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An inkjet printing compatible platform for sensitive detection of dengue virus via gel based LAMP

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Thesis

AN INKJET PRINTING COMPATIBLE PLATFORM FOR SENSITIVE DETECTION OF DENGUE VIRUS VIA GEL BASED LAMP

by

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DEDICATION

To my wonderful family, Mamma, Papa and Bhaiya for their endless love and sacrifice.

This work is also dedicated to all the heroes of the current pandemic, the healthcare workers, grocery store workers and other volunteers who are fighting on the frontlines to ensure safety of others. Words fail to thank them enough.

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First, I would like to express my gratitude towards my research advisor Prof. Catherine Klapperich, for her continual support and guidance throughout my project. I have been a part of her group since September 2018, and her wisdom, mentorship and patience have always been a source of inspiration. Additionally I would like to thank my co-advisor, Dr. Christine McBeth who has been my biggest pillar of support ever since I started working with her. She has always helped me understand the nuances of my project through her thoughtfulness and I have learned so much from our weekly discussions. I want to thank her for her belief in my skills and motivating me whenever things seemed tough. This work would not have been possible without my advisors.

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Finally, I would like to send love to all my family back home, my friends here Anish, Pawas and my girlfriend Emily and for helping me get through this!

AN INKJET PRINTING COMPATIBLE PLATFORM FOR SENSITIVE DETECTION OF DENGUE VIRUS VIA GEL BASED LAMP

RISHABH SINGH

ABSTRACT

In these current times of the COVID-19 pandemic, the need for a widely available diagnostic platform for diagnosis of viral infections cannot be overstated. Such a platform that can be easily manufactured on a large scale would be beneficial both for making clinical decisions as well as provide new tools to epidemiologists broadly screening the population during epidemic threats. Fraunhofer USA CMI has focused on manufacturability of point-of-care (POC) diagnostics as a strategic point of development that can reduce costs and close the gap between research efforts and getting devices to the market. Here we report an inkjet printed platform that has the potential to increase the sensitivity of the molecular assay, be compatible with mass manufacturing methods and allow for reagent storage on-chip. Isothermal methods such as loop-mediated isothermal amplification (LAMP) have been used for rapid disease diagnosis in low resource settings due to their increased sensitivity and lack of thermal cycling. Fluorescent based LAMP readout techniques like QUASR also lend themselves to easy deployment in field settings via smartphones. However, rather than taking the standard approach, we developed a hydrogel based LAMP (gel-RTLAMP) assay by incorporating an engineered hydrogel, highly methacrylated gelatin (GM_{10}). In this study we show that this hydrogel is compatible with both the LAMP reaction and piezoelectric inkjet printing. Furthermore, via limit of detection studies we also show that this gel-RTLAMP assay is 100 fold more sensitive than

a standard RT-LAMP assay and results in clinically relevant detection of DENV RNA in analytical samples (10 copies per reaction). Unfortunately we could not characterize the final inkjet printed platform due to time limitations and other issues, but instead we report a proof-of-principle study to show that this gel-RTLAMP assay can be adapted in a microarray format via inkjet printing and has the potential for rapid multiplexing and digitization of the assay.

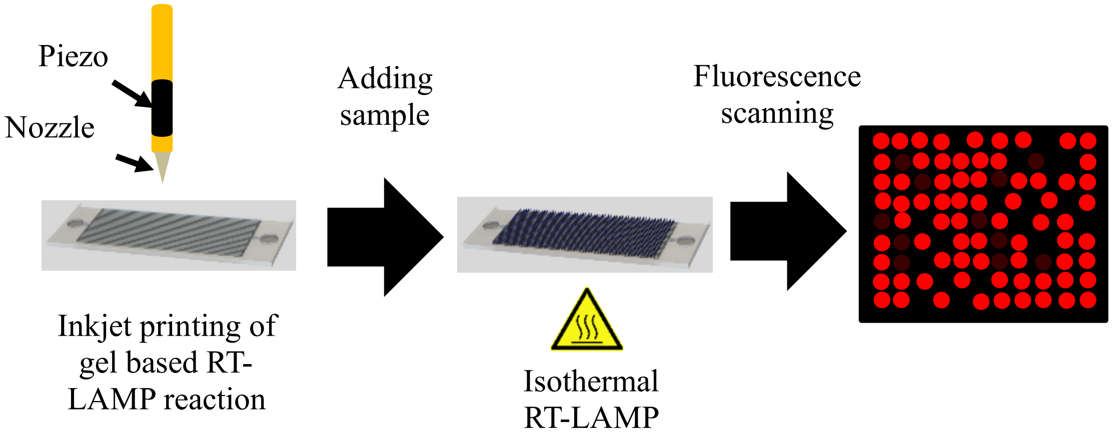

Figure 1: Graphical abstract: : Block diagram depicting the proposed diagnostic platform workflow. Following printing (piezo dispensary capillary shown in yellow) of RT-LAMP regents on top of a crosslinked hydrogel (GM_{10}) matrix on the substrate, the hydrogel spots will be interacted with sample. After isothermal heating, average fluorescent intensity of each well will be plotted and a threshold will be set to make a distinction between positive (target amplification) and negative wells.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
COP	Cyclic Olefin Polymer
DENV	Dengue Virus
DNA	Deoxyribose Nucleic Acid
dNTP	Nucleoside Triphosphate
ECM	Extracellular Matrix
HIV	Human Immunodeficiency Virus
LAMP	Loop Mediated Isothermal Amplification
LED	Light Emitting Diode
NAAT	Nucleic Acid Amplification Test
NCR	Non Coding Region
NTC	No Template Control
PCR	Polymerase Chain Reaction
POC	Point Of Care
QUASR	Quenching of Unincorporated Amplification Signal Reporters
RNA	Ribonucleic Acid
RT-LAMP	Reverse Transcriptase Loop Mediated Isothermal Amplification
TMSPMA	3-(Trimethoxysilyl) Propyl Methacrylate
WHO	World Health Organization

CHAPTER ONE

Introduction

The need for rapid, low-cost detection of diseases cannot be overstated. Both developed and developing countries face immense challenges in accurately diagnosing and responding to diseases (St John, et al. 2014). Limited access to centralized labs and highly trained staff in developing regions in addition to recent severe infectious outbreaks of diseases like Covid-19, Ebola, Zika, dengue (Pai, et al. 2012), highlight the need for a diagnostic platform that can provide quick, simple screening and can be readily manufactured on a large scale for distribution. Emerging lab-on-a-chip technologies like microfluidic and paper-fluidic approaches (Kolluri, et al. 2017) show good promise for developing diagnostics that satisfy the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, deliverable to end users) criteria published by WHO (Drain, et al. 2014). However, there is a significant gap between the development and implementation of these POCs in developing regions. We believe that a major barrier for the commercialization of POC diagnostic devices is their incompatibility with established large-scale manufacturing techniques (Brecher, et al. 2015, Senkbeil, et al. 2016, Becker, H. 2009). Processing costs can increase rapidly if device materials cannot facilitate commonly used techniques like printing, injection molding or a roll-to-roll production line. In order to enable large scale implementation of POC devices we need to work within the constraints present in low resource settings such as lack of funding, advanced infrastructure for manufacturing and skilled labor (Land, et al. 2019). Motivated by the ability to bridge this gap, we aim to investigate printing technologies as a way to fabricate POC diagnostic devices that can realize the ASSURED criteria as well as be accessible in resource limited settings.

Background

Dengue is the one of the most prevalent viral disease in humans, with 3.6 billion people living in areas with a significant risk of disease transmission and an estimated 96 million dengue cases annually (Bhatt, et al. 2013). DENV outbreaks in between 2006 and 2013, in countries like India, China, Singapore, Malaysia and Portugal (Lopez-Jimena, et al. 2018), highlight the necessity of rapid virus detection to identify DENV as the cause, in order to manage and control virus spread. However, the diagnosis of dengue virus infections cannot rely solely on clinical manifestations, since many patients are asymptomatic. Therefore, rapid, accurate, relatively low-cost diagnostic tools for DENV are critical for effective disease management and control via mass screenings, especially in developing countries with limited and inaccessible health care resources. As recommended by the WHO Special Program for Research and Training in Tropical Diseases (TDR) (WHO, 2009), the specifications of an ideal dengue test are that it should (i) distinguish between dengue and other diseases with similar clinical presentations (such as malaria, chikungunya, and other flaviviruses), (ii) be highly sensitive, (iii) provide rapid results, (iv) be inexpensive, (v) be easy to use, and (vi) be stable at temperatures above 30°C for usage in the field and in primary health care settings, usually with very limited/no optimal storage options. NAATs are one such category of tests that have the potential to satisfy these conditions.

Loop-mediated isothermal amplification (LAMP) based NAATs are very popular tools for rapid POC diagnostics because of their simplicity, rapid nature, specificity, sensitivity (Notomi, et al. 2000) and cost-effectiveness, as no special equipment is needed (just a heating block or water bath capable of maintaining a constant temperature between 60°C to 67°C). LAMP can amplify up to 10^9 copies of DNA in less than 1 hour under isothermal conditions (65°C). Reactions can be visualized by monitoring either the turbidity or the fluorescence by visual inspection under UV lamp when using an intercalating dye or by color change. In this thesis, we propose to use the technique called quenching of unincorporated amplification signal reporters (QUASR) which enables bright single step, closed tube detection (Ball, et al. 2016). It is based on inclusion of a dye-labeled primer that is incorporated into the target-specific amplicon if the target is present. A short, complementary quencher hybridizes to unincorporated primer upon cooling down to room temperature at the end of the reaction, thereby quenching fluorescence of any unincorporated primer. Equipped with a simple LED excitation source and a colored plastic gel filter, we can easily discriminate between positive and negative QUASR reactions, which produce a difference in signal of approximately 10:1 without background subtraction. Priye et.al (2017) have described a POC NAAT device which combines QUASR multiplexed RT-LAMP assay with a novel smartphone-based detection system, allowing simultaneous analysis for Zika, Chikungunya and dengue which share overlapping symptoms and epidemiology (Figure 2). One of the key features of this work was that they demonstrated their LAMP reagents could function in dried formats and are compatible with different sample matrices (blood, saliva, urine), which minimizes the necessity for complex sample prep and simultaneously incorporated the versatility of an ordinary smartphone to provide an alternative to advanced molecular diagnostics. However, the assay reaction housing module of the device requires manual setup and additional processing that is not compatible with conventional manufacturing techniques. Nonetheless, their assay shows great potential for use in a POC setting.

