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

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Pre-amplification is a basis for numerous polymerase chain reaction (PCR) protocols but bears severe contamination risks due to handling of high-copy DNA samples. Therefore we developed a self-contained centrifugal microfluidic system comprising pre-stored reagents; it enables pre-amplification of specific DNA sequences prior to automated aliquoting and real-time PCR in a modified commercial thermocycler.

Testing a DNA sample for a number of specific genetic sequences (*e.g.* for typing of pathogenic bacteria) usually requires the sample to be aliquoted into separate microtubes in which a specific amplification reaction can take place. However, if the number of relevant target sequences exceeds the number of initial DNA copies in the sample, appropriate aliquoting is no longer feasible. There are three workarounds to this limitation: firstly, to restrict analysis to samples with a sufficiently high DNA concentration; secondly, to perform a multiplex (*i.e.* simultaneous) amplification of multiple loci in the same reaction tube; or thirdly, to perform a primary amplification comprising a few thermocycles with subsequent aliquoting of the pre-amplified sample into separate reaction tubes followed by a specific amplification.[1,2]

All three procedures pose severe disadvantages. The first approach is insufficient since DNA concentration in (usually unknown) samples is hardly controllable prior to analysis. The second approach of multiplexing makes high demands on assay design[3] and (in the case of real-time fluorescence detection) is limited to typically three to six reporter dyes due to spectral overlap. Finally, the pre-amplification approach is occasionally used in laboratories but requires opening of the reaction tube after the primary PCR for aliquoting the pre-amplified sample into new reaction tubes for secondary PCR. This PCR protocol is applied in a number of *nested PCR* formats like *consensus PCR*[4] or *tandem PCR*[5] because specificity and sensitivity are significantly improved. However, handling of pre-amplified (potentially high-copy) samples bears an extreme risk of DNA contamination and carry-over.[6] Therefore, protocols comprising a primary and secondary PCR are either banned from laboratories or may even require use of separate laboratory buildings for the primary PCR on the one hand and the secondary PCR on the other hand.[7]

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Though microfluidic integration of two successive DNA amplifications has been suggested before,[8,9] it has not been investigated so far. Therefore, the objective of this work is to demonstrate microfluidic implementation of a fully integrated and self-contained laboratory protocol including the following steps: specific primary PCR, dilution of the pre-amplified sample by a secondary PCR mix, aliquoting into 14 reaction wells, and there, specific amplifications by secondary real-time PCR with pre-stored primers and probes. The function of the secondary PCR mix is to reduce concentrations of primary PCR primers and thereby to prevent undesired side reactions in the secondary PCR.

The laboratory protocol is integrated into a microfluidic foil disk that is operated in a slightly modified commercial thermocycler (Rotor-Gene 2000, Corbett Research Pty. Ltd., now Qiagen GmbH) allowing for centrifugal liquid control.[10] The system performance is mainly validated by processing samples of the Exfoliatin A gene from the methicillin-resistant *Staphylococcus aureus* (MRSA).

The experimental setup and particularly the disk design (Fig. 1a) emerge from our previously reported work.[11] Among the multiple options for liquid actuation in microfluidic assay implementations,[12] we selected centrifugal microfluidics[13–15] because it excellently allows to process discrete samples by centrifugal forces. The disk is circularly shaped and comprises two identical and independent microfluidic structures. Each structure has an inlet for the pre-amplification PCR mix and one for the diluting secondary PCR mix (Fig. 1b). Both inlet chambers are connected to a larger mixing chamber by 950 μm wide capillary siphon valves. This width prevents clogging of the channels due to bubble formation during thermocycling (Fig. S1, ESI†). A 380 μm wide siphon valve (operated at room temperature) follows together with an aliquoting structure comprising 14 metering chambers, 14 reaction wells and a large waste reservoir. Air displacement through liquid transport is achieved by a closed venting loop. An air vent which is covered by a hydrophobic filter membrane is provided in each structure in order to allow sufficient pressure balance during thermocycling.[11]

Disks are based on microstructured films[16] made of cyclic olefin polymer (COP) and fabricated as previously described[11] including microthermoforming by soft lithography, laser cutting, attachment of filter membranes onto the vent opening, dehydration of primers and probes in the outer reaction wells and finally sealing with pressure sensitive adhesive tapes (Methods, ESI†).

