

MICROFLUIDIC APP FEATURING NESTED PCR FOR FORENSIC SCREENING ASSAY ON OFF-THE-SHELF THERMOCYCLER

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ABSTRACT

Microfluidic Apps are a novel class of microfluidic chips designed to inbuilt process automation into standard laboratory instruments [1]. This approach circumvents costly robotic liquid handling systems. We demonstrate a new *Microfluidic App* for automation of forensic screening assays using an off-the-shelf centrifugal real-time thermocycler. This instrument is used for nucleic acid testing by polymerase chain reaction (PCR). Compared to previous approaches, which enabled centrifugal microfluidic liquid handling by control of rotational frequency [2] our novel *App* excels by implementation of a multistep liquid handling protocol at constant rotational frequency using centrifugo-thermopneumatic actuation [3]. Automation of the forensic assay integrates the following steps into a microfluidic *LabDisk* segment referred to as *GeneSlice*: PCR pre-amplification of the DNA-sample and a no template-control, followed by aliquoting of the pre-amplified DNA into 15 reaction wells ($5.1 \pm 0.4 \mu\text{l}$, CV 6.9 %, N=4), in which amplification by nested PCR including internal positive- and extraction-controls with subsequent melting curve analysis is performed. Discrimination of human-, dog-, and red deer-DNA could be successfully demonstrated. The novel *Microfluidic App* enables process automation at low cost and avoids the risk of cross-contaminations typically found in non-integrated nested PCR analyses.

KEYWORDS: Microfluidic App, centrifugal microfluidics, forensics, nucleic acid testing, real-time PCR, thermocycler

INTRODUCTION

Forensic DNA samples often contain low and/or limited amounts of DNA, which is challenging when a variety of targets have to be detected, especially if multiplexing is not possible. Regarding species determination, nested PCR can solve this problem by combining a universal pre-amplification (PA) prior to specific main-amplification (MA) [4]. Multiple required manual handling steps make nested PCR in forensics susceptible to contamination. A self-contained microfluidic integration of nested PCR steps highly reduces this risk.

For the first time, we present a suitable automation as *Microfluidic App* that can be operated on an unmodified thermocycler (Rotor-Gene Q, QIAGEN GmbH, Hilden, Germany) enabling implementation into any laboratory (Figure 1) [2]. We have greatly expanded a proof-of-concept [3] to actuate fluids at the low (400 rpm) and constant rotational frequency of the Rotor-Gene using centrifugo-thermopneumatic unit operations.



Figure 1: *Microfluidic Apps* for nested PCR feature low contamination risk in a self-contained *Lab-on-a-Chip* system, which can be processed in an unmodified standard laboratory real-time PCR thermocycler (Rotor-Gene Q, Qiagen, Germany). A special rotor holder enables insertion of four *GeneSlices* per run.

FUNCTIONAL PRINCIPLE

The fluidic design fits on a *LabDisk* segment referred to as *GeneSlice* (Figure 2). The *GeneSlice* is microthermoformed by the HSG-IMIT Lab-on-a-Chip Design- & Foundry Service (www.loac-hsg-imit.de/en/design-foundry-service) according to [5] and features two inlet/PA chambers for a DNA sample (100 μ l) and a no template control (NTC, 40 μ l), respectively. The PA is performed under constant rotation. After PA, rotation is halted allowing for capillary siphon valving. A subsequent MA run, which is automatically executed by a software macro, includes two temperature steps under constant rotation: Centrifugation splits volumes into 14 (sample) and 1 (NTC) times 5 μ l by filling metering fingers. Heating causes expansion of enclosed gas volumes inside downstream reaction wells. By subsequent cooling, the retained aliquots are sucked from the metering fingers into the reaction wells. Afterwards, the temperature cycling protocol for the MA and melting curve analysis is performed within the same run.

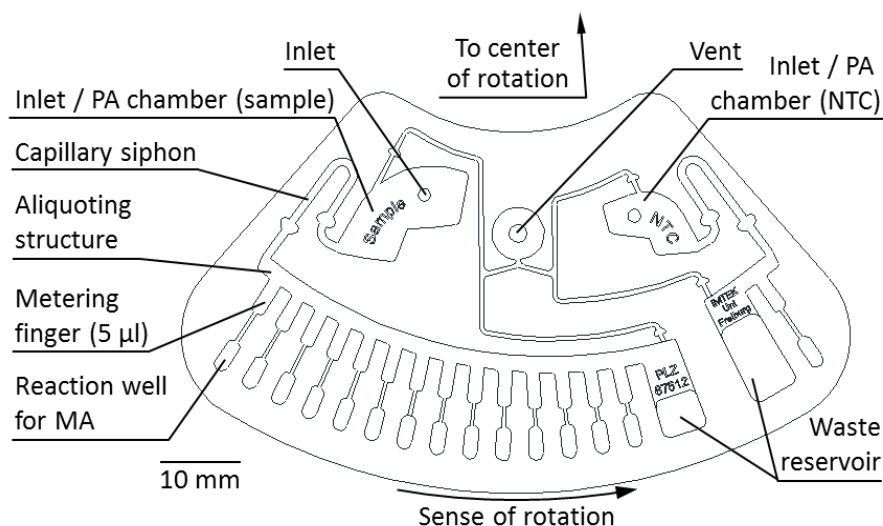


Figure 2: Schematic overview of *GeneSlice* design. The *GeneSlice* comprises two inlet chambers for pre-amplification (PA) of sample and no template control (NTC) respectively. Capillary siphon valves transfer liquids after PA into an aliquoting structure. Centrifugo-thermopneumatic aliquoting is achieved at constant and low (400 rpm) frequency. Reaction wells contain pre-stored primers for specific main amplification (MA), template and primers for internal positive control, and universal primers for extraction control in an alternating manner. Melting curve analysis is subsequently performed in same reaction wells.

