling. A complete amplification including 40 cycles can be performed within 5 min. Instead, Auroux et al. used a glass capillary mounted on copper heating blocks [21]. The sample was sandwiched between two mineral oil plugs and transported back and forth using a syringe pump.

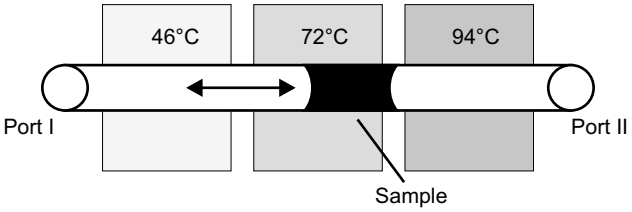

Fig. 5. Transport scheme of an oscillating PCR system. The sample is introduced into the channel and pumped back and forth. Three heaters are maintained at constant temperatures regarding the PCR protocol

On the basis of this concept, a microfluidic chip with an integrated peristaltic micropump has been introduced by Bu et al. [37]. Besides a theoretical model of the pump, a thermal analysis by FEM regarding the temperature uniformity and the location of the heaters has been provided. An oscillating-flow PCR chip based on silicon was presented by Wang and co-workers [8]. They also have theoretically analyzed and simulated the thermal performance of the chip. Chiou and co-workers presented a further actuation principle [60]. By applying different pressure values to the capillary outlets a 1 µl sample was transported between different heating zones. The capillary in which the sample was placed was completely filled with oil. Once the sample was loaded, the capillary was pressurized to 5 psi to prevent sample degassing. By applying a pressure increase of 2.5 psi to one capillary outlet, the sample plug was set in motion. The sample position was identified by additional photodiodes in combination with a laser beam that was coupled into the channel. Instead of three separated heating zones at constant temperatures, Cheng et al. introduced a sample plug that was shuttled along a temperature gradient [84]. The circular microfluidic chip comprising the channel structure was made of PMMA and clamped to a glass heater chip. The heater chip itself consisted of an outer and inner heater ring, which generated a radial temperature gradient of $2° \text{ K mm}^{-1}$. The denaturation, extension, and annealing segments of the microchannel were specifically placed in relation to the temperature gradient to provide a homogenous temperature distribution, cf. Fig. 6a.

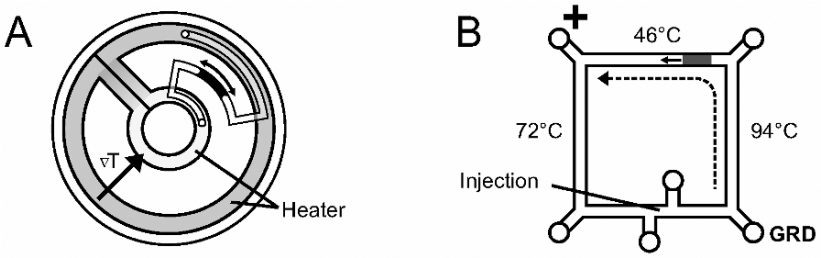

Fig. 6. (a) PCR chip with temperature gradient provided by one heating element. The sample is shuttled back and forth by a connected pump performing amplification. (b) Electrokinetically driven sample plug. After injection, the sample is transported by switching the voltage over to specific reservoirs positioned around the loop

Instead of pressure-driven flow for continuous flow-through or oscillating PCR systems, Chen et al. introduced a polycarbonate microchip using electronically driven pumping [68]. The chip consisted of one circular microchannel, 0.5 µL in volume, which was completely filled with a PCR reaction mix. The DNA templates were injected through a double T-injector by applying voltage to connected reservoirs. By switching the voltage to additional reservoirs, the plug that comprises the DNA target was transported around the loop through three independent temperature zones, cf. Fig. 6b. Completing 27 cycles required approximately 18 minutes at an electric field strength of 300 V cm^{-1}. A PCR system renouncing moving parts or external pumps has been presented by West and co-workers [89]. Here, AC magnetohydrodynamic actuation is used to circulate the PCR mixture through different temperature zones. Such a setup can be advantageous for pumping strong electrolytes or ionic liquids.

After Pollack et al. had demonstrated that droplets containing PCR reagents can be actuated by electrowetting without having a negative effect on a subsequent DNA amplification [100], Griffith et al. presented a computational approach to design a digital microfluidic system in the form of a PCR analysis array [101, 102]. The algorithm is supposed to coordinate the motions of hundreds of droplets simultaneously and chemical reactions accordingly. Furthermore, Zhang et al. presented a performance comparison with SystemC, a software program suitable for high level modeling of digital systems, between continuous flow systems (pressurized flow) and droplet-based systems based on electronically driven liquid handling, e.g., electrowetting [103]. The comparison, which takes into account the system throughput, system correction capacity, and system design complexity,

results in higher performance and less setup complexity of the droplet-based microfluidic system.

Buoyancy forces allow fluid transport without any moving parts and can be induced by only one heating source placed inside a chamber. In this case, a constant temperature gradient creates laminar thermal convection circulating reagents along stationary paths, cf. Fig. 7a. The simplest way of convective polymerase chain reaction has been presented by Henning and Braun [94]. They preheated a chamber to annealing temperature and dipped an immersion heater into 20 µL of the reaction volume. The temperature of the heater stayed constant and generated a continuing recirculation of the reagents between the hot and the cold region, thereby amplifying DNA strands. Previously, Braun and co-workers introduced a similar PCR system [104]. Instead of using a simple heated wire, the thermal convection was induced by an infrared beam that was focused on the center of the chamber. The DNA was replicated 100,000-fold within 25 min.

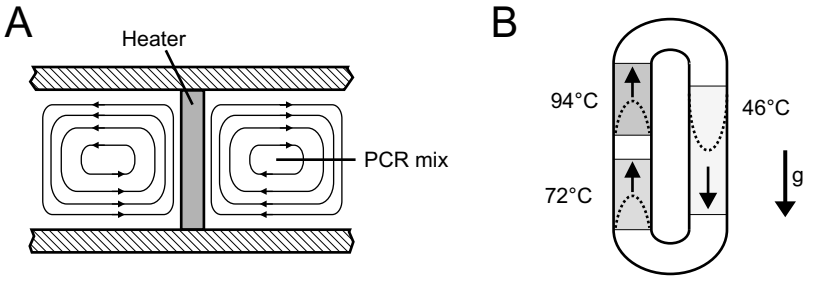

Fig. 7. (**a**) Convective flow within PCR chamber. Only one heating element is necessary to perform DNA amplification. (**b**) PCR chamber using buoyancy forces and gravity (g) to circulate reagents in a closed loop continuously

Krishnan et al. developed a similar system based on Rayleigh-Bérnard convection [70]. It consisted of two heating plates that were constantly heated up to 61°C (top plate) and 97°C (bottom plate). In between a 35-µL Rayleigh-Bérnard cell was placed. As soon as the fluid layer is heated from below, the density of the fluid near the bottom plate decreases compared to the fluid density in the upper part of the chamber. As a result, the top region of the fluid becomes unstable and convective motion occurs. This leads to circulating flow forming convection cells. Thus, amplification conditions were provided and also different chamber designs have been examined by simulation and flow visualization [105]. A different development was introduced by Wheeler et al. [86]. Their transportable convection-based thermocycler utilized natural convective forces to circulate PCR reagents in a closed loop. It consisted of two heating zones set at

94°C and 57°C, respectively. This device was later modified by Chen and coworkers [56]. They added a third heater responsible for the primer extension process, cf. Fig. 7b. Additionally, they presented some modeling in order to predict the fluid velocity and the temperature distribution around the loop using a one- and three-dimensional model. A comparable droplet-based set-up was presented by Walsh et al. [106] and Daly et al. [57]. It consisted of nanoliter sample droplets, which were filled into a loop, embedded in an immiscible carrier liquid. The heated carrier liquid was transported by active pumping and buoyancy forces dragged the sample droplets along the loop.

Further approaches toward miniaturized PCR systems are microreactors in the shape of water-in-oil emulsion [107] which have been used for, e.g., amplification of single DNA molecules by Nakano et al. [108]. Also, amplifications inside single cells have been reported for which the microfluidic technology could provide numerous application areas [109–112]. So far, most miniaturized systems have utilized polymerase chain reaction for target amplification. But an isothermal amplification, called NASBA, has also been presented in the form of a microfluidic system for real-time cancer marker detection [50, 83].

An overview of different realization methods is given in Table 2. Further review articles with regard to continuous flow-through systems [6] or PCR microsystems in general [121–127] can be found in the literature.

