

Point-of-Care Diagnostic Device Development for Multiplexed Detection of Infectious Diseases

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Abstract

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The COVID-19 pandemic highlighted major weaknesses in the global healthcare system for patient accessibility to diagnostics. The pandemic led to a significant increase in demand for respiratory disease testing to facilitate treatment and limit transmission, demonstrating in the process that most existing test options in centralized facilities were too complex and expensive to perform in point-of-care settings. While laboratory-based nucleic acid amplification test (NAAT) assays remained the gold standard of clinical diagnosis, their reliance on complex equipment, cold-chain storage, and trained personnel initially limited their accessibility to testing in well-equipped labs and kept them out of low-resource or home environments. As transmission of SARS-CoV-2 led to national lockdown orders around the world, remote and home testing for infectious pathogens emerged as the new standard in patient care and clinical research. There was a major demand for multi-disease detection tools that could be implemented in point-of-care or home settings and could readily be adapted for new variants or different diseases entirely. In this thesis, I explored the design and development of multiple integrated point-of-care devices to perform simultaneous detection of multiple infectious diseases from a single patient sample. First, we

demonstrated a point-of-care, paper-based rapid analysis device that could simultaneously detect multiple viral RNAs (for COVID-19 and influenza A) that had been spiked onto a commercial nasal swab. This compact and affordable device, enabled by novel valving innovations, not only enabled fast, sensitive detection of both SARS-CoV-2 and influenza A viruses from a single sample, but also opened the possibility for simple applications in other nucleic acid-based detection systems. Next, we adapted the multiplexing UbiNAAT platform for screening of two sexually transmitted illnesses (gonorrhea & chlamydia) in vaginal lysate samples from female clinical patients. We demonstrated the device's compatibility with different assays and sample types, while incorporating additional processing steps for clinical sample treatment. The results highlighted the UbiNAAT's potential for multiplexed screening of clinical samples across a multitude of infectious diseases. Lastly, we explored the development of an at-home testing platform for early-stage HIV using fingerstick blood samples. Toward this end, a novel point-of-care device with onboard plasma separation, chemical lysis, target concentration, isothermal amplification, and an innovative pull-tab valving system was integrated alongside extensive assay development for fluorescence and colorimetric detection. In future work, we anticipate that the findings and technologies developed in this thesis could be employed for a multitude of other targets, sample types, and detection systems that may enable large-scale, sensitive point-of-care testing.

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1. Chapter One - Introduction

1.1 The COVID-19 Pandemic and Limitations in the Diagnostics Landscape

The COVID-19 pandemic was one of the worst pandemics in human history. By the time the World Health Organization (WHO) declared an end to the global COVID public health emergency in May 2023, there were over 768 million official confirmed cases of COVID-19 and 7 million official COVID-related deaths (1). The pandemic's impact was immediately evident worldwide, from nationally imposed lockdowns and commercial shutdowns to expedited vaccine distribution and hesitancy over travel (2,3). The many variants of the SARS-CoV-2 virus that emerged (most notably B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and B.1.1.529 (Omicron)) (**Figure 1.1**) resulted in unprecedented rates of disease transmissions worldwide (4).

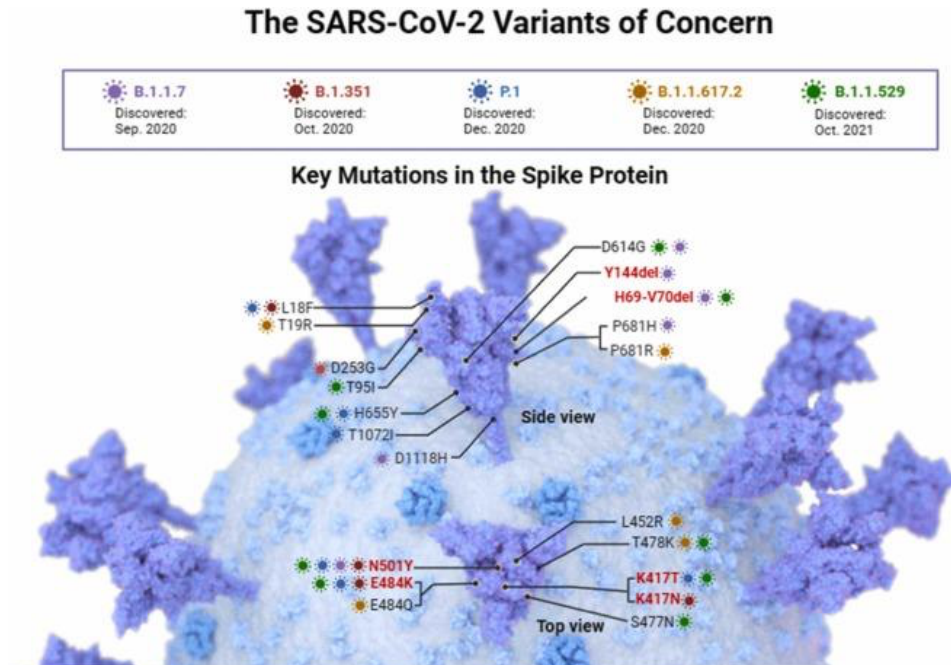


Figure 1.1. Variants of Concern for SARS-CoV-2.

Image from Senevirathne et al. (2024) (4).

Efforts to contain COVID-19 transmission proved especially challenging for healthcare systems globally. Neither healthcare providers nor policy makers were prepared for the strain that the pandemic would impose on even the most well-developed of healthcare systems (5). Even with the vast majority of available resources devoted towards the diagnosis and treatment of SARS-CoV-2, healthcare systems were not able to effectively contain the rate of transmission over the first two years of the pandemic; whereas July 2020 saw an average of approximately 200,000 new daily cases, January 2022 saw over 3.6 million new daily cases as new viral variants continued to emerge (1,5). Amid widespread uncertainty and chaos, national lockdown mandates negatively affected the physical and mental health across populations (6,7). Healthcare workers, such as doctors, nurses, and technicians, reported record prevalence of anxiety, depression, and stress due to massive workloads, inadequate support, and extensive media coverage (6). Similarly, students of all ages also suffered as the switch to online learning, altered means of communication, and uncertainty regarding future developments impacted years of education (7).

Critically, the COVID-19 pandemic exposed major weaknesses in the healthcare system's preparedness and efficacy in providing timely, accessible diagnostics. It became apparent that despite immense resources being poured into rapid development of test kits capable of identifying SARS-CoV-2's many variants, there remained large gaps in adapting these testing options in order to properly address public health needs and improve accessibility; due to the combination of inadequate healthcare staffing and lockdown-imposed regulations requiring negative test results to attend work or school, patients would often wait hours or even days to be tested at hospitals and clinics (8). In the time that a clinic required to test and then notify COVID-positive patients of their status, transmission may have already occurred (**Figure 1.2**) (9). The insufficiency of

centralized testing led to increased demand for accessible testing options, bringing a major shift to the diagnostic landscape.

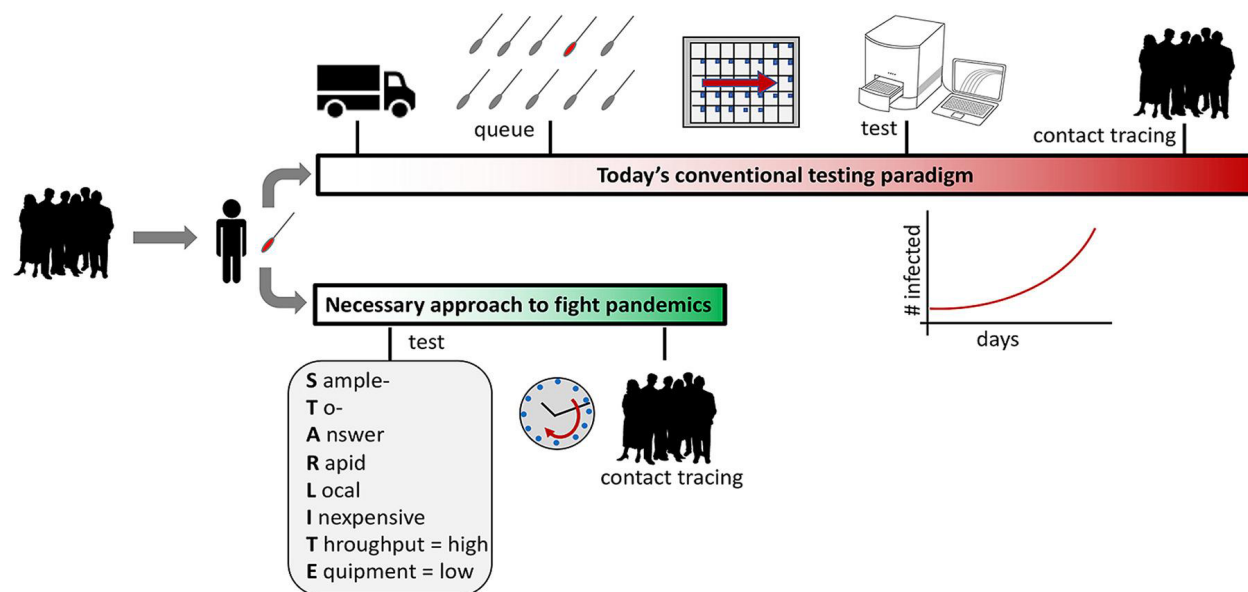


Figure 1.2. Workflow and Limitations of Conventional Centralized Testing

Image from Everitt et al. (2021) (9)

1.2 Demand for Point-of-Care Diagnostics

The need for accessible, fast testing options that could be implemented in both developed and low-resource communities led to a significant increase in demand for point-of-care diagnostic options. While quantitative polymerase chain reaction (qPCR) remained the gold standard of molecular testing, its long time-to-result, complex operations, and dependence on advanced equipment heavily limited screening throughput testing (10). As centralized testing was hindered by prolonged turn-around times and relied on trained personnel, point-of-care testing emerged as a necessary alternative to provide earlier disease detection, reach remote populations, and allow effective isolation of test-positive individuals (9,11). The demand for point-of-care testing was

evident as hospitals and clinics became overwhelmed with COVID-positive patient admits, which potentially endangered healthy individuals as they waited to be tested (11).

During the pandemic, record healthcare investments were made by venture capitalists, large companies, and federal governments to improve COVID screening and treatments. In 2020, healthcare venture capital investments totaled nearly \$17 billion, which more than doubled investments made in 2015 and was nearly 10X the investments made in 2010 (12). Similar efforts were made by U.S. federal agencies such as Biomedical Advanced Research and Development Authority (BARDA), which invested \$160 million by the end of 2020 for both COVID diagnostic and therapeutic development (13). The global effort to facilitate development of diagnostics tools for greater accessibility and outreach led to significant advances in the point-of-care testing capabilities for both COVID and other pathogens. As more tests were developed, the need for testing tools to be simple in operation, high sensitivity, and disease-agnostic in translation became increasingly emphasized.

1.3 Current State of Point-of-Care Diagnostics Testing

During the pandemic, significant efforts were dedicated towards developing point-of-care diagnostics technologies. Antigen-based immunoassays, in particular lateral flow tests (LFTs), steadily rose in popularity due to their ease-of-use in home environments, affordability, and fast time-to-result (14,15). These factors resulted in large-scale distribution of immunoassay test kits by various companies, such as Abbott's BinaxNow COVID-19 Ag card and the Ellume antigen tests (16,17). While antigen-based immunoassays incentivized users to self-test from home, there remained considerable hesitancy over these assays' sensitivity and false positive rates, especially

among non-symptomatic COVID patients (18). Despite their high popularity, antigen-based immunoassays were ineffective for early onset disease detection and still required users to self-quarantine for days before providing a reliable test result.

While antigen-based immunoassays grew in popularity due to their cost-effectiveness and ease-of-use, nucleic acid amplification test (NAAT) assays, notably qPCR, remained the gold standard in regard to sensitivity and accuracy. With financial support from both federal agencies and venture capital funding, many companies have developed NAAT-based point-of-care detection platforms for SARS-CoV-2. Cepheid and Roche both released rapid qPCR kits early in the pandemic as a part of the GeneXpert and COBAS platforms respectively, however the complexity of the test instruments made it difficult for such tests to be performed in home environments (19,20). Visby Medical developed a three-assay rapid RT-PCR respiratory panel disposable device that could simultaneously detect COVID, influenza A, and influenza B (21,22). Other companies employed isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP); LAMP's isothermal activity, fast time-to-result, and high specificity from the use of 4-6 primers made it a prime candidate for point-of-care implementation (23). Lucira Health developed a colorimetric RT-LAMP test kit, the LUCIRA, that enabled fast, sensitive testing of SARS-CoV-2, and also adapted the platform to screen for influenza A and B (24). Cue Health also released an expensive LAMP-based test kit that was shown to have similar sensitivity to the Cepheid GeneXpert (25). The international enthusiasm for point-of-care test innovation, however, was not without controversy; notably, Cue Health's COVID test was later recalled by the FDA due to a significant likelihood of false results (26). Furthermore, while many of these test kits offered a viable solution for remote testing, the high cost and complex instructions of available NAAT-based point-of-care test kits have hindered their widespread adoption. To address current point-of-

care testing needs, detection systems must not only be accurate, sensitive, and specific, but also affordable, simple-to-use, and multiplexable for disease-agnostic applications.

1.4 Growing Trend of Multiplexed Disease Testing

During the pandemic, similarities in symptoms between COVID and other respiratory diseases such as influenza and respiratory syncytial virus (RSV) led to an emphasis on the development of multiplexed disease detection technologies (27). With many illnesses sharing common symptoms, it is critical that patients can properly identify their disease to receive appropriate treatment and self-quarantine if necessary. Following the end of the COVID-19 public health emergency, testing strategies turned to distinguishing SARS-CoV-2 infection from the rising transmission of influenza and other respiratory illnesses (28). Toward this end, many companies have invested in developing multiplexed detection systems capable of identifying multiple illnesses from a single patient sample. Recently, Visby Medical's three-assay rapid RT-PCR respiratory panel for detecting COVID, influenza A, and influenza B received FDA clearance and CLIA certificate of waiver (29). BD (Beckton, Dickinson and Company) developed a 3-assay antigen-based test, the Veritor, that demonstrated high specificity for each assay (30). The trend towards multiplexed detection systems has further influenced early-stage diagnostic companies. Anavasi Diagnostics, a UW spinoff company that launched through NIH RADx funding, developed a fast, low-cost, tube-based RT-LAMP assay that could detect SARS-CoV-2 within 20 minutes, but aimed to adapt its platforms towards other viral and bacterial diseases such as influenza (flu) and strep throat (31,32). As point-of-care testing solutions progress, it is critical that new platforms embrace multiplexed, multiplexed diagnostic approaches that could readily differentiate between multiple pathogens from a single patient sample.

1.5 Summary of Issues Addressed in This Work

In summary, point-of-care testing development during the COVID-19 pandemic has led to significant advances in the current diagnostic landscape. Nevertheless, many solutions continue to be limited in their application and adoption due to high cost, complexity, and challenges with multiplexing. It is important for detection tools to be simple and low-cost so that diagnostic applications can be readily implemented in remote or home environments with minimal user intervention and multiplexing capabilities. The remainder of this dissertation will focus on the development of technologies that enable low-cost, integrated, portable, multiplexable, and autonomously operated detection of infectious diseases.

Chapter 2 presents a point-of-care, paper-based rapid analysis device that simultaneously detects viral RNAs from two common respiratory viruses (SARS-CoV-2 and influenza A) spiked onto a commercial nasal swab. Chapter 3 provides an in-depth analysis of valve implementation in paper-based microfluidic systems through the design and integration of two unique and adaptable thermally actuated valves for application in a point-of-care rapid analysis device. Chapter 4 describes the detection of two common bacterial sexually transmitted illnesses, *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG), from clinical vaginal swab samples in an inexpensive point-of-care integrated device. Chapter 5 illustrates assay development and device design in an ongoing research project to develop an at-home HIV test device that utilizes sample concentration to enable sensitive viral detection from a fingerstick sample. Chapter 6 explores challenges, troubleshooting, and other lessons learned from past and ongoing experimental work with the aim of providing guidance for future work. Finally, Chapter 7 outlines conclusions, impact, open-ended questions that have yet to be answered, and future directions. I anticipate that the collection of technologies, methods, and detection capabilities presented here will facilitate the

progression of next-generation point-of-care diagnostics and offer valuable guidance towards translation to remote or home applications.

In this thesis, I will also be referencing price points of current diagnostic testing, such as rapid antigen tests and NAAT platforms. Having previously worked as an engineer at Cepheid, an in vitro diagnostic company, prior to starting my PhD studies, I initially expected that pricing for COVID test kits would be based on research and development, manufacturing, marketing, and other internal costs. However, the COVID pandemic had set unprecedented market trends with regards to price points, as many companies produced COVID test kits in response to the skyrocketing demand for test development, record investments into healthcare, and facilitated regulation approval through the use of emergency use authorizations. Price points for many commercial tests were also difficult to determine. This is due to many federal governments providing subsidies and contracts to test makers, resulting in faster product development and wider distribution to patients worldwide (3,5,8). Therefore, it is possible that price points for test distribution in regions with governmental financial assistance were different than regions without governmental assistance. Additionally, many commercial test kits, particularly NAAT-based platforms such as the Visby Medical test kit, were not approved by the FDA for home use (21,22). These test kits were distributed for use by health professionals only, and were implemented in public spaces, such as airports. Therefore, while development costs certainly could have contributed to the high price points of many current testing products, it is important to consider the international context of governmental subsidies under which these tests were distributed that may not be applicable in future public health crises.

2. Chapter Two - UbiNAAT: Multiplexed Point-of-Care Nucleic Acid Diagnostic Platform for Rapid At-Home Pathogen Detection

The work in this chapter was published in Lab on a Chip (Jiang *et al.*, 2024) (33) and is reprinted with permission from the Royal Society of Chemistry.

2.1 Abstract

The COVID-19 pandemic increased demands for respiratory disease testing to facilitate treatment and limit transmission, demonstrating in the process that most existing test options were too complex and expensive to perform in point-of-care or home scenarios. Lab-based molecular techniques can detect viral RNA in respiratory illnesses but are expensive and require trained personnel, while affordable antigen-based home tests lack sensitivity for early detection in newly infected or asymptomatic individuals. The few home RNA detection tests deployed were prohibitively expensive. Here, we demonstrate a point-of-care, paper-based rapid analysis device that simultaneously detects multiple viral RNAs; it is demonstrated on two common respiratory viruses (COVID-19 and influenza A) spiked onto a commercial nasal swab. The automated device requires no sample preparation by the user after insertion of the swab, minimizing user operation steps. We incorporated lyophilized amplification reagents immobilized in a porous matrix, a novel thermally actuated valve for multiplexed fluidic control, a printed circuit board that performs on-device lysis and amplification within a cell-phone-sized disposable device. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) products are visualized *via* fluorescent dyes using a modified cell phone, resulting in detection of as few as 10^4 viral copies per swab across both pathogens within 30 minutes. This integrated platform could be commercialized in a form

that would be inexpensive, portable, and sensitive; it can readily be multiplexed to detect as many as 8 different RNA or DNA sequences, and adapted to any desired RNA or DNA detection assays.

2.2 Introduction

During the COVID-19 pandemic, remote and home testing for infectious agents emerged as the new standard in patient care and clinical research. While medical laboratories and clinics continue to address testing demands through quantitative polymerase chain reaction (qPCR) assays, these tests have not so far been scalable to meet the need for extensive public health screenings due to the need for complex equipment and trained personnel (34,35). As commercial point-of-care testing options that offer high sensitivity and specificity developed during the pandemic, these test options were costly and inaccessible for frequent use with limited readiness to be multiplexed with additional assays (36,37). For patients who are pre-symptomatic or asymptomatic for respiratory illnesses, there is a continuing need for a multiplexable diagnostic platform that is more sensitive than antigen tests, adaptable to new pathogens, and nearly as affordable as lateral flow tests for daily use in the home. Here, we describe a new platform that meets these needs, and demonstrate its ability to detect at least two respiratory pathogens (including influenza A and SARS-CoV-2) from a nasal swab. This isothermal nucleic acid amplification platform can be readily expanded to include 8 or more assays, with samples in the form of various human bodily matrices. Quantitative polymerase chain reaction (qPCR) assays remain the gold standard for molecular testing, yet their limitations have bottlenecked the global demand for rapid, multiplexable testing (34,35,38,39). While highly accurate and sensitive, qPCR tests suffer from long sample-to-result time, low throughput, complex operation, and high cost (40–42). The highest sensitivity for COVID testing has been achieved using in-lab qPCR and digital droplet PCR (ddPCR) kits at <10 copies RNA

per test sensitivity (43). However, in-lab PCR protocols require trained personnel and complex equipment, and researchers have also noted that COVID-19 qPCR tests may not detect new viral strains (40,44,45). As cases of other endemic respiratory illnesses such as the flu, common cold, and RSV begin to rise worldwide, there also emerged a growing need to distinguish between respiratory illnesses with common symptoms.

Significant efforts have been dedicated towards implementing point-of-care diagnostic technologies in home and remote settings. Antigen-based immunoassays, such as the Abbott BinaxNOW COVID-19 Ag card and the Ellume antigen tests, steadily rose in popularity due to their ease of use at home and affordability, despite substantially poorer sensitivity than qPCR tests (16,18,46). When a viral load lower than 10^5 SARS-CoV-2 RNA copies per mL is applied, antigen tests perform at less than 40% sensitivity (47). BD's 3-assay antigen-based test, the Veritor system, was shown to have high specificity for each assay but demonstrated significantly lower sensitivity compared to qPCR systems (48). Other groups explored continuous monitoring options, such as the use of electrochemical biosensors for antibodies or identifying single nucleotide polymorphism through CRISPR-Cas12 (49,50). Isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and isothermal strand displacement amplification (iSDA) have emerged as attractive alternatives to qPCR (19,20,42). LAMP has been shown to be capable of highly sensitive and specific amplification of target DNA and, with the addition of a reverse transcriptase, RNA; this is achieved by utilizing four to six primers. Its isothermal activity, which eliminates the need for thermal-cycling, plus the fact that the basic LAMP assay is off-patent, have made it a prime candidate for low-cost diagnostics and analysis for point-of-care implementation (23,24,51,52). While LAMP assay

sensitivity is generally lower than that of qPCR, its compatibility with point-of-care device design has encouraged many groups to pursue LAMP-based diagnostics for infectious diseases (53,54).

Many companies released commercial molecular test kits during the COVID-19 pandemic for clinical and point-of-care (POC) use through the FDA's emergency use authorization (EUA); these systems served as early testing benchmarks (19–22,55). While Cepheid's PCR-based GeneXpert system is widely regarded as a leader in POC testing and serves as the benchmark for other test development, other companies have also released test kits for single-assay and multi-assay detection (19,20,55). Cue Health's isothermal nucleic acid amplification tests (NAATs) was initially demonstrated to yield similar sample sensitivity compared to Cepheid's GeneXpert, yet have been limited in widespread adoption, in large part because of the high cost of their disposable (25). Lucira Health initially developed an RT-LAMP POC platform for COVID testing, before expanding their test platform into a multiplexed platform that simultaneously detects SARS-CoV-2, influenza A, and influenza B (24,56). Visby Medical's 3-assay POC respiratory panel for COVID, influenza A, and influenza B enabled qualitative detection of multiple respiratory illnesses from a single patient sample, which could be operated in a CLIA-waived setting (21,22). These leading COVID POC tests currently enable 10³ viral copy detection from a human nasal swab within 30 minutes, which has set the standard for COVID testing (37,40,57). However, these tests are often expensive, ranging from \$75-\$175 per test, and may include complex instructions for its operation (56). Paper-based NAATs have emerged as an affordable, scalable platform for point-of-care diagnostics, with many groups utilizing porous matrix to control flow, concentrate reagents, and enable sequential delivery within an assay without the need for complex equipment (51,52,58–62). Researchers have also explored methods for enabling long-term storage of reagents

for POC application, such as lyophilization (60,63,64), and vitrification (51,65). With nasal matrix and saliva serving as standard sample matrices for COVID testing (44), inactivation of inhibitors, such as RNases, became critical, with proteinase K (66–69) and RNase inhibitors (e.g. RNasin Plus) (23,70,71). To facilitate portable, quantitative measurements, cell phones have emerged as ideal tools for providing low-cost, high image quality for both fluorescence and colorimetric-based assays, though color gradient, sensor capabilities, and gamma correction differences have been noted (64,72–74). While laboratory PCR tests and rapid antigen assays supported a large demand for testing during the COVID-19 pandemic, there is a growing need for a platform that can readily be expanded to incorporate new assays as new pathogens appear, and numerous respiratory illnesses circulate in parallel. Rapid and decentralized solutions that are inexpensive, simple, and scalable enough for frequent screenings of large numbers of asymptomatic individuals will continue to be needed (75).

In our lab's previous work, Lafleur *et al.* (2016) demonstrated the MAD NAAT (for multiplexable autonomous disposable nucleic acid test), an instrument-free rapid diagnostic tool using isothermal strand displacement amplification (iSDA), and Kumar *et al.* (2020) showed that amplification reagents can be freeze-dried and stored long-term under ambient conditions (63,76). The lab also developed the MD NAAT, a version of the MAD NAAT platform using USB-powered printed circuit board (PCB) and 2D paper network (2DPN) for bacterial lysis and amplification that could be coupled with a cell phone reader that is compact and simple to use (76–81). (the A in MAD was removed because this device was not truly “autonomous”). The incorporation of paper-based networks, storage of amplification-based reactions, and cell phone readers has also been utilized by other research groups, who developed POC platforms for detecting various infectious diseases (42,51). This thesis describes an integrated sample-to-result paper-based UbiNAAT (Ubiquitous

NAAT) platform with a USB-powered PCB for onboard lysis, fluid control, and amplification that is accurate, fast, and sensitive. We demonstrate its ability to detect at least two respiratory pathogens (including influenza A and SARS-CoV-2) from a nasal swab through LAMP amplification and signal captured through a cell phone-based reader. This addresses a current limitation in multiplexed testing (82–84), as there does not currently exist an inexpensive, quantitative platform that could be administered in home environments. This platform does not require sample preparation by the end user. To inactivate endogenous RNases, we have developed an automated, in-device protocol based on the HUDSON method (85,86). To achieve our point-of-care aims, the UbiNAAT has the following four characteristics: 1) no sample preparation by the user following nasal swab insertion, 2) dried storage of RT-LAMP reagents in a glass fiber, Quartz microfiber grade A (QMA) matrix, 3) autonomous control of the fluid from a blister for dissolution of the nasal matrix, sample lysis, and amplification, and 4) a compact form factor that fits in the palm of one's hand. While our current lab-based device iteration relies on a cell phone for creation of an image stack for monitoring amplification, a laptop for power, and MATLAB script for analysis, future versions can be built around a standalone, wall-powered imaging hub.

2.3 Materials and Methods

The RT-LAMP assay reagents consist of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), SYTO-82 fluorescent intercalating dye (Thermo Fisher Scientific, Waltham, MA), hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA), trehalose (Life Sciences Advanced Technologies), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham, MA), and assay-specific LAMP primers. Six primers were used for COVID-19, respiratory syncytial virus (RSV), and POP7 human DNA LAMP assays, and

8 primers were used for influenza (Flu) A RT-LAMP assays, with all sets of primer sequences acquired from literature (Integrated DNA Technologies, Skokie, IL) (87–90). (Primer sequences shown in **Tables 1.1-1.4**). Purified synthetic genomic RNA for SARS-CoV-2 (Twist Biosciences, San Francisco, CA) and Flu A (ATCC, Manassas, VA) were purchased. SARS-CoV-2 virus was heat-inactivated by ATCC before purchase. Dilutions for RNA and heat-inactivated virus conditions were performed with TE buffer (10 mM Tris, 0.1 mM EDTA). For the HUDSON (“heating unextracted diagnostic samples to obliterate nucleases”) solution, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Millipore Sigma) and ethylenediaminetetraacetic acid (EDTA) (Invitrogen by Life Technologies) were acquired to prepare an inactivation solution for nasal swab matrix RNases (86).

Reverse transcription loop-mediated isothermal amplification (RT LAMP) assay in-tube and 5-pad QMA matrix

Commercially purified genomic RNA stocks for SARS-CoV-2 and Flu A were each diluted to 10^5 copies per microliter, and heat-inactivated viral stocks were each diluted to 10^4 copies per microliter. Reaction “master mixes” were prepared in PCR tubes at 20 μ L volume. For in-tube reactions, 1 μ L of template was combined with 19 μ L of master mix (**Table 2.5**). Once combined and homogenized by vortex mixer, the PCR reaction tubes were placed into a BioRad Real-Time Thermocycler (CFX Opus) and RT-LAMP was performed at 63°C for 60 minutes. For in-tube reactions, signal liftoff times were determined using the CFX Maestro, which applied a multivariable, nonlinear regression model to determine initial amplification. For in-pad reactions, signal liftoff times were determined by steep-slope amplification signal rising above a defined fluorescence threshold.

To evaluate the performance of RT-LAMP in porous matrices, devices were built that enclosed 5 identical porous pads side-by side. RT-LAMP was performed in separated Whatman quartz fiber membranes (QMA) from Cytiva (VWR, Radnor, PA) inside of 33 mm x 25 mm x 1/16 in polymethyl methacrylate (PMMA) devices (“5-pad devices”). For these and all other amplification devices including the UbiNAAT prototypes, the major tool for machining polymer pieces, films, and paper-like materials was a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ). The bottom PMMA shell held 5 parallel channels (16.7 mm x 3.3 mm) that had been laser-etched to a depth of 600 µm; the top enclosure part had a vent at each end of the channel to allow insertion of fluids and escape of air. Polydimethylsiloxane (PDMS) pressure-sensitive tape (Valley Industrial Products, Huntington, NY) was applied between the top and bottom tray parts and one QMA pad was placed in each channel. The top PMMA casing was then applied, and the device was pressed together using a clamp. QMA membranes (1851-150) were laser-cut to 15 mm x 3 mm strips with a fluid capacity of 25 µL. They were blocked by soaking in a solution of 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) in nuclease-free water for an hour before being drained of fluid and dried overnight in a 40°C oven. After filling with 25 µL of solution (Table S5), the vents at both end of each fiber-pad-filled channel were sealed using Microseal B adhesive sealer (BioRad, Hercules, CA) and the sealed devices were placed into a custom-made, dual-sided heating oven with an ITO glass top heater (**Figure 2.6**) and incubated at 64°C for 60 minutes.

RT LAMP Lyophilization and Storage

Lyophilization protocols were performed as described in our previous work (63). In short, 1% BSA, 0.1% Tween-20-coated QMA pads, held in a 6-well plate, were each hydrated with 24 µL

of RT-LAMP reaction mix (no template) that contains 10% trehalose and 0.5% dextran (500 kDa). The apparatus was quickly frozen by insertion in liquid nitrogen for 1 minute before being placed into a fast-freeze flask (Labconco) and attached to a FreeZone 2.5 liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, Mo) for overnight drying with the system cold finger set at -47°C and the vacuum at 0.03 mbar. For lyophilizing reaction tubes, PCR tubes containing 19 µL reaction mix with holes poked into the caps were dipped into liquid nitrogen for 1 minute, then placed into a 96-well aluminum tube holder (Qiagen) prior to attachment to the lyophilizer.

Nasal Swab Collection

Nasal material was self-collected from the anterior nares by healthy volunteers using Rhinostic polypropylene swabs (Rhinostics, Boston, MA) (66,91) following University of Washington institutional review board study protocol 00011163. These swabs were chosen for their small fluid volume retention of 20-25 µL; previously used flocked swabs (e.g. Copan ClassiQ swabs) retained 130-150 µL fluid, which unnecessarily diluted the samples. Each volunteer collected two swabs per collection event, one from each naris. One swab was screened for the presence of SARS CoV2 RNA using either 1) qRT-PCR (Invitrogen RNA Ultrasense one-step kit) or 2) RT-LAMP kit (NEB) and compared against positive COVID RNA standards. When SARS CoV2 RNA was detected (Ct prior to 34 cycles for qRT-PCR or prior to 50 minutes for RT-LAMP) from the control swab, the other swab from the same volunteer was discarded. SARS CoV2 detection events were not reported to volunteers. Prior to testing in the UbiNAAT device, swabs were stored at 4°C.

Cell Phone Fluorescence Reader and Image Acquisition

Similarly to the protocol described in Shah *et al.* (2023), a Google Nexus 5X phone was fitted with a custom black PMMA fixture holding two interference filters: a FES0550 emission short-pass

filter (Thorlabs) and BP 587/25 excitation filter (Zeiss) (80). A 12 mm wide, 12 mm focal length plano-convex lens (Edmund Optics) was placed 6 mm from the light-emitting diode to tighten the focus of the excitation light (**Figure 2.7**). The bandpass and short-pass filters were chosen to optimize detection using SYTO-82. The phone was fixed to a stand that was 8.5 cm above the amplification device. Images were taken every 60 seconds using the phone's incandescent white balance setting, 1/5 aperture, 200 ISO, and was manually focused to 8.5 cm from the lens. Image stacks collected from each run ("image stacks") were sent *via* USB connection to a desktop computer for analysis.

PCB Temperature Control and Resistive Heating

The PCB was previously developed by the Yager lab for application to bacterial lysis and subsequent nucleic acid amplification (80). Here, it was programmed using TeraTerm (Tera Term Project, Japan) to perform a series of automated heating steps that lysed the sample, fluidically transported the sample to the amplification pads, and heated the amplification pads to LAMP operating temperatures. First, the lysis heaters were activated at 100% power for 7 min, which was determined to heat the sample to above 95°C for 5 min. Next, all heaters were turned off for a 3-minute cooling step that allowed the sample to cool to approximately 65°C; this was instituted to prevent denaturing enzymes when the sample enters the amplification pads. Then, the valve heater was activated at 100% power for 55 seconds, which was determined to consistently melt the wax in that valve and allow the sample to flow into the amplification pads. Finally, the amplification heater was run using a PID loop to maintain a temperature setpoint of 71.5°C, which was determined to maintain an amplification pad temperature of 63°C. Thermal validation was monitored via T-Type thermocouples (Omega, Norwalk, CT) in the amplification zone, as shown in **Figure 2.14**.

2-plex UbiNAAT assembly and operation

The UbiNAAT disposable device (**Figure 2.1**) is composed of 3 major components: the RT-LAMP microfluidic components, 3D printed enclosure, and custom printed circuit board. A labelled schematic for the enclosure and internal device is shown in **Figure 2.2**. In our laboratory tests we reuse both the enclosures and the printed circuit boards, which do not become contaminated with amplicons in use. The RT-LAMP microfluidic system device consists of a lysis chamber, buffer-

holding plastic blister, 2D paper network, amplification pads, and valve in a PMMA casing. The lysis chamber was assembled by bonding three layers of 3/8 in. PMMA (McMaster-Carr, Elmhurst, IL) with methylene chloride (Fisher Scientific, Hampton, NH), and is shrouded with copper tape to facilitate heat conduction. The blister was assembled by vacuum forming 0.25 mm thick PETG (Vaquiform, Burbank, California), then filled with 540 μ L of TE buffer containing 2.5 mM TCEP and 1 mM EDTA. The 2D paper network is made up of 0.02% Tween-20-blocked 8950 glass fiber (“glass fiber connector”) (Ahlstrom, Helsinki, Finland); it connects the lysis chamber to the amplification pads. A fluidic shunt, made of untreated QMA membrane, sits perpendicular to the middle of the glass fiber connector; it is included to capture un-lysed sample fluid and prevent it from entering the amplification zones. The two amplification pads, consisting of QMA membranes that have been blocked by 1% BSA and 0.1% Tween, were lyophilized after wetting with RT-LAMP master mix for either COVID or Flu A assays. At the terminal end of the amplification pads sits a valve composed of dental wax (melting temp 65°C) (Electron Microscopy Sciences, Hatfield, PA), termed an “air spring valve,” which confines air within the 2D paper network to block fluid flow into the amplification pads prior to valve thermal activation and drainage of the wax into a QMA-based wick. To prevent reagent leakage and hold in air heated during operation, the 3D printed shell fully encloses the internal device, and the region between the amplification pads and the wax valve is covered with Tyvek membrane (DuPont, Wilmington, DE), which prevents viral lysate solution from moving towards the air vents (**Figure 2.13**). The glass fiber network, fluidic shunt, amplification pads, valve, and wax wick sit between two layers of PMMA that are bonded by PDMS tape.

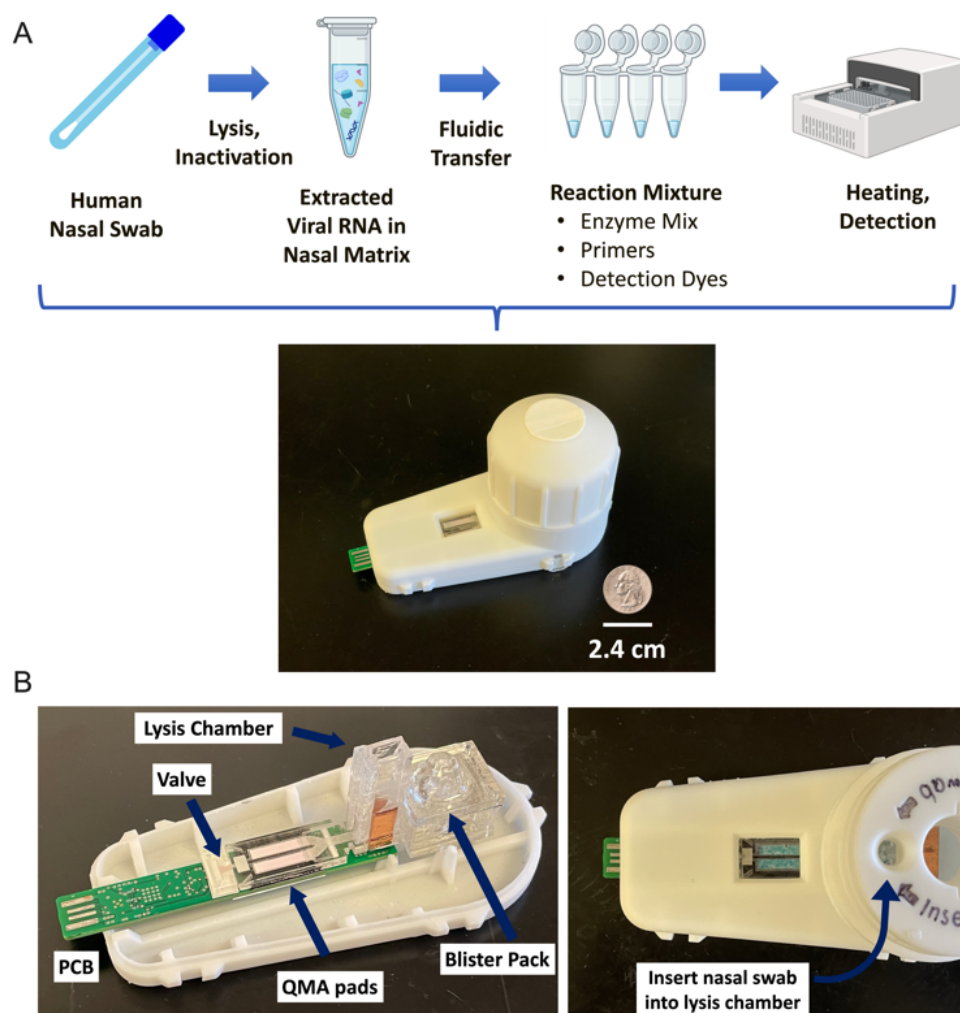


Figure 2.1 Translation of benchtop diagnostic assay to integrated device.

(A) Incorporating standard lab assay protocol into a portable, automated device (B) Device without top enclosure (left) and device without cap, with opening for swab insertion into lysis chamber (right)

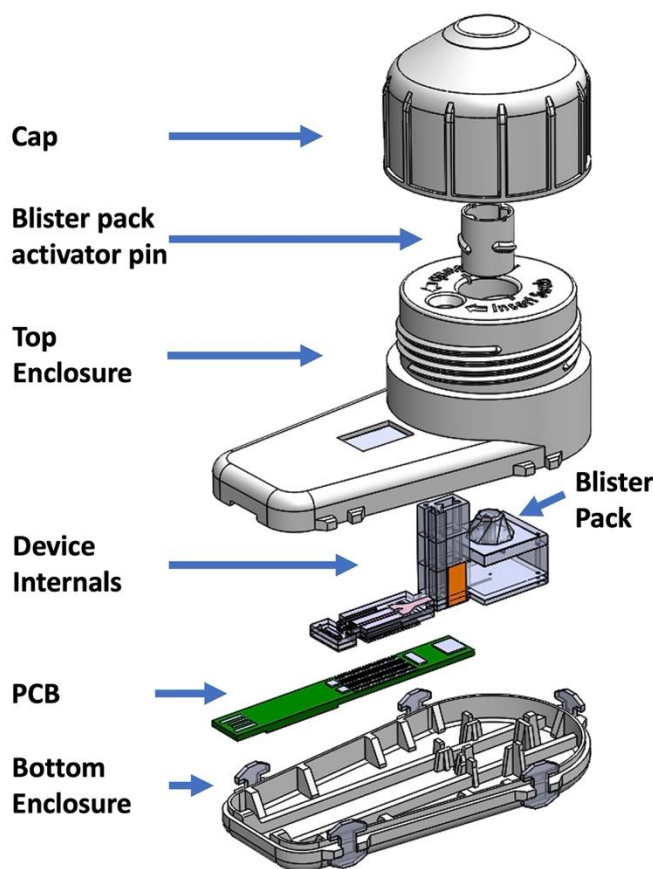


Figure 2.2 Schematic of full prototype UbiNAAT device, including outer enclosure assembly and device internal components. (Figure by Steven Bennett)

The device enclosure consists of 3 major components (cap, top tray, and bottom tray) that were fabricated using a stereolithographic (SLA) 3D printer (Formlabs, Form 2) employing their standard white resin (Formlabs), with a layer height of 0.05 mm. A 10 mil thick Mylar window (Fralock, Valencia, CA) was affixed to the top tray with PDMS tape to allow imaging the amplification pads while limiting heat and vapor loss. After the cap is unscrewed, a self-sampled nasal swab was inserted into the designated opening (see **Figure 2.1B**) and the cap was reattached. The cap held an activator pin that moved with the rotation of the cap, compressing the blister when the cap was unscrewed and pushing 300 μ L of solution into the lysis chamber (see **Figure 2.11**).

The device was then transferred to a dark room, placed under the cell phone reader, and connected to a laptop *via* a USB cable. A preloaded PCB script was initiated using TeraTerm. First, the device underwent 7 minutes of lysis chamber heating set at 100°C, followed by 3 minutes of passive cooling. The wax valve was then activated *via* resistive heating, during which the swab lysate fluid wicked through the glass fiber pathway and rehydrated the QMA reaction pads. As each pad held ~25 μ L volume, there was a 10-fold dilution of the swab's initial 10^4 viral load, resulting in 10^3 viral copies per reaction pad. Upon QMA pad rehydration and reagent resuspension, the phone was manually initiated to start imaging at one-minute intervals. Following 60 minutes of imaging, the device was disconnected from the USB cable. The cell phone was then connected to a desktop computer, where the image stack was exported for data analysis.

Preliminary Testing of 8-plex PMMA Device

Similarly to the 5-pad QMA matrices, the 8-plex device was fabricated using 33 mm x 25 mm x 1/16 in polymethyl methacrylate (PMMA) layers (see **Figure 2.5A**) that were laser-cut on a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ) and bonded together using PDMS pressure-sensitive tape (Valley Industrial Products, Huntington, NY). The bottom PMMA shell held 8 separate channels that had been laser-etched to a depth of 600 μ m; the top enclosure part had a vent at each end of the channel to allow insertion of fluids and escape of air. QMA membranes (1851-150) were laser-cut to trapezoidal strips with a fluid capacity of 25 μ L. They were blocked by soaking in a solution of 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) in nuclease-free water for an hour before being drained of fluid and dried overnight in a 40°C oven. RT and DNA-based LAMP master mixes were individually lyophilized into the one of the assay pads in the 8-plex

PMMA bottom layer for COVID, RSV, influenza (Flu) A, or POP7 human gene assays (n=2 per assay). Template solutions containing 2×10^4 copies of SARS-CoV2 inactivated viruses, RSV inactivated viruses, flu viruses, and human DNA were prepared in 500 μ L TE buffer, lysed at 95°C for 5 minutes in a BioRad thermocycler, and used to simultaneously resuspend all eight pads through a single opening that uses an eight-channeled 8950 glass fiber connector to reach each reaction pad. The inclusion of the POP7 human gene served as an internal processing control to ensure adequate swabbing of the anterior nares. The device was sealed using Microseal B adhesive sealer (BioRad, Hercules, CA) and the sealed devices were placed into a custom-made, dual-sided heating oven with an ITO glass top heater (**Figure 2.6**) and incubated at 64°C for 60 minutes.

Data Analysis

Data analysis was performed using a custom MATLAB script previously described in Shah *et al.* (2022) (81). The intensity of selected regions in each QMA pad during amplification were quantified by measuring the intensity changes from frame to frame of the top 1% of pixel intensity within the selected pad's region of interest (ROI) (80,81). The average background intensity from minutes 5-10 (presumably before any possible liftoff) was subtracted from each image. At each minute over the total one-hour period, the top 1% of the most rapidly brightening pixels within each ROI image were converted to a fluorescence intensity value to generate curves of the fluorescence intensity over time ("amplification curves"). Per Shah *et al.*, the liftoff time was defined as the time-to-threshold (set at 0.02 RFU) for both positive and negative samples in QMA-based RT-LAMP assays (81).

2.4 Results and Discussion

2.4.1. UbiNAAT Design Optimization

While many devices detect SARS-CoV-2 in both centralized and point-of-care settings, decreasing COVID transmission numbers worldwide have brought increased focus to rising infection rates of other respiratory illnesses with similar symptoms, such as influenza and RSV. The demand for increased multiplex functionality has encouraged numerous companies to produce multi-assay detection tools, yet high costs in development and multiplexing limit their applications in low-resource communities. To address this issue, we developed the UbiNAAT prototype using cost-effective materials and technologies that enables complex fluidics control and affordable multiplex expansion, as shown in **Tables 2.6-2.7**. To minimize user steps and potential errors, the device integrates and automates a standard assay operation, as shown in **Figure 2.1A**: (1) resuspending a nasal swab in a fluid, (2) extracting viral RNA from the virions *via* heat or chemical lysis, (3) mixing the freed RNA with amplification reagents, and (4) supporting the amplification and allowing visualization of the detection chemistry. In doing so, the device addresses current centralized testing limitations, such as the need for sample preparation, complex equipment, and protocol-trained personnel. While the results of this study were collected in a controlled laboratory environment and included manual operation of the cell phone reader, laptop, and data analysis software, we intend to integrate these procedures into a low-cost imaging hub for future designs to collect and transmit images for remote analysis. The prototype has far more parts than a commercial version would have, and many laser-cut and 3D printed parts would be consolidated and converted to injection-molded parts.

