A Tube with Thermally Removable Barrier for Single Tube, Two-Stage Polymerase Amplification

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***Abstract*— The Hepatitis C Virus (HCV) is one of the leading causes of chronic liver disease that is commonly spread by needle sharing. The current standard for diagnosing HCV comprises an initial screening for HCV antibodies and followed, when positive, by a nucleic acid amplification test designed to detect the presence of HCV RNA. However, this time-consuming and expensive method is something that developing countries with a higher prevalence of HCV cases are unable to afford and is susceptible to patients failing to follow up and receive treatment. As a result, the spread of the virus is not controlled. In this paper, we focus on an HCV detection apparatus and method that is rapid, inexpensive, and simple to perform – a few of the conditions for point-of-care (POC) diagnostics. We developed a new polymerase chain reaction tube combined with the utilization of a two-stage isothermal amplification process (comprised of loop-mediated isothermal amplification and recombinase polymerase amplification) that together create a suitable POC device to test for HCV. Detecting the amplified template HCV target using visual colorimetric detection, our device is biocompatible with the amplification processes and can accurately diagnose controlled samples. With a simple two-stage process contained in a single tube, our device is a feasible tool for POC diagnostics in developing countries for the detection of HCV.**

***Index Terms*—**3D-printing, colorimetric detection, hepatitis C virus, loop-mediated isothermal amplification, point-of-care, recombinase polymerase amplification

I. Introduction

Hepatitis C is a viral infection caused by the hepatitis C virus (HCV). There is currently no vaccine for HCV, however, depending on its severity, hepatitis C can pose as a short-term infection, possibly one without any symptoms, or progress to a chronic liver infection. Today, HCV is commonly transmitted through infectious blood often via injection drug usage or blood transfusions [1].

In the most recent reported data from the US Center for Disease Control and Prevention (CDC) in 2019, a total of 4,136 hepatitis C cases (1.3 cases per 100,000 people) were reported in the United States. This had been a 14% increase from 2018 and a 133% increase from 2012 [2]. To adjust to case under-ascertainment and underreporting, the CDC estimates a total of 57,500 acute infections in 2019 [2]. In addition, an estimated 3% of the world population, roughly over 180 million people, are infected by HCV – making HCV a significant contributor to morbidity and mortality worldwide [1].

To this date, screening for HCV is a two-stage process. However, being a two-stage process has a few disadvantages that several studies aim to improve upon. Because screening requires two tests, it can become a time-consuming process just to confirm the state of the HCV infection - namely a few days to a few weeks for the results to come back from a lab. In addition, although being a standardized nucleic acid amplification technique, the use of the reverse transcription-polymerase chain reaction (RT-PCR) assay to amplify a given sample is very complex. PCR requires expensive equipment, namely a thermocycler, and generally takes multiple hours to amplify the DNA or RNA, as PCR requires cycling through 3 different temperatures to properly amplify the target. After amplification, PCR then requires a complicated target detection method that only trained personnel can conduct [3, 4].

We have chosen to focus on the higher prevalence of HCV cases in developing countries as those countries often lack the equipment and personnel required to perform today's standardized screening for HCV [1]. Therefore, with a potential higher rate of transmission as well as an inadequate way to control or minimize the spread of HCV, hepatitis C becomes a major problem for developing countries.

To resolve the issue of HCV screening being time-consuming, complex, and expensive, there have been a few constant temperature (i.e., isothermal) amplification techniques developed that simplify the amplification process to become simpler as well as require less specialized equipment. As developed in [5], here we use a two-stage isothermal amplification process via loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). Two-stage isothermal amplification was discovered to be 10 times more sensitive than performing one stage isothermal amplification [5]. This makes for the possibility of a reliable early detection technique for diagnosing HCV.

The previous literature provides the procedure and materials needed for performing the two-stage amplification process allowing for this paper to extend that work. Our focus was to create a more efficient method for performing this two-stage

amplification. In the prior work, each stage was performed separately in two tubes before having to be manually combined into one tube to produce the final amplified product [5]. Performing the stages separately poses the risk for open air contamination as well as a higher susceptivity to human error. This is where we worked to create an apparatus that would allow this two-stage amplification process to happen all in a single tube. Our proposed method for two-stage amplification in a single tube is illustrated in Fig 1. Using 3D-printing to fabricate our modified PCR tube, a thermally removable barrier can be created to establish a lower chamber and an upper chamber within the tube to hold each amplification process. With the addition of this thermally removable barrier, our tube can house both amplification solutions separately while also allowing for a systematic process for the two-stage amplification.

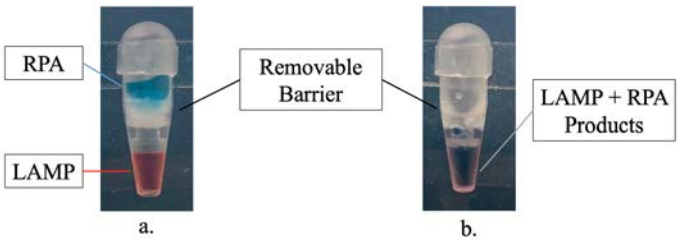


Figure 1 *Visual interpretation of the two-stage amplification solutions in our 3D-printed tube. Using dye for ease of visualization, the blue dye represents the RPA solution, and the red dye represents the LAMP solution. After the docosane barrier liquifies, the two solutions can mix.*
a. 3D-printed tube at initial insertion
b. 3D-printed tube after the docosane barrier is set to liquify allowing for the two solutions to mix. The docosane barrier remains on top of the mixture.

