

Microfluidics for biological analysis: Triumphs and hurdles of CD platforms

JONATHAN SIEGRIST¹, RÉGIS PEYTAVI², and MARC MADOU^{3,*}

¹*Department of Biomedical Engineering, University of California, Irvine, CA 92697*

²*Centre de Recherche en Infectiologie de l'Université Laval, Centre Hospitalier Universitaire de Québec (Pavillon CHUL), Sainte-Foy, Québec, Canada, G1V 4G2*

³*Department of Mechanical and Aerospace Engineering, University of California, Irvine, CA 92697*

**e-mail: marcmadou@me.com*

Research on microfluidic technologies has become extensive, particularly in regards to the development of sample-to-answer platforms focusing on protein and nucleic acid diagnostics. However, as of yet there have been no such systems to reach the market with as much success as, for example, the handheld blood glucose meter. Many of the hurdles yet to be overcome in the nucleic acid diagnostics field revolve around preparation of the biological sample, a subject often ignored or only taken into account as an afterthought. Here, the steps typically involved in a nucleic acid diagnostic system are presented with an emphasis on sample preparation. Centrifugal microfluidic platforms based on the compact-disc format are discussed, along with the advantages they hold to tackling each of the steps involved in molecular diagnostics. Finally, an overview of the challenges that remain to the development of a microfluidic nucleic acid diagnostics system are presented, with solutions and a forward-looking description of a system that can be used both in and out of a standard laboratory setting.

1. Introduction

While the field of microfluidics is expansive, the focus is often on molecular diagnostics. Microscale phenomena can be taken advantage of to efficiently prepare biological samples for the detection of nucleic acids (NA) and proteins [1]. Indeed, the 'holy grail' in this field is the development of a single system that can accept a wide range of biological samples, such as blood or saliva, perform the required sample preparation and analysis, and quickly output an answer with little or no user input required, save for the initial sample introduction. There are few successful examples of such sample-to-answer systems for nucleic acid and protein diagnostics in research or development, and even fewer examples that are commercially available, if any [2,3]. In contrast, there are notable sample-to-answer successes for measuring simpler

analytes in biological samples, such as finger-stick blood-glucometers, Abbott's blood gas and electrolytes panels, and urine-based pregnancy tests [4].

Over the last 10 years, research towards implementation of NA and protein-based diagnostic tests using microfluidics has skyrocketed, driven in large part by the rapid progress made in the field(s) of molecular biology and molecular diagnostics [5]. 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. Often the emphasis is placed on the amplification of the NA analyte or on a more sensitive and/or selective detection of the NA or protein. Commonly, little or no emphasis is placed on preparing the sample itself, or taking into consideration how and from where the sample is obtained [6].

Keywords. Microfluidics; centrifugal; molecular diagnostics; sample-to-answer.

The neglect of sample preparation is one of the most important pitfalls that has prevented a more widespread success of portable, integrated, microfluidic system for molecular diagnostics, and NA diagnostics in particular. Here, the authors attempt to logically break down and define the general steps required in such a sample-to-answer NA system, with an emphasis on sample preparation and technology description and clarification. The argument is made for centrifugal microfluidic devices, and the authors present their vision of the future for this field using centrifugal platforms as an example. Finally, challenges and solutions are presented that reach beyond just centrifugal platforms.

2. Background

There is a myriad of terminologies used in the field of microfluidics to describe an integrated system that can accept a sample and perform a complete analysis: micro total analysis system (μ TAS), lab-on-a-chip (LOC), and, when referring to centrifugal platforms, lab-on-a-CD. Other terms include point-of-care (POC) and sample-to-answer. Sample-to-answer and μ TAS are approximate synonyms referring to the methods and systems discussed in this paper. LOC and lab-on-a-CD refer to particular steps or processes adapted onto microscale devices and centrifugal microscale devices, respectively, but do not necessarily indicate a complete system. Point-of-care refers most commonly to diagnostic tests performed at the patient's location, as opposed to sending a sample to a lab. In practice, all these terms are often used as synonyms. Here, the authors will use the terms μ TAS, POC, and sample-to-answer interchangeably when discussing complete, microfluidic, molecular diagnostic systems.

A common and general sample-to-answer process flow for NA diagnostics (1) is as follows: 2) sample collection, 3) sample preparation, 4) amplification, and 5) detection (figure 1). Variations in these steps are dictated by the type of sample collected. For example, a highly concentrated NA sample or a very sensitive detection scheme may negate the need for amplification. In the discussion that follows, however, all these steps are addressed.

2.1 Analyte

The first and most obvious step when performing NA diagnostics is collection of the sample. However, before discussing what types of samples can be collected, it is worth briefly mentioning what, physically, the tests hope to detect. NA tests will target either DNA or RNA as the analyte, as shown

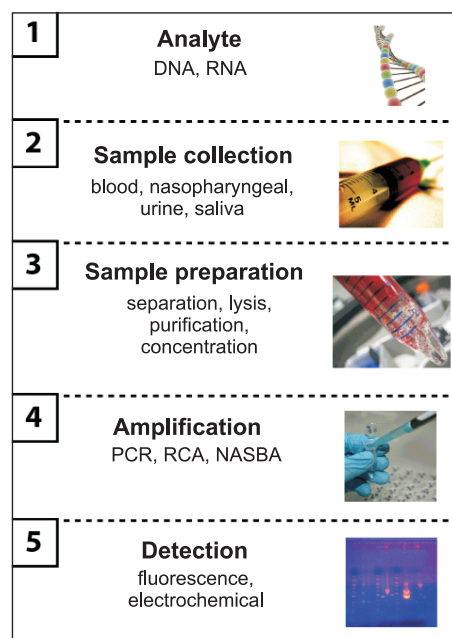


Figure 1. Process flow of a typical nucleic acid analysis, listing common examples for each step.

in table 1. In the case of DNA, one can detect the presence of foreign DNA (e.g., the DNA of a bacteria causing infection), or one can examine changes in genomic DNA (e.g., mutations that may be the source of cancer).

When targeting RNA, there are many different types to consider, including messenger RNA (mRNA) and ribosomal RNA (rRNA). rRNA has shown a high degree of evolutionary stability, and is present in relatively high concentrations inside cells [7]. For example, detection of 16S rRNA can be a good choice for identification of pathogenic strains present at low concentrations [8].

In the case of an extremely low pathogen concentration, such as with sepsis, mRNA can be a good choice for detection. One can choose to look for a specific host gene-marker expression response, elicited by the presence of a microbe, by profiling mRNA production of the infected organism. This allows for testing with significantly smaller sample volume sizes, which is more compatible with microfluidic disposables [9,10]. mRNA detection can also be an excellent option when one wants to genetically profile, for example, tumor tissue.

2.2 Sample collection & characteristics

The biological sample is almost always collected with a means independent of the microfluidic device that performs the analysis. Sample collection methods are not discussed in detail here, but it is important that the reader has an overall

Table 1. Analysis-relevant contrasts between DNA and RNA, listing common characteristics of each.

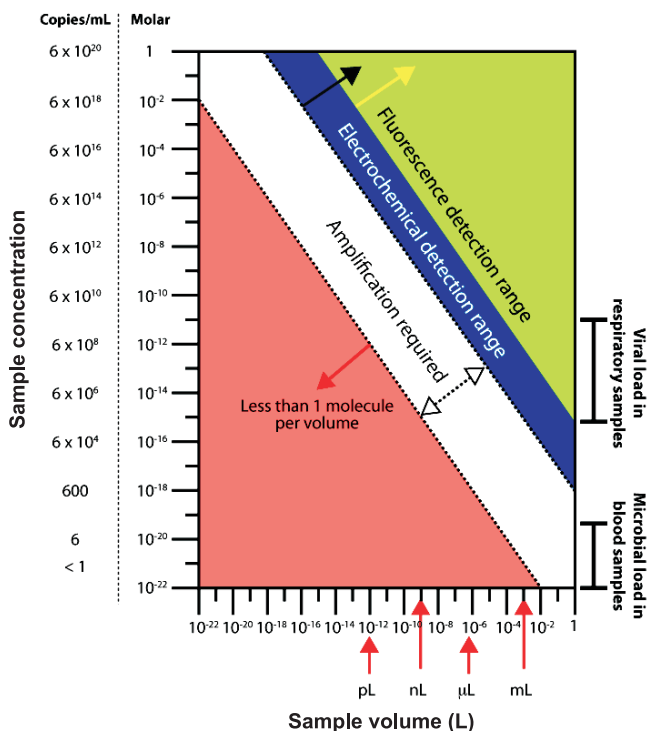
Characteristic	DNA	RNA
<i>Primary structure</i>	Double stranded (including linear and circular) – requires denaturation or digestion to obtain single strand, often required for amplification and/or detection	Single stranded; secondary structure is often present, requiring denaturation for amplification and/or detection
<i>Type</i>	Genomic, plasmidic	mRNA, rRNA, as well as genomic (viral)
<i>Concentration</i>	Relatively low concentrations, but always present	Can be in relatively higher concentrations, but run the risk of having none present if not currently being expressed
<i>Location</i>	Inside cells and/or viruses	Inside cells and/or viruses
<i>Amplification</i>	Can be directly amplified by most methods	Must sometimes be reverse-transcribed into DNA before amplification (e.g., PCR); can also be directly amplified (e.g., NASBA)
<i>Diagnosis</i>	Can detect the presence of an analyte	Can detect the presence of an analyte as well as the condition of the analyte (e.g., are certain proteins being expressed)

understanding and appreciation for how a given sample is collected, and the impact this can have on further analysis. Without going into details about specific conditions and diseases, one can also gain an appreciation for sample types by listing the most common clinical samples collected: blood, urine, nasopharyngeal (including direct swabs and aspirates), tissue, fecal, saliva, and interstitial fluid to name a few. The reader is referred to the following reference for a more comprehensive overview of sample collection methods [11].

