2 Material and Methods

2.1 Material

2.1.1 Primers and Oligos

Table 1: Primers and oligos: T_A – PCR annealing temperature of the primer. In column 'Misc' additional information like labels or the orientation of primers is given. FOR – forward PCR primer, REV – reverse PCR primer

Name	T_A	5'— 3' Sequence	Misc	Template
FAM-oligo		10-mer with random sequence	3'FAM-dye labeled	
NOD2_SNP8_for	60°C	CAGATCACAGCAGCCTTCCT	FOR	hgDNA
NOD2_SNP8_rev	60°C	CAACTTGAGGTGCCCAACATT	REV	hgDNA
PECAM_for	60°C	CATTTTGCATTTCTCTCCACC	FOR	hgDNA
PECAM_rev	60°C	GCAGGGCAGGTTCATAAATAAG	REV	hgDNA
RNAseP_Flank_for	60°C	TCGCTATGTGTTCTGGGAAA	FOR	hgDNA
RNAseP_Flank_rev	60°C	CGGAGGAGAGTGGTCTGAAT	REV	hgDNA
MU5_for	58°C	GTGTTGATGTGTATATTCAAATAT	FOR	hgDNA
MU5_rev	58°C	GAAACCTGCAATACTTGCTGAA	REV	hgDNA
RNAseP_for	60°C	– confidential –	FOR	hgDNA
RNAseP_rev	60°C	– confidential –	REV	hgDNA

2.1.2 Chemicals

Table 2: Chemicals

Name	Manufacturer
KCl	Sigma-Aldrich
$MgCl_2$	Sigma-Aldrich
$(NH_4)_2SO_4$	Sigma-Aldrich
TrisHCl	Sigma-Aldrich
Cresol Red, CAS: 1733-12-6	Sigma-Aldrich
QuantiTect SybrGreen® PCR Kit	Qiagen
SybrGreen® PCR Master Mix	Applied Biosystems
Mineral Oil	Sigma-Aldrich
Polydimethylsiloxane CAS:107-51-7	ABCR GmbH
SybrGreen® I nucleic acid gel stain	Molecular Probes
Tatrazine, CAS 1934-21-0	Sigma-Aldrich
hgDNA	Promega
Amplitaq Gold	Applied Biosystems

2.1.3 Consumables

Table 3: Consumables

Name	Manufacturer
Optical Adhesive Covers	Applied Biosystems
VIEWseal® film	Greiner BioOne
EASYseal® film	Greiner BioOne
POWERseal® film	Greiner BioOne
384-well microtiterplate	Eppendorf
AB-0558	AB-Gene
Soft Sealing	MG Research
384 Well Clear Flat Bottom UV-Transparent Microplate	Corning
Microplate, 384 well, PS, small volume	Greiner BioOne

2.1.4 Software used

Table 4: Special software that was used for instrument control, data retrieval and analysis

Name	Version	Manufacturer
Alpha Ease FC^{TM}	4.0.0	Alpha Innotech Corporation
R - A language and environment	2.4.0	www.r-project.org
Sciclone Workstation Software	3.5.69	Caliper Life Sciences
SciFLEXARRAYER	2.06.006	Scienion AG
SDS	2.1	Applied Biosystems

2.1.5 TaqMan gene expression assays

Table 5: TaqMan® gene expression assays were directly ordered from Applied Biosystems and came premixed in a 20 × concentration.

Gene	Description	Accession no.	Species
RNAse P	TaqMan RNAse P Detection Reagents Kit	ENSG00000195681	human
Actb	actin, beta, cytoplasmic	NM_007393	mouse
Cacna1g	calcium channel, voltage-dependent T type α	NM_009783	mouse
Fol1H	folate hydrolase	NM_016770	mouse
Odf2	outer dense fiber of sperm tails 2	NM_013615	mouse
Pttg1ip	pituitary tumor-transforming gene protein binding	NM_145925	mouse

2.1.6 TaqMan SNP genotyping assays

Table 6: SNP Genotyping TaqMan® assays. The first 10 assays were purchased separately as oligos (4 oligos per assay), the last 9 assays were directly ordered from Applied Biosystems and came premixed in a 20 × concentration.