A summary of our proposed method for HCV screening is illustrated in Fig. 2.

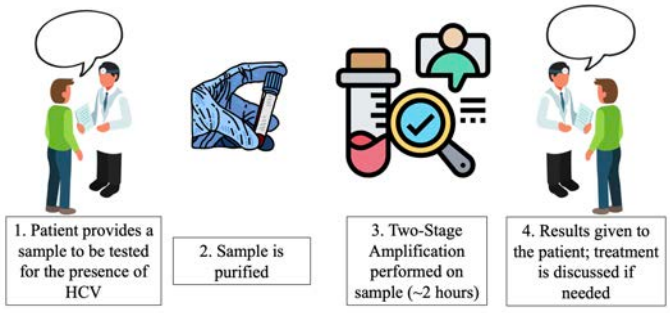


Figure 2 *Illustration of an HCV test with the use of a two-stage amplification operation. In this paper, we focus on developing the procedure for step 3 in the above illustration. As seen in our method, the patient can receive results within the same visit.*

For developing countries, a molecular test must be simple and inexpensive for an infection like HCV to be controlled. With our device and the two-stage molecular test from [5], we have created a point-of-care diagnostic tool that can be easily deployed to those where HCV is a major public health concern.

II. Background

A. Hepatitis C Virus (HCV) Screening

The standard HCV screening used today consists of two stages. The first stage is an antibody test (anti-HCV test) where a positive test result indicates that an individual has been infected with HCV at some point. In other words, a positive antibody test does not indicate whether an individual currently has hepatitis C. However, following a positive result from the anti-HCV test, another test is then needed. As the second stage for testing for HCV infection, the nucleic acid test (NAT) for HCV RNA commonly uses the reverse transcription-polymerase chain reaction (RT-PCR) assay to amplify the target in the sample. Since HCV RNA can be detected as early as 1 week after an individual has been exposed, HCV NAT has been determined to be reliable in diagnosing a current HCV infection [1].

This two-stage process is feasible for developed countries that have access to the necessary equipment and trained personnel. However, these specifications for HCV screening, specifically for performing the HCV NAT, pose issues for developing countries. This is where we look to isothermal amplification processes as a rapid and inexpensive alternative.

B. Loop-Mediated Isothermal Amplification

First discovered in 2000 by Notomi *et al* [6, 7], loop-mediated isothermal amplification (LAMP) is an alternative nucleic acid amplification technique. As an isothermal amplification technique, LAMP is capable of amplifying DNA under a single temperature – around 60-65°C [8]. This allows LAMP to eliminate the need for expensive and complex equipment usually required for PCR-based amplification. All that is needed is a simple heating block or heated water bath that can provide a fixed temperature to the samples [9]. LAMP is also highly sensitive to even lower amounts of nucleic acid as it is reported that it can detect even as low as 6 copies of the target in a sample [7]. This is crucial to its potential for detecting HCV as a higher sensitivity test can provide a reliable method for early detection of an HCV infection as well as a condition to minimize false-negative results [5, 9]. Lastly, LAMP can utilize fluorescent or colorimetric detection to display an easily identifiable presence of the target pathogen. This detection technique is the simplest and least timely way to evaluate the results from the LAMP process. Therefore, it is a feasible method to easily detect HCV when placing such a test in developing countries [6-9].

Many studies over the last decade have confirmed that LAMP is extremely versatile and can detect many pathogens and bacteria ranging from those that invoke food-borne illnesses to ones from common viruses [6]. Also, LAMP can be used to detect either RNA or DNA targets which allows for RT-LAMP to be a suitable technique for detecting the presence of HCV RNA [3, 9].

C. Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA) is another isothermal nucleic acid amplification technique. Operating at a steady low temperature anywhere between 31 and 43°C [4, 5,

11], RPA likewise requires minimal equipment to maintain this temperature during amplification. For detection of the target, RPA uses either real-time, target specific fluorescent probes or lateral flow strip detection [10, 11]. RPA is also able to detect the target typically in less than 30 minutes, making it a suitable rapid point-of-care test. In this work, our test is based on LAMP, however, together with RPA, the sensitivity is 10 times more sensitive than LAMP or RPA alone [5]. The manufacture TwistDx currently holds a patent on this amplification process. We will be using their kit to perform RPA in our lab.

D. Thermally Removable Barrier Material: Docosane

We use a water-insoluble solid called docosane as the material for the removable barrier that is placed inside our 3D-printed tubes. Docosane is white when solid and transparent when liquid. In addition, docosane is less dense than water which makes the placement of this material into the tube simple. With the melting point of docosane within 42-45°C, we can systematically perform our two-stage amplification process for the detection of HCV all in one tube [12].

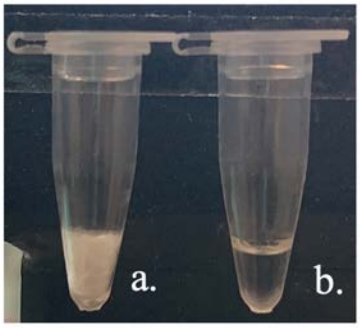


Figure 3 *Docosane in its solid (a.) and liquid form (b.)*

E. Visual Colorimetric Detection of LAMP

To get rapid results from our two-stage amplification process, we use visual colorimetric detection. One significant advantage of using visual detection is that it allows our process to be suitable for testing in developing countries. There is no extra instrumentation or trained personal needed to diagnose for HCV using this detection method [5].