Table 2. Overview of different realization methods

Microchamber PCR	10–13, 17, 22, 25, 26, 28–31, 34–36, 38, 39, 42–44, 49, 53–55, 62, 64–67, 69, 71–73, 77–80, 82, 87, 88, 100, 113–117
Open droplet PCR	18, 96
Continuous flow-through PCR	5, 6, 19, 20, 23, 24, 27, 33, 40, 41, 45–47, 57, 58, 63, 74, 75, 118
Oscillating PCR	7, 8, 21, 37, 60, 84
Convective PCR	56, 70, 86, 94, 104, 106, 119
Microarray PCR	14, 15, 16, 61, 76, 85, 90, 120
Electrokinetically driven PCR	68

3.3. Surface Treatments

Although microfluidic systems for biochemical analysis offer the great possibility of reducing the sample amount and reagent consumption, the control of surface chemistry and protein adsorption becomes much more important, as already mentioned above. By decreasing the dimension of the device, the surface-to-volume ratio increases inversely proportional to its size (square-cube law). A fluidic system possessing a characteristic length of 1 m results in a surface-to-volume ratio of 1 m^{-1}. In the case of a length scale of 1 μm, the surface-to-volume ratio increases to 10^6 m^{-1}. That means that the molecules in a microfluidic system are influenced by the wall surface nearly all the time. Target molecules or analytes may adsorb on-the-wall surfaces completely and nonspecifically [128, 129]. These bindings are mainly noncovalent and based on H-bonding, electrostatic, and hydrophobic interaction [130]. Accordingly, different contact angles of the used substrate material and sample can influence the protein adsorption [131]. Because of the size and three-dimensional structure of a protein, the wall absorbance can lead to physical or chemical transformations. These transformations can cause protein modifications that again may result in inactivity or reduced activity of the protein.

Protein adsorption and its prevention are particularly critical problems for DNA amplification in microsystems. Here, nonspecific adsorption can either lead to loss of reaction efficiency resulting in decreased yield or to no amplification at all. Different groups observed that especially Taq polymerase immobilized on the channel surfaces and denatured [7,52]. In this context, Erill et al. presented an analysis of different glass–silicon chips with respect to reagents binding [52] and Felbel et al. presented the inhibit behavior of silicon powder [132]. The loss of separation efficiencies, especially when using polymeric material, is also known from capillary electrophoresis devices [133].

To prevent protein adsorption, a distinction is drawn between static and dynamic wall coatings. The first type often consists of covalently linked coatings or coatings that are attached to the channel wall by covalent chemical bonds. But pretreatments based on different deposition techniques also have the character of static passivation. The static coatings are usually more time-consuming and complex. Often these types of coatings require several chemical reaction steps but provide a higher potential and better performance. The second type is dynamic coatings at which polymers or molecules, e.g., enzymes, adhere to the wall surface only by adsorption [134]. Dynamic coatings are attractive and easy to create. In most cases, a specific amount of coating material is dissolved in a fluid that is introduced into a chamber or flushed through a microchannel for some minutes.

Because of the weak binding energy of the adsorbed material, parts of the coating material can go again into solution during the PCR process. Dynamic coating can be also performed during DNA amplification itself by adding additional chemical substances to the PCR mixture. These additives can be used as a complementary protection against nonspecific adsorption of proteins without any former wall coating process. Additionally, definite types of additives can also stabilize the activity of Taq polymerase enzymes.

A well-known covalently linked static coating is the silanization of surfaces. Mostly silicon or glass-based miniaturized PCR systems have been coated by silanization. The process was first introduced by Hjertén and makes surfaces hydrophobic [135]. Silanization is performed at moderate temperature and pressure and is achieved by a self-assembly process from an organic solution of silane molecules. By wet chemical treatment, hydroxyl groups are formed on oxidized surfaces that act as covalent binding sites for alkylsilane. The inhibitory effect of silicon-based materials on DNA amplification was already mentioned by Northrup et al., who presented one of the first micro-PCR systems [10]. They found silanization as an adequate method to hold up reaction processes. This was also confirmed by Shoffner and co-workers [51], even though there have been doubts on sufficient reproducibility of efficiency [51,136]. Munro et al. presented an evaluation of different silica coatings, especially silanization, for PCR systems tested on reliability in electrophoretic microchips. The preferred coating consisted of a silanization with chlorodimethyloctylsilane dissolved in toluene and combined with a polymer layer of poly(vinylpyrrolidone) [137]. The silanization treatment was also applied to continuous flow-through amplification systems [5, 20, 23, 24] as well as oscillating systems [8]. Another method to create biocompatible environments is the use of silicon dioxide-coated silicon chips also allowing an DNA amplification in microchambers [14, 35, 36]. Static coating with polyethylene glycol (PEG) and its positive effect of preventing protein adsorption have been proven by different groups. Holmberg and co-workers have grafted PEG chains to polystyrene [138]. Österberg et al. used the same substrate material but, additionally, they compared PEG with dextran and differentiated between end-on and side-on polymer configurations [139]. They observed a more effective reduction of protein adsorption using end-on PEG and side-on dextran coatings. Furthermore, packing density seems to be more important in preventing protein adsorption than layer thickness. Surface modifications of PDMS have been performed by Hu and co-workers [140]. In a one-step procedure, they covalently linked different polymers to the channel walls of PDMS microdevices. A so far uncommon surface passivation has been presented by Shin et al. [75]. They

covered the PDMS wall surface of a PCR microchip with parylene. The polymer is known as an inert coating for medical devices and was used here to inhibit protein adsorption as well as sample evaporation.

In contrast, mainly three different substances have been associated with dynamic coatings of miniaturized PCR systems. The most frequently used is the protein bovine serum albumin (BSA), which sticks to almost all surfaces and is typically used as a blocking agent to reduce nonspecific binding in enzyme-linked immunosorbent assay (ELISA) systems [141]. It acts as a stabilizer for other proteins and enzymes and as protein base controls and calibrators. The molecular weight of BSA is 66 kDa, its density 1.36 g cm^{-3} [142], and the isoelectric point approximately pH 4.9 [143]. The concentration of BSA solutions used for pretreatment of microchambers or channels varies from 2 mg mL^{-1} [47] to 10 mg mL^{-1} [64, 77]. Often these solutions also contain additional compounds like Tween [33, 64], polyethylene glycol (PEG) [94], or PCR buffer itself [33, 63]. However, either the solution is flushed through the device [63] or the microstructures are flooded for 2 [30] or up to 30 min before use [77]. An additional temper step for 30 min at 80°C during the BSA coating process has been performed by Zou et al. [47]. Not specifically examined for PCR systems but performed at different flow rates, Chun and co-workers studied the protein adsorption on PEG-modified surfaces [130]. It was determined that specific proteins were adsorbed more at higher flow rates compared to lower flow rates. This could be important for oscillating or continuous flow-through PCR systems. A reduced adsorption of Taq polymerase due to PEG pretreatment of polymeric microchannels was also presented by Münchow et al. and Hennig et al. [7, 94]. To create a more dense PEG molecular brush-like coating on PMMA surfaces, Bi and co-workers modified PEG with butyl methacrylate (BMA), which also led to less nonspecific protein adsorption [144]. A PEG vapor-phase coating of glass surfaces and its reducing effect on nonspecific bindings has been presented by Popat et al. [145]. Epoxy polymers coatings have been used for either capillary electrophoresis or microchamber PCR systems [31,146]. Ferrance et al. have coated their glass chambers by flushing them with 1M NaOH solution for 10 min and a subsequent flush with 0.4% (w/v) epoxy-polydimethylacrylamide (EPDMA) solution [31]. In systems based on glass, EPDMA is only effective as a pretreatment when adsorbed to the wall surface before use. The coating does not work when only dissolved in the PCR mixture itself [136].

Although dynamic coatings can be performed as an initial operation step, surface treatments can also be applied during the PCR process itself. Once again, BSA represents one of the most used PCR mixture additives preventing inhibition of PCR processes by dynamic coating. Compared to

a pretreatment solution, the BSA concentration within the PCR mixture is reduced and varies between 0.2 ng mL^{-1} [61] and 2.5 mg mL^{-1} [46]. The average concentration lies between 0.1 and 0.5 mg mL^{-1} [19, 56, 59, 62]. The addition of BSA has also been combined with Triton X-100, PEG, and Tween [19, 59, 62, 90]. For Erill et al., the addition of BSA became essential for obtaining a PCR product [46]. Kaack and co-workers presented a three times higher yield of PCR product once BSA was added [61] and Nagai et al. also reported a strong increase of fluorescence intensity corresponding to the amplified DNA amount after adding BSA [14]. Overall, the application of BSA as a surface coating has been presented for all kinds of miniaturized PCR systems consisting of silicon, glass, or polymers. However, polyvinylpyrrolidone (PVP) is also known as a supporter of PCR reactions and has been used for dynamic coating of glass. An applied concentration of 2.5% (w/v) in the PCR mixture leads to 78% of the product amount relative to the amount observed in standard polypropylene inserts used for conventional thermocyclers [136]. For SiO_2 microstructures, Krishnan et al. also make use of Teflon coating or magnesium added to the reaction mixture [53].

Not specifically examined for PCR but for continuous biochemical flow-through systems in serial mode (droplets within a carrier liquid), Roach and co-workers presented the use of surfactants influencing the protein adsorption at the liquid–liquid interface. The adsorption of proteins can be either prevented or induced [147].

Concluding, a further enhancement of preventing protein adsorption can be reached by the combination of surface pretreatment and PCR mixture additives [5, 11, 14, 20, 23, 24, 30, 33, 63, 64, 77]. A good overview with regard to polymer wall coatings is given by Horvath et al. [148].

3.4. Detection of Amplified DNA

Still, the most famous target detection procedure is to remove the reaction mixture from the microchip after amplification and analyze it by gel [7, 20, 33] or capillary electrophoresis (endpoint detection) [12, 55, 61]. Because of utilized sieving matrices, these techniques allow a separation by size and the ability to identify different DNA segments of a PCR product. Visualization is performed by intercalators, a type of dye molecule that binds to double-stranded DNA (dsDNA) and inserts itself into the DNA structure. After binding, the intercalators show a strong increase in fluorescence intensity. Because of the transparent nature of glass and different kinds of polymers, the use of intercalators also enables real-time detection during the amplification process itself [43, 96]. However, since the intercalators do not

only bind to specific but also to nonspecific products, there will be no differentiation between them. The most popular examples for intercalators are ethidium bromide and SYBR® Green.

