Spring 5-15-2021

POINT OF CARE INFECTIOUS DISEASE DIAGNOSIS VIA ISOTHERMAL NUCLEIC ACID AMPLIFICATION INTEGRATED INTO A SAMPLE TO ANSWER DEVICE

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POINT OF CARE INFECTIOUS DISEASE DIAGNOSIS VIA ISOTHERMAL NUCLEIC ACID AMPLIFICATION INTEGRATED INTO A SAMPLE TO ANSWER DEVICE

BY

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A Dissertation submitted to the Faculty of Keck Graduate Institute of Applied Life Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Life Sciences.

Keck Graduate Institute, Claremont 2021

Approved by:

03/26/2021

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Updated September 2018

ABSTRACT OF THE DISSERTATION

Point of Care Infectious Disease Diagnosis via Isothermal Nucleic Acid Amplification Integrated Into a Sample to Answer Device

By

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Keck Graduate Institute of Applied Life Sciences: 2021

Sexually transmitted infections caused by Chlamydia trachomatous (CT) and Neisseria gonorrhea (NG) are often under-diagnosed, miss-diagnosed, and not properly treated, leading to long term complications such as ectopic pregnancy and infertility, and to emergence of drug resistance. Dengue virus (DENV) infections can cause dengue fever (DF), which if not properly managed can lead to Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), causing significant morbidity and mortality. In both cases, early and accurate detection at the point of care is critical to facilitate proper patient care and disease management. Nucleic acid amplification tests (NAATs) provide suitable sensitivity and specificity, but are often complex, expensive, and difficult to implement at the point of care, especially in low resource settings. We have developed a prototype system consisting of a cartridge and compact instrument that can execute sample preparation, isothermal Loop Mediated amplification (LAMP) and lateral flow detection of these pathogens. For CT and NG, we have established singleplex and duplex LAMP assays, in liquid and paper form, with lateral flow detection. We have performed inhibition testing, demonstrated full process execution outside the cartridge, then implemented the

process in the cartridge and device, demonstrating detection down to 100 Elementary Bodies (EB)/mL for CT and 100 colony forming unit (CFU)/mL NG in urine. For DENV, we established a pan-serotype singleplex RT-LAMP assay and a duplex assay with MS2 as an internal amplification control, in liquid and paper form. We demonstrated that the master-mix in paper form is stable upon storage at RT and 40^{o}C over 8 weeks. For sample preparation starting from whole blood, we performed plasma separation coupled to amplification, with inhibitor testing. The next step is a front to back experiment and implementation in a modified cartridge for the detection of all DENV serotypes. The herein described assay processes and prototype systems can be further developed to enable point of care diagnosis for these and other pathogens in low resource settings.

DEDICATION

I dedicate this research and thesis to my family: my American parents Tom and Sharon who always believed in me and continued to support me throughout years of self-learning as a first-generation in college and years of challenges and doubts. Also, to my mom, my dad, and my siblings who supported me and sacrificed a lot for me to be in the U.S. in a safe place to be able to build my own future. Also, I dedicate this thesis work to my fiancé Patrick, who has been a constant source of support during the challenges of graduate school lifestyle. Lastly, to Gandalf for sending me flowers after every milestone achievement. It is true it takes a village to raise a child. I am grateful to everyone who helped me be the person I am today.

BIBLIOGRAPHY

I was born in Iraq on March 5, 1990. My family and I escaped Iraq at the age of 7 years old and moved to Jordan. While living in Jordan illegally with fear of being deported back to Iraq, my mother sought our help through the United Nations. Three months before graduating high school in Jordan, we came to the United States as refugees and were placed in Kent, WA. Within a week of our arrival, I was enrolled at Kent-Meridian High School, where I restarted my junior and senior years of high school with ESL classes to learn English. Two years later, I enrolled at Eastern Washington University and graduated in 2016 with my Bachelor of Science in Biology and minor in organic chemistry. I searched for pre-medical summer programs when I found Keck Graduate Institute (KGI) in Claremont, CA which had the one-year post-baccalaureate pre-medical program. During my first year at KGI, I joined Dr. Rachita Sumbria's lab and enjoyed the research. Therefore, I continued my education at KGI by completing my masters in 2018 and started my PhD immediately after with Dr. Angelika Niemz. My research was concentrated on the isothermal amplification reaction for LAMP and RT-LAMP coupling with lateral flow detection in a system that automates and integrates pathogen lysis, isothermal DNA amplification and lateral flow detection in a compact, robust, and low-cost design.

ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. Angelika Niemz, who has been my mentor throughout my research, the development of the KGI Ph.D. program, my Ph.D. committee: Jim Osborne, Sakamuri Rama Murthy, Travis Schlappi, and the PhD program director Jim Sterling. I would also like to thank all of the past and present members of the Niemz Lab who have made this possible. I would also like to thank the Naval Medical Research Center (NMRC) in Silver Springs, MD for their collaboration in the project

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INTRODUCTION

Chlamydia trachomatis and Neisseria Gonorrhea Infections

Chlamydia trachomatis (CT) and Neisseria gonorrhoeae (NG) are the two most common genital infections worldwide and the most common reported sexually transmitted diseases (STIs) in the United States.[1] In 2012, the World Health Organization (WHO) projected 130.9 million cases of chlamydia and 78.3 million cases of gonorrhea. However, the number of cases continues to increase. In the US, between 2013 and 2017, there was a 22% increase in cases of chlamydia and 67% increase in cases of gonorrhea.[2,3,4] Both infections are mostly asymptomatic and can lead to long term complications if left untreated, such as pelvic inflammatory disease, infertility, chronic pelvic pain and tubal pregnancy.[5,2] Therefore, timely treatment is crucial to prevent further transmission and complications to the individuals and their partners. The Centers for Disease Control and Prevention (CDC) recommends azithromycin or doxycycline as a first-line treatment for chlamydia. A dual treatment of ceftriaxone and azithromycin should be taken together as a first line treatment for NG.[6-7] However, there is an alarming increase in antimicrobial resistance, especially in NG and CT. There is an urgent need for rapid and accurate detection of these infections to ensure effective and timely treatment with the correct antibiotic, in order to prevent health complications and stop the spread.

Both infections are not fatal and can be cured with early detection and treatment. However, there is lack of resources available for early detection, namely diagnostics. The diagnostics currently available are cell culture and nucleic acid amplification testing (NAAT), which both require a central lab setting. Cell culture has a high specificity (~99%) but is limited by low

sensitivity (60-80%), extended turn-around time and high labor intensity. NAAT methods became the diagnostic gold standard with both high sensitivity (~98%) and specificity (~99.5%), however, the involved instruments are expensive and for central lab use only. In contrast, CT/NG can be detected at the point of care via direct microscopy or antigen detection lateral flow assays.[8] The direct microscopic exam has low sensitivity (17-74%) and high specificity (~99%). Lateral flow antigen detection has low sensitivity ranging from 53.1- 62.5% and a specificity of 98.0%.[8-9]

Rapid NAATs, such as the Cepheid CT/Neisseria gonorrhoeae (NG) GeneXpert assay, have equivalent performance characteristics to traditional lab-based NAATs (Gaydos et al., 2013), but was originally categorized as moderate complexity, requires a central lab setting, and has a 90 min test-run time which is too long for integration into the standard workflow of many healthcare settings. A more recent molecular point-of-care platform is the Binx Health io system. The device provides results in 30 minutes combining testing and treatment in a single patient visit. However, the Binx io requires a bulky benchtop instrument.

There is an unmet need with the current diagnostics. Therefore, we are working on a rapid, accurate, and affordable molecular diagnostic device to detect CT and NG from urine and swab samples at the point of care.

Dengue Virus

Epidemic Dengue is a mosquito-borne disease transmitted by the Aedes aegypti mosquito. Dengue virus (DENV) is an enveloped single positive-strand RNA virus of the Flaviviridae family. DENV has five different serotypes: DENV1, DENV2, DENV3, DENV4, and DENV 5. DENV5 was recently discovered.[10] The serotypes are antigenically distinct and share approximately 65% of their genomes.[10-11]

Approximately 390 million dengue infections occur annually, of which 96 million clinically manifest as Dengue fever (DF) or severe Dengue, which includes Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS).[12] The virus is mostly spread in tropical urban countries. Mosquito vectors spread even more in areas where there is free standing water and a lack of sanitation. [13]

The virus hijacks the endosome cell forming process to enter the body and tricks the cells into allowing the virus to penetrate into the cell. There are two ways the virus can exit the endosome, when the cell environment is acidified, and the endosomal membrane will gain a negative charge. Once the virus exits the endosome, it is released into the cytoplasm and replicates itself. This viral complex makes more copies of the viral RNA that translate into more viral proteins that can infect other cells.

One in four people infected with DENV will experience illness. The most common type of DENV infection in adults is DF, which causes mild symptoms that can be misdiagnosed for other diseases. Symptoms can start as fever, eye pain, joint pain, muscle pain, bone pain, headache, rash and vomiting.[11] Warning signs for severe dengue include bleeding from the nose or gums, hematemesis, hematochezia and/or restlessness and irritability. There are 0.5 million individuals who are hospitalized for DHF, of which mostly are children under the age of five.[11, 14]

A dengue patient who is infected with one serotype will develop a lifelong immunity to the infecting serotype, although there is no cross protective immunity to the other serotypes. However, if the patient gets infected with a different serotype there is an increased risk of developing severe dengue illness due to antibody mediated enhancement, meaning these antibodies can act as Trojan horse and can enable the virus to be more infectious.[10,15]

Clinical symptoms for Dengue fever resemble general febrile illness, but differential diagnosis is important due to the risk of complications. If a patient is discharged prematurely, they may develop severe dengue, which has a mortality rate as high as 20% if left untreated, compared to 2-5% mortality with proper treatment.[11] Treatment involves oral or intravenous hydration therapy, bed rest, paracetamol for fever reduction, and avoidance of blood thinners such as aspirin.[16-17]

Current diagnostic methods for Dengue virus include molecular tests, antigen tests, serological tests and viral cell culture. The acute phase of the disease is the first 1-7 days after symptom onset, with the virus located in blood or blood-derived fluids (serum or plasma). During this phase, the dengue virus RNA can be detected with molecular tests (NAAT) and NS1, the non-structural protein, can be detected with NS1 antigen tests. The virus secrets this protein during the acute phase of the infection. During the acute phase NS1 tests are as sensitive as molecular tests, but after the first week the CDC does not recommend NS1 tests. [14, 18] NS1 tests do not provide serotype information, while molecular testing does (Figure 1). The second phase, the convalescent phase, is more than seven days post symptom onset. During the convalescent phase, patients develop a primary antibody response that includes an increase in IgM antibodies. The IgM antibodies are detectable in 50% of patients by day 3-5 and 99% of patients by day 10.[19,20] However IgM antibodies reach peak levels at two weeks, then decline two weeks after symptom onset. IgM antibodies remain detectable for up to three months.[20] The fourth biomarker is the anti-dengue serum IgG. The IgG is detectable at low titers starting at day 7 of the illness, then its concentration increases slowly and can be detected months or years after illness. During a secondary dengue infection (when a patient get infected with dengue virus for the second time), the IgG titer is at a high level and can be detected even during the acute phase and may last in the host body for months and sometimes for life. Furthermore, the IgM levels are lower during the acute phase of the

secondary dengue infection compared to the primary infection, allowing for tests to use the ratio of IgM/IgG antibodies to distinguish if the patient has a primary or secondary infection.

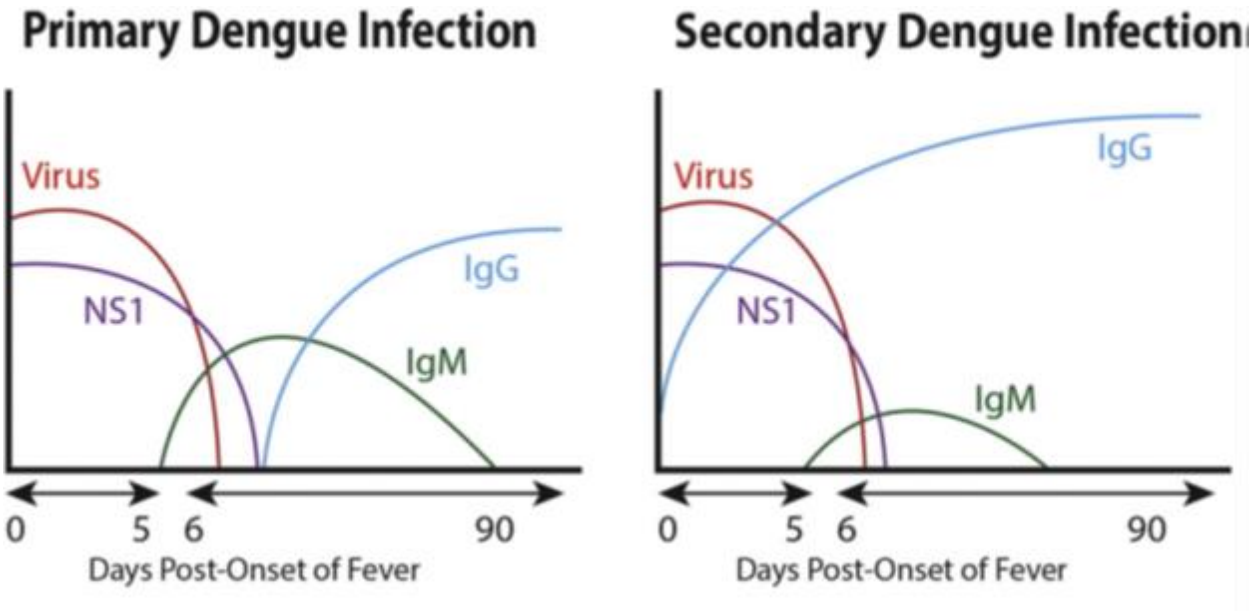


Figure 1. Dengue Virus Biomarkers. Viral markers IgG, IgM, and NS1.[21]

Nucleic acid amplification tests (NAATs) became the main tool for the detection of the virus. Reverse transcription polymerase chain reaction (RT-PCR) provides high sensitivity and specificity, featuring RNA extraction, reverse transcription, purification, amplification and detection of the target RNA. This method is best used for early detection of the virus. [22,23,24] However, most available NAATs tests are expensive and require an expert to use laboratory equipment.

TECHNICAL BACKGROUND

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) makes million to billions of copies of specific DNA regions from different sources. The main steps in PCR are shown in (Figure 2). This technique is used in a variety of applications including gene cloning, infectious diseases diagnosis, and genetic screening .[25] Initially, researchers used agarose gel electrophoresis and image analysis to visualize the bands of DNA when ethidium bromide fluoresces. [19, 20] However, PCR with endpoint detection gives qualitative and/or semi-quantitative analysis only. Often times the qualitative analysis was not sensitive enough to verify if the target amplified. Real-time quantitative PCR (qPCR) is a more advanced version of PCR that can measure the product in every cycle. Reverse transcription polymerase chain reaction (RT-PCR) is used to detect viral RNA by reverse transcribing the RNA to DNA prior to amplification. qPCR measures fluorescence after every cycle, bypassing the need for gel electrophoresis to review the results. Optionally, melting curve analysis is available to verify the presence of the correct amplicon. qPCR can use intercalating DNA binding dyes (e.g., SYBR green or EvaGreen) or probes for the detection of the target. This method is easy to use, simple and quick.

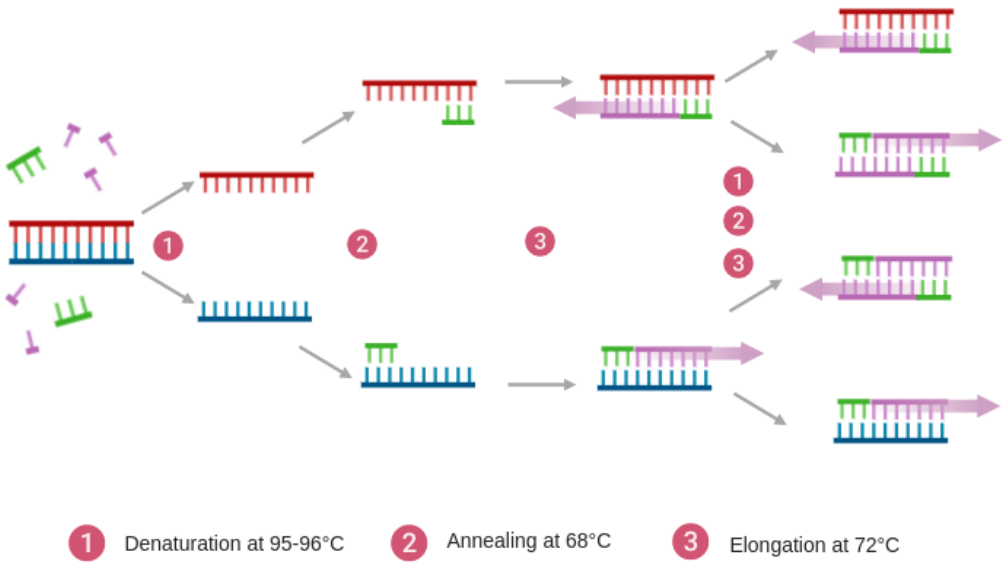


Figure 2. Illustration showing the main steps in the polymerase chain reaction (PCR). PCR technique that uses DNA polymerase to synthesize millions of new DNA copies via a template DNA strand. Step one: Denaturation: As in DNA replication, the two strands in the DNA double helix need to be separated. The separation happens by raising the temperature of the mixture, causing the hydrogen bonds between the complementary DNA strands to break. This process is called denaturation. Step two: Annealing: Primers bind to the target DNA sequences and initiate polymerization. This can only occur once the temperature of the solution has been lowered. One primer binds to each strand. Step three: Extension, in which DNA polymerase extends the 3′ end of each primer along the template strands.[27]

Isothermal Nucleic Acid Amplification

Isothermal nucleic acid amplification provides a rapid, sensitive, specific, and simple means for the detection of nucleic acids from samples that require less expensive instrumentation. It does not require any thermal cycling and therefore is easier to operate with less energy required compared to PCR. Furthermore, isothermal amplification techniques are used in infectious diseases diagnosis and are suitable for point of care settings.

There are many types of isothermal nucleic acid amplification methods which are categorized by the principle of the reaction. Some technologies are based on RNA transcription such as nucleic acid sequence-based amplification (NASBA) and Transcription-mediated amplification (TMA).

[28,29] Another reaction principle method is based on DNA replication with enzymatic duplex melting/primer annealing such as recombinase polymerase amplification (RPA) and Helicase Dependent Isothermal Amplification (HDA).[28] Other methods are based on polymerase extension/strand displacement, such as loop-mediated amplification (LAMP), one of the most commonly used isothermal amplification methods. In some cases amplification includes a single strand cutting event such as in strand displacement amplification (SDA).

LAMP has been widely used in both laboratory and point of care diagnostic applications. LAMP is more resilient to inhibitors than PCR, thus simplifying the DNA purification step. LAMP requires a set of four to six primers that bind to six or eight different regions on the target gene making LAMP highly specific.[30] The LAMP primer set must include two outer primers (F3 and B3) and two inner primers (forward inner primer (FIP) and backward inner primer (BIP)) that can recognize six distinct regions within the target DNA. Optionally, loop primers (loop forward and loop backward) can be added to speed up the amplification and detection efficiency. In the first step of the amplification reaction (Figure 3), the FIP primer hybridizes to F2c in the target DNA and is extended using a polymerase with displacement activity such as Bst polymerase. The outer primer F3 hybridizes to F3c in the target DNA and is extended, which leads to displacement of the product obtained from FIP extension. The same set of reactions occurs using BIP and B3 on the product of FIP. These reactions then form a dumbbell-like structure essential for isothermal amplification. The single strand can be annealed by FIP or BIP leading to elongated products with various copies of the target sequence. [31-32] LAMP requires careful primer design and optimal reaction conditions to avoid false positives.

LAMP product detection can be performed by adding dyes for fluorescence or colorimetric determination, based on turbidity changes from pyrophosphate precipitation. Other options include

agarose gel electrophoresis or lateral flow detection. However a more improved detection method is the use of fluorescent labeled probes and primers.

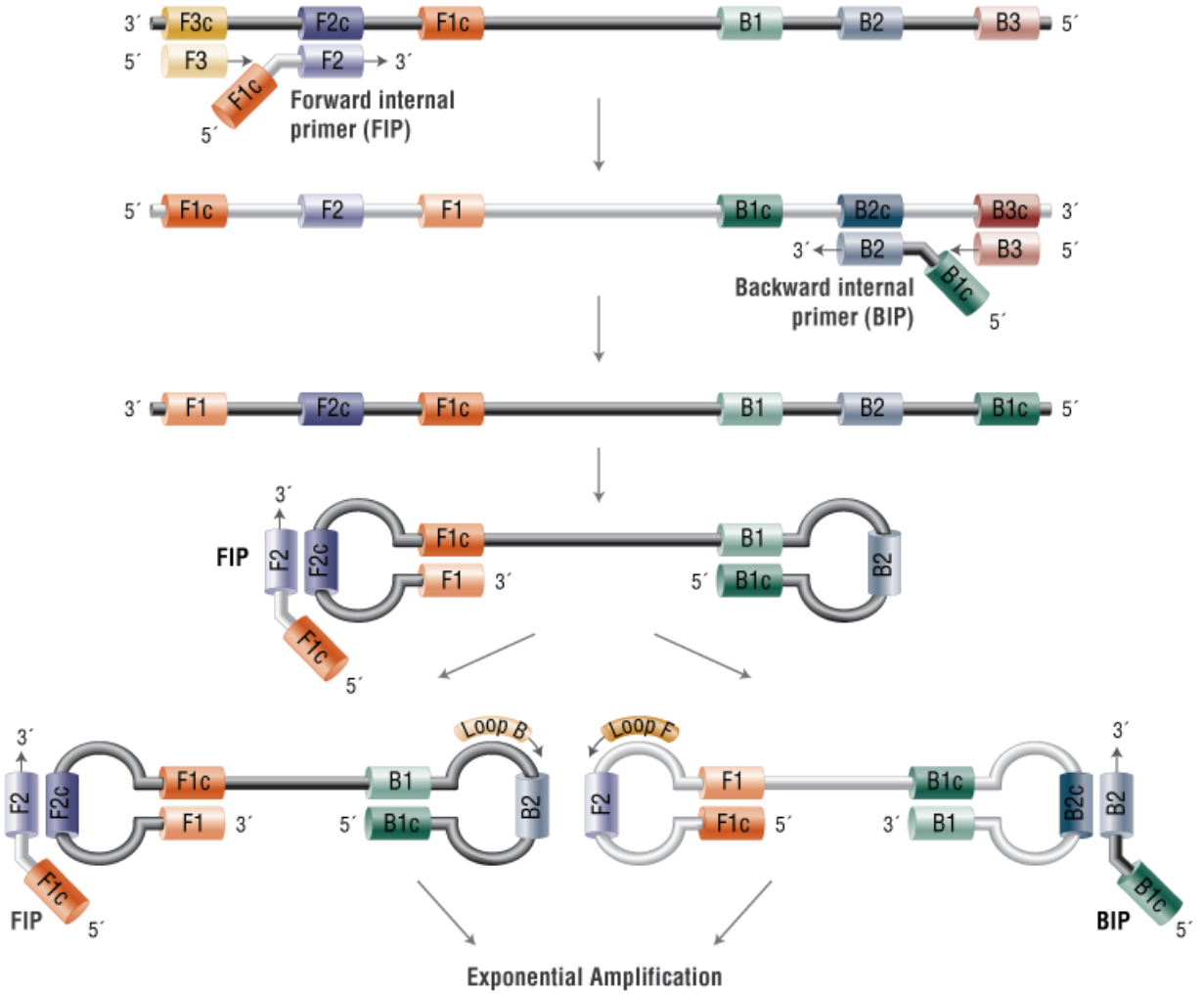


Figure 3. Graphic illustration of LAMP reaction. The Forward Inner Primer (FIP) consists of a F2 region at the 3'-end and an F1c region at the 5'-end; Forward Outer Primer (F3 Primer) consists of a F3 region which is complementary to the F3c region of the template sequence; Backward Inner Primer (BIP) consists of a B2 region at the 3'-end and a B1c region at the 5'-end. Backward Outer Primer (B3 Primer) consists of a B3 region, which is complementary to the B3c region of the template sequence. The reaction starts with the F2 region of FIP hybridizing to the F2c region of the target DNA and initiates complementary strand synthesis. Next the F3 primer hybridizes to the F3c region of the target DNA and extends, displacing the FIP linked complementary strand. This displaced strand forms a loop at the 5'-end, which serves as a template for BIP. B2 hybridizes to the B2c region of the template DNA. Then, B3 hybridizes to B3c region of the target DNA and extends, displacing the BIP linked complementary strand. Dumbbell-shaped DNA is formed. [33]

Infectious disease caused by RNA viruses can be detected via Reverse Transcription Loop-mediated isothermal Amplification (RT-LAMP). This technology combines LAMP with reverse transcription. RT-LAMP requires a reverse transcriptase, or a DNA polymerase with intrinsic reverse transcriptase activity. There are multiple enzymes that are used for RT-LAMP in order to convert RNA to cDNA prior to DNA amplification such as using a combination of *GspM* with Avian Myeloblastosis Virus (AMV RT) or Gsp SSD2 pol (OptiGene, UK) alone.

Paper Based Devices

Paper based devices for infectious disease diagnosis have potential for use in a point-of-care setting. Paper can be used for multiple purposes ranging from reagent storage to fully integrated paper based devices that control fluidics through printing wax on paper to create different reaction zones[34,35], by folding the paper, [34,36] and through layered devices.[37,38] We herein focus on the use of paper as a substrate for the master-mix component and reagent addition.

Nucleic acid amplification tests (NAAT) have become widely implemented in paper-based device formats. Many groups were successful in using paper as a substrate for isothermal amplification methods such as RPA[34,38,39], HDA[40,41] LAMP[35,37]and iSDA.[42]

A number of groups have demonstrated RPA by adding primers on paper, and using enzymes commercially purchased as freeze-dried pellets.[38,43] The ultimate goal would be to store all reagents on paper and add a reconstitution buffer containing the target DNA or RNA.

Cellulose chromatography paper is the most common used for separation and wicking[34,38,40,42] glass fiber for reagents release[37,38], nitrocellulose, polyethersulfone (PES) for hybridization and protein binding. [38,40]. Another key factor is paper pretreatment to avoid irreversible adsorption of reagents or the target. Some investigators chose not to pre-treat the papers prior to dispensing the

reaction mix[34,41] although this resulted in a significantly higher LOD (10^7 copies/uL) or reduced stability, respectively. In some cases, initial attempts to complete the amplification reactions without pretreatment proved unsuccessful[40]. Common pre-treatment reagents include BSA[40,42] and Tween 20[42]. Seok 2017 tested multiple pre-treatment reagents and found that PVA3% was most successful in maximizing fluorescent signal detection for LAMP. [37]

Although some paper based nucleic acid amplification methods have been developed, none of the studies have demonstrated the thermostability and sensitivity of the paper-based dry reagent master mixes. Shetty 2016 showed stable dried enzyme pads in ambient temperatures for 34 days. Magro 2017 stored for one month at room temperature with no light or humidity, showed 20% success of devices after 3 months. Tang 2017 showed greater stability with addition of BSA treatment for up to 30 days at 25ºC. [34,37]

Nucleic Acid Lateral Flow based Detection

Lateral Flow Strips (LF) are assay materials used for the detection of designated antigens by capillary action; composed of porous nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad; abbreviated to LF. The sample Pad component of LF Strip, contains pretreatment, method of application of sample to be tested and conjugate pad component of LF strip, contains colloid used as the method of detection. Lateral flow strips contain avidin functionalized gold nanoparticles on the conjugate pad, anti-FAM antibodies on the test line and anti-Dig antibodies on the control line purchased from USTAR (Hangzhou, China). To allow detection of the target, we utilized biotinylated primers labeled with digoxigenin (DIG) or FAM (Figure 4) depending on the assay target.

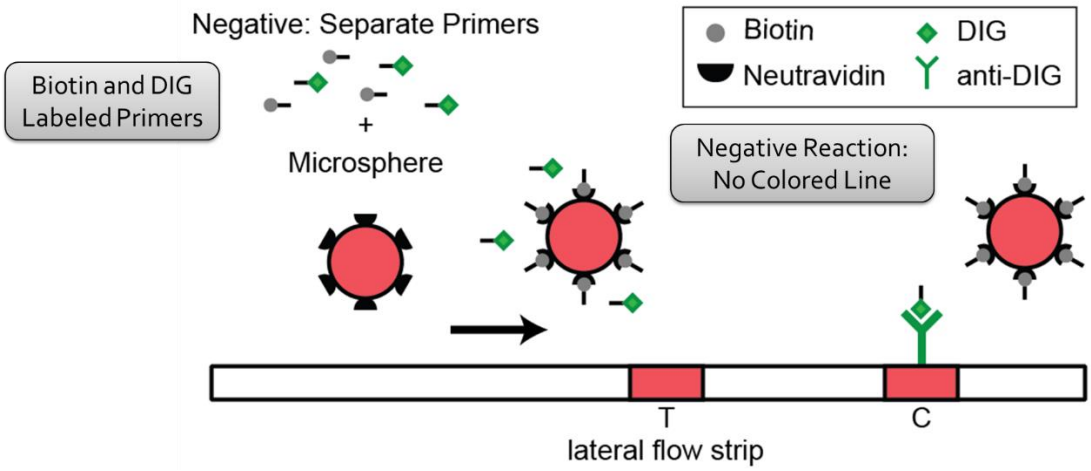


Figure 4. Lateral Flow Assay is designed to detect amplified DNA/RNA sequence which is labeled with biotin and a small molecule hapten. The lateral flow strip consists of a sample pad with buffers, a conjugate pad containing gold nanoparticles conjugated to steptavidin (GNPs), a nitrocellulose membrane containing capture antibodies specific to the hapten, and an absorbent pad. Target RNA labeled with biotin is dispensed onto the sample pad, which flows to the conjugate pad and binds to the Streptavidin on the GNPs. The target RNA – GNP complex flows down the strip and binds to the target capture antibody, while unbound GNPs bind to the positive control. The signal of the conjugate bound to the capture antibodies is proportional to the number of copies captured.

Herein we have characterized the limit of detection and thermostability of these dry reagent LAMP and RT-LAMP paper pads, as further described in the methods section. We tested the long-term storage capability of the RT- LAMP assay reagents performing a thermostability study. The goal is for the assay to be stable at room temperature and at 40^{o}C for eight weeks showing no false negative results. This result can affect the overall cost of the NAAT device that our lab is building to be able to serve low-resource settings.

CHLAMYDIA AND GONORRHEA

AMPLIFICATION AND DETECTION

MATERIALS AND METHODS

Reagents

We purchased Chlamydia trachomatis genomic DNA, and the plasmid pFEN207 that contains the kdo gene from ATCC (ATCC® VR-886D). Quantified genomic DNA of Neisseria gonorrhoeae was purchased from ATCC (ATCC® 700825DQ™, Manassas, VA, USA). All CT, NG, and Bsub primers were purchased from Integrated DNA Technologies (IDT). Tris and other general reagents were purchased from (Sigma Aldrich, St. Louis, MO, USA). For CT quantification: Calf DNA (Qiagen) and Quant-iT™ PicoGreen™ dsDNA Assay Kit was purchased from ThermoFisher (Waltham, MA, USA). 5M Betaine solution, bovine serum albumin, Deoxynucleotide Set 100 mM (DNTP100A-1KT), Magnesium sulfate solution (MgSo4) and trehalose are obtained from Sigma Aldrich (St. Louis, MO, USA). Bst 2.0 warmstart glycerol free polymerase was obtained from New England Biolabs (Ipswich, MA, USA). SYBR Green was purchased from Life technologies (Carlsbad, CA, USA). All the reagent solutions were prepared using nuclease free water prepared by Promega (Madison, WI, USA). CF1 100% cotton linter sample pads (176 µm thickness, 22 mm width, and 50 m length) were purchased from GE Lifesciences (Marlborough, MA, USA).

Chlamydia plasmid standard quantification via Pico Green

A standard curve was obtained using calf thymus DNA (Type I; Sigma, St. Louis, MO, USA), dissolved in Tris-EDTA buffer solution (Sigma Aldrich, St. Louis, MO, USA) and serially diluted to concentrations ranging from 5–40 ng/mL as shown in Table (1). We allowed PicoGreen to warm up to room temperature, then prepared a 200x dilution by adding 12.5µL of PicoGreen to 2,487.5µL of TE buffer in a plastic 50mL tube wrapped in foil or placed in the dark. Triplicate

aliquots of 100µL DNA standard and unknown plasmid DNA, plus 100µL of 200x PicoGreen added to each replicate resulting in 200µL/well, were mixed by shaking for 5 seconds. Absorbance measurements were taken in a SPECTRAmax PLUS plate reader with excitation at 480 nm and emission at 520 nm. We subtracted the reagent blank fluorescent reading from each sample, plotted the standard curve of concentrations against fluorescence, and used the standard curve to determine the unknown concentration of the plasmid.

