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Plant pathogen detection on a lab-on-a-disc using solid-phase extraction and isothermal nucleic acid amplification enabled by digital pulse-actuated dissolvable film valves

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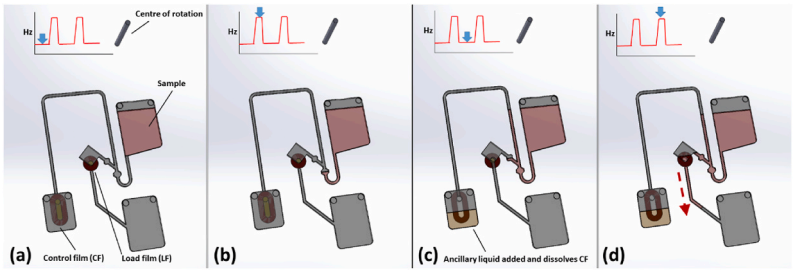
HIGHLIGHTS

- An novel mechanism of centrifugal flow control permitting valve actuation by 'digital pulses' in disc-spin rate.
- Digital Pulses determine when valves open and disc architecture determines the sequence.
- Functional demonstration with complex multi-step assay including solid-phase DNA purification and on-disc LAMP amplification.

GRAPHICAL ABSTRACT



ABSTRACT

By virtue of its ruggedness, portability, rapid processing times, and ease-of-use, academic and commercial interest in centrifugal microfluidic systems has soared over the last decade. A key advantage of the LoaD platform is the ability to automate laboratory unit operations (LUOs) (mixing, metering, washing etc.) to support direct translation of 'on-bench' assays to 'on-chip'. Additionally, the LoaD requires just a low-cost spindle motor rather than specialized and expensive microfluidic pumps. Furthermore, when flow control (valves) is implemented through purely rotational changes in this same spindle motor (rather than using additional support instrumentation), the LoaD offers the potential to be a truly portable, low-cost and accessible platform. Current rotationally controlled valves are typically opened by sequentially increasing the disc spin-rate to a specific opening frequency. However, due lack of manufacturing fidelity these specific opening frequencies are better described as spin frequency 'bands'. With low-cost motors typically having a maximum spin-rate of 6000 rpm (100 Hz), using this 'analogue' approach places a limitation on the number of valves, which can be serially actuated thus limiting the number of LUOs that can be automated. In this work, a novel flow control scheme is presented where the sequence of valve actuation is determined by architecture of the disc while its timing is governed by freely programmable 'digital' pulses in its spin profile. This paradigm shift to 'digital' flow control enables automation of multi-step assays with high reliability, with full temporal control, and with the number of LUOs theoretically only limited by available space on the disc. We first describe the operational principle of these valves followed by a demonstration of the

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capability of these valves to automate complex assays by screening tomato leaf samples against plant pathogens. Reagents and lysed sample are loaded on-disc and then, in a fully autonomous fashion using only spindle-motor control, the complete assay is automated. Amplification and fluorescent acquisition take place on a custom spin-stand enabling the generation of real-time LAMP amplification curves using custom software. To prevent environmental contamination, the entire discs are sealed from atmosphere following loading with internal venting channels permitting easy movement of liquids about the disc. The disc was successfully used to detect the presence of thermally inactivated *Clavibacter michiganensis. Michiganensis (CMM)* bacterial pathogen on tomato leaf samples.

1. Introduction

There is a need for point-of-need/point of care systems for rapid and accurate detection of pathogens in humans, animals, and plants [1–7]. Point-of-care (human) diagnostic testing can offer results faster than a centralised hospital lab (e.g. for sepsis and meningitis) [8,9], can provide results where testing infrastructure is not well developed (e.g. neglected tropical diseases) [10], and can support infectious disease management and suppression strategies (e.g. COVID-19) [11,12]. Furthermore, food safety monitoring can prevent foodborne disease and limit foot waste [13].

Climate change is expected to result in outbreaks of plant pathogens which, if not timely managed, may result in famine and undermine global food security [11–13]. Historically, such famines have included potato blight in Ireland in the 1840s and the Great Bengal Famine of 1943 [13]. Famine is particularly likely to occur when subsistence farmers rely on a monoculture. Increased globalisation of agriculture, where plants are grown where they are unlikely to have evolved resistance to local pathogens, is also a concern. Today, approximately 20% of pre-harvest annual crop is lost to plant pathogens while an additional 10% is lost post-harvest [14]. Furthermore, there is increasing pesticide resistance emerging among plant pathogens [15]. The ability to detect plant pathogens at early stages of an outbreak can support farmers in choosing the best control strategies [16].

This can include providing a farmer on-site information to decide if tailored pesticide use (which may prevent future pesticide resistance) or culling of a crop is the best option.

A technology, which has emerged as particularly beneficial for automating point-of-use tests, and particularly those using nucleic acid methods, is Lab-on-a-Chip. The ability for automation of lab processes has been well demonstrated by the scientific community and, despite some challenges, is increasingly available as a commercial technology [17]. Indeed, microfluidics and Lab-on-a-Chip has been increasingly applied to applications in agriculture, food safety, and plants [7,18–20]. This has included diagnostics but also an increasing interest in a soil-on-a-chip/plant-on-a-chip approach for more holistic analysis [21,22]. Focussing on diagnostics, a variety of different approaches have been implemented in microfluidics chips including ELISA assays [23], microarrays [24,25], colorimetric detection [26,27], absorbance detection [28], electrochemical detection [29], and nucleic acid methods [30,31].

One microfluidic platform that has great potential for applications at point-of-care and point-of-use is the lab-on-a-disc (LoaD) [32–35]. Here, microfluidic cartridges are similar to CDs and DVDs and are rotated about their axis to induce a (centrifugal) pumping force on the liquid. Key advantages include low-cost instrumentation using a spindle motor rather than specialized pumps, enhanced sterility through disposable microfluidic chips which can be completely sealed from the surrounding environment, samples and reagents not contacting/contaminating any part of the supporting instrumentation, inherent centrifugation to support sample preparation, and easy loading (world-to-chip interfacing) through pipetting or directly loading a sample into the disc [36,37]. Perhaps even more important, the LoaD has an unparalleled ability transfer laboratory protocols from ‘on bench’ to ‘on chip’ using ‘unit operations’ [34], such as mixing, metering, and aliquoting. This greatly reduces the cost overhead related to bioassay optimisation during Lab-on-a-Chip assay development.