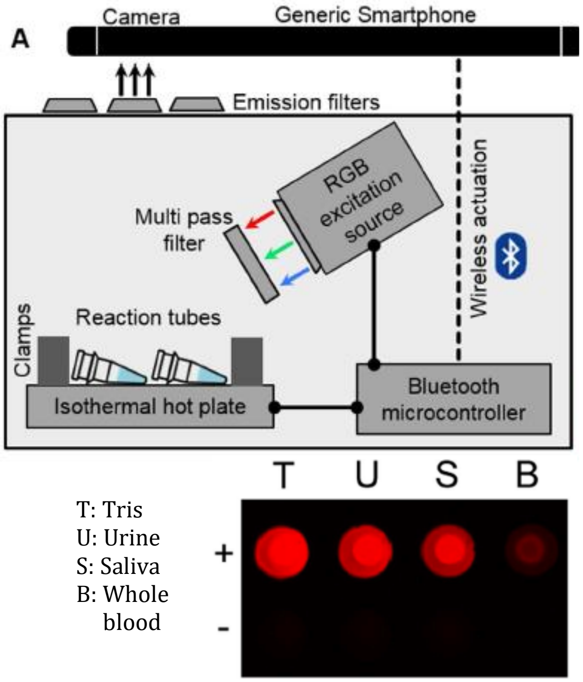


Figure 2: Smartphone based QUASR detection in complex sample matrices (Priye, et al. 2017). (A) Schematic of the RT-LAMP detection setup depicting the isothermal heater with reaction tubes, LED excitation source and Bluetooth microcontroller (Arduino Uno), (B) QUASR detection in RT-LAMP preserves sensitivity in crude matrices. Image shown here depicts positive and negative ZIKV detection in mentioned matrices

There can be many instances in healthcare where a quantitative readout from an assay is helpful in designing a treatment plan. For example, it has been shown that quantifying viral loads in HIV patients helps monitor efficiency of drug therapy and improve clinical outcomes (Fu, et al. 2014). Hence, quantitative readout has clinical utility, and would be beneficial in low-resource settings. Digital LAMP (dLAMP) is one such absolute quantitation method which provides greater sensitivity and accuracy than standard assays (Sanders, et al. 2011). In dLAMP, a sample containing target DNA is diluted into many discrete volumes which initially contain either zero, or one or more, DNA molecules, and the DNA is amplified.

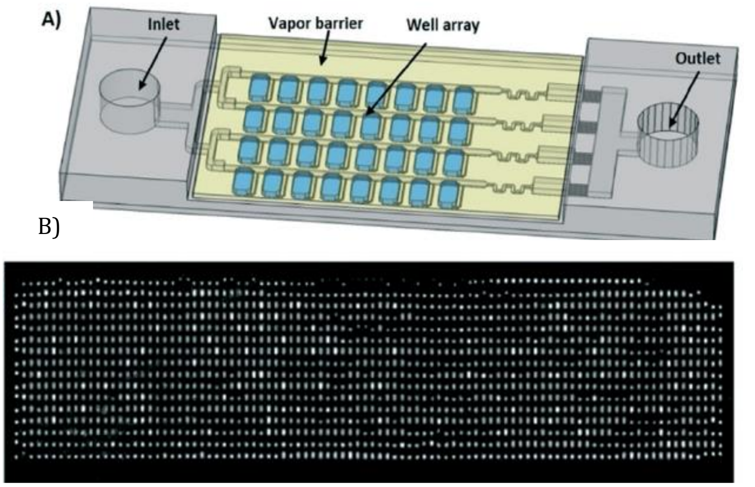


Figure 3: The self-digitization (SD) chip designed by Kreutz, et al. (2019). (A) The SD chip consists of an array of channels and wells that spontaneously compartmentalizes aqueous samples into defined volumes for digital nucleic acid quantification assays. (B) Fluorescent image taken after the dLAMP assay. The arrays consisted of 1536 wells with a well volume of 6.5 nL. Positive wells in which amplification occurred are more brightly fluorescent than negative wells.

Volumes that initially contain one or more molecules can result in DNA amplification and produce a fluorescent signal. The concentration of target DNA in the original sample is quantified based on the number of positive wells using Poisson statistics (Kreutz, et al. 2019). Methods to digitize samples include using droplets within emulsions and microvalve based arrays of reaction chambers. Though these platforms offer favorable performance, a simple and cost-effective method is still highly desired, especially in a POC setting. Kreutz, et al. (2019) have developed a self-digitization (SD) chip (Figure 3) – a microfluidic device that partitions samples into a large array of small chambers based on viscoelastic fluid phenomena which is controlled by the geometric properties of the channels and chambers in the chip. Compared to other methods, SD technology results in digitization without the need for precise and complex external equipment and instrumentation. But, it has been noted in literature that photolithographic techniques required for fabrication of such precise microfluidic chips can often become very expensive on a large scale and require high upfront costs (Becker, H. 2009). We believe that approaching a dLAMP assay via inkjet printing techniques can pave the way for a POC platform that is accessible for manufacturing in low resource countries.

Our collaborators at Fraunhofer IGB have developed novel gelatin derivatives for applications in cell and ECM inkjet printing (Hoch, et al. 2012). For inkjet printing, the viscosity of ink should be low, typically between 1 mPa s and 10 mPa s (Hoch, et al. 2011). It is known that solutions of non-modified gelatin and gelatin with a low degree of methacrylation, hold comparatively high viscosities, even above their melting temperature. While the degree of methacrylation determines the stiffness of the crosslinked hydrogels, additional acetylation can be used to control the rheological behavior of the gelatin solutions simultaneously. Our collaborators have demonstrated that inks based on such gelatin derivatives (like highly methacrylated GM_{10}), might be suitable for printing experiments using a piezoelectric inkjet printer (Hoch, et al. 2012). In this thesis, we will demonstrate the GM_{10} ink is compatible with LAMP assays and inkjet printing and hence suitable for the proposed platform.

CHAPTER TWO: MATERIALS AND METHODS

DNA Stocks and RNA translation

We utilized the DENV target sequence first reported by Lau, et al. (2015) which codes for the 3’NCR sequence of DENV-1 Western Pacific strain (Genbank: U88535.1). The nucleotide sequence was purchased (GenScript) cloned in a pcDNA3.1 plasmid. Standard heat shock transformation was used to insert the plasmid into NEB® 5-alpha Competent E. coli cells (NEB). The control DENV-1 plasmid was extracted using a QIAprep® Spin Miniprep Kit (Qiagen). The DENV-1 plasmid was linearized with SmaI (NEB) prior to downstream usage. For RT-LAMP assays, the linearized DENV-1 plasmid was reverse transcribed and purified following instructions on MEGAscript™ T7 Transcription Kit (Invitrogen) and MEGAclear™ Transcription Clean-Up Kit (Invitrogen).

The quality of purified DNA/RNA was estimated and quantified via NanoDrop™ 2000c Spectrophotometer (ThermoFisher Scientific). The DENV-1 DNA plasmid used in this study is 5624 bp in length and this corresponds to a molecular weight of about 3475 kDa. Along with the concentration of plasmid (ng/µL), we estimate 6.15 pg of target to correspond to 10^6 copies. The transcribed RNA product is long and has a molecular weight of 451 kDa. We estimate 74.4 pg of DENV RNA target to have 10^6 copies.

All DNA/RNA stocks were stored in aliquots of 10^6 copies/µL at -20°C.

Sensitivity and specificity of DENV RT- LAMP assay with QUASR detection

We adapted our QUASR LAMP assay from Meagher, et al. (2018) which reported optimized primer sets for targeting DENV-1 and also compatible with the QUASR technique. All primer sequences used are listed in Table 1.

Primer name	Sequence
DENV-1 F3	TGGGGTAGCAGACTAGTGG
DENV-1 B3	TCTGTGCCTGGAATGATGC
DENV-1 FIP	CCACCAGGGTACAGCTTCCC GACCCCTCCCAAAACACAA
DENV-1 BIP Cy5	Cy5-AGAGGTTAGAGGAGACCCCCC CAGGATCTCTGGTCTCTCCC
DENV-1 LoopF	TGGTGTTGGGCCCCGCT
DENV-1 LoopB	AAACAGCATATTGACGCT

Table 1: Complete set of primers used for DENV RT-LAMP assay taken from Meagher, et al, (2018). Underlined sequences within the FIP and BIP primers reflect the F1c and B1c regions of those primers

RT-LAMP reactions were either carried out in 8 strip PCR tubes or 96-well plates with 1 µL of template and 4/9 µL of master mix solution. The fresh master mix solutions (optimized concentrations) were prepared with 1X Isothermal Amplification Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20, pH 8.8 @25 °C, NEB), 1.4 mM of each nucleotide (dNTP mix, NEB), 8 mM MgSO4 (NEB), 1X CYGREEN Nucleic Acid Dye (Enzo), 0.6 µM each F3 and B3, 0.8 µM each LF and LB, 4.8 µM each FIP and Cy5 labelled BIP and 7.2 µM of quencher primers (Table 1), 1.6 units of WarmStart® RTx Reverse Transcriptase (NEB), and 3.2 units of Bst 2.0 WarmStart® DNA Polymerase (NEB). Remaining volume in the reaction was made up with nuclease free water (NEB) or with 57.91% (w/v) of GM_{10} for a final concentration of 10% (w/v) in case of hydrogel-based RT-LAMP. Samples were incubated at 65°C for 50 minutes in a thermocycler or a hotplate (Eppendorf). Limit of detection studies were performed using serially diluted RNA (10_6 - 10_0 copies per reaction). For specificity study, equal amounts of both ZIKV/DENV RNA were used (10_6 copies per reaction) at the same conditions as above.

For real-time monitoring of the reaction, fluorescent intensity was collected every 1 minute through FAM channel during incubation at 65 °C for 50 minutes and then reaction was brought down to 25°C for 5 minutes and fluorescent intensity was again measured though Cy5 channel using QuantStudio5 thermocycler (ThermoFisher Scientific).

Gel electrophoresis/Imaging for target

Gel electrophoresis was used to analyze success of DENV plasmid purification, linearization of DENV plasmid and resulting LAMP products. For longer DNA like plasmids, 1.5% agarose gels poured in house were run with 1X TBE buffer (VWR). 5µL of plasmid with 6X loading dye (NEB) were added per well and gels were run for 1.5 hours at 150 V. For analyzing LAMP products, 6% Novex® pre-cast TBE Gels (Invitrogen) were run following manufacturer specifications for 1 hour at 90 V. All gels were stained after completion of run with SYBR green for 30 mins and imaged with a Versadoc Imager (BioRad) or a UV trans-illuminator (Invitrogen).

All LAMP assays both in tube/plate and on chip were imaged at end point with an inverted fluorescence microscope (Olympus IX-70, Center Valley, PA) for the acquisition of the fluorescence images. Cy5 fluorescence measurement was done at an excitation wavelength of 635 nm. Each image covered a 250-µm by 250-µm area of the sample. Image data was collected by a monochrome CCD camera (Hammatsu, Japan) and Azzure Sapphire Biomolecular Imager (BU Proteomics Imaging Core).

