

AUTOMATED SAMPLE-TO-ANSWER NUCLEIC ACID TESTING WITH FREQUENCY CONTROLLED REAGENT RELEASE FROM CARTRIDGE INTEGRATED STICKPACKS

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ABSTRACT

For the first time we demonstrate an automated centrifugal Lab-on-a-Disk system for sample-to-answer point-of-care testing of multiple nucleic acid targets that features pre-storage of all required liquid reagents for nucleic acid extraction as well as primers and probes and magnetic beads. Highly wetting and thus hardly controllable liquid buffers were pre-stored in stickpacks with frequency controlled on-demand reagent release enabling automated addition of binding buffer after sample lysis. The self-contained Lab-on-a-Disk system automates all necessary assay steps for PCR-based pathogen detection: RNA extraction, aliquoting of the RNA and geometrically multiplexed real-time RT-PCR. As a proof-of-principle, we demonstrated detection of as little as 15 plaque forming units (pfu) of RNA bacteriophage MS2 in a 200 μ L sample in 3.5 hours.

KEYWORDS

LabDisk; centrifugal microfluidics; Lab-on-a-Chip; Nucleic acid testing; Point-of-care; Sample-to-answer; Nucleic acid detection; Polymerase-Chain-Reaction; Point of care testing (POCT); short turnaround time (STAT); Pathogen detection; infectious diseases

INTRODUCTION

There is a growing demand for fast, small and easy-to-use diagnostic tests for infectious diseases e.g. respiratory tract infections - the third most common cause of death by disease or injury. State-of-the-art analysis in central laboratories often leads to turnaround times in the order of many hours up to days due to shipping of the sample. Thus, a fast and affordable solution for the automated point-of-care diagnosis has a high benefit for patient safety. The Lab-on-a-Chip [1,2] development is encouraged to automate and integrate new assays and facilitating ease of use for fast diagnostics in laboratories [3] or at the point of care[4].

The fully automated Lab-on-a-Disk (LabDisk) system (Figure 1) combines reagent prestorage and portability with fast turnaround times. It enables untrained users to conduct complex medical tests. This could decrease diagnostic delays in medical practice and help to apply pathogen-specific drugs.



Figure 1: The LabDisk platform (former fluidic layout) with a portable point-of-care prototype LabDisk player for automation of all necessary assay and liquid handling steps.

MATERIAL AND METHODS

LabDisk fabrication

A 3D-CAD drawing (SolidWorks, Dassault Systèmes SolidWorks Corp., France) of the microfluidic structure is transferred to an ultra-precision milling machine (Kern EVO, KERN Microtechnik GmbH, Germany) to process a 6.0 mm thick polymethylmethacrylate (PMMA) master. The PMMA master is cast with polydimethylsiloxane (PDMS) (Elastosil RT-607, Wacker Chemie AG, Germany) and cured at 80 °C for 2 hours. Subsequently the PDMS mold with the elevated (positive) microfluidic structures is post-cured at 200 °C for one hour. This procedure is done due to prevention of outgassing of remaining monomers of the PDMS during the following thermoforming process. Subsequently the PDMS mold is placed in a hot embossing machine (HEX01, Jenoptik AG, Germany) for fabrication of the LabDisk (diameter = 130 mm) by micro-thermoforming of 188 μ m thick cyclic olefin polymer foil (COP, ZF14, TOPAS Advanced Polymers GmbH, Germany). High transparency, mechanical and thermal stability as well as biological inertness are the beneficial properties approving COP as fabrication material for LabDisk production [5].

Liquid reagents for nucleic acid extraction are pre-stored in stickpacks [6] - aluminum pouches allowing long-term liquid storage and featuring frangible seals, which are opened due to liquid pressure under centrifugation.

