AUTOMATED DNA PURIFICATION AND MULTIPLEXED LAMP ASSAY PREPARATION ON A CENTRIFUGAL MICROFLUIDIC "LAB-ON-A-DISC" PLATFORM

David J. Kinahan[1], Lourdes A.N. Julius[1], Cor Schoen[2], Tanja Dreo[3] and Jens Ducrée[1]
[1]Dublin City University, Dublin, IRELAND
[2]Wageningen University Research, Wageningen, THE NETHERLANDS
[3]National Institute of Biology, Ljubljana, SLOVENIA

ABSTRACT

This work presents a rotational-pulse actuated microfluidic cartridge enabling automated detection of plant pathogens on a compact device towards point-of-use monitoring of food safety. This highly integrated "Lab-on-a-Disc" (LoaD) system first runs the sample over a stationary phase of silica beads, followed by ethanol (EtOH) wash and final elution of DNA. The eluate is then homogenized using 'shake mode' agitation, accurately metered and then mixed with reagents for loop-mediated isothermal amplification (LAMP). We successfully purify plant DNA and demonstrate on-disc quantitative LAMP amplification.

INTRODUCTION

Centrifugal microfluidic systems [1] have been established as a preferred means for high-value and complex point-of use testing. The platform can enable greater complexity and sensitivity than typical paper-based [2] or lateral flow platforms while also offering significantly enhanced robustness and usability compared with conventional microfluidic lab-on-a-chip devices. A wide range of bioanalytical protocols have been automated in a sample-to-answer fashion for applications in biomedical point-of-care diagnostics [3], cell analysis [4], environmental monitoring and quality control of industrial processes.

The platform comprises of a disposable cartridge, typically of a similar geometry as optical data storage media (e.g. CD or DVD), which is rotated about its axis to generate a centrifugal field; the resulting pressure head pumps liquid from the centre of the disc to its periphery. With proper design, these discs can automate common Laboratory Unity Operations (LUOs) such as metering, mixing and washing.

The "Lab-on-a-Disc" (LoaD) platform offers numerous benefits for point-of-care or point-of-use applications. The innate capability of centrifugation supports particle separation, e.g. for blood-based bioanalytical testing [5]. The self-stabilising, rotational actuation by a simple spindle motor eliminates the need for costly precision pumps and their cumbersome and error-prone fluidic 'world-to-chip' interfaces based on physical connectors. The system can further be loaded by a common pipette, and operated while staying open to atmosphere.

As the centrifugal force acts on all liquid residing on the rotor, flow-control constitutes a key enabling technology for the LoaD platform. Typically, valving is categorized into rotational and externally actuated techniques. In the case of latter case, additional, instrument-based modules transfer energy to the rotor, e.g. via electromagnetic radiation, heat, gas pressure or mechanical setups.

However, the most ubiquitous valving class are the former, rotationally controlled valving schemes; these typically rely on the balance of the rotationally modulated hydrostatic pressure head and counteracting effects. Common, "low-pass" siphon valves are axially primed at reduced spin rate by the dominant axial capillary force. On the other hand, "high-pass" valves yield upon an increase of the spin rate, for instance common capillary burst and the centrifugo-pneumatic dissolvable film (DF) barriers [6,7,8] used in this work.

The rather poor fidelity of their burst frequency due to unavoidable manufacturing tolerances poses a serious challenge to these rotationally controlled valving architectures. The burst frequencies hence must be separated to ensure the correct sequence of LUOs. The practical limitation of the maximum spin rate thus restricts the number of sequential operations which can be concatenated on a single disc. Various strategy have been proposed to circumvent this constraint; for example, by combining high-pass capillary valves with low-pass siphoning.

In this paper we use 'digital pulse-actuated' dissolvable-film valves to automate nucleic acid purification and LAMP amplification on a custom instrument incorporating modules for heating and fluorescent detection (Figs. 1 and 2).

In this approach, 'digital-like' pulses in the spin frequency trigger each step of the protocol. Compared with implementation by conventional burst valves, the number of LUOs is only limited by the radial extension of the disc. Additionally, as the valves open at constant spin rate, thus notably lowering the hardware requirements.

Nucleic acid analysis on the LoaD systems has enormous potential, in particular for decentralised, on-site applications spanning fields such as cancer diagnostics and food quality monitoring. Related laboratory protocols typically require cell lysis, nucleic acid extraction, clean up to remove inhibitors, target amplification and detection.