Identifier	Primer 1 & Primer 2	Probe 1 & Probe 2
rs1050152	GGGTAGTCTGACTGTCCTGATTG	AAGGGTGAGGATTC
	TCTGGAAGAGTCATTCCCAAACTTTC	AAGGGTGAAGATTC
rs2073838	GGAGGAAGATGGGAGGATGTG	AAACACTAAGTCTGCATTG
	GTGTTGCAAGCCCTGAGTCA	AAACACTAAATCTGCATTGA
rs2631367	GCGGCTGGCCTTACATAGG	CAGGCCCGGAACC
	GGGCCTCAGGTGCACT	CAGGCCCGCAACC
05IGR2096a	TCTGAGACAGGAGCCACTAGAG	CATGTCACTTCTCTTTAAAA
	CACAGCATCCAGAGTGATCCT	ATGTCACTTCTCTGTAAAA
2011	CCACTCCTTAGTCGAGGCAAGAC	CCTCGGTTCGGCG
	GGCCGGCGTGGACTG	CCTCGCTTCGGCG
2063	ACAGACTTTACTTCCCTGAGGGAG	AAATAATCACATGGATCTC
	GCACCCTCTCGTAGGAGCTATG	AAATAATGACATGGATCTC
2230	TGCAGGCAGAACAGCCATACT	AAATACACCCTAAATGGCTAA
	GGCCACAGAACTTTCATTAAAGTAGGA	ACACCCTAAGTGGCTAA
3236	CCAGGGTGCCTATAAGAGGAAAC	CCACATCAACCTCTC
	AAGCACTGGCATGAAGTAACCA	CCACATCACCCTCTC
IGR2198a	GGGTTGCATGAGCATTAAGTTTCAA	TATTTTGCATGGTTCGTC
	CCACATCAAGGATAAGACTGCTAAATACT	TATTTTGCATCGTTCGTC
X100	CTTGTGGAACCAGCGTTGTTTT	CCCGTAATAATTCG
	AGGGTCCCGTTTGTTGAATTAGTT	CCGTGATAATTCG
rs4279384	Pre-mixed (no sequence information available); ABI Assay-ID: C__25651201_10	
rs3822356	Pre-mixed (no sequence information available); ABI Assay-ID: C__25932305_10	
rs778584	Pre-mixed (no sequence information available); ABI Assay-ID: C___3221082_20	
rs2862717	Pre-mixed (no sequence information available); ABI Assay-ID: C__15836062_10	
rs3087616	Pre-mixed (no sequence information available); ABI Assay-ID: C__15794148_10	
rs9007	Pre-mixed (no sequence information available); ABI Assay-ID: C___3220494_10	
rs919744	Pre-mixed (no sequence information available); ABI Assay-ID: C___7538336_20	
rs7195236	Pre-mixed (no sequence information available); ABI Assay-ID: C___7711033_10	
rs4776116	Pre-mixed (no sequence information available); ABI Assay-ID: C___1484868_10	

2.2 Standard procedures

Standard molecular biological procedures such as the determination of DNA concentration, agarose and polyacrylamide gel-electrophoresis, gel staining and analysis were done according to Sambrook et al.[99]

2.2.1 Standard PCR

A typical PCR reaction mix for 10 µl contained the following components: 20mM TrisHCl, 16mM $(NH_4)_2SO_4$, 25mM KCl, 2mM $MgCl_2$, 25 µM each dNTP, 5U Taq Polymerase (inhouse, R. Pawlik, Max-Planck-Institute for Molecular Genetics), 0.5 µM

forward and reverse primer with varying template concentrations. PCR was performed in a 384 well microtiterplate (Eppendorf) with changing annealing temperatures according to the primer pair used: 2 min denaturation at 95℃ 40 cycles of [20 sec 95℃ ,30 sec ...℃ (varying annealing temperatures), 1 min 72℃] and finally 15℃ until removal from the cycler.

2.2.2 Standard SybrGreen assay

SybrGreen assays were performed using either the SybrGreen® PCR Master Mix from Applied Biosystems or the QuantiTect® SybrGreen PCR Kit from Qiagen, both in a concentration of 1×, along with 0.5 µM each primer. DNA was used in different concentrations. The following cycling program was subjected: 95℃ for 10 min, 40×[95℃ for 20 sec and 60℃ for 1 min].

2.2.3 Standard TaqMan assay

A typical reaction mix contained 1×Universal TaqMan® PCR Mastermix from Applied Biosystems, 1×primer and probe concentration, deionized water and template in varying amounts. Thermocycling programs and data retrieval varied depending on the cycling unit used.

10 µl reactions
Real-Time PCR using a 384 well format was performed in the ABI Prism 7900HT Sequence Detection System. Cycling parameters were initially 10 min at 95℃ followed by 40 cycles of 15 sec at 95℃ and 1 min at 60℃Ȧmplification plot and respective Ct-values were obtained with the Sequence Detection Software (SDS 2.1, PE Applied Biosystems).

Miniaturized reactions with real-time detection
Quantitative PCRs of 200 nl volumes and below were processed in the µPCR chip using the custom-made real-time cycling system. Cycling conditions were 96 ± 2℃ for 10 min, 45×[96 ± 2℃ for 15 sec and 58 ± 2℃ for 45 sec]. The temperature gradient between the heated lid and the thermal block was 5℃ during denaturation phases and 20℃ during annealing-elongation steps (for details see 2.7.3).

Miniaturized reactions with endpoint detection
Non-quantitative TaqMan assays of 200 nl volumes and below were performed in the custom-made ABI Sandwich Cycler (2.7.1) and in the Hydro Sandwich Cycler (2.7.2). Cycling conditions of the ABI cycler were 95 ± 2 ℃ for 10 min, 45×[95 ± 2 ℃ for 15 sec and 58 ± 2℃ for 45 sec]. Thermocycling conditions for the Hydro Sandwich Cycler were identically, except that before each temperature stage, a preliminary phase of 60 sec was inserted, to reach the nominal temperature.

2.2.4 Standard TaqMan assay for genotyping

SNP genotyping was performed using 1×Universal TaqMan® PCR Mastermix from Applied Biosystems, 1 µM forward and reverse primers and 0.2 µM each probe for assays with separately stored oligos (see upper part of Table 6). The premixed SNP genotyping assays (assays-on-demand, which contain primers and probes mixed) were used in a 1×concentration (see lower part of Table 6). Human genomic DNA was used in a final concentration of 0.5 ng/µl unless specified. SNP genotyping in nanoliter volumes was performed in the custom-made Hydro Sandwich Cycler (see 2.7.2). Cycling conditions were 96 ± 2℃ for 10 min, 40×[96 ± 2℃ for 20 sec and 56 ± 2℃ for 40 sec].