However, a disadvantage of the Lab-on-a-Disc platform is all liquid on the disc is subject to the same centrifugal pumping force; hence the need to implement flow-control using valves. In their simplest form, these valves are often local narrowing or widening of microfluidic channels to use the capillary force as a valve [38,39]. The capillary force prevents the liquid flowing through the valve; by increasing the speed the disc rotates (and thus the body forces acting on the liquid), the liquid can be forced through the valve. Another simple valving scheme uses siphon valves which actuate by slowing down the disc [40] and combining capillary valves with siphon valves has also been demonstrated [41]. However, for complex multi-step assays, these ‘open channel’ valves are not fit for purpose due to their dependence of both high-fidelity manufacturing and the interfacial tension between the liquid and disc material; they are often unreliable and rarely are more than 4 discrete valving steps automated [42]. This has led to development of more advanced rotationally actuated valves, some of which have been used to great effect, including centrifuge-pneumatic siphon valves [43,44], burstable foils [45], deformable membranes [46], and dissolvable films [47].

In an alternative approach, many researchers have developed ‘instrument supported’ valving schemes where supporting instrumentation is used to open valves on the LoaD platform [48]. These approaches have included connection of the chips to external pumps [49], use of IR light sources to melt or ablate membranes/phase change materials/channel walls [50–53], physical manipulation [54–56], secondary rotation of the chip [57], co-rotating wireless electronics [58], and magnetic interaction [59]. In addition, discs are often simply manually loaded step-by-step and indeed of late there is a trend towards interfacing the LoaD with liquid handling robotic systems. The greatest concern with ‘instrument supported’ LoaD platforms is their ruggedness and portability for point-of-use/in the field applications such as plant-pathogen detection. Table 1 compares some of the key technologies with the DF valves.

The approach of embedding dissolvable films (DFs) [47] in a LoaD for rotationally actuated valving is based on placing the DF in a recessed gas/air pocket. Increasing the disc spin-rate resulted in liquid pushing into the air pocket against the trapped gas; when the disc reached a critical spin frequency the liquid contacts the DF, dissolves it, and lets liquid pass through the valve. The entry of liquid into the gas pocket is easily predicted using Boyle’s law and so the actuation frequencies can be easily tuned [42,47]. Mishra *et al.* [42] demonstrated automation of 8 valve actuations through stepped increases in disc spin-rate.

This DF based approach expanded through the development of ‘Event-Triggered Dissolvable Films’ [36]. These valves function in a manner similar to a single-use electrical relay; the valve is composed of two DFs (called the Load Film (LF) and the Control Film (CF)). Due to the valve architecture, and connecting pneumatic channels, wetting and dissolvable the CF induces the release of a liquid through the LF. Correctly configured, this can result in domino-like effect on the LoaD where release of one sample/reagent triggers the release of the next sample/reagent. This technology has been applied to nucleic acid based assays [36,60,61], blood processing [58,62], antibody based assays [44,63,64] and colorimetric assays [55,55]. However, a major drawback to these valves is that the time between sequential valve actuation is dependent on the dissolve time of the DFs; this is typically of order 5–40s and so does not enable long on-disc incubations to take place. This has been circumvented somewhat through the integration of a siphon valve to control timing of reagent release [44,63,65] or through the use of supporting instrumentation [55,58].

Table 1
Comparison of common valve types and dissolvable film-based valves to digital pulse actuated valves.

Valve Type	Advantages	Disadvantages	Exemplar References
Capillary Valve	Inherently part of the disc design	Highly dependent on manufacturing tolerances. Typically, a limited number of unit operations can be implemented.	[38,39]
Siphon Valves	Inherently part of the disc design	Highly dependent on manufacturing tolerances. Highly dependent on fluid properties/surface coatings/contact angle. Typically, a limited number of unit operations can be implemented.	[40]
Serial Siphon Valves	Inherently part of the disc design. Enables actuation based on pulsed increased/decreases	Highly dependent on manufacturing tolerances. Highly dependent on fluid properties/surface coatings/contact angle. Takes up significant space on the disc.	[41,42]
Rotationally actuated dissolvable films	Opening spin frequency of valves can be tuned.	DFs must be placed into the disc during manufacture which can increase cost. Maximum practical disc spin speed will inherently limit number of LUOs which can be automated.	[47]
Event-triggered dissolvable films	Operate independently of disc spin-speed so number of LUOs only limited by available space on the disc.	DFs must be placed into the disc during manufacture which can increase cost. Timing of LUOs is dependent on dissolve time of membranes.	[36]
Instrument controlled valves (general)	Typically, more reliable and permits a greater number of LUOs.	Additional instrumentation beyond spindle motor can be heavy, expensive, and power-hungry. Often, additional active components (ferro-wax, ablatable films etc) must be placed in the discs increasing their cost of manufacture.	[48]
Digital Pulse Actuated Dissolvable Film Valves	Only two spin-speeds are required for operation leading to inherent simplification of instrumentation. Number of LUOs implemented on-disc only limited by available disc real-estate.	DFs must be placed into the disc during manufacture which can increase cost of manufacture.	This paper

In this work, we present a novel implementation of dissolvable film valves henceforth referred to as 'pulse actuated' (PA) valves. These valves combine the best features of the rotationally actuated DF valves with those of the event-triggered DF valves. These valves are designed such that the sequence/order of valve actuation is determined by the disc architecture (per the event-triggered DF valves) but the timing of valve actuation is determined by stepped changes in the disc spin-rate (per the rotationally actuated DF valves). This allows us to decouple the number of valves on the disc from both disc manufacturing fidelity (as the valves remain closed at low frequencies and open at high frequencies) and from the maximum available spin-rate (again as only two spin frequencies are required). With this technology, the number of valving steps which can be integrated into the LoaD platform is now only limited by available space on the disc.

The Lab-on-a-Disc has become a popular platform for the automation of multiplex molecular/nucleic acid diagnostic assays [66] for a range of application including foodborne pathogens [67–70] pathogens and detection of genetically modified plants [71]. The LoaD has also been applied to a range of other applications in point-of-care and point-of-use testing. Often, solid phase extraction (SPE) will be integrated [36,61,67,69,72–77] with amplification methods such as PCR [74,78], LAMP [71,79–81], NASBA [82], and RPA [83–86].

To demonstrate the capabilities of these new pulse-actuated valves, we demonstrate plant-lysate to answer automation of plant-pathogen detection on a Lab-on-a-Disc based on solid phase extraction (SPE). We leverage prior work on SPE which demonstrated an extraction efficiency of ~32% on a Lab-on-a-Disc [61,87]. We first implement sold-phase extraction of plant and pathogen DNA from the sample, then mix the eluate with LAMP reagent, and meter this across six chambers containing LAMP primers for a positive control for tomato leaf (*cytochrome oxidase gene* (COX)) [88] and five common tomato leaf pathogens. These are a bacteria *Clavibacter michiganensis. Michiganensis* (CMM) [89], and RNA virus *Pepino mosaic virus* (PepMV) [90], a fungal blight *Phytophthora infestans* (P. Inf) [91], a viroid *Potato spindle tuber viroid* (PSTVd) [92] and fungus *Botrytis cinerea* (BOTRY) [93] which is the causal agent for grey mold. Following disc loading and sealing from atmosphere, the protocol is entirely automated on a custom spin-stand with thermal blocks to heat amplification chambers to 70 °C and a custom fluorescence sensor to measure amplification in real-time. We tested this system against lysed tomato leaf, which was artificially spiked with heat-inert *CMM* bacteria at a range of different concentrations. Overall, the disc took ~20 min to prepare the DNA for amplification and, depending on concentration; spiked CMM was detected between 25 min and 60 min following start of LAMP amplification.

