Centrifugal microfluidic system for primary amplification and secondary real-time PCR

Maximilian Focke,[a] Fabian Stumpf,[a] Günter Roth,[a,b] Roland Zengerle,[a,b] Felix von Stetten[a,b]
[a] *Laboratory for MEMS Applications, Department of Microsystems Engineering (IMTEK), University of Freiburg, Georges-Koehler-Allee 106, 79110 Freiburg, Germany.*
[b] *HSG-IMIT, Wilhelm-Schickard-Straße 10, D-78052 Villingen-Schwenningen, Germany*

SUPPLEMENTARY INFORMATION

Content

1) Supplementary methods

a. Fabrication

Microfluidic cartridges are microthermoformed by soft lithography with cyclic-olefin polymer foils (COP, ZF14, Zeon Chemicals L.P., USA). Film thickness is 188 µm. Process regime is extensively explained in the cited literature.
Laser cutting (PLS 3.60, Universal Laser Systems Inc., USA) is applied to trim the microthermoformed foil disks in circular shape (diameter 130 mm) and to cut openings for air vents and sample inlets into the foil disks. The process set point parameters for the laser cutting process are 90 % power, 2.5 % speed, 700 PPI at a set focal height of 3.0 mm. The same parameters were applied for cutting air holes into the foil disks after sealing with a pressure sensitive adhesive tape (no. 900320, HJ-Bioanalytik GmbH, Germany). The air holes are required for sufficient heat convection as this is the thermocycling principle in the applied Rotor-Gene 2000 thermocycling instrument.
Finally, hydrophobic filter membranes are applied on the openings for air vents (GORE^{TM} Medical Membranes MMT-314, W. L. Gore & Asscociates Inc., USA).

b. Surface treatment

Each siphon channel is spilled with a solution of Vistex 111-50 (Filmspecialities, Inc., USA) in 2-propanol (1:15 v/v) using a standard pipette. The first two wider siphons receive 9.8 µl each, the smaller siphon is coated with 3.2 µl of the solution. When the solvents evaporate, the agent remains in the siphons and is cured for 60 minutes at 120 °C in a laboratory oven. Measurement of static contact angles of the applied PCR mix on treated (or un-treated) COP films was carried out with a OCA 15+ (DataPhysics Instruments GmbH, Germany).

c. PCR conditions

The dilution ratio of the pre-amplified mix with the secondary amplification mix is 1:9. The compositions of the primary and secondary PCR mixtures are found in Supplementary tables 1 and 2. Bovine serum albumine (BSA) and salmon sperm DNA act as sacrificial molecules to prevent undesired absorption of polymerase or target DNA on surfaces of the microfluidic channels and reservoirs.
For pre-storage of dry PCR reagents, target-specific primers and TaqMan probes are spotted in each reaction well and dehydrated for 1 h at room temperature in the dark. Each reaction well comprises primers and probes for exactly one specific DNA target (Supplementary table 3). The concentrations are 300 nM for target-specific forward and reverse primers each as well as 200 nM target-specific TaqMan probe (biomers.net GmbH).

Supplementary table 1. Primary PCR mixture

Component	Supplier	Amount
2.5x RealMasterMix	5Prime GmbH, Germany	8 µl
Forward primers	biomers.net GmbH, Germany	300 nM
Reverse primers	biomers.net GmbH, Germany	300 nM
BSA	Carl Roth, Germany	2 µl (0.3 % final concentration)
Salmon sperm DNA	Sigma Aldrich, Germany	2 µl (1 ng/µl)
DNA sample	Genomic Research Laboratory Geneva	2 µl (varying DNA concentrations)
H_2O	Invitrogen GmbH, Germany	Fill up to 20 µl

Supplementary table 2. Secondary PCR mixture

Component	Supplier	Amount
2.5x RealMasterMix	5Prime GmbH, Germany	72 µl
BSA	Carl Roth, Germany	16 µl (0.3 % final concentration)
Salmon sperm DNA	Sigma Aldrich, Germany	16 µl (1 ng/µl)
H_2O	Invitrogen GmbH, Germany	Fill up to 160 µl

Supplementary table 3. Sequences of primers and probes (5'-3')

