

A Novel Point of Care Diagnostic for Sexually Transmitted Infections

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Abstract

The sexually transmitted infection gonorrhea poses a grave threat to populations in both developed and developing countries because even though it is treatable, it is highly underdiagnosed. In order to improve lives and prevent its spread, an accurate diagnosis of the causative pathogen *Neisseria gonorrhoeae* (NG) must be made at the point-of-care. Using the power of DNA detection, we began the development of a credit card-sized chip with molecular diagnostic capabilities, ultimately creating a novel and more efficient technology. In order to enable NG detection at the point-of-care, we developed a device to capture, amplify, and detect NG DNA from patient samples such as urine. The microfluidic chip minimizes sample loss and contamination through the use of a passive valving system, targets specific NG amplification through the use of helicase dependent amplification (HDA), and visually detects amplified DNA by a lateral flow strip. We have successfully transitioned from many separate off-chip processes to one fully integrated, on-chip process. Taken as a whole, the successful development of our microfluidic chip decreases the need for expensive diagnostic equipment, minimizes sample exposure to outside reagents, and shortens the turnaround time of laboratory results - all of which are improvements over current clinical practice paradigms.

Significance and Background

Gonorrhea is the second most commonly reported notifiable disease in the United States [1], having an estimated 700,000 new NG infections occur each year [2]. On a global scale, approximately 106 million new gonococcal infections occur each year [3]. As a result, the public health benefits of a rapid and accurate NG point-of-care diagnostic will be universal since people, no matter where they live, will not seek medical treatment until an accurate diagnosis is made. One of the greatest risks NG carries is that it is frequently asymptomatic until serious complications arise. If left undetected and untreated, the long-term effects of NG infection include pelvic inflammatory disease (PID), infertility, and ectopic pregnancy in women [3]. In addition, maternal infections can lead to severe infection and blindness in infants at birth. NG infection can also increase a person's risk of contracting HIV by at most five-fold [4]. Furthermore, infected individuals may spread the infection unknowingly to others, promoting a vicious pandemic cycle. The properties of NG, its worldwide prevalence, and its asymptomatic tendencies, all demonstrate how critical an early, accurate, and fast diagnosis is to global health.

A nucleic acid amplification test (NAAT) for the detection of NG is the most commonly employed diagnostic test in use today. For a NAAT, a urogenital swab or a urine sample is obtained from the patient; the DNA is amplified using polymerase chain reaction (PCR); a particular gene of NG is targeted; and a fluorescence-based assay is run to quantitatively measure the presence and amount of infectious DNA. Compared to other methods of detection such as bacterial culture, NAATs are highly sensitive and accurate (i.e. fewer false positives). There are many technical difficulties with NAATs, however, such as the high cost and quality control [5]. Furthermore, there is a time delay of approximately 2 weeks between testing and initial treatment, which increases the chance of transmission by infected individuals [6]. Because of these issues, a point-of-care diagnostic is the ideal test for NG detection. While new point-of-care diagnostics are currently being developed for NG

detection, these tests have been hindered by low sensitivity and specificity compared with clinical testing methods such as NAAT [7].

Design Innovation

As mentioned previously, primary healthcare clinics often lack diagnostic tools on-site to determine proper treatment of infectious diseases. A compact molecular diagnostic device would be groundbreaking in helping to detect infectious diseases on a large-scale, especially for infections that tend to be asymptomatic like gonorrhea. With a point-of-care (POC) microfluidic chip, health organizations and clinicians across the globe will be able to diagnose and thus treat more patients. A simple molecular diagnostic device would obviate the need for clinicians to receive special training or lab support [8] and would result in fewer errors in diagnostic procedure as well as quicker turnaround time to patients [9]. Additional advantages of complete on-chip sample treatment are low fluid volumes and, therefore, less reagents, resulting in lower test costs and less biohazardous waste. The complete diagnostic test, from sample collection to readout, will take less than one hour [10]. Because the microfluidic diagnostic chip lowers manufacturing costs, is disposable, and can be mass produced, it will enable vital efforts to promote global health by allowing those with NG to be accurately diagnosed, therefore allowing them the chance to receive medical treatment and to prevent spreading the infection to others.

This project began with the preliminary chip design as seen in **Figure 1**, which had been developed as the result of previous proof of concept studies. While this chip contained the three critical parts of a diagnostic chip, a main channel, waste chamber, and lateral flow strip, it was not yet optimized for practical use and contained no valving system to control fluid flow.