As indicated in **Figure 2.1**, the UbiNAAT prototype incorporates lysis, sample prep, fluidic transport, and amplification into a compact form (see **Figure 2.1A** for scale). When the cap was unscrewed, the fluid-containing blister pack beneath the circular cap was burst and compressed, pushing into the lysis chamber 300 μ L of lysis buffer with HUDSON reagents (2.5 mM TCEP, 10 mM Tris, 1.1 mM EDTA). As illustrated in literature and **Figure 2.12B**, this HUDSON solution has been shown to adequately inactivate endogenous ribonucleases and associated RT-LAMP inhibitors from nasal and other human matrices, enabling amplification of recovered viral RNA (85,86). As shown by Rabe *et al.* (2020), the addition of TCEP and EDTA to nasal matrix and heating to 95°C denatures proteins, reduces disulfide bridges and sequesters divalent cations; as a result, RNase enzymes are rendered inert (86). After the swab is inserted into the designated opening, it is lowered directly into the lysis chamber, which holds lysis buffer from the blister pack. As shown in **Figure 2.12**, HUDSON solution remains stable for over 2 weeks at room temperature at working concentration without noticeable loss of efficacy for inactivation of nasal RNases compared to 4°C-stored HUDSON solution in Flu A RT-LAMP, allowing for flexible storage of the device under ambient conditions. This is particularly favorable for point-of-care application compared to methods such as proteinase K and RNasin Plus, which require freezer storage. While this study only evaluated ambient storage of HUDSON up to 15 days, it is possible that HUDSON solution may remain viable for longer periods at ambient conditions.

Every part of the prototype device, aside from the vacuum formed fluid container and PCB, was produced by either laser cutting or 3D printing. The fluidic path from the lysis chamber to the amplification pads is composed of glass fiber pads. Lysed sample is conducted by 8950 glass fiber to the two amplification pads by diverging into a Y-shape, as shown in **Figure 2.13**. We chose

8950 glass fiber for its low capillary pressure, allowing liquid to be released rapidly into the two QMA amplification pads. In the prototype demonstrated here, there were only two pads used, one each for SARS-CoV-2 and Flu A assays. We were limited to 2 pads (and assays) by the width of the PCB—widening it and the area of its heating zone to accommodate more independent amplification pads would allow the UbiNAAT to perform several more assays.

2.4.2 RT-LAMP assay optimization

In freshly prepared in-tube reactions, both RT-LAMP assays lifted off from baseline fluorescence within 15 minutes of heating at 63°C while using a 96-well thermocycler (CFX, BioRad) at a relatively high RNA load of 10^4 copies per reaction. As shown in **Figure 2.3A**, both assays ($n=3$) showed early signal liftoff, with COVID RT-LAMP showing average signal detection at 15.5 mins and Flu A RT-LAMP showing average signal liftoff at 11.7 mins. While freshly prepared reactions are feasible for centralized lab operation, lyophilizing amplification reagents enables the assay device to be portable and implemented where refrigeration is not always possible, such as in homes and in low-resource settings. Time-to-result is also critical, as signal detection within 30 minutes is considered a benchmark of COVID diagnostics. As this lab has previously demonstrated, addition of 10% trehalose and 2.5% dextran (500 kDa) to isothermal nucleic acid amplification reagent solutions preserves the amplification reagents during and after lyophilization, and provides for controlled rehydration by a sample; the dextran slows rehydration, which allows for relatively uniform distribution of those reagents across a fibrous pad for amplification (63). Addition of trehalose and dextran to the amplification mix does not inhibit the performance of fresh controls. In contrast, lyophilizing the reaction mix without adding sugar completely denatures RT-LAMP enzymes and results in no amplification. As observed in **Figure 2.9A**, the addition of sugars (trehalose, dextran) enabled lyophilized RT-LAMP signal liftoff at 13.7 ± 0.2 mins (*mean, SD*).

This liftoff time is close to the liftoff time of a freshly prepared, standard laboratory RT-LAMP at 12.3 ± 0.4 mins, indicating reagent preservation during lyophilization. To improve the signal-to-background ratio for SYTO 82-based fluorescence detection, the dye HNB was added to the reaction. HNB, a metal cation indicator, has been shown to lower background fluorescence when combined with intercalating dyes (52). Notably, amplification speed was improved by HUDSON inhibitor inactivation during the viral lysis step. HUDSON inactivation of nasal inhibitors allowed for significantly earlier amplification liftoff compared to non-treated nasal matrix in COVID RT-LAMP. As shown in **Figure 2.12B**, HUDSON-treated nasal matrix enabled RT-LAMP reactions ($n=3$) to reach signal liftoff at 14.6 ± 0.5 mins (*mean, SD*), while non-treated nasal matrix did not show consistent signal liftoff within 60 minutes across $n=3$ replicates. As the HUDSON method (95°C for 5 mins) could be integrated into the lysis workflow, this allowed for seamless integration into the UbiNAAT workflow alongside stability at ambient conditions. To evaluate assay specificity between COVID and Flu A RT-LAMP, each assay was spiked with three templates aside from its designated viral target at 10^3 copies per reaction (**Figure 2.9C, 2.9D**). Neither assay amplified in the absence of the appropriate target. The lyophilized RT-LAMP assays also exhibited good sensitivity at 100 copies of viral RNA per reaction, as shown in **Figure 2.9E**.

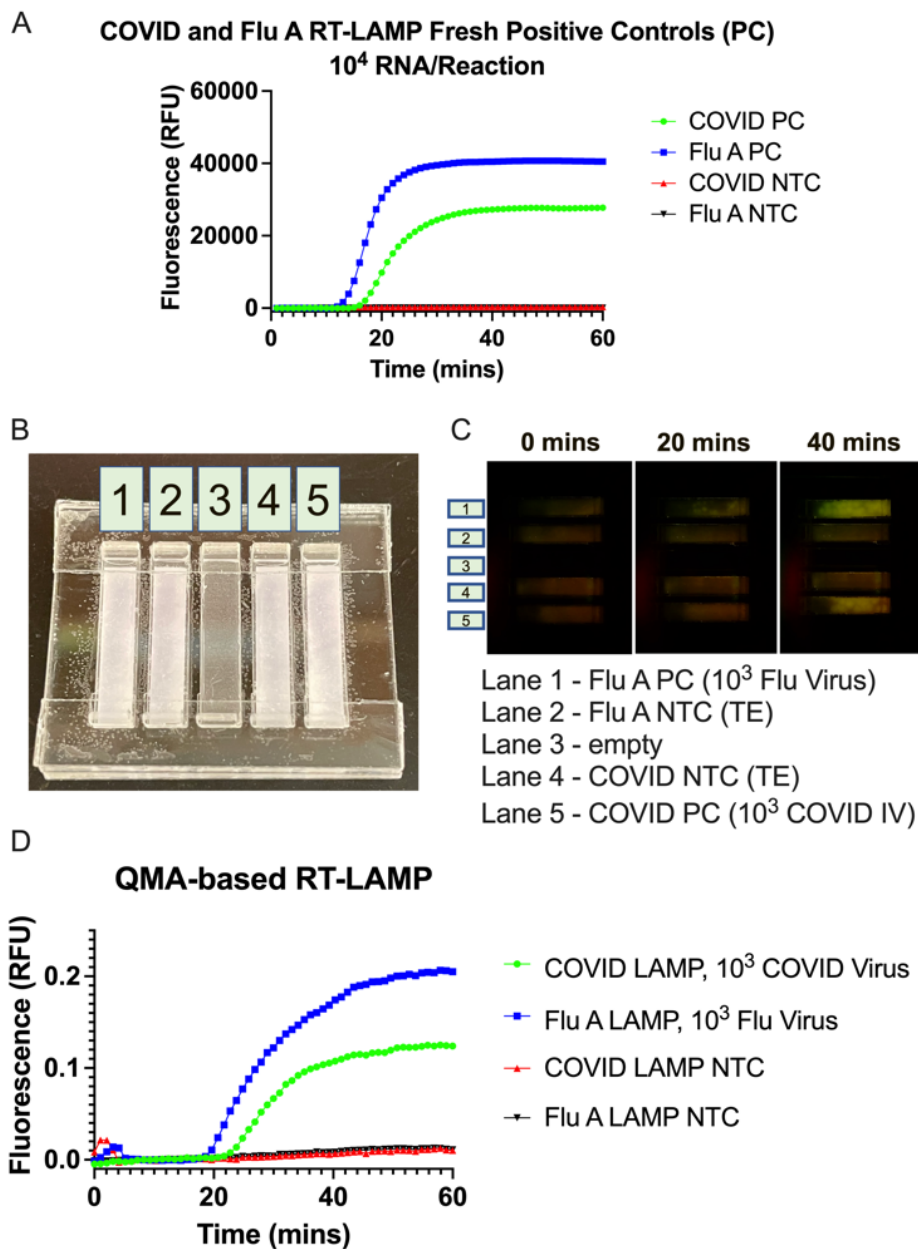


Figure 2.3. 5-pad test device running lyophilized RT-LAMP on QMA membrane.

(A) Averaged fluorescence signal for freshly prepared controls at 10^4 RNA copies per reaction for COVID and Flu A RT-LAMP. Across $n=3$ replicates, COVID RT-LAMP positive controls showed liftoff times of 15.5 ± 0.8 mins, while Flu A RT-LAMP showed liftoff times of 11.7 ± 0.9 mins (mean, SD) (B) 5-pad PMMA tray holding 4 QMA membranes with rehydrated RT-LAMP reagents (C) Fluorescence signal amplification in-paper as imaged by cell phone reader showing fluorescent signal liftoff by 40 minutes (D) Quantified COVID and Flu A RT-LAMP at 10^3 viral copies per reaction results from QMA membrane image stacks ($n=1$)

2.4.3 QMA-based RT-LAMP amplification

The fabrication by hand of the full UbiNAAT prototype is somewhat time-consuming, and since any contamination of a device by amplicons leads to false positives, the parts in contact with the amplification reaction could only be used once. To test the ability of the assays to be dried and used on the QMA pads, we manufactured a “test rig” that was much easier and faster to make. A 5-pad “test device,” as shown in **Figure 2.3B**, was designed to optimize RT-LAMP in the QMA membrane. Two lyophilized QMA RT-LAMP membranes for each assay (one positive sample and one negative sample) were embedded into the 5-pad device, which was then placed into our custom oven and incubated at 64°C for 60 mins. Image acquisition was performed in a dark room to ensure low ambient lighting. As shown in **Figure 2.7**, the cell phone reader can capture low fluorescence signals at a focal distance of 8.5 cm. **Figure 2.3C** shows real-time fluorescence signal as collected by the cell phone reader from each reaction pad over the course of an hour. Quantified curves, as seen in **Figure 2.3D**, indicate that both COVID and Flu A RT-LAMP reactions show signal liftoff by 20 minutes.

It is important to note that RT-LAMP reactions performed in QMA pads show later liftoff times, as compared to tube-based reactions (**Figures 2.3, 2.4**). This phenomenon was previously observed in Lafleur *et al.* (2016) in the application of iSDA (76), and may be due to any of several factors. First, while commercial CFX instruments raise sample temperature to setpoint prior to starting the first cycle of data acquisition, the first cell phone image for QMA-based assays was taken as soon as possible after the 5-pad device was placed onto the oven. Therefore, the time needed for the QMA sample pads to reach the temperature setpoint is incorporated into the hour of data acquisition. In addition, the PMMA layer between the QMA pads and the heater surface is significantly thicker compared to the width of a PCR reaction tube wall. The coating of the QMA

membrane by the blocking solution of BSA and Tween 20 may also cause minor amplification inhibition. Despite these challenges, amplification liftoff by 20 minutes was consistently observed in both SARS-CoV-2 and Flu A RT-LAMP in QMA pads with addition of 10^3 viral copies per pad, indicating that QMA is a suitable material for quick paper-based RT-LAMP amplification with high signal-to-background contrast.

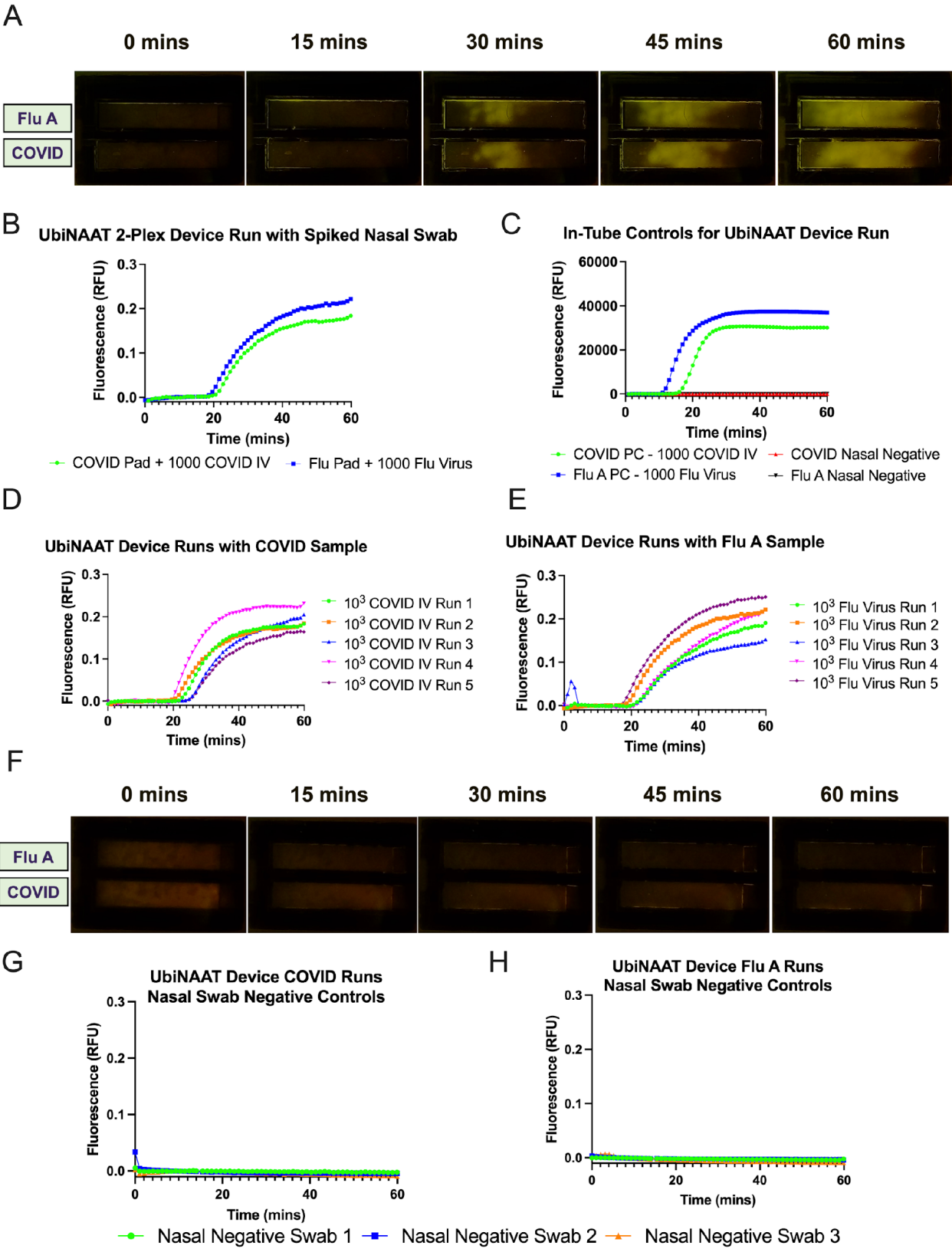


Figure 2.4 UbiNAAT 2-plex RT-LAMP detection of COVID inactivated virus (IV) and Flu virus spiked onto human nasal swab samples.

All device and tube reactions were run with human nasal matrix. (A) Real-time RT-LAMP fluorescence signal of COVID and Flu A over an hour (B) Quantified fluorescence from 2-plex UbiNAAT shows ability to simultaneously detect both COVID and Flu A viruses (C) In-tube RT-LAMP nasal controls show clear positive signals and flat NTC curves (D) Compiled results for COVID detection using UbiNAAT (n=5) (E) Compiled results for Flu A detection using UbiNAAT (n=5) (F) NTC samples using non-spiked human nasal swab on UbiNAAT device shows no fluorescence signal over an hour (G) Compiled results for COVID NTC evaluation using UbiNAAT (n=3) (H) Compiled results for Flu A NTC evaluation using UbiNAAT (n=3).

2.4.4 UbiNAAT 2-plex prototype device operation

While RT-LAMP assay compatibility with human nasal matrix and paper-based application were demonstrated in PCR tubes and the 5-pad device, the prototype of a user-friendly device for point-of-care use required incorporation of viral lysis and timed fluidic movement between multiple chambers. Using the PCB heater developed in our lab as described by Shah *et al.* (2023), the UbiNAAT onboard viral lysis at 100°C was shown to be functionally equivalent to viral lysis at 95°C for 5 mins in a BioRad thermocycler, as shown in **Figure 2.8** (80). The PID loop that controlled the PCB amplification heater thermistor was programmed to enable consistent heating at 63°C across both pads, as shown in **Figure 2.14** across multiple PCBs.

To prevent swab lysate from prematurely leaving the lysis chamber, a novel “air spring” valve¹ actuated by melting dental wax was placed downstream of the amplification zone to completely block air mobility along the fluidic path. In such a valve, the liquid tries to fill the sealed chamber

¹ Note that the air spring valve concept was first developed jointly in ~2014 between the Yager group at UW and a team at PATH that were collaborating on the DARPA MAD NAAT project. However, at that time the channel air pressure was contained by a closed silicone rubber tube that was pinched to prevent air escape, and that pinching was relieved by wetting a strip of “spy paper” that was wetted by a controlled flow of buffer to that point.

formed by the sealed channel that surrounds each of amplification pads. When the air pressure equals the capillary pressure, flow stops. In this case, the system was set so that flow did not proceed much further than the area below the lysis chamber. The terminal air spring valve approach was chosen ahead of traditional passive and piezo valves (82,92), because of its simplicity, reliability, ease of actuation, and the fact that we already had a PCB capable of heating that region in controlled fashion. No pumps or piezoelectric actuators are required for opening the valve. While other devices have used wax valves along the fluidic channel (76), the placement of the air spring valve post-amplification pads in the UbiNAAT ensures that the valve material does not leach into the amplification zone, which could lead to contamination or assay inhibition. With a wax melting point of approximately 65°C, the valve remains stably closed at ambient temperatures, completely blocking fluidic flow into the distal end of the 2DPN until the valve is opened by PCB resistive heating, which allows air flow out of the distal end of both amplification zones. Following lysis, the swab lysate solution was cooled for 3 minutes before the resistive heater under the wax valve was heated, melting the wax, which itself flowed into an adjacent channel away from the main path, which allowed air to move through to the amplification zones. The lysate then quickly and fully rehydrates both the COVID and Flu A RT-LAMP pads, as shown in **Figure 2.13** and **Supplemental Movie** (refer to Jiang *et al.* (2024) (33)). The script then automatically turned on the resistive heater below the amplification pads; the PCB-based heater had higher thermal conductivity compared to the heater oven due to the placement of two layers of thermal and aluminum tape to evenly distribute heating across the amplification zone. As shown in **Figure 2.14**, the PID loop based on a thermistor surrounded by the heating coil ensured that both amplification pads were held close to 63°C over the course of an hour (80). Image acquisition *via* cell phone reader utilized the same parameters as the 5-pad device.

In this study, nasal swabs from eight healthy volunteers were tested on the full UbiNAAT platform (five “positive” spiked swabs and three negative swabs). Each volunteer donated two nasal swabs, one to be used for device testing and the other for in-tube controls. To simulate a positive patient, 1.5×10^4 copies of both COVID and Flu A viruses in TE solution were pipetted onto a nasal swab that had been self-sampled by volunteers. Negative swab samples (NTC, or “no template control”) runs were performed using a nasal swab that was not spiked with either respiratory virus. The swab was then inserted into the UbiNAAT device. Following QMA pad rehydration, each reaction pad contained 1250 copies of both viral RNA. **Figure 2.4A** shows the real-time fluorescence images collected by the cell phone reader, where both pads showed fluorescence signal liftoff before 30 minutes. The fluorescence curves were quantified using MATLAB for the top 1% of pixel intensity at each minute, as shown by the fluorescence curves in **Figure 2.4B**. To ensure that the reagents were not contaminated during the lyophilization procedure, in-tube RT-LAMP control master mixes for both assays were simultaneously lyophilized alongside each set of QMA reaction pads and rehydrated with both COVID inactivated viruses (IV) and Flu virus lysed in nasal matrix to ensure proper reagent functionality, as shown in **Figure 2.4C**.

To demonstrate robustness of the UbiNAAT assay platform, five different prototype devices were tested on five different “positive” nasal swab samples, one for each. As summarized in **Figures 2.4D** and **2.4E**, the UbiNAAT can reliably detect 1.5×10^4 viral copies for each assay from a single nasal swab. Statistical analyses of assay performance across devices indicated that COVID RT-LAMP showed average liftoff of 24.6 ± 3.0 mins, while Flu A RT-LAMP showed average liftoff of 22.3 ± 2.0 mins, as summarized in **Figure 2.10**. Lower viral loads resulted in signal liftoff in excess of 30 minutes (data not shown). In-tube nasal controls for the reactions, shown in **Figure 2.4C**, showed noticeably faster amplification liftoff of 12.1 ± 0.3 mins (Flu, n=2) and 16.4 ± 0.3

mins (COVID, n=2). This may be attributed to slower heating in PMMA and QMA coating due to a thicker PMMA layer between the assay pad and heat source (compared to reaction tubes in a thermocycler); we have found that BSA and Tween treatment is necessary to prevent non-specific enzyme binding to the QMA and ensure wicking of fluids into the membrane.

Three negative swab samples were tested using human nasal swabs without viral loads in three separate prototype devices. Across three NTC device runs, none of the pads crossed the defined threshold in fluorescence signal when the top 1% of fluorescent pixels were evaluated. As seen in **Figure 2.4F**, the fluorescence signal remains low over the course of 60 minutes. Quantified fluorescence curves (**Figures 2.4G and 2.4H**) show fluorescence curves that consistently stay below 0.02 RFU. The low background fluorescence observed in the NTC pads may be attributed to the combination of utilizing HNB, which lowers fluorescence intensity, as well as QMA membranes, which were observed to have low autofluorescence.

While fluorescence intensity as detected by the cell phone reader provided a dynamic range sufficient for distinguishing between positive and negative swab samples, there were considerable variability of the peak fluorescence intensity in both tube and QMA- based RT-LAMP reactions (**Figures 2.3, 2.4, 2.9**). The variability may be contributed by the usage of fluorescent intercalating dyes, such as SYTO-82, whose peak fluorescence changes considerably depending on the concentration of double stranded DNA produced during the reaction (93) Intercalating dyes are known to bind to any double stranded DNA, including human DNA from the human nasal swab. Therefore, the varying concentrations of human DNA from nasal swabs could have contributed significantly to the varying peak fluorescence.

2.4.5 UbiNAAT fluidic cartridge design enables 8-plex multiplexing

The UbiNAAT platform is not limited to two amplification zones; it is a low-cost, paper-based platform that can readily be expanded to include additional amplification pads for eight or more assays, as shown in **Figure 2.5A**. Lyophilized membranes containing LAMP reagents for COVID, Flu A, RSV, and POP7 human gene detection were placed into 8-pad arrays, with 2 replicates of each assay. The devices were heated using the heater oven at 64°C for 60 minutes. Image acquisition was performed using the same cell phone setup as the 2-plex UbiNAAT. Interestingly, while fluidic resuspension of the 2-plex device was noted at less than 15 seconds, the rate at which each pad in the 8-plex device resuspended appeared to be more variable.

Using a single spiked sample containing all three respiratory viruses and human DNA templates at 10^3 copies per reaction pad, fluorescence amplification was detected across all eight lanes simultaneously over the course of an hour. **Figure 2.5B** shows real-time fluorescence across all eight lanes as amplification occurs, with **Figure 2.5C** showing quantified curves of eight RT-LAMP reactions. Notably, all four assays amplified prior to 30 minutes, though distribution of fluorescence signal across the eight pads differ significantly. This demonstrates the potential of using the UbiNAAT platform to assess up to eight different assays concurrently. The PCB's amplification heat trace could readily be expanded to encompass all eight pads.

It is important to note that while amplification was enabled in all eight reaction pads, there was significant non-uniformity in the intensity of fluorescence signals as seen in both Figure 5B and 5C. This phenomenon was previously observed in Shah *et al.* (2022), where the distribution of fluorescence intensity decreases as the reaction pads deviate from the center axis of the cell phone reader's interference filters (80). As the 8-plex design's outer reaction pads were significantly farther away from the center of the cell phone reader, the fluorescence intensity from these reaction pad decreased compared to pads near the center. While the quantified results show lower peak

fluorescence at the outer edges compared to the center of the device, these signals can be normalized through the placement of fiducial markers. The problem is likely caused by the fact that at the steep angles of illumination and detection required for this larger device, the interference filters used are no longer operating at the optimal wavelengths for the SYTO-82 dye. The multiplexing capabilities of the UbiNAAT can be achieved affordably due to the ease of 2DPN fluidic movement. Importantly, expansion to 8 pads does not change the upstream sample processing or lysis. Furthermore, the use of these interference filters was a legacy from earlier experiments in our lab—in the future less inexpensive filters without this angular dependency will be employed.

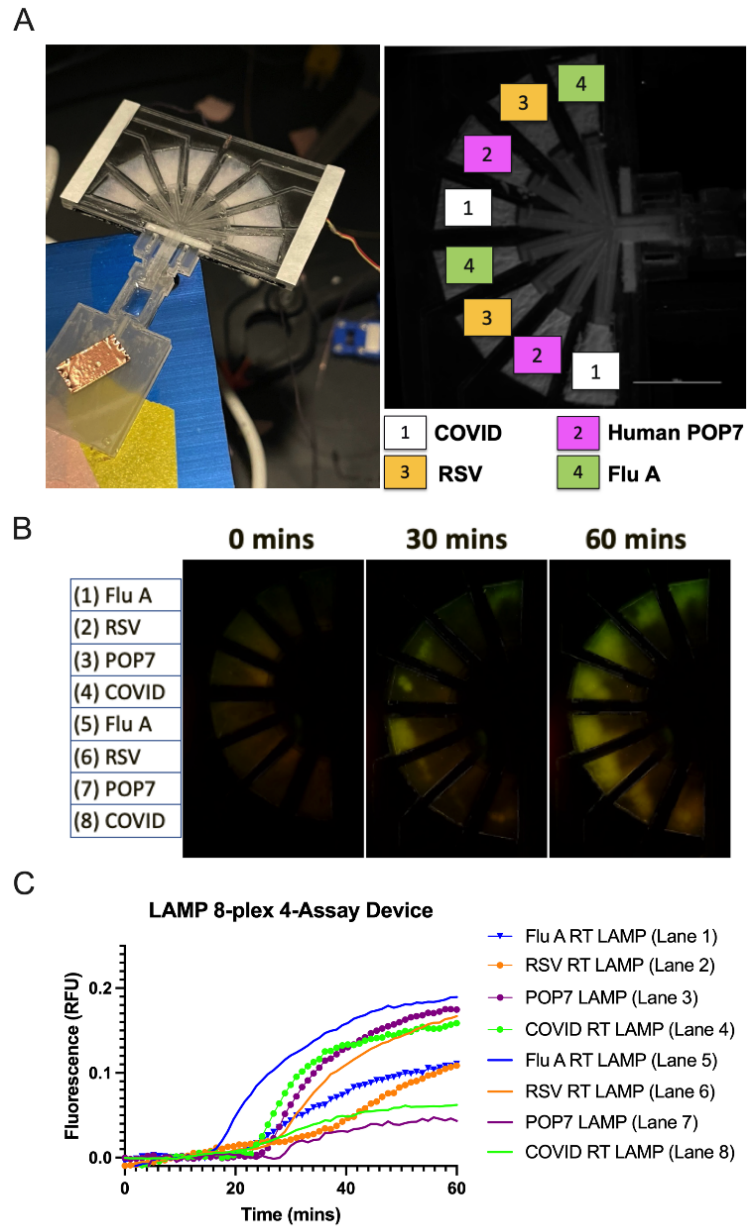


Figure 2.5. 8-plex RT-LAMP showing simultaneous detection of nucleic acids from SARS CoV2 inactivated virus, Influenza A virus, RSV virus, and human DNA (POP7).

(A) 8-plex device and assigned assay lanes (B) Fluorescence amplification of all four LAMP assays simultaneously across 8-plex device enables real-time detection of four distinct RNA and DNA targets ($n=2$) (C) Quantified fluorescence results on 8-plex device show target detection across all four assays within 30 minutes.

Adaptation for home or remote use

In its current iteration, the UbiNAAT is an early-stage lab prototype that enables simple, affordable multiplexing. While many point-of-care testing options, including multiplexed platforms, exist commercially, many of the NAAT options are limited by high cost when additional assays are added. To be successful for home or remote use, the per-use cost must be low while enabling simultaneous detection of multiple pathogens. For this goal, the commercial version of the UbiNAAT platform is envisioned to be easily expandable, inexpensively produced, and compatible with common technology such as cell phone readers. As shown in **Tables 2.6-2.7**, the consumables cost per device is ~\$3, while the reusable component cost could be significantly decreased through component integration. We envision the UbiNAAT disposable prototype, as demonstrated here, to be redesigned for improved manufacturability to allow multiplexing of up to eight different assays in a home environment. In addition to homes, such a system could be applicable to workflows in clinics or hospitals in the developed and developing worlds. As new assays could be developed by switching out the primer set, it would be simple to build kits for different assays without modifying the device design. Note that work is ongoing to apply this single-use disposable technology for detection of several STIs and HIV (as discussed in later chapters in this thesis).

2.5 Conclusion

We have demonstrated an accurate, fast, sensitive, paper-based prototype for a commercial disposable device that was optimized for extremely simple use that could be paired with an inexpensive imaging permanent “hub”. The prototype accepts a swab directly and performs onboard lysis and amplification; it is portable and only requires power from a laptop USB connection. In this study, we have demonstrated the feasibility of extracting and detecting viral

RNA across two respiratory diseases from a single nasal swab at 1.5×10^4 copy viral sensitivity per swab. While the UbiNAAT sensitivity is currently less than that of qPCR or some commercial NAAT products, its fast, reliable detection of 10^4 viral load compares favorably to antigen test sensitivity of <40% at the same viral load (47). Its design also facilitates a simple, more affordable multiplexing for higher numbers of reaction pads. In future iterations, device sensitivity could be improved using larger amplification pad volume or by concentrating RNA in porous matrix. The device is notable for its automated process from sample addition to amplification completion, requiring minimal input by the user. By storing lyophilized LAMP master mix within the amplification pads, the device eliminates the need for cold storage, increasing its portability and application in home and point-of-care settings.

While the current UbiNAAT prototype was demonstrated on detection of only two respiratory pathogens, we have shown that the platform can readily be expanded to feature at least 8 assays. By modifying the primers to target different bacterial and viral genes, the device can become an at-home screening panel for sexually transmitted infections, respiratory infections such as with SARS-CoV-2 and influenza virus, or be used to monitor different genes within the same pathogen to prevent false negatives due to genetic variability among strains. This integrated and automated device addresses the needs of low-resource communities that lack laboratory or clinical facilities, providing a promising alternative to current gold standard testing. It combines molecular diagnostic techniques with robustly engineered fluidics and heating control to deliver a point-of-care solution for complex diagnostic problems.

2.6 Acknowledgements

This work was principally funded by generous gift support from the Mercatus Foundation through an Emergent Ventures Fund Fast Grant to the Yager Laboratory at UW. We also wish to thank the Washington Research Foundation (WRF) for their gift through a Phase I Technology Commercialization Grant. Special thanks to healthy volunteers who donated human nasal swabs to this study.

Special acknowledgement to and in memory of Max Wheeler for all his contributions towards NAAT device developments in our laboratory.

2.7 Supplemental Information

Table 2.1. Nucleotide sequences of LAMP primers that target the pandemic influenza A gene.

Primer	Sequence (5' – 3')	Final Concentration
F3-1	GACTTGAAGATGTCTTTGC	0.1 μ M
F3-2	GACTGGAAAGTGTCTTTGC	0.1 μ M
B3-1	TRTTATTTGGGTCTCCATT	0.1 μ M
B3-2	TRTTGTTTGGGTCCCCATT	0.1 μ M
FIP	TTAGTCAGAGGTGACARRATTGCAGATCTTGAGG CTCTC	1.6 μ M
BIP	TTGKTTTCACGCTCACCGTGTTTGGACAAAGCGTC TACG	1.6 μ M
Loop F	GTCTTGTCTTTAGCCA	0.4 μ M
Loop B	CMAGTGAGCGAGGACTG	0.4 μ M

Table 2.2. Nucleotide sequences of LAMP primers that target the SARS-CoV-2 N gene.

Primer	Sequence (5' – 3')	Final Concentration
F3	TGGCTACTACCGAAGAGCT	0.2 μ M
B3	TGCAGCATTGTTAGCAGGAT	0.2 μ M
FIP	TCTGGCCCAGTTCCTAGGTAGTCCAGACGAATTCG TGGTGG	1.6 μ M

BIP	AGACGGCATCATATGGGTTGCACGGGTGCCAATGT GATCT	1.6 μ M
Loop F	GGACTGAGATCTTTCATTTTACCGT	0.4 μ M
Loop B	ACTGAGGGAGCCTTGAATACA	0.4 μ M

Table 2.3. Nucleotide sequences of LAMP primers that target the respiratory syncytial virus (RSV) gene.

Primer	Sequence (5' – 3')	Final Concentration
F3	GCTGTTCAATACAATGTCCTAGA	0.2 μ M
B3	GGTAAATTTGCTGGGCATT	0.2 μ M
FIP	TCTGCTGGCATGGATGATTGGAGACGATGATCCTGCAT CA	1.6 μ M
BIP	CTAGTGAAACAAATATCCACACCCAGCACTGCACTTCT TGAGTT	1.6 μ M
Loop F	ACATGGGCACCCATATTGTAAG	0.4 μ M
Loop B	AGGGACCTTCATTAAGAGTCATGAT	0.4 μ M

Table 2.4. Nucleotide sequences of LAMP primers that target the POP7 gene.

Primer	Sequence (5' – 3')	Final Concentration
F3	TTGATGAGCTGGAGCCA	0.2 μ M
B3	CACCCTCAATGCAGAGTC	0.2 μ M
FIP	GTGTGACCCTGAAGACTCGGTTT- TAGCCACTGACTCGGATC	1.6 μ M
BIP	CCTCCGTGATATGGCTCTTCGTTT- TTTTCTTACATGGCTCTGGTC	1.6 μ M
Loop F	ATGTGGATGGCTGAGTTGTT	0.4 μ M
Loop B	CATGCTGAGTACTGGACCTC	0.4 μ M

Table 2.5. In-tube and QMA-based LAMP master mix

Reagent	Final Concentration (In-tube)	Final Concentration (QMA)
WarmStart LAMP Kit	1X	1X
SYTO-82	7.5 μ M	7.5 μ M

Hydroxynaphthol Blue	40 μ M	40 μ M
Assay specific primers (See Tables S1-S2)	varies	varies
Trehalose	10%	10%
Dextran, 500 kD	0.5%	0.5%
Sample	1 μ L in volume	1 μ L in volume
Nuclease-Free H ₂ O	Fill up to 20 μ L	Fill up to 25 μ L

Table 2.6. Cost analysis of amplification assay reagents

	Component	Manufacturer	Cost/Device
RT-LAMP Reagents	WarmStart RNA/DNA LAMP Kit	New England Biolabs	\$1.50
	SYTO-82	Thermo Fisher	<\$0.01
	Hydroxynaphthol Blue	Millipore Sigma	<\$0.01
	Trehalose	Life Sciences	<\$0.01
	20X Primer Mix	Integrated DNA Technologies	\$0.05
	Dextran, 500 kDa	Millipore Sigma	<\$0.01
	TCEP	Millipore Sigma	<\$0.01
	EDTA	Life Technologies	<\$0.01
	Tris-HCl	Invitrogen	<\$0.01
Subtotal			\$1.55

Table 2.7. Cost analysis of UbiNAAT device components

	Component	Manufacturer	Cost/Device
UbiNAAT Device (Non- Reuseable)	Poly methyl methacrylate (PMMA), 1/16"	McMaster-Carr	\$0.25
	Poly methyl methacrylate (PMMA), 3s/8"	McMaster-Carr	\$0.06
	Poly methyl methacrylate (PMMA), 1/8"	McMaster-Carr	\$0.24
	Polydimethylsiloxane (PDMS) tape-silicone transfer	Valley Industrial Products	\$0.90
	Glass fiber 8950	Ahlstrom	<\$0.01
	Tyvek	Du Pont	<\$0.01

	Dental Wax	Electron Microscopy Sciences	<\$0.01
	Quartz microfiber (QMA)	Cytiva (VWR)	<\$0.01
	3M™ Copper Conductive Tape	Ted Pella	<\$0.01
	Aluminum Foil	Reynolds	<\$0.01
	Thermal Tape	Newark	\$0.05
	Aluminum Foil Tape (Black)	Advance Tapes	<\$0.01
UbiNAAT Enclosure (Reusable)	White Resin	Formlabs	\$18.00
	Poly methyl methacrylate (PMMA), 1/16"	McMaster-Carr	\$0.10
	Mylar (0.004" thickness)	Fralock	<\$0.01
Cell Phone Fluorescent Filters (Reuseable)	Emission filter FES0550 - Ø1" Shortpass Filter, Cut-Off Wavelength: 550 nm	Thorlabs	\$1360
	Excitation filter BP 587/25 (HE)	Zeiss	\$80
Subtotal			\$1459.60
Subtotal (Reusable)			\$1458.10

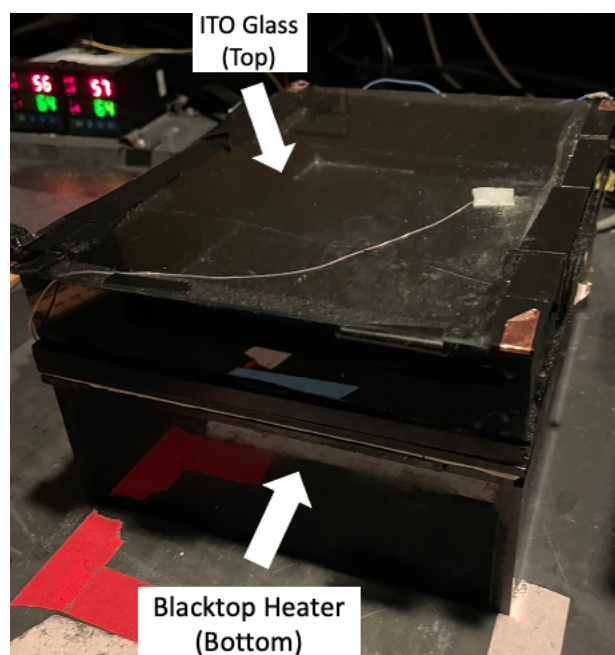


Figure 2.6. Custom dual-sided heater for testing of QMA-based RT-LAMP assays in 5-pad devices.

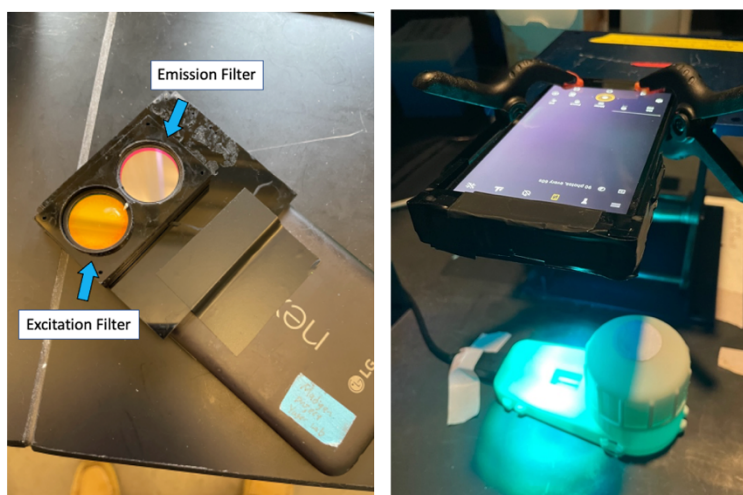


Figure 2.7. Cell Phone Fluorescence Reader

(left) Google Nexus 5 cell phone fitted with a custom PMMA fixture holding an excitation filter (Zeiss Bandpass 587/25 (HE)) and emission filter (Thorlabs Ø1 in. Shortpass Filter, Cut-Off Wavelength: 550 nm). (right) Cell phone reader fixed on stand 8.5 cm above device amplification pad region. Camera settings are: (1) Incandescent white balance (2) Manual focus with a focal length of 8.5 cm between lens and image target (3) 200 ISO (4) 1/5 second shutter speed (5) Time interval of 1 image per minute for 60 minutes.

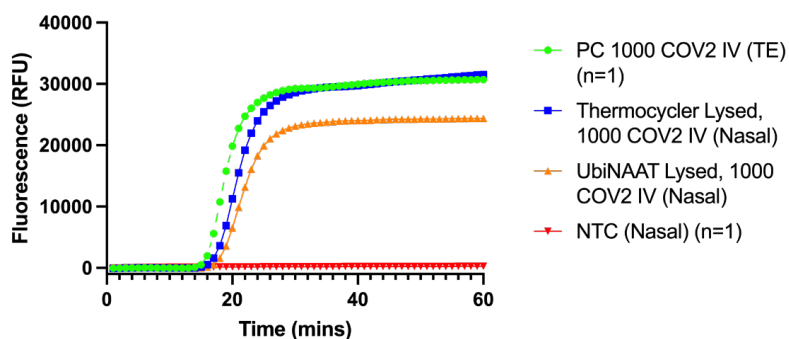


Figure 2.8. UbiNAAT Lysis Evaluation

SARS-CoV-2 RT LAMP evaluation of UbiNAAT lysis compared to thermocycler lysis (BioRAD MJ Mini 48-well) of 1000 copies of COVID inactivated virus (ATCC) (averaged fluorescence signal across n=3 replicates). BioRAD thermocycler lysis set to 95 °C for 5 minutes, and UbiNAAT lysis set to 100% power for 7 minutes (100 °C for 3 mins).

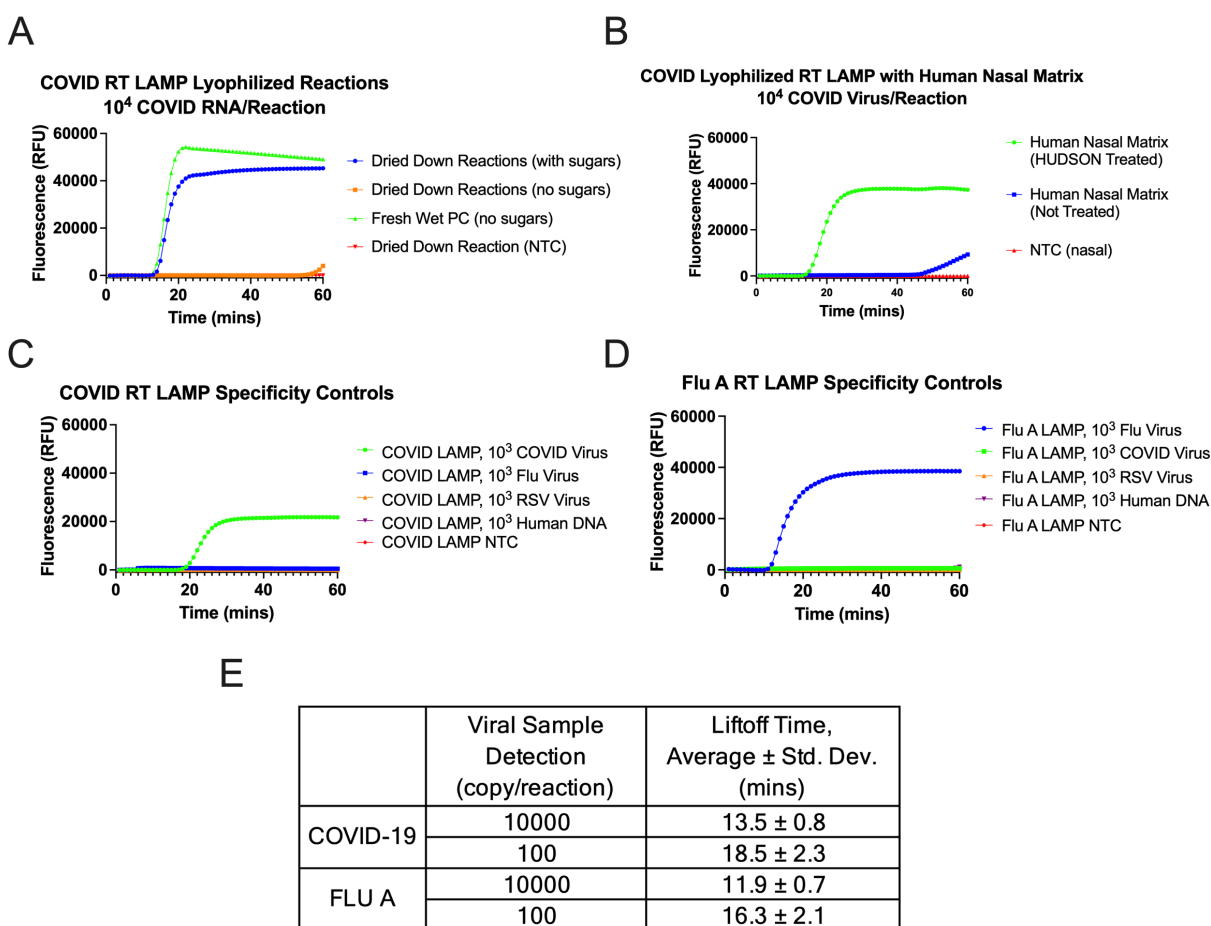


Figure 2.9. COVID and Flu A RT-LAMP in-tube development

In-tube RT-LAMP assay development for COVID-19 and Influenza A detection (*mean $\pm$ SD*) for (A) At 10⁴ RNA copies per reaction, COVID lyophilized RT-LAMP reactions (13.7 $\pm$ 0.2 mins, n=3) show similar liftoff times to freshly prepared RT-LAMP reactions (12.3 $\pm$ 0.4 mins, n=3). Lyophilized reactions without sugars and non-template controls (NTC) showed no significant signal liftoff in 60 minutes of amplification. *Note that LAMP Fluorescent Dye (NEB) was used instead of SYTO-82 in these results.* (B) HUDSON-treated nasal matrix RT-LAMP reactions show early signal liftoff (14.6 $\pm$ 0.5 mins, n=3) while untreated nasal matrix reactions do not show reliable amplification across replicates (C) COVID RT-LAMP is specific against human DNA, Flu, and RSV viruses (n=1) (D) Flu A RT-LAMP is specific against human DNA, COVID, and RSV viruses (n=1) (E) Fluorescence signal liftoff times for lyophilized COVID and Flu A RT-LAMP assays showing pre-20 minute signal liftoff across viral copy range (n=3)

UbiNAAT 2-Plex Device Assay Signal Liftoff Times

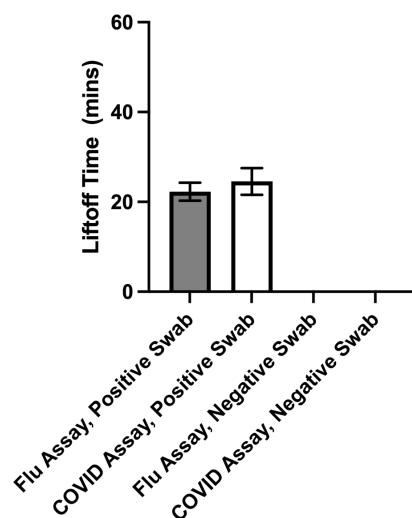
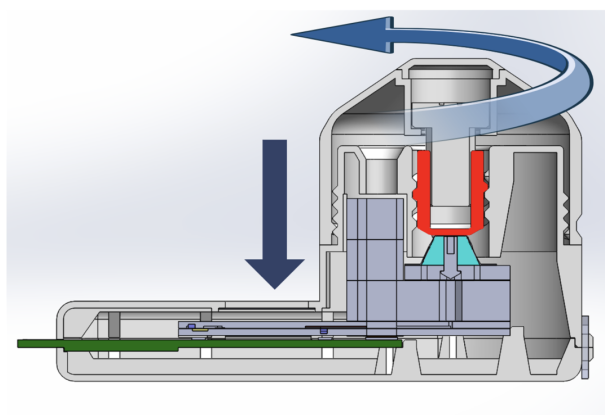
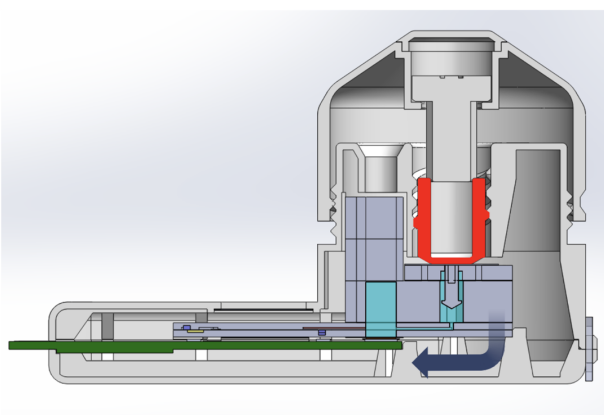


Figure 2.10. UbiNAAT Sample Detection Liftoff Times



Step 1: As cap is removed by turning counter-clockwise, an internal activator pin (*red*) is permanently lowered to compress the blister pack (*light blue*)



Step 2: The compressed blister pack is punctured by an internal sharp, and pushes buffer into the lysis chamber through a channel

Figure 2.11. Schematic of Blister Pack Operation.