2.3 Material tests

2.3.1 Test for impact on PCR performance (biocompatibility)

Slivers of test samples for plate or sealing material were included in a 10 µl standard PCR (see 2.2.1) using primer set NOD2_SNP8 (for details see Table 1). Template used was commercially available human genomic DNA (Promega) in a final concentration of 0.5 ng/µl. Size (289 bp) and integrity of the amplified DNA were checked on an 1.5 % agarose gel.

2.3.2 Background fluorescence & thermal stability

Fluorescence background intensity was measured with the custom-made detection system (see 2.7.3) with an excitation wavelength of 480 ± 40 nm. Emission was measured at 535 ± 50 nm. To examine thermal stability, chip material samples were heated up five times to 95℃ and cooled down to 60℃, after which they were checked for deformation.

2.3.3 Tested materials

A spectrum of plastic materials was tested which were suitable for one of two promising plate fabrication technologies used by our cooperation partners. ThinxX GmbH uses highly-developed injection molding methods. MicroTEC (Bad Dürkheim, Germany) is specialist in Rapid Micro Product Development® based on photolithographic methods for fabrication of micro structured devices. Different conventional materials as well as prototypes made of photo-polymerisable metacrylates were tested. Additionally several commercially available materials for adhesive covering of high-density microwell arrays (HD-MWA) were tested.

Materials for injection-molding

- transparent polypropylene (PP)
- black coloured PP
- transparent cycloolefine-copolymere (COC)
- black coloured COC
- black polycarbon (PC)
- transparent PC

Materials from photolithographic fabrication

- prototype 6EB (black)
- prototype 6EB (transparent)
- prototype 6C7 (black)
- prototype 6C7 (transparent)
- prototype 6C8
- prototype 6A9
- prototype 6C8
- prototype 77D
- prototype 1B06B/744

Materials for adhesive covering

- Optical Adhesive Covers®(Applied Biosystems, #4311971)
- VIEWseal® film (Greiner BioOne, #676070)
- EASYseal® Sealer(Greiner BioOne, #676001)
- POWERseal® (Greiner BioOne, #676080)
- AB-0558 (ABGene)
- Soft Sealing (MG Research)

2.4 The µPCR chip

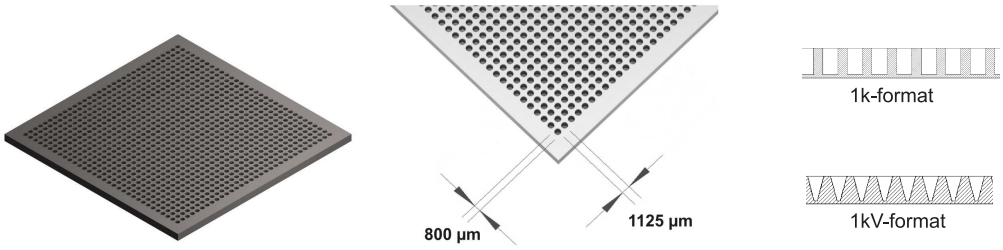
The µPCR chip is made of black colored PP. Presently, three different chip formats for different volume ranges are available. The designs of all formats consider sharp edges along the upper border of each well to keep the width of the partition walls as large as possible for efficient sealing.

2.4.1 1k-format

On a 40×40 mm area the 1k-format of the µPCR Chip has a total of 1024 (32×32) wells. The distance between neighboring wells(pitch) measures 1125 µm. Well diameter and bottom width are 800 µm and 150 µm, respectively. Each well has a volume capacity of 500 nl, with a working volume ranging between 200 nl and 400 nl.

2.4.2 1k/V-format

The 1k/V-format of the µPCR chip has identical proportions. Due to the V-shape of the wells the total volume capacity is reduced to 200 nl with working volumes between 50 nl and 150 nl.



(a) The chip in total, 1024 wells on 40×40 mm

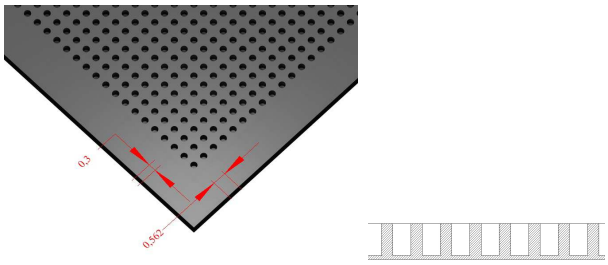
(b) Selection with dimensions of diameter and pitch

(c) Side view of 1k-format and 1k/V-format

Figure 8: 1k-format and 1k/V-format of the µPCR Chip.

2.4.3 4k-format

Further reduction of the reaction volume enables the 4k-format. On the same area of 40×40 mm with a pitch of 562.5 µm and a diameter of 300 µm, the total capacity per well is reduced to 25 nl. The chip comprises 4096 wells and the working volume ranges between 10 – 18 nl.