Specifically for detection of LAMP, we are using hydroxy naphthol blue (HNB) colorimetric dye. The LAMP solution contains many Mg^{2+} ions that during incubation, react to form a magnesium pyrophosphate biproduct [8]. This suggests that during incubation, the Mg^{2+} concentration in the LAMP solution gradually decreases. This property is how the HNB dye, also classified as a metal indicator, can accurately signal the presence of the HCV target. If there is no HCV target in the solution for the LAMP process to amplify, the solution will exhibit a violet hue. On the contrary, if there is HCV target in the solution, the LAMP process will amplify the HCV RNA and cause the solution to exhibit a blue hue. HNB colorimetric detection is shown in Fig. 4.

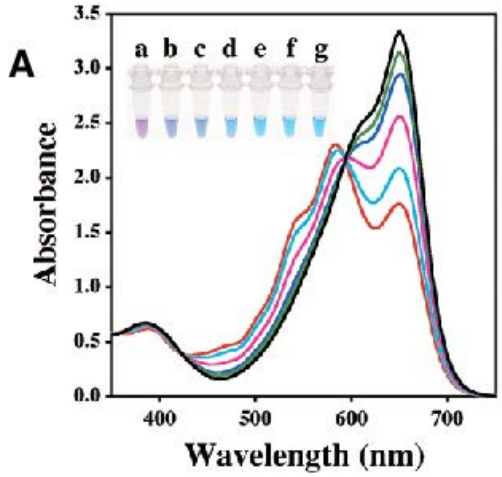


Figure 4 *Visual colorimetric detection using hydroxy naphthol blue (HNB) [8]. This figure details the color spectra of HNB due to varying concentrations of Mg^{2+} ions. Tube a, 8mM Mg^{2+} ions without dNTPs; tube b, 8mM Mg^{2+} ions with dNTPs; tube c, 7mM Mg^{2+} ions; tube d, 6mM Mg^{2+} ions; tube e 5mM Mg^{2+} ions; tube f, 4mM Mg^{2+} ions; tube g, 0mM Mg^{2+} ions. In our experiments, we have used a concentration of 8mM Mg^{2+} ions.*

Alternatively, we have also used pH sensitive dyes as a method for visual colorimetric detection of the target template. This method of amplification utilizes the significant pH change from an initial alkaline pH to an acidic pH after incubation as observed from the LAMP reaction [13]. When there is a target template that invokes amplification, the solution will change from its initial pink/red hue to a yellow hue. With this color change only occurring upon amplification, pH sensitive dyes provide an additional reliable colorimetric detection method.

Figure 5 *Visual colorimetric detection using pH sensitive dye. With a clear color difference between positive (pink) and negative (yellow) presence of the target template, pH sensitive dyes are a valuable visual detection method. Image from: https://www.neb.com/products/m1800-warmstart-colorimetric-lamp-2x-master-mix-dna-rna#*

F. Point-of-Care Tests

Point-of-care (POC) tests can be suggested as an ideal diagnostic test as they align with the World Health Organization's suggestion that those diagnostic tests be highly sensitive, rapid, require minimal equipment or personnel, and inexpensive [14]. Due to its rapidity, results can often be given to a patient within a short period and therefore can give a patient the necessary treatment without needing the patient to come back for another visit. Since the standardized HCV screenings

are conducted in two stages, it is extremely beneficial if this screening can be improved and shortened to just one stage. In addition, due to POC tests being inexpensive and simple to perform, these tests can be easily transported to any site. This gives it the advantage of being able to be utilized in developing countries [14].

III. Materials and Methods

In this section, we have described the proposed materials and methods needed for detecting HCV using our alternative PCR tube design. To create our device, this project focuses on experimentation and is guided through 3 main steps: design, fabrication, and testing.

A. Design

We began with the alternative PCR tube design, created using the 3D modeling capabilities of the SolidWorks software. This alternative PCR tube has an outer shell that directly replicates the outer shell of a standard PCR tube as seen in Fig. 6. This was intended as we needed to make sure that our alternative PCR tube was versatile enough to be tested in the same machines that a standardized PCR tube would normally be placed in (for example, a thermocycler).

The inside of our tube differs from a standard PCR tube. As shown in Fig. 7b, our alternative tube design has a partition fabricated to create two vertical vias inside the tube on one side and on the other, a main channel with a ledge that connects the lower and upper chambers. The vertical vias are available to access the lower chamber after the docosane barrier is placed in the main channel.

A cap was also designed to fit snugly around the top of the tube. Though a simple design, this cap showed to be suitable for keeping the samples contained within the tube during the amplification processes.