(Figure by Steven Bennett)

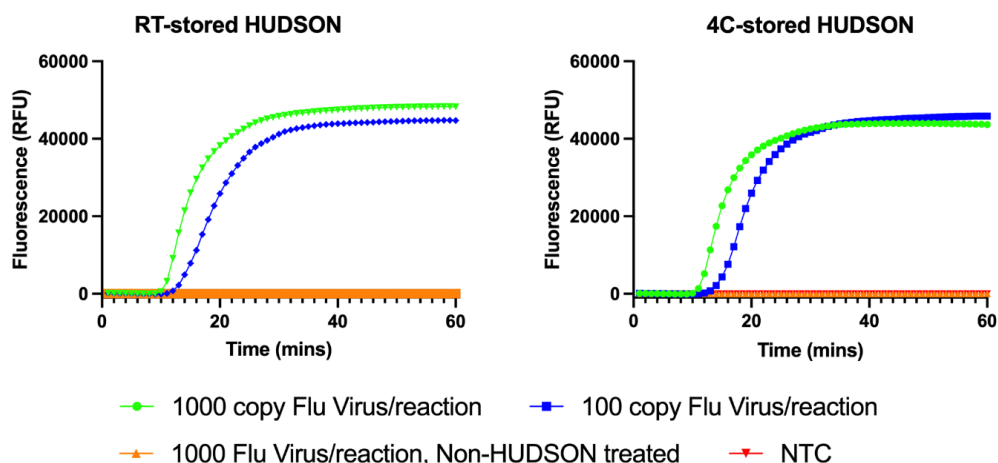


Figure 2.12. HUDSON Impact on RT-LAMP with Nasal Matrix

Flu A RT LAMP indicating HUDSON solution stability for inactivation of human nasal matrix following 15 days of storage at either ambient temperature (RT) or 4C refrigerator (averaged fluorescence signal across $n=3$ samples). Solutions were freshly prepared at 1X working concentration (2.5 mM TCEP, 1 mM EDTA, pH 8.0) prior to storage.

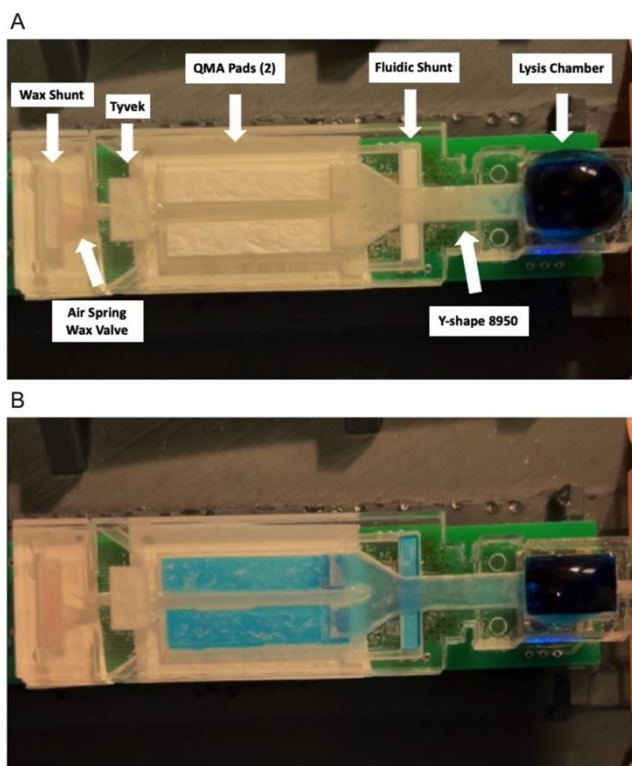


Figure 2.13. Fluidic flow path within UbiNAAT device, as controlled by the air spring terminal wax valve

Fluidic path is shown with food coloring in (A) labeled fluidic path from lysis chamber to QMA pads, and is enabled by PCB resistive heating of wax valve at 80°C to (B) fully resuspended device. Tyvek is added between the amplification pads and the wax valve to prevent lysate fluidic flow towards the air vents.

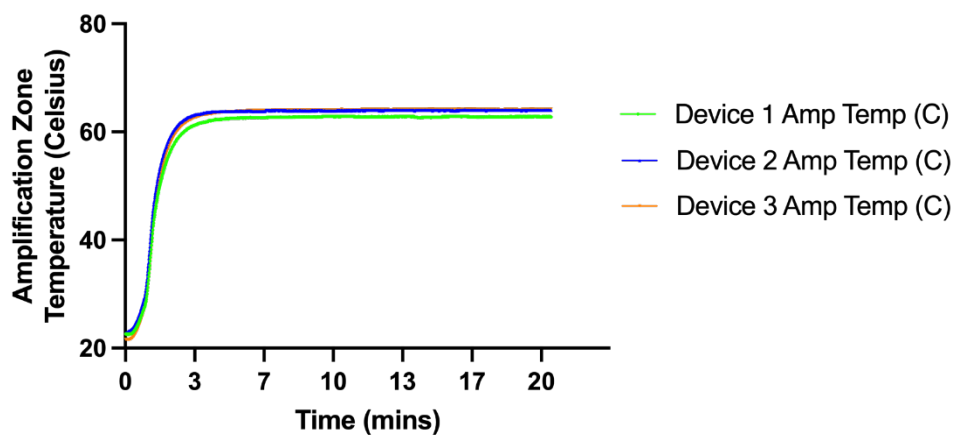


Figure 2.14. Verification of UbiNAAT QMA pad temperature being within target range (62-65°C) during amplification heating.

In-pad temperature across three separate UbiNAAT devices collected via T-type thermocouple (Omega) inserted into amplification zone.

3. Chapter Three - Heat-Actuated Valving to Enable Infectious Disease Detection in a Point-of-Care, Paper-Based Microfluidic Device

3.1 Abstract

Paper-based microfluid devices have great potential to perform complex assays like nucleic acid amplification tests (NAATs) in point-of-care (POC) and low resource settings, but have not often succeeded because there is frequently need for complex fluidics and sample delivery without risk of contamination. These issues are exacerbated when multiple sample processing procedures must be automated by valve integration in the absence of advanced supportive instrumentation or trained personnel. For NAAT application in remote or home environments, valving within a POC device must facilitate precise fluidic movement and physically separate each chemical processing step. Ideally, the valve should also be robust in varying climate environments, reliable after long storage, affordable, and non-reactive with chemical reagents or samples. Here, we report the design and integration of two adaptable thermally-actuated valves for application in a point-of-care, paper-based rapid analysis device. Both valves are designed to facilitate the fluidic movement and control necessary to enable reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) reactions in a paper-based device. Each valve can be used independently or alongside the other valve to control fluidic movement at specific locations along the paper fluidic pathway. The valves differ in their material composition, fabrication, and mechanisms for fluidic control, but both are affordable, durable, and simple to use. We demonstrate that each valve could be used to enable

RT-LAMP reactions in a paper-based device for detecting respiratory infectious diseases (COVID and Flu A) from human nasal swabs.

3.2 Introduction

Paper-based microfluidics is a rapidly growing field in analytical system development, especially for point-of-care (POC) applications. Compared to conventional microfluidics, paper-based microfluidics offers numerous benefits, such as low cost of materials, simple fabrication, and lacking the need for traditional pumps or fluidic routers, which control the direction of flow (94,95). Paper microfluidics have been used across many industries, ranging from diagnostics to environmental safety; notably, paper-based analytical devices (PADs) have emerged as powerful tools for diagnostics development (94,96–98). Their affordability, enabling of complex fluid flow without the need for pumps, as well as the storage of dried reagents within paper membranes make the technology especially valuable for remote and home testing. However, their translation to commercial POC platforms has been hindered by limitations in multi-step, automated fluidic control to integrate sample processing and target detection procedures (99–101). Here, we describe the design, fabrication, and implementation of two novel thermally-actuated valves that are integrated into a multi-step, multiplexed, paper-based rapid analysis tool that simultaneously detects multiple respiratory pathogens (COVID and influenza A) from a single nasal swab. Both valves offer unique advantages in addressing the need for adaptable fluid control in multi-step detection systems.

Many types of diagnostic assays have been implemented with paper-based analytical devices, ranging across lateral flow tests (LFTs), electrochemical sensing, nucleic acid amplification tests, and more. The demand for effective valving increases with assay performance. Lateral flow tests

are used in large-scale testing for clinical and public health applications given their low-cost, rapid readout, simple protocol, and compatibility with self-testing in decentralized settings (102,103). By leveraging pre-stored chemical reagents in paper membranes to rapidly immobilize and detect target analytes with only a single user step, LFTs allow patients to quickly self-test for in remote or home environments (102). During the COVID-19 pandemic, LFTs for detecting SARS-CoV-2 and other respiratory diseases were distributed globally by the billions, resulting in unprecedented scale of global outreach (14,104–107). However, the conventional LFT's single-step procedure limits its assay sensitivity compared to other techniques (106,107). Electrochemical sensing has been integrated with paper-based microfluidics to enable highly sensitive devices, but often require multiple sample clean-up and processing steps (108,109). The potential of these systems for both infectious disease (e.g. COVID) and chronic disease (e.g. cancer, cardiovascular diseases) detection at point-of-care scale is therefore reliant on efficient fluidic control via valving (110–113).

Nucleic acid amplification tests (NAAT) are the current gold standard of molecular testing (114). Complex fluidic control has been critical in the translation of NAAT to POC platforms, as they require multiple processing steps such as sample lysis, inhibitor inactivation, assay rehydration, and amplification (35,114). Isothermal amplification techniques, notably loop-mediated isothermal amplification (LAMP), are prime candidates for low-cost diagnostics to be used in POC implementation given their isothermal activity, high sensitivity, and high specificity (23,24,51,52). LAMP, which uses four to six primers to rapidly and specifically amplify target nucleic acids, is also compatible with dried storage, which makes it portable and compatible with implementation in low-resource settings (33,51,63). Fluidic control *via* valves to separate the individual steps is necessary in the absence of complex, expensive pumps and routers. Automating valve actuation

would further benefit the user by minimizing possible operator errors. To be compatible with POC paper-based diagnostic devices, valves must not only be effective at blocking and controlling fluid flow, but also be affordable, automatically actuated, compatible with assay reagents, and robust across varying storage and use climates.

While many groups have developed effective valves for a multi-step assay device integration, there has yet to be a valve approach that addresses all the needs of a point-of-care (POC) platform. Phillips *et al.* demonstrated a thermally-actuated wax-ink valve on nitrocellulose membranes that could be sequentially opened or closed in both LFT and LAMP-based devices, but this valve utilized proprietary wax and could be actuated at a relatively low ambient temperature 41°C (51,115). Kim *et al.* employed a solenoid-driven pressure valve that could be activated repeatedly; however, it required a dedicated pressure driven system and would be costly to implement at POC scale (116). Iwasaki *et al.* utilized a thermo-responsive polymer valve that could expand and shrink around a critical temperature of 32°C and was shown to be compatible with various types of reagents (117). However, its low operating temperature limited its transportability and functionality in warmer climates. Adedokun *et al.* developed a ball-based valve which blocks a hole at the bottom of a reagent well to prevent fluid flow; this approach is limited to manual actuation by the user across multiple assay steps (118). While many existing valve systems present promising solutions to specific operating conditions, their inability to be adaptable to POC conditions limits their translation. Coupled with each assay's demands for different reagents and materials, there remains a need for an adaptable valve design that could address the fluidics needs of multi-step POC diagnostic systems.

In previous work from this lab, we demonstrated the UbiNAAT (Ubiquitous NAAT), an integrated sample-to-result paper-based microfluidic device with a USB-powered printed circuit board (PCB)

for heating, two-dimensional paper network (2DPN) for fluidic transport, and paper-based RT-LAMP assay pads for simultaneous separate detection of SARS-V-CoV-2 and influenza A (33). The device operated on an automated script controlling heaters for lysis, valve actuation, and amplification while paired with a cell phone fluorescence reader for real-time image collection of amplification detection. To control fluidics, a novel “air spring” wax valve was integrated on the terminal end of the 2DPN; the valve operates by stopping air flow out of the fluidic pathway, thereby blocking fluid flow into the farthest end of the 2DPN prior to thermal valve actuation. Lafleur *et al.* previously demonstrated a thermally-actuated beeswax valve striped further upstream within the 2DPN, but it required complex manufacturing (76). The air spring valve’s simple fabrication and integration into the UbiNAAT enabled multiple critical LAMP steps (lysis, sample inhibitor inactivation, assay pad rehydration by the sample, and amplification) to be performed automatically in sequence. As a result, we demonstrated the UbiNAAT’s ability to detect multiple pathogens from a single human nasal swab through paper-based RT-LAMP (33). The low-cost and durable air spring valve further allowed the UbiNAAT to be produced affordably.

Here, we show the development, fabrication, and mechanisms of the two thermally-actuated valves that we have separately integrated into the UbiNAAT, and that could be adapted for other paper-based detection platforms. The first, denoted herein as the “in-path” valve, is a heat-shrink valve composed of polyvinyl chloride (PVC) film that could be placed in the middle of a two-dimensional paper network to directly block fluid flow from one segment to the next. The second, the air spring valve, is a wax-based valve placed at the terminal end of a 2DPN pathway to block fluid entry into the segment of the 2DPN upstream of it. We demonstrate that both valves could

be assembled using low-cost materials, reliably actuated at reasonable temperatures *via* thermal resistive heating, and enable LAMP detection of multiple respiratory pathogens from a single nasal swab using the UbiNAAT platform. These valves, individually or in combination, present adaptable solutions for complex fluidic systems that require multiple processing steps. We also present the advantages and disadvantages of each valve type in device design and implementation to illustrate the various use cases where each valve could be appropriate.

3.3 Materials and Methods

Valve Fabrication

In-Path Valve Fabrication

A film of polyvinyl chloride (PVC) (Uline, Pleasant Prairie, WI), a heat-shrink polymer, was cut on a Silhouette Cameo knife plotter (Silhouette America, Lindon, Utah) into strips of 5 mm x 13 mm, with an additional 2 mm line cut into the middle of the strip (see **Figure 3.7**). With the knife blade the slit penetrated through the PVC sufficiently to allow air to slowly pass, but did not form a substantial opening in the film. To form an airtight seal, polycaprolactone (PCL) (InstaMorph, Murray, Utah) was dissolved in toluene (Sigma Aldrich, St. Louis, Mo) at a 1% w/v ratio. Three μL of dissolved polycaprolactone solution was pipetted onto the 2 mm line until the line was fully covered by a circle of polycaprolactone. If 3 μL does not fully cover the 2 mm line, 1-2 additional μL of solution could be added. The valve was then dried at ambient temperature for 30 minutes before storage.

To investigate possible negative consequences of this method of sealing the slit with PCL in this valve, leachate solution from PCL dissolution was collected by submerging a prepared in-path valve in 100 μ L of TE buffer (10 mM Tris, 0.1 mM EDTA) in an Eppendorf tube (Eppendorf, Hamburg, Germany), then heating at 100 °C for 5 minutes on a heat plate (VWR, Radnor, PA). Solution from three separately prepared in-path valves were then collected *via* centrifugation at 5000 RCF for 1 minute and used to prepare template solution for rehydrating lyophilized reaction mixes.

Air Spring Valve Fabrication

The wax valve holder was fabricated alongside the fabrication of the UbiNAAT internal device (see device fabrication below), with a wax volume of approximately 1.1 mm³ (see **Figure 3.7**). The holder was fabricated using a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ) by rastering (formation of a patterned depression in the in the surface of a material through scanned laser ablation). Dental wax (Electron Microscopy Sciences, Hatfield, PA) was melted with a soldering iron and dropped directly into the valve holder section of the UbiNAAT internal device. Once hardened, excess wax was scraped off to the level of the PMMA surface surrounding the valve.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay

The RT-LAMP assay was described in our previous work (33). Briefly, the reagents consist of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), SYTO-82 fluorescent intercalating dye (Thermo Fisher Scientific, Waltham, MA), hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA), trehalose (Life Sciences Advanced Technologies), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham,

MA), and assay-specific LAMP primers (**Table 3.1**). Six primers were used for COVID-19 and 8 primers were used for Flu A RT-LAMP assays, with all sets of primer sequences acquired from literature (Integrated DNA Technologies, Skokie, IL) (**Tables 3.2-3.3**) (87,88). For inactivating nasal swab matrix ribonucleases (RNases), the HUDSON solution (“heating unextracted diagnostic samples to obliterate nucleases”) was adapted from literature; it was prepared with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Millipore Sigma) at 2.5 mM and ethylenediaminetetraacetic acid (EDTA) (Invitrogen by Life Technologies) at 1 mM (85,86). Purified synthetic genomic RNA for SARS-CoV-2 (Twist Biosciences, San Francisco, CA) was purchased. SARS-CoV-2 virus (heat-inactivated) (ATCC, Manassas, VA) and Flu A viruses (ATCC) were purchased, then diluted in TE buffer.

RT-LAMP reactions were performed in both PCR tubes (20 μ L volume) and on Whatman quartz fiber membranes (QMA) (25 μ L volume) from Cytiva (VWR, Radnor, PA) (33), as described in **Table 3.1**. In-tube reactions were performed on a 96-well thermocycler (CFX Opus, Biorad) at 63°C, while QMA-based reactions were performed in device at 64°C. QMA membranes were blocked by soaking in a solution of 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) in nuclease-free water for an hour before being drained of fluid and dried overnight in a 40°C oven. Once lyophilization solution (containing no target) was added to both tubes (placed in a 96-well aluminum tube holder (Qiagen)) and QMA membranes (placed in a sterile 6-well plate (Costar)) at their respective volumes, both were lyophilized following protocols described in our previous work (33,63). In short, each apparatus was quickly frozen by insertion in liquid nitrogen for 1 minute before being placed into a fast-freeze flask (Labconco) and attached to a FreeZone 2.5 liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, Mo) for overnight drying with the system cold finger set at -47°C and the vacuum at 0.03 mbar.

Nasal Swab Collection

The nasal swab collection protocol was described in our previous work (33). In short, nasal matrix was self-collected from healthy volunteers using Rhinostic polypropylene swabs (Rhinostics, Boston, MA) following a University of Washington institutional review board (IRB) approved protocol (IRB Study 00011163). Each healthy volunteer provided informed consent for their sample through a signed consent form. During testing, swabs were screened for SARS-CoV-2 via RT-LAMP, and COVID-positive samples were discarded. Prior to testing, the negative nasal swabs were stored at 4°C.

UbiNAAT Device Assembly and Operation

The UbiNAAT device and assembly process were detailed in our previous work (33). Briefly, the UbiNAAT consists of three major components: an RT-LAMP internal device, a custom printed circuit board, and a 3D printed enclosure. The 3D enclosure had a marked site for nasal swab insertion. The internal device consisted of a lysis chamber, solution blister, and a 2DPN consisting of several different porous materials surrounded by a PMMA housing held together by PDMS pressure-sensitive tape (Valley Industrial Products, Huntington, NY). The 2DPN included two RT-LAMP amplification pads and one valve. The lysis chamber was externally sheathed in copper tape to facilitate even heating of the entire swab sample during lysis. The solution blister held 540 μL of HUDSON solution and was activated via a pin in the 3D enclosure lid that pushed down when the lid was closed. Pin actuation on the blister pushed 300 μL of HUDSON solution into the lysis chamber to fully submerge the nasal swab. The 2D paper network was made up of 0.02% Tween-20 blocked 8950 glass fiber “connector pads” that connects the lysis chamber to the

lyophilized QMA amplification pads, as shown in **Figure 3.1**. Air vents were added to the top PMMA layer of the internal device and covered with Tyvek membrane (DuPont, Wilmington, DE) to enable air flow. The internal device was attached to a custom printed circuit board via thermal tape (Newark Electronics, Chicago, IL), then enclosed by the 3D-printed enclosure.

To begin a device run, each nasal swab sample was spiked with 1.5×10^4 copies of both heat-inactivated COVID virus and influenza A virus. As each QMA pad can hold no more than a 25 μL volume, there was a 12-fold dilution of the swab's initial viral load, resulting in approximately 1250 viral copies per reaction pad. The swab was inserted into the designated opening on the 3D printed enclosure and the lid was closed, activating the push pin on the blister to push 300 μL solution into the lysis chamber. The device printed circuit board was then transported into a dark room and connected *via* USB cable to a laptop, from which TeraTerm (Tera Term Project, Japan) was used to initiate the automated heating protocol. A cell phone fluorescence reader was fixed above the device to begin image collection per minute over the course of an hour (80).

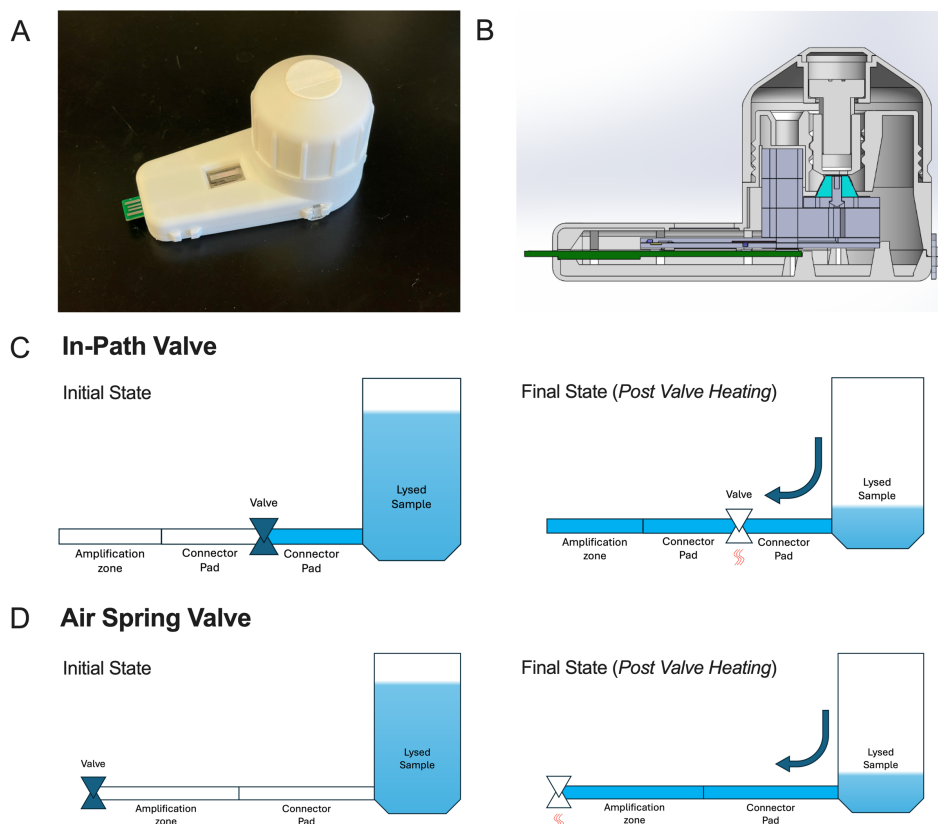


Figure 3.1. Operational Schematic for In-Path Valve and Air Spring Valve

(A) UbiNAAT integrated device (B) Side view of UbiNAAT schematic, including internal device display (C) In-Path Valve Functionality. In the initial state, the in-path valve is closed and blocks fluid flow from reaching the amplification zone. Once the valve is opened via resistive heating, lysed sample flows past the valve and fully rehydrates amplification pads. (D) Air Spring Valve Functionality. In the initial state, the valve is closed, with pressure from the compressed air in the two-dimensional paper network (2DPN) restricting fluid flow out of the lysis sample chamber. Once the valve is opened via resistive heating, the air pressure is released and lysed sample flows out of the lysis chamber and fully rehydrates the 2DPN including amplification pads.

(Figure by Kevin Jiang and Paul Yager, PhD)

Two iterations of the UbiNAAT were developed for valve testing. **Figure 3.2** shows the in-path valve variant of the device, with an amplification zone width of 20.3 mm. The connector pad was divided into two segments, with the in-path valve placed in between. The in-path valve was held to the bottom layer of the PMMA casing via PDMS tape. In **Figure 3.3**, the air spring valve device

variant placed the valve at the terminal end of the amplification zone in the internal device. Only a single, longer connector pad was needed, and the width of the amplification zone was reduced to 13.0 mm. Additional air gaps were introduced between the amplification pads and the air spring valve segments of the internal device to reduce heat dissipation.

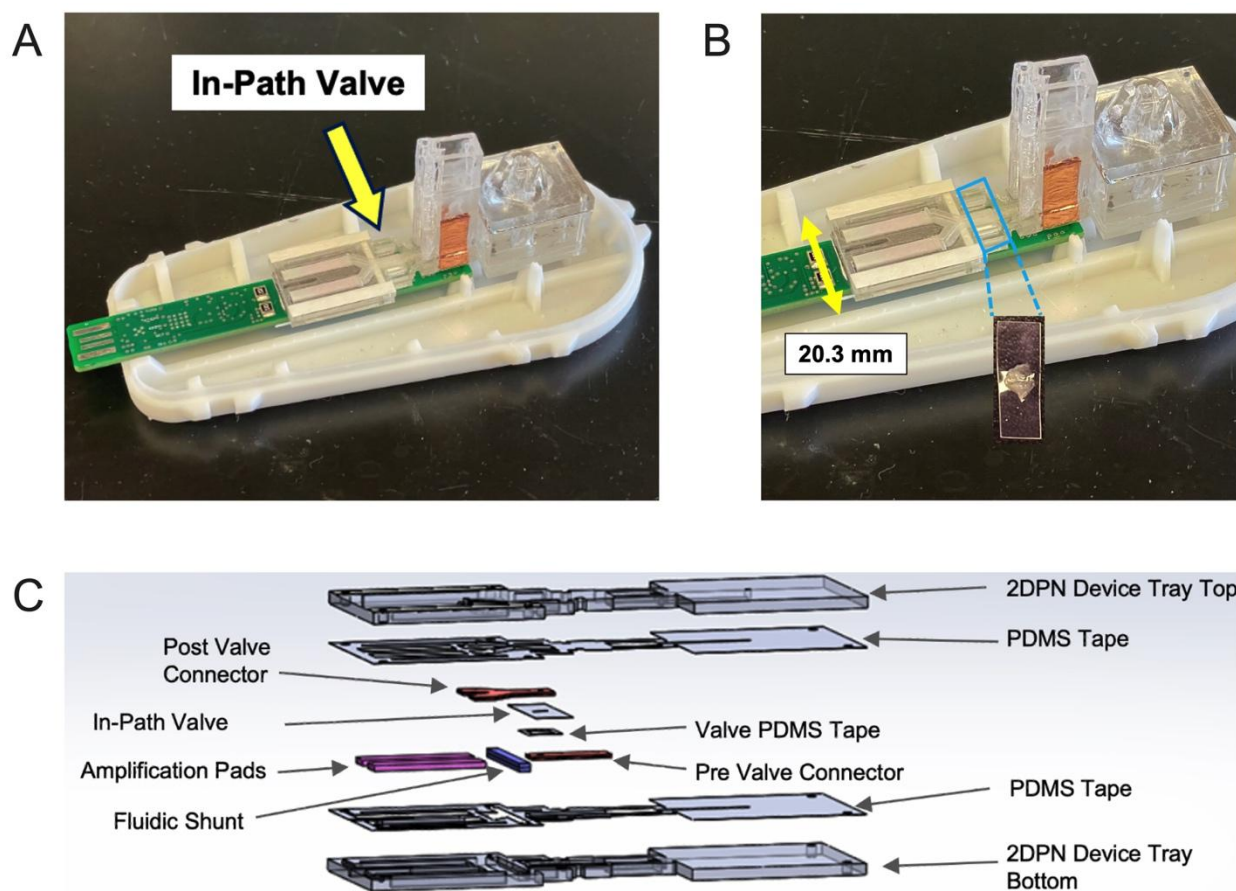


Figure 3.2. In-Path Valve Integration in the UbiNAAT Device.

(A) Valve location in the UbiNAAT device (arrow). (B) Zoomed in view showing the valve (polycarbonate film with dried polycaprolactone covering) and its orientation. (C) Exploded view of UbiNAAT internal device schematic showing valve placement between other components.

(Figure by Kevin Jiang and Steven Bennett)

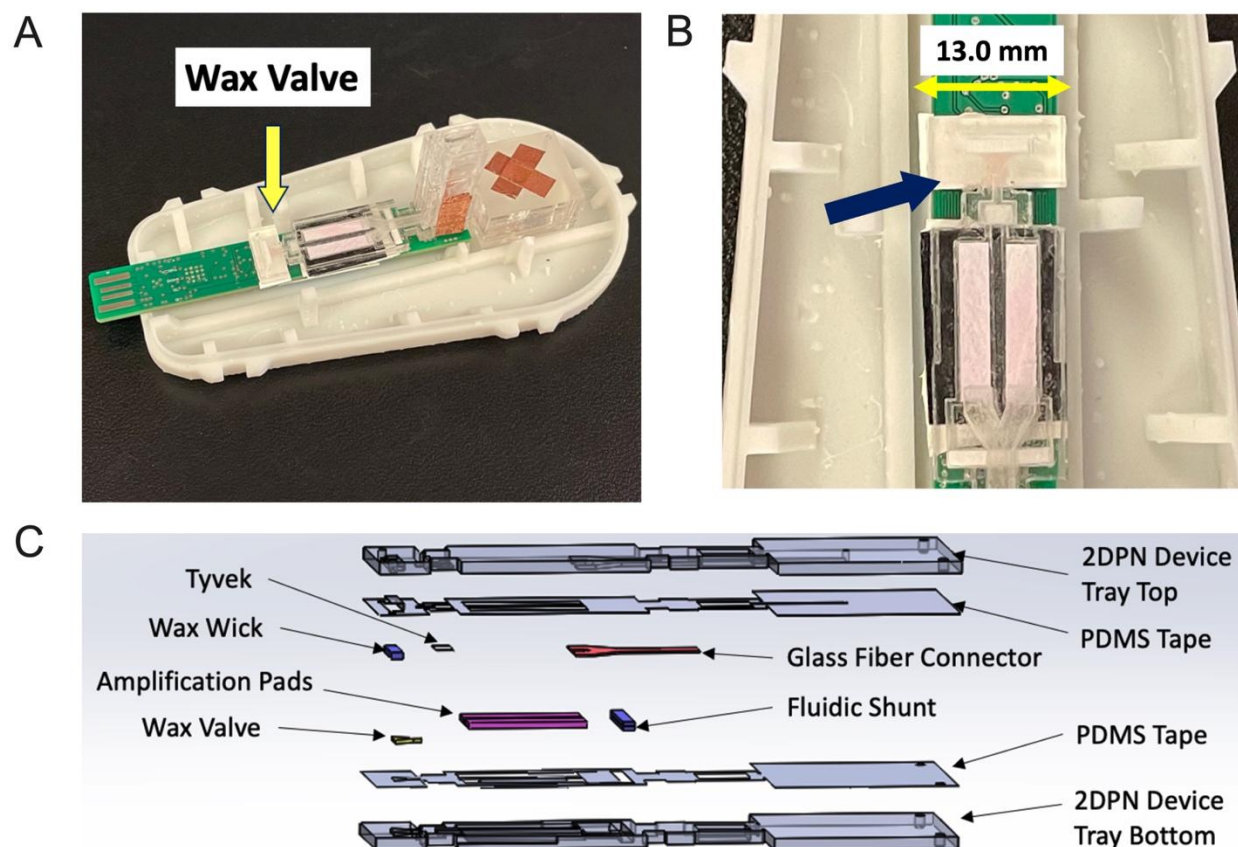


Figure 3.3. Air Spring Valve Integration in the UbiNAAT Device.

(A) Valve location in the UbiNAAT device (arrow). (B) Zoomed in view showing the valve and its placement on the terminal end of the device. (C) Exploded view of UbiNAAT internal device schematic showing wax valve placement between other components.

(Figure by Kevin Jiang and Steven Bennett)

Cell Phone Fluorescence Reader, Image Acquisition, and Data Analysis

As described in previous works, a Google Nexus 5X phone was fitted with a custom black polymethyl methacrylate (PMMA) (McMaster-Carr, Elmhurst, IL) fixture that held a 12 mm wide, 12 mm focal length plano-convex lens (Edmund Optics) and two interference filters: a FES0550 emission short-pass filter (Thorlabs) and BP 587/25 excitation filter (Zeiss) (33,80). The phone was held on a stand 8.5 cm above the amplification device. Images were taken every 60 seconds

using the phone's incandescent white balance setting, 1/5 aperture, 200 ISO, and manually focused to 8.5 cm between lens and device. Images were collected and sent via USB connection to a desktop computer for analysis.

The time at which significant fluorescence appeared (amplification signal liftoff time) for in-tube RT-LAMP were calculated using CFX Maestro, which applied a multivariable, nonlinear regression model to determine initial amplification. For in-pad reactions, a custom MATLAB script was used to quantify the top 1% of pixel intensity within the selected pad's region of interest (ROI), as shown in previous works (33,81). Average background intensity from minutes 5-10 (prior to any signal liftoff) was subtracted the ROI pixel intensity of each image. For every minute over the course of an hour, the top 1% of the most rapidly brightening pixels in each ROI image were quantified using a MATLAB script to a fluorescence intensity value and plotted to generate an amplification signal curve. Signal liftoff times were determined by steep-slope amplification signal rising above a defined fluorescence threshold of 0.02 RFU for both positive and negative samples in QMA-based RT-LAMP assays.

Printed Circuit Board (PCB) Heater

As previously demonstrated, the PCB microprocessor was programmed using TeraTerm to perform automated and timed heating steps (33,80). First, lysis heaters were activated at 100% power for 7 minutes, which warmed the sample to ~95°C for 5 minutes. The heater was then turned off for 3 minutes to allow the sample to cool to between 55 and 60 °C. The valve heater was then activated at 100% for 55 seconds, which opens the valve to allow sample to flow into the amplification pads. This setting was used for both the in-path and air spring valves. Lastly, the amplification heater was run via PID loop control to maintain a heat setpoint of either 74°C (for

in-path valve) or 71.5°C (for air spring valve), which corresponded to an amplification pad temperature of 64°C.

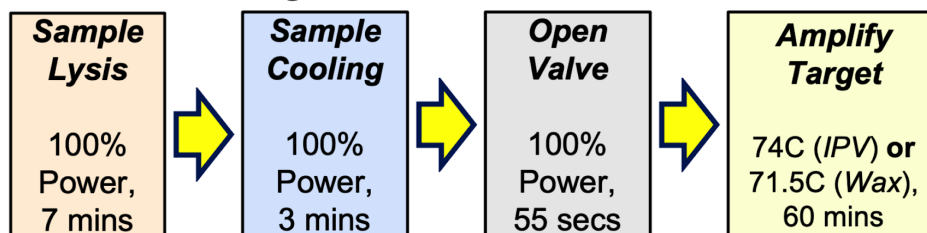
3.4 Results

3.4.1 Thermal Validation of In-Path Valve and Air Spring Valve

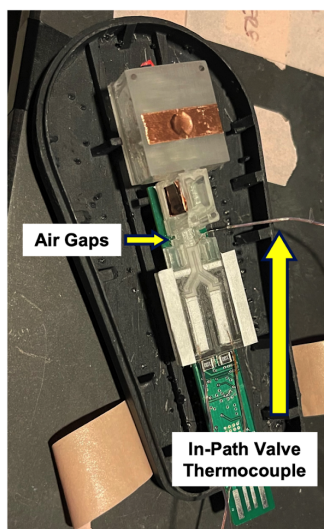
As shown in **Figure 3.1**, the two valve types were thermally actuated through different mechanisms. In its initial state, the in-path valve prevents fluid flow past the valve (**Figure 3.1C**). Once opened through heating, fluid flowed past the valve to rehydrate the rest of the 2DPN. The air spring valve, as seen in **Figure 3.1D**, blocked fluid flow in its initial state by allowing the column of fluid in the lysis chamber and the capillary suction of the fluid of the 2DPN upstream of the valve to compress air within the 2DPN until the pressures were equal. This prevented fluid flow substantially past the lysis chamber, and stopped it well before the fluid could reach the amplification pads. Once the wax was heated and wicked into an adjacent membrane, the valve opened and the pressure in the amplification zones reached ambient through air flow out terminal vents, allowing fluid flow into all of the 2DPN.

We measured the actuation temperature of both the in-path valve and air spring valve to ensure that both valves would open reliably and quickly upon thermal heating. The total actuation time for both valves was set at 55 seconds, with the acceptable time-to-open being defined as less than or equal to 35 seconds. The remaining 20 seconds of both valves' actuation time enable sample rehydration of the connector and amplification pads. **Figure 3.4A** illustrates the full heating protocol as programmed on the printed circuit board (PCB) for lysis, cooling, valve actuation, and amplification. Nasal matrix RNase inactivation via the HUDSON method occurred at the same temperature as lysis, as previously demonstrated in device (33).

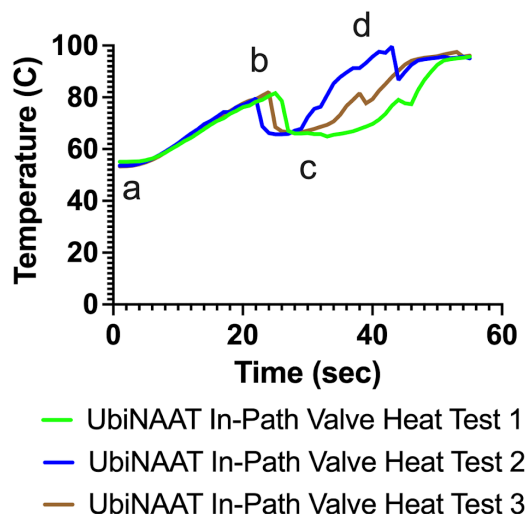
A Device Heating Protocol via Printed Circuit Board



B



C UbiNAAT In-Path Valve Activation



D

Lysis Heat Characterization for In-Path Valve

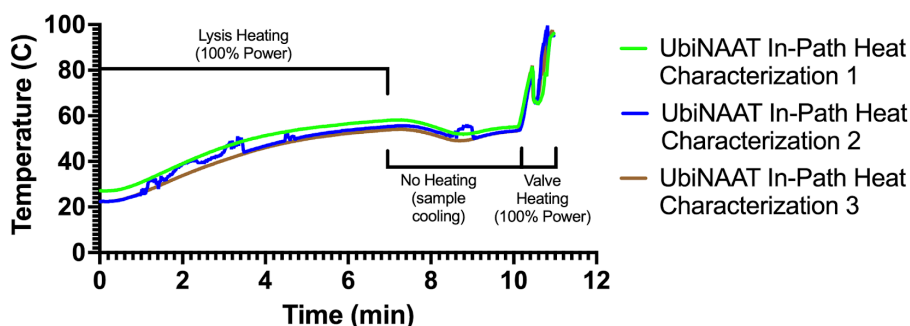


Figure 3.4. Thermal testing of valve actuation.

(A) Heating script as programmed on the printed circuit board (PCB) for automated lysis, cooling, valve actuation, and amplification. Parameters and duration of each step are listed across various PCB resistors. (B) Measurement of temperature at in-path valve during valve actuation via T-type thermocouple, which is embedded in a rastered channel directly beneath the valve. (C) Recorded

temperature at in-path valve during valve actuation. Valve actuation begins with turning on the valve resistors to 100% (a) resulting in temperature increasing until the valve opens (b), resulting in temperature falling from fluidic flow through (c) until temperature increases following full rehydration of the two-dimensional paper network (d). The slight dip at (d) likely indicates filling of air channels surrounding the pads post 2DPN rehydration. (D) Recorded temperature at in-path valve during sample lysis (100% power for 7 minutes) and sample cooling (no heating for 3 minutes).

To monitor the average time and consistency of the in-path valve actuation, we measured the valve temperature during actuation via a T-type thermocouple (Omega, Norwalk, CT) that was placed in a rastered channel directly below the in-path valve (see **Figure 3.4B**). The temperature needed to be monitored to ensure that the solution temperature entering the amplification pads did not exceed 65°C to avoid damaging dried LAMP reagents. The heating protocol was started and continued through the end of valve actuation, with **Figure 3.4C** showing valve temperature during the 55 seconds of actuation. Across three device tests, the average in-path valve actuation time was 23.7 ± 1.2 seconds. **Figure 3.8** illustrates the in-path valve actuation.

In addition to actuation, temperature of the valve was measured via thermocouple during sample lysis and cooling steps, as listed in **Figure 3.4A**. The valve temperature profiles from protocol initiation through valve actuation across the three characterization tests are shown in **Figure 3.4D**. Starting at ambient temperature, the valve thermocouple reading reaches between 55-57°C following 7 minutes of lysis and stays at approximately 55°C during the following 3 minutes of sample cooling. Once the valve heater is activated, the valve thermocouple reading exceeds the threshold. No premature valve leakage was observed during either sample lysis or cooling steps across any of the characterization tests.

Since the polycaprolactone (PCL) on the in-path valves dissolves during actuation, its potential impact on RT-LAMP efficacy during rehydration was tested. Leachate solution was collected from three separate in-path valves, and used to prepare template solution along with 1000 copies of spiked SARS-CoV-2 RNA. The template solution was then used to rehydrate lyophilized reaction tubes to perform RT-LAMP on a 96-well thermocycler. Compared to the positive control (liftoff time of 13.3 mins), each PCL leachate replicate showed no significant difference in liftoff time (13.9 ± 0.4 mins) and yielded higher peak fluorescence (**Figure 3.9**). This result indicated no significant negative impact of PCL leaching into RT-LAMP reaction mixes.

Valve actuation temperature for the air spring valve was evaluated via external heating. With a melting point of approximately 65°C, the wax valve actuation rate was tested on a heat plate (VWR) on top of an aluminum sheet (which represented thermal tape under the internal device). **Figure 3.10** illustrates the extent of a 0.5 cm x 0.5 cm block of dental wax melting at 65°C, 75°C, and 100°C for 60 seconds each. While the 65°C and 75°C heating conditions still showed residual solid wax after a minute of heating, the 100°C heating resulted in fully melted wax after 60 seconds. The air spring valve actuates when the valve is heated at 100% power (reaching approximately 110 °C) (for video, refer to Jiang *et al.* (2024) (33)). The higher temperature used to actuate the air spring wax is less critical given its location far away from the amplification pads on the distal end of the path, ensuring that the LAMP reagents would not be damaged during valve heating.

3.4.2 In-Path Valve Integration in 2-Plex UbiNAAT Operation

To demonstrate the in-path valve compatibility in a point-of-care (POC) device, we integrated it into the UbiNAAT 2-plex device to simultaneously detect SARS-CoV-2 and influenza A viruses that had both been spiked onto a single human nasal swab. To meet this goal, the valve must not

only block fluid flow up to the moment of actuation, but also enable RT-LAMP detection of both SARS-CoV-2 and influenza A post-rehydration. As shown in **Figure 3.5**, both COVID and influenza A (Flu A) assay pads showed signal detection of its respective viral targets from the human nasal swab. **Figure 3.5A-3.5B** illustrates the RT-LAMP fluorescence signal and quantified results for both assays. QMA-based RT-LAMP in the UbiNAAT device showed detection at 53.7 minutes in the COVID assay pad and at 46.5 minutes in the Flu A assay pad. In-tube lyophilized RT-LAMP controls were run in parallel with spiked nasal matrix at 1000 viral copies per reaction for both assays. **Figure 3.5C** shows the amplification signal curves from both positive and negative nasal controls, with the COVID positive control showing amplification by 17.1 minutes and the Flu A positive control showing amplification by 14.2 minutes. Neither assay's negative nasal control showed detectable signal amplification by the end of an hour.

