Journal Name

Dynamic Article Links ▶

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Rapid and Multiplex Detection of Legionella's RNA using Digital Microfluidic

Amir M. Foudeh,‡ [a] *Daniel Brassard*,‡ [b] *Maryam Tabrizian* [a,] and *Teodor Veres* *[a, b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Abstract

Despite recent advances in the miniaturization and automation of biosensors, technologies for on-site monitoring of environmental water are still at an early stage of development. Prevention of outbreaks caused by pathogens such as *Legionella pneumophila* would be facilitated by the development of sensitive and specific bioanalytical assays that can be easily integrated in miniaturized fluidic handling systems. In this work, we report on the integration of an amplification-free assay in digital microfluidics (DMF) for the detection of Legionella bacteria based on 16s rRNA marker. We first review the design of the developed DMF devices, which provides the capability to manipulate up to one hundred nl-size droplets simultaneously, and discuss the challenges involved with on-chip integration of the RNA-based assay. By optimizing the various steps of the assay, including magnetic capture, hybridization duration, washing steps, and assay temperature. a limit of detection as low as 1.8 attomoles of 16s rRNA was obtained, which is at least 250 times less than reported for similar amplification-free detection systems reported to date. Finally, we demonstrate the specificity of the developed assay by performing multiplex detection for a pathogenic and a non-pathogenic species of Legionella. We believe the developed DMF devices combined with the proposed detection system offers new prospects for the deployment of rapid and cost-effective technologies for on-site monitoring of pathogenic bacteriae.

1. Introduction

Water-related diseases are responsible for more than 3.4 million deaths annually.[1] Among these diseases, Legionellosis, an acute form of pneumonia, is a major concern mainly due to unpredictable outbreaks such as recent incidents reported in Canada, U.S.A., Norway, and Germany.[2-4] *Legionella*, the causative agent of this disease, was responsible for more than 30% of water borne disease outbreaks in USA between 2001-2006.[5].Legionellosis outbreaks are associated with high mortality rates (15 to 20%)[6], which can reach up to 50% for individuals with a compromised health condition.[7] *Legionella* is found in most natural and man-made water systems[8] such as cooling towers, air conditioners, hot tubes and showerheads. These systems not only provide optimal growth conditions, but can also propagate Legionella through aerosol.[9] Presently, more than 50 *Legionella* species have been identified with approximately half of these species being associated with human disease.[10] [11] To have an accurate and reliable evaluation of the water risk assessment it is thus crucial to design detection systems that can distinguish between pathogenic and non-pathogenic *Legionella*. In order to monitor the water systems routinely to predict any potential outbreaks, development of on-site systems that are portable, automated, cost-effective and rapid.. A biosensor for detection of *Legionella* should be specific and sensitive with capability of multiplex detection of different bacteria's species. Today, detection of *Legionella* continues to rely on the conventional culturing method which is very time-consuming and expensive. molecular methods such as polymerase chain reaction (PCR), DNA microarray and immunology based methods are also been utilized for detection of the *Legionella* in laboratory settings but are less practical for on-site monitoring.

Miniaturization of pathogen detection methods and their integration in microfluidic devices has been gaining much attention as it can not only lead to the reduction of reagent consumption and analysis time but can also automate the multiples handling steps of chemical and biological assays.[12] Digital microfluidics (DMF) has recently arisen as a promising and versatile platform for chemical and biological applications. In DMF, as opposed to continuous flow microfluidics, individual droplets (of pL- to μL) are manipulated independently by applying electric potential to an array of electrodes. Multiple droplets containing different reagents can be manipulated simultaneously and the operation scheme can be reprogrammed without the need to change the device design. Each droplet can thus act as microreactor from which independent tests can be

performed concurrently in a confined environment. Therefore, DMF is a promising candidate for applications involving complex and multistep assays.[13] Also, compared to conventional continuous flow microfluidic devices using fixed channel arrangements, the very high reconfigurability of DMF can help improving assay optimization and decrease development costs. On the other hand, until recently, most DMF devices were primarily designed and utilized for simple assays requiring only a few steps and limited number of droplets. The developed devices thus typically lacked the complexity required to perform multiplexed bioassays in which numerous tests must performed concurrently.

Different bioassays have been performed using DMF, including as immunoassays[14], cell culture[15], DNA hybridization[16] , PCR[17] and isothermal amplification[18]. However, most attempts to detect pathogens in DMF were based on immunoassay or DNA hybridization and PCR amplification. The PCR and other amplification techniques although provide rapid results with high sensitivity, require expensive reagents and enzymes that are also susceptible to the presence of inhibitors in the environmental samples. In addition, the DNA based (e.g PCR) and immunoassay detection techniques have a major drawback because of its inability to distinguish between live and dead bacteria. This is a major concern because of the false-positive results especially after the water treatments in the environmental water settings. In contrast, targeting ribosomal RNA (rRNA) is a viable alternative that overcomes the aforementioned shortcomings. Indeed, since RNA expression level is directly correlated to the microbial activity, it provides a more reliable and accurate target for detection of live *Legionella*.[19]

There have been only few attempts to develop detection assays in DMF based on RNA. For example, Jebrail *et al.* [20] demonstrated the feasibility of RNA extraction from blood using magnetic beads within a DMF device. In another recent work, Rival et al. [21] performed single cell analysis using micro RNA from human HaCaT cells followed by mRNA capture on magnetic beads, mRNA conversion to DNA and RT-PCR amplification. The use of RT-PR, even if it provides high sensitivity, can require elaborated sample preparation steps, expensive enzymes and reagents, and precise control of the temperature, making this method less desirable for on-site applications.