Figure 6 *Our 3D-printed tube design*
a. standard PCR tube strip
b. 3D-printed tube (with docosane barrier)
c. 3D modeled tube in SolidWorks (front view, with cap)

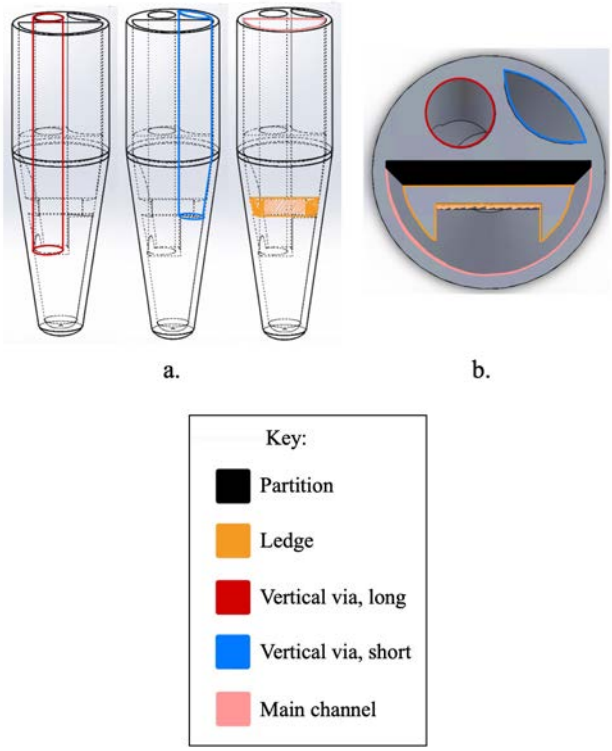


Figure 7 *Model of our 3D-printed tube design and the terms used to describe the components of the inside of the tube. All models were designed in SolidWorks.*
a. Wireframe, front view
b. Top view

B. Fabrication

We have chosen to utilize 3D-printing technologies as a quick and inexpensive (rapid prototyping) fabrication technique to create our tube. In our lab, we use the Formlabs Form 3 3D-printer - which is built on stereolithography (SLA) 3D-printing technology. Using Formlabs's Clear V4 resin material, our fabricated tube can remain partially transparent. After the part has been printed, we followed Formlabs's SLA basic finishing steps. This included washing the part in the company's Form Wash machine, post-curing the parts under UV light in the company's Form Cure machine, and lastly letting the part sit in an oven (set to 45°C) overnight. However, after complications with a pH test using our 3D-printed tubes, we discovered that the Clear V4 resin was not compatible with the pH visual colorimetric detection method.

We hypothesize that the tube's resin may contain additional OOH groups that release protons into the solution and as a result, cause a change in the pH of the solution. To counteract this incompatibility of the 3D-printed tubes, we treated the inner surface of the tubes with an alkaline solution. This surface modification involved setting the 3D-printed tubes (after having been properly cleaned and post-cured) in 1M NaOH solution for 24 hours. After 24 hours, the tubes were rinsed with deionized water and placed in an oven set to 45°C overnight. For our experiments, we treated all our tubes with NaOH solution. Alternatively, we found that treating the tubes with a combination of 1M NaOH and 1% bovine serum albumin (BSA) was also a way to modify the surface of the tubes. This

surface modification could be done by treating the tubes with 1M NaOH, as previously detailed, but additionally placing BSA into the tube. The BSA filled tubes would sit for 3 hours before being emptied and placed in an oven set to 45°C overnight.

For the thermally removable barrier, we have used docosane as the material for the barrier. For inserting the barrier, we first placed approximately 60μL of deionized water into the main channel of the 3D-printed tube. We then inserted 23μL of docosane (liquified beforehand on a hot plate at 75°C) into the main channel. To create a barrier that was uniformly distributed above the ledge in the main channel, the tube was dipped into a warm water bath (set to about 80°C) for 30-40 seconds to allow for the docosane to re-liquify. Observations with the naked eye indicate that because docosane is less dense than water, the docosane would slowly liquify but remain on top of the deionized water in the tube. After 30-40 seconds passed, the tube was taken out of the warm water bath and placed into a slot within a Styrofoam cube. The Styrofoam acts as an insulator that allowed for the tube and the docosane to uniformly cool. After cooling for 5 minutes, the deionized water remaining underneath the barrier was taken out through the long vertical via while the short vertical via allowed air to enter the lower chamber. The tube, now only containing a solid docosane barrier, was then left to rest for an hour to allow for the docosane to fully harden. Steps for the creation of the thermally removable barrier are summarized in Fig. 8.

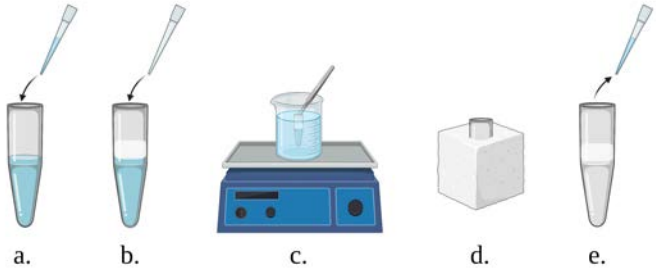


Figure 8 *Fabrication procedure for the thermally removable barrier*
a. insert deionized water into the tube
b. insert docosane into the tube
c. dip tube into warm water bath
d. uniformly cool tube in Styrofoam
e. remove deionized water out from under the docosane barrier
[Figure created with BioRender.com]

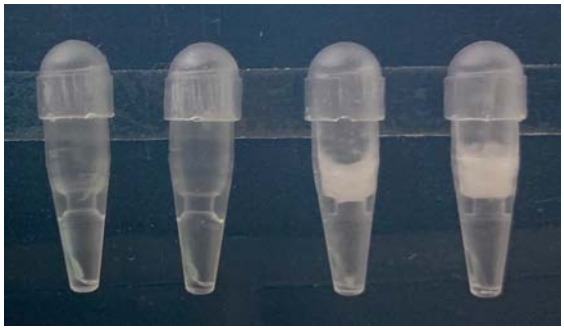


Figure 9 *3D-printed tubes before and after the insertion process of the docosane*

C. Testing

After allowing the docosane barrier to rest, the tubes are ready for experimentation. We began with testing the structural integrity of the docosane barrier using a dye test. Since the docosane barrier was meant to establish an upper and lower chamber, this barrier needs to be able to hold an amplification solution without letting any pass through the solid barrier. We also aimed to use the minimum amount of docosane needed to establish that solid barrier. We had identified 23μL of docosane was the minimum amount of docosane needed. However, oftentimes incomplete coverage or the surface tension of the inside of the tube creating a concave meniscus may cause a 23μL docosane barrier to be faulty. As seen in Fig. 10a, this docosane barrier has formed a concave meniscus after cooling while Fig. 10b shows the creation of a docosane barrier that does not have a meniscus. This is where we can use the dye test to verify these barriers' structural integrity.

