Validation of a centrifugal microfluidic sample lysis and homogenization platform for nucleic acid extraction with clinical samples†

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The applications of microfluidic technologies in medical diagnostics continue to increase, particularly in the field of nucleic acid diagnostics. While much attention has been focused on the development of nucleic acid amplification and detection platforms, sample preparation is often taken for granted or ignored all together. Specifically, little or no consideration is paid to the development of microfluidic systems that efficiently extract nucleic acids from biological samples. Here, a centrifugal microfluidic platform for mechanical sample lysis and homogenization is presented. The system performs sample lysis through a magnetically actuated bead-beating system followed by a centrifugal clarification step. The supernatant is then transferred for extraction using a unique siphon. Several other new microfluidic functions are implemented on this centrifugal platform as well, including sample distribution, a unique hydraulic capillary valve, and self-venting. Additionally, the improved system has features with a small footprint designed specifically for integration with further downstream processing steps. Biological validation of the platform is performed using *Bacillus subtilis* spores and clinical samples (nasopharyngeal aspirates) for respiratory virus detection. The platform was found to be as efficient as in-tube bead-beating lysis and homogenization for nucleic acid extraction, and capable of processing 4 samples in batch to near PCR-ready products in under 6 min.

1. Introduction

Over the last several years, research towards implementation of nucleic acid (NA) *in vitro* diagnostic tests using microfluidics has skyrocketed, driven in large part by the rapid progress made in the fields of molecular biology and molecular diagnostics.[1] When developing microfluidic systems for molecular diagnostics, the problem is usually parsed into the development of separate pieces of equipment that tackle single steps within the total sample-to-answer process. These steps typically involve biological sample preparation followed by NA amplification, and finally NA detection. Often the emphasis is placed on amplification of the NA analyte or on more sensitive detection methods. Commonly, little or no emphasis is placed on preparing the biological sample itself, and specific characteristics of the sample are often not taken into account.[2] The neglect of sample preparation remains one of the most significant pitfalls that have prevented the widespread success of integrated, microfluidic systems for NA diagnostics.

Biological sample characteristics such as analyte and inhibitor concentrations as well as fluidic characteristics such as surface energy and viscosity must be taken into consideration when designing a microfluidic NA diagnostics device. Analysis and characterization of the sample will determine which preparation steps are required. Direct processing of raw blood samples remains a challenge for NA diagnostics, as these samples pose large hurdles due to their high concentration of inhibitors and their very low concentration of analyte.[3] For example, sepsis-related microbe concentrations in blood can be <10 NA molecules per mL when directly targeting microbial DNA.[4] In contrast, respiratory samples can contain much higher analyte concentrations with fewer inhibitors. Such is the case for viral respiratory samples that can exhibit $>10^5$ NA copies per mL. Thus, sample characteristics must be kept in consideration during the development of any sample preparation system.

One of the most essential components of sample preparation is lysis, which serves to release DNA or RNA from the cells/viruses of interest to facilitate further analysis. Various forms of benchtop lysis systems are used, and can be roughly classified into two main groups: chemical/biological methods and physical methods.[5] The chemical/biological methods, which include lysis by chemical detergents or biological enzymes, are the simplest to implement in terms of hardware requirements, as they use little or no power and need activation only by mixing or reconstitution of reagents. However, a major limitation in these methods is that they may leave behind residual substances that can inhibit amplification processes (*e.g.*, polymerase chain reaction or PCR) and so must often be removed or filtered out. This can pose problems of increased complexity for integrated microfluidic systems. Physical lysis methods, which include manual grinding, freeze/thaw cycles, sonication, and mechanical disruption, among others, require additional instrumentation. However,

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such systems leave behind little or no residual chemicals, and are often faster and more efficient than chemical/biological techniques. Indeed, mechanical lysis is the most effective method for breaking down cells that have thick cell walls, such as Gram-positive microbes, and for successfully extracting intact DNA.[6,7] One mechanical lysis method known as bead-beating is the most efficient method in this respect, and functions by combining the sample with an agitated mixture of milling beads.[8,9]

1.1. Microfluidic sample lysis

There are numerous demonstrations of the benefits gained by moving from a typical wet-bench set-up to a microfluidic device. Some of these benefits obtained include reduced reagent use, decreased total processing time, increased abilities for parallel processing, and reduction in process variability *via* automation, among others.[10,11] To create a complete, microfluidic NA analysis system, the sample preparation and lysis components must be miniaturized as much as possible, remaining within the constraints of the required sample volume, and adapted for integration with further downstream processing steps.[5] Microfluidic lysis systems are required for transition from large, stand-alone processing units to individual sample-to-answer systems. While examples in the literature of R&D efforts on microfluidic sample preparation devices are relatively few, several groups have worked on novel microfluidic lysis methods and are worth reviewing. For brevity, emphasis here is placed on microfluidic systems for physical disruption of biological samples for NA analysis.

1.1.1. Electro-lysis. Electro-lysis utilizes electric fields to create pores in cell membranes for NA extraction. Cheng *et al.* used electro-lysis to extract NA from *Escherichia coli* and then performed detection using a separate array chip.[12] This method shows promise for research applications, yet requires high voltage sources, adding expense and complexity to the system while limiting integration and throughput abilities. While electro-lysis has been shown for simple-to-lyse cells such as *E. coli*, it is often presented in conjunction with additional lysis mechanisms, and not as a stand-alone method.[12] These systems are better suited for single cell analysis, which is currently not adequate for most NA clinical applications.

1.1.2. Laser lysis. While the exact mechanism for lysis has not been confirmed, the rupture of cell membranes likely occurs from the instantaneous change in temperature induced by the laser.[5] These systems are extremely fast and have proven that after lysis, PCR-ready samples can be isolated from difficult-to-lyse microbial spores.[13] However, limitations include expensive and complex laser systems difficult for parallel processing and integration. Lee *et al.* created a portable laser-based lysis system that incorporated magnetic beads to aid in lysis and fluidic processing for PCR, and successfully implemented lysis of Gram-positive bacterial cells.[3,14] The viability of using this system with clinical samples remains unknown.