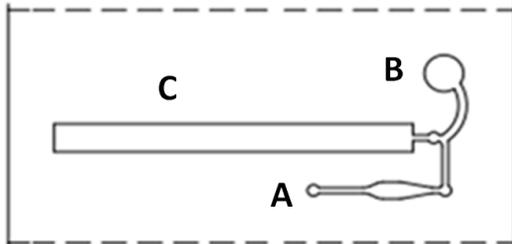


Figure 1. A CAD drawing of the initial credit card-shaped chip design with the (A) main channel containing the site for the cell filter, also known as a porous polymer monolith (PPM), (B) waste chamber, and (C) lateral flow strip compartment.

Chip Design

The largest problem presented by the initial chip design was a propensity for pre-polymer solution wicking during insertion. This led to inadequate polymerization of the cell filter inside the main channel, thus preventing NG cell capture. It was then determined that wicking was occurring purely along the chip walls, so surface tension was never able to form across the channel. This observation led to the development of our final chip design, which is displayed in **Figure 2**. This design physically prevents the pre-polymer solution from wicking along the walls with curved protrusions on either side of the main channel outlet. Any fluid that tries to wick along the walls is trapped and thus holds the rest of the pre-polymer solution in place. The waste chamber geometry was also adjusted to help ease the chip fabrication process.

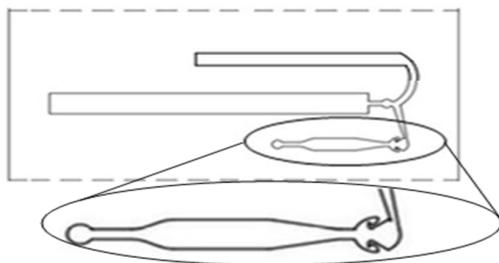


Figure 2. (A) A CAD drawing of the final chip design iteration containing wicking “trap” protrusions in the main channel outlet. (B) A close-up view of the main channel outlet protrusions.

Valving System

Within our diagnostic chip, we have successfully implemented a passive valve that will manipulate fluid flow as shown in **Figure 3**. The passive valve is not a physical barrier preventing fluid flow but merely a patch of a hydrophobic material. For our chip, hydrophobic ink is placed on the branch point leading up to the waste chamber and the lateral flow strip compartment. The hydrophobic material presents a barrier to the urine, forcing it to flow into the waste chamber. Once the chamber is filled, additional fluid is now able to overcome the hydrophobic barrier because of the fluid's incompressibility. A washing buffer then pushes the amplified DNA onto the lateral flow strip through the opened valve. Other hydrophobic materials such as wax and toner were tested but were not as easily implemented or as successful as ink from a permanent marker.

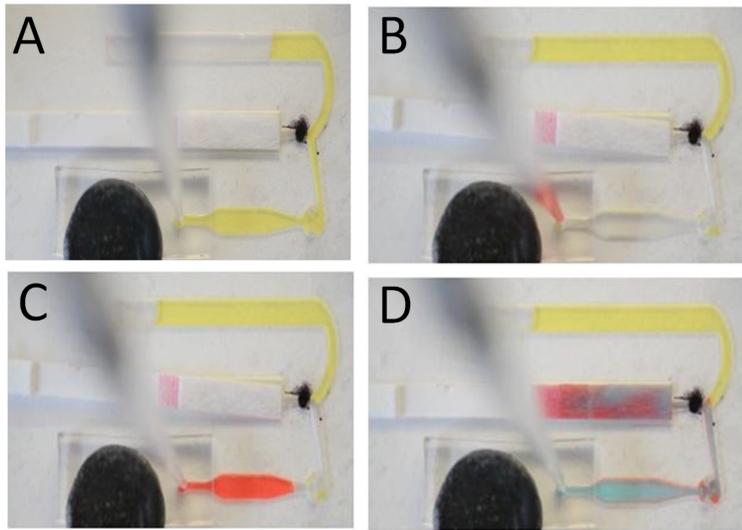


Figure 3. Still images taken from a passive valve demonstration. **A.** Water with yellow dye is inserted to simulate a patient urine sample. **B.** Air is used to push the remaining “urine” solution into the waste chamber. **C.** Water with red dye is inserted into the main channel to represent the HDA mastermix solution. **D.** A final solution of water with blue dye, representing the elution buffer, is used to wash the “DNA amplicon” onto the lateral flow strip for detection.