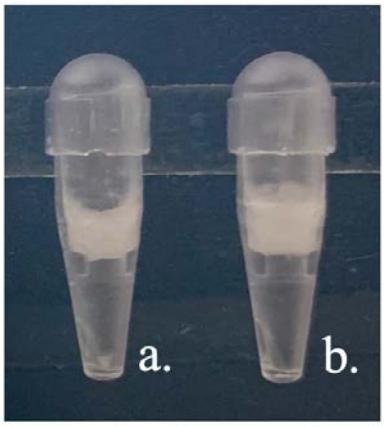


Figure 10 *The effect of surface tension on the structural integrity of the docosane barrier*
a. Case where a concave meniscus has formed after the barrier has cooled
b. Case where no meniscus has formed after the barrier has cooled

To mimic the placement of an amplification solution in our dye test, we placed 25μL of food coloring on top of the docosane barrier. We then observed the tube periodically for 60 minutes to verify the structural integrity of the barrier. However, we later determined that the dye test was destructive to the LAMP process and resorted to testing only one tube from each batch of tubes made. The tube that was chosen for the dye test was then discarded while the remaining tubes moved on to the experimentation with the amplification processes.

As per the prior literature that this paper extends, this paper uses the two-stage amplification process termed RAMP. We will break down the RAMP process in the following sections.

1) RT-LAMP Conditions (Part 1 of 2 for RAMP)

RT-LAMP is a modified form of LAMP that uses a reverse

transcriptase to make complimentary DNA to then be amplified. Since we are working with a HCV RNA target, RT-LAMP is necessary for amplification. The total volume of the RT-LAMP solution was kept to 20µL by including 10µL of 2X composition of reaction mixture (manually created in the lab using the protocol detailed in [3]), 1µL of HCV primer solution, 1µL of DNA polymerase, 1µL of target HCV template (for positive controls), 1µL of hydroxy naphthol blue (HNB) colorimetric dye and 6µL of molecular water. With the inclusion of the HNB colorimetric dye, this LAMP mixture would utilize the metal indicator for visual detection of the target.

Alternatively, to use the pH sensitive dye for visual detection, we used 10µL of WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs), 1µL of HCV primer solution, 1µL of target HCV template (for positive controls), and 8µL of molecular water. The total volume of the LAMP solution was likewise kept to 20µL.

This LAMP solution is created first as it will occupy the lower chamber in the tube. The above composition is inserted using a vertical via. Only one visual detection method, either the metal or pH indicator, is used per experiment.

2) RPA Conditions (Part 2 of 2 for RAMP)

RPA reactions were assembled according to the manufacturer's instructions (TwistDx, TwistAmp Basic Kit). Additionally, we have used the HCV primer sequences used in the previous work that developed the two-stage RAMP process. This solution is created second as it will occupy the upper chamber and can be inserted in the main channel directly on top of the docosane barrier.

After creating the RAMP solution, our tube is prepared for the two-stage amplification process. At this point, we can record the initial color of the RAMP solution. In the upper chamber holding the RPA mixture, the solution is clear. In the lower chamber holding the LAMP mixture, a violet hue can be observed. After recording the initial color and confirming its state, we begin the two-stage amplification of the solution. With a lower amplification temperature of around 38°C, the first-stage RPA solution in the upper chamber of the tube can be incubated in a heat block set to 38°C for 30 minutes. The heating source is then set to 50°C for 5 minutes, to allow for the docosane barrier to fully liquify. After the docosane barrier has liquified and allowed the RPA and RT-LAMP volumes to mix, the heat block is set to 63°C for 45-60 minutes for the second-stage LAMP reaction.

At the end of the incubation process, the tube is placed on ice for 5 minutes to halt amplification. The naked eye can then observe the color of the RAMP solution. If using HNB colorimetric detection, a violet hue signals a negative detection of HCV while a blue hue signals a positive detection of HCV. If using the pH colorimetric detection, a pink hue signals negative detection while a yellow hue signals positive detection.

A summary of the entire methodology explained in this section is illustrated in Fig. 11.

