1 Introduction

Deciphering the genomic sequence is a first and important step to a better understanding of the transduction from the genomic information to the phenotypic appearance.[1] For the human genome the number of coding genes is currently estimated to approx. 22,000 genes.[2] The total number of transcripts is even higher, currently estimated to approx. 49,000 (Ensembl, Genebuild Aug 2006).[3] Investigating the circumstances under which a particular gene is up- or down-regulated provides important clues about gene function and is one central component of functional genomic analysis. Facing this high number of genes and transcripts, high throughput technologies were needed for comprehensive functional genomic analyzes. This led to the development of highly parallel working technologies such as microarrays, which allow to analyze many genes simultaneously.[4] Meanwhile microarrays emerged as a powerful and cost-effective tool for large-scale analysis of gene expression or genetic variation.[5–9] Shortcomings of this technology are a relatively low sensitivity and dynamic range of quantification, as well as a high susceptibility against biased signal intensities,[10] which make the detection of e.g. small changes in expression levels difficult.[11] For expression analysis the quantification ability of microarrays is low compared to other technologies such as real-time PCR. Microarrays are normally used for finding candidates, which are then validated with other methods. A highly parallel and cost-effective working platform for functional genomic analyzes with minimal sample and reactant consumption and a better accuracy and sensitivity could help to save precious samples and would accelerate comprehensive gene expression studies.[12] This led to the idea of a miniaturized high-density open-well based platform for more accurate and sensitive PCR based methods to investigate gene expression and genetic variation at high-throughput, good flexibility and moderate costs.

1.1 Miniaturization of bioanalytical systems

The development of miniaturized analytical systems has been an ongoing research for almost 30 years. First reports were shown on the field of chromatography.[13] A first instrument for nucleic acid analysis (capillary electrophoresis) was introduced to the market almost 8 years ago by Caliper/Agilent Technologies. The last decade was characterized by developments on the field of microarrays,[12] which became available on the market. Miniaturization of liquid assays especially microfluidic research still lacks a significant number of commercialized solutions in bioanalytics. Research on miniaturization of bioanalytical systems is motivated by the reduction of needed volumes of sample and reagents, the creation of relatively high surface-to-volume ratios, the possibility of manufacturing portable or hand-held devices and enabling screening with high throughput at moderate costs. Miniaturization allows a higher data density, shorter

processing times and shorter analysis times, which are central requirements for complex analyzes in functional genomics.

For the applicability of miniaturized systems in bioanalytics it is essential that they become integrable into a laboratory by capable interfacing between the macro- and microscale.[13,14] This led to the idea of highly integrated "Lab-On-A-Chip" systems, which are also called micro total analysis systems (μTAS).[15–22] Lab-On-A-Chip systems predominantly belong to the static and chamber based solutions for miniaturized systems. Chamber based solutions are the most prevalent form of miniaturized systems currently in development for biochemical analysis using liquid assays. Reactions are performed in parallel each in a confined space.[23]

As an alternative to parallel processing, sequential flow-through systems were shown to be appropriate for miniaturizing biochemical assays, too. Samples are kept in capillaries or channels and subjected to a continuous flow where they pass e.g. thermal zones or sensors for signal read-out.[16,24–30] More recently approaches were shown using miniemulsions (e.g. water in oil) of distinct droplets for the segmentation of reaction compartments.[31,32] For all approaches mentioned it is crucial to separate samples efficiently from each other by defining stable compartments, which makes them all capable of performing PCR based applications.

1.2 The Polymerase Chain reaction

The polymerase chain reaction (PCR) is a very widely used and powerful technique in molecular biology for the highly specific and efficient amplification of desoxyribonucleic acids. More than 20 years ago it was introduced by Mullis and coworkers[33] and evolved to the 'gold standard' for the amplification of DNA. In principle, the reaction is based on repeated cycles of three steps. First step is the denaturation at a temperature of approx. 95℃. The double stranded template DNA is separated into two complementary single strands. In the second step, the elongation, temperature is lowered to the hybridization temperature of the primers. Primers are two oligonucleotides mostly with a length of 18 – 25 nucleotides each complementary to one strand. After hybridization of the oligos to the DNA strand a polymerase starts to elongate the primers on their 3'-end by attaching additional nucleotides complementary to the DNA strand. The polymerase used is a heat-resistant DNA polymerase from a thermophile bacterium such as *Thermus aquaticus*. Elongation is strengthening the bonding between the primers and the DNA. Thus, in the third step the temperature can be increased to the temperature optimum of the enzyme such as 72℃to achieve optimal product yield. These steps are repeated 30–40 times. After each cycle the number of copied DNA molecules doubles and therewith the number of available targets as well. This exponential nature of the reaction enables amplification rates of 10^6 to 10^8. Therefore and because of sensitivity and specificity, PCR has many applications in genetics, clinical medicine, forensic

science or evolutionary biology.

Currently PCR is performed in standard volumes of 50 µl down to 20 µl using tubes or 96-well microtiter plates. With the application of the 384-well plate, the most widely used microtiter plate format in molecular biology, the reaction volume could be reduced to 10 µl. Even microtiter plates with 1536 wells are already available, capable of dropping down the volume to 1 – 2 µl. However, they are only rarely used because of a lack of appropriate processing devices. To perform biochemical assays in small reaction volumes it is crucial to develop miniaturized formats as well as appropriate processing and detection devices.