Isothermal Amplification

The method of DNA amplification we decided to use was helicase dependent amplification, or HDA. HDA can be performed at a constant temperature of 65°C in a time period of approximately twenty minutes [11], allowing a patient to get diagnostic test results in the same visit. This is in stark contrast to the standard practice of sending a sample to a central laboratory and waiting weeks for the results to be sent back [12], which the patient may never return to find out. HDA works like the popular polymerase chain reaction (PCR), but has the significant advantage of being able to function at a single temperature; this eliminates the need for expensive thermocycling lab equipment, requiring only a single heat source for NG amplification and detection.

Results

Genomic NG DNA Amplification in Chip

Amplification of genomic NG DNA in the chip was tested before experiments of DNA extracted from NG whole cells was performed. To prove that amplification could occur in our plastic chip, we first tried to amplify 10^5 copies of DNA, a quantity that reliably amplifies while using traditional lab equipment. We then analyzed the results by using both a polyacrylamide gel and lateral flow strips for detection. Looking at the gel image in **Figure 4A**, the band at approximately 100 base pairs shows that NG successfully amplified. Looking at the strips in **Figure 4B**, the red test line further confirms the successful amplification of NG.

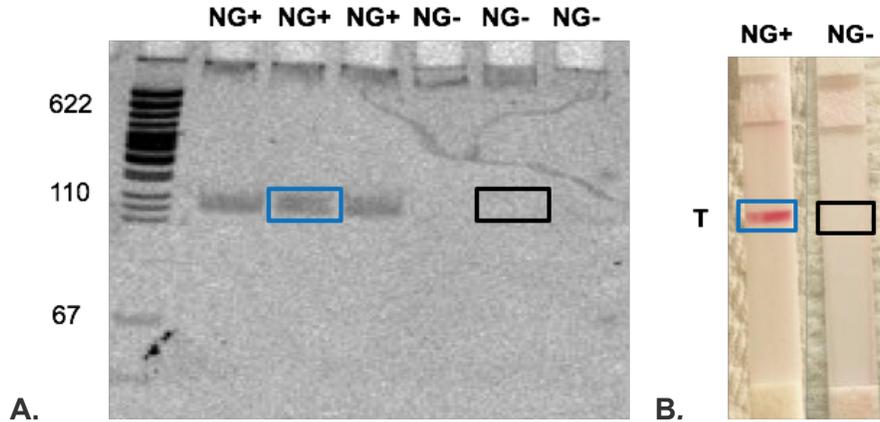


Figure 4. Confirmation of NG genomic DNA amplification on chip by **(A)** polyacrylamide gel and **(B)** lateral flow strips for $n=3$ samples. *Note: The lateral flow strips in this figure and throughout the paper will only show a single amplification test band (T) without a second control band since the optimization of the control reaction in addition to NG amplification requires HDA multiplexing and is the focus of a separate project in the Klapperich laboratory.*

NG cell lysis and DNA amplification in chip

After successfully amplifying genomic DNA on chip, we then progressed to testing cell lysis and amplification of the cell DNA. The HDA reaction occurs at 65 degrees Celsius, which provides adequate heat to lyse the cells without the need of a lysis buffer, allowing for cell lysis and amplification to occur in the same step. We confirmed NG DNA amplification from cells with the use of a polyacrylamide gel. Using 10-fold dilutions, we explored total cell concentrations ranging from 10^5 to 10. Looking at the gel images in **Figure 5A**, the bands at approximately 100 base pairs indicate NG DNA amplification. **Figure 5B** is plot representative of the gel band intensity as a function of cell count. Note that the lower limit of detection is 100 cells as it is the smallest concentration statistically significant from the negative control. The clinically relevant NG concentrations vary between 10^2 – 10^6 cells/mL and our limit of detection falls in the lower spectrum of this range.