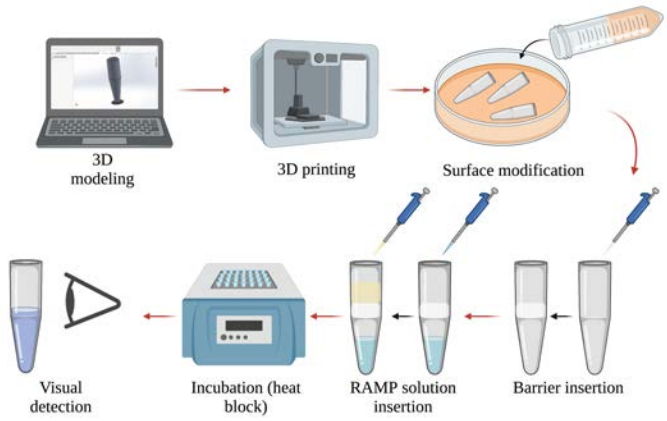


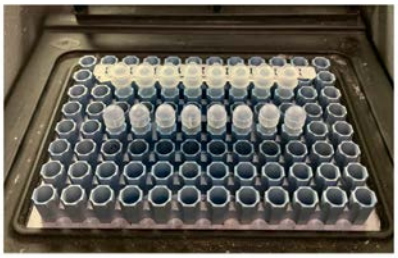
Figure 11 *Flow chart illustration of the process used in this project for the creation of a POC device for HCV detection.* [Figure created with BioRender.com]

IV. Results and Discussion

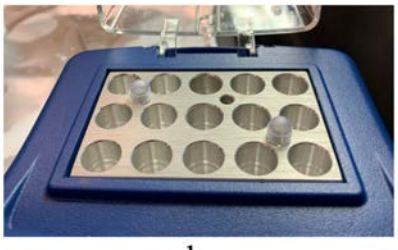
To develop a POC device that would be suitable for diagnosing HCV, the device would not only have to meet the structural conditions for replacing a standard PCR tube but would also need to not interfere with the biological amplification processes. Here we discuss how our new PCR tube performed in the lab.

A. Device: Structural Integrity

The first necessary experiments done with our 3D-printed tubes aimed to test the structural integrity of the tube and the docosane barrier inside. Being an alternative design to a standard PCR tube, our tube was made to directly match the outer shape and size of a standard tube. In addition, it was made sure that our device would be able to fit within the spaces in either a heat block for 0.5mL tubes or a thermocycler.



a.



b.

Figure 12 *3D printed tubes compatibility with the potential heating sources*
a. Thermocycler with an 8-tube PCR strip and eight 3D-printed tubes
b. VWR Advanced Mini Dry Block Heater with two 3D-printed tubes (note: we did not have a heat block for 0.5mL tubes in our lab. The heat block shown is for 1.5mL tubes but has slots that can fit our 0.5mL printed tubes)

After designing a device that replicated the outer shape of a standard PCR tube, we moved on to testing the structural integrity of the docosane barrier. In our two-stage amplification process, one amplification solution must lie on top of the barrier and incubate on its own before letting it mix with the solution in the chamber underneath. Here we used the dye test to observe whether the barrier would be able to hold up a volume of solution. We concluded the minimum amount of docosane needed to make the barrier in our tubes was 23μL. This amount not only created a solid barrier in the tube but also withstood the dye test for an average of 90 minutes – well past the necessary time needed for performing RPA.

After performing a dye test on a select few tubes per batch of tubes created at a time, our 3D-printed tubes would be ready for the next experiments.

B. Device: Biocompatibility

Verifying the structural integrity of our design was the first part of creating a suitable POC device. The next necessary experiments done with our 3D-printed tubes then focused on biocompatibility. The 3D-printed tubes would be housing nucleic acids, enzymes, buffers, etc. that would be necessary for the amplification processes to take place. Therefore, we needed to perform controlled experiments directly in our tube to confirm that our tubes would not inhibit the amplification processes.

We began with verifying the biocompatibility of our 1M NaOH surface modification to the inside of the 3D-printed tubes. As shown Fig. 13b, we observed that this surface modification was able to successfully chemically modify the inner surface of the tube so that our 3D-printed tubes would not affect the pH of the solutions. Verifying the pH of the 3D-printed tubes was done using 10μL of WarmStart Colorimetric LAMP 2X Master Mix (New England BioLabs) and 10μL of molecular water. An expected decrease of volume can be observed after incubation due to evaporation. Since the solution in the tubes remain the same color before and after incubation, we confirmed that our surface modification prevents our 3D-printed tubes from modifying the pH of the reaction mix.

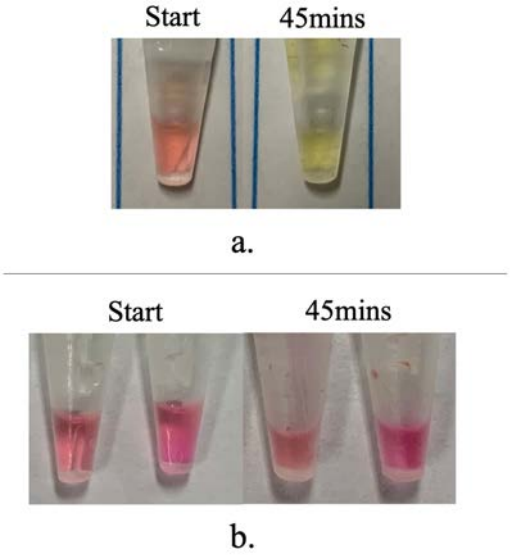


Figure 13 *Before and after the surface modification of the 3D-printed tubes*
a. 3D-printed tubes before a surface modification. Note that there is a spontaneous color change to yellow after a 45-minute incubation in the absence of target templates.
b. 3D-printed tubes after a 1M NaOH surface modification. Note that there is no color change after a 45-minute incubation in the absence of target templates. It is also interesting to note that the colors of the LAMP mixture at the start have a significantly pinker hue for the tubes with a surface modification. This further shows the NaOH solution can chemically make the inside of the tubes more basic.

