4 Discussion

The development of a miniaturized analytical system is not just a transfer of conventional assays to the micro- or nanoscale. Changed geometrical dimensions make surface properties of miniaturized devices more important. The transportation of liquids in nanoliter volumes needs more advanced technologies, the workflow is much harder to control, and additionally the smaller assay volumes require highly sensitive detection. This is hardly achievable with conventional systems and requires the development of appropriate systems for processing and detection as well as the development of assays.[13]

Miniaturization is close to material science, since surface related effects become greatly enhanced by shrinking reaction volumes and consequently increasing the surface-to-volume ratio.[22] For this project it was of pivotal importance to select an appropriate substrate for the fabrication of a micro-structured analysis device. Since the platform was intended to the application of PCR based assays, the PCR compatibility of the material was central requirement. To keep the whole platform cost-effective at a high throughput, the price for a potential consumable was also of concern. Thus plastic materials were preselected as potential substrates. Plastic chips are disposable, inexpensive, optional optically transparent and biocompatible,[106] which makes them excellent candidates for mass produced devices at low costs.

4.1 Material tests for the µPCR Chip

Due to the expected impact of surface properties potential materials for chip fabrication were tested regarding PCR compatibility, thermo stability and background fluorescence. PCR compatibility was evaluated by investigating the enzymatic activity, yield of the expected PCR fragment and the degree of dimerisation in the PCR material test (2.3). Materials were excluded, which showed a slight reduction of product yield or a higher accumulation of primer dimerisation products. Furthermore materials were rejected that had a high fluorescence background or were thermally instable.

With respect to PCR compatibility transparent samples of COC and PP performed best. No inhibition effects were observed. Especially the COC sample showed excellent optical properties such as a low background, which is in good agreement with other reports.[47,107] Due to the small dimensions of high density well plates signal crosstalk between neighboring reactors has to be taken into account. In a study of Irawan et al. crosstalk was reported in up to 6 mm distanced fluorescein filled glass capillaries.[108] To avoid a potential signal crosstalk between different wells, black colored variants of COC and PP were tested. With respect to the PCR compatibility and thermostability materials were similar to the transparent material samples, the background fluorescence was significantly reduced and comparable for both materials.

The observed reduction in product yield for some materials is probably caused

by lower enzyme activity or adhesion effects on other reactants. Hydrophobicity of polymer surfaces was reported to be mainly responsible for the increased adsorption of proteins,[13,109] which would apply to the polymerase as well and could reduce the enzyme activity. However, with the 'PCR material test' it was not possible to distinguish between adsorption of enzyme or the adsorption of other reactants, but it was a straight forward method to select PCR compatible materials. Although COC and PP turned out to be equally appropriate materials with respect to PCR compatibility, first test using oil showed that COC is non-resistamt against highly polar solvents. Thus PP was chosen for fabrication of the µPCR chip, since it was fully PCR compatible with no limitations such as COC.

4.2 The µPCR chip

A main challenge for the design of plastic reactors is to achieve rapid thermal cycling and even heat dissemination, despite poor thermal conductivity of plastic materials.[110] Northrup et al.[35] showed the advantages of silicon reaction chambers in respect of thermal conductivity and thermal uniformity. They also showed, that the PCR reaction fidelity could be significantly enhanced by various polymer liners. PP turned out to be the most appropriate one, which corroborates the results from material testing. The design of the polypropylene-based µPCR chip (L. Nyarsik) possesses a very thin bottom for optimal heat transfer. Three different formats were developed for an reaction volume range from 18 to 200 nl: first, the flat bottomed 1k-format, which allows assay volumes down to 200 nl, second the 1k/V-format and third the 4k-format. Flat bottomed reaction vessels are limited to the volume at which the bottom surface is not fully covered anymore. This leads to optical irritations or signal intensity imbalances, caused by a reduced or biased accessibility for the excitation source or the detector. The V-shaped reaction wells of the 1k/V-µPCR chip circumvent this and allow assay volumes down to 50 nl. For further scaling down the flat bottomed 4k-µPCR chip with a total volume of 25 nl was developed.

All formats are fabricated by injection molding, which allows a cost effective production of the chips. It is an advantage of master based fabrication strategies such as embossing or injection molding, that the difficult fabrication steps like lithography and electroplating are performed only once. After obtaining the master plastic micro-devices can be highly reproducible fabricated in a very cost effective manner.[111] Therewith the three µPCR chip formats on basis of PP represent cost-effective and appropriate substrates for miniaturized PCR based assays in a reaction volume range from 18 to 200 nl.

4.3 Workflow for a miniaturized open well based platform

The µPCR chip is the central micro-structured component of the platform. By a specific workflow the micro-chip is integrated into an application dependent system of processing units. Thus it is also possible to adapt the µPCR platform to multiple applications. The workflow developed for gene expression analysis as well as SNP genotyping combines nanoliter liquid handling, thermal processing and sensitive fluorescence detection. In the following the parts of the workflow with the respective processing units in use are discussed.