EXPERIMENTAL

A real-time PCR based forensic screening assay for common European mammals and human [4] has been modified to run on *GeneSlices*. The universal PA (10 cycles) targets two gene fragments (*12S rRNA* and *cytochrome b*), and is followed by animal-family-specific MA (26 cycles). MA requires inactivation of PA primers, which is achieved by primer modification with inosine and C3-spacing blocking residual PA primers. Lyophilized PCR beads containing DNA polymerase, dNTPs and the intercalating dye EvaGreen (Jena Bioscience, Jena, Germany) were used together with universal primers (Biomers, Ulm, Germany) in the PA chamber. Reagents in the MA reaction wells (additional EvaGreen (Jena Bioscience), specific primers, template and primers for the internal positive control (IPC), and universal 12S rRNA primers as extraction control (EC)) were pre-stored.

Here, as a proof-of-concept, DNA from three targets (human, dog (*canidae*), 500 pg each; red deer (*cervidae*), 100 pg) as well as the synthetic IPC and EC have been detected in three *GeneSlice*-runs.

RESULTS AND DISCUSSION

Aliquoting has been optically quantified after processing in the Rotor-Gene: *GeneSlices* were transferred to a stroboscopic spin-stand and pictures were taken at 400 rpm. Images were post-analyzed via ImageJ (National Institutes of Health, MD, USA) optically measuring the filling height of each reaction well. Volumes were then modeled and calculated using SolidWorks prior to PCR tests. Aliquoting was found to be 5.1 ± 0.4 μ l (CV 6.9 %, N = 4) and thus comparable to aliquoting using variable frequencies [5]. The melting curve analyses of the PCR tests revealed a successful detection of all processed targets and controls by accordant peaks within corresponding temperature ranges (bins, see Figure 3).

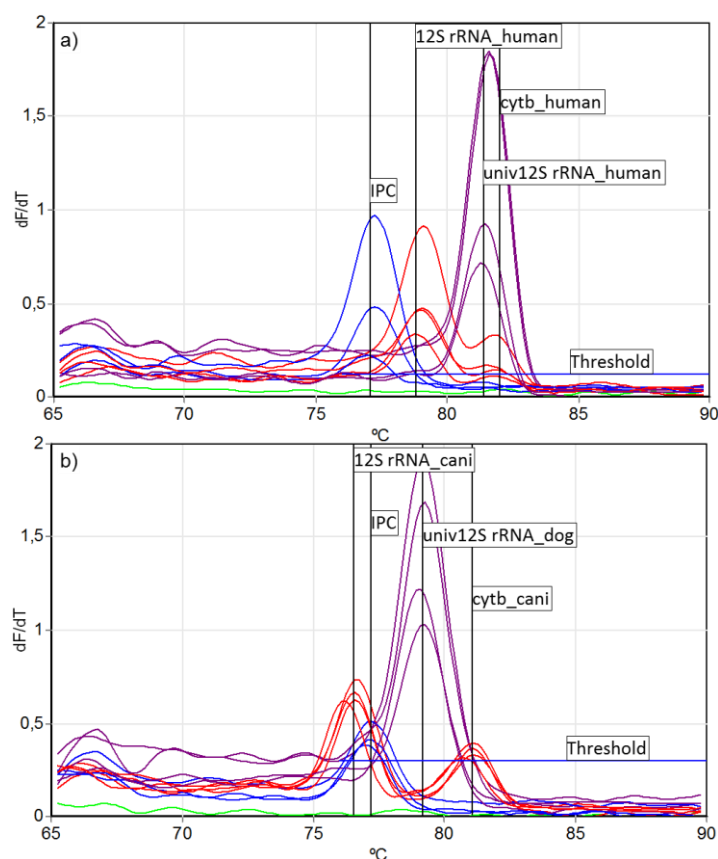


Figure 3: Melting curves (first derivative dF/dT of fluorescence signal) of specific amplicons (red) of four targets (temperature threshold for melting peak detection: $76.0\text{ }^{\circ}\text{C}$): human (a, dF/dT threshold: 0.12) and dog (b, canidae, dF/dT threshold: 0.30) together with extraction control (EC, purple), internal positive control (IPC, blue) and no template control (NTC, green). Red deer (cervidae) not shown. No cross-contaminations between wells and no peaks in NTCs are detectable.

CONCLUSION

A ready-to-use forensic *GeneSlice* as *Microfluidic App* for centrifugal real-time thermocyclers is introduced. It reduces the risk of contamination in forensic nested PCR analyses. The *GeneSlice* could be operated in any off-the-shelf Rotor-Gene and is ready-to-use by pre-stored reagents. Fluidic actuation is achieved by centrifugo-thermopneumatic unit operations applying regular capabilities of the Rotor-Gene, which comprise constant centrifugation at 400 rpm as well as heating and cooling. *Microfluidic Apps* for forensic analyses combine advantages of microfluidic automation with ease of integration into existing laboratory instruments and workflows.

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