Hydrogel preparation and inkjet printing

Freeze dried hydrogels (GM_{10} - the suffix denotes the molar excess of the reagent used with respect to free amino groups) in sealed packets were obtained from our collaborators at Fraunhofer IGB (Hoch, et al 2012). To crosslink and dissolve the gel, required weight of freeze-dried gels were dissolved in nuclease free water containing 1% (w/v) of Irgacure-2959 (Sigma) at 80°C. After dissolving, gels were given a quick spin and filtered via 0.2 µm syringe filters.

For inkjet printing, filtered hydrogel was dispensed in 500 pL droplets at defined spots onto a target substrate using a piezoelectric noncontact printer (SciFLEXARRAYER S3, Scienion AG, Berlin, Germany). Spotted substrates were dried for 10 mins inside the humidified chamber before being transported to be UV crosslinked. For printed RT-LAMP reactions, the reaction mix mixed with template was dispensed using the above printer at the same location of the hydrogel and transferred to a sealed well plate for heating.

Hydrogel adherence and glass substrate treatment

Standard glass slides were cleaned with 100% ethanol via sonication for 30 minutes, followed by 30 minutes of sonication with homemade nuclease removal solution (10% bleach, 10% NaOH, 1% Alconox®) and another 30 minutes of sonication with nuclease free water. After slides were dried with a compressed air gun, they were treated with 1% (v/v) TMSPMA (Sigma) in methanol for 30 minutes. Any unbound TMSPMA was rinsed off with 100% ethanol and let to oven dry at 37°C before use.

For adherence tests, 1 µL of prepared hydrogel was pipetted onto the treated glass slide in discrete spots and then crosslinked by UV with a peak wavelength of 365 nm for specified intensity and time. After crosslinking, slides were immersed in 1X PBS (VWR) for one hour and number of adhered spots were recorded. The optimized crosslinking time used for downstream assays was 2 minutes at an intensity of 9 mWcm^{-2}.

Hydrogel reagent storage

5 µL of freshly prepared hydrogels were pipetted into PCR tubes and UV cured for 2 minutes at an intensity of 9 mWcm^{-2}. 2.13 µL of reaction mix -1 containing only the primers, dNTPs, polymerase and dyes were added on top of the hydrogels in tubes, given a quick spin and let to dry in laminar air flow for 30 minutes. Prepared tubes were then sealed in sterilized pouches and stored at either room temperature or at 37°C away from light for required amount of time. To reconstitute the reaction, 1.87 µL of reaction mix -2 containing only Isothermal Amplification Buffer and $MgSO_4$ were added to each tube

along with 1 µL of template and 2.13 µL of nuclease free water to make up the volume to 5 µL. Tubes were given a quick spin before running them on the thermocycler.

Device fabrication

Acrylic sheets of 1.5 mm thickness (McMaster-Carr) and double-sided adhesive tape (ARseal™ 90880), were laser cut by a Epilog Zing laser cutter (35 W) as per the input CAD drawing. The cutting parameters for 1.5mm thick acrylic sheets were 30% speed, 50% power and 500 Hz frequency. For the double-sided adhesive tape, cutting parameters were 80% speed, 5% power and 500 Hz frequency. All device materials were rinsed with 100% ethanol and homemade RNAase AWAY. After device materials were air dried, they were assembled by carefully by aligning the layers of acrylic sheets and tape. The cover of the device was attached once the hydrogels containing the RT-LAMP assay reagents were added to the wells and crosslinked. The sample was added manually via a 100 µL pipette tip.

Statistical analyses

The limit of detection was determined as the lowest DNA/RNA concentration that yielded 100% amplification rates detectable by Cy5 fluorescence in all three replicate trials. A reaction was considered positive if the average Cy5 fluorescent intensity was twice as intense as the corresponding negative control. This threshold was set by observing trends in real time plots. Chips and benchtop reactions with detectable Cy5 fluorescence in no template controls were removed from analysis due to possible contamination in the common reaction mix. All microscopic images were analyzed with ImageJ (NIH). Any fluorescent intensity measurements were measured using inbuilt ImageJ functions. Real time LAMP plots and Cy5 fluorescence intensity measurements for in tube assays were recorded via the QuantStudio (ThermoFisher) software. All plots were graphed using Microsoft Excel (Microsoft). Error bars depict standard deviation values. All figure preparations were done using Inkscape. Probit analysis was done with custom written code (Justin Rosenbohm) in MATLAB.

CHAPTER THREE: DENV RT-LAMP ASSAY DEVELOPMENT

As mentioned earlier, standard RT-PCR assays are harder to implement in low resource settings to detect viral infections. Instead of requiring precision equipment for thermal cycling for amplification of DNA/RNA we adapted the LAMP technique which can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions and with greater specificity. This increased specificity is due to the fact that LAMP uses 6 set of primers to target 6 distinct sequences initially and then by 4 distinct sequences onwards. (Notomi, et al. 2000)

However one of the common drawbacks of LAMP is the lack of suitable closed tube detection techniques. The color changes in a colorimetric closed tube detection LAMP based assay maybe too subtle to detect and not color blind friendly. While other nucleic acid binding dyes require elevated temperatures for distinction between positive and negative samples. Hence, we decided to adapt a novel detection technique called QUASR (Ball, et al. 2016) which enables closed tube fluorescent detection of specific amplicons. The QUASR technique relies on introducing a fluorescent reporter at the 5’ end of one of the LAMP primers (usually FIP) and also the incorporation of a short 3’ complementary quencher probe. The quencher probe is designed in such a way that the quencher probe is dissociated during amplification so that it doesn’t interfere with amplification or bind to the complementary primer. As the temperature of the reaction drops down after the amplification run, the quencher probe binds to any free labelled primer present that has not been incorporated in the amplicon. This results in the quenching of the fluorescent probe on the primer. However the incorporated labelled primer results in fluorescently tagged amplicons which can be detected upon laser excitation (Figure 4). This technique allows us to discriminate well between positive and negative tubes by clearing out any excess fluorescence and recognizing target amplicons (Ball, et al. 2016).

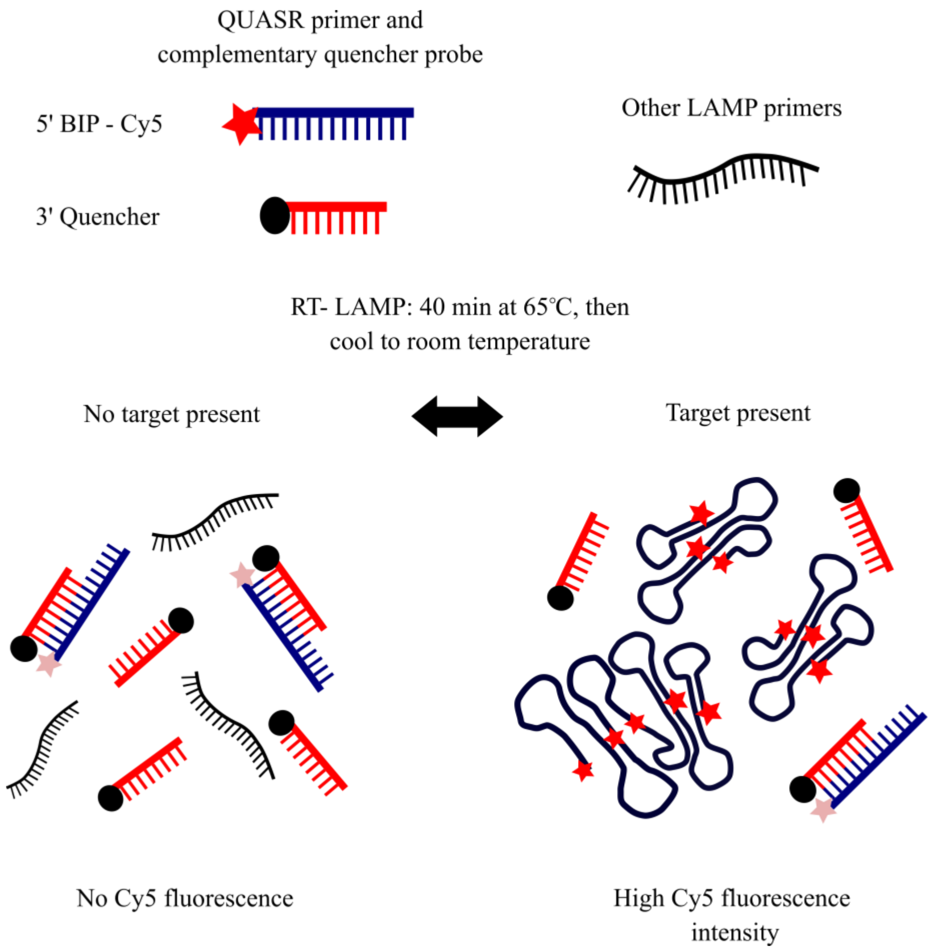


Figure 4: Principle of QUASR detection in RT-LAMP. The reaction mixture has one of the inner primers (FIP or BIP) labeled with a fluorescent dye and a short complementary quencher probe which is in relative excess. As reaction cools down to room temperature, the unincorporated fluorescent primers are quenched while amplicon remain highly fluorescent due to incorporated primers.

To validate the efficacy of the DENV QUASR RT-LAMP assay developed by Meagher, et al. (2018) in our platform, primer and reagent concentrations present in the reaction mix were optimized along with the establishing a reduced volume setup, assay sensitivity and specificity to common symptomatic diseases. An assay was considered optimized if the limit of detection was below the range of clinically reported DENV loads in serum.

We report that we could reduce the volume of the reaction by half without affecting the lowest detection limit (shown in a LAMP assay with DNA target). A reduction in volume also resulted in a decrease of time to positivity (Ct). By increasing the inner primer concentrations and adjusting Mg^{++} ions, we were able to detect 10^3 copies per reaction or more. This detection limit is suitable for diagnosing infections in DHF and DF on day 3-4 of illness when viral loads in serum are higher than detection limit (Teoh, et al. 2015). We did not further work on pushing the detection limit and planned to do so after incorporating hydrogel in the assay. We were also able to confirm specificity of the optimized assay for DENV against other closely related viruses.

Reduced volume setup of DENV LAMP assay

Since our proposed platform would incorporate nano-liter LAMP reactions, we wanted to investigate the effects of reducing total reaction volume. Reducing the volume of a LAMP reaction can affect the efficiency of amplification and in some cases either decrease or increase the limit of detection (Gaines, et al. 2002). Following the reaction mix listed in Meagher, et al. (2018), we ran the LAMP assay with DENV DNA target for an end point readout in two volume setups one being 10 μL (standard) and other 5 μL (lowest possible volume to ensure reliable pipetting). Figure 5 shows the fluorescent end point readout of this assay with serially diluted copies of DNA target ranging from 10_6 copies per reaction to 1 copy per reaction. With a reduced volume setup (5 μL) we observed a 10-fold increase in limit of detection (n=3). From the real time plots, we also observed lower Ct values for the reduced volume setup (Appendix Table A1). We believe that this could be because of increased interaction of primers and target and subsequent self-priming LAMP amplicons. Thus reducing volume could help increase limit of detection as well as decrease time to positivity although significant testing would be needed before validating this claim.