When designing downstream steps, it is essential that the minimum expected concentration

of analyte be known and kept in consideration. The scaling relation between sample sizes and concentrations can be seen in figure 2. With NA diagnostics, the analyte of interest is often present in very low concentrations and in some cases, such as sepsis, can be as dilute as <10 DNA molecules per mL of sample when directly targeting microbes [13]. This represents a concentration on the order of zeptomolar (10^{-21} M). In such a case, a very specific and powerful amplification coupled with a sensitive detection is required. This also dictates how much sample needs to be collected. If, for example, the test requires at least 100 copies of DNA for valid detection, then in the sepsis case above, 10 mL of blood is required at minimum. Somewhat contradictory, the microfluidic disposable must then be able to handle and process this macro amount of fluid. On the other hand, a much smaller sample volume will likely be required if processing respiratory samples for virus detection, as viruses can be present in concentrations as high as 10^9 per mL (10^{-11} M).

Other important sample parameters include fluidic properties, such as viscosity, surface tension, and density. These properties and, more importantly, the variation in these properties among different samples can have a significant effect on the microfluidic function of a given disposable. Additionally, biological inhibitors in the sample must be taken into consideration. For example, it is well known that the hemoglobin in red blood


Figure 2. Scaling of sample concentrations and volumes (based on [12]).

cells inhibits polymerase chain reaction (PCR), the most common DNA amplification method [14]. Interfering compounds must be identified and neutralized or removed. Finally, the physical and biological homogeneity of the sample must be considered, as this will largely dictate what types of sample preparation steps are required. For example, respiratory samples will need to be thoroughly homogenized to release and spread the analyte out evenly, and to remove large solids that could interfere with the microfluidics.

There are many factors to consider, and several of them are closely linked. While average sample characteristics can often be deduced or even extracted directly from the literature, the authors wish to emphasize that it is extreme deviations from the average that cause a system to fail. Importantly, the sick patients for whom the system is used represent those extreme cases. For example, the disposable may well be able to process blood from a healthy patient with a normal hematocrit level (red blood cell count). However, the disposable may fail when hematocrit levels are particularly high or low, as is the case with many ill patients for which the test is designed. As another example, ill patients have particularly viscous nasopharyngeal samples, which can easily clog even the largest of microchannels in a fluidic device. Only with a keen understanding of the sample and its characteristics can the downstream analysis steps be robustly designed.

2.3 Sample preparation

Once the sample has been collected and introduced into the analytical system, the first task is to prepare the sample for analysis. The most crucial aspect of sample preparation is lysis. The NA must be released from the cells and/or viruses to make it available for amplification and/or detection. Again, it is necessary to know in detail the characteristics of the sample from which the NA will be extracted. For example, if the analyte is RNA, it may be present in viruses, host cells (e.g., white blood cells), foreign cells (e.g., bacterial cells), or a combination thereof.

Various forms of lysis have been developed and are in use, including enzymatic lysis (digestion via enzymes), chemical lysis (breakdown via detergents), plasma lysis (disruption via electrical charge pulses), and mechanical lysis (breakdown via physical means). The two most common types of lysis are chemical and mechanical [15]. Chemical lysis methods can leave behind residual substances that must often be removed or filtered out. However, these methods require little or no power and can be activated just by mixing or reconstitution of reagents. Mechanical disruption

requires more energy, but leaves behind no residual chemicals. However, it often requires the sample to come in contact with some energy transfer medium (e.g., beads). Nucleic acids may adsorb to this medium, decreasing the concentration and ultimately degrading amplification and detection steps.

Mechanical lysis is the most effective method for breaking down cells that have thick cell membranes, such as Gram-positive microbes, and for successfully extracting intact DNA [16,17]. One mechanical lysis method known as bead beating is the most efficient method in this respect, and functions by combining cells with an agitated mixture of milling beads [18,19]. This method can be well controlled by altering the bead size and the extent of agitation.

The mechanical lysis methods mentioned are not selective; all cells and/or viruses are disrupted. In some cases, especially when preparing blood samples, it may be necessary to perform a separation step before lysis. Separation steps usually occur on the cellular or viral level. When processing blood, the red blood cells, white blood cells, and plasma can be separated using a centrifugal density gradient. Often times the host NA present in white blood cells can severely hinder amplification or detection methods, and so must be removed or avoided all together by processing only the plasma. Antibodies can also be used to capture particular cells or viruses of interest. As mentioned above, there may be inhibitors within the sample that can hinder downstream steps. Separation steps are usually used when this is the case.

While separation steps can greatly reduce the amount of interfering compounds, it may also be necessary to perform a purification step on the NA, in which the NA of interest is isolated. This step occurs after cell lysis, and usually accompanies a concentration step in which beads functionalized with NA capture probes gather the NA of interest as the lysed sample flows through. Then, the captured NA is eluted (e.g., by heating or pH change) into a defined buffer volume. This solid-phase capture technique serves to both purify and concentrate.

In theory, most or all of these steps should be used to ensure that a very concentrated and clean sample is delivered to the amplification and/or detection steps. In today's practice, this often means a very complex, lengthy, and costly system. Imagine a microfluidic system designed for blood-based microbe detection that accomplishes the following tasks: it accepts a blood sample (say with a volume on the order of several mLs), performs a centrifuge-based separation step to isolate the plasma (or perhaps the white blood cells and plasma), performs mechanical cell lysis step on the

plasma to release the microbial DNA, flows the resulting lysate over a column of microbeads functionalized with capture probes that hybridize only with the microbial DNA of interest, and finally washes an elution solution (with a volume on the order of μL) over the same capture beads to release only the microbial DNA. The result is a much smaller volume of solution containing a clean, pure, and concentrated DNA sample. The reader is encouraged to envision what such a complex system might look like, and to recognize this does not even include the amplification and detection steps to come.

2.4 Amplification

Once the sample prep steps are completed, the next required step is usually amplification of the NA of interest (i.e., the target). This most often takes the form of polymerase chain reaction (PCR), in which DNA is amplified by the use of DNA polymerase, or reverse transcriptase-PCR (RT-PCR), in which RNA is first reverse-transcribed into DNA, and then amplified using PCR. The authors will not discuss in detail how these methods work, but instead will focus on existing alternate methods and terminology issues that arise when working with NA amplification.

While PCR requires the sample to be thermocycled between at least 2 different temperatures, other NA amplification methods exist that allow for amplification at a single temperature – isothermal amplification. Most isothermal amplification methods are enzymatic-based and amplify, much like PCR, the nucleic acid target. Examples of these alternatives include helicase dependant amplification (HAD) [20], recombinase polymerase amplification [21], and Nucleic Acid Sequence Based Amplification (NASBA) [22]. If methods such as these are used, the amplified target can be detected using the same detection systems for PCR product analysis. Other isothermal enzymatic amplification technologies do not amplify the targets themselves, but instead amplify the primers. Such is the case for rolling circle amplification (RCA) [23] and Expar, which can produce a 10^6 -fold signal amplification in 5 minutes as detected by nanobead agglutination [24]. Other isothermal amplifications are not enzymatic-based: branched DNA amplification [25] relies only on hybridization, Nanosphere’s technology uses gold nanoparticles to catalyze silver deposition when a specific NA is present [26], and fluorescent chain reaction (FCR) uses polythiophene polymers to detect the NA of interest in very small quantities [27]. The reader is encouraged to review the referenced publications to appreciate the variety of amplification mechanisms available.

While PCR provides a very specific and intense amplification, it can be very time and power consuming, as many temperature transition cycles must be completed. In the case of many analytes, a non-PCR method may be ideal, as it could provide time and power benefits. When deciding on which amplification method to use, or indeed if one is required at all, it is necessary to fully characterize the sample and the conditions of amplification desired. Characterization of NA amplification abilities is often an extremely difficult task.