In this work, we report the multiplex and amplification-free detection of 16s rRNA from *Legionella* bacteria using a DMF device contains multiple reservoir and several hundred electrodes which is capable of handling complex assays. The DMF device proposed herein , demonstrate simultaneous manipulation of multiple droplets on-chip and investigate the optimal hybridization conditions and limit of detection for *L. pneumophila* 16s rRNA. We additionally demonstrate that the developed assay, which is based on two sets of DNA as capture and detector probes, can achieve a high degree of selectivity by showing the multiplex detection of rRNA from two different species of *Legionella*, one pathogenic (*L. pneumophia*) and one non-pathogenic (*L. israelensis*). We believe the proposed DMF device combined with the proposed detection system have great potential for rapid, high-throughput, multiplex, and inexpensive detection of pathogens with minimal sample and regent volume.

2. Design of the DMF devices

The integration of multiplex protocols in DMF requires the development of devices that can manipulate and store multiple droplets simultaneously to perform the dilution, mixing and analysis steps required by the assay. Unfortunately, it is challenging to design and fabricate digital microfluidic devices containing enough active electrodes to handle complex protocols while simultaneously keeping fabrication cost and process complexity low enough for typical biomedical applications. To simplify the fabrication of the devices, we have developed a process where negative SU-8 photoresists is used directly as the dielectric for the fabrication of advanced DMF requiring multiple levels of metallization.[24] We have indeed found that SU-8 not only offers excellent electrical properties (such as a high dielectric breakdown ~4MV/cm and high dielectric constant of about 4), but also ease of deposition and patterning, long term resistance to humid environment and saline buffers, resistance to scratches and pinhole formation, and good temperature stability.

The design of the DMF devices is shown in Figure 1a. The device contains 560 active electrodes, 7 reservoirs, 1 waste area, mixing regions, and sample preparation regions. The device also includes enough storage regions to hold up to 100 individual droplets, as we have found that the maximum assay complexity that can be integrated in DMF is often limited by the maximum number of droplets that can be stored simultaneously on-chip. It is noteworthy that the DMF device shown in Figure 1 is capable of handling assays even more complex that those demonstrated in this paper. This was done on purpose to so as to take full advantage of the very high reconfigurability of DMF, where only one chip can be easily designed to handle the needs of various different assays by simply changing the droplet programming sequence.

To limit the complexity of the electronic circuits and facilitate electrical connection to the device, we have limited the number of independent electrical inputs to only 24. Thus, each electrical input is connected simultaneously to multiple active electrodes by using connection lines placed on a different metallization level. The assignment of the electrical inputs to each active electrode has to be cleverly designed to avoid as much interferences as possible when multiple droplets are on the DMF devices simultaneously. As shown in Figure 1b, to minimize unwanted interactions between the fluidic operations, the input-to-electrode assignment has been divided into partitions[25] according to the function of the electrodes: 8 electrical input pins where assigned for dispensing (blue), 5 pins for transportation (green), 6 pins for preparation (red), 4 pins for storage (purple), and 1 pin is connected to top plate (not shown). As shown in Figure 1c, the pin assignment within each partition has also been optimized to maximize interdependence of fluidic operations when multiple droplets are located in the same partition. For example, to move only the droplet marked with an arrow from the sample preparation to the transportation partition, the electrical input pins would be actuated as follow: 2 – 5 – 1 – C. The input pins are also assigned in a similar manner in the storage region, except that smaller active electrodes (labelled □ and □) are used to minimize the real estate of the device. Finally, the distribution of the 8 independent electrical pins within the reservoir partition (blue color in Figure 1b), ensures that a droplet can be dispensed

independently from each reservoir.

4. Assay design and optimization

Due to presence of many species of *Legionella* which half of them are not pathogenic, it is critical to design assays with a high selectivity capable of differencing pathogenic from non-pathogenic species. As shown schematically in Figure 2a, we have developed an assay based on the hybridization of Legionella's 16s rRNA on magnetic beads. In order to achieve high specificity, two DNA probes were designed for each target. One probe served as a capture probe and was immobilized on magnetic beads while the second probe, in addition to ensuring the high specificity, is used as a detector probe functionalized with a fluorescent dye (Figure 2a).

Before integrating the assay in the DMF device, different critical parameters were evaluated to obtain the highest hybridization efficiency (signal-to-noise ratio with minimum analyte consumption, and the shortest assay time. The following factors were also considered: hybridization buffer composition, temperature, reaction volume, and the incubation time. Among these factors, buffer composition and temperature played an important role in specificity and sensitivity of the hybridization. We previously[19] demonstrated that the 600 mM salt concentration in the neutral pH buffer at 37 °C for the *L. pneumophila* RNA-DNA hybridization resulted in the highest specificity. As discussed in the material and methods section, all on-chip assays have thus been performed at a temperature of 37°C.

To validate the on-chip 16s rRNA hybridization protocol and optimize the speed of on-chip assays, we have first performed a series of simple on-chip measurements to assess the effect of incubation time on hybridization efficiency. For on-chip tests, *L. pneumophila* 16s rRNA and the detector probes were first mixed together off-chip. Then, for each incubation times reported in Figure 2b, one droplet of a 100 nM RNA solution was dispensed and mixed on-chip with one droplet containing magnetic beads coated with immobilized capture probes. As described more in details in Section 5, the mixed droplet was washed six times and fluorescent measurements were carried out immediately. As can be seen in Figure 2b, the intensity of fluorescence increased from one minute up to 20 minutes after which fluorescent signal is seen to saturate. Therefore, we chose 20 minutes as the optimal incubation time for further experiments.