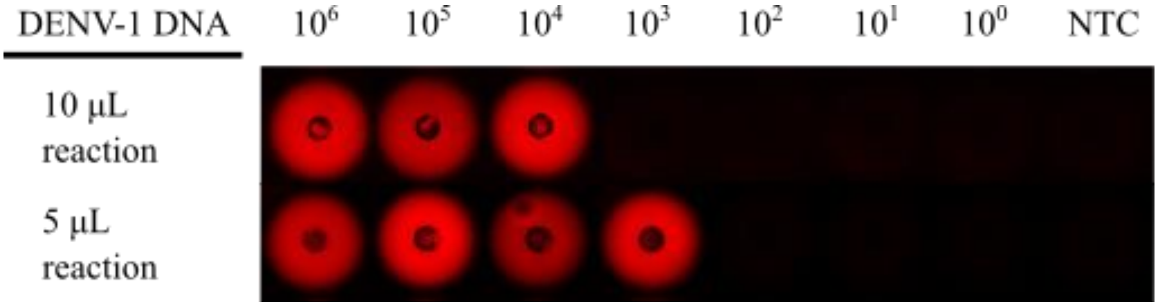


Figure 5: Reduced volume LAMP assay. The above figure shows the endpoint fluorescent readout in Cy5 channel for respective wells after amplification cycles have ended (n=3). We observe that the limit of detection increases by 10 fold when we decrease the total reaction volume by half. Data representative of 3 trials.

RT-LAMP assay sensitivity and limit of detection in tube

To develop a robust RT- LAMP assay that would result in a clear readout between positive and negative DENV samples while still detecting low viral concentrations in clinical samples, we determined if the assays limit of detection was below reported clinical viral loads. The clinical DENV RNA loads found in serum of infected patients range from as low as 10^{4} copies/mL to about 10^{12} copies/mL (Gurukumar, et al. 2009). With the current master mix we could not detect even 10^{3} copies per reaction in 100% of trials (3 positive in 6 cases) and hence we decided to optimize the assay conditions further by modifying concentrations of primers and Mg^{++} ions such that we could have detection around 10 copies per reaction (equivalent to 10^{4} copies per mL). The inner primers are involved in the initial stages of the amplification cycle and are necessary in binding to target and creating amplicons that self-initiate amplification (Notomi, et al. 2000). So we increased the concentration of primers 1.5x times and also increased concentration of Mg^{++} ions. Results from this assay are shown in Figure 6. Gel electrophoresis analysis of products is shown in Appendix Figure A1.

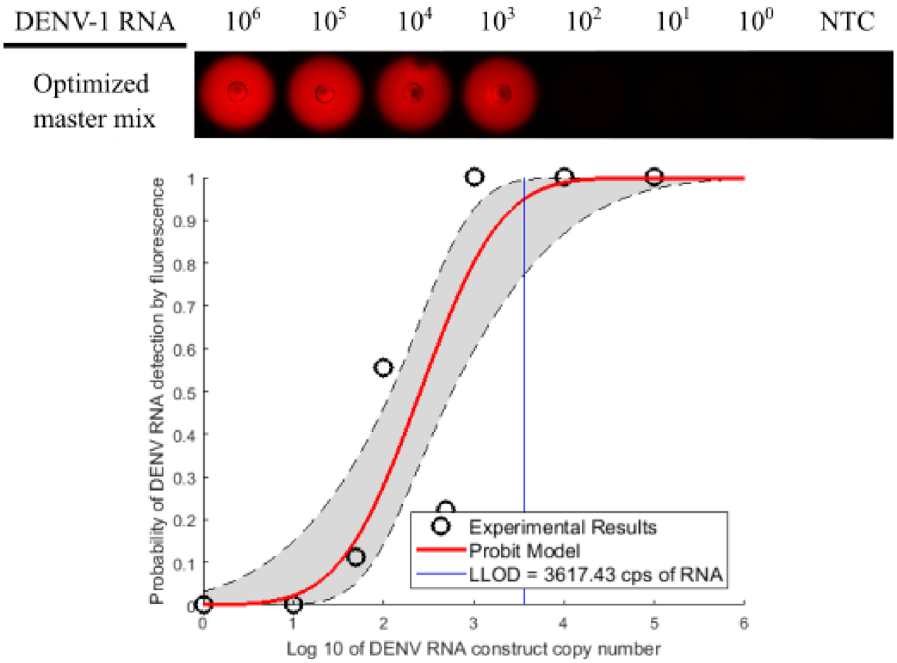


Figure 6: Limit of detection of DENV RT-LAMP assay. Top row shows the endpoint fluorescent readout in Cy5 channel after amplification cycles of serially diluted RNA has ended (50 min). The limit of detection observed from fluorescent readout is 10^{3} copies per reaction. The results from all trials (n=9) were also fitted to a probit curve with shaded region indicating 95% confidence interval.

The lowest concentration that indicates Cy5 fluorescence in 100% of trials is reported as the limit of detection. Increasing the primer concentration resulted in a detection limit of 10^3 copies per reaction (n=9). We were also able to detect 10^2 copies per reaction in 55% of trials. The probit model predicts the limit of detection with 95% positivity to be 3.6 x 10^3 copies per reaction. Even though we are not able to detect lowest reported clinical loads yet with our assay, our limit of detection is well below the mean viral loads reported in early days of infection (greater than 10^4 copies per reaction on day 3). We finalized the RT-LAMP reaction mix recipe for subsequent reactions and hypothesized that we could instead increase limit of detection by incorporating the hydrogel in the assay further on.

We also tested the effect of betaine in reaction mix. Betaine has been reported in some cases to improve amplification efficiency and decrease threshold times (Wang, et al. 2015). But we noted that this was not the case in our assay setup and in fact we noticed a lower amplification rate with betaine and hence decided not include betaine in the assay. To further verify the chosen conditions, we also varied the temperature of the reaction to 63°C and 70°C and noted that 65°C was the optimal temperature.

Assay selectivity against ZIKA and other common viruses

To confirm selectivity for DENV specific 3’ NCR region, we tested our RT-LAMP assay against other viral infections (ZIKV, JEV, SINV, CHIKV) that have similar symptoms to DENV and can confound diagnosis. By testing our assay against ZIKV, we screened for non-specific amplification when high concentrations of DNA is present. Lau et al. (2015)

reported their screening test (Table 2) and found this assay to be highly specific to DENV RNA. Since, we had increased the concentration of primers in our reaction mix we wanted to find out if that increased the chances of non-specific amplification. From our results against ZIKV, we conclude that our optimized reaction mix did not affect the selectivity of the assay.

Type of Virus	Primer reactivity	References
Dengue virus - 1 (DENV – 1)	+	Lau, et al. 2015
Dengue virus - 2 (DENV – 2)	+	Lau, et al. 2015
Dengue virus - 3 (DENV – 3)	+	Lau, et al. 2015
Dengue virus - 4 (DENV – 4)	+	Lau, et al. 2015
Japanese Encephalitis virus (JEV)	-	Lau, et al. 2015
Chikungunya virus (CHIKV)	-	Lau, et al. 2015
Sindbis virus (SINV)	-	Lau, et al. 2015
Zika virus (ZIKV)	-	This study

Table 2: Specificity data for DENV RT-LAMP assay. Most of the data is reported from Lau et.al which uses the same set of primers. The assay was tested against DENV1-4, Japanese encephalitis virus (JEV), Chikungunya virus (CHIKV), and Sindbis virus (SINV). All dengue positive samples showed positive results while other viruses (in patient samples) were all negative. We also reported specificity for DENV against Zika virus (ZIKV). Data representative of 3 trials.

Additional questions about the DENV RT LAMP assay with QUASR detection

The Cy5 fluorescent intensity is a direct measure of the amount of target amplicons present in each tube at the end of the reaction due to their incorporation of labelled primers. We would expect, higher input RNA concentrations to lead to higher Cy5 fluorescent values. But instead we observe the highest fluorescent Cy5 intensities in the lowest detected RNA concentration of 10_3 copies per reaction (Figure 7). We do not see a particular trend in the mean Cy5 fluorescent values as we vary the input RNA concentrations from high to low. This suggests that kinetics of our reduced volume LAMP reaction vary from a traditional LAMP assay where we would either get a linear standard curve or a log-linear standard curve. Since we were not interested in extracting quantitative information about input RNA from the fluorescent intensity readout we did not investigate this further.

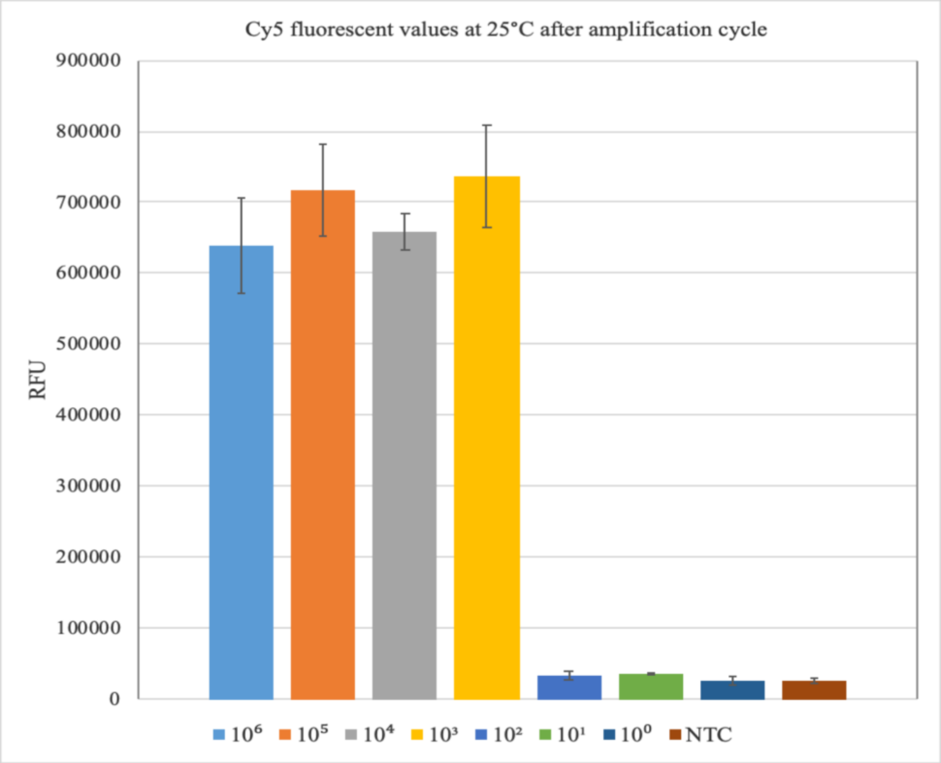


Figure 7: End point readout of Cy5 fluorescent values of DENV RT-LAMP assay at 25°C. Serially diluted RNA was amplified via RT-LAMP for 50 minutes and then brought down to an ambient temperature of 25°C (n=3). Fluorescent intensity was recorded by QuantStudio5 via Cy5 channel at endpoint and mean values at each concentration were plotted. Error bars depict standard deviation values. We observe highest fluorescent intensity for the lowest positively detected RNA concentration.