Additionally, the capillary siphon valves are coated locally with a hydrophilic surface agent (Vistex 111-50, Filmspecialities Inc.). The coating is usually applied as scratch-resistant anti-fog coating for goggles but proved feasible in our applications of microfluidics and biochemistry. Surface modification is applied prior to sealing and reagent pre-storage (Methods, ESI†). The static contact angle ($N = 10$) of the applied PCR dilution buffer on Vistex-coated COP was

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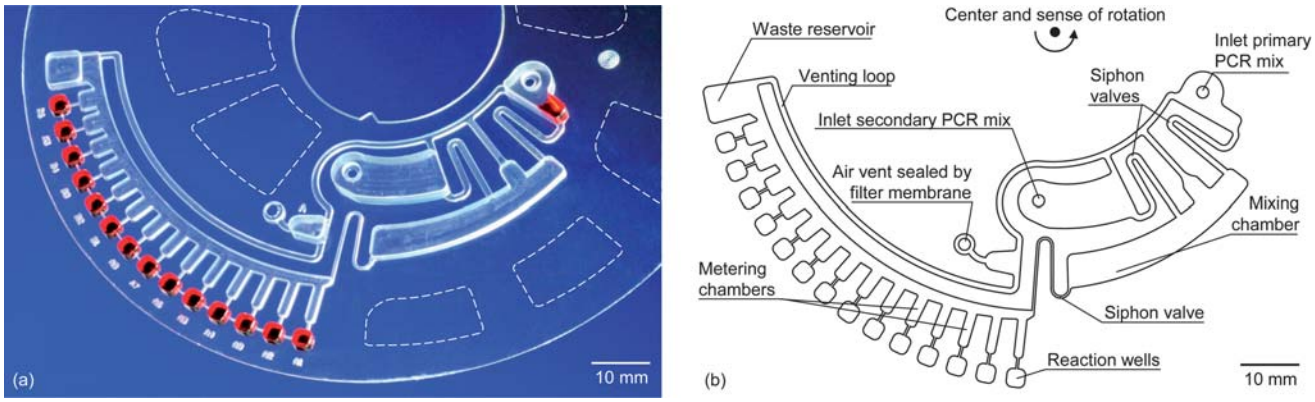

Fig. 1 Design of foil disk. (a) Photograph of one of two microfluidic structures on the foil disk. The chamber for primary PCR and reaction wells for secondary PCR are highlighted with dyed liquid. Dashed lines indicate through holes for sufficient heat convection during cycling. (b) Scheme of microfluidic elements in one segment.

19.3° ± 1.4° whereas the contact angle on untreated COP was 53.8° ± 1.1° (Fig. S2 and S3, ESI†).

Liquids are transported by capillary action and centrifugal forces that are induced through rotation of the disk in the modified thermocycling instrument.[11] Its modification refers to a custom-made chuck for the foil disks (Fig. S4, ESI†) and an additional relay to increase the rotational speed discretely from standard 6.6 Hz to 27.2 Hz.

In order to show technological feasibility of microfluidics and PCR implementation, we only employed one specific DNA target sequence for primary and secondary PCR and proved its parallel detection in all reaction wells (though a specific sequence would be detected in only one reaction well in regular genotyping applications). We chose Exfoliatin Toxin A, a sequence of a subtype of the methicillin-resistant *Staphylococcus aureus* (MRSA). The conditions for amplification by PCR are found in the Methods, ESI†.

Primary PCR was performed with primers that are slightly shorter than the primers of the secondary PCR. This implies lower annealing temperatures and thus inactivation of primary PCR primers during secondary PCR. The specific primers and the reporter dye were dehydrated[11] in 11 out of 14 wells. The remaining three wells contain primers and a respective probe corresponding to a different genetic sequence to obtain a base signal as well as negative controls.

Initially, the DNA sample is mixed with the primary PCR mix off-disk. Then, 20 µl of this mixture and 160 µl of the secondary PCR mix are filled into their respective chambers in the disk (Fig. 2a). The inlet holes are sealed by small adhesive tapes, and the disk is placed into the thermocycling instrument. Then the microfluidic protocol starts (Fig. 2b–h) and spins the disk at 27.7 Hz accompanied by thermocycling for primary PCR (Methods, ESI†). After ten cycles the fluid portions are transferred to the mixing chamber where the pre-amplified PCR mix is diluted with the secondary PCR mix in a unidirectional shake mode mixing protocol.[17] Afterwards the liquid is aliquoted into 14 separate reaction wells where it rehydrates the pre-stored primers and probes for specific secondary PCR and real-time detection (Fig. S5–S7, ESI†).

The microfluidic protocol was performed with 104 structures (comprising a total of 52 disks with two independent microfluidic structures per disk). 312 of 312 siphon valves worked well and primed in less than 5 seconds (950 µm wide siphons) and 35 seconds (380 µm siphons), respectively. Those siphon channels exposed to high temperatures during thermocycling were not clogged by gas bubbles due to their exceptional width.