1.3 Microchip PCR systems

Attempts to miniaturize PCR led to a wide spectrum of solutions. The majority of developments has been made on the basis of static chamber and channel like systems. Differences exist regarding the accessibility of the reaction vessels. Closed channel/chamber based systems are accessible via capillaries that direct to the reaction vessel. Open well solutions are accessible from top and have to be sealed after filling. Closed systems were more prevalently used to realize a "lab-on-a-chip" platform, whereas open well based solutions were shown for screening purposes. Advantages of closed systems are the lower contamination risk and the stringently defined interfaces between different steps in the workflow. First examples like simple PCR reaction chambers[34] have meanwhile been replaced by rather complex solutions of PCR with integrated downstream analyzes such as gel-electrophoretic analysis on the chip.[17] Shortcomings of complex closed systems are low flexibility in use, required complex surface rendering according to the properties of the liquids used and a high risk of clogging.

Systems that contain open wells are more appropriate to the requirements of miniaturized high-throughput screening platforms. Attractive features of this approach are a potentially large number of individual reactors, the random and multiple access to individual vessels, which supports flexibility and no carry-over problems like in sequential flow reactors. Challenging for the open well approach are the higher contamination risk from the outer environment, evaporation losses and the robust liquid transfer in the nanoliter range.[23]

1.3.1 PCR chip materials and fabrication strategies

Stimulated by the technological developments in microelectronics the first miniaturized devices were made from silicon or glass.[16–18,34,35] Although these materials are excellently suited for for microfabrication, especially in the case of silicon-based PCR chips partial inhibition of PCR amplification has been repeatedly reported.[36–38] Two complementary mechanisms were proposed to account for this, the straight chemical

inhibition[36] and surface adsorption.[36,38] Enzyme adsorption has been proposed as the most probable cause for inefficient PCR performance, but this might also apply to the availability of other reagents.[37] Adsorption effects could be especially relevant for miniaturized systems due to the high surface-to-volume ratio. For glass, only minimal inhibitory effects on the PCR exist.[37] Meanwhile several coating and surface passivation procedures to render the surfaces of microchips 'PCR compatible' have been developed.[35–37] Silicon oxide and some polymer based coatings for silicon as well as alternative substrates led to good amplification results.[37,39] Apparent inconsistencies in literature to the effect of several polymer based coatings demonstrate probably more the challenge of an consistent coating of small dimensioned devices, than effects of the coating itself.

Polymeric materials are actually a very good alternative to silicon or glass for fabrication of micro-structured PCR chips. To achieve a micro-structuring of polymers comparable with silicon or glass, new fabrication technologies had to be developed. Within the last years technologies like molding or embossing became more sophisticated by developing new fabrication tools. These methods have been complemented by further technologies such as photolithography.[40,41] Meanwhile structures can be created down to the nanometer scale using polymers. During the last few years devices from various polymeric materials like polyimide,[42] polydimethylsiloxane-glass (PDMS),[43,44] polymethylmethacrylate (PMMA),[45] polycarbonate,[46] parylene[39] and cyclic olefine copolymere (COC)[47] were shown to be appropriate for several bio-analytical assays.

Except parylene, which was used as a coating for PCR-devices, and PDMS which was used in combination with glass, all materials appeared to be possible substrates for devices of PCR applications. With respect to PCR based application materials primarily have to fulfil the following requirements: biochemical and especially PCR compatibility, thermal stability up to at least 100℃, good thermal conductivity for good heat transfer as well as an even heat dissemination, cost effectiveness and low background in respect of the detection method used.

1.3.2 Thermocycling of PCR chips

Thermal processing of PCR can be done either using contact heating methods or non-contact heating methods. For contact heating methods the heating source for a microchip PCR can be an external heating block, which is currently in most cases done by peltier elements, or heaters directly integrated in the chip (e.g. tungsten or platinum films).[48–50] One of the non-contact heating methods used is infrared radiation.[42,51] Cooling can be done by forced air using e.g. a fan or by means of a coolable heating block like e.g. a peltier cooler-heater element.[34,52] A more sophisticated thermal control of microchips was developed by the implementation of specific structural features in the chip, such as grooves and air spaces.[49,53] These features were designed to isolate

the PCR chamber and minimize lateral heat transfer from the chamber to the bulk of the microchip.