CHAPTER FOUR: HYDROGEL BASED DENV RT-LAMP ASSAY

Hydrogels are polymeric materials which swell up in the presence of water and are able to hold a distinct three-dimensional shape. They were among the first biomaterials developed for human use (Kopeček, et al. 2007) and have widely been used in various tissue engineering applications. They are promising biomaterials for the detection of various biomolecules including RNAs, because of their biocompatibility and the ease of adding various physical and (bio)chemical functionalities (Choi, et al. 2018). Hydrogels have been also been used as a long-term storage matrix for PCR reagents and as a filter to identify pathogens in whole blood (Beyer, et al. 2016). Inspired by work by on *in situ* polyacrylamide-based PCR (Mitra et al., 1999), we hypothesized that by integrating hydrogel with the DENV RT-LAMP reaction we could; (a) help store LAMP reagents on chip by mixing them in a gel, (b) restrict diffusion of viral DNA/RNA across the crosslinked gel which in turn could increase sensitivity of the gel based LAMP assay.

Another reason why we chose hydrogels for this particular application is because of their suitability with contact free, high throughput inkjet printing. Researchers have demonstrated production of DNA/protein microarrays using inkjet printing on non-porous substrates, such as glass and plastic (Mujawar, et al. 2014). Inkjet printing allows us to precisely dispense picolitres of volume in predefined locations thus allowing us to run large numbers of parallel amplification assays on a single chip leading to more accurate detection. We first tested different formulations of highly methacrylated gelatin GM_{10} for compatibility with inkjet printing and observed optimum results for a 10% (w/v) GM_{10}

hydrogel. We further characterized the UV conditions required for crosslinking and attaching the gel to a glass-based substrate and were able to achieve a stable gel attachment for at least 12 hours. We also show that it is possible to dry LAMP reagents on top of crosslinked hydrogels and store them for a period of 30 days at 37°C without losing on enzyme activity (with the exception of reverse transcriptase). In our reagent diffusion studies we found out that lower molecular weight components like primers and polymerases were free to diffuse across crosslinked gels while higher molecular weight DNA targets were found to be localized along the hydrogel spot boundaries but further investigation regarding mesh sizes of crosslinked hydrogels will be needed before we can claim this. By integrating the hydrogel in our RT-LAMP assay, we were also able to increase our limit of detection by 100-fold to 10 copies per reaction.

Hydrogel ink and piezoelectric printing

Piezoelectric inkjet printing is an attractive non-contact technique and drop-on-demand method for creating microarrays on a variety of substrates (Jung, et al. 2018). The ink required for this application needs to be both printable and also incorporate hydrogel precursors, which help provide the ability to crosslink into stable hydrogels after printing. Thus, ink development requires adjusting solution properties such as viscosity and surface tension. For inkjet printing, the viscosity of the ink should be on the lower side typically between 1 mPa s and 10 mPa s (Hoch, et al. 2013) as the power generated by the piezoelectric dispenser can be limited. Our collaborators at Fraunhofer IGB, have developed novel systemically modified gelatin-based matrices with suitable printing capability. These modified methacrylated gelatin hydrogels result in viscosities and gelling behavior that is expected to be inkjet printable.

Since our collaborators used a different dispensing system (Nanoplotter™ 2.1, GeSiM mbH, Germany) than what we had available (sciFLEXARRAYER s3, Scienion AG, Germany), we wanted to make sure their hydrogel formulations were compatible with our system too. To verify this we tested different formulations of the hydrogel with varying weight percentages and composition on the spotter (Table 3).

Hydrogel ink formulation	Stable printing process
GM_{10} - 5% (w/v)	Yes
GM_{10} - 10% (w/v)	Yes
GM_{10} - 15% (w/v)	No
GM_{10} - 10% (w/v) in 50% (v/v) glycerol	No
GM_{10} - 5% (w/v) in 50% (v/v) glycerol	No

Table 3: Stability of the printing process of GM10 hydrogel ink formulations with varying mass fractions and viscosities. We believed that adding glycerol to the ink could help us prevent sample evaporation during a LAMP reaction. But glycerol made the ink too viscous for inkjet printing and was unable to result in a stable printing process.

We observed that high weight percentages of all the modified gels (10-15% w/v) were either not printable at all or exhibited irregular drop formation. After including a further filtration and spinning down step during hydrogel preparation we were able increase our consistency and get reliable drop formation with most inks (Table 3). By consistent and reliable drop formation we imply that the volume of drop should not change drastically (±

20 pL) in between multiple dispensing runs (measured by Autodrop function in Scienion software) and across repetitions (n=3). As reported by our collaborators (Hoch, et al. 2013), the 5% (w/v) GM_{10} and 10% (w/v) GM_{10} hydrogel formulations both resulted in a stable printing process. We could not get 15% (w/v) GM_{10} to print reliably without observing satellite drop formation in between runs or clogging the nozzle. From their analysis (Hoch, et al. 2013) of degree of swelling and mechanical strength data, we know that higher mass fractions lead to an increase in mechanical strength and a decrease in degree of swelling.

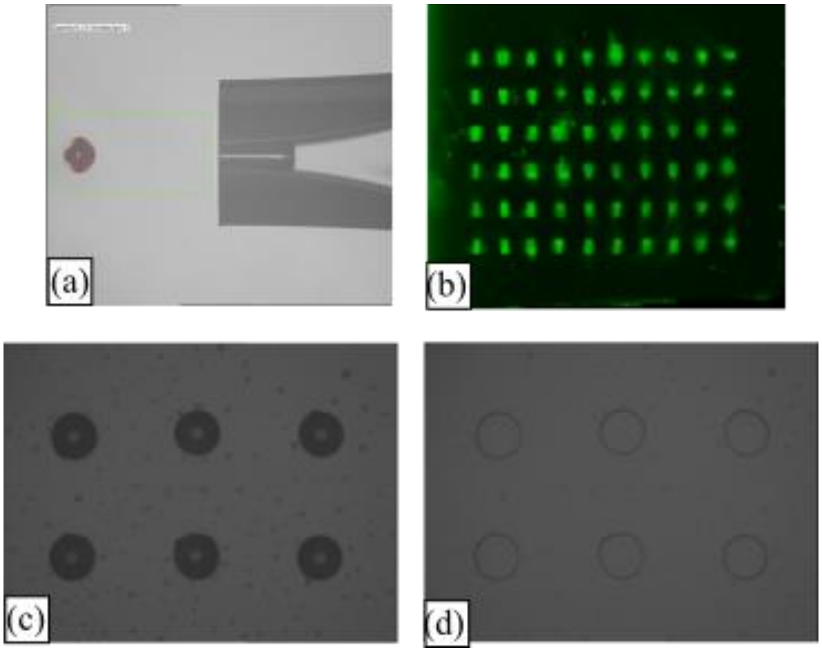


Figure 8: Drop formation of 10% (w/v) GM_{10}. (a) Snapshot of drop indicates regular drop formation at the center of the nozzle and without any satellite drops. (b) An array of 10% (w/v) GM_{10} mixed with SYBR green labelled DENV plasmid DNAs spots printed on TMSMPA treated COP strip. (c) 50 drops per spot of 10% (w/v) GM_{10} right after printing and (d) after drying in humidified chamber for 30 min. Data representative of 3 trials

For our application, we wanted our hydrogel ink to have a lower swelling ratio (smaller mesh size) so it could facilitate entrapment of higher molecular weight LAMP amplicons while allowing diffusion of lower molecular weight LAMP components. Hence as shown in figure below (Figure 8), 10% (w/v) GM_{10} was chosen as the hydrogel ink on which we would build our RT-LAMP assay. To make things easier for us when scaling down the reaction, we wanted to first characterize our inkjet printed assay at the highest possible reaction volumes that could be inkjet printed. The printing nozzle that we used resulted in a droplet volume of around 500 pL and the highest number of drops per spot we attained without interfering with the dispensing nozzle was 50 drops per spot (anymore and the nozzle would end up touching the dispensed liquid on the substrate).

Hydrogel crosslinking and attachment to device substrate

After establishing our hydrogel formulation, we wanted to characterize the UV parameters required for crosslinking and attaching it to the device. For these experiments both glass slides and COP sheets were used as the device substrate. To facilitate adhesion of the hydrogel to a surface, the substrate was treated with 3-(Trimethoxysilyl) propyl methacrylate (TMSPMA) which creates a silanized layer.

UV parameters	Number of adhesion spots remaining after buffer wash
9 mW cm^{-2} for 10 s	0/3
9 mW cm^{-2} for 30 s	0/3
9 mW cm^{-2} for 2 min	3/3
39 mW cm^{-2} for 10 s	0/3
39 mW cm^{-2} for 30 s	3/3

Table 4: UV parameters for hydrogel crosslinking and attachment to device substrate (n = 3).

The settings that use the lowest power intensity and take the shortest time to crosslink would be considered as the optimal UV parameters. This was done so that we could eliminate the need of specialized, high-powered UV sources. From Table 4, we observe ideal attachment for a number of different settings. Based on our criteria mentioned above, we chose a UV intensity of 9 mW cm_{-2} for 2 minutes as our final parameters.

Diffusion of LAMP reagents within hydrogel matrix

Ideally, a hydrogel matrix should allow free diffusion of small molecules with molecular weight (MW) such as water, ions, primers (<50 bp, MW < 15.5 kDa), and enzymes (Bst 2.0 WarmStart® Polymerase: 97 kDa, WarmStart® RTx Reverse Transcriptase: 71 kDa) but restrict the diffusion of heavier DNA/RNA templates (> 451 kDa) and subsequent LAMP concatemers. To verify that all LAMP reagents will be free to diffuse within the UV crosslinked hydrogel, we used fluorescently tagged BSA (MW = 67 kDa) to model the behavior of polymerase and reverse transcriptase enzymes (MW = 71-97 kDa) as their molecular weights are close. We observed that the diffusion of BSA was very fast in bulk solution (at 65°C) as the spots were submerged in isothermal amplification buffer while it was very nominal for the DNA target (Figure 9). The apparent loss of fluorescence implies that BSA is diffusing into the solution from within the gel while we observe fluorescent rings of DNA target around the hydrogel spots. We believe this implies that the current mesh size of crosslinked hydrogels should allow free diffusion of RT-LAMP reagents and not cause any steric inhibition to the RT-LAMP assay while also collecting DNA around its surface. We would like to further explore how our DNA/RNA target interacts with the crosslinked hydrogel and whether our crosslinked hydrogel can immobilize DNA/RNA from a sample. For this study, we plan on evaluating DNA/RNA capture efficiency of mini hydrogel posts (5 µL) and inkjet printed hydrogel spots (50 nL) by tracking fluorescent intensity changes as we add sample to the chip reservoir and perform subsequent washes to observe how efficiently DNA/RNA can get immobilized around hydrogel spots.