2. Valve design and operation

Using nomenclature adapted from event-triggered valves [36], the basic pulse-actuated (PA) valve configuration is based on a pneumatic chamber sealed by two DFs; a Load Film (LF) and a Control Film (CF) (Fig. 1). In the novel scheme presented here, a PA valve will remain closed at any (reasonable) spin rate while the CF is still intact. However, once the CF is dissolved, the valve will open when the spin rate exceeds a critical opening, or burst, frequency. For convenience, we term this the valve 'ready state' (the more suitable term, primed, is, in centrifugal microfluidics, already used for siphon-valves)

As with the event-triggered type, the PA valves can be concatenated serially in a way where liquid released from one valve dissolves the CF of the following valve and puts it in a ready state. In Fig. 2, and ESI Movie 1, we demonstrate this concept using a simplified disc which is composed of one 'burst valve' [42,47] followed by two of the new pulse-actuated valves. Initially, the disc is spun below a critical burst frequency. In this design, the critical burst frequency is 40 Hz and the disc is rotated at between 20Hz and 30 Hz during the experiment. Approximately 45s into this test (see ESI Movie 1), the disc spin-rate is increased (digital pulse) to 50 Hz for an ~10s duration. This wets DF1 on the first valve, and it subsequently opens about 20 s later. Referring to ESI Movie 1, and Fig. 2(e), a second pulse opens Valve #2 at 110s into the video and Valve #3 is opened at 150s into the video (Fig. 2(g)). Note that in ESI Movie 1, a digital pulse at ~80s was ineffective, as CF2 had not yet dissolved; this was a result of operator error.

By actuating valves using these short pulses in the spin rate (where the disc is decelerated before the next CF in sequence is dissolved), the timing of each valve actuation can be controlled entirely by the motor. This in turn can enable automation of complex bio-assays as the number of unit operation is theoretically only limited by available disc real-estate while requiring just two operating frequencies (above and below the critical frequency). This can greatly simplify the control software used for a spindle motor and allow use of lower-cost equipment. Additionally, as the lower operating frequency is usually well spaced from the critical opening frequency (in Fig. 2 spaced by 10Hz) this in turn makes these valves extremely robust and tolerant of manufacturing flaws.

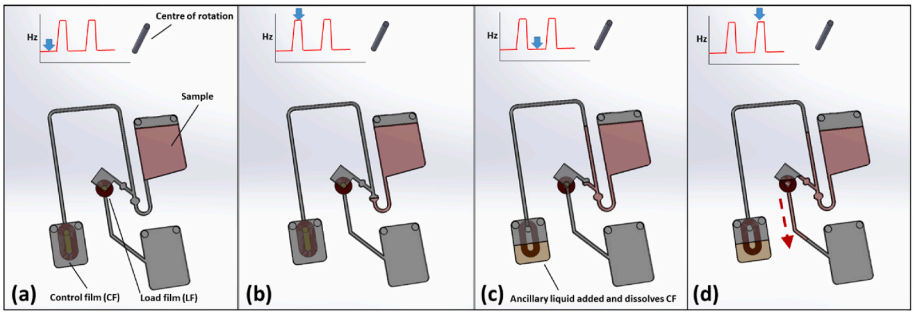

Fig. 1. Schematic illustration of the operation of the digital pulse actuated dissolvable film valve (a) the sample is loaded and both CF and LF films are sealed (b) the motor is pulsed to a higher spin-rate and the valve remains sealed (c) the addition of an ancillary liquid dissolves the CF. This permits the sample to enter the valve. However, as the LF is recessed in a pneumatic chamber it is not wetted. This is the valve placed in a 'readied' state. (d) Increasing the spin-rate pushes the sample into the dead-end chamber, wetting the LF and opening the valve.