After first theoretical and experimental studies it became obvious that miniaturization will enable much faster cycling than it is now possible with conventional devices. Thermal mass is the key for fast thermocycling, and this is predominantly determined by the used heating device for miniaturized substrates. Microsystems allow low volumes of samples and reagents, coupled with very effective heat dissipation and transfer, which leads to faster and more specific reaction kinetics.[37]

1.3.3 Detection of amplification product

Most of the PCR based assays in a miniaturized format as well as larger volumes, include a step, when the integrity of the product is tested. Basically this can be done two ways: first, the separation of products after performing PCR to control for the expected fragment size, and second, by using probes to tag the expected fragments either during or after the PCR. Separation is mainly done by electrophoresis, but alternative such as chromatographic methods, isoelectric focussing based methods or separation according to the diffusion coefficient were also developed.[54] For the probe based approach the detection system used depends on the marker used. Most prevalent in literature are fluorescent dyes, but also UV radiation, chemiluminescence, electrochemical detection and mass spectrometry have been used for the detection of PCR products.[54]

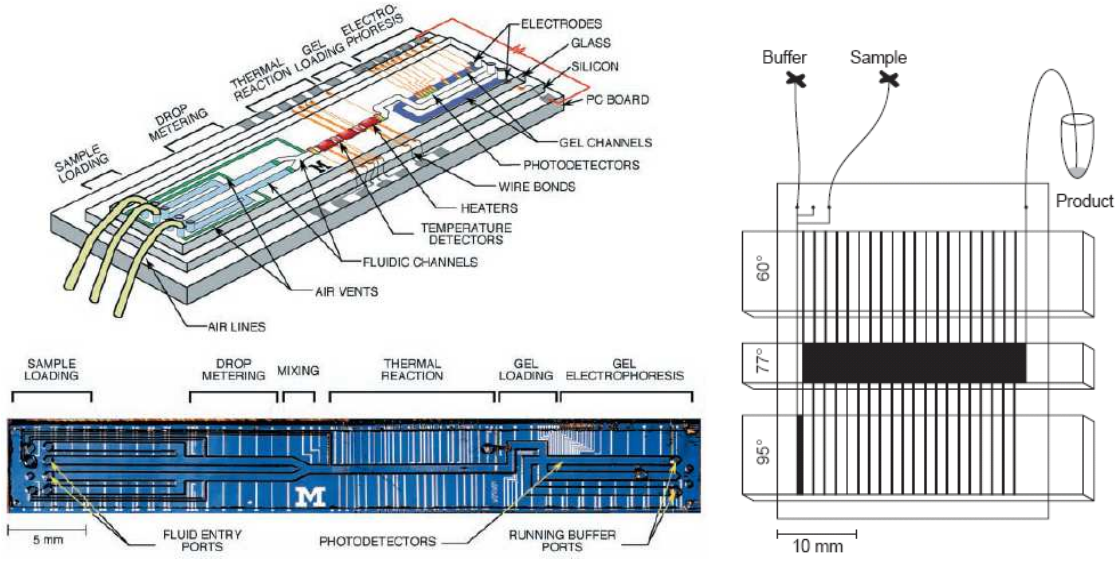
For excitation of fluorescent dyes lamp/filter based systems are used by illuminating the chip with light corresponding to the used dye, or with laser light that is focused to the individual reaction chambers. The emitted light is collected by camera objectives,[47] microscope objective lens[30,44] or photomultipliers.[18] Some systems are able to measure continuous spectra, others use a filter system to measure at particular wavelengths.

1.4 Advances in miniaturization of PCR

Miniaturization of PCR volumes is driven by the following motives: reduction of dimensions to enable highly integrated µTAS systems, the increase of data density, saving of costs and biological material, enabling mobile and hand-held analytical devices, shorter processing times and the ability of single molecule applications. Within the last decade combinations of almost every kind of substrate material, chip architecture and detection approach were shown. In the following an overview is given regarding the developments in miniaturized PCR using closed chamber based, flow through and open well based devices.

1.4.1 Closed chamber based devices

First attempts to miniaturize the PCR were made in the early nineties with closed chamber based devices. Chambers were usually filled via channels by capillary action. Wilding et al.[34] were the first who reported a miniaturized PCR with integrated capillary electrophoresis (CE) in one miniaturized device, using a silicon/glass hybrid device to perform 5–10 µl PCR. The functional integration of PCR and capillary electrophoresis with focus on the development of portable PCR analysis devices was introduced by Woolley et al.[18] and Northrup et al.[35] In the beginning PCR was performed with a rather large volume of 20 µl, but already with processing times of less than 20 min. Meanwhile one can find reports of PCRs in the low nanoliter range for portable devices with processing times of less than 5 min.[42,55] A more complex solution for performing miniaturized PCR was developed by Burns et al.,[17] who integrated a simple sample mixing, PCR and gel-electrophoretic analysis into one device. The reaction was performed with a volume of 120 nl. Figure 1 a shows the relatively complex chip, with samples being loaded onto the chip, mixed, thermally processed and gel-electrophoretically analyzed.



(a) Schematic of integrated device with two liquid samples and electrophoresis gel present.[17] The only electronic component not fabricated on the silicon substrate, except for control and data-processing electronics, is an excitation light source placed above the electrophoresis channel.

(b) Schematic for flow-through-PCR chip with three well-defined thermal zones for denaturation (95℃), hybridisation (60℃) and elongation (77 ℃).[16]

Figure 1: Examples for miniaturized closed-chamber and flow-through device.

Recently Marcus et al. showed a complex concept of integrated mRNA isolation, cDNA synthesis and gene expression measurement[56] for single cells on one chip. The maximal reduction of volumes for closed chamber based systems was shown 2003 by Liu et al.[30,57] In a PDMS chip PCR was performed with a volume down to 3 nl.