1.1.3. Mechanical lysis. Mechanical lysis has been performed by sonication in a microfluidic system, and likely occurs due to lysis *via* gaseous cavitation. Sonication forces can disrupt cells by forming and collapsing air pockets from dissolved gases, creating high pressure and temperature micro-environments.[15] Several systems have been developed with microfluidic cartridges, and micro-sonicators and have been used to lyse difficult models such as bacterial spores with resulting PCR-ready NA.[16] Additionally, Belgrader *et al.* developed a sonicator system that utilizes glass beads to increase the surface area for cavitation and thus increase the number of cavitation events.[15] However, there remain problems with integration and clinical sample analysis and the need for additional sonication hardware, such as piezoelectrics. Sonication also has the inherent problems of cavitation-induced foaming and aerosols.

Microfluidic mechanical lysis has also been demonstrated that involves the forcing of cells through a microfluidic filter lined with nano-barbs, where the barbs act like an array of blades that disrupt cells.[17] Lastly, the Madou group has previously developed two mechanical bead-beating systems, both based on compact-disc (CD)-like centrifugal microfluidics.[18,19] Both CD platforms adopted forms of rapid universal cell lysis and nucleic acids preparation (RUCLANAP), one of the most efficient in-tube, bead-beating sample preparation methods.[20] In both platforms, lysis was accomplished by combining cell samples with grinding media (milling beads) and exposing the system to intense mixing within the lysis chambers. Lysis in the earlier system developed occurred due to collisions between milling beads and cells, and took place when alternating the CD spin direction causing impaction and friction due to Coriolis effects in the lysis chambers. The second system developed replaced the Coriolis-induced lysis with magnetic-assisted bead-beating. Strong magnetic lysis disks inside the CD were actuated during rotation by strategically placed magnets on a stationary platform, causing impaction and lysis in the radial direction.[18] Both systems were validated using a range of cell types from *E. coli* to the more difficult model of yeast. However, there remains a need for microfluidic sample preparation and lysis systems amenable to integration towards sample-to-answer systems that focus on NA extraction.

1.2. Centrifugal microfluidics

When developing fluidic devices of any kind, the controlled movement of liquids is of primary concern. This general problem can be summarized as the need for two related technologies: pumps and valves. The centrifugal (CD) platform provides elegant, simple, and effective modes of pumping and valving in the microfluidic domain.

Fluid propulsion on the CD is performed by centrifugally induced pressure on the fluid as the CD spins; Madou *et al.* and Duffy *et al.* have characterized this type of flow extensively.[21,22] The volumetric flow rate is dependent in part on the speed at which the disc spins, the distance the liquid is from the center of the disc, the geometry of the fluidic channels, and the fluidic properties (density, viscosity, and surface energy) (Fig. 1A). By using combinations of different channel geometries and spin speeds, flow rates ranging from 5 nL s^{-1} to 0.1 mL s^{-1} can be achieved with a high degree of accuracy and precision. Typical fluid-pumping rotation speeds used can range from 300 to 3000 revolutions per minute (rpm).

Centrifugal pumping has many advantages over other typical microfluidic pumping techniques as outlined by Madou *et al.*[21]

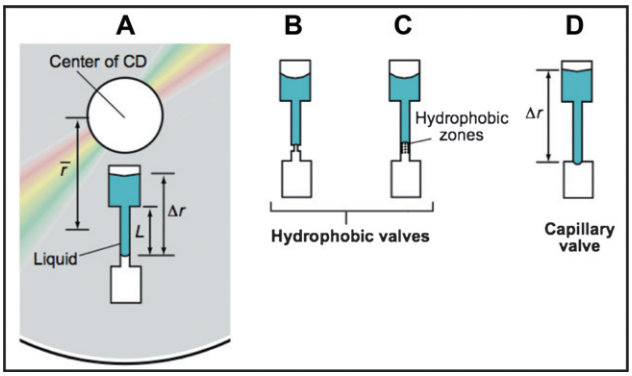

Fig. 1 Schematic of microchannels on a CD. (A) Two reservoirs connected by a single channel, (B) hydrophobic valve made by a channel restriction in a hydrophobic material, (C) hydrophobic valve made by functionalization of the channel surface with hydrophobic material and (D) capillary valve made by a channel widening in a hydrophilic material (adapted from ref. 21).

Namely, centrifugal pumping eliminates the need for large power supplies and/or pumps to drive fluids (only a low-power motor is needed), and centrifugal pumping is not strongly dependent on the pH or ionic strength of the fluid. Centrifugal pumping also provides forces across the entire length of a fluid element, allowing smooth and controlled flow. In addition, several individual systems can be placed on a single CD, making parallel processing and integration towards sample-to-answer systems feasible.

Valving on the CD is commonly performed using two main valve types: hydrophobic and capillary (Fig. 1B–D). Hydrophobic valves can take on two different forms: one utilizing changes in channel geometries (Fig. 1B) and the other utilizing surface modification (Fig. 1C). In both cases, the fluid can be forced past the hydrophobic valve by increasing the spin frequency above a critical value. The capillary valve is also commonly used in CD platforms, and is a result of the balance between centrifugal and surface tension forces in a hydrophilic material (Fig. 1D). When fluid being pumped through a narrow channel by centrifugal forces reaches an abrupt widening, a large surface tension force develops at that widening. If the surface tension force is greater than that of the centrifugal force, then the fluid flow will stop even though the CD continues to spin. At a certain spin speed, known as the burst frequency, the centrifugal forces will overcome the surface tension forces and the fluid will continue down the channel. By designing microfluidic structures with channels of varying capillary valve sizes, control of when a valve "opens" can be achieved simply by increasing the rotational speed of the CD.