The volume of the RNA sample on which the detection experiment is performed is another key factor that can affect the results of the detection assay. Indeed, by integrating the assay into In conventional laboratory experiments the reaction volume that is needed, is more than tens of µL, in this DMF device, we were able to reduce the volume required for one hybridization assay to 30 nL which is even smaller by a factor of 10 to 100 times compared with other reported reaction volumes in DMF[13, 18, 21]. The developed integrated assay thus offers the interesting prospect to significantly decrease both the reagent consumption and minimal sample volume. In particular, the reduced consumption of streptavidin coated magnetic beads to only 15 nL per hybridization assay (about 3600 particles) offers the potential to reduce the cost of each assay. On the other hand, the reduced sample volume can obviously impact the ultimate limit of detection of the assay. In order to evaluate the limit of detection, there is a need to perform all the measurement for different concentrations in the same condition. Since in the top section of the EWOD chip there are six different sample preparation area, six different concentrations was chosen to manipulate simultaneously.

5. Results and discussion

On-chip serial dilution and hybridization

To evaluate the limit of detection of the assay in DMF devices, we have performed on-chip the protocol shown schematically in Figure 3. Figure 4 shows sequential images of various steps required to implement in DMF the exponential dilution protocol described schematically in Figure 3. The first steps, which are summarized in Figure 3a and Figure 4a, involve the generation of sample droplets containing a series of different concentrations. One droplet from the RNA reservoir is first dispensed and transported to the mixing area. Next, another droplet is dispensed from the buffer reservoir and transferred to the same mixing area. In the mixing area, the two droplets are mixed with rapid circular movements and split into two identical daughter droplets, one of which is move either to the storage area for later use or to the waste reservoir (depending on the targeted concentration profile). The other daughter droplet is kept at the mixing area for another dilution step with a droplet from the buffer reservoir. In this way, an exponential dilution series of the original droplet is obtained. For the developed assay, droplets having concentrations of about 500 nM, 125 nM, 8 nM, 1.0 nM, 0.5 nM and 0.12 nM were analyzed.

As shown in Figure 3b-c and Figure 4b, each droplet from the dilution series is then actively incubated with magnetic beads. To that end, one droplet from the reservoir containing the magnetic beads functionalized with *L. pneumophila* CP probe is first dispensed and transferred to the adjunct mixing area. In the next step, one of the droplets from the dilution series of *L. pneumophila*'s RNA is transferred from the storage area to the same mixing area. After mixing, the new larger mixed droplet is transferred to sample preparation area. Subsequently, all of the six *L. pneumophila*'s RNA concentrations are mixed with magnetic beads and transferred to the sample preparation area. The droplets are incubated for around 20 minutes during which they are slowly moved on the sample preparation area to create fluid recirculation, minimize sedimentation and maximize the hybridization efficiency.

Finally, as shown in Figure 3d-e and Figure 4c, the magnetic particles are captured and washed to remove the un-hybridized RNA. To capture the magnetic beads, two 2.5 mm diameter cylindrical neodymium rare-earth magnets are positioned on top of the DMF chip (each magnet is located in the center top of the three sample preparation electrodes - see Figure 4c). The magnets are positioned to attract and concentrate the magnetic beads on the top part of the droplet. After capture of the magnetic beads, all the six droplets are split simultaneously into the two daughter droplets and the droplets containing the supernatant are transferred to the waste reservoir. The magnets are then removed temporarily and each droplet containing the magnetic beads are washed by (i) transferring them one at a time to the mixing area located on top of the chip and (ii) mixing them with one droplet from the buffer reservoir. The mixed droplet is then transferred

back to its previous location in the sample preparation area. The capture and wash sequence of the magnetic beads is repeated for a total of six times.

In general, to capture and separate magnetic beads in a droplet, the magnetic force should be sufficient enough to capture the magnetic particles but not too strong as to cause irreversible particle aggregation.[13, 26-27] As described, the two permanent magnets placed on top of the DMF allowed to concentrate efficiently the magnetic beads on top of the droplets, remove supernatant and perform several washes. On the other hand, we observed that sedimentation of the magnetic particles at the bottom of the droplet could make capturing the magnetic beads difficult. In order to alleviate this issue, we implemented a new strategy for capture and separation of the magnetic beads. In this strategy, the droplet was spread on two electrodes on top of the sample preparation area by activating both electrodes in the presence of magnets (Figure 4-7). This was followed by switching on and off only the top electrode while the bottom electrode was kept activated. The frequency of the on/off switching was optimized to achieve two goals simultaneously. The first was to ensure that the pellet of magnetic beads remained intact, and the second is to facilitate the re-capture of sedimented magnetic beads. To achieve acceptable separation, a frequency around 7 Hz was used for turning the top electrode on and off. We hypothesize that the capillary wave[28] is the dominant phenomenon here and causes the sedimented particles resuspend into the droplet and to be captured by magnet. In addition, the use of Pluronic F-127 in the buffer solutions was also found to improve not only droplet manipulations but also the re-suspension of the particles after magnetic capture.