Gene	Primary PCR forward primer	Primary PCR reverse primer	Secondary PCR forward primer	Secondary PCR reverse primer	Probe sequence	Dye
exfoliatin A	CCATATG GAGAGTA TGAAGTC	TGAAACA CCGTTTT GATC	CCATATG GAGAGTA TGAAGTC AAAGAAA	TGAAACA CCGTTTT GATCTGG TT	CCATTTG GTGCAGG TGTTGAT TTAGCAT TAA	FAM
exfoliatin B (*)	GAGGTAG GAAACTC TGGAT	TCTATTG AAAAACA CTCCTATT	GAGGTAG GAAACTC TGGATCA GGTAT	TCTATTG AAAAACA CTCCTATT GGAAGA	AGGTATT CACAGTG GTAAAGG CGGACAA CA	FAM
TSST-1 (*)	TCATCAG CTAACTC AAATACA	TGTGGAT CCGTCAT TCATTGTT	TCATCAG CTAACTC AAATACA TGGATTA	TGTGGAT CCGTCAT TCATTGTT	TCCAATA ACCACCC GTTTTATC GCTTGA	FAM
SCCmec type II (*)	AACGAGA CGTGCCC A	CATCAGT TCATGTTT ACTATTA G	AACGAGA CGTGCCC AAGAAG	CATCAGT TCATGTTT ACTATTA GGTATTT TGTC	ATTTGCC GCTGGGC T (**)	FAM

(*) These sequences were only applied in the multiplexing experiment (Supplementary Figure 9).
(**) Minor groove binder

The thermocycling protocol for primary PCR comprises an activation step at 95 °C (110 sec), followed by 10 thermocycles including denaturation at 95 °C (30 sec), annealing at 50 °C (30 sec) and extension at 71 °C (30 sec). The protocol for secondary PCR comprises activation at 95 °C (110 sec), followed by 50 thermocycles including denaturation at 95 °C (20 sec) and annealing at 57 °C (40 sec) with real-time fluorescence readout of the reporter dye. All temperatures are setpoint values.

d. Microfluidic protocol

Primary PCR takes place under rotation at 27.2 Hz. This rotational speed prevents full priming of the capillary siphons (Fig. 2b). After ten thermocycles, the disk is stopped to allow instant priming of the first two siphons (Fig. 2c).

Acceleration to 27.2 Hz transfers both liquid portions into the mixing chamber where they are mixed by a uni-directional shake-mode protocol [M. Grumann et al., *Lab Chip*, 2005, vol. 5 (5), 560] at 6.6 to 27.2 Hz with 10 alternations (Fig. 2d). Then, the disk is stopped to allow priming of the third capillary siphon (Fig. 2e). The liquid is subsequently distributed at 6.6 Hz through the sloping feed channel (Fig. 2f) into 14 finger-like metering chambers with a nominal volume of 10 µl each and an adjacent waste liquid chamber (Fig. 2g). Then, the aliquots are transferred into the reaction wells at alternating rotational frequencies of 6.6 to 27.2 Hz (10 alternations) which is well above the burst frequencies of the passive valves [D. Mark et al., *Lab Chip*, 2009, vol. 9 3599]. Finally, the aliquots dissolve the pre-stored primers and probes for the secondary PCR which starts under rotation at 6.6 Hz (Fig. 2h).

2) Clogging bubbles

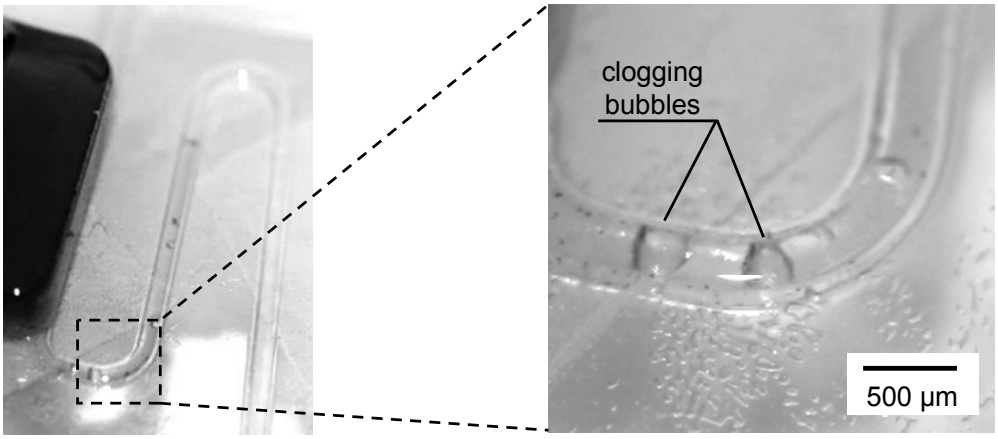

Fig. S1. Bubbles generated during thermocycling are clogging a siphon channel. Such conventional standard siphon channels were 380 µm wide and 200 µm deep and proved insufficient for the application. The PCR mixture was dyed with black ink for better visibility.