1.3. Sample preparation CD

Here, a new system for sample lysis and homogenization on a centrifugal (CD) microfluidic platform is presented. The system includes a stationary stand with permanent magnets placed beneath the CD and the CD itself, which contains ferromagnetic lysis disks and zirconium/silica lysis beads. As the CD spins over the stationary magnets, each lysis chamber is subjected to a circumferential, oscillating magnetic field and in turn, the magnetic lysis disks are oscillated inside the CD's lysis chambers. The movement of the ferromagnetic disks creates a snap and drag motion, resulting in forces between the chamber walls and the lysis beads that create mechanical impaction and shearing of the sample; further sample disruption is obtained *via* Coriolis forces. The resulting forces disrupt cells/viruses and homogenize the sample based on the bead-beating method.

The lysis CD platform can perform bead-beating lysis and homogenization of 4 separate samples in parallel, each 90–95 μL maximum volume, or a single 360–380 μL sample that is then metered among the 4 chambers. This is followed by a centrifugal-based clarification step that separates solid particulates/debris and leaves nucleic acid suspended in the supernatant. After a volume definition step, a unique siphon is used to transfer the clarified sample containing nucleic acid for removal.

The sample lysis system was developed for use with viral respiratory samples, and designed to deliver a processed NA sample for amplification and subsequent detection. Thus, both bacterial spores for their known resistance to lysis and nasopharyngeal aspirates (NPA) to demonstrate heterogeneous matrices were used for biological validation of the CD system's lysis and homogenization abilities.

2. Materials and methods

2.1. CD fabrication

The CD devices presented here consist of multi-layer structures made of inexpensive polycarbonate plastic and double-sided, pressure-sensitive adhesive (PSA). Using a relatively simple computer-numerical control (CNC) machine (T-Tech, GA, USA-QuickCircuit 5000), channel widths down to 1 mm are machined into stock polycarbonate plastic (McMaster-Carr, CA, USA). A cutter-plotter (Graphtec, Japan-Graphtec CE-2000) is used to cut channel widths as narrow as 200 μm in thinner materials such as 100 μm thick PSA (FLEXcon, MA, USA-DFM 200 Clear V-95 150 POLY H-9 V-95 4). Once the appropriate pieces have been designed and machined, they are aligned centrally and radially and laminated together using the PSA layers.

The microfluidic CD presented here consists of 6 layers: (1) top polycarbonate CD (~1 mm thick) with CNC-machined sample loading, sample removal, and air venting holes (sealed using a thin adhesive film during operation), (2) pressure-sensitive adhesive with microchannel features cut using a plotter, (3) middle

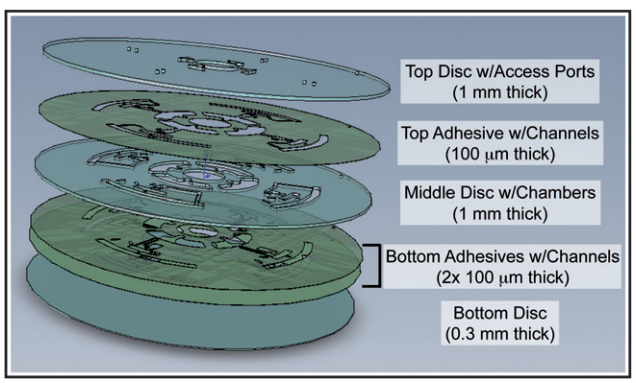

Fig. 2 Schematic showing assembly of the microfluidic CD, consisting of polycarbonate and pressure-sensitive adhesive layers.

polycarbonate CD (~1 mm thick) with CNC channel features, (4 and 5) pressure-sensitive adhesive with microchannel features cut using a plotter, doubled-up to obtain a higher thickness, and (6) solid bottom polycarbonate CD (~300 μm thick) to seal off the channels (Fig. 2). When assembled, the microfluidic CD allows more complex 3D fluidics through the use of multiple layers.

Often, additional materials and devices are placed inside CDs during fabrication, such as beads to aid in lysis, lyophilized reagents, or filters. In addition, surface treatments like exposure to O_2 plasma or functionalization with bovine serum albumin (BSA) can be used to create hydrophilic and hydrophobic surfaces, respectively. The CD devices presented rely on O_2 plasma treatment to render the polycarbonate surface hydrophilic, essential for the siphon (explained in detail below). A Technics 500II Asher was used at an O_2 pressure of 200 mTorr with 200 W of power for 2.5 min for hydrophilization of the unassembled polycarbonate CD layers. This step also serves to sterilize the parts in preparation for biological testing. During assembly, pre-autoclaved circular ferromagnetic disks (5.08 mm diameter, 0.635 mm thick) were placed inside each lysis chamber (V&P Scientific, CA, USA-VP721F).

After assembly, an aliquot of lysis-bead slurry was applied to each lysis chamber. The lysis-bead slurry consisted of 100 μm diameter (average) zirconia beads (BioSpec Products, Inc, OK, USA-11079110zx). The slurry was made by adding a 1% (w/w) solution of polyvinylpyrrolidone (PVP) (BASF, Ludwigshafen, Germany-Luviskol® K90) in DI water to the beads. The slurry was aspirated into the bottom of a vertical Teflon tube (1/8″ od, 1/16″ id) and the beads were allowed to settle (*via* gravity) in the tube. This was done to ensure the dispensed volume had the maximum number of beads (*i.e.*, bead packing) and achieved a high precision of bead dispensing. Each dispensed volume into the lysis chambers was 125 μL (±2%). After dispensing of the bead slurry into the CDs, a vacuum was applied overnight to accelerate evaporation of water from the slurry. After complete evaporation, a cake of lysis beads remained bound together with the PVP, also immobilizing the magnetic lysis disk until the addition of sample. Finally, adhesive tabs were placed on exposed loading and venting holes.