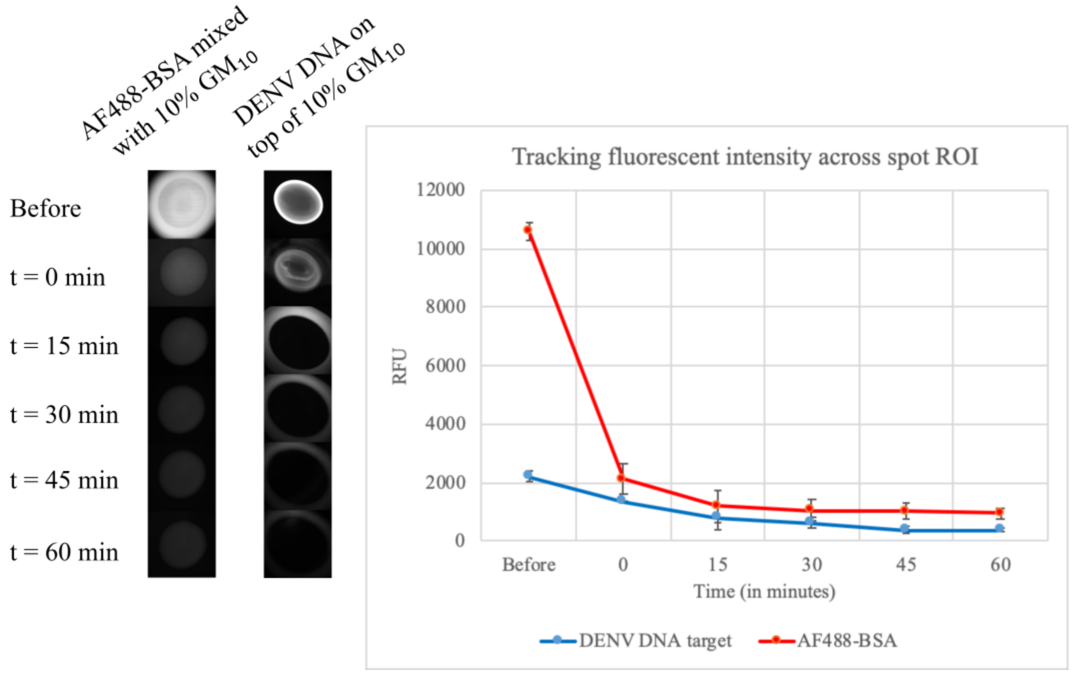


Figure 9: Diffusion of fluorescently labelled BSA and DENV DNA target within 10% GM10 at 65°C to model diffusion of LAMP reagents within the hydrogel matrix. 5 µL spots of 10% GM_{10} mixed with AF488-BSA were crosslinked and attached to a TMPSMA treated glass slide while SYBR green treated DENV DNA was added on top of 10% GM_{10} spots. Fluorescent images were taken before adding IAB in the reservoir (marked as before) and after every 15 minutes. The corresponding fluorescent intensity profiles across the spots at each timepoint are plotted on the right. Data representative of n = 3 trials.

Limit of detection of gel based DENV RT-LAMP assay

With the hydrogel formulation and diffusion of LAMP reagents characterized, now we needed to test whether the hydrogel could itself inhibit the RT-LAMP reaction. To verify this, we performed the previously optimized RT-LAMP assay in a tube but replaced water with GM_{10} hydrogel such that the final weight percentage of GM_{10} is 10% in the reaction. We observed that we could indeed observe corresponding Cy5 fluorescence in tubes containing the DENV RNA target. To test the integration efficiency of 10% GM_{10} a limit of detection study was also performed. Again, as previously mentioned, the lowest DENV RNA concentration that amplified in 100% of trials (n = 6) was considered the assays limit of detection in a hydrogel matrix.

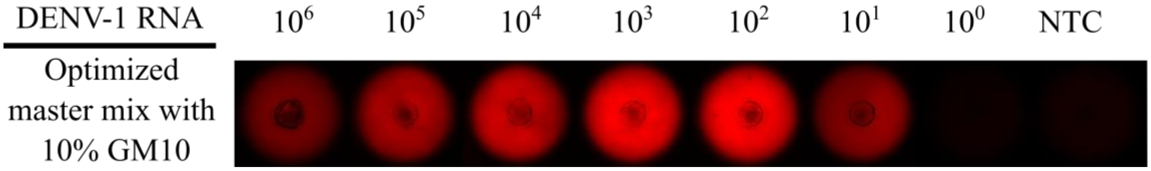


Figure 10: Limit of detection of gel based DENV RT-LAMP assay. Panel shows the endpoint fluorescent readout in Cy5 channel after amplification cycles of serially diluted RNA has ended (50 min). The limit of detection observed from fluorescent readout is 10 copies per reaction (n=6) Higher concentrations have lower Cy5 fluorescent intensity, indicating lower efficiency of amplification. Data is representative of n = 6 trials.

When the RT-LAMP assay was transferred into a hydrogel matrix, the limit of detection was increased 100-fold to 10 copies per reaction (Figure 10). This limit was well below clinically reported DENV RNA loads in serum (Gurukumar, et al. 2009) and is very close to the sensitivity of highly optimized LAMP reactions reported in literature. We believe this could be because of restricted diffusion of large molecular weight LAMP concatemers within the hydrogel which increases the chances of primer binding and self-initiation. Similar to DENV RT-LAMP, we also notice lower Cy5 fluorescent intensities for higher concentrations of RNA which is indicative of a lower number of amplicons. This suggests that the efficiency of the assay at high concentrations is lower when higher concentrations of sample are present. One reason for this occurrence could be the effect of reducing the reaction volume on the kinetics of the assay. But since we were note interested in developing standard curves and gaining quantitative information about the sample from the Cy5 fluorescent intensities we chose not to investigate this further.

Storage of LAMP reagents in hydrogel matrix

We were also interested in investigating stability of LAMP reagents such as polymerase, primers, dNTPs and reverse transcriptase when stored in 10% (w/v) GM_{10} hydrogel. These LAMP reagents are usually stored at -20°C for preserving their efficiency and this necessitates a cold chain requirement for any field deployment of a diagnostic LAMP assay. It has previously been shown that Bst 2.0 polymerase is relatively stable at 37°C for a period of 30 days (Thekisoe, et al. 2008). We believed that we could preserve the reagents by drying them on top of UV crosslinked hydrogels (detailed protocol in Materials and Methods section). To validate this, we reconstituted LAMP reactions that were previously air dried on crosslinked hydrogels placed in PCR tubes by adding the template and buffers to make up the volume. We could successfully determine positive amplification of the DENV-1 DNA target (Figure 11) for a duration of at least 30 days (n = 3). Unfortunately, we could not repeat these results in any of the trials (n = 3) when WarmStart® RTx Reverse transcriptase enzyme was dried on crosslinked hydrogels for 30 days to evaluate storage efficiency of RT-LAMP assay at 37°C. In future work, we will instead repeat the RT-LAMP storage experiments for shorter time intervals (1 - 15 days) to understand at what time period we lose the activity of the enzyme. We also did not test detection for low concentrations of target (at limit of detection) so we cannot claim yet whether storage of LAMP reagents on hydrogel would preserve the sensitivity of the assay and we would like to explore this further in future work.

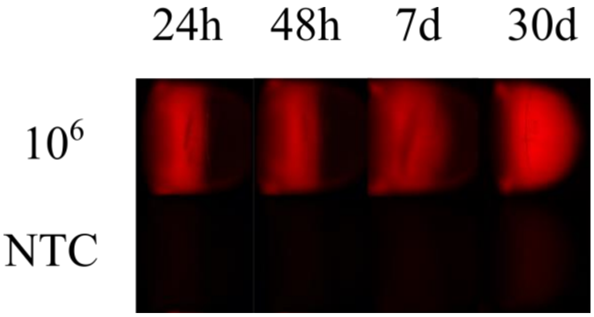


Figure 11: LAMP reagents dried onto 10% (w/v) GM_{10} hydrogels for long term storage at 37 °C. The LAMP reaction mix containing only dNTPs, primers, dyes and polymerase was added on top of crosslinked gels, dried under laminar airflow and stored in sterilized packets at 37 °C. The panel above shows Cy5 fluorescence readout of target amplicons at endpoint. We were successfully able to reconstitute the LAMP assays by adding buffers and DNA template for a period of at least 30 days. Data is representative of n = 3 trials.

CHAPTER FIVE: INKJET PRINTED PLATFORM FOR HYDROGEL BASED RT-LAMP ASSAY

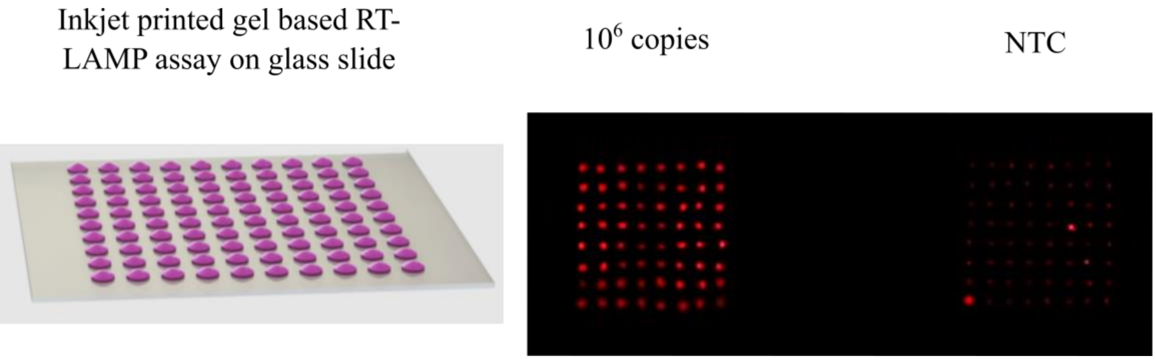
After validating the hydrogel based DENV RT-LAMP assay *in vitro*, we were ready to adapt the assay to an inkjet printed platform. In the previous chapter we have already characterized the inkjet printing properties of the chosen hydrogel formulation (10 % w/v GM_{10}) and its adhesion to a glass or COP substrate. We hypothesized that we could use the precise nature of piezoelectric inkjet printing to spot a microarray of hydrogel based DENV RT-LAMP reactions. This would allow us to, (a) reduce the volume of RT-LAMP reaction to nanoliters which helps reduce the cost of reagents and number of amplification cycles required for positive detection (Dahl, et al. 2007), (b) approach a digitized readout because of discretization of reactions and template, and (c) allow multiplex detection on the same device by printing microarrays of RT-LAMP reaction containing respective primer sets alongside each other.