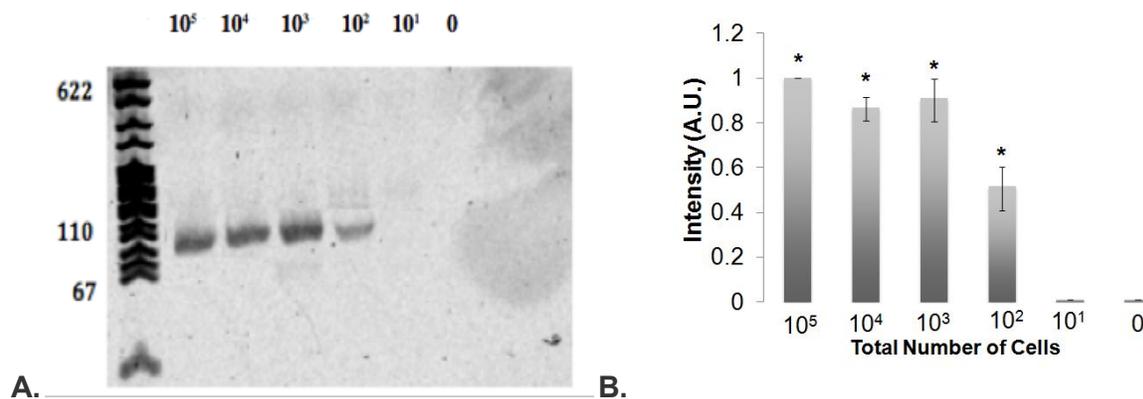


Figure 5. (A) Polyacrylamide gel of HDA reaction for varying NG cell concentrations. **(B)** Quantification of polyacrylamide gel band intensities. * denotes $p < 0.05$ compared to negative control, $n=5$.

NG cell capture, lysis, and amplification in chip with PPM

Our final experimental step was to determine whether our chip could successfully capture NG cells from a liquid sample and subsequently amplify its DNA. In order to do this, we performed preliminary experiments with 0.8 μm red fluorescent beads, which are similar in size to NG cells. **Figure 6A** shows a main channel with a PPM. The corresponding SEM image shows its sponge-like structure that allows for fluid to flow through while trapping the cells. A phase contrast image of the PPM and the subsequent fluorescent image that shows the fluorescent beads captured at the PPM interface are displayed in **Figure 6B and 6C**, respectively. After determining that our PPM has the ability to capture NG cell-sized objects, we moved onto flowing fluorescently stained gonorrhea cells through the PPM. The green area in the center of the image in **Figure 6D**, demonstrates NG cells were successfully captured by the PPM.

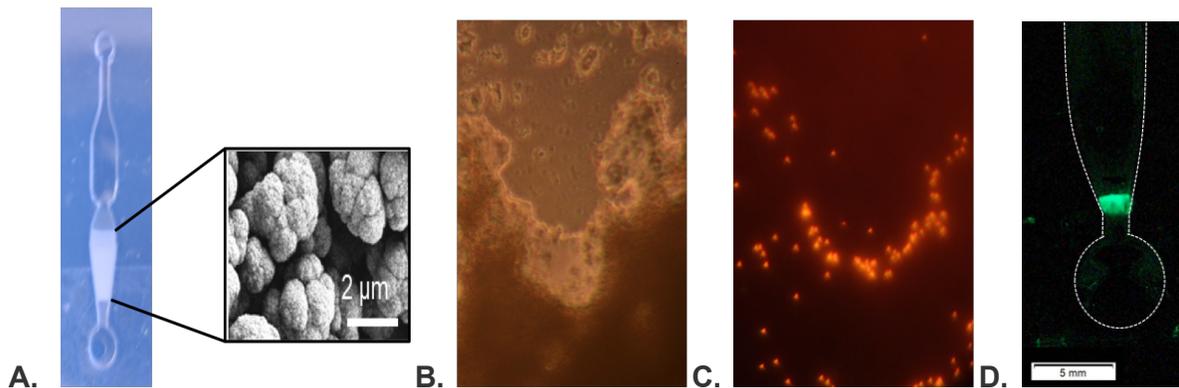


Figure 6. (A) PPM image with accompanying SEM image of the microscopic structure. (B) A 40x phase contrast image of the PPM. (C) A 40x fluorescent image of the PPM with trapped 0.8 μm red fluorescent beads. (D) A 2x fluorescent image of the PPM with trapped fluorescent NG cells.

After determining that we could capture NG cells, we moved on to testing our amplification assay on these cells. The gel in **Figure 7A** shows the successful amplification in the range from 3000 to 30 cells. Further testing of the resulting amplicon on lateral flow strips confirmed the limit of detection to be 30 cells (**Figure 7B**). Finally, quantitative analysis of the gel results, determined by comparing the amplification band intensities at each cell count, revealed each sample to be statistically significant from the negative control (**Figure 7C**).