4.3.1 Advanced nanoliter liquid handling

Crucial requirement for the developed system is an efficient and robust liquid handling. Both dispensing technologies, piezo nanodispensing with the SciFlexArrayer and valve nanodispensing with the custom-made station as well as the commercial iNL10, demonstrated a good volume precision with CV values not above 5% for volumes down to 100 nl.

The piezo nanodispenser (SciFlexArrayer) was optimal to be used for the volumes down to 18 nl. It is the device with the highest droplet resolution down to 0.4 nl using nozzles with 70 µm orifice and allows very precise positioning (±1 µm). This becomes crucial for high dense formats like the 4k-format of the µPCR chip. Limiting for the piezo dispenser was the high susceptibility against differing liquid properties. Even slight changes in viscosity or surface tension led to loss of performance. Since a higher viscosity of sample solutions can be caused by higher concentrations of genomic DNA, fragmentation of the DNA was tried to reduce viscosity. This is especially important for the very low volume range such as 18 nl, when a relatively high template concentration is used. Fragmentation reduced the viscosity and improved the stability of the piezo dispensing. The procedure itself had no effect on PCR efficiency, but reduced the sensitivity of the real-time PCR methodology. This might become limiting for some applications, when a high sensitivity is needed or low abundant targets have to be detected. Further disadvantage of fragmentation is a complication of the workflow by introducing an additional step. DNA fragmentation can be used for stabilization of piezo nanodispensing, but with respect to a simple workflow and a high sensitivity it should be avoided.

The second dispensing technology tested was the valve nanodispensing technology. Although valve nanodispensing is limited to a minimal reliable dispensing volume of approx. 20 nl, the good robustness of the system against changing liquid properties and higher viscosities makes it suitable for high-troughput applications. This robustness allowed to work with oil (PMDS), which was used as a separation layer between system liquid and sample. With pre-aspiration of oil (1 µl PMDS) observed mixing between system liquid and sample could be efficiently avoided, and the death volume, which

is the sample liquid volume additionally needed to the altogether dispensed amount, was noticeably reduced. The introduction of an oil separation layer for the valve dispensers was a prerequisite for the progress made in reduction of variation for dispensed components.

Non-contact dispensing is only one possibility to actualize a macro-to-micro interface to an open well. Alternatively contact dispensing could be used for filling wells. Contact dispensing is a rather long known technology, which is mainly used for microarray spotting. Spotted volumes are determined by the pin geometry and properties of the liquid. Changing liquid properties have direct impact on the volume. Slightly increased viscosities can easily lead to poor reproducibility or even clogging (pers. comm. C. Hultschig). Since this considerably restricts the spectrum of potentially used liquids, we a-priori had opted for non-contact nanodispensing.

Stringent definition of interfaces between working steps is especially important to avoid evaporation.[112] For the µPCR chip platform this is solved by optimized and standardized nanodispensing protocols. In closed chamber- or channel-based devices steps of the workflow are serially connected with highly defined interfaces on the chip itself. According to evaporation or the risk of cross contamination this is one of the advantages of such closed systems,[13] but makes them less flexible in use. Furthermore, in particular surface properties such as hydrophobicity, which can cause problems by filling with aqueous solutions,[113] have to be harmonized to the expected liquids in microfluidic devices via surface treatments, which can be limiting for some applications. The open well approach allows more flexibility with respect to the possible spectrum of liquids used.

Non-contact dispensing is one of the most capable approaches for a flexible macro-to-micro interface for high-throughput platforms. It allows a relatively high flexibility in what liquid and where to it has to be transferred. Although both nanodispensing technologies allowed precise nanoliter handling, a robust non-contact nanodispensing, which performs highly parallel with a high speed, is challenging. The low robustness of the piezo dispenser used might be limiting for high-throughput applications and parallelization, but it is the more suitable device for volumes below 50 nl. Parallelization is necessary to achieve the throughput needed and to minimize loss of fluid by evaporation in open systems like the µPCR chip platform. This was achieved with the valve nanodispensing technology. Valve nanodispensing demonstrated to be the more robust method at a good precision and reproducibility.

4.3.2 Evaporation and Sealing

Beside short dispensing times, evaporation loss was reduced by the order of liquid handling. Drying down of the preloaded samples sample in the desiccation step, which showed no impact on PCR performance, avoided an influence of sample volumes on

the final reaction volume, because all wells were fully desiccated. This limited the evaporation sensitive step to the following mix dispensing, which was performed under cooled conditions. Alternatives such as the supplement of high-boiling components like glycerol[114] or triethylene glycol[115] to reduce the vapor pressure of the reaction mix and therefore reducing the evaporation, were not applied, because of the high viscosity of these liquids, which presumably would complicate or even impede nanodispensing.

After filling with reaction mix the wells are immediately sealed. Efficient sealing was accomplished using polypropylene based adhesive sealing covers combined with permanent application of a moderate pressure from top. The application of moderate pressure was challenging for the realtime detection unit. Here the application of pressure from top, an even heat dissemination of the lid heating and the optical detection had to be combined. Application of pressure is crucial because the sealing material itself as well as the glue on it change their properties at different temperatures. Although 60% of the upper 1k-µPCR chip surface is available for adhesive sealing, the high number of individual reactors leads to a thin width of the partition walls between neighboring wells down to 325 µm. Moreover, the upper reaction liquid level is quite close to the sealing favoring capillary effects, which could lead to cross-contamination between neighboring wells. This was observed during de-sealing of µPCR chips after thermocycling. Capillary and electrostatic forces immediately pulled the little volumes out of the wells.