There are many reports of the nucleic acid purification on the LoaD platform. Solid phase purification using silica [8,9] is almost ubiquitously used. For example, silica based solid phase extraction methods achieved about 45% DNA recovery from lysed blood [9].

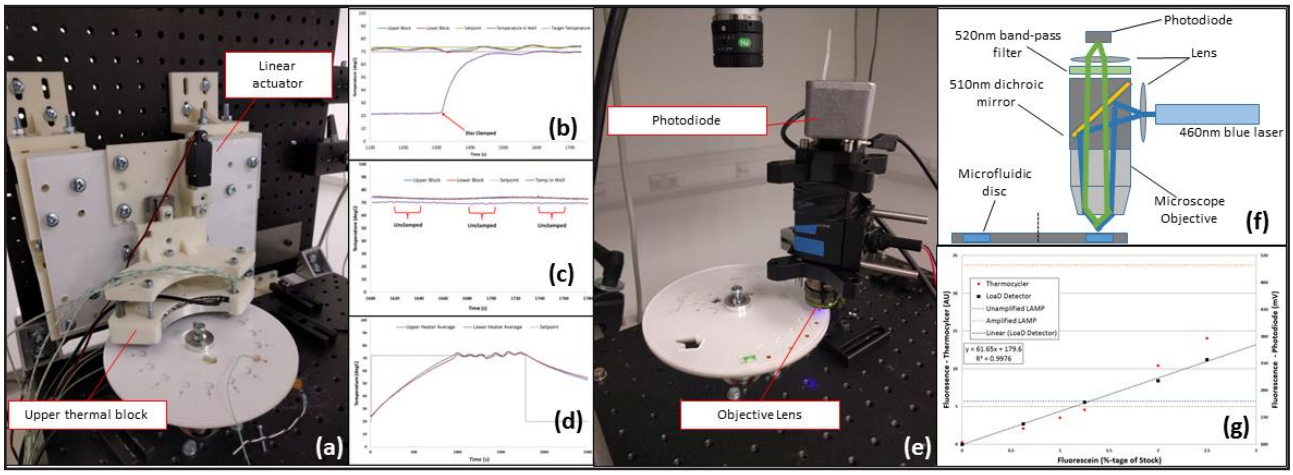

Figure 1: Instrumentation with (a) Heating module. (b) Warmup time measured inside disc (using micro-thermocouple) amounts to approximately 5 minutes. (c) Thermal stability inside read-chamber during fluorescence measurement (unclamp and rotate disc to detector). (d) Long-term heating, stability and cool-down of the heating blocks. (e) Fluorescent detection system, (f) detector configuration and (g) demonstration of system sensitivity. Graph shows serial dilution of fluorescein (measured on a commercial thermo-cycler and the LoaD based detector). The dashed lines show intensity of amplified and non-amplified LAMP samples measured on the LoaD based detector.

Along with purification, there have also been reports of LoaD systems for LAMP amplification. Indeed, many recent advances incorporate sample lysis, nucleic acid purification and subsequent isothermal nucleic acid amplification to identify specific markers. For example, the major food-borne pathogen *Salmonella enterica* was identified [10]. External heating via laser diode actuated the valves and the isothermal nucleic acid amplification method, recombinase polymerase amplification (RPA), was used for identification. Rather than a conventional fluorescent detection, the group used colour change on a lateral flow strip to indicate the presence of the pathogen.

In this work, we present an automated microfluidic LoaD system. Lysed plant pathogen samples are first purified and then mixed with five plant pathogen targets common to the tomato plant and one positive control (identifying the presence of tomato plant DNA)

MATERIALS AND METHODS

Disc Manufacture

The microfluidic cartridge (Fig. 2a) was assembled using multi-lamination methods from four layers of Poly(methyl methacrylate) (PMMA) and four layers of PSA (Pressure Sensitive Adhesive, Adhesives Research, Limerick, Ireland). Voids in the 86-µm thick PSA layers representing microchannels and other small features were defined by a commercial knife-cutter (Graphtec, Yokohama, Japan). Larger features such as reservoirs for reagent storage and sample collection were created in 1.5-mm thick PMMA layers using a laser cutter (Epilog Zing, USA).