Two other factors should also be considered when separating the un-hybridized RNA and detector probes from the magnetic beads. First, the magnetic force should be enough to prevent the magnetic particles pulling into the supernatant droplet after splitting. The second factor that should be considered is the choice of the washing protocol and number of washes. In our experiments, we observed that a total of six washes with 1:1 ratio of buffer to sample was sufficient in removing the supernatant from magnetic particles before fluorescence measurement. This number of washes is also in accordance with a similar reported protocol.[13] In this method, the magnet was manually removed after the 'capture and separation' step and the droplet containing magnetic beads was re-suspended in wash buffer droplet in the mixing area (Figure 4-9). The removal of the magnet ensures that there won't be any entrapment of the unhybridized RNAs and detector probes in the pellet of the captured magnetic beads. We hypothesize that this is advantageous compared to other previously reported methods where the magnet was at the same place throughout the whole washing process. For example, when the magnet position is kept constant, is has been reported that up to 18 washes are required[37] when the buffer to sample ratio is of 1:1 and 5 washes[34] for a buffer to sample ratio of 5:1.

Determination of on-chip limit of detection for L. pneumophila's RNA

As described earlier, six different concentrations of the *L. pneumophila*'s RNA ranging from 0.5 µM to 122 pM were made on the DMF chip and hybridized with functionalized magnetic beads for twenty minutes at 37°C. After six times washing with buffer, the fluorescent intensity for each droplet was measured directly on-chip and subtracted from the negative control. As can be seen in Figure 5, the developed system could successfully detect 16s rRNA at concentrations as low as 122 pM in less than 30 minutes. Considering the 15 nl volume of the RNA droplet, this amount is equivalent to 1.8 attomoles of 16s rRNA. Due to the very low dead volumes offered by the proposed system, the LOD in terms of absolute amounts is thus around 250 to 10,000 times less than the LOD reported for 16s rRNA using amplification-free detection systems such as SPRi,[19] and electrochemical[29] techniques Respectively. Moreover, with a total analysis time of only 30 minutes, the system provides a measurement 6 times faster than the aforementioned methods. One of the limiting factors in our sensitivity was the auto-fluorescence of the electrodes, which interfered with the signal obtained from the droplet at low concentrations. We believe that, by alleviating this problem (for e.g., by designing a new set of electrodes with an opening window in the center), the signal-to-noise ratio and the LOD could even be increased further. Finally, it is also worthwhile noting that the developed assay offers a rather large dynamic range, providing a regular signal increase for more than three orders of magnitude of RNA concentration (Figure 5).

Multiplex detection of pathogenic and non-pathogenic Legionella

As described in the introduction, the multiplex detection and ability to distinguish the pathogenic from non-pathogenic bacteria is a critical feature required for monitoring environmental water samples. Thus, in addition to *L. pneumophila*, we designed a series of capture and detector probes targeting the 16s rRNA from *L. israelensis* as a non-pathogenic *Legionella* species, since there is no report of human disease from this species.

In order to perform the multiplex detection of these two target RNAs, the detector probe specific to *L. israelensis* was functionalized with Cy5 dye in contrast to the *L. pneumophila's* detector probe which was tagged with Cy3 dye. Two sets of functionalized MB with a concentration of 2.4 x 10^8 particles/mL were also prepared, each with one of the two capture probes (*L. pneumophila* MB and *L. israelensis* MB).

For the multiplex protocol, the on-chip incubation, magnetic separation, and washing steps were performed in a similar manner to the exponential dilution protocol discussed before (see Figure 4). However, in this case, RNA concentration was fixed at 100 nM and two additional reservoirs were used for the *L. israelensis* MBs and for *L. israelensis* RNAs. Also, instead of performing a dilution series, fluidic operations were such that the two different types of functionalized magnetic beads (i.e., *L. pneumophila* MB and *L. israelensis* MB) were each hybridized with three different RNA samples prepared by mixing (i) a *L.p* droplet with a buffer droplet, (ii) a *L.i* droplet with a buffer droplet and (iii) a *L.p* with a *L.i* droplet. A total of six different hybridization measurements were thus performed to evaluate the specificity of the developed assay.

Figure 6 shows the resulting measured fluorescence intensity for the six hybridization tests for both the Cy3 and Cy5 filters (corresponding respectively to the dyes of *L.p* and *L.i* detector probes). As expected, the reaction of only *L.p* RNA with *L.p* MB resulted in a significant fluorescent signal only with Cy3 filter, indicating that only *L.p* detector probes hybridized significantly

to the beads. The opposite trend was observed for the reaction of *L.i* RNA with *L.i* MB, which resulted in a strong signal only in Cy5 filter (i.e., only *L.i* detector probe was hybridized). On the other hand, much smaller signals were measured in both Cy3 and Cy5 filters when *L.p* RNA was incubated with *L.i* MB or when *L.i* RNA was incubated with *L.p* MB, indicating that neither the *L.p* detector probes nor the *L.i* detector probes were hybridized to the beads. Finally, for the mixed sample containing both *L.p* and *L.i* RNA, the normalized fluorescent intensities for Cy3 and Cy5 filters were in the same level as those obtained for *L.p* RNA with *L.p* MB and *L.i* RNA with *L.i* MB respectively. In summary, these results confirm that the developed assay based on two sets of independent capture and detector probes can achieve a specificity high enough to discriminate between RNA from two *Legionella* species.