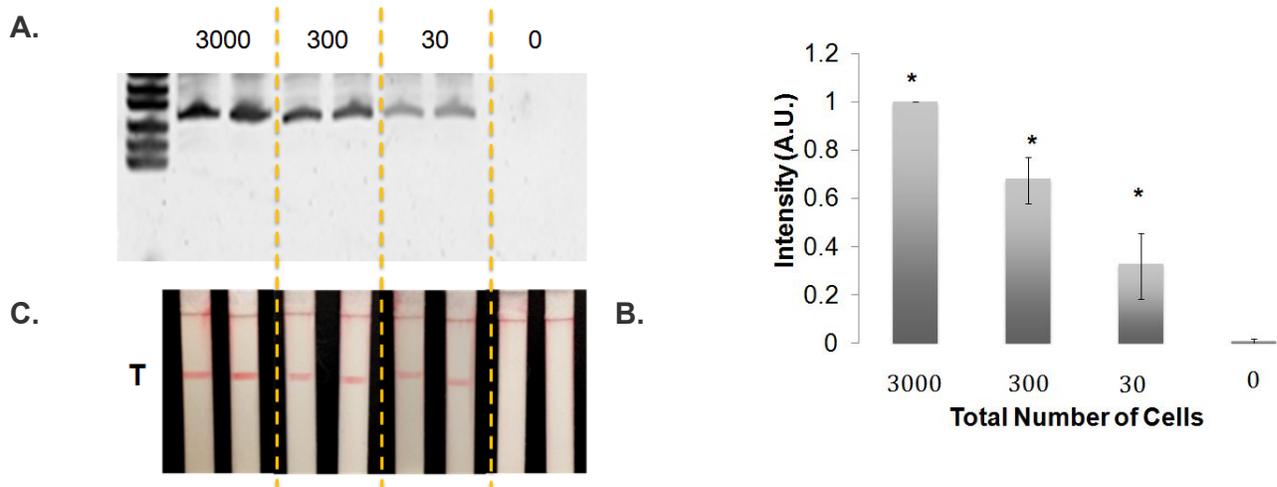


Figure 7. (A) Polyacrylamide gel of HDA reactions for varying NG cell concentrations flowed into the PPM. **(B)** Lateral flow strip confirmation for varying NG cell concentrations flowed into the PPM **(C)** Quantification of polyacrylamide gel band intensities. * denotes $p < 0.05$ compared to negative control, $n = 4$.

Final Integration

After optimizing each individual component, including the control of directed fluid flow, the gonorrhea cell capture in the PPM, and the HDA amplification reactions, we combined the steps in our diagnostic for the final integration process. Just one hour later, we were able to successfully detect gonorrhea at a clinically relevant concentration on our lateral flow strip and then confirm the results with a gel as shown in **Figure 8**.

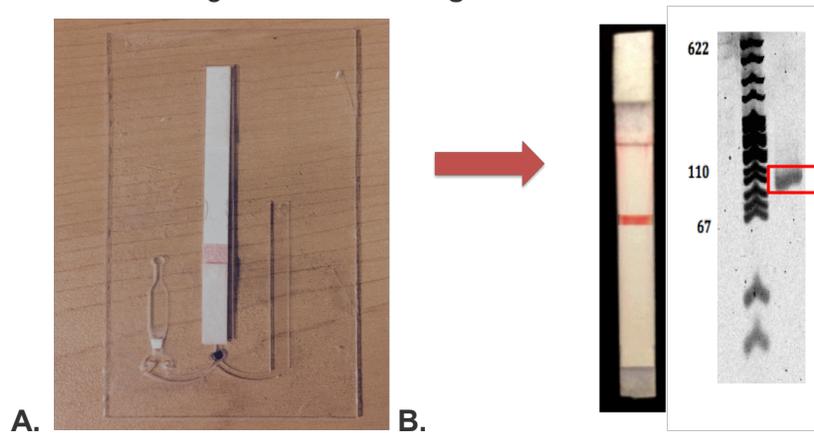


Figure 8. (A) Image of the completed chip prototype which includes the PPM, hydrophobic valve, and lateral flow strip. **(B)** Lateral Flow strip and polyacrylamide gel results of final prototype amplification.

In conclusion, we have developed a microfluidic diagnostic that can capture gonorrhea cells through the use of a porous polymer monolith and extract the DNA using heat lysis; it can amplify NG DNA through an isothermal HDA reaction; and it can detect NG at the lower limit of clinical relevancy, all the while using a simple and innovative valving system to safely handle sample waste. Our portable, credit-card sized diagnostic is over 100x faster than existing diagnostic methods and has the potential to change the diagnostic paradigm in clinics all over the world.

Appendix

Hyperlink of Video Detailing How Our Diagnostic Works:

<https://www.youtube.com/watch?v=41Yf5QPzjP4&feature=youtu.be>

References

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