Besides the above-mentioned sequence-independent detection methods, there have been several approaches applying sequence-dependent detection methods. Mainly these methods utilize the ssDNA–ssDNA hybridization (ssDNA, single-stranded DNA). After DNA is heated to denaturation temperature and has formed single strands, these single strands will reform to double helices at regions of sequence complementarity after cooling. This technique has been used for real-time PCR [149] as well as for subsequent detections on microarrays. Another example for sequence-dependent real-time detection is the use of TaqMan® probes consisting of a fluorophore, an oligonucleotide, and a fluorescence quencher, which are added to the PCR mixture. They are designed to hybridize between the two primers, meaning that a third part of the target sequence is scrutinized. During PCR, when the Taq polymerase replicates a template on which a TaqMan® probe is bound, the 5′ nuclease activity of the polymerase cleaves the probe that leads to the separation of the quencher and fluorophore. The corresponding fluorescence signal, which is generated during the amplification and increased in each cycle, indicates the total amount of the target sequence. Further real-time PCR chemistries are Molecular Beacons and Scorpions®, all allowing multiple DNA species to be measured in the same sample (multiplex PCR). Therefore, fluorescence dyes with different emission spectra are attached to different probes.

A detection method in combination with capillary electrophoresis (CE), which is not based on fluorescence, has been presented by Abad-Villar et al. [150]. With an external contactless conductivity detector, they not only detected DNA but also peptides or proteins.

General overviews with regard to DNA detection are given by various authors [113, 121, 151], especially Kubista et al., who presented a broad overview of real-time polymerase chain reactions [152].

3.5. Integrated Micro-PCR Systems

The polymerase chain reaction is only one part in a row of steps toward a complete genetic analysis. In the first step, the nucleic acids have to be extracted from cells. In most cases, the released DNA strands have to be purified before subsequent amplification steps. After amplification, the PCR products have to be purified again before using them in CE or real-time detection systems. Depending on the assay, there are also differences in the number of necessary analytical steps. For example, in the case of a slab gel

electrophoresis, the second purification step can often be omitted. In general, this kind of conventional genetic analysis lasts up to 12 h [153]. Using appropriate microsystems, this time can be reduced down to between 20 and 70 min. The great challenge of fully integrated microsystems is to combine various analytical steps which are prior and subsequential to the PCR process. The advantages of integrated systems are the reduction of analysis times, sample and reagents volumes, as well as elimination of manual transfer steps between individual analysis steps. Hence, integrated systems require microfluidic connections between different microfluidic units as well as chip-to-world connections for sample loading and removal. Additionally, the directed transport of the sample has to be carried out by devices such as micropumps and microvalves. Corresponding microsystems can be classified according to existing analytical steps, i.e., systems including a subsequent detection based on, e.g., separation or hybridization, systems including presteps like sample preparation, and finally fully integrated systems that include both. But even the embedding of microheaters and sensors, which was presented for most microchamber-based systems, represents the first step toward integrated PCR systems.

In most cases, PCR devices have been first extended by a detection unit. Here, one possibility is real-time fluorescence measurement during PCR in microchambers [18, 27, 43, 69, 154] or microarrays [14, 16, 76] via addition of a DNA fluorescence dye. An online laser-induced fluorescence detection system combined with a segmented continuous flow-through PCR system was presented by Curcio et al. [58]. The combination of PCR and a subsequent capillary electrophoresis is another alternative for an integrated detection and was already presented by Woolley et al. in 1996 [87]. Here, a PCR microchamber was attached to a CE chip made of glass. After 15 min of amplification, the sample was electrokinetically injected directly into the CE chip and analyzed within 120 s. A further example for integrated systems including PCR and CE has been performed by Lagally and co-workers [22]. Also, microfabricated heaters, temperature sensors, valves, and hydrophobic vents were included, which allowed fast heating and cooling rates and sensorless positioning of samples. The amplification and integrated detection of samples has been achieved within 15 min and was later prepared for pathogen detection and genotyping [29]. The PCR device for nanoliter samples presented by Burns et al. also included heaters and CE channels [54]. The sample loading itself was controlled by capillary forces, integrated hydrophobic stop valves, and air vents.

To reach a higher level of integration, optical components also find their way into integrated PCR systems. Schabmueller and co-workers [45] positioned a diode detector inside a PCR microchamber. An optical fibre guided the light of a HeNe laser into the chamber, and resulting fluores-

cence emission has been detected by a built-in detector. The integrated system presented by Huang and co-workers consists of not only a PCR chamber with a connected CE channel, but also a chip-integrated optical fiber for on-line detection [80]. Further optical detection systems, not associated with PCR but with microfluidic bioanalysis systems, have been presented by several authors [155–158]. An alternative label-free PCR product quantification, also applicable for integrated PCR systems, has been introduced by Hou and co-workers [159]. The microelectronic sensor is based on field effect measurements and can monitor the product concentration at various stages of PCR. The readout is similar to real-time fluorescent measurements.

Besides real-time fluorescence measurement inside a microchamber or subsequent capillary electrophoresis, hybridization is a further possibility to detect amplified DNA fragments and enable sequence-specific analysis. A polycarbonate PCR chip including such a hybridization channel was introduced by Liu et al. [65]. After successful PCR, the amplicons were pumped through an attached hybridization channel by a syringe pump where oligonucleotides were previously deposited. After hybridization, the readout can be performed by laser scanning operating with desired wavelengths. A hybridization system that was not based on fluorescence but on sequence-specific electrochemical detection was presented by Lee et al. [44]. After reaction, single-stranded and amplified target DNA hybridized to probes immobilized on a working electrode, that was also located inside the PCR microchamber. The binding of additional indicators led to an electrochemical transduction and to a final detection.

The highest level of integration is reached as soon as only the raw sample has been injected followed by final results after a specific time span. Hence, to minimize the number of analysis steps done off-chip, further tasks, especially sample preparation, have to be integrated. Yuen and coworkers designed a microdevice containing a filter structure inside the PCR microchamber, which retained white blood cells from whole blood samples with volumes below 3 μL [71]. After filtration, the chamber was flooded with PCR reagents to complete the cell isolation process and amplification was carried out. A dielectrophoresis (DEP) sample pretreatment enabling cell capture and simultaneous removal of PCR inhibiting components has also been integrated in PCR systems [88]. After purification, the cells were transported into the microreaction chamber and PCR was performed. A system consisting of a cell lysis reactor, different reservoirs for reagents, an active mixer, and PCR chamber was presented by Lee et al. [79]. After thermal lysis of the introduced cells, the extracted DNA samples as well as the PCR reagents were driven electroosmotically into the mixing section. By applying a high external voltage to shielded electrodes,

the solution was electrokinetically mixed and driven into the PCR chamber where the final amplification was performed. One of the first PCR systems, which combined preceding as well as subsequent genetic analysis steps, was performed by Waters and co-workers in 1998 [115]. The chip design was based on the previous work of Woolley et al. but included a thermal step for cell lysis [87]. The PCR chamber was adapted to a CE chip. After amplification, the products were loaded electrokinetically into the separation path and an additional reservoir provided DNA markers for electrophoretic separation. A strong integrated system, which combined sample preparation from whole blood (including bead-based target cell capture, cell preconcentration, purification, and cell lysis), PCR and a final DNA microarray detection, was presented by Liu and co-workers [66]. Additional microfluidic mixers, valves, pumps, and heaters completed the self-contained analysis system. A different device but also containing multiple genetic analysis steps was presented by Anderson [67]. It allowed the extraction and enrichment of nucleic acids, amplification, and a final hybridization. The device was similar in size of a credit card and can handle more than 60 sequential operations. A capillary-based system offering an analysis directly from cheek cells has been presented by He and co-workers [113]. The device included all necessary DNA analysis steps followed by the separation and detection of the PCR product.

An overview of integrated systems is given in Table 3. Additional reviews can also be found in the literature [162–164].

Table 3. Overview of integrated PCR systems

PCR + capillary electrophoresis	29–31, 54, 78, 80, 82, 87, 115, 116
PCR + fluorescence detection of PCR mixture (including real-time)	18, 34, 43, 45, 58, 69, 96, 100, 104, 154, 160
PCR + hybridization	44, 65–67, 96
PCR + other detection methods	159
sample preparation + PCR	71, 79, 161
sample preparation + PCR + detection	66, 67, 115, 116

4. Alternative Protocols to PCR

Very often, amplification is equated with PCR. But PCR represents a class of amplification methods and does not, by far, include all amplification principles. In the following, the large field of PCR and further possibilities for amplifications are presented, without being exhaustive.

A variant of PCR, called *multiplex PCR*, enables the simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. First presented by Chamberlain et al. in 1988 [165], the method has been applied to many areas of DNA testing. Sometimes the target sequence is not well known. In these cases, degenerated primers, meaning a set of primers whose base sequence varies within the unknown sequence section of the target, are used. This technique is called degenerate oligonucleotide-primed PCR (*DOP PCR*) and can also be applied to whole genome amplification if random primers are used [166–168]. Human Alu sequences are short interspersed elements that are found at about one million sites in our genomes. If the goal of amplification is a genetic fingerprint of bands from an uncharacterized human DNA, these Alu sequences are amplified using PCR primers specific to them. The corresponding PCR is called *Alu-PCR* [169].