These layers were aligned on a custom assembly jig. Between each alignment step, the attachment of layers was reinforced using a hot-roll laminator (Hot Roll Laminator, Chemsultant Int., US). Each DF (KC35, Aciello Corporation, Japan) was mounted on a PSA tab using previously described methods and then manually positioned within the disc during manufacture.

Experimental Test Stand

All discs in this study were imaged using a test and development tool commonly referred to as a "spin stand". Discs are mounted on a computer controlled motor (FESTO, Germany) and spun at user-defined rates (Fig. 1) using a custom control program (LabVIEW, National Instruments, USA). The discs were imaged by a stroboscopic light source (Drelloscop 3244, Drello, Germany) and a sensitive, short-exposure time camera (Baser Ace ac-2040u, Basler, Germany) in synchrony with the motor. Custom hardware results in acquisition rate of 5 frames per second.

In addition, a custom thermal heating unit (Fig. 1) heats the disc for LAMP amplification. Aluminium thermal blocks were machined and are fitted using thick-film resistor heaters. The temperature is set via PID control from a custom LabVIEW program. The thermal blocks are spring-mounted on positioning stages. For heating the LAMP reagents, the disc is stopped and the thermal blocks clamp the disc, using linear actuators (Firgelli L12-10-210-12-S, Active Robots, UK). Liquid inside the disc reach the temperature after approximately 5 minutes; due the favourable insulating of the PMMA, the disc be unclamped for up to a minute without a measurable decrease in temperature (Fig. 1b-d) to allow rapid re-positioning of the disc for fluorescent measurements.

Fluorescent detection was via a custom system (Fig. 1e-g) composed of a 10× objective lens, a dichroic mirror and band-pass filter (Edmund Optics, UK), a 450-nm laser diode (Egismos Technology Corporation, Taiwan) and photodiode (P/N S7686, Hamamatsu, UK).

To automate the protocol, the disc was spun at 30 Hz and then increased for 12 seconds to 50 Hz to open the rotational-pulse actuated valves. Mixing steps were implemented by rapidly cycling the spin rate, in 4-second intervals, from 30 Hz to 20 Hz. All acceleration and deceleration was implemented at 12.5 Hz s^{-1}. The full spin protocol is shown in Figure 2c.