Table 12. Protocol of preparing standard curve via PicoGreen Assay.

Standard	Volume (µL) of TE	High Concentration	Sample	Final Volume (µL)	Final DNA Concentration
Stock					6630 ng/mL
S1	795 µL	5µL	Stock	800 µL	40 ng/mL
S2	400 µL	400µL	S1	800 µL	20 ng/mL
S3	400 µL	400µL	S2	800 µL	10 ng/mL
S4	400 µL	400µL	S3	800 µL	5 ng/mL
S5	400 µL	400µL	N/A	800 µL	0 ng/mL
					6630 ng/mL

CT Genomic DNA Quantification via quantitative polymerase chain reaction (qPCR)

A standard curve was prepared by serial dilution of the quantified plasmid sample to four different concentrations ranging between 1e5-1e2copies/reaction diluted in Tris-EDTA buffer. A set of two primers was purchased from (Integrated DNA Technologies, Coralville, IA, USA). Each reaction contained a final concentration of 0.9 µM each of the forward primer (GAGCTTCTGTGGGGGAAGTC) and reverse primer (ATACAAACGACGAGCGGTGT). 8.5µL of 2X PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). 0.2X of SYBR Green and 11.5µL of DNA sample. QPCR amplification curves were analyzed using the Roche LightCycler® 96 instrument with software version 3.5 (Roche Diagnostics). After ensuring there is good amplification for the standard and the unknown, we

made sure the efficiency of amplification of the control template is within 90–110% (meaning slope is within -3.58 to -3.10) and that the R2 >0.9. Then we calculated the initial concentration of CT unknown DNA based on the standard curve generated from the quantified control plasmid DNA.

Loop Mediated Isothermal Amplification (LAMP)

LAMP primers:

We designed CT LAMP primers targeting the CT CDS2 gene and NG LAMP primers targeting the NG 16S rRNA gene via Primer Explorer software (Eiken Chemical Co., Tokyo, Japan). BLAST analysis was used to check any cross reactivity of the primers. Final primer sequences are shown in Table 2, where FIP and BIP are the forward and backward inner primers, F3 and B3 are the forward and backward outer primers, and LF and BF are the forward and backward loop primers.

Table 13. Primers for Chlamydia trachomatis CDS2 gene, Neisseria gonorrhoeae 16S rRNA gene and Bacillus subtilis

Primer Name	Sequence (5′–3′)
CT1 F3	GAGCGAGTTACGAAGACAA
CT1 B3	CAGACAATGCTCCAAGGA
CT1 FIP 56-FAMN	ACCGTCAGACAGAAAAGAGGATTATCCTCTTCGTTGACCGATG
CT1 BIP	GCTGGGAGAAAGAAATGGTAGCGTAAACGCTCCTCTGAAGT
CT1 LF	TCCTCAGAAGTTTATGCACT
CT1 LB 5BiodT	CAAATCTGACTAATCTCCAAGC
NG1 F3	AGGGAAGAAAAGGCCGTTG
NG1 B3	TTCAGAACGCAGTTCCCG
NG1 FIP	GCTGCTGGCACGTAGTTAGCCAATATCGGCGGCCGATGA
NG1 BIP 5DiGN	CGTAGGGTGCGAGCGTTAATCGGGGGATTTCACATCCTGCTT
NG1 LF 5BiodT	GGTGCTTATTCTTCAGGTACCG
NG1 LB	AATTACTGGGCGTAAAGCGGGC
Bsub F3	GAAGAGGATATGCCTTACCTTC
Bsub B3	CGACAGATACACGGTTATCAA
Bsub FIP	GGTAACGAGCGGCCATACCTACCATCACGTATGAACATCG
Bsub BIP	TGGCATTCACATTGCATCTCCTCCGGCTTCTTCAAGTGTT
Bsub LF	CATGTGAAGTTCCAATACCTGC
Bsub LB	GCGAGAAGAGGATGTCTGG

Reaction Mixture for Liquid LAMP Assay:

The LAMP protocol was designed for a total of 25µL per reaction. The singleplex liquid assay for CT and NG LAMP reactions contained a final concentration of 0.8µM each of FIP and BIP, 0.2µM each of F3 and B3 and 0.4µM each of LF and LB. However, the singleplex Bsub liquid

assay contained a final concentration of 1.6µM each of FIP and BIP, 0.2µM each of F3 and B3 and 0.8µM each of LF and LB. CT, NG and Bsub liquid assay also contained 0.4mM dNTPs, 2mM MgSO4, 1M betaine, 0.20mg/mL BSA, 1x Isothermal amplification buffer (IAB) made as shown in Table 3, 0.1x of SYBR green and 8U Bst DNA polymerase large fragment (New England Biolabs). The LAMP master-mix liquid solution was prepared on ice without target DNA. After adding all reagents, we added 1µL of target DNA to make a total volume of 25µL per reaction for the positive control in a 1.5mL eppendorf tube. For the negative control, 1µL of nuclease free water was added to make a total volume of 25µL per reaction for the negative control in a 1.5mL eppendorf tube while on ice. The mixture was pipetted into LightCycler® 8-Tube strips (Bio-Rad, Hercules, CA) with 25µL in each well and amplified using the Roche LightCycler® 96 for 45–60 min at 63 °C.

Table 14. Composition of 10X Isothermal Amplification Buffer

Chemical	Stock conc (M)	Target conc (M)	Vol (mL)
Tris	1	0.2	2
pH 8.5			0.54
pH 9			1.46
(NH4)2SO4	3.2	0.1	0.3125
KCl	2	0.5	2.5
MgSO4	1	0.02	0.2
Triton	10	1	1
Water			4.00

NG LAMP Liquid Taguchi

We used the Taguchi method to test for the best combination of NG LAMP primer concentrations to improve NG DNA amplification. The NG primers were selected as the independent factors, with multiple concentrations for each primer (levels). Each factor had five

levels of concentrations as shown in Table 4. All parameters were distributed in a balanced orthogonal array of 25 experimental combinations. To fit all replicates in one 96 well plates, we ran 24 out of these 25 combinations. It was important to run all combinations at the same time, in one plate, to avoid any outside variables. For each combination we ran two positive replicates (772 copies/reaction NG DNA) and two negative replicates with nuclease free water. The mixture was pipetted into a LightCycler® 96 well plate (Bio-Rad, Hercules, CA) with 25µL in each well and amplified using the Roche LightCycler® 96 for 60 min at 63 °C. We analyzed the data using the Taguchi module in the software suite DesignExpert (Stat-Ease) to find the main effects of each primer and interaction effects between primers and how significant these effects are on the LAMP amplification and detection of NG DNA.

To confirm the results of the Taguchi experiment, we ran the best four combinations of primer concentrations obtained from the Taguchi experiment (Table 5). We tested each combination with three replicates of positive NG DNA at 772 Copies/reaction and three replicates of negative control with nuclease free water. The mixture was pipetted into LightCycler® 96 well plates (Bio-Rad, Hercules, CA) with 25µL in each well and amplified using the Roche LightCycler® 96 for 60 min at 63 °C.

Table 15. Factors and levels (concentrations) evaluated in the Taguchi experimental design for the NG LAMP assay.

Primer	Level 1	Level 2	Level 3	level 4	level 5
B3	0.3uM	0.25uM	0.2uM	0.15uM	0.1uM
FIP	1.2uM	1uM	0.8uM	0.6uM	0.4uM
BIP	1.2uM	1uM	0.8uM	0.6uM	0.4uM
FLP	0.6uM	0.5uM	0.4uM	0.3uM	0.2uM
BLP	0.6uM	0.5uM	0.4uM	0.3uM	0.2uM

Table 16. Primer concentration combinations from the Taguchi design experiment.

	Original Concentration	Combination 1	Combination 2	Combination 3	Combination 4
NG1 FIP	0.8uM	0.8uM	0.8uM	1.0uM	0.6uM
NG1 BIP	0.8uM	1.0uM	1.0uM	1.0uM	0.8uM
NG1 F3	0.2uM	0.2uM	0.2uM	0.2uM	0.2uM
NG1 B3	0.2uM	0.3uM	0.2uM	0.2uM	0.2uM
NG1 LF	0.4uM	0.4uM	0.6uM	0.5uM	0.5uM
NG LB	0.4uM	0.3uM	0.3uM	0.5uM	0.6uM

Multi-parametric Optimization via Fractional Factorial Design Experiments

Eight conditions were used to optimize the detection of CT and NG DNA. Factors and Levels: Temperature: low = 60, high = 63 degrees Celsius. Mg^{+}: low = 2mM, high = 2.5 mM and DMSO: low = 0%, high = 5%, low = 0, high = 1. This 2^3 full factorial design resulted in 8 reaction conditions representing all possible combinations of these parameters. For each condition we ran LAMP reactions containing 3e3copies/reaction of Chlamydia DNA, 3e3copies/reaction of NG DNA, and the negative NTC control. Samples were prepared as described earlier. Samples were pipetted into Roche LAMP strips (Bio-Rad) with 25µL in each well and amplified using the Roche LightCycler® 96 for 45–60 minutes at 63 °C. Results were analyzed via the statistical tool design expert to understand the interactions between the different independent variables.

CT and NG Multi-parametric Optimization

Two factors and two levels per factor were tested to optimize CT/NG amplification: polymerase: low = 0.2mM, high = 0.4mM. Mg+: low = 2mM, high = 3mM. Five LAMP mixtures were prepared as described above in a reaction mixture of LAMP session. The experimental design model showed statistical design and data analysis via Design Expert Software (version 7.0). Two independent effective variables, Polymerase concentration and Mg concentration were tested. Amplification curves were generated using the "Abs quant" analysis in the LightCycler 96 software. Amplification curve colors distinguish each condition.

Internal Control (Bsub) Liquid Assay LOD

The liquid LAMP protocol was designed as described above for a total of 25µL per reaction. The Bsub LAMP reactions contained a final concentration of 0.8µM each of FIP and BIP, 0.2µM each of F3 and B3 and 0.4µM each of LF and LB of Bsub primers. In addition to 0.4mM dNTPs, 1M betaine, 0.20mg/mL BSA, 1x Isothermal amplification buffer (IAB) made as shown in Table 3, 0.1x of SYBR green and 8U Bst DNA polymerase large fragment (New England Biolabs). The LAMP master-mix liquid solution was prepared on ice without a target DNA. After adding all reagents, we added 1µL of Bsub DNA to make a total volume of 25µL per reaction, with target DNA present at 1e3copies/reaction, 5e2copies/reaction and 250copies/reaction. For the negative control, we added 1µL of nuclease free water to make a total volume of 25µL per reaction. The mixture was pipetted into LightCycler® 8-Tube strips (Bio-Rad, Hercules, CA) with 25µL in each well and amplified using the Roche LightCycler® 96 for 45–60 minutes at 63 °C.

Dry LAMP Assay Reagent Preparation

Pre-Treatment Process

Cellulose fiber cotton pads are used to support the dry master-mix reagents and are purchased from GE Lifesciences (Marlborough, MA, USA). The pads were cut to a round shape (176 µm thickness, 22 mm width, 50 m length), then we placed individual pads into a 24-well plate. Pads were pre-treated with a solution consisting of 0.01M Tris pH 8, 0.50M Betaine, 0.40mg/mL BSA, and 3% Trehalose (all Sigma Aldrich). We vortexed, then spun-down the pre-treatment solution, and pipetted 9µL onto each pad. We slightly covered the 24-well plate and placed it into a desiccator for a minimum of four hours.

Master-Mix Process

The master-mix solution was prepared immediately after the pre-treatment process was over. We made two different solutions to generate enzyme and primer pads. The final concentration of Bst2.0 WS polymerase was 0.40U/µL for the enzyme pads. For the NG primer pads we added 1x IAB buffer, 0.80mM dNTP, 4mM MgSO4, 0.80µM FIP and BIP, 0.20µM F3 and B3, and 0.40µM LF and LB. For the CT primer pads as a final concentration upon reconstitution to a final volume of 100 µL. For the CT/NG duplex primer pads, we added 9.96µL. We pipetted 8.5µL of concentrated primers mix or enzyme mix onto the respective pads, covered the plate, and placed in a -80°C freezer for one hour minimum. An hour later we used the lyophilizer from Labconco (6 Liter Console Freeze Dry System freeze dry) to dry the reagents for a minimum four hours.

Dry Reagents Quality Control Check

As standard quality control, we tested each batch of CT and NG master-mix papers for use in loop-mediated amplification (LAMP) after lyophilization. We reconstituted the dry reagents

present on a set of enzyme and primer papers by adding 100uL of 25mM Tris pH 9.2, 1mM of EDTA, 0.1x of SybrGreen with target DNA at a concentration as indicated in the results section. We combined one enzyme paper with one primers paper in an Eppendorf tube, and then placed the tubes on ice. We soaked the dry reagents paper in the solution and let them sit on ice for one minute. We vortexed the soaked dry reagents in a tube three times, one second each, which were discarded after. The liquid solution from the soaked papers were pipetted into PCR strips to test for amplification using the Roche LightCycler® 96 instrument.

Lateral Flow Detection

To enable the detection of LAMP amplicons via lateral flow, the forward loop primers (FLP) of the CT and NG LAMP assays were biotinylated at the 5' end. The backward inner primer (BIP) of the NG LAMP assay was 5'-labeled with digoxigenin, while the BIP of the CT LAMP assay was labeled with FAM. During testing outside the cartridge, for lateral flow detection of LAMP amplicons from liquid master-mix, 10 μL amplified master-mix was combined with 90 μL LF running buffer (USTAR), and then applied to the sample pad of the LF strip. For lateral flow detection of LAMP amplicons from reconstituted dry reagent master-mix, 90 μL amplified master-mix was directly applied to the sample pad of the LF strip. In either case, the sample was allowed to migrate along the strip, and results were read after 10 minutes. Lateral flow strip results were considered positive if a visible line appeared at the appropriate location, depending on whether a DIG or FAM labeled primer was used.

Multiplex Liquid LAMP Assay Optimization

To identify CT and NG in the same assay reaction, along with an appropriate internal control, we established multiplexed isothermal amplification coupled with lateral flow detection. The internal control is co-extracted and co-amplified to identify false negatives. We established a LAMP assay for the internal control, *Bacillus subtilis* (*B.sub*), and performed preliminary studies on a duplex assay to detect CT and NG in liquid master-mix and as dry reagent paper assay.

CT/NG Duplex Labeled Liquid Assay

The CT/NG liquid assay master-mix was prepared as described above. The primers were prepared to a final concentration of 0.80µM FIP and BIP, 0.20µM F3 and B3, and 0.40µM LF and LB. We tested four conditions, one condition containing NG at 5e3copies/reactions only, one condition containing CT 9e3copies/reaction only, one condition containing both NG DNA at 5e3copies/reactions and CT DNA at 9e3copies/reaction, and one for the NTC. We amplified three replicates of 25µL per condition, then analyzed the amplified master-mix via lateral flow as discussed previously.

CT/NG Duplex Labeled Paper Assay

The duplex dry reagents papers were prepared as described above. The primer pads were prepared to yield a final concentration of 0.80µM FIP and BIP, 0.20µM F3 and B3, and 0.40µM LF and LB, 0.80mM dNTPs, and 4mM $MgSO_4$ upon reconstitution to a final volume of 100 µL, The 10x IAB buffer was split 75% and 25% between the enzyme and primer pads to yield a final 1x concentration of 20mM Tris pH 9.2 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 50 mM KCl, and 0.1% Triton. NG primers were labeled with DIG and CT primers labeled with FAM as described above.

We tested three papers, each with two LAMP amplification replicates, of the dry reagent paper master-mix at 9e3copies/reaction of CT DNA only. A second set of three dry reagent papers were tested at 5e3copies/reactions of NG only, then a third set of three papers (six replicates) was tested at 5e3copies/reactions NG DNA and 9e3copies/reaction CT DNA. The fourth set of three papers was for the NTC, with no DNA added. The amplified master-mix was analyzed via lateral flow strips as previously discussed.

Dry LAMP reagent optimization process:

Pathogen Lysis

We tested two different methods to lyse NG cells: mechanical versus heat lysis (Figure 5). Mechanical lysis was performed using the Claremont Bio OmniLyse bead blender (ClaremontBio, CA). Each condition was tested at two different concentrations (1e3CFU/mL and 1e4CFU/mL). We tested nine replicates for heat lysis at 1e4CFU/mL and nine replicates at 1e3CFU/mL. In addition, we tested nine replicates for the OmniLyse bead blender at 1e4CFU/mL and nine replicates at 1e3CFU/mL all in one experiment, along with an NTC for each condition. We prepared master mix contained a final concertation of 0.9uM each of NG primers F and B, 0.2uM of SybrGreen and 8.5uL of PowerUp™ SYBR™ Green Master Mix (ThermoFisher, MA) to make a total of 25uL per reaction. NG cells samples were diluted in 1X PBS to make the target concertation for each condition. For heat lysis, NG samples were placed into an incubator (Benchmark Scientific, NJ) at 95^{o}C for five minutes. After that the samples were mixed with the master-mix. For mechanical lysis, the NG cells in 1XPBS were pumped through the OmniLyse bead blender at ~4 mL/min flow rate. The mixture was pipetted into LightCycler® 8-Tube strips

(Bio-Rad, Hercules, CA) with 25µL in each well and amplified using the Roche LightCycler® 96 for 45–60 minutes at 95 °C. The data was analyzed by comparing the Cq values of samples processed via mechanical versus heat lysis.

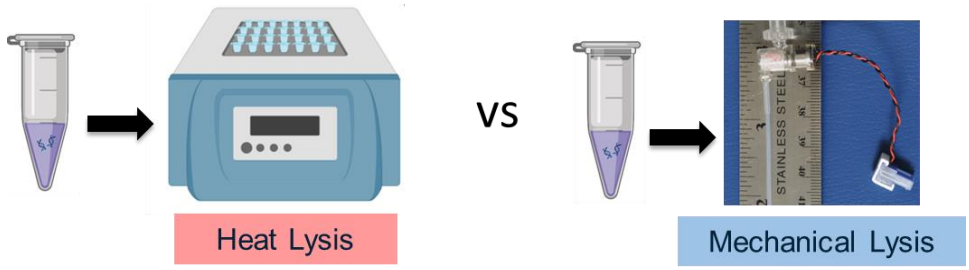


Figure 5. NG Lysis: Heat lysis verse Mechanical lysis.

NG Automated Process Execution outside of the Cartridge

We initially tested the process including mechanical lysis, isothermal LAMP, and lateral flow detection outside of the cartridge and instrument. As the sample matrix, we used the supernatant of 25mL human urine (Lampire Laboratory, Encinitas CA) that was spun at 800 rpm for 5 min. We used NG clean titer at the original stock concentration (4.8e8 CFU/mL) from Zeptomatrix (Buffalo, NY) to make a 1e6CFU/mL NG working solution in 1X sterile PBS. From that we performed a serial dilution in urine to obtain 3mL each of spiked urine samples with 1e4, 1e3, 1e2 and 1e1CFU/mL. For the NTC, we used urine without NG. We performed the full process execution of three replicates of one mL each for the positive samples, and four replicates of 1 mL each for the negative control. These samples were pushed through a Claremont Bio OmniLyse bead blender operating at 6 V, at a flow rate of ~4 mL/min. Of the lysed samples, 100 µL was

diluted in 300 µL TE buffer pH 9.2. For each sample, a pair of NG LAMP dry reagent papers, prepared as previously described, were rehydrated with 99 µL of the diluted sample and 1uL of 10X SybrGreen, then analyzed in duplicates of 45µL in each well on the Roche LightCycler® 96, with amplification for 60 min at 63 °C. Finally, we combined the two technical replicates of the amplified master-mix into a new microcentrifuge tube and used USTAR disposable nucleic acid detection strips (Hangzhou, China) to detect the target as previously discussed.

Material compatibility: NG Inhibition Studies

We tested LAMP assay inhibition potentially caused by the reaction insert, the inlet valves, the polypropylene film vs aluminum film, and by heat sealing the master-mix papers into the reaction insert. We used the NG liquid assay to test the inlet valves in our reaction inserts. We fabricated 3 reaction inserts with and 3 without inlet valves. We filled each reaction insert with TE buffer pH9.2 and incubated each insert in the oven at ~63°C for 1 hour. Each inhibition test included three replicates of the positive at 3e3copies/reaction NG DNA and negative control nuclease free water. In another series of testing, the reaction inserts were incubated with TE buffer pH 9.2 for an hour minimum. The TE buffer that was flushed through the reaction insert was collected to reconstitute NG unlabeled papers at previously described at 5e4copies/reaction. To test any inhibition caused by either the polypropylene film or the Aluminum film used to heat seal the reaction insert in the cartridge, we flushed the heat sealed reaction inserts with TE buffer pH 9.2, and used that solution to reconstitute NG papers with unlabeled primers. We ran 3 paper replicates each for the aluminum and polypropylene films, with two positive and one negative replicate each. The positive controls contained 5e3copies/reaction NG DNA. We also tested heat sealed NG papers. The papers were heat sealed into the reaction insert, then the reaction pouch was cut open to remove the papers

again. These papers and control papers that were not heat sealed were reconstituted at 3e3copies/reaction NG DNA.

NG Labeled Liquid Assay Inside the cartridge

To demonstrate that amplification and detection works inside the cartridge, we used the NG liquid assay with labeled primers to test the cartridge designed by the engineering team. The engineering team made four cores cartridges. We prepared NG Liquid master-mix with labeled primers and divided it into three tubes. Two tubes contained 192μL of the master-mix and 8μL NG DNA at a concentration of 5e4copies/reactions. The third tube contained 192μL of master-mix and 8μL of nuclease free water for the NTC. All three master-mix tubes were handed to the engineering team and pumped into the cartridge by manual pressing. The engineering team cut holes into the cartridge body and attached tubing to inject the master-mix samples. The samples ran through the OmniLyse® device into the reaction insert, then followed by amplification at 63C for 60 minutes, and pumped into the lateral flow strip chamber.

Automated Process Execution inside of the Cartridge

Our engineering team developed an integrated cartridge plus associated instruments to automate the process execution described above for detection of CT (singleplex), NG (singleplex) and CT/NG (duplex). We prepared CT, NG, and CT/NG LAMP dry reagents papers as previously described. The respective reagent pads were then integrated into the reaction insert of the cartridge. The cartridge further incorporated an OmniLyse blender motor, 300 μL of 9.2pH elution buffer, and a LF strip.

We prepared human urine samples spiked with NG only, CT only, and CT plus NG at concentrations indicated in the results section. Of these samples, 80 µL was introduced into the cartridge. The sample in the cartridge was diluted with 240 µL TE buffer pH 9.0, lysed via the omniLyse blender motor, and amplified in the reaction insert in contact with a heater in the instrument. The amplified master-mix was then pumped into the lateral flow strip chamber in the cartridge for detection. Images of the developed lateral flow strip in the cartridge were taken using a Canon DC 8.1v camera under uniform lighting. The signal intensity of the test line was then analyzed using image processing software suite imageJ [44]. Briefly, each image was cropped to obtain consistent registration features and sizes. Two regions of interest were defined: one region at the respective test line to signify presence or absence of either CT or NG in the sample, and a second separate region just upstream of that test line to obtain the background intensity. Presence of a red test line relative to background was determined based on a decrease in the imageJ green channel intensity, (i.e. less green light was reflected at the test line due to absorbance by the gold nanoparticles on the test strip, which makes the line appear red). A common cut-off value was used to differentiate positive from negative results.

RESULTS AND DISCUSSION

CT/NG Front to back process Overview

Figure 6. CT and NG Full Process Overview, which starts with sample preparation in urine or swab samples followed by LAMP isothermal amplification and lateral flow detection.

The full process execution of CT and NG requires three main steps starting with sample preparation, isothermal amplification and later flow detection. The work presented herein is part of a team effort (Figure 6). However, the sample preparation work presented in this thesis was performed by me with the guidance from the former lab postdoctoral Sakamuri Rama Murthy. The device and engineering side of the project was designed by Hsiang Wei Lu and other members of our engineering team. I am focusing on isothermal amplification and coupling thereof with upstream sample preparation and downstream lateral flow detection. The work described herein constitutes my contributions to this effort unless otherwise noted.

CT DNA Quantification

We purchased genomic DNA of Chlamydia trachomatis grown inside mammalian host cells, which means that the CT DNA is present in a background of host DNA and therefore needs to be quantified. To quantify the amount of CT genomic DNA in this sample, we used the plasmid pFEN207 that contains the kdo gene, a single copy CT gene, as reference standard. We used the PicoGreen assay to quantify the plasmid relative to a Calf thymus DNA standard curve. The PicoGreen assay was used to quantify the plasmid due to its high sensitivity, specificity, and linear range. We obtained a suitable trend line with an R 2 value of 0.999, from which we calculated the final concentration of the plasmid standard to be 1.69E+07 copies/µL (Figure 7 A).

qPCR was used to quantify the CT DNA concentration in a background of host DNA relative to the plasmid standard (Figure 7 B). We calculated the DNA concentration via the standard curve by making a plot of Ct vs log concentration. The CT DNA is 1E+07 per µL (Figure 7 C).

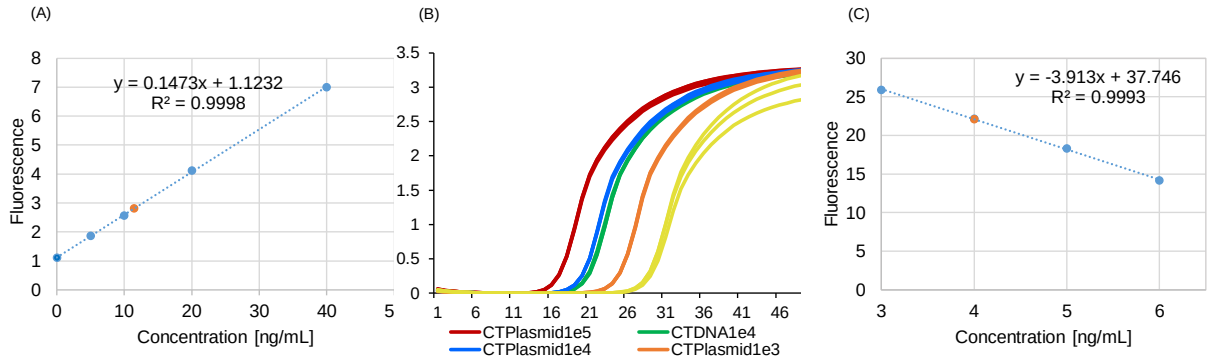


Figure 7. Quantification of Chlamydia Genomic DNA via qPCR. (A) PicoGreen assay standard curve obtained with calf thymus DNA relative to plasmid DNA. (B) The amplification of standard CT plasmid dilutions made by serial dilution of CT Plasmid and one sample of genomic DNA and plasmid DNA. (C) Standard curve obtained with quantified plasmid DNA relative to genomic DNA.

LAMP Process Optimization Goals

Key components of the master-mix include dNTP, Mg^{2+}, BSA, betaine, primers, and polymerase, and temperature (Figure 8). We did experiments to test the effect of each component on the assay.

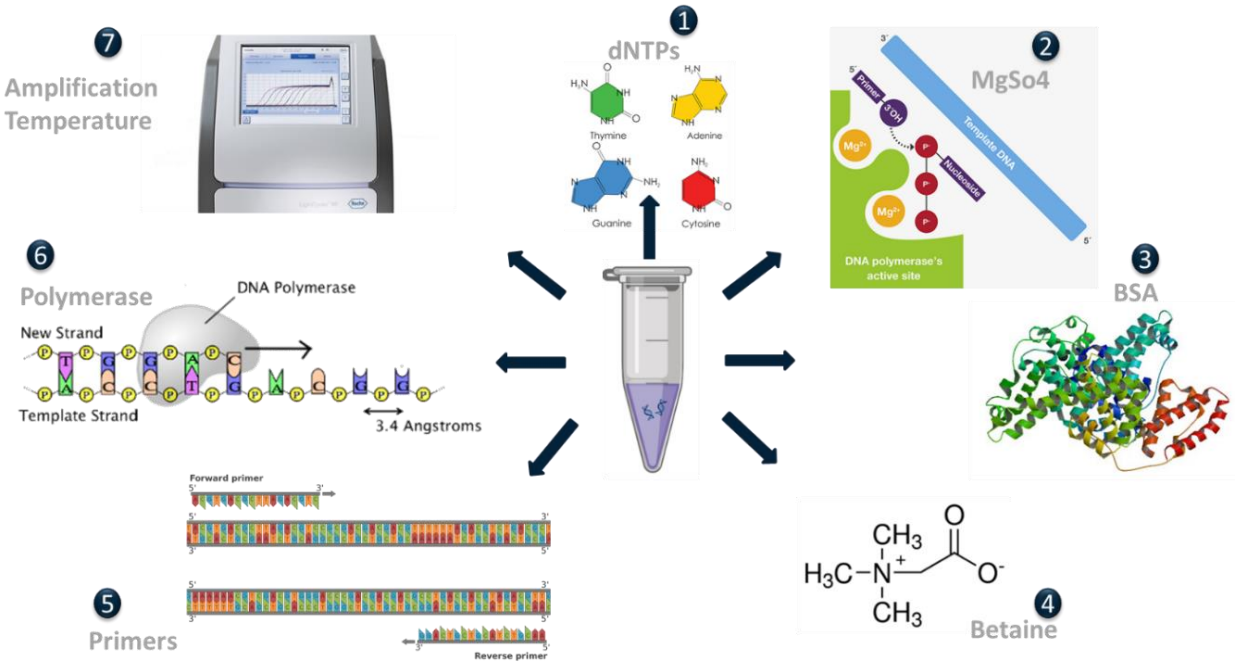


Figure 8. The Main Parameters for Assay Optimization. Parameters include: 1) deoxyribose nucleotide triphosphate **(**dNTPs). dNTPs comes with Extension (68c to 72c) of primers acting as building blocks to

the strand by DNA polymerase. 2) MgSo4: Mg2+ helps to coordinate interaction between the 3′-OH of a primer and the phosphate group of an incoming dNTP in DNA polymerization. 3) BSA increases PCR yields from low purity templates. 4) Betaine used to enhance amplification of GC rich sequences. 5) Primers are DNA strands designed to target conserved regions within the target DNA. 6) Polymerase enzyme makes a new strands of target DNA, using existing strands as templates. 7) Temperature at an isothermal condition (63 to 65°C) gives greater efficiency of DNA amplification.

Our goal is to get consistent amplification of the positives with no amplification of the NTC (Figure 9A). Sometimes we get late and inconsistent amplification (Figure 9B), or NTC amplification (Figure 9C), which is what we want to avoid via further optimizing.

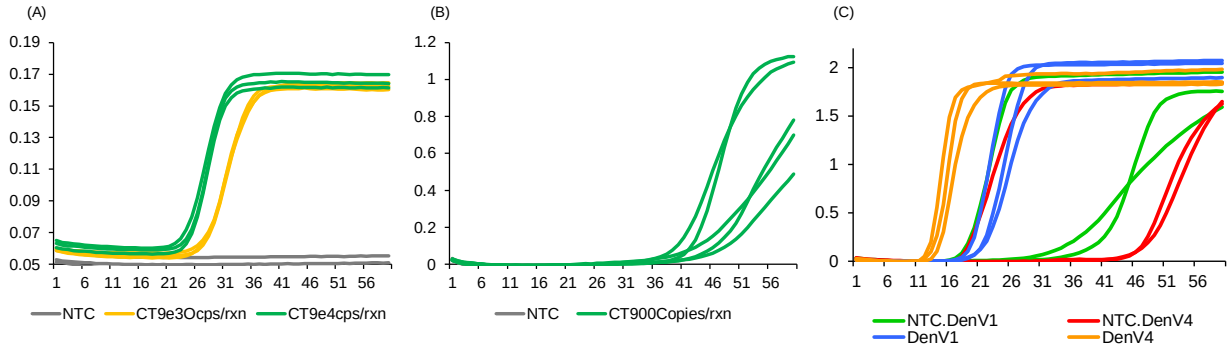


Figure 9. Examples of varying Amplification Time and Consistency of LAMP and RT-LAMP Assay. (A) Early and consistent amplification of CT DNA. (B) Late amplification of CT DNA. (C) Inconsistent and false positive amplification of DENV RNA.