Another variation of amplification uses two pairs of primers, both of which belong to the same gene. The amplification starts, as usual, with only one primer pair amplifying a large amplicon. After 15–20 cycles, the second pair of primers (nested primers) is added. During the following 20–25 cycles, these primers bind within the first amplified amplicon and produce a second PCR product that will be shorter than the first one. As a result, this *nested PCR* has a higher specificity and sensitivity.

One last example of the variability is represented by the *asymmetric PCR*. This kind of PCR is used to preferentially amplify one strand of the original DNA. Compared to normal PCR, the concentration of primers for the chosen strand is significantly higher, or the second primer is not added at all. Accordingly, either the forward or backward reaction is highly enhanced, which is especially interesting for, for example, a subsequent microarray hybridization where the presence of only two complementary strands is ideal. It is noteworthy that the advantage of an exponential amplification disappears, at least in later cycles, after the limiting primer type has been consumed and the reaction passes into a linear amplification.

However, another central ingredient, the polymerase, also offers quite some variability. There are more than four different polymerases to choose from: Taq-, Tth-, Pwo-, and Pfu-polymerase. Tth- and Taq-polymerase

show a high 5′-3′-activity in addition to a reverse transcriptase activity. This allows a good forward and backward amplification as well as RNA generation. Besides their 5´-3´-activity, Pwo- and Pfu-polymerase also possess proof-reading activity (5´-3´-exonuclease-activity). Thus, the choice of the polymerase type leads to differences in the performance that can be decisive for an application.

To prevent nonspecific amplification during the setup of PCR, hot-start polymerases have been developed. This special kind of polymerase is activated only after a certain temperature is exceeded. An alternative option is to use thermally activated primers developed by TriLink Bio Technologies Inc.

If the initial template is not DNA but RNA, the amplification process starts with the translation of RNA into cDNA using reverse transcriptase, e.g., MMLV-RTase, AMV-RTase, or Tth-polymerase. This type of PCR is called reverse transcriptase PCR (*RT-PCR*).

For whole genome amplification, in particular, there are a number of other methods, such as ligase-mediated PCR (*LM-PCR*) [170–173], linker-adaptor PCR (*LA-PCR*) [174–177], primer extension preamplification PCR (*PEP-PCR*) [169, 178, 179] or *Tagged-PCR* [180], all of which use different approaches according to primers. In the first two approaches, sequences are ligated to the DNA allowing specific primers to anneal. The other two approaches, PEP-PCR and Tagged-PCR, are characterized by special random primers. However, it must be noted that the above-mentioned methods are not only limited to whole genome amplifications.

An alternative amplification method to PCR, which also uses high temperature for denaturation and a lower temperature for hybridization, is the ligase chain reaction (*LCR*). This reaction achieves a similar sensitivity compared to PCR [181]. After denaturation, two oligonucleotides, called probes, are hybridized to the single-stranded DNA and then ligated by a thermally stable ligase. After ligation, the cycle is completed and starts again with the denaturation step. A more elaborated method is multiplex ligation probe amplification (*MLPA*) [182]. Utilizing the same denaturation, hybridization, and ligation steps, the difference lies in the applied probes ending with a sequence that is used for PCR amplification afterward. One of them also includes a stuffer sequence between the primer and hybridization sequence and is used for product determination. Therefore, the assay can easily be multiplexed using different hybridization sequences all equipped with stuffer sequences of varying length but with the same primer. The reason for this is that a subsequent PCR can be performed with only one primer pair and the different amplified and ligated probes can be separated using capillary electrophoresis.

So far, all presented amplification methods use thermal cycling by some means, but isothermal methods have also been developed. The first isothermal transcription-based amplification system was described by Guatelli imitating the retroviral replication [183]. Two commercial variants of this basic methodology are the nucleic acid sequence-based amplification (*NASBA*) and the transcription-mediated amplification (*TMA*) [184]. NASBA requires Ribonuclease H as well as reverse transcriptase and T7-RNA-polymerase, whereas TMA only needs two different kinds of enzymes, namely reverse transcriptase and T7-RNA-polymerase. Furthermore, these two methods are very similar since they both rely on the same principal amplifying RNA. Compared to RT-PCR methods, the amount of product shows an exponential increase leading to much higher amplifications [185].

Another isothermal amplification method is the strand displacement amplification (*SDA*) [186]. This method works at 40°C and results in an amplification of eight orders of magnitude within 2 h. The technique is based upon the ability of restriction endonuclease to nick a hemimodified recognition site and of exonuclease-deficient DNA polymerase to displace a downstream DNA strand during replication. SDA is a cyclic process in which modified target sequences are repeatedly nicked. The strand is displaced and the displaced strands are primed [186–188].

Besides the above-mentioned methods using PCR for whole genome amplification, the strand displacement, used by SDA, can also be applied for whole genome amplification. The technique is called multiple strand displacement (*MSD*) and uses phi29, a highly processive mesophillic DNA polymerase, to replicate up to 100,000 bp without dissociating from the genomic DNA [189, 190]. The first step is a random priming followed by the phi29 DNA polymerase movement along the DNA template displacing the complementary strand at 30°C. The displaced strand becomes a template for replication allowing for the generation of high yields of high-molecular-weight DNA.

Supported by its processivity, the same phi29 polymerase can also be used for the rolling cycle amplification (*RCA*) amplifying circular DNA, e.g., plasmids [191, 192]. The use of a single primer leads to a linear amplification that can be transformed into an exponential method by adding a second primer.

During helicase-dependent amplification (*HDA*), the DNA is denaturated by the enzyme helicase operating at a constant temperature of 37°C. After a primer pair anneals to each end of the target DNA, it is extended by a polymerase leading again to double-stranded DNA representing the starting point for the next cycle [193].

A further amplification method is the Q-beta-replicase amplification using the homonymous enzyme q-beta-replicase. This enzyme has the unique feature not to amplify a target DNA sequence but to replicate RNA having a specific secondary structure. This RNA hybrid consists of a sequence called MDV-1 and a target probe complementary to the DNA sequence of interest. After binding to the DNA, the RNA hybrid contorts activating the replicase that starts to copy the MDV-1 and its tag-along RNA sequence [194]. As the RNA products serve as new templates, this method allows a target amplification of nine orders of magnitude within 30 min.

All these amplification methods presented demonstrate the wide range of possibilities to choose from. Methods that have not been mentioned here are methods normally based on the variation of process parameters like temperature, e.g., touchdown PCR [195], and methods subsequent to the amplification, like melting curve analysis or microarrays.

Corresponding to the aforementioned scaling properties nonisothermal methods always have a certain potential to be accelerated by miniaturization. On the other hand, for many applications, e.g., point of care, and relating to power consumption of micro devices, isothermal amplifications might be more advantageous compared to thermal cycling.

5. Conclusion

Since the first miniaturized PCR devices were presented in the early 1990s, miniaturized PCR systems represent one of the most important applications of lab-on-chip technologies. Showing a huge range of variations, they have developed from feasibility studies toward microchip-based products [116, 149, 160, 196] and handheld systems [197–199]. Continuous flow devices in particular have been commercialized by different companies, e.g., IQuum Inc., Thermal Gradient Inc., and microfluidic ChipShop GmbH. Current efforts primarily aim bioanalytical point of care systems, which also comprises additional analytical steps, such as sample preparation and target detection. But, in addition to that, the automation of microfluidic control including sample loading and liquid handling (e.g., fluid transport, mixing, and valving) has to be addressed to obtain reliable and easy-to-use systems in the future. Micro-PCR systems, which allow reduction of amplification time and less reagent consumption, can provide, together with other analytical processes, a new level of standardization of high throughput gene analysis.

