DISSERTATION

MOLECULAR DIAGNOSTIC PLATFORMS FOR POINT-OF-NEED PATHOGEN DETECTION

Submitted by

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ABSTRACT

MOLECULAR DIAGNOSTIC PLATFORMS FOR POINT-OF-NEED PATHOGEN DETECTION

Rapid, accurate, reliable nucleic acid testing (NAT) platforms are essential in the diagnosis and management of diseases. The inherent complexity associated with NAT requires that such testing be performed in centralized laboratories by highly trained personnel. Modified molecular technologies that can be used at the point-of-care (POC) are needed to improve the turnaround times of results and lower the global burden of infectious diseases. To help address this urgent need, we have developed a nucleic acid sensor platform utilizing nuclease protection and lateral flow detection for rapid, point-of-need nucleic acid analysis. We have also improved the analytical performance of the assay by pairing it with isothermal padlock rolling circle amplification (RCA). RCA is one of the simplest and most versatile isothermal amplification techniques as it only requires one primer and a strand-displacing polymerase. Utilizing our rolling circle amplification lateral flow platform, we have developed assays for beta-lactamase resistance genes for antimicrobial resistance monitoring and severe acute respiratory virus coronavirus 2 (SARS-CoV-2). We have also explored the use of exponential isothermal amplification to further improve the assay limit of detection. We also propose a microfluidic device to rapidly detect the RCA amplicons. The device allows programmable sequential delivery of reagents to a detection region, reducing the number of user steps. With further development, such microfluidic devices can be used to develop fully integrated sample-to-result molecular diagnostic platforms that integrate sample pretreatment, amplification, and detection in an easy-to-use, point-of-need nucleic acid

sensor platform. Chapter 1 presents a brief review of the nucleic acid testing landscape, the challenges associated with the development of point-of-need nucleic acid sensors and recent successes utilizing paper-based devices for fully integrated sample-to-result sensors. Chapters 2 and 3 discuss the development of the nuclease protection lateral flow assay and padlock probe-based rolling circle amplification lateral flow assay. Chapter 4 describes our work on the use of exponential RCA to improve the limit of detection of the SARS-CoV-2 assay. In Chapter 5, we present our work on a paper-plastic microfluidic device for the rapid detection of the RCA amplicon. We believe that such devices can be used for the development of integrated molecular diagnostic sensor platforms that can be used at the point-of-need in resource-limited settings.

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Chapter Overview

Rapid, accurate, reliable nucleic acid testing (NAT) platforms are essential in the diagnosis and management of communicable and non-communicable diseases. The inherent complexity associated with NAT requires that such testing be performed in centralized laboratories by highly trained personnel. Modified molecular technologies that can be used at the point-of-care (POC) are needed to improve the turnaround times of results and lower the global burden of infectious diseases. Typical nucleic acid analysis requires the extraction and preconcentration of nucleic acids from the sample of interest, followed by a target-specific amplification, and downstream detection using fluorescent or colorimetric methods. While several sample-to-result, molecular diagnostic platforms have been proposed, the integration of sample pretreatment, nucleic acid amplification and detection into an affordable, reliable, and easy-to-use POC device remains challenging. This chapter provides an overview of NAT technology focusing on isothermal amplification techniques. We compare the different isothermal amplification methods for their analytical performance, robustness, turnaround times and adaptability for POC use. We conclude with a review of sample-to-result/integrated systems that have been reported to date, focusing on microfluidic and paper-based platforms which provide a low-cost substrate for the development of POC nucleic acid sensors.

INTRODUCTION

Nucleic Acid Testing (NAT)

Nucleic acid testing (NAT) is rapidly replacing conventional culture-based techniques for pathogen detection in the developed world due to their superior analytical performance and speed.[1] However, the cost and complexity of NAT have essentially prohibited their use in low-and-middle income countries (LMIC) where the need for such testing capability is the highest.[1-3] Culture remains the gold standard for pathogen detection in the developing world, which due to its lack of sensitivity and long turnaround time to result, fail to provide actionable diagnostic information in a timely manner.[1-3] In the absence of rapid, accurate and reliable molecular diagnostics patients exhibiting symptoms of infection are diagnosed and treated using symptomology or local prevalence of the disease which complicates patient care and contributes to the spread of anti-microbial resistance.[4-7] Nucleic acid testing is also critical in the diagnosis of non-communicable diseases, such as cancer and genetic diseases, and in monitoring the effectiveness of therapeutics.[8-11] Modified molecular technologies that can be used at the point-of-care (POC) without the need for high-end instrumentation or significant laboratory infrastructure are urgently needed to lower the burden of disease in the developing world.[1, 12-19] NAT involves three main steps, the extraction and preconcentration of nucleic acids from the sample, the amplification of the nucleic acid sequence of interest, and detection using fluorescent or colorimetric methods.[20-26] While there are non-amplification based nucleic acid testing methods such as hybridization protection assays, nucleic acid analytes are typically present at low concentrations in biological samples, requiring an amplification step. While polymerase chain reaction (PCR) has become the gold standard for NAT, the cost and complexity associated with PCR have limited its use to centralized laboratories largely in the developed world. The threat of emerging infectious diseases

along with the rapid spread of antimicrobial resistance have made the need for accessible molecular diagnostics even more urgent and great. Point-of-care molecular diagnostics that can be used in resource-limited settings without significant instrumentation and infrastructure would vastly improve access and speed of diagnosis and help lower the global burden of disease.[1, 2, 17]

Integration of extraction, amplification and detection into an easy to use platform remains one of the biggest hurdles to POC applications of NAT.[27-29] While several devices have been proposed in the literature, only a handful of devices have made it past feasibility phase and into clinical use (shown in Figure 1.1).[27-35] Notable examples of commercially available platforms are GeneXpert® by Cepheid Inc, (Sunnyvale, CA, USA) and Idylla™ by Biocartis NV (Belgium) which integrate sample preparation, PCR and fluorescent detection into a benchtop instrument that can be used in resource-limited settings.[36-40] These instruments have been pivotal in improving access to oncology and infectious disease molecular diagnostics, though the instrument costs range between $20,000 - $25,000 with an additional ~$10/test.[37]