6. Conclusion:

We have shown the successful integration of a multiplex RNA assay in DMF for the specific detection of *Legionella* species using 16s rRNA targets. An advanced DMF platform was designed to integrate the developed assays, which offered the possibility to perform on-chip complex fluidic manipulations with multiple droplets was used and a customized protocol for this specific application was implemented. The various steps of the assays, including magnetic capture, hybridization duration, washing steps, and assay temperature were optimized. The advanced fluidic capabilities of the platform were first used to perform exponential dilutions so as to evaluate simultaneously in the same assay and under the identical condition, .the signal from multiple RNA concentrations. We have shown that, by integrating the assay in DMF devices, we were able not only to reduce drastically reagent and magnetic beads consumption, but also to decrease the minimum amount of RNA required to achieve positive sample identification to about only 1.8 attomoles, which is at least 250 times less than reported for 16s rRNA amplification-free detection systems. Finally, we have shown that specific detection for a pathogenic and a non-pathogenic specie of *Legionella* can be achieved by using capture and detector DNA probes for each 16s rRNA target. We have thus demonstrated a proof of concept for the automated multiplex detection of pathogenic and non-pathogenic *Legionella* in DMF. We believe that this proposed detection system could be used as a versatile tool for high-throughput and multiplex detection of several types of bacteria with minimum reagent consumption. reaching having the goal standard to 1 CFU improve the ultimate limit to meet the

7. Materials and methods

7.1 Chemical and reagents

BioMag Streptavidin coated superparamagnetic beads were purchased from Bangs Laboratories (Fishers, IN, U.S.A). Pluronic F-127 was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.). SSPE buffer (20X buffer is 3.0 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA at pH 7.4.), was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Silicone oil (viscosity of 2 cSt) was purchased from Clearco (Bensalem, PA U.S.A), SU8 photoresists from Gersteltec (Pully, Switzerland) and Teflon AF from Dupont (Mississauga, ON, Canada).

7.2 DMF device fabrication

The DMF devices were fabricated by first depositing and patterning, by standard lithography, layers of 10 nm thick Cr and 100 nm thick Au on a borosilicate glass wafer to form a network of contact pads and 200 □m wide connection lines. A first layer of about 5 □m thick SU8 dielectric was then deposited by spin-coating and UV exposed through a mask to open interconnection vias in specific locations. A second layer of Cr and Au was then patterned on top of the first dielectric layer to form the 500x500 □m active electrodes and reservoirs of the devices. The electrodes were finally covered with a second layer of about 2.5 □m thick SU8 dielectric and a thin 30 nm layer of hydrophobic coating based on Teflon AF. The top plate of the devices was made by covering ITO-coated plate (Delta technologies, Stillwater, MN, USA) with the same hydrophobic coating. As a final step, the DMF devices were finally post-baked at 200ºC for 2h.

7.3 Microfluidic platform and DMF device operation

The DMF devices were powered with a home-developed AC voltage source capable of amplifying the 5 V DC voltage from a USB connection to a 0.3 to 3 kHz square-wave of 0 to 150 V. The use of AC voltage minimizes the amount of charge trapping occurring inside the dielectric of the devices compared to DC voltage, thus improving both the reliability of droplet displacement and DMF lifetime. A typical operation voltage of about 85V RMS at 1 kHz was used for droplet displacements, which was found to provide reliable droplet displacement at a speed of 10 electrodes per seconds. The 24 independent electrical inputs of the devices were contacted with a custom clip made from spring-loaded pogo-pins. A home-developed software providing advanced sequence programming capabilities have been developed to control the electrical inputs and associated droplet displacements.

The devices were filled by dispensing droplets of about 1 □l on the bottom electrodes forming the reservoir of the DMF devices using a pipette. Before reservoir filling, a small amount (i.e., < 0.1 □l) of silicone oil was applied on the reservoir by touching the device with a the tip of a pipette As discussed elsewhere [22], the oil naturally forms a thin shell around the droplets, which has been shown to facilitates droplet displacements and improves device reliability. The top plate of the device is then electrically grounded and put in place along with a spacer providing a constant gap of about 70 □m. Individual droplets of about 15 nL are then dispensed from the reservoirs of the devices by applying a sequence of voltage on the electrode of the DMF devices. The temperature was controlled by mounting the DMF devices on a thermoelectric element connected to an H-bridge electrical circuit controlled by an Arduino microcontroller in communication with a computer. While performing the RNA assay, the temperature in the DMF devices was kept constant at 37ºC to favor hybridization. To minimize the evaporation of the small 15 nl droplets, DI water was dispensed around the edge of the DMF devices. In this configuration, only marginal evaporation was observed for the duration of the assay (about 30 min). No significant evaporation of the thin oil shell around the droplet was

observed.Many regents used in biological applications such as proteins are susceptible to non-specific adsorption to the hydrophobic layer of the DMF devices, increasing dragging forces and eventually preventing droplet displacement[23]. In our experiments, we have found that the droplets containing the streptavidin-coated paramagnetic beads could not be manipulated reliably despite the presence of an oil shell around the droplet. Reliable droplet displacement was obtained by adding 0.1% (v/v) Pluronic F127 to the solutions

7.4 DNA probe design and hybridization condition

DNA capture probes (CP), complementary to *L. pneumophila* and *L. israelensis's* 16s rRNA, were designed using bioinformatics software packages from Cardiff University, England. Particular features such as loops and hairpins, were checked for and avoided. The specificity of these probes was confirmed using the Check Probe program and Ribosomal Database Project (RDP). In terms of detection probes, a fluorescent tagged DNA probe with zero base pair gap between the capture and detection probes (DP) for each target RNA sequence was designed. Cy3 (excitation at 550 nm, emission at 570 nm) and Cy5 (excitation at 649 nm, emission at 670 nm) dyes were used for *L. pneumophila* and *L. israelensis* detector probes respectively. The length of each detector probe was determined to ensure similar melting temperatures while avoiding cross-reactivity and hybridization to any capture probes. The cross reactivity of these detector probes was tested against the capture probe, revealing no significant interaction (data not shown). Two RNAs (60bp in length) from the *L. pneumophila and L. israelensis's* 16S rRNA, which contains complementary sequences for the designed capture and detector probes, were synthesized by Integrated DNA Technology (Table 1).