Optimization of sealing parameters during stickpack fabrication (SBL50, Merz-Verpackungsmaschinen GmbH, Germany) enables protocol defined release of highly wetting binding buffer at $70 \text{ Hz} \pm 3 \text{ Hz}$ after the lysis procedure while all other liquid reagents are previously released at $50 \text{ Hz} \pm 3 \text{ Hz}$. Magnetic beads (Agrobiogen GmbH, Germany), primers and fluorescence probes (RealAccurate Quadruplex Respiratory qPCR panels, PathoFinder B.V., Netherlands) are mixed with trehalose (Carl Roth GmbH & Co. KG, Germany) as stabilizing agent (final trehalose concentration: 200 mM for magnetic beads, 56 mM for primer and fluorescence probes) and air-dried into the LabDisk for 12 h. The LabDisk was finally sealed with an adhesive foil (Polyolefin-foil, HJ-Bioanalytik GmbH, Germany) with an attached $0.2 \mu\text{m}$ pore size PTFE membrane filter (Merck Millipore KGaA, Germany) covering the venting hole.

Processing device

Processing was conducted in a prototype LabDisk player (Qiagen GmbH, Germany) featuring PCR-thermocycling, fluorescence detection and the possibility to run predefined centrifugal protocols. Due to the size and weight of the portable processing device ($178 \times 283 \times 150 \text{ mm}^3$; 2 kg) the system is suitable for use at the point-of-need.

Microfluidic layout

The microfluidic layout of the sample-to-answer LabDisk is shown and described in detail in Figure 2. The LabDisk consists of inlet chambers for sample and PCR buffers, space-saving overlapping stickpack chambers, the nucleic acid magnetic bead extraction structure, fluid transfer channels, the aliquoting structure and the reaction cavities. The stickpack chambers possess the maximum structure height of 3.55 mm.

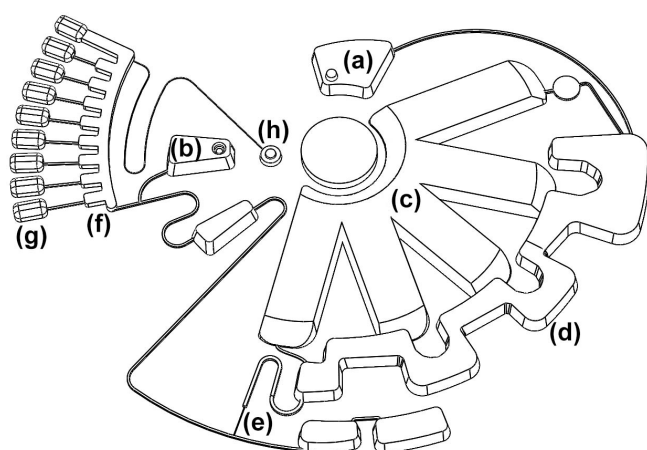


Figure 2: CAD drawing of the microfluidic structure for sample-to-answer nucleic acid detection consisting of inlet chambers for the $200 \mu\text{l}$ sample (a) and $100 \mu\text{l}$ RT-qPCR-buffer (b). Liquid reagents are prestored in stickpacks and placed into the stickpack chambers (c) which are connected

to the nucleic acid extraction chamber (d) where the magnetic beads are transferred through the different extraction buffers after frequency induced release of the extraction buffers. The channels and pressure chambers of section (e) are used for inward pumping of the eluate into the aliquoting structure (f) and subsequently to the reaction cavities (g). The venting system (h) is covered by a membrane providing aerosol dissemination and ensuring the self-containment of the LabDisk.

Microfluidic process flow

The microfluidic process flow consisting of a defined sequence of different rotation frequencies is illustrated and described in detail in Figure 3.