There were three essential elements that needed to be validated for our device could be finalized. We needed to figure out (a) if the reduced volume (100 nL) hydrogel based DENV RT-LAMP assay would still result in detectable Cy5 fluorescence intensities at the end of amplification cycle, (b) how to deliver sample to the printed RT-LAMP reaction spots, and (c) how to prevent evaporation of reaction and sample volume when the device is placed on a hotplate for the amplification cycle. We report that we were able to record positive Cy5 fluorescence in printed hydrogel-based RT-LAMP reactions on a glass substrate when the template was premixed with the reaction. Although we observed an increased rate of false positives in this setup, we believe this was due to lack of aseptic RNA handling techniques. We also noticed that the reaction spots printed on glass substrate did not dry out during amplification when they were placed and sealed in a well plate. To manually deliver sample to printed RT-LAMP reaction spots, we fabricated reservoirs (25 µL volume) with drilled inlet and outlet ports using acrylic sheets and adhesive tape. Unfortunately we were not able to perform the printed hydrogel-based RT-LAMP assay with manual sample delivery or characterize the printed RT-LAMP assay for sensitivity. Due to an error, the glass tip of the PDC nozzle used to print was damaged and could not be replaced in time.

We instead report a different workflow strategy as a proof-of-concept study for delivering samples to the hydrogel spots containing the LAMP reagents on chip. Instead of having a printed microarray, we used acrylic sheets to create wells for manually pipetting hydrogel spots (5 µL) containing the LAMP reagents. We demonstrate amplification via endpoint Cy5 fluorescence detection in manually pipetted spots containing the hydrogel-based LAMP assay (DNA target was used instead to eliminate errors due to RNA contamination/degradation on device materials).

Array of inkjet printed gel based DENV RT-LAMP reactions

To test if we could we could adapt the hydrogel based DENV RT-LAMP assay to a 50 nL volume, we first printed an array (8x8) of 50 nL spots of 10% (w/v) GM_{10} on a TMSPA treated glass slide. After crosslinking these hydrogel spots, the DENV RT-LAMP reaction mix along with the corresponding template was printed on top of the hydrogel spots and let to dry in a humidified chamber for 30 minutes. We observed bright Cy5 fluorescence spots after amplification at the printed locations indicating a positive readout (Figure 12). One of the issues with this experiment was the high rate of false positives that we observed (in 2 out 4 cases). We believe this was due to introduction of residual contamination, as printed substrates required unnecessary exposure to uncontrolled environment in between the printing and crosslinking processes as the crosslinking chamber and printing chamber were not the same. We believe if we can eliminate exposure to outside environment during the crosslinking phase, we should be able to reduce any false positives.


Inkjet printed gel based RT-LAMP assay on glass slide

10^6 copies

NTC

Figure 12: Array of inkjet printed gel based DENV RT-LAMP reactions. On the left of the figure, a graphical representation of 10% (w/v) GM$_{10}$ spots containing the DENV RT-LAMP (in pink) printed on a TMSPMA treated glass slide is shown. On the right of the figure, we can see positive Cy5 fluorescence readout of target amplicons at endpoint when 10$_6$ copies of DENV RNA were added to the reaction mix. We also observe bright fluorescent spots in some of the spots in negative control and this could be due to local RNA contamination. Data representative of 2 of 4 trials.

Revised workflow for hydrogel based DENV RT-LAMP on chip

To imitate our proposed inkjet printed device for DENV RT-LAMP, we used laser cut acrylic sheets bonded to glass slides to create wells which were filled with 10% (w/v) GM_{10} and crosslinked to the glass. As described in Chapter 4, LAMP reaction mix containing only the primers, dNTPs and polymerase were dried on top of these gels in laminar air flow. For ease of handling, we only had two wells per device but this could be easily increased by increasing the size of the device to incorporate more wells. Positive (10^5 copies/µL) samples resulted in target amplification in only 1/2 cases as confirmed by endpoint Cy5 fluorescence readout (Figure 13).

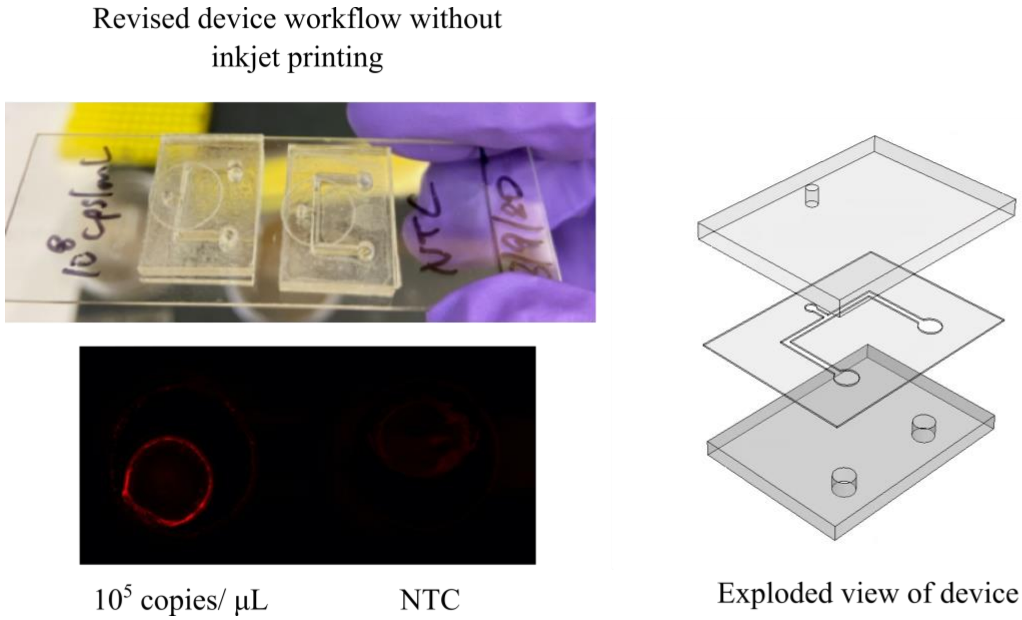


Figure 13: Revised workflow for hydrogel based DENV LAMP assay without inkjet printing. Top panel shows a photograph of the assembled device with inlet closed with PCR sealing tape. On the right the exploded view of the device shows its three layers; the bottom acrylic layer with wells for holding the hydrogel based LAMP reaction, sample delivery channel made with double sided adhesive tape and an acrylic cover with a sample delivery port to seal the device. In the bottom panel, we can see a Cy5 fluorescent ring around the crosslinked gel indicating positive detection of target amplicon. Data representative of 1 of 2 trials

Though it has been shown by other research groups that acrylic, glass and adhesive tape do not inhibit LAMP reactions themselves, we believe this low positive amplification rate could be due to improper handling techniques while assembling the device. Further work is required to solve these issues.

Design for proposed platform using inkjet printed hydrogel based RT-LAMP assays

One of the main challenges that we faced while adapting our assay in a device format was figuring out a way to deliver sample to the printed hydrogel spots containing the RT-LAMP assay. In Chapter 4 we concluded that all LAMP reagents like polymerase, primers and buffers can freely diffuse from inside the crosslinked hydrogel into the bulk solution. This meant that if a large sample volume interacts with the printed reactions, we risk diluting the assay components and making it very inefficient. While the ideal way to run the assay would be to incorporate sample while printing the reaction as shown in Figure 12, it will be unsuitable for in house clinical or POC testing. To overcome this, we propose printing a microarray of 250 spots (50 nL reaction volume each) and surround it with a reservoir that can contain a volume of 25 µL. This will ensure that the bulk solution volume will not dilute the concentration of the reagents and instead act as one pot of 25 µL RT-LAMP reaction. Any DNA/RNA target present in the sample should get immobilized around the printed hydrogel spots resulting in bright fluorescent rings indicating target amplification. Figure 14 indicates a graphical representation of this device. The gel-based RT-LAMP reactions (50 nL reaction volume) will be printed on a TMSPMA treated glass slide (75 x 25 mm). This printed slide will be then transferred to a UV crosslinking chamber to crosslink and attach the gel-based RT-LAMP spots to the COP sheet. Using adhesive tape (100 µm thickness), a microfluidic channel will be fabricated to create a reservoir for the sample (25 µL volume). An acrylic cover with sample delivery ports drilled in will be used to seal this device. To prevent evaporation of sample during isothermal heating, delivery ports will be sealed with PCR grade sealing tape after adding the sample in via a micropipette. A readout will be obtained via a fluorescent scanner and the number of positive spots will indicate the accuracy of detection. The microarray format would also help us in extracting semi-quantitative information about the sample by comparing the percentage of positive spots with information obtained from a standard curve established with known concentrations of sample.

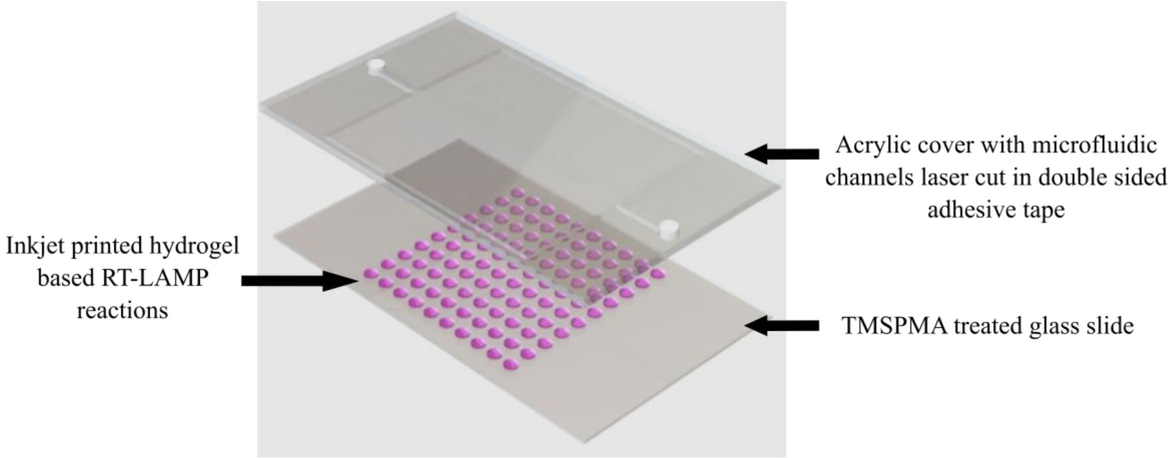


Figure 14: Proposed platform for hydrogel based DENV RT- LAMP assay on device

CONCLUSION

Current POC devices use various techniques — such as advanced microfluidics, nanomaterials and microarray technology — which can be expensive to implement and create barriers in field implementation. Here we adapt piezoelectric inkjet printing, which is compatible with large scale manufacturing and roll-to-roll processing to develop a platform that can sensitively detect DENV RNA via fluorescence. An inkjet printing approach to a simple and robust RT-LAMP assay was accomplished by integrating it with an engineered highly methacrylated (GM_{10}) hydrogel. Based on a previously published DENV RT-LAMP assay, we demonstrated that a reduction in total reaction volume could improve sensitivity. The assay was then optimized and characterized for the in tube, reduced volume setup with the limit of detection being 10^3 copies per reaction which was below the range of DENV RNA loads reported after day 3 of infection.

