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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Lab-on-card based sensor for DNA detection using Loop-mediated isothermal amplification

Ashwaq Zain

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. ir. Ronald THOELEN

MENTOR :

De heer Frederik VREYS

2018
2019

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List of abbreviations

POC	**point of care**
LAMP	**Loop-mediated isothermal amplification**
Bst polymerase	**Bacillus stearothermophilus polymerase**
PCR	**polymerase chain reaction**
WHO	**World Health Organisation**
PET	**polyethylene terephthalate**
PCB	**printed-circuit-board**
SG	**SYBR Green I**
PBS	**phosphate buffered saline**
LOChip	**Lab-On-Chip**
LOC	**Lab-on-card**
DW	**Distilled water**
MTBC	**Mycobacterium tuberculosis complex**
TB	**Tuberculosis**
No°	**Number**
LOD	**Limit of detection**

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Zain Ashwaq

09-06-2019

1. Abstract

Nucleic acid-based diagnostics are extremely attractive tools for pathogen detection and early diagnosis. However, the conventional molecular diagnostics are unsuitable for resources-limited point of care (POC) due to the heavy expenses, and the lack of suitable equipment and well-trained labors. Therefore, miniaturizing lab devices towards a Lab-on-card (LOC) is extensively useful in POC testing. Consequently, this project aims to translate macroscale lab-techniques towards a single LOC sensor for cell lysis and DNA extraction, amplification and detection using Loop-Mediated isothermal amplification (LAMP). Indeed, LAMP is highly sensitive, cheap, rapid and don't need a thermocycler which makes it very appealing for POC biosensors. Therefore, we hypothesized that **LAMP can be incorporated for the development of a LOC sensor for DNA extraction, amplification and detection.**

The work focused on the following main objectives to be tested on the card: cell extraction out of sputum, mixing up the samples on the card, cell lysis and DNA extraction, and optical detection of DNA labelled by SYBR Green I (SG) using a photosensor. A card with a micro-sized channel is designed by a laser cutter to test each step separately.

The functionality of the different subcomponent of the card sensor was tested in a microfluidic system. Artificial sputum with yeast cells is prepared then liquified using hot water. Cells were extracted from sputum on the card using a Whatman filter paper. On a separate card, thermal cell lysis was tested using a microheater and the DNA extraction was estimated by the Nanodrop.

A micro-mixer is tested successfully on the card to mix, first, the extracted DNA, then the amplified DNA with the LAMP master-mix, and the SG respectively. Later, the detection of the SG-labelled DNA amplicons was performed, a UV-LED was employed to excite the SG and a micro-photosensor was able to detect the emitted light (522nm) that corresponds to the amount of DNA.

The success of developing a cheap, simple, portable and rapid DNA diagnostic card is of great value for early disease detection in remote settings and poorly equipped POC.

In this project we provide cards that can extract cells in a sputum sample, rupture the cells and extract the DNA thermally, mix sample and reagents, detect the DNA by an optical photosensor. Each of these steps was tested on a card separately. LAMP reaction was already tested by microfluidics.

To conclude, each step of the DNA card sensor was already performed by LOC. Hence, the development of a single card for DNA detection is very promising.

2. Introduction

2.1. Background

Diagnosis of the causative pathogen should always be the first step to define the cause of symptoms and the treatment of suspected infectious diseases. In order to eliminate the spread of infectious disease, it's important to do the diagnosis step rapidly as well. Several types of molecular diagnostic assays are available nowadays, majorly based on protein, metabolite and nucleic acid analysis and quantification.
DNA diagnostics are quite appealing tools since they aim to detect a specific DNA sequence that acts as a fingerprint of the pathogen. Actually, the detection of DNA is an important diagnostic approach for the confirmation of the presence of viruses, parasites, bacterial infections, and food contamination(1).

Nevertheless, most of the classical nucleic acid analytical approaches are time-consuming and only available in hospitals or central laboratories. In addition, the increased cost and the complicated protocols are all challenges for using the conventional DNA detection techniques in resource limited-point of care (POC)(2, 3). Besides, the amount of DNA, in most cases, is too low to be detected which makes its amplification a need. In this viewpoint, attempts have been made to develop more sensitive methods for nucleic acid detection from a limited genetic sample(4).

Polymerase chain reaction (PCR) took the lion's share between the other amplification methods of DNA. Yet, PCR has its own limitations as a candidate for resource-limited point of care (POC). Some of these limitations include the laborious thermal cycling, sophisticated laboratory equipment, the need for highly trained personnel and the large volume of reagents.

In resource-limited POC it is important to reduce the cost and refine the quality of health care(5). According to the World Health Organisation (WHO), diagnostic tools should have features that meet the needs of the developed and under-developed world. These features are abbreviated as **"ASSURED"** criteria which stand for: Affordable, Sensitive, Specific, User-friendly (simple to perform), Robust and rapid, Equipment-free and Deliverable to those who need them(6).

2.2 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is an excellent alternative to PCR. LAMP is a highly sensitive, specific, single step isothermal method used for nucleic acid amplification. The reason for the high specificity of LAMP is that it's performed using a set of four or six specially designed primers. These primers can recognize a total of six to eight distinct sequences on the target DNA. A

polymerase with a strand displacement property (usually the *Bacillus stearothermophilus* (Bst) polymerase) is used resulting in the separation of the non-template strand from the template DNA with no need for thermal cycles. This method can amplify a few copies of DNA to 10^9 in less than an hour due to the addition of the loop primers(7). Another benefit of LAMP is that it works under isothermal (constant temperature) conditions (60–65°c) and the result is a mixture of loop DNAs with different sizes(8).

LAMP has several **advantages including:**

- Cheap (fewer instruments required for the amplification).
- Highly sensitive (equal to or higher than that of PCR).
- Highly specific (four to six primers target six to eight regions on the target DNA).
- Rapid (less than 1 hour in microfluidics).
- Isothermal low temperature is required so no need for a thermocycler.
- LAMP is less affected by inhibiting substances.
- LAMP reagents can be freeze-dried.
- LAMP can amplify unprocessed and/or partially processed samples.

These advantages made the LAMP more attractive to implement DNA amplification and drew the attention at the possibility of developing a miniaturized LAMP sensor (microfluidic) for DNA detection for resources-limited POC(9-11).

2.3 Microfluidic Lab-0n-Card

Since the start of microfluidic technologies, especially Lab-On-Chip (LOChip) in the 90s, their use in remote settings has been observed as one of the most powerful applications by taking advantage of their portability, low volume requirement for samples, rapid analysis and the automation in a cheap device without the need for a skilled operative(12, 13).

It's important to know that LOChip is a part of the **microfluidic chip**. The latest refers to a set of micro-channels engraved or melded into a material (glass, silicon or polymer such as Polydimethylsiloxane). The micro-channels forming the microfluidic chip are connected together in order to achieve the desired features (mix, pump, sort, or control the biochemical environment). This network of micro-channels trapped into the microfluidic chip is connected to the outside by inputs and outputs pierced through the chip, as an interface between the macro- and micro-world. Compared to the classical laboratory approaches, microfluidic chip has numerous advantages like smaller reagent or sample volumes and low cost.

An alternative to LOChip that has gathered much attention recently is **Lab-0n-Card** (LOC). LOC incorporates thin film and/or paper as a substrate to construct microfluidic devices for use in rapid diagnostic tests. Paper, is biocompatible, provides a simple matrix for cells and DNA capture and isolation, and it can transport liquids passively through capillary action or wicking which eradicates the need for pumps or other fluid regulatory equipment (14).