3) Surface coating and channel appearance

Fig. S2. Filling of siphon channels with a solution of the surface agent Vistex to achieve a hydrophilic coating.

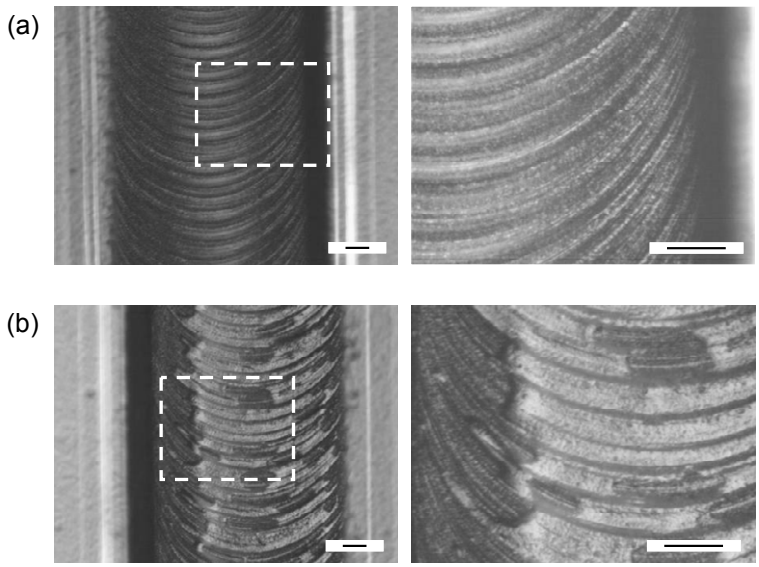

Fig. S3. Deposition of Vistex agent. (a) Plain siphon channel in foil disk without coating. (b) Coated channel. Scale bars are 100 µm.