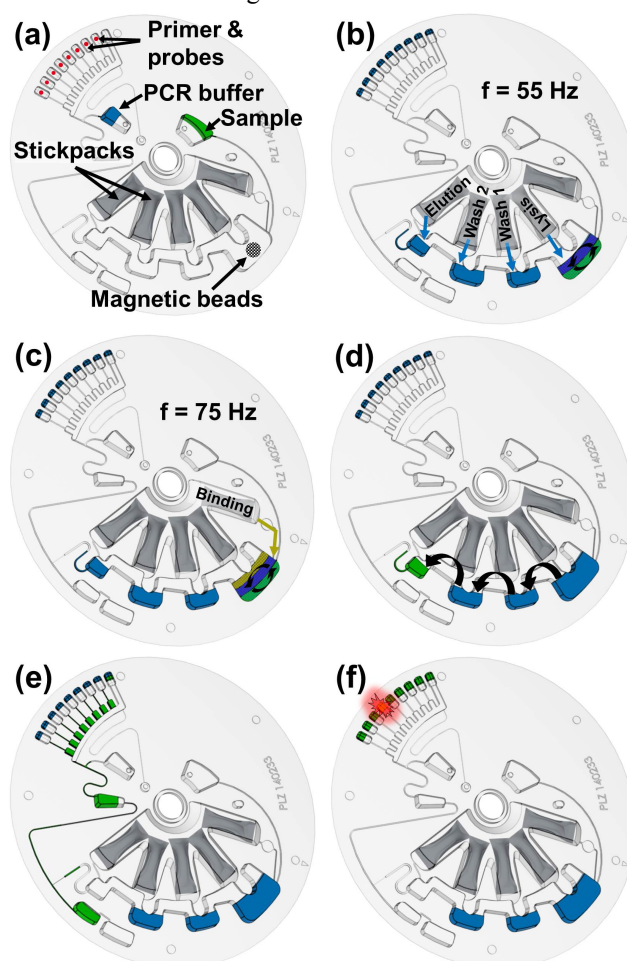


Figure 3: Fluidic process-flow: (a) The $200 \mu\text{l}$ sample and $100 \mu\text{l}$ RT-qPCR buffer are pipetted into the LabDisk. Then, the automated protocol starts: Sample and reagents are pumped radially outwards by centrifugation. (b) At the frequency of 55 Hz the RNA extraction buffers (except the binding buffer) are released out of the stickpacks, magnetic beads gets rehydrated and sample lysis is started while mixing through frequency alteration between 2 Hz and 14 Hz. Also, the RT-qPCR buffer is aliquoted à $10 \mu\text{l}$ into the final reaction cavities, rehydrating the primer and fluorescence probes. (c) Binding buffer is added to the lysed sample at a frequency of 75 Hz enabling RNA binding to the

magnetic beads. (d) Magnetic beads are transferred through the washing buffers into the elution buffer by magnetic actuation [7]. (e) 120 μL of the RNA eluate is transferred and aliquoted to 10 μL into the reaction cavities where the aliquots are combined with RT-qPCR buffer to the final reaction volume of 20 μL per reaction cavity. Subsequently, the RT (10 min @ 50 °C) and 45 qPCR thermocycles (20 sec @ 95 °C; 45 sec @ 60 °C) with fluorescence signal readout are performed.

EXPERIMENTAL

200 μL of a model sample (RNA bacteriophage MS2, 75 pfu/ml) and 100 μL of qScript One-Step RT-qPCR buffer (Quanta BioSciences, Inc., USA) were pipetted into the corresponding inlet chambers. After pipetting the inlet holes are sealed with adhesive foil. The automatic processing was started as described in Figure 3. Sample lysis is followed by RNA extraction which was performed by subsequently transferring magnetic beads through the extraction buffers, using the automated magnetic beads transfer method [7]. After RNA elution, 120 μL of eluate was transferred radially inwards [8] into the reaction cavities (10 μL each) which are already filled with RT-qPCR buffer (10 μL each) as well as initially air-dried and by RT-qPCR buffer already rehydrated primers and fluorescence probes. Finally the RT-qPCR starts with the RT step holding the temperature at 50 °C for 10 minutes. Subsequently the 45 PCR thermocycles with denaturation at 95 °C for 20 seconds and annealing and extensions step at 60 °C for 45 seconds with fluorescent signal readout per thermocycle are performed. Within the initial experiments each reaction cavity of the LabDisks was provided with the same primers and fluorescence probes (RealAccurate Quadruplex Influenza qPCR panel, PathoFinder B.V., Netherlands) so that a positive signal is expected in each reaction cavity due to the fact that the RNA bacteriophage MS2 acts as positive control for the Influenza qPCR panel. For the secondary experimental procedure each reaction cavity of the LabDisk was provided with different primers and fluorescence probes from the RealAccurate Quadruplex Respiratory qPCR panels (PathoFinder B.V., Netherlands). Internal control primers and fluorescence probes are missing in two of the panels. Thus, only six of the eight reaction cavities contained primers and fluorescence probes for the internal control so that a positive signal is expected in six and respectively a negative signal in two of the cavities.