We ran the liquid assay for CT and NG at different DNA concentrations to establish the current detection limit. CT can be detected around 2.5e3copies/reaction with early and consistent amplification (Figure 10 A). However, when we tested CT DNA at lower DNA concentrations (600copies/reaction) we failed at detecting our target. Likewise, the liquid NG LAMP assay can detect NG DNA as low as 500copies/reaction with early and consistent amplification (Figure 10 B). However, we are still working on optimizing the assay to lower the concentration of DNA. The goal is to be able to detect CT at 50 copies / reaction, and NG at 100 copies/reaction.

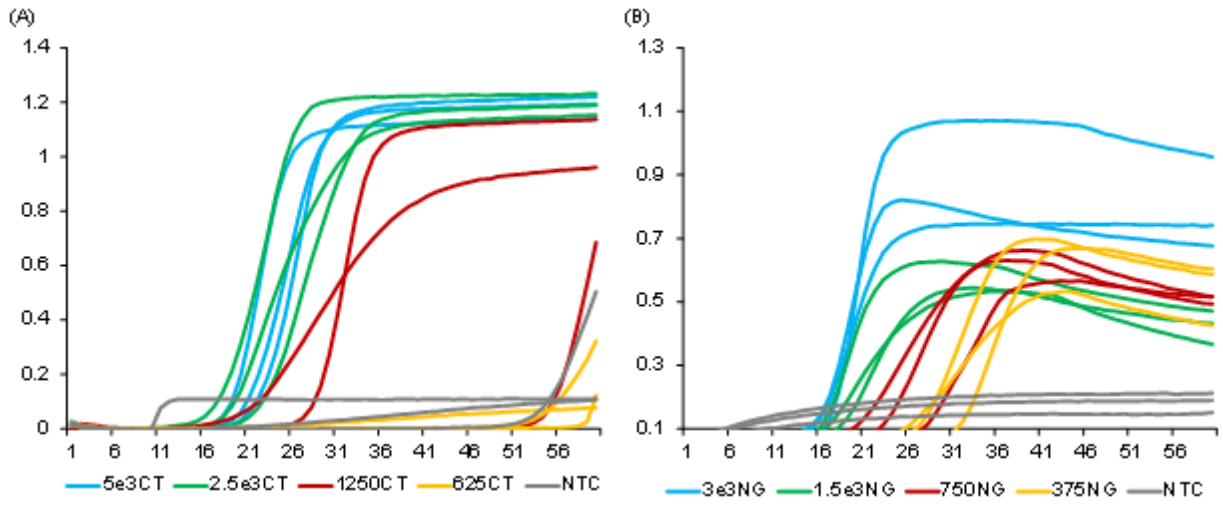


Figure 10. Amplification and Detection of Liquid LAMP Assay for CT and NG at Variant DNA Concentration. (A) Amplification of CT DNA at different DNA concentrations. (B) Amplification of NG DNA at different DNA concentrations.

NG LAMP Liquid Taguchi

The Genichi Taguchi method is a simple statistical method used in process optimization that uses an orthogonal array to test multiple variables and conditions for statistically significance to improving the process of interest.[45] We used the Taguchi technique to optimize the NG LAMP assay using combinations of different NG primer concentrations as described in the methods session. The goal is to detect NG DNA as early as possible with consistent amplification at a low DNA concertation. Figure 11A shows the times to onset of amplification (amplification times) for all 24 conditions labeled with the amplification time. Figure 11B shows that there is no NTC amplification observed in the negative replicates for each condition. The amplification times for the positives allow us to see which primer combinations amplified earlier than the control combination1. The best combinations of primer concentrations are highlighted with orange boxes. The final concentrations of each primer at each of the four combinations is shown in Table 5 in the Method section. We then re-tested these combinations via liquid NG LAMP.

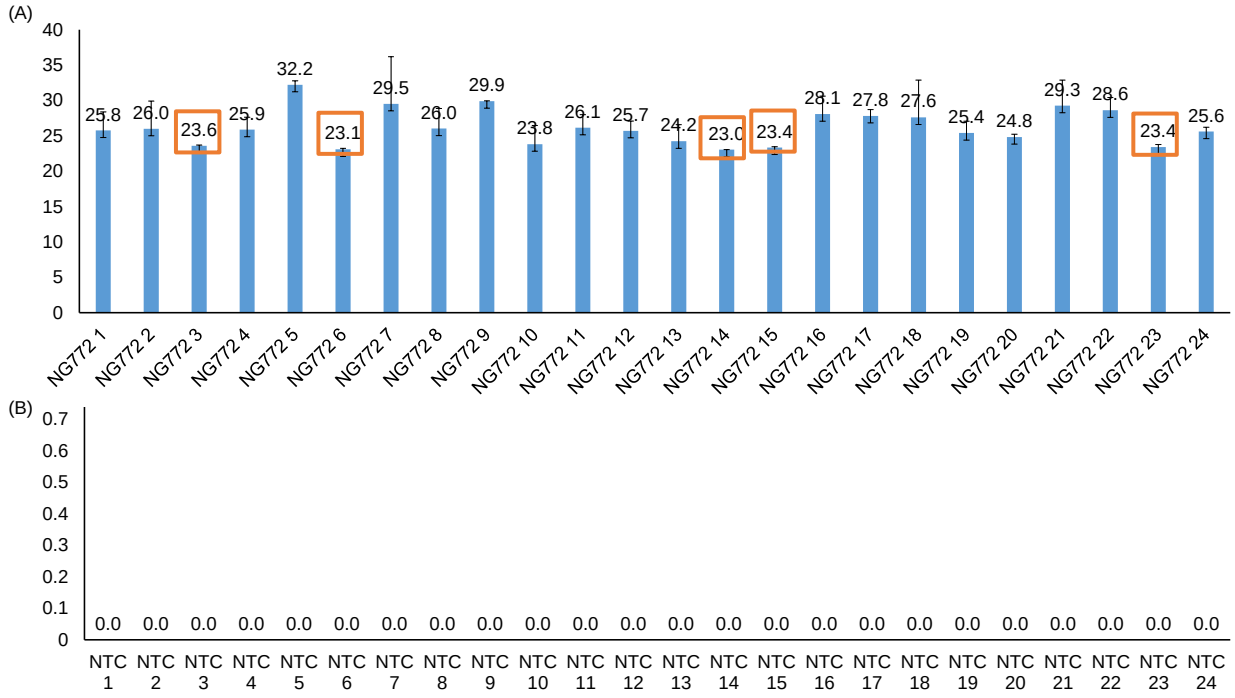


Figure 11. Taguchi NG LAMP Liquid Assay. A) The amplification times of the positive condition of NG LAMP Liquid Taguchi assay at 772Copies/reaction of NG DNA. B) Represent the negative control for each condition.

Figure 12 shows that the earliest and most consistence amplification was observed for combination 1 at an amplification time value of 25.3. For this condition none of the negative controls amplified. However, the results don't provide statistical significance compared to the original condition. We reran the Taguchi experiments multiple times and still showed no statistical significance and we ran the combinations multiple times and showed our original condition was the best combination.

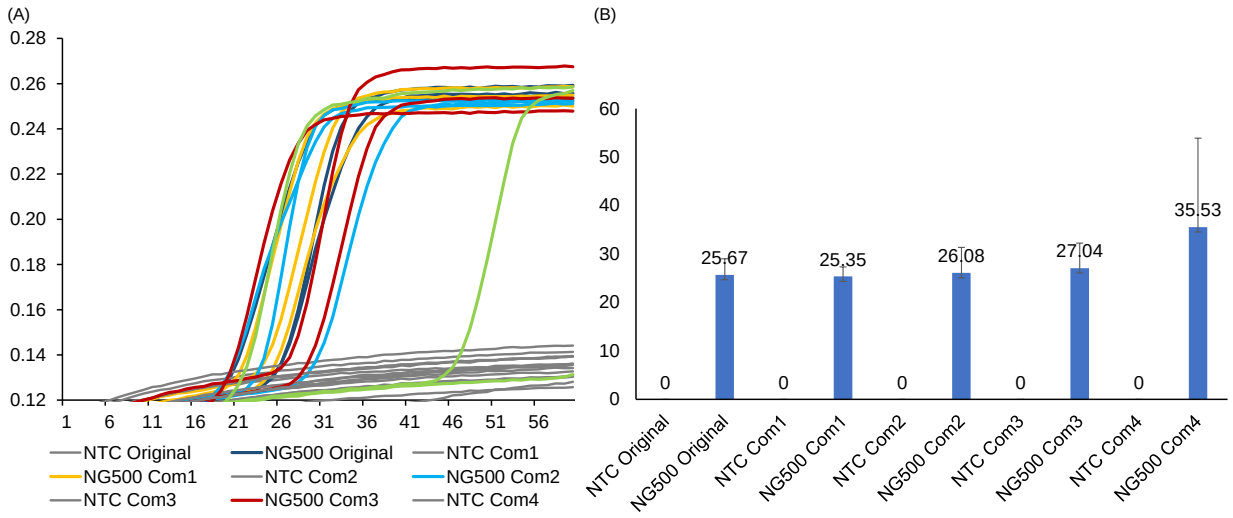


Figure 12. NG Liquid Assay to Test the best combination of primers concentration. A) Real time amplification curves of each replicate at 500Copies/reaction and NTC curves. B) The amplification time of each condition labeled with the amplification time.

Multi-parametric Optimization via Design of Experiments:

CT/NG full 3x3 Fractional Factorial Design

We ran multi-parametric optimization using an approach called design of experiments. In one experiment, we investigated the effect of three factors: amplification temperature, Mg2+ concentration, and DMSO as an additive. We ran a 2^3 full factorial experiments, with two levels for each factor, referred to as 0 for the low level and 1 for the high level, resulting in a total of 8 conditions (Table 6). Condition 5 represents our current parameters. The best detection shown at the earliest and most consistent amplification with our standard combination of parameters (Figure 13B and C). Which means there is no need to change the concentration of DMSO and Mg+ nor the temperature for future further optimization. However, we will be testing other parameters such as the type of polymerase to use and the primers sequences.

Table 17. The eight different conditions used for multi-parametric optimization.

Conditions	Temp	Mg^{2+}	DMSO
1	0	0	0
2	0	0	1
3	0	1	0
4	0	1	1
5	1	0	0
6	1	0	1
7	1	1	0
8	1	1	1

Levels: Temp 0=60C, 1=63C; Mg 0=2mM, 1=2.5mM; DMSO 0=0%, 1=5%

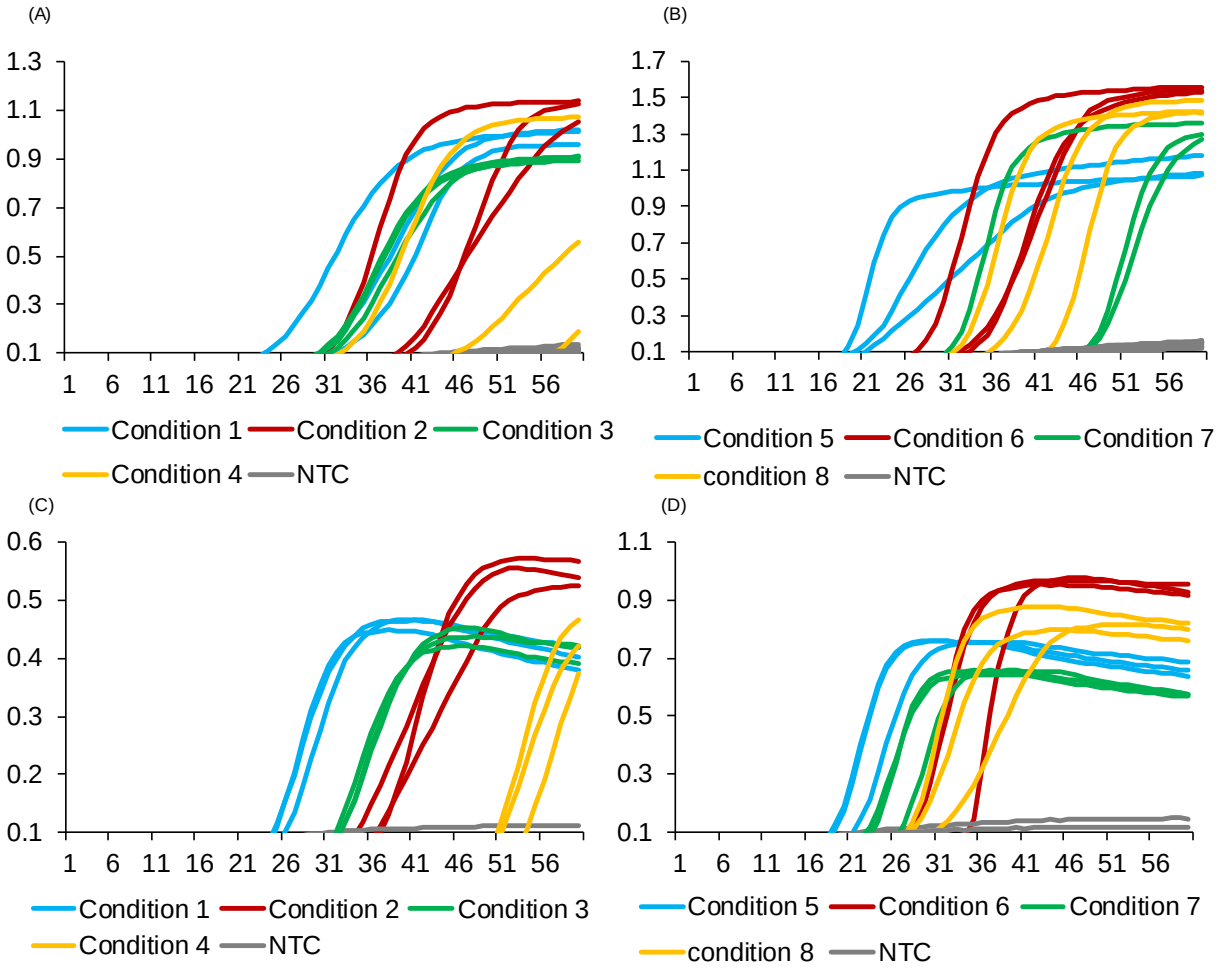


Figure 13. Multi-parametric Optimization via 2^3 full Factorial Design of Experiments. Approach for: LAMP (A) CT at 60°C. (B) CT at 63°C and (C) NG at 60°C (D) NG at 63°C.

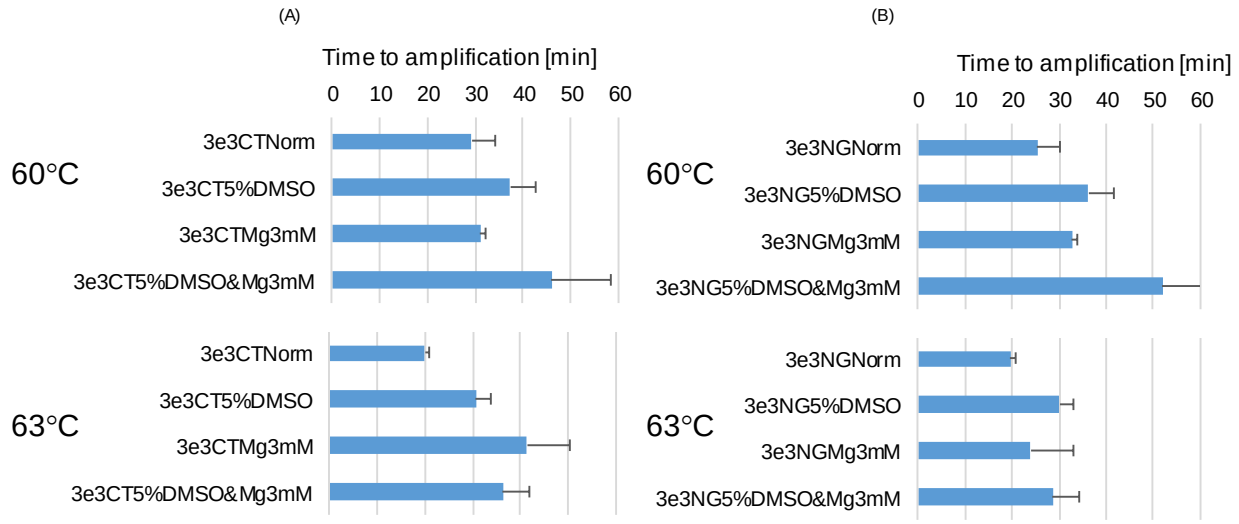


Figure 14. Onset time of amplification for multi-parametric optimization via Fractional Factorial Design of Experiments approach for (A) CT LAMP and (B) NG LAMP. Triplicate measurements for each condition.

Multi-parametric Design of Experiments:

Polymerase and Mg+ Concentration. CT

We tested the Mg+ and the polymerase in liquid LAMP assay at different concentrations separately and together. We learned from this experiment that adding an extra 0.2mM on polymerase did improve the amplification of CT DNA at 5e3copies/reaction, as shown in (Figure 15 A and B) below.

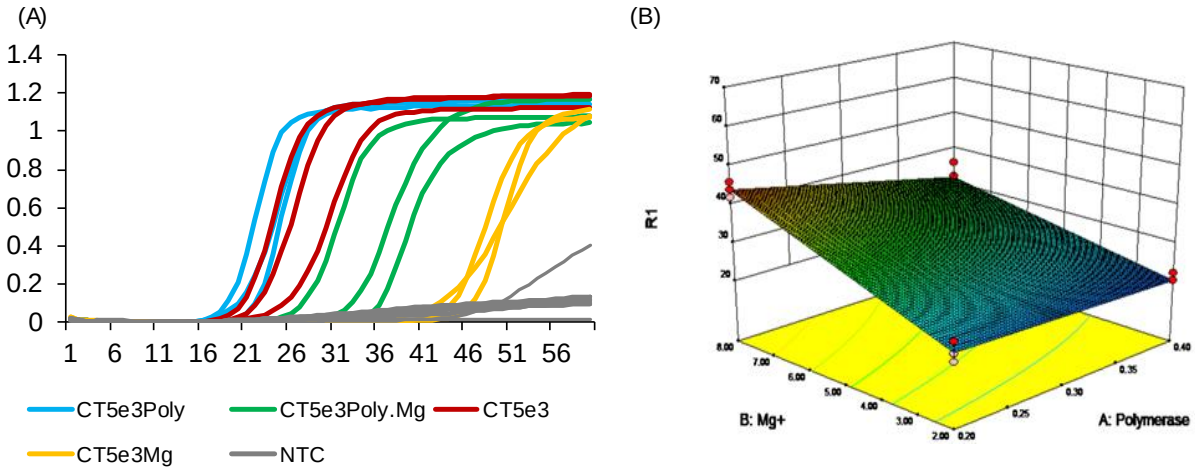


Figure 15. Multi-parametric Optimization of Chlamydia DNA via Isothermal Amplification Method. (A) CT DNA amplification at varying conditions. (B) Response surface graph of model with interaction via Design Expert graph.

Liquid LAMP Assay vs Dry Reagents LAMP Assay

We formulate our LAMP master-mix in dry reagent form to improve thermostability and to facilitate integration of the assay components into the cartridge. Dry reagents are obtained by lyophilizing the master-mix onto pre-treated cellulose paper pads.

As shown in (Figure 16A) the amplification of CT liquid assay at 5e3copies/reaction is consistence and early. However, (Figure 16B) shows the dry reagents amplification assay is slower and inconsistent at a DNA concentration of 9e3copies/reaction.

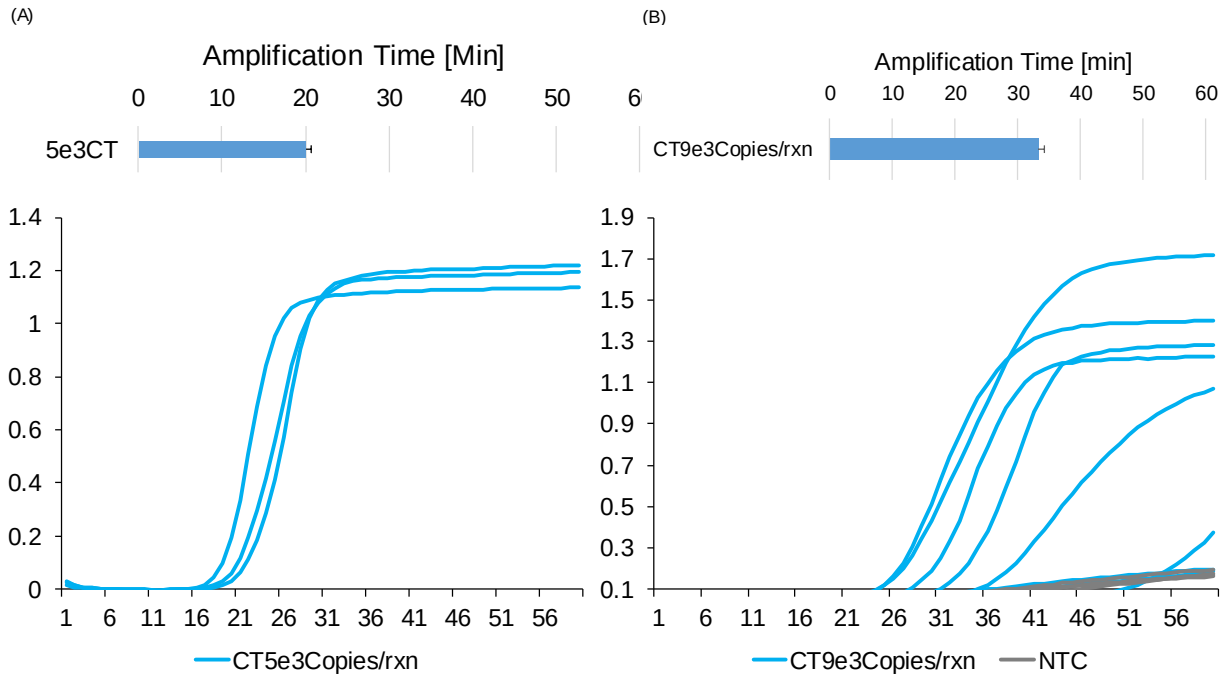


Figure 16. The Amplification and Detection of CT Liquid Assay vs Dry Reagents Assay. (A) Consistent and early amplification of CT DNA via liquid LAMP assay at 5e3copies/reaction. (B) Inconsistent and delayed Amplification of CT DNA via dry reagent paper assay at 9e3copies/reaction.

Likewise, as shown in (Figure 17A) the amplification of the NG liquid assay at 3e3copies/reaction is consistent and early. However, Figure (Figure 17B) shows that the dry reagents amplification assay is slower and inconsistent. Further optimization of the dry reagent assay is in progress.

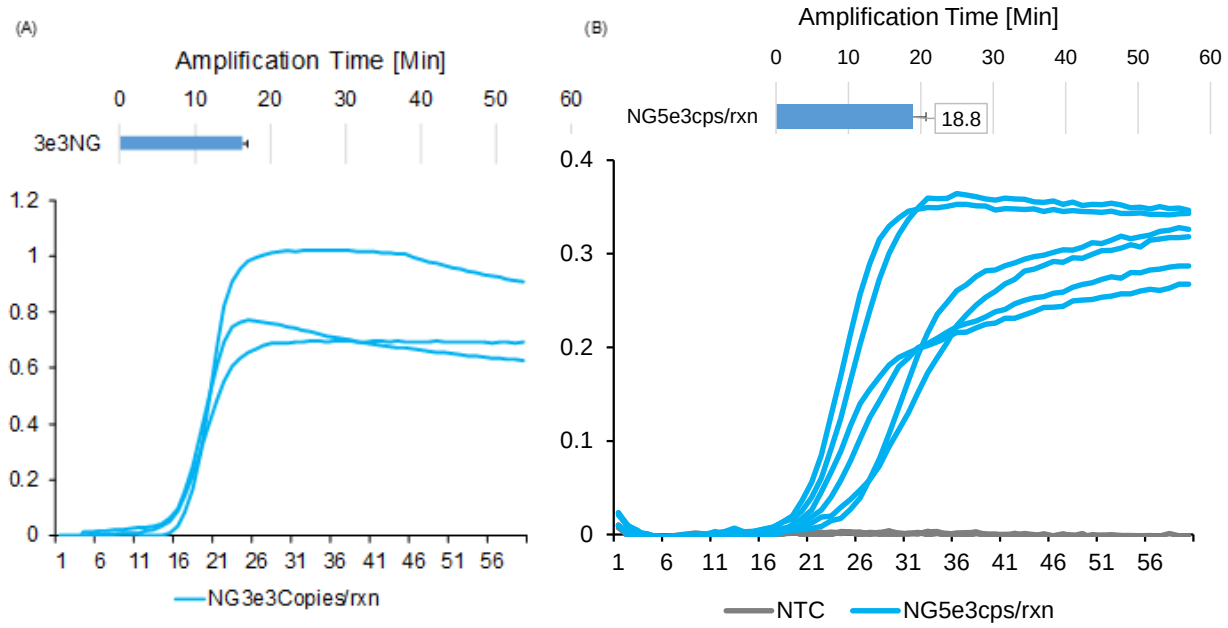


Figure 17. The Amplification and Detection of NG Liquid Assay vs Dry Reagents Assay. (A) Consistent and early amplification of NG DNA via liquid LAMP assay at 3e3copies/reaction (B) Inconsistent and delayed Amplification of NG DNA via dry reagent paper assay at 9e3copies/reaction.

Internal Amplification Control

Bacillus subtilis (Bsub) has been used as an internal amplification control (IAC). The IAC DNA is co-extracted and co-amplified to identify false negatives. For our technology to be approved by the FDA, we are required to have an internal control. We chose Bsub as IAC because it is a bacterial organism that can be sporulated, thus is very stable and hard to lyse, which makes it a stringent control for the mechanical lysis part of the process execution.

We tested the detection of our internal amplification control (IAC) Bsub using our liquid and dry reagent paper assay. As shown in (Figure 18A and B) below we successfully detected Bsub in the liquid assay format, and in the dry reagent paper format we had a few Bsub positive replicates that were negative (Figure 18B).

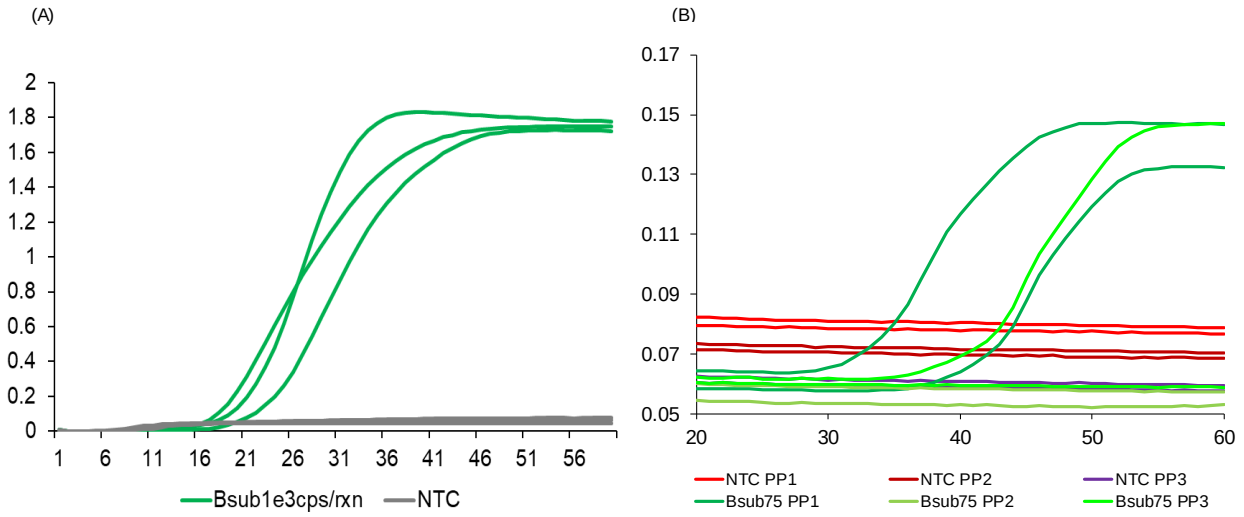


Figure 18. The Amplification and Detection of Bsub Liquid Assay and Dry Reagents Assay. (A) Consistent and early amplification of Bsub DNA via liquid LAMP assay at 1e3copies/reaction (B) Amplification of Bsub DNA via dry reagent paper assay.

To use Bsub as an internal amplification control for CT and NG detection, we need to detect Bsub in a robust manner at a suitably low concentration via LAMP using liquid master-mix and dry reagent paper master-mix. We tested the limit of detection of Bsub DNA via liquid LAMP as shown in Figure 19 A and B, and successfully detected Bsub DNA at a concentration of 1e3copies/reaction. The goal of adding Bsub to the assay is to identify any false negatives, meaning negative results even though NG and/or CT are present. Detecting Bsub at a low concentration is not as critical, since we can control how much to add to the sample. More important is designing the assay such that Bsub does not compete and suppress the amplification of CT and NG if they are present at low concentration.

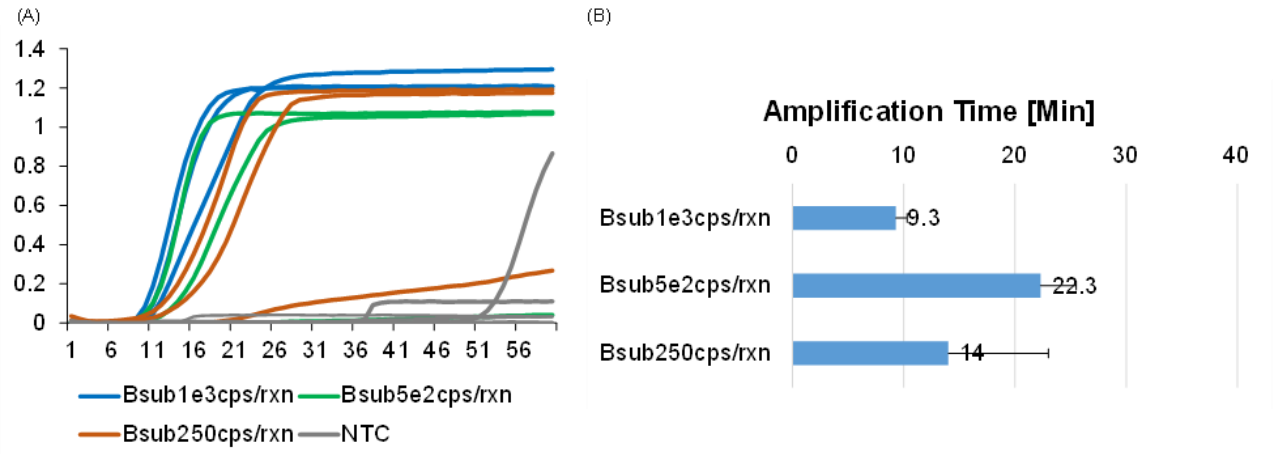


Figure 19. Bsub Limit of Detection Liquid Assay. A) Real-time amplification curves of each Bsub concentration for each replicate. B) Cq values for each Bsub concentration at a real time amplification value.

Lateral Flow Detection

We have coupled our LAMP assays with lateral flow detection to facilitate a simple visual read-out compatible with integration into a compact point of care device. This process involves primers labeled with Biotin/FAM or DIG binds to the Neutravidin on the microsphere. For example, the dig labeled primers binds to the antibody on the line of the LF strip. If there is no target present, those primers are separate from each other and there will be no line detected. (Figure 20A) However, if there is DNA present then an amplicon is created connecting Biotin and DIG or FAM and there will be a visible colored line on the LF strip (Figure 20B).