4) The slightly modified thermocycling device

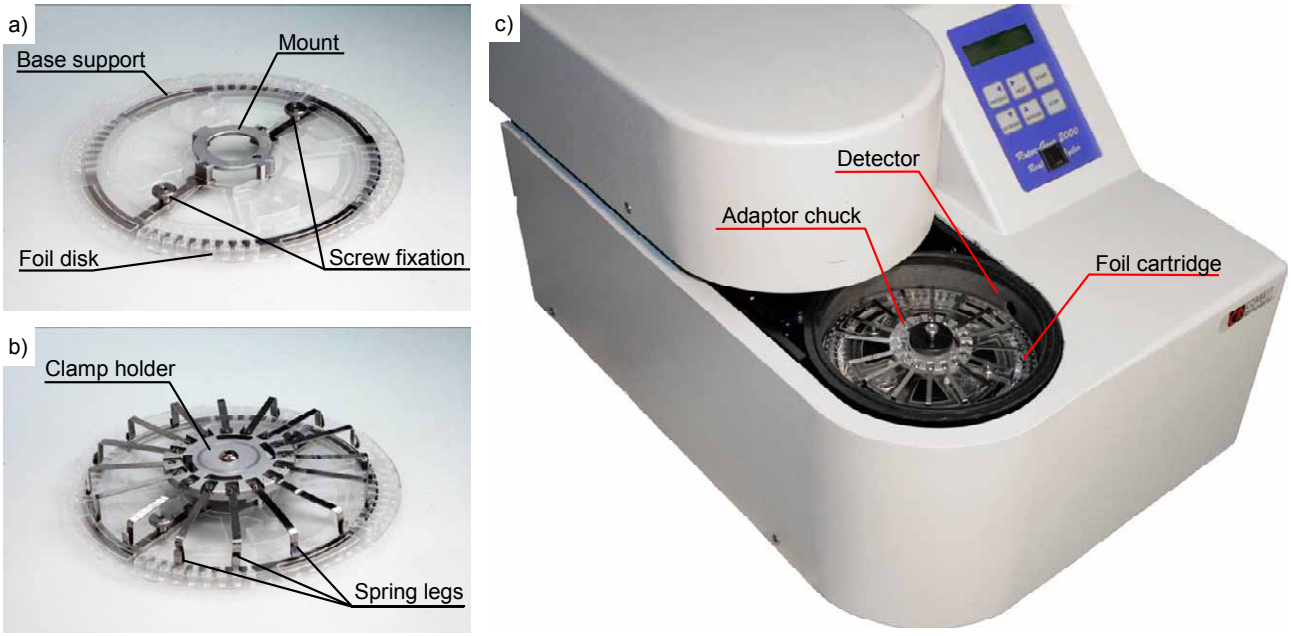

Fig. S4. Fixation of foil disks. (a) Disks are screwed onto a base support with a mount compatible to the Rotor-Gene 2000. (b) The disk is firmly pressed onto the base support by a clamp holder. (c) Assembly in the thermocycling instrument Rotor-Gene 2000 (open top cover position).

5) Microfluidics

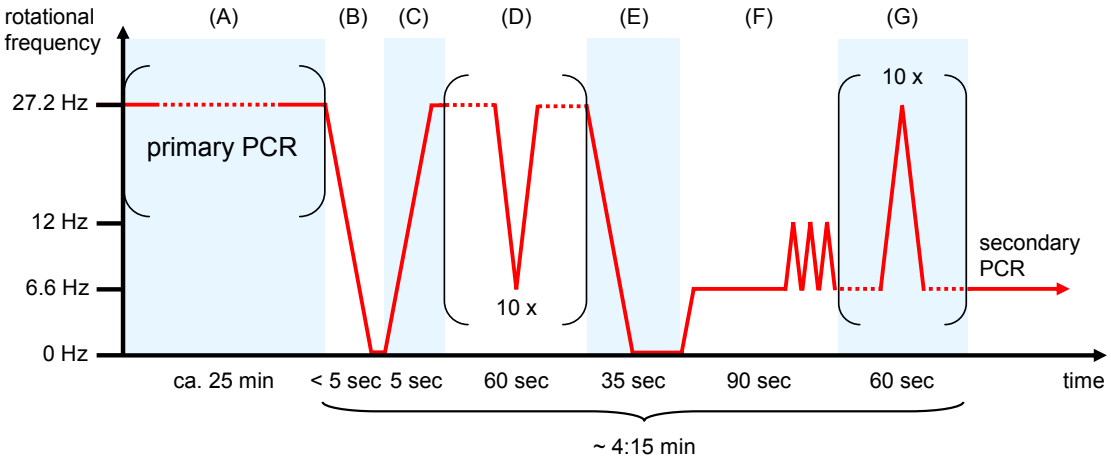

Fig. S5. Chronological sequence of microfluidic protocol. (A) Primary PCR. (B) Capillary priming of the first two siphon valves. (C) Transfer of reagents into mixing chamber. (D) Shake-mode mixing. (E) Capillary priming of third siphon valve. (F) Aliquoting with some short rotational impulses in order to assure proper liquid flow. (G) Burst of centrifugo-pneumatic valves for liquid transfer into reaction wells.