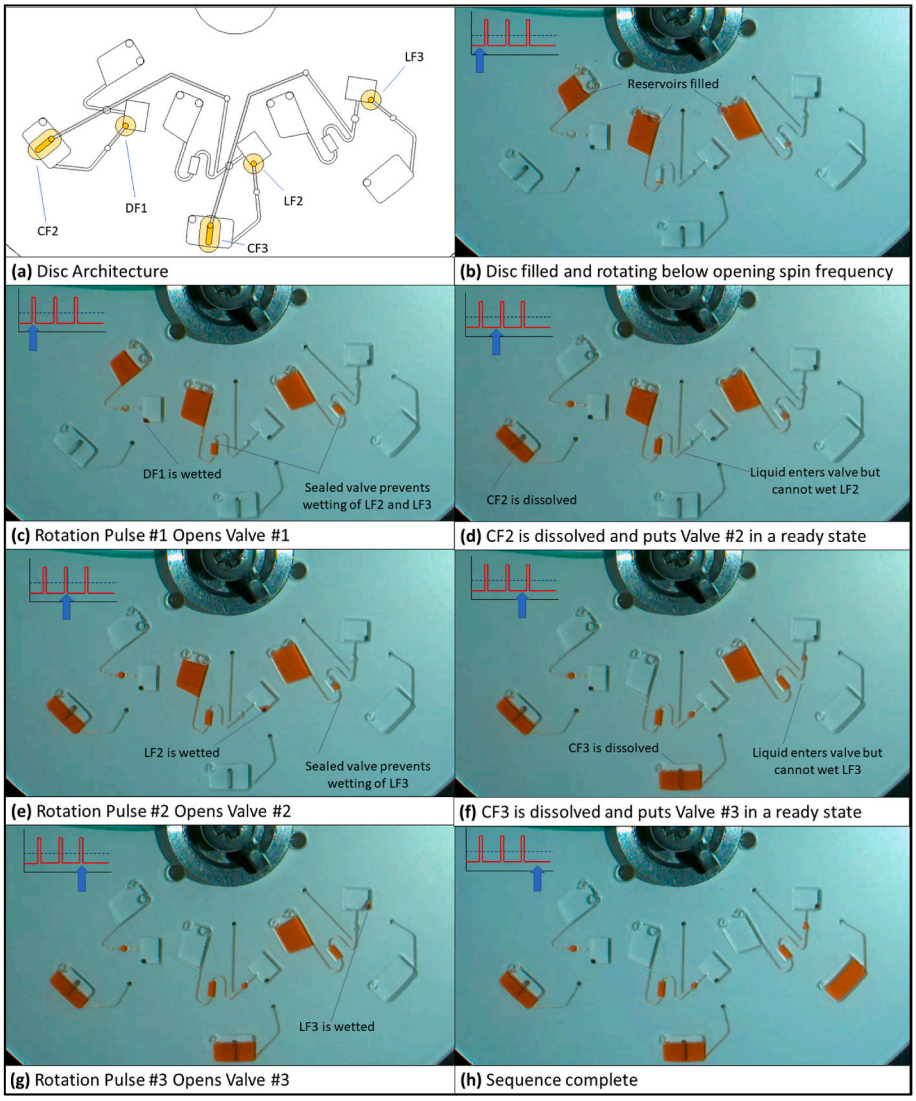

Fig. 2. Concatenation of pulse valves in a pre-defined sequence on a simple disc. This disc is composed of a 'burst valve' followed by two pulse-actuated valves (a) shows a schematic of the disc architecture. DF valves are highlighted in yellow and connecting channels on the lower levels of the disc (not visible in subsequent frames) are shown in grey (b–h) shows the sequence of valve actuation using pulses. See also ESI Movie 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Materials and methods

3.1. Plant pathogen assay

3.1.1. Primer selection and design

Five common pathogens for tomato leaf, and one positive internal control, were identified. The primers for the internal control (*cytochrome oxidase gene* (COX)) were taken from Tomlinson *et al.* [88] The primers for bacteria *Clavibacter michiganensis. Michiganensis* (CMM) were taken from Yasuhara-Bell *et al.* [89], RNA virus *Pepino mosaic virus* (PepMV) from Hasiow-Jaroszewska *et al.* [90], *Potato spindle tuber viroid* (PSTVd) from Lenarcic *et al.* [92] and fungus *Botrytis cinerea* (BOTRY) from Tomlinson *et al.* [93]. The primers for fungal blight *Phytophthora infestans* (P. Inf) were modified from those described by Hansen *et al.* [91] The full primers used in this study are provided in ESI. Primers were acquired from IDT (Leuven, Belgium) and were validated against G-Blocks (also provided in ESI). The optimised concentration of primers are also provided in ESI.

For on bench protocol for target detection, 25 μL volumes were created from primers (4 μL), LAMP reagent ISO-100 (Optigene, UK) (15

μL); for positive control, 6 μL for DNA extracts; for negative controls 6 μL nuclease-free water. The nuclease free water had bovine serum albumin (BSA, Sigma Aldrich, UK) added such that the final concentration in the reaction was 1% for disc-based testing. The presence of the BSA blocked the plastic in the microfluidic devices and promoted DNA amplification; for consistency, the BSA was also used in benchtop controls.

When loaded on-disc, the total volume of the reactions was 30 μL (10 μL pre-loaded in the reaction chamber and 20 μL delivered from metering structure). Each 20 μL volume was composed of LAMP reagent (16 μL) and sample (4 μL). The 10 μL was made up of primers (9 μL) and mineral oil (1 μL) with BSA added to the chambers are part of the LAMP reagent with a final concentration of 1%.

3.1.2. Lysate sample preparation

The tomato leaves are prepared using the DNEasy Plant Mini Kit (Qiagen). The full protocol used is provided in ESI. Briefly, 70 mg of plant tissue is cooled in liquid nitrogen and ground to powder using a mortar and pestle. At this point, if spiking the sample with thermally inactivated CMM bacteria (IPO-3208), 53 μl volume (diluted to the appropriate concentration) is added. Next, a lysis buffer and RNAse are added, and the sample is incubated for 10 min at 65 °C. A second buffer is added, and the sample is incubated on ice for 30 min. The sample is then further processed through spin-columns, provided as part of the kit, using supplied buffers. Because the DNEasy kit includes addition of an RNAse, amplification from PepMV and PSTVd (which are an RNA virus and viroid respectively) was not expected even when RT-LAMP was used on disc. For on bench controls, isolation of the plant material/lysate sample preparation took place using the protocol described above. Next, sample purification (Qiagen QiaQuick) was performed as per manufacturer's instructions. Isothermal amplification was performed on a commercial qPCR instrument (Qiagen Rotorgene, Manchester, UK) (60 min, 65 °C, SYBR Green acquisition every 10 s) followed by melt curve analysis.

3.1.3. Centrifugal test stand

The centrifugal test stand was composed of a spindle motor (Festo EMME-AS-55-M-LS-TS, Esslingen, Germany). The motor outputs a trigger signal to synchronise it with a stroboscopic light source (Drelloscop 3244, Drello, Germany) and a scientific camera (Basler Ace 2040-90uc, Basler, Germany). This trigger signal is filtered by custom hardware such that acquisition will only acquire 5 frames-per-second no matter how fast the disc rotates. This synchronisation allows each frame to be acquired at the same angular location; thus the disc appears stationary and liquid flow on the disc can be seen clearly.

The centrifugal test-stand is further modified with the addition of a heating unit and a fluorescence detection system (See ESI Figure SI1). The design and performance of these systems has been previously described [94]. Briefly, the heating unit is designed to heat part of the disc (containing reaction/amplification chambers) up to 65 °C. This is composed of two aluminium blocks which are mounted on a spring-loaded clamping system. The clamping system is enabled by linear actuators (Firgelli L12-10-210-12-S, Active Robots, UK). Liquid inside the reaction chambers will reach 65 °C after ~5 min. Due to PMMA's low thermal conductivity, the disc can be unclamped for up to a minute with minimal (~1 °C) temperature drop This allows rapid re-positioning of the disc for fluorescent measurements.

A custom system was developed for fluorescence detection. This was composted of 450 nm laser diode (Egismos Technology Corporation, Taiwan) for excitation and an amplified photodiode (P/N S7686, Hamamatsu, UK) for detection. The 450 nm laser beam is expanded/diffused by a convex lens. It is then reflected by a 510 nm dichroic mirror (Edmund Optics, UK) and the diffuse light is focused onto the sample using a 10× microscope objective lens. As the laser beam is expanded and then focused, the light reaching the detection chamber, though attenuated, is largely uniform in intensity. Fluorescent signal is focused by the microscope objective, passes through the 510 nm dichroic mirror, and is filtered by a 520 nm high-pass filter (Edmund Optics, UK). A second concave lens focusses the light onto the photodiode. As described previously [94], the sensor was characterized relative to a commercial qPCR machine (Rotorgene) and showed equal sensitivity and greater linearity detecting fluorescence across the relevant signal ranges.