Beside contamination issues hermetic closing of the reaction chamber is prerequisite for a constant reaction volume over time, which has a direct impact on the variation of signals. Changes in volume lead to changed concentrations of reactants and dye, which influences the kinetics of the reaction and the overall signal intensity, respectively.[116] Thus, the application of moderate pressure from top was crucial for the µPCR chip platform, to achieve this efficient sealing solution using a conventional sealing material.

4.3.3 Thermal processing of the µPCR chip

As mentioned thermal processing with integrated fluorescence detection for real-time applications is a challenging task. This was achieved by a transparent lid heating, which was integrated into the real-time cycling unit. Currently the lid heating is implemented in the real-time detection unit by a ring shaped heating around the detection window. The Lid heating can be separately controlled, which allows a higher temperature of the lid and consequently a thermal gradient from top to bottom resulting in a directed condensation during the cycling process. This keeps the reaction volume joined and avoids optical irritations on the well ceiling. The achieved detection area of 20 × 20 mm was suitable for performing real-time PCR assays with an acceptable heat dissipation across the covering glass. Extending the detection area to the full µPCR chip area or

even larger is probably not possible with the setup described. Since the lid heating also contributes to the thermal cycling of the assay volumes, a thermal gradient across the cover glass would cause a temperature gradient on the µPCR chip. This could be circumvented by a new plate geometry, which contains zones for the placement of heating modules from top.

It is a general challenge for the construction of cycling units with integrated real-time detection to achieve even heat dissemination across the whole plate. Due to thermal inhomogeneities large variations (factor of 4) can occur in PCR amplification.[117] Differences of thermal response and uniformity were for example reported for integrated heaters onto silicon chips.[35] Non-contact approaches such as infrared mediated thermocycling[42] seem to be promising and allow optical detection from top, but depend on the plate material used and have to be harmonized with the detection optics used. Heating from both sides of the chip helps to avoid condensation, if the wells are not completely filled. Wells that are completely filled could be processed without lid heating, but would rise the problem of PCR compatibility of the glue used with the sealing material.

For the µPCR chip platform precise cycling was achieved with the developed cycling modules. The even heat dissemination across the µPCR chip was reached with and without real-time detection. Due to the application of moderate pressure efficient sealing was accomplished using conventional adhesive sealing covers.

4.3.4 Signal detection, data retrieval and analysis

Currently the real-time unit is able to monitor one dye by a CCD camera and a dye equivalent system of filters for excitation and emission. This is sufficient for performing real-time PCR, but does not allow the use of a passive reference such as the dye ROX. A passive reference is used, to normalize for non-PCR-related fluctuations in fluorescence signal. Integration of multiple dye measurements is planned for the future and will enable ROX normalization. However, due to the precise nanoliter dispensing this was not limiting for our studies. The GT detection unit allows endpoint measurements of two dyes, also with a CCD camera and a system of several filter combinations. These are necessary for SNP genotyping with the allele calling algorithm developed.

The read-out of fluorescence intensities is a manual procedure. Possible bias was minimized by applying defined grids to whole series of pictures belonging together. Further analysis was done with the R statistics environment,[101] because it allows to easily extend an analysis procedure to almost arbitrarily large data sets. This applies to real-time PCR and SNP genotping analysis. The data analysis software for real-time PCR analysis was developed according to the principle of the Ct-value method.[66,104] Concordance between CT-values calculated from ABI and the custom-made analysis module on basis of raw intensity data from the µPCR chip platform showed clearly the good capability of the software developed (not shown). The quality of the de-

veloped allele calling algorithm for SNP genotyping is discussed later (4.6.1). Via an R written program data was displayed and threshold setting for failing reactions and the determination of clusters was manually done. This analysis step is also easily extendable to higher numbers of samples per SNP. It is also conceivable to integrate an automatic clustering algorithm such as K-means into the program, to further reduce manual processing of data.

In both cases, real-time and endpoint analysis, a random distribution of dust particles was observed on the sealed chip surface, probably caused by electrostatic interactions. This can cause optical irritations but could be circumvented by working under dust-free conditions. A dust-free environment would further decrease the risk of contamination due to dust particles.