2.2. Magnetic platform fabrication

The stationary magnetic platform was built utilizing polycarbonate material (McMaster-Carr, CA, USA) milled with the CNC machine. The platform consisted of a polycarbonate plate with embedded strong permanent magnets (McMaster-Carr, CA, USA-nickel plated, neodymium-iron-boron, 3/8 in. diameter, 1/4 in. thickness). Four magnets were arranged with equal spacing on a radius similar to that of the lysis chambers on the CD (Fig. 3). Additionally, plastic spacers were added between the spin-stand (where the control motor was mounted) and the magnetic platform in order to minimize the gap between the magnets on the platform and CD bottom. This provided optimal coupling of the magnetic field to ensure maximum actuation and lysis efficiency.

2.3. Experimental set-up

Microfluidic testing of the sample preparation CDs was carried out by strobe-imaging of the CDs during operation. Volumes of various buffer solutions containing a contrast agent (McCormick, MD, US-Neon food dye) were placed inside the CD, and the CD placed on a spin-stand equipped with a rotational motor (Pacific Scientific Servo Motor) and an amplifier/controller (PAC SCI Programmable Servo Drive) which enables various rotational profiles and precise positioning. The servo drive uses a graphical user interface program, ToolPAC, to easily configure and program the motor for specific applications. The CDs to be tested were placed on an aluminium chuck coupled to the motor shaft and locked in place. An imaging system was used that allows viewing of a sequence of color images of an area of interest on the CD in real time (while it is rotating) and storage of the captured frames on a computer. The imaging system is composed of: a camera (Basler A301bc, 640 × 480 pixels, 80 fps max., 10× zoom lens mounted), a strobe light (PerkinElmer MVS-4200, 6 μs duration), and a retro-reflective fiber-optic sensor (Banner D10 Expert Fiber-Optic Sensor). The strobe light, with a 100 Hz maximum repetition frequency, is employed to reduce blurry images of the fast moving CD. In order to generate synchronized signals, a reflective marker (~2 mm × 2 mm) is placed on the surface of the CD and aligned with the fiber-optic sensor. When the sensor detects the marker, a signal pulse is sent to the video capture board, triggering the camera and strobe light to acquire one image frame per CD revolution.

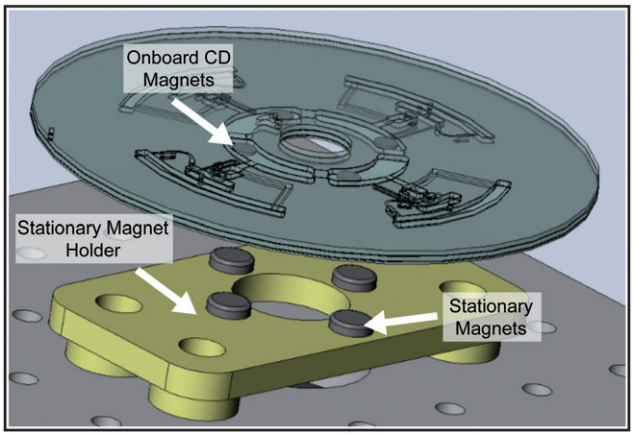

Fig. 3 Schematic showing the disposable CD and the permanent magnetic hardware system (motor for centrifugal control not shown).

2.4. Biological testing

As a test for the biological lysis and homogenization functionality of the CD platform, two different types of samples/analytes were chosen: bacterial spores and viral respiratory clinical samples. Bacterial spores are known to be particularly difficult-to-lyse, and so are a good test for lysis efficiency. Additionally, clinical nasopharyngeal aspirate (NPA) samples containing respiratory viruses were chosen both to show lysis efficiency capabilities on real-world patient samples and also to show homogenization capabilities, as NPA can have varying degrees of mucus that requires homogenization. The gold standard used for lysis and homogenization comparison was the RUCLANAP in-tube, bead-beating lysis protocol.[20]

2.4.1. Bacterial spore preparation and lysis. As a difficult-to-lyse model, *Bacillus subtilis* subsp. *subtilis* 168 was grown according to a previous study[20,23] and sporulation obtained according to the protocol of Laflamme *et al.*[24] 70 μL of DI water containing ~1000 spores μL^{-1} were subjected to lysis using both the standardized RUCLANAP protocol and the CD platform. For the on-CD method, the sample was pipetted into the CD, the loading and venting holes sealed with adhesive tape, and the CD subjected to the lysis spin profile, described in detail below.

After lysis, the samples were removed (either from the tube or the CD), subjected to a 95 °C heating step to inactivate PCR inhibitors, and then used in a real-time PCR assay.

2.4.2. Bacterial spore real-time PCR assay. Two μL of spore lysate containing ~2000 lysed spore genome copies were amplified in a 25 μL real-time PCR reaction volume assay as a measure of lysis efficiency. The real-time PCR assay used for the detection of *B. subtilis* spores, including specific primers, was performed based on a previous protocol by Picard *et al.*[23] Real-time PCR threshold cycle values (C_t) were recorded for samples processed with both the RUCLANAP and the CD methods, as well as non-lysed samples as a control.

2.4.3. Clinical specimen collection and preparation. The clinical samples (targeted for respiratory viruses) were authorized by the ethics committee of the Centre Hospitalier Universitaire de Québec. The eight samples in this study (*i.e.*, 8 patients) were collected according to standard procedures at the hospital. Each frozen NPA sample, up to 200 μL in volume, was thawed and subjected to the same lysis protocols (RUCLANAP and on-CD) as used for the *B. subtilis* spores (see Section 2.4.1, above). As above, the samples were removed after lysis (either from the tube or the CD), subjected to a 95 °C heating step to inactivate PCR inhibitors, and then used in a PCR assay.