Confusion abounds when engineers and molecular biologists discuss NA amplification methods. This can be attributed both to nomenclature confusion and unqualified statements about amplification capabilities. Standard PCR is singled out for the sake of this discussion.

Molecular biologists refer to running a PCR experiment or assay as ‘running a reaction’ (often abbreviated ‘rxn’), and refer to the number of copies of starting analyte DNA in a reaction – e.g., they would say ‘we are running a rxn with 100 copies.’ This statement does not include important qualifiers that describe the conditions of the assay, such as the liquid volume of this reaction (are those initial 100 copies dissolved in 50 pL or 50 μL ?), which makes a huge difference in terms of the initial concentration of DNA. This has severe implications for the sample preparation steps, as it could mean the difference between requiring a run-of-the-mill DNA concentration step or a very intense concentration step.

Scientists and engineers are also guilty of confusing the terms limit-of-detection (LOD) and sensitivity when discussing PCR. Limit of detection refers to the smallest amount of material one is able to detect (viz., the low limit-of-detection). Sensitivity refers to the slope of the calibration curve for a given detection system. In the case of fluorescence detection, if the amount of emitted fluorescence changes only a small amount, but the detection system signal changes by a large amount, then the detection system is sensitive. If the detection system does not output a significant signal when the fluorescence concentration is less than 10^{-12} M, then the LOD of the system is 10^{-12} M.

When discussing PCR, the term sensitivity is often incorrectly used in place of LOD. The statement ‘this PCR assay has a sensitivity of 10 copies’ provides no real information. The intent of the statement is to convey that the LOD of this specific PCR assay under a given set of conditions (which remain unstated) is 10 copies. This means that for this given PCR assay, in a given volume, under given conditions, no signal is detected when less than 10 copies of starting DNA analyte are used. Keep in mind that this statement also encompasses the capabilities of the detection

method – is the detection based on a fluorescence or electrochemical signal? Each comes with its own inherent limitations.

Engineers have allocated a lot of resources to developing systems that can run faster PCR reactions in smaller volumes. A source of confusion in this area stems from statements such as ‘the system can run PCR in 5 minutes.’ Again, no qualifiers are given. Often, this statement refers only to the pure thermocycling capabilities of the system, with disregard for the actual PCR assay capabilities. A system with rapid thermocycling capabilities that can heat and cool a volume of fluid from $60^{\circ}\text{C} \rightarrow 72^{\circ}\text{C} \rightarrow 95^{\circ}\text{C} \rightarrow 60^{\circ}\text{C}$ (a typical PCR thermocycle) within 1 second will likely give very poor PCR results (i.e., the LOD will be very poor). In fact, it has been shown that slower, more uniformly controlled heating and cooling rates greatly increase the LOD of a PCR reaction [28]. Moreover, each PCR assay is different – under a given set of conditions, a PCR assay designed to detect a single analyte may exhibit a LOD of 10 copies. A similar assay designed to detect an additional analyte in a multiplex fashion (i.e., two different analytes are simultaneously amplified within a single PCR reaction volume) may show a LOD of 100 copies for the original analyte and 500 for the additional one. This often occurs as different multiplexed analytes compete for PCR chemicals (e.g., polymerase enzyme and nucleotides).

Finally, engineers’ attempts to run PCR in very small volumes usually ignore realistic sample concentrations. For example, a system with a single molecule LOD in a pL size droplet has a starting analyte concentration of $\sim 1 \times 10^{-12}$ M. For comparison, a standard yet robust $25\ \mu\text{L}$ PCR assay may exhibit a LOD of 10 starting copies. This translates to a starting concentration of $\sim 5 \times 10^{-19}$ M, a difference of 7 orders of magnitude better LOD as compared to the droplet PCR system! The reader should now have an appreciation for the complexity of characterizing a PCR system, and should realize that all conditions and qualifiers should be taken into account. Once amplification has taken place (if it was required at all), the final step is detection.

2.5 Detection

The final step in NA analysis is detection of the analyte. The detection step can be incorporated during amplification, and the most common method of this is real-time PCR (not to be confused with RT-PCR, which strictly refers to reverse-transcriptase PCR, as discussed above). Real-time PCR comes in various forms, all of which involve the monitoring of an increased fluorescence signal as amplification progresses (figure 3a–c). Once the

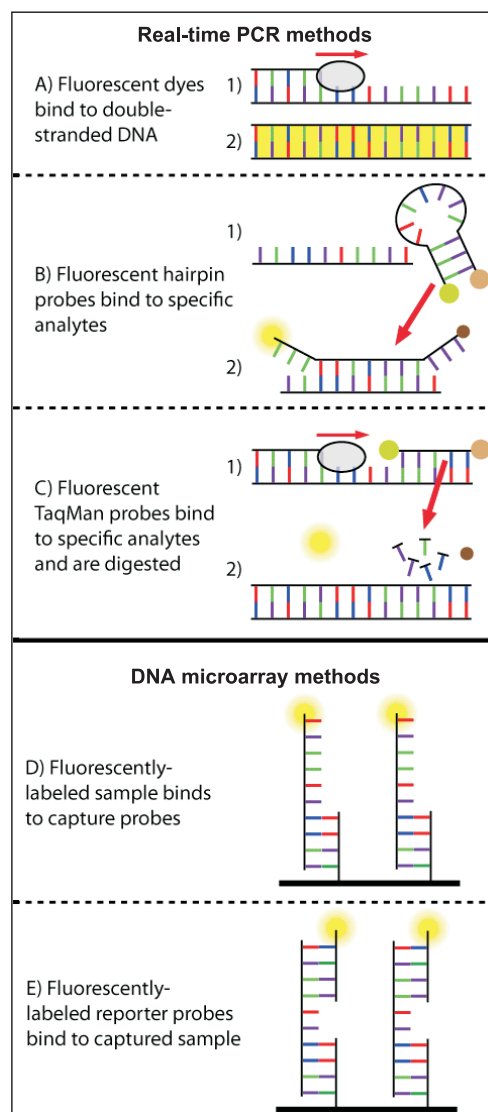


Figure 3. Schematic showing the most common forms of optical nucleic acid detection. a) Real-time PCR using intercalating dyes, b) real-time PCR using hairpin FRET probes that emit fluorescence upon analyte binding, c) real-time PCR using TaqMan FRET probes that release the FRET pairing upon digestion by a polymerase, d) DNA microarray using a labeled sample, and e) DNA microarray using a capture probe-analyte-reporter probe sandwich structure.

accumulated fluorescence signal increases above a certain background fluorescence, or threshold, amplification is verified. The cycle number at which this verification occurs is often referred to as C_t (threshold cycle). When references are included in a multiplexed fashion (e.g., 10 copies of reference A and 1000 copies of reference B), a starting-concentration calibration-curve can be created based on C_t values. This can then be used to back-calculate the starting concentration of the analyte(s) of interest. In this case, the real-time PCR becomes quantitative, and is referred to as qPCR.

The various forms of fluorescence labeling for real-time PCR include the use of dyes that bind to double-stranded DNA and become fluorescent (figure 3a). An example of this is the commonly used SYBR green dye (SYBR Green I). These have the disadvantage of not being specific – they bind to all double-stranded DNA.

As an alternative, fluorescently-labeled DNA probes can be used to provide specificity. These have the additional advantage of allowing multiplexing, as different fluorophores can be used for each analyte. However, they remain expensive. These use Förster resonance energy transfer (FRET) probes (i.e., a reporter and a quencher) to minimize fluorescence until a binding or polymerase-extension event occurs. In the former case, a hairpin DNA probe is often utilized with FRET quenching near the probe's end until bound with the analyte (figure 3b). In the latter, the probe is digested by the polymerase, thus separating the FRET probes. This is currently the most common real-time PCR method used, and goes by the trade name of TaqMan (viz., TaqMan probes) (figure 3c).

Limitations on PCR chemistry and fluorescent probes prevent robust multiplexing beyond 4–6 analytes at a time. When more analytes are of interest, optical DNA microarrays are often used for detection (figure 3d–e). Capture probes designed to hybridize with the NA of interest are immobilized (usually with the use of a spotter) on a substrate. The sample is fluorescently labeled (usually by using PCR to incorporate fluorescently labeled primers) and is applied to the substrate, followed by a wash and/or rinse to remove nonspecifically-bound and adsorbed analyte (figure 3d). Alternatively, the sample (non-labeled) can bind to the capture probes, and in a second step a fluorescently-labeled reporter probe binds to the captured sample, forming a sandwich structure. This has the advantage of added specificity by the use of dual probes, but the disadvantages of increased complexity (figure 3e). Capture probe spots that become fluorescent indicate successful hybridization and detection.