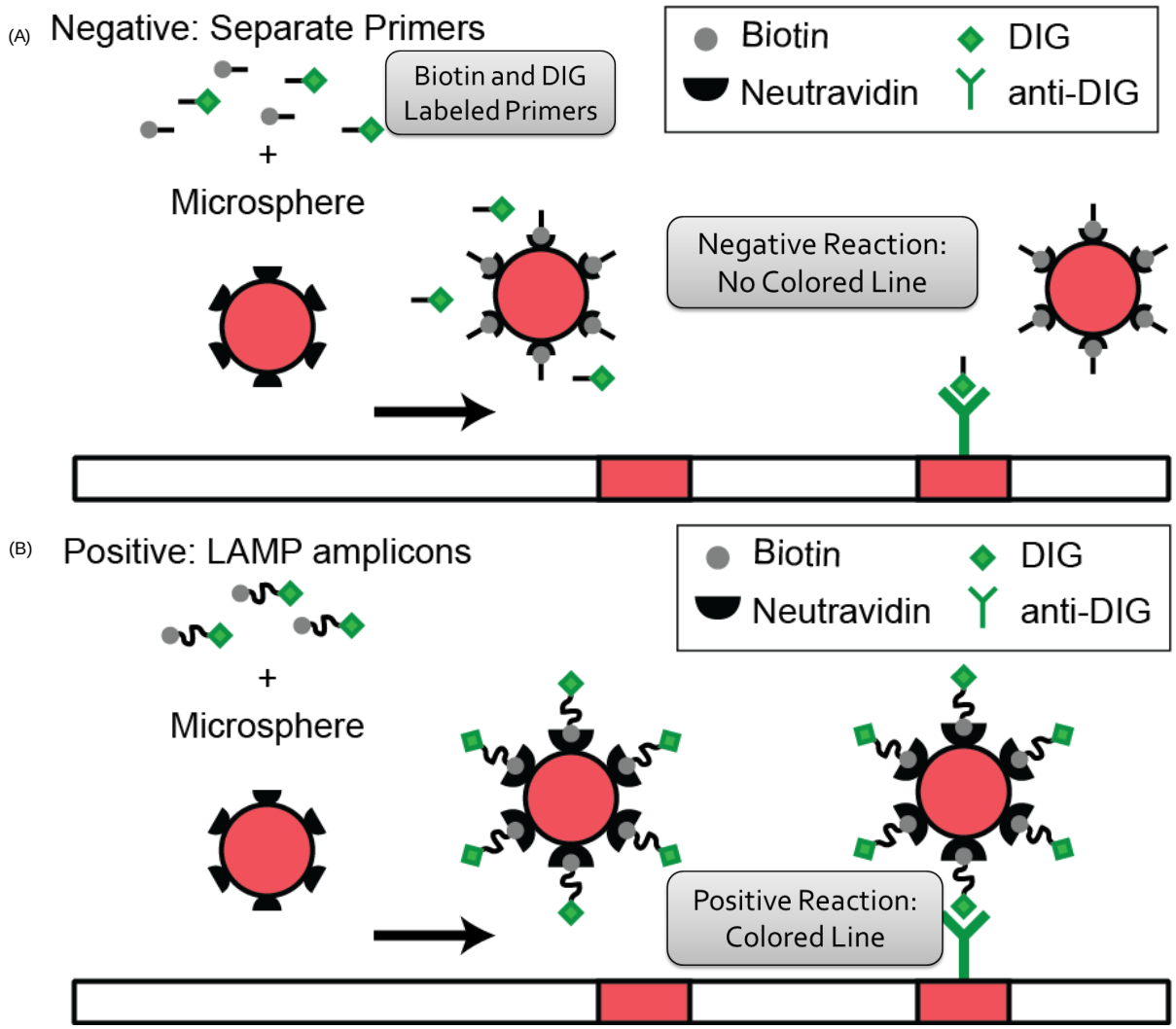


Figure 20. Lateral Flow Assay is designed to detect amplified DNA/RNA sequence which is labeled with biotin and a small molecule hapten. The lateral flow strip consists of a sample pad with buffers, a conjugate pad containing gold nanoparticles conjugated to steptavidin (GNPs), a nitrocellulose membrane containing capture antibodies specific to the hapten, and an absorbent pad. Target RNA labeled with biotin is dispensed onto the sample pad, which flows to the conjugate pad and binds to the Streptavidin on the GNPs. The target RNA – GNP complex flows down the strip and binds to the target capture antibody, while unbound GNPs bind to the positive control. The signal of the conjugate bound to the capture antibodies is proportional to the number of copies captured.

Multiplexing Assay

CT/NG Duplex Labeled Liquid and Paper Assay

We used multiplexing to detect CT and NG at the same time in one reaction. For this, we used a LAMP assay that combines all 12 primers for CT and NG detection, wherein the BIP primer

of NG is 5'-DIG labeled, and the BIP primer of CT is 5'- FAM labeled, to enable differentiation on the two line lateral flow strip. The duplex liquid assay of CT and NG was amplified containing 9e3copies/reaction for CT DNA and 5e3copies/reactions for NG DNA, separately and together. We collected the amplified product and introduced them into the lateral flow strips. Figure 21 shows lateral flow strips with two lines representing NG on the top part of the strip and CT on the lower part of the strip, respectively, one line for NG DNA only and one line for CT DNA only.

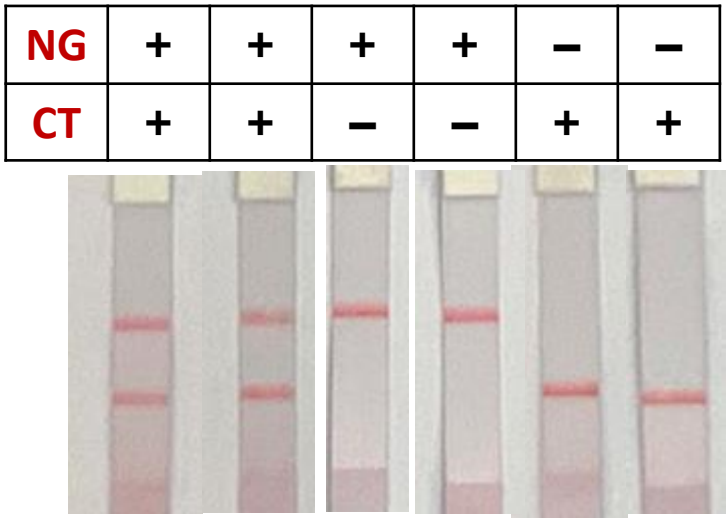


Figure 21. Duplex CT/NG Amplification and Detection via liquid LAMP combined with lateral flow detection, wherein the top line represents NG and the bottom line represents CT.

We then implemented the CT and NG duplex assay in dry reagent paper form with all 12 primers on a single paper. Figure 22 shows a duplex assay for CT at 9e3copies/reaction and NG at 5e3copies/reaction, separately and together. In some similar experiments we preformed, one line was either faint or nonexistent, giving us a false negative. CT and NG target DNAs tend to compete with one another, so if CT was the first target to amplify it will use up most of the amplification reagents, leaves NG with little to none. This problem requires further optimization to be able to detect both targets robustly with a strong later flow readout signal.

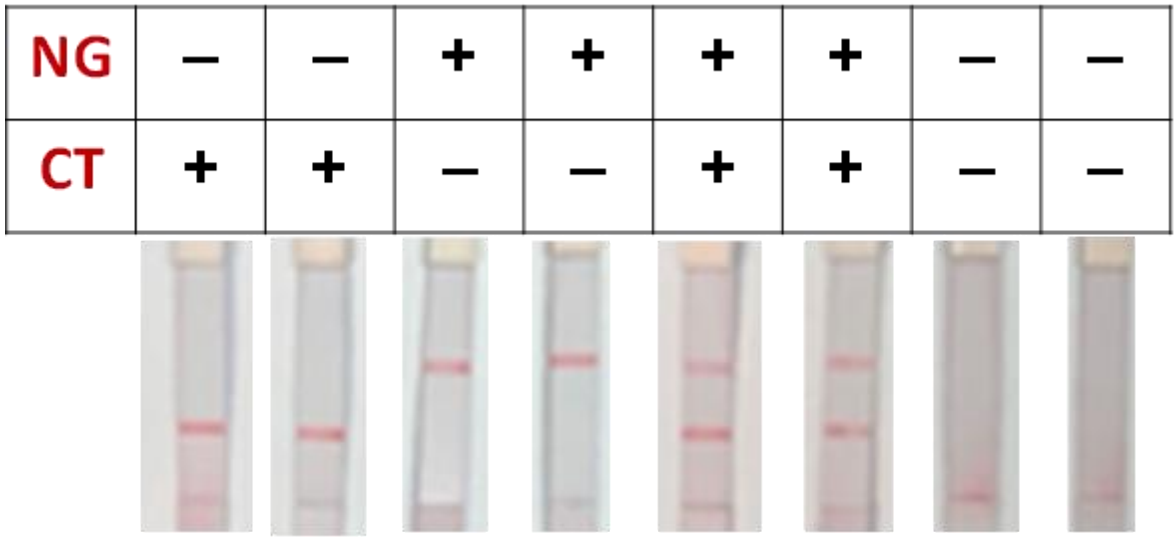


Figure 22. Duplex CT/NG Amplification and Detection via Paper LAMP combined with lateral flow detection, wherein the top line represents NG and the bottom line represents CT. The FAM tag is specific to amplicon products that attach to the anti-FAM test line.

CT/NG Full Process Execution

Mechanical Lysis vs Heat Lysis

We ran an experiment to compare mechanical lysis using the Claremont Bio OmniLyse bead blender versus heat lysis of CT and NG to liberate the DNA (Figure 23). The experiment was performed twice with two different concentrations and 30 replicates per concentration, and both times results were consistent. Mechanical lysis (blue curves) provided earlier amplification and thus superior performance compared to heat lysis (red curves). Furthermore, mechanical lysis can be readily implemented in a point of care device. Compared to heat lysis, mechanical lysis is faster. The device already contains one heater to amplify the reaction at 63°C, so adding a second heater to the device to reach 95ºC step would in principle be feasible but would complicate the cartridge design because proper insulation is required to separate the two temperature zones, since the high

temperature for lysis might interfere with master-mix stability and the amplification step. In addition, heating to 95°C requires more power than running the blender motor for mechanical lysis.

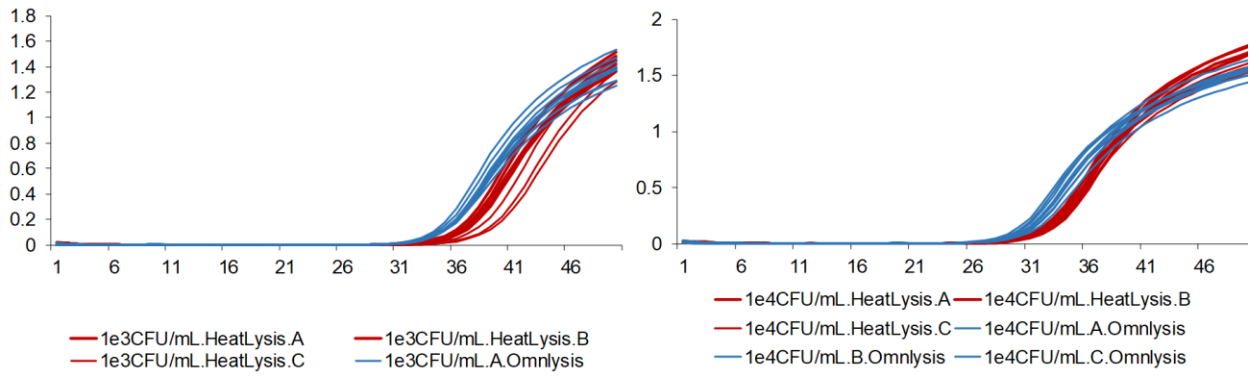


Figure 23. Heat Lysis vs Mechanical Lysis. Amplification of three replicates of each condition of NG DNA at two different concentrations 1e3CFU/mL and 1e4CFU/mL.

Front to back experiment for detecting NG in urine

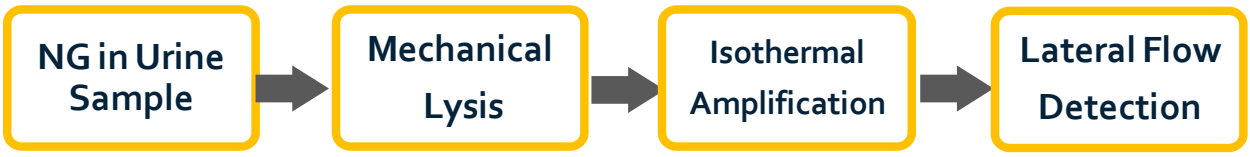


Figure 24. Full Process Execution of NG in Urine. Starts with sample preparation spiking NG in urine then mechanical lysis the sample followed by LAMP isothermal amplification and lateral flow detection.

We initially established the full process execution to detect NG in urine samples outside of the cartridge (Figure 24). Urine was spiked with NG at various concentrations. The sample was then mechanically lysed as discussed above, followed by isothermal amplification with dry NG LAMP reagent papers, and finally lateral flow detection. As shown in (Figure 25) we were able to detect NG at concentrations as low as 10 CFU/mL in urine.

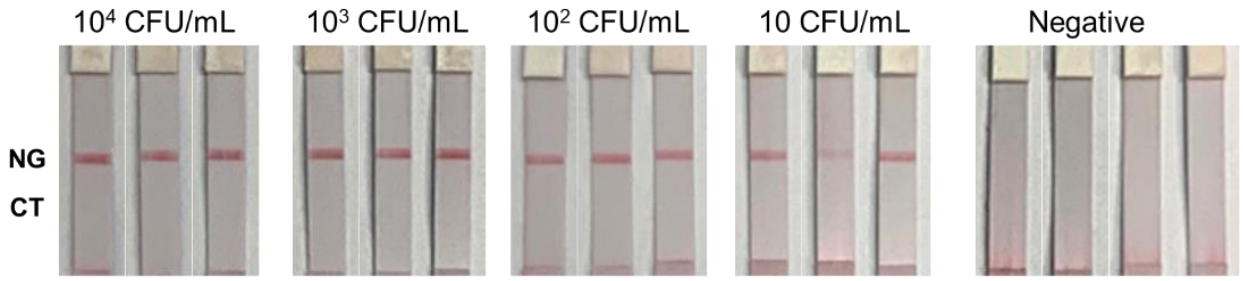


Figure 25. Full Process Execution of NG in Urine. Strong detection of NG in urine at different of concentrations.

It is important to note that one CFU NG does not equal one genome equivalent of NG. At Zeptomatrix, NG quantification was performed through serial dilution plating of NG grown in culture on chocolate II agar plates. NG has a tendency to clump, so one CFU may correspond to more than one bacterium. Furthermore, NG has limited viability in culture, so it is likely that dead bacteria are present in solution. A precise conversion factor for NG CFU/mL to NG genome equivalents/mL could not be determined.

Material compatibility: NG Inhibition Studies

In developing the cartridge, we needed to make design choices for materials. One important requirement was ensuring that none of the components in contact with the reaction fluid would be inhibiting LAMP. In this experiment we tested if the type of film used to seal the reaction insert is inhibiting the assay by flushing buffer pH 9.2 though reaction inserts sealed with a Polypropylene (PP) film versus aluminum laminated polyethylene (AL) film. Figure 26A and B show the amplification curves of reaction inserts sealed with PP and AL film, respectively, versus the control condition of NG dry reagent assay paper QC, each at 5e3copies/reaction NG DNA. Figure 26C shows the time to amplification for all conditions indicating no significant difference in amplification time for all three conditions. The results show that AL and PP films did not cause significant inhibition to the assay because the amplification time is around the amplification time of the positive NG control with no film introduced. The engineering team decided to use the PP

film when building the device because it is clear compared to aluminum that blocks what's under with its silver color.

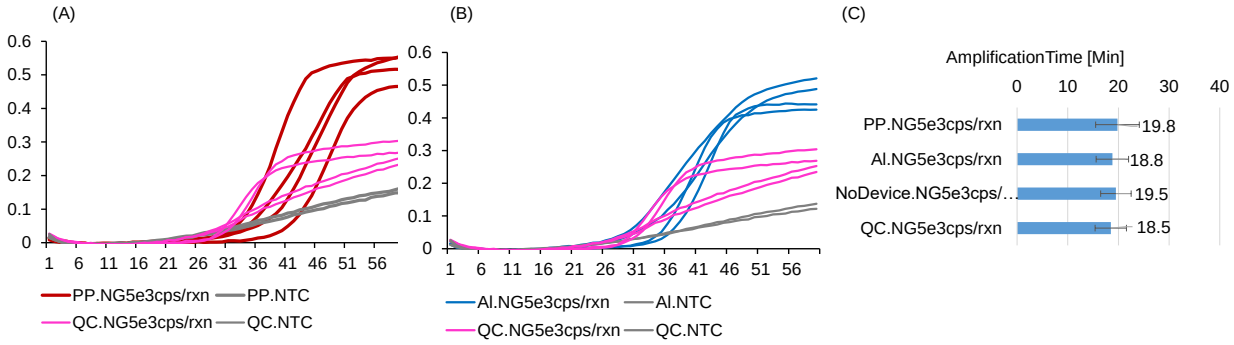


Figure 26. NG Liquid LAMP Assay Inhibition Testing of PP Film vs AL Film. A) Real time amplification curves of NG LAMP liquid assay with Polypropylene film and negative control replicates compared to standard NG liquid LAMP. B) Real time amplification of NG LAMP liquid assay with Aluminum film compared to standard NG liquid assay. C) Amplification time of PP film condition, AL film, no device and control assay amplification.

To determine if the inlet valve we use in the reaction insert is inhibiting LAMP, we ran NG liquid LAMP with buffer passed through six reaction inserts, three made with the inlet valve and three without. Figure 27A represents one sample amplification curves of Valve 3 compared to the control NG assay. Figure 27B shows the time to amplification for all conditions, with no significant difference observed between amplification times for buffer passed through reaction inserts made with and without valves, and the positive control.

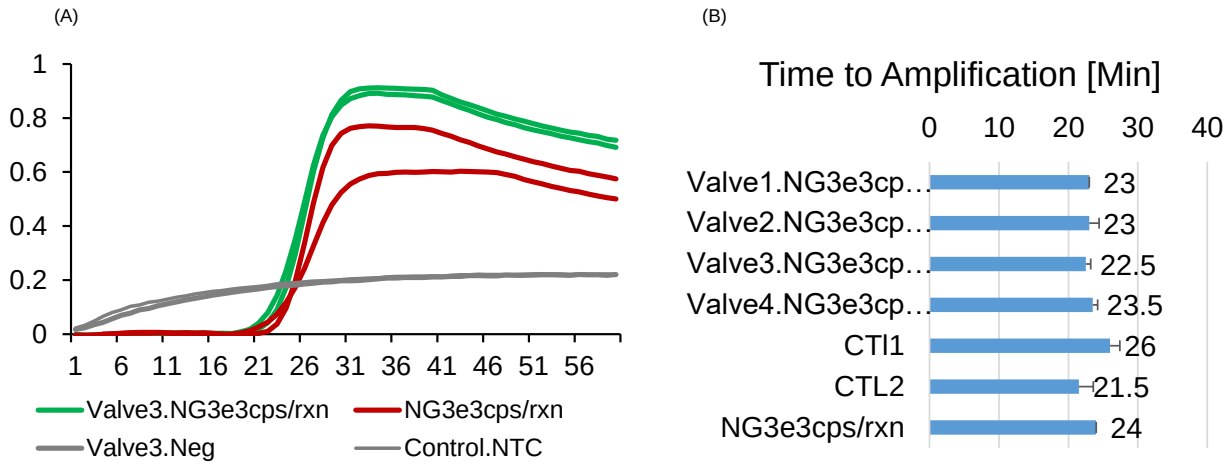


Figure 27. NG Liquid LAMP Assay Inhibition Testing of Inlet valves. A) Real time amplification curves of NG LAMP liquid assay with Valve 3 at 3e3copies/reaction NG DNA and negative control replicates compared to standard NG liquid LAMP assay at the same DNA concentration. B) Amplification time of all 4 reaction inserts with valves that been tested, the two reaction inserts without valves and the positive control of NG assay.

To test for inhibition caused by the reaction insert, we reconstituted NG dry reagent papers at 5e3copies/reaction NG DNA using TE buffer pH 9.2 that was injected into the reaction chamber of the insert and incubated there for one hour minimum. Figure 28A shows no significant difference between this condition and the control.

Also, we tested if heat sealing the master-mix papers in the reaction insert might be causing LAMP inhibition. We heat sealed NG dry reagents paper with unlabeled primers into the reaction insert, then removed the papers and reconstituted them with TE buffer containing 5e3copies/reaction NG DNA or negative control. Figure 28B shows one out of the six papers that were heat sealed. All papers show similar results indicating insignificant difference in amplification time.

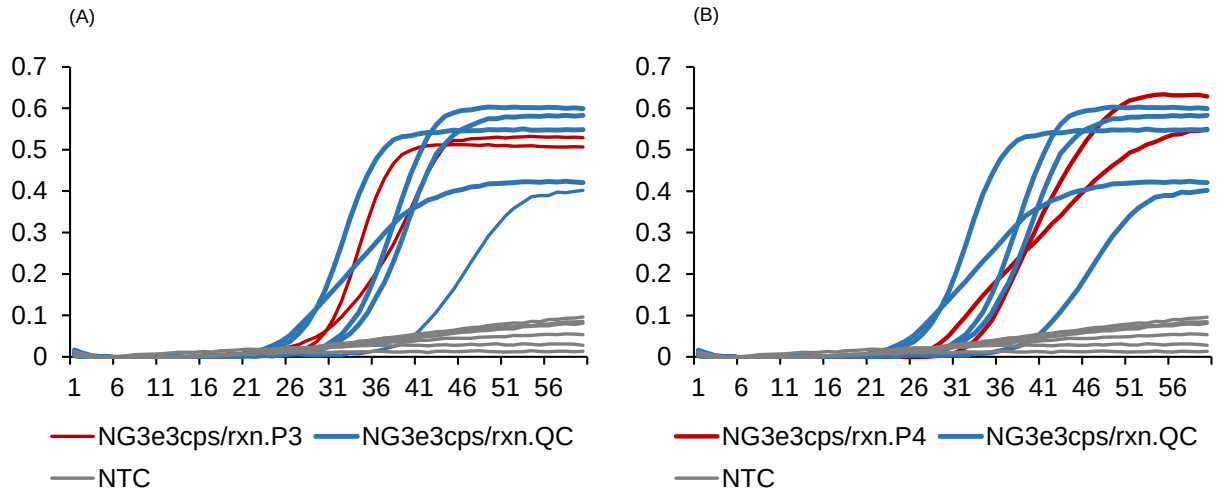


Figure 28. Reaction inserts inhibition tests. A) Real time amplification of NG LAMP dry reagents assay papers (Paper3:P3) heat sealed into the reaction insert compared to standard NG dry reagents assay with negative control. B) Real time amplification of NG LAMP dry reagents assay papers (Paper4:P4) heat sealed into the reaction insert compared to standard NG dry reagents assay with negative control.

NG labeled liquid master-mix for inside the cartridge

Our big goal is to execute the assay process in the cartridge from sample into lateral flow detection. We started with performing the liquid assay inside the cartridge to demonstrate that amplification and detection works with liquid master-mix. Figure 29A shows real time amplification of NG DNA positive control run outside the cartridge, plus the lateral flow strip images of the control positive at 5e4copies/reaction. Figure 29B shows an actual image of the same master-mix amplified and detected in the cartridge. We successfully detected the NG DNA sample using the liquid assay method, the next step is to detect the NG spiked urine samples using the dry reagents paper assay.

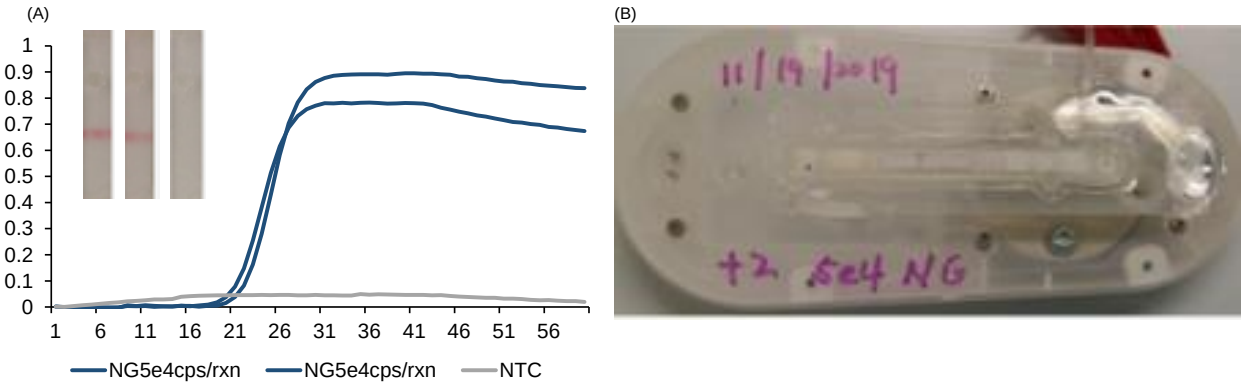


Figure 29. Testing of NG LAMP inside the cartridge with labeled liquid master-mix. A) Real time amplification curves of NG LAMP liquid assay at 5e4copies/reaction and negative control replicates. B) A picture of the cartridge top with casing removed and without the sample chamber cover lid. The cartridge showing the positive NG sample at 5e4copies/reaction.

Automated Process Execution inside of the Cartridge

To be able to diagnose infectious diseases at the point of care and in low resource settings, our engineering team developed a prototype system that consists of an integrated cartridge, shown in Figure 30, approximately the size of a mobile phone, in conjunction with a compact instrument. This system can execute sample preparation, isothermal DNA amplification and lateral flow-based detection in a fully automated manner.

Figure 30. A picture of our prototype device including the instrument and cartridge.

The engineering team fabricated eighteen identical cartridges to detect NG spiked into urine at various concentrations. Figure 31 shows example cartridges from this experiment with the top casing removed.

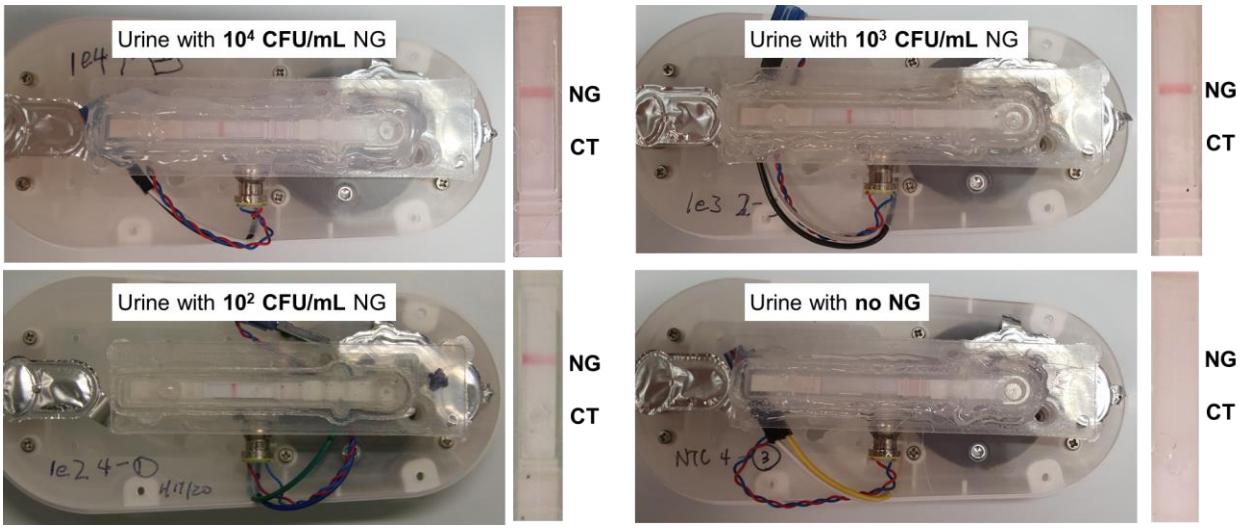


Figure 31. Automated Process Execution inside the Cartridge of NG spiked urine samples at 1e4, 1e3, 1e2 CFU/mL and negative eluates. Pictures of selected cartridges with the top casing removed, showing the LF strip signal line in positive samples and no line in the negative sample.

Cropped images of the lateral flow strips inside each cartridge are shown in Figure 32. The figure confirms the detection of NG spiked into urine at different concentrations. However, some of the lateral flow strips show weaker signal compared to others in the same concentration.

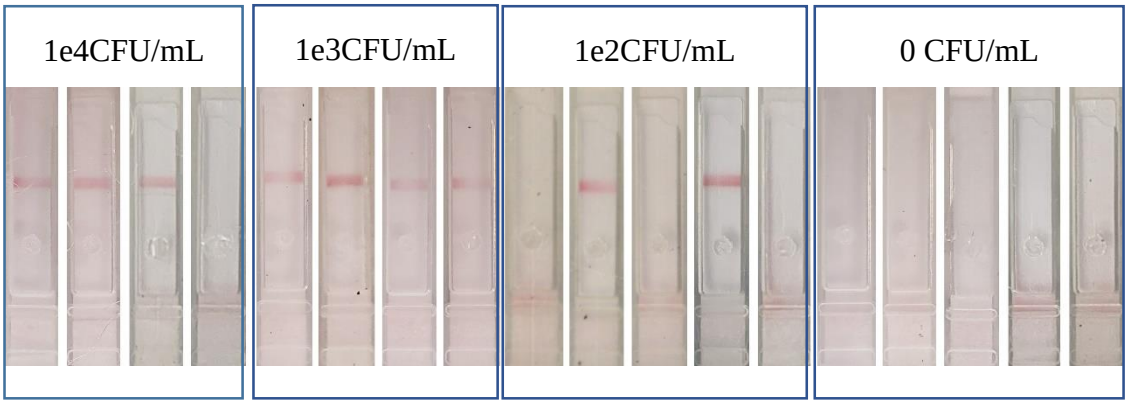


Figure 32. Cropped images of the lateral flow strips inside each of the cartridge used for automated detection of NG in urine at various concentrations.

Table 18. Detection of NG cells in urine at various concentrations plus negative sample, with number of positive results per total number of cartridges run at each concentration.

NG CFU/mL urine	Positive / Total
10,000	3/4
1,000	4/4
100	2/5
0	0/5

We were able to detect 3 out of 4 samples at 1e4CFU/mL, 4 our 4 NG samples at 1e3CFU/mL, and 2 out 5 samples at 1e2CFU/mL as shown in table 7. The negative samples show no lateral flow signal meaning no detection. The cartridge was more successful at detecting NG samples at higher concentrations. We will need to optimize the assay or the cartridge to allow detection at lower concentrations. However, this was the first experiment of full process executed inside the cartridge.

CT Automated Process Execution inside of the Cartridge

A second set of experiment was performed to detect CT elementary bodies spiked in urine at various concentrations inside the cartridge and automatically performed by the instrument. Figure 33 shows example cartridges from this experiment with the cartridge top casing removed. Cropped images of the lateral flow strips inside each of the cartridges run in this experiment are shown in Figure 34.

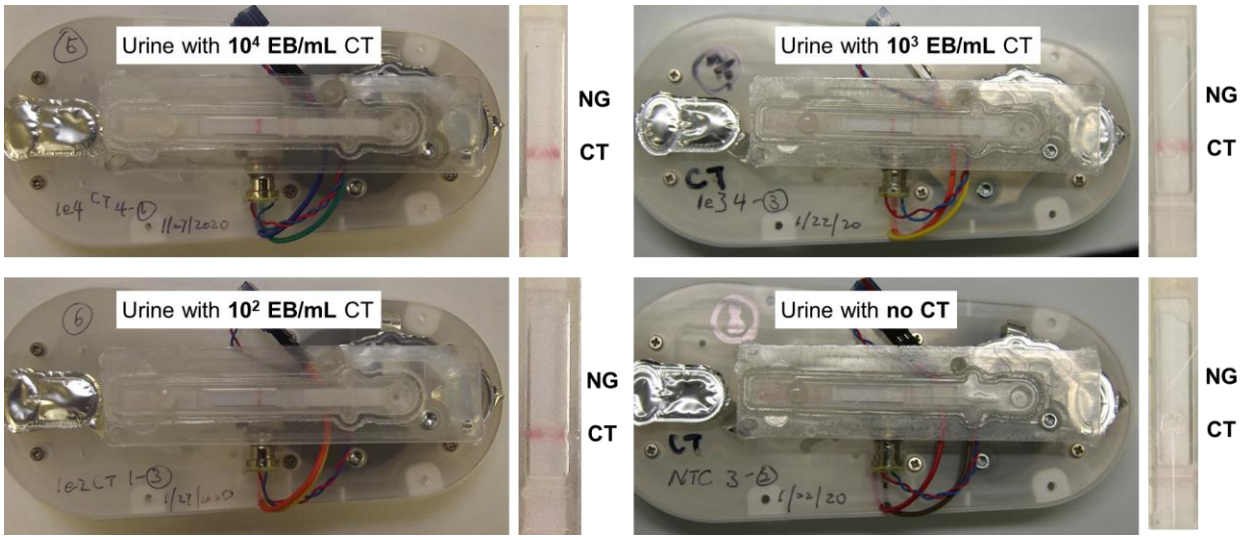


Figure 33. Automated Process Execution inside the Cartridge of CT spiked urine samples at 1e4, 1e3, 1e2EB/mL and negative eluates. Pictures of the cartridge top with casing removed showing the LF strip signal line in positive samples and no line in the negative sample.