The objective of employing microfluidic card for DNA testing is to transfer the DNA detection from laborious macroscale to a simple, rapid, equipment-free micro-scale to suit the underdeveloped POC. Thus, minimizing the labor and equipment needed for the DNA detection starting by the sputum sample processing and ending by the gel electrophoresis (Figure 1).

Figure 1: DNA testing: from lab macro-scale to a LOC micro-scale

A: The traditional steps and equipment for DNA detection in laboratories including sputum sample collection, sample processing and centrifugation, cell lysis (kit), DNA extraction and purification (kit), PCR thermocycler for DNA amplification and gel electrophoresis for DNA detection. B: Steps performed in micro-scale on our final equipment-free LOC. LOC= Lab-on-card, SG= SYBR Green I, LAMP= Loop-mediated isothermal amplification.

2.4 Previous studies

Several studies have reported success in individual step devices for DNA detection or extraction but not a fully integrated card for DNA extraction, amplification, and detection (15, 16).

Previous studies developed several analytical POC devices for DNA detection. For example, paper-based DNA extraction and amplification disk was developed for the diagnosis of rotavirus A, where reagents need to be added with a comparatively high limit of detection(17). Wang *et al*. developed a microfluidic device combining on-chip PCR amplification with lateral flow detection of *B. cereus* DNA in saliva with no on-chip DNA extraction step(18). Another study described a LAMP device using heating blocks and cell phone camera for detection, another used a battery-less LAMP technique (19, 20). Bentaleb et al. established a device for DNA amplification out of chemically fluidized sputum prior to the amplification(21). Most of these devices need external reagents to be added after the running of the device or include a complicated procedure that is hard to follow by inexperienced individuals not mentioning the complexity of manufacturing.

2.5 Hypothesis and objectives

This project focuses on **developing a simple, rapid microfluidic card sensor for DNA extraction, amplification, and detection using the LAMP technique**. The final LOC DNA sensor is in the size of a credit-card holding a net of micro-canals, permeable filter papers, micromixer, LAMP reaction zone, heating chambers, detection zone and inlet/outlet ports for the samples and/or reagents (Figure2). For this purpose, several cards were specifically designed to meet the different objectives and will be assembled in one single card eventually. Here, we seek to fabricate a disposable card that is cheap, portable and apposite for in-field usage for the detection of tuberculosis (TB) caused by Mycobacterium tuberculosis complex (MTBC) in a sputum specimen. Although prevention and treatment have greatly improved recently, TB is still an actual public health problem, foremost in low to middle revenue countries. Globally 10 million people developed TB in 2017 and it is registered as one of the top 10 causes of death worldwide second only to HIV(22). This is majorly related to the lack of proper, fast, specific diagnostic tools, especially in poorly equipped POC. That being the case, the fabrication of such a diagnostic card is urgently required.

The success of the development of this miniaturized, simple, rapid and low-cost molecular detection sensor would save labor, cost and time. The production of such a sensor will have an impact in the developing world where early detection is made difficult by a lack of suitable screening methods. In addition, this biosensor can also be used in small-scale laboratories, minor hospitals as well as private clinics.

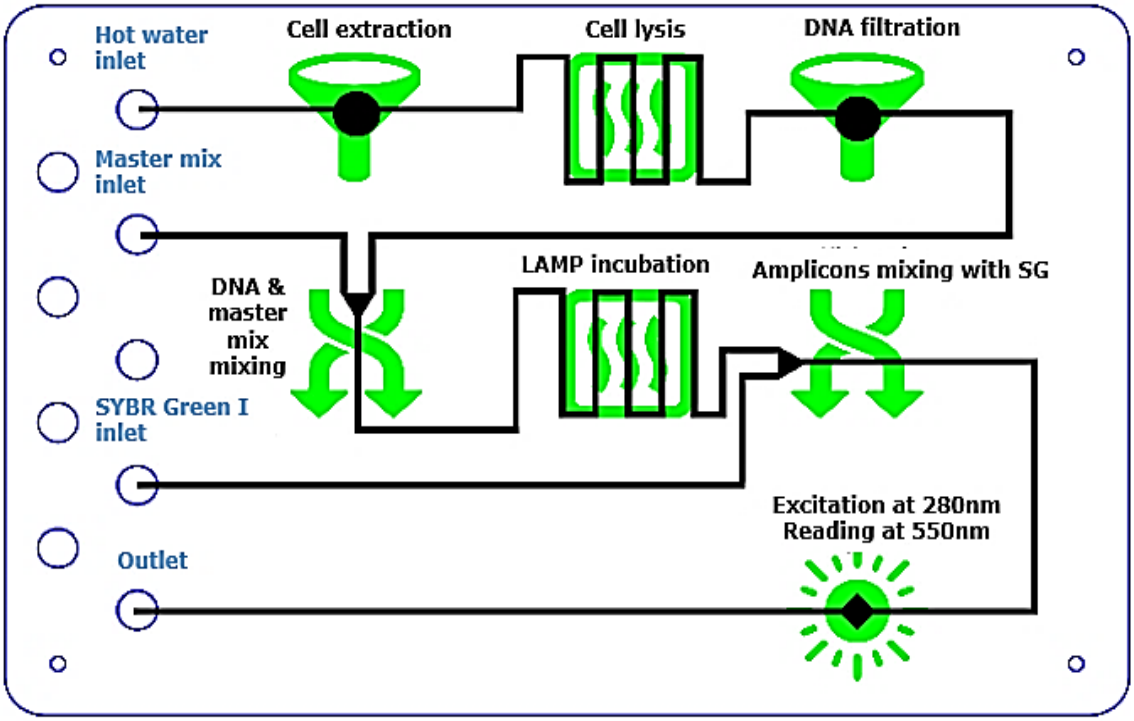


Figure 2: LOC concept for DNA detection

The sample, sputum liquifying hot water, master mix and SG are introduced through the inlet ports, pass through microchannels (black lines) and collected at the outlet. Different steps performed on the card include: cell extraction out of sputum, cell lysis & DNA extraction by a heater, DNA filtration by a filter paper, mixing the extracted DNA with the LAMP master mix, incubating the mixture at 65°C to start the LAMP reaction, mixing the amplified DNA with SG, SG-labelled DNA excitation by a UV-LED and detecting the SG emission using a photosensor incorporated with the card. LOC= Lab-on-card, SG= SYBR Green I, LAMP= Loop-mediated isothermal amplification.

We **hypothesized** that:

LAMP can be incorporated for the development of a LOC sensor for DNA extraction, amplification, and detection.

To check this hypothesis the following **objectives** were established:

Detection of DNA on the card, on card cell extraction in a sputum sample, on card cell lysis and DNA extraction, performing LAMP on the card and finally developing a single LOC sensor.

3. Materials and methods

3.1 Microfluidic Card design and fabrication

The design of the card and the channel patterns can be promptly drawn using graphic design software (Inkscape. Ink and Silhouette Studio 3.3.451). Silhouette CAMEO 3® (desktop cutting plotter) and LASER CUTTER (Trotec 100R) can be used to cut out the card. The cards were made-out of polyethylene terephthalate (PET) sheets, double-sided adhesive film and Whatman filter paper number (No°) 113 with a 30µm pore size. Whatman paper was used since it has a uniform composition and a small pore size, besides, it lacks additives that affect the flow rate. The double-sided adhesive film was used for stacking the layers and making channels by cutting through the adhesive part according to the design. The cutter made the channel pattern of 0.2 to 1mm wide (according to the design) by engraving or cutting through the layers to create a microfluidic channel for sample distribution. The top layer of the card sealed off the channels except for the inlet and outlet holes to prevent sample leaking and evaporation. A printed-circuit-board (PCB) which is a micro-electrical heater was incorporated and tested in the card to provide the heat for the thermal cell lysis and LAMP reaction. A thermocouple used as a temperature sensor for controlling the heater temperature. The card is thermally sealed using heat press (Lotus LTS 38) for two minutes at 100°C to prevent leaking. The card has the same dimensions as a credit-card which are 85*55 mm. To accurately stack the different layers of the card, the previously cut substrate materials are aligned using a fixation station with pins that fit precisely with the border holes of the card.