References

1. Zhao R (2005) From single cell gene-based diagnostics to diagnostic genomics: Current applications and future perspectives. Clin Lab Sci 18: 254–262
2. Hodinka RL (1998) The clinical utility of viral quantitation using molecular methods. Clin Diagn Virol 10: 25–47
3. Mueller JD, Pütz B, Höfler H. (1997) Self-sustained sequence replication (3SR): An alternative to PCR. Histochem Cell Biol 108: 431–437
4. Wuesten JJAM, Carpay WM, Oosterlaken TAM, van Zuijlen MCA, van de Wiel PA (2002) Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. Nucleic Acids Res 30: e26
5. Kopp MU, deMello AJ, Manz A (1998) Chemical amplification: Continuous-flow PCR on a chip. Science 280: 1046–1048
6. Schneegaß I, Köhler JM (2001) Flow-through polymerase chain reactions in chip thermocyclers. Rev Mol Biotechnol 82: 101–121
7. Münchow G, Dadic D, Doffing F, Hardt S, Drese KS (2005) Automated chip-based device for simple and fast nucleic acid amplification. Expert Rev Mol Diagn 5: 613–620
8. Wang W, Li ZX, Luo R, Lü SH, Xu AD, Yang YJ (2005) Droplet-based micro oscillating-flow PCR chip. J Micromech Microeng 15: 1369–1377
9. Sasaki N, Izawa M, Shimojo M, Shibata K, Akiyama JI, Itoh M, Nagaoka S, Carninci P, Okazaki Y, Moriuchi T, Muramatsu M, Watanabe S, Hayashizaki Y (1997) A novel control system for polymerase chain reaction using a RIKEN GS384 thermocycler. DNA Res. 4: 387–391
10. Northrup MA, Ching MT, White RM, Watson RT (1993) DNA amplification with a microfabricated reaction chamber. Proceedings of Transducers '93: 924–926
11. Daniel JH, Iqbal S, Millington RB, Moore DF, Lowe CR, Leslie DL, Lee MA, Pearce MJ (1998) Silicon microchambers for DNA amplification. Sensor Actuator Phys 71: 81–88
12. Northrup MA, Gonzalez C, Hadley D, Hills RF, Landre P, Lehew S, Saiki R, Sninsky JJ, Watson R, Watson R Jr (1995) A MEMS-based miniature DNA analysis system. Proceedings of Transducers '95: 764–767
13. Poser S, Schulz T, Dillner U, Baier V, Köhler JM, Schimkat D, Mayer G, Siebert A (1997) Chip elements for fast thermocycling. Sensor Actuator Phys 62: 672–675
14. Nagai H, Murakami Y, Morita Y, Yokoyama K, Tamiya E (2001) Development of a microchamber array for picoliter PCR. Anal Chem 73: 1043–1047
15. Nagai H, Murakami Y, Yokoyama K, Tamiya E (2001) High-throughput PCR in silicon-based microchamber array. Biosens Bioelectron 16: 1015–1019
16. Nagai H, Murakami Y, Wakida S, Niki E, Tamiya E (2001) High throughput single cell PCR on a silicon microchamber array. Proceedings of Micro Total Analysis System 268–270

17. Felbel J, Bieber I, Pipper J, Köhler JM (2004) Investigations on the compatibility of chemically oxidized silicon (SiO_x)-surfaces for applications towards chip-based polymerase chain reaction. Chem Eng J 101: 333–338
18. Neuzil P, Pipper J, Hsieh TM (2006) Disposable real-time microPCR device: Lab-on-a-chip at a low cost. Mol BioSyst 2: 292–298
19. Obeid PJ, Christopoulos TK (2003) Continuous-flow DNA and RNA amplification chip combined with laser-induced fluorescence detection. Anal Chim Acta 494: 1–9
20. Sun K, Yamaguchi A, Ishida Y, Matsuo S, Misawa H (2002) A heater-integrated transparent microchannel chip for continuous-flow PCR. Sensor Actuator Chem 84: 283–289
21. Auroux PA, Day PJR, Niggli F, Manz A (2003) PCR micro-volume device for detection of nucleic acids. Proceedings of Nanotec, 55–58
22. Lagally ET, Emrich CA, Mathies RA (2001) Fully integrated PCR-capillary electrophoresis microsystem for DNA analysis. Lab Chip 1: 102–107
23. Li S, Fozdar DY, Ali MF, Li H, Shao D, Vykoukal DM, Vykoukal J, Floriano PN, Olsen M, McDevitt JT, Gascoyne PRC, Chen S (2006) A continuous-flow polymerase chain reaction microchip with regional velocity control. J Microelectromechyst S 15: 223–236
24. Park N, Kim S, Hahn JH (2003) Cylindrical compact thermal-cycling device for continuous-flow polymerase chain reaction. Anal Chem 75: 6029–6033
25. Friedmann NA, Meldrum DR (1998) Capillary tube resistive thermal cycling. Anal Chem 70: 2997–3002
26. Oda RP, Strausbauch MA, Huhmer AFR, Borson N, Jurrens SR, Craighead J, Wettstein PJ, Eckloff B, Kline B, Landers JP (1998) Infrared-mediated thermocycling for ultrafast polymerase chain reaction amplification of DNA. Anal Chem 70: 4361–4368
27. Baker J, Strachan M, Swartz K, Yurkovetsky Y, Rulison A, Brooks C, Kopf-Sill A (2003) Single molecule amplification in a continuous flow lab-chip device. Proceedings of Micro Total Analysis Systems, 1335–1338
28. Legendre LA, Bienvenue JM, Roper MG, Ferrance JP, Landers JP (2006) A simple, valveless microfluidic sample preparation device for extraction and amplification of DNA from nanoliter-volume samples. Anal Chem 78: 1444–1451
29. Lagally ET, Scherer JR, Blazej RG, Toriello NM, Diep BA, Ramchandani M, Sensabaugh GF, Riley LW, Mathies RA (2004) Integrated Portable Genetic analysis microsystem for pathogen/infectious disease detection. Anal Chem 76: 3162–3170
30. Khandurina J, McKnight TE, Jacobson SC, Waters LC, Foote RS, Ramsey JM (2000) Integrated system for rapid PCR-based DNA analysis in microfluidic devices. Anal Chem 72: 2995–3000
31. Ferrance JP, Wu Q, Giordano B, Hernandez C, Kwok Y, Snow K, Thibodeau S, Landers JP (2003) Developments towards a complete mircototal analysis system for Duchenne muscular dystrophy diagnosis. Anal Chim Acta 500: 223–236

32. Zhang Q, Wang W, Zhang H, Wang Y (2002) Temperature analysis of continuous-flow micro-PCR based on FEA. Sensor Actuator Chem 82: 75–81
33. Schneegaß I, Bräutigam R, Köhler JM (2001) Miniaturized flow-through PCR with different template types in a silicon chip thermocycler. Lab Chip 1: 42–49
34. Zhao Z, Cui Z, Cui D, Xia S (2003) Monolithically integrated PCR biochip for DNA amplification. Sensor Actuator Phys 108: 162–167
35. Schabmueller CGJ, Lee MA, Evans AGR, Brunnschweiler A, Ensell GJ, Leslie DL (2000) Closed chamber PCR chips for DNA amplification. Eng Sci and Educ J 9: 259–264
36. Cheng J, Shoffner MA, Hvichia GE, Kricka LJ, Wilding P (1996) Chip PCR. II. Investigation of different PCR amplification systems in microfabricated silicon–glass chips. Nucleic Acids Res 24: 380–385
37. Bu M, Melvin T, Ensell G, Wilkinson JS, Evans AGR (2003) Design and theoretical evaluation of a novel microfluidic device to be used for PCR. J Micromech Microeng 13: S125-S130
38. Yoon DS, Lee YS, Lee Y, Cho HJ, Sung SW, Oh KW, Cha J, Lim G (2002) Precise temperature control and rapid thermal cycling in a micromachined DNA polymerase chain reaction chip. J Micromech Microeng 12: 813–823
39. Wilding P, Shoffner MA, Kricka LJ (1994) PCR in a silicon microstructure. Clin Chem 40: 1815–1818
40. Köhler JM, Dillner U, Mokansky A, Poser S, Schulz T (1998) Micro channel reactors for fast thermocycling. Proceedings of 2nd International Conference on Microreaction Technology, 241–247
41. Poser S, Ehricht R, Schulz S, Uebel S, Dillner U, Köhler JM (1999) Rapid PCR in flow-through Si chip thermocyclers. Proceedings of 3rd International Conference on Microreaction Technology, 411–419
42. Poser S, Schulz T, Dillner U, Baier V, Köhler JM, Mayer G, Siebert A, Schimkat D (1997) Temperature-controlled chip reactor for rapid PCR. Proceedings of 1st International Conference on Microreaction Technology, 294–301
43. Oh KW, Park C, Namkoong K, Kim J, Ock KS, Kim S, Kim YA, Cho YK, Ko C (2005) World-to-chip microfluidic interface with built-in valves for multichamber chip-based PCR assays. Lab Chip 5: 845–850
44. Lee TMH, Carles MC, Hsing IM (2003) Microfabricated PCR-electrochemical device for simultaneous DNA amplification and detection. Lab Chip 3: 100–105
45. Schabmueller CGJ, Pollard JR, Evans AGR, Wilkinson JS, Ensell G, Brunnschweiler A (2001) Integrated diode detector and optical fibres for in situ detection within micromachined polymerase chain reaction chips. J Micromech Microeng 11: 329–333
46. Erill I, Campoy S, Rus J, Fonseca L, Ivorra A, Navarro Z, Plaza JA, Aguiló J, Barbé J (2004) Development of a CMOS-compatible PCR chip: Comparison of design and system strategies. J Micromech Microeng 14: 1558–1568