(a) Selection of the 4k chip

(b) Profile 4k

Figure 9: 4k µPCR Chip (a) and side view (b) of the flat bottom shaped reaction wells

2.5 Functionalization by low pressure gas-discharge plasmas

A couple of µPCR chips of the 1k-format were functionalised by our collaborator Dr. Asmus Meyer-Plath from the Federal Institute for Materials Research and Testing using low pressure gas-discharge plasma. The chips were functionalised with allylamine (AAM) and glycidyl methacrylate (GMA) to investigate the effects of positively (amino) and negatively (epoxy) charged surfaces, respectively. At a process pressure of 20 Pa and a capacity of 30 W a radio frequency excited (13.56 MHz) anisothermal plasma

was used to generate a layer thickness of approximately 50 nm. Beside the target functionality the generated surfaces contain further functional groups. For allylamine a density of 6 primary amino groups per 100 carbon atoms was achieved. Secondary amino groups and amides increase the number of nitrogen atoms per 100 carbon atoms to 15. Moreover the surface has 10 oxygen containing groups per 100 carbon atoms including peroxy, hydroperoxy, hydroxyl, carboxyl and carbonyl functionalities. The GMA surface contains approximately 10 epoxy groups per 100 carbon atoms and 5 – 10 additional oxygen containing groups.

2.6 Liquid handling

For liquid handling two non-contact nanodispensing technologies were used. With the SciFlexArrayer from Scienion a commercial available piezo-dispenser was used. The second dispensing technology was the valve nanodispensing technology (Seyonic / Switzerland). On the basis of the valve technology a custom made nanodispensing unit (see figure 11a) and the commercially available Staccato nanodispensing system with the iNL10 nano-dispensing head from CaliperLS (see figure 11b) were used.

2.6.1 Piezo dispensing - SciFlexArrayer

The SciFlexArrayer is based on an open fluidic channel system, combining high flexibility and very small volume dispensing (down to 0.4 nl). A fluidic jet is created through an electro-kinetically induced pressure on the wall of a capillary. Under high speed (> 1 m/s) a single droplet is formed via a nozzle with an aperture of 70 µm and a 400 pl droplet resolution. Performance is visually controlled via a head-mounted microscope with integrated video chip connected to a PC.

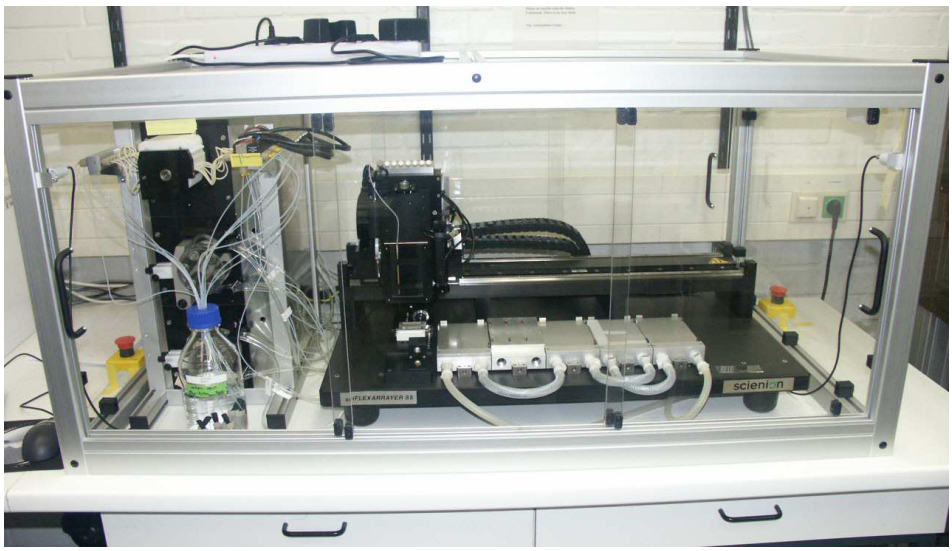
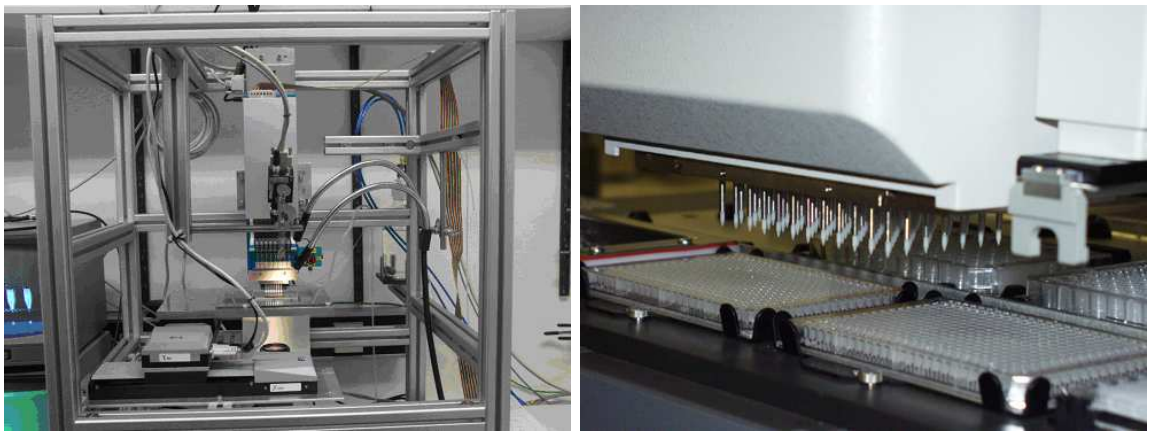


Figure 10: SciFlexArrayer - piezo dispensing station

2.6.2 Valve dispensing - ValveProto

The ValveProto (Figure 11a) is a custom-made 8-channel nanodispenser where the valve dispensing technology from Seyonic was integrated. It allows dispensing volumes down to 10 nl. In contrast to the piezo approach, the valve nanodispensing technology uses a pressure driven liquid system flow with feedback control. A sensor signal is integrated to measure the aspirated or dispensed volume. This information is directly used for feedback control of the valve. The use of high-speed solenoid valves ensure efficient non-contact dispense of liquids, contained in the dispenser tip.