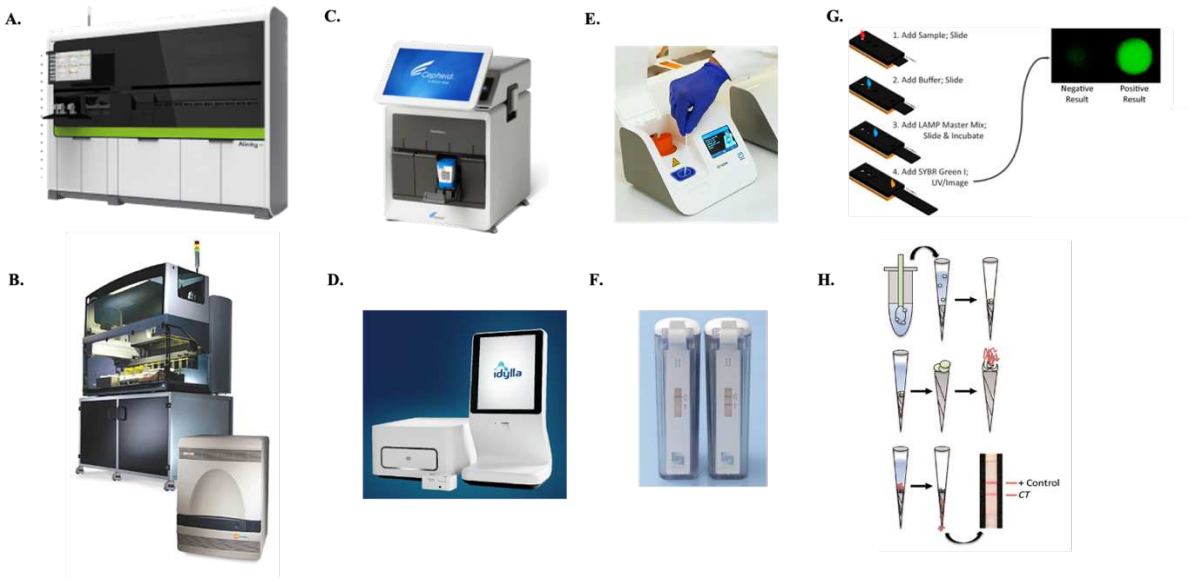

Figure 1.1: Nucleic acid testing platforms A. Alinity m high-throughput molecular diagnostic platform (Abbott laboratories, Chicago IL) B. m2000 real-time system (Abbott laboratories, Chicago IL) C. GeneXpert™ (Cepheid Inc., Sunnyvale CA) PCR-based NAT platform. D. Idylla™ Platform (Biocartis NV, Belgium). E. Abbott ID NOW® for rapid SARS-CoV-2 detection F. BESt™ cassette – Type II (BioHelix Inc., Beverly, CA) G. 'Paper machine' for rapid, point-of-need NAT (Diagnostics For All Inc., Cambridge, MA). H. Paper-based molecular diagnostic for *Chlamydia Trachomatis* (Klapperich et al.).

One way to lower the cost and complexity of instrumentation is to utilize isothermal amplification techniques instead of PCR which requires precise temperature control and cycling. Isothermal amplification techniques perform amplification at a constant lower temperature and therefore require less power. Several isothermal amplification techniques like loop-mediated isothermal amplification (LAMP),[41-48] recombinase polymerase amplification (RPA),[49-54] nucleic acid sequence-based amplification (NASBA),[55-58] helicase-dependent amplification (HDA),[59-63] strand displacement amplification (SDA), [64-68] and rolling circle amplification (RCA) [69-75] have been reported. These techniques in most cases can match the analytical performance of PCR while reducing instrumentation complexity.[23] PCR is also prone to inhibition from compounds present in unprocessed biological, environmental and food samples, requiring significant sample matrix-

specific pre-processing steps.[76-79] Isothermal techniques have been shown to be more robust and resilient to the presence of common PCR inhibitors, and therefore have the potential to reduce the overall complexity involved in NAT by using unprocessed or minimally processed samples.[23, 25, 80, 81] Furthermore, using colorimetric lateral flow detection for end-point detection of amplification reactions instead of fluorescence can further reduce the need for instrumentation. As a result, lateral flow assays have been paired with several of the isothermal amplification techniques to develop 'instrument-free' detection systems.[50, 82-84] Other instrument-free methods of amplicon detection include turbidity-based measurements from the formation of magnesium pyrophosphate during the amplification reaction[85, 86] and dot blot hybridization assays.[87]

Recently, several microfluidic devices have been reported that partially or fully integrate the extraction, amplification, and detection of nucleic acids into a disposable POC device format.[27-35, 62, 88, 89] Microfluidics enable directed fluid flow in channels impregnated with reagents to carry out complex chemical assays. These devices have the potential to eliminate or significantly reduce the need for equipment and semi-automating NAT to enable POC use. Paper microfluidics, i.e. microfluidic devices constructed from porous substrates like paper, have gained significant interest from researchers developing POC use diagnostic devices.[90-94] The World Health Organization (WHO) recommends that diagnostics developed for use in the developing world follow the 'ASSURED' criteria, which states that diagnostic devices be affordable, sensitive, specific, user-friendly, robust, equipment-free and deliverable to those in need. The availability, ease-of-manufacturing, biodegradability, cost-effectiveness, and ability to transport fluids through wicking have made paper an attractive platform for developing ASSURED diagnostics.[90, 91, 95, 96] Paper-based devices have also been applied in the development of sample-to-result molecular

diagnostics. These devices have yet to be properly validated in field conditions, though they show that paper-based, sample-to-result NAT is feasible.[27, 30, 83, 97-99]

Given the need for sample pretreatment before amplification and the unique reaction conditions during each of the extraction, amplification and detection processes, many researchers have employed multi-layered paper-based, paper-plastic hybrid devices [27, 83, 100], origami-based [30, 101], paper-substrate embedded in microcapillaries [35], magnetic sliding strips [97] and pipette tips [83, 97] to separate the reactions. These devices are a great step toward the development of ASSURED nucleic acid testing platforms and have the potential to vastly improve access to molecular diagnostics in low-and-middle income countries. Improving the ease-of-use, robustness to environmental conditions during testing, reliability, shelf-life, diagnostic accuracy and reducing operator time and tasks are some challenges that remain to be addressed for such devices to reach the global markets and positively impact global healthcare access.