2.4.4. Clinical specimen PCR assay. After lysis, 2 μL of the processed clinical NPA sample were used in a 20 μL RT-PCR reaction as a measure of both lysis and homogenization efficiency. The specific assay used, including primers, was based on the application patent by Bergeron[25] designed to target and detect common human respiratory viruses, including human metapneumovirus (HMPV) (RNA-based), enteroviruses (RNA-based), and adenoviruses (DNA-based). As with the spores, RUCLANAP was used as the gold standard for lysis comparison.

3. Results and discussion

3.1. CD system performance

3.1.1. CD microfluidics. The CD system presented provides implementation of several new centrifugal microfluidic functions. In the interests of clarity, functionality is presented in the order that fluids are processed through the CD. Fig. 4 is used as a reference for the majority of this discussion, and the spin profile utilized during microfluidic and biological testing can be seen in Fig. 5.

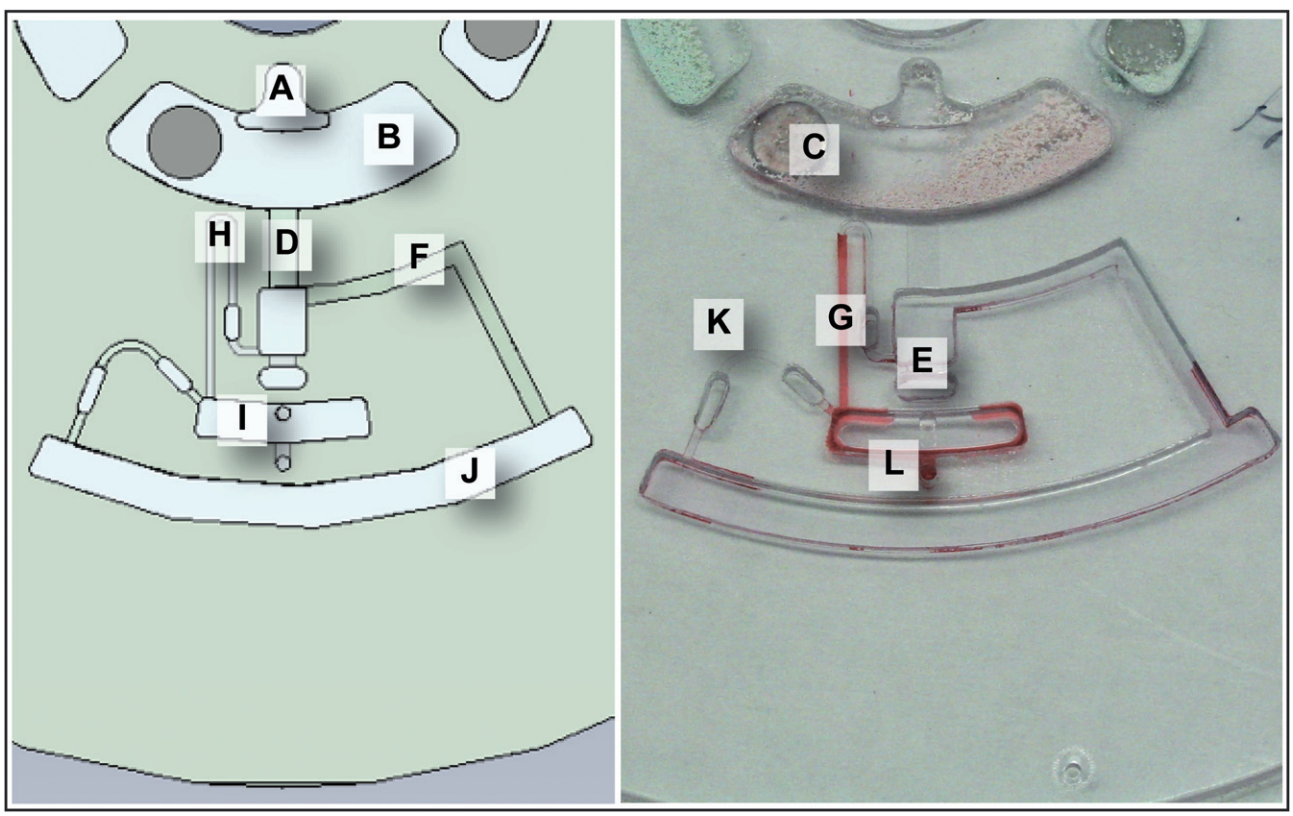

Fig. 4 Schematic (left) and photo (right) of a single lysis device on the CD (presented in duplicate for clarity). A—sample inlet port, B—lysis chamber, C—magnetic lysis disk, D—hydraulic capillary valve, E—clarification (upper) and capture (lower) chambers, F—volume definition channel, G—siphon capillary valve, H—siphon, I—collection chamber, J—waste chamber, K—self-venting channels, and L—sample collection port.

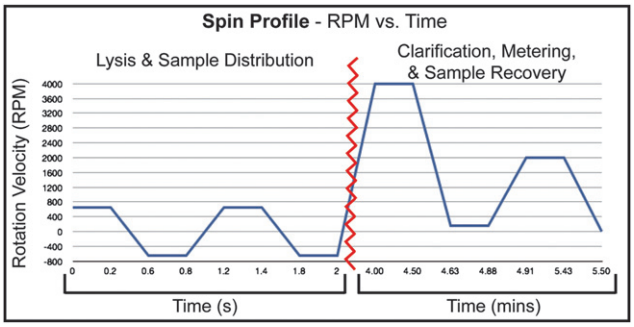

Fig. 5 Spin profile for the CD system. Lysis and sample distribution involve oscillation of the CD in a clockwise, counter-clockwise motion at ±650 rpm. This is repeated for a total of 4 min. The next step involves clarification (4000 rpm), followed by siphon priming (150 rpm), and sample recovery (2000 rpm).