(a) ValveProto - custom made valve dispensing unit

(b) Staccato system from CaliperLS

Figure 11: The valve dispensing platforms
a) Experimental setup of the ValveProto nanodispenser. 1) Central element is the 8-channel valve nanodispensing module from Seyonic. 2) Plate adjustment is done by a XYZ positioning table and enables positioning of the PCR chip with a precision down to ± 1 µm. 3) The dispensing process can be monitored by a CCD camera with a telecentric objective. Software to integrate all system compartments and for operating the nanodispenser was developed using the LabView software development architecture (version 5.1). **b)** Selection of the 96-nozzle dispensing array of the Staccato workstation.

2.6.3 Valve dispensing - Staccato workstation with iNL10

The Staccato inL10 from CaliperLS (Figure 11b) allows dispensing from 10 nl to 10 µl. Valuable feature of the system is the independent pipetting control over each channel in the 96 channel pipetting head, which is based on the feedback control technology from Seyonic described above. Performance parameters are monitored in real time and facilitate "on the fly" adjustments to compensate for variations like temperature, viscosity, and residual volume that compromise the performance of other approaches, particularly at low volumes. The solenoid valves in the inL10 do not receive time-based instructions, but open and close in a dynamic fashion based on closed loop reporting of the actual volume being aspirated or dispensed per channel.

2.6.4 Protocols for nano-liquid handling

Piezo dispensing - SciFlexArrayer
The SciFlexArrayer was operated in single nozzle modus. Every morning a routine washing was performed for the whole system.

Routine washing

- Flush aqueous wash station (2 min)
- Flush waterering/Neutralreiniger wash station (2 min)
- Circulate System at 10V (5min)
- Flushing nozzles dipped into aqueous wash bath combined with ultrasonic at 5V (10min)
- Wash5sFlush10sNozzles
- Wash5V15sNozzlesW2
- Automatic drop detection, image is saved for performance control

For dispensing the nozzles were operated at approx. 100 V with a pulse of 50 Hz. The voltage may vary depending on different nozzles used. Each nozzle has its own performance optimum, which was determined by visual analysis of the camera based spot control. Parameters were adjusted to get a perfectly shaped droplet leaving the nozzle orifice with a straight-lined stream. Dispensing of mix as well as samples was done as follows:

Standard dispensing protocol for the piezo dispenser

- Wash5sFlush3sNozzlesUltrasonic
- Start loop
- Aspiration of sample/mix at 3 V for 10 sec (uptake of approx. 10 µl)
- Dip into water
- Check drop manually
- Dispense sample / mix
- Wash5sFlush10sNozzlesUltrasonic
- Check drop manually
- End loop

Valve dispensing - ValveProto
The ValveProto was operated with an aspiration pressure of -50 mbar and an dispensing pressure of 500 mbar. Before working with samples the nozzle performance was checked by dispensing 100 nl volumes of system liquid (water). The residual volumes were checked to be in the range of -15 nl – 10 nl. This was repeated after each dispensing run. If the residuals were out of the range, the system was newly primed. Priming of the ValveProto nanodispenser system was done according to the instructions from Seyonic. The duration of CO_2 – purging was 45 min at a pressure of 0.5 bar. Priming was combined with helium sparging of the system liquid for 15 min at a pressure of 500 mbar. The ValveProto nanodispensing system was operated without oil separation.

Aspiration pressure was set to -50 mbar and dispensing pressure to 500 mbar. The standard dispensing protocol was as follows:

Standard dispensing protocol for the ValveProto

- Aspiration of samples – volume: [number of wells × dispensing volume] + 2 µl, total ≤ 9 µl
- 2 × predispense with 100 nl volumes
- Positioning of nozzles 1 - 4
- Stepwise dispensing of final volumes with nozzle 1 - 4
- Repositioning of nozzles 5 - 8
- Stepwise dispensing of final volumes with nozzle 5 - 8
- Flushing of nozzles for 5 sec
- Aspiration of 100% IPA – volume: same as in sample aspiration step
- Flushing of nozzles for 10 sec
- Aspiration of 100% IPA – volume: same as in sample aspiration step
- Flushing of nozzles for 10 sec

Valve dispensing - Staccato workstation with iNL10

For the Staccato workstation (2.6.3) dispensing and washing procedures were developed with the device control software from CaliperLS. The system was operated with an aspiration pressure of -125 mbar and a dispensing pressure of 500 mbar. Nozzle performance was controlled by monitoring residual volume, dispensed volume and dispensing time. Residuals were kept to be in a range of -15 nl – 10 nl, the dispensed volume ± 5 nl of the set dispensing volume. The dispensing time should not differ more than 20% of the morning performance check value, which was done before any dispensing. Two versions of dispensing protocols were used. Depending on the needed configuration, nozzles were enabled or disabled and the protocol was applied to the active nozzles.