3.2 Cell extraction out of sputum:

3.2.1 Artificial sputum preparation

Sputum is an aqueous solution and it owes its viscoelastic, hydrating and lubricant properties to the mucin which is mainly made of glycoprotein(like saccharide polymer attached to amino acids)(23). Using this information, artificial sputum with high viscosity is prepared as follow:

0.1g of Agar-agar (E406) is mixed with 9ml of boiling water with continuous stirring. In a separate tube, 0.18 g (1%) of dried yeast is mixed thoroughly with 3ml of tab water then added to the Agar-agar mixture and stirred. 6ml of light corn syrup (Karo light corn syrup) is poured to the yeast mixture with light stirring. The final volume is 18ml. The artificial sputum mixture is then kept at 4°C till use. Although artificial sputum was previously prepared for biological purposes, yet, most of these methods were not as viscus as real sputum(24). Here, we prepared artificial sputum with a similar viscosity of that of natural sputum. The objective

in this step is to mimic clinical sputum specimen of TB patients, then sputum needs to be processed prior to cell extraction.

3.2.2 Artificial sputum liquification and cell extraction

First, Yeast (*Saccharomyces cerevisiae, Dr.Oetker*) solution (1%) is prepared by dissolving 0.5 g of Baker's yeast in 50ml phosphate buffered saline (PBS), (Thermo SCIENTIFIC, ref 28372). According to estimations, 1g of yeast can yield around 200 billion cells which means that the estimated concentration of our yeast solution is $2*10^6$ cells/ml.

Different types of filter papers (Whatman No°1 (11µm), Whatman No°4 (20-25µm) and Whatman No°113 (30µm)) were tested. The cell solution is pipetted into an Eppendorf tube which was then sealed by one of the Whatman filter papers each at a time via an adhesive film. The tube is then turned upside-down on a microscope slide and the filtrate is visualized with a microscope (Axiovision LE64 microscope). Although all the filter papers were able to filtrate yeast cells, Whatman No°113 was employed since it has the biggest pore size and can handle the pressure. Second, a card is designed especially with Whatman filter paper No°113 for cell extraction out of a liquified sputum sample. In short, the card consists of three layers: a top made of PET (2mm thick), a middle made of the double-sided adhesive laminating film which includes the channels. The channels had a width of 0.9 mm and 42mm length. A sink-like bowl made of a non-porous Polydimethylsiloxane (PDMS) was used as a sample container and fixed on top of the inlet port (Figure 3A). A 100 µl of sputum that has been liquified by boiling water (see section 4.2 optimization of sputum liquefaction) is then employed in the PDMS part which was used as a container for the sputum. A peristaltic pump was used to create negative pressure under the filter paper forcing cells filtration towards the outlet. The filtrates are collected and visualized under the microscope.

3.3 Microfluidic mixing

Several designs are developed and tested using two colors (red, blue) of water as a sample. The main goal of all the mixing designs is to induce a turbulent flow that can enhance different fluids mixing.

First designs were based on an article where it used an acoustic buzzer and sharp mixing edges to induce oscillating mixing(25). The buzzer can cause mechanical vibrational waves on the fluid inducing a turbulent effect. The idea was to fixate the buzzer underneath a thin cover slide that the fluid can flow through the mixing channel directly on top of that slide. The mixing channels varied between 0.3 to 4 mm wide with a teeth-like structure surrounding the inner edges of the channels.

The mixing canals for each card were made by cutting the design through the adhesive film that is glued on top of the cover slide.

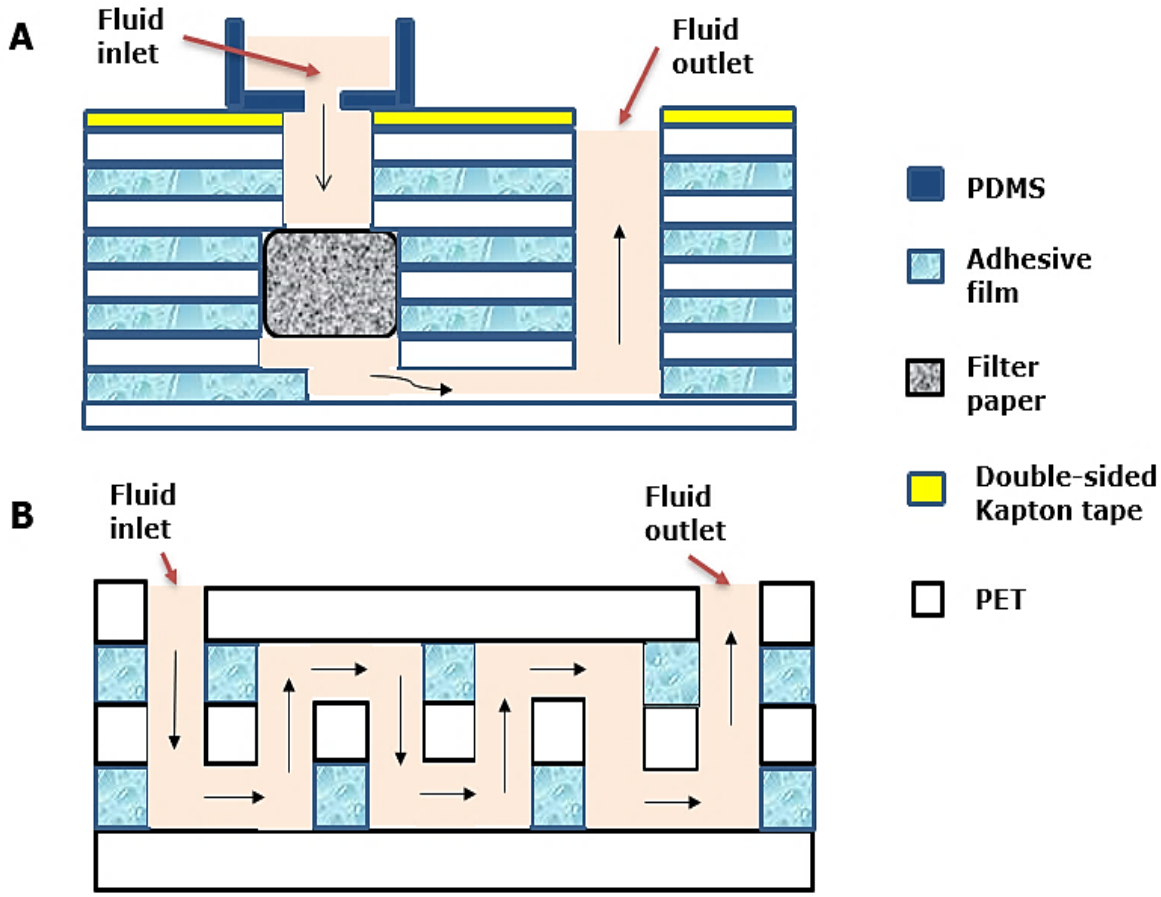


Figure 3: Schematic side-view of cell extraction card and mixing card

A: A schematic side view of the cell extraction card. B: A schematic side view of the micromixer card. Both show the material used and the way of stacking the layers, the black arrows represent the flow direction of the sample solution inside the microfluidic channel cavity. PET= polyethylene terephthalate, PDMS= Polydimethylsiloxane.