The first unique fluidic function demonstrated is sample distribution. Two versions of the CD system presented were designed and tested: version 1 provides multiplexed processing of 4 independent samples each through a single device, while version 2 provides multiplexing of a single sample distributed to 4 separate devices (Fig. 6). In the 1st version (non-connected), the implementation is relatively straight-forward: 4 distinct lysis chambers are provided, each with its own sample loading inlet allowing the introduction of 4 separate 90–95 μL samples (Fig. 6A). In the connected version, there is only 1 sample inlet port provided, yet 4 distinct lysis chambers remain (Fig. 6B); the lysis chambers are connected through a radial channel in the upper adhesive layer, which is only 100 μm thick. This allows liquid communication between the 4 chambers, yet prevents the magnets and lysis beads (beads 100 μm in diameter) from traveling between the 4 chambers and re-distributing themselves. The end result is a single sample, with a volume ranging from 360–380 μL that is distributed and processed into 4 distinct volumes, such that each of the 4 subsamples can be subjected to, for example, 4 different downstream processing steps after lysis and homogenization.

The liquid sample distribution occurs *via* two means. The first is centrifugal force applied during lysis. Rotation at 650 rpm forces the liquid towards the bottom of the chamber, and allows excess to spill through the 100 μm connection channel into adjacent lysis chambers. Note also the presence of the small lip in the lysis chamber beneath the connection channel; this ensures that, as the sample is distributed, each lysis chamber captures and retains the minimal volume necessary for processing (Fig. 6B).

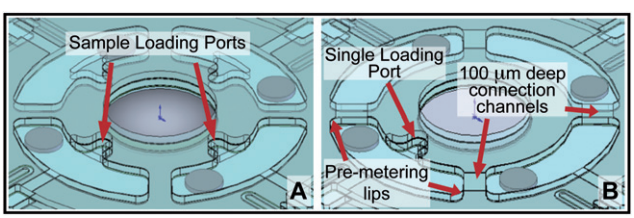

Fig. 6 Schematic showing the 4 unconnected lysis chambers for processing of 4 separate samples (A) and the 4 lysis chambers connected for processing of a single sample (B). The connected version allows distribution and pre-metering of the sample while keeping the beads (not shown) and magnets isolated.

The second distribution method is *via* motion of the magnets and beads, which helps distribute the sample circumferentially around the CD and overcome any capillary valve effects present at the exits of the connection channels. During sample distribution and lysis, the CD spin direction is alternated. This creates additional distribution and mixing *via* the Coriolis force. The sample distribution and lysis functions occur simultaneously.

The second unique fluidic function is a novel valve that relies both on capillary valve forces and on hydraulic forces to retain the fluid inside the lysis chamber during lysis and homogenization (Fig. 4D). Once the sample inlet port(s) is sealed after loading, there is no connection to the atmospheric environment behind the liquid. This means that, in addition to the normal capillary forces present on the capillary valve at the exit of the lysis port (Fig. 4D), there is a low-pressure environment behind that liquid, further preventing the sample from moving on and into the clarification chamber (Fig. 4E) during lysis. This double-force, hydraulic capillary valve allows higher rotation speeds (*viz.*, burst frequency) to be achieved as compared to a normal, single-force capillary valve. This translates to the ability to perform more efficient lysis at higher speeds without worry of the sample bursting into the clarification chamber prematurely. The downside of the capillary hydraulic valve is that a much higher rotation speed must be achieved in order to reach the burst frequency and move the liquid into the clarification chamber. In this system, however, the centrifugal force required for clarification (4000 rpm) far exceeds the burst frequency of the hydraulic capillary valve, thus adding no additional requirements to the hardware system.

After magnetic lysis, to be described further below, the system is sped up to 4000 rpm, causing the hydraulic capillary valve to burst and the sample to move into the clarification and capture chambers (Fig. 4E). During this step, volume definition occurs, with excess sample moving out through the volume definition channel (Fig. 4F) and into the waste chamber (Fig. 4J). This leaves a total of 25 μL of sample combined in the clarification and capture chambers, siphon capillary valve (Fig. 4G), and partially in the siphon (Fig. 4H). As the CD continues to spin at 4000 rpm, any viral/cell lysis debris present in solution is pelleted at the bottom of the capture chamber, with NA remaining in solution. It is worth noting the *g*-force experienced during this clarification step, whose relation to the rpm and distance from the CD center is given as:

$$\times g = \bar{r}f^2(1.118 \times 10^{-5}) \quad (1)$$

where $\times g$ is the force in terms of times gravity, $\bar{r}$ is the radius from the CD center in cm, and f is the CD spin frequency in rpm. Given the rpm value of 4000 and the $\bar{r}$ value of 2.94 cm (average value for the capture and clarification chamber fluid element), the *g*-force experienced is 526 $\times g$. The capture chamber also serves to entrap any particulates from the sample (*e.g.*, respiratory mucus) and escaped lysis beads. The connection channel between the clarification chamber and capture chamber is 200 μm deep to facilitate transfer of this material into the capture chamber.

After clarification, the CD speed is reduced to 150 rpm, allowing capillary forces in the hydrophilic siphon to overcome the centrifugal forces, thus priming the siphon valve, as follows.

Capillary forces draw the liquid through the entire length of the siphon, up and over the siphon crest, and to a point diametrically "lower" than the clarification chamber, at the entrance of the sample collection chamber (Fig. 4I). The CD is then slowly sped up to 2000 rpm, resulting in siphon action pumping only the supernatant from the clarification chamber and siphon capillary valve into the sample collection chamber. 20 μL of sample are deposited into the sample collection chamber, and the "dirty" sample remains in the capture chamber. At this stage, the sample, still containing active PCR inhibitors, is removed from the CD and subjected to a 95 °C heating step to inactivate remaining inhibitors. This completes the lysis and homogenization, clarification, and supernatant extraction steps.