With the real-time PCR unit a sensitive monitoring of the amplification in combination with thermal processing was achieved. The requirement of a two dye detection for TaqMan based SNP genotyping was accomplished with the developed endpoint analysis unit. For data processing and analysis appropriate software modules were developed using an open source development environment.[101]

4.4 Nano-PCR on a plastic substrate

4.4.1 PCR-based assays on a nanoliter scale

To investigate the feasibility of PCR down to few nanoliters (10 nl), a preliminary experimental setup was used. In PP microcaps successful amplifications down to 18 nl were achieved. Although recently the feasibility of PCR could be shown down to picoliter volumes[59,60] this experiment was necessary to assure the appropriateness of PP for the miniaturized format. The picoliter PCRs mentioned above were performed on a silicon-based device. To my knowledge no data about PCR in a miniaturized PP substrate has been shown so far. An example for successful amplification down to few nanoliters using a polymeric device was recently shown in a closed channel based PDMS device.[57] Except the reports from Xiang[44] (PDMS) and Morrison[118] (steel) all open well based devices for PCR in nanoliter volumes were made from silicon or glass.[59,61]

Due to the small volumes no gelelectrophoretic analysis could be applied to test for successful amplification. Therefore an intercalating dye (SybrGreen) was used for staining of amplification product. Non-template controls of the nanoliter PCR showed relatively high fluorescence intensities caused by primer dimerisation. Primer dimerisation is known to be influenced by the target concentration, the primer design and the quality of the enzyme used.[119,120] No reports about a correlation between the reaction volume and the degree of primer dimerisation are known. Miniaturization leads to an increased surface-to-volume ratio, which could have an effect on primer-dimer accumulation. A relatively large hydrophobic surface of the polymer used, could for ex-

ample reduce polymerase activity and specificity, since the enzyme needs a hydrophilic environment for its proper function.

Therefore a comparison of SybrGreen and TaqMan assays of different volumes between 10 and 0.5 µl was performed. Results for different template concentrations (see 3.4.3) did not show any effect of the reduced assay volumes on the degree of dimerisation or PCR efficiency. Microcaps are not optimized for being cycled like a reaction vessel and it can be assumed that an influenced thermocycling profile led to the increased accumulation of primer dimerisation products. Further aspect might be the enzyme used, which hasn't been a hotstart polymerase in the microcap experiment. Recently it was reported that chemically modified Taq polymerases show lower enzyme activity before thermal activation compared to non-hotstart or antibody modified hotstart polymerases. Enzymatic activity of the polymerase before activation (start of the PCR cycling) was shown to be an enhancer of primer dimererization.[121]

Beside hydrophobicity effects the charge of the surface might also have an impact on the availability or accessibility of PCR mix components. To study effects of the surface charge, µPCR chip surfaces were functionalized to carry mainly negative (epoxy) or positive (amino) charges. For the amino functionality no predictions can be made, because of the total PCR inhibition by the used coating chemical allylamine. The negatively charged surface lead to a clearly reduced sensitivity of the real-time PCR methodology, but had no significant effect on PCR efficiency. This might be due to electrostatic interactions. Recently, Cardenas et al.[109] showed, that hydrophobic interactions dominate over electrostatic ones and constitute the driving force for DNA adsorption. However, not only DNA or the polymerase have an impact on PCR. All components can be influenced and therefore have impact on dimerisation or PCR performance. Further studies are necessary to really understand the effect of miniaturization on the availability of the different reactants and its role in dimerisation.

Nevertheless the data clearly shows the feasibility of PCR down to few nanoliters in a PP based reaction vessel. Primer dimerisation was identified as a problem for endpoint analysis increasing the risk of false positives. Although a melting curve analysis allows to detect the formation of primer dimerisation artifacts at the endpoint, further manual handling considerably increases the degree of non-automated handling - a possible bottleneck for performing assays in high throughput. Thus the probe based assay approach was chosen. Although probe based solutions mostly result in higher assay costs, they allow a more straight forward analysis of results. This is especially important for high-troughput applications, as analysis can considerably contribute to final assay costs as well.

4.4.2 Sensitivity of the platform

Reducing the reaction volume at constant concentrations considerably lowers the absolute amounts of PCR components. This especially applies to the number of target molecules. At low template concentrations the specificity of the PCR plays an increasingly important role, since it enables to selectively amplify a single target sequence from a large background.[20] Therefore the sensitivity of the developed platform was studied.

Successful reports of single molecule amplifications were shown for µl volumes[69,122] and recently also for nanoliter volumes in a silicon made open well device.[61] Own DNA dilution series experiments using piezo dispensing showed for concentrations near 1 initial target molecule per well unexpected low rates of successful amplifications. With an initial target number of 1 molecule per well only 53% of the wells showed successful amplification. First assumptions were, that this is caused by adhesion effects inside of the dispensing nozzles. After careful evaluation of the dispensing, which has been discussed before, this results occurred to be mainly caused by mixing effects. A second experiment using valve nanodispensing with oil separation could confirm this (see 3.5.1). Percentages of approx. 25% and 50% for theoretical 0.2 and 0.5 initial target molecules per well, respectively, were corresponding to the expected values. 74% successful amplifications for 1 initial target molecule are not due to a reduced sensitivity of the system, but caused by a non-normal distribution of target molecules. This is in good agreement with findings by Morrison and colleagues (77% for single target assays).[118] With a given concentration of 1 molecule per dispensing volume a distribution of one molecule per dispensing step (and well) is less likely then having stochastic fluctuations between more then one molecule and zero molecules in a dispensing series. At target molecule numbers below 1 the probability of dispensing more than 1 molecule per dispense decreases. This results in a lower percentage of wells with more than 1 molecule, which matches to our data for 0.5 an 0.2 molecules. In light of these results a single molecule detection sensitivity can be confirmed for the µPCR chip system.