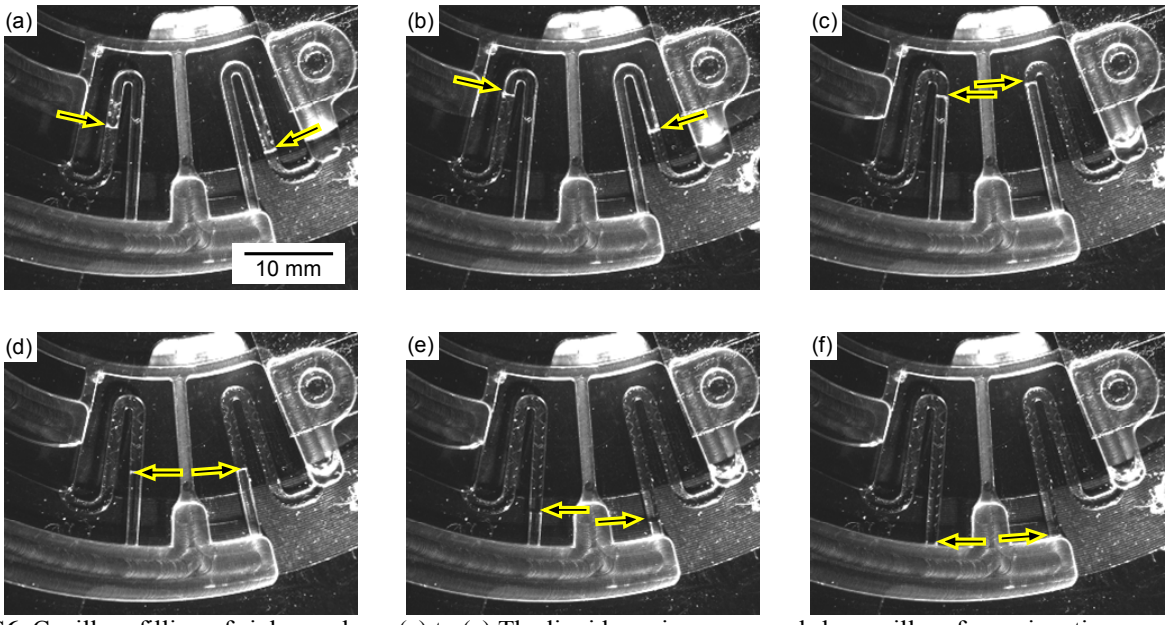

Fig. S6. Capillary filling of siphon valves. (a) to (e) The liquid meniscus proceeds by capillary forces in a time span of less than 5 seconds. The arrows point at the respective location of the meniscus. The disk is at rest. (f) Once the meniscus pins at the exit port, the chambers can be emptied by rotation (not shown).

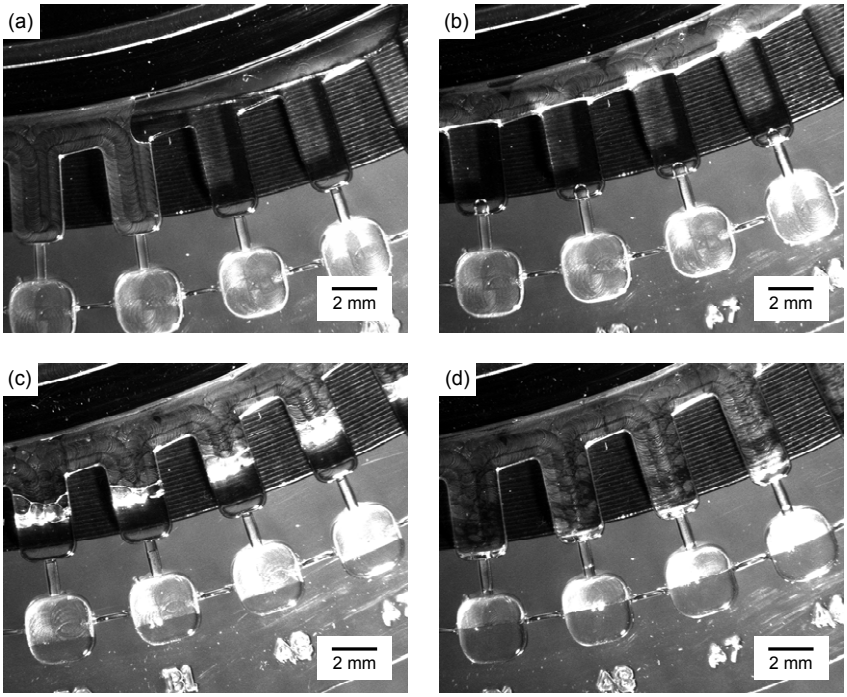

Fig. S7. Aliquoting scheme. (a) The metering chambers are filled through the distribution channel. (b) All volumes are metered and held by the centrifugo-pneumatic valves. (c) Emptying the metering chambers at rotational speed around 27 Hz. (d) Final aliquots of nominally 10 µl in the reaction wells.