47. Zou ZQ, Chen X, Jin QH, Yang MS, Zhao JL (2005) A novel miniaturized PCR multi-reactor array fabricated using flip-chip bonding techniques (2005) J Micromech Microeng 15: 1476–1481
48. Kim SH, Noh J, Jeon MK, Kim KW, Lee LP, Woo SI (2006) Micro-Raman thermometry for measuring the temperature distribution inside the microchannel of polymerase chain reaction chip. J Micromech Microeng 16: 526–530
49. Pal D, Venkataraman V (2002) A portable battery-operated chip thermocycler based on induction heating. Sensor Actuator Phys 102: 151–156
50. Gulliksen A, Solli L, Karlsen F, Rogne H, Hovig E, Nordstrøm T, Sirevåg R (2004) Real-time nucleic acid sequence-based amplification in nanoliter volumes. Anal Chem 76: 9–14
51. Shoffner MA, Cheng J, Hvichia GE, Kricka LJ, Wilding P (1996) Chip PCR. I. Surface passivation of microfabricated silicon-glass chips for PCR. Nucleic Acids Res 24: 375–379
52. Erill I, Campoy S, Erill N, Barbé J, Aguiló J (2003) Biochemical analysis and optimization of inhibition and adsorption phenomena in glass-silicon PCR-chips. Sensor Actuators Chem 96: 685–692
53. Krishnan M, Burke DT, Burns MA (2004) Polymerase chain reaction in high surface-to-volume ratio SiO_2 microstructures. Anal Chem 76: 6588–6593
54. Burns MA, Johnson BN, Brahmasandra SN, Handique K, Webster JR, Krishnan M, Sammarco TS, Man PM, Jones D, Heldsinger D, Mastrangelo CH, Burke DT (1998) An integrated nanoliter DNA analysis device. Science 282: 484–487
55. Giordano BC, Ferrance J, Swedberg S, Hühmer AFR, Landers JP (2001) Polymerase chain reaction in polymeric microchips: DNA amplification in less than 240 seconds. Anal Biochem 291: 124–132
56. Chen Z, Qian S, Abrams WR, Malamud D, Bau HH (2004) Thermosiphon-based PCR reactor: Experiment and modeling. Anal Chem 76: 3707–3715
57. Daly J, Davies M, Dalton T (2005) A fully controlled natural convection polymerase chain reaction device. Proceedings of ECI International Conference on Heat Transfer and Fluid flow in Microscale
58. Curcio M, Roeraade J (2003) Continuous segmented-flow polymerase chain reaction for high-throughput miniaturized DNA amplification. Anal Chem 75: 1–7
59. Nakano H, Matsuda K, Yohda M, Nagamune T, Endo I, Yamane T (1994) High speed polymerase chain reaction in constant flow. Biosci Biotech Biochem 58: 349–352
60. Chiou J, Matsudaira P, Sonin A, Ehrlich D (2001) A closed-cycle capillary polymerase chain reaction machine. Anal Chem 73: 2018–2021
61. Kaack RM, Jung A, Wenz HM, Zengerle R, Daub M (2004) PCR-slide: A modular and cascadable platform for DNA sample processing with integrated nanolitre dosage. Proceedings of 17th IEEE International Conference on Micro Electro Mechanical Systems MEMS, 338–342

62. Yang J, Liu Y, Rauch CB, Stevens RL, Liu RH, Lenigk R, Grodzinski P (2002) High sensitivity PCR assay in plastic micro reactors. Lab Chip 2: 179–187
63. Hashimoto M, Chen PC, Mitchell MW, Nikotopoulos DE, Soper SA, Murphy MC (2004) Rapid PCR in a continuous flow device. Lab Chip 4: 638–645
64. Wang J, Chen Z, Corstjens PLAM, Mauk MG, Bau HH (2006) A disposable microfluidic cassette for DNA amplification and detection. Lab Chip 6: 46–53
65. Liu Y, Rauch CB, Stevens RL, Lenigk R, Yang J, Rhine DB, Grodzinski P (2002) DNA amplification and hybridization assays in integrated plastic monolithic devices. Anal Chem 74: 3063–3070
66. Liu RH, Yang J, Lenigk R, Bonanno J, Grodzinski P (2004) Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection. Anal Chem 76: 1824–1831
67. Anderson RC, Su X, Bogdan GJ, Fenton J (2000) A miniature integrated device for automated multistep genetic assays. Nucleic Acids Res 28: e60
68. Chen J, Wabuyele M, Chen H, Patterson D, Hupert M, Shadpour H, Nikitopoulos D, Soper SA (2005) Electrokinetically synchronized polymerase chain reaction microchip fabricated in polycarbonate. Anal Chem 77: 658–666
69. Lee DS, Park SH, Yang H, Chung KH, Yoon TH, Kim SJ, Kim K, Kim YT (2004) Bulk-micromachined submicroliter-volume PCR chip with very rapid thermal response and low power consumption. Lab Chip 4: 401–407
70. Krishnan M, Ugaz VM, Burns MA (2002) PCR in a Rayleigh-Bénard convection cell. Science 298: 793
71. Yuen PK, Kricka LJ, Fortina P, Panaro NJ, Sakazume T, Wilding P (2006) Microchip module for blood sample preparation and nucleic acid amplification reactions. Genome Res 11: 405–412
72. Niu ZQ, Chen WY, Shao SY, Jia XY, Zhang WP (2006) DNA amplification on a PDMS-glass hybrid microchip. J Micromech Microeng 16: 425–433
73. Liao CS, Lee GB, Liu HS, Hsieh TM, Wang CH, Fan CL, Luo CH (2004) Micro reverse-transcription polymerase chain reaction system for clinical diagnosis. Proceedings of Micro Total Analysis Systems, 87–89
74. Liu J, Enzelberger M, Quake S (2002) A nanoliter rotary device for polymerase chain reaction. Electrophoresis 23: 1531–1536
75. Shin YS, Cho K, Lim SH, Chung S, Park SJ, Chung C, Han DC, Chang JK (2003) PDMS-based micro PCR chip with parylene coating. J Micromech Microeng 13: 768–774
76. Yu X, Zhang D, Li T, Hao L, Li X (2003) 3-D microarrays biochip for DNA amplification in polydimethylsiloxane (PDMS) elastomer. Sensor Actuator Phys 108: 103–107
77. Prakash AR, Adamia S, Sieben V, Pilarski P, Pilarski LM, Backhouse CJ (2006) Small volume PCR in PDMS biochips with integrated fluid control and vapour barrier. Sensor Actuator Chem 113: 398–409

78. Lee CY, Lee GB, Liu HH, Huang FC (2002) MEMS-based temperature control systems for DNA amplification. Int J Nonlinear Sci Numer Simul 3: 215–218
79. Lee CY, Lee GB, Lin JL, Huang FC, Liao CS (2005) Integrated microfluidic systems for cell lysis, mixing/pumping and DNA amplification. J Micromech Microeng 15: 1215–1223
80. Huang FC, Liao CS, Lee GB (2006) An integrated microfluidic chip for DNA/RNA amplification, electrophoresis separation and on-line optical detection. Electrophoresis 27: 3297–3305
81. Nakayama T, Kurosawa Y, Furui S, Kerman K, Kobayashi M, Rao SR, Yonezawa Y, Nakano K, Hino A, Yamamura S, Takamura Y, Tamiya E (2006) Circumventing air bubbles in microfluidic systems and quantitative continuous-flow PCR applications. Anal Bioanal Chem DOI 10.1007/s00216-006-0688-7
82. Hong JW, Fujii T, Seki M, Yamamoto T, Endo I (2001) Integration of gene amplification and capillary gel electrophoresis on a polydimethylsiloxane-glass hybrid microchip. Electrophoresis 22: 328–333
83. Gulliksen A, Solli LA, Drese KS, Sörensen O, Karlsen F, Rogne H, Hovig E, Sirevåg (2005) Parallel nanoliter detection of cancer markers using polymer microchips. Lab Chip 5: 416–420
84. Cheng JY, Hsieh CJ, Chuang YC, Hsieh JR (2005) Performing microchannel temperature cycling reactions using reciprocating reagent shuttling along a radial temperature gradient. Analyst 130: 931–940
85. Zou Q, Miao Y, Chen Y, Sridhar U, Chong CS, Chai T, Tie Y, The CHL, Lim TM, Heng CK (2002) Micro-assembled multi-chamber thermal cycler for low-cost reaction chip thermal multiplexing. Sensor Actuator Phys 102: 114–121
86. Wheeler EK, Benett B, Stratton P, Richards J, Christian A, Chen A, Weisgraber T, Ness K, Ortega J, Milanovich F (2003) Convectively driven polymerase chain reaction thermal cycler. Proceedings of Micro Total Analysis Systems, 1133–1135
87. Woolley AT, Hadley D, Landre P, de Mello AJ, Mathies RA, Northrup MA (1996) Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA Analysis Device. Anal Chem 68: 4081–4086
88. El-Ali J, Perch-Nielsen IR, Poulsen CR, Jensen M, Tellemann P, Wolff A (2003) Microfabricated DNA amplification device monolithically integrated with advanced sample pre-treatment. Proceedings of Transducers '03: 214–217
89. West J, Karamata B, Lillis B, Gleeson JP, Alderman J, Collins JK, Lane W, Mathewson A, Berney H (2002) Application of magnetohydrodynamic actuation to continuous flow chemistry. Lab Chip 2: 224–230
90. Leamon JH, Lee WL, Tartaro KR, Lanza JR, Sarkis GJ, deWinter AD, Berka J, Lohman KL (2003) A massively parallel PicoTiterPlate™ based platform for discrete picoliter-scale polymerase chain reactions. Electrophoresis 24: 3769–3777