Table 1: Oligonucleotide sequences employed in the experiments

Name	Sequence 5'--3'
L. pneumophila CP	/Biotin/TTTTTTTTTTCAGGTCGCCCCTTCGCCGCC
L. israelensis CP	/Biotin/TTTTTTTTTTGCGCCAGGCCATAAGGTCCC
L. pneumophila DP	CTCTGTATCGGCCATTGTAGCTTTTTTTTTT/Cy3/
L. israelensis DP	CAGCTTTACTCCAAAGAGCATATGCGGTTTTTTTTTT/Cy5/
L. pneumophila's RNA	UACACACGUGCUACAAUGGCCGAUACAGAGGGCGGCGAAGGGGCGACCUGGAGCAAAUCC
L. israelensis RNA	CTAATACCGCATATGCTCTTTGGAGTAAAGCTGGGGACCTTATGGCCTGGCGCTTTAAGA

7.5 Microparticle preparation and signal measurement

The hybridization buffer was chosen based on previously reported work[19]. Briefly all the reagents were diluted in 4X SSPE buffer containing 600 mM NaCl and hybridization experiments were carried out at 37°C inside the DMF chip.

Before the start of the assay, the streptavidin coated superparamagnetic particles (MB) were washed off-chip three times with 4X SSPE buffer containing 0.01% pluronic F-127 and were concentrated to the final concentration of 2.2 mg/mL (2.4 x 10^8 particles/mL). In order to immobilize the biotin capture probes on magnetic beads, an excess amount of DNA capture probe (4 µL of 100µM) was incubated off-chip with 100 µL of the magnetic bead solution for 15 min at room-temperature. This was followed by three times washing with 4X SSPE buffer. The same protocol was used for the preparation of the MB used in the capture of *L. pneumophila* and *L. israelensis.* The functionalized beads were kept at 4°C before use.

An inverted fluorescence microscope (Nikon TE 2000-E) was used for measurement of the fluorescence intensity of the droplets inside the chip. All images were captured using a CCD camera and analyzed by ImageJ (National Institutes of Health, Bethesda, MD). The Fluorescent measurements were carried out on the chip by locking at the target droplet under the microscope. All measurements were subtracted by the intensity obtained from a negative control. The negative control droplet was contained magnetic beads with the detector probe and washed six time same as other droplets. For the multiplex detection of RNA, the fluorescent intensities for each sample were normalized for each filter independently by the positive control (the mixture of the magnetic bead, RNA and proper detector probe).The lower detection limit was defined as the smallest concentration of an analyte, calculated as the blank signal plus or minus three standard deviations. All data were expressed as the mean ± standard deviation.

Notes and references

[a] *3775 University Street, Department of Biomedical Engineering, Faculty of Medicine, McGill University H3A 2B4, Montreal (QC), Canada.*
[b] *National Research Council Canada, 75 Boul. de Mortagne, J4B 6Y4Boucherville, QC, Canada. Tel (450) 641-5232 Cell (514) 449 3370 Fax (450) 641-5105 E-mail : teodor.veres@cnrc-nrc.gc.ca*
‡:These two authors made equal contributions.