6) Utility of primary PCR and proof of sufficient homogeneity of mixing

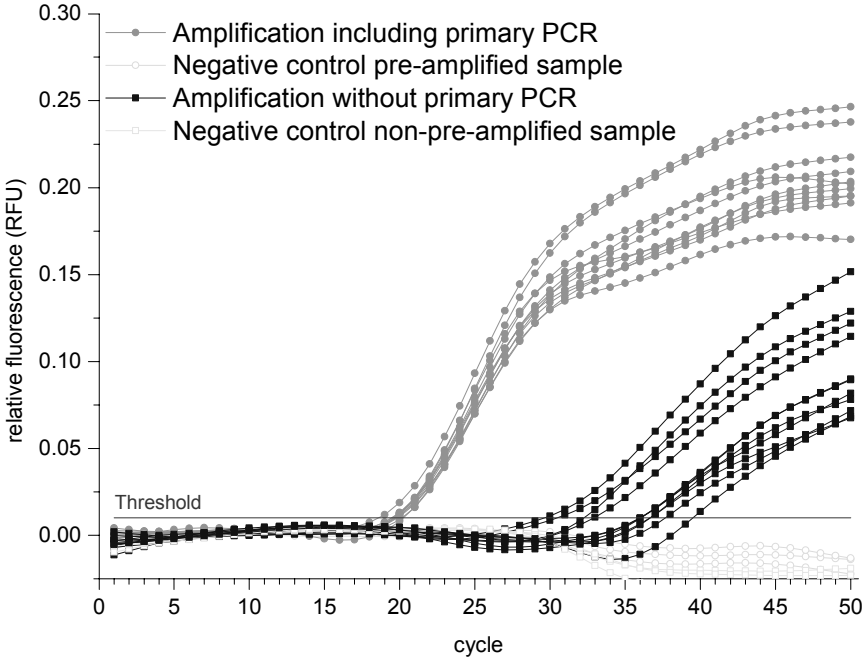

Fig. S8. Comparison of a pre-amplified and a non-pre-amplified sample in a foil cartridge. The fluorescence signal of the pre-amplified sample (threshold cycle at a threshold of 0.01 RFU is 20.2 +/- 0.4 cycles, s.d.) is far more homogenous than the signals of the non-pre-amplified sample (threshold cycle at a threshold of 0.01 RFU is 35.6 +/- 2.8 cycles, s.d.). The homogenous fluorescence signals of the pre-amplified sample also show that microfluidic mixing prior to aliquoting leads to a sufficiently homogenous mixture.

7) Example of multiplexing capabilities

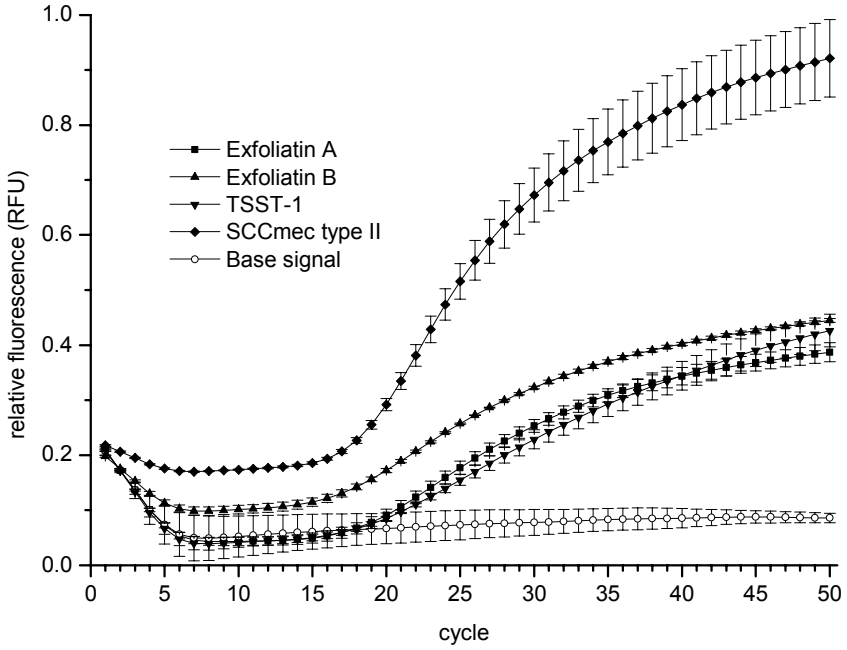

Fig. S9. Real-time amplification plot of four different targets after multiplex pre-amplification. A sample containing specific sequences of the genes Exfoliatin A and B, TSST-1 and SCCmec type II were pre-amplified and subsequently detected during real-time PCR in the reaction cavities. Each target was detected in three separate reaction wells in which respective primers were pre-stored. The base signal is generated in two further reaction wells in which primers of a dissimilar genetic sequence were dehydrated.