Another unique function provided on this CD platform is self-venting. Before operation, the sample inlet port(s) and the sample removal ports (Fig. 4L) are all sealed using adhesive film. This means the entire system is closed, with no exposure to the outside environment. The practicality-of-use implications are that the CD can be used in any standard molecular biology lab or clinic without concerns of contaminating the area with direct liquid samples or aerosols. Once the hydraulic capillary valve is breached after lysis, air from the system moves back up into the lysis chamber, thus achieving air-pressure equilibrium. The self-venting channel (Fig. 4K) then allows air to continue through the system and replace the liquid behind it as it is processed, keeping the entire system in equilibrium and ensuring that no negative or positive pressures develop. To recover the sample, the adhesive tape is removed from the sample collection port and the vent hole directly above it. This again allows back flow of air into the sample collection chamber as the sample is removed.

The final novel fluidic function is related to problems often encountered with siphon valving on centrifugal platforms. When extracting liquid using a siphon, and especially in systems where liquid is intentionally left behind (*e.g.*, cell/viral debris in the capture chamber), remaining liquid tends to gather at the base of the siphon, and can re-prime the siphon once the CD speed is lowered again. This clogs the system fluidics and prevents liquid from moving further downstream. This has severe implications in integrated systems, where many subsequent high and low spin speed cycles are required to continue processing fluids. Channels can become clogged upstream at the siphon, and worse yet, the re-primed liquid can continue downstream, contaminating sensitive assay steps (*e.g.*, PCR). This problem has been elegantly solved by placing a capillary valve inline with the siphon. This prevents liquid from re-priming the siphon at low speeds and disrupting further fluidic functions.

The CD system presented here provides many unique fluidic implementations for a rapid and robust sample lysis and homogenization system. In addition, the system has been designed from the ground up specifically for integration with further downstream steps (*viz.*, nucleic acid amplification and detection). The extremely compact footprint of the device provides extensive real estate for further fluidics, the siphon and capillary features have been designed to eliminate integration problems, and the entire CD is self-vented to maintain a closed system.

3.1.2. Magnetic actuation. The magnetically actuated lysis system presented here combines friction created by Coriolis effects on samples with milling beads and mechanical impaction due to magnetic actuation, creating an efficient lysis platform. A visualization of the magnetic actuation sequence can be seen in Fig. 7. As the lysis chamber rotates, a magnetic coupling is created between the on-CD magnetic lysis disk and the off-CD stationary magnet (Fig. 7A). Once the lysis chamber moves over the next stationary magnet, the ferromagnetic lysis disk inside the lysis chamber snaps through the CD and hits the wall nearest the next magnet, inducing sample damage by hitting the wall and propelling milling beads between it and the wall (Fig. 7B). As the CD continues to spin, the on-CD magnetic lysis disk is pulled along the chamber wall, and grinding of the sample occurs between the magnetic lysis disk, the milling beads, and the wall (Fig. 7C). Finally, the magnetic lysis disk hits the outer edge of the chamber causing another impaction, and putting it in a position to repeat the actuation sequence (Fig. 7D). During high-speed rotation, this impaction occurs very quickly, causing mechanical disruption of the biological sample due mainly to shear forces in the system. Additionally, the CD spin direction is oscillated between clockwise and counter-clockwise directions. The resulting forces (including Coriolis) help to lyse and also homogenize the sample. The result is a multi-force system

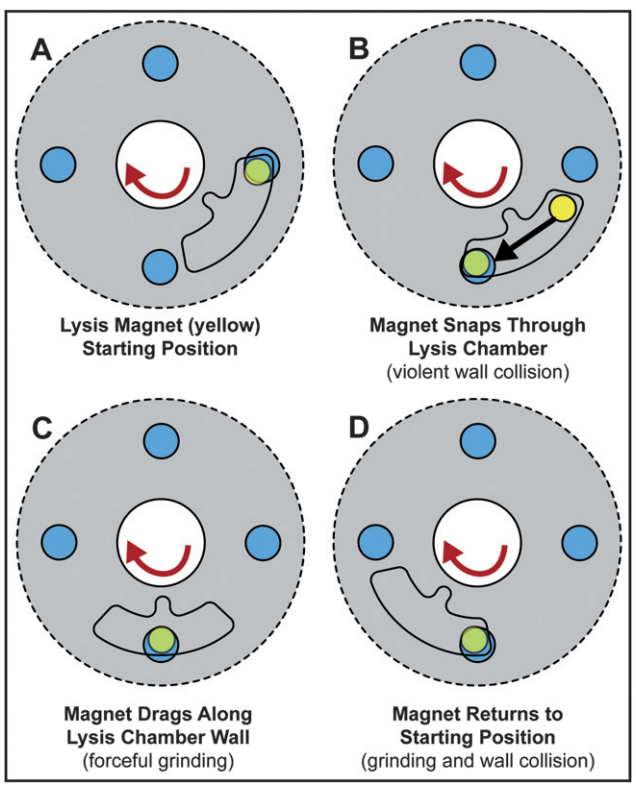

Fig. 7 Schematic showing the magnetic snap–drag lysis motion as the on-CD lysis magnet (yellow) interacts with the stationary off-CD magnet (blue). For clarity, only a single lysis chamber is shown. (A) Lysis magnet starts at the edge of the lysis chamber, attracted to a fixed magnet beneath, (B) upon exposure to the magnetic field of another fixed magnet, the lysis magnet snaps through the lysis chamber, (C) as the CD continues to spin, the magnetic attraction between the lysis and fixed magnet remains, and (D) the lysis magnet returns to the starting position, so the actuation sequence can be repeated.

capable of performing efficient sample lysis and homogenization. Moreover, no additional power is required beyond that of the control motor, and the off-CD magnetic platform can be re-used. The CD and the on-CD magnetic lysis disks and beads are the only disposables of the system.

