AN INTEGRATED LAB-ON-A-CHIP SYSTEM WITH DNA EXTRACTION, PRE- AND MAIN PCR AMPLIFICATION FOR AUTOMATED DETECTION OF LOW CONCENTRATED PATHOGENS

G.Czilwik[1*], O. Strohmeier[1], I.Schwarz[1], N. Paust[1], S. Zehnle[1], F. von Stetten[1,2], R. Zengerle[1,2,3] and D. Mark[1]

[1] *HSG-IMIT - Institut für Mikro- und Informationstechnik, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*

[2]*Laboratory for MEMS Applications, IMTEK - Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*

[3]*BIOSS – Centre for Biological Signalling Studies, University of Freiburg, 79110 Freiburg, Germany*

ABSTRACT
We developed an automated Lab-on-a-Disk system "LabDisk", for a PCR-based, point-of-care sepsis test. The self-contained LabDisk features all necessary assay steps for pathogenic genotyping: Sample preparation, DNA eluate metering, subsequent PCR pre-amplification and final geometrically multiplexed real-time PCR. Conducting the test solely requires loading of a sample and liquid reagents. As a proof-of-principle, we demonstrated detection of 6.6×10^2 genome equivalents of *Staphylococcus aureus* within 4 hours. The parallel detection of a full neonatal sepsis panel in multiple reaction cavities is currently under development.

KEYWORDS: Microfluidic, Lab-on-a-Chip, LabDisk, DNA extraction, Polymerase-Chain-Reaction, pathogen detection

INTRODUCTION
For many infectious diseases, there is a growing demand for fast, small and easy-to-use diagnostic tests. E.g. for neonatal sepsis, delays during the identification of causative pathogens are one of the major reasons for unacceptably high mortality rates (35-70%) [1]. State-of-the-art technologies, such as time intensive blood culturing or the use of bulky molecular diagnostic workstations, are unsuitable for rapid point-of-care diagnostics. Fully automated Lab-on-a-Disk systems combine portability with fast turnaround times and enable untrained users to conduct complex medical tests. This could decrease reporting hold-ups in diagnosis of infectious diseases and help to apply pathogen-specific, narrow-spectrum antibiotics.

MATERIALS AND METHODS
The LabDisk was fabricated by the HSG-IMIT Lab-on-a-Chip Design- & Foundry-Service [2]. In short, 188 µm COP foils (COP ZF 14, Zeon Chemicals, USA) were microthermoformed [3]. The DNA extraction chambers (Figure 1, yellow) were hydrophobically coated using Teflon AF (DuPont, USA) in Fluorinert FC77 (3M, Belgium). The pre-amplification chamber (Figure 1, green) was loaded with lyophilized PCR beads (illustra mix RTG, GE Healthcare) after primers were previously dried-up directly in the same cavity (final concentration 0.5 µM). The LabDisk was finally sealed with a pressure sensitive adhesive foil (#900 320, HJ Bioanalytic, Germany). The liquid PCR buffer for the main amplification was previously prepared in a reaction tube.

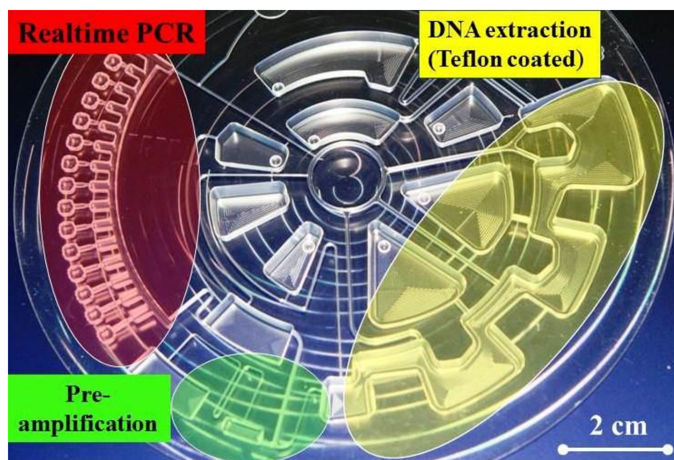

Figure 1 - LabDisk structure including DNA extraction (yellow), pre-amplification (green) and realtime PCR amplification (red) modules.

Figure 2 – LabDisk Player prototype enabling full process control (frequency, acceleration, thermocycling ,fluorescence read-out)

Processing of the LabDisk was performed in the LabDisk player (QIAGEN Lake Constance, Stockach, Germany), depicted in Figure 2. The portable device with a size of approximately 18 x 28 x 15 cm³ and a weight of approx. 2 kg features fluorescence detection (e.g. FAM, ROX, Cy5 etc.), PCR-thermocycling and the possibility to run predefined protocols with high flexibility on defined rotational frequencies (0 Hz - 90 Hz), accelerations (0.1 Hz s^{-1} – 50 Hz s^{-1}) and a precise positioning accuracy (< 0.1°).