91. Hühmer AFR, Landers JP (2000) Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. Anal Chem 72: 5507–5512
92. Fermér C, Nilsson P, Larhed M (2003) Microwave-assisted high-speed PCR. Eur J Pharm Sci 18: 129–132
93. Chaudhari AM, Woudenberg TM, Albin M, Goodson KE (1998) Transient liquid crystal thermometry of microfabricated PCR vessel arrays. J Microelectromech Syst 7: 345–355
94. Hennig M, Braun D (2005) Convective polymerase chain reaction around micro immersion heater. Appl Phys Lett 87: 183901
95. Chen Z, Wang J, Quian S, Bau HH (2005) Thermally-actuated, phase change flow control for microfluidic systems. Lab Chip 5: 1277–1285
96. Guttenberg Z, Müller H, Habermüller H, Geisbauer A, Pipper J, Felbel J, Kielpinski M, Scriba J, Wixforth A (2005) Planar chip device for PCR and hybridization with surface acoustic wave pump. Lab Chip 5: 308–317
97. Litborn E, Curcio M, Emmer Å, Roeraade J (2000) Synthesis and analysis of chemical components in nanoscale. Proceedings of Micro Total Analysis Systems, 447–454
98. Barrett B, Sirr N, Sayers M, Dalton T, Davies M (2005) Liquid properties for droplet or plug-flow microfluidic polymerase chain reaction devices. Proceedings of ECI International Conference on Heat Transfer and Fluid flow in Microscale
99. Gui L, Ren CL (2006) Numeric simulation of heat transfer and electrokinetic flow in an electroosmosis-based continuous flow PCR chip. Anal Chem 78: 6215–6222
100. Pollack MG, Paik PY, Shenderov AD, Pamula VK, Dietrich FS, Fair RB (2003) Investigation of electrowetting-based microfluidics for real-time PCR applications. Proceedings of Micro Total Analysis Systems, 619–622
101. Griffith EJ, Akella S (2005) Coordination multiple droplets in planar array digital microfluidic systems. Int J Rob Res 24: 933–949
102. Griffith EJ, Akella S, Goldberg MK (2006) Performance characterization of a reconfigurable planar-array digital microfluidic system. IEEE Trans Comput Aided Des Integrated Circuits Syst 25: 340–352
103. Zhang T, Chakrabarty K, Fair RB (2002) System performance evaluation with SystemC for two PCR microfluidic systems. Proceedings of Nanotech, 48–53
104. Braun D, Goddard NL, Libchaber A (2003) Exponential DNA replication by laminar convection. Phys Rev Lett 91: 158103
105. Krishnan M, Agrawal N, Burns MA, Ugaz VM (2004) Reactions and fluidics in miniaturized natural convection systems. Anal Chem 76: 6254–6265
106. Walsh EJ, King CR, Grimes RH, Davies MR, Dalton TM (2005) Passive movement and manipulation of microfluidic using buoyancy. Proceedings of ECI International Conference on Heat Transfer and Fluid flow in Microscale
107. Leamon JH, Link DR, Egholm M, Rothberg JM (2006) Overview: Methods and applications for droplet compartmentalization of biology. Nat Methods 3: 541–543

108. Nakano M, Komatsu J, Matsuura SI, Takashima K, Katsura S, Mizuno A (2003) Sigle-molecule PCR using water-in-oil emulsion. J Biotechnol 102: 117–124
109. Haase AT, Retzel EF, Staskus KA (1990) Amplification and detection of lentiviral DNA inside cells. Proc Natl Acad Sci USA 87: 4971–4975
110. Komminoth P, Werner M (1997) Target and signal amplification: Approaches to increase the sensitivity of in situ hybridization. Histochem Cell Biol 108: 325–333
111. Brehm-Stecher BF, Johnson EA (2004) Single-cell microbiology: Tools, technologies, and applications. Microbiol Mol Biol Rev 68: 538–559
112. Bagasra O, Harris T (2006) Latest developments in in situ PCR. Methods Mol Biol 334: 221–240
113. He Y, Zhang YH, Yeung ES (2001) Capillary-based fully integrated and automated system for nanoliter polymerase chain reaction analysis directly from cheek cells. J Chromatogr A 924: 271–284
114. Lagally ET, Simpson PC, Mathies RA (2000) Monolithic integrated microfluidic DNA amplification and capillary electrophoresis analysis system. Sensor Actuator Chem 63: 138–146
115. Waters LC, Jacobson SC, Kroutchinina N, Khandurina J, Foote RS, Ramsey JM (1998) Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal Chem 70: 158–162
116. Raisi F, Blizard BA, Shabari AR, Ching J, Kintz GJ, Mitchell J, Lemoff A, Taylor MT, Weir F, Western L, Wong W, Joshi R, Howland P, Chauhan A, Nguyen P, Petersen KE (2004) Human genomic DNA analysis using a semi-automated sample preparation, amplification, and electrophoresis separation platform. J Sep Sci 27: 275–283
117. Liao CS, Lee GB, Wu JJ, Chang CC, Hsieh TM, Huang FC, Luo CH (2005) Micromachined polymerase chain reaction system for multiple DNA amplification of upper respiratory tract infectious disease. Biosens Bioelectron 20: 1341–1348
118. Wang H, Chen J, Zhu L, Shadpour H, Hupert ML, Soper SA (2006) Continuous flow thermal cycler microchip for DNA cycle sequencing. Anal Chem 78: 6223–6231
119. Agrawal N, Ugaz VM (2006) A buoyancy-driven compact thermocycler for rapid PCR. JALA 11: 217–221
120. Marcus JS, Anderson WF, Quake SR (2006) Parallel picoliter RT-PCR assays using microfluidics. Anal Chem 78: 956–958
121. Auroux PA, Koc Y, deMello A, Manz A, Day PJR (2004) Miniaturised nucleic acid analysis. Lab Chip 4: 534–546
122. Krishnan M, Namasivayam V, Lin R, Pal R, Burns MA (2001) Microfabricated reaction and separation systems. Curr Opin Biotechnol 12: 92–98
123. Vilkner T, Janasek D, Manz A (2004) Micro total analysis systems. Recent developments. Anal Chem 76: 3373–3386
124. Lee SJ, Lee SY (2004) Micro total analysis system (μ-TAS) in biotechnology. Appl Microbiol Biotechnol 64: 289–299

125. Kricka LJ, Wilding P (2003) Microchip PCR. Anal Bioanal Chem 377: 820–825
126. Obeid PJ, Christopoulos TK (2004) Microfabricated systems for nucleic acid analysis. Crit Rev Clin Lab Sci 41: 429–465
127. Zhang C, Xu J, Ma W, Zheng W (2006) PCR microfluidic devices for DNA amplification. Biotechnol Adv 24: 243–284
128. Lassen B, Malmsten M (1997) Competitive protein adsorption at plasma polymer surfaces. J Colloid Interface Sci 186: 9–16
129. Sukhishvili SA, Granick S (1999) Adsorption of human serum albumin: Dependence on molecular architecture of the oppositely charged surface. J Chem Phys 110: 10153–10161
130. Chun CJ, Lenghaus K, Riedel L, Bhalkikar A, Hickman JJ (2006) A behaviour of protein adsorption on poly(ethylene glycol)-modified surfaces under flow conditions at relatively low concentrations for microfluidics systems. Proceedings of Nanotech 2: 674–677
131. Kai J, Sohn YS, Ahn CH (2002) Study on protein (IgG) adsorption in terms of surface modification of cyclic olefin copolymer (COC) for protein biochip. Proceedings of Micro Total Analysis Systems, 419–421
132. Felbel J, Bieber I, Köhler JM (2002) Chemical surface management for micro PCR in silicon chip thermocyclers. Proceedings of SPIE 4937: 34–40
133. Lacher NA, de Rooij NF, Verpoorte E, Lunte SM (2003) Comparison of the performance characteristics of poly(dimethylsiloxane) and Pyrex microchip electrophoresis devices for peptide separations. J Chromatogr A 1004: 225–235
134. Vreeland WN, Barron AE (2002) Functional materials for microscale genomic and proteomic analyses. Curr Opin Biotechnol 13:87–94
135. Hjertén S (1985) High-performance electrophoresis. Elimination of electroendosmosis and solute adsorption. J Chromatogr 347: 191–198
136. Giordano BC, Copeland ER, Landers JP (2001) Towards dynamic coating of glass microchip chambers for amplifying DNA via the polymerase chain reaction. Electrophoresis 22: 334–340
137. Munro NJ, Hühmer AFR, Landers JP (2001) Robust polymeric microchannel coating for microchip-based analysis of neat PCR products. Anal Chem 73: 1784–1794
138. Holmberg K, Bergström K, Brink C, Österberg E, Tiberg F, Harris JM (1993) Effects on protein adsorption, bacterial adhesion and contact angle of grafting PEG chains to polystyrene. Contact Angle, Wettability and Adhesion, Ed. Mittal KL, 813–827
139. Österberg E, Bergström K, Holmberg K, Schuman TP, Riggs JA, Burns NL, van Alstine JM, Harris JM (1995) Protein-rejecting ability to surface-bound dextran in end-on and side-on configurations: Comparison to PEG. J Biomed Mater Res 29: 741–747
140. Hu S, Ren X, Bachman M, Sims CE, Li GP, Allbritton N (2002) Surface modification of poly(dimethylsiloxane) microfluidic devices by ultraviolet polymer grafting. Anal Chem 74: 4117–4123