4.5 Real-time PCR based expression analysis with the µPCR chip platform

The initially intended application of the developed platform was real-time PCR based gene expression analysis. Inspired by the existing shortcomings of conventional microarrays such as long incubation times, rather low detection sensitivity, a narrow dynamic range for quantification, and a fairly poor quantification precision,[11,111] it arised the idea of an high density array of liquid real-time PCR reactions to improve the quantification accuracy in gene expression studies. Real-time PCR, which developed to the 'gold standard' for accurate gene expression analysis,[11] could enable researchers to obtain expression profiles in large scale with a much better sensitivity and precision.[12]

4.5.1 Quantification capability

Since high sensitivity of the platform was proven, next step was to study the quantification capability using the preferred probe based assays. Experiments were performed in 200 nl with a template concentration gradient from 100 down to a single initial target molecule. A good correlation between measured Ct-values and the prepared template concentration gradient was achieved ($R^2 = 0.99$). Expected fold-changes between assays with different target concentrations were confirmed. Only for the two lowest concentrations (5 and 1 initial target molecules) underestimation of the fold change was observed. This was due to distributional effects of target molecules. The average number of initial target molecules per well for these low concentrations is higher, because molecules for wells that remained empty due to an unsteady distribution, were distributed to other wells. This increased average of molecules per well resulted in an underestimation of the mean Ct-value, which consequently led to the observed underestimation of the fold-change.

The good relative quantification accuracy was confirmed in the experiment to evaluate the dynamic range of target concentrations for precise quantifications (3.5.4). 200 nl reactions from 40 up to 4.3×10^5 initial target molecules were performed. A good relative accuracy for quantification with a correlation coefficient of 0.995 was demonstrated and approves a dynamic range of reliable quantification over at least 4 orders of magnitude. Variation of the Ct-values observed for the individual template concentrations clearly showed a correlation between the reduced numbers of targets and the found variation in Ct-values (see 3.5.2). This is in agreement to studies using larger volumes. Canales et al. found increased CVs with decreasing abundance of transcripts below target concentrations of 6000 molecules per 10 µl assay.[62] Distributional effects start to dominate at reduced target numbers whereas events such as the distribution of few target molecules follow the poisson distribution.[20]

The absolute precision of the µPCR chip platform was evaluated by looking at the standard deviations of the Ct-values for replicates.[62] Since the Ct-value has an exponential scale, standard deviations were transformed into percent initial target molecules[104] (Results Table 12). Conventionally performed 10 µl assays showed a variation up to ±7% in initial target molecules. 200 nl volumes showed approx. 20 – 30% deviation in initial template molecules for assays with at least 10 initial target molecules. Below this threshold a deviation in initial target molecules of 64% calculated from the assays with 5 initial target molecules and 142% for assays with 1 molecule was observed. This reflects more the impact of molecule distribution during sample dispensing than the precision of the system. Recently a study of real-time PCR based expression analysis in 33 nl was published.[118] The system used was a through-hole array made from stainless steel, which was rendered PCR compatible by coating with polyethylene glycol (PEG). Using 1 ng cDNA equivalent per well, 72 – 78% of the assays performed

with a standard deviation in Ct-values < 0.5 (quadruplicates). This corresponds to a deviation up to 41% in initial target molecules. Comparing only one assay on one chip with in average 500 target molecules per well showed a lower deviation of 12% in target molecule numbers. This confirms our results, that the initial target number has strong impact on the reproducibility of the quantification result in nanoliter volumes.

Correlation analysis of 200 nl assays between the gradient of target molecules and the obtained Ct-values demonstrated a good relative quantification accuracy of the µPCR chip platform. Due to distributional effects reliable quantification seems to be limited to a lower threshold number of initial molecules, which is in agreement with recent finding by others.[62,118] For the µPCR chip this threshold is expected to be between 10 to 50 initial target molecules for 200 nl assays. The dynamic range of 200 nl assays for reliable quantification was approved to be at least 4 orders of magnitude.

4.5.2 Inter-chip reproducibility

Comparison of identically processed µPCR chips to assess the inter-chip reproducibility showed good correlation ($R^2 = 0.95$). Only for higher Ct-values (Ct>30) reproducibility was significantly reduced. Reasons are forexample effects on the enzyme, differences caused by the biological sample or slight variations in the temperature control, which are different strong for different wells. These differences are also amplified during the PCR reaction, which becomes obviously in the later cycles and thus applies especially to late Ct-values. Additionally, distributional effects are much higher on these low concentrated targets and add further variation to the data.[62] It might be practical to set a threshold in the range between 30–32 cycles from where the data are not used anymore. This limits the sensitivity of the system, but will ensure a good intra- and inter-chip reproducibility.

4.5.3 Data quality vs. number of replicates

Data from the concentration gradient experiment between 100 and 1 initial target molecules to assess the quantification capability of the platform (3.5.2) was used to investigate the effect of the number of replicates on the data quality, and to explore a minimal number of replicates needed for a certain data quality for the µPCR chip platform. Ct-values of 30 or 60 replicate 200 nl TaqMan assays of the varying target concentrations were taken as base data set.