Sample Pretreatment

One of the biggest hurdles to improved molecular diagnostics access is that nucleic acid amplification methods generally require purified nucleic acids to operate reliably, requiring extensive sample pretreatment steps and adding significant cost, complexity, and time for analysis. Nucleic acid amplification using PCR requires several sample preparation steps, which include lysis of the cells in the sample, separation of nucleic acids from proteins and other potential interferents, and preconcentration of nucleic acids. There are several chemical and mechanical lysis methods, each with their own set of advantages and disadvantages. Ionic, nonionic and zwitterionic detergents such as Triton X-100 or sodium dodecyl sulfate (SDS) are common

chemical lysis reagents which break down the cellular membranes by disrupting lipid-lipid, lipid-protein, and protein-protein interactions. Mechanical lysis methods include freeze/thaw, homogenization, mechanical grinding, bead beating and electrical lysis. Chemical lysis reagents can often inhibit downstream amplification and detection reactions while mechanical lysis methods are chemical-free but require additional specialized instrumentation. Assay developers must therefore carefully select a lysis method that does not negatively affect downstream reactions and provides high lysis efficiency in the target sample type. A combination of chemical and mechanical lysis methods may be required for complex sample types like animal tissue.

Nucleic acid purification and extraction methods can be divided into liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods. These methods utilize electrostatic, hydrophobic or hydrogen bonding interactions to separate, purify and preconcentrate nucleic acids. Liquid-liquid extraction utilizes two immiscible solvents to separate and concentrate nucleic acids into one of the phases.[102,109] Examples of LLE methods include phenol-chloroform based extraction and Trizol® based extraction where NAs are separated into the aqueous phase, lipids in the organic phase and proteins at the interface. These techniques utilize toxic organic solvents and require several manual steps that can affect reproducibility of results. Solid-phase extraction methods utilize solid absorbent membranes such as silica, or glass beads or magnetic particles to capture nucleic acids from a lysed sample. The captured nucleic acids are washed with alcohol and eluted under low ionic strength buffer conditions.[102,109] A number of LLE and SPE commercial kits are available to perform nucleic acid extraction from complex biological samples, though they require significant auxiliary equipment to use. A brief summary of the sample pretreatment process is shown in figure 1.2.

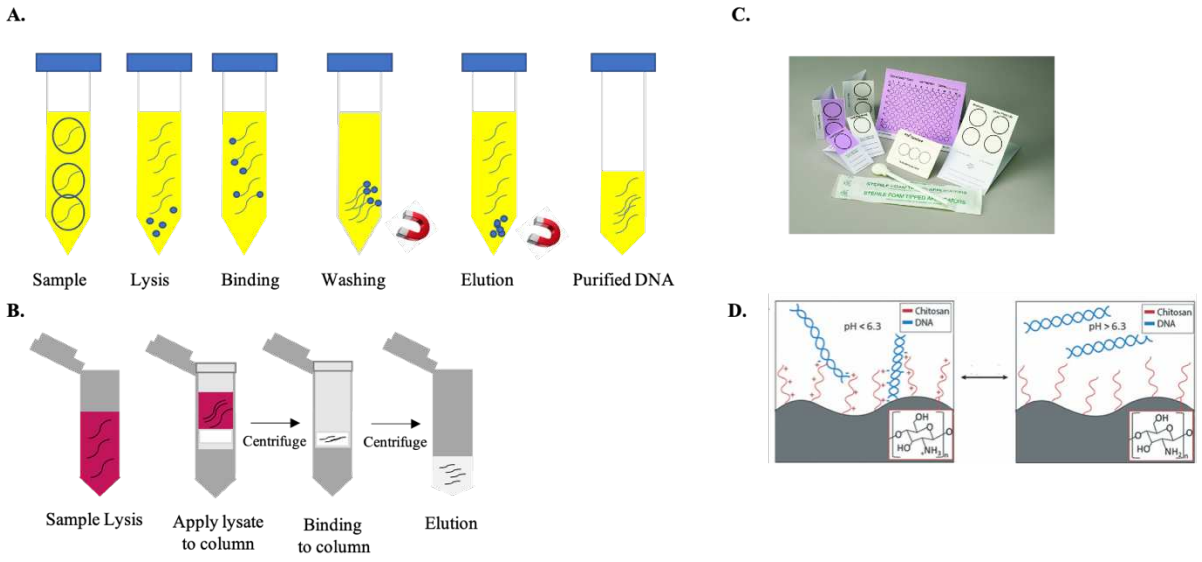

Figure 1.2 Sample Pretreatment methods A. Magnetic bead-based method for purification of nucleic acids. B. Silica or membrane-based method for the purification of nucleic acids. C. Whatman FTA cards for sample collection, transport, and storage. Nucleic acids remain detectable in FTA cards for decades allowing later analysis. Chitosan based nucleic acid isolation utilizes the pH dependent affinity of chitosan for nucleic acids.

Sample pretreatment adds significant cost, complexity and time to the nucleic acid testing process prohibiting use in resource-limited settings or at the point-of-care. Samples collected in remote areas must therefore be transported to a centralized laboratory for analysis delaying diagnosis and treatment. Paper has been used in dried blood spot sample collection, and several commercially available paper products like the Whatman® FTA cards allow the isolation, purification, and storage of nucleic acids for later analysis. These paper products are treated with proprietary chemistry to lyse the sample, denature proteins, and protect nucleic acids in the sample from degradation during transport and storage. Nucleic acids stored in FTA cards remain detectable by PCR for upwards of a decade therefore these products are an excellent alternate to conventional sample collection, transport, and storage. They also reduce the cost of sample extraction from USD $25 per sample to ~USD $0.10 per sample, therefore ideal for use in

resource-limited settings. Paper-based devices treated with chitosan, a naturally derived polymer which exhibits a pH dependent affinity for nucleic acids, have also been reported for sample purification and preconcentration.[102-104] Paper-based nucleic acid extraction and purification methods offer simple, and cost-effective alternatives to conventional sample pretreatment methods though further development is needed to improve the extraction efficiency, applicability for diverse sample types, and ease of use of such devices. Devices capable of on-board sample pretreatment, or ones that can perform NAT in relatively crude samples would be highly desirable in POC and resource-limited settings.

Nucleic Acid Amplification

Nucleic acid biomarkers are generally present at low concentrations in biological samples, requiring an amplification step for reliable detection. While PCR has been widely utilized and accepted as the gold standard for nucleic acid amplification, isothermal techniques have the potential to reduce the cost and complexity associated with nucleic acid analysis. The various nucleic acid amplification methods each have their own set of advantages and disadvantages. These benefits and limitations along with target sequence-specific and potential testing environment constraints need to be considered when designing point-of-need nucleic acid sensors. In this section, we briefly describe the advantages and disadvantages of each nucleic acid amplification methods. Several excellent reviews have been published comparing the analytical performance, amplification time, operating temperatures, and robustness of the various isothermal amplification methods.[22, 23, 25] In this section, we focus on the adaptability of the various isothermal amplification methods for POC molecular diagnostic devices. We present the parameters that must be considered in choosing an appropriate isothermal amplification technique for POC sensor

platform development. Table 1.1 shows a comparison of the analytical performance, operating conditions, amplification time, amplification power and suitability for POC use of the various isothermal amplification techniques. Common isothermal amplification methods include but are not limited to LAMP, RPA, HDA, NASBA, RCA and SDA. Figure 1.3 shows a brief overview of the working principle of the common isothermal amplification techniques.