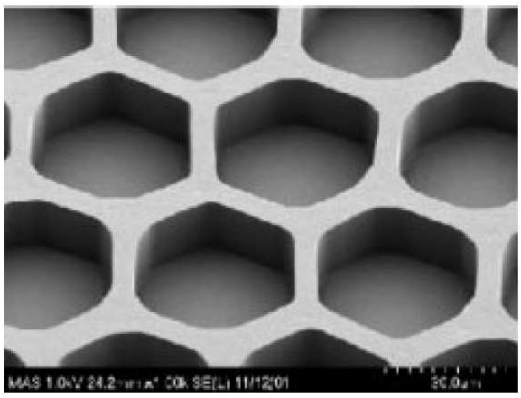
1.4.2 Flow-through devices

In a continuous-flow amplification system the reaction mixture is pumped through the chip with several thermal zones. PCR is performed by repeatedly passing the temperature zone for denaturation, annealing and elongation. This approach is characterized by a low thermal mass of the thermal processing, as the thermal heating block does not require fast temperature changes like in conventional cyclers, and thus does not contribute to the thermal mass. This allows extremely fast thermal processing, which is determined by the flow rate. The first example of a flow-through device in smaller dimensions was given by Kopp et al.[16] As shown in Figure 1 b three thermal zones were passed by capillaries to perform PCR with 20 cycles in a time ratio of 4:4:9 for denaturation, annealing and elongation, respectively. Final times were determined by the flow rate, allowing successful PCRs with a total processing time of 1.5 – 2 min. The continuous flow approach bares a high risk of contamination since different samples have to pass the same reaction channel. Obeid and coworkers[25] were one of the first who showed a continuous-flow PCR system able to process different samples in a serial manner. Surprisingly only a combination of an air bubble and a water plug were suitable to wash between two samples to avoid cross-contamination.

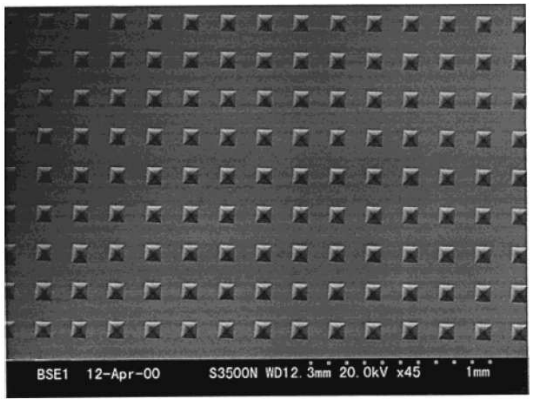
1.4.3 Open well based devices

Open well systems are mainly plate formats with multiple reactors, which are accessible from top. If necessary the system can be closed for processing by adequate sealing methods. The open well approach is one of the most suitable approaches to achieve highest densities of reaction vessels and low reaction volumes. A 1536–well system introduced by Sasaki et al.[58] in 1997 already enabled reaction volumes below 1 µl and addressed high-throughput by miniaturization. Nagai and coworkers[59] used an silicon-based open well solution to determine the minimal volume for performing PCR (Figure 2 b). They could reduce the volume down to 86 pl. Below this volume adhesive effects between the substrate and DNA as well as Taq polymerase were determined as the responsible factors for the failing amplification. Later the possible volume of PCR on a silicon device could be further reduced by Leamon and colleagues down to 39.5 pl, which seems to be the minimal reported volume of a PCR up to date (Figure 2 a).[60] With an average fold amplification of 2.36×10^6 a result comparable to conventional volumes was reached with the 39.5 pl reactions.

To ensure successful amplification in nano- and picoliter volumes the initial target concentration must be rather high. Low concentrations can easily lead to failing reactions due to distributional effects on target molecules. This distributional effect was used by Matsubara[61] et al. for quantifying the initial number of target molecules. They performed PCR in 40 nl and quantified the initial number of molecules by counting reaction chambers with high fluorescence signal. The ratio between failing and successful



(a) PicoTiterPlate from 454.com[60]



(b) Microchamber array from Nagai et al.[59]

Figure 2: Two examples for high density well plates **a)** The PicoTiterPlates from 454.com are made by etching into silicon and have a diameter of approx. 40 µm with depths ranging between 26 – 76 µm. **b)** The microchamber array from Nagai et al., also made from silicon wafers, comprise wells of 80 × 80 µm.

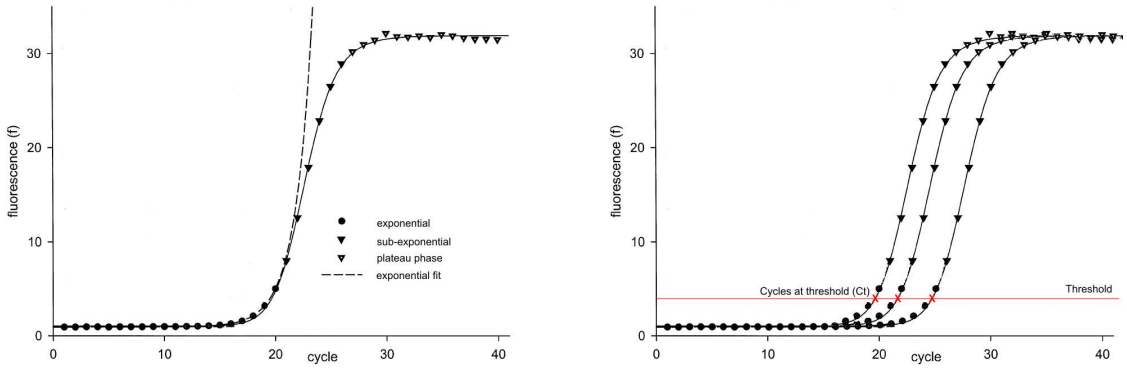
reactions was used to determine the initial number of molecules. This quantification method has only a low dynamic range of one order of magnitude. The methodology of real-time PCR (see 1.5) offers a much wider dynamic range of more than 8 orders of magnitude.[62] Real-time PCR was recently used on a PDMS fabricated open well device to quantify initial amounts of DNA in a volume down to 900 nl.[44] This is the only example of an open well based approach using plastics and real-time analysis in combination.