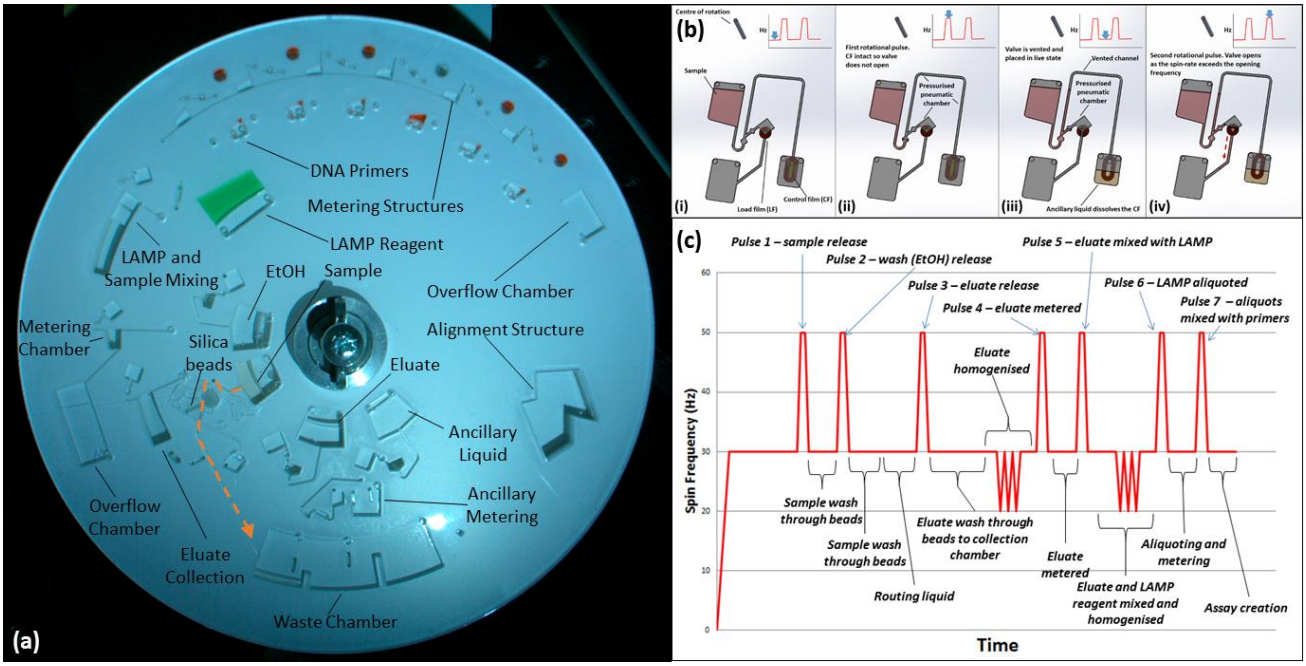

Figure 2: Operation of the disc. (a) Disc design with labelled reservoirs. (b) Function of the pulse actuated valves. (c) Spin protocol enabling disc operation. Each pulse can occur at an arbitrary time while disc geometry defines the liquid handling sequence.

Valve Function

The digital pulse valves [8] (Fig. 2b) represent a hybrid of previously introduced DF burst valves [6] and event-triggered valves [7]. The valves feature two DFs, a control film (CF) and a load film (LF). While the CF is still intact, the burst frequency is designed to clearly exceed maximum spin rate of the motor. However, dissolution of the CF vents the valve and therefore critically reduces the opening frequency of the valve (in this work to 40 Hz). Therefore, any 'digital pulses' which occur when the CF is intact will not open a valve, but if the CF is dissolved the valve will open at that 'digital pulse.

The disc architecture (Figure 2a) is defined such that liquid released from the first digital pulse will disintegrate the CF of any subsequent valve in the protocol. Therefore, each digital pulse is associated
with actuation of a valve. As the DFs typically take 30 seconds to fully dissolve, there is ample time to open a valve and then decelerate the disc to 30 Hz to prevent accidental triggering of subsequent steps. In addition, LUOs which require rapid changes in spin rate, such as mixing, can be implemented without actuating valves providing the spin rate does not exceed 40 Hz.

PURIFICATION AND AMPLIFICATION

We first characterized the DNA purification, using a 2 µg µl^{-1} DNA standard and obtained a DNA purification yield of 27.8% ± 4.9% (N = 3). To demonstrate our system, we first processed DNA (150 µl of G-Block DNA (at 2 µg µl^{-1}) from the common plant fungus Botrytis Cineria (Boty)) [11] entirely on-disc except performing the amplification off-disc in a commercial PCR thermocycler. The DNA was purified on-disc by washing through a packed column of acid washed beads, washing these beads with an organic liquid (200 µl of 75% ethanol EtOH),

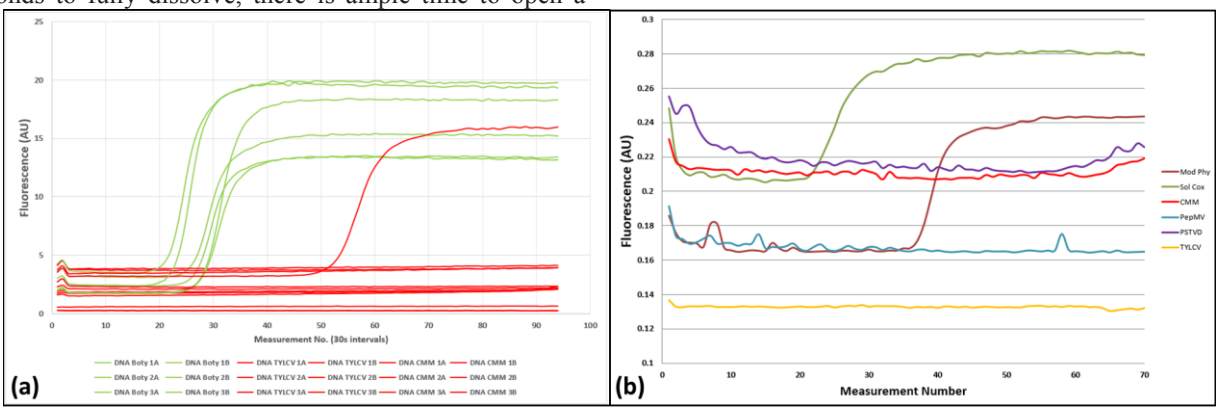

Figure 3: LAMP amplification curves. (a) Results from purifying Boty DNA (3 discs). Samples were pipetted off-disc and amplified on a commercial thermocycler. Expected amplification and non-amplification marked in green and red, respectively. A false positive was identified by benchtop testing as a result of reagent contamination. (b) Amplification curves obtained using the LAMP enabled spin stand screening SolCox DNA or NTCs against array of primers (n=1). Measurements are at 60-s intervals. SolCox amplification (in green) can be seen after approx. 20 minutes. Amplification of Mod Phy is a result of contamination.