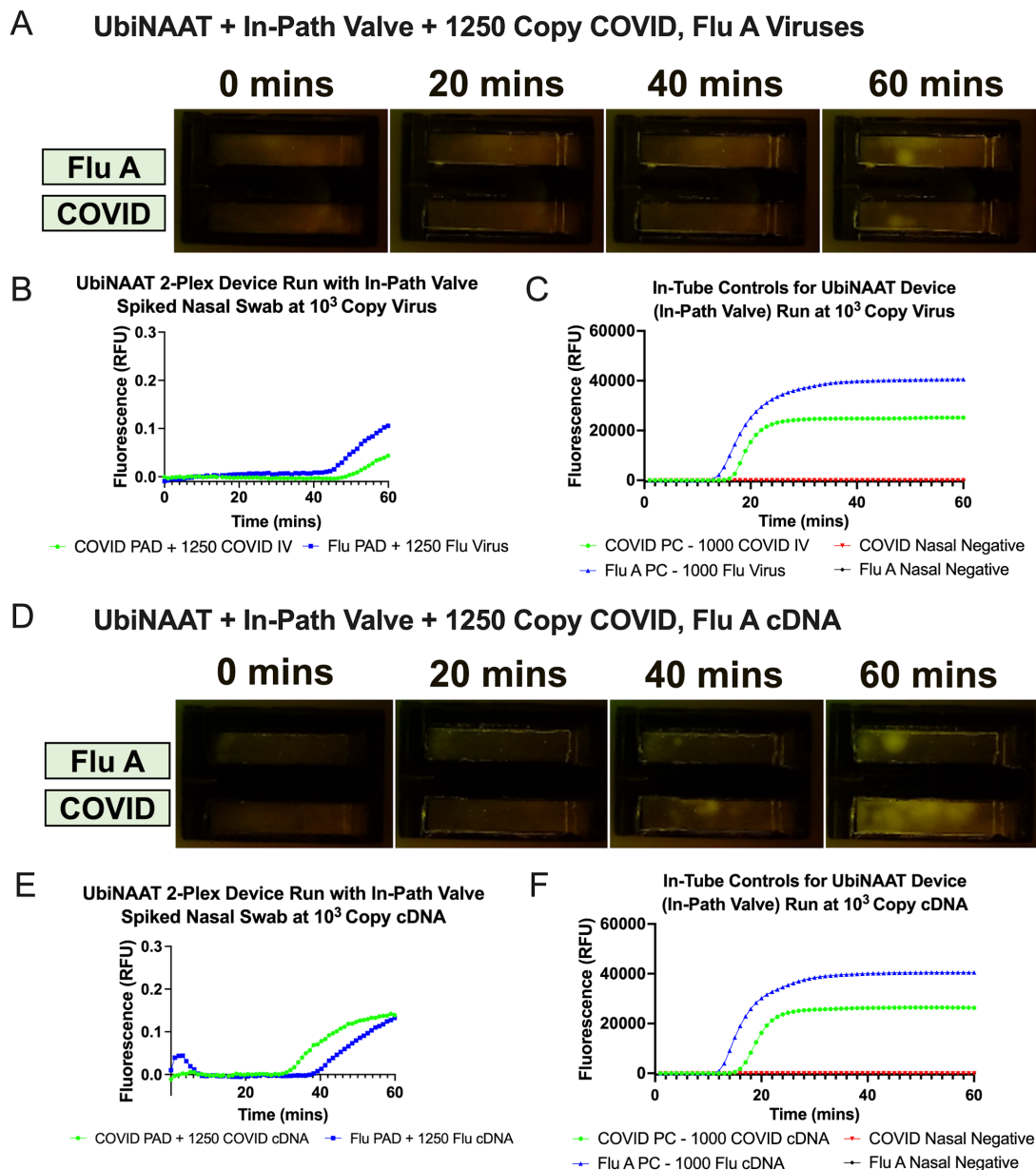


Figure 3.5. In-Path Valve integrated UbiNAAT 2-plex RT-LAMP detection of COVID inactivated virus (IV) and Flu virus spiked onto human nasal swab samples.

All device and tube reactions were run with human nasal matrix. (A) Real-time RT-LAMP fluorescence signal of COVID and Flu A viruses over an hour. (B) Quantified fluorescence from 2-plex UbiNAAT to detect both COVID and Flu A viruses simultaneously. (C) In-tube RT-LAMP nasal controls show clear positive signals from viral samples and flat NTC (no template control) curves. (D) Real-time LAMP fluorescence signal of COVID and Flu A cDNA over an hour. (E) Quantified fluorescence from 2-plex UbiNAAT to detect both COVID and Flu A cDNA

simultaneously. (F) In-tube RT-LAMP nasal controls show clear positive signals from cDNA samples and flat NTC curves.

We further tested the in-path valve's compatibility with UbiNAAT operations using cDNA samples for both SARS-CoV-2 and Flu A to evaluate whether DNA targets could be detected at better sensitivity compared to RNA targets. As DNA is more robust than RNA, it could be less susceptible to heat damage during valve actuation. In place of a viral sample, 1.5×10^4 copies of cDNA for both pathogens were spiked onto a single human nasal swab, before proceeding with UbiNAAT operations. As shown in **Figure 3.5D**, both COVID and Flu A assay pads showed signal detection of its respective cDNA targets from the human nasal swab. **Figure 3.5E** indicates that QMA-based RT-LAMP showed detection of cDNA at 41.3 minutes in the COVID assay pad and at 34.1 minutes in the Flu A assay pad. For in-tube controls, the COVID positive cDNA control at 1000 copies showed signal liftoff at 16.4 minutes, while the Flu A positive cDNA control at 1000 copies showed signal liftoff at 12.6 minutes. Neither assay's negative nasal control showed detectable signal amplification by the end of an hour.

3.4.3 Air Spring Wax Valve Integration in 2-Plex UbiNAAT Operation

The air spring wax valve's compatibility with the UbiNAAT 2-plex device detection of SARS-CoV-2 and influenza A viruses from a single human nasal swab was also tested. Similarly to the in-path valve device tests, both pathogen viruses were spiked onto a single swab. However, two different viral loads for each pathogen were tested; the first test was performed at 1.5×10^4 copies of each virus per reaction, while the second test was performed at 7500 copies of each virus per reaction. For the two tests, rehydrated QMA pads held up to 1250 and 625 copies of each virus, respectively. As shown in **Figure 3.6**, both COVID and Flu A assay pads showed signal detection

of its respective viral targets from the human nasal swab. **Figure 3.6A** illustrates the amplification fluorescence signal for both assays at 1250 viral copies per reaction. **Figure 3.6B** shows the quantified fluorescence curves, which indicated detection at 20.6 minutes in the COVID assay pad and at 17.5 minutes in the Flu A assay pad. In-tube lyophilized RT-LAMP controls were run in parallel with spiked nasal matrix at 1000 and 500 viral copies per reaction for both assays. **Figure 3.6C** shows the amplification signal curves from both positive and negative nasal controls, with the COVID positive control showing amplification by 17.4 ± 0.1 minutes (n=2) and the Flu A positive control showing amplification by 15.1 ± 0.5 minutes (n=2). Neither assay's negative nasal controls (n=2) showed detectable signal amplification by the end of an hour.

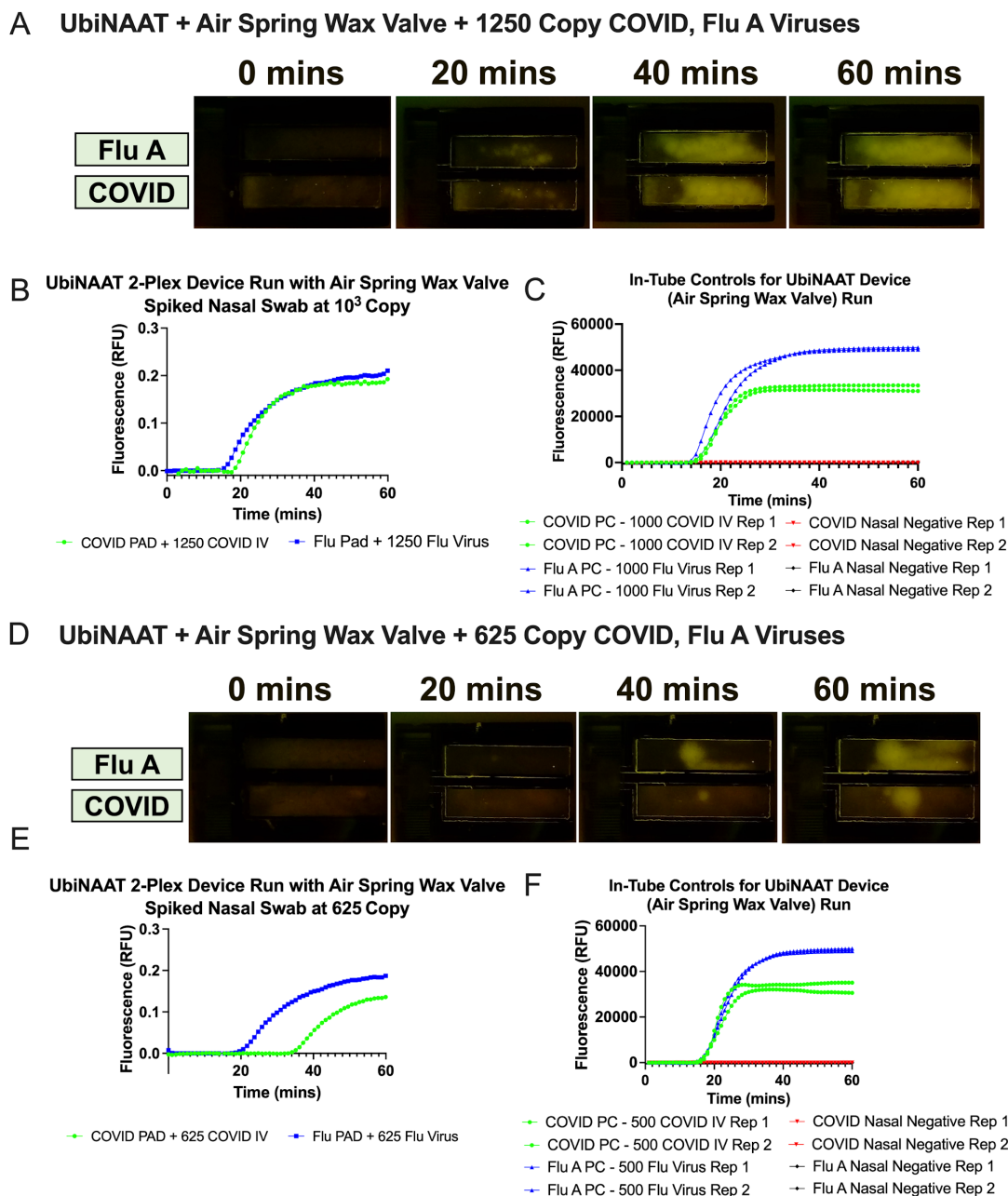


Figure 3.6. Air Spring Valve integrated UbiNAAT 2-plex RT-LAMP detection of COVID inactivated virus (IV) and Flu virus spiked onto human nasal swab samples.

All device and tube reactions were run with human nasal matrix. (A) Real-time RT-LAMP fluorescence signal of COVID and Flu A viruses at 10^3 copies per swab over an hour. (B) Quantified fluorescence from 2-plex UbiNAAT to detect both COVID and Flu A viruses at 10^3 copies per swab simultaneously. (C) In-tube RT-LAMP nasal controls show clear positive signals from viral samples at 10^3 copies and flat NTC (no template control) curves ($n=2$). (D) Real-time

LAMP fluorescence signal of COVID and Flu A cDNA at 625 copies per swab over an hour. (E) Quantified fluorescence from 2-plex UbiNAAT to detect both COVID and Flu A viruses at 625 copies per swab simultaneously. (F) In-tube RT-LAMP nasal controls show clear positive signals from viral samples at 500 copies and flat NTC curves (n=2).

When tested with 7500 viral copies of both SARS-CoV-2 and influenza A per nasal swab, the air spring wax valve integrated device also enabled early detection of each pathogen in their respective assay pad. As shown in **Figure 3.6D**, both COVID and Flu A assay pads showed signal detection of 625 viral copies. The quantified fluorescence curves in **Figure 3.6E** indicated that QMA-based RT-LAMP showed detection of cDNA at 37.2 minutes in the COVID assay pad and at 22.7 minutes in the Flu A assay pad. For in-tube controls, the COVID positive viral nasal control at 500 copies showed signal liftoff at 16.1 ± 0.3 minutes (n=2), while the Flu A positive viral nasal control at 500 copies showed signal liftoff at 17.8 ± 0.2 minutes (n=2). Neither assay's negative nasal controls (n=2) showed detectable signal amplification by the end of an hour.

3.5 Discussion

While the two types of valves operate through different mechanisms, both valves successfully blocked fluid flow in the device prior to the completion of lysis and sample inactivation. Their differing mechanisms of actuation enable them to serve different functions. For the in-path valve, its mechanism consisted of simultaneously melting and dissolving the polycaprolactone and heat-shrinking the PVC film. The PVC film is designed to shrink once heated above a critical temperature ($\sim 65^{\circ}\text{C}$), which in this case pull the middle slit apart to create an enlarged opening (not shown). Once the valve was open, fluid moved from one connector pad through the opening to the next, rehydrating the 2DPN. This method allowed for fluids to travel up to but not past the

valve, allowing for additional sample processing steps to be incorporated in future iterations. While the high temperature used to actuate the valve was important for fast actuation, there was noted concern for sample stability as high heat introduced significant variability to the solution temperature as it entered the amplification zones. We hypothesize that this heat step could have caused potential damage to the nucleic acids, which may be the reason for worsened nucleic acid amplification performance. Alternatively, the air spring valve functioned by confining air within the 2DPN prior to actuation, resulting in nearly all of the fluid staying within the lysis chamber once its pressure (primarily due to gravity) has equaled the pressure of the compressed air ahead in the 2DPN. Its placement on the terminal end of the 2DPN not only avoided direct and uncontrolled heating of the sample, but also prevented the valve components from interacting with the sample and RT-LAMP reagents, eliminating the possibility of contamination or wax inhibition. The ability to use high temperature to actuate the air spring valve allowed for fast operation without risking sample or reagent damage. While this method of using a single valve is compatible with critical RT-LAMP steps in our device (lysis, sample inactivation, and amplification), other procedures (e.g. sample concentration, filtration) will likely require additional valves along the fluidic pathway to separate the additional chemical processing steps.

The air spring valve device allowed early detection of both SARS-CoV-2 and influenza A viruses at both spike levels on the human nasal swab. By removing interaction with all reagents and membranes upstream of the amplification sites, the air spring valve eliminates possibility of contamination while still allowing for complete control of fluidic movement within the flow path. As observed in **Figure 3.6**, all test conditions in device showed strong fluorescence signal amplification across the entirety of the pads and early liftoff times, except at the lower copy COVID assay condition. Early positive control detection and flat negative controls indicated little

to no contamination or human error (**Figure 3.6C**). The decrease in amplification rate for the paper-based RT-LAMP assay may have been exacerbated by QMA blocking treatments or inadequate storage of the lyophilized pads, as mentioned in previous studies (33,63). With little interaction with the 2DPN and an isolated PMMA holder section, the air spring valve prevented additional heating of the lysed sample prior to rehydrating the amplification pads. Its design offers two distinct advantages. First, the air spring mechanism can be performed with any material that could be thermally actuated, as its lack of interaction with the rest of the 2DPN eliminates the risk of contamination or cross-reactivity in each assay for unspecific targets. Second, the air spring valve's presence at the end of a fluid path eliminates unnecessary heating of the sample and could be easily integrated with other paper-based assay designs. Due to these advantages, we have incorporated the air spring valve into a larger sample testing of spiked human nasal swabs for greater validation of the UbiNAAT's 2-plex detection of SARS-CoV-2 and influenza A (33). In future work, we intend to explore integration of the air spring valve into other assay designs in addition to NAAT.

Notably, the in-path valve performed worse compared to the air spring valve when integrated into the UbiNAAT for 2-plex detection of 1.5×10^4 SARS-CoV-2 and influenza A viruses from a nasal swab. As shown in **Figure 3.5B**, both the COVID and flu A assay pads both showed only small regions of fluorescence signal growth and signal liftoff times past 46 minutes, which is past the acceptable threshold of 30 minutes time-to-result for commercial in-vitro COVID tests (14,103). Early signal detection for in-tube positive control across both assays and flat negative control signal curves suggested that the late amplification was not caused by assay contamination (**Figure 3.5C**), and is further supported by the polycaprolactone leachate not significantly impacting RT-LAMP performance (**Figure 3.9**). When the nasal swab sample was spiked with 1.5×10^4 copies

of cDNA for each pathogen, amplification results significantly improved across both assays in signal liftoff time and, in the COVID pad, a greater fluorescence signal across the entire pad. This suggested that the thermal actuation of the in-path valve may have damaged the sample prior to its rehydration of the amplification pads. With temperature at the valve reaching up to 95°C, the lysed RNA sample may have been degraded, whereas cDNA remained more robust to heating. The heated sample may have similarly denatured enzymes upon rehydration, which worsened the assay's performance. Additionally, the valve heating step may have also caused increased binding of RNA to the glass fiber pad, which has been previously observed (63). While the in-path valve offered distinct advantages of being easily integrated in the middle of a 2DPN for fluidic control, its actuation may have limited its compatibility with RNA-based amplification system. This issue could be addressed by reducing the size of the valve or replacing polycaprolactone with another material to reduce the temperature required for actuation. In future iterations, we intend to explore the in-path valve's application with other assay types.

For both valves, material composition was selected for reliable thermal actuation and robust durability in normal operating conditions. A key advantage of thermal actuation is the widespread availability of both automated and manual methods, including but not limited to printed circuit boards, heat plates, heat packs, and water baths. For the in-path valve, the polycaprolactone covering the PVC film slit requires higher than 60°C heating to dissolve, while the PVC valve itself does not open until heated above 65°C. Similarly, the dental wax employed in the air spring valve only consistently starts melting above 65°C. Stability up to at least 65°C allows either valve-device variant to be capable of being transported and utilized in warm climates, with portability made even more possible by the compact form of the UbiNAAT itself. Additionally, lysis heating only raises the temperature at the in-path valve site by a consistent 30°C over the course of 7

minutes despite being powered at 100% (with resistive heaters reaching up to 130°C). This was achieved by minimizing heat transfer between the lysis chamber and in-path valve through the addition of air gaps (**Figure 4B**) and isolating device contact with the PCB heater using thermal tape. This approach allows the device to be implemented in climates reaching up to 35°C without concerns of premature valve leakage. In climates above 35°C, the device could be readily adapted by shortening the lysis heating time due to less time needed to reach lysis temperature or by swapping the valve film or wax with more robust materials. With both valves being affordable and manufacturable with automated processes such as liquid injectors and heat pressing, they can readily be produced in large scale in (and for use in) low resource settings.

To accommodate each valve's design, the UbiNAAT's internal device amplification zone dimensions needed to be adapted. With a width of 13.0 mm, the in-path valve required an amplification zone width of 20.3 mm (**Figure 3.2B**). The greater width caused higher heat dissipation towards the outer edges of the amplification zone, which required a higher PCB amplification setpoint of 74°C. The air spring valve, with a smaller width of only 3.0 mm, enabled the UbiNAAT amplification zone to be narrowed to 13.0 mm. As a result, the air spring valve device's amplification zone is only slightly wider than the PCB heat trace, resulting in less heat loss on the horizontal plane. To prevent heat loss along the length of the amplification zone, air gaps were introduced to further divide the air spring valve from the amplification pads, allowing for the use of a high valve actuation temperature (**Figure 3.3B**). This noticeably improved heat uniformity throughout the amplification zone, where a PCB resistive heating setpoint of 71.5°C enabled 64°C within the amplification pads. The higher amplification setpoint used for the in-path valve device may have also contributed to slower amplification, as a higher heat gradient likely

caused uneven heating along the entire assay pad. This may be mitigated by reducing the size of the in-path valve and the connector pads to equalize the width of the two devices.

3.6 Conclusions

We have demonstrated two adaptable designs for thermally-actuated valves to enable complex fluidic control, and demonstrated both in a multiplexed, paper-based rapid analysis tool that simultaneously detected multiple respiratory pathogens (COVID and influenza A) from a nasal swab. Both valves showed reliable thermal actuation, are robust in their functionality, and are simple to manufacture. The flexibility to insert a valve in the middle or end of a paper-based system enables compatibility with different assay types depending on reagent or heating compatibility. The heat durability of the materials used in both valves also encourages application in POC settings and diverse climates. We believe that these valves can be implemented across many microfluidic systems (including those without microporous materials forming the fluidic channels) to address multi-step fluidic needs. Future work from our group will report on demonstration of multi-step assay devices incorporating complex, valve-enabled sample processing of human samples for highly sensitive detection of RNA or DNA-based pathogens in a sample-to-result point-of-care platform.

3.7 Acknowledgments

This work reported here was principally funded by generous gift support from the Mercatus Foundation through an Emergent Ventures Fund Fast Grant to the Yager Laboratory at UW. We also wish to thank the National Institute of Allergy and Infectious Diseases (NIAID) for their

support through Award Number FA215700/A177358. Special thanks to healthy volunteers who donated human nasal swabs to this study.

We would like to acknowledge the contributions of the research team at PATH, which had co-developed along with the Yager Lab an earlier, mechanically-actuated version of the air spring valve (unpublished prior to this manuscript). This first iteration utilized silicone tubing pinched by a lever to similarly trap air at the end of the fluidic pathway, with dissolvable “spy paper” holding the lever in place. To open the valve, fluid flow along a secondary pathway dissolved the spy paper and released the lever, which then released the silicone tubing, allowing the fluid pathway to reach ambient pressure, allowing flow along the primary flow path. This earlier design inspired the current air spring wax valve, which is composed of affordable dental wax and employs thermal heating for actuation. This prior development of the air-spring valve was performed under our MAD NAAT grant HR0011-11-2-0007 from DARPA from 2011 to 2016.

We also want to thank Mike Roller for his work on developing the in-path valve and other components that integrated into the UbiNAAT device (80).

I want to acknowledge Steven Bennett, who is a co-first author on this manuscript. This manuscript is currently in review with PLOS One.

3.8 Supplemental Information

Table 3.1. Reverse transcription loop-mediated isothermal (RT-LAMP) assay reagents for tube and paper-based recipes

Reagent	Final Concentration (In-tube)	Final Concentration (QMA)
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WarmStart LAMP Kit	1X	1X
SYTO-82	7.5 μ M	7.5 μ M
Hydroxynaphthol Blue	40 μ M	40 μ M
Assay specific primers (See Tables S1-S4)	varies	varies
Trehalose	10%	10%
Dextran, 500 kD	0.5%	0.5%
Sample	1 μ L in volume	1 μ L in volume
Nuclease-Free H ₂ O	Fill up to 20 μ L	Fill up to 25 μ L

Table 3.2. Nucleotide sequences of LAMP primers that target the pandemic influenza A gene.

Primer	Sequence (5' – 3')	Final Concentration
F3-1	GACTTGAAGATGTCTTTGC	0.1 μ M
F3-2	GACTGGAAAGTGTCTTTGC	0.1 μ M
B3-1	TRTTATTTGGGTCTCCATT	0.1 μ M
B3-2	TRTTGTTTGGGTCCCCATT	0.1 μ M
FIP	TTAGTCAGAGGTGACARRATTGCAGATCTTGAGG CTCTC	1.6 μ M
BIP	TTGKTTTCACGCTCACCGTGTTTGGACAAAGCGTC TACG	1.6 μ M
Loop F	GTCTTGTCTTTAGCCA	0.4 μ M
Loop B	CMAGTGAGCGAGGACTG	0.4 μ M

Table 3.3. Nucleotide sequences of LAMP primers that target the SARS-CoV-2 N gene.

Primer	Sequence (5' – 3')	Final Concentration
F3	TGGCTACTACCGAAGAGCT	0.2 μ M
B3	TGCAGCATTGTTAGCAGGAT	0.2 μ M
FIP	TCTGGCCCAGTTCCTAGGTAGTCCAGACGAATTCG TGGTGG	1.6 μ M
BIP	AGACGGCATCATATGGGTGTCACGGGTGCCAATGT GATCT	1.6 μ M
Loop F	GGACTGAGATCTTTCATTTTACCGT	0.4 μ M
Loop B	ACTGAGGGAGCCTTGAATACA	0.4 μ M

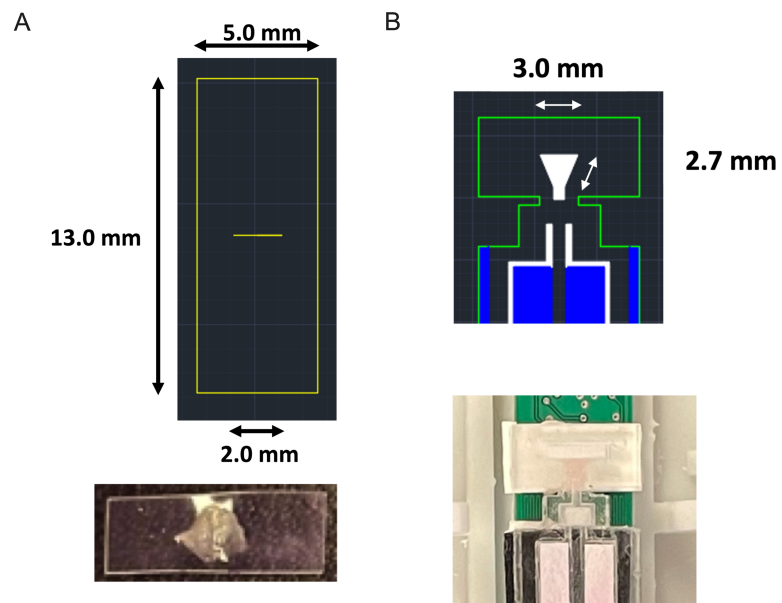


Figure 3.7. Valve dimensions and assembly

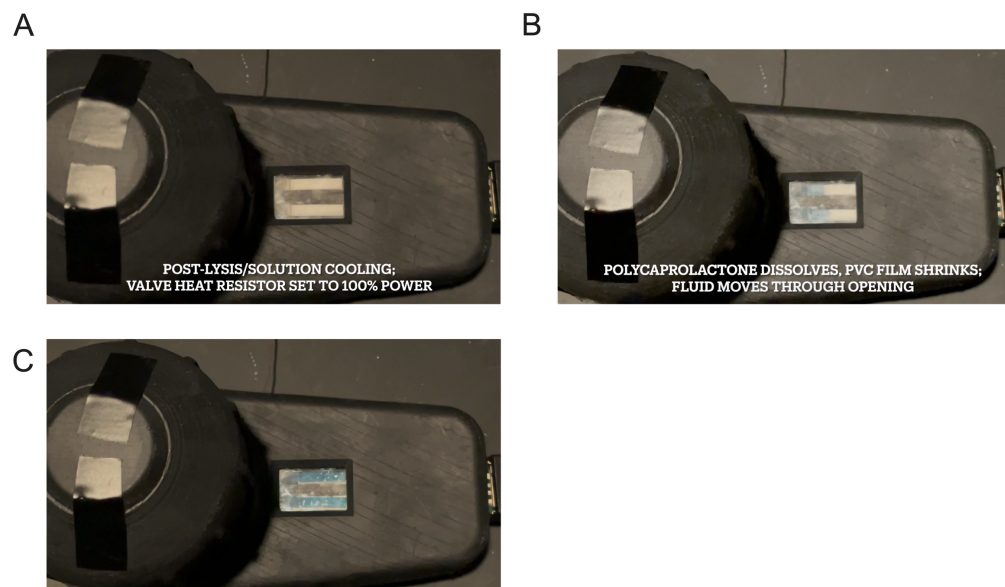


Figure 3.8. Diagram of In-Path Valve Actuation

(A) Post-lysis, the valve heater is activated at 100%. (B) Polycaprolactone dissolves and PVC film shrinks, enabling fluid to move from lysis chamber through opening in valve and across paper network. (C) Fluid fully rehydrates amplification pads.

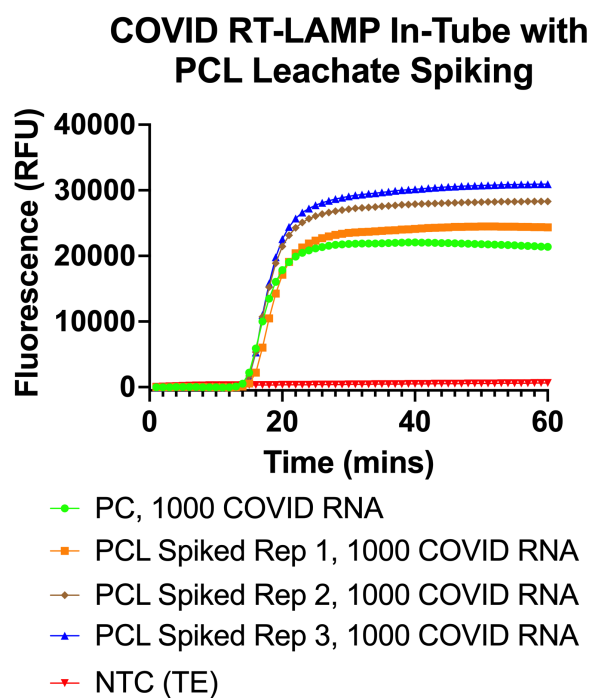


Figure 3.9. Impact of polycaprolactone leachate on RT-LAMP efficacy.

Leachate extracted from heating in-path valve shows no impact on RT-LAMP fluorescence signal compared to positive control.

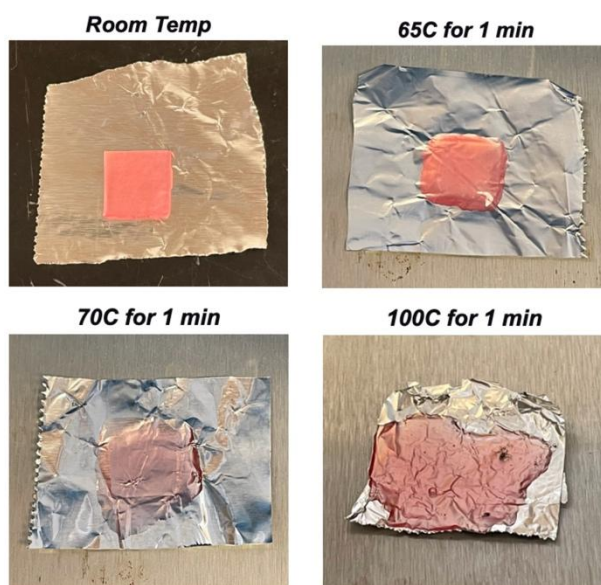


Figure 3.10. Thermal testing of dental wax used in air spring valve.

0.5 cm x 0.5 cm wax blocks were prepared at room temperature. Wax blocks were separately heated for 60 seconds at 65°C, 70°C, and 100°C to evaluate extent of melting.

4. Chapter Four - A low-cost point-of-care device for simultaneous detection of bacterial sexually transmitted pathogens in vaginal swab samples

4.1 Abstract

Curable sexually transmitted infections (STIs) caused by the bacteria *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) are widespread globally. These infections are particularly dangerous for female patients, causing pelvic inflammatory disease, infertility, and increased risk for HIV acquisition. Vaginal self-swab sampling can improve access to STI screening but is still subject to treatment delays due to centralized processing. A low-cost point-of-care (POC) device capable of detecting these bacteria from a self-collected vaginal swab could address this delay and allow for more timely treatment. In this work, vaginal swab material from patients infected with CT or NG required a filtration step before lysis and loop mediated isothermal amplification (LAMP) detection on the UbiNAAT device. We have shown a simple, low-cost sample preparation method that supports rapid DNA detection from NG and CT in vaginal swab samples from clinical female patients using our POC UbiNAAT platform.

4.2 Introduction

There are over 1 million new curable sexually transmitted infections (STIs) globally *every day* (119). The most common bacterial STIs are caused by *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), and *Treponema pallidum* (TP), in decreasing prevalence; together these bacteria accounted for 2.5 million new infections in the United States in 2022 (120). Despite a

large portion of CT and NG infections being asymptomatic, they can still cause adverse outcomes, especially in female patients, including pelvic inflammatory disease, infertility, and an increased risk of HIV acquisition (121,122). Worryingly, strains of NG that are antimicrobial resistant (AMR) or even multi-drug resistant (MDR) have increased in prevalence recently (123), prompting changes in treatment guidelines. Since 2020, the recommended treatment for uncomplicated NG has been a single antimicrobial, ceftriaxone (124). When NG strains resistant to ceftriaxone emerge, clinicians may run out of suitable treatment options.

One of the key strategies suggested by the World Health Organization (WHO) for controlling STIs is increased access to screening (119). Barriers to widespread screening for STIs include social stigma, worries about confidentiality, and cost (125). Currently, NG and CT are diagnosed mainly by nucleic acid amplification testing (NAAT, e.g. PCR); while culture or gram staining can be used, NAATs are considered more accurate, especially for asymptomatic infections (126). For female patients, urine samples have been widely used for diagnosis, and intriguing devices have been developed for the use of urine at the point of care (127). However, as a sample, urine suffers from sensitivity variations (e.g., clean-catch *versus* first-catch sampling protocols, degree of hydration of the subject) and low pathogen concentrations. A recent meta-analysis of vaginal swab samples shows them to be more sensitive than urine samples (128). One way to lower screening barriers is to implement self-collection of the clinical sample. Several studies have demonstrated the feasibility of using self-collected vaginal swab samples for the diagnosis of CT and NG (120,129–131). Any differences in sample self-collection *versus* clinician-collected samples have been outweighed by the benefits of increased screening, including better reach to first-time testers (131).

However, self-sampling alone cannot guarantee better patient outcomes. Part of the problem is the time it takes to receive a result: up to 9 days in a typical healthcare system (132). On-site use of the Cepheid GeneXpert rapid qPCR CT/NG test instead of processing in an off-site hospital laboratory reduced the time from testing to treatment from a median of 10 days to 2 days (133). A 30-minute point-of-care (POC) test for CT/NG (Binx Health *io*) was shown to reduce time to treatment, as well as reduce unnecessary antibiotic treatment through improved specificity, but also increased the cost of testing (134). The Visby Medical PCR point-of-care disposable diagnostic device was shown to be sensitive and specific for CT, NG, and *Trichomonas vaginalis* (TV) (135). Though the Visby device is an exciting development in the field, it is complex and expensive, especially for a single-use test. While it tackles the issue of long wait times between sample and result, its cost could hamper its effectiveness in low-resource settings. Non-NAAT rapid diagnostics are less expensive but have been shown to lack the sensitivity and specificity required for impactful screening and treatment of patients (132,136). Therefore, the diagnostic with the most impact would 1) analyze self-collected vaginal swab samples, 2) be a sensitive and specific NAAT, 3) be widely available and inexpensive, 4) be easy to use, and 5) provide results in 30-60 minutes, ideally before a patient leaves the clinic. It is conceivable these technologies could also be used in the home for speed and to avoid social stigma, but home testing may have public health logistics that need to be addressed before its widespread use (137).

Increasingly, isothermal amplification NAATs have been investigated for their use in low-resource settings; their need for only a single incubation temperature (as opposed to PCR's temperature cycling protocol) reduces the complexity of running these assays. One of the most commonly used isothermal methods is loop mediated isothermal amplification (LAMP); it uses 4 to 6 primers, 2 of which create looped products that allow exponential amplification by a strand displacing

polymerase (138,139). This method is fast, sensitive, has greater resistance to inhibitors than PCR (140), and can be coupled to reverse transcriptase (RT) activity for detecting RNA (141,142). During the SARS-CoV-2 pandemic, LAMP technology advanced rapidly since the speed and technical ease of using the assay was particularly attractive for product development (143,144). LAMP has been incorporated into microfluidic devices, allowing for the future detection of pathogens outside the lab (145).

We envision the above workflow for a commercialized version of this device (**Figure 4.1**). When a patient checks into their clinic, they are provided with a swab and instructions for self-sampling. Then, the clinician inputs the swab to the device, which runs for <60 minutes. After the run has completed, the clinician checks the result, counsels the patient, and supplies antimicrobial treatments (if needed) before the patient leaves the clinic. With sufficient ease of use, this technology would also be appropriate for the diagnosis of STIs in a home setting, with results transmitted to clinicians for treatment decisions.

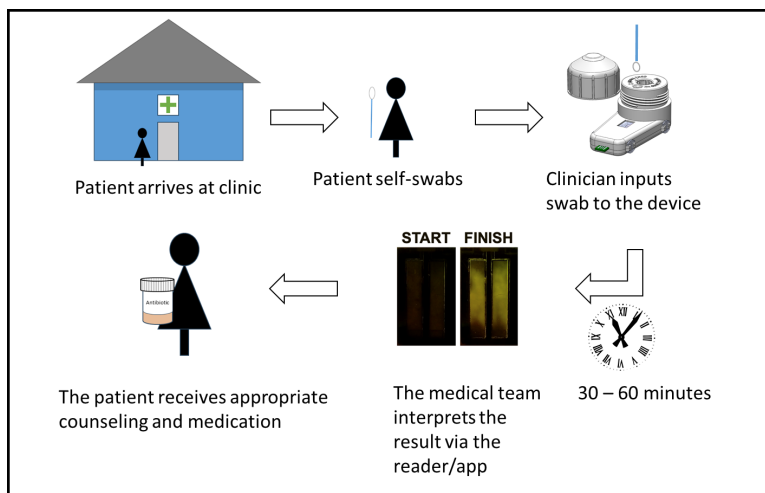


Figure 4.1. Proposed future workflow for increased CT/NG screening using UbiNAAT in a point-of-care setting.

(Figure by Erin Heiniger)

In this work, we demonstrate the detection of CT and NG DNA from clinical vaginal swab samples in an inexpensive point-of-care integrated device based on our previous work demonstrating a swab-to-result respiratory panel diagnostic on the low-cost (\$~3 in single-use disposable parts/reagents) UbiNAAT platform (33). Briefly, a patient swab is inserted into the device and agitated in a nuclease-inactivating lysis buffer. Then, the device is activated and automatically heats the sample to lyse the cells and release the nucleic acids. Lysate is allowed to flow into porous membranes carrying lyophilized LAMP reagents. The amplification regions are heated, and amplification is detected *via* a fluorescence increase using a cell phone. The device was successfully adapted to detect CT and NG DNA from clinical vaginal swabs after filtration.

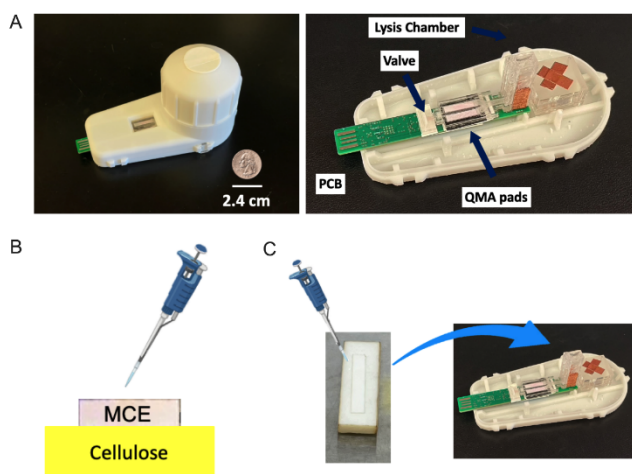


Figure 4.2. UbiNAAT integrated device

(Figure by Kevin Jiang)

(A) Fully assembled device (left) and device without top enclosure (right) (B) Mixed cellulose ester (MCE) filtration setup with cellulose wicking membrane (C) Transfer of MCE membrane post-filtration and rinsing into lysis chamber on UbiNAAT device. (B) and (C) not to scale.

4.3 Results and Discussion

In this paper we show vaginal swab samples from patients contain amplification inhibitors that can be excluded from the sample by filtration. The filter captures pathogenic bacteria, and can be input to our UbiNAAT device for cell lysis followed by LAMP fluorescence detection of pathogen DNA in a POC-compatible manner.

4.3.1 Commercial swabs spiked and treated with HUDSON

For initial development of the proposed diagnostic device, we purchased “normal” (non-disease-state) vaginal swab samples from a commercial source, then spiked them with CT or NG bacteria as described above. Spiked samples were then used to rehydrate LAMP reagents in QMA porous pads and incubated at 63°C for amplification. Our previous work showed that nasal swab samples could be treated in this manner and supported amplification of viral RNA with no further sample preparation.²⁸ We observed amplification from the NG-spiked sample when NG primers were used (**Figure 4.3A-B**). Likewise, the CT-spiked sample amplified when CT primers were used. Cross reactivity of the LAMP primers was not observed, as NG-spiked samples did not amplify when CT primers were used and *vice versa*. Non-spiked samples did not amplify when either primer set was used, confirming that these samples were negative for both CT and NG before spiking. These results led us to believe vaginal swab samples could be used on devices with this simple sample preparation method. A simple protocol is ideal for delivering a diagnostic device at the lowest possible cost.

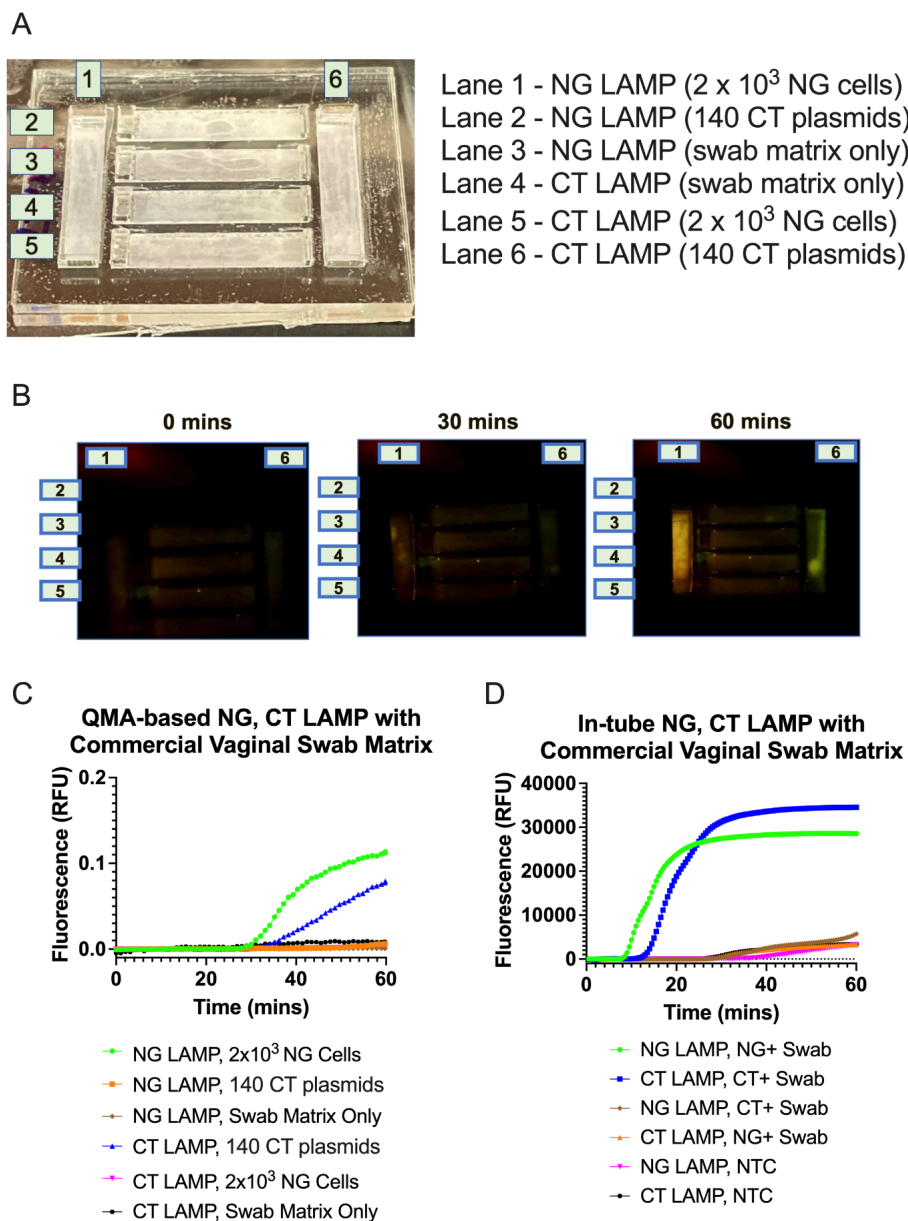


Figure 4.3. Six-pad test device running lyophilized LAMP on QMA membrane with commercial vaginal swab lysate spiked with *Neisseria gonorrhoeae* (NG) or *Chlamydia trachomatis* (CT) bacterial cells

(A) 6-pad PMMA tray holding 6 QMA membranes with rehydrated NG or CT LAMP reagents (B) Representative images of fluorescence for signal amplification in-paper as imaged by cell phone reader showing fluorescence signal growth across 60 minutes (C) Quantified NG and CT LAMP results from QMA membrane image stacks. Results are representative of reproduced replicates (not shown). (D) In-tube NG and CT LAMP processed on BioRAD CFX thermocycler with

commercial vaginal swab lysate spiked with Neisseria gonorrhoeae (NG) or Chlamydia trachomatis (averaged fluorescence signal across n=3 replicates)

(Figure by Erin Heiniger, PhD and Kevin Jiang)

In the QMA pads, we observed amplification liftoff at 30-35 minutes after the start of amplification heating (**Figure 4.3C**). When the sample was used to rehydrate LAMP reagents in PCR tubes, we observed liftoff at 10-15 minutes while retaining specificity (**Figure 4.3D**). These results are consistent with our previously reported observation that amplification in our porous membranes has been slower than in PCR tubes (33). We speculate that heating conditions and membrane treatment—perhaps the BSA coating required to prepare the membrane for lyophilization—interfered with the speed of the reaction, but we have no definitive evidence of the latter hypothesis.

4.3.2 Surprisingly, JHU clinical swabs inhibited amplification

We initially believed clinical swab samples provided by JHU could be processed in the same way as those from the commercial source because they used almost identical buffers and similar swabs. For initial testing of the JHU swab material, we took a portion of several samples, added TCEP to a final concentration of 2.5 mM, and used a thermal lysis protocol. However, when these samples were used to rehydrate LAMP reagents lyophilized in tube, we noted that many of them changed the color of the solution (**Figure 4.4A**). Hydroxynaphthol blue (HNB), which is used in the UbiNAAT LAMP amplification protocol to reduce background fluorescence and improve signal-to-noise ratio (33), is purple in the presence of higher concentrations of divalent cations, such as Mg^{2+} and Ca^{2+} , and blue in lower concentrations (146,147). When DNA in buffer was used to rehydrate the same reagents, the solution remained blue.