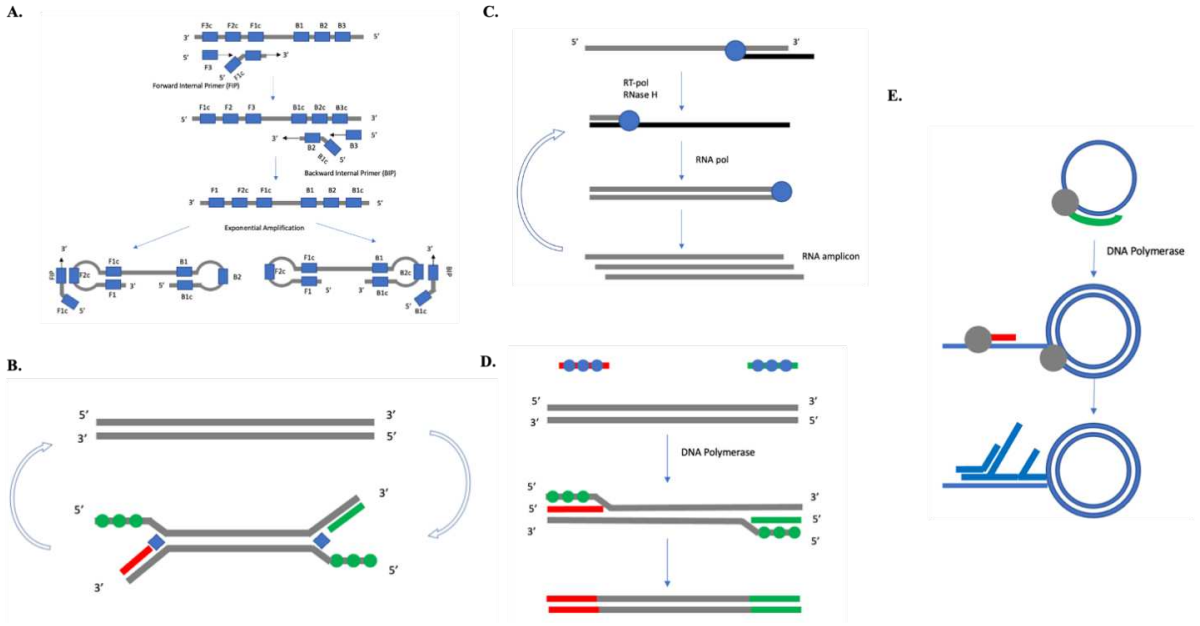

Figure 1.3: Isothermal Amplification techniques working principles A. Loop-mediated isothermal amplification (LAMP) uses 4-6 primers to target 6-8 regions of the target DNA. Strand-displacing polymerase and loop primers initiate synthesis of a long, concatenated (>20kb) DNA product that can be detected using gel electrophoresis, intercalating agents, fluorescent probes, turbidimetric and lateral flow detection methods. B. Helicase dependent amplification uses 2 sets of primers (red and green), single-stranded DNA binding proteins (green circles) and helicase (blue diamond) to separate the target DNA strands, allowing primer annealing and extension by a strand displacing polymerase. C. Nucleic acid sequence-based amplification (NASBA) utilizes two primers and an enzyme cocktail of T7 RNA polymerase and Reverse Transcriptase to generate cDNA intermediates from RNA targets. RNase H activity of reverse transcriptase degrades the RNA in the DNA-RNA hybrids and synthesizes the complementary DNA strand, which is in turn transcribed into RNA by the T7 RNA polymerase resulting in exponential amplification of the target RNA. D. Recombinase polymerase amplification (RPA) utilizes recombinase enzyme (blue circles) to form complexes with oligo primers to invade the dsDNA, single-stranded DNA binding proteins are used to create D-loop structures that initiates amplification by a strand-displacing polymerase. E. Rolling circle amplification (RCA) utilizes a short primer and strand-displacing polymerase to amplify a circular template. Additional primers can initiate branched RCA increasing the sensitivity and speed of amplification.

Table 1.1: Comparison of existent isothermal amplification techniques

Method	Sensitivity	Primers	Target (ssDNA, dsDNA, RNA)	Operating temp. (°C)	Reaction Time (min)	Reaction Product	No. of Steps	Compatible Detection Methods
LAMP	~5 copies	6 - 8	ssDNA, dsDNA, RNA (RT-LAMP)	60 – 65	60	Concatenated DNA	2	Intercalating dyes, fluorescence, lateral flow assay, turbidimetric
RPA	1 copy	2	dsDNA	37 – 42	20-30	DNA	1	Fluorescence, lateral flow assay
NASBA	1 copy	2	RNA	41	60	RNA	2	Fluorescence, lateral flow assay
HDA	1 copy	2	dsDNA	64	100	dsDNA	2	Intercalating dyes, fluorescence, lateral flow assay
SDA	10 copies	2	ssDNA	37 – 42	120	dsDNA	1	Fluorescence
RCA	10 copies	1	Circular ssDNA	30 – 37	90	Concatenated ssDNA	2	Fluorescence, intercalating dyes, lateral flow assay

LAMP, the most commonly used technique utilizes 4-6 primers to target 6-8 distinct regions of the target DNA. Strand-displacing polymerase and loop primers initiate the synthesis of a long, concatenated (>20kbps) amplicon DNA which can be detected using agarose gel, fluorescence, turbidimetric and colorimetric methods. LAMP can match or exceed the analytical performance of PCR and allow ultrasensitive nucleic acid detection with detection limits as low as 5 copies of target per reaction. LAMP can also be combined with reverse transcription to detect RNA targets. Given the need for 6-8 primers, the primer design is much more complex in LAMP than other isothermal amplification methods. LAMP also requires a higher operating temperature of approximately 65°C which increases the cost and complexity of instrumentation. One of the main advantages of LAMP is that the amplification time is relatively short compared to other isothermal amplification techniques, enabling rapid, POC nucleic acid detection.[41, 43, 48, 85]

Helicase-dependent amplification, HDA utilizes 2 primers, single-stranded DNA binding protein and helicase to invade the target DNA, enabling primer annealing and extension by a strand-displacing polymerase. HDA provides single-copy detection limits within 100 mins of amplification time. HDA is also a single-step reaction, and compatible with fluorescence and lateral flow detection methods. HDA reactions are performed at 64 °C which complicates heating equipment needed to maintain the reaction temperature.[59-61] Nucleic acid sequence-based amplification (NASBA) utilizes two primers and an enzyme cocktail of T7 RNA polymerase and Reverse Transcriptase to generate cDNA intermediates from RNA targets. RNase H activity of reverse transcriptase degrades the RNA in the DNA-RNA hybrids and synthesizes the complementary DNA strand, which is in turn transcribed into RNA by the T7 RNA polymerase

resulting in exponential amplification of the target RNA.[55, 57, 58] NASBA provides single-copy detection within 60 mins of amplification time at 41°C.