Second, to make mixing more efficient on our card and to eliminate the need for a buzzer, a card with a special design is developed. The micromixer built in this car is shown in Figure 10. In short, the mixer card was made of three PET layers (top, middle connecting and bottom layer) and two adhesive layers with cut-through channels to stack the PET. The mixer channels were composed of 15 mixing cells made of V shapes (cut on the top adhesive layer) and Y shapes (cut on the bottom adhesive layer) and are connected by holes made on the PET middle layer. The mixer channel size is roughly 0.24mm wide and 113mm length over the two planes.

3.4 Thermal cell lysis and DNA extraction

Cell lysis is essential for extracting the DNA to be amplified. By the same token, an important part of the LAMP reaction is the incubation of the DNA and LAMP Master-mix for approximately an hour at 60-65°c(8). Nevertheless, since the volumes used on the card are less than these used in macro-scale LAMP setup, the time of incubation is expected to be less as well. For this step a card with a heater incorporated was designed with a channel dimension of 0.45mm wide and 1160mm length. The design included a sharp zigzag-like channel shape to increase the time needed for cell lysis and/or LAMP incubation.

First 0.15g of yeast dissolved in 3ml Milli Q water (5% yeast solution). 300µl of the prepared (5%) cell sample was employed on the lysis cards via the inlet port by a peristaltic pump at a low flow rate (33µl/minute). The samples were directly heated up to 100°C and 90°C respectively on two separate cards for 5 minutes. Its noticed that high concentration of cells caused cells to clump inside the channels on top of the heater leading to reduced cell flow. Thus, cells will be trapped and heat up for a longer period and this may cause the extracted DNA to start degrading. Then, to attain effective lysis of the cells and to avoid cell clumping, only 10µl of 1% yeast suspension was tested on another card at the same flow rate at 100°C.

All the lysates were directly collected at the outlets and kept at –20°C for 10 minutes then centrifuged at 14000 g for 5 minutes (Sigma laborzentrifugen, 3-3OK, Germany). The supernatant was recovered as an extracted DNA solution. Nanodrop 2000 was used to determine DNA yield and purity by measuring the absorbance at a certain wavelength.

3.5 On-card optical detection of DNA

In this project, the developed DNA detection system consists of a micro-photosensor (Adafruit AS7262 6-Channel spectral sensor) connected to an Arduino board (70*55mm, ARDUINO UNO, Italy) and then to a PC.
In order to detect DNA in real-time in situ, a card is designed with one inlet and outlet with a channel dimension of 0.8mm wide, 113mm long, 11µm depth and a 3mm detection circle. The card and the detection zone are fitted on top of a UV-LED and the photosensor (Figure 5).
After the amplification step, the sample containing the amplified DNA bind to SG. When this sample passes over the detection zone the SG is excited by the UV-LED that is incorporated with the photosensor. The excited SG will emit green light at 522nm that is detected by the photosensor. The photosensor output is processed and displayed as numerical data on the PC by home-build software (LabVIEW 2017, National Instruments company).

To characterize the photosensor in this project, the card was fixed on top of the Arduino. The whole setup is shielded inside a corrugated cartoon box to reduce the noise and interference of ambient light. Samples included 10µl of colored water (green, blue, red), 5µl of negative and positive samples of MTBC DNA (supplied by MASciR) that are already amplified by LAMP and SG stained according to manufacturer instructions.

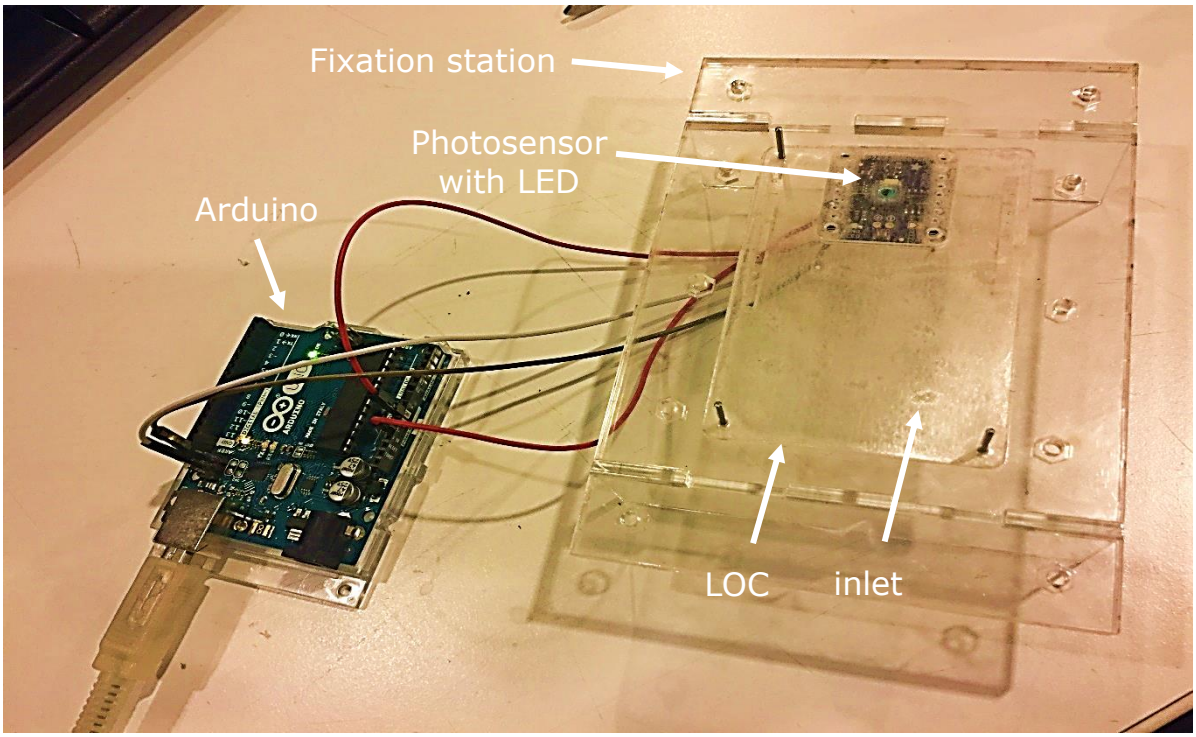


Figure 4: Optical detection setup

The photograph shows a card (LOC) fixed by the fixation station on top of the photosensor with a UV-LED incorporated. The photosensor is connected to the Arduino which transfer the results to the PC. LOC= Lab-on-card.

4. Results and Discussion

This project focuses on performing the following steps on the card: cell extraction from sputum sample, sample mixing, cell lysis and DNA extraction, DNA detection by SG. These steps are in favor of developing a card sensor for MTBC DNA detection eventually. For each step, a card with different design is developed and tested according to the objectives. Yeast cells were employed as a biological sample instead of TB cells since they are highly contagious. First, artificial sputum that includes the cells is prepared to mimic the viscosity of natural phlegm then fluidified by boiling water. Next, cells were extracted out of the liquified sputum by a Whatman filter No°113 incorporated in a specially designed card. After that, cells were erupted thermally to extract the DNA. For that, a heater was combined and tested on a card to heat up the sample during cell lysis and LAMP incubation. On another card, a mixer was incorporated and tested to mix-up the sample and reagents when needed. Also, a DNA detection of SG stained samples (5µl) was performed by employing a UV-LED and a photosensor on the card.

4.1 Optimising reaction volume and time

One of the many benefits of exploiting microfluidics in the biological analysis is the ability to maneuver sample volume, hence minimizing the costly reagents needed and preserving the sample. Similarly, reducing the time needed for biological diagnosis can slow down the propagation of diseases and speed up the treatment.