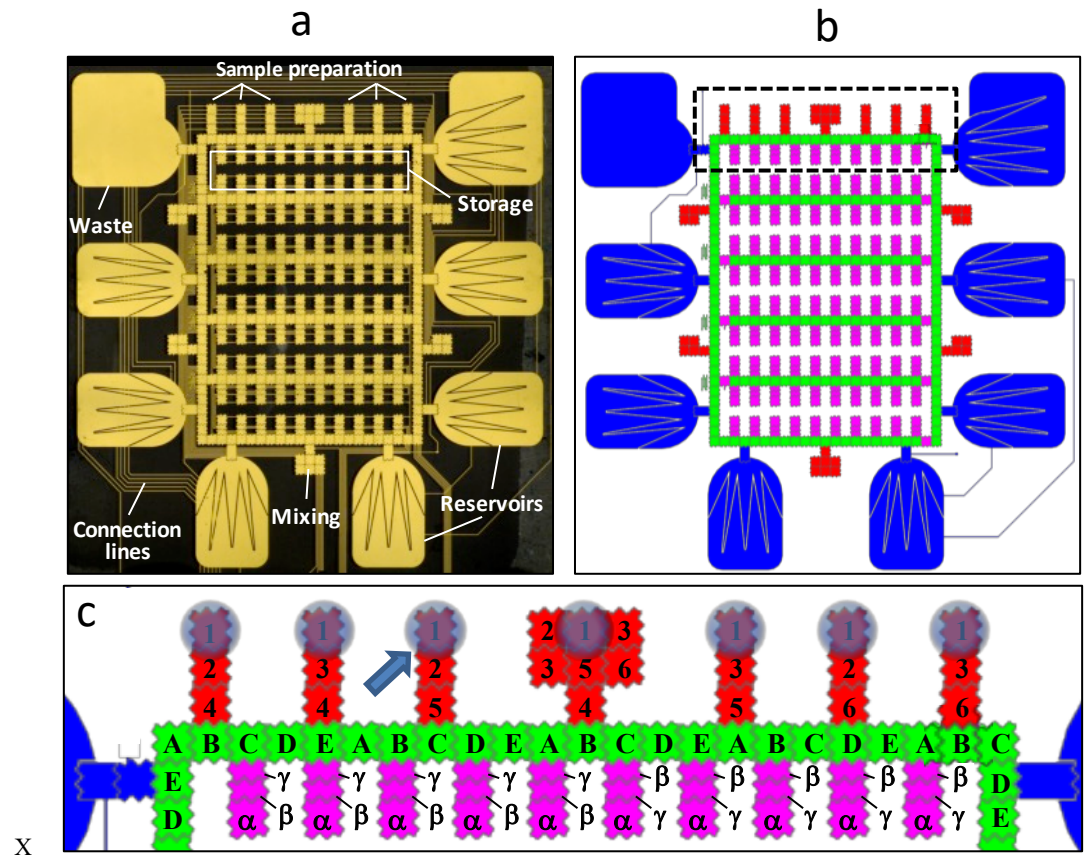

Figure 1: a) Top view of the developed digital microfluidic device. b) Schematic of electrical input pin to EWOD electrode assignment. Each color represents a partition to which specific input pins are assigned. The partitions were defined according to the functions of the EWOD electrodes: dispensing (blue), transportation (green), preparation (red), and storage (purple). c) Example of pin assignment in top section of the chip, each number/letter representing a specific electrical input.

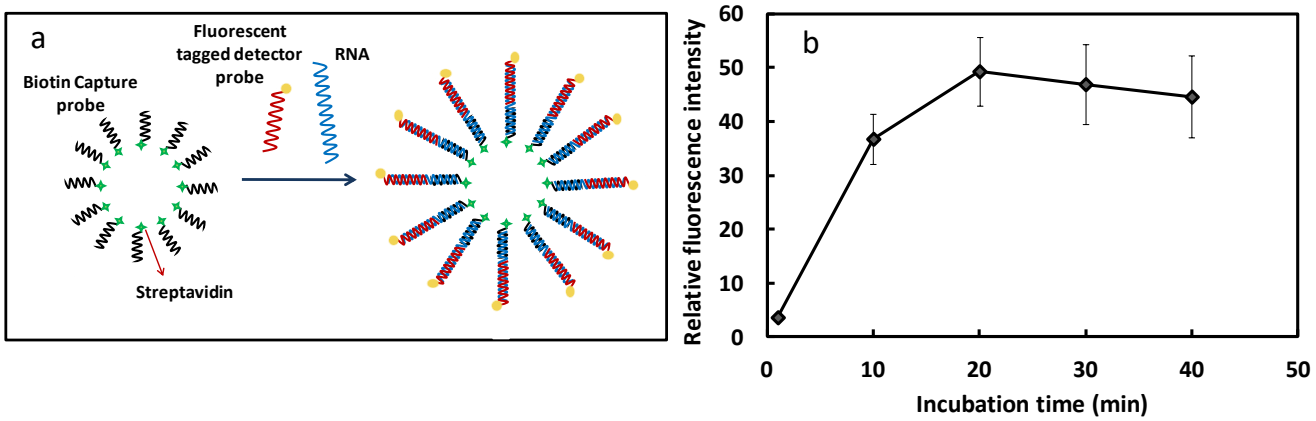

Figure 2: a) Schematic of hybridization of target RNA on the magnetic beads using designed capture and detector probes b) Hybridization of 100 nM target RNA with superparamagnetic beads. Particles were washed six times to remove any excess RNA.

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

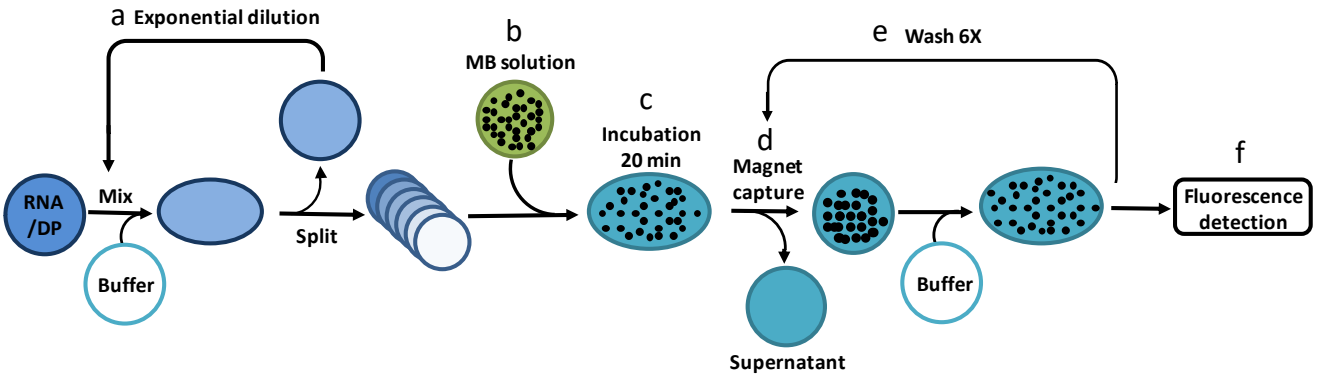

Figure 3: Protocol schematics for the serial dilution and hybridization of 16s rRNA on the DMF devices. a) Creation of the exponential dilution of the RNA sample into six concentrations. b) Mixing of the diluted RNA droplets with the magnetic beads. c) Incubation of the magnetic beads with six concentrations of 16s rRNA. d) Capture of magnetic beads and separation of supernatant e) Six times washing of magnetic beads. f) Fluorescent measurement.