RPA utilizes recombinase enzyme to form complexes with primers to invade the target DNA strands. Single-stranded DNA binding protein allow the formation of D-loop structures which initiates amplification by a strand-displacing polymerase. RPA provides single copy detection limits, and excellent specificity. RPA is one of the fastest techniques as detection of RPA reactions is possible in just 20 mins.[49, 50] Rolling circle amplification (RCA) utilizes a short primer and strand-displacing polymerase to amplify a circular template. Additional primers can initiate branched RCA increasing the sensitivity and speed of amplification. Hyper-branched RCA has been shown to provide 10 copies detection limit within 90 mins of amplification time at 30-37°C. RCA is one of the only techniques that provides a concatenated ssDNA amplicon (>100kb) which can be detected using fluorescence and lateral flow detection methods.[69, 71]

The analytical performance, target NA type, number of user steps, amplification reaction temperature, amplification time and compatible detection methods should be carefully considered in designing POC nucleic acid sensor platforms based on isothermal amplification methods. Another consideration in the selection of an appropriate isothermal amplification technique is multiplexing or at least the target and an internal control. LAMP and RPA have been most commonly utilized in the development of integrated disposable NAT platforms because of their fast result turnaround time (<60 min).[97] The need for 6-8 primers to target 4-6 distinct regions of the target DNA makes LAMP primers difficult to design. RCA is one of the simpler and more versatile isothermal amplification techniques as it only requires one primer and a strand-displacing polymerase to detect both RNA, DNA, and protein targets, but lacks the sensitivity and speed of

LAMP. A comprehensive review of primer design rules, and introduction of software to help researchers design and analyze primers for the various isothermal amplification techniques would vastly improve the speed of assay and nucleic acid sensor platform development.

Nucleic Acid Detection

In conventional clinical labs, fluorescence is used to detect PCR amplicons. Fluorescence also allows real-time monitoring of the amplification reactions, allowing quantitation of the copy number in the sample. Real-time fluorescence measurements require complex equipment which is difficult to operate and maintain in resource-limited settings. Alternate methods for nucleic acid detection include colorimetric visual detection, lateral flow assays, dot blot hybridization and turbidimetric detection. Lateral flow assays are most frequently paired with the various isothermal amplification methods and provide a simple colorimetric method for the visualization of results.[82-84, 105] Lateral flow detection paired with nucleic acid tests fall under two categories, nucleic acid lateral flow assays (NALF) and nucleic acid lateral flow immunoassays (NALFIA), which use nucleic acids and immunolabels respectively, to capture and detect the amplification reaction product. The appearance of a test line indicates the presence of the sequence of interest, semi-quantitation of the copy number is possible through the analysis of the signal color intensity. This provides simple, visual, and instrument-free readout of results facilitating adaptation into POC devices. An example of such a system is the BESt^{TM} cassette – Type II by BioHelix Inc. (Beverly, CA, Figure 1F) which allows visual colorimetric detection of HDA and PCR amplicons in a POC device and have been applied in the detection of human immunodeficiency virus type - I (HIV-1) and herpes simplex virus (HSV).[23, 106] LAMP reactions provide a high yield of amplicon DNA which results in the production of pyrophosphate as the amplification reaction proceeds which

reacts with magnesium ions to produce magnesium pyrophosphate precipitate increasing turbidity. As a result, turbidimetric and visual end-point colorimetric detection using pH sensitive dyes is feasible with LAMP reactions.[107, 108] Isothermal amplification techniques compatible with facile and easy-to-use detection methods offer several advantages over conventional PCR and can facilitate the development of fully integrated NAT platforms.

Integrated Nucleic Acid Sensor Platforms

Integration of sample preparation, amplification and detection methods in a rapid and easy-to-use NAT platform is one of the biggest hurdles to improved molecular diagnostic access in the developing world. Several microfluidic devices have been reported in the literature that partially or fully integrate sample preparation, amplification, and detection reactions into a PoN sensor platform. Several reports have shown that fully integrated paper-based NAT was feasible (Figure 1.4). The Whitesides group at Harvard University and Diagnostics For All Inc. have developed a fully integrated paper-based microfluidic device using magnetic sliding strips that enables a paper substrate to slide into different positions to carry out sample preparation, LAMP, and end-point detection in resource-limited settings.[97]