EXPERIMENTAL

200 µL of a `model` sample (human serum spiked with 2.0 pg genomic DNA of *Staphylococcus aureus*, magnetic beads and Proteinase K) was pipetted into sample inlet chamber (Figure 3, i1). Binding buffer (450 µL), lysis buffer (350 µL), two washing buffers (490 µL each) and 195 µL of elution buffer (all from Instant MP extraction Kit, Analytik Jena, Germany) were loaded into the corresponding inlets (Figure 3, i2-i6). The automatic processing was started as described in Figure 3. DNA extraction was performed by subsequently transporting magnetic beads through the extraction buffers, using the automated magnetic beads transfer method described in [4]. After DNA elution, 135.0 µL of real-time PCR buffer was loaded (Figure 3, i7), constituting the only manual handling step besides initial extraction buffer and sample loading.

All additional processing steps (DNA eluate metering, pre-amplification, transfer of pre-amplification product to mixing chamber, mixing and aliquoting of reaction mix, realtime PCR) were carried in the LabDisk player.

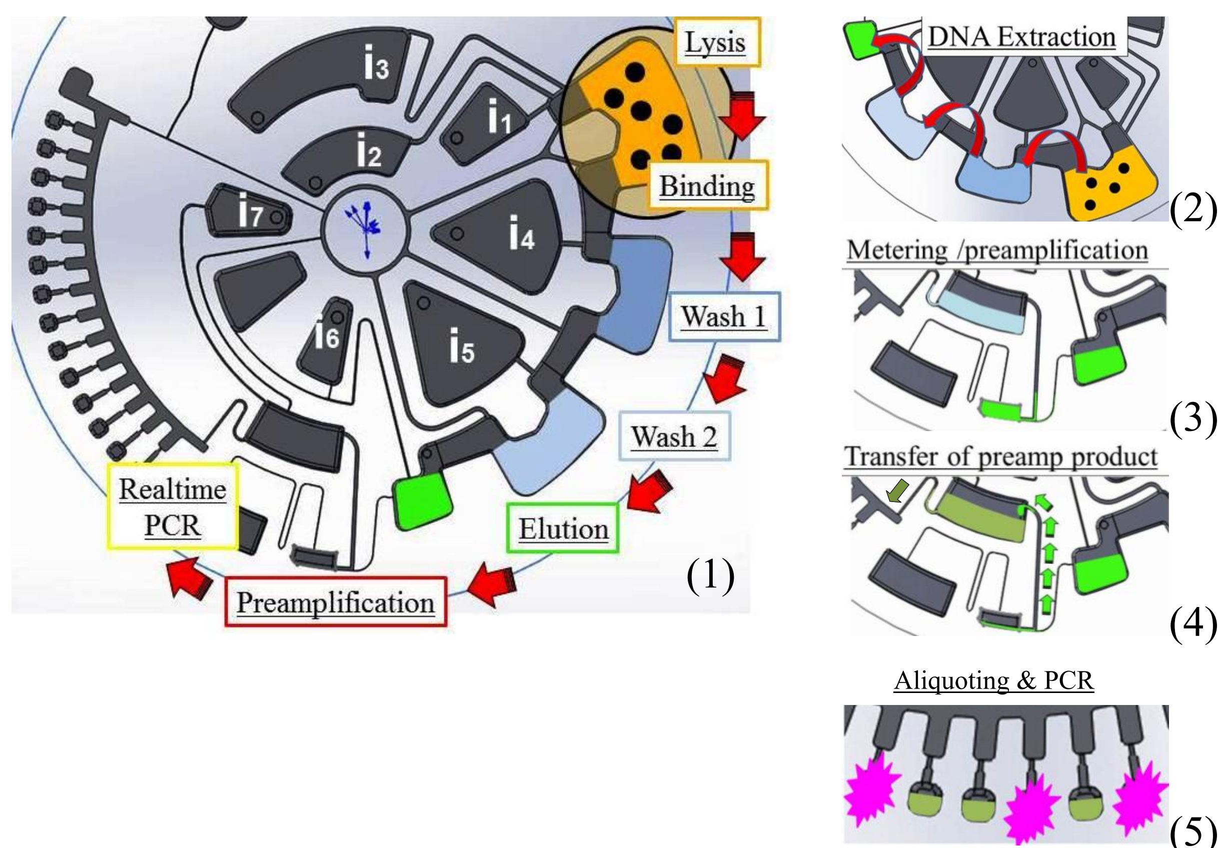

Figure 3 - Fluidic process-flow: (1) Sample with magnetic beads, lysis-, binding-, elution- and washing buffers are pipetted into inlets i1-i6. Then the automated protocol starts: Reagents are pumped radially outwards by centrifugation. (2) Cells are lysed, beads bind the DNA and are transported through the washing buffers into the elution buffer by magnetic actuation. 135.0 µl of PCR buffer for real-time amplification are loaded at inlet i7. (3) 40 µL of the DNA eluate are metered and transferred into the pre-amplification chamber. Amplification is performed by applying 12 thermocycles (15 s, 95 °C; 60 s, 52°C; 30 s, 70°C) at a constant rotational frequency of 30 Hz. (4) The pre-amplification product is transferred to a mixing chamber by pneumatic action [5] at 7 Hz and 60°C. Here, the pre-amplification product is mixed with the main amplification PCR buffer, which was pumped into the mixing structure by centrifugal force during the pre-amplification step. (5) The solution is finally aliquoted into the final reaction chambers (10 µL aliquots) and thermocycling with fluorescent real-time readout is performed.