1.5 PCR based expression analysis

The analysis of gene expression is an essential element of analytics in the field of functional genomics. Although there are several methods available, nowadays RNA expression analysis mainly builds on DNA microarrays.[4] However, regarding the less accurate quantification capability of microarray based methods, real-time PCR based expression profiling is currently the 'gold standard' for precise monitoring of gene expression.[63] The technology possesses a large dynamic range of above 8 orders of magnitude. This means, that it enables quantification of initial molecules up to target number differences ranging from X to $X \times 10^8$ molecules.[62] This and its high sensitivity are perhaps the main reasons why real-time PCR is ubiquitously applied for expression analysis in molecular biology.

Real-time PCR is a refinement of the original PCR method invented by Mullis and co-workers.[33,64] Since normally PCR reaches a plateau phase at the end of the reaction when the amount of amplicon saturates, precise quantification can not be performed by simply looking at an endpoint measurement. Early attempts to perform a quantitative analysis of PCR were based on the quantification of PCR products at an empirically

determined number of cycles[65] before all reactions reached the product saturation. PCR was stopped, loaded on a gel, stained and intensities were compared. In 1993 Higuchi and coworkers[66] introduced the basic concept of the real-time PCR with their 'Kinetic PCR Analysis'. They continuously monitored the amplification by using a CCD camera to measure fluorescence from an intercalating dye that was introduced into the reaction mix (see schematic view in Figure 3 a). Thus fluorescence corresponds to the amount of



(a) Schematic view of PCR phases

(b) Concept of cycle at threshold in real-time PCR

Figure 3: Time course of product accumulation during PCR (figure modiefied after Tichopad et al.[67]) **a)** Trajectory of the PCR reaction by plotting amplification product yield over time. **b)** Cycle at threshold concept, introduced by Higuchi et al.[66] Threshold has to be set within the exponential phase of the PCR.

amplification product. The time or PCR cycle when the fluorescence signal significantly increases above background is in proportion to the initial amount of starting material. This is the key aspect differentiating the kinetic PCR from former methodologies. In the early cycles of PCR the amount of product accumulates exponentially, described by the following equation:

$$P = I \cdot E^n \tag{1}$$

P is the amount of product, I the initial amount of target, E the efficiency of the reaction, ranging between 1 and 2, and n the number of cycles.
At later reaction stages the reaction trajectory significantly diverges from the exponential type.[67] A phase with sub-exponential product accumulation starts and goes over to the last PCR phase, the plateau phase, where the product yield levels off and saturates. This is mainly because the reaction is running out of some critical components like primers, the reporter, or the dNTPs. Also the number of polymerase molecules can be limiting. In this case the exponential amplification goes over to linear amplification.[68] Furthermore saturation of the amplification is caused by the rapid reannealing of product strands. normally at a concentration of 10^9 product molecules/µl.[69]

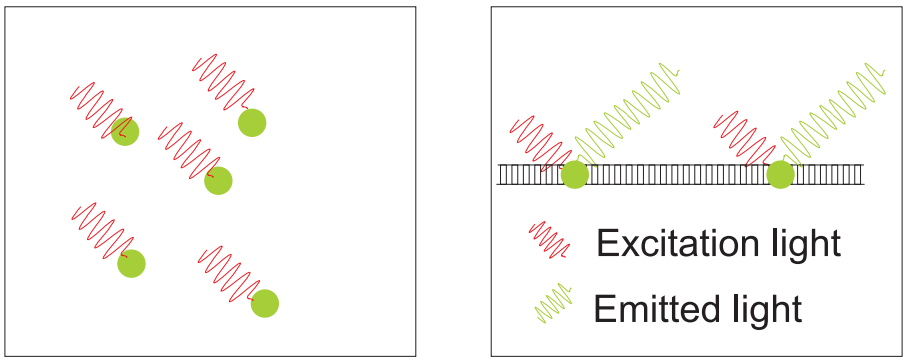
The target copy number is determined by the fractional cycle at which a threshold amount of amplicon DNA, represented by a threshold fluorescence intensity, is reached

(threshold cycle or Ct). The sooner the amplification trajectory crosses the set threshold (within the exponential phase), the more starting material was present (see Figure 3 b). This allows to quantify the amount of initial target DNA with the advantage that sensitivity is independent of copy number. For kinetic or real-time PCR a possible dynamic quantification range of 7 to more than 8 orders of magnitude could be shown.[62,70–72]

During the last years a wide spectrum of detection chemistries including alternatives to the initially used intercalating dyes has been developed. Basically assay methods can be divided into specific or nonspecific methods. Nonspecific methods are used to detect the amount of amplicon, but do not provide any sequence related information. These methods, as for example intercalating dyes, are prone to 'false-positives', since also primer-dimer-artifacts and spurious amplicons lead to an increase of signal intensity.[73] Therefore, very often tedious optimization of the reaction conditions and repeated primer design are necessary. In contrast, specific methods use sequence specific probes with attached fluorophores to detect only amplicons of the desired target. In the following the principle of the intercalating chemistry and some examples for probe based chemistries are shown, which both are typically used for real-time PCR.