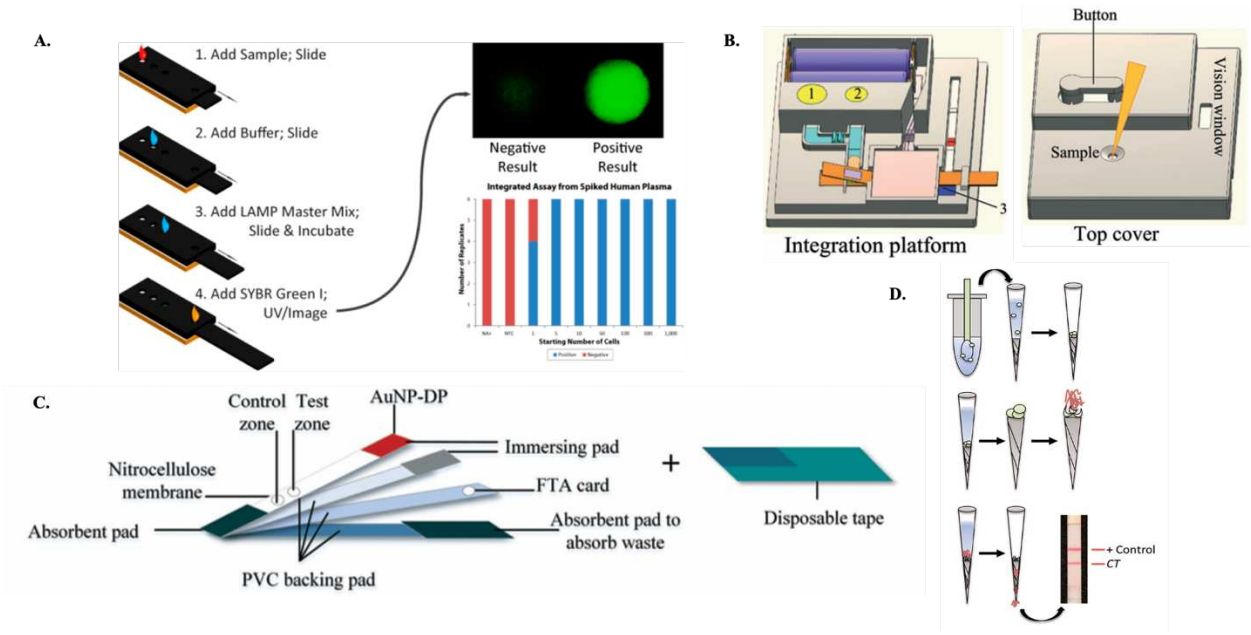

Figure 1.4: Integrated Paper-based NAT platforms A. 'Paper-machine' for molecular diagnostics (Diagnostics For All Inc, Cambridge, MA). B. Fully integrated NAT platform based on HDA and lateral flow detection. C. Paper-based NAT platform based on HDA and lateral flow detection. D. Paper-based molecular diagnostic for *Chlamydia trachomatis*

Xu et al., reported a multi-layered, paper-based sample-to-result device that incorporates sample preparation, amplification, and lateral flow detection. The paper layers were separated by hydrophobic polyvinyl chloride films to create 'valves' separating the sample preparation, amplification and detection regions.[27] Tang et al., reported a fully integrated and disposable paper-based NAT platform that integrates nucleic acid extraction, helicase-dependent amplification and lateral flow detection that can be performed by minimally trained personnel.[100] Klapperich et al., reported a paper-plastic hybrid device using paper substrate embedded in a micropipette tip to perform sample preparation, HDA and lateral flow detection. These devices show that paper-based NAT platforms are feasible and could provide rapid, low-cost, portable alternative to conventional methods.

Challenges, Opportunities and Future Outlook

Paper-based devices NAT platforms could significantly improve access to molecular diagnostics especially in resource-limited settings. These devices, however, are not without limitations. These limitations include, poor analytical performance, multiple user steps, inability to multiplex targets and need for cold-storage of off-chip reagents. Furthermore, these devices are yet to be properly validated in field conditions by intended users of the technology to show clinical utility. Improving amplification time, shelf-life and minimizing the number of user steps are key to improving adoption of such devices in the developing world. An ideal molecular diagnostic platform for use in resource-limited settings must provide rapid and reliable results, in an easy-to-use and cost-effective device. One way to lower the cost of NAT platforms is to employ isothermal amplification paired simple detection methods like the lateral flow assay, turbidimetric or colorimetric detection methods. Some isothermal amplification techniques like LAMP and RCA have been shown to be more robust and resilient to the presence of common PCR inhibitors, therefore such techniques can be performed in relatively crude samples. Eliminating or minimizing the need for sample pretreatment steps would significantly improve the turnaround time of results and enable rapid, point-of-care diagnosis and management of infectious diseases. This dissertation describes the development of a non-amplification based molecular diagnostic platform based on nuclease protection and lateral flow detection, covered in Chapter 2. This platform is easy-to-use and rapid with results within 30 min, though lacks the sensitivity needed for infectious disease diagnostics. Chapter 3 describes the use of isothermal rolling circle amplification with the nuclease protection lateral flow assay to improve the assay sensitivity. Chapter 4 explores the use of exponential mode rolling circle amplification to further improve assay limit of detection and clinical utility for the diagnosis of Severe acute respiratory syndrome coronavirus, the novel

coronavirus discovered in 2019 (SARS-CoV-2). Chapter 5 describes the use of low-cost substrates like patterned transparency sheets to develop microfluidic devices which perform sequential delivery of reagents to the lateral flow strip for colorimetric detection. The devices allow dried reagent storage in paper pads embedded in channels. With further development such devices can be used to fully integrate the NAT steps of sample pretreatment, nucleic acid amplification and detection on an easy-to-use, disposable device. Such a device could provide a highly portable, low-cost, and point-of-need NAT platform that can be deployed in resource-limited settings or for home-use testing applications.

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CHAPTER 2: PAPER-BASED NUCLEASE PROTECTION ASSAY FOR POINT-OF-NEED PATHOGEN DETECTION

Chapter Overview

Pathogen detection is crucial for human, animal, and environmental health; crop protection; and biosafety. Current culture-based methods have long turnaround times and lack sensitivity. Nucleic acid amplification tests offer high specificity and sensitivity. However, their cost and complexity remain a significant hurdle to their applications in resource-limited settings. Thus, point-of-need molecular diagnostic platforms that can be used by minimally trained personnel are needed. The nuclease protection assay (NPA) is a nucleic acid hybridization-based technique that does not rely on amplification, can be paired with other methods to improve specificity, and has the potential to be developed into a point-of-need device. In traditional NPAs, hybridization of an anti-sense probe to the target sequence is followed by single-strand nuclease digestion. The double-stranded target-probe hybrids are protected from nuclease digestion, precipitated, and visualized using autoradiography or other methods. We have developed a paper-based nuclease protection assay (PB-NPA) that can be implemented in field settings as the detection approach requires limited equipment and technical expertise. The PB-NPA uses a lateral flow format to capture the labeled target-probe hybrids onto a nitrocellulose membrane modified with an anti-label antibody. A colorimetric enzyme-substrate pair is used for signal visualization, producing a test line. The nuclease digestion of non-target and mismatched DNA provides high specificity while signal amplification with the reporter enzyme-substrate provides high sensitivity. We have also

developed an on-chip sample pretreatment step utilizing chitosan-modified paper to eliminate possible interferents from the reaction and preconcentrate nucleic acids, thereby significantly reducing the need for auxiliary equipment. This work was performed in collaboration with Dr. Eka Noviana, and a co-first authored journal article was published in the Analytical and Bioanalytical Chemistry journal.