RESULTS AND DISCUSSION

For proof-of-principle demonstration of the automated workflow, 6.6 x 10^2 genome equivalents of *S. aureus* were successfully detected in approximately 3 ¾ hours (~ 35 min. lysis and DNA extraction; ~ 45 min. pre-amplification; ~ 150 min. realtime PCR amplification). Figure 4 shows the detection signals for the first eight real-time PCR cavities. The signals for the remaining cavities were not acquired.

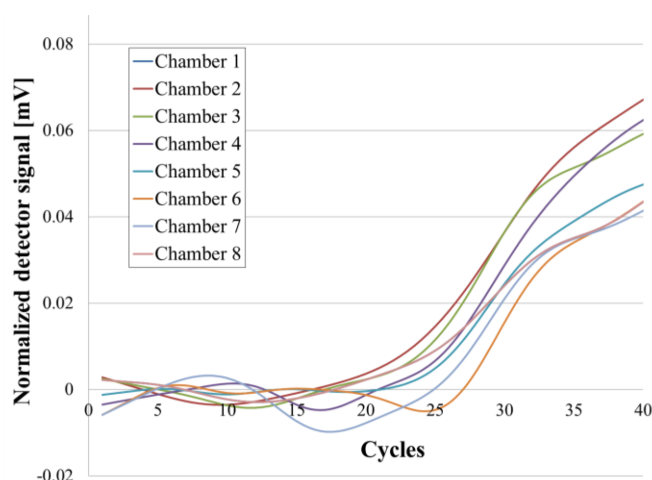

Figure 4 – Result of sample-to-answer analysis showing successful amplification of a gyrase B gene sequence of Staphylococcus aureus using the LabDisk Player. The signals were acquired after the annealing step in the PCR chambers.

CONCLUSION AND OUTLOOK

We demonstrated DNA-based sample-to-answer pathogen detection of *S. aureus*. Small dimensions of the LabDisk Player and the self-contained LabDisk design ideally meet the requirements of point-of-care testing, where a high degree in automation is required. In future work, the system will be equipped with additional primer sets in the amplification chambers for detection of a full panel of sepsis associated pathogens. Furthermore, by prestorage of liquid reagents in stickpacks [6], removal of all manual handling steps (except loading of the sample) will be targeted on. Testing with ´real´ clinical samples will assess the clinical utility of this device for sepsis diagnostics.

ACKNOWLEDGEMENTS

The results are part of the ASCMicroPlat project, funded by the European Union FP 7 (Grant Agreement 258759).

REFERENCES

[1] Vergnano, Sharland M, Kazembe P, and Mwansambo C. *"Neonatal sepsis: an international perspective."* Arch Dis Child Fetal Neonatal Ed, 90, 220-224, **(2005)**

[2] HSG-IMIT Lab-on-a-Chip Design- & Foundry Service; http://www.loac-hsg-imit.de/

[3] M. Focke, D. Kosse, D. Al-Barmani, S. Lutz, C. Müller, H. Reinecke, R. Zengerle and F. von Stetten, „*Microthermoforming of microfluidic substrates by soft lithography (µTSL): optimization using design of experiments*", JMM, 21, 11502, **(2011)**

[4] O. Strohmeier, A. Emperle, G. Roth, D. Mark, R. Zengerle, F. von Stetten *"Centrifugal gas-phase transition magnetophoresis (GTM) – a generic method for automation of magnetic bead based assays on the centrifugal microfluidic platform and application to DNA purification"* Lab On A Chip, 13, 146 – 155, **(2013)**

[5] S. Zehnle, F. Schwemmer, G. Roth, F. von Stetten, R. Zengerle, N. Paust **"***Centrifugo-dynamic inward pumping of liquids on a centrifugal microfluidic platform"* Lab on a Chip, 12, 5142 – 5145 **(2012)**

[6] T. van Oordt, Y. Barb, R. Zengerle and F. von Stetten*, "Miniature Stick-Packaging – An industrial technology for pre-strorage and release of reagents in Lab-on-a-Chip systems"*, Proc. of µTAS, 437-439, **(2011)**

CONTACT

*G. Czilwik, tel: +49-761-20373231; gregor.czilwik@hsg-imit.de