Randomly generated sub-groups of Ct-values (N = 1 – 9) were taken from the original data sets and compared to them (each concentration separately). As an attribute of quality for the resulting mean Ct-value for the sub-group the respective value was compared to the mean Ct-value of the original data set. Sub-groups with a mean Ct-value laying in the confidence interval (CI) around the mean of the original data set

were accounted as being comparable to the original data set with N=60. Additionally the median of each sub-group was calculated and compared as described.

The CI was chosen as the interval of trust around the mean, since it is a very stringent parameter. For N = 60 and a confidence level of 95% it means, by repeating a measurement with 60 replicates the probability is 95% that the mean of the new measurement is within the borders of the CI. For maximal comparison between all concentrations (two concentrations were only done with 30 replicates), the confidence interval was equalized to a original sample size of N=60 by mathematical transformation. The CI needs a normal distribution. This is only partially given, since rare events follow a poisson distribution. Thus this approach is only an estimation, but provides a good basis for the evaluation of different numbers of replicates.

Best results were obtained for sub-groups with 9 replicates and an initial target number of ≥ 50 molecules. More than 70% of the 9-er sub-groups show a comparable result to the original data. Although a replicate number of 9 is quite high, no consolidation of the quality parameter can be observed. For replicate numbers between 1 – 9 the data quality increases almost linear. The percentage is decreasing with a reduced number of initial target molecules as well as smaller sized sub-groups. At a number of 6 replicates still 60% of the reactions accomplish comparable results to a 60-fold measurement.

Using the median of sub-groups and comparing it to the original data set resulted in comparable results to the mean calculations. Only for assays with low concentrated target ($\leq$50 molecules) the values with the median-based calculation were better. The median is an outlier non-sensitive measure. Outliers and extreme values, such as observed in low target number assays, affect results using the mean calculation more than using the median. Thus the median is a possible alternative to the mean calculation for measurement with few replicates.

Due to the shown impact of outliers on the final result using the mean the detection of outliers in groups of few values might be advantageous. However, the verification of different large groups of replicates showed, that a number of 6 – 9 replicates results in a good concordance to an data set with 60 replicates.

4.5.4 Reduction of the assay volume below 200 nl

Continuous assay volume reduction results in reduced absolute numbers of target molecules. Assays with few target molecules show increased variation and more extreme values. Nevertheless, reliable quantification results for TaqMan based real-time PCR were achieved down to 50 nl using the 1k/V µPCR chip (3.6.1).

Experiments in 100 nl and 50 nl showed a correlation between target concentration and resulting Ct-values of 0.995 and 0.983, respectively. This affirms a good relative quantification capability in both volumes, despite increased variation of Ct-values for

lower assay volumes. The increase of variation is caused by declined dispensing reproducibility for smaller volumes (see 3.2) and stronger distributional effects. Generally the target concentration and therewith distributional effects on target molecules seem to have the largest impact on the reproducibility. This corresponds to data from Morrison and colleagues who reported reduced real-time PCR reproducibility below 1000 molecules per 33 nl SybrGreen assay.[118]

The kinetics of a reaction depends on the concentration of the reactants. Thus, the efficiency of a reaction in a microchamber should be comparable to conventional volumes at comparable concentrations.[59,123] However, with respect to thermal processing the increased surface-to-volume ratio of a miniaturized device can be beneficial for PCR application. More efficient cycling due to the improved thermal energy transfer can result in a higher assay efficiency.[22] In 50 nl and 100 nl experiments PCR efficiency was rather low with 62% and 61%. One reason could be the thicker bottom of the 1kV µPCR chip. Due to the V-shape each reaction chamber of a 1kV chip is surrounded by thicker polypropylene walls as reaction chambers of the flat bottomed 1k µPCR chip. In the shown experiment the same thermal profile as for 200 nl assays was applied. The poor thermal conductivity of plastic reactors is often discussed as a disadvantage of plastic materials,[110] and could reason the reduced assay efficiency. This can be circumvented by an appropriate chip design, as shown with the 1k µPCR chip, which enables optimal heat dissemination, and by optimization of the thermal profile applied. The increased PCR failure rate at an initial target number of N=10 for 100 nl as well as for 50 nl is presumably caused by the reduced efficiency, than due to the reduced assay volume.

With the 4k µPCR chip further reduction of the assay volume down to approx. 18 nl was possible. For this volume range no valve dispensing could be applied, since the resolution for reproducible liquid distribution of the device lays between at approx. 20 nl. Using the piezo dispenser distinct mixing between dispenser system liquid (water) and sample fluid as reported above has to be taken into account (3.2.3). No clear correlation between target concentration and resulting Ct-values was found, which is mainly due to mixing of sample and system fluid of the dispenser. A more reliable distribution of target molecules will significantly reduce variation and allow quantification. Sporadically appearing fluorescence intensity peaks were caused by imbalances in the power supply, which will be solved by implementation of a controlled power supply for smooth curve progression.