After confirming the surface modification would cease the chance of the 3D-printing resin from affecting the pH of the solution, we then tested for the biocompatibility of the tube with the amplification processes. Although we aim to use our tube for the two-stage amplification process, we first needed to test the compatibility of the two stages (LAMP and RPA) independently and without the presence of the docosane barrier.

We started with testing the LAMP procedure in the 3D-printed tubes using a hepatitis B virus (HBV) as the target template for one experiment and HCV for another. We used a concentration of 250 copies per reaction of HBV and 3.0×10^5 copies per reaction of HCV. In Fig. 14, we have four total 3D-printed tubes with two tubes set up as a negative control and two tubes set up as a positive control. After incubation, we can observe that the HNB indicator remains violet for the negative controls and changed to blue for the positive controls.

We concluded with visual observation of the distinct color difference between the negative and positive controls that our 3D-printed tubes are biocompatible with the LAMP process.

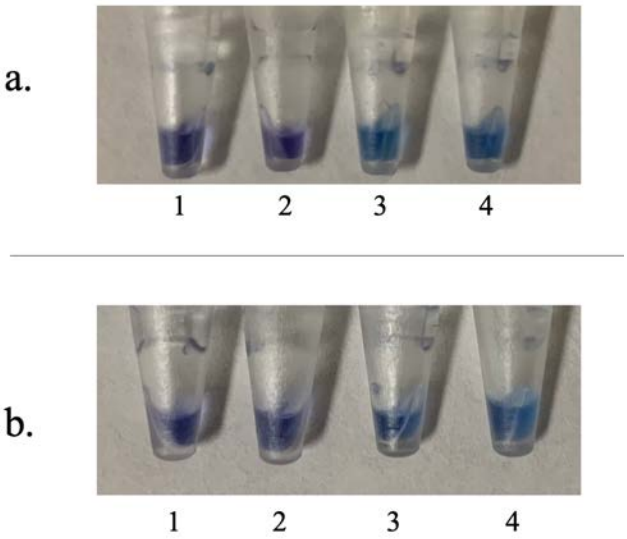


Figure 14 *Testing the LAMP procedure in the 3D-printed tubes without docosane present. Using HNB colorimetric detection to verify the presence of the target template. Tubes 1-2: negative control (no target). Tubes 3-4: positive control (target present).*
a. Target template: HBV.
b. Target template: HCV.

Due to time constrains and complications that will be explained in the next section, we were unable to test the RPA procedure in the 3D-printed tubes. Consequently, the two-stage RAMP process was also not tested.

C. Ongoing Complications

After confirming that the tube without docosane was biocompatible with the LAMP process, we planned to test the biocompatibility of the tube and docosane with LAMP. However, when performing this combination in our tube, we observed an inconsistency with what we expected in the negative and positive control groups. Since we had determined that the 3D-printed tubes without docosane were biocompatible with LAMP, we concluded that docosane might be the source of the unexpected results.

There was now the possibility that somehow the docosane was chemically interfering with the LAMP process. We then moved to testing the docosane alone with the LAMP process in standard PCR tubes. One experiment conducted was comprised of LAMP 2X buffer and varying amounts of docosane. With no target in any of the tubes, we expected all the tubes to remain violet when using the HNB indicator. As seen in Fig. 15a, this hypothesis was disproved. In the tube with 10µL of docosane, there was a noticeable color change of the HNB indicator to blue while all other amounts of docosane remained violet. Additionally, we performed another experiment with docosane but now added a target template as a positive control. We expected that all negative controls would remain violet while all positive control would change to blue. As seen in Fig. 15b, this hypothesis was also disproved.

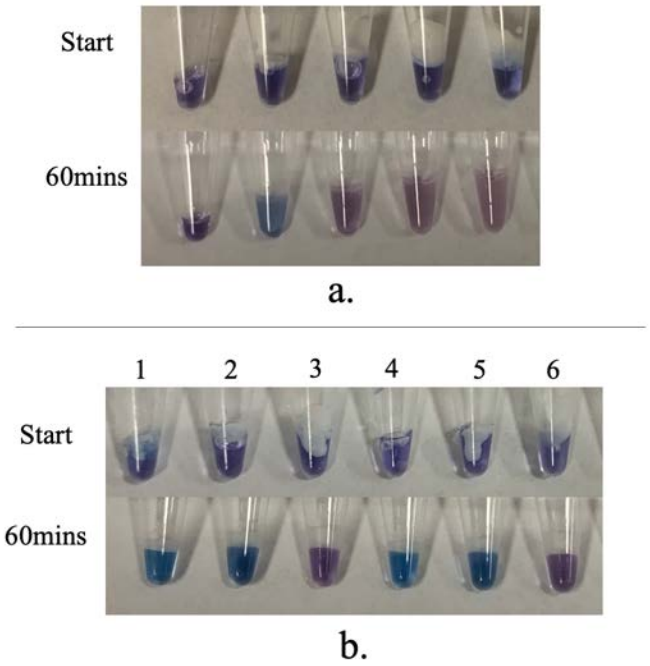


Figure 15 *Docosane testing series using the HNB indicator on the LAMP process*
a. 2X LAMP buffer and varying amounts of docosane. (From left to right, 0µL, 10µL, 20µL, 30µL, 40µL of docosane)
b. Varying amounts of docosane tested with negative and positive control groups. (Tubes 1-3: negative control, from left to right, 5µL, 10µL, 20µL of docosane. Tubes 4-6: positive control, from left to right, 5µL, 10µL, 20µL of docosane).