3.2. Biological performance

There are few, if any, reports in the literature showing the dual capabilities of a microfluidic lysis and homogenization platform for cells as well as viruses. In both cellular and viral processing cases presented here, the CD showed equivalent lysis and homogenization efficiency to the standard, in-tube RUCLANAP method, which already has commercial applications (BD GeneOhm™ Lysis Kit). It is known that bacterial spores are the most difficult samples to lyse, and that NPA viral respiratory samples, while easier to lyse, can suffer performance from a lack of homogenization. Over 50 experiments were run using bacterial spores, providing a robust validation and excellent indicator of experimental repeatability for lysis applications. Moreover, the rare report of testing the microfluidic system with real-world samples from real patients showed the viability of this system for lysis and homogenization applications; 8 such patient samples were processed, showing excellent proof-of-concept for the system as a clinical tool.

3.2.1. Bacterial spore lysis performance. Lysis efficiency testing using bacterial spores as a model of hard-to-lyse micro-organisms is shown in Fig. 8. The CD platform processing yields, after heating, PCR amplifiable material equivalent to the bead-beating tube method. The C_t values for both methods (each $n = 24$) are statistically identical, thus validating and displaying equivalent lysis performance of the CD platform. The no-lysis control group had a higher C_t to be expected from residual DNA adsorbed on the spores upon sporulation.

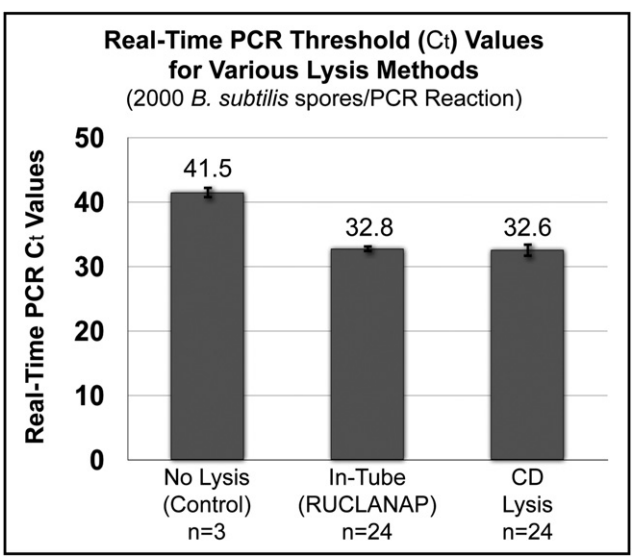

Fig. 8 Comparison of bacterial spore lysis efficiency between the CD platform and lysis in a tube (RUCLANAP). Real-time PCR amplification levels are expressed as cycle threshold values (lower value indicates higher lysis efficiency). The CD system shows *B. subtilis* lysis efficiency equivalent to that of the in-tube, gold standard.

Table 1 Comparison of two lysis and homogenization methods using eight NPA clinical samples. Detection of respiratory viral targets was identical in the RT-PCR assay for both methods, showing equivalent lysis and homogenization function (entero: enterovirus, adeno: adenovirus, and HMPV: human metapneumovirus)

Sample	In-tube	On-CD
H0607-145	Negative	Negative
H0607-154	Negative	Negative
H0607-161	+Entero	+Entero
H0607-163	Negative	Negative
H0607-165	+Entero, adeno	+Entero, adeno
H0607-182	+HMPV	+HMPV
H0607-183	+Entero, HMPV	+Entero, HMPV
H0607-187	Negative	Negative

3.2.2. Clinical specimen lysis and homogenization. In order to verify the usefulness of such a lysis and homogenization system for integration in a NA analysis device for *in vitro* diagnostics, 8 clinical samples from real patients were tested. Table 1 shows the diagnosis results of these samples as scanned for 3 different respiratory viruses or group of viruses. The diagnosis results of the in-tube method are identical to the on-CD method, thus validating the lysis and homogenization capabilities and demonstrating that the CD platform is suitable for integration towards a respiratory virus sample-to-answer detection device. It is worth noting that lysis and homogenization have been shown for real-world clinical samples containing both RNA and DNA-based viral targets.

3.3. Practicaltiy of use

While efficient lysis and homogenization for nucleic acid extraction have been shown, it is worth discussing some of the practical implications and limitations of this system. The disposables of the CD system have been kept low cost, at <$2 US per CD, translating to a low cost per test of only 50¢; this can be further reduced in a mass-manufacturing setting. As discussed, operation of the system is simple, requiring only addition of the specimen and then removal of the prepared sample 6 min later. Automation of the system translates to less user-introduced error and contamination, thus reducing the percentage of false positives. Moreover, automation has obvious cost advantages in terms of reduction in labor.

The system presented is not a high-throughput system, but it is rapid and easy-to-use. As mentioned, the CD-based lysis and homogenization platform have been designed to be part of a sample-to-answer system for clinical diagnostics, and not to replace larger, higher-throughput methods.

While this system was tested and validated using spores and clinical viral respiratory samples, it should be noted that the system has further potential applications beyond those of clinical diagnostics, such as for the analysis of food samples for bacterial contamination and perhaps water testing as well. However, the CD does have some clinical limitations, insofar as the system would not be able to prepare PCR-ready samples directly from whole blood; an additional centrifugation and blood-plasma extraction step would have to be added upstream of the lysis and homogenization steps.

4. Conclusions

Even with the development of a compact sample lysis and homogenization CD, obstacles remain to creating a sample-to-answer system for nucleic acid analysis. For such a device, multiple analysis steps must be performed, including sample preparation, amplification, and detection. In order to successfully develop such a device, each step must be designed with future integration in mind. The authors have done this here by designing a new system with a smaller footprint amenable to integration. By changing the experimental setup from a radial length system to one that works in the inner radius of the CD, space has been created for additional analysis steps. Several new centrifugal microfluidic functions have been implemented, and considerations for practical use in the clinic have been kept in mind. The microfluidic engineering and biological functions of the system have been proven, and the device is ready for use in the clinic. Next steps will include incorporation of the heating step, and integration with additional processing steps such as NA amplification and detection, towards a sample-to-answer system for NA *in vitro* diagnostics.

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