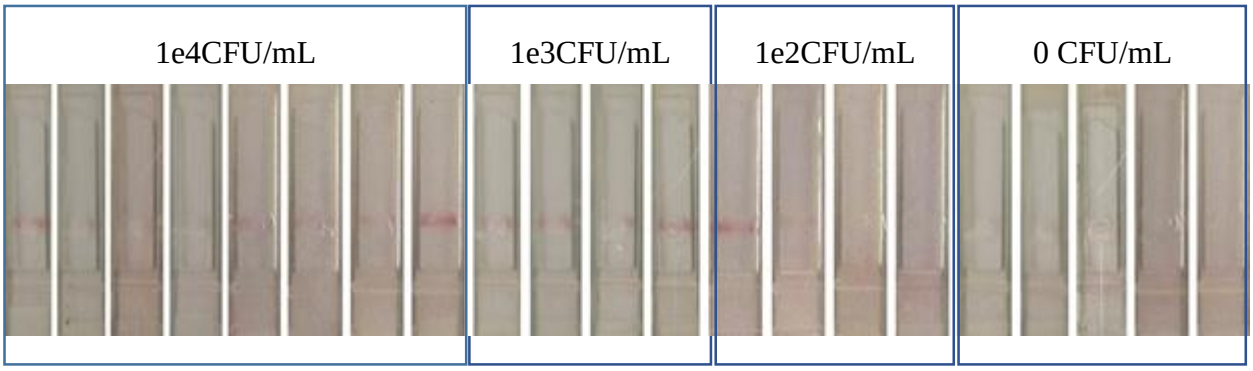


Figure 34. Images of lateral flow strips in the cartridge of the NG automated process execution inside of the Cartridge at various concentrations.

Table 19. The detection of CT elementary bodies in urine at various concentrations, with number of positive results per total number of cartridges run at each concentration.

CT EB/mL urine	Positive / Total
10,000	6/8
1,000	4/4
100	2/4
0	0/5

In Figure 35 it can be seen that the CT lateral flow strip line is obstructed by a white dot, a plastic protrusion caused by a design flaw of the lateral flow strip chamber injection molding. The sprue of the mold is where the liquid materials pass through to solidify to the form part. In this case the sprue of the lateral flow strip chamber mold was located exactly where the CT lateral flow strip line is located, which it is causing the white dot on top of the CT line lateral flow strip readout.

Even at high CT EB concentrations, the lateral flow strips readout shows very weak to no lines. Whether a signal is considered positive is subject to interpretation. As shown in Table 8, if weak signals are considered positive, then we were able to detect 6 out 8 samples using our integrated cartridge and instrument system at 1e4EB/mL, 4 out of 4 samples at 1e3EB/mL, 2 out of 4 CT samples at 1e2EB/mL, and the negative controls showed no detection. Overall, the CT results are not as promising as the NG results. The next step is optimizing the cartridge by moving the sprue of the lateral flow strip chamber to a different location. Then we need to further optimize the assay outside the cartridge to be able to robustly detect CT EB using dry reagents papers at a lower concentration, by itself and as part of the front to back experiment. Then finally we can move to implementing an optimizing the process inside the cartridge.

CT/NG Duplex Automated Process Execution inside of the Cartridge

We ran a third set of experiment with fully automated process inside the cartridge using duplex dry reagent papers with all primers for detection of CT and NG. We tested urine spiked with just CT, just NG, and both CT and NG together. Table 8 shows the concentrations that were run for each target pathogen by itself and in combination, along with the results.

Figure 35 shows images of some of the cartridges that were tested. The results show a clear readout lateral flow strips lines for the duplex and singleplex fully automated system. However, not all cartridges were successful at detecting the duplex assay.

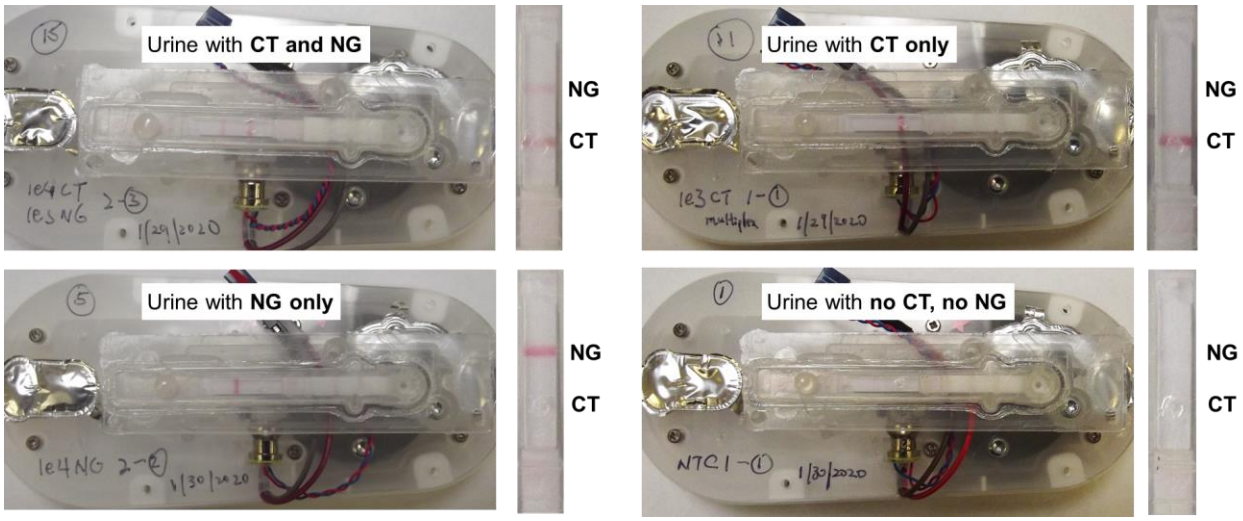


Figure 35. Automated Process Execution inside the Cartridge for the duplex assay to detect CT and NG-spiked urine samples at NG 1e4CFU/mL with CT 1e4EB/mL, NG 1e3CFU/mL with CT 1e3EB/mL, NG 1e3CFU/mL with CT 1e4EB/mL, NG 2e3CFU/mL with CT 1e4EB/mL and negative eluates. Pictures of the cartridge top with casing removed showing the LF strip signal line in positive samples and no line in the negative sample.

Table 20. CT/NG Duplex Automated Process Execution inside of the Cartridge at various concentrations.

NG Concentration (CFU/mL)	CT Concentration (EB/mL)	NG Positive	CT Positive
1.00E+03	1.00E+03	3/3	0/3
1.00E+04	1.00E+04	3/3	0/3
1.00E+03	1.00E+04	2/2	2/2
2.00E+03	1.00E+04	4/5	2/5
NTC	NTC	1/4	0/4

We ran the duplex assay for CT and NG at varying concentrations. The NG assay performs more robustly and tends to out-compete the CT assay. Therefore, when both are present at the same concentration (e.g. both 1e4 and both 1e3), then only NG amplifies as seen in the data shown in (Figure 36). However, when CT is present at a higher concentration than NG (CT 1e4 and NG 1e3) then both amplified (Figure 36). The NG control showed positive amplification on the NG

line in both replicates, while only one replicate of the CT produced a positive lateral flow strip readout. One of the negative controls showed amplification on the NG line.

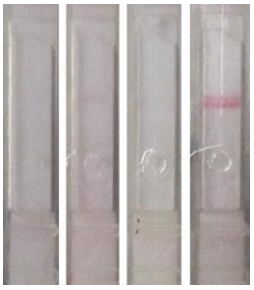
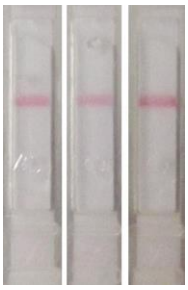
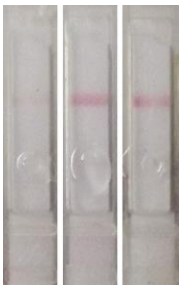
NG	1e3 CFU/mL	1e4 CFU/mL	0 CFU/mL	0 CFU/mL	1e3 CFU/mL	1e4 CFU/mL
CT	1e4 EB/mL	0 EB/mL	1e3 EB/mL	0 EB/mL	1e3 EB/mL	1e4 EB/mL
						

Figure 36. Images of lateral flow strips in the cartridge of the duplex NG and CT automated process execution inside the Cartridge at various concentrations.

CONCLUSIONS:

The goal of the project is to develop a rapid, accurate and affordable point of care diagnostic device for detecting CT and NG in urine and swab samples. For this, we need to develop a process on the bench consisting of sample preparation, amplification, and lateral flow (LF) detection that can be implemented and automated in a cartridge in conjunction with a robust and portable instrument.

For CT/NG I worked on amplification and detection, and on full process execution. For amplification and detection, we established singleplex assays for CT, NG, Bsub using dry a reagent master-mix, coupled to LF detection. We demonstrated execution of CT/NG duplex and CT/NG/Bsub triplex assays using two-line LF strips. The next steps are executing the triplex assay with three-line lateral flow strip detection, as well as further optimization of the LOD attainable with the dry reagent master-mix, along with master-mix thermo-stability. We will further validate the assay's inclusivity, i.e. ability to detect all relevant CT serovars and NG reference strains, and

exclusivity, i.e. lack of cross-reactivity with other pathogens. For full process execution, we accomplished the detection of NG in urine down to 10 CFU/mL through front to back process execution, as well as proof of principle for the front to back assay process using contrived swab samples. We have established amplification in the current prototype cartridge and instrument, then refined the prototype and demonstrate fully automated process execution in the cartridge controlled by the instrument. We were able to detect CT and NG via full process execution inside the cartridge, although the process for CT still requires further refinement as the detection signals were very weak. We will optimize the detection of CT via full process execution outside and inside the cartridge to detect CT EBs with more consistency. We also had some success in duplex detection of CT and NG in the same reaction, although one target often out-competes the other. We have worked towards a solution to this problem that involves geometric multiplexing, which entails locating the primers in different regions of the reaction chamber. Once we have suitably optimized the assay process inside the cartridge and instrument, we will test a set of clinical samples to determine the clinical sensitivity and specificity.

DENGUE VIRUS

AMPLIFICATION AND DETECTION

DENGUE ASSAY MATERIALS AND METHODS

Reagents

We purchased MS2 Bacteriophage RNA (ATCC® 15597-B1™) from ATCC (Manassas, VA, USA). MS2 RNA was purchased from Sigma Aldrich (St. Louis, MO, USA). DENV1-4 RNA was obtained from virus reference strains individually propagated in Vero cells from African green monkey kidney. Bacillus subtilis (Bsub) DNA. Both MS2 and DENV primers were purchased from Integrated DNA Technologies (IDT). Tris and other general reagents were purchased from (Sigma Aldrich, St. Louis, MO, USA). 5M Betaine solution, bovine serum albumin, Deoxynucleotide Set 100 mM (DNTP100A-1KT), Magnesium sulfate solution (MgSo4) and trehalose are obtained from Sigma Aldrich (St. Louis, MO, USA). GspSSD 2.0 large fragment DNA polymerase with reverse transcriptase activity was obtained from OptiGene Ltd (Horsham, UK). SYBR Green was purchased from Life technologies (Carlsbad, CA, USA). All the reagent solutions were prepared using nuclease free water prepared from Promega (Madison, WI, USA). CF1 100% cotton linter sample pads (176 µm thickness, 22 mm width, and 50 m length) were purchased from GE Lifesciences (Marlborough, MA, USA). Reagent alcohol 95%, Fetal Bovine serum, heat inactivated (sigma), PowerUp Sybr Master Mix (life technologies Corporation), Pierce BCA Protein Assay UE, PBS, RNASEOUT Recomb, Yeast tRNA 500 UL (life technologies Corporation).

Samples and Genomic RNA:

At the Naval Medical Research Center (NMRC), DENV1-4 virus reference strains were individually propagated in Vero cells from African green monkey kidney, and viral concentrations in PFU/mL in the cell culture supernatant were determined via plaque assays. To obtain genomic

DENV RNA to develop and optimize our DENV RT-LAMP assay, NMRC sent us nucleic acids extracted from the cell culture supernatant of DENV grown inside mammalian host cells; therefore, the DENV RNA is mixed with mammalian host DNA/RNA.

Escherichia coli bacteriophage MS2 was obtained from ATCC® (15597B1) in pre-quantified dry form and was rehydrated according to the manufacturer's instructions. Quantified MS2 genomic RNA was obtained from Sigma Aldrich.

G-blocks Template for In Vitro Transcription to obtain DENV RNA standards

To create a template for in vitro transcription, we used a synthetic G-block DNA sequence representing the 3' UTR region of DENV1/3, DENV2, DENV4 (Table 4). DENV g-blocks were cloned into a TOPO plasmid (Twist Bioscience, San Francisco). Then we preformed E. coli transformation to introduce the DenV plasmid DNA into bacterial cells to allow us to replicate the plasmid of interest in the bacteria. First we aliquoted the E. coli DH5 alpha Chemical Competent cells (ThermoFisher) once they were delivered but before usage, to avoid multiple freezing and thawing cycles that would reduce competent cell viability; then they were stored in -80°C. We thawed the required amount of cell by placing them on ice for 30 minutes. Then we introduced the plasmid by adding 1µL of plasmid to the melted cells on ice and mixed them by flicking with a finger a couple of times. Samples were again left on ice for 30 minutes. Then samples were placed in a heat shock bath at 42°C for 45 seconds. We placed the samples back on ice for 2 minutes. The change of temperature increases the permeability of the bacterial cell membrane and allows the plasmids to enter the cells. We added 1mL of SOC medium (Sigma-Aldrich) and closed tight. The S.O.C. Medium is used in the final step of bacterial cell transformation to obtain maximal transformation efficiency of E. coli. S.O.C. Samples were placed horizontally on a shaker at 37°C

for an hour. Samples were vortexed and we pipetted 50µL of each sample to plate them. LB agar plates must contain an antibiotic to which the plasmid of interest will provide resistance. In our case the plasmids are ampicillin resistance, therefore we platted 1 plate of LB agar with 100 µg/ml of ampicillin (Sigma-Aldrich) per strain, a total of 4 plates including the negative control. We labelled the plates and incubated overnight at 37°C. The next day we picked a few colonies of each strain and incubated them into a tube with 5mL of LB broth and 100 µg/ml of ampicillin liquid media and left overnight to grow on a shaker at 225 rpm at 37°C. The next day we collected the grown cells and stored in sterilized glycerol stock at -80°C for long-term storage of transformed bacterial cells containing the plasmids. In order to extract the plasmid, we used a QIAprep Spin Miniprep Kit (Qiagen). Then we linearized the plasmids by digestion using BamHI (New England BioLabs) and XbaI (New England BioLabs) in CutSmart Buffer (New England BioLabs) for 3 hours at 37°C. We prepared agarose gel in 1X TAE buffer and 5µL of ethidium Bromide and let it sit for 30 minutes to harden. Then we added 6µL of 6x DNA loading dye into 60µL of sample and used 100bp ladder (New England BioLabs). Samples were run on gel at 100 Volts for 3hours. After observing the fragments of interest on gel, we cut them and extracted them using GIAquick Gel Extraction Kit (Qiagen). Once DNA is extracted from gel, it needs to be purified. We used a Zymo purification kit. (Thermo Scientific).

Table 21. G Block Sequences for DENV Serotypes Inserted into Topo Vectors

DENV Serotypes	G Block Sequence
DENV 1/3	3′−CGGCGGTGTGAGCGGATAACAATTCCCGGATCCTAATACG ACTCACTATAGGGGTAAAATGAAGTCAGGCCGAAAGCCACGG TTTGAGCAAACCGTGCTGCCTGTAGCTCCATCGTGGGGATGTA AAAACCCGGGAGGCTGCAAACCATGGAAGCTGTACGCATGGG GTTGCAGACTAGTGGTTAGAGGAGACCCCTCCCAAGACACAA CGCAGCAGCGGGGCCCAACACCAGGGAAAGCTGTACCCTGGT GGTAAGGACTAGAGGTTAGAGGAGACCCCCCGCATAATAATA AACAGCATATTGACGCTGGGAGAGACCAGAGATCCTGCTGTC TCTACAGCATCAATCCAGGCACAGAGCACCAGAAAATGGAAT GGTGCTGTTGAATCAACAGGTTCTTCTAGACCGCTGAGCAATA ACTAGCATCGCGCC-5’
DENV 2	3’-CGGCGGTGTGAGCGGATAACAATTCCCGGATCCTAATACG ACTCACTATAGGGGTTAAAAGAAGTCAGGCCATTACAAATG CCATAGCTTGAGTAAACTATGCAGCCTGTAGCACCACCTGA GAAGGTGTAAAAAATCTGGGAGGCCACAAACCATGGAAGCT GTACGCATGGCGTAGTGGACTAGCGGTTAGAGGAGACCCCTC CCTTACAAATCGCAGCAACAATGGGGGCCCAAGGTGAGATGA AGCTGTAGTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCC CCCCAAAACAAAAAACAGCATATTGACGCTGGGAAAGACCAG AGATCCTGCCGTCTCCTCAGCATCATTCCAGGCACAGAACGCC AGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCTTCTAGAC CGCTGAGCAATAACTAGCATCGCGCC-5’
DENV 4	3’-CGGCGGTGTGAGCGGATAACAATTCCCGGATCCTAATACGA CTCACTATAGGGATTGAAGTCAGGCCACTTGTGCCACGGTTTG AGCAAACCGTGCTGCCTGTAGCTCCGCCAATAATGGGAGGCGT AATAATCCCCAGGGAGGCCATGCGCCACGGAAGCTGTACGCGT GGCATATTGGACTAGCGGTTAGAGGAGACCCCTCCCATCACTG ATAAAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTG TACTCCTGGTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAA CACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTG CTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAAGATG GATTGGTGTTGTTGATCCAACAGGTTCTTCTAGACCGCTGAGCA ATAACTAGCATCGCGCC-5’

In Vitro Transcription RNA

We used the Promega IVT kit and the purified plasmid as a DNA template for in vitro transcription, according to the manufacturer's instructions. Then we purified the IVT RNA by adding 1ml of Trizole and 200µl Chloroform, vortexed samples and centrifuged at full speed for 15 minutes. Then we removed 600ul upper aqueous phase (contains RNA & DNA) into a new tube and added 1:1 volume 4M Lithium Chloride LiCl then placed on ice for 2 hours then centrifuged at full speed for 15 minutes and removed supernatant (contains DNA) carefully without disturbing pellet (invisible) into a new tube. Then we added 250µl of nuclease-free water to the pellet that contains the RNA. We then added 500µl RNA binding buffer plus 250µl Ethanol to the 250µl re-suspended RNA pellets and moved to the Zymo Spin column for a wash. Lastly, we added 30µl Nuclease Free water to the column and elute the RNA.

Use IVT RNA to quantify DENV 1-4 stock from NMRC

To quantify the IVT RNA standards we used the Quant-iT™ RiboGreen™ RNA Assay Kit (ThermoFisher). For a standard curve, we used 16S ribosomal RNA. We prepared a low-range standard curve ranged between 1- 50 ng/mL. Then we determined the RNA concentration on the basis of absorbance at 260 nm (A260) in a plate.

The quantified IVT RNA standards were then used to quantify the DENV1-4 RNA from NMRC via RT-qPCR. We used 3 reactions for each serotype. We made four different standard concentrations and started with 1E8copies/reaction, 1E7, 1E6 and 1E5 copies/reaction. We purchased the itaq universal SYBER Green Kit. (Bio-Rad). For each reaction, we added 10µL of the Itaq Universal SybreGreen Mix, 0.4µL of F3 and B3 primers, 0.2µL of Iscript Reverse Transcriptase, 8µL of nuclease free water and 1µL of the target RNA.

RT-LAMP Primers

To detect MS2 bacteriophage, we used primers that target the replicase gene, described by Chander et al.[46] To detect DENV RNA, we used pan-serotype LAMP primers, described by Teoh et al, targeting the 3' untranslated region (3'UTR) of the DENV1-4 genome. [47] Primer sequences are shown in Table 5, where FIP and BIP are the forward and backward inner primers, F3 and B3 are the forward and backward outer primers, and LF and BF are the forward and backward loop primers. Primers were purchased from Integrated DNA technologies (San Diego, CA, USA) and Eurofins (Louisville, KY, USA).

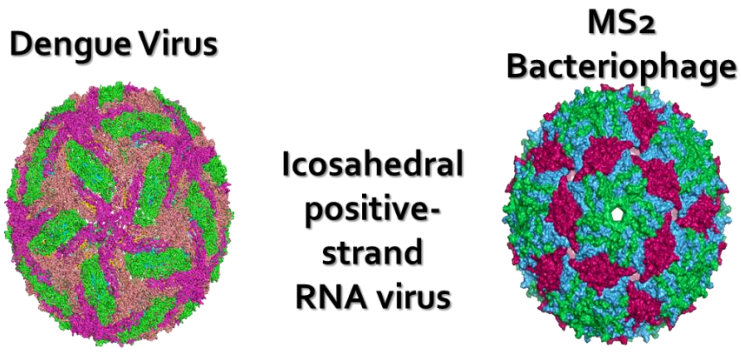


Figure 37. Dengue virus versus MS2 Bacteriophage

Table 22. RT-LAMP Primer Sequences for detecting DENV[47] and MS2[46]

Primer Name	Sequence (5′–3′)
DENV F3/134	CAAACCGTGCTGCCTGT
DENV F3/2	TGAGTAAACTATGCAGCCTGT
DENV B3/123	ACCTGTTGATTCAACAGCACC
DENV B3/4	ACCTGTTGGATCAACAACACC
DENV FIP/123	AGGGGTCTCCTCTAACCRCTAGTCTTTCAAACCRTGGAAGCTGTACGC
DENV FIP/4	AGGGGTCTCCTCTAACCRCTAGTCTTTTTTGCCACGGAAGCTGTACGC
DENV BIP/123	ACAGCATATTGACGCTGGGARAGACGTTCTGTGCCTGGAATGATGCTG
DENV BIP/4	ACAGCATATTGACGCTGGGARAGACGCTCTGTGCCTGGATTGATGTTG
DENV BLP/1234	CAGAGATCCTGCTGTCTC
MS2 F3	TGTCATGGGATCCGGATGTT
MS2 B3	CAATAGAGCCGCTCTCAGAG
MS2 FLP	CCAGAGAGGAGGTTGCCAA
MS2 BLP	TGCAGGATGCAGCGCCTTA
MS2 FIP	GCCCAAACAACGACGATCGGTAAAACCAGCATCCGTAGCCT
MS2 BIP	GCACGTTCTCCAACGGTGCTGGTTGCTTGTTCAGCGAACT

DENV RT-LAMP Liquid Assay

After quantifying the DENV RNA of all four serotypes, we tested our liquid assay method using the quantified RNA. The RT-LAMP protocol was designed for a total of 25µL per reaction. The master-mix reactions contained a final concentration of 0.10µM each of F3/134, F3/2, B3/123 and B3/4, and a final concertation of 0.8µM each of FIP/123, FIP/4, BIP/123, BIP/4 and 0.5µM each of BLP/1234. In addition, the master-mix contained 1.4mM dNTPs, 4mM MgSO4, 800mM betaine, and 1x Isothermal amplification buffer (IAB, prepared as shown in Table 3), 0.1x of SYBR green and 0.50 U/µL of GspSSD2.0 DNA Polymerase was obtained from OptiGene Ltd (Horsham, UK). The RT- LAMP master-mix was prepared on ice without target RNA. After

adding all reagents, we added 1µL of target RNA to make a total volume of 25µL per reaction for the positive control and 1µL of nuclease free water to make a total volume of 25µL per reaction for the negative control. The mixture was pipetted into Roche LAMP strips (Bio-Rad) with 25µL in each well and amplified using the Roche LightCycler® 96 for 45–60 min at 63 °C.

DENV Primers Optimization in Liquid RT-LAMP

We optimized the primer concentrations in the liquid RT-LAMP assay to obtain earlier detection of DENV, targeting in particular DENV serotype 2 and 3. We tested the selected primers shown in table 6 at three different concentrations. We tested 9 different conditions (Table 8), for each we created positive master-mix containing 5e3 copies/reaction of DENV 2 or 3, along with master-mix for the negative NTC controls. We tested three positive replicates and three negative replicates. The mixture was pipetted into Roche LAMP strips (Bio-Rad) with 25µL in each well and amplified using the Roche LightCycler® 96 for 60 min at 63 °C as described above.

Table 23. DENV Primers Liquid RT-LAMP Optimization

Primer Name	B3/123	FIP/123	BIP/123
Condition 1	0.10 µM	0.6 µM	0.6µM
Condition 2	0.2 µM	0.80 µM	0.8µM
Condition 3	0.3 µM	1 µM	1µM
Original	0.10 µM	0.8µM	0.8µM

DENV Liquid RT-LAMP Assay with NMRC RNA

We tested the liquid RT-LAMP assay for all four serotypes of NMRC quantified RNA. The Master-mix was made as described above with a final concentration of 0.10µM each of F3/134, F3/2, B3/123 and B3/4, and a final concertation of 0.8µM each of FIP/123, FIP/4, BIP/123, BIP/4 and 0.5µM each of BLP/1234. We ran three replicates for each serotype at 1e5copies DENV RNA/reaction. Next, we ran a liquid RT-LAMP master-mix as described above to perform an LOD study for all four serotypes at 1e4, 1e3 and 1500 copies DENV RNA /reaction. Due to the positive amplification of NTC controls shown on real time amplification, we ran another liquid DENV LAMP assay with DIG labeled FIP/123 primer for DENV serotype 1, 2 and 3 at 1e4copies/reaction plus 3 NTCs to differentiate false positives due to primer dimer amplification or contamination.

DENV RT-LAMP Dry Reagents

DENV RT-LAMP concentrated master-mix was likewise prepared in two parts, of which ~10ul were dispensed onto pre-treated paper pads that were later reconstituted in 100uL buffer as described below. Upon reconstitution in 100uL, the Part 1 master-mix (primer paper) yields final 1x concentrations of 1.40 mM dNTPs, 6.00 mM $MgSO_4$, 0.10uM of F3/134, F3/2, B3/123 and B3/4, 0.80uM of DIG-labeled FIP/123, DIG-labeled FIP/4, BIP/123 and BIP/4, and 0.50uM of biotinylated-BLP/1234, while the Part 2 master mix (enzyme paper) yields a final 1x concentration of 0.7 U/uL GspSSD2.0 DNA polymerase. Of the 10x isothermal amplification buffer (IAB), 10% was included in the primer paper, while 90% was included in the enzyme paper. The final 1x IAB concentration was 20 mM Tris-HCl pH 9.2, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, and 0.1.% Triton X-100.

One primer paper and one enzyme paper were placed in a 1.5 mL microfuge tube, and master-mix reagents were reconstituted by adding 160ul of reconstitution buffer (25mM Tris pH 8.5 and 1mM EDTA) with 1X SYBR green. For positive reactions, DNA or RNA was added to the reconstitution buffer at the concentration indicated below. The mixture was incubated for at least 1 min, vortexed briefly, centrifuged for 30 sec in a desktop microfuge, and the final master mix solution was transferred to fresh tubes and kept on ice until testing. From each reconstitution solution, two 60uL replicates were pipetted into PCR strips or plates, and isothermal amplification was performed at 63°C for 80 min using a Roche LightCycler 96, with fluorescence detection at 488 nm every minute. The threshold time for isothermal amplification onset was determined using LightCycler 96 V1.1 software automated parameter for Cq (base line correction- where the amplification curve crosses the threshold value). Real time fluorescence results were considered positive if at least one amplification replicate had a threshold time ≤ 60 min. Each batch of the paper-based reagents were tested for quality control (QC) with three negatives NTC and three positives.

MS2 Liquid RT-LAMP Assay

The LAMP protocol was designed for a total of 25µL per reaction. The MS2 master-mix reactions contained a final concentration of 0.10µM each of F3 and B3, and a final concertation of 0.3µM each of FL and BL and 0.5µM each of BIP and FIP. In addition, the master-mix contained 1.4mM dNTPs, 4mM MgSO4, 800mM betaine, and 1x Isothermal amplification buffer (IAB, prepared as shown in Table 3), 0.1x of SYBR green and 0.50U/µL of GspSSD2.0 DNA Polymerase that was obtained from OptiGene Ltd (Horsham, UK). The RT- LAMP master-mix was prepared on ice without target RNA. After adding all reagents, we added 1µL of target RNA

to make a total volume of 25µL per reaction for the positive control and 1µL of nuclease free water to make a total volume of 25µL per reaction for the negative control. The mixture was pipetted into Roche LAMP strips (Bio-Rad) with 25µL in each well and amplified using the Roche LightCycler® 96 for 60 min at 63 °C.

MS2 Dry Reagents

The MS2 RT-LAMP dry reagent master-mix was prepared similar to the LAMP reagent pad preparation as described above. Part 1 of the master-mix (primer paper) contained a final concentration of 1.40 mM of dNTP mix, 6.00 mM of MgSO4, 0.10 µM of F3 and B3, 0.40 µM of FLP and BLP, and 0.70 µM of FIP and BIP 200 mM of Tris-HCl pH 8.5, 100 mM of (NH4)2SO4, 500 mM of KCl, 20 mM of MgSO4, 1% of Triton X-100. Part 2 of the master mix (enzyme paper) includes 0.70 U/uL GspSSD2.0 DNA polymerase in IAB buffer 200 mM of Tris-HCl pH 8.5, 100 mM of (NH4)2SO4, 500 mM of KCl, 20 mM of MgSO4, 1 % of Triton X-100. In the preparation for the primer and enzyme papers, 17.68 µL Part 1 was aliquoted on primer paper and 17.68 µL part 2 was aliquoted on each enzyme paper respectively. Enzyme papers and primer papers in 24 well tissue culture plates were frozen at -80oC for 1 hour before freeze drying using a Freezone 4.5 lyophilizer (Labconco) for 4 hour at <200mbar pressure. The freeze-dried reagent papers were stored at -20°C in airtight aluminum pouches with desiccant and oxygen scavenger bags until further use.

Protocols for rehydration of the dry RT-LAMP reagent pads are as described above. Two 45 µL replicates were pipetted into PCR strips or plates, and isothermal amplification was performed at 68°C for 60 min using a Roche LightCycler 96. Then it detected via later flow detection strips.

Inhibition Studies

Hemoglobin Inhibitor Studies

To quantify the amount of hemoglobin in the plasma eluate via UV-VIS absorbance, we prepared a standard curve containing various hemoglobin concentrations from 0-2 mg/mL. The hemoglobin stock solution was prepared in phosphate buffer pH 7 containing 0.5mg/mL ascorbic acid. This solution was incubated at 4°C overnight to reduce met-hemoglobin to the ferrous hemoglobin form [48]. Our hemoglobin is in the methemoglobin (contains Fe 3+) form and we need to convert it to hemoglobin containing Fe2+ with the use of 0.5mg/mL ascorbic Acid. To evaluate the effect of hemoglobin on the RT-LAMP assay performance, we ran MS2 RT-LAMP reactions at 1e3copies/reaction spiked with 0.1mg/mL, 0.25mg/mL, or 0.5mg/mL hemoglobin in triplicate following the MS2 liquid RT-LAMP protocol described above.

To determine the amount of hemoglobin introduced by the plasma separation device into the plasma obtained from the device, we pooled the plasma eluates from 4 devices and then measured the average hemoglobin concentration present in the sample based on the absorbance at 500 nm, relative to a standard curve of known hemoglobin concentrations using a SpectraMax plus 384 plate reader.

Protein Inhibition and Removal Studies:

We utilized the BCA assay Sigma Aldrich (St. Louis, MO, USA) to quantify the protein concentration in 10% plasma and in the eluates obtained from the plasma separation device, these samples were incubated on ice for 30 seconds, then vortexed for 30 seconds and spun down for 1 minute. The supernatant was collected and used for the BCA assay. Mixtures of BCA reagent and either standards (20-2,000 μg/mL BSA) or unknown samples in triplicate were prepared following

the manufacturer's microplate procedure instructions, and the absorbance at 562nm were recorded using a SpectraMax plus 384 plate reader.

Plasma Inhibition Studies

To test how much plasma the RT-LAMP assay can tolerate, we did a series of dilutions containing 0%, 10%, 20%, 30% and 40% plasma in the elution buffer. These plasma dilutions were then used to reconstitute the MS2 dry reagent papers. We prepared 3 dry reagent MS2 papers for each plasma percentage in the master-mix. We added RNAseout, an RNAse inhibitor, to each condition prior to adding the MS2 RNA. All positive conditions were tested at 1e3copies/reaction MS2 RNA and to the negative control we added nuclease free water. The master-mix was amplified at 63ºC for 80min. The amplified products were run on the lateral flow strips.

DENV 1 Thermo-stability

For the DENV1 thermostability study, we freeze dried (lyophilized) the primers on a total of 30 porous cellulose papers and freeze dried (lyophilized) the enzyme on another 30 porous cellulose papers (Whatman No. 1 chromatography). We combined one primer paper with one enzyme paper in a 1.5 mL eppendorf tube to make a total of 30 papers pairs in 30 tubes. We stored 6 tubes of 6 pairs of papers in a foil pouch with desiccant and oxygen scavenger at room temperature (25ºC). A total of 5 pouches were labeled to be tested and analyzed weekly started at week 1 then week 2, 3, 4 and 5. Each week papers were analyzed via isothermal amplification and real time fluorescence detection followed by lateral flow detection. Three papers were positive (1e5copies/reaction) and three papers were negative NTC. We did a second thermo-stability study, were we made 36 pairs of papers divided to 6 pouches. Each pouch contained 6 pairs of papers in with desiccant and oxygen scavenger stored at room temperature (25ºC). Papers were analyzed immediately started at week0 then followed by week 2, week 4, week 6 and 8.