Oil free dispensing protocol

- Aspiration of sample – volume: [number of wells × dispensing volume] + 2 µl, total ≤ 9 µl
- Dip into water
- 2 × predispense with 100 nl volumes
- Positioning of 1^{st} half of the activated nozzles (in maximum 4 × 4 nozzles on one µPCR chip)
- Stepwise dispensing of final volumes with first set of activated nozzles
- Positioning of 2^{nd} half of the activated nozzles (in maximum 4 × 4 nozzles on one µPCR chip)
- Stepwise dispensing of final volumes with second set of activated nozzles
- Flushing of nozzles for 6 sec
- Aspiration of 100% IPA – volume: same as in sample aspiration step
- Flushing of nozzles for 6 sec
- Wash procedure: 25 sec ultrasonic treatment while nozzles are dipped into the watherbath
- Emptying of watherbath and refill with water

To separate system liquid and sample solution a polydimethylsiloxane oil pre-aspiration step was integrated in the dispensing protocol. This also required adjustments of the washing procedures.

Oil separation dispensing protocol

- Aspiration of 1 µl oil
- Dip into IPA
- Aspiration of sample – volume: [number of wells × dispensing volume] + 2 µl, total ≤ 8 µl
- Dip into water
- 2 × predispense with 100 nl volumes
- Stepwise dispensing of final volumes with activated nozzles
- Flushing of nozzles for 6 sec
- Aspiration of 100% IPA – volume: same as in sample aspiration step
- Flushing of nozzles for 6 sec
- Wash procedure: 25 sec ultrasonic treatment while nozzles are dipped into the watherbath
- Emptying of watherbath and refill with water

2.6.5 Volume Measurements

The dispensed volumes were checked by dispensing aqueous solutions of a FAM dye labeled 10-mere (FAM-oligo) or a aqueous tartrazine solution. The amount of dispensed FAM-oligo and tartrazine was determined by fluorescence and absorbance measurements, respectively. Fluorescence was measured at standard FAM dye settings in the real-time detection unit (see 2.7.3). For tartrazine the aqueous solution was dispensed into a with 40 µl water pre-filled Corning® 384 Well Clear Flat Bottom UV-Transparent Microplate. After shaking and short centrifugation dye intensities were measured in a spectrophotometer (SpectraMax Plus384, Molecular Devices).

2.7 Cyclers

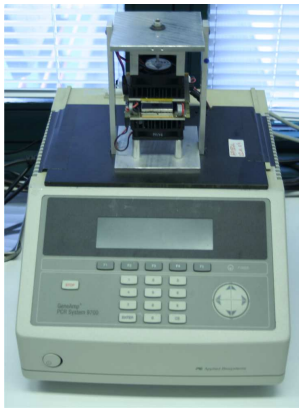
Several thermocyclers were used for different purposes. Thermocycling procedures for standard PCRs were carried out in a MJ PTC 200 Thermocycler obtained from Biozym. Real-time PCR experiments in standard 384 well format were carried out with the 7900HT Fast Real-Time PCR System from Applied Biosystems. Furthermore three custom-made thermocyclers were in use.

2.7.1 ABI Sandwich Cycler

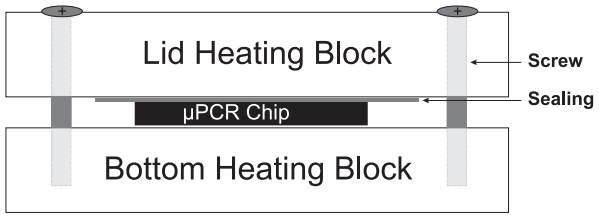
This custom-made cycler was provided by our cooperation partner Applied Biosystems. Basis is a PE GeneAmp 9700 PCR System with removed lid and heating block(Figure 12a). Instead, two sandwich like oriented peltier elements are mounted on the device. Both heating blocks are controlled over the device software and can be actively cooled via fans. The blocks can be pressed together to ensure tight contact for good heat transfer to the chip.

2.7.2 Hydro Sandwich Cycler

The custom-made Hydro Sandwich Cycler has two heating blocks which can be operated with 4 different temperatures. A valve system connects the blocks to four pretemperated thermo baths. Temperature is controlled with a custom made LabVIEW (version 5.1) application. Each block has only a 500 µm thick surface layer which enables efficient thermal transfer to the chip. Figure 12b shows the arrangement of the blocks for efficient cycling. To keep the sealing intact the blocks are moderately squeezed together using two screws in opposite orientation.



(a) ABI Sandwich Cycler



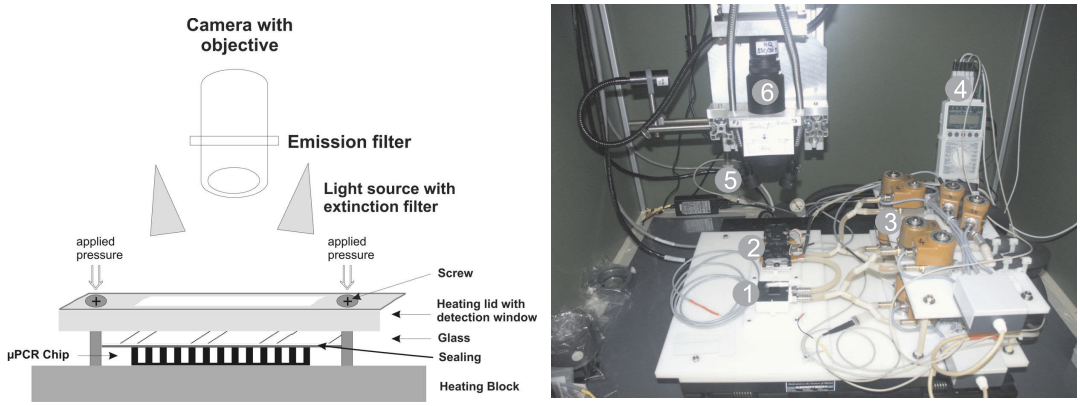
(b) Schematic view of the Hydro Sandwich Cycler