The entire system was controlled using a custom LabVIEW program with commands including disc speed, disc acceleration, moving the disc to specific angular positions, clamping and unclamping the heaters, turning the laser on, and acquiring fluorescence. The program also ran human readable/editable scripts which ran a pre-defined list of these commands; thus permitting the amplification to take place autonomously. The software also logged temperature, motor position and speed, and fluorescence during the experiment. Finally, the software plotted fluorescence signal from the six reaction chambers in real-time during a run to enable real-time interpretation of data. The test-stand running a simple script may be seen in ESI Movie 2.

3.1.4. Disc design, fabrication and operation

In order to demonstrate the operation of the PA DF valves and their potential to robustly automate complex multistep bio-assays, a microfluidic disc was designed to automate solid phase extraction of DNA/RNA from tomato leaf plant lysate, to mix this purified DNA/RNA with LAMP reagent, and to then screen this against six biologically relevant targets (five common plant pathogens and one positive control present in tomato leaf). The workflow is shown in Fig. 3, the spin profile used to automate the protocol is shown in Fig. 4, and an image of the disc loaded with reagents is shown in Fig. 5.

This disc, which is 160 mm diameter, was initially designed in SolidWork (Dassault System) to be manufactured and assembled from 8 separate layers (four of knife-cut Pressure Sensitive Adhesive (Adhesives Research, Ireland) and four of laser cut PMMA) as described previously [36]. Briefly the layers are as follows.

- Layer 1 is 0.5 mm PMMA and contains loading vents. It is the top layer of the disc.
- Layer 2 is PSA and contains the majority of micro-channels
- Layer 3 is 1.5 mm PMMA and contains reservoirs for liquids
- Layer 4 is PSA and seals dissolvable films tabs in place
- Layer 5 is PSA and provides a support layer for the dissolvable film tabs. This layer, along with Layer 4, also includes some micro-channels.
- Layer 6 is 1.5 mm PMMA and contains further reservoirs
- Layer 7 is PSA and contains microchannels
- Layer 8 is 0.5 mm PMMA and acts as the lower base of the disc.

Following design in SolidWorks, the layers are extracted as 2D dxf files and transferred to AutoCAD (AutoDesk) for minor editing to make them suitable for knife and laser cutting. The 2D files are provided in ESI. The PSA and PMMA layers are then cut on a cutter plotter (Graphtec CE5000 (Graphtec)) or a laser cutter (Epilog Xing (Epilog)).

Prior to assembly, the PMMA layers are washed/sonicated using detergent and then washed/sonicated in DI water as previously described [36]. The disc is then assembled manually with each layer aligned atop the next using a custom assembly jig. Between addition of each layer, the disc is rolled on a laminator (HL-100, Cheminstrument, USA). Between Layers 4 and 5, DF tabs, manufactured as described previously [36], are inserted into the disc. All film used was KC35 (Aciello, Japan) with a dissolve time of 30–40s in DI water [36] except the valves corresponding to the release of the PE buffer. Here, custom film referred to as AR-film (Adhesives Research, Ireland) was used as this was specifically tailored to dissolve in high concentrations of PE buffer/EtOH. Valves for aliquoting structures were PVA films with 5 s dissolution time. Following assembly, the disc was sealed in an air-tight heat-sealable bag.

This disc is designed to automate an adapted version of the SPE protocol described used by the Qiagen QiaQuick kit. Reagents from the

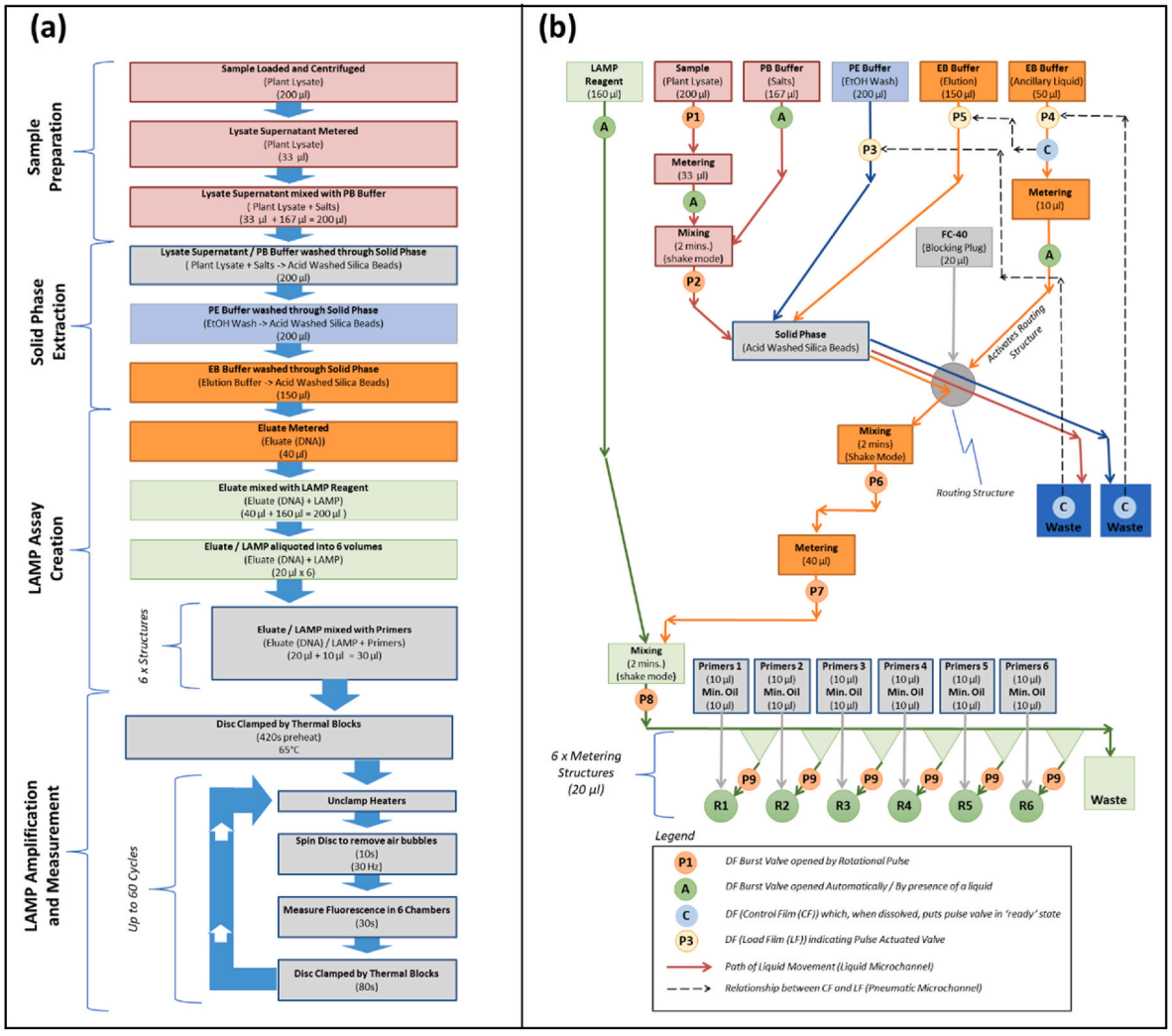

Fig. 3. Schematic describing (a) the detection and analysis steps implemented for on-disc SPE and DNA/RNA LAMP amplification and (b) the internal architecture and the operation of the pulse actuated Lab-on-a-Disc.