As with optical microarrays, electrochemical microarrays can achieve similar results, but often with less expensive hardware. However, they often require complex electrical multiplexing, as each detection spot will require sensing electrode(s). These detection schemes often utilize enzymatic labels, and the enzymatic products are detected electrochemically. For example, DNA can be labeled with horseradish peroxidase via a biotin-streptavidin chemistry. These methods have the benefit of built-in signal amplification, and may negate the need for NA amplification in some cases.

Microarrays allow virtually simultaneous screening of many analytes at once. In contrast to real-time PCR, they may dictate the need for a less-specific PCR assay – one that amplifies many different analytes at once. Specificity is then achieved through the microarray capture probes. If signal amplification can be built into the detection scheme, then NA amplification may be eliminated, such as with FCR and Nanosphere's technology, referenced above.

3. Centrifugal microfluidics

Microfluidic platforms hold much promise to tackle and integrate the NA diagnostics steps discussed here. 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 include reduced reagent use, significantly decreased total processing time, and increased abilities for high-throughput and/or parallel processing [1,29].

As with any field, microfluidics does not come without its particular set of challenges. Foremost is the issue of laminar flow typical for the low-Reynolds number conditions in many microfluidic conduits. Mixing, required for fast reactions, in low-Reynolds number conditions remains challenging. Other problems include pumping and the ever-present experimentalist's enemies – surfactants, which can cause many valves to fail, and channel-clogging air bubbles. The authors believe that centrifugal (CD) microfluidic platforms provide many elegant solutions to overcome some of these limitations inherent to microfluidic disposables.

3.1 CD advantages

While developing fluidic devices of any kind, the main concern is how to get liquids to and from the areas of interest in a controlled manner. This general problem can be summarized as the need for two technologies: pumps and valves. The centrifugal compact-disc (CD) platform provides elegant, simple, and effective modes of pumping and valving.

Fluid propulsion on the CD is performed by centrifugally induced pressure on the fluid as the CD spins, and Madou *et al* and Duffy *et al* have characterized this type of flow extensively [30,31]. The volumetric flow rate is dependent on the speed at which the disc spins, the distance from the center of the disc, the geometry of the fluidic channels, and the fluidic properties (namely density, viscosity, and surface tension) (figure 4a). By using combinations of different channel geometries and spin

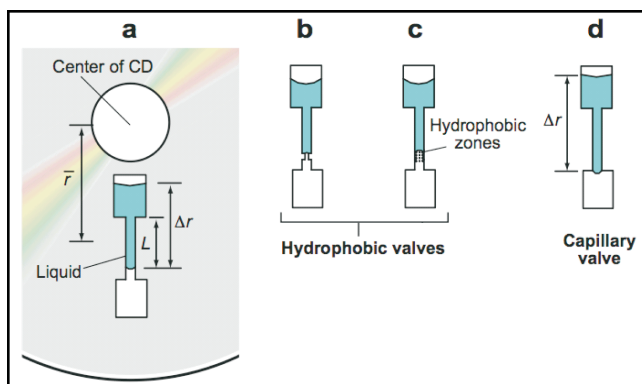


Figure 4. Schematic of microchannels on a CD. a) Two reservoirs connected by a single channel, b) hydrophobic valve made by a channel restriction in a hydrophobic material, c) hydrophobic valve made by functionalization of the channel surface with hydrophobic material, and d) capillary valve made by a channel widening in a hydrophilic material (adapted from [33]).

speeds, flow rates ranging from 5 nL/s to 0.1 ml/s can be achieved with a high degree of accuracy and precision. Typical fluid-pumping rotation speeds used range from 300 to 2000 RPM.

Centrifugal pumping forces on the CD provide many advantages over some of the other pumping methods available, such as syringe, peristaltic and electroosmotic pumping. Today's pressure-driven syringe and peristaltic pumps provide very good control over large flow rates, but can be unwieldy when trying to miniaturize and/or process in parallel [32]. In addition, the pressures needed to move fluids through microchannels scale disadvantageously as $1/r^4$, so they become very large in the microdomain. This makes implementation into small, high-throughput platforms difficult. Electroosmotic pumping methods overcome these problems, as they scale more advantageously in the microdomain and can be easily adapted into microchannels using microfabrication. However, these methods are highly dependent on pH and ionic strength of the fluid being pumped [32]. The high-voltage power supplies ($\gg 1$ kV) required in these systems make them expensive and rather impractical. Centrifugal forces, however, do not need large power supplies (only a low-power motor), are not dependent on pH or ionic strength of the fluid, and do not need fluidic interconnects or tubing for force application. They also provide forces across the entire length of a fluid element, providing smooth and controlled flow. This has additional benefits, as bubbles that develop have less chance of disrupting downstream fluidic processes. In the authors' experiences, bubbles have been a negligible issue on CD platforms. In addition, many individual systems can be placed on a single CD, making parallel processing easy.

When combined with the simple valving solutions available on the CD, powerful platforms can be developed.

Valving on the CD is performed using two main valve types: hydrophobic and capillary (figure 4b–d). Hydrophobic valves can take two different forms: one utilizing changes in channel geometries (figure 4b) and the other utilizing surface modification (figure 4c). In both cases, the fluid can be forced past the hydrophobic valve by increasing the spin frequency past a critical value. The capillary valve is commonly used in CD platforms, and is a result of the balance between centrifugal and surface tension forces in a hydrophilic material (figure 4d). 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 higher frequency, 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. This has in fact been implemented to obtain sequential control of valve openings.

For a more comprehensive overview of CD platforms, consult the literature [33]. The advantages of CD platforms discussed above have led to the development of several platforms geared towards NA diagnostics. When combined with the fabrication methods described below, centrifugal microfluidic platforms can be prototyped with relative ease.

3.2 CD fabrication

Most commonly, CDs for microfluidic applications consist of multi-layer structures made of inexpensive polycarbonate plastic and pressure-sensitive adhesives (PSA). Using relatively simple CNC machines, channel widths down to 1 mm are machined into stock polycarbonate plastic. A cutter-plotter is used to cut channel widths as narrow as $200\ \mu\text{m}$ in thinner materials such as $100\ \mu\text{m}$ -thick PSA or thin plastic films. Once the appropriate pieces have been designed and machined, they are aligned centrally and radially and laminated together using the PSA layers.

Even the simplest, microfluidic CD used in the Madou research group consists of no less than 5 layers: (1) top polycarbonate CD with CNC-machined sample loading, sample removal, and air venting holes (sealed using a thin adhesive film

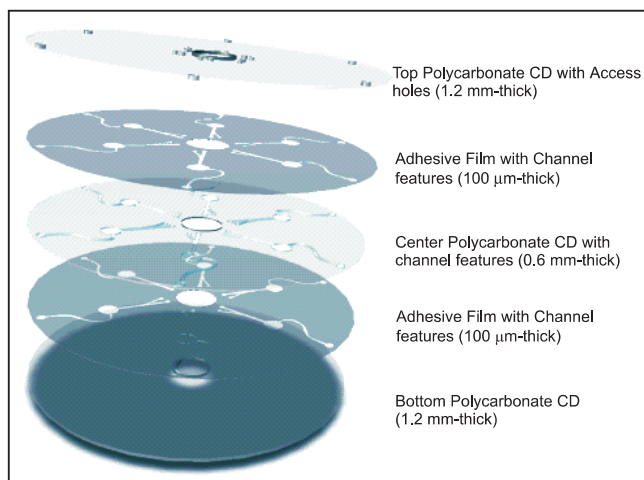


Figure 5. Schematic showing the assembly of a typical 5-layer microfluidic CD.

during operation), (2) pressure-sensitive adhesive with microchannel features cut using a plotter, (3) middle polycarbonate CD with CNC channel features, (4) pressure-sensitive adhesive with microchannel features cut using a plotter, (5) solid bottom polycarbonate CD to seal off the channels (figure 5). Microfluidic CD platforms can involve more layers to accommodate more complex fluidics. Moreover, different devices and substances can be placed inside the CD during fabrication, such as beads, lyophilized reagents, or filters. The CDs can also be exposed to O_2 plasma treatment or functionalized with bovine serum albumin (BSA) to create hydrophilic and hydrophobic surfaces, respectively. The fabrication process usually ends with running the CDs through an industrial press to ensure excellent adhesion and sealing between all CD layers.

While the majority of CD platforms developed by the Madou group utilize standard macro-machining processes, microfabrication is easily integrated onto the CD platform. This usually takes the form of creating microfluidic PDMS molds on 6" Si wafers using multi-level, thick-resist lithography. Once created, these soft-lithography PDMS parts can be placed on a polycarbonate CD.