However, with the µPCR chip platform reliable quantification down to 50 nl was achieved. This was accomplished with the 1kV-format of the µPCR chip. Further reduction of assay volumes was shown with the 4k-format, which allowed processing of assays down to 18 nl.

4.5.5 Expression analysis in mouse tissues using the µPCR platform

To access system performance using biologically relevant samples, an analysis of tissue specific expression in mouse was performed using 200 nl TaqMan assays. Comparison to results of in parallel performed 10 µl TaqMan assays using a commercial platform showed concordance between both volumes. The genes *Cacna1g* and *Odf2* are clearly more strongly expressed in brain, whereas *Folh1* and *Pttg1ip* have the highest expression in kidney and liver, respectively. These results corroborate earlier findings where *Cacna1g*, *Odf2*, *Folh1* were shown to be predominantly expressed in brain, testis, kidney, respectively.[124,125] For all targets studied, a high correlation was observed between the 10 µl and 200 nl assays. Trends of over/under expression were concordant. Standard deviations of 200 nl assays were comparable to the previously performed experiments with the RNAse P TaqMan assay and ranged from 0.1 – 0.4 cycles (6 replicates). *Actb*, which was used for normalization, showed slightly higher standard deviations at comparable Ct-values. Variation was observed to be dependent on the assay and is a crucial factor for the reliable quantification in the low volume range, especially with the quantification method used. Relative quantification with the $\Delta\Delta$Ct method[72] uses three individual Ct-vales to calculate the difference in expression between two samples. This results in an accumulation of the respective measurement errors. Thus, not only an even efficiency across all assays performed,[71] but also an excellent assay performance is important for a reliable quantification result. Excellent assay performance is indicated by a normal shaped real-time course and a low variation of technical replicates, which was verified for the assays used.

Reduction of volumes increased variation of Ct-values, as seen by the standard deviations. This was demonstrated to be of special concern for studying the expression of low abundance genes, since a good reproducibility is needed for reliable quantification. However, results of the platform investigations and the presented expression analysis demonstrate, that reducing the reaction volume by 50 folds and presumably more, the ability to quantify accurately was conserved using the µPCR chip platform.

4.6 TaqMan based SNP genotyping using the µPCR chip platform

This promising results for real-time PCR based expression analysis were convincing to go a step forward and establish a further PCR based application on the µPCR chip platform. Due to its wide application in SNP fine mapping and association studies[126,127] and the proven function of the TaqMan assay on the µPCR chip platform, TaqMan based SNP genotyping was chosen as a second application. Because no post-PCR processing or label-separation steps are required, the TaqMan assay is a straight forward method, useful for high-throughput genotyping. For application in conventional volumes (10

µl) the costs for probes were somehow limiting for real high-throughput application.[87] This could be improved by a significant reduction of assay volumes using the µPCR chip platform.

4.6.1 Detection and allele calling algorithm

TaqMan based SNP genotyping requires detection of at least two dyes, each for one allele.[81] Therefore a custom-made SNP detection unit was built up, to enable parallel detection of two dyes and thereby an allelic discrimination. According to absorption and emission spectra of the dyes FAM and VIC, the filter used for excitation and emission were chosen. An discriminating INDEX value was introduced (Equation 9), which allows to discriminate the three genotypes using signal intensities of the $\mathrm{FAM}_{Excitation}/FAM_{Emission}$ and $\mathrm{FAM}_{Excitation}/VIC_{Emission}$ filter combinations. Signal intensity of the $\mathrm{VIC}_{Excitation}/VIC_{Emission}$ filter combination was not necessary for calculation the INDEX value, but it turned out to be perfect for the detection of failing reactions, which clustered together with heterozygous samples.

In contrast to a system working with continuous spectra such as the ABI Prism 7900 HT, a filter based system delivers only few signal intensities obtained from available filter combinations. Thus, quality of allele calling highly depends on the chosen filter sets and the respective allele calling algorithm. The validation of the developed allele calling algorithm showed the capability of the algorithm to discriminate between all genotypes using the signal intensities obtained with the chosen filter sets. Observed miscalling was caused by the performance of individual assays, not by the algorithm. A poor performance was given, when the VIC/VIC signal intensities of samples did not discriminate from the signal intensities of non-template controls. Differences in assay quality/performance were shown to have more impact in the nanoliter volumes, than in the conventional 10 µl volumes. The introduction of the new GT TaqMan reaction mix from Applied Biosystems was the major contributor to the improvement of allele calling in small volumes.

With the developed allele calling algorithm robust allele calling was achieved. To avoid misclassifications of failing reactions an introductory step was integrated to detect failing reactions, which lead to improved results.

4.6.2 DNA quality and quantity on the µPCR chip platform

Although PCR based methods helped to reduce the required amount of sample, with the increasing number of possible targets, genomic DNA sample quantity can be a limiting resource in large SNP genotyping studies. Whole genome amplification(WGA) is often used to amplify the precious samples before further analysis.[128–130] Thus, hgDNA and WGA-DNA samples were investigated for limiting amounts of initial template.