Figure 12: Custom-made cyclers without real-time detection
a) ABI sandwich cycler – The µPCR chip can be placed between the two heating blocks and moderate pressure is applied. Temperature control is done via the device control software. b) Hydro sandwich cycler – The µPCR chip is placed between two heating blocks. Moderate pressure is applied with two screws in opposite orientation.

2.7.3 Real-time Hydro Cycler

The custom-made real-time cycling system is basically the bottom heating block of the Hydro Sandwich Cycler and an electrically heating lid with integrated detection window to allow optical detection from top. Figure 13 shows the system (B) and a schematic view (A) of the real-time processing unit. The sealed PCR chip is covered with a thermo glass to enable efficient heat transfer from the ring-shaped lid heating across the whole plate. During the whole cycling process moderate pressure is applied by squeezing down the lid heating with two screws in opposite orientation. Synchronization between block and lid heating is done via an in-house developed software using the LabVIEW software development architecture (version 5.1). For optical read out two ACE light sources (Polytec) are used for excitation with an HQ 480/40 filter (AHF Analysentechnik). During cycling, fluorescence was recorded at the end of each annealing-elongation step using a coolSamBa HR-320 CCD camera (Sensovation AG) with an integrated emission filter Q 535/50 (AHF Analysentechnik). Generated images

were used for semi-automated read out of intensities (Alpha Imager^{TM}2000 software, Documentation & Analysis System, Version 4.0;Alpha Innotech).



(a) Schematic view of the real-time cycling unit.

(b) Real-time cycling unit in darkroom

Figure 13: The custom-made real-time cycler station
a) Structure of the custom-made real-time cycling system. **b)** 1 and 2 show the bottom and lid heating, respectively. The valve system (3) has an integrated temperature control (4) which is controlled by a self-developed software. Two light sources with integrated filter(5) are positioned at the lower end of the objective(6).

2.8 Fluorescence detection

For fluorescence detection two custom-made systems were used. First the already mentioned optical detection unit of the real-time cycling system (2.7.3), and for genotyping a second detection unit with several filters (see Table 7) was used.

Table 7: Filters used in SNP genotyping detection unit

Reporter dye	Excitation [nm]	Emission [nm]
FAM	470 ±20	515 ±15
VIC	500 ±18	550 ±20

2.8.1 Detection unit for genotyping

The genotyping detection unit (Figure A) consists of the sensitive CCD camera Andor iXonEM^{+} (Andor Technologies)(see Figure B), two custom-made filter changers for excitation and emission filters (for filters from AHF Analysentechnik see Table 7) and a microscope X/Y–positioning table(Figure D). Light source for excitation was an ACE light source (Polytec). Fluorescence was measured by several excitation / emission filter combinations. After positioning of the µPCR chip, images were taken with the camera specific software iXion Version 1.1.37.1, converted into the TIFF format and quantified

using the Alpha Imager^{TM}2000 software, Documentation & Analysis System, Version 4.0;Alpha Innotech).

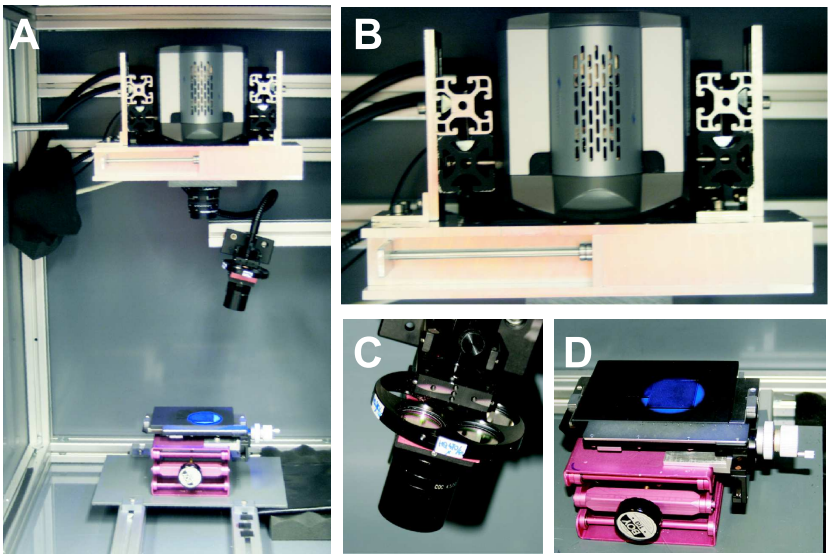


Figure 14: Genotyping detection unit
A) Darkroom with whole assembly of optical detection unit for SNP genotyping b) CCD camera Andor iXonEM^{+} with emission filter changer just below the camera. C) Light source with filter changer for excitation. D) X/Y–positioning table with placed 1k-µPCR chip.