1.5.1 Detection using intercalating dyes

Intercalating dyes are frequently used because the dye is independent from the primer design. This makes the assay design easy. The genotoxic ethidium bromide,[74] which was used by Higuchi and colleagues,[66] has almost completely been replaced by other less toxic and more sensitive dyes such as SybrGreen I. In Figure 4 the function principle of intercalating dyes is shown: As long as no double stranded DNA (dsDNA) is present,



(a) Free intercalating dye in solution

(b) Intercalating dye bound to dsDNA

Figure 4: Principle of intercalating dyes **a)** Free intercalating dyes in solution, without any double stranded DNA. No light is emitted. **b)** Intercalating dyes bound to the PCR amplicon. Excitation leads to emitted light.

the amount of free dye is large and no fluorescence is emitted. If dsDNA is present,

intercalating dyes bind without or little sequence specificity to the dsDNA. Thereby each dye has its own stoichiometry e.g. ethydiumbromide at one dye molecule per 4 – 5 base pairs DNA.[75] Once these dyes are bound to dsDNA, their fluorescence is 10-fold enhanced.[76] The resulting signal intensity is proportional to the amount of all double-stranded DNA present in the reaction.

For verification of the product identity a melting curve analysis can be performed at the end of the PCR. If the fluorescence is monitored continuously throughout a temperature gradient, starting below the annealing temperature up to the denaturation temperature of 95℃, product denaturation can be observed as a loss of signal intensity near the denaturation temperature.[77] Shape and peak position of a melting curve are functions of the GC/AT ratio, the length and sequence of the amplicon and can therefore be used to separate products by melting temperatures differing less than 2℃.[78]

1.5.2 Probe based detection chemistries

For the probe based approach different detection chemistries were developed using e.g. hydrolysis probes, hybridisation probes or molecular beacons.

Hydrolysis probes

The hydrolysis probe chemistry (TaqMan) depends on the 5'–3'-exonuclease activity of Thermus aquaticus DNA polymerase. In Figure 5 the basic principle of the TaqMan assay is shown. A single stranded DNA probe is designed to hybridize within an am-

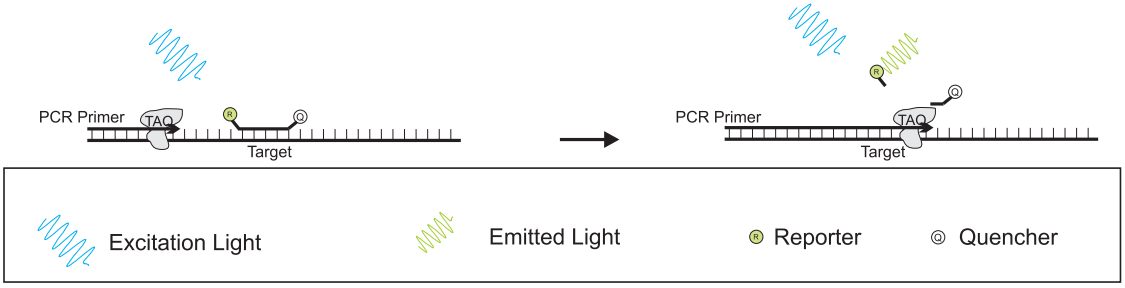
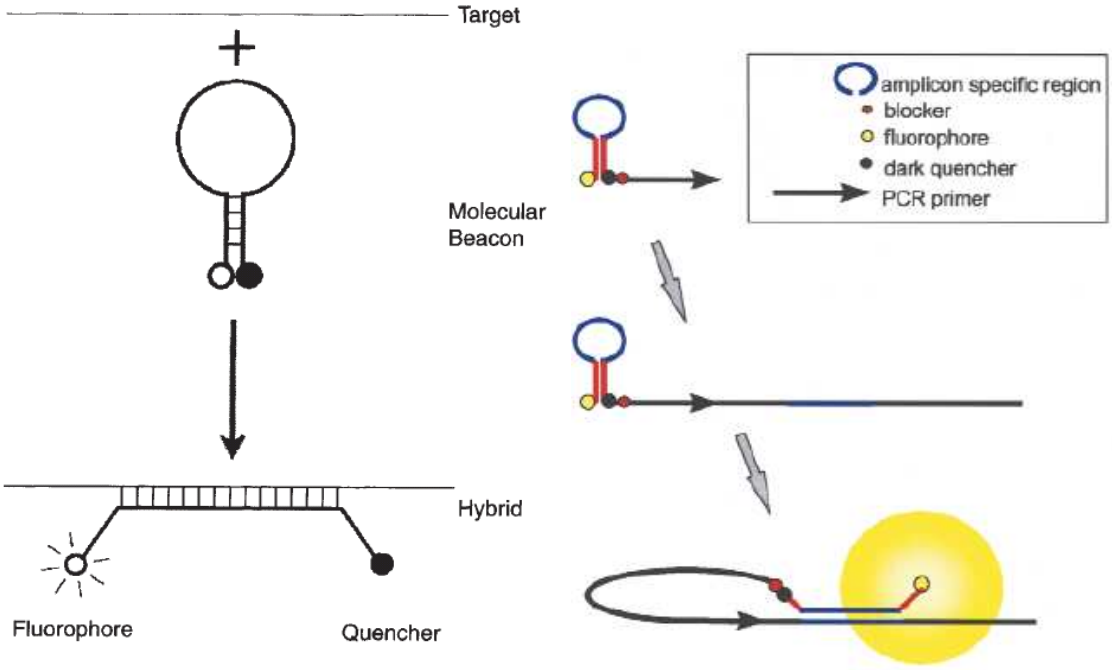