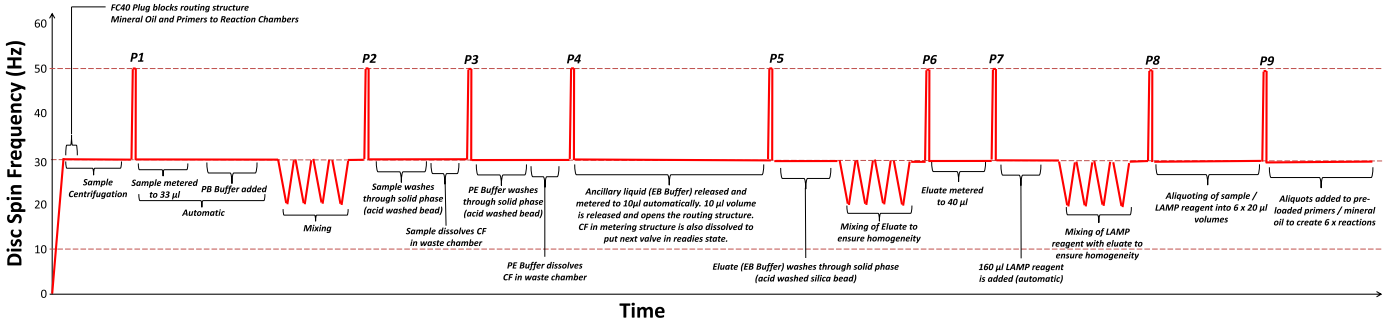

Fig. 4. Schematic describing the spin protocol used to automate the on-disc assay. From sample centrifugation to reaction creation takes ~20 min.

QiaQuick kit are prepared according to the instructions from this kit (e.g. addition of ethanol to PE buffer etc).

Prior to use, the disc was removed from the sealed bag and the solid-phase is added (acid washed glass beads, (Sigma-Aldrich)). First, the end of a1000 µL plastic pipette tip is removed using a scalpel. This is placed, as a funnel, into the chamber on the disc, and glass beads are added. The pipette tip is gently tapped and beads are gravity fed into the disc; once the SPE chamber is filled the pipette is removed and this chamber is sealed with transparent PSA (Adhesives Research, Ireland).

As described in Fig. 3, the disc is initially loaded with 195 µL of LAMP reagent with 32.5 µL 1% BSA solution (ISO-100 (Optigene, UK).

The disc is then loaded with 200 µL of PB buffer (chaotropic salts), 200 µL of PE buffer for washing the solid-phase, 200 µL of EB Buffer for elution, and 130 µL of EB Buffer to act as an ancillary liquid. As each reagent is loaded, the chamber is sealed with transparent PSA. Next, 9 µL of primers, corresponding to plant pathogen targets or positive controls,

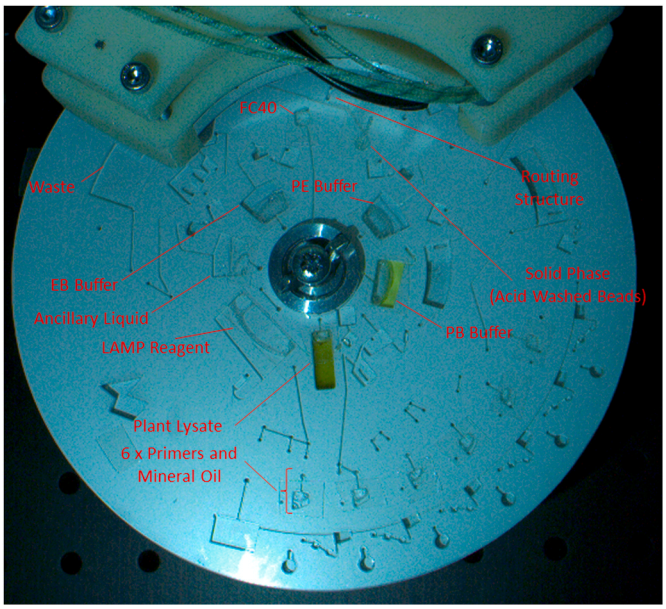

Fig. 5. Labelled image of the centrifugal disc, fully loaded with reagents and sealed from atmosphere, just prior to initiating the protocol.

are loaded into 6 chambers along with 1 μL of mineral oil to prevent any evaporation. Again these chambers are sealed with PSA. Finally, 10 μL of FC-40 and 200 μL of plant lysate are loaded and these chambers sealed with PSA. It should be noted that, at this stage, the entire disc is sealed from atmosphere and so environmental contamination with amplified DNA should be prevented. A network of internal venting channels permits air to recirculate around the disc as liquid moves and so prevents air-locking of parts of the disc.

The centrifugal test stand is now activated and the following operations take place (see also Figs. 3 and 4 and ESI Movies 2-4).