RESULTS

For demonstration of the automated workflow, 15 pfu of RNA bacteriophage MS2 initially contained in the 200 μL sample were successfully detected in approximately 3.5 hours. Sample lysis, nucleic acid extraction and eluate transfer is finished after less than 60 minutes. The RT-qPCR procedure lasts approximately 150 minutes. Figure 4 illustrates the positive fluorescence detection signals of the real-time RT-qPCR of one of the experiments with the equal primers and fluorescence probes in each reaction cavity.

Figure 5 illustrates the positive fluorescence detection signals of the real-time RT-qPCR with different primers and fluorescence probes in each reaction cavity.

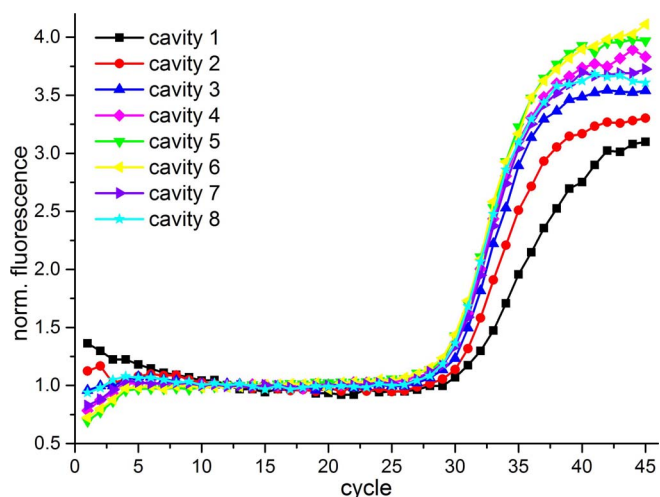


Figure 4: Fluorescence signal for parallel RT-qPCR reactions acquired after the annealing step in each reaction cavity. Each cavity contained equal primers and fluorescence probe so that the positive signals were expected in each cavity.

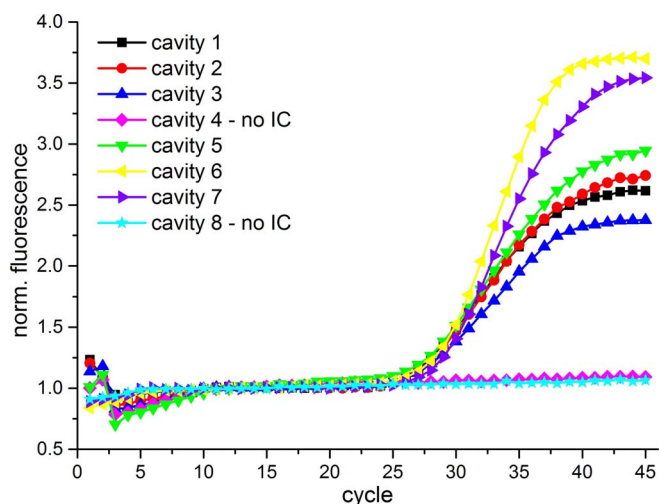


Figure 5: Fluorescence signal for parallel RT-qPCR reactions acquired after the annealing step in each reaction cavity. Except reaction cavity 4 and 8 each cavity contained primers and fluorescence probe also detecting RNA bacteriophage MS2 as internal control. Missing primer and fluorescence probes for internal control led to the negative signal in these two reaction cavities.

The six positive signals of the cavities containing primers and fluorescence probes also for the internal control and the two negative signals of the cavities with nonexistent primers and probes for the internal control are demonstrating the success of the experiments.

CONCLUSION AND OUTLOOK

We demonstrated RNA-based sample-to-answer detection of RNA bacteriophage MS2. The portable LabDisk Player and the self-contained LabDisk cartridge ideally meet the requirements of point-of-care testing, where a high degree of automation is required. In future work, the system will be equipped with additional primers and fluorescence probes in the amplification cavities for multiplex detection of panels with up to 26 nucleic acid targets, e.g. respiratory pathogens and validated with clinical patient samples. Furthermore the addition of liquid PCR mastermix by the operator will be circumvented by prestorage of lyophilized PCR beads. This way addition of the sample will remain as the only manual handling step.

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