Several cards were designed to minimize the time and volume of fluid that can be used. All the first measurements were based on using different filter paper dimensions and channel sizes. We first tested the time required for the fluid (water) to spread with different sizes of Whatman filter paper. Then, different designs and channel sizes were employed. Alongside, we tested the effect of heat (65,90,100°C respectively) on the rate of flow speed and on the card integrity using PCB flexible microheater (PCBway, China). The heating step is important for both thermal cell lysis and the LAMP incubation steps. We noticed that the volume of fluid needed varied depending on the size of Whatman paper and the channel width. In other words, the cards with a smaller filter paper and/or channel size had a higher flow rate and required less volume and time for spreading. The heating increased the speed flow and no leaking was triggered by the heating since the card was already heat-sealed before use. However, some cards did show some leaking when put under high pressure and heat (>90°C) for more than 10 minutes.

These findings indicate that the width of the channel and the size of filter paper are inversely proportional to the flow rate of fluid on the card. In addition, heating up the sample on the card reduced the time of spreading which is correlated with the flow speed. Employing all these results we were able to use as low as 10µl of sample on the card.

4.2 Optimisation of sputum liquefaction:

During disease and inflammation, the airways tend to secrete a juicy fluid known as phlegm. The moment that the phlegm is expectorated by coughing it becomes sputum. Sputum is a complex clinical specimen composed of a complicated linking network of mucin, actin, proteoglycans, elastin fibers, DNA and cell debris. Entrenched within this mesh are bacteria and viruses, which are the target for diagnostic methods. With TB the case is more complicated as mycobacteria tend to clump together causing unequal distribution of bacteria in the specimen (26). For this reason, effective fluidization is necessary to breakdown the sputum network and release the bacterial cells to be extracted efficiently.

The traditional way of sputum processing involves the use of 4% NaOH for sputum decontamination and fluidisation. In this step, 4% (W/V) of NaOH (as a final concentration) is added to the needed volume of the yeast-sputum mixture then shake-up vigorously for one minute. The liquefaction step is important to enhance cell extraction out of the thick, viscous sputum.

To avoid using chemicals for the fluidization of sputum, the fluidization step of using NaOH was optimized by using boiling water with the sputum in a 1.5:1 ratio respectively with vigorous shaking for one minute. Sputum was completely fluidized by NAOH. However, boiling water is cheaper and more suitable for POC settings. Thus, the optimization of sputum fluidization using boiling water is more attractive. Indeed, the sputum was fully liquified after the addition of boiling water and shaking (Figure 6).

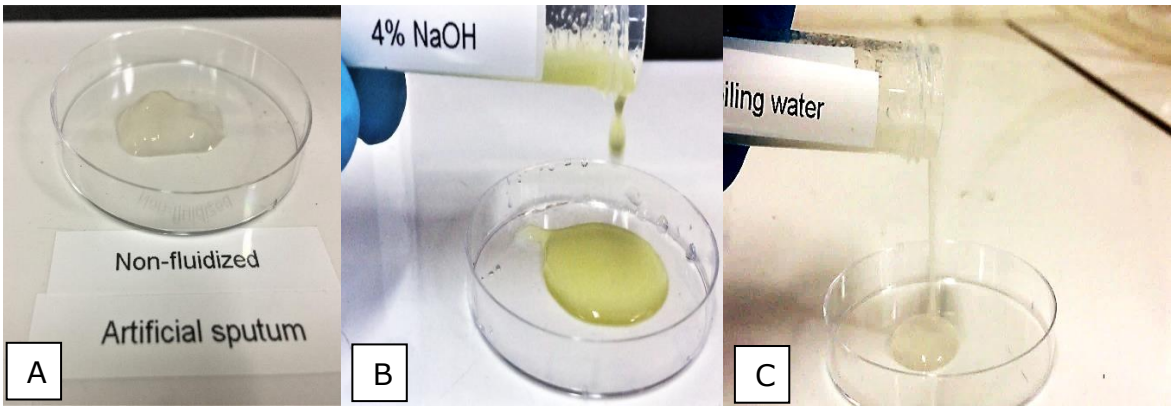
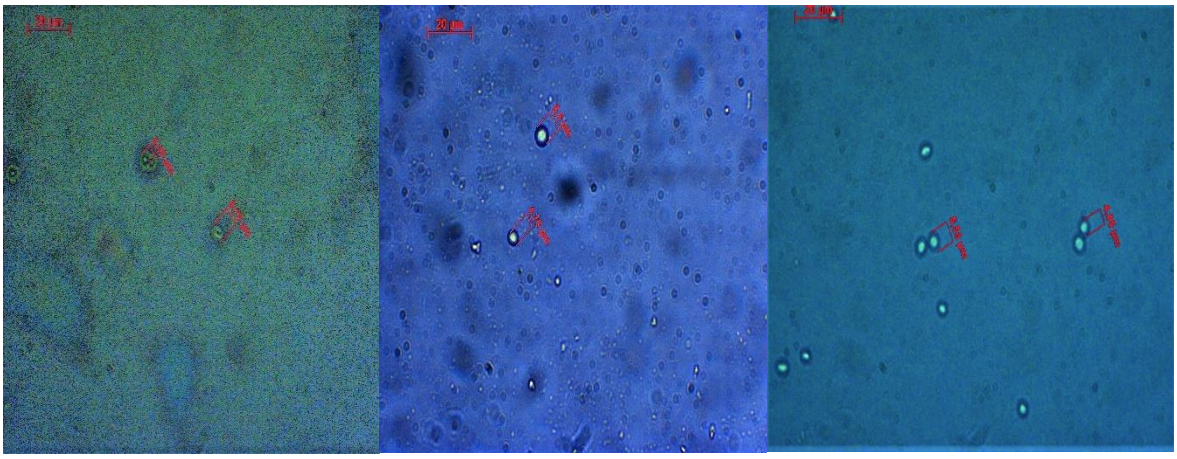


Figure 5: Artificial sputum liquification

A: non-liquified highly viscous artificial sputum. B: sputum liquified by 4% NaOH. C: sputum liquified by boiling water.

4.3 On-card Cell extraction in sputum by Whatman paper

The non-invasive clinical specimen is always preferred to reduce patient inconvenience. With that being the case, we pursue to extract the MTBC cells out of sputum specimen for further analysis. However, since Tuberculosis (TB) is highly contagious and it's not allowed to work with contagious cells at the IMO, Saccharomyces Cerevisiae (Baker's yeast) is used as a cell model in our experiments instead of MTBC. Yeast has a size of 3-4µm which is close to the size of MTBC (2-4µm in length and 0.2-0.5µm in width) and they both tend to clump making yeast an acceptable biological sample instead of MTBC for sputum-cell extraction testing. However, since we don't have a real sputum specimen, artificial sputum is prepared. In order to choose the appropriate type of filter paper for yeast extraction out of cell solution rather than artificial sputum which is more viscous. Three types of Whatman filter paper were first tested with an Eppendorf tube (as mentioned above), which are Whatman No°1 (pores size 11µm), Whatman No°4 (pores size 20-25µm) and Whatman No°113 (pores size 30µm). Then the filter paper with the most appropriate results and characteristics was selected to be used in the final card. All three types of filter paper were able to filtrate cells (Figure 7). However, Whatman filter paper No°113 is considered to be the best choice compared to the other filter papers. Whatman 113 has high wet strength due to the addition of a small quantity of chemically stable resin, ultra-high loading capacity with a 30 µm particle retention and its ideal for use with gelatinous or highly viscous precipitates like sputum. All these advantages made it more proper to be employed in our final card.