Introduction

Point-of-need pathogen detection has wide ranging applications in medical diagnostics,[1] food and water safety,[2] agriculture,[3] air quality and environmental monitoring,[4] biosafety,[5] evolutionary biology and epidemiology.[6] Conventional culture-based methods widely employed in pathogen detection have long turnaround times (days to weeks), low sensitivity (300-3000 colony-forming units (CFU) per mL) and a prevalence of false-negatives.[1] Moreover, many pathogens cannot be cultured using standard methods or require high-containment culture facilities.[7] Molecular diagnostic methods such as blotting- and hybridization-based techniques, next generation sequencing (NGS), and nucleic-acid amplifications tests (NAATs) have become very common for pathogen detection in clinical and research labs across the developed world. NAATs include polymerase chain reaction (PCR) and isothermal amplification techniques, both of which are known to offer high sensitivity (~3 CFU/mL) because the target of interest is amplified exponentially. However, each step of PCR is performed at a different temperature requiring a thermocycler which adds significant cost and limits portability. To simplify instrumentation needs, newer isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP),[8,9] template-mediated amplification (TMA),[10] helicase-dependent amplification (HDA),[11] and rolling circle amplification (RCA)[12] have been used for

nucleic-acid analysis. While these techniques reduce the need for instrumentation by enabling amplification at a fixed temperature, the need for several distinct primers for amplification, significant laboratory infrastructure, and highly trained personnel make their use in the developing world cost prohibitive. Furthermore, NAATs can produce false negative results due to the presence of inhibitors in complex samples and therefore require extensive sample preparation steps.[13]

Nuclease protection (NPAs) or hybridization protection assays have been in used in RNA mapping and determining gene transcription levels for decades.[14] Unlike NAATs, NPAs do not require nucleic acid amplification, which reduces assay time, cost, and complexity. Traditional NPAs involve hybridizing a labeled probe complementary to the target sequence followed by digestion of unhybridized and non-target nucleic acids using a nuclease which selectively cleaves single-stranded oligomers. Thus, only perfectly matched probe-target hybrids remain intact and can be detected using colorimetric or radiological methods.[14-17] NPAs have been integrated with sandwich hybridization (NPA-SH) to detect harmful algae in aqueous samples.[15-18] Recently, a nuclease protection enzyme-linked immunosorbent assay (NP-ELISA) method has also been developed for the detection of Zika and Kunjin virus sequences with colorimetric, chemiluminescent and electrochemical detection motifs.[14] However, these NPA strategies require external readers which are sometimes expensive and not accessible in resource-limited settings.

Lateral Flow Assays (LFAs) are frequently used in point-of-care diagnostics, the most ubiquitous of which are the over-the-counter pregnancy tests. LFAs have also been used in direct and indirect detection of pathogens such as *Plasmodium falciparum* and proteins such as Diphtheria Toxin.[19,20] LFAs can significantly reduce the need for instrumentation and provide

user-friendly result readouts, typically with the appearance of a test line when the target is present. This easy-to-read platform offers value as an end-point detection method and has been paired with PCR [21] and isothermal amplification reactions [22-24] to create low-cost nucleic acid sensors. Lateral flow nucleic acid sensors can be divided into two categories, i.e., nucleic acid lateral flow assays (NALF) and nucleic acid lateral flow immunoassays (NALFIA). NALFs involve direct detection of nucleic acids using oligonucleotide probes immobilized on lateral flow strips while NALFIAs use hapten or other biomolecule-labeled probes and antibodies to capture them in an immunoassay format.[25]

We have developed a paper-based nuclease protection assay (PB-NPA) for the rapid detection of nucleic acids at the point-of-need (Figure 2.1). The assay involves hybridization of a 5'-digoxigenin and 3'-biotin-labeled oligonucleotide probe to the target, followed by digestion with P1 nuclease to cleave unhybridized probe, target and other single-stranded non-target DNA. The protected probe-target hybrids are captured using an anti-digoxigenin antibody immobilized on nitrocellulose membrane and visualized with streptavidin-conjugated horseradish peroxidase (Strep-HRP) and a colorimetric substrate solution (tetramethylbenzidine (TMB) and hydrogen peroxide). A blue line appears in the detection region to indicate the presence of the target DNA. The use of a reporter enzyme allows for signal amplification from the captured protected probes, providing a sensitive method for detection without performing complex nucleic acid amplification reactions. The assay provides simple yes/no readout with the appearance of a test line when the target sequence is present in the sample above the detectable limit. The assay combines the high specificity of the NPA with the user-friendly lateral flow strip platform for end-point detection.

When quantification of the target is needed, the colorimetric signal can be captured and analyzed using a cellphone camera and image analysis software.

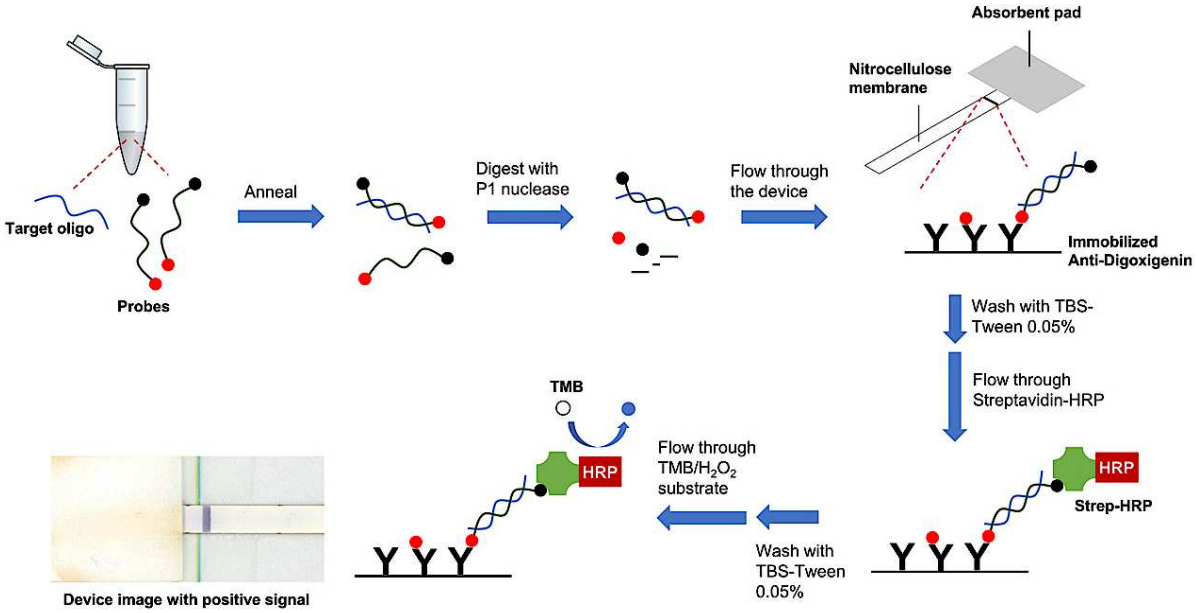

Figure 2.1. Schematic representation of the paper-based Nuclease Protection Assay