The authors have found that using these fabrication methods allows for a manageable transition when moving from prototyping to mass production of injection molded plastic disposables. The ability to produce injection molded parts with microscale features is of great importance to success, and is discussed further in section 4.1.

3.3 Current CD platforms

The Madou group along with many others has developed several CD platforms geared towards

NA diagnostics and the analysis steps described above. While certain platforms will be highlighted here, this section is by no means an exhaustive review of all microfluidic CD achievements.

Above, the reader was encouraged to envision a blood sample preparation system that involves almost all available preparation steps. Along these lines, Ko *et al* from Samsung have developed a system designed to extract pathogens from μL -sized samples of whole blood [34]. Using CD fabrication methods from the Madou group as described above, a system was developed that works as follows: whole blood is separated via centrifugation, specific capture of cells/viruses from plasma using antibody-labeled beads is performed, the cells/viruses are eluted to perform concentration and purification, and finally lysis is performed. The entire platform runs in ~ 12 mins. This achievement is an excellent example showing the feasibility of combining several NA analysis steps onto one CD platform. As expected, however, the platform is complex, requiring several different components and technologies. Ferrowax (a mixture of wax and magnetic nano-particles) is used as a valve, which requires placement of the ferrowax inside the CD during assembly, a laser for heating, and a moving magnetic platform to actuate the wax once melted. Cell lysis is performed using the same laser, minimizing hardware requirements. However, the system was not able to prepare PCR-ready materials from whole blood. The authors assume this is because cell lysis is performed as the last step, possibly releasing inhibitors. This means yet another sample-preparation step would be required in a product-ready NA diagnostics μTAS system. This again underscores the complexity of sample preparation and the non-triviality of integrating sample preparation steps.

The most crucial aspect of sample preparation is lysis, and Madou *et al* have developed a stand-alone microfluidic cell lysis CD that relies on a bead-beating method powered by magnetic forces [35]. It utilizes small magnetic disks placed inside each lysing chamber that oscillate while spinning by interaction with stationary magnets on a static CD platform. This oscillation causes shear forces that result in lysis. Once lysed, solids are removed by centrifugation, and the supernatant (containing DNA) is extracted using a unique siphon valve. The platform is capable of lysing cells with thick walls and membranes while preserving the extracted DNA. Moreover, the platform has proven efficient for viral lysis and sample homogenization as well [36]. Validation of the CD platform showed that it can accept a raw sample of up to $60\ \mu\text{L}$ and deliver a clarified sample from both whole *E. coli* and yeast cells within ~ 5 mins. This system requires a magnetic platform (although stationary), and

placement of materials inside of the CD during assembly. It is also worth noting that this CD delivers only a clarified sample, and does not perform NA concentration or purification.

A common next step is amplification via PCR. Madou *et al* have developed a microfluidic PCR card system designed for future CD integration capable of rapid and efficient amplification [37]. Peltier thermoelectric devices were chosen to perform active heating and cooling of the PCR cards. Keeping future CD integration in mind, it was realized that not only will a liquid valve need to be incorporated, but a vapor valve will be required during thermocycling as well. Thermocycling causes vapors to accumulate as the liquids come close to boiling temperature (95°C). Large pressures build up as a result causing the samples to expand, and so the entire chamber needs to be sealed and isolated to avoid loss of sample. With Peltier devices already integrated onto the PCR platform, a novel ice-valve scheme was chosen in which Peltier devices are used to freeze a small plug of liquid at each end of the reaction chamber, thus preventing the movement of both liquid and vapor during thermocycling.

The PCR platform achieved rapid heating and cooling ramping speeds of up to 10° C/s. Because the Peltier devices allow active heating and cooling based on polarity of the current used, shorter thermocycling times were achieved as compared to standard PCR systems that rely on active heating but feature passive cooling only. Validation of the system's PCR abilities was performed by amplifying an *E. coli* gene in a 25 μ L reaction volume. A 40-cycle amplification was conducted using a temperature profile of 7s, 15s, and 15s (ramping times included) at 95°C, 60°C and 72°C, respectively. Using gel electrophoresis, the limit of detection observed was 10 copies for the particular assay and conditions chosen, but was completed in less than half the time taken on a standard benchtop thermocycler. This system provides an elegant solution to several PCR system problems, namely thermocycling abilities and valving, but requires a large amount of power both for thermocycling and ice-valve actuation. However, all hardware is left out of the PCR card, making for a good example of partitioning of functions between the disposable and the instrument.

The final step in NA analysis is detection. Madou *et al* have developed a rapid, flow-through DNA hybridization disposable designed for detection via optical DNA microarray [38,39]. The rate-limiting step when performing analysis with DNA microarrays is the time for hybridization to occur, which can take upwards of 18–24 hours. Enough time must be given to the DNA of interest to passively diffuse through the entire solution and be

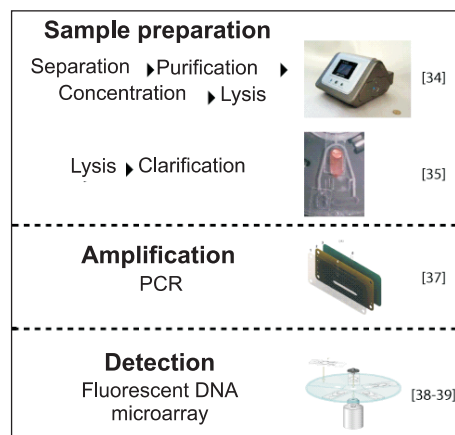


Figure 6. Sampling of current microfluidic platforms designed to tackle the steps of nucleic acid diagnostics.

exposed to every capture probe on the surface. By using thin and narrow microfluidic chambers, hybridization time can be significantly reduced by making the diffusion distances much smaller. Moreover, by incorporating active flow, mass-transport of sample DNA to the capture probes can be enhanced.

Using microfabrication, a PDMS microfluidic disposable was developed that provides equivalent or better performance (*viz.*, signal intensities and SNR) to standard array kits, and with a processing time of only 15 mins. Glass slides were spotted with capture probes, and the PDMS part with embedded fluidics passively adhered to the slides, which were then mounted in a centrifugal holder. Serial capillary valves were used to sequentially release the sample (fluorescently-labeled), a wash solution, and then a rinse solution based on increasing spin speeds. The slides were then spun dry, the PDMS removed, and the fluorescence scanned using a standard glass-slide scanner.

The system was able to detect the differences between several bacteria of interest, and the specificity was high. Moreover, the signal intensities were high, verifying that efficient hybridization did occur due to the gains in mass transport from the rapid, microfluidic flow-through system.

The microfluidic systems described here can be seen in figure 6, showing each platform and the steps it accomplishes. As should be apparent to the reader, robustly integrating those platforms listed into a single μ TAS for POC NA diagnostics is no small task. Many hurdles have yet to be overcome, including those inherent to centrifugal platforms.

3.4 CD challenges

While the CD indeed provides elegant pumping and valving options, the method is not without limitations. It is worth noting that few of the

CD valving schemes presented above act as vapor valves – they perform liquid valving only. Thus, liquid reagents could not be stored for long periods of time as evaporation and cross-chamber contamination could occur. In addition, high-temperature heating steps (e.g., PCR) would cause the sample to escape and evaporate. This would result in a failed step as well as possible channel clogging once vapors condense downstream. Such conditions require a valve that not only holds back liquids but prevents the movement of vapors as well, similar to the ferrowax valve as shown in the Samsung work, referenced above.

Pumping on the CD still requires energy to create the centrifugal forces. Other pumping methods, such as manually-driven pumps or lateral-flow, do not require onboard power sources. Perhaps the largest obstacle of centrifugal pumping is that it is unidirectional – fluids flow only from the center of the CD radially outward. In addition, the surface energy of the fluids being pumped greatly affects fluidic behavior, especially when implementing capillary valves. Thus, it becomes essential that a CD be designed and tested from the outset using the same biological fluids and samples that will ultimately be used in the final disposable. As mentioned above, the test fluids used must represent both the average and extreme cases of relevant fluidic properties.

The problems discussed so far are limited to CD platforms in particular, but there are many broader issues that will affect any microfluidic device designed for NA diagnostics. With an in-depth understanding of some of the biological and microfluidic issues facing μ TAS systems for NA diagnostics today, the reader can now better appreciate a discussion of these broader problems that will follow below.

4. A vision for tomorrow

The ideal POC NA diagnostics disposable of tomorrow must be as simple to manufacture and to use as handheld glucometers are today. POC glucometers can be used with little or no training, cost less than \$100 US to manufacture, work with small, standard batteries, and use disposables that cost mere cents to manufacture. An equivalent system for NA testing does not exist today because the methods used are far more complex than those needed for detection and quantification of glucose molecules. It is worth briefly contrasting the two platforms to gain an appreciation for the challenges that lie ahead.