The unexpected color of the HNB in the clinical sample assay tests not only indicated higher than expected divalent cation concentrations, but was also accompanied by an inhibition of LAMP amplification (**Figure 4.4B**). Of the samples tested, there was variable inhibition of amplification, and this inhibition was not limited to swabs from NG- or CT-infected people, as the *negative samples were also inhibitory* (data not shown). Because HNB is a metal cation indicator, we hypothesized that the inhibitory element was likely an excess of ions in the clinical solutions provided by JHU. LAMP has some tolerance to increased Mg^{2+} concentration but becomes inhibited at concentrations above 8 mM in a dose-dependent manner (**Figure 4.10**). The inhibition by the clinical samples was relieved by a 1:4 dilution (**Figure 4.9**), which suggests the concentration of the unknown factor was reduced to below its inhibitory threshold.

4.3.3 Inhibition of clinical swab amplification overcome by MCE filtration

To remove soluble inhibition factors like divalent cations, we applied the JHU samples to an MCE membrane (**Figure 4.2**), which we previously showed to be capable of capturing NG and CT-infected epithelial cells with high efficiency (manuscript in preparation). We washed the captured cells and then transferred the MCE membrane bearing the cells into lysis buffer (TE/TCEP). After heating to lyse the cells, bacterial DNA was found in the solution by qPCR analysis. When we used this lysate solution to rehydrate LAMP reagents in a PCR tube, amplification was restored (**Figure 4.4B**). This indicates that the inhibitory element was removed by the filtration and washing process.

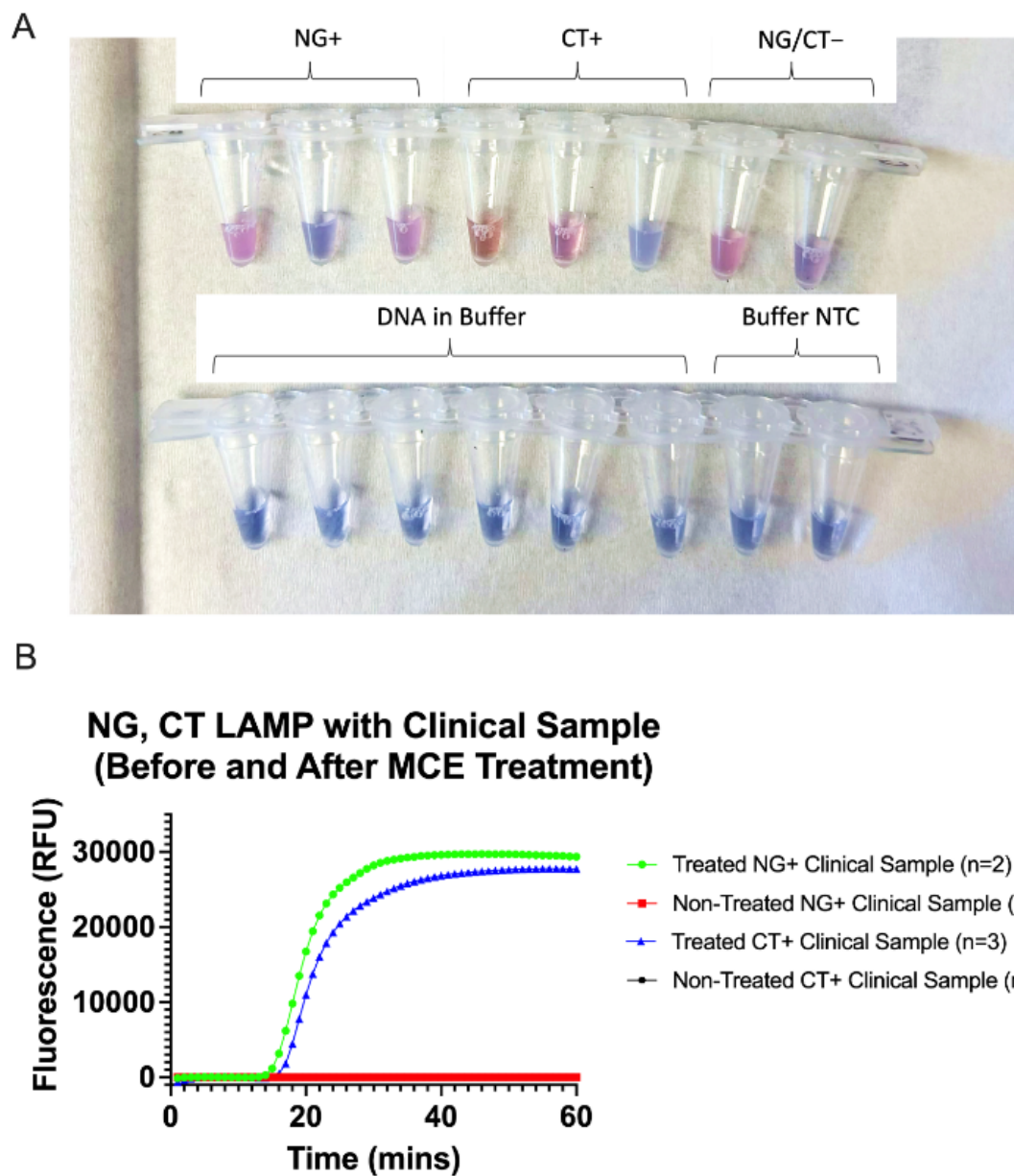


Figure 4.4. Filtration of clinical vaginal sample enabled in-tube LAMP reactions

(A) Comparison of lyophilized LAMP reagents rehydrated with clinical vaginal sample (top row) and buffer samples (bottom row) (B) Quantified averaged signal *Neisseria gonorrhoeae* (NG) (n=2) and *Chlamydia trachomatis* (CT) (n=3) LAMP results from QMA membrane comparing treated and non-treated clinical vaginal samples. (Figure by Erin Heiniger, PhD and Kevin Jiang)

We do not currently understand the ways in which the Lee Bioscience vaginal swab samples differ from those obtained from JHU. As we described above, the Lee Bioscience samples were obtained using BBL Culture Swabs, which are then frozen and shipped dry. Upon use, the swab material was eluted into TE/HUDSON lysis buffer (10 mM Tris, 1.1 mM EDTA, 2.5 mM TCEP) by manual agitation/swirling for 10 seconds. The JHU swab material was self-collected at the subject's home using a Copan FLOQ swab, shipped dry to JHU, then eluted into 10 mM Tris, 1 mM EDTA. Then, they were frozen and shipped to our UW lab in Seattle. In previous work, we showed that FLOQ swabs are compatible with LAMP amplification (data not shown), so it is unlikely they are the source of the amplification inhibitor found in the JHU samples. One hypothesis is that the JHU population of women could have a significantly higher Mg^{2+} and/or Ca^{2+} concentrations in vaginal mucus than another population. It may be that sample site preparation, sample storage, and/or patient education could prove impactful to the accuracy of a vaginal swab diagnostic device. We are pursuing the answer to these questions.

4.3.4 LAMP assay of MCE filtered clinical sample in QMA membranes

Six-pad devices were designed to test the compatibility of QMA-based LAMP assays with vaginal swab clinical samples for NG and CT. Each six-pad device held three lyophilized QMA LAMP pads for each of NG and CT LAMP assays (one positive sample, one negative sample, and one non-specific sample that tested a sample containing the other pathogen).

For paper-based reactions, prior to filtration of the clinical samples through the mixed cellulose ester (MCE) pads, both NG and CT LAMP assays in QMA membranes also showed no amplification over the course of an hour; this was true for each assay's positive, negative, and cross-reactivity with non-specific target conditions. As seen in **Figure 4.5A**, none of the assay pads showed a fluorescence amplification signal during 60 minutes of heating. Quantified

amplification curves, shown in **Figure 4.5B**, indicated no amplification liftoff following background subtraction. The lack of difference between each assay's positive, negative, and cross-reactivity with non-specific target conditions suggested strong inhibition of LAMP activity by the clinical samples across both assays. Repeated testing with additional clinical samples yielded similar lack of amplification.

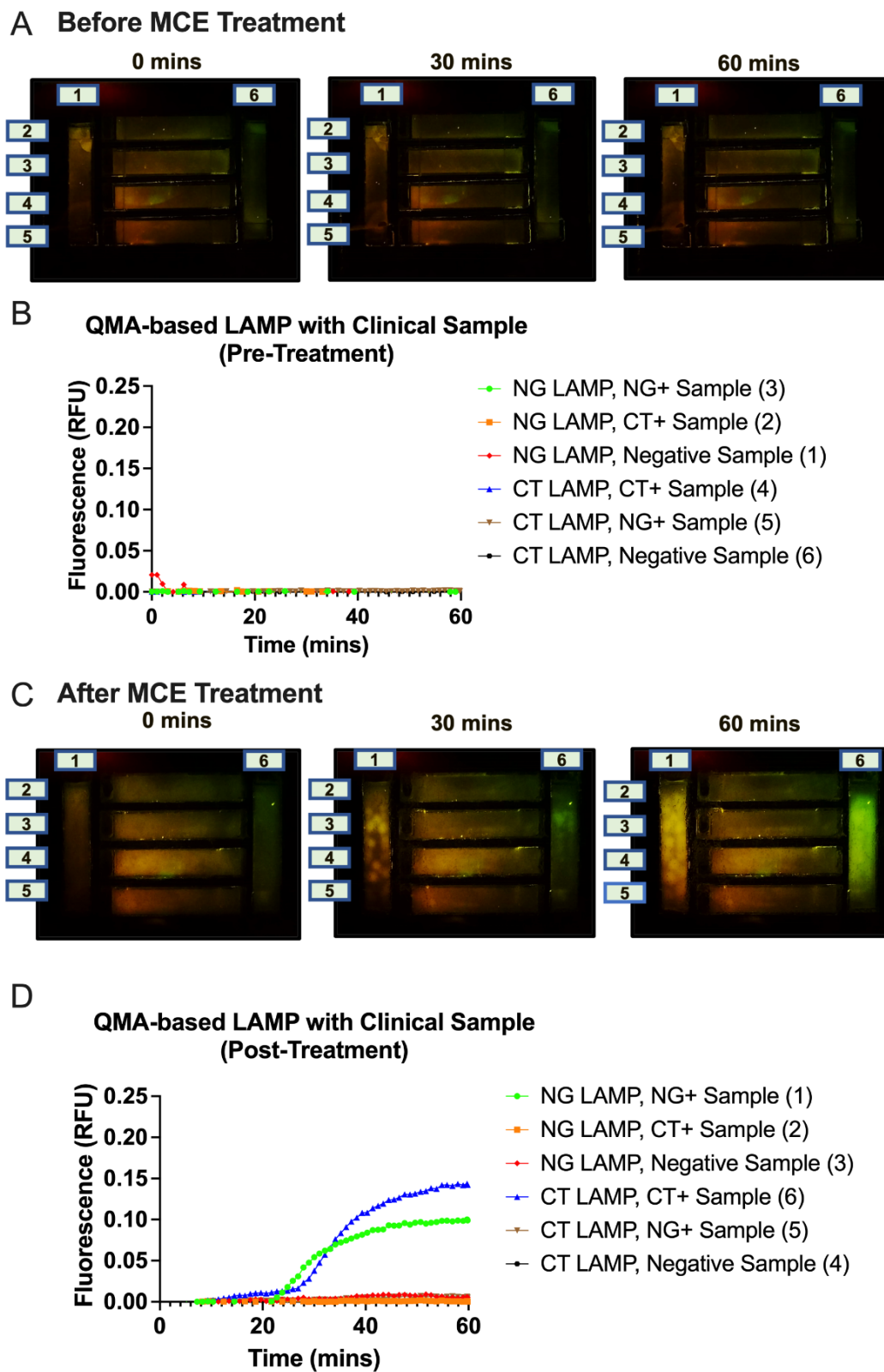


Figure 4.5. Filtration of clinical vaginal sample enabled QMA-based LAMP reactions.

*A cross-reactivity amplification test (sample containing pathogen(s) not targeted by the specific assay) was included for both *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) assays. (A) Rehydrated LAMP reactions with non-treated clinical vaginal samples. Lane number indicated in parentheses in legend. (B) Quantified fluorescence curves showed flat signal curves across positive, negative, and non-specific samples ($n=1$). (C) Rehydrated LAMP reactions with treated clinical vaginal samples. (D) Quantified fluorescence curves show clear positive signals for both NG and CT assays ($n=1$). Cross-reactivity against non-specific target and negative controls showed no fluorescence signal over an hour ($n=1$). (Figure by Erin Heiniger, PhD and Kevin Jiang)*

Following mixed cellulose ester filtration of clinical samples, amplification was enabled across both NG and CT LAMP assays in QMA membranes. Both the NG-positive and CT-positive samples showed clear amplification for their respective LAMP assays, with strong fluorescence signals across the entirety of the assay pad (**Figure 4.5C**). Quantified curves showed early signal detection for both assays, with amplification liftoff achieved by 30 minutes for both NG and CT LAMP (**Figure 4.5D**). Cross-reactivity test samples (samples containing pathogen(s) not targeted by the specific assay) negative clinical samples across both assays showed no amplification after an hour of heating, with quantified amplification curves remaining flat.

4.3.5 MCE treated STI clinical samples amplified in full 2-plex UbiNAAT device

Fifteen clinical vaginal samples were processed on the UbiNAAT platform (five positive NG samples, five positive CT samples, and five double-negative samples). To determine bacterial load in each sample run and ensure that reagents were not contaminated, qPCR in PCR reaction tubes for each assay was performed in parallel, with results shown in **Table 4.4**. For the NG-positive and CT-positive clinical samples, successful detection of each pathogen was achieved using the UbiNAAT platform. Fluorescence images for a NG-positive clinical sample run showed sample

detection in the NG assay pad only (**Figure 6.6A**), with quantified results indicating signal liftoff by 30 minutes (**Figure 6.6D**). **Figure 6.6B** showed strong fluorescence amplification across the entirety of the CT assay pad for a CT-positive clinical sample, with the quantified curve indicating signal detection by 20 minutes (**Figure 6.6E**). Neither the NG nor CT-positive samples showed non-specific amplification in the other assay pad, which indicated no contamination. The negative clinical sample showed no detectable fluorescence increase throughout the 60 minutes of amplification (**Figure 6.6C**), with quantified results indicating flat signal curves (**Figure 6.6F**).

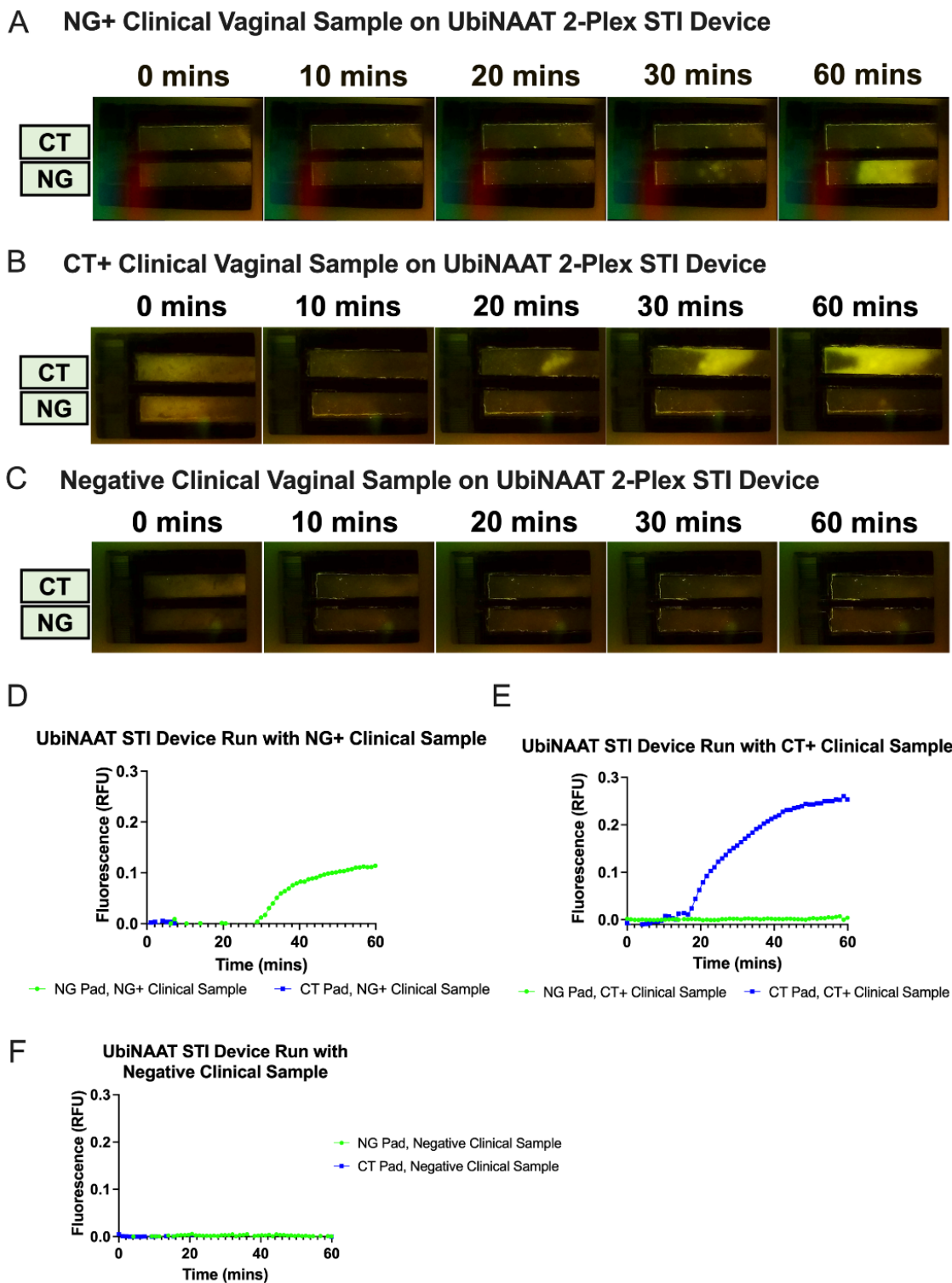


Figure 4.6. UbiNAAT 2-plex LAMP detection of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) clinical vaginal samples.

(A) Representative images of paper-based LAMP reaction with non-treated clinical vaginal samples. Lane number indicated in parentheses in legend. (B) Quantified fluorescence curves showed flat signal curves across positive, negative, and non-specific samples. Results are representative of reproduced replicates (not shown). (C) Representative images of paper-based LAMP reactions with treated clinical vaginal samples. Lane number indicated in parentheses in legend. (D) Quantified fluorescence curves show clear positive signals for both NG and CT assays. Cross reactivity and negative controls showed no fluorescence signal over an hour. Results are representative of reproduced replicates (not shown). (Figure by Erin Heiniger, PhD and Kevin Jiang)

Results across the 15 clinical sample runs demonstrated the UbiNAAT's reproducibility and high sensitivity across samples of different bacterial loads, with compiled results showing consistent performance for both assays. All 5 NG-positive samples were successfully detected on the NG LAMP assay pad, with no non-specific amplification in the CT assay pad across each run (**Figure 4.7A**).

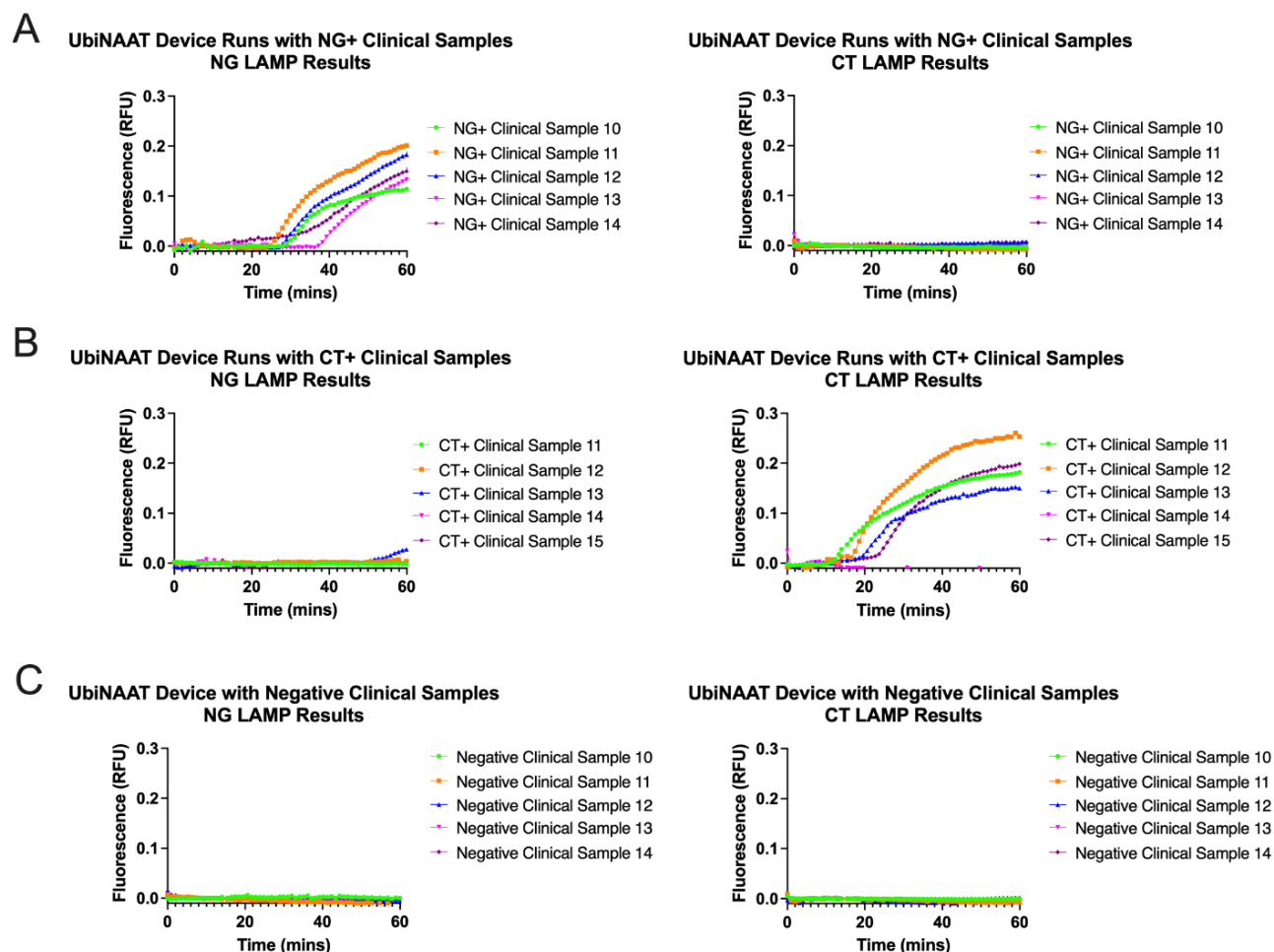


Figure 4.7. Compiled results of UbiNAAT assessment of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) clinical vaginal samples.

(A) UbiNAAT assessment of NG-positive (NG+) clinical vaginal samples across NG LAMP (left) and CT LAMP (right) ($n=5$). (B) UbiNAAT assessment of CT-positive (CT+) clinical vaginal samples across NG LAMP (left) and CT LAMP (right) ($n=5$). (C) UbiNAAT assessment of negative clinical vaginal samples across NG LAMP (left) and CT LAMP (right) ($n=5$). (Figure by Erin Heiniger, PhD and Kevin Jiang)

For CT-positive LAMP in the UbiNAAT, 4/5 CT-positive clinical samples showed amplification liftoff prior to 25 minutes, with no non-specific NG assay signal liftoff prior to 55 minutes (**Figure 4.7B**). The average clinical sample signal liftoff time for UbiNAAT NG LAMP was 31.8 ± 4.6

minutes across n=5 samples, while CT LAMP showed an average liftoff time of 19.2 ± 4.0 minutes across n=4 samples (**Figure 4.8**).

These results indicated that none of the clinical patients was positive for both pathogens, with minimal non-specific activity showing high device specificity. Across n=5 negative clinical samples, neither the NG nor CT LAMP assays showed notable fluorescence amplification across 60 minutes (**Figure 4.7C**). It was noted that 1 of the 5 CT-positive clinical samples resulted in no amplification due to a mechanical failure of the UbiNAAT air spring valve prior to lysis. Despite a high bacterial load in the sample, the lack of a lysis step likely resulted in insufficient recovery of DNA from the patient sample. Subsequent observation of the device did not reveal errors in device assembly. The malfunction may have been the result of a one-time damaged wax material in the air spring valve.

The 2-plex UbiNAAT assessment of clinical patient samples that were positive for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection, combined with previous demonstration of RT-LAMP to detect viral pathogens (29), suggests that the platform can be readily adapted for any nucleic acid-based pathogen detection. The performance of each assay for clinical samples of varying bacterial loads indicates compatibility with a sample to result time within 60 minutes, with high specificity for each assay and low risk of contamination. Assay sensitivity and operation time could be improved in future device iterations with integration of the MCE treatment step into the device design and the incorporation of a sample concentration step.

We envision a possible future workflow for this device as shown in **Figure 4.1**. When the patient arrives at the clinic, they will be given a swab and instructions for self-sampling. The clinician would then take the swab and insert into the device, where all sample preparation and nucleic acid

amplification would occur within 30-60 minutes. The medical team would interpret the results and send the patient home with the appropriate treatment.

4.4 Conclusions

We have shown here a proof of concept for an inexpensive NAAT device for STI detection using clinical vaginal swab samples. Our success in detecting CT and NG from vaginal swab samples suggests we may have similar success with swab samples from other physiological sites. It has been shown that self-collected penile-meatal swabs are a possible alternative to first-void urine samples for the diagnosis of CT and NG in male patients (148). Our previous success with nasal swab samples, coupled with our success here with vaginal swabs suggests that a similar filtration and deactivation strategy could work for any number of swab samples. Swabs from multiple anatomical sites could be taken from one patient and input to the same low-cost disposable diagnostic UbiNAAT device, maximizing the probability of detecting NG infections. Additionally, modeling shows that the rapid identification of the correct antimicrobial treatment for each patient could extend the lifetime of previously abandoned first-line antimicrobial drugs against NG (149). Future work will involve further multiplexing of the device to include NAAT for the identification of antimicrobial resistance markers. We will also incorporate the pre-lysis MCE filtration step into the autonomous device to reduce user steps and improve reproducibility. With the addition of a filtration step, all positive and negative NG and CT clinical samples were correctly identified, save one mechanical failure.

4.5 Materials and Methods

PCR quantification of bacterial DNA

Bacterial cell copy numbers in clinical samples were determined using conventional quantitative PCR. Sequences of the PCR primers and probes used are found in **Table 4.1**. For quantifying NG chromosomes, we used primers and probe from Hjelmevoll *et al.* (2006) (150), adapted for use with the Sensifast Probe No-ROX kit (BIO-86050; Meridian Bioscience, Memphis, TN, USA). The qPCR reaction included 1X kit master mix, 400 nM each primer, 100 nM probe, and 1-5 μ l template per 20 μ l reaction. Reactions were run on either of two qPCR thermocyclers, a CFX-90 Touch or a CFX Opus (Bio-Rad, Hercules, CA, USA). Purified, quantified NG genomic DNA (#19424DQ; ATCC, Manassas, VA, USA) was used to build a standard curve. DNA from clinical samples was purified (DNeasy blood and tissue kit, Qiagen, Hilden, Germany) before qPCR determination of copy number. NG DNA starting concentration was calculated by the CFX Manager software (Bio-Rad), which uses a multivariable, nonlinear regression model to calculate amplification threshold.

Two different qPCR assays were used to quantify CT plasmid DNA. CT assay 1 (**Table 4.1**) was modified from Dhawan *et al.* (2014) performed as above, using the Sensifast Probe kit (151). The standard curve was built using quantified DNA (VR-348BD; ATCC), which contained a mixture of chlamydia and human DNA. We were unable to quantify the number of CT cryptic plasmids in the purchased standard, so we engineered a plasmid sequence to serve as a known standard (see Supplemental Methods for plasmid construction details). Quantified plasmid was compared to mixed CT and human genomic DNA using the Sensifast SYBR qPCR kit (Meridian Bioscience). Forward and reverse primers from CT assay 2 were used at 400 nM each. We found an average of 3 cryptic plasmid copies per fg mixed DNA standard. Using this value, we converted our measured

fg DNA equivalents to plasmid copies for all CT concentrations. DNA from clinical samples was purified (DNeasy kit, Qiagen) before qPCR determination of copy number.

LAMP assay

The LAMP assay reagents consisted of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), SYTO-82 fluorescent intercalating dye (Thermo Fisher Scientific, Waltham, MA), hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA), trehalose (Life Sciences Advanced Technologies), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham, MA), and assay-specific LAMP primers (Integrated DNA Technologies (IDT), Coralville, IA). Primer sequences are shown in **Table 4.2**. Final concentrations for all components can be found in **Table 4.3**.

LAMP mixes were prepared at 20 µl volumes inside of PCR reaction tubes, homogenized on a vortex mixer, and heated on a BioRAD Real-Time Thermocycler (CFX Opus) at 63°C for 60 minutes. Signal liftoff times were determined using CFX Maestro software to set the amplification threshold as described above.

NG and CT cultivation and use

NG cells were purchased from ATCC (19424) and stored frozen at -80°C in 25% glycerol. Before use in experiments, frozen material was streaked on a Chocolate Agar II plate (#221267; BD Diagnostics, Franklin Lakes, NJ, USA) and incubated at 37°C in a high-CO₂ bag (GasPak EZ CO₂ Pouch, # 260684; BD Diagnostics) for 48 hours. Then, a single colony was picked and spread on a new Chocolate Agar plate, and incubated as above for 24-48 hours. After incubation, cells were scraped from the surface of the plate and resuspended in 10 mM Tris, 0.1 mM EDTA buffer (TE).

Absorbance at 600 nm was measured and compared to a standard curve of known cell concentrations to estimate cell concentration (Spectramax iD3, Molecular Devices, San Jose, CA, USA). Cells were diluted in TE to the desired concentration.

CT-infected epithelial cells were gifted to us by collaborators, who estimated cell concentration by microscopy and delivered cells to us on ice. After receipt, infected cells were frozen in small aliquots and stored at -80°C. Before use, cells were thawed and gently mixed.

Commercial swab spiking and lysis

Purchased vaginal swabs (991-25-S-3; Lee Biosolutions, Maryland Heights, MO, USA) were shipped frozen and dry (without buffer). The BD™ BBL™ CultureSwab™ Sterile, Media-free Swabs are used for this product. Lysis buffer was based on the HUDSON (“heating unextracted diagnostic samples to obliterate nucleases”) solution protocol. Briefly, 2.5 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP) (Millipore Sigma), 10 mM Tris-HCl pH 8.0 (Invitrogen by Life Technologies), and 1 mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen by Life Technologies) were combined and used with heating (95°C for 5 minutes) to inactivate nucleases and other interfering proteins in the vaginal swab matrix (86).

On the day of use, each swab was equilibrated to room temperature, then submerged into 460 µl lysis buffer. The swab was agitated by twirling for 10 seconds before removal from the buffer. Buffer was squeezed from the swab by pressing it against the side of the tube. For each tested sample, 114 µl swab material in lysis buffer was mixed with 6 µl NG cells or CT-infected epithelial cells for a 120 µl sample. Spiked samples were heated to 95°C for 5 minutes in a heat block (Digital dry block heater, VWR, Radnor, PA, USA). After heating, samples were allowed to cool to room temperature. Then, a portion was used to rehydrate lyophilized LAMP reagents. The remaining sample was used as template for qPCR to quantify the NG or CT DNA content.

Clinical swab source

As a benefit of our funding mechanism, we received vaginal swab exudates from self-collected vaginal swabs (Copan FLOQ swabs) that had been sent dry to clinical collaborators at the POCTRN center at Johns Hopkins University. A portion of each swab exudate was analyzed by the Hologic STI system at JHU and determined to be positive or negative for both NG and CT. The remaining exudate (expressed into TE buffer (10 mM Tris, 1 mM EDTA)) was frozen and then shipped to University of Washington on dry ice. Samples were stored at -80°C until use, when they were thawed and gently mixed before use.

Mixed cellulose ester (MCE) filtration

Mixed cellulose ester filters with a 0.22 μm average pore size (0.22 μm MCE Membrane, Merck Millipore) were cut to 3 mm x 15 mm using a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ). Each filter was placed on a 22 mm x 16 mm x 2.5 mm cellulose wicking pad (**Figure 4.3B**). Then, 200 μl of expressed clinical vaginal swab sample was pipetted onto the MCE and allowed to wick through. Next, 200 μl TE (10 mM Tris and 0.1 mM EDTA) was pipetted onto the upper surface of the filter. After allowing five minutes for fluid to flow through the membrane, the MCE filter was removed from the cellulose using forceps, then put into a 1.5 ml microfuge tube (#3451, Thermo Fisher Scientific, Waltham, MA, USA) and covered with 200 μl TE/HUDSON buffer. Alternatively, the filter was placed directly into the UbiNAAT device (described below). CT or NG DNA was released into the buffer phase by heating to 95°C for 5 minutes.

Lyophilization in tube and in pad

LAMP reagent lyophilization protocols were performed as described in our previous works (33,63). In short, Whatman quartz fiber (QMA) pads that had been blocked in 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) were held in a 6-well plate and hydrated with 24 μ L of LAMP reaction mix (no template) (see Table S3). The plate was quickly flash-frozen in liquid nitrogen before being connected to a FreeZone 2.5-liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, MO, USA) through a fast-freeze flask (Labconco) and vacuum-dried overnight.

Amplification pad test devices

The assembly of devices and preparation of porous membranes for testing porous matrix-based reactions was previously described in Jiang *et al.* (2024) (33). Briefly, devices were built that enclosed six identical porous pads (“six-pad devices”). The six pads were oriented to be fully enclosed within the cell phone fluorescence reader field-of-view, with four pads side-by-side and one pad placed perpendicularly on each end (see Figure 3A). Each LAMP reaction was performed in an individual Whatman quartz fiber membrane (QMA) from Cytiva (VWR, Radnor, PA) inside of a 33 mm x 26 mm x 1/16 in polymethyl methacrylate (PMMA) device (McMaster-Carr, Elmhurst, IL, USA). The bottom PMMA shell held six channels (16.7 mm x 3.3 mm) that were laser-etched to a 600 μ m channel depth, while the top shell placed a vent at the end of each channel to allow for fluid addition and air venting. The top and bottom enclosure of the six-pad devices were bonded together using polydimethylsiloxane (PDMS) pressure-sensitive tape (Valley Industrial Products, Huntington, NY, USA). All porous materials, PMMA pieces, and films were machined and etched on a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser

Systems, Scottsdale, AZ). After each QMA reaction pad was filled with 25 μ L of template solution, the PMMA top enclosure vents were sealed using Microseal B adhesive sealer (BioRad, Hercules, CA), and the sealed device was placed inside the custom-made, dual-sided (ITO glass top) heater oven at 64 °C for 60 minutes to enable LAMP.

UbiNAAT device fabrication

The 2-plex UbiNAAT device (**Figure 4.2**) assembly protocol and fabrication of device components (microfluidic internal device, 3D printed enclosure, custom printed circuit board) were described previously (33,78,79,81,80). In this study, the device was modified by removing the blister that would normally contain the sample inactivation solution, and the blister fluidic path was covered with adhesive copper tape. At the start of a sample process run, the post-capture MCE membrane was inserted into the lysis chamber using tweezers and pushed to the bottom of the lysis chamber (see **Figure 4.2**). 200 μ l of HUDSON inactivation solution was pipetted through the top of the lysis chamber to ensure full submersion of the MCE membrane.

Fluorescence detection with cell phone

QMA pad-based LAMP reactions were visualized *via* a cell-phone-based fluorescence reader as described in Jiang *et al.* (2024); the reader consisted of a Google Nexus 5X with two interference filters: an FES0550 emission short-pass filter (Thorlabs) placed over the phone's camera lens and a BP 587/25 excitation filter (Zeiss) placed over the phone's light-emitting diode (LED). The filters were held in a custom black PMMA fixture, with a 12 mm wide, 12 mm focal length plano-convex lens (Edmund Optics) placed 6 mm from the phone LED (before the excitation filter) (33).

The cell phone reader was fixed at 8.5 cm above the amplification device, with images taken at 60-second intervals using the phone's incandescent white balance, 1/5 aperture, 200 ISO, and manual focus settings. Image stacks from each 60-minute run were transferred to a desktop computer *via* a USB connection.

As previously described by Shah *et al.* (2022), a custom MATLAB script was used for fluorescence image analysis (81). Similar to the analysis method in Jiang *et al.*, the top 1% of pixel intensity within a selected region-of-interest (ROI) for each pad was used to determine fluorescent intensity changes across each minute to generate a fluorescence amplification curve over time (80,81). Background subtraction was performed by subtracting the average fluorescence intensity from minutes 5-10 from each image, and a 0.02 RFU fluorescence threshold was used to determine amplification liftoff time for distinguishing positive and negative clinical samples in each LAMP reaction (33,81)

UbiNAAT diagnosis from clinical samples

As previously demonstrated in Jiang *et al.*, the UbiNAAT detection device is capable of incorporating lysis, fluidic movement, and simultaneous amplification detection for two assays in its current configuration (33). Here, we utilized the same platform to assess vaginal swab clinical samples for *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT). The device utilized the PCB heater and PID loop previously developed in the lab and described by Shah *et al.* (2023), which was shown to be functionally equivalent to benchtop equipment for performing lysis and amplification heating (33,79). Fluidic control was enabled using a terminal “air spring valve”, which was activated by resistive heating after completion of the lysis step (33). Each 2-plex

UbiNAAT device contained one lyophilized QMA LAMP pad for both NG and CT assays, with amplification heating performed at 63°C across both pads.

At the start of each run, the vaginal clinical sample was treated using MCE filtration and bacterial capture, which was shown in-tube and on QMA membranes as necessary to remove amplification inhibitors. Following clinical sample flow-through and washing of the MCE filter, the membrane was then transferred to the UbiNAAT lysis chamber *via* tweezers and pushed to the bottom of the chamber. Addition of TE-HUDSON solution *via* pipette was chosen to ensure that the MCE membrane was fully submerged in liquid, as the membrane was not fixed to any part of the lysis chamber and could be pushed against the chamber walls through introduction of solution from below. PCB lysis heating then followed at a 100°C setting for 7 minutes, followed by 3 minutes of cooling. The terminal air spring valve was then opened to enable flow of the lysed clinical sample through the device to rehydrate both assay pads. Amplification heating began following assay pad rehydration; images of both pads were acquired by the cell phone fluorescence reader at 60-second intervals for 60 total minutes. Fluorescence images were quantified as described above.

Construction of quantitated plasmid engineered CT standard

The primers for the CT assay 2 were used with MyTaq Red master mix (BIO-25043; Meridian Bioscience) to PCR amplify a 430-nucleotide product containing a KpnI restriction enzyme digestion site at one end, and a HindIII restriction site at the other. The PCR product and the pUC19 plasmid were digested with both enzymes (KpnI-HF, HindIII-HF; New England Biolabs, Ipswich, MA, USA). Insert and vector backbone were ligated together at a 3:1 molar ratio using the Quick Ligase kit (NEB) and transformed into *E. coli* DH5 α chemically competent cells using the manufacturer protocol (NEB). After selection on Luria Bertani (BD Diagnostics) agar (containing

100 μ M ampicillin, 200 μ M IPTG, and 20 μ g/ml X-gal), white colonies were screened for insert using PCR. Purified plasmid was quantified via spectroscopy (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA) and copy number per μ l was calculated.

4.6 Acknowledgments

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I want to thank Erin Heiniger, PhD, with whom I am an equally contributing author on this manuscript. This manuscript is currently being prepared for submission.

4.7 Supplemental Information

UbiNAAT 2-Plex STI Device Assay Signal Liftoff Times

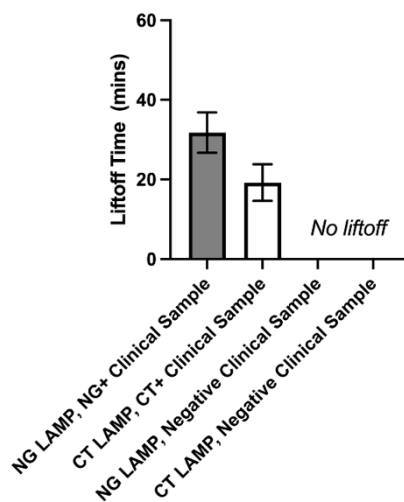


Figure 4.8. Liftoff times for positive and negative clinical vaginal samples as processed in UbiNAAT device (mean $\pm$ standard deviation) after filtration using mixed cellulose ester (MCE).

Filtration removes unknown soluble amplification inhibitor. Positive clinical samples for *Neisseria gonorrhoeae* assay (31.8 ± 4.6 mins, n=5) and *Chlamydia trachomatis* assay (19.2 ± 4.0 mins, n=4) both showed clear signal detection, while none of the negative clinical samples (n=5) showed signal liftoff within 60 minutes of amplification. (Figure by Kevin Jiang)

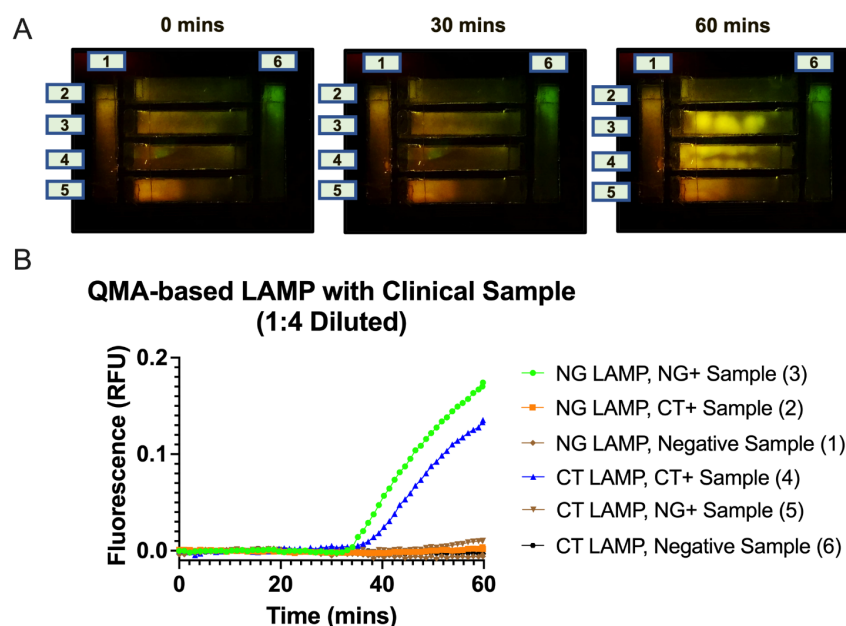


Figure 4.9. Sample dilution eliminates amplification inhibition.

The six-pad test device running lyophilized LAMP on QMA membrane with buffer-diluted clinical vaginal sample (**A**) Rehydrated LAMP reactions with 1:4 diluted clinical vaginal samples. Lanes in legend (**FIX**) (**B**) Quantified LAMP results show signal detection across both *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) assays with diluted positive clinical samples (n=1). Non-specific and negative samples showed no signal detection over an hour (n=1). (Figure by Kevin Jiang)

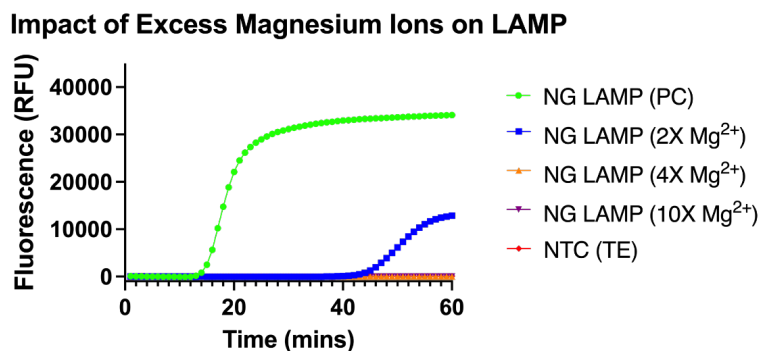


Figure 4.10. NG LAMP activity is inhibited when concentration of magnesium ions in solution exceeds the intended final concentration (8 mM).