RESULTS AND DISCUSSION

DENV Front to back process Overview

Figure 38. DENV Full Process Execution. Starts with whole blood sample preparation followed by filter plasma via plasma separation device then RT-LAMP isothermal amplification and lateral flow detection.

Another key effort in our lab pertains to detection of Dengue Virus, and RNA virus, starting from whole blood. In this process, we use blood sample, filter plasma via plasma separation device, reverse transcribed- isothermal amplification and lateral flow detection. This was a team effort, with Elizabeth Celaya working on sample preparation process, Hsiang Wei working on the plasma separation device and other engineering components; I am focusing on reverse transcribed isothermal amplification and coupling with upstream sample prep and downstream lateral flow detection, and Katie Wilson working on custom lateral flow strip production.

DENV Genomic RNA:

To optimize our assay, we need genomic RNA for all 4 DENV serotypes (DENV1-4). As mentioned above, we have obtained DENV RNA extracted from cell culture supernatant from our collaborators at the naval medical Research Center (NMRC), but this RNA contains host DNA and RNA. We need to quantify the amount of DENV RNA present in the sample.

Template for In Vitro Transcription

To quantify the amount of DENV RNA present in the sample we are using a quantified RNA standard generated through In Vitro Transcription (IVT). To generate this RNA standard, we started by creating a DNA template. We cloned synthetic G-block DNA sequences representing the 3’ UTR regions of DENV1/3, DENV2, and DENV4 with upstream T7 RNA polymerase

promoter regions into TOPO vectors that were then transformed into *E.coli*. Figure 39 shows the colonies we obtained from E.coli transformation experiments for all four serotypes.

Figure 39. DENV Bacterial Transformation. Grown colonies of all four DENV serotypes in ampicillin resistance agar plate.

In Vitro Transcribed RNA

We collected the grown colonies from bacterial transformation, expanded them in liquid medium, and then purified the plasmid. We then digested the plasmid with BamH1 and Xba1 to obtain a linearized DNA template for in vitro transcription (Figure 40).

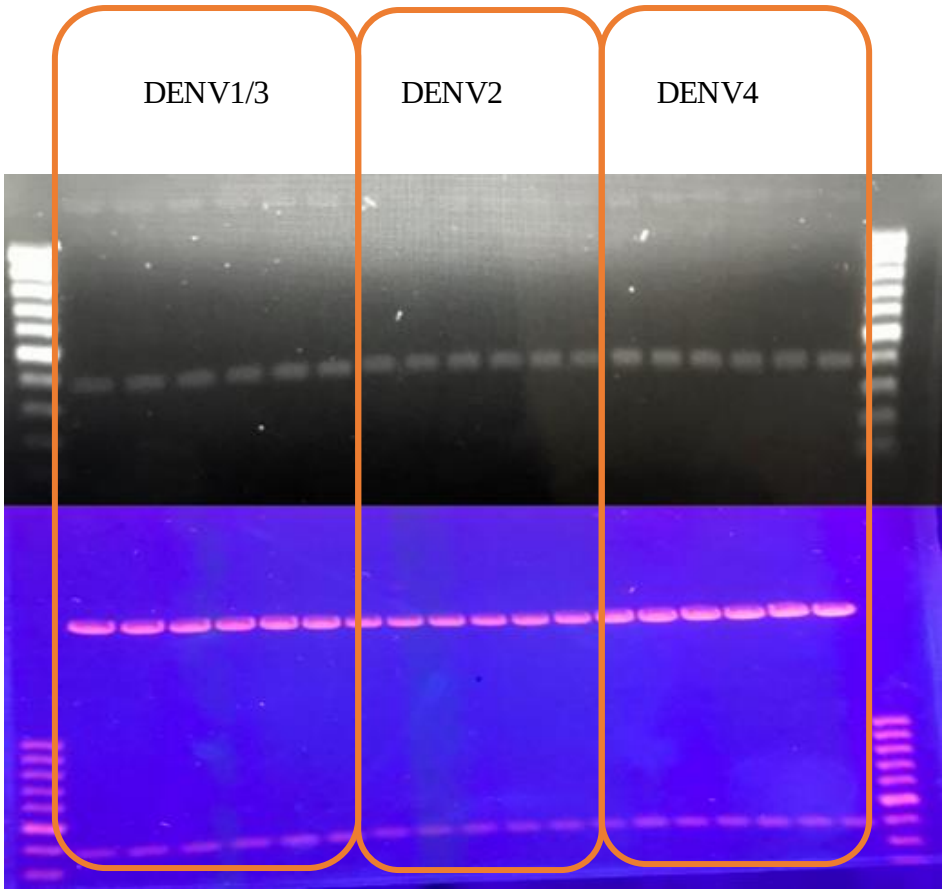


Figure 40. DNA template for DENV IVT obtained via plasmid digestion, showing the fragments of all four serotypes of DENV. Each serotype is around 396 base pair.

We purified the fragments representing the IVT template and used these to run the in vitro transcription assay. The product was run on a gel for each serotype to confirm the presence of RNA. (Figure 41).

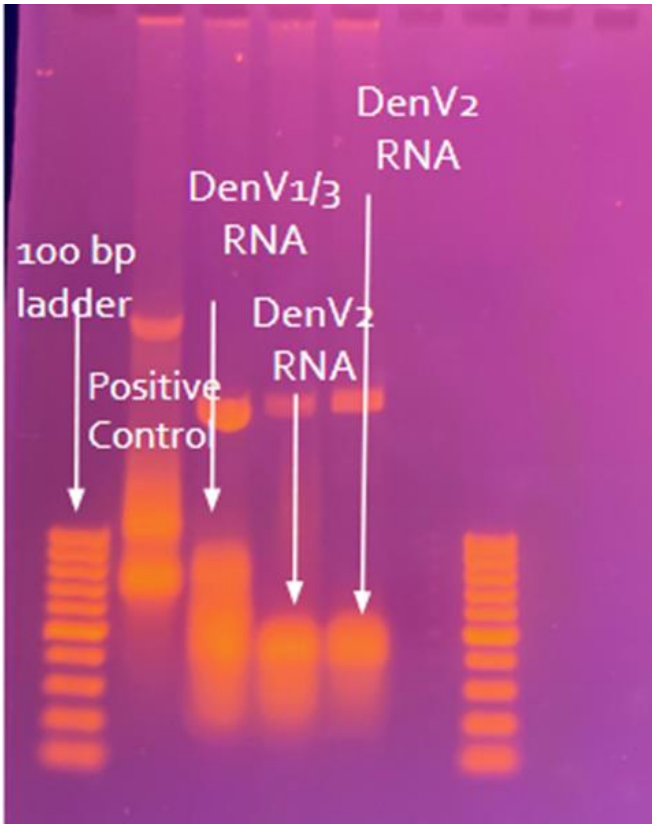


Figure 41. In vitro transcription product ran on gel to confirm the RNA present including a positive control.

We washed and purified the product of in vitro transcription then ran q-PCR to confirm our RNA product is clean from any DNA template contamination. In qPCR that does not contain a reverse transcriptase, only DNA but not RNA is amplified. Therefore, if amplification is observed in qPCR, then there is remaining g-block template in the sample. Figure 42 shows no amplification for sample RNA, but amplification for the positive, containing the g-block template DNA.

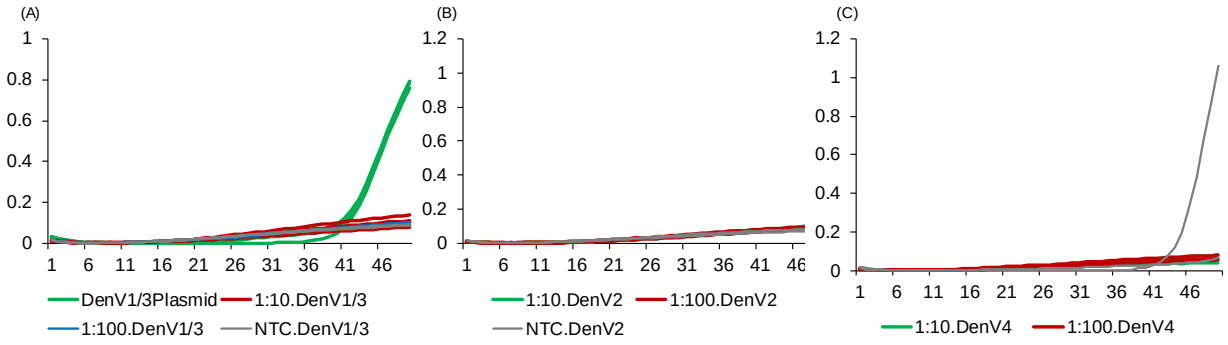


Figure 42. Amplification of in vitro transcribed RNA for all four serotypes at two different concentration to confirm no contamination in all samples at 1:10 and 1:100 dilution via qPCR (A) amplification of DENV1/3 g-blocks as positive control and no other amplification from the DENV1/3 sample (B) no amplification from the DENV2 sample which confirms sample is clean and (C) no amplification of DENV 4 IVT RNA sample means sample is clean.

We also ran RT-qPCR and confirmed that there is in vitro transcribed RNA after purification (Figure 43).

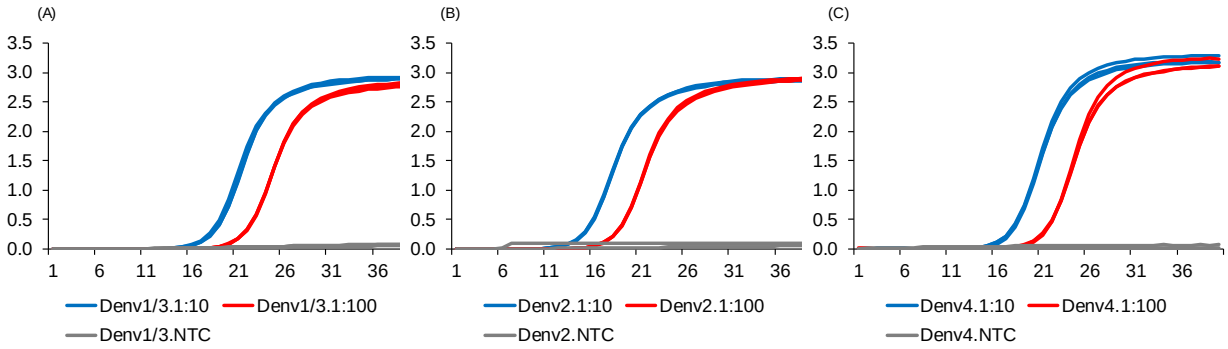


Figure 43. Amplification of in vitro transcribed RNA for all four serotypes at two different concentration, 1:10 and 1:100 dilution via RT-qPCR (A) amplified DENV1/3 IVT RNA (B) amplified DENV2 IVT RNA and (C) amplified DENV 4 IVT RNA.

Now that we confirmed that there is only RNA in our sample, we moved forward to quantify the in vitro transcribed RNA to use as a standard. We used the RiboGreen assay with pre-quantified ribosomal RNA as standard as shown in (Figure 44).

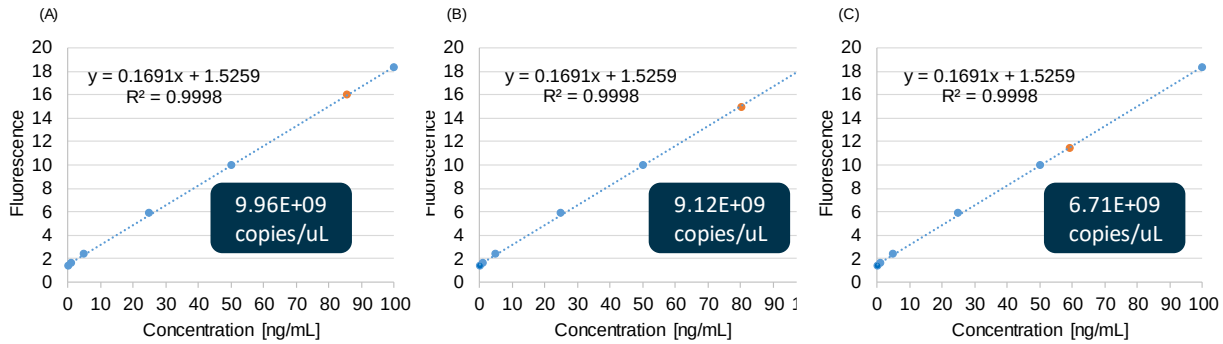


Figure 44. Quantification of IVT RNA standards using the RiboGreen Assay. The blue dots represent tRNA standards and the orange dot is our in vitro transcribed RNA, with absolute concentration indicated in each graph (A) DENV1/3 IVT RNA quantification and calculated to 9.9E9copies/ µL. (B) DENV 2 IVT RNA quantification and calculated to 9.1E9copies/ µL and (C) DENV4 IVT RNA quantification and calculated to 6.7E9copies/reaction.

In Vitro Transcribed RNA to quantify DENV 1-4 stock

After quantifying the standards, we used them to quantify the DENV RNA obtained from NMRC via RT-qPCR. We used the IVT RNA standards to generate a standard curve from which we calculated the concentration of DENV RNA serotype 1 within the sample. As shown in (Figure 45 A) below, the concentration of unknown RNA serotype 1 amplified within our range. Using the standard curve calculation as shown in (Figure 45 B) the unknown RNA concentration for DENV1 is 3.00E+05copies/µL.

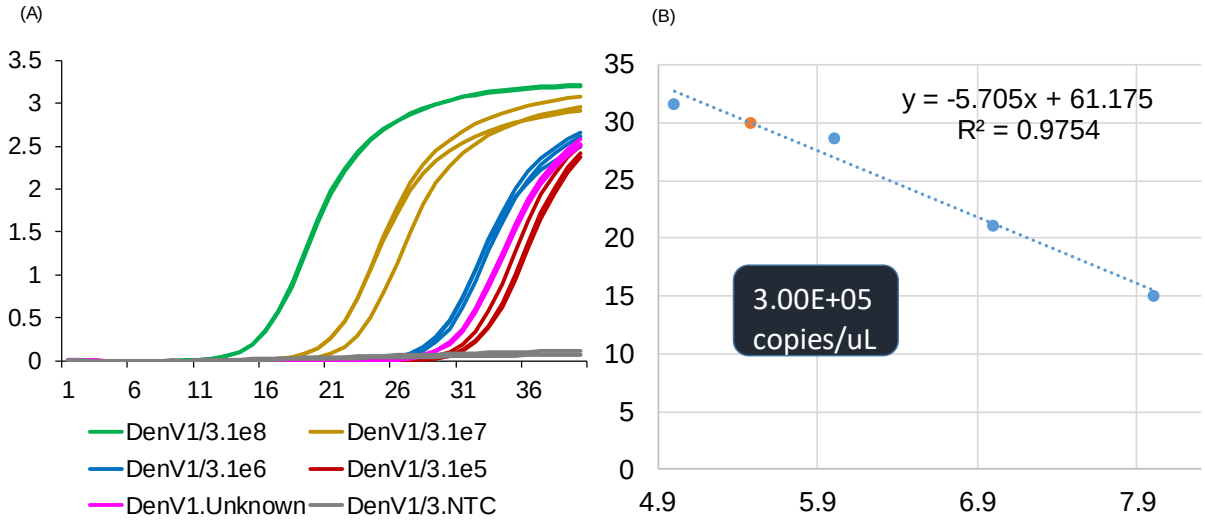


Figure 45. Quantifying DENV RNA serotype 1 via RT-qPCR (A) the amplification of standards curves made by serial dilution of quantified IVT RNA serotype 1 and unknown RNA. (B) Standard curve obtained with quantified IVT RNA relative to unknown RNA.

.

Also, we calculated the concentration of DENV RNA serotype 2 within the sample. As shown in (Figure 46 A) below, the concentration of unknown RNA amplified within our range. Using the standard curve calculation as shown in (Figure 46 B) the unknown RNA concentration for DENV2 is 2.20E+04copies/µL.

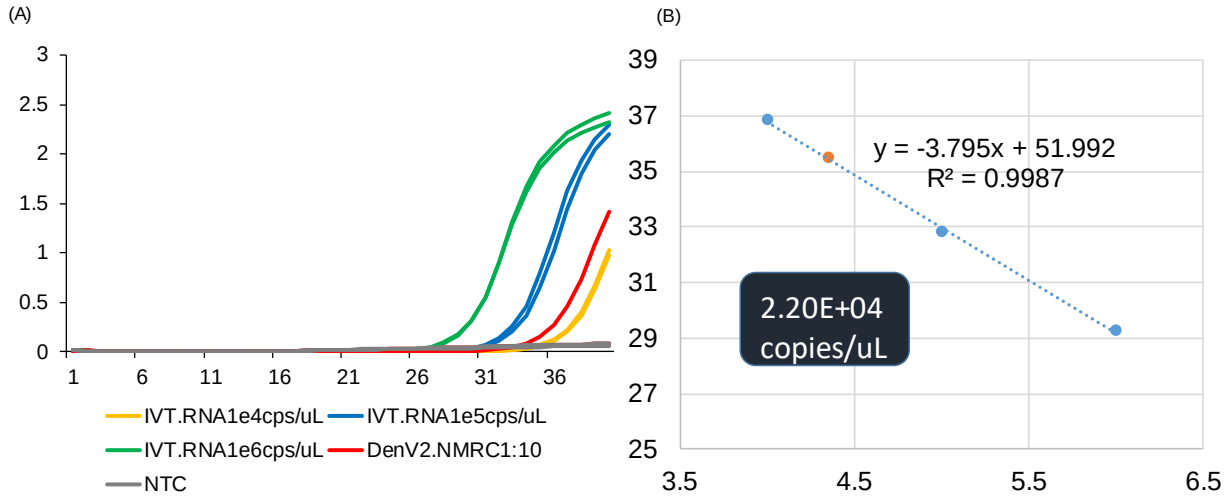


Figure 46. Quantifying DENV RNA serotype 2 via RT-qPCR (A) the amplification of standards curves made by serial dilution of quantified IVT RNA serotype 2 and unknown RNA. (B) Standard curve obtained with quantified IVT RNA relative to unknown RNA.

We quantified DENV RNA serotype 3 within the sample. As shown in (Figure 47 A) below, the concentration of unknown RNA amplified within our range. Using the standard curve calculation as shown in (Figure 47 B) the unknown RNA concentration for DENV3 is 3.73E+05copies/µL.

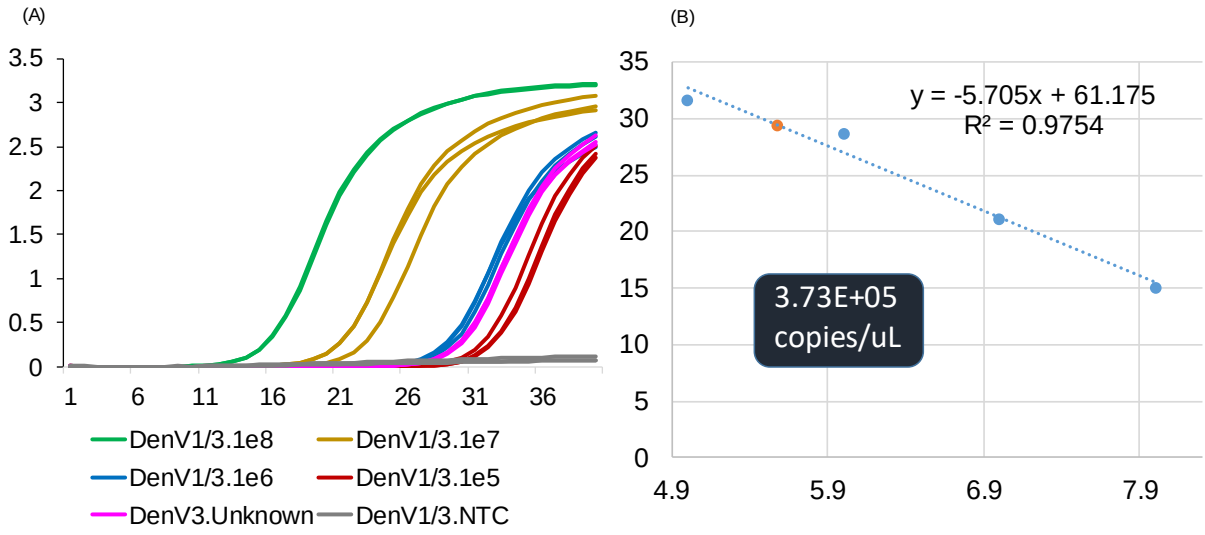


Figure 47. Quantifying DENV RNA serotype 3 via RT-qPCR (A) the amplification of Standard curves made by serial dilution of quantified IVT RNA serotype 3 and unknown RNA. (B) Standard curve obtained with quantified IVT RNA relative to unknown RNA.

DENV RNA serotype 4 shown in (Figure 48 A) below, the concentration of unknown RNA amplified within our range. Using the standard curve calculation as shown in (Figure 48 B) the unknown RNA concentration for DENV4 is 2.45E+06copies/µL.

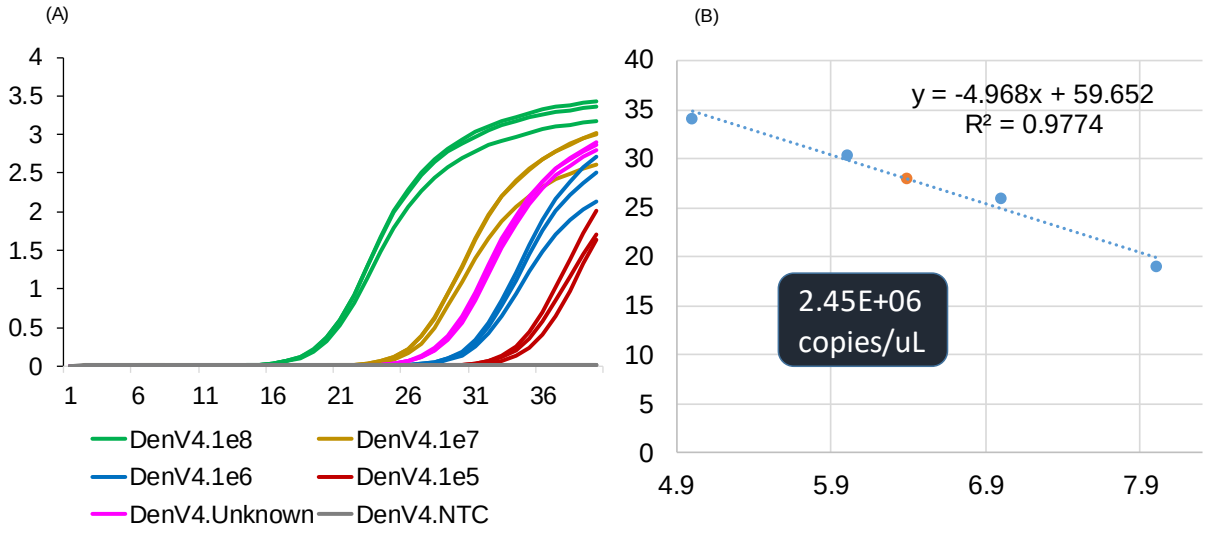


Figure 48. Quantifying DENV RNA serotype 4 via RT-qPCR (A) the amplification of Standard curves made by serial dilution of quantified IVT RNA serotype 4 and unknown RNA. (B) Standard curve obtained with quantified IVT RNA relative to unknown RNA.

DENV RT-LAMP Coupled to LF Detection

We have implemented in our lab a pan-serotype RT-LAMP assay for detecting DENV1-4 RNA, based on primers reported in the literature.[49] We used previously quantified DENV4 RNA to test our RT-LAMP assay with liquid and dry reagents paper assay. We were able to detect the target at 1e3copies/reaction early and consistently as shown in (Figure 49 A) and obtained a strong signal on lateral flow strips using the dry master mix reagents (Figure 49 B)

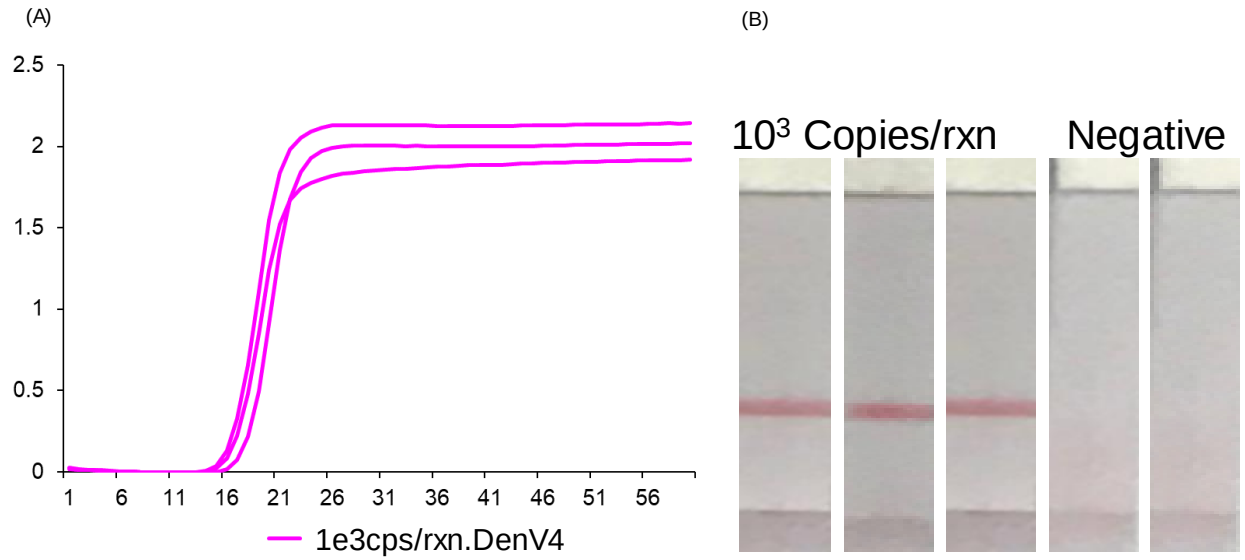


Figure 49. DENV4 RT-LAMP Liquid and Dry Reagents Assay. (A) Real time fluorescence detection of DENV4 RT-LAMP via liquid assay combined with (B) lateral flow detection of DENV4 at 10E3copies/reaction.

DENV Primers Optimization in Liquid RT-LAMP

We have optimized the assay starting by changing the concertation of three main primers: B3/123, BIP/123, and FIP123, as shown in the method section, targeting DENV2 and DENV3 RNA. Figure 50 showed the amplification of DENV2 with three concentrations of B3/123, where 0.1µM is the original concentration. The earliest amplification time of 25.7 minutes shows at a final concentration of 0.3µM of B3/123 in the master-mix. However due to the variability between replicates the difference in amplification time between the primers concentrations is not statistically significant (T-test p-values ≥0.698). Our hypothesis is that the negative replicates that amplify are primer dimers due to the large number of primers combined in one master mix, and we will confirm this hypothesis in a later experiment.

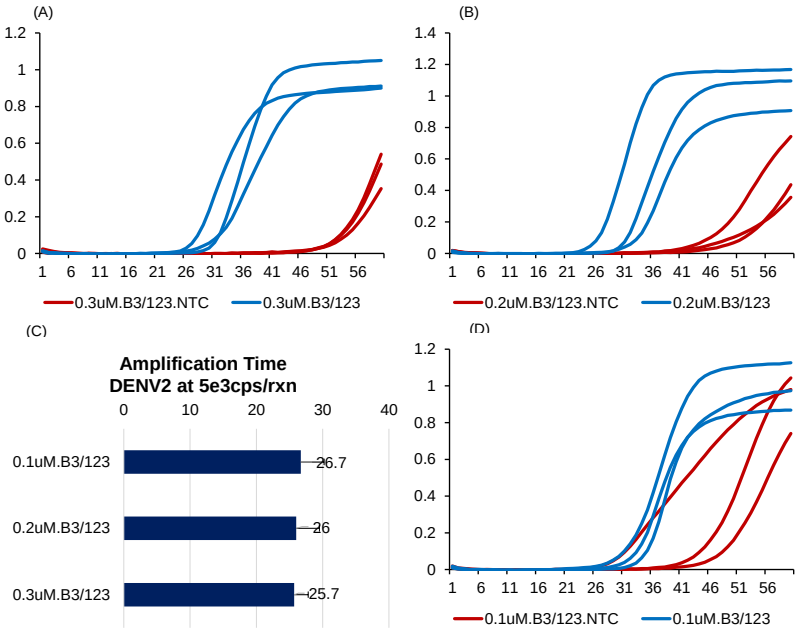


Figure 50. DENV2 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV2 RNA at 5e3copies/reaction via liquid assay with a final B3/123 concentration of A) 0.3µM, B) 0.2µM, and D) 0.1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.698).

Figure 51 shows the amplification of DENV3 with three concentrations of B3/123. The amplification and detection of DENV3 was delayed and inconsistent for all three primer concentrations. We will need to optimize the assay, but also we need to confirm that there is not degradation or inconsistency between aliquots of the NMRC RNA stock. In other studies, we have ran some DENV3 and DENV4 liquid RT-LAMP experiments that were repeated using different NMRC tubes for the same serotype and concentration, but have observed different results. Another possibility is that through operator error RNAses were introduced into the master-mix, so that the DENV RNA degrades quickly. However, not all DENV serotypes show delayed amplification Perhaps some DENV serotypes RNA are more or less stable than other DENV serotypes. We again hypothesize that amplification of negative replicates entails primer dimers.

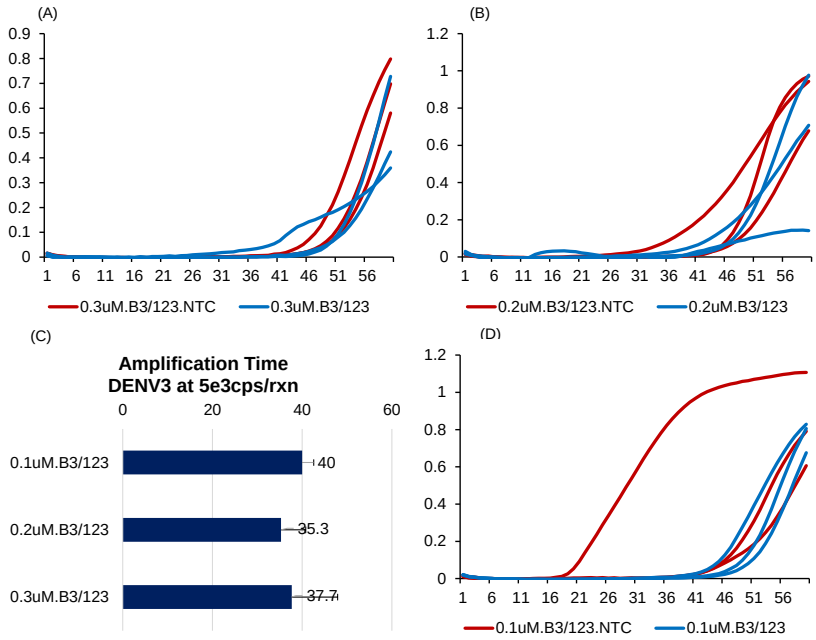


Figure 51. DENV3 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV3 RNA at 5e3copies/reaction via liquid assay with a final B3/123 concentration of A) 0.3µM, B) 0.2µM, and D) 0.1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.734).

Figure 52 shows the RT-LAMP amplification with three concentrations of BIP/123 targeting DENV2 RNA. The earliest amplification (22.7 minutes) was observed at a final concentration of 0.1µM of BIP/123 in the master-mix. The original concentration of BIP/123 is 0.8 µM.