A **B** **C**

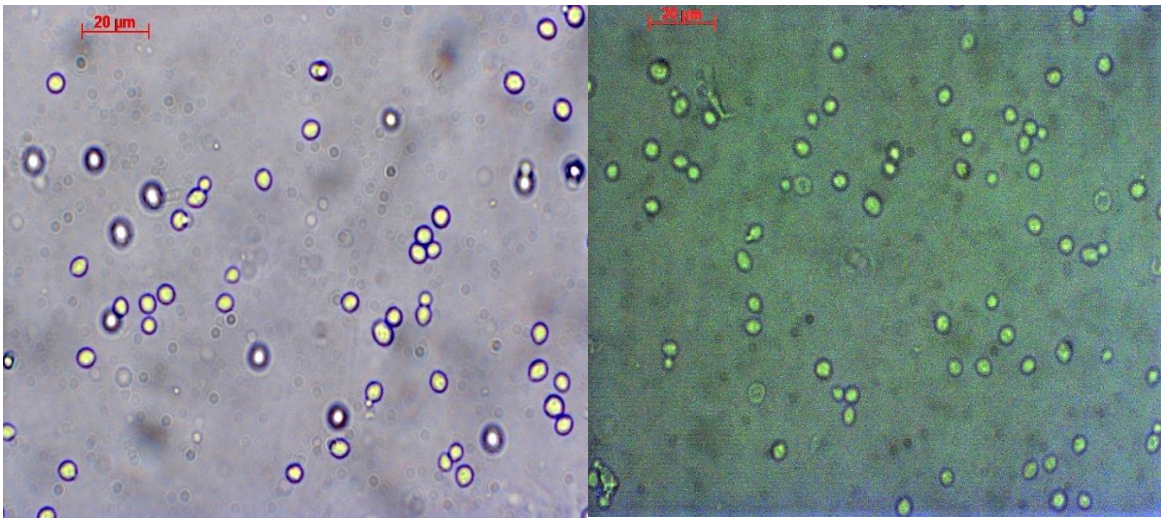
Figure 6: Cell extraction by different Whatman paper

A: Extraction of yeast by Whatman filter paper number 1. B: Extraction of yeast by Whatman filter paper number 4. C: Extraction of yeast by Whatman filter paper number 113. Filtrated cells are visualized under the microscope in all the filtrates of the three Whatman papers.

Filtrate collected from the outlet of the filtration card was directly visualized under a microscope (Axiovision LE64 microscope). Yeast Cells were detected under the microscope in the collected filtrate (Figure 8). Although, the filtrate of the Whatman paper showed a high concentration of cells. However, extracted cells were not counted since the yeast, in this case, were continuously budding which make it hard to have a right estimation of the cells that were extracted. This indicates that cells have passed the filter. These findings suggested that Whatman No°113 can handle the pressure of the pump and the viscosity of the sputum alongside filtrating the cells.

4.4 DNA extraction by Thermal cell lysis

With a view to amplify the DNA by LAMP, the DNA needs to be extracted out of the cellular components first. The method of DNA extraction should be convenient for POC and applicable by LOC. Both electrical and thermal cell lysis are very appealing methods in LOC. In this study, we investigated the thermal cell lysis for DNA extraction on the card. Thermal lysis has numerous advantages since it necessitates neither chemicals nor separation equipment. In microfluidics, the heat is dispersing rapidly due to the low volume to surface ratio leading to effective cell lysis in a short time. Mycobacterium Tuberculosis was reported to break down at 80°C for 20 minutes(27, 28). Direct cell lysis and DNA extraction are also performed by the high temperature during PCR (95°C)(29).



A **B**

Figure 7: On-card cell extraction by Whatman number 113

A: Cells in fluidized sputum before filtration on the card. B: Cells extracted on the card by Whatman filter paper number 113, a high concentration of cells was extracted.

In this step, we perused to perform DNA extraction on the card out of yeast at a high temperature and in a short time. The isolated DNA can be amplified later by the LAMP master mix on the same card. Thermal cell lysis is performed on the card by employing an electrically resistive heater or PCB (PCBway, China).

First 0.15g of yeast dissolved in 3ml Milli Q water (5% yeast solution). 300µl of the prepared (5%) cell sample was employed on the lysis cards via the inlet port by a peristaltic pump at a low flow rate (33µl/minute). The samples were directly heated up to 100°C and 90°C respectively on two separate cards for 5 minutes. Its noticed that high concentration of cells caused cells to clump inside the channels on top of the heater leading to reduced cell flow. Thus, cells will be trapped and heat up for a longer period and this may cause the extracted DNA to start degrading. Then, to attain effective lysis of the cells and to avoid cell clumping, only 10µl of 1% yeast suspension was tested on another card at the same flow rate at 100°C. All the lysates were directly collected at the outlets and kept at −20°C for 10 minutes then centrifuged at 14000g for 5 minutes (Sigma laborzentrifugen, 3-3OK, Germany) and the supernatant that contain the DNA was collected. Nanodrop 2000 was used to determine DNA yield and purity by measuring the absorbance at a certain wavelength (results are shown in Table 1). Absorbance measurement is the most common method because it's simple and requires only a UV-equipped spectrophotometer device. Absorbance readings are performed at 260nm where DNA absorption of light is the strongest. The number produced is used to estimate the DNA concentration in the solution.

260/280 ratio is used to determine protein contamination of the nucleic acid sample. The optimal ratio is 1.8 and 2.0 for DNA and RNA respectively. A DNA sample with a ratio of less than 1.7 is considered highly contaminated with a protein.

Table 1: DNA yield determined by Nanodrop 2000.

sample	DNA concentration (ng/µl)	260/280 absorbance ratio	260/230 absorbance ratio
5% yeast-300µl at 100°C	**1101**	**22.02**	**1.33**
5% yeast-300µl at 90°C	**1243.8**	**2.32**	**1.40**
1% yeast-10µl at 100°C	**542.4**	**1.94**	**1.10**

A high value of 260/280 ratio for the samples of lysis at 100°C and 90°C severally (Table 1) can estimate that the DNA sample is highly pure. While according to other literature this can be signaled by DNA degradation and the presence of free nucleotide(30). However, the time of heating was too short and the lysis at 90°C

also had a high absorbance value at the 260/280 ratio. Another factor that can increase the ratio by 0.2-0.3 is the increased basicity of the DNA sample(31). Since DNA and RNA are so similar, it's difficult to use Nanodrop for detecting the contamination of DNA by RNA and vice versa which could explain this high ratio. Using lower volume and concentration (10µl of 1% yeast) gave a slightly lower absorbance ratio of 260/280 compared to the other measurements (Table 1).