- The disc is rotated at 30 Hz. The sample (plant lysate) is centrifuged and some plant material sediments to the base of its chamber. The primers and mineral oil enter the corresponding reaction chambers. FC-40 (Sigma-Aldrich), which is a dense immiscible fluorocarbon with a specific gravity of ~1.7, forms a blocking plug in a routing structure which functions as previously described [61]. Briefly, the FC-40 plug prevents the routing structure opening until an ancillary liquid is used to open a dissolvable film.
- Pulse 1 opens a DF burst valve. As the valve uses KC35 film, as long as the pulse in disc spin-rate lasts less than ~30s, the disc will be back below the critical burst frequency (40 Hz) by the time the valve opens. In this work, default disc acceleration is 40 Hz s^{-1} and rotational pulses typically last 10 s. Opening this valve releases ~50 μL of plant lysate to enter a metering structure. This structure is previously described [36] and is automatic; here excess liquid in an overflow structure triggers the release of the metered volume. This volume, 36 μL, is transferred to a mixing chamber where the addition of 200 μL of PB buffer solution (as per the QiaQuick protocol) prepares the same for the solid-phase (i.e. addition of chaotropic salts to enhance DNA binding to the solid-phase). The disc is then rapidly accelerated and decelerated (between 5 Hz and 15Hz) to use the Euler force to enhance mixing [95].
- Pulse 2 opens a DF burst valve and allows the sample to wash through the solid phase; RNA and DNA bind to the acid washed glass beads while the sample continues to the waste chamber. In the waste chamber, the sample dissolves a DF which puts the next valve in series in a 'ready' state
- Pulse 3 opens the PA DF valve and releases the PE buffer to wash through the beads. This removes contaminants/amplification inhibitors but leaves the DNA/RNA attached to the glass beads. The PE buffer washes through to the waste where it dissolves a DF and puts the next valve in a 'ready' state.
- Pulse 4 release a volume (130 μL) of EB Buffer which acts as an ancillary liquid. This liquid flows into a metering chamber where it is automatically metered to 10 μL. The overflow 120 μL dissolves a DF which puts the next valve in a readied state. The 10 μL volume continues to a routing structure where it dissolves a DF. This removes the FC-40 plug, which is captured in a side chamber, and opens a route for the elution buffer
- Pulse 5 opens the valve and releases 200 μL of EB buffer which washes through the acid washed beads and is routed away from the waste chamber (via the now open routing structure) to a collection chamber. This buffer elutes RNA and DNA from the solid-phase. After elution, shake mode mixing homogenises the eluate to ensure it contains an even concentration of DNA/RNA.
- Pulse 6 opens a DF burst valve and transfers the eluate to a metering chamber where the volume is reduced to 40 μL.
- Pulse 7 opens a DF burst valve and transfers the 40 μL. of eluate to a mixing chamber. Here, a DF is dissolved which release 227.5 μL. of LAMP reagent to mix with the sample to create a total volume of 267.5 μL. Again, rapid shake-mode mixing is used to homogenise the sample.
- Pulse 8 opens a DF burst valve and transfers the sample to a metering/aliquoting structure. The sample is split into 6 × 20 μL volumes and the remainder flows into a waste chamber.
- Pulse 9 opens the 6 DF valves simultaneously and transfers the aliquoted samples into the 6 reaction chambers where they mix with target specific primers.

With the assays created, the first reaction chamber is aligned with the fluorescence detector and this location is set as zero. A pre-programmed script is activated in the custom software and operator leaves the laboratory (which is left in darkness except for the fluorescence excitation source). Following the script, the disc is clamped by the heaters for 420 s to heat up the reaction chambers. Next, the measurement sequence starts; this takes 110 s per measurement. The disc is unclamped and the disc is rotated at 30 Hz for 10 s to remove any air bubbles which may have appeared in the chamber. Next, the disc stops at each of the 6 reaction chambers chamber and the fluorescent signal is measured. This takes 4 s per chamber. Finally, the disc rotates to align the read chambers with the thermal blocks and it is clamped once again. In total, the disc is unclamped for 30s during measurement and clamped/heated for 80s. Due to the low thermal conductivity of the PMMA plastic, measurements show that the temperature in the reaction chambers drops ~1 °C in this 30s measurement window and recovers to the nominal temperature in the 80s heating window. The measurement cycle is repeated for a maximum of 60 cycles (total run time of ~117 min).

4. Results

This work focused on the detection of CMM (bacterial plant pathogen) DNA extracted from thermally inactivated CMM sample, which was spiked onto a tomato leaf sample prior to chemical lysis. Integrated into this setup is a positive control in the form of COX present in tomato plant leaves. The ability to screen against 6-plex targets is additionally demonstrated (Fig. 6).

Next, the discs were loaded with primers to screen against COX (two chambers), CMM (two chambers) and no primers (two chambers). Here, the system extracted. Here, the LoaD was tested against tomato leaf samples over a range of CMM colony forming units (CFU) concentrations (10^4, 10^5, and 10^6 CFU/mL) (Fig. 7). Note that these experiments were crosschecked in parallel using a benchtop gold standard PCR system (Rotor-gene) (see ESI - Figure SI2 for a representative PCR instrument-based control test).

During these experiments, tomato leaf was acquired from local

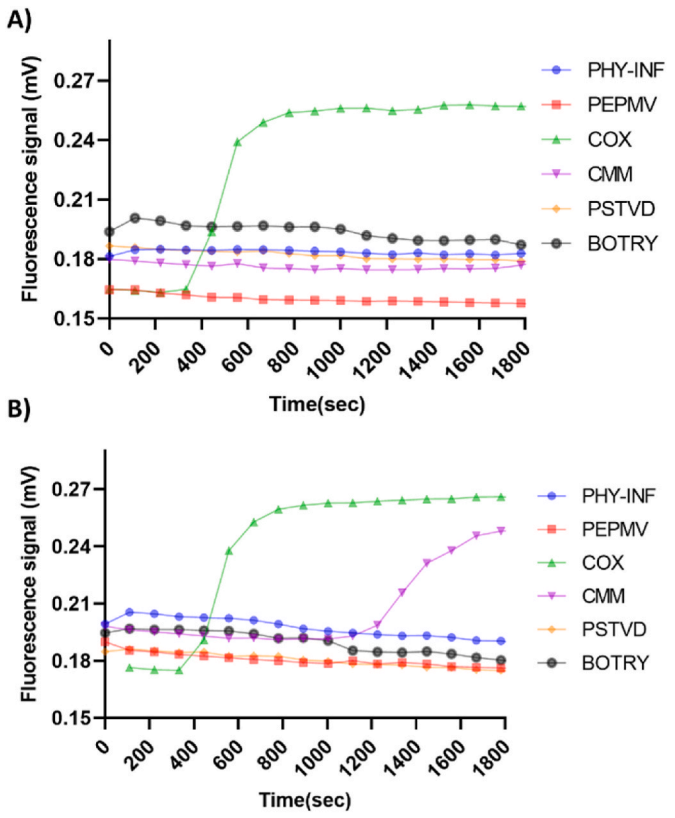

Fig. 6. Representative LAMP amplification curves acquired from the custom laboratory instrument on the Lab-on-a-Disc (a) shows result from a tomato-leaf lysate sample (with the COX positive control indicating the presence of tomato leaf) and (b) shows results from a tomato leaf lysate spiked to 10^5 CFU/mL of thermally inactivated CMM bacteria. Note the 420s pre-heat is not included in this data.

florists as required. On one supply of leaves, Botrytis cinerea (BOTRY) was detected in both on disc and gold standard benchtop methods (in samples both spiked and free of thermally inactivated CMM). The BOTRY was only detected in this particular supply of leaves; providing a fortuitous demonstration of the capability of the system (Fig. 8). Note that we believe the elevated fluorescence at the start of this experiment is due to poor thermal contact between our heater blocks and disc during this particular test. This resulted in it taking longer for the LAMP assays to reach temperature. As fluorescence is highly dependent on temperature, the (background) fluorescence will drop until the assays reach 65 °C [96].