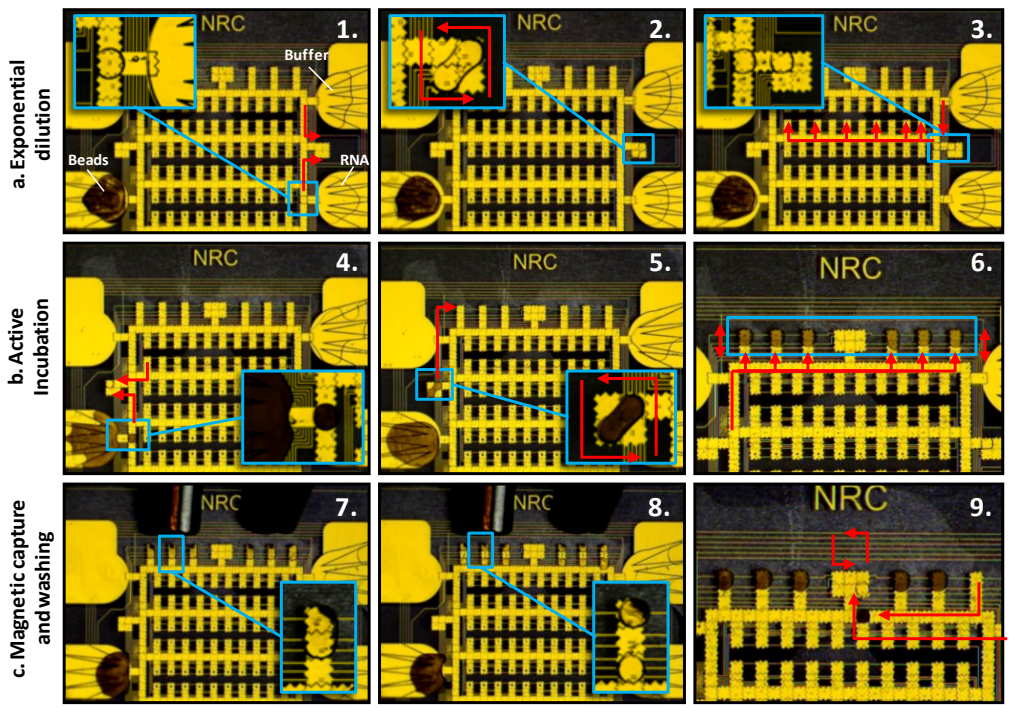

Figure 4: Top view image sequence showing the digital microfluidic protocol used for the RNA serial dilution and hybridization assay. a) Creation of the exponential dilution profile of the RNA sample into 6 droplets (1. to 3.). b) Mixing of the diluted RNA droplets with the magnetic beads and incubation (4. to 6.). c) Magnetic capture and washing of the incubated droplets (7. to 9.).

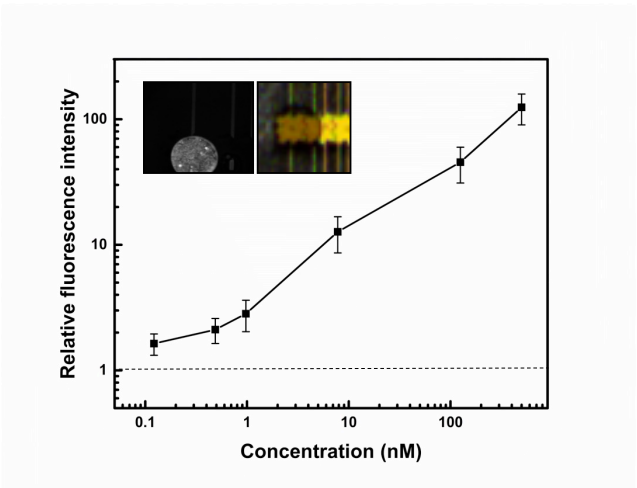

Figure 5: Measured relative fluorescent intensity versus *L. pneumophila's* RNA concentration using superparamagnetic beads and Cy3 fluorescent tagged detector probe. Inset: A brightfield and fluorescent images of a droplet containing captured RNA onto the magnetic beads

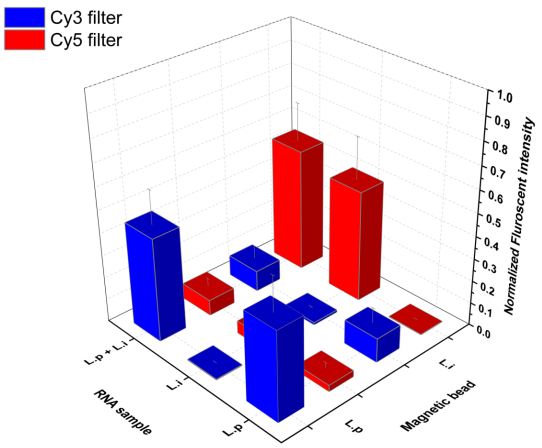

Figure 6: Multiplex detection of *Legionella* 16s rRNAs including pathogenic, *L. pneumophila* (*L.p*) and non-pathogenic *L. israelensis* (*L.i*). Detector probe specific to *L.p* RNA sample was tagged with Cy3 dye while the detector probe specific to *L.i* RNA sample was tagged with Cy5 dye. Three RNA samples including *L.p*, *L.i* and mixture of *L.p* and *L.i* were incubated with two types of magnetic beads functionalized with either *L.i* or *L.p* capture probes. The fluorescent measurements were carried out with Cy3 and Cy5 filters for each droplet.