Figure 5: During elongation the hybridized probe is hydrolyzed by the 5'–3'-exonuclease activity of Thermus aquaticus DNA polymerase. The quenched reporter is liberated and excitation leads to the dye specific emission of light, which can be detected.

plicon. The 3'-end of the probe is phosphorylated, to keep the oligo nonextendable.[79] The probe is labeled with a fluorescent reporter dye and a quencher at opposite ends of the sequence. Because of close proximity between quencher and reporter for the intact probe no fluorescent signal is emitted. During PCR the probe is hydrolyzed, which liberates dye and quencher.[80] Fluorescence of the reporter increases proportionally to the quantity of produced amplicon.[81]

Molecular Beacons

A further probe based detection chemistry are Molecular Beacons (MB). MBs are single-stranded nucleic acid molecules that possess a stem-and-loop structure (see Figure 6 a). The loop portion of the MB is a probe sequence complementary to the target. The stem is formed by the annealing of two complementary sequences, which are unrelated to the target. A fluorescent moiety is attached to one part and a nonfluorescent quenching moiety is attached to the other end. Over the stem both moieties are kept in close proximity, which leads to an effective quenching of the fluorophore by resonance energy transfer.[82] If the probe encounters a target, a more stable MB-target-complex than the original stem-loop-conformation is formed, which separates fluorophore and quencher and leads to fluorescence.[83]



(a) Principle of molecular beacons (Tyagi et al.[83]) Molecular Beacons are based on single-stranded nucleic acid detector molecules, which possess a stem-loop-structure and carry a quenched reporter dye. A change in conformation by hybridization to the target leads to the abolishment of the quenching.

(b) Principle of Scorpion probe (Whitcombe et al.[73]) Scorpion probes are also based on a stem-loop-structure. The Scorpion probe comprises the PCR primer. This transforms the interaction of primer, target and probe into an unimolecular event.

Figure 6: Selection of probe based approaches

Scorpions

A third example for a probe based chemistry are Scorpion probes. Similar to MBs they possess a stem-loop-structure with dye and quencher, but with an attached PCR primer. Between the 5'–end of the primer and the attached probe element a hexethylene glycol (HEG) monomer is included to block the amplification of the probe element. After one PCR cycle a loop is formed by hybridization of the newly formed amplicon with

the attached probe element (Figure 6b). This leads to separation of dye and quencher inducing a rise in fluorescence. The Scorpion technology combines the intermolecular action of primer, target and probe to an unimolecular event, making the reaction kinetics extremely fast.[73,84]

Hein et al.[85] showed that sensitivity and reproducibility are broadly comparable for assays with intercalating chemistry (SybrGreen I) and hydrolysis probe based assays (TaqMan). The request to speed up the assays led to continuous innovation and new technologies such as the scorpion probes.[84] Probe based assays allow closed-tube applications with no downstream analyses, which further reduces the time needed.

1.6 PCR based SNP genotyping

Single Nucleotide Polymorphisms (SNP) are the most prevalent form of variation in the human genome. A SNP is a DNA sequence variation when a single nucleotide in the genome differs between members of a species. Per definition those positions are called SNP, if the frequency of the lower abundant allele (minor allele) is above 1% within the population.[86,87] SNPs are highly abundant and have been initially estimated to appear in average at one of thousand bases, equivalent to 3 million SNPs in the human genome.[88–90] More recently this number of SNPs was estimated to be higher at a total of approx. 10 million SNPs.[91] Currently public databases such as the NCBI SNP database already contain almost 12 million (5.5 million validated) SNP markers for human, and their number is still growing (NCBI dbSNP Build 126).[92]

Concerning their position SNPs are distributed across all regions of the genome. Depending on the regions function this can have different consequences. SNPs that are for example found in regulatory elements can cause changes in the transcription level of a gene.[93] If they are positioned in the coding sequence, SNPs can lead to protein structure changes by amino acid exchange.[87] Alternative splicing can be due to SNP, that are localized in splice cites. SNPs are also located in non-coding regions, where their role is not fully understood. Those SNPs were seen to be useful as markers for population genetics or evolutionary studies.[94,95] Recent research on non-coding RNAs led to a change in the understanding of the meaning of noncoding regions within the genome,[96,97] which might change the assumptions on the importance of SNPs in these regions in the near future.