Through these experiments, we noticed that there is the possibly of a pattern that correlates the amount of docosane to the incompatibility to the LAMP process. In all cases where less than 10µL of docosane were tested, the LAMP solution changed to blue. This was regardless of whether there was a target template present. On the contrary, in all cases where more than 20µL of docosane was used, the LAMP solution remained violet. Likewise, regardless of the presence of a target template. (We would also like to note that the range of 10µL to 20µL of docosane was not tested).

Despite the setback with the HNB indicator testing method, we also attempted to use the pH sensitive dye indicator for a possible alternative way to continue testing our 3D-printed device. However, due to docosane's hypothesized slightly acidic property, the pH sensitive dye prematurely changed colors despite the absence of a target template when tested in a standard PCR strip (as seen in Fig. 16).

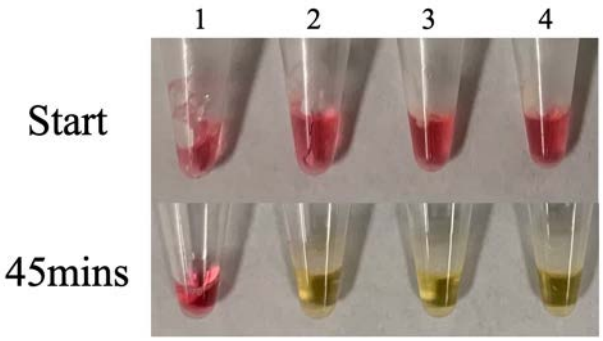


Figure 16 *Docosane testing using the pH indicator LAMP buffer in a standard 8-tube PCR strip. Tube 1: no docosane. Tube 2-4: varying amounts of docosane (from left to right, 10μL, 20μL, 30μL of docosane). No tubes contain a target template. After a 45-minute incubation, tubes 2-4 changed to yellow.*

Upon noticing the acidic nature of docosane, we recalled that our 3D-printed tubes were currently having their surface modified with the 1M NaOH solution to chemically manipulate the surface to be more basic. We then performed another test with the docosane and the pH indicator LAMP 2X buffer but placed the solution directly into the 3D printed tubes before incubating. After incubation, as seen in Fig. 17, the tube with 10μL of docosane did not cause the pH indicator to prematurely change color. This was an intriguing result as the same amount of docosane tested in the standard PCR strip had caused a color change whereas there was no color change in a surface modified tube. We hypothesize that possibly after the application of the surface modification, some NaOH is left over that inherently causes an increase in the pH of the reaction mix. As a result, this may be enough to counteract the alleged acidity of the docosane for small amounts of docosane used. This finding now poses a possibility for some form of biocompatibility between the docosane and the LAMP process.

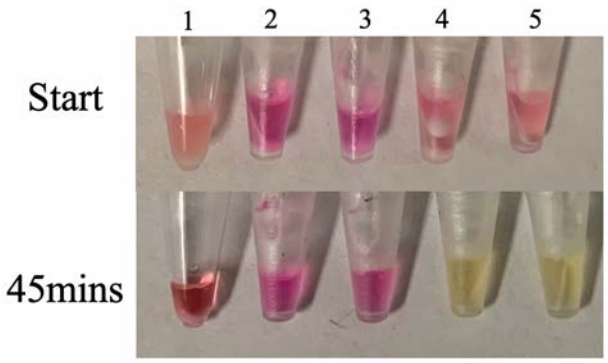


Figure 17 *Docosane testing using the pH indicator LAMP buffer in 3D-printed tubes with 1M NaOH surface modification. Tube 1: standard PCR tube, no docosane. Tube 2: 3D-printed tube, no docosane. Tubes 3-5: 3D-printed tubes, varying amounts of docosane (from left to right, 10μL, 20μL, 30μL of docosane). No tubes contain a target template. After a 45-minute incubation, tubes 4 and 5 changed to yellow while tube 3 (containing 10μL of docosane) remained pink.*

From these experiments testing with docosane, we can conclude that there are some possible chemical or biological complications with the biocompatibility of the docosane material with the LAMP process. Unfortunately, time restrictions did not allow for further experimentation.

V. Conclusion and Future Directions

In this work, we propose an alternative PCR tube design that can be utilized in developing countries for diagnosing HCV. While using the two-stage amplification process termed RAMP, as described in the literature that this work extends, we have determined that our 3D-printed tube design is biocompatible with the first-stage LAMP process. Additionally, through an inner surface modification of the tube that chemically adjusts the pH of the tube to remain basic, our 3D-printed tube can accommodate the usage of either an HNB metal indicator or a pH indicator to detect the presence of the target HCV template. This allows the 3D-printed tube on its own to be flexible enough to provide accurate results for whichever visual colorimetric detection method is preferred or available.

As noted previously, we had run into a few roadblocks concerning the biocompatibility of docosane with the LAMP reaction process. However, we did observe that when our 3D-printed tubes were modified with an alkaline solution, certain amounts of docosane did not invoke a color change when a target template was not present. Although this was specifically characteristic with the pH indicator test, this suggests that additional chemical modifications could counteract the hypothesized acidic docosane. We suggest adding a higher concentration of Tris-HCl buffer (pH 8.8) to the LAMP 2X reaction mixture to see if this additional alkaline volume can halt the docosane from prematurely changing the pH of the solution.

If such an additional chemical modification can be created to counter the chemical nature of docosane, we believe that our 3D-printed tube has significant potential for use as a rapid and inexpensive POC device.

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