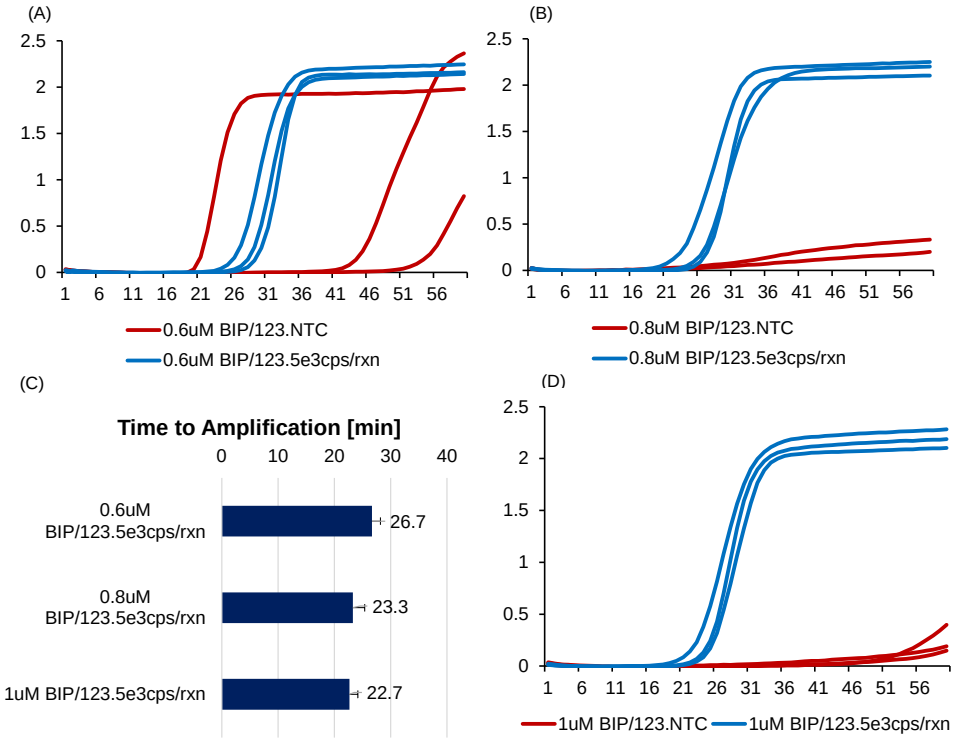


Figure 52. DENV2 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV2 RNA at 5e3copies/reaction via liquid assay with a final BIP/123 concentration of A) 0.6µM, B) 0.8µM and D) 1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.679).

Continuing with the primer optimization experiment, Figure 53 below shows the amplification with three concentrations of BIP/123 targeting DENV3 RNA. The earliest amplification (23 minutes) was observed for a final concentration of 0.6µM of BIP/123 in the master-mix. The original concentration of BIP/123 is 0.8 µM, and that master-mix amplified at 31.3 minutes, so the difference is quite significant (T-test p-values ≥0.050). Furthermore, this experiment showed much better amplification of DENV3 RNA than observed in Figure 51, which supports our hypothesis that the DENV3 RNA in some of the aliquots may have been degraded.

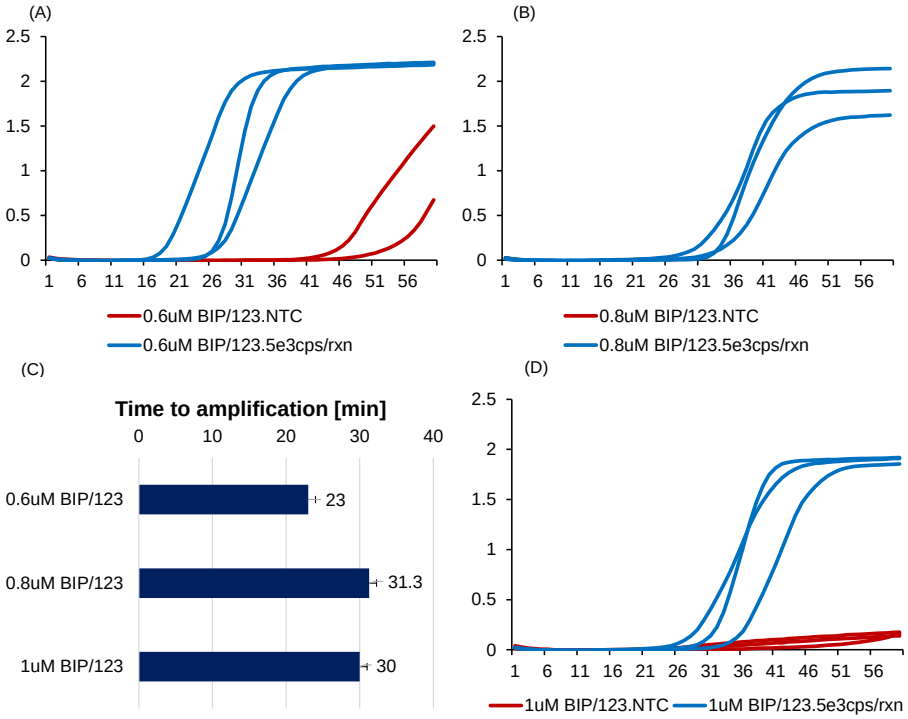


Figure 53. DENV3 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV3 RNA at 5e3copies/reaction via liquid assay with a final BIP/123 concentration of A) 0.6µM, B) 0.8µM, and D) 1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.050).

Figure 54 below shows the amplification with three concentrations of FIP/123 targeting DENV2 RNA. We did not observe statistically significant differences in amplification times for the different primer concentrations.

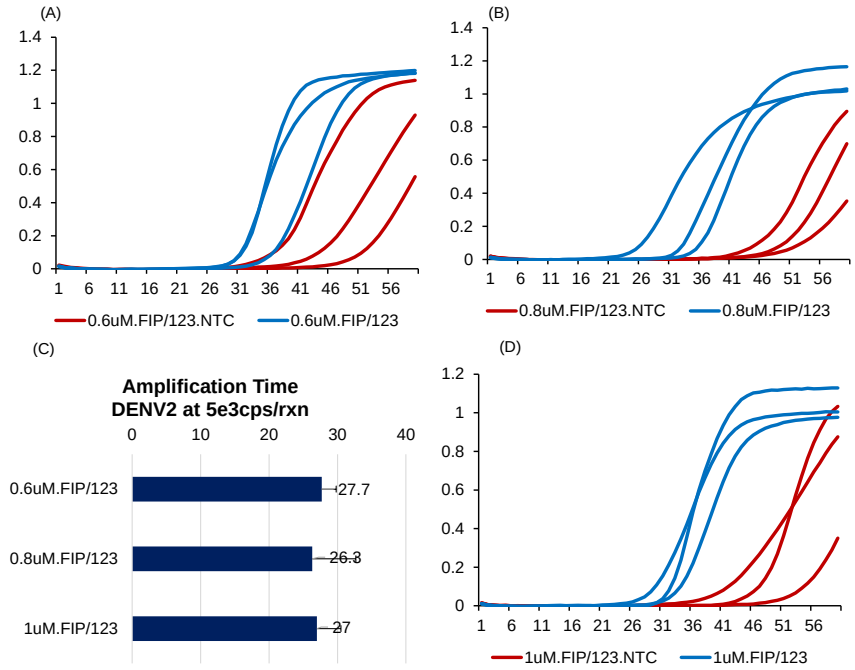


Figure 54. DENV2 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV2 RNA at 5e3copies/reaction via liquid assay with a final FIP/123 concentration of A) 0.6µM, B) 0.8µM, and D) 1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.884).

Figure 55 below represent the results for the last condition of the primer optimization experiment: The RT-LAMP liquid assay with three concentrations of FIP/123 targeting DENV3 RNA. The results were very inconsistent, so again a problem with amplifying DENV3 RNA was observed, and any differences are not statistically significant.

In summary, regarding the optimal primer concentration we observed different results for DENV2 and DENV 3, and it seemed if we change one primer concentration to benefit one serotype then it will negatively impact another serotype. Some results showed no major difference in amplification time while others showed earlier amplification time with the original concentration such as detecting DENV2 with 0.8µM concentration of FIP/123. Therefore, based on the results of the primer optimization experiment, we decided to continue our RT-LAMP assay optimization experiments with the original primer concentrations.

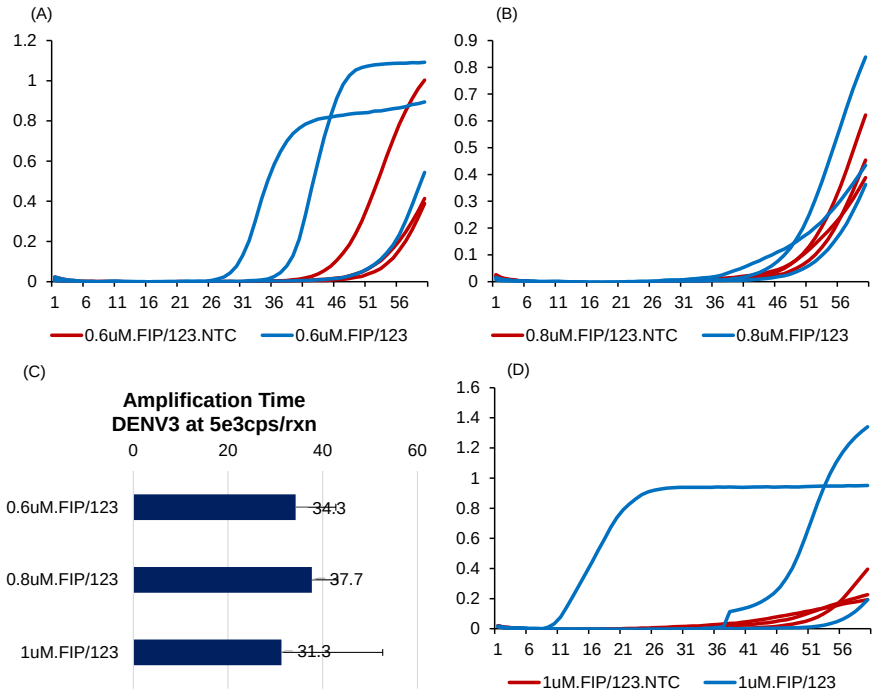


Figure 55. DENV3 RT-LAMP Liquid Primer Optimization. Real time amplification detecting DENV3 RNA at 5e3copies/reaction via liquid assay with a final FIP/123 concentration of A) 0.6µM, B) 0.8µM, and D) 1µM. C) Average amplification times for each master-mix with standard deviations, showing no statistically significant difference in amplification time (T-test p-values ≥0.663).

In the previous primer optimization experiments we only tested DENV 2 and DENV3. To confirm robust amplification of all four serotypes using the final primer concentrations, we ran a liquid RT-LAMP assay targeting all four DENV serotypes. The results show early amplification to all four serotypes. DENV 1, 2 and 3 RNA were detected at 1e5copies/reaction and DENV4 RNA at 1e7copies/reactions. We used a higher concentration of DENV4 RNA due to multiple inconsistence and delayed amplified results from previous experiments. Figure 56 shows the negative reactions did show on real time amplification; however, our next step was to use labeled primers and test the amplified product on lateral flow strips.

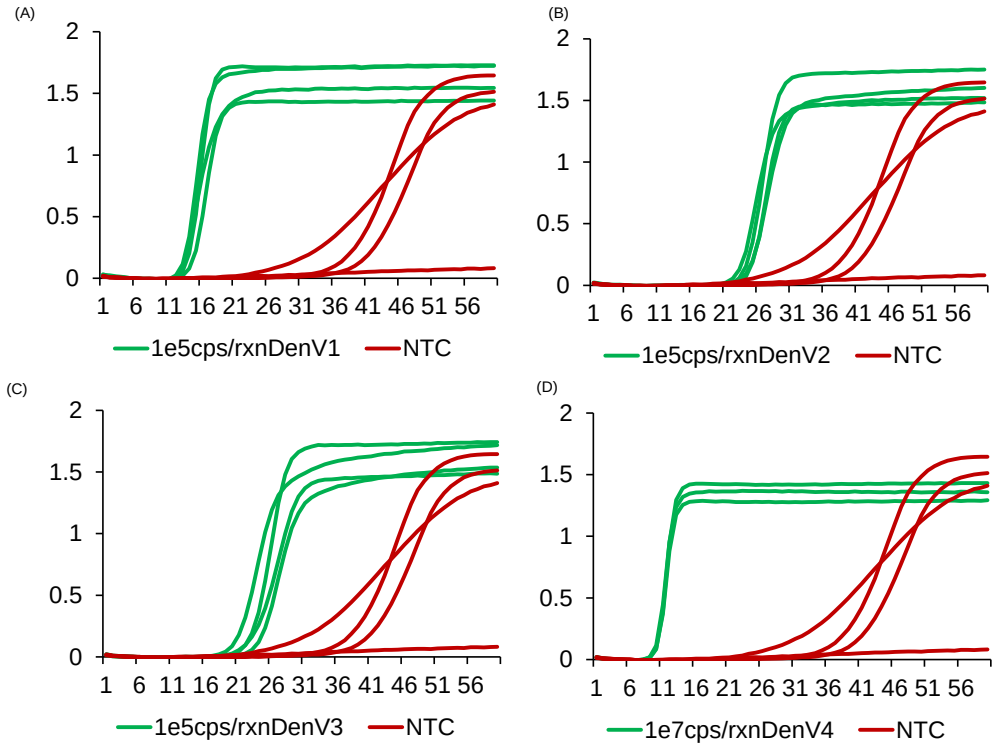


Figure 56. RT-LAMP liquid assay. Real time amplification detecting DENV1,2,3 and 4 RNA at 1e5copies/reaction via liquid assay

We needed to confirm the cause of the real time amplification of the negative controls. We tested DENV1, 2, 3 and 4 RNA with DIG labeled primer FIP/123 and DIG labeled primer FIP/4. Figure 57 shows the positive and the negative replicates of each target amplifying on the real time amplification, however when we ran the amplified samples on the later flow strips, we do not see the negatives on the LF strip line. The results confirmed our hypothesis: the real time amplification of negatives is not due to contamination but is due to primer dimer amplification since there are nine primers all in one master-mix. We will need to further optimize the assay to avoid the real time false positive amplification. Meanwhile we can move on to testing the DENV serotypes on the dry reagents paper assay.

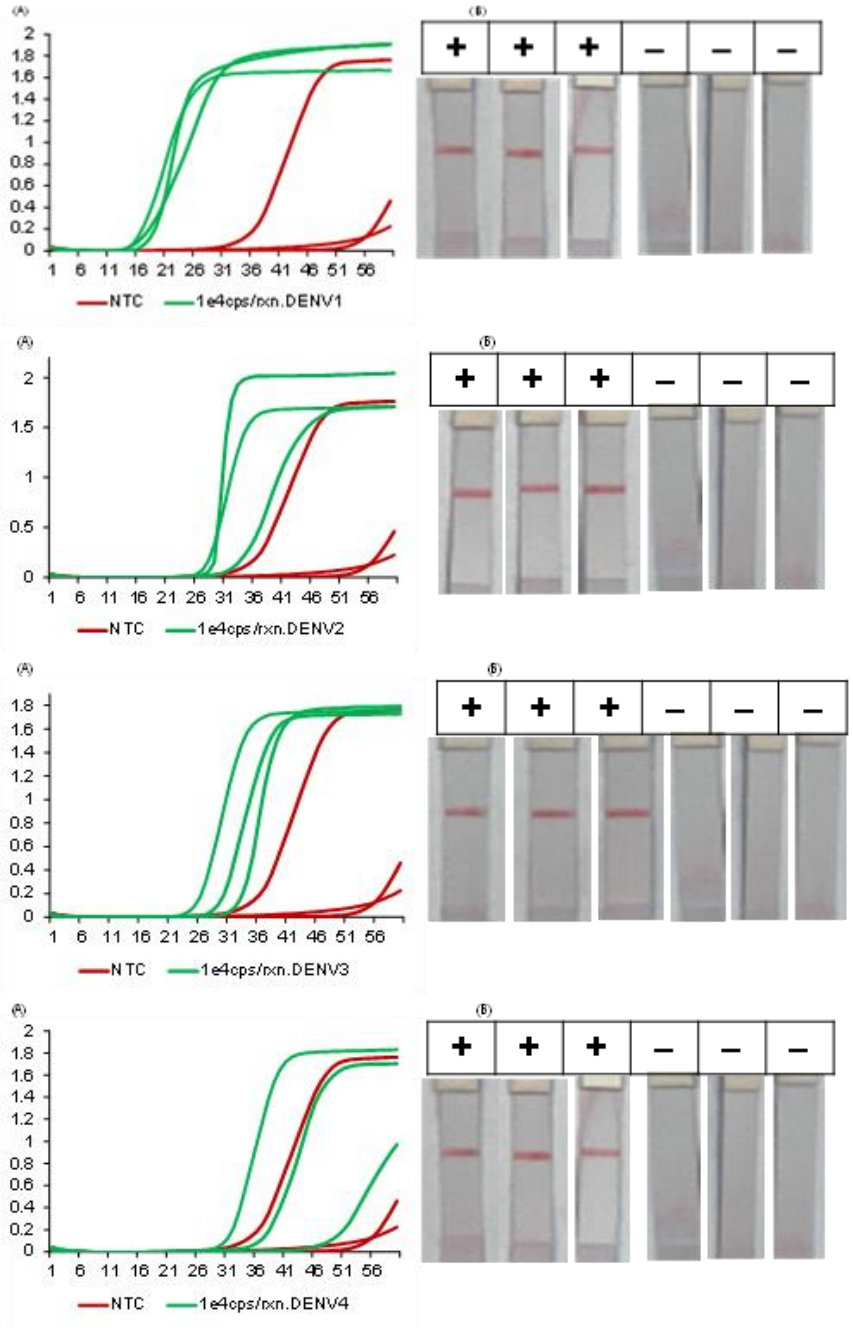


Figure 57. RT-LAMP Liquid Labeled Primers Assay Optimization. Real time amplification detecting DENV1, 2, 3 and 4 RNA at 1e4copies/reaction via labeled primers liquid assay combined with lateral flow detection, wherein the top line represents DENV RNA detection.

DENV RT-LAMP Dry Reagents

We moved on to test DENV RT-LAMP amplification on dry reagent papers at two different concentrations of DENV RNA: 1e5 and 1e4 copies/reaction. Figure 58 below shows the amplification curves for all four serotypes. At the higher concentration (1e5 copies/reaction) we were able to detect DENV 1, 2, and 3 reasonably early and consistent. The amplification was more variable at 1e4 copies/reaction. However, a good improvement was that the negative controls did not show amplification. For DENV4, the results in Figure 58 D show that only three out of 12 replicates amplified, and amplification times are inconsistent. The lack of detection of DENV4 NMRC RNA has been observed in other studies as well. We used different NMRC tubes for the same serotype and concentration but have observed different results. This requires further optimization and perhaps a new DENV4 RNA preparation at NMRC followed by re-quantification and quality control through stability studies.

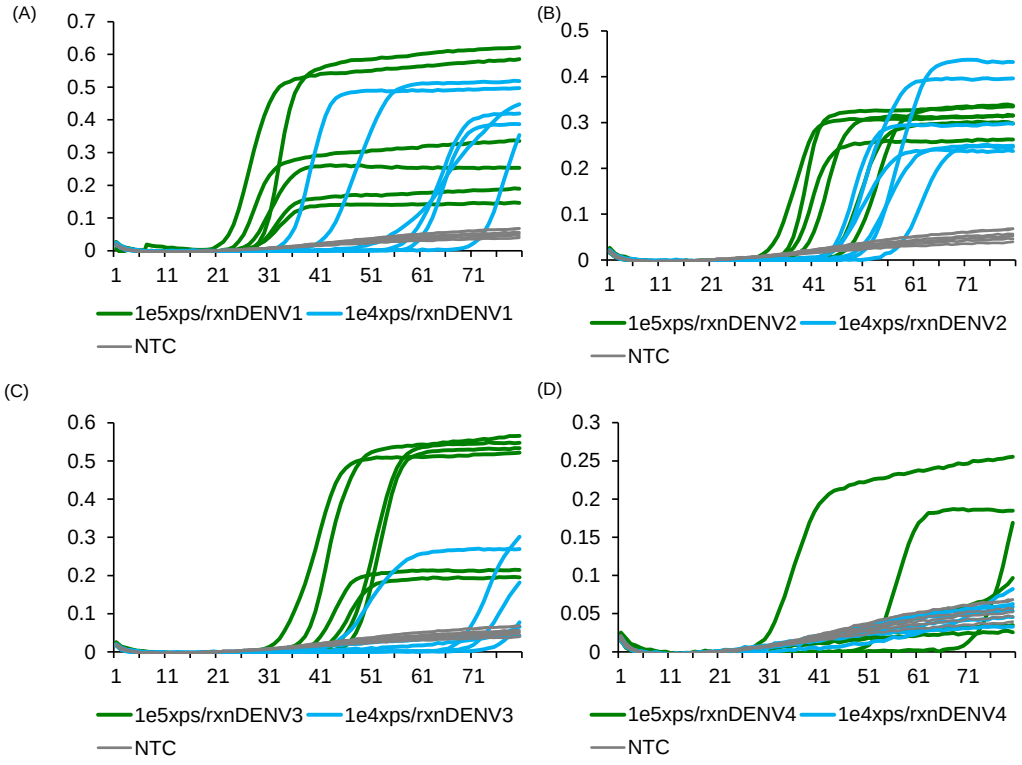


Figure 58. RT-LAMP Paper Assay LOD. Real time amplification detecting DENV1, 2, 3 and 4 RNA at 1e4 and 1e5 copies/reaction using unlabeled primers.

DENV (MS2) Full Process Execution

Bacteriophage MS2 is used as internal amplification control in our assay, and we are also using it to establish the front to back assay process, since it is a BSL1 organism. We have implemented an MS2 RT-LAMP assay using primers reported in the literature.[49]

We tested the MS2 RT-LAMP assay via liquid and dry reagents. (Figure 59) shows early and consistence amplification with real time and lateral flow strip detection.

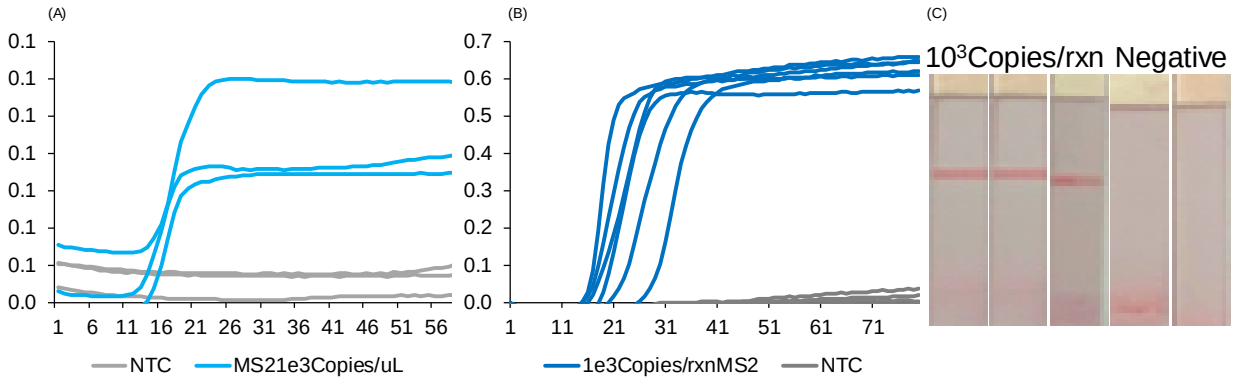


Figure 59. MS2 RT-LAMP via Liquid and Dry Reagent Assay. (A) Real time fluorescence detection of MS2 RT-LAMP via liquid assay and (B) real time fluorescence detection of MS2 RT-LAMP via paper assay combined with (C) lateral flow detection of MS2 at 10E3copies/reaction.

Inhibition Studies

After running the MS2 full process execution multiple times we recognized that the eluates contain inhibitors which prevent us from detecting the target. Therefore, we ran multiple studies to learn what might be causing the inhibition. The plasma separation device' eluates contain hemoglobin, which is a known polymerase inhibitor. To calculate the amount of hemoglobin in the eluates we compared the sample against a standard curve derived from hemoglobin that was converted into the Fe2+ form via citrate reduction. (Figure 60) shows that we have on average between 0.5 and 1mg/mL hemoglobin in our eluates.

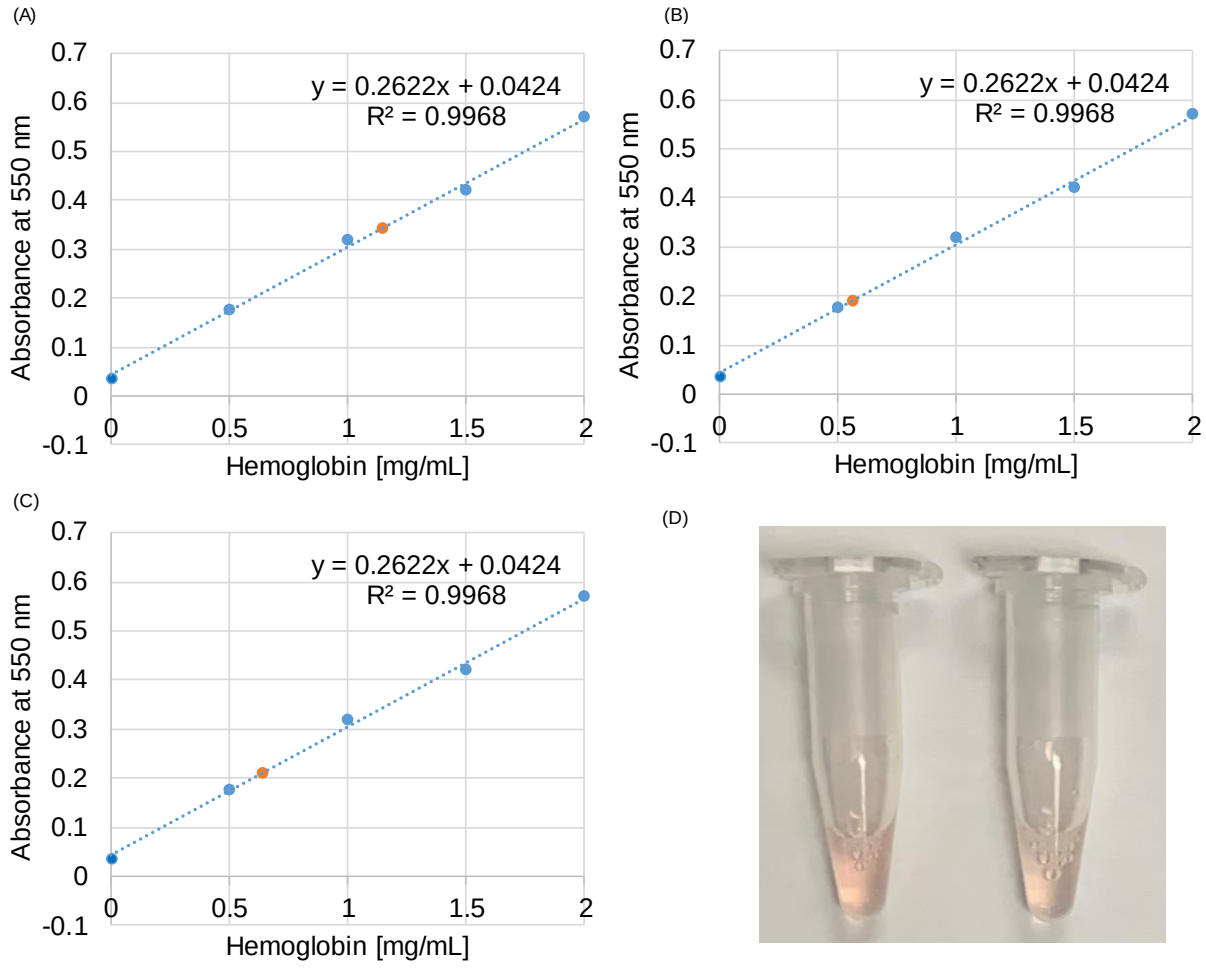


Figure 60. The Amount of Hemoglobin in MS2 Eluates via BCA Assay. (A, B and C) represent the hemoglobin concentration at three different eluates from three separate plasma separation devices. (D) The color of the samples.

To check what concentration of hemoglobin would be inhibiting the assay, we ran an MS2 liquid RT-LAMP assay with different concentrations of hemoglobin spiked into the master-mix. (Figure 61) shows the impact of hemoglobin on amplification time. The higher the concentration of hemoglobin the slower the reaction. However, spiking 0.5 to 1 mg/mL hemoglobin into the master-mix delayed the amplification by 7-8 minutes.

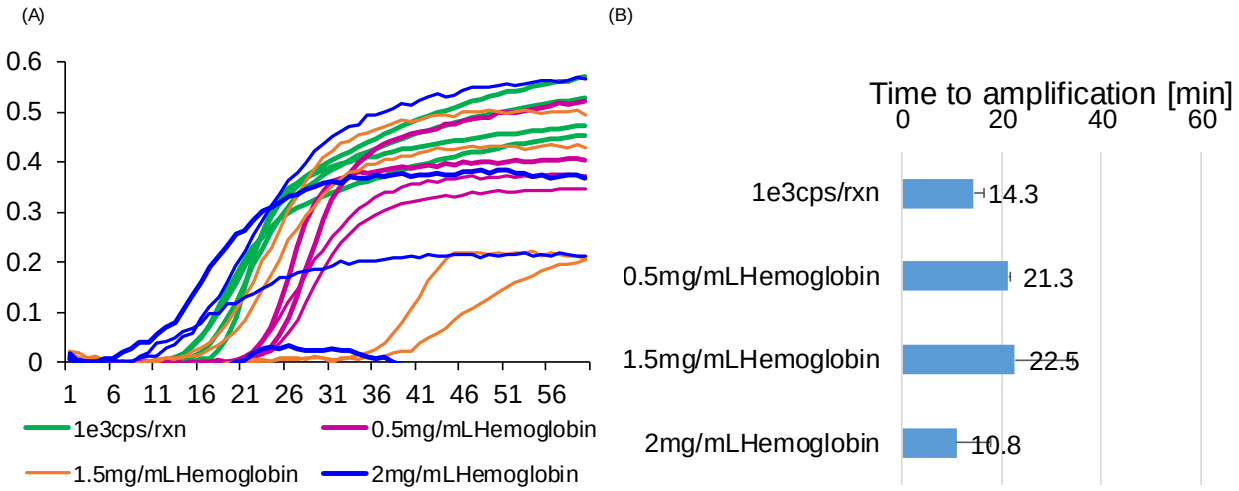


Figure 61. Hemoglobin impacts on MS2 RT-LAMP Liquid Assay with Spiked Hemoglobin at different Concentrations. (A) Real time fluorescence detection of MS2 amplification after spiking the master-mix with three different concentration of hemoglobin plus a positive control with no hemoglobin in master-mix with (B) the time of amplification for each condition.

The second inhibitor we tested was the amount of protein in the plasma separation device elutes. We used the BCA assay as shown in (Figure 62) to determine the concentration of protein, which was found to be between 50-550 μg/mL, unexpectedly low. Further investigations are underway.

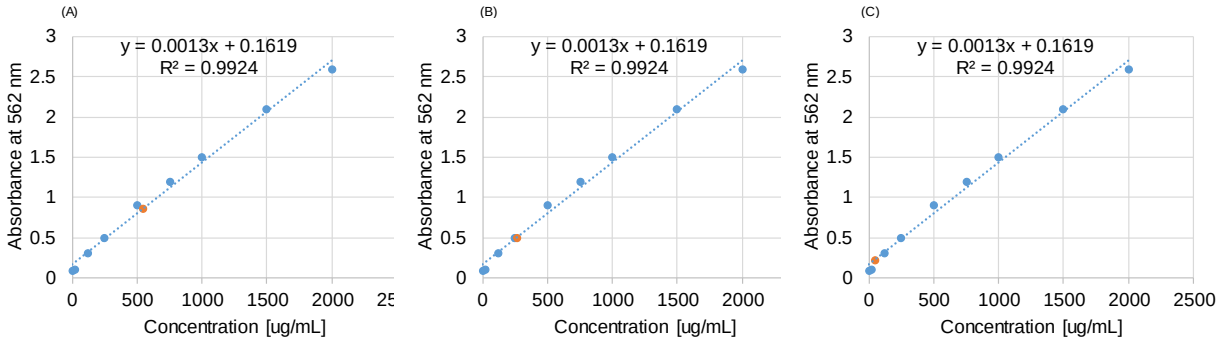


Figure 62. The Amount of Protein in MS2 Eluates via BCA Assay.

The next step was to test the effect of protein on the assay process. We ran the MS2 RT-LAMP liquid assay with different concentrations of BSA. The results in (Figure 63) show that the higher

the concentration of protein the slower the reaction. However, amplification was only delayed by ≤5 minutes, which means that protein inhibition likely is not substantial enough to completely inhibit the reaction.