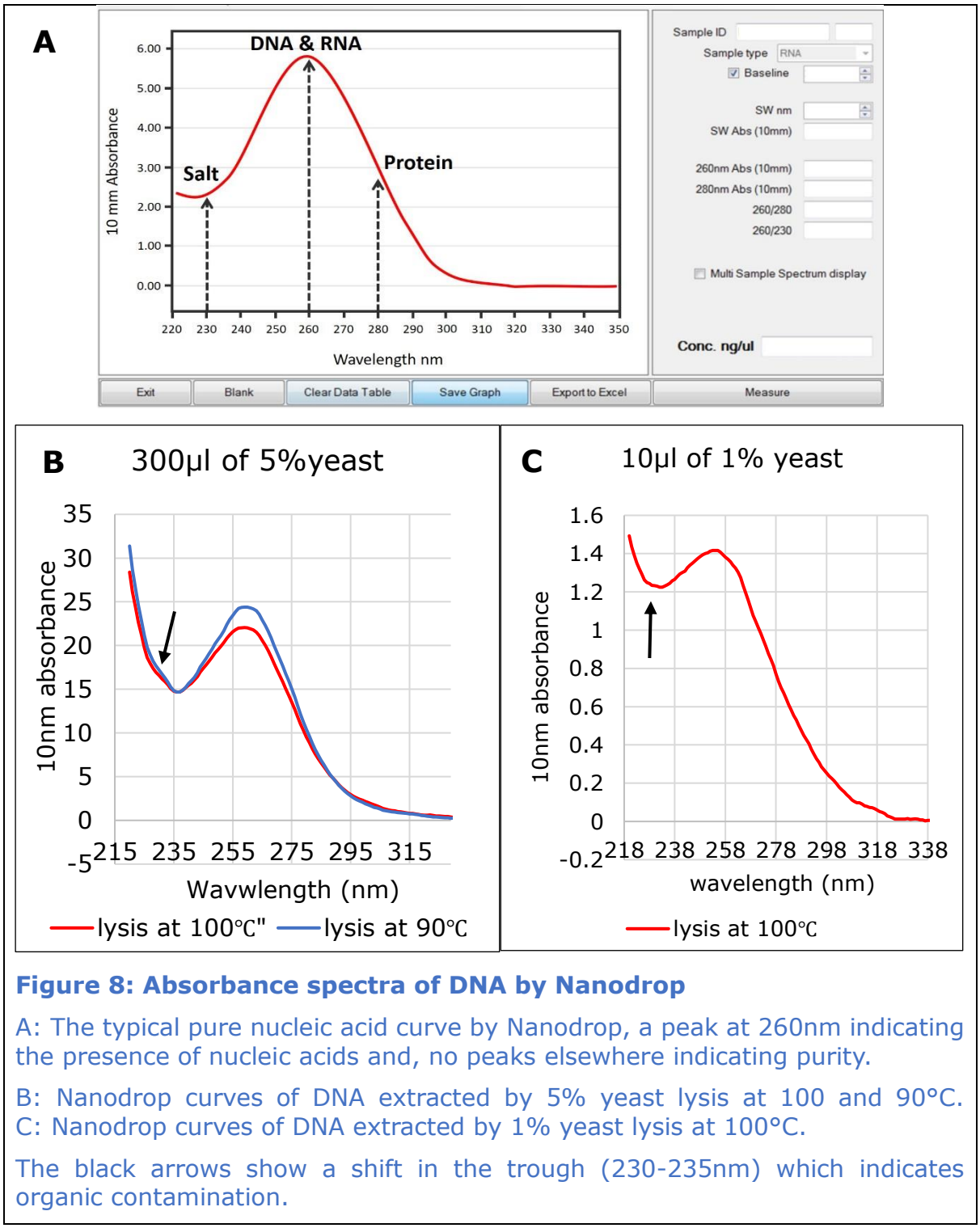


Figure 8: Absorbance spectra of DNA by Nanodrop

A: The typical pure nucleic acid curve by Nanodrop, a peak at 260nm indicating the presence of nucleic acids and, no peaks elsewhere indicating purity.

B: Nanodrop curves of DNA extracted by 5% yeast lysis at 100 and 90°C. C: Nanodrop curves of DNA extracted by 1% yeast lysis at 100°C.

The black arrows show a shift in the trough (230-235nm) which indicates organic contamination.

Thermal lysis on-card showed that using a small volume of sample is favored. This is because the channel of the card will not be blocked by the accumulation of cells on top of the heater for a long time that may affect DNA integrity.

260/230 ratio is used to determine the organic contamination. In a pure DNA sample, the optimal 260/230 ratio should be close to 2.0. If the ratio is lower than 1.8 this indicates the presence of organic contamination like isopropanol, ethanol and other aromatic compounds that could be used with some equipment in the lab which can explain the low ratio of all our samples. The curve given by the Nanodrop is also informative. The shifts in the trough (compared to the typical DNA sample spectrum) of the Nanodrop graph of the samples also suggest the contamination with organic compounds with all the samples (32, 33)(Figure 9).

Importantly, to assess the performance of our microfluidic thermal DNA extraction, we need to compare the DNA yield results with the results of the commercially available kit (like yeast DNA extraction kit) following the manufacturer's protocol. In addition, the thermal cell lysis on the card needs further investigation with attention to contaminating materials. The DNA concentration and integrity can also be verified by gel electrophoresis which can give better results than Nanodrop(34). Together the findings indicate that DNA was extracted by thermal lysis on the card. Although some signs may suggest RNA contamination, yet, thermal cell lysis and DNA extraction were achieved on the card.

4.5 On-card mixing

Mixing in micro-fluidics represent a problem due to the laminar flow of fluids. In this project, it’s important to make sure that the sample and master mixed well for the LAMP reaction to occur. On the other hand, it’s essential to have everything on the card as simple as possible. To fulfill this goal, several designs were tested using a special buzzer adapted from a publication where they used it to liquify the sputum on the card(35). All the designs that incorporated a buzzer underneath or on top of the mixing channels did not cause any visible mixing on our card. Although, the design was optimized several times and different buzzer (Murata Electronics N° 81-7BB-27-4L0) was used. None of the buzzer designs showed any visible mixing on our card

On the other hand, the mixing by the developed micromixer was clearly visible. The mixing in this micromixer is based on the folding flow of fluids in two planes by splitting and recombining streams(36, 37). So, the fluids tend to flow in two planes repeatedly to create the turbulence effect. The two colors were completely mixed at the end of the mixer as shown in Figure 10.

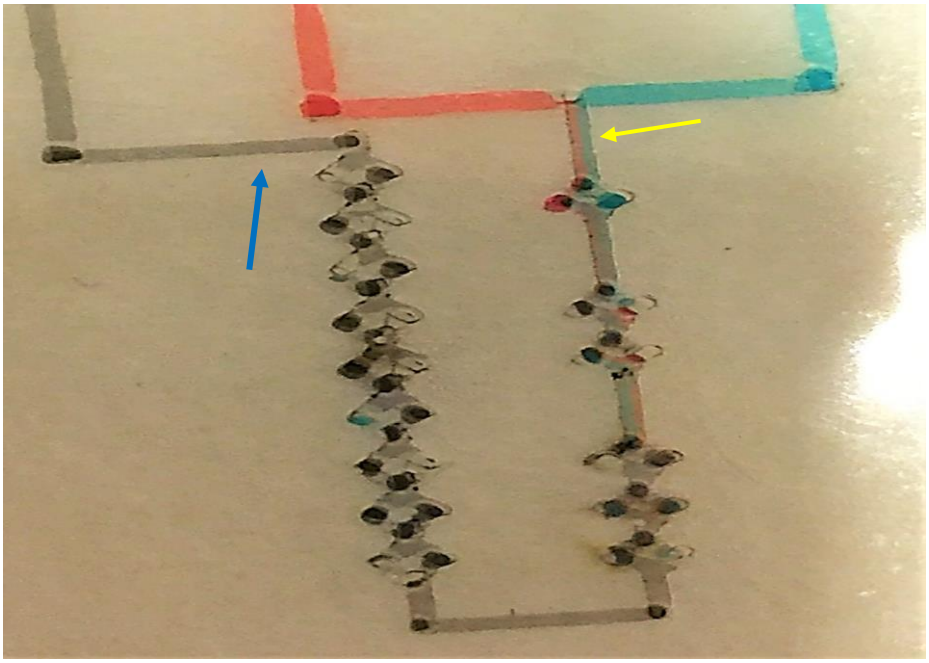


Figure 9: On-card micromixer

A visible linear flow of two colors (yellow arrow) at the inlet channel. At the end of the mixer design, the colors are merged into one color (blue arrow) indicating fluid mixing.