One of the biggest differences between glucose sensing and NA detection is that glucose is present in much higher concentrations (on the order of

mM) as compared to NA. In addition, glucose is freely present in the blood – it is not bound up inside cells or viruses. This greatly simplifies sample preparation, as no lysis is required. In fact, no sample preparation steps at all are required when using a glucometer. Finally, the sole detection of NA molecules is insufficient to give an informative answer. A POC NA system cannot simply detect the presence of NA – it must detect a specific sequence of NA.

For all these reasons, NA assays are much more complex, requiring procedures such as lysis, purification and concentration, amplification, labeling, and specific sequence detection. Naturally, it is the well-established yet complex procedures carried out in benchtop equipment that have first been adapted into POC systems. This has led to complex, high cost systems that are a far cry from a simpler glucometer. To be successful, the POC NA system of tomorrow will have to be redesigned from the start as a simple, integrated instrument with a disposable cartridge, capable of low cost, low complexity, and low power operation. Moreover, the system must be ‘field-deployable.’ This means it must function in harsh conditions and under extreme temperatures outside of a clean, controlled laboratory setting. To achieve this, completely new strategies in terms of design, manufacturing, reagent storage, and power use will have to be established and developed in light of biological needs and constraints. Engineers and biologists will need to work together at each step to tackle each problem. No longer can obstacles be labeled as either the engineers’ problem or the biologists’ problem.

4.1 *Design and manufacturability*

While futuristic designs are often portrayed as complex, the successful products we know today are usually designed to be as simple as possible. This same strategy is needed for NA diagnostics. The system must be simplified as much as possible, on both the biological and microfluidic fronts, to reduce cost and increase robustness. In addition, the disposable must be able to make the transition from lab prototype to mass-manufactured part while remaining low-cost.

Complex biological assays can be very sensitive to changes in the environment in which they are run. Assays often show different results when performed by equally skilled scientists using the same equipment and lab space. The biological steps required for the μ TAS system of the future will need to be re-designed to be robust, simple, and far less sensitive to environmental changes. For example, current amplification assays such as PCR usually require very clean, pure DNA samples

for successful amplification with an optimal low LOD. It is unrealistic to expect a μ TAS system to deliver a perfectly pure sample to downstream steps, such as amplification and detection, with every use. Moreover, the purity of the sample delivered to these steps will likely vary, depending in part on initial sample characteristics. Thus, steps downstream of sample preparation must be designed to be robust, even in non-pure, ‘dirty’ solutions.

Optimized biological approaches can also help to reduce the required sample size. For conditions such as sepsis, the microbial load can be very low, requiring a large volume for detection, as discussed above. However, this is only true if the presence of the pathogen NA is directly detected. Alternatively, techniques could be used that focus on the host’s response to the pathogen, which would utilize detectable analytes that occur in much higher concentrations. An assay such as this would require much less sample volume and likely avoid an amplification step.

To minimize both the biological and microfluidic complexity, as many steps as possible should be combined. For example, performing lysis in the presence of beads functionalized with robust capture probes could combine lysis and concentration/purification steps. Real-time PCR is an excellent example where two steps, amplification and detection, have been elegantly combined into one. Perhaps NA amplification could be run in the presence of capture beads, again eliminating the need for a separate, dedicated microfluidic chamber for amplification. Tradeoffs and compromises between the assay and the microfluidics must be made.

The microfluidics of the disposable itself must also remain simple. To that end, the total number of chambers, channels, and valves required must be minimized. It will likely be valves and volume-dependent fluidic functions that cause the disposable to fail. If their number can be minimized and the parts made more robust, the disposable will have a much higher chance for success. There are a variety of valve technologies available, and if a single method of valving that can accomplish both vapor and liquid valving can be utilized, this will further reduce complexity.

Another large source of complexity comes from the need to place materials inside the microfluidic disposable for added functionality. Examples of this from the preceding descriptions include magnets, glass beads, filters, and other parts. These numbers should again be minimized, although their complete elimination will likely not be feasible, as they are a convenient way of adding functionality.

In order to make the POC disposable economically feasible, it will have to be mass-manufacturable. PDMS has become a well-established material for fabricating microfluidic devices in the laboratory. However, the material has severe limitations for the applications discussed here, namely small molecule absorption and permeability to water vapor, preventing it from being used for longer-term applications outside of the research laboratory (e.g., shelf-life for a commercial product must be more than 6 months) [40]. Moreover, under applied pressure, PDMS parts can change size, which can be detrimental to many applications. It must also be stated that the truly microscale features as enabled by soft-lithography will likely not be required. As has been discussed extensively, the volumes that the future μ TAS platform handles range from μ Ls to mLs. This translates to fluidic conduit sizes on the order of 100s of μ m to mms. Micro and nanoscale features designed to handle nanoliter volumes and smaller will likely not be necessary. Thus, the authors believe that soft-lithography PDMS and silicon-based MEMS microfabrication techniques are not viable manufacturing candidates for this application.

Today, there are few manufacturing techniques as economically viable as injection molding of plastics. The authors believe this manufacturing technology of today will be the one used to make the μ TAS disposables of tomorrow. However, some unique challenges are newly introduced, as the disposable must contain macro and microscale features on the same plastic part. The injection molding methods used must exhibit a large dynamic range of manufacturable sizes in order to deal with the widely varying dimensions and scales. Towards reduction in complexity, the device should also be made out of as few different materials as possible. Ideally it should be a monolithic structure made of 1 material only. The device more realistically will be made of at least 2–3 parts, and some parts may require bonding via thermal, chemical, or ultrasonic means.

Bonding of different parts is an incredibly important yet seemingly simple manufacturing step. The injection-molded microfluidic disposables will likely have initially exposed channels open for insertion of materials and reagents. Then, a featureless cover layer of plastic will be applied to close and seal the disposable. At this point the disposable will have sensitive components onboard, such as reagents, reviewed in detail next, and perhaps surface treatments. The bonding process used must not be too disruptive to the disposable. If chemical bonding is used, residues cannot be left behind that may interfere with analysis. If thermal bonding is used, the temperature may

not exceed $\sim 100^\circ\text{C}$, as this will likely inactivate any enzymes and other reagents onboard. Finally, the bonding method must also not disrupt or distort any of the microfluidic channels. Small imperfections, such as gaps or overhangs, can cause catastrophic failure in a microfluidic device, especially if the material is hydrophilic, such as after oxygen-plasma treatment.

4.2 Liquid storage and lyophilization

While the manufacturing process should be kept as simple as possible, the one perhaps unavoidable complication is the introduction of reagents into the disposable for storage. A collection of different reagents such as NA probes, reaction buffers/salts, enzymes/polymerases, lysis salts, etc. will likely be required for the μTAS disposable to function as a standalone-alone unit. It would greatly increase the complexity of use to have the user manually load all of these reagents before use, and so most if not all of these reagents must be stored on the disposable itself.

The μTAS disposable will be mass-produced, shipped to a location, and then stored until use. The disposable must have a shelf life of at least 6–12 months, and this means that the reagents required need to be preserved on the device for that same period of time. In addition, the preserved reagents must be able to withstand a range of temperatures that occur during shipping and storage. A common and well-accepted method of reagent storage is lyophilization (i.e., freeze-drying), in which reagents are formed into powders that then reconstitute when they come into contact with liquids.

A drawback of lyophilization is that liquid is required for re-constitution and that reconstitution in a confined space does take time. However, it will be essential that the disposable contain its own liquid reagents, as most of the assay steps required occur in aqueous form. Relying on the introduction of water from the user introduces a huge source of variability, as the introduction of water from unclean, contaminated sources could cause assay failure. In addition, assays are often sensitive to concentrations of reagents. While assay robustness will be an important contributor to success, the volume of liquids used will need to be well controlled by storing defined volumes inside the disposable.

Liquids can be stored long-term in glass ampoules or in plastic or metal pouches. These pouches can be pierced or broken open by physical pressure from the user or a mechanical actuator as part of the instrumentation. Packets or pods of liquid can be manufactured independent of

the disposable, and placed inside during disposable fabrication.

4.3 Environmental and power requirements

In order to meet operational requirements outside of the laboratory, the instrument must be low power and relatively unaffected by environmental conditions such as heat, humidity, and dirt.

Currently, NA amplification methods such as PCR are the most power demanding and lengthy processing steps. For example, the microfluidic PCR card developed by the Madou group uses over 150 Watt-hours of energy to power the Peltier thermocyclers and ice-valves during a standard PCR run. So, the authors believe there needs to be a movement away from PCR towards robust, low-temp, isothermal amplification schemes. This will greatly reduce the power requirements of the platform. Moreover, the authors believe that a more robust amplification method needs to be developed that does not amplify the NA directly, but rather another ‘reporter’ molecule. This new method would likely still rely on hybridization and/or polymerase extension events for initiation, but the result would be amplification of a reporter much easier to detect, without the need for power.