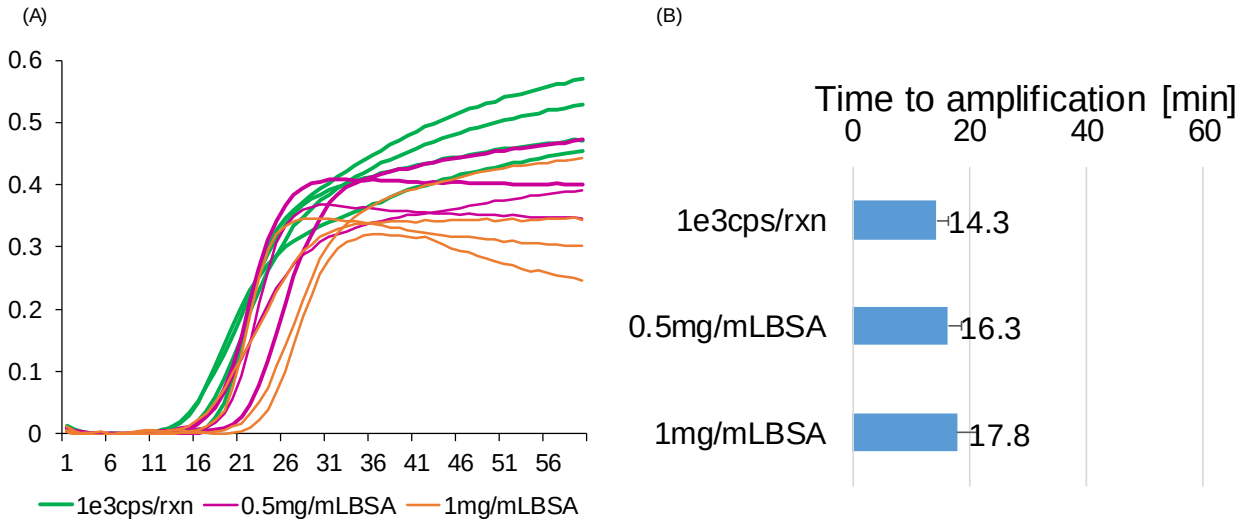


Figure 63 BSA impacts on MS2 RT-LAMP Liquid Assay with Spiked BSA at different Concentrations. (A) Real time fluorescence detection of MS2 amplification after spiking the master-mix with two different concentration of BSA plus a positive control with no BSA in master-mix with (B) the time of amplification for each condition.

Another set of inhibition study was adding plasma to the MS2 RT-LAMP liquid master-mix at different percentage. The results in (Figure 64) show the higher the percentage of plasma present in master-mix the slower the amplification, in fact even at 10% plasma we see a delay and inconsistency in the amplification of the target RNA. However, this concentration appeared to be a good compromise between avoiding inhibition and ensuring that a sufficient amount of target virus in plasma makes it into the amplification reaction. The engineering team has optimized the size of the absorbent pad in the plasma separation device and the amount of elution buffer used to wash the plasma separation device to keep the amount of plasma at 10% in the eluate that reconstitutes the dry reagent master-mix for amplification.

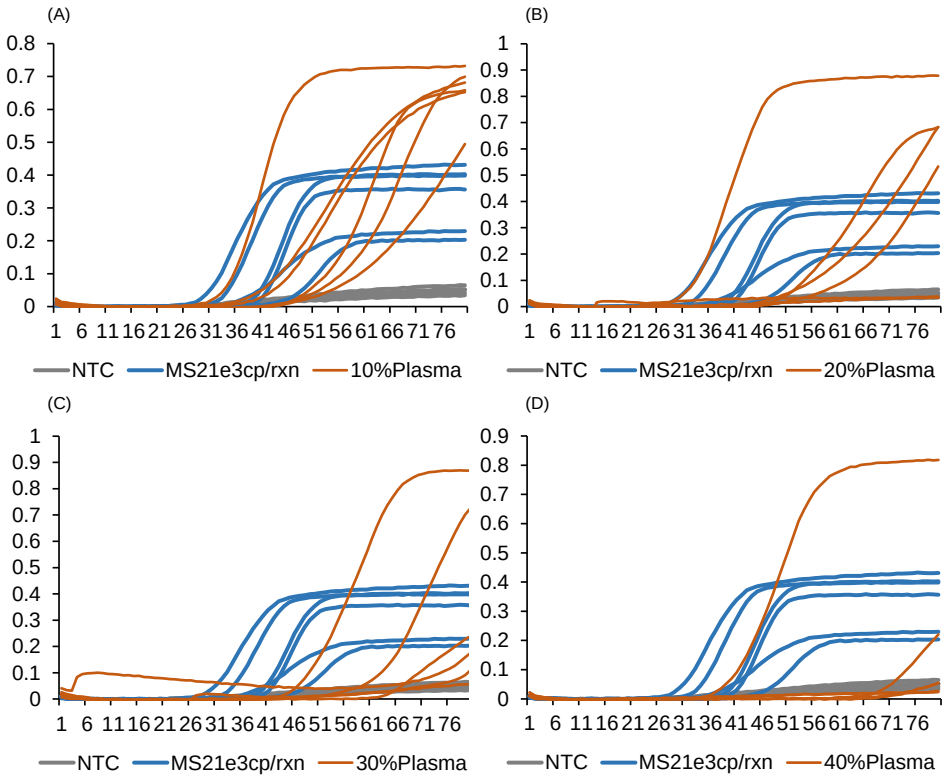


Figure 64. MS2 RT-LAMP Liquid Assay Plasma Inhibition study. Real time amplification detecting MS2 RNA at 1e3copies/reaction via liquid assay with a plasma percentage in master-mix of A) 10%, B) 20%, and C) 30%. D) 40%.

DENV RT-LAMP Thermostability:

Through prior experiments we found that the RT-LAMP dry reagent master-mix is not stable upon storage at room temperature or elevated temperature if using Bst polymerase and a separate enzyme, AMV reverse transcriptase. Therefore, we changed the master-mix to using Gsp-SSD2, a polymerase with intrinsic reverse transcriptase activity that exhibits good thermo-stability.

We examined the stability of the dry reagent RT-LAMP master-mix papers stored at room temperature via two separate studies, and for storage at 40ºC through a third study. In each case, we used the real time amplification of DENV1 RNA at 1e5 copies/ reaction followed by lateral flow detection as indicator for master-mix stability. To assess the stability upon storage at room

temperature, the first study tested the papers on a weekly basis over five weeks total and the second study tested the papers on upon storage for 1, 2, 4, 6, and 8 weeks. The third experiment tested the dry reagent master-mix stability upon storage at 40ºC on a weekly basis over five weeks. The compiled results are shown in Table 13, Figure 65, and Figure 66. In each case we observed that the dry reagent RT-LAMP master-mix papers are stable at room temperature and 40ºC for the duration of each study with strong lateral flow signals as shown below. The amplification onset time within the first study at room temperature over 5 weeks stayed relatively constant, while a delay in the amplification onset time was observed for the second study over 8 weeks at room temperature. For the 5-week study at 40ºC, the amplification onset time was delayed compared to the first week. For the real time detection, we did observe NTC amplification, but for lateral flow detection all replicates except for week 5 at room temperature. Although further optimization of RT-LAMP dry reagent master-mix thermo-stability is required, these results are very promising.

Table 24. DENV RT-LAMP Thermostability Data.

	Positive			Negative NTC		
	Threshold Time[(a)]	Fluorescence Positive[(b)]	Lateral Flow Positive[(c)]	Threshold Time[(a)]	Fluorescence Positive[(b)]	Lateral Flow Positive[(c,d)]
Weeks	First Run: Storage at Room Temperature					
1	27.3 ± 5.3 (6/6)	(3/3)	3	69.4 ± 2.3 (4/6)	(0/3)	0
2	38.4 ± 5.2 (6/6)	(3/3)	3	68.4 ± 5.5 (2/6)	(0/3)	0
3	39.4 ± 8.6 (6/6)	(3/3)	3	65.4 ± 5.3 (2/6)	(0/3)	0
4	41.3 ± 4.9 (6/6)	(3/3)	3	75.9 ± 7.9 (1/6)	(0/3)	0
5	32.1 ± 4.7 (6/6)	(3/3)	3	51.9 (2/6)	(1/3)	1
Weeks	Second Run: Storage at Room Temperature					
0	38.7 ± 6.2 (6/6)	(3/3)	3	74.4 ± 7.7 (3/6)	(0/3)	0
1	33.3 ± 6.5 (6/6)	(3/3)	3	57 ± 30.3 (3/6)	(0/3)	0

2	38.6 ± 11.5 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
4	34.5 ± 5.6 (6/6)	(3/3)	3	59.8 (1/6)	(0/3)	0
6	41.7 ± 9 (6/6)	(3/3)	3	73.2 ± 1.9 (2/6)	(0/3)	0
8	46 ± 6.4 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
Weeks	Third Run: Storage at 40°C					
0	43.8 ± 15.5 (5/6)	(3/3)	3	78.6 ± 0.2 (2/6)	(0/3)	0
1	33.9 ± 5 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
2	36.1 ± 6.1 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
3	45.4 ± 10.5 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
4	42.4 ± 4.1 (6/6)	(3/3)	3	NA (0/6)	(0/3)	0
5	49.1 ± 3.7 (6/6)	(3/3)	3	77.5 (1/6)	(0/3)	0

(a) Average ± Standard Deviation across all replicates (amplified/total) (b) Threshold time ≤ 60 minutes for at least one amplification replicate (c) and (d) Lateral flow strips positive signal.

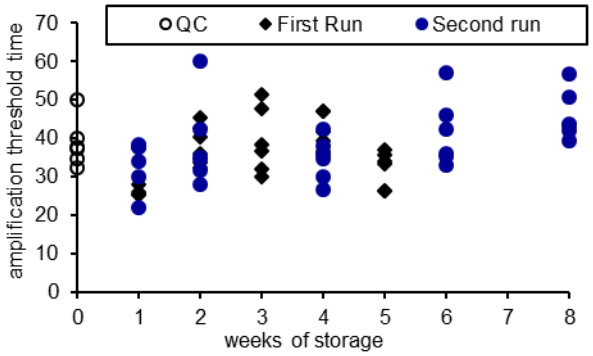


Figure 65. Thermostability study involving the storage of RT-LAMP DENV1 master-mix papers at room temperature (RT). All amplification replicates are plotted.

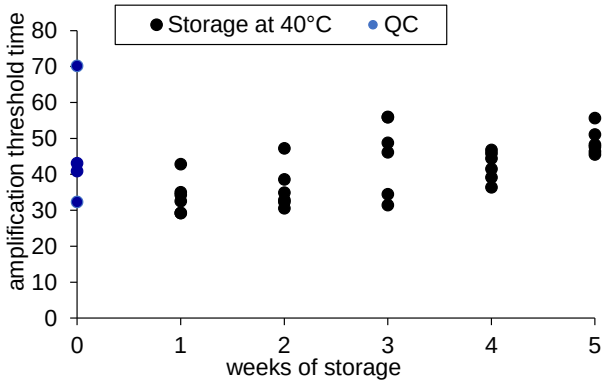


Figure 66. Thermostability study involving the storage of RT-LAMP DENV1 master-mix papers stored at 40ºC. All amplification replicates are plotted.

Real time fluorescence amplification and lateral flow images of the first week of dry reagent thermostability testing shown Figure 67. The results show early amplification for week1, with real time fluorescence amplification of the negative control. However, it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

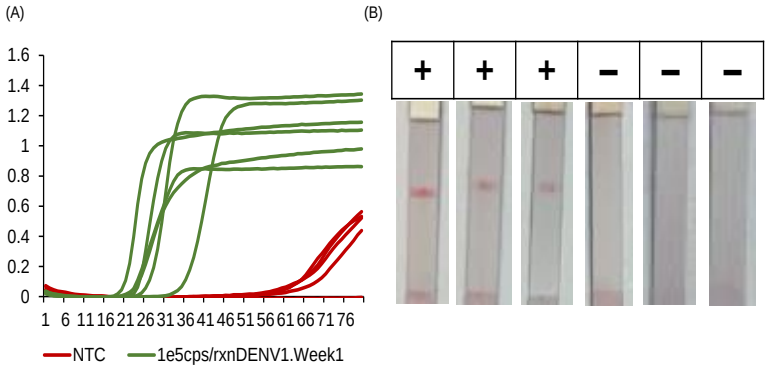


Figure 67. Week 1 Data of the First Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of the second week of dry reagent thermostability testing shown in Figure 68. The results show early amplification for week 2, with real time fluorescence amplification of the negative control. However, it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

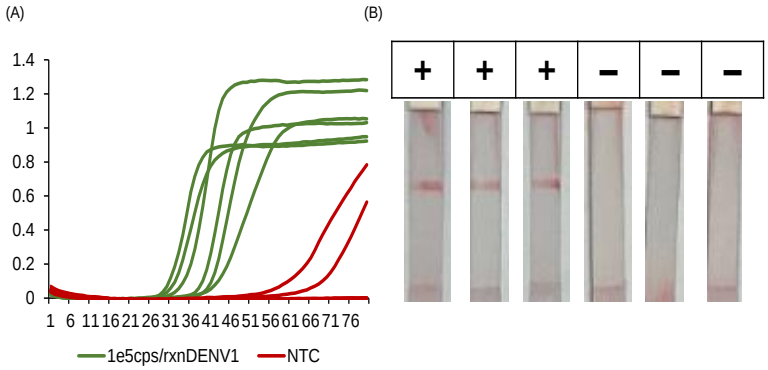


Figure 68. Week 2 Data of the First Run DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of the third week of dry reagent thermostability testing shows delay in amplification (Figure 69) however all positive replicates amplified, with real time fluorescence amplification of the negative control. However,

it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

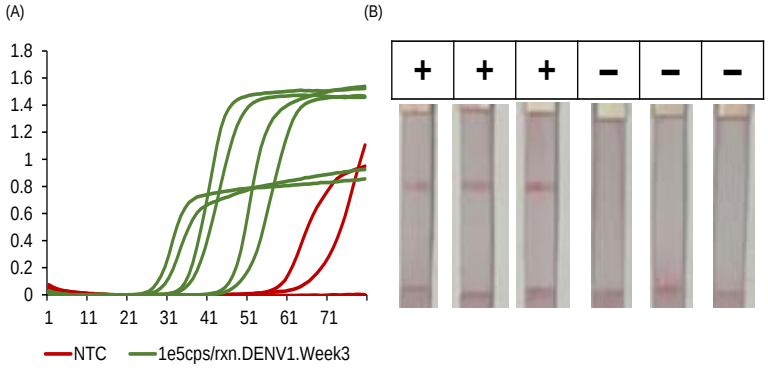


Figure 69. Week 3 Data of the First Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 4 shown in (Figure 70). The results show amplification of DENV1 at 1e5copies/reaction, with real time fluorescence amplification of the negative control of one replicate. However, the one replicate does not show on the lateral flow images, which is thought to be due to non-specific amplification.

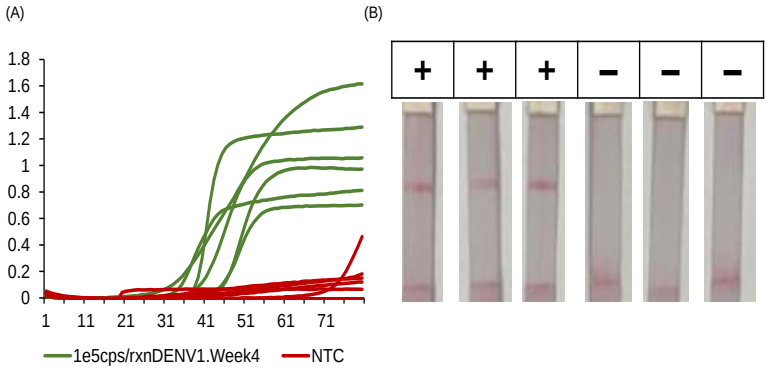


Figure 70. Week 4 Data of the First Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 5 shows a negative control amplification in real time fluorescence and lateral flow strips (Figure 71) perhaps due to cross contamination,

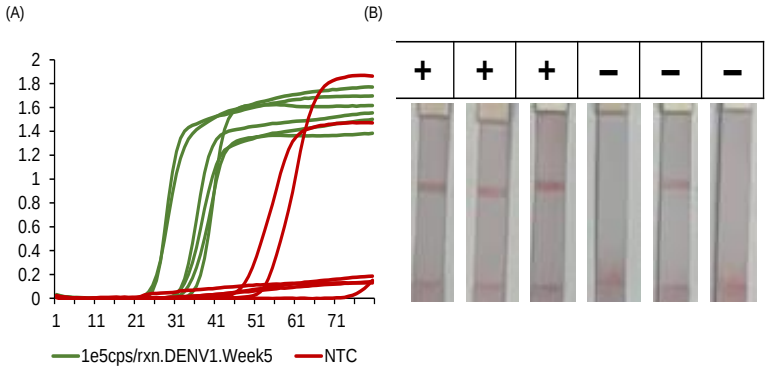


Figure 71. Week 5 Data of the First Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 0 of the second run of the dry reagent thermostability testing shown Figure 72. The results show early amplification for week 1, with real time fluorescence amplification of one replicate of a negative control. However, it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

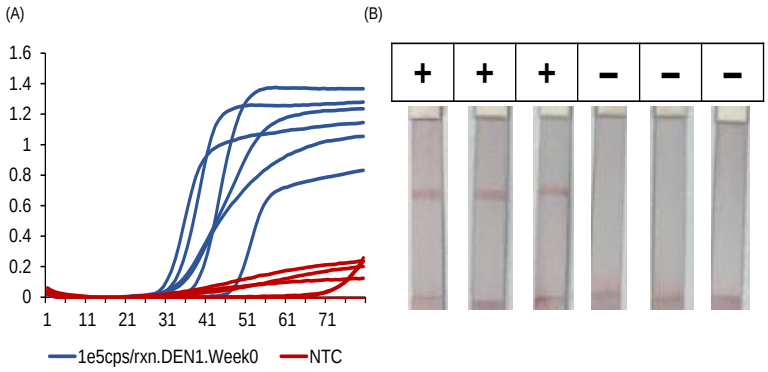


Figure 72. Week 0 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 1 of the second run of the dry reagent thermostability testing shown Figure 73. The results show early amplification for week1, with no real time fluorescence amplification of the negative control.

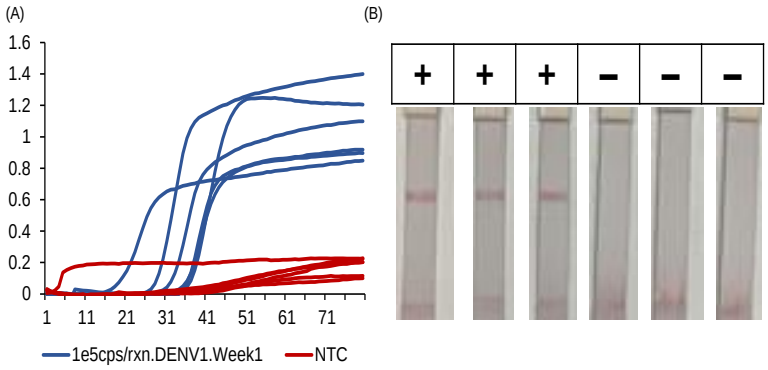


Figure 73. Week 1 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 2 of the second run of the dry reagent thermostability testing shown Figure 74. The results show early amplification for week1, with no real time fluorescence amplification of the negative control.

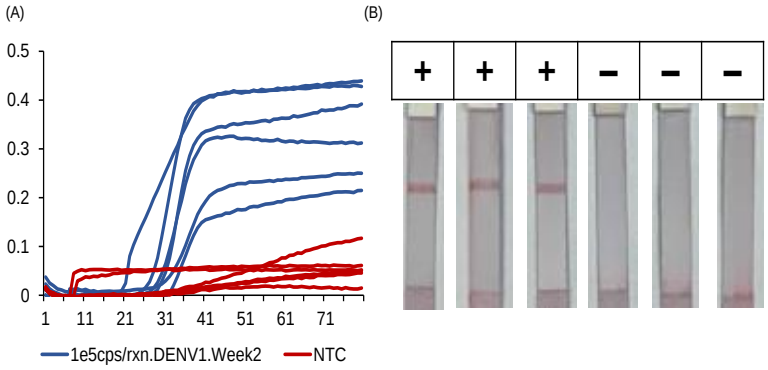


Figure 74. Week 2 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

In the second study we stored the dry reagents for a longer period of time, we tested the dry regents at week 4. Real time fluorescence amplification and lateral flow images of week 4 of the second run of the dry reagent thermostability testing shown Figure 75. The results show somewhat

consistence early amplification for week4, with one real time fluorescence amplification of the negative control that does not show on the lateral flow images.

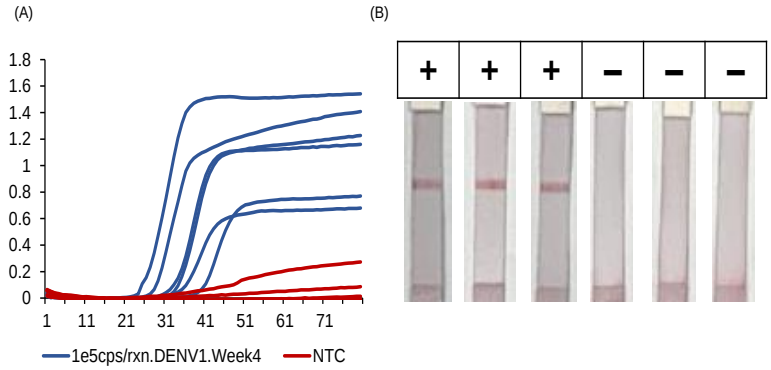


Figure 75. Week 4 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 6 of the second run of the dry reagent thermostability testing shown Figure 76. The results show delay in amplification for week 6, with real time fluorescence amplification of one replicate of a negative control. However, it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

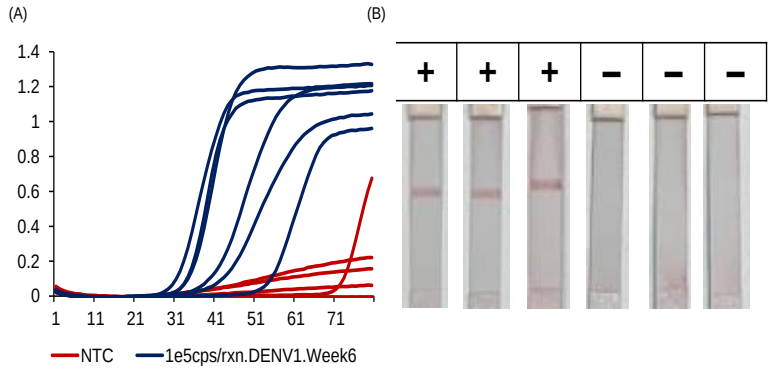


Figure 76. Week 6 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

The second study of the thermostability we stored the dry reagents up to 8 weeks. Results show slightly delayed real time fluorescence amplification of week 8 Figure 77. However no real time fluorescence amplification of a negative control and all positive amplified on the lateral flow strips.

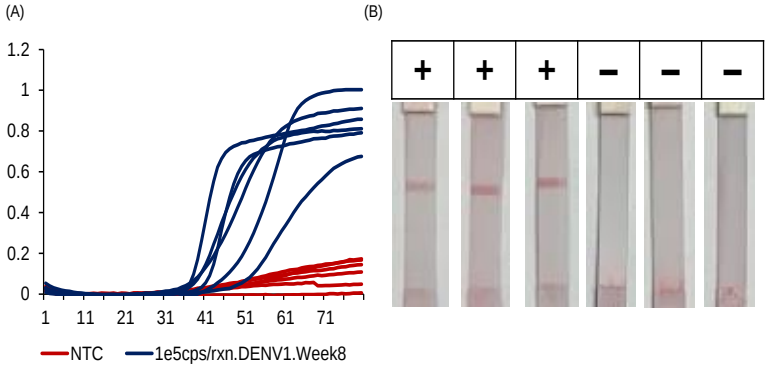


Figure 77. Week 8 Data of the Second Run of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at Room Temperature. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

The third thermostability study we stored the dry reagents at 40ºC up to five weeks. Real time fluorescence amplification and lateral flow images of week 0 shown Figure 78. The results show delay and inconsistency in the amplification. However, all the positive replicates amplified on the lateral flow strips.

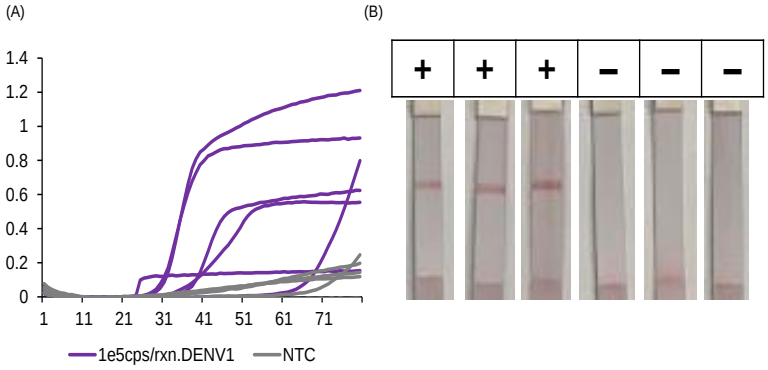


Figure 78. Week 0 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 1 of the dry reagent thermostability testing shown in Figure 79. The results show early amplification for week1, with no real time fluorescence amplification of the negative control.

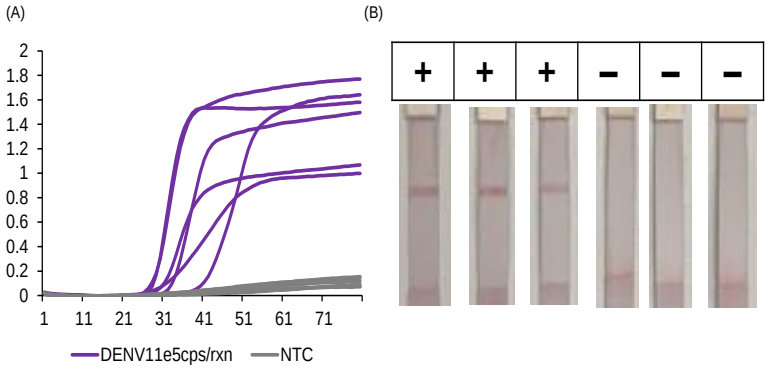


Figure 79. Week 1 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 2 of the dry reagent thermostability testing shown Figure 80. The results show early amplification and lateral flow signals for week 2.

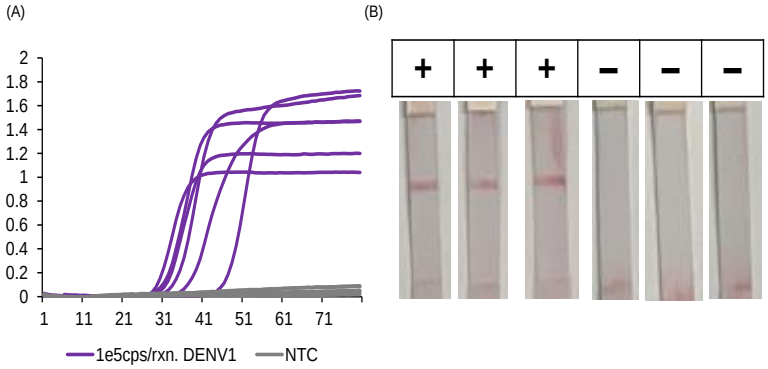


Figure 80. Week 2 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 3(Figure 81). The results show a delay in the amplification for week 1, with real time fluorescence amplification of one

replicate of a negative control. However, it does not show on the lateral flow images, which is thought to be due to non-specific amplification.

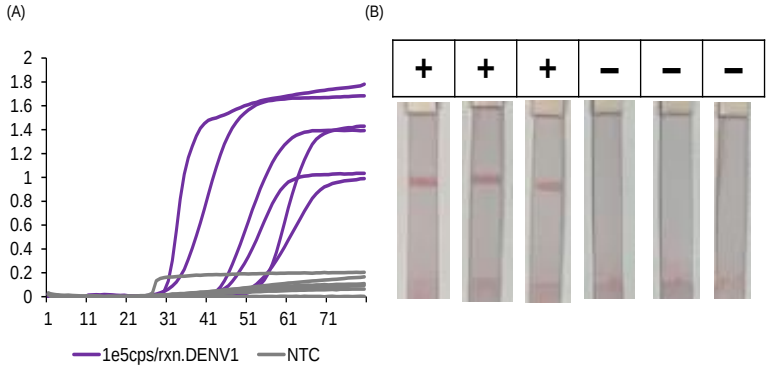


Figure 81. Week 3 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

Real time fluorescence amplification and lateral flow images of week 4 of the dry reagent thermostability testing shown Figure 82. The results show earlier amplification compared to the previous week, with no real time fluorescence amplification of a negative control.

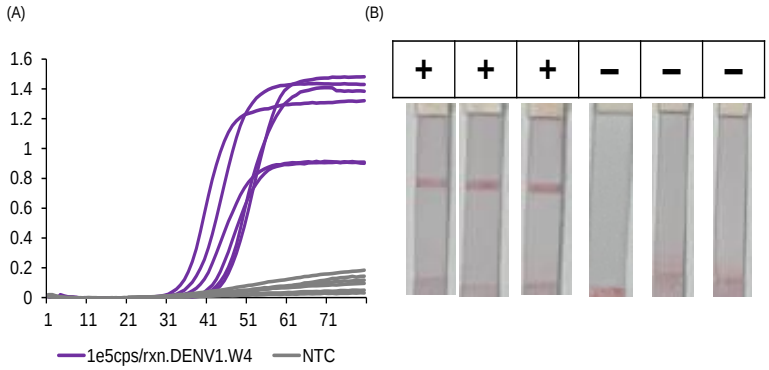


Figure 82. Week 4 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

The last week of the dry reagent thermostability testing shown in Figure 83. An early real time fluorescence amplification and lateral flow images.

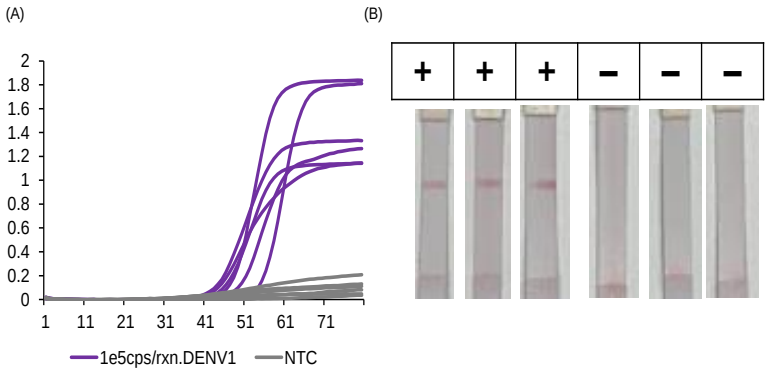


Figure 83. Week 5 Data of DENV1 Thermostability RT-LAMP via Dry Reagent Assay stored at 40ºC. (A) Real time fluorescence detection of DENV1 RT-LAMP via paper assay combined with (B) lateral flow detection of DENV1 at 10e5copies/reaction.

In summary the dry reagent papers are stable at both room temperature and 40ºC until 8 weeks, when tested using 1e5copies/reaction of DENV1. Storing the dry reagent papers at room temperature or 40ºC has no increase in the non-specific amplification in either the real time or LF assays. Although the mean Cq values are higher after 3 weeks at both RT and 40ºC when compared to the starting values of the dry reagent papers at -20ºC, all positive real time reactions were positive on the LF strips.

CONCLUSIONS

Our other major effort is DENV detection in blood; in this effort I focused on amplification of DENV RNA, and full process execution using MS2 as a surrogate. Regarding amplification and detection, I accomplished quantification of DENV RNA via in vitro transcribed standards and proof of principle for a pan-serotype RT-LAMP assay coupled to LF for the detection of DENV4 RNA. In addition, we established an RT-LAMP assay for the detection of MS2 bacteriophage in both liquid and dry reagent format, coupled to LF detection. MS2 will serve as the internal amplification control for our assay. Through a thermo-stability study, we demonstrated that our RT-LAMP dry reagent papers are stable upon storage at room temperature for up to 8 weeks and at 40ºC for up to 5 weeks, with a delay in onset of amplification. The next goals regarding DENV amplification and detection are to optimize the DENV RT-LAMP assay (liquid and dry reagent) and establish suitable LODs for all 4 DENV serotypes. Furthermore, we need to optimize the DENV/MS2 duplex assay (liquid and dry reagent) to attain suitable LODs for DENV and ensure that MS2 amplifies in the absence of DENV. Regarding full process execution, we designed a plasma separation device compatible with integration into the cartridge and ran multiple inhibitor characterization studies. The next step entails full process execution, wherein MS2 is spiked into whole blood which is then processed through the plasma separation device. The plasma is then eluted and used to reconstitute the dry reagent master-mix, followed by amplification and detection. Once this process is suitably optimized, we will transfer the technology to NMRC, who will perform the same experiment with DENV spiked into blood. Next we will design a cartridge that incorporates the plasma separation device, for fully integrated and automated process execution, first with MS2, then with DENV, by itself and as duplex assay with MS2. Eventually,

we will perform studies with clinical samples to determine the attainable clinical sensitivity and specificity.

We believe that the herein described assay processes and prototype systems can make an impact through enabling point of care diagnosis for these and other pathogens in low resource settings.

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