Amplification is significantly delayed at 2X Mg²⁺ concentration (16 mM), and fully inhibited at 4X Mg²⁺ (32 mM) and 10X Mg²⁺ (80 mM) concentrations. (Figure by Kevin Jiang)

Table 4.1. qPCR primers

Assay	Primer description	Primer Sequence	Source
CT cryptic plasmid 1	Forward	5'-CATGAAAACTCGTTCCGAAATAGAA -3'	(151)
	Reverse	5'-GTTTCAGCGGCAGCATTCA -3'	
	Probe	56-FAM/TCGCATGCA/ZEN/AGATATCGA/3IABkFQ	Modified from (151)
CT cryptic plasmid 2	Forward	CAT <u>GGT ACC</u> TTT AGG CTA TAT TAG AG	This study
	Reverse	CAT <u>AAG CTT</u> GGA GAT TAG TCA GAT TTG	This study
NG <i>porA</i>	Forward	5'-CCGGAACCTGGTTTCATCTGATT-3'	(150)

Table 4.2. LAMP primers

Assay	Primer description	Primer Sequence	Final concentration (μM)	Source
CT cryptic plasmid	F3	5'-AATATCATCTTTGCGGTTGC-3'	0.2	(152)
	B3	5'-TCTACAAGAGTACATCGGTCA-3'	0.2	
	FIP	5'-TCGAGCAACCGCTGTGAC-GACCTTCATTATGTCTGGAGTC-3'	0.8	
	BIP	5'-GCAGCTTGTAAGTCCTGCT-TGAGTCTTCGTAACCTCGCTCC-3'	0.8	

	Loop-F	5'-TACAAACGCCTAGGGTGC-3'	0.4	
	Loop-B	5'-CGGGCGATTTGCCTTAAC-3'	0.4	
Assay	Primer description	Primer Sequence	Final concentration (μM)	Source
NG <i>porA</i>	F3	5'-CCATTGATCCTTGGGACAG-3'	0.2	(153)
	B3	5'-CAGACCGGCATAATACACAT-3'	0.2	
	FIP	5'-GGGAATCGTAACGCACGG-AAATAATGTGGCTTCGCAATTG-3'	1.6	
	BIP	5'-AGCGGCAGCATTCAATTTGTT-CCTGATTACTTTCCAGCGTGA-3'	1.6	
	Loop-F	5'-ATACCGTCGTGGCGTTTG-3'	0.8	
	Loop-B	5'-CGCCTATACGCCTGCTAC-3'	0.8	

Table 4.3. LAMP reagent recipe

Reagent name	Manufacturer	Product number	Final concentration
Warmstart LAMP Kit	New England Biolabs	E1700L	1X
Trehalose	Life Sciences	TS1M-100	10%
Dextran 500kDa	Millipore Sigma	D8802-50ML	0.5%
SYTO-82	ThermoFisher	S11363	7.5 μM
HNB	Millipore Sigma	1045930025	40 μM
Primer mix	Assay dependent	N/A	1X
Nuclease Free water	Hyclone	SH30538.03	N/A

Table 4.4. Clinical sample DNA target concentration by qPCR

(Table by Erin Heiniger)

Sample name	JHU diagnostic PCR result	NG chromosomes per ml	CT plasmids per ml
CT + #11	CT positive	ND	1.2×10^5
CT + #12	CT positive	ND	7.1×10^7
CT + #13	CT positive	ND	6.5×10^5
CT + #14	CT positive	ND	3.5×10^7
CT + #15	CT positive	ND	3.1×10^7
NG + #10	NG positive	ND	ND
NG + #11	NG positive	4.0×10^6	ND

NG + #12	NG positive	3.7×10^3	ND
NG + #13	NG positive	6.1×10^3	ND
NG + #14	NG positive	2.4×10^5	ND
Neg #10	CT and NG negative	ND	ND
Neg #11	CT and NG negative	ND	ND
Neg #12	CT and NG negative	ND	ND
Neg #13	CT and NG negative	ND	ND
Neg #14	CT and NG negative	ND	ND

ND = not detected

5. Chapter Five - Development of an At-Home, NAAT-based HIV Screening Device

5.1 Introduction

The human immunodeficiency virus (HIV) epidemic remains a critical global health challenge. While overall HIV incidences and mortality rates have steadily declined worldwide since 2010 (154), many countries continue to face difficulties in limiting their rate of annual infections, including the United States (rising rates of infection among ages 25–34-year-olds from 2009-2018) and the Philippines (418% increase in new HIV infections between 2010-2022) (155,156). As of 2025, nearly half of all new annual infections occur in sub-Saharan Africa, with twice as many infections occurring in eastern and southern Africa compared to western and central Africa (154,157). While the trend of fewer infections is encouraging, the current annual infection rate of 1.3 million falls well short of the UNAID's goal of reducing annual infection rates to 370,000 by 2025 (154). Additionally, most countries have yet to meet the UNAID's 2025 goal of a worldwide 95-95-95 plan, in which 95% of all HIV patients know of their status, 95% of all HIV patients are receiving antiretroviral therapy (ART), and 95% of ART-recipient patients have achieved viral suppression (158). Critically, nearly half of the 38 million people currently living with HIV do not yet know of their status (158,159). At this time, the recent funding cuts to USAID is widely believed to have the potential to threaten much of the progress that has been made in the last decade in suppressing HIV infections across the world (160).

In the global effort to end the HIV/AIDS epidemic, two important interventions contributed greatly to the decline of HIV transmission. First, the widespread adoption of anti-retroviral therapy has dramatically lowered HIV/AIDS-related mortality rates and allow people living with HIV to

maintain undetectable viral loads while sustaining a relatively normal lifestyle (159). Notably, the use of ART medication (such as tenofovir or emtricitabine) as pre-exposure prophylaxis (PrEP) by uninfected but at-risk individuals has significantly decreased HIV infection rates, with an effectivity rate of 99% by PrEP users (157). Secondly, advances in point-of-care (POC) diagnostics have enabled earlier detection of HIV infections. While early ELISA (enzyme-linked immunosorbent assay) antibody-detection assays were limited in large-scale testing due to requiring complex laboratory infrastructure and trained personnel, the development of rapid antibody test kits, such as the OraQuick ADVANCE Rapid HIV 1/2 Antibody test and the INSTI HIV-1/2 antibody test, have enabled detection HIV IgM antibodies by 30 days following initial infection (161,162). The ability to privately self-test has dramatically increased the percentage of HIV patients who are aware of their condition and, in conjunction with ART, which can lower the risk of sexual HIV transmission by up to 96% (157,163).

While existing HIV rapid antibody tests are a valuable resource in enabling patient detection while preserving privacy, there remains an urgent need for improved test sensitivity. As nucleic acid amplification tests (NAATs) are considered the gold standard of molecular testing, the World Health Organization (WHO) has set the current undetectable viral load threshold at 1000 copies per milliliter of plasma (164). Below this threshold, the risk of sexually transmitting HIV is near zero (165,166). By enabling as early detection of HIV infection as possible, high-sensitivity POC test options allow for patients to receive immediate ART intervention and minimize HIV's impact on their daily lives. However, achieving high-sensitivity NAAT testing in a point-of-care or home environment has been challenging. The current best market solution for POC NAAT HIV testing is the Cepheid GeneXpert platform and its Xpert HIV VL assay, which could detect down to 40 copies of HIV RNA/mL plasma; its use has significantly reduced the turnaround times for HIV

testing in sub-Saharan African countries (167,168). However, its feasibility as a true point-of-care tool for remote and home testing is limited by the need for trained personnel to operate the instrument, the high cost of the GeneXpert, and its requirement of centrifugation of the patient sample prior to addition to the test cartridge (166,168). To fully enable high sensitivity testing in a point-of-care or home environment, there remains a need for improving and simplifying current POC test functionalities to support large-scale screening of early-stage HIV.

Here, we present a point-of-care microfluidic device that enables sensitive, multiplexed NAAT detection of the gene for the HIV p24 gene from a fingerstick blood sample. The prototype device incorporates blood-plasma separation using MF1 membrane (51,169), proteinase K chemical lysis (67,68), lyophilized paper-based RT-LAMP (33,170), sample concentration, and an innovative pull-tab valving system for fluidic control. To enable lyophilization-compatible sample concentration in a POC device, we incorporate DNA probe capture and magnetic bead concentration to concentrate HIV viral RNA from separated plasma using, which has been shown previously to significantly enhance assay sensitivity (171,172). For detection, two iterations of the assay were tested, with one variant using fluorescence-based readout to be coupled with a cell phone reader used alongside the device and the second variant using a colorimetric readout. In early prototype testing, device heating was enabled by a hot plate.

At the time of writing this thesis, the HIV project is ongoing and has yet to reach full device testing with either contrived or clinical samples. With protocol characterization being divided among multiple group members, the work described here will incorporate only my own designs and experimental work. Therefore, this chapter will describe results related to RT-LAMP assay optimization, current assay sensitivity for both fluorescence and colorimetric readouts, device design and fabrication, as well as fluidics control *via* valving. Results and methods regarding

magnetic bead sample concentration, proteinase K lysis, and RNA elution will not be covered outside of their membrane design in device.

5.2 Materials and Methods

HIV p24 RT-LAMP Assay in-tube and porous matrices

Two variants of the RT-LAMP assay were used in this study. The fluorescence-based RT-LAMP assay was described in our previous work (33). Briefly, the reagents consist of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), SYTO-82 fluorescent intercalating dye (Thermo Fisher Scientific, Waltham, MA), hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA), sucrose (Sigma-Aldrich, St. Louis, Mo), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham, MA), and assay-specific LAMP primers (**Table 5.1**). For the colorimetric assay variant, Eriochrome Black T (Sigma-Aldrich, St. Louis, Mo) replaced SYTO-82 and HNB in the reaction mix (**Table 5.1**). Six primers were used for HIV p24 RT-LAMP assay, with primer sequences acquired from literature (Integrated DNA Technologies, Skokie, IL) (**Table 5.2**) (173). In-tube reactions were performed on a 96-well thermocycler (CFX Opus, Biorad) at 63°C for 60 minutes.

To evaluate the performance of RT-LAMP in porous matrices, devices were built that enclosed 5 identical porous pads side-by side. RT-LAMP was performed in separated Whatman quartz fiber membranes (QMA) from Cytiva (VWR, Radnor, PA) inside of 33 mm x 25 mm x 1/16 in polymethyl methacrylate (PMMA) devices (“5-pad devices”). For these and all other amplification devices including the UbiNAAT prototypes, the major tool for machining polymer pieces, films, and paper-like materials was a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ). The bottom PMMA shell held 5 parallel channels (16.7 mm x 3.3 mm)

that had been laser-etched to a depth of 600 μm ; the top enclosure part had a vent at each end of the channel to allow insertion of fluids and escape of air. Polydimethylsiloxane (PDMS) pressure-sensitive tape (Valley Industrial Products, Huntington, NY) was applied between the top and bottom tray parts and one QMA pad was placed in each channel. The top PMMA casing was then applied, and the device was pressed together using a clamp. QMA membranes (1851-150) were laser-cut to 15 mm x 3 mm strips with a fluid capacity of 25 μL . They were blocked by soaking in a solution of 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) in nuclease-free water for an hour before being drained of fluid and dried overnight in a 40°C oven. After filling with 25 μL of solution (Table S5), the vents at both end of each fiber-pad-filled channel were sealed using Microseal B adhesive sealer (BioRad, Hercules, CA) and the sealed devices were placed into a custom-made, dual-sided heating oven with an ITO glass top heater (**Figure 2.6**) and incubated at 64°C for 60 minutes.

RT LAMP Lyophilization and Storage

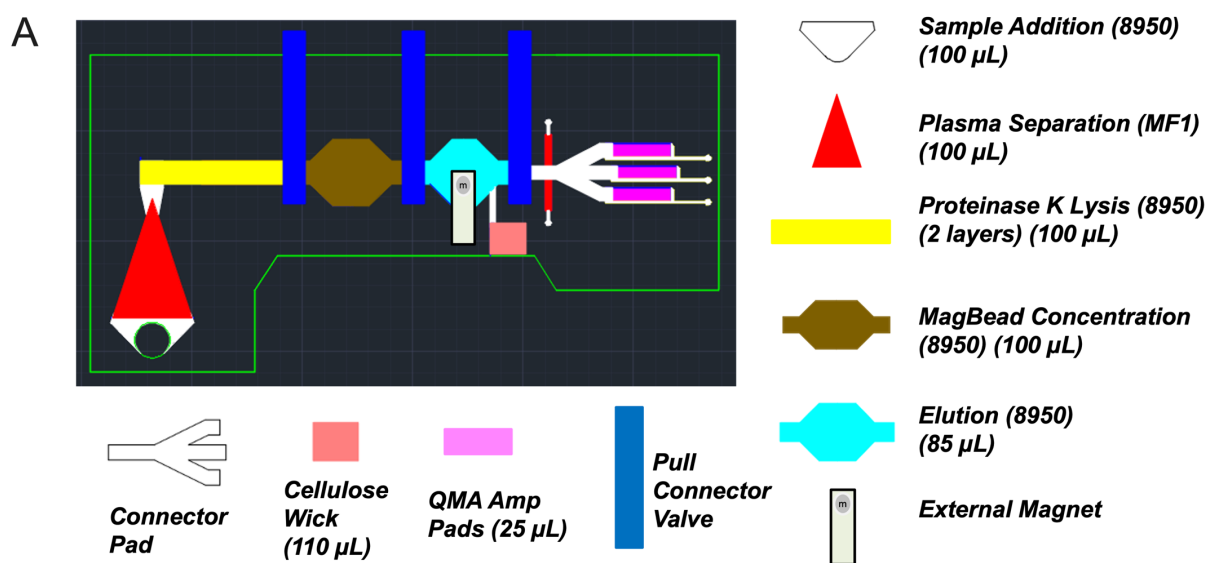
Lyophilization protocols were performed as described in our previous work (63). In short, 1% BSA, 0.1% Tween-20-coated QMA pads, held in a 6-well plate, were each hydrated with 24 μL of RT-LAMP reaction mix (no template) that contains 10% sucrose and 0.5% dextran (500 kDa). The apparatus was quickly frozen by insertion in liquid nitrogen for 1 minute before being placed into a fast-freeze flask (Labconco) and attached to a FreeZone 2.5 liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, Mo) for overnight drying with the system cold finger set at -47°C and the vacuum at 0.03 mbar. For lyophilizing reaction tubes, PCR tubes containing 19 μL reaction mix with holes poked into the caps were dipped into liquid nitrogen for 1 minute, then placed into a 96-well aluminum tube holder (Qiagen) prior to attachment to the lyophilizer.

For the colorimetric RT-LAMP assay, the Eriochrome Black T was removed from the lyophilization mixture. The reagent would be added to the rehydration template buffer post-lyophilization.

Device Fabrication and Assembly

The HIV internal microfluidic device was fabricated using 1/16 in. polymethyl methacrylate (PMMA) layers that were laser-cut on a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ) and bonded together using PDMS pressure-sensitive tape (Valley Industrial Products, Huntington, NY). Between the two layers of PMMA sits a 2D paper network (2DPN) fluidic pathway. The fluidic pathway consists of a unidirectional flow path with multiple sample processing steps integrated. First, the sample is added to a sample addition 8950 pad (Ahlstrom, Helsinki, Finland) that evenly distributes the sample to an MF1 blood-plasma separation pad (GE Healthcare, Chicago, IL). The MF1 pad connects to a rectangular 8950 pad that would hold lyophilized proteinase K reagents for chemical lysis and DNA probes for HIV RNA capture. The “proteinase K pad” then connects to an 8950 membrane containing lyophilized magnetic beads for HIV RNA capture. The “magnetic bead pad” is connected to an 8950 elution pad for eluting RNA from the magnetic beads. From the elution pad, an 8950 strip connects to a cellulose wicking pad, and another three-pronged 8950 connector pad connects to the three amplification pads made of Whatman quartz fiber membranes (QMA) from Cytiva (VWR, Radnor, PA). A fluidic shunt sits below the connector pad to catch non-target containing buffer. Three manually-actuated “pull-connector valves” made from polytetrafluoroethylene (PTFE) (McMaster-Carr, Elmhurst, IL) membrane separates the fluidic pathway, with one valve in between the proteinase K/magnetic bead pads, the magnetic bead/elution pads, and the elution/connector pads. A diagram of the device is illustrated in **Figure 5.1A**. All glass fibers

(8950, MF1, QMA) were fabricated on a high-powered CO₂ laser cutter (VLS3.60 CO₂ laser, Universal Laser Systems, Scottsdale, AZ). QMA membranes were blocked by soaking in a solution of 1% BSA (GeminiBio, Sacramento CA) and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Mo) in nuclease-free water for an hour before being drained of fluid and dried overnight in a 40°C oven. All 8950 membranes were similarly blocked using 0.1% Tween 20 solution only. The fully assembled HIV internal device without chemical reagents is shown in **Figure 5.1B**.



B

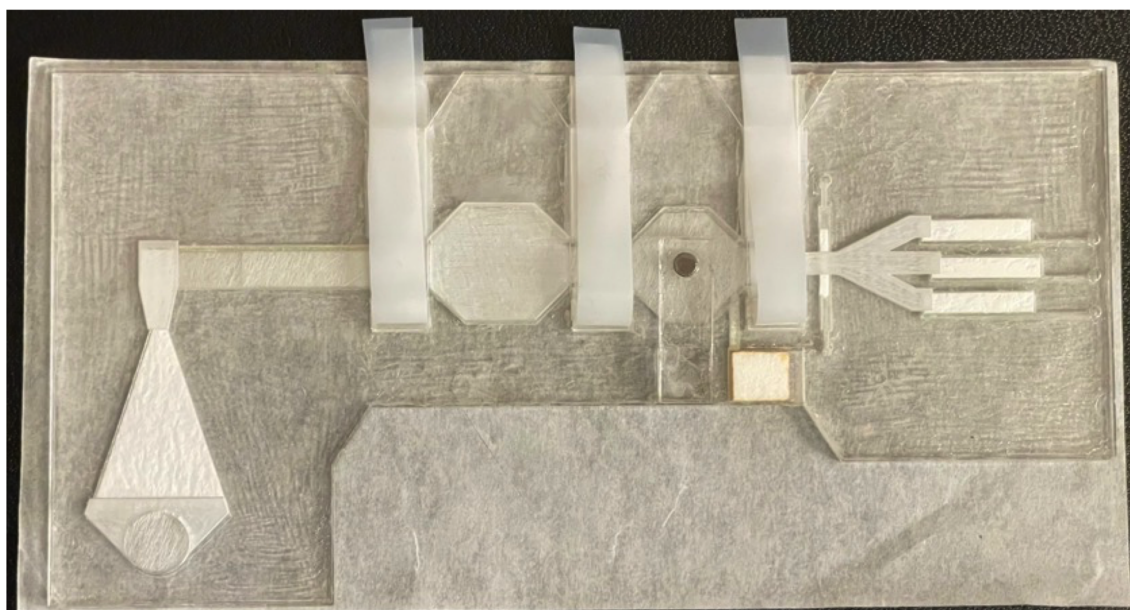
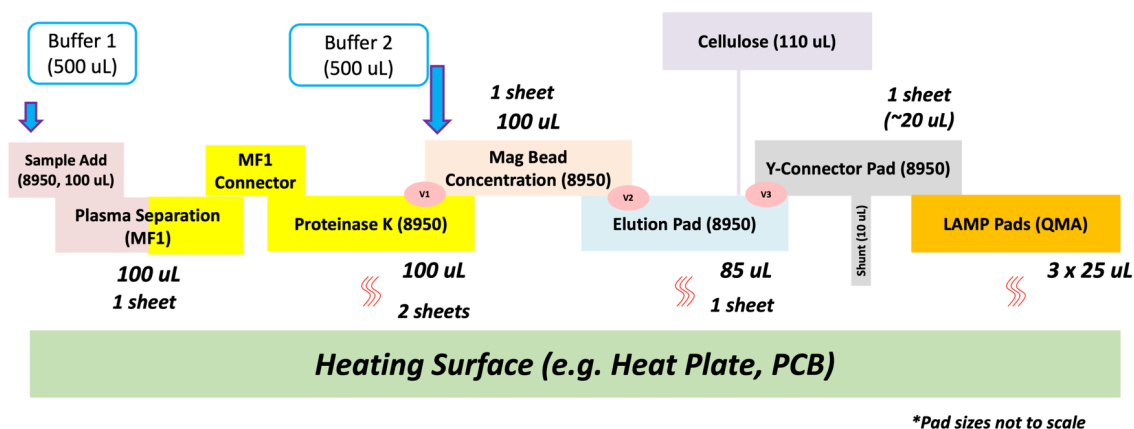


Figure 5.1. Schematic of HIV internal device, with membrane functions and volume capacity.

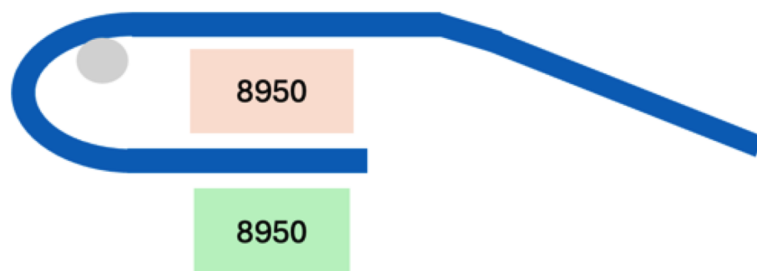
(A) Schematic of HIV device and component layout (B) Assembled HIV internal device (no reagents in membranes)

Figure 5.2A illustrates the layout of the membranes in the vertical plane. Each membrane is oriented either in the device upper layer or lower layer, primarily due to the need for heating during a sample processing step. The PTFE pull-connector valve sits in between membranes at the designated locations, and is manually activated after each incubation step is completed. Each valve is fabricated *via* sterilized scissors or razor blade on a PTFE membrane whose dimensions have been marked. The movement path of the pull-connector valve is shown in **Figure 5.2B**. To enable immobilization of magnetic beads within the device, an external PMMA holder containing a 3 mm diameter neodymium magnet (K&J Magnetics, Pipersville, PA) was added on top of the upper layer of the HIV device. As shown in **Figure 5.3C**, the PMMA magnet holder could be readily attached or detached *via* sliding and is positioned to immobilize magnetic beads from upstream.

A



B



C

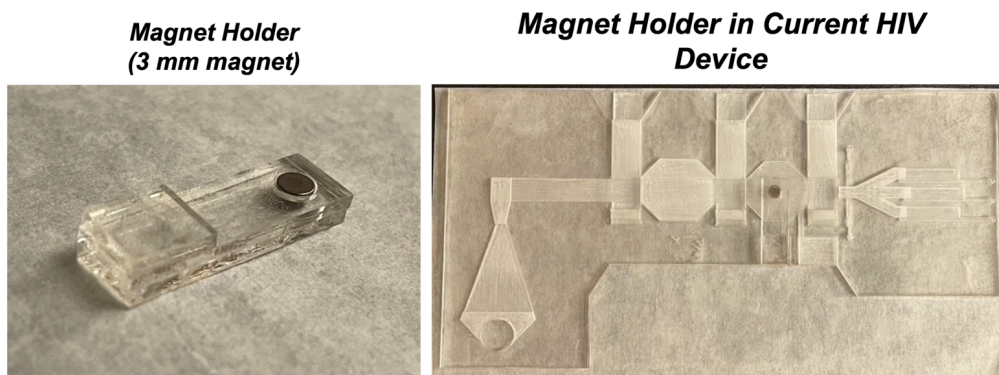


Figure 5.2. HIV Device Component Vertical Layout and Descriptions.

(A) Vertical arrangements of sample processing pads. Membranes on the bottom layer (proteinase K lysis, magnetic bead capture, elution) require heating. (B) PTFE pull-connector valve is placed between two membranes and looped around a PMMA holder (gray circle) in device. Pulling the valve (towards right) connects the two membranes due to a downward valve pull direction (C) Magnet holder shown independently (left) and in device (right).

5.3 Results and Discussion

5.3.1 Fluorescence RT-LAMP Optimization in-tube and porous matrices

In freshly prepared (“fresh”) and lyophilized PCR tube-based (“in-tube”) reactions, fluorescence-based HIV p24 RT-LAMP assays lifted off from baseline fluorescence within 20 minutes of heating at 63°C while using a 96-well thermocycler (CFX Opus, BioRad) at a relatively low RNA load of 100 copies per reaction. 10% sucrose replaced 10% trehalose in the master mix after testing in QMA showed higher assay sensitivity (see **Figure 5.8**). For optimized in-tube testing, 10% sucrose without dextran showed the best signal liftoff and sensitivity results. As shown in **Figure 5.3A**, fresh in-tube reactions showed early signal liftoff prior to 20 minutes down to a limit-of-detection (LOD) of 50 RNA copies per reaction. However, 0/2 replicates at 10 RNA copies per reaction showed signal liftoff prior to an hour, while fresh positive controls (without sugars) showed early signal liftoff at 10 RNA copies condition. In lyophilized in-tube reactions, the 10% sugar condition showed early signal liftoff down to 10 RNA copies per reaction. As shown in **Figure 5.EB**, 2/2 replicates at 10 RNA copies per reaction showed signal liftoff prior to an hour, with both replicates reaching peak fluorescence by 30 minutes. NTC (no template control) signal curves stayed flat through the 60 minutes of amplification.

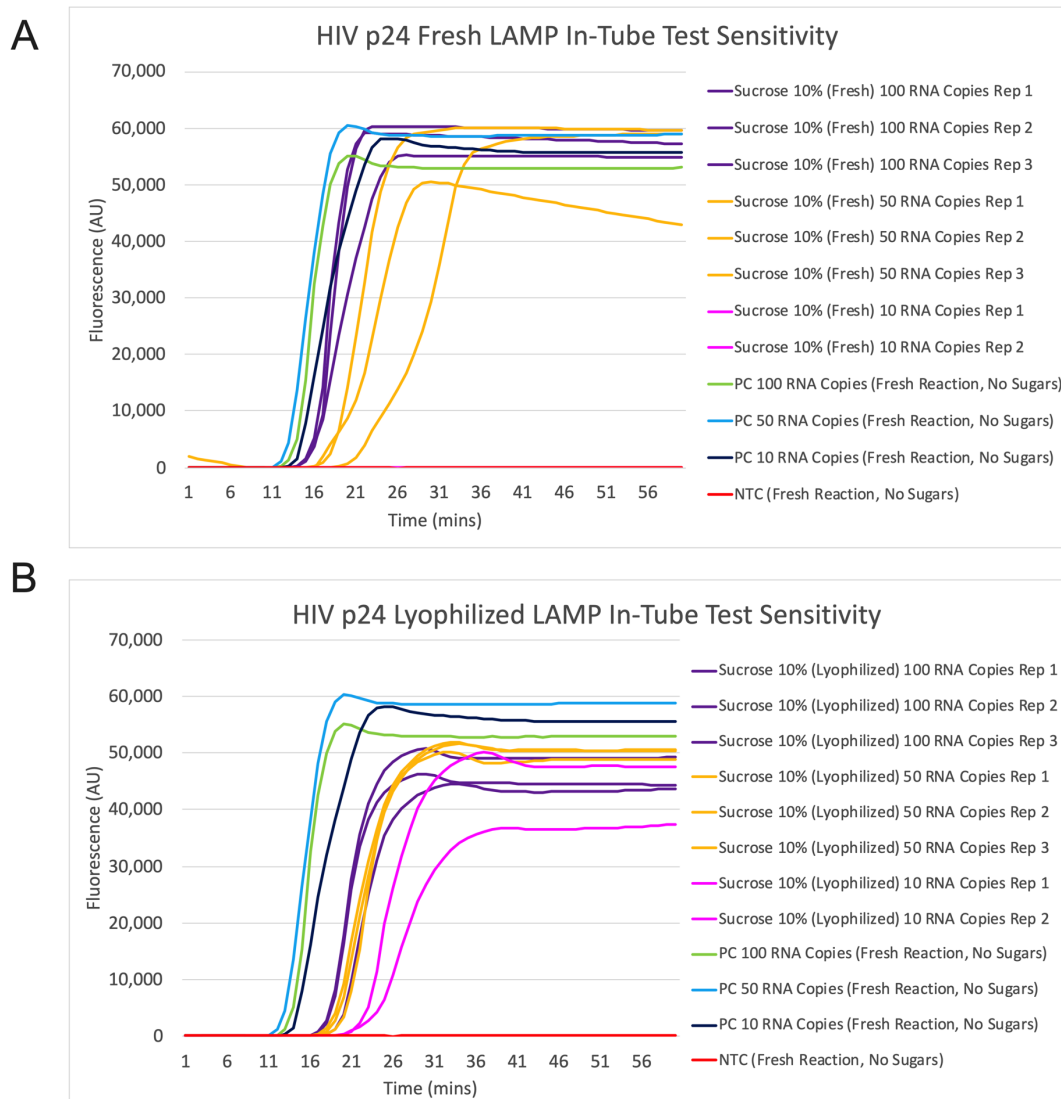


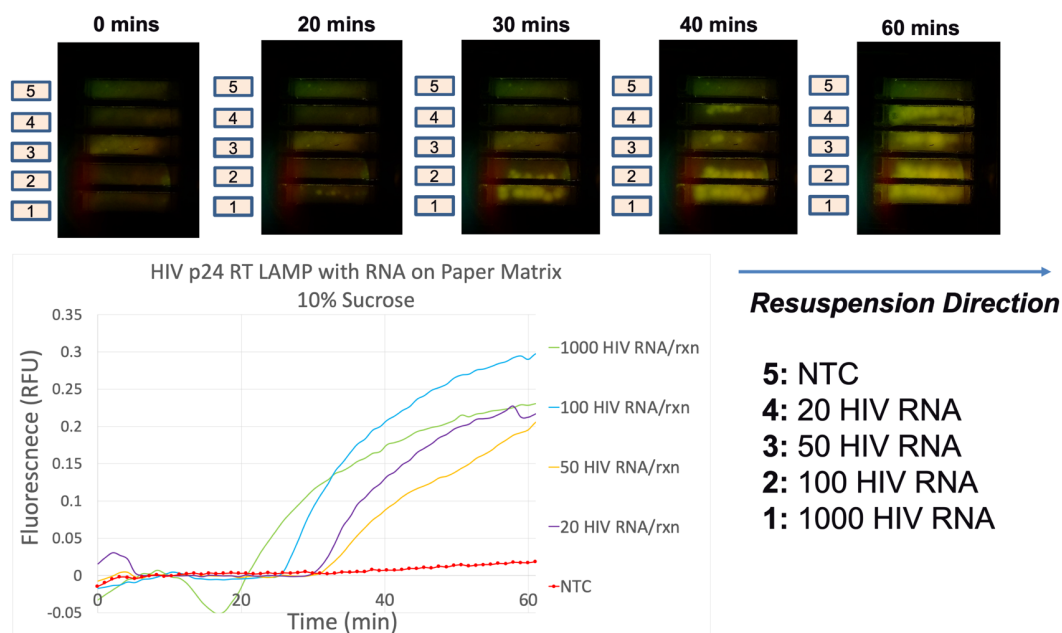
Figure 5.3. Fluorescence HIV RT-LAMP In-Tube Limit-of-Detection for HIV RNA

(A) HIV RT-LAMP fresh reaction limit-of-detection of 50 RNA copy per reaction reaction in-tube with 10% sucrose (B) HIV RT-LAMP lyophilized reaction limit-of-detection of 10 RNA copy per reaction reaction in-tube with 10% sucrose

For QMA-based RT-LAMP reactions, 0.5% dextran was added to the lyophilization reaction mix to enable gradual rehydration of the lyophilized pads upon sample rehydration in-device, thereby reducing concentration gradients (33,63).

To test the translation of the fluorescence HIV RT-LAMP onto QMA pads, a “5-pad device” (previously shown in Chapter 2) was used to first test the translation of the HIV RT-LAMP with 10% sucrose mixture in HIV RNA LOD testing. As shown in **Figure 5.4A**, the QMA-based fluorescence HIV RT-LAMP showed LOD for HIV RNA of 20 copies per reaction ($n=1$). A “6-pad device” was used to first test the translation of the HIV RT-LAMP with 10% sucrose mixture in HIV RNA LOD testing, such that an additional replicate of the lowest RNA copy condition could be added. As shown in **Figure 5.4B**, the QMA-based fluorescence HIV RT-LAMP showed LOD for HIV RNA of 25 copies per reaction ($n=2$). Notably, the sucrose + dextran RT-LAMP mixture showed slower time-to-signal liftoff compared to the sucrose-only condition. This may have been due to the dextran significantly increasing the master mix’s viscosity, resulting in slower kinetics within the solution, but this is speculation. While time-to-result is slower with the addition of dextran, its inclusion results in a more even reagent concentration profile across the pad (as previously observed in Chapter 2), which could be beneficial for consistent detection of an amplification signal.

A HIV RT-LAMP Limit-of-Detection in QMA with 10% Sucrose



B HIV RT-LAMP Limit-of-Detection in QMA with 10% Sucrose + 0.5% Dextran

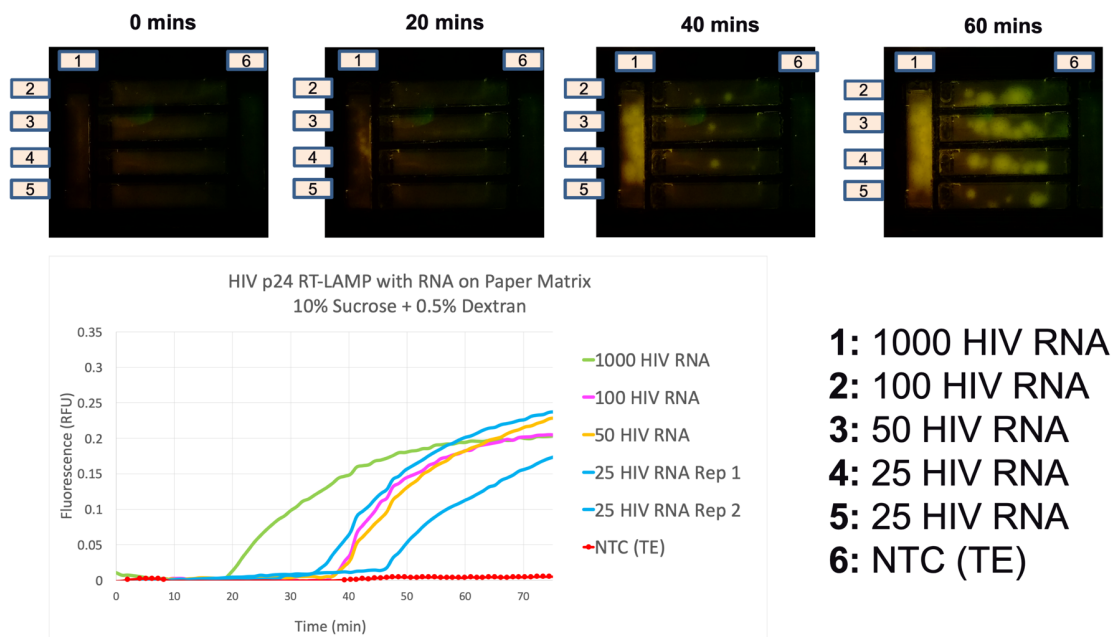


Figure 5.4. HIV Fluorescence RT-LAMP Limit-of-Detection for RNA in QMA

(A) HIV RT-LAMP limit-of-detection of 20 RNA copy per reaction in QMA with 10% sucrose (B) HIV RT-LAMP limit-of-detection of 25 RNA copy per reaction in QMA with 10% sucrose + 0.5% dextran

5.3.2 Colorimetric RT-LAMP Optimization in-tube and porous matrices

To enable a more affordable and simple version of the assay by using colorimetric detection in the HIV RT-LAMP, SYTO-82 and HNB were replaced with 160 μ M Eriochrome Black T (Erio T), a metal cation indicator that significantly changes color in the presence of Mg^{2+} ions (**Figure 5.9**) and has been previously studied in LAMP assays (174). In freshly prepared (“fresh”) and lyophilized tube-based (“in-tube”) reactions, colorimetric-based HIV p24 RT-LAMP assays were performed on a 96-well thermocycler (CFX Opus, BioRad) from high to low RNA LOD conditions. Colorimetric evaluations were performed by eye at endpoint, with images taken pre- and post-amplification. As shown in **Figure 5.5**, both fresh and lyophilized in-tube reactions showed signal liftoff at a limit-of-detection (LOD) of 50 RNA copies per reaction. Noticeably, the colorimetric profile between the starting and endpoint colors of the reaction mixes differed notably between the fresh and lyophilized conditions. As shown in **Figure 5.5A**, the starting color of the fresh mix is a bright red, while the endpoint is a purple. In **Figure 5.5B**, the starting color of the lyophilized mix is already purple, while the endpoint is a dark blue color. We hypothesize that the cause of the colorimetric differences is caused by the precipitation of magnesium-pyrophosphate during the lyophilization protocol, where the removal of moisture from the reaction mix results in concentration of reagent and facilitating co-precipitation. This phenomenon is further explored in Chapter 6. Despite the differences in colorimetric output, there is a clear difference between the start and end profile for the in-tube reactions.

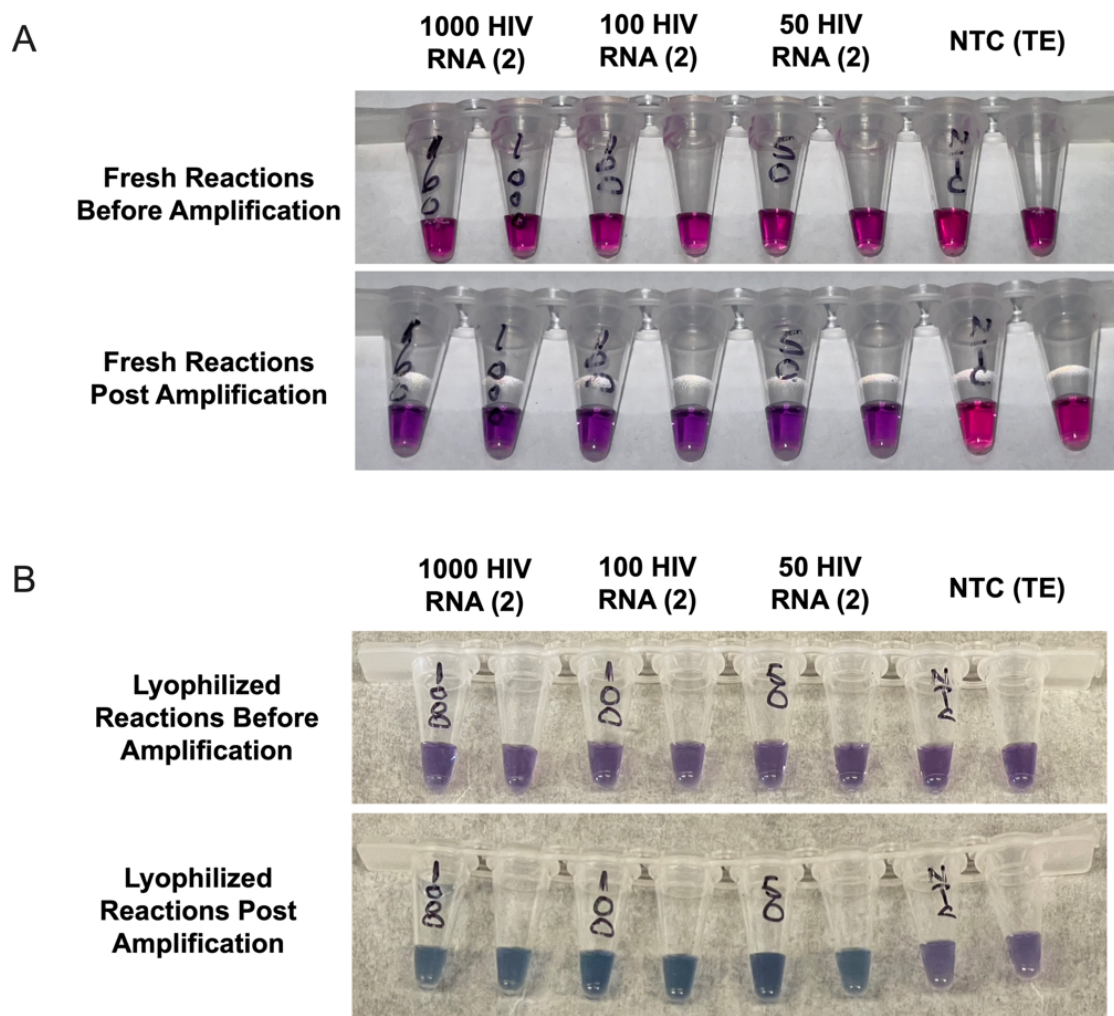


Figure 5.5. Colorimetric HIV RT-LAMP limit-of-detection (LOD) reactions in-tube under fresh and lyophilized conditions

(A) *Fresh in-tube colorimetric HIV RT-LAMP LOD reactions* (B) *Lyophilized in-tube colorimetric HIV RT-LAMP LOD reactions.*

The colorimetric HIV RT-LAMP was performed in QMA pads using 5-pad devices, following similar protocol to the fluorescence RT-LAMP in QMA testing. As shown in **Figure 5.6A**, the fresh colorimetric HIV RT-LAMP in QMA showed vibrant colorimetric shift at high RNA copy number conditions. Across 60 minutes of amplification, the positive control conditions shifted from red to blue-purple, while the negative control stayed red throughout. However, attempts at

colorimetric detection using lyophilized RT-LAMP reaction mixes proved challenging. As shown in **Figure 5.6B**, directly lyophilizing the entire reagent mix onto the QMA membrane followed by rehydration *via* RNA-containing buffer results in an unexpected immediate shift of each pad to purple. Post-amplification, the positive control condition pads appear blue, but only slightly more so than the dark purple color in the negative control condition pads. When observed by eye under ambient lighting or with phone flash, it was difficult to distinguish between positive and negative control QMA pads post-reaction. Notably, the white streaks appearing in the middle of pads at the start of a reaction is caused by compression of the pad against the PMMA cartridge; as liquid evaporates out of the pads during amplification, the white streak becomes less obvious by the end of the reaction.

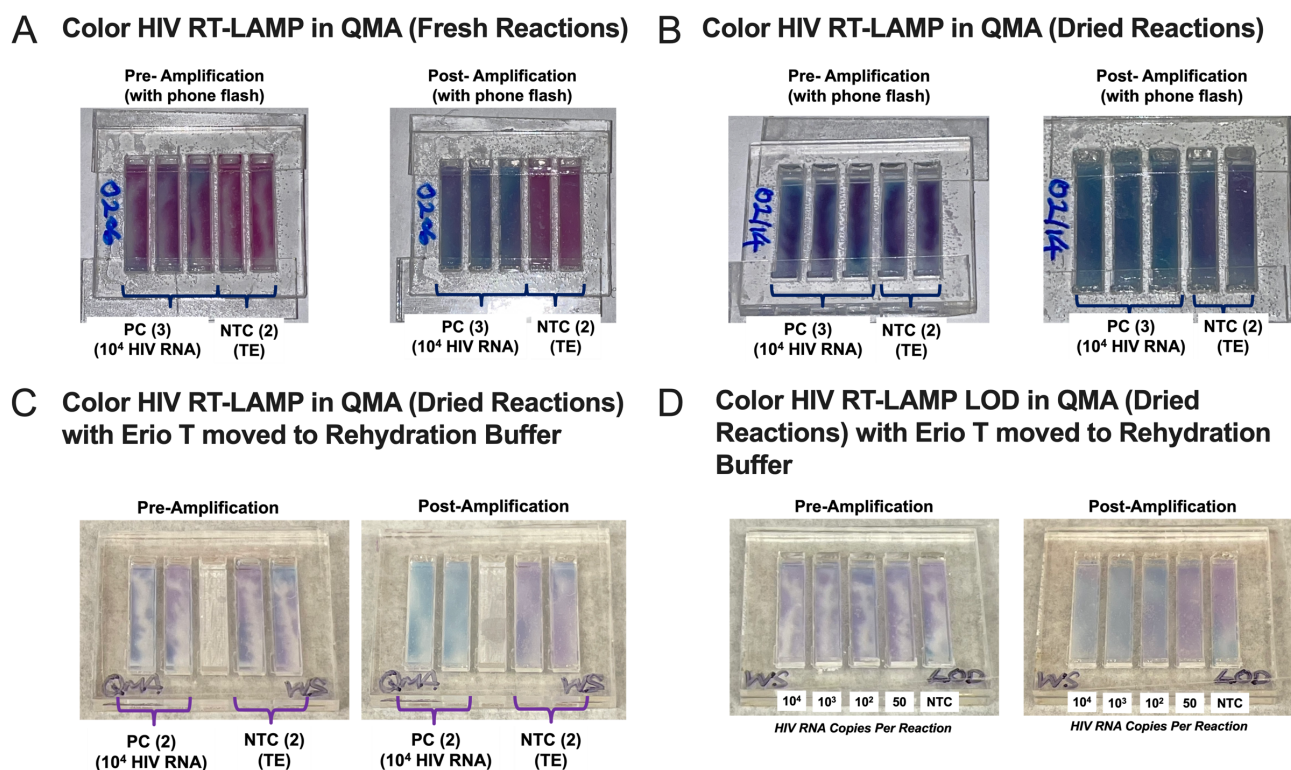


Figure 5.6. Colorimetric HIV RT-LAMP in QMA under fresh and lyophilized conditions

(A) Fresh colorimetric HIV RT-LAMP LOD reactions in QMA (B) Lyophilized colorimetric HIV RT-LAMP LOD reactions in QMA (C) Lyophilized colorimetric HIV RT-LAMP reactions in QMA, with Erio T moved to rehydrating template solution (D) Lyophilized colorimetric HIV RT-LAMP LOD reactions in QMA, with Erio T moved to rehydrating template

To enhance the colorimetric shift of lyophilized Erio T HIV RT-LAMP in QMA, the 160 μ M Erio T was transferred to the rehydration buffer containing either RNA or negative control buffer. This adjustment is possible because Erio T is stable at ambient temperature and could be stored in solution form in a blister instead of needing to be lyophilized. As shown in **Figure 5.6C**, moving Erio T to the template buffer significantly improved the colorimetric shift in the positive control from start to finish. The positive control changes from purple to blue, while the negative control stays purple throughout the amplification. **Figure 5.6D** shows an LOD evaluation of the new colorimetric assay protocol, which shows reliable detection of colorimetric amplification signals at high RNA copy positive control conditions only. At 100 and 50 copy conditions, there is noticeable separation in the colorimetric profile, with both blue and purple appearing on the same pad. This likely indicates incomplete amplification across the entirety of the pad, which makes determining whether a pad is positive or negative difficult. This observation is further explored in Chapter 6. NTC pads stayed purple throughout the reaction, though some blue spots did appear indicating chromatographic separation. Therefore, the colorimetric method currently appears viable for only high-copy target conditions.

5.3.3 HIV Fluidic Device Operations

The HIV fluidic device prototype has been designed to incorporate multiple sample processing steps into a single platform. The device operation is intended to proceed as follows (**Figure 5.7**): 100 μ L of human patient blood is to be added to the sample addition pad, followed by 500 μ L of

high salt push buffer. The blood will be pushed through the MF1 plasma separation pad, where plasma would be separated from red blood cells. The plasma would be pushed through the proteinase K pad, where it would be incubated for chemical lysis at 55°C for 5 minutes while a pull-connector valve blocks forward fluid flow. The pad also holds biotinylated DNA probes that will bind onto the RNA as lysis occurs. After incubation, the valve will be actuated and the lysed sample will move forward into the magnetic bead pad, where streptavidin-coated magnetic beads bind to the biotinylated probes over 10 minutes of ambient incubation while a second valve blocks flow. Once the second valve is actuated, the bead-probe-RNA stack will move to the elution pad, where a magnet immobilizes the beads. Here, a washing step with a second, low-salt solution will be performed; the buffer will push the high-salt buffer into a cellulose wicking pad, after which the magnet will be removed during a 95°C heating step for 5 minutes to elute the RNA from the DNA probe while a third valve blocks forward fluid flow. Post-elution heating, the third valve will be actuated so that the detached RNA flows forward into the amplification pads. Please note that operational schematic in **Figure 5.7** was assembled using a test device that employed valves made from Tyvek membranes (DuPont, Wilmington, DE); the design has since switched to using PTFE valves that operate *via* the same mechanism.