Following in tube characterization, we validated our hydrogel formulations (10% w/v GM_{10}) as inkjet printing compatible. We were able to show reliable and precise printing of 10% (w/v) GM_{10} (50 nL spots) using our precision dispensing system. With the help of fluorescently labelled proteins of similar molecular weight as LAMP reagents, we confirmed free diffusion of the reagents within the crosslinked hydrogel while the heavier DENV DNA target localized around the hydrogel boundaries . Further work is needed to understand this size-based exclusion by investigating the pore sizes of the crosslinked hydrogel. By incorporating the hydrogel to develop a gel-based RT-LAMP assay (5 µL volume), we were able to increase the limit of detection by 100-fold to 10 copies of DENV RNA per reaction. This made our assay highly sensitive and suitable for diagnosis of early onset and asymptomatic DENV infections. In a small time-scale study, we were also able to show the stability of the gel-based LAMP assay for up to 30 days at 37°C implying its suitability for on chip storage. However, we were unable to repeat the results with the addition of reverse transcriptase enzyme and further studies are needed to understand how long-term storage affects the sensitivity of the assay.

Lastly, we demonstrated positive amplification of DENV RNA in inkjet printed gel-based RT-LAMP assay (50 nL volume). Unfortunately due to equipment errors, we were unable to characterize the inkjet printed assay for sensitivity and time to positivity but we believe that our approach of inkjet printing will help us drastically reduce the cost as well as the time to positivity (from 50 min to as low as 10 min) making it a very rapid assay. Since we could not fabricate and validate our inkjet printed platform which incorporated sample delivery, we instead demonstrated our workflow in a manually printed platform with increased reaction volume (5 µL). Further development of our platform would involve adapting the inkjet printed, gel-based RT-LAMP assay to a microarray format on the proposed device and approach digitization of the readout to extract quantitative information from the assay. We hope we can build on this diagnostic platform and prove its efficacy with clinical samples.

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APPENDIX

For reduced volume reaction setup of DENV LAMP assay, we compared limit of detection and real-time amplification plots. Figure A1 compares the time to positivity (Ct) values obtained for different concentrations of DNA target. Figure A2 shows the molecular weights of the DENV RT-LAMP assay amplicons via gel electrophoresis and as expected it's a mix of concatemers of varying sizes. Figure A3 displays the device used in diffusion studies used for Figure 9. Figure A4 is a representation of the inkjet printing process on a glass slide.

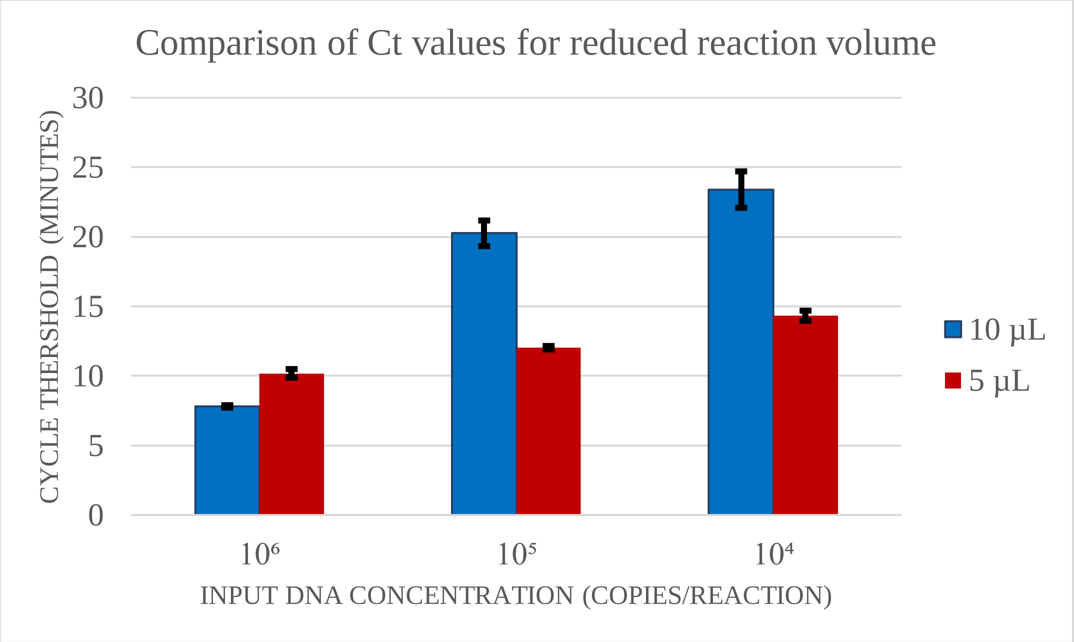

Figure A1: Effect of reduction of reaction volume on time to positivity (Ct) values for DENV LAMP assay. Above values were obtained from real time DENV LAMP assay on the QuantStudio (n=3) and are for the concentrations of DNA target that resulted in a positive readout. We note that by reducing the volume in half we were able to reduce the time to positivity. Another important thing to mention is that Ct values were not actually higher for higher concentrations of DNA (10_6 copies) when volume of the reaction was reduced.

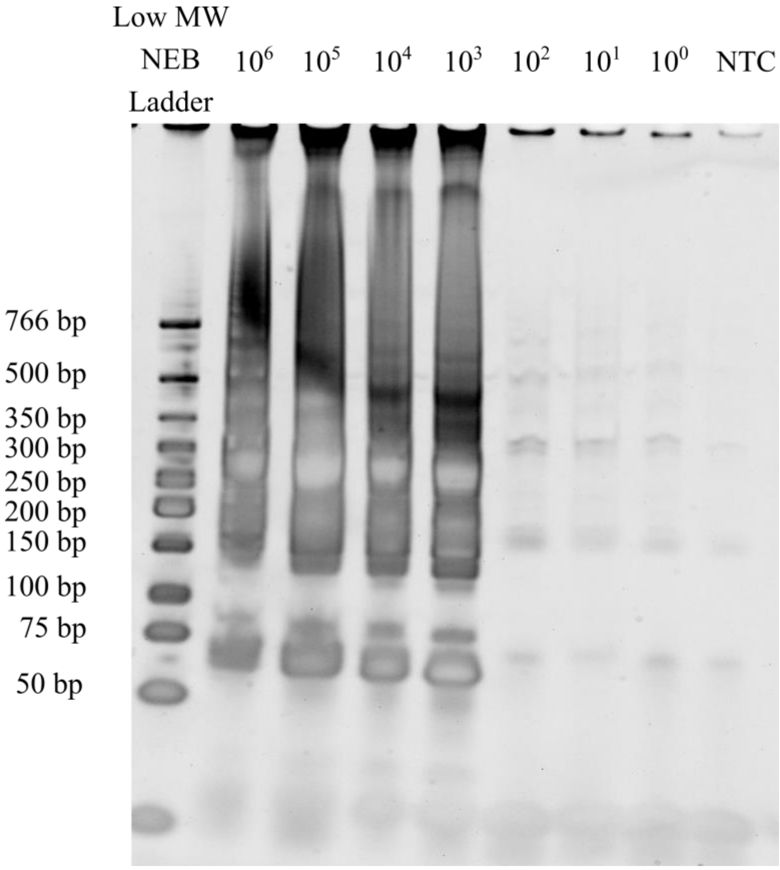

Figure A2: Gel electrophoresis of optimized DENV RT-LAMP products.

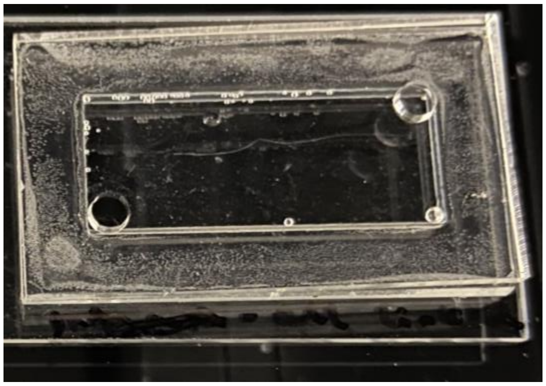

Figure A3: Acrylic device used to create a reservoir for investigating diffusion of RT-LAMP reagents within crosslinked hydrogel.

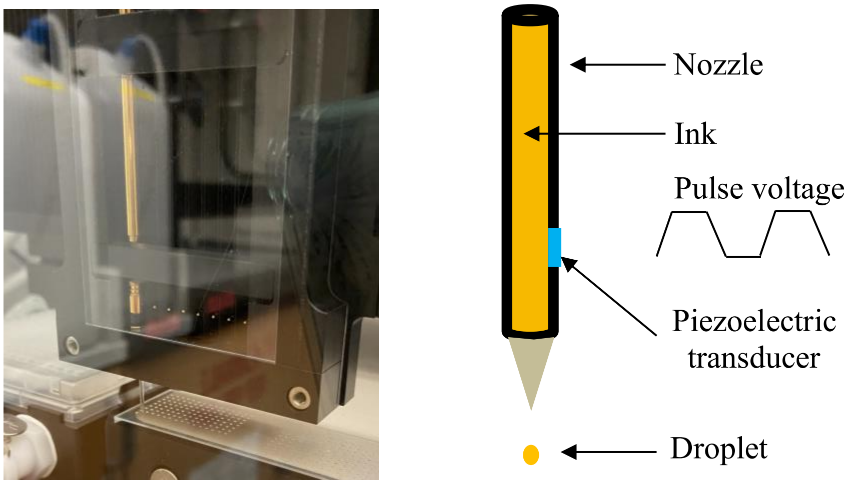

Figure A4: Automated piezoelectric inkjet printing in process with the schematic on the right depicting how the piezoelectric dispensing nozzle operates.

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