To assess the genetic variation by genotyping SNPs a wide variety of technologies have been developed: hybridization with allele specific oligonucleotides, allele specific PCR, single nucleotide extension assays, allele specific ligation, the Invader technology or restriction site cleavage.[87] Although specially microarray technologies are available for performing studies on a genome-wide scale, flexible genotyping methods such as the PCR-based methods are still needed, e.g. for subsequent follow-up fine mapping of candidate regions in association studies. Before the invention of PCR the detection of

a single base mismatch in a complex matrix like the human genome was a demanding task. Wallace et al.[98] were the first who showed the detection of a single base pair mismatch using allele specific hybridization probes. Since the invention of PCR it has been possible to amplify the target region to reduce the complexity of the genome, which allowed the development of more efficient genotyping methods. In Figure 7 again the principle of the TaqMan assay is shown, but here for PCR based SNP genotyping:

Two allele specific hybridization (TaqMan) probes are designed to hybridize within

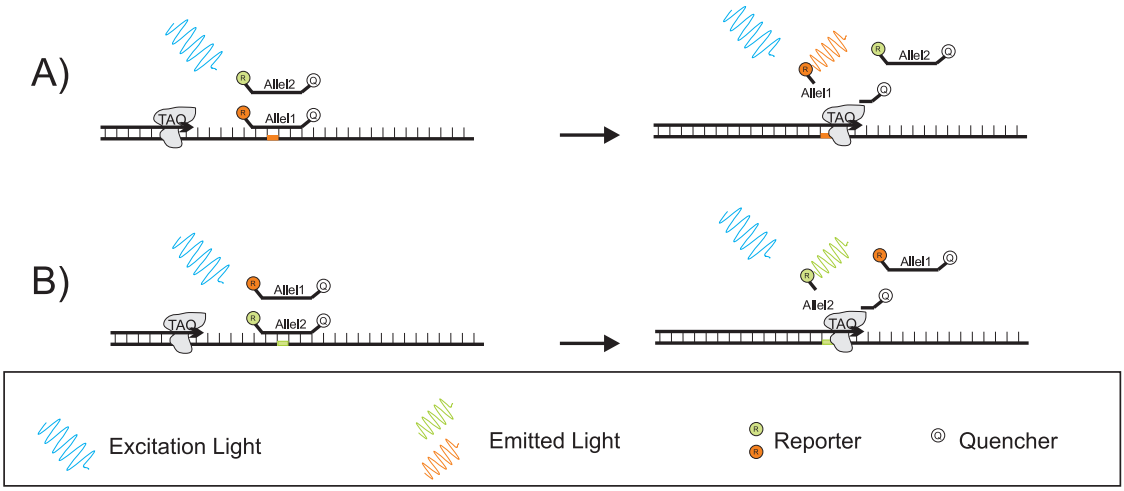


Figure 7: Principle of TaqMan SNP genotyping assay **a)** Allele specific hybridization of Probe 1 leads to a fluorescent signal of the reporter 1. **b)** Allele specific hybridization between allele 2 and the appropriate probe leads to fluorescence by reporter 2.

an amplicon. Both carry different reporters that are distinguishable from each other by their spectrum. Similar to the gene expression TaqMan assay the hybridizing probe is hydrolyzed by the exonuclease activity of the Taq DNA polymerase. In case of homozygosity only the reporter 1 or reporter 2 signal is detected. Heterozygous samples show rising fluorescence intensities for both reporters.[80] Allele calling can be done by an endpoint measurement of intensities for both reporters, no separation step is needed. This makes such one-step assays easy and quickly to perform, which might be one of the reasons why TaqMan assays are broadly used for SNP fine mapping genotyping studies.

1.7 Aim of this work

Aim of this work was to develop a miniaturized platform for performing PCR-based assays on a nanoliter scale for gene expression analysis and SNP genotyping. The approach of a high-density open well platform was chosen, since it allows a high density of reaction vessels and a good flexibility in the configuration of screening scenarios. An arrangement of liquid assays in a dense array like format would allow the combination of the advantages of the array format such as parallelized processing with the advantages of liquid PCR based assays like a high sensitivity and specificity.

At first an appropriate and cost-effective substrate was required. Materials had to be studied concerning their bio and in particular PCR compatibility. Prerequisite for the performance of nanoliter PCRs was the development of an advanced liquid handling, which allows reliable distribution of assay components in nanoliter volumes. For the changed dimensions with their increased surface-to-volume ratio an robust assay format for real-time PCR based expression analysis as well as SNP genotyping needed to be found. Candidates had to be evaluated with respect to a miniaturized high-throughput setting. Parameters of interest were reliability, robustness, sensitivity, reproducibility, comparability to conventional volumes, required amount of biological material and cost efficiency. Developed units for processing of the assays such as thermocycling and optical detection had to be evaluated and required procedures for data handling and analysis should be developed. Finally validation studies for both applications in the low nanoliter range were intended to demonstrate the applicability of the platform in functional genomics.