Starting with an amount of 0.75 ng (225 molecules hgDNA) per 200 nl assay, the clustering quality was studied down to initial 0.017 ng (approx. 5 molecules) per well. With the conventional TaqMan reaction mix no clear clustering for any WGA-DNA template concentration could be achieved. For hgDNA template amounts of approx 40 – 50 molecules per 200 nl assay were limiting. Such significant loss of genotyping performance for WGA-DNA samples was not observed in conventional volumes. Only a slightly but significantly reduced SNP determination rate for WGA-DNA samples compared to genomic DNA samples was reported for the TaqMan assay.[128]

Observed problems with WGA-DNA were solved by using the new GT TaqMan reaction mix. With the new mix the limiting amount of template was reduced to 0.03 ng genomic DNA and WGA-DNA. This was unexpected as it means that TaqMan based SNP genotyping can be successfully performed on initial 5 molecules hgDNA per allele. However, to keep the procedure robust the initial target number in 200 nl assays should not be below 50 molecules hgDNA or the equivalent amount WGA-DNA, respectively. A locus bias during WGA amplification can result in lower quantities of the particular target,[128] which could make it necessary to increase the initial amount of template for some regions.

With the µPCR chip successful allele calling could be demonstrated down to 5 initial target molecules in a 200 nl TaqMan assay. Observed differences in allele calling quality between hgDNA and WGA-DNA template could be equalized with the introduction of the new GT TaqMan reaction mix.

4.6.3 Validation study

For validation of TaqMan based SNP genotyping in nanoliter volumes a study in 200 nl was performed comprising 16 SNPs and 60 patients on one 1k-µPCR chip. Identically configured chips were processed, rates of failing reactions and the concordance to the data from Kiel University (provided the samples as well) were evaluated.

For the majority of assays concordance rates close to 100% were achieved (in average 95%). For few samples (5) an accumulation of missclassifications was observed. This was due to dispensing problems. Removing these samples from the data set increased the concordance to approx. 98%. The missclassifications observed as well as the found failing rates of in average 3% were significantly higher, than in large volumes.[127] Since failing reactions were only rarely observed in expression profiling at the same volume range, this is probably caused by the quite time consuming sample pre-loading procedure. Each patient sample was distributed 16 times across the plate in a more or less serial manner. Thus, samples are kept relatively long in the nanodispenser nozzle, which might cause irregularities during dispensing. A configuration with more patients and fewer SNPs per chip will allow more parallelized dispensing with shorter dispensing times and therefore eliminate this problem.

PCR based SNP genotyping methods are known to be very sensitive against cross-contamination.[131,132] Single molecules can entail a false genotyping result. With the µPCR chip platform contaminations can occur from neighboring dispenser nozzles due to reduced dispensing performance or by residuals in the nozzle from the sample before. Tracking of the nanodispenser performance, efficient washing such as in the washing routine used and a reduction of the dispensed sample volume were used to successfully reduce the contamination risk.

The results of this study lead to the conclusion, that with introduction of the new GT TaqMan reaction mix, assay dependent differences in genotyping performance could be almost eliminated. Liquid and sample handling seems to have a strong impact on allele calling quality for TaqMan based SNP genotyping in nanoliter volumes. With the ability of advanced performance tracking of the valve nanodispensing system, which has not been integrated yet, further optimization of the workflow is possible, making the µPCR platform a very reliable technology for high-throughput SNP genotyping studies on a nanoliter scale.

4.7 PCR based assays on a nanoliter scale at high throughput

The data for gene expression analysis as well as SNP genotyping demonstrates the µPCR platform as a capable system for PCR based assays on a nanoliter scale using a cost-efficient polypropylene based open well format. With respect to flexibility the open well approach was shown to be adaptable to different applications via an appropriate workflow.

The workflow developed, comprises capable advanced non-contact dispensing technologies, which allow a balancing between parallelized processing and flexibility. Compared to the microarray technology, which per se follows an one sample against multiple targets screening pattern,[4] the µPCR chip allows more flexibility. Final screening patterns of targets vs. samples are mainly determined by the time required for distributing samples or assay mixes. Limiting are only complex loading patterns of samples or assays, which result in very long dispensing times. As discussed in the thermocycling section, PCR was shown with processing times below 30 min. This is a big advantage of this technology. Long hybridization times such as with microarrays are not needed. Short processing times and a standardized read-out without any post-processing are possible.[73] Data analysis is straight forward and standardizable, which makes it appropriate for high-throughput platforms.

Miniaturized systems are always discussed to be very promising for the reduction of screening costs.[13] Although more complex and therefore more expensive processing devices, the cost reduction per assay has tremendous impact on the costs in large scale screening. Compared to a 10 µl experiment with 4 replicates, the volume for a 200 nl assay is 50–fold reduced. The cost per target for expression analysis in 200 nl reactions

were reduced by a factor of 33, because a 50% higher number of replicates (6). For SNP genotyping down to 100 nl cost reduction was even 100–fold. This displays the potential saving of costs in large scale screening using the µPCR platform.

In conclusion, it was demonstrated that the developed platform enables biological studies with PCR based methods in nanoliter volumes. For the shown applications gene expression analysis and SNP genotyping a good precision and sensitivity was achieved.