141. Eteshola E, Leckband D (2001) Development and characterization of an ELISA assay in PDMS microfluidic channels. Sensor Actuator Chem 72: 129–133
142. Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell J (1996) Molekulare Zellbiologie, Berlin, de Gruyter
143. Putnam FW (1975) The Plasma Proteins: Structure, Function and Genetic Control. Vol. 1, New York, Springer-Verlag
144. Bi H, Meng S, Li Y, Guo K, Chen Y, Kong J, Yang P, Zhong W, Liu B (2006) Deposition of PEG onto PMMA microchannel surface to minimize nonspecific adsorption. Lab Chip 6: 769–775
145. Popat KC, Desai TA (2004) Poly(ethylene glycol) interfaces: An approach for enhanced performance of microfluidic systems. Biosens Bioelectron 19: 1037–1042
146. Chiari M, Cretich M, Damin F, Ceriotti F, Ceriotti L, Consonni R (2000) New adsorbed coatings for capillary electrophoresis. Electrophoresis 21: 909–916
147. Roach LS, Song H, Ismagilov RF (2005) Controlling nonspecific protein adsorption in a plug-based microfluidic system by controlling interfacial chemistry using fluorous-phase surfactants. Anal Chem 77: 785–796
148. Horvath J, Dolnik V (2001) Polymer wall coatings for capillary electrophoresis. Electrophoresis 22: 644–655
149. Lee JG, Cheong KH, Huh N, Kim S, Choi JW, Ko C (2006) Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification. Lab Chip 6: 886–895
150. Abad-Villar EM, Kubáň P, Hauser PC (2005) Determination of biochemical species on electrophoresis chips with an external contactless conductivity detecto. Electrophoresis 26: 3609–3614
151. Kan CW, Fredlake CP, Doherty EAS, Barron AE (2004) DNA sequencing and genotyping in miniaturized electrophoresis systems. Electrophoresis 25: 3564–3588
152. Kubista K, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Aspects Med 27: 95–125
153. Lagally ET, Soh ET (2005) Integrated genetic analysis microsystems. Crit Rev Solid State Mater Sci 30: 207–233
154. Belgrader P, Benett W, Hadley D, Richards J, Stratton P, Mariella R Jr, Milanovich F (1999) PCR detection of bacteria in seven minutes. Science 284: 449–450
155. Namasivayam V, Lin R, Johnson B, Brahmasandra S, Razzacki Z, Burke DT, Burns MA (2004) Advances in on-chip photodetection for applications in miniaturized genetic analysis systems. J Micromech Microeng 14: 81–90
156. Roulet JC, Völkel R, Herzig HP, Verpoorte E, de Rooij NF, Dändliker R (2002) Performance of an integrated microoptical system for fluorescence detection in microfluidic systems. Anal Chem 74: 3400–3407
157. Chabinyc ML, Chiu DT, McDonald JC, Stroock AD, Christian JF, Karger AM, Whitesides GM (2001) An integrated fluorescence detection system in

poly(dimethylsiloxane) for microfluidic applications. Anal Chem 73: 4491–4498
158. Kamei T, Paegel BM, Scherer JR, Skelley AM, Street RA, Mathies RA (2003) Integrated hydrogenated amorphous Si photodiode detector for microfluidic bioanalytical devices. Anal Chem 75: 5300–5305
159. Hou CSJ, Milovic N, Godin M, Russo PR, Chakrabarti R, Manalis SR (2006) Label-free microelectronic PCR quantification. Anal Chem 78: 2526–2531
160. Cho YK, Kim J, Lee Y, Kim YA, Namkoong K, Lim H, Oh KW, Kim S, Han J, Park C, Pak YE, Ki CS, Choi JR, Myeong HK, Ko C (2006) Clinical evaluation of micro-scale chip-based PCR system for rapid detection of hepatitis B virus. Biosens Bioelectron 21: 2161–2169
161. Wilding P, Kricka LJ, Cheng J, Hvichia G, Shoffner MA, Fortina P (1998) Integrated cell isolation and polymerase chain reaction analysis using silicon microfilter chambers. Anal Biochem 257: 95–100
162. Burke DT, Burns MA, Mastrangelo C (1997) Microfabrication technologies for integrated nucleic acid analysis. Genome Res 7: 189–197
163. Paegel BM, Blazej RB, Mathies RA (2003) Microfluidic devices for DNA sequencing: Sample preparation and electrophoretic analysis. Curr Opin Biotechnol 14: 42–50
164. Erickson D, Li D (2004) Integrated microfluidic devices. Anal Chim Acta 507: 11–26
165. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16: 11141–11156
166. Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BA, Tunnacliffe A (1992) Degenerate oligonucleotide-primed PCR: General amplification of target DNA by a single degenerate primer. Genomics 13: 718–725
167. Pich U, Houben A, Fuchs J, Meister A, Schubert I (1994) Utility of DNA amplified by degenerate oligonucleotide-primed PCR (DOP-PCR) from the total genome and defined chromosomal regions of field bean. Mol Gen Genet 243: 173–177
168. Liu B, Segal G, Vega JM, Feldmann M, Abbo S (1997) Isolation and characterization of chromosome-specific DNA sequences from a chromosome-arm genomic library of common wheat. Plant J 11: 959–965
169. Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N (1992) Whole genome amplification from a single cell: Implications for genetic analysis. Proc Natl Acad Sci USA 89: 5847–5851
170. Mueller PR, Wold B (1989) In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246: 780–786
171. Schlissel M, Constantinescu A, Morrow T, Baxter M, Peng A (1993) Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'-phosphorylated, RAG dependent, and cell cycle regulated. Genes Dev 7: 2520–2532

172. Staley K, Blaschke AJ, Chun J (1997) Apoptotic DNA fragmentation is detected by a semiquantitative ligation-mediated PCR of blunt DNA ends. Cell Death Differ 4: 66–75
173. Liu D, Liu C, DeVries S, Waldman F, Cote RJ, Datar RH (2004) LM-PCR permits highly representative whole genome amplification of DNA isolated from small number of cells and paraffin-embedded tumor tissue sections. Diagn Mol Pathol 13: 105–115
174. Ludecke HJ, Senger G, Claussen U, Horsthemke B (1989) Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification. Nature 338: 348–350
175. Kao FT, Yu JW (1991) Chromosome microdissection and cloning in human genome and genetic disease analysis. Proc Natl Acad Sci USA 88: 1844–1848
176. Klein CA, Schmidt-Kittler O, Schardt JA, Pantel K, Speicher MR, Riethmüller G (1999) Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc Natl Acad Sci USA 96: 4494–4499
177. Zhang FY, Yin WB, Shi R, Hu YK, Yan YM, Chen YH, Zhou YH, Hu J, Wang R, Hu ZM (2005) Construction and characterization of chromosome 1b specific DNA library of wheat. Can J Plant Sci 85: 309–316
178. Snabes MC, Chong SS, Subramanian SB, Kristjansson K, DiSepio D, Hughes MR (1994) Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci USA 91: 6181–6185
179. Barrett MT, Reid BJ, Joslyn G (1995) Genotyping analysis of multiple loci in somatic cells by whole genome amplification. Nucleic Acids Res 23: 3488–3492
180. Grothues D, Cantor CR, Smith CL (1993) PCR amplification of megabase DNA with tagged random primers (T-PCR). Nucleic Acids Res 21: 1321–1322
181. Wu DY, Wallace RB (1989) The ligation amplification reaction (LAR)-amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4: 560–569
182. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acid Res 30: e57
183. Guatelli JC, Whitfield KM, Kwoh DY, Barringer KJ, Richman DD, Gingeras TR (1990) Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 87: 1874–1878
184. Kwoh DY, Davis GR, Whitfield KM, Chappelle HL, DiMichele LJ, Gingeras TR (1989) Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 86: 1173–1177

185. Chan AB, Fox JD (1999) NASBA and other transcription-based amplification methods for research and diagnostic microbiology. Rev Med Microbiol 10: 185–196
186. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP (1992) Strand displacement amplification – An isothermal, in vitro DNA amplification technique. Nucleic Acids Res 20:1691–1696
187. Persing DH, Smith TF, Tenover FC, White TJ (1993) Diagnostic Molecular Microbiology Principles and Applications. Americal Society for Microbiology, Washington D.C.
188. Walker GT, Little MC, Nadeau JG, Shank DD (1992) Isothermal in vitro amplification of DNA by restriction enzyme/DNA polymerase system. Proc Natl Acad Sci USA 89: 392–396
189. Lizardi PM (2000) Multiple displacement amplification. United States Patent 6124120
190. Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS (2002) Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 99: 5261–5266
191. Nelson JR, Cai YC, Giesler TL, Farchaus JW, Sundaram ST, Ortiz-Rivera M, Hosta LP, Hewitt PL, Mamone JA, Palaniappan C, Fuller CW (2002) TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. Biotechniques 32: S44–S47
192. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet 19: 225–232
193. Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5: 795–800
194. Lizardi PM, Guerra CE, Lomeli H, Tussie-Luna I, Kramer FR (1988) Exponential amplification of recombinant-RNA hybridization probes. Bio/Technology 6: 1197–1202
195. Hecker KH, Roux KH (1996) High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. Biotechniques 20: 478–485
196. Conoci S, Di Pietro P, Petralia S, Amore MG, San Biagio F, Alaimo G, Iacono G, Alessi E, Ricceri D, Di Trapani G, Di Francesco F, Palmieri M (2006) Fast and efficient nucleic acid testing by ST's In-Check™ lab-on-chip platform. Proc Nanotech: 562–565
197. Higgins JA, Nasarabadi S, Karns JS, Shelton DR, Cooper M, Gbakima A, Koopman RP (2003) A handheld real time thermal cycler for bacterial pathogen detection. Biosens Bioelectron 18: 1115–1123
198. Lee TMH, Hsing IM (2006) DNA-based bioanalytical microsystems for handheld device applications. Anal Chim Acta 556: 26–37
199. Belgrader P, Smith JK, Weedn VW, Northrup MA (1998) Rapid PCR for identity testing using a battery-powered miniature thermal cycler. J Forensic Sci 43: 315–319