and then eluting the DNA from the beads (using 100 µl buffer). Reagents used for these steps were taken from a widely used DNA purification kit (Qiagen QiaQuick, Qiagen, UK).

Next, the eluted DNA was homogenized through 'shake-mode' mixing and metered to 40 µl. The DNA was then transferred to the next chamber and mixed with 160 µl of LAMP reagent. This mixture of LAMP reagent and DNA was then distributed, on-disc, into six 15-µl aliquots.

As a final step, these aliquots were transported to reaction chambers pre-loaded with primers targeting common tomato plant pathogens Boty (n = 2), Tomato leaf curl virus (TYLCV) (n = 2) and bacteria *Clavibacter michiganensis (CMM)* (n = 2). The samples, which had been created entirely on disc, were then pipetted into microtubes and amplified using a benchtop thermocycler (Corbett RotorGene) (Fig. 3a).

Integrated purification, aliquoting and real-time monitoring of LAMP amplification was then demonstrated. Here, we loaded our disc with G-Block DNA for Cyto Chrome C oxidase (SolCox); a positive control for the tomato plant. The purified samples were then screened against a positive control (SolCox primers) and primers for five pathogen markers (CMM and TYLCV described above and additionally Pepino mosaic virus (PepMv), a ssRNA virus, and Potato spindle tuber viroid (PSTVd), and Phytophthora infestans (fungus) (Mod Phy). The presence of the Tomato DNA was successfully identified (Fig. 3b) while amplification in the Mod Phy channel was confirmed in benchtop experiments to be a result of contamination.

CONCLUSION

In this work, we present a disc architecture which can purify lysed DNA samples, mix them with LAMP reagent and perform a spatially multiplexed (6x) detection. The on-disc process is exclusively controlled by short "digital" pulses in the spin rate. In addition, we incorporated heating and fluorescent detection into an instrument, thus providing a fully automated solution.

This LoaD platform was designed for screening pathogens extracted from complex matrices such as, in this case, plant leaf. With this in mind, the lysis step remains 'off-disc' as the cartridge is therefore stays compatible with other common food matrices such as milk. However, it is also clear that this platform could be adapted, through the inclusion of further upstream preconditioning modules, to address automated nucleic acid based detection from other matrices such as blood.

ACKNOWLEDGEMENTS

This work was supported by the European Union (FP7-KBBE-2013-7-613908-DECATHLON), and Science Foundation Ireland (SFI) and Fraunhofer Gesellschaft under its Strategic Partnership Programme Grant Number 16/SPP/3321.

REFERENCES

[1] S Smith *et al., Micromachines*, 7(2), pp. 22, 2016.
[2] P Lisowski *et al., Chromatographia*, 76(19-20), pp.1201-1214, 2013.
[3] R Gorkin *et al., Lab on a Chip*, 10(14), pp.1758-1773, 2010.
[4] R Burger *et al., Current Opinion in Chemical Biology*, 16(3), pp.409-414, 2012.
[5] DJ Kinahan *et al., PloS one*, 11(5), p. e0155545, 2016.
[6] R Gorkin *et al., Lab on a Chip*, 12(16), pp.2894-2902, 2012.
[7] DJ Kinahan *et al.*, *Lab Chip*, 14 (13), pp 2249-2258, 2014.
[8] DJ Kinahan *et al.*, *Proc. µTAS 2014,* pp. 1437-1439.
[9] KR Jackson *et al., Analytica Chimica Acta*, *937*, pp.1-10, 2016.
[10] TH Kim *et al., Analytical Chemistry*, 86(8), pp.3841-3848, 2014.
[11] DWY Chung *et al.*, *Proc. µTAS 2016,* pp. 848-849.

CONTACT

*D.J. Kinahan; +353-1-700-6433; david.kinahan@dcu.ie
*J. Ducrée; +353-1-700-7658; jens.ducree@dcu.ie