Moreover, some isothermal amplification methods can take less time than PCR. Time and power trade-offs must be thoroughly examined to minimize power requirements. In addition, different heating methods must be examined. While thermoelectric heating can provide precise control and rapid temperature changes, it requires intimate contact with the disposable and large amounts of power. Infrared heating is an excellent alternative that can provide good precision without requiring contact. Chemical heating by use of exothermic reactions requires no power, but can be difficult to control.

Thermal management of the disposable must also be considered. Just as the temperature of a computer processor must be controlled within a working range, the chamber holding the disposable will have to be heated and/or cooled to remain reliable. NA assay steps, such as hybridization, can rely on specificity by being at a constant, usually slightly elevated temperature (e.g., 37°C). The hardware inside the platform, for example circuitry, light sources, and detectors, will also require the temperature to remain within a working range. This will clearly require large amounts of power, as a platform functioning in the tropics where temperatures can reach 44°C or higher must be cooled, and a platform in cool environments will need to be heated. These thermal management requirements will exist on top of the requirements for specific

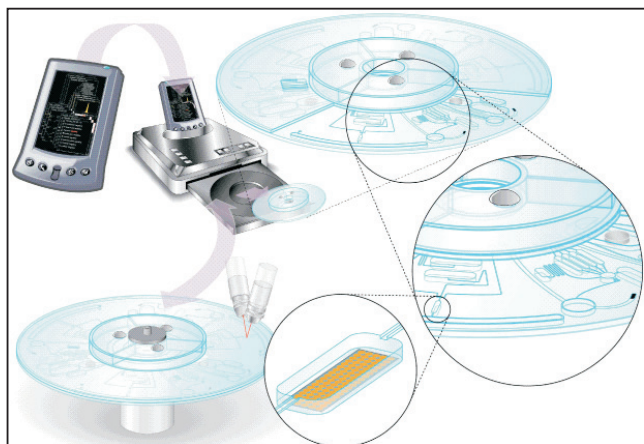


Figure 7. Artist's rendition of a CD system for NA diagnostics, showing a center-hub for large-volume sample preparation and optical detection via microarray [41].

heating and cooling steps for the assays, further adding to the complexity of the system.

The platform must also be impermeable to environmental factors such as dirt and humidity. Dirt and humidity, along with temperature, are common factors that rarely have to be dealt with in a laboratory setting. However, they can greatly affect performance of, say, amplification enzymes or specificity of DNA capture probes. Ignoring these real-world factors will lead to a system limited to use only inside well-controlled laboratories, and prevent the system from reaching those who need it most.

Finally, the disposable must be environmentally friendly to use and discard. In the case of infectious diseases, the samples will contaminate the entire disposable, making it a biohazard. Steps must be taken to ensure that, in the case of improper disposal, risk of infections from used disposables remain minimal. This will become extremely important following success of a system, as its use will become widespread and waste will become a dangerous problem.

4.4 *CD of the future*

With a firm understanding of the many hurdles that must be overcome to develop a truly integrated μ TAS system for POC NA diagnostics, the reader can fully appreciate the authors' vision for the future. With the many advantages that centrifugal platforms hold over standard microfluidic devices, the disposable of the future will be CD-based (figure 7). It will be disposable, and have all reagents (lyophilized and liquid) stored on the CD. The CD itself will be stored in a vacuum-sealed pouch impermeable to outside conditions, but will not require refrigeration for storage. The CD platform will be a larger-sized handheld instrument,

similar in scale to a portable radio, and will be able to process 3 patients per CD at minimum. The instrument will contain a single, small battery used to retain internal settings (such as calibration), but will derive its main power from 1 of 3 attachable options: hand-crank generator, rechargeable battery or fuel cell, or power cord. The CD platform itself will also include a handheld wireless device that stores all results and information. When a connection is available, wired or wireless, the device will upload results to a server. This will allow for an organized database of information to track the spread of diseases and possible deadly outbreaks.

The CD itself will be injection molded out of a single type of plastic – polycarbonate. It will consist of 3 different plastic pieces: a monolithic CD containing fluidics for amplification and detection as the middle layer, a solid, featureless bottom disc, and one of many various sample preparation hubs placed on top. As has been emphasized, the largest source of variability in NA diagnostic processing will come from the type of sample being processed. Thus, the authors believe it will be essential to have different sample preparation modules for different samples. Modular sample preparation hubs will be designed for different sample types: blood samples will require hubs capable of processing larger volumes, while respiratory samples will require smaller hubs. These various sample preparation hubs can be snapped into the standard CD bases designed for amplification and detection; this may be done during manufacturing, but to retain optimal flexibility and modularity, the end user may select the appropriate sample preparation hub.

The majority of reagents will be lyophilized and placed inside the CD during manufacturing. The remaining reagents will be in liquid form and stored in metalized pouches that will be placed inside the sample prep hubs during manufacturing. The CD will include beads for lysis and solid-phase concentration/purification. The CD will utilize DNA microarrays for detection, and so capture probes will be spotted onto the CD during manufacturing as well. Finally, the bottom disc of the CD will be thermally bonded to the top disc, ensuring no disruption of the components inside the CD occurs due to extreme bonding temperatures.

The system prepares samples using a combination of chemical lysis, and light low-power mechanical agitation. Concentration and purification steps are performed using solid-phase extraction, but this step is combined with lysis to reduce complexity. The system performs amplification using a low-temperature, isothermal, nucleic acid amplification method. Heating on the platform, for steps including isothermal amplification and

hybridization, is performed using a small infrared lamp in combination with exothermic chemical heating. A field-deployable μ TAS system for NA detection will likely not scan for only a few analytes, but rather a large panel of analytes. Thus, detection will be performed with standard, fluorescent DNA microarrays using low-power, inexpensive optical hardware (i.e., LED or laser-diode light sources and photodiode detectors).

Operation of the CD platform is as follows, using respiratory samples as an example: **(1)** user snaps in a fresh battery to the bottom of the platform, selects an appropriate sample preparation hub for respiratory samples and then attaches it to the standard amplification & detection CD base, **(2)** respiratory sample for each of the 3 patients is added to the hub, and the hub is closed, **(3)** user manually pushes 3 buttons on the sample prep hub that pierce the internal metal pouches, thus releasing the stored liquid reagents, **(4)** the CD is loaded into the platform, the appropriate program is verified from the interface screen, and the test is started. The user can then focus on other tasks, such as more sample collection, as the test runs. During this time, the sample is lysed, clarified and purified, and sent to the amplification chamber; these steps take 5–10 mins and a relatively small amount of power. Amplification takes 30–35 mins, still the largest user of power, and the sample is then flowed across the microarray, taking an additional 10 mins. Finally, the detection optics scan the array and upload the image to the diagnosis software, where the result is output to the screen in an easy-to-interpret, digital diagnosis (infected or not) for each of the analytes. The CD is then ejected from the platform and discarded by the user. The CD exits the platform with all ports and openings permanently sealed, making the chances of contamination and infection very low for those who later come across the used disposable. The entire analysis takes less than an hour and is easy to use.

This is a close approximation of how the μ TAS system for NA diagnostics of the future will operate. It is still somewhat complex, and the CD instrument itself will not be as low-cost as desired. However, much effort has been put into simplifying and combining steps as well as minimizing power requirements. There is still clearly room for improvement, but importantly, the user experience is easy and streamlined, the results robust and reliable, and the CD disposable parts inexpensive.

5. Conclusions and perspectives

There are many obstacles in the development of a successful μ TAS system for POC NA diagnostics.

As the authors have stressed, many of these barriers to commercialization can be overcome. First, closer attention must be paid to the characteristics of the sample being processed, a subject often overlooked. Variations in surface tension, viscosity, and homogeneity of the sample can have drastic effects on both microfluidic and biological parameters. As discussed, biological samples from ill patients can exhibit extreme variations of these characteristics, further adding to the challenge. Sample volume must also be carefully considered. Enough sample must be collected to have a statistically relevant number of analyte molecules for downstream steps, such as amplification and detection. This often means the microfluidic disposable must be able to handle quite larger sample volumes, on the order of several mLs. Moreover, the downstream steps must be designed with all of the sample characteristics in mind. There is little point in developing amplification steps that require a minimal analyte concentration of 10^{-12} M when the starting concentration of analyte in the sample is only 10^{-18} M.

A second need to overcoming commercialization barriers is clarification of differing jargon between biological and engineering disciplines. To this point, the authors have attempted to provide a clear view on some important issues and confusions that affect both disciplines. When characterizing, for example, NA amplification reactions, engineers rarely have an interest in knowing only how many initial analyte DNA molecules are present in a reaction tube. It is essential to also describe the volume conditions, so that the starting concentration can be known. Similarly, biologists have little interest in knowing that a thermocycling system can heat and cool at record speeds. It is more important to know the quality of the PCR reaction that results when using this system – what is the lower LOD, and how reproducible is it?