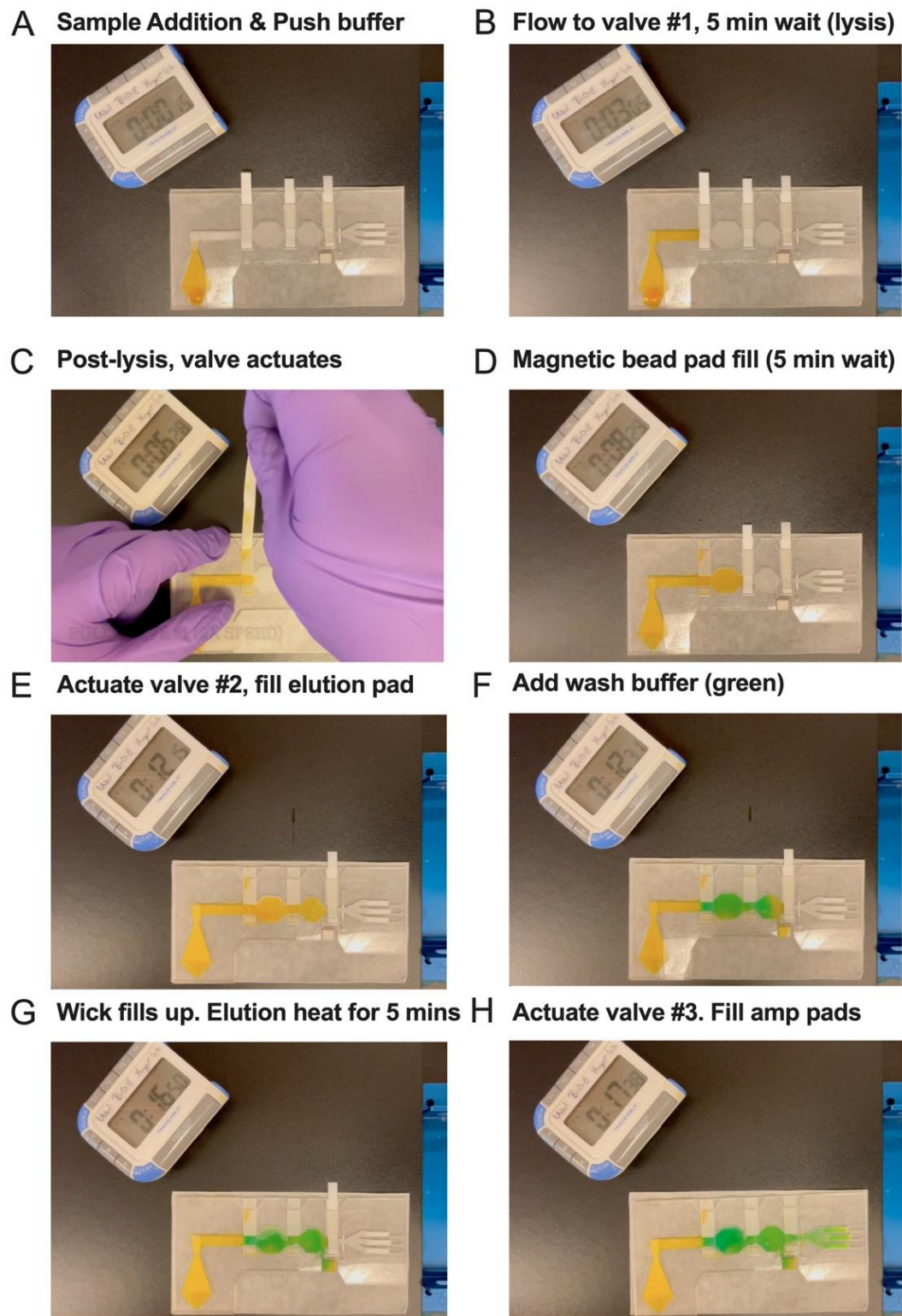


Figure 5.7. HIV Test Device Operational Flow Chart

(A) Sample (yellow) is added followed by push buffer (yellow) (B) Fluid flows through proteinase K pad and is stopped by valve #1. 5 minute wait for lysis (C) Pull valve #1 post-lysis to connect the proteinase K pad to magnetic bead pad (D) Magnetic bead pad rehydrates, followed by 5 minute wait for capture (E) Pull valve #2 to connect and rehydrate elution pad (F) Add wash buffer (green) to displace high salt buffer (yellow) into wicking pad (G) Full buffer displacement, followed by elution heating (H) Post elution, pull valve #3 to connect and rehydrate amplification pads. In future device iterations, the manual steps in this device will be replaced with heat-actuated valves and automated operation via a custom printed circuit board, as shown in Chapters 2-4.

The device fluid flow path is enabled by the pull-connector valve, so named for its dual function of blocking flow followed by its ability to connect a preceding lower membrane with a following upper membrane. The valve functions due to it being situated between two membranes; the movement of the valve (**Figure 5.2B**) pulls the upper membrane downward such that the two membranes are connected. In the simulated testing shown in **Figure 5.7**, the sample and push buffer (yellow) flow through the 2DPN as controlled by the valve. No premature leaking is observed prior to valve actuation. At each step requiring incubation, a 5-minute waiting time was implemented, though this may change during actual clinical device testing. The washing buffer (green buffer) illustrates complete buffer exchanges in the elution pad, as the yellow buffer is completely pushed into and saturates the wicking pad. Once the last valve is actuated, the solution rehydrating the amplification pads is primarily green, indicating full buffer exchange. In upcoming testing with contrived and clinical samples, the device will be tested with reagent membranes and RT-LAMP amplification pads.

5.4 Conclusion

Here, we present fluorescence and colorimetric-visualized HIV RT-LAMP assays. Both variants of the RT-LAMP assay have been characterized in PCR tubes and QMA membranes under both

fresh and lyophilized conditions, with fluorescence detection enabled by SYTO-82 and colorimetric detection enabled by Eriochrome Black T. We have also designed a microfluidic device with integrated pull-connector valves to facilitate fluidic control across multiple sample processing steps. Through test operations, we demonstrate that the device could support sample flow-through, washes, and fluid retention without leakage. In future testing, we intend to use this type incorporate contrived and clinical HIV samples for both fluorescence and colorimetric evaluations while further optimizing the design for point-of-care applications. Manual steps in the current device will be replaced with automated and timed operations via a custom printed circuit board and heat-actuated valves, as shown in Chapter 2-4.

5.5 Acknowledgments

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I want to thank Erin Heiniger, PhD, who is the project lead on this manuscript in preparation.

5.6 Supplemental Information

Table 5.1. In-tube and QMA-based HIV LAMP master mix

Reagent	Fluorescence RT-LAMP Final Concentration (In-tube, QMA)	Colorimetric RT-LAMP Final Concentration (In-tube, QMA)
WarmStart LAMP Kit	1X	1X
SYTO-82	7.5 μ M	-
Hydroxynaphthol Blue	40 μ M	-
Eriochrome Black T	-	160 μ M
Assay specific primers (See Table 5.1)	1X	1X
Sucrose	10%	10%

Dextran, 500 kD	0.5%	0.5%
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Table 5.2. HIV p24 RT-LAMP primers

Source: Curtis *et al.* (2024) (173)

Primer sequence	Primer name	Final concentration (1X)
ATTATCAGAAGGAGCCACC	F3	0.2 µM
CATCCTATTTGTTTCCTGAAGG	B3	0.2 µM
CAGCTTCCTCATTGATGGTTTCTTT TTAACACCATGCTAAACACAGT	FIP	1.6 µM
TGTTGCACCAGGCCAGATAATTTT GTACTGGTAGTTCCTGCTATG	BIP	1.6 µM
TTTAACATTTGCATGGCTGCTTGAT	LoopF	0.8 µM
GAGATCCAAGGGGAAGTGA	Loop B	0.8 µM

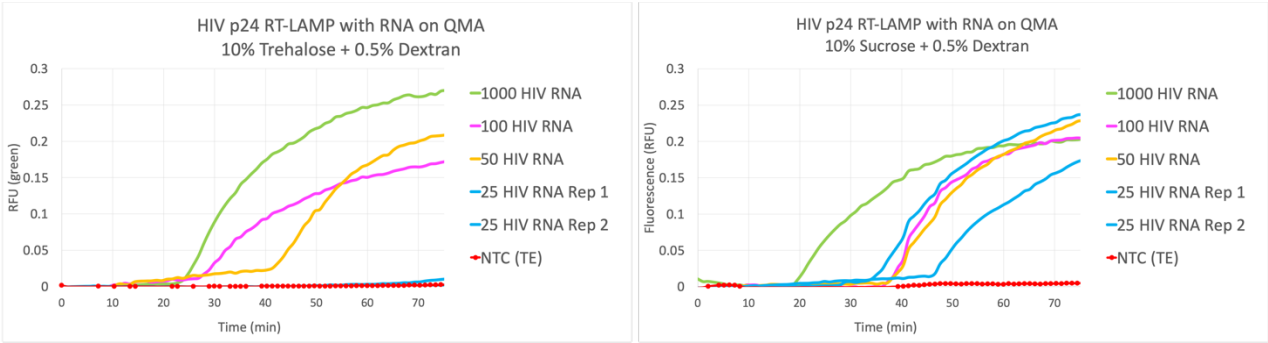


Figure 5.8. HIV RT-LAMP comparison between trehalose + dextran vs sucrose + dextran conditions in QMA-based reactions

10% trehalose + 0.5% dextran RT-LAMP (left) showed lower sensitivity at detection of 25 HIV RNA copy per reaction than 10% sucrose + 0.5% dextran RT-LAMP (right).

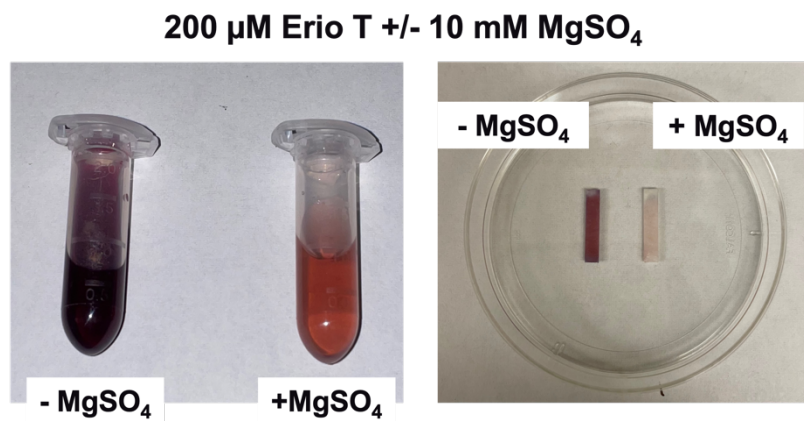


Figure 5.9. Eriochrome Black T +/- MgSO_4 in-tube (left) and in QMA (right)

6. Chapter Six – Lessons Learned

Over the course of my PhD studies, there were many challenges and troubleshooting efforts that would not appear in talks or publications. These lessons proved to be highly educational as we tackled other projects. This chapter describes some of the most critical lessons learned during my PhD. I anticipate that these findings may contribute towards both assay and device design and implementation for point-of-care diagnostic technology advancements in the long-term.

6.1 Delayed amplification in porous matrices compared to in-tube reactions

6.1.1 Introduction

As shown in Chapter 2.4, QMA-based LAMP reactions reach amplification signal liftoff considerably later than their in-tube forms. Across multiple replicates, the time-to-signal in QMA-based reactions appears to be 5-10 minutes longer than in in-tube reaction, even at high RNA copy conditions. This disparity was also observed in prior porous matrix testing on Std17 before switching to QMA. To evaluate this disparity, we analyzed material treatment differences that could have affected RT-LAMP efficacy between the two modes of testing, and also compared differences in the instruments used for reagent heating.

6.1.2 Materials and Methods

Leachate Collection from Treated and Untreated Porous Matrices

Leachate solutions from both 1% BSA, 0.1% Tween 20 blocked and unblocked Whatman Std17 membranes (GE Healthcare, Chicago, IL) were collected by submerging the membranes in 100 μ L of TE buffer (10 mM Tris, 0.1 mM EDTA) in Eppendorf tubes (Eppendorf, Hamburg, Germany); tubes were then heated at 63°C for 30, 60, or 90 minutes on a heat plate (VWR, Radnor,

PA). Solutions from multiple Std17 membranes were then collected *via* centrifugation at 15000 RCF for 5 minutes and used to prepare template solution for rehydrating lyophilized reaction mixes. Reactions were performed on a 96-well BioRad thermocycler (CFX Opus).

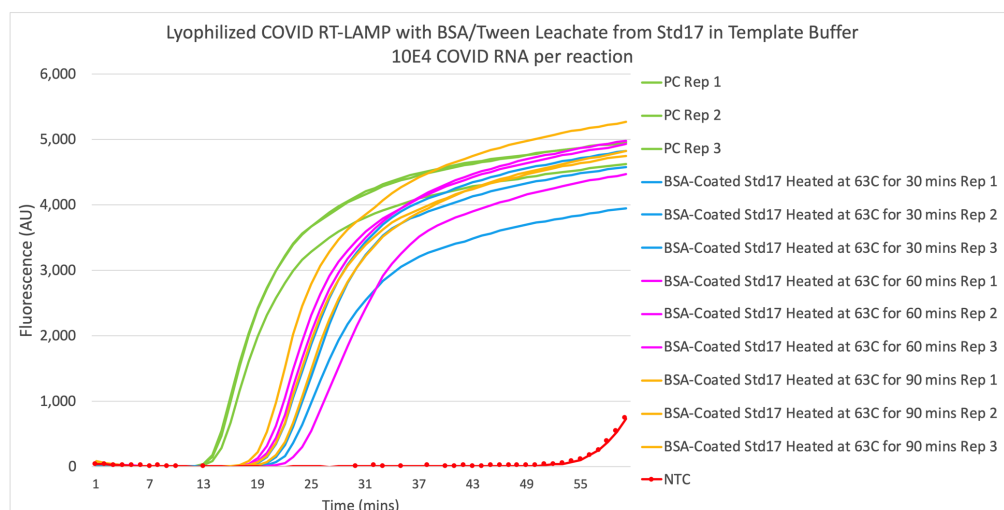
RT-LAMP Assay Mixture

As previously indicated in Chapter 2.3 and **Table 2.5**, the COVID RT-LAMP assay reagents consist of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), acridine orange fluorescent intercalating dye (Sigma-Aldrich, St. Louis, Mo), hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA), trehalose (Life Sciences Advanced Technologies), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham, MA), and COVID LAMP primers (Integrated DNA Technologies, Skokie, IL) (88). For fluorescence detection, 120 μ M acridine orange (Sigma-Aldrich, St. Louis, Mo) was used in place of 7.5 μ M SYTO-82.

6.1.2 Results and Discussions

To prepare leachate solution, blocked and untreated Std17 membranes were submerged in TE buffer and heated at 63°C for 30-90 minutes to simulate RT-LAMP heating conditions. Leachates from the blocked and unblocked versions of the Std17 would indicate the impact of the potential “poisonous” materials leaching off from both iterations that could be impacting RT-LAMP. The impact of both leachate solutions is shown in **Figure 6.1**.

A Leachate from 1% BSA, 0.1% Tween 20 blocked Std17



B Leachate from Untreated Std17

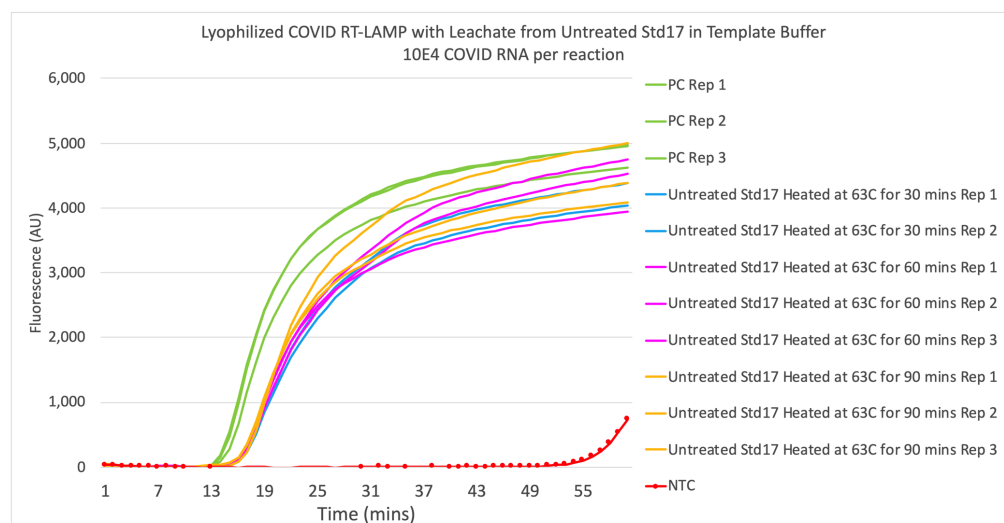


Figure 6.1. Impact of Leachate from Blocked and Untreated Porous Matrices on RT-LAMP

(A) Impact of 1% BSA, 0.1% Tween 20 blocked Std17 leachate on RT-LAMP (B) Impact of untreated Std17 leachate on RT-LAMP

As shown in **Figure 6.1**, both the blocked and untreated Std17 yielded leachate solutions that worsened the observed RT-LAMP time-to-result. **Figure 6.1A** indicates that BSA and Tween 20

blocking has a significant delaying effect on in-tube COVID RT-LAMP time-to-result compared to positive control, with all replicates showing 5-10 minutes delays. This indicates that the BSA and Tween treatment, while necessary for keeping RT-LAMP enzymes viable on the porous matrices, may be inhibiting the performance of the enzymes themselves. As all porous matrices, including both Std17 and QMA, were treated with both BSA and Tween, this effect would be observed in all paper-based RT-LAMP results. Figure 6.1B shows that while the untreated Std17 leachate also delays time-to-result for in-tube COVID RT-LAMP, the delay is noticeably shorter compared to the BSA-Tween blocked variant. Therefore, the BSA-Tween treatment has a significant impact on causing the longer signal liftoff times as observed in porous matrices.

Additionally, there are noticeable differences in the instruments used for in-tube and in-paper based reactions. All in-tube experiments were performed using a 96-well BioRad thermocycler, while paper-based reactions were performed in a custom dual-sided oven heater. The 96-well thermocycler is programmed to have its lid and sample temperature reach setpoint prior to beginning its hour-long timer. As shown in **Figure 6.2**, the time that it takes from starting a heating protocol to starting the first cycle in the hour-long amplification takes an average of 2.5 to 3.5 minutes for the sample to first reach 63°C. In addition, the optical unit for fluorescence evaluation takes an addition 10-12 seconds to operate following each cycle. Therefore, each 60 second cycle in the CFX data output takes 72 seconds of real-time data collection. In contrast, the custom oven heater timer begins right after the device is placed onto the heater and the cell phone reader is initiated. Therefore, this times difference is also contributing to the delayed time-to-result as observed in paper-based RT-LAMP systems.

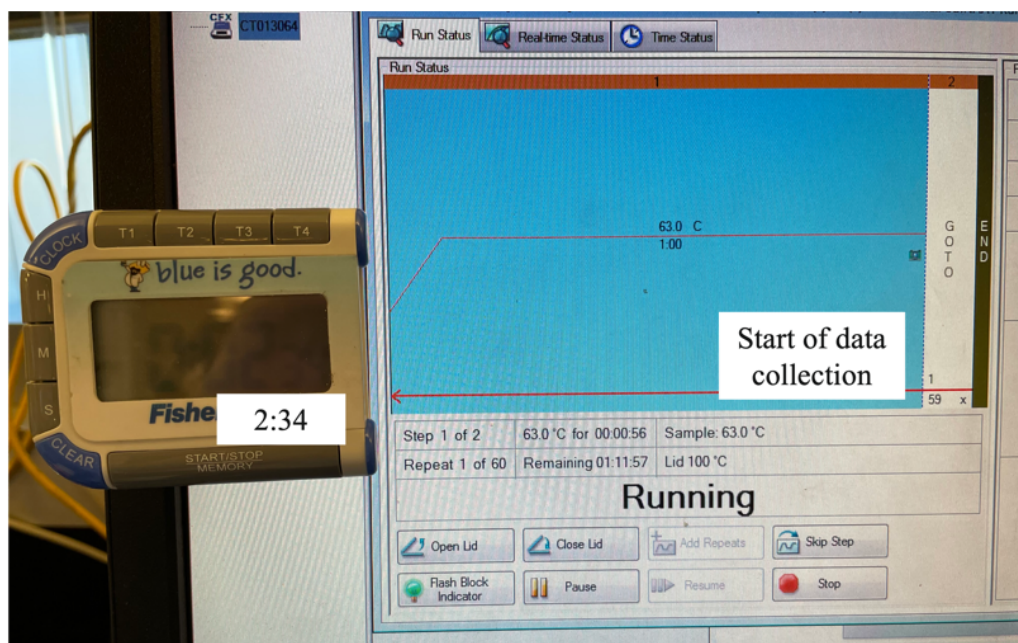


Figure 6.2. BioRAD (CFX) 96-well thermocycler takes at least 2.5 minutes for samples to reach set temperature before starting first amplification cycle

6.2 Magnesium-pyrophosphate precipitation during RT-LAMP assays

6.2.1 Introduction

The precipitation of magnesium-pyrophosphate plays a prominent role during RT-LAMP, and has been used by itself and in conjunction with metal cation indicators and as its own visualization method for evaluating amplification (52,175). Understanding the fundamental cause for metal cation indicators' color change was vital in our attempts to adapt to the reaction mixture color change against clinical vaginal lysate samples in Chapter 4 and to develop a colorimetric HIV RT-LAMP assay in Chapter 5. In both cases, we hypothesized that metal cation indicator color change is caused by the change in free magnesium ion concentration in solution. Currently, we do not have a definitive explanation for why lyophilization caused a change in the magnesium concentration in lyophilized reactions compared to fresh reactions. One of our leading hypotheses

is that lyophilization causes damage to dNTPs, possibly from overconcentration or drying. This could cause dNTPs to release pyrophosphate ions that would precipitate with free magnesium ions, resulting in a lower magnesium ion in solution upon rehydration. Another possibility is that lyophilization causes dNTPs to bind irreversibly to magnesium, resulting in a lower concentration of free magnesium in solution. If magnesium-pyrophosphate precipitation is the primary mechanism for metal cation indicator color change, it would further elucidate how paper-based amplification systems must be adapted to optimize lyophilized visualizations of colorimetric RT-LAMP. To test our hypothesis, we performed the following experiments to evaluate magnesium-pyrophosphate precipitation in solution and measure its direct impact on the color state of metal cation indicators (hydroxynaphthol blue, Eriochrome Black T).

6.2.2 Materials and Methods

RT-LAMP Assay Component Testing

To isolate the role of magnesium-pyrophosphate precipitation on metal cation color change during amplification, the colorimetric RT-LAMP reaction mix was simplified across two experiments. First, the lyophilization mix in PCR tubes consisted only of nuclease-free water (Thermo Fisher Scientific, Waltham, MA), magnesium sulfate (MgSO_4) (New England Biolabs (NEB), Ipswich, MA), (New England Biolabs (NEB), Ipswich, MA), and hydroxynaphthol blue (HNB) (Millipore Sigma, Burlington, WA) with the variable of deoxynucleotide triphosphates (dNTPs), shown in **Table 6.1**. The variable of adding dNTPs allowed for identification of whether magnesium ions would stay in or spontaneously precipitate out of solution during lyophilization.

Table 6.1. Mixture for testing magnesium pyrophosphate precipitation during lyophilization

Reagent	Lyophilization Mixture (Added dNTPs)	Lyophilization Mixture (No dNTPs)
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Nuclease Free Water	1X	1X
MgSO ₄	8 mM	8 mM
Hydroxynaphthol Blue	40 μ M	40 μ M
dNTPs	1.4 mM	-

Next, a fresh reaction mix was prepared in PCR tubes consisting only of TE buffer, magnesium sulfate (MgSO₄) (New England Biolabs (NEB), Ipswich, MA), and Eriochrome Black T (Erio T) (Sigma-Aldrich, St. Louis, Mo), shown in **Table 6.2**. Sodium pyrophosphate (Thermo Fisher Scientific, Waltham, MA) was added as a variable to induce magnesium-pyrophosphate precipitation.

Table 6.2. Color Reaction Mixture Using Sodium Pyrophosphate

Reagent	Fresh Mixture (no sodium pyrophosphate)	Fresh Mixture (added sodium pyrophosphate)
TE Buffer	1X	1X
MgSO ₄	8 mM	8 mM
Erio T	120 μ M	120 μ M
Sodium Pyrophosphate	-	10 mM

PCR Tube Reaction Lyophilization

For the tube-based reaction mixture shown in **Table 6.1**, lyophilization protocols were performed as described in our previous work (33,63). For lyophilizing reaction tubes, PCR tubes containing 19 μ L mixture volume with holes poked into the caps were dipped into liquid nitrogen for 1 minute, then placed into a 96-well aluminum tube holder (Qiagen) prior to attachment to a FreeZone 2.5 liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, Mo) for overnight drying with the system cold finger set at -47°C and the vacuum at 0.03 mbar.

6.2.3 Results and Discussions

The phenomenon of magnesium-pyrophosphate precipitation and its impact on both HNB and Erio T color change was evaluated across lyophilization and induced precipitation. During lyophilization, we hypothesized that magnesium would precipitate out of solution along with pyrophosphate from dNTPs when over-concentrated during drying, thereby causing the rehydrated mixture to reflect blue color in the metal cation indicator due to less free magnesium in solution. This explanation could also explain the difference between lyophilized and fresh reactions, as the concentration of magnesium and dNTP is the same across both. Therefore, any differences observed in the rehydrated Erio T color would likely come a change in the free magnesium concentration in the lyophilized condition post-rehydration. We tested this phenomenon using a simplified mixture of only water, MgSO_4 , and HNB while dNTP was either added or left out of mixture as a variable. Both the lyophilized pellet and rehydrated solution of both conditions are shown in **Figure 6.3**.

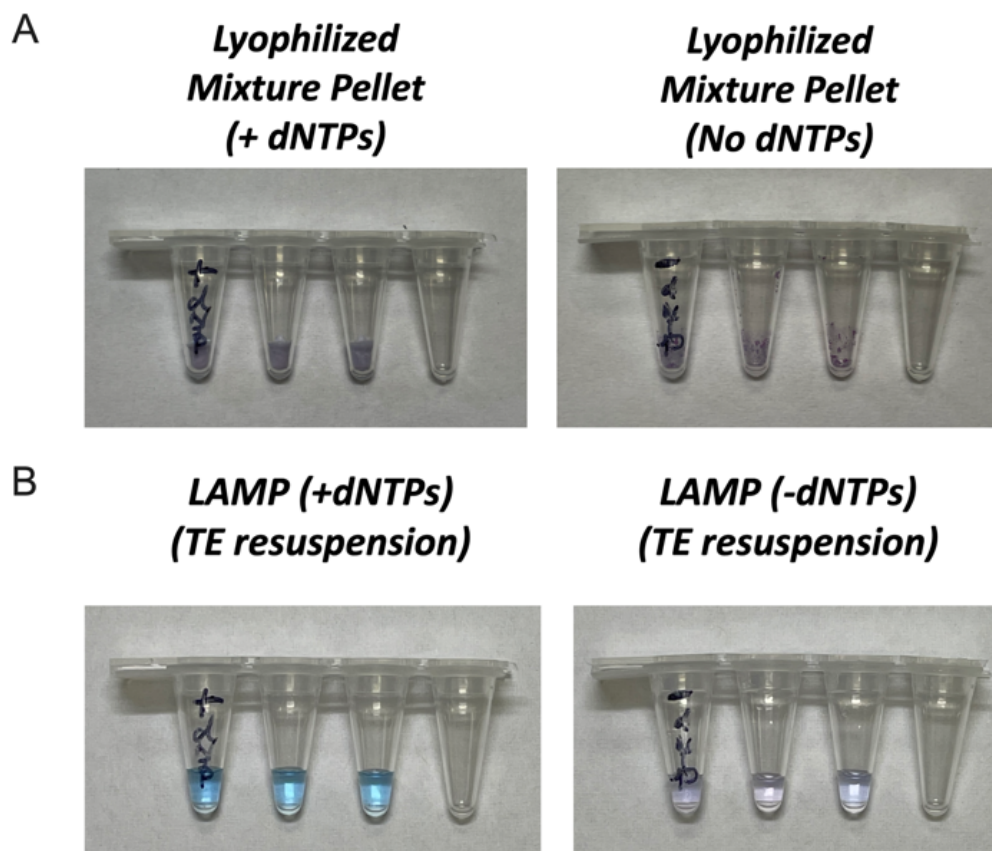


Figure 6.3. Lyophilized HNB-magnesium mixture with and without dNTPs

(A) Lyophilized pellets for both +dNTP and -dNTP conditions (B) Rehydrated solutions for both conditions

As shown in **Figure 6.3A**, the presence of dNTPs had a noticeable impact on the shape of the pellet, as the +dNTP replicates appear to be collected at the bottom of the tube, while the non-dNTP pellets appeared scattered and flaky. Once the pellets were rehydrated with TE buffer in **Figure 6.3B**, there was a noticeable difference in solution color with the +dNTP replicates appearing blue and the non-dNTP conditions appearing purple. As evident from Chapter 6, HNB is natively blue and shifts towards purple in the presence of magnesium ions. In the +dNTP replicates, both magnesium and dNTP were present during lyophilization, meaning that magnesium-pyrophosphate likely precipitated out of solution and stayed precipitated post-

rehydration. Meanwhile, in the -dNTP condition, magnesium did not have pyrophosphates to co-precipitate and likely stayed in solution, resulting in a purple HNB color as more magnesium ions were present post-rehydration.

Another test using Erio T was performed to evaluate magnesium precipitation when sodium pyrophosphate is directly added to solution. This experiment sought to verify whether Erio T changes color through a similar mechanism as HNB. A solution of TE buffer, 8 mM MgSO_4 , and 120 μM Erio T was first prepared across four replicates (**Figure 6.4**), with 10 mM sodium pyrophosphate then added to only replicates 3-4. The tubes were then vortexed and spun down before evaluation.

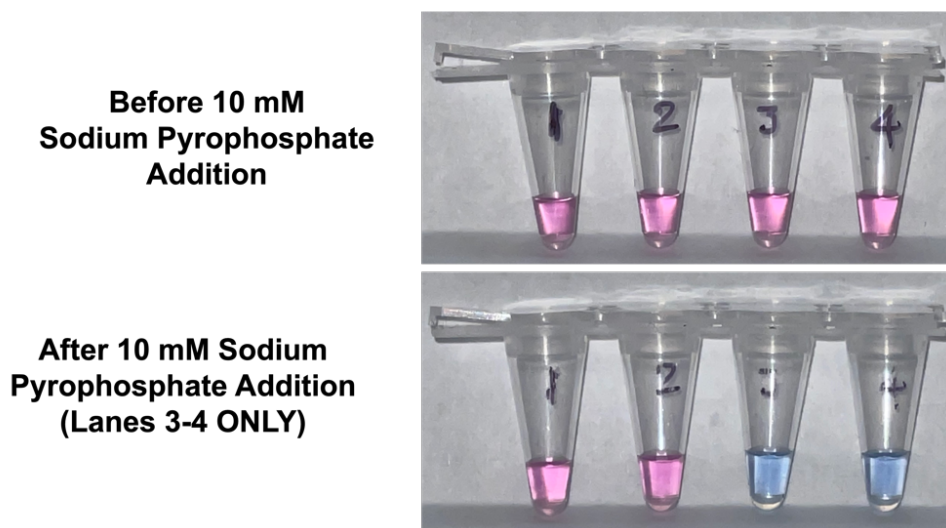


Figure 6.4. Impact of added pyrophosphate on Erio T-magnesium solution

As shown in **Figure 6.4**, all four solutions with only Erio T and MgSO_4 initially appeared pink, which is the expected color of Erio T in the presence of MgSO_4 as observed in Chapter 5. Upon addition of 10 mM sodium pyrophosphate in replicates 3-4, the solution color changes to a distinct blue. This indicates that magnesium ions likely precipitated out of solution along with pyrophosphate ions, resulting in less magnesium in solution. Inspection of replicates 3 and 4 also

showed small precipitates on the bottom of the tube. Thus, it is evident that Erio T changes color based on the availability of free magnesium ions, which changes through co-precipitation with pyrophosphate.

6.3 Color separation in colorimetric paper-based RT-LAMP

6.3.1 Introduction

As briefly mentioned in Chapter 5.3, the development of a lyophilized colorimetric HIV RT-LAMP assay in porous matrices was limited by a noticeable separation of color across the QMA membrane. Notably, Eriochrome Black T's initial in-paper color showed both purple (its intended starting color in the presence of higher magnesium concentration) and blue (which should only be its color state at the end of amplification once magnesium concentration had been depleted). This color separation hinders our ability to distinguish between a positive and negative signal, as signal detection by eye is reliant on color uniformity across the entirety of the pad. This contrasts with fluorescence RT-LAMP development in porous matrices, where the fluorescence signals could be readily distinguished against a dark background. Here, we demonstrate various methods in attempt to resolve the colorimetric separation issue, including membrane surface treatments,

6.3.2 Materials and Methods

During development, two approaches were used in rehydrating lyophilized QMA assay pads to optimize the extent of Erio T color change during RT-LAMP. In the first approach, as shown in **Table 5.1**, the colorimetric HIV RT-LAMP assay consist of WarmStart LAMP Kit (New England Biolabs (NEB), Ipswich, MA), Eriochrome Black T (Erio T) (Sigma-Aldrich, St. Louis, Mo), sucrose (Sigma-Aldrich, St. Louis, Mo), dextran 500 kDa (Sigma-Aldrich, St. Louis, Mo), nuclease-free water (Thermo Fisher Scientific, Waltham, MA), and assay-specific LAMP primers

(**Table 5.1**). Notably, Erio T was removed from the lyophilization mixture and instead added to the rehydration template buffer. This approach was used to generate the colorimetric results shown in chapter 5.

In the second approach (Approach 2), the WarmStart LAMP mixture was manually assembled using individual reagents instead of the commercial kit. This mixture consisted of Isothermal Amplification Buffer pack, MgSO₄, dNTP Mix, WarmStart Bst 2.0 DNA polymerase, and WarmStart RTx Reverse Transcriptase, with all reagents being purchased from New England Biolabs (Ipswich, MA). The remaining reagents were similar to the first approach, as summarized in **Table 6.3**. In this approach, Erio T as well as 6 mM MgSO₄ were both removed from the lyophilization mix and instead added to the rehydration template buffer.

Table 6.3. Approach 2 recipe for colorimetric RT-LAMP in QMA

Reagent	Approach 2 (Final Concentration)
<i>Lyophilization Mixture</i>	
Isothermal Amplification Buffer (contains 2 mM MgSO ₄)	1X
dNTP Mix	1.4 mM
WarmStart Bst 2.0 DNA polymerase	8 units
WarmStart RTx Reverse Transcriptase	7.5 units
HIV p24 RT-LAMP specific primers (See Table 5.2)	1X
Sucrose	10%
Dextran, 500 kD	0.5%
<i>Template Solution (Rehydrate lyophilized pads)</i>	
MgSO ₄	6 mM
Eriochrome Black T	160 μM

As previously described in Chapters 2-5, QMA pads were tested using 5-pad PMMA “test devices.” For device assembly, refer to chapter 2.3. Post device assembly and pad rehydration with template solution, the 5-pad device was heated on a custom oven at 64°C for 45 mins.

QMA-based Reaction Mix Lyophilization

To lyophilize the QMA-based reactions using the mixture shown in **Table 6.3**, 1% BSA, 0.1% Tween-20-coated QMA pads, held in a 6-well plate, were each hydrated with 24 µL of RT-LAMP lyophilization mix that contained 10% sucrose and 0.5% dextran (500 kDa). The apparatus was quickly frozen by insertion in liquid nitrogen for 1 minute before being placed into a fast-freeze flask (Labconco) and attached to a FreeZone 2.5 liter freeze-drying benchtop lyophilizer (Labconco, Kansas City, Mo) for overnight drying with the system cold finger set at -47°C and the vacuum at 0.03 mbar.

6.3.3 Results and Discussion

During development of the colorimetric HIV RT-LAMP assay, different approaches were explored to optimize Erio T’s color shift from pink-purple to blue in QMA membranes. To optimize this shift, we developed approach (here termed “Approach 2”) to utilize individual reagents to make up the WarmStart LAMP mix; the goal was to minimize magnesium-pyrophosphate precipitation in the QMA during lyophilization, which could not be performed using the WarmStart LAMP mix due to MgSO₄ already being a part of the commercial mix. It should be noted that the isothermal amplification buffer still contained 2 mM MgSO₄. We hypothesized that the rehydration buffer’s mixture of 6 mM MgSO₄ and 160 µM Erio T mixture would yield the optimal starting color for RT-LAMP, and with results from this iteration of testing shown in **Figure 6.5**.

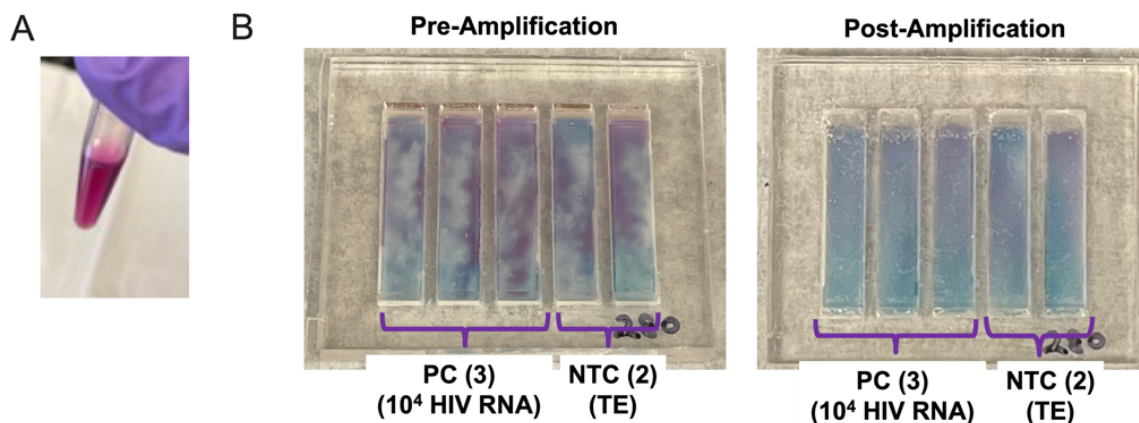


Figure 6.5. Colorimetric HIV RT-LAMP on QMA membranes with Erio T, MgSO₄ in rehydration solution

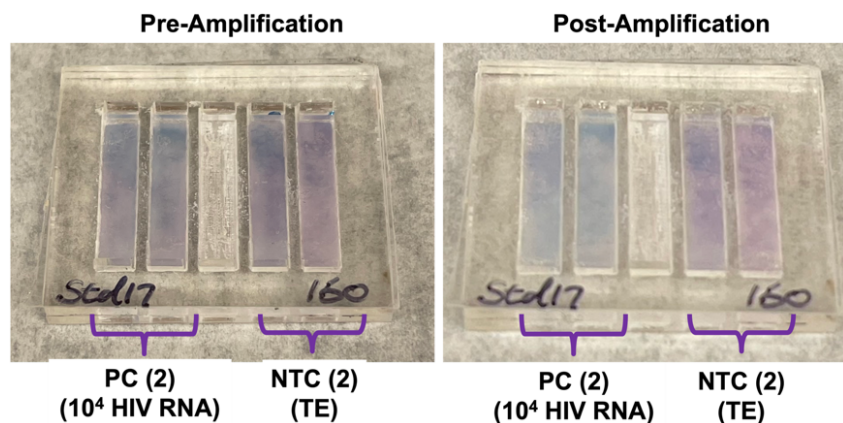
(A) Rehydration solution appears red-pink with Erio T, MgSO₄ (B) Amplification results for before (left) and post-amplification (right)

As shown in **Figure 6.5**, the colorimetric HIV RT-LAMP showed significant color separation in QMA membranes both before and after amplification. The rehydration solution, with 6 mM MgSO₄ added, showed a clear red-pink color but was separated into blue and purple once pipetted into the pads. Even at high HIV RNA copy, it was difficult to distinguish between the positive and negative control pads due to the severity of the color separation. Strangely, the color separation did not seem to follow a pattern. None of the pads showed clear separation of purple and blue on distinct regions of the pad, indicating that separation may be dependent on how the pad was lyophilized.

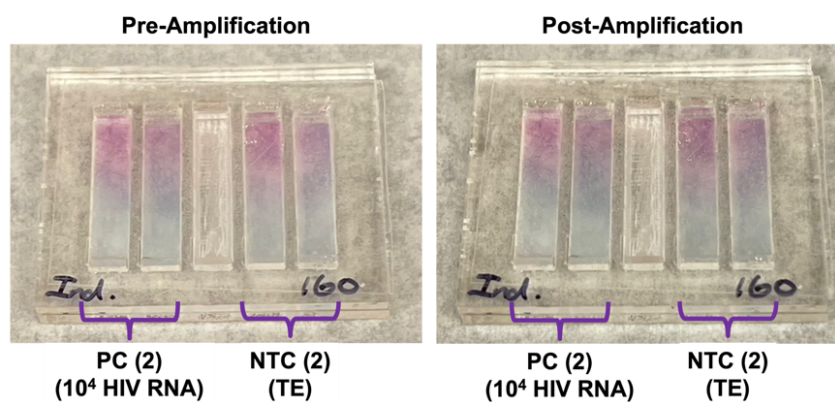
To troubleshoot this issue, multiple strategies were explored. First, Std17 was used in place of QMA to identify whether the color separation issue was exclusive to QMA. Next, dextran was removed from the reaction mix in Std17 to determine whether the higher viscosity that it produces may have contributed to increased color separation due to a slower rehydration rate. Then, a higher

concentration of Erio T at 280 μM was also tested to evaluate whether a higher starting color intensity could overcome the color gradient. Results are shown in **Figure 6.6**.

A Troubleshooting 1: Std17 replaced QMA as porous matrix



B Troubleshooting 2: Removed dextran 500 kD from RT-LAMP reaction mix



C Troubleshooting 3: Used higher Erio T concentration (280 μM)

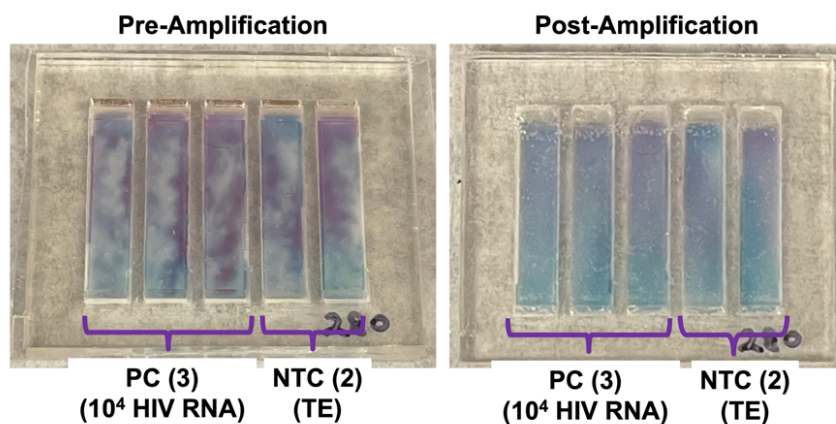


Figure 6.6. Strategies for troubleshooting color separation issue in paper-based colorimetric HIV RT-LAMP

(A) Colorimetric RT-LAMP on Std17 membrane (B) Colorimetric RT-LAMP in Std17, with dextran 500 kD removed from reaction mix (C) Colorimetric RT-LAMP on QMA membrane with 280 μ M Erio T in solution

As illustrated in **Figure 6.6**, various strategies were implemented to try and minimize color separation before and post RT-LAMP in porous matrices. Across multiple conditions and replicates, it became evident that color separation began at pad rehydration. Over the course of amplification, the colors largely stayed separated across the membrane, indicating that reagent concentration across the membrane (likely magnesium and dNTPs) were unevenly distributed during lyophilization and rehydration.

The various strategies for minimizing color separation failed to adequately resolve the issue. In **Figure 6.6A**, the replacement of QMA for Std17 sought to speed up rehydration rate using a higher pore-size membrane, as QMA was only available in 2.2 μ m pore size. However, increasing flow rate did not noticeably affect the color separation as the Std17 reaction pad still exhibited patches of pink and blue in both the positive and negative control pads. A similar strategy of removing dextran 500 kD, as shown in **Figure 6.6B**, was employed to both speed up rehydration rate and reduce reaction viscosity. We hypothesized that lowering the solution viscosity may also reduce the over-concentration of reagents into specific regions on the membrane during lyophilization. However, this did not noticeably impact the resulting color distribution, with even more evident color separation in the Std17 membranes post-dextran removal. We then returned to using QMA membranes and tried adding Erio T to the mixture for a higher final concentration (280 μ M). We hypothesized that the greater starting color intensity may overcome the color separation by reducing blue visualization at the start of amplification. **Figure 6.6C** indicates that while higher

Erio T did result in a stronger intensity of purple in QMA at the start of the reaction, the prevalence of blue was just as evident. Given these results, we proceeded in Chapter 5 with using Approach 1, which yielded noticeably less color separation but also weaker color contrast between positive and negative signals.

Given these results, we hypothesize that the cause for color separation in porous matrices is likely caused by local variations in the concentration of RT-LAMP reagents in specific regions across the membrane during lyophilization or rehydration. Upon rehydration, the magnesium and pyrophosphate ions could remain unevenly redistributed, resulting in different color profiles across the pads. This likely also occurs during fluorescence-based RT-LAMP, as observed in results shown in Chapters 2-5 where fluorescence signals can appear in spots across the membrane. While it is feasible to measure only the brightest of fluorescence signals against a dark background, this observed phenomenon of uneven concentrations is consequential in colorimetric detection by eye that requires signal uniformity. Surface analysis of reagent distribution across the membrane post-lyophilization may provide guidance on surface treatments and drying techniques that improves colorimetric RT-LAMP signal profile.

7. Chapter Seven - Conclusion

Here, I present assay development, device integration, and technological innovation for enabling multiplexed point-of-care detection of infectious diseases from a single patient sample. This dissertation has demonstrated that the integration of isothermal amplification, paper-based fluidics, thermally-actuated valves, and compact device design could facilitate affordable, simple, and sensitive disease-agnostic detection platforms. First, we presented the feasibility of extracting and detecting viral RNA from two respiratory diseases from a single nasal swab in the automated multiplexed UbiNAAT device. Next, we discussed innovations in thermally-actuated valve design that supports low-cost and reliable fluidic control for point-of-care implementation. We then showed the UbiNAAT's adaptability for disease-agnostic application by detecting *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) from clinical vaginal swab samples, while discussing strategies for incorporating additional sample processing steps. Finally, we described a point-of-care device approach for enabling sensitive detection of HIV viral load from fingerstick blood samples through the integration of multiple, complex sample procedures. In summary, the findings for both assay optimization and device integration presented here facilitates multiplexed detection of infectious diseases from patient samples and mark an important step for future translation of affordable, disease-agnostic diagnostic technologies.