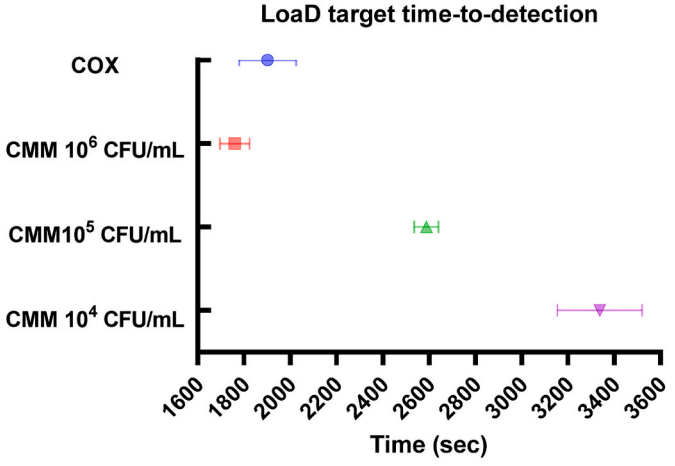

Fig. 7. On-disc time-to-detection are shown for tomato leaf samples spiked with thermally inactivated CMM bacteria with concentrations of 10^4 CFU/mL (n = 3), 10^5 CFU/mL (n = 4) and 10^6 CFU/mL (n = 3). Time-to-detection of the corresponding COX positive control from these tests is also shown (n = 10). Note the 420s preheat is not included in this data.

5. Conclusions

The work shown in this paper demonstrates the use of a novel rotationally actuated digital valve design for lab-on-a-disc (LoaD) and describes its fundamental principles of operations. The valves have proven to be compatible with a relatively low-cost, low-fidelity manufacturing method (multilayer assembly of laser cut PMMA/knife-cut PSA). The valves are demonstrably robust as they require only two operating spin-rates (above and below the critical burst frequency) and so are compatible with low-cost spindle motors. As the valves can be tuned to operate at relatively high spin-rates (compared with other common LoaD valves), they function largely independent of fluid properties such as interfacial tension and viscosity. In fact, assuming all reagents on the disc have a density similar to water, the critical burst frequency is entirely dependent on the geometry and radial location of the valve/reservoir [42]. With the sequence of valve actuation determined by the disc architecture, and the timing of valve actuation determined by the disc spin-profile, these valves can automate complex multi-step assays, in a highly robust and reliable manner, without requiring any instrumentation beyond a spindle motor. This is demonstrated in this paper where 9 rotational pulses are used to control 15 discrete unit operations. The robust nature of these valves means they can be applied to a range of manufacturing techniques such as the multi-layer approach here; indeed, this multilayer approach can easily be translated into roll-to-roll manufacturing to drastically reduce the cost of disc fabrication.

Additionally, this paper also demonstrates a capability use these valves to automate a complex lysate-to-answer protocol incorporating solid-phase purification of DNA, mixing of purified sample with LAMP reagent, and multiplex detection on a sealed integrated system. Although LAMP is typically used as an end-point detection method, the ability of this platform to monitor fluorescence during amplification enables pseudo-quantitative measurement CMM concentration on the tomato leaf samples (Fig. 7). This offers the potential to ensure positive signals actually indicate the presence of pathogens rather than deriving from environmental contamination. It should be noted that the use of the DNEasy Plant Mini Kit (Qiagen) for sample preparation, and particularly the use of RNase in the protocol, along with the use of LAMP

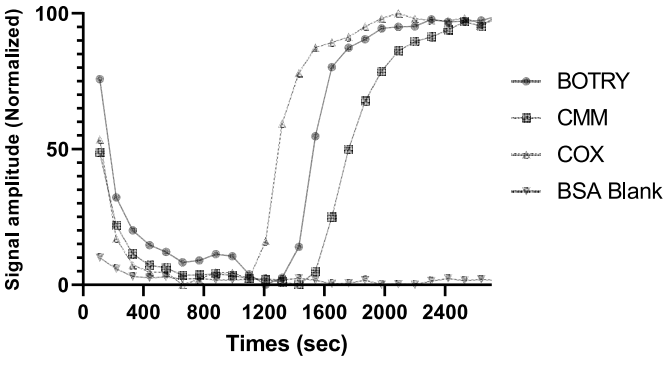

Fig. 8. LAMP amplification curves acquired from a multiplexed Plant Pathogen detection Lab-on-a-Disc. The presence of both CMM and BOTRY pathogens is detected rather fortuitously since only the CMM pathogen was artificially spiked on to locally obtained tomato leaves while the presence of BOTRY on the leaves was confirmed by laboratory benchtop methods. In contrast, previous tomato leaf sample used in the detection scheme in Fig. 6 does not show the presence of the BOTRY pathogen on-disc or with benchtop methods.

reagent, means that the RNA targets screened on this platform (PepMV and PSTVd) would not be detected and in effect these wells served as blanks/baselines for fluorescence signal. However, replacing the DNA lysis kit with one compatible with both DNA and RNA, and replacing the LAMP reagent with RT-LAMP reagent, would enable this platform to screen against both RNA and DNA based plant pathogen targets without any modification of the disc architecture.

It should be noted that this work was conducted in a laboratory environment using a modification of an existing centrifugal test-stand; this instrument is not suitable for deployment in the field. However, as these valves only require a low-cost spindle motor for operation, if supported by an appropriately designed instrument they can easily operate at point-of-use/point-of-care.

Most importantly, the fundamental architecture described in this work, which is enabled by the pulse-actuated valves, can be modified (or indeed simplified) to use the newest generation of lysis reagents (which often do not require solid phase purification), and to use conventional PCR or other isothermal reagents. Thus, it can be see that these pulse-actuated valves have the potential to enable robust, minimally instrumented, sample-to-answer detection of a range of genetic targets taken form a range of sample matrices.

Credit author statement

The manuscript was written by RM, DJK, GH and NPMcD with contributions of all authors. All authors have given approval to the final version of the manuscript. Project conceptualization: DJK and JD. Project Funding: JD. Project management / directed research: RM, DJK and NPMcD. Valve conception and disc architecture: DJK. Disc optimization: DJK and LANJ. Instrument control software: DJK. Fluorescence detection system: LANJ and DJK. Heating system: FM and DJK. Primer selection and preliminary assay optimization: CS and TD. Assay optimization and sample preparation: KM, PP, DJK, NPMcD, PLE, LANJ and RM. Disc manufacturing and testing: PP, JC, MD PLE, RM, DJK, NPMcD, and LANJ. Provision of thermally inactivated bacteria: CS.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341070.

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