Thirdly, the entire system must be designed to be simple, with as few different materials, different technologies, and number of parts as possible. The disposable must be kept extremely inexpensive, and the instrument must be able to handle all conditions, in and outside of the laboratory. Thermal and power management will be a key component of this, and environmental impact of the system must also be considered.

A fourth factor to engender faster commercialization is to prototype disposables using technologies that have a realistic chance of being transferred and scaled-up to mass manufacturing. This is the only way to make the microfluidic disposables economically viable and the entire system successful. A movement away from standard silicon micromachining and away from soft-lithography PDMS devices is essential. Disposables must be

prototyped with technologies and methods that are easily transferred to a plastic injection-molding method. For the applications presented, a system that works perfectly but costs \$100 US per test will be a non-starter. The system must perform tests for less than ~\$20 US per test.

There are many microfluidic technologies available as candidates for creating a successful NA diagnostic disposable. Centrifugal platforms in particular hold some unique advantages, including low-power pumping, robust valving schemes, and ease of parallel processing. Several commercialization barriers have already been overcome in the development of systems for microfluidic NA diagnostics using CD platforms. Using any type of new microfluidic platform, one needs to design from the ground up, with an open mind of how to develop diagnostics outside of the norm (and outside of the lab). Only with a vision of the entire problem can a successful disposable and instrument be made. Not all problems can be solved simultaneously, and indeed the problems must be prioritized and tackled one at a time. However, having a holistic knowledge of future problems to come can help steer decision making early on, thus avoiding major pitfalls.

Engineers and biologists will need to work closely together, and when μ TAS systems do become successful, it will be because the two disciplines have learned to work as integrated teams. Once available, these systems will help many people across the world while simultaneously being a huge technological and scientific achievement.

Acknowledgements

The authors wish to thank NSF, NIH, DARPA, Indo-US, and Genome-Québec for funding. Many thanks also go to Jim Zoval, Horacio Kido, RotaPrep, and Dr. Michel Bergeron and his entire group in Québec, Canada.

References

- [1] Manz A, Graber N and Widmer H 1990 Miniaturized total chemical analysis systems: A novel concept for chemical sensing; *Sensors Actuators B* **1** 244–248.
- [2] <http://www.cepheid.com/>
- [3] <http://www.iquum.com/>
- [4] St-Louis P 2000 Status of point-of-care testing: Promise, realities and possibilities; *Clinical Biochemistry* **33(6)** 427.
- [5] Bissonnette L 2006 Next revolution in the molecular theranostics of infectious diseases: Microfabricated systems for personalized medicine; *Expert Rev. Mol. Diagnostics* **6(3)** 433.
- [6] Yager P 2008 Point-of-care diagnostics for global health; *Ann. Rev. Biomed. Engg.* **10(1)** 107.
- [7] Fey A 2004 Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism; *Applied and Environmental Microbiol.* **70(6)** 3618.
- [8] Starke E M 2007 Saliva-based diagnostics using 16S rRNA microarrays and microfluidics; *Ann. New York Acad. Sci.* **1098(1)** 345.
- [9] Chinnaiyan A M 2001 Molecular signatures of sepsis multiorgan gene expression profiles of systemic inflammation; *Am. J. Pathol.* **159(4)** 1199.
- [10] Prucha M 2004 Expression profiling: Toward an application in sepsis diagnostics; *Shock* **22(1)** 29.
- [11] Hosek J 2006 Methods of mycobacterial DNA isolation from different biological material: A review; *Veterinary Medicine* **51(5)** 180.
- [12] Petersen K, McMillan W, Kovacs G, Northrup A, Christel L and Pourahmadi F 1998 The promise of miniaturized clinical diagnostic systems; *IVD Technol.* **4** 43.
- [13] Brecher M E 2001 Evaluation of an automated culture system for detecting bacterial contamination of platelets: An analysis with 15 contaminating organisms; *Transfusion* **41(4)** 477.
- [14] Rådström P *et al* 2004 Pre-PCR processing; *Mol. Biotech.* **26(2)** 133.
- [15] Aldous W K 2005 Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative real-time PCR; *J. Clin. Microbiol.* **43(5)** 2471.
- [16] Taskova R M, Zorn H, Krings U, Bouws H and Berger R G 2006 A comparison of cell wall disruption techniques for the isolation of intracellular metabolites from *Pleurotus* and *Lepista* sp.; *Zeitschrift Fur Naturforschung C-A J. Biosci.* **61** 347.
- [17] Jarrell K F, Faguy D, Hebert A M and Kalmokoff M L 1992 A general-method of isolating high-molecular-weight DNA from methanogenic Archaea (Archaeobacteria); *Canadian J. Microbiol.* **38** 65.
- [18] Zoetendal E G, Ben-Amor K, Akkermans A D L, Abee T and de Vos W M 2001 DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract; *Systematic and Applied Microbiol.* **24** 405.
- [19] Van Burik J A H, Schreckhise R W, White T C, Bowden R A and Myerson D 1998 Comparison of six extraction techniques for isolation of DNA from filamentous fungi; *Med. Myco.* **36** 299.
- [20] Vincent M 2004 Helicase-dependent isothermal DNA amplification; *EMBO Rep.* **5(8)** 795.
- [21] Piepenburg O, Williams C H, Stemple D L *et al* 2006 DNA detection using recombination proteins; *PLoS Biol.* **4(7)** e204.
- [22] Compton J 1991 Nucleic acid sequence-based amplification; *Nature* **350** 91.
- [23] Fire A 1995 Rolling replication of short DNA circles; *Proc. Nat. Acad. Sci. USA* **92(10)** 4641.
- [24] Tan E, Wong J, Nguyen D *et al* 2005 Isothermal DNA amplification coupled with DNA nanosphere-based colorimetric detection; *Analytical Chemistry* **77(24)** 7984.
- [25] Collins M L 1997 A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml; *Nucleic acids Res.* **25(15)** 2979.

- [26] Park S J *et al* 2002 Array-based electrical detection of DNA with nanoparticle probes; *Science* **295** 1503.
- [27] Boudreau D *et al* 2006 Protein detecting arrays based on cationic polythiophene–DNA–aptamer complexes; *Advanced materials* **18(20)** 2703.
- [28] Kim Y H, Yang I, Bae Y-S and Park S-R 2008 Performance evaluation of thermal cyclers for PCR in a rapid cycling condition; *Biotechniques* **44** 495.
- [29] Beebe D J, Mensing G A and Walker G M 2002 Physics and applications of microfluidics in biology; *Ann. Rev. Biomed. Engg.* **4** 261.
- [30] Duffy D C, Gills H L, Lin J, Sheppard N F and Kellogg G J 1999 Microfabricated centrifugal microfluidic systems: Characterization and multiple enzymatic assays; *Analytical Chemistry* **71(20)** 4669.
- [31] Madou M J and Kellogg G J 1998 *The LabCD™: A centrifuge-based microfluidic platform for diagnostics, systems and technologies for clinical diagnostics and drug discovery* (eds) Cohn G E and Katzir A **3259** (San Jose, California: SPIE) 80.
- [32] Madou M J 2002 *Fundamentals of microfabrication* (Boca Raton, London, New York, Washington DC: CRC Press) 2nd edition.
- [33] Madou M, Zoval J, Jia G Y, Kido H, Kim J and Kim N 2006 Lab on a CD; *Ann. Rev. Biomed. Engg.* **8** 601.
- [34] Ko C 2007 One-step pathogen specific DNA extraction from whole blood on a centrifugal microfluidic device; *Lab on a chip* **7(5)** 565.
- [35] Kido H *et al* 2007 A novel, compact disk-like centrifugal microfluidics system for cell lysis and sample homogenization; *Colloids and Surfaces* **58(1)** 44.
- [36] Unpublished.
- [37] Jia G *et al* 2007 A low-cost, disposable card for rapid polymerase chain reaction; *Colloids and Surfaces* **58(1)** 52.
- [38] Jia G 2006 Dynamic automated DNA hybridization on a CD (compact disc) fluidic platform; *Sensors and Actuators. B: Chemical* **114(1)** 173.
- [39] Peytavi R, Raymond F R, Gagné D *et al* 2005 Microfluidic device for rapid (<15 min) automated microarray hybridization; *Clinical Chem.* **51(10)** 1836.
- [40] Toepke M W 2006 PDMS absorption of small molecules and consequences in microfluidic applications; *Lab on a chip* **6(12)** 1484.
- [41] Gilles Chabot, Centre de Recherche en Infectiologie de l'Université Laval, Centre Hospitalier Universitaire de Québec.