2.9 Samples and sample preparation

2.9.1 RNAse P TaqMan assay flanking PCR product

Primers were designed to amplify a PCR fragment of 423 bp spanning the RNAse P TaqMan® assay amplicon (app. 100 bp). For primer sequences see Table 1. Multiple standard PCRs were performed and pooled afterwards. The concentration of pooled PCR product was measured using the ND-1000 Spectrophotometer (Nanodrop Technologies). Finally concentration were adjusted to 10 ng/µl to be used as stock solution.

2.9.2 Genotyping samples

The human samples for genotyping were provided by the University of Kiel (S. Schreiber); samples of hgDNA in a quantity of 100 µl and a concentration of 50 ng/µl, whole genome amplified DNA (WGA-DNA) in 20 µl aliquotes of a 250 ng/µl concentration. The WGA-DNA was produced using the multiple displacement approach.[100] Working solutions were prepared with ddH_2O in a concentration of 5 ng/µl. Genotype information from Kiel for comparison is listed under Appendix A.3.2.

2.9.3 Preparation of DNA dilution gradients

DNA concentrations of the stock solutions were checked with the ND-1000 Spectrophotometer (Nanodrop Tech.). Concentration gradients were made by serial dilution.

2.9.4 DNA fragmentation

Fragmentation of hgDNA was done by applying ultrasonic. The aqueous hgDNA sample was kept in a 1.5 ml tube. For the duration of ultrasonic treatment the tube was totally dipped into the system liquid of the ultrasonic bath.

2.9.5 DNA desiccation

Drying of samples was done by applying a vacuum during centrifugation at room temperature in a SpeedVac® SC210A (Savant/Thermo) until complete desiccation. Dried samples were stored at 4℃.

2.9.6 Total RNA extraction from mouse

Total RNA of mouse was extracted from frozen tissues of C57BL6/J adult female mouse using Trizol reagent (Invitrogen), following the manufacturers instructions. RNA was treated with RNase-free DNase I, quantified by UV spectrophotometry, and its integrity was verified by gel electrophoresis. RNA was transcribed into cDNA using SuperscriptII reverse transcriptase (Invitrogen) and random hexamers. In total, 8 µg of total RNA for each sample were converted into cDNA in 8×1 µg reactions, then pooled and diluted to 12.5 ng/µl equivalent total RNA.

2.10 Data analysis

2.10.1 Read-out of intensities by image analysis

The fluorescence intensities in the obtained images were quantified by image analysis using the Alpha Imager^{TM} 2000 software, Documentation & Analysis System, Version 4.0 (Alpha Innotech). For intensity quantification, a circular equally sized area was defined around each well. Numeric values for the averaged fluorescence intensity of each single circular spot were used for further analysis.

2.10.2 Control for successful amplification by endpoint measurement

For normalization purpose the average intensity of non-template controls was subtracted for each individual reaction. PCRs with an intensity of above 25% of the maximal normalized intensity were considered as successful amplifications.

2.10.3 Processing of raw intensity data from real-time experiments

The raw intensities of one image were summarized using unix functionalities and structured by using perl scripts (see A.4.1). To normalize the raw intensities of the 200 nl reactions a background correction step was applied to the data. For each individual reaction a background level was calculated as the average of the intensities in the

baseline range. This baseline range spanned the cycles where no increase of the signal intensity was detected. Each individual intensity value was then adjusted according to the well specific background. Data analysis was primarily done with Microsoft®Excel, but due to the high number of data points, finally the R statistics environment[101] was used for comprehensive analysis. R has limitations concerting interactivity during the analysis process, because it is based on scripts, but it is very adaptable to the needs of the analysis.

2.10.4 Categorisation of outliers in real-time experiments

Outliers were single reactions with clearly diverging Ct-values of the rest of the performed replicates. Reactions which had a deviating Ct-value of more than 15 % from the median of all replicates were treated as outliers and removed from the calculations.

2.10.5 Relative quantification of expression levels in mouse study

Threshold cycles were obtained using the method established by Higuchi et al.[66] To select a suitable reference gene, four housekeeping genes were tested in 10 µl reactions on sample cDNA. The most stable gene across all samples was identified using the geNorm method.[102] Beta-actin was selected as reference gene for relative quantification with the $\Delta\Delta$Ct method used.[103]

2.10.6 Real-time PCR standard curve and PCR efficiency

PCR efficiencies were calculated using the standard curve method according to Rutledge and Cote.[104] Standard curves were obtained by measuring the product for the RNAse P assay on serial dilutions of various DNA templates. For each dilution, Ct values were plotted against the Log value of the input DNA. The real-time PCR efficiencies (E) were derived from the respective slopes using the following equation:

$$E = 10^{-Slope} - 1 \tag{2}$$

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2.10.7 Calculation of the confidence interval and other statistical tests

Calculation of the CI was done using the function boxplot.stats of the R statistics environment.[101] The confidence level was set to 95%. Further functions used in R were: *hist, boxplot, qqnorm, mean* and *median*.

Definition of the CI

For a population parameter such as mean is an interval between two numbers with an associated probability p (confidence level), which is generated from a random sample of an underlying population, such that if the sampling was repeated numerous times and the confidence interval recalculated from each sample according to the same method, a proportion p of the confidence intervals would contain the population parameter in question.