The sensitivity of the system was tested with varying DNA concentrations in the range of 7 to 7000 copies *per sample*. The primary PCR was set to 10 thermocycles. In Fig. 3 the corresponding

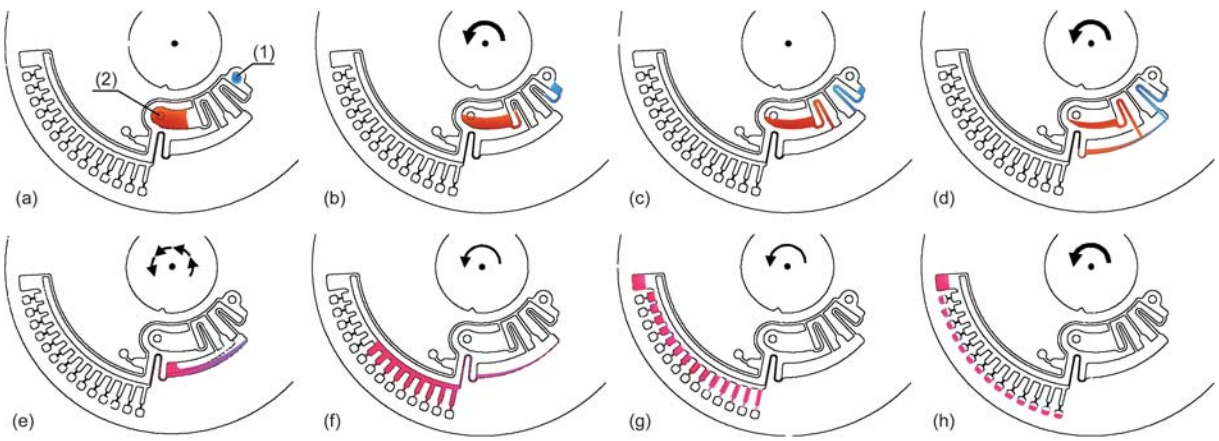

Fig. 2 Centrifugal microfluidic protocol. (a) Insertion of primary PCR mix (1) and secondary PCR mix (2). (b) Primary PCR. (c) Priming of siphons. (d) Transfer to adjacent chamber. (e) Shake mode mixing. (f) Distribution. (g) Metering of aliquots. (h) Filling of reaction wells and secondary PCR.

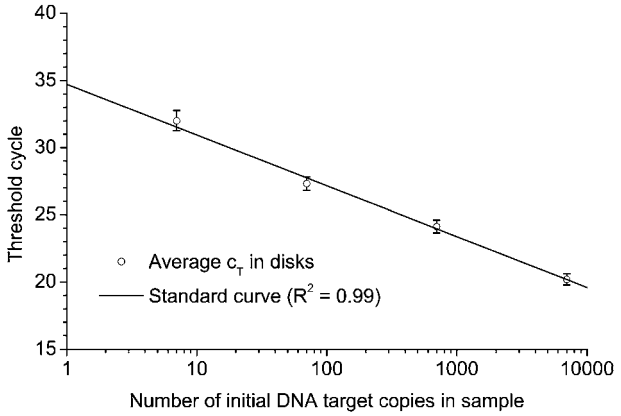

Fig. 3 Threshold cycles of the secondary PCR according to the DNA copy number in the respective sample. The 10 thermocycles of the primary PCR are not included. Data from 110 reactions on 5 disks. Error bars are standard deviations.

threshold cycles of the secondary PCR are depicted. Efficiency of amplification was 85%. The respective threshold cycles of reactions in different wells showed acceptably small standard deviations of up to 0.74 cycles. Negative controls did not generate any signals. It was possible to generate specific real-time signals from all reaction wells even if the sample contained only ~7 DNA copies in a total of 44 out of 44 reactions.

Pre-amplification stabilises the subsequent real-time PCR and leads to more homogenous fluorescence signals. Samples of DNA that were pre-amplified feature a considerably lower deviation in threshold cycles when compared to not pre-amplified samples (Fig. S8, ESI†).

Sometimes samples harbouring more than one target sequence must be analysed. Therefore, capability to conduct multiplex PCR during pre-amplification is an important criterion for the universality of the system. Feasibility of quadruplex primary PCR was shown in a separate experiment in which four target genes (ExfA, ExfB, TSST-1 and SCC*mec* type II) could successfully be detected during real-time secondary PCR (Fig. S9, ESI†).

Cross-contamination from well to well was not observed during all experiments which corresponds to our earlier studies.[11] Hence, the self-contained system drastically improves fail-safe sample processing. Opening of reaction vials after pre-amplification (containing critically high amounts of DNA) is fully circumvented by microfluidic automation and system integration into an existing laboratory instrument suitable for centrifugal microfluidics and thermocycling.

Both microfluidics and PCR assay are performed in a single disposable disk allowing reliable real-time detection and exhibiting extraordinary robust handling. Thus, the system provides prospect to convenient genotyping assays in the broad application fields of clinical pathogen detection, single nucleotide polymorphism (SNP) analyses, food analyses, forensics and any other genotyping assay that is based on real-time PCR or melt curve analysis.

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