4.6 Optical detection of DNA

The optical detection method hired on this card is based on using a micro-photosensor to detect DNA molecules stained with nucleic acid fluorescence dye. The use of this micro photodetector excludes the need for the sizable DNA discovery equipment and make the device easily portable and applicable anywhere. However, to understand the principle of the photosensor it's important to understand the detection mechanism of fluorescence dyes first. Fluorescence dyes like SG are favored for their extraordinary selectivity and sensitivity attributed to the fluorescence activity of these dyes. SG is a nucleic acid- an intercalating dye that binds preferably to double-stranded DNA and to single-stranded DNA at a lower extent to form the DNA-SG complex(38). This complex absorbs blue light and gets excited at a maximum excitation wavelength of 497nm and at a secondary peak near 280nm. The excited SG will emit green light at a maximum emission wavelength of 520nm, which can be detected by the photodetector. Since SG is known for its compatibility with the LAMP amplification. In our samples, SG was used to stain the target DNA after performing the LAMP reaction independent from the card. Then, the samples were used to test the detection of DNA on the card by the photosensor. The photosensor used can measure the green fluorescence intensity of SG through an integrated green light filter. When the negative DNA sample (amplified sample of Non-target MTBC DNA)

passed over the detection zone of the card, the intensity of green light was slightly increased (results are shown in Figure 11). This can be explained by the ambient noise and the reflection of the source light that contain green light and not by the presence of DNA. On the other hand, the sample that contained a high concentration of the target positive DNA (amplified sample of target DNA of MTBC) possessed a higher fluorescence intensity of green light (> 20 times) compared to the negative sample. This indicates that the photosensor on our card has a high signal-to-noise ratio. In addition, the speed of detecting the changes in the intensity of light propose that the photosensor is highly sensitive to the changes of the DNA in a sample and the LOC can be used to quantitively and qualitatively monitor DNA amplification by LAMP. Consequently, the sensitivity and the limit of detection of the LOC sensor with the photosensor can be assessed in the future by using a serial dilution of the template DNA (to obtain a standard series from 10^6 to 1copy). These dilutions are then to be amplified by the LAMP and detected on the card by the photosensor.

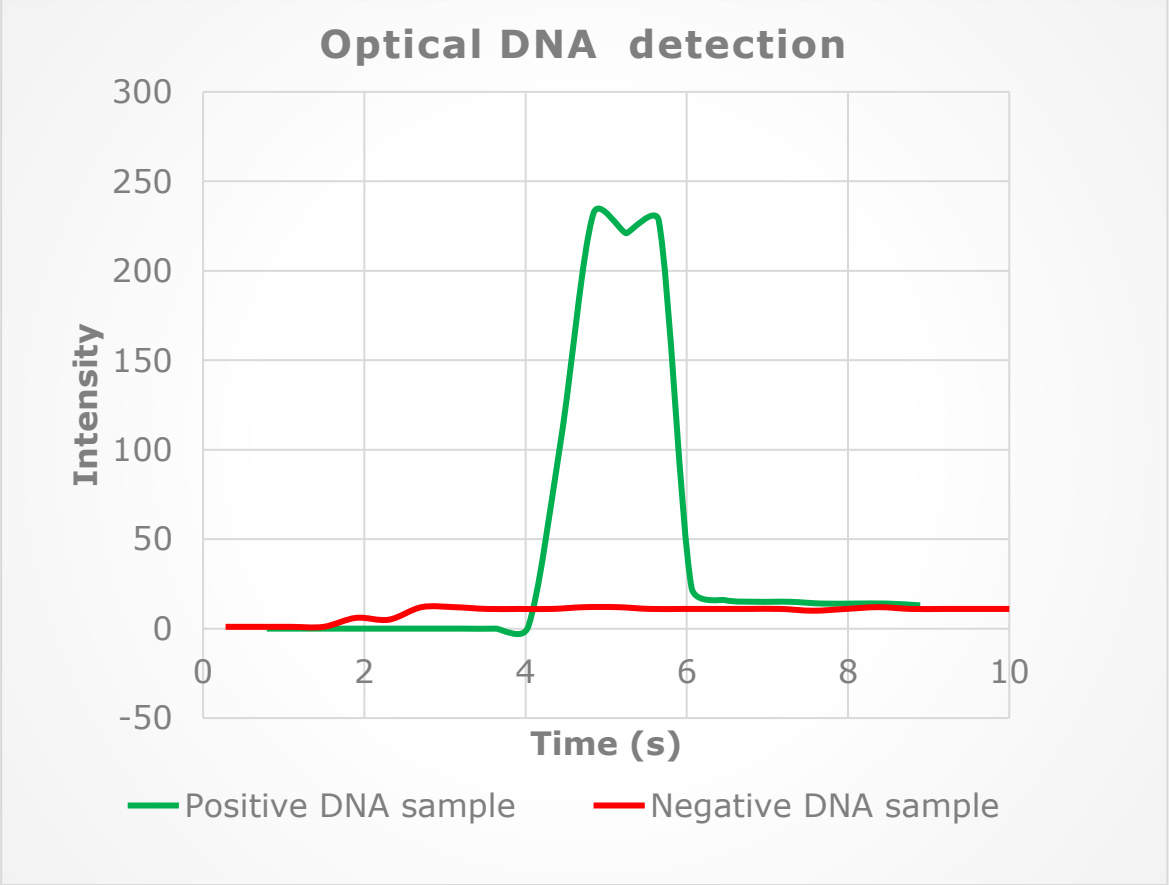


Figure 10: Optical DNA detection by the photosensor

The graph represents the intensity of light emitted by a sample of positive DNA and negative DNA detected by the photosensor on the card in a period. The graph shows an increase in the intensity with the positive sample (>20 times compared to the negative sample). The graph shows a very slight increase in the intensity of negative DNA sample caused by ambient noise.

Positive DNA sample= Mycobacterium tuberculosis DNA amplified by LAMP and stained by SG. Negative DNA sample is the result of a sample free of Mycobacterium tuberculosis DNA amplified by LAMP and stained by SG. S= seconds, SG= SYBR Green I.

5. Conclusion

In summary; we were able to perform several steps toward the development of the DNA LOC sensor. Each step of the LOC sensor was tested and approved on a differently designed card separately. Eventually, the cards developed were simple, cheap and credit-card sized. The extraction card was able to extract the cells out of water-fluidized sputum by simply using a filter paper. While, another card was able to heat up the sample by an on-card developed flexible heater that induced cell rupture and led to DNA extraction. Mixing of different fluids on the card was achieved by a specially designed microfluidic mixer which excluded the need for an external mixing device and reduced the extra cost. On the other hand, DNA detection was attained successfully on the card through the use of maximum 10 µl of sample and a micro photosensor that was capable of detecting the intensity of light emitted by SG-labelled DNA which corresponds to the amount of DNA. Indeed, the LAMP step has already been tested by other researches on microfluidics(39). Hence, the integrational development of a single DNA card sensor is a matter of time. These results harmonize with the initial goal of developing a sensor that is compatible with the ASSURED criteria and with the POC needs.

Our future perspectives of this project are to start with performing the LAMP on the card the as the superior perspective. Optimization of the thermal cell lysis is also an important step to have an optimal DNA yield. Testing electrical cell lysis on cad as well. Testing the limit of detection (LOD), sensitivity and specificity of the card.

As any other study, this study has some limitations. Although, fluorescence detection is considered as one of the most sensitive detection technologies available. However, not using an excitation filter with the detection device can influence the sensitivity of the detection signal by the background noise. A Bandpass excitation filter can be used with the light to solve this issue. Another drawback is the possibility of card blockage by the filter paper being clogged by cells or debris.

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