7.1 Impact

While considerable progress has been made in point-of-care diagnostics over the course of the COVID-19 pandemic, there remains a critical need for affordable, accessible, simple-to-use, multiplexed detection platforms that could be implemented in low resource or home environments. Many academic groups and companies have developed sophisticated systems that incorporate

well-studied and validated assays, such as lateral flow tests, NAATs, and other approaches. However, the feasibility of these platforms in low-resource or home-based testing remains limited owing to factors such as test sensitivity, complexity, high cost, or need on trained personnel. The next-generation of point-of-care diagnostics must be facilitated by advancements in assay sensitivity, fluidic control, and incorporation of complex sample processing protocols through low-cost, simple approaches.

This dissertation explores many of the current critical limitations in point-of-care diagnostic development and presents integrated technologies that enable accurate, simple, multiplexed disease detection. Through simple fabrication techniques and affordable materials, we have demonstrated complex fluidics control in paper-based system, accurate detection capabilities through lyophilized isothermal amplification, and complex sample processing that require minimal user intervention. Additionally, we present novel methods of enabling storage of RT-LAMP reagents in porous matrices, integrated sample processing, and multiplexable technologies that could readily be expanded up to 8-plex arrays or more. Through working with different samples, we describe strategies for integrating additional sample treatment of both clinical samples and materials for adaptation to different diseases and assay types. While fully integrated devices were developed for testing both contrived and clinical patient samples across multiple types of infectious diseases, the approaches and innovation discussed here could facilitate the characterization and optimization of other assays, mechanics, and hardware designs for acute diagnostics or chronic illness screening. I anticipate that these contributions will provide both toolkits and guidance for enabling advancements in future point-of-care diagnostic capabilities and commercial translations.

7.2 Open-Ended Questions

There remain many open-ended questions that should be considered in future development of assays, materials, and integrated systems. These questions include, but are not limited to:

- How can we improve assay sensitivity in point-of-care systems while still enabling ambient storage and automated operation?
- What is the best approach for addressing assay inhibitors in different human sample types?
- Could porous matrices be improved in fluidics, assay performance, and target recovery through surface treatments, or is there a better material that could outperform in all of these categories?
- How does lyophilized chemical reagents in porous matrices get spatially distributed across the membrane, and could this be leveraged for better assay efficacy and sensitivity?
- What is the best approach to facilitate large-scale testing in future epidemics or pandemics, and how could home-based point-of-care testing improve to prevent the next pandemic?

Exploration of these questions and more could significantly impact not only how we build future pathogen screening tools but also how such tools could be optimally used in future public health emergencies.

7.3 Future Directions

In future work, assay sensitivity and porous matrix treatment should be further optimized for device testing in clinical setting. While the use of fluorescent intercalating dyes and cell phone readers is viable for point-of-care applications, the development of either an integrated hub

housing heating and optical units or adaptable optical filter housing for different phone types could make operating the UbiNAAT more readily viable. While the UbiNAAT has enabled accurate, simple, and multiplexed detection of infectious diseases, its current design could be further simplified for easier assembly. The incorporation of additional sample processing steps will also require incorporations of additional valves and customization of the printed circuit board. The spatial distribution of chemical reagents across paper membranes during lyophilization, blocking, and other treatments should be studied to better understand reagent stability, uniformity, and performance. As assays and device become more compact, digital amplification techniques should be explored for enhancing sensitivity and accuracy. Data analytical approaches via software should readily adapt in evaluating different materials, biomarkers, and visualization methods. I anticipate that these future directions will provide a better understanding of how to overcome current point-of-care assay limitations and facilitate next-generation diagnostics innovations.

8. References

1. Organization WH. Global spending on health: coping with the pandemic. World Health Organization; 2024. 86 p.
2. Del Riccio M, Caini S, Bonaccorsi G, Lorini C, Paget J, van der Velden K, et al. Global analysis of respiratory viral circulation and timing of epidemics in the pre-COVID-19 and COVID-19 pandemic eras, based on data from the Global Influenza Surveillance and Response System (GISRS). *International Journal of Infectious Diseases*. 2024 Jul 1;144:107052.

3. Lazarus JV, White TM, Wyka K, Ratzan SC, Rabin K, Larson HJ, et al. Influence of COVID-19 on trust in routine immunization, health information sources and pandemic preparedness in 23 countries in 2023. *Nat Med*. 2024 Jun;30(6):1559–63.
4. Senevirathne TH, Wekking D, Swain JWR, Solinas C, De Silva P. COVID-19: From emerging variants to vaccination. *Cytokine & Growth Factor Reviews*. 2024 Apr 1;76:127–41.
5. Rathnayake D, Clarke M, Jayasinghe VI. Health system performance and health system preparedness for the post-pandemic impact of COVID-19: A review. *International Journal of Healthcare Management*. 2021 Jan 2;14(1):250–4.
6. Vizheh M, Qorbani M, Arzaghi SM, Muhidin S, Javanmard Z, Esmaeili M. The mental health of healthcare workers in the COVID-19 pandemic: A systematic review. *J Diabetes Metab Disord*. 2020 Dec 1;19(2):1967–78.
7. Liyanage S, Saqib K, Khan AF, Thobani TR, Tang WC, Chiarot CB, et al. Prevalence of Anxiety in University Students during the COVID-19 Pandemic: A Systematic Review. *International Journal of Environmental Research and Public Health*. 2022 Jan;19(1):62.
8. Peeling RW, Sia SK. Lessons from COVID-19 for improving diagnostic access in future pandemics. *Lab Chip*. 2023 Mar 1;23(5):1376–88.
9. Everitt ML, Tillery A, David MG, Singh N, Borison A, White IM. A critical review of point-of-care diagnostic technologies to combat viral pandemics. *Analytica Chimica Acta*. 2021 Feb 15;1146:184–99.

10. Yelin I, Aharony N, Tamar ES, Argoetti A, Messer E, Berenbaum D, et al. Evaluation of COVID-19 RT-qPCR Test in Multi sample Pools. *Clinical Infectious Diseases*. 2020 Nov 19;71(16):2073–8.
11. Rezaei M, Razavi Bazaz S, Zhand S, Sayyadi N, Jin D, Stewart MP, et al. Point of Care Diagnostics in the Age of COVID-19. *Diagnostics*. 2021 Jan;11(1):9.
12. Bellavitis C, Fisch C, McNaughton RB. COVID-19 and the global venture capital landscape. *Small Bus Econ*. 2022 Oct 1;59(3):781–805.
13. Torreele E, Wolfe D, Kazatchkine M, Sall A, Ruxrungtham K, Fitchett JRA, et al. From private incentives to public health need: rethinking research and development for pandemic preparedness. *The Lancet Global Health*. 2023 Oct 1;11(10):e1658–66.
14. Budd J, Miller BS, Weckman NE, Cherkaoui D, Huang D, Decruz AT, et al. Lateral flow test engineering and lessons learned from COVID-19. *Nat Rev Bioeng*. 2023 Jan;1(1):13–31.
15. Hsiao WWW, Le TN, Pham DM, Ko HH, Chang HC, Lee CC, et al. Recent Advances in Novel Lateral Flow Technologies for Detection of COVID-19. *Biosensors*. 2021 Sep;11(9):295.
16. Perchetti GA, Huang ML, Mills MG, Jerome KR, Greninger AL. Analytical sensitivity of the Abbott BinaxNOW COVID-19 ag card. *Journal of Clinical Microbiology*. 2021;59(3).
17. Jean S, Burnham CAD, Chapin K, Garner OB, Pant Pai N, Turabelidze G, et al. At-Home Testing for Infectious Diseases: The Laboratory Where You Live. *Clinical Chemistry*. 2022 Jan 1;68(1):19–26.

18. Kost GJ. Home Antigen Test Recall Affects Millions: Beware False Positives, but Also Uncertainty and Potential False Negatives. *Archives of Pathology and Laboratory Medicine*. 2022;146(4).
19. Moran A, Beavis KG, Matushek SM, Ciaglia C, Francois N, Tesic V, et al. Detection of SARS-CoV-2 by use of the Cepheid Xpert Xpress SARS-CoV-2 and Roche Cobas SARS-CoV-2 assays. *Journal of Clinical Microbiology*. 2020;58(8).
20. Serei VD, Cristelli R, Joho K, Salaru G, Kirn T, Carayannopoulous MO, et al. Comparison of Abbott ID NOW COVID-19 rapid molecular assay to Cepheid Xpert Xpress SARS-CoV-2 assay in dry nasal swabs. *Diagnostic Microbiology and Infectious Disease*. 2021;99(4).
21. Katzman BM, Wockenfus AM, Kelley BR, Karon BS, Donato LJ. Evaluation of the Visby medical COVID-19 point of care nucleic acid amplification test. *Clinical Biochemistry*. 2021;
22. Renzoni A, Perez F, Ngo Nsoga MT, Yerly S, Boehm E, Gayet-Ageron A, et al. Analytical evaluation of Visby Medical rt-PCF portable device for rapid detection of SARS-CoV-2 *Diagnostics*. 2021;11(5).
23. Qian J, Boswell SA, Chidley C, Lu Z Xiang, Pettit ME, Gaudio BL, et al. An enhanced isothermal amplification assay for viral detection. *Nature Communications*. 2020;11(1).
24. Zahavi M, Rohana H, Azrad M, Shinberg B, Peretz A. Rapid SARS-CoV-2 Detection Using the Lucira™ Check It COVID-19 Test Kit. *Diagnostics*. 2022 Aug;12(8):1877.

25. Phan T, Cravener Z, McCullough M, Mays A, Gribschaw J, Wells A. Clinical evaluation of the Cue's COVID-19 diagnostic test to detect SARS-CoV-2 in the upper respiratory tract. *Journal of Medical Virology*. 2022;94(8).
26. Health C for D and R. UPDATE: Do Not Use Cue Health's COVID-19 Tests Due to Risk of False Results: FDA Safety Communication. FDA [Internet]. 2024 Oct 16 [cited 2025 Mar 9]; Available from: <https://www.fda.gov/medical-devices/safety-communications/update-do-not-use-cue-healths-covid-19-tests-due-risk-false-results-fda-safety-communication>
27. Sockrider M, Jamil S, Santhosh L, Carlos WG. COVID-19 Infection versus Influenza (Flu) and Other Respiratory Illnesses. *Am J Respir Crit Care Med*. 2020 Nov 15;202(10):P27–8.
28. Angeletti PM, Marchi S, Trombetta CM, Altobelli E. Flu vaccine administration in the period before SARS-CoV-2 infection and its outcomes: An umbrella review. *Preventive Medicine Reports*. 2024 Feb 1;38:102575.
29. Medical V. Visby Medical™ Receives FDA Clearance and CLIA Waiver for Point-of-Care Respiratory Health Test [Internet]. [cited 2025 Mar 8]. Available from: <https://www.prnewswire.com/news-releases/visby-medical-receives-fda-clearance-and-clia-waiver-for-point-of-care-respiratory-health-test-302385373.html>
30. Christensen K, Ren H, Chen S, Cooper CK, Young S. Clinical Evaluation of BD Veritor SARS-CoV-2 and Flu A+B Assay for Point-Of-Care System. *Microbiology Spectrum*. 2022;10(2).

31. Soong R, Bradford E, Yeh KB, Olinger Jr G. Using a Target Product Profile (TPP) to guide innovation for future In Vitro Diagnostic (IVD) developments in the United States. *Global Security: Health, Science and Policy*. 2023 Dec 31;8(1):2244561.
32. Fast, Lower Cost, Environmentally Friendly Viral Testing Device | Seed [Internet]. [cited 2025 Mar 9]. Available from: <https://seed.nih.gov/portfolio/stories/Anavasi>
33. Jiang KP, Bennett S, Heiniger EK, Kumar S, Yager P. UbiNAAT: a multiplexed point-of-care nucleic acid diagnostic platform for rapid at-home pathogen detection. *Lab Chip*. 2024 Jan 30;24(3):492–504.
34. Liang LL, Tseng CH, Ho HJ, Wu CY. Covid-19 mortality is negatively associated with test number and government effectiveness. *Scientific Reports*. 2020;10(1).
35. Weissleder R, Lee H, Ko J, Pittet MJ. COVID-19 diagnostics in context. *Science Translational Medicine*. 2020 Jun 3;12(546).
36. Asci Erkocyigit B, Ozufuklar O, Yardim A, Guler Celik E, Timur S. Biomarker Detection in Early Diagnosis of Cancer: Recent Achievements in Point-of-Care Devices Based on Paper Microfluidics. *Biosensors*. 2023 Mar;13(3):387.
37. Mercer TR, Salit M. Testing at scale during the COVID-19 pandemic. *Nature Reviews Genetics*. 2021;22(7).
38. Madariaga A, McMullen M, Sheikh S, Kumar R, Liu FF, Zimmermann C, et al. COVID-19 Testing in Patients with Cancer: Does One Size Fit All? *Clinical Cancer Research*. 2020;26(18).

39. Smyrlaki I, Ekman M, Lentini A, Rufino de Sousa N, Papanicolaou N, Vondracek M, et al. Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. *Nat Commun.* 2020 Sep 23;11(1):4812.
40. Lai CKC, Lam W. Laboratory testing for the diagnosis of COVID-19. *Biochem Biophys Res Commun.* 2021 Jan 29;538:226–30.
41. Smyrlaki I, Ekman M, Lentini A, Rufino de Sousa N, Papanicolaou N, Vondracek M, et al. Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. *Nature Communications.* 2020;11(1).
42. Wu X, Chen Q, Li J, Liu Z. Diagnostic techniques for COVID-19: A mini-review. *Journal of Virological Methods.* 2022;301.
43. Lu R, Wang J, Li M, He J, Wang Y, Dong J, et al. Retrospective quantitative detection of SARS-CoV-2 by digital PCR showing high accuracy for low viral load specimens. *The Journal of Infection in Developing Countries.* 2022 Jan 31;16(01):10–5.
44. Oh H, Ahn H, Tripathi A. A Closer Look into FDA-EUA Approved Diagnostic Techniques of Covid-19. *ACS Infect Dis.* 2021 Oct 8;7(10):2787–800.
45. Bustin S, Mueller R, Shipley G, Nolan T. Covid-19 and diagnostic testing for SARS-CoV-2 by RT-qPCR—facts and fallacies. *International Journal of Molecular Sciences.* 2021;22(5).
46. Khandker SS, Nik Hashim NHH, Deris ZZ, Shueb RH, Islam MA. Diagnostic Accuracy of Rapid Antigen Test Kits for Detecting SARS-CoV-2: A Systematic Review and Meta-

Analysis of 17,171 Suspected COVID-19 Patients. *Journal of Clinical Medicine*. 2021 Jan;10(16):3493.

47. Wagenhäuser I, Knies K, Hofmann D, Rauschenberger V, Eisenmann M, Reusch J, et al. Virus variant–specific clinical performance of SARS coronavirus two rapid antigen tests in point-of-care use, from November 2020 to January 2022. *Clinical Microbiology and Infection*. 2023 Feb 1;29(2):225–32.
48. Johnson-Arbor K, Reid N, Smolinske S. Human toxicity from COVID-19 rapid home test kits. *American Journal of Emergency Medicine*. 2022;57.
49. Bracaglia S, Ranallo S, Plaxco KW, Ricci F. Programmable, Multiplexed DNA Circuits Supporting Clinically Relevant, Electrochemical Antibody Detection. *ACS Sens*. 2021 Jun 25;6(6):2442–8.
50. Huang Z, Tian D, Liu Y, Lin Z, Lyon CJ, Lai W, et al. Ultra-sensitive and high-throughput CRISPR-powered COVID-19 diagnosis. *Biosensors and Bioelectronics*. 2020 Sep 15;164:112316.
51. Phillips EA, Moehling TJ, Ejendal KFK, Hoilett OS, Byers KM, Basing LA, et al. Microfluidic rapid and autonomous analytical device (microRAAD) to detect HIV from whole blood samples. *Lab on a Chip*. 2019;
52. Kong JE, Wei Q, Tseng D, Zhang J, Pan E, Lewinski M, et al. Highly Stable and Sensitive Nucleic Acid Amplification and Cell-Phone-Based Readout. *ACS Nano*. 2017;

53. Aoki MN, de Oliveira Coelho B, Góes LGB, Minoprio P, Durigon EL, Morello LG, et al. Colorimetric RT-LAMP SARS-CoV-2 diagnostic sensitivity relies on color interpretation and viral load. *Sci Rep.* 2021 Apr 27;11(1):9026.
54. Inaba M, Higashimoto Y, Toyama Y, Horiguchi T, Hibino M, Iwata M, et al. Diagnostic accuracy of LAMP versus PCR over the course of SARS-CoV-2 infection. *Int J Infect Dis.* 2021 Jun;107:195–200.
55. Tsang HF, Leung WMS, Chan LWC, Cho WCS, Wong SCC. Performance comparison of the Cobas® Liat® and Cepheid® GeneXpert® systems on SARS-CoV-2 detection in nasopharyngeal swab and posterior oropharyngeal saliva. *Expert Review of Molecular Diagnostics.* 2021 May 4;21(5):515–8.
56. CLIawaived Inc. [Internet]. [cited 2023 Oct 18]. COVID-19. Available from: <https://cliawaived.com/covid-19-related-supplies.html>
57. Crozier A, Rajan S, Buchan I, McKee M. Put to the test: Use of rapid testing technologies for Covid-19. *The BMJ.* 2021;372.
58. Liang T, Robinson R, Houghtaling J, Fridley G, Ramsey SA, Fu E. Investigation of Reagent Delivery Formats in a Multivalent Malaria Sandwich Immunoassay and Implications for Assay Performance. *Anal Chem.* 2016 Feb 16;88(4):2311–20.
59. Bradbury DW, Azimi M, Diaz AJ, Pan AA, Falktoft CH, Wu BM, et al. Automation of Biomarker Preconcentration, Capture, and Nanozyme Signal Enhancement on Paper-Based Devices. *Anal Chem.* 2019 Sep 17;91(18):12046–54.

60. Jawla J, Kumar RR, Mendiratta SK, Agarwal RK, Singh P, Saxena V, et al. A novel paper based loop mediated isothermal amplification and lateral flow assay (LAMP-LFA) for point-of-care detection of buffalo tissue origin in diverse foods. *Journal of Food Safety*. 2023;43(3):e13038.
61. Scherr TF, Gupta S, Wright DW, Haselton FR. An embedded barcode for “connected” malaria rapid diagnostic tests. *Lab Chip*. 2017 Mar 29;17(7):1314–22.
62. Magro L, Escadafal C, Garneret P, Jacquelin B, Kwasiborski A, Manuguerra JC, et al. Paper microfluidics for nucleic acid amplification testing (NAAT) of infectious diseases. *Lab Chip*. 2017 Jul 11;17(14):2347–71.
63. Kumar S, Gallagher R, Bishop J, Kline E, Buser J, Lafleur L, et al. Long-term dry storage of enzyme-based reagents for isothermal nucleic acid amplification in a porous matrix for use in point-of-care diagnostic devices. *Analyst*. 2020 Oct 26;145(21):6875–86.
64. Damhorst GL, Duarte-Guevara C, Chen W, Ghonge T, Cunningham BT, Bashir R. Smartphone-Imaged HIV-1 Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) on a Chip from Whole Blood. *Engineering*. 2015 Sep 1;1(3):324–35.
65. Hayashida K, Kajino K, Hachaambwa L, Namangala B, Sugimoto C. Direct Blood Dry LAMP: A Rapid, Stable, and Easy Diagnostic Tool for Human African Trypanosomiasis. *PLOS Neglected Tropical Diseases*. 2015 Mar 13;9(3):e0003578.
66. Weil AA, Luiten KG, Casto AM, Bennett JC, O’Hanlon J, Han PD, et al. Genomic surveillance of SARS-CoV-2 Omicron variants on a university campus. *Nat Commun*. 2022 Sep 6;13(1):5240.

67. Clarke E, Robinson R, Laurentius LB, Porter MD. Proteinase K Pretreatment for the Quantitative Recovery and Sensitive Detection of the Tuberculosis Biomarker Mannose-Capped Lipoarabinomannan Spiked into Human Serum. *Anal Chem.* 2023 Jun 20;95(24):9191–8.
68. Sritong N, Medeiros MS de, Anthony Basing L, C. Linnes J. Promise and perils of paper-based point-of-care nucleic acid detection for endemic and pandemic pathogens. *Lab on a Chip.* 2023;23(5):888–912.
69. Delgado-Diaz DJ, Sakthivel D, Nguyen HHT, Farrokzhad K, Hopper W, Narh CA, et al. Strategies That Facilitate Extraction-Free SARS-CoV-2 Nucleic Acid Amplification Tests. *Viruses.* 2022 Jun;14(6):1311.
70. Kline EC, Panpradist N, Hull IT, Wang Q, Oreskovic AK, Han PD, et al. Multiplex Target-Redundant RT-LAMP for Robust Detection of SARS-CoV-2 Using Fluorescent Universal Displacement Probes. *Microbiology Spectrum.* 2022 Jun 16;10(4):e01583-21.
71. Dong Y, Zhao Y, Li S, Wan Z, Lu R, Yang X, et al. Multiplex, Real-Time, Point-of-care RT-LAMP for SARS-CoV-2 Detection Using the HFman Probe. *ACS Sens.* 2022 Mar 25;7(3):730–9.
72. Nixon M, Outlaw F, Leung TS. Accurate device-independent colorimetric measurements using smartphones. *PLoS One.* 2020 Mar 26;15(3):e0230561.
73. Lin T, Lin D, Hou L. 4 - Fluorescence measurements, imaging and counting by a smartphone. In: Hussain C, editor. *Smartphone-Based Detection Devices* [Internet]. Elsevier;

2021 [cited 2023 Jul 14]. p. 57–72. Available from:

<https://www.sciencedirect.com/science/article/pii/B978012823696300012X>

74. Wood CS, Thomas MR, Budd J, Mashamba-Thompson TP, Herbst K, Pillay D, et al. Taking connected mobile-health diagnostics of infectious diseases to the field. *Nature*. 2019 Feb;566(7745):467–74.
75. Baker RE, Park SW, Yang W, Vecchi GA, Jessica C, Grenfell BT. The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. *Proceedings of the National Academy of Sciences of the United States of America*. 2020;117(48).
76. Lafleur LK, Bishop JD, Heiniger EK, Gallagher RP, Wheeler MD, Kauffman P, et al. A rapid, instrument-free, sample-to-result nucleic acid amplification test. *Lab Chip*. 2016 Sep 21;16(19):3777–87.
77. Shah KG, Yager P. Wavelengths and Lifetimes of Paper Autofluorescence: A Simple Substrate Screening Process to Enhance the Sensitivity of Fluorescence-Based Assays in Paper. *Analytical Chemistry*. 2017;89(22).
78. Shah KG, Singh V, Kauffman PC, Abe K, Yager P. Mobile Phone Ratiometric Imaging Enables Highly Sensitive Fluorescence Lateral Flow Immunoassays without External Optical Filters. *Analytical Chemistry*. 2018;90(11).
79. Shah KG, Kumar S, Singh V, Hansen L, Heiniger E, Bishop JD, et al. Two-Fluorophore Mobile Phone Imaging of Bplexed Real-Time NAATs Overcomes Optical Artifacts in Highly Scattering Porous Media. *Analytical Chemistry*. 2020;92(19).

80. Shah KG, Roller M, Kumar S, Bennett S, Heiniger E, Looney K, et al. Disposable platform for bacterial lysis and nucleic acid amplification based on a single USB-powered printed circuit board. *PLOS ONE*. 2023 Apr 26;18(4):e0284424.
81. Shah KG, Kumar S, Yager P. Near-digital amplification in paper improves sensitivity and speed in biplexed reactions. *Sci Rep*. 2022 Aug 26;12(1):14618.
82. Natsuhara D, Saito R, Aonuma H, Sakurai T, Okamoto S, Nagai M, et al. A method of sequential liquid dispensing for the multiplexed genetic diagnosis of viral infections in a microfluidic device. *Lab on a Chip*. 2021;21(24):4779–90.
83. Kim H, Chung DR, Kang M. A new point-of-care test for the diagnosis of infectious diseases based on multiplex lateral flow immunoassays. *Analyst*. 2019;144(8):2460–6.
84. Song M, Hong S, Lee LP. Multiplexed Ultrasensitive Sample-to-Answer RT-LAMP Chip for the Identification of SARS-CoV-2 and Influenza Viruses. *Advanced Materials*. 2023;35(10):2207138.
85. Myhrvold C, Freije CA, Gootenberg JS, Abudayyeh OO, Metsky HC, Durbin AF, et al. Field-deployable viral diagnostics using CRISPR-Cas13. *Science*. 2018 Apr 27;360(6387):444–8.
86. Rabe BA, Cepko C. SARS-CoV-2 detection using isothermal amplification and a rapid, inexpensive protocol for sample inactivation and purification. *Proceedings of the National Academy of Sciences*. 2020 Sep 29;117(39):24450–8.

87. Nakauchi M, Yoshikawa T, Nakai H, Sugata K, Yoshikawa A, Asano Y, et al. Evaluation of reverse transcription loop-mediated isothermal amplification assays for rapid diagnosis of pandemic influenza A/H1N1 2009 virus. *Journal of Medical Virology*. 2011;83(1):10–5.
88. Zhang Y, Tanner NA. Development of multiplexed RT-LAMP for detection of SARS-CoV-2 and influenza viral RNA. *medRxiv*. 2020;
89. Broughton JP, Deng X, Yu G, Fasching CL, Singh J, Streithorst J, et al. Rapid Detection of 2019 Novel Coronavirus SARS-CoV-2 Using a CRISPR-based DETECTR Lateral Flow Assay. *medRxiv*. 2020 Mar 27;2020.03.06.20032334.
90. Mahony J, Chong S, Bulir D, Ruyter A, Mwawasi K, Waltho D. Development of a Sensitive Loop-Mediated Isothermal Amplification Assay That Provides Specimen-to-Result Diagnosis of Respiratory Syncytial Virus Infection in 30 Minutes. *Journal of Clinical Microbiology*. 2020 Dec 21;51(8):2696–701.
91. Hartigan DR, Adelfio M, Shutt ME, Jones SM, Patel S, Marchand JT, et al. In Vitro Nasal Tissue Model for the Validation of Nasopharyngeal and Midturbinate Swabs for SARS-CoV-2 Testing. *ACS Omega*. 2022 Apr 12;7(14):12193–201.
92. Ju T. Working Principle and Applications of Active and Passive Microfluidic Valves. *J Phys: Conf Ser*. 2022 Mar;2230(1):012013.
93. Lakowicz JR, Masters BR. *Principles of Fluorescence Spectroscopy*, Third Edition. *J Biomed Opt*. 2008;13(2):029901.

94. Noviana E, Ozer T, Carrell CS, Link JS, McMahon C, Jang I, et al. Microfluidic Paper-Based Analytical Devices: From Design to Applications. *Chem Rev.* 2021 Oct 13;121(19):11835–85.
95. Anushka, Bandopadhyay A, Das PK. Paper based microfluidic devices: a review of fabrication techniques and applications. *Eur Phys J Spec Top.* 2023 Jun 1;232(6):781–815.
96. Hou Y, Lv CC, Guo YL, Ma XH, Liu W, Jin Y, et al. Recent Advances and Applications in Paper-Based Devices for Point-of-Care Testing. *J Anal Test.* 2022 Sep 1;6(3):247–73.
97. Tseng CC, Kung CT, Chen RF, Tsai MH, Chao HR, Wang YN, et al. Recent advances in microfluidic paper-based assay devices for diagnosis of human diseases using saliva, tears and sweat samples. *Sensors and Actuators B: Chemical.* 2021 Sep 1;342:130078.
98. Yang SM, Lv S, Zhang W, Cui Y. Microfluidic Point-of-Care (POC) Devices in Early Diagnosis: A Review of Opportunities and Challenges. *Sensors.* 2022 Jan;22(4):1620.
99. Modha S, Castro C, Tsutsui H. Recent developments in flow modeling and fluid control for paper-based microfluidic biosensors. *Biosensors and Bioelectronics.* 2021 Apr 15;178:113026.
100. Chen C, Meng H, Guo T, Deshpande S, Chen H. Development of Paper Microfluidics with 3D-Printed PDMS Barriers for Flow Control. *ACS Appl Mater Interfaces.* 2022 Sep 7;14(35):40286–96.

101. Liu Y, Shen H, Yang X, Kang S, Cai L, Tian T, et al. Recent progress in microfluidic biosensors with different driving forces. *TrAC Trends in Analytical Chemistry*. 2023 Jan 1;158:116894.
102. Di Nardo F, Chiarello M, Cavallera S, Baggiani C, Anfossi L. Ten Years of Lateral Flow Immunoassay Technique Applications: Trends, Challenges and Future Perspectives. *Sensors*. 2021 Jan;21(15):5185.
103. Sachdeva S, Davis RW, Saha AK. Microfluidic Point-of-Care Testing: Commercial Landscape and Future Directions. *Front Bioeng Biotechnol* [Internet]. 2021 Jan 15 [cited 2025 Mar 4];8. Available from: <https://www.frontiersin.org/journals/bioengineering-and-biotechnology/articles/10.3389/fbioe.2020.602659/full>
104. Liu Y, Zhan L, Qin Z, Sackrison J, Bischof JC. Ultrasensitive and Highly Specific Lateral Flow Assays for Point-of-Care Diagnosis. *ACS Nano*. 2021 Mar 23;15(3):3593–611.
105. Boehringer HR, O'Farrell BJ. Lateral Flow Assays in Infectious Disease Diagnosis. *Clinical Chemistry*. 2022 Jan 1;68(1):52–8.
106. Ardekani LS, Thulstrup PW. Gold Nanoparticle-Mediated Lateral Flow Assays for Detection of Host Antibodies and COVID-19 Proteins. *Nanomaterials*. 2022 Jan;12(9):1456.
107. Kahn M, Schuierer L, Bartenschlager C, Zellmer S, Frey R, Freitag M, et al. Performance of antigen testing for diagnosis of COVID-19: a direct comparison of a lateral flow device to nucleic acid amplification based tests. *BMC Infect Dis*. 2021 Aug 10;21(1):798.

108. Deroco PB, Wachholz Junior D, Kubota LT. Paper-based Wearable Electrochemical Sensors: A New Generation of Analytical Devices. *Electroanalysis*. 2023;35(1):e202200177.
109. Ataide VN, Mendes LF, Gama LILM, Araujo WR de, Paixão TRLC. Electrochemical paper-based analytical devices: ten years of development. *Anal Methods*. 2020 Feb 27;12(8):1030–54.
110. Shi D, Zhang C, Li X, Yuan J. An electrochemical paper-based hydrogel immunosensor to monitor serum cytokine for predicting the severity of COVID-19 patients. *Biosensors and Bioelectronics*. 2023 Jan 15;220:114898.
111. Ehsan MA, Khan SA, Rehman A. Screen-Printed Graphene/Carbon Electrodes on Paper Substrates as Impedance Sensors for Detection of Coronavirus in Nasopharyngeal Fluid Samples. *Diagnostics*. 2021 Jun;11(6):1030.
112. Prasad KS, Cao X, Gao N, Jin Q, Sanjay ST, Henao-Pabon G, et al. A low-cost nanomaterial-based electrochemical immunosensor on paper for high-sensitivity early detection of pancreatic cancer. *Sensors and Actuators B: Chemical*. 2020 Feb 15;305:127516.
113. Boonkaew S, Jang I, Noviana E, Siangproh W, Chailapakul O, Henry CS. Electrochemical paper-based analytical device for multiplexed, point-of-care detection of cardiovascular disease biomarkers. *Sensors and Actuators B: Chemical*. 2021 Mar 1;330:129336.
114. Yu CY, Chan KG, Yean CY, Ang GY. Nucleic Acid-Based Diagnostic Tests for the Detection SARS-CoV-2: An Update. *Diagnostics*. 2021 Jan;11(1):53.

115. A. Phillips E, Shen R, Zhao S, C. Linnes J. Thermally actuated wax valves for paper-fluidic diagnostics. *Lab on a Chip*. 2016;16(21):4230–6.
116. Kim TH, Hahn YK, Lee J, van Noort D, Kim MS. Solenoid Driven Pressure Valve System: Toward Versatile Fluidic Control in Paper Microfluidics. *Anal Chem*. 2018 Feb 20;90(4):2534–41.
117. Iwasaki W, Toda H, Morita N, Motomura T, Fujio Y, Takemura K, et al. A thermoresponsive valve to control fluid flow in microfluidic paper-based devices. *Microfluid Nanofluid*. 2022 May 30;26(6):47.
118. Adedokun G, Sidhu G, Alipanah M, Wang GP, Fan ZH. A handheld HIV detection platform using paper-based sample preparation and real-time isothermal amplification. *Microsyst Nanoeng*. 2024 Nov 29;10(1):1–11.
119. Global health sector strategies on HIV, viral hepatitis and sexually transmitted infections for the period 2022-2030 [Internet]. [cited 2025 Mar 9]. Available from: <https://www.who.int/publications/i/item/9789240053779>
120. Muljadi M, Cheng CM, Yang CY, Chang TC, Shen CJ. A pilot clinical validation study of a self-collected vaginal swab device for the detection of chlamydia trachomatis in women. *Front Bioeng Biotechnol* [Internet]. 2022 Oct 4 [cited 2025 Mar 9];10. Available from: <https://www.frontiersin.org/journals/bioengineering-and-biotechnology/articles/10.3389/fbioe.2022.1008761/full>

121. Tang W, Mao J, Li KT, Walker JS, Chou R, Fu R, et al. Pregnancy and fertility-related adverse outcomes associated with *Chlamydia trachomatis* infection: a global systematic review and meta-analysis. *Sex Transm Infect.* 2020 Aug 1;96(5):322–9.
122. Malekinejad M, Barker EK, Merai R, Lyles CM, Bernstein KT, Sipe TA, et al. Risk of HIV Acquisition Among Men Who Have Sex With Men Infected With Bacterial Sexually Transmitted Infections: A Systematic Review and Meta-Analysis. *Sexually Transmitted Diseases.* 2021 Oct;48(10):e138.
123. Ewers EC, Curtin JM, Ganesan A. Challenges in Managing Gonorrhea and New Advances in Prevention. *Infectious Disease Clinics of North America.* 2023 Jun 1;37(2):223–43.
124. Cyr SS. Update to CDC’s Treatment Guidelines for Gonococcal Infection, 2020. *MMWR Morb Mortal Wkly Rep* [Internet]. 2020 [cited 2025 Mar 9];69. Available from: <https://www.cdc.gov/mmwr/volumes/69/wr/mm6950a6.htm>
125. Hull S, Kelley S, Clarke JL. Sexually Transmitted Infections: Compelling Case for an Improved Screening Strategy. *Population Health Management.* 2017 Sep;20(S1):S-1.
126. Tuddenham S, Hamill MM, Ghanem KG. Diagnosis and Treatment of Sexually Transmitted Infections: A Review. *JAMA.* 2022 Jan 11;327(2):161–72.
127. Jue E, Schoepp NG, Witters D, Ismagilov RF. Evaluating 3D printing to solve the sample-to-device interface for LRS and POC diagnostics: example of an interlock meter-mix device for metering and lysing clinical urine samples. *Lab Chip.* 2016 May 10;16(10):1852–60.

128. Aaron KJ, Griner S, Footman A, Boutwell A, Pol BVD. Vaginal Swab vs Urine for Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*: A Meta-Analysis. *The Annals of Family Medicine*. 2023 Mar 1;21(2):172–9.
129. Masek BJ, Arora N, Quinn N, Aumakhan B, Holden J, Hardick A, et al. Performance of Three Nucleic Acid Amplification Tests for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by Use of Self-Collected Vaginal Swabs Obtained via an Internet-Based Screening Program. *Journal of Clinical Microbiology*. 2009 Jun;47(6):1663–7.
130. Knox J, Tabrizi SN, Miller P, Petoumenos K, Law M, Chen S, et al. Evaluation of Self-Collected Samples in Contrast to Practitioner-Collected Samples for Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* by Polymerase Chain Reaction Among Women Living in Remote Areas. *Sexually Transmitted Diseases*. 2002 Nov;29(11):647.
131. Vialard F, Anand A, Leung Soo C, de Waal A, McGuire M, Carmona S, et al. Self-sampling strategies (with/without digital innovations) in populations at risk of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: a systematic review and meta-analyses. *Sex Transm Infect*. 2023 Sep;99(6):420–8.
132. Brook G. The performance of non-NAAT point-of-care (POC) tests and rapid NAAT tests for chlamydia and gonorrhoea infections. An assessment of currently available assays. *Sex Transm Infect*. 2015 Dec 1;91(8):539–44.

133. Wingrove I, McOwan A, Nwokolo N, Whitlock G. Diagnostics within the clinic to test for gonorrhoea and chlamydia reduces the time to treatment: a service evaluation. *Sex Transm Infect.* 2014 Sep 1;90(6):474–474.
134. Huntington S, Weston G, Adams E. Assessing the clinical impact and resource use of a 30-minute chlamydia and gonorrhoea point-of-care test at three sexual health services. *Ther Adv Infect Dis.* 2021 Dec 2;8:20499361211061645.
135. Morris SR, Bristow CC, Wierzbicki MR, Sarno M, Asbel L, French A, et al. A Cross Sectional Study of Performance of a single use rapid point-of-care PCR device for the detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis*. *Lancet Infect Dis.* 2021 May;21(5):668–76.
136. Zhou Y, Jiang TT, Li J, Yin YP, Chen XS. Performance of point-of-care tests for the detection of chlamydia trachomatis infections: A systematic review and meta-analysis. *EClinicalMedicine.* 2021 Jun 18;37:100961.
137. Cannon CA, Piraino AK, Golden MR, Barbee LA. Sexually Transmitted Infection Testing Using Online Companies: Benefits, Drawbacks, and Call for Official Guidance. *Sexually Transmitted Diseases.* 2021 Nov;48(11):e168.
138. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 2000 Jun 15;28(12):e63.
139. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes.* 2002 Jun 1;16(3):223–9.

140. Francois P, Tangomo M, Hibbs J, Bonetti EJ, Boehme CC, Notomi T, et al. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology & Medical Microbiology*. 2011 Jun 1;62(1):41–8.
141. Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus. *Journal of Clinical Microbiology*. 2004 Jan;42(1):257–63.
142. Sen S, Bhowmik P, Tiwari S, Peleg Y, Bandyopadhyay B. Versatility of reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) from diagnosis of early pathological infection to mutation detection in organisms. *Mol Biol Rep*. 2024 Jan 25;51(1):211.
143. Mostafa M, Barhoum A, Sehit E, Gewaid H, Mostafa E, Omran MM, et al. Current trends in COVID-19 diagnosis and its new variants in physiological fluids: Surface antigens, antibodies, nucleic acids, and RNA sequencing. *TrAC Trends in Analytical Chemistry*. 2022 Dec 1;157:116750.
144. Ngoc LTN, Lee YC. Current Trends in RNA Virus Detection via Nucleic Acid Isothermal Amplification-Based Platforms. *Biosensors*. 2024 Feb;14(2):97.
145. Wang Y, Chen J, Yang Z, Wang X, Zhang Y, Chen M, et al. Advances in Nucleic Acid Assays for Infectious Disease: The Role of Microfluidic Technology. *Molecules*. 2024 Jan;29(11):2417.

146. Goto M, Honda E, Ogura A, Nomoto A, Hanaki KI. Colorimetric Detection of Loop-Mediated Isothermal Amplification Reaction by Using Hydroxy Naphthol Blue. *BioTechniques*. 2009 Mar 1;46(3):167–72.
147. Ito A, Ueno K. Successive chelatometric titration of calcium and magnesium using Hydroxy Naphthol Blue (HNB) indicator. *Bunseki kagaku*. 1970;19(3):393–7.
148. Berry L, Stanley B. Comparison of self-collected meatal swabs with urine specimens for the diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in men. *Journal of Medical Microbiology*. 2017;66(2):134–6.
149. Turner KM, Christensen H, Adams EJ, McAdams D, Fifer H, McDonnell A, et al. Analysis of the potential for point-of-care test to enable individualised treatment of infections caused by antimicrobial-resistant and susceptible strains of *Neisseria gonorrhoeae*: a modelling study. *BMJ Open*. 2017 Jun 14;7(6):e015447.
150. Hjelmevoll SO, Olsen ME, Sollid JUE, Haaheim H, Unemo M, Skogen V. A Fast Real-Time Polymerase Chain Reaction Method for Sensitive and Specific Detection of the *Neisseria gonorrhoeae* porA Pseudogene. *J Mol Diagn*. 2006 Nov;8(5):574–81.
151. Dhawan B, Rawre J, Ghosh A, Malhotra N, Ahmed MM, Sreenivas V, et al. Diagnostic efficacy of a real time-PCR assay for *Chlamydia trachomatis* infection in infertile women in north India. *Indian J Med Res*. 2014 Aug;140(2):252–61.
152. Jevtuševskaja J, Uusna J, Andresen L, Krölov K, Laanpere M, Grellier T, et al. Combination with antimicrobial peptide lyses improves loop-mediated isothermal

amplification based method for *Chlamydia trachomatis* detection directly in urine sample.

BMC Infect Dis. 2016 Jul 13;16:329.

153. Liu ML, Xia Y, Wu XZ, Huang JQ, Guo XG. Loop-mediated isothermal amplification of *Neisseria gonorrhoeae* porA pseudogene: a rapid and reliable method to detect gonorrhea.

AMB Express. 2017 Dec;7(1):48.

154. Cowan FM, Shahmanesh M, Revill PA, Busza J, Sibanda EL, Chabata ST, et al.

Preventing HIV in women in Africa. Nat Med. 2025 Feb 13;1–10.

155. Sullivan PS, Johnson AS, Pembleton ES, Stephenson R, Justice AC, Althoff KN, et al.

Epidemiology of HIV in the USA: epidemic burden, inequities, contexts, and responses. The Lancet. 2021 Mar 20;397(10279):1095–106.

156. Cordero Jr DA. Exploring the HIV Epidemic in the Philippines: Initiatives and

Challenges. J Int Assoc Provid AIDS Care. 2025 Jan 1;24:23259582241312294.

157. Harries AD. Ending the TB epidemic – what can we learn from the HIV epidemic?

IJTL D OPEN. 2025 Feb 1;2(2):61–3.

158. Frescura L, Godfrey-Faussett P, Feizzadeh A, El-Sadr W, Syarif O, Ghys PD. Achieving the 95 95 95 targets for all: A pathway to ending AIDS. PLoS One. 2022 Aug

4;17(8):e0272405.

159. Menéndez-Arias L, Delgado R. Update and latest advances in antiretroviral therapy.

Trends in Pharmacological Sciences. 2022 Jan 1;43(1):16–29.

160. McClure M, Gandhi M. Threat of HIV and tuberculosis drug resistance after US funding cuts. *The Lancet Infectious Diseases*. 2025 May 1;25(5):e256–7.
161. Adams S, Luo W, Wesolowski L, Cohen SE, Peters PJ, Owen SM, et al. Performance evaluation of the point-of-care INSTI™ HIV-1/2 antibody test in early and established HIV infections. *Journal of Clinical Virology*. 2017 Jun 1;91:90–4.
162. Human anti-HIV IgM detection by the OraQuick ADVANCE® Rapid HIV 1/2 Antibody Test [PeerJ] [Internet]. [cited 2025 Mar 10]. Available from: <https://peerj.com/articles/4430/>
163. Weld ED. Limits of Detection and Limits of Infection: Quantitative HIV Measurement in the Era of U = U. *The Journal of Applied Laboratory Medicine*. 2021 Jan 12;6(1):324–6.
164. Organization WH. The role of HIV viral suppression in improving individual health and reducing transmission: policy brief. World Health Organization; 2023. 16 p.
165. Broyles LN, Luo R, Boeras D, Vojnov L. The risk of sexual transmission of HIV in individuals with low-level HIV viraemia: a systematic review. *The Lancet*. 2023 Aug 5;402(10400):464–71.
166. Pinar SS, Manak M, Saravanan S, Imami N, Kibirige C. Point-of-care nucleic acid testing – a step forward in controlling the HIV epidemic: A review. *HIV Medicine* [Internet]. [cited 2025 Mar 10];n/a(n/a). Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/hiv.13757>
167. Nyirenda-Nyang'wa M, Manthalu G, Arnold M, Nkhoma D, Hosseinipour MC, Chagomerana M, et al. Costing and cost-effectiveness of Cepheid Xpert HIV -1 Qual Assay

- using whole blood protocol versus PCR by Abbott Systems in Malawi. *J Glob Health Econ Policy*. 2022;2:e2022013.
168. Garrett NJ, Drain P, Werner L, Samsunder N, Karim SSA. Diagnostic Accuracy of the Point-of-care Xpert® HIV-1 Viral Load Assay in a South African HIV clinic. *J Acquir Immune Defic Syndr*. 2016 Jun 1;72(2):e45–8.
 169. Burgos-Flórez F, Rodríguez A, Cervera E, De Ávila M, Sanjuán M, Villalba PJ. Microfluidic Paper-Based Blood Plasma Separation Device as a Potential Tool for Timely Detection of Protein Biomarkers. *Micromachines*. 2022 May;13(5):706.
 170. A. Phillips E, J. Moehling T, K. Ejendal KF, S. Hoilett O, M. Byers K, Anthony Basing L, et al. Microfluidic rapid and autonomous analytical device (microRAAD) to detect HIV from whole blood samples. *Lab on a Chip*. 2019;19(20):3375–86.
 171. Oreskovic A, Lutz BR. Ultrasensitive hybridization capture: Reliable detection of <1 copy/mL short cell-free DNA from large-volume urine samples. *PLOS ONE*. 2021 Feb 26;16(2):e0247851.
 172. Yan AJ, Olson AM, Weigel KM, Luabeya AK, Heiniger E, Hatherill M, et al. Detection of *Mycobacterium tuberculosis* from tongue swabs using sonication and sequence-specific hybridization capture. *PLOS ONE*. 2024 Aug 15;19(8):e0308235.
 173. Curtis KA, Rudolph DL, Owen SM. Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). *J Virol Methods*. 2008 Aug;151(2):264–70.

174. Neshani A, Zare H, Sadeghian H, Safdari H, Riahi-Zanjani B, Aryan E. A Comparative Study on Visual Detection of Mycobacterium tuberculosis by Closed Tube Loop-Mediated Isothermal Amplification: Shedding Light on the Use of Eriochrome Black T. *Diagnostics* (Basel). 2023 Jan 2;13(1):155.
175. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation. *Biochemical and Biophysical Research Communications*. 2001 Nov 23;289(1):150–4.