Materials and Methods

Materials

Nitrocellulose FF120HP A4-sized sheets, Whatman Grade 1 filter paper and Whatman Grade 4 filter paper were purchased from GE Healthcare (Chicago, IL). Anti-digoxigenin monoclonal antibody was purchased from Abcam (Cambridge, MA). Trehalose dihydrate and formamide was purchased from EMD Millipore (Burlington, MA). StabilGuard® Immunoassay Stabilizer (BSA-free) was purchased from Surmodics, Inc. (Eden Prairie, MN). P1 Nuclease (from *P. citrinum*) and 10X P1 nuclease reaction buffer were purchased from New England Biolabs, Inc. (Ipswich, MA). Glycerol was purchased from Mallinkrodt (Staines-upon-Thames, United Kingdom). Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), Sodium chloride, Ethylenediaminetetraacetic disodium salt acid, 2-(N-Morpholino)ethanesulfonic acid hydrate

(MES), Tris(hydroxymethyl)aminomethane (Tris), Tween® 20 and chitosan oligosaccharide lactate (average M_n 5,000) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Streptavidin conjugated to Horseradish Peroxidase (Strep-HRP) was purchased from R&D Systems, Inc. (Minneapolis, MN). Pierce™ 1-Step Ultra TMB-Blotting Solution was purchased from Thermo Scientific (Waltham, MA). Table 2.1 gives the NPA probe and DNA oligonucleotide sequences used in the study; all DNA sequences were ordered from Integrated DNA Technologies, Inc. (Coralvile, IA).

Table 2.1. NPA probe and DNA sequences

Sequence Name	**DNA Sequences**
NPA Probe	5'biotin/GTGATTGACGATGGGGCCCAA/3'digoxigenin
Complementary Target	5'/TTGGGCCCCATCGTCAATCAC/3'
1-base mismatch	5'/TTGGG**T**CCCATCGTCAATCAC/3'
2-base mismatch	5'/TTGGG**T**CCCAT**G**GTCAATCAC/3'
3-base mismatch	5'/TT**T**GG**T**CCCAT**G**GTCAATCAC/3'
4-base mismatch	5'/TT**T**GG**T**CCCAT**G**GTCAAA**A**CAC/3'
Fluorescently labeled oligo	5' Amine modifier C6/AGGTATGTAGAGGCA/3' 6-FAM (Fluorescein)

Device fabrication

A 100 μL anti-digoxigenin Ab solution (1 mg/mL) was mixed with 2.5 μL of 2 M trehalose and 10 μL of 50% glycerol. The solution was then striped twice onto a nitrocellulose (NC) membrane at 0.036 μL/mm deposition rate using an Automated Lateral Flow Reagent Dispenser

(ALFRD)^{TM} from ClaremontBio (California, USA) equipped with a syringe pump (0.15 μL/s flow rate; 4.2 mm/s striping rate). The membrane was dried at 37°C for 1 h. To block the surface of the NC membrane from non-specific binding, StabilGuard® Immunoassay Stabilizer solution was added to fully wet the membrane. The membrane was then air dried at room temperature for 30 min. After that, the membrane can be immediately used to fabricate devices or stored in a closed container for later use.

To construct the device, the Ab-striped NC membrane was cut using a Zing CO_2 laser cutter from Epilog (Colorado, USA) to create 20 mm × 4 mm strips with the deposited antibody located 5 mm from one edge of the strip (i.e., downstream edge). The NC strip was placed on a transparency sheet (as a backing material) using double-sided adhesive. Two pieces of 20 mm × 15 mm laser cut Whatman No. 4 filter paper were stacked together and placed on the downstream edge of the NC strip using a tape such that there was 2 to 3 mm overlapping region between the materials. The Whatman grade 4 qualitative filter paper was used as an absorbent pad.

Paper-based nuclease protection assay

Nuclease protection assays were performed based on a protocol described by Filer et al.[14] with some modifications. For a triplicate experiment, 3 μL probe oligo solution was mixed with 3 μL target oligo solution, 2.4 μL 10X hybridization buffer (0.4 M PIPES pH 6.8, 4 M NaCl, 0.02 M EDTA) and 15.6 μL DI water in a tube. Formamide was added at 0-80% total concentrations by reducing the volume of DI water to accommodate the volume of formamide added without changing the total volume of the solution. The solution was then heated at 95°C for 2 min and at 53°C for 10 min. The solution was subsequently placed in ice bath and a 6 μL mixture of P1 nuclease 1 U/μL-10X P1 buffer (1:1 v/v) was added to the solution. To start digestion, the solution

was incubated in a water bath at 37°C for the indicated time. A 10 μL aliquot from nuclease digestion was flowed through the device, followed by an addition of 10 μL TBS-Tween 0.05% wash buffer pH 8.6. The following solutions: 10 μL Streptavidin-HRP (1:200 diluted), 10 μL TBS-Tween 0.05% wash buffer, and 2x 10 μL TMB/H_2O_2 substrate solution were then added subsequently to develop the colorimetric signal.

Testing DNA interaction with chitosan-modified paper

A Xerox® ColorQube 8870 solid-ink printer was used to print wax pattern on Whatman grade 1 filter paper. Wax patterned paper were placed on a hot plate set to 150°C for 1 min to allow the wax to melt and permeate the thickness of the paper, forming a hydrophilic zone surrounded by hydrophobic wax barrier. To study the interaction of DNA with chitosan, fluorescently labeled oligo DNA (fluorescein, Ex_{max} = 495nm, Em_{max} = 520nm) was flowed through chitosan-modified wax patterned filter paper discs (printed diameter of 3mm) in a vertical flow format with an absorbent pad at the bottom to wick the sample (n = 9 per layer). A fluorescence microscope (Eclipse TE2000-U Inverted Microscope, Nikon Inc, Tokyo, Japan) was used to image the chitosan layer and the absorbent pad. ImageJ image processing software was used to quantify the mean intensity in each of the layers. To determine the optimal conditions for the elution of captured DNA, varying volumes (3 – 6 μL) of Tris buffer with pH (7.5, 8, 8.5 and 9) were flowed through the chitosan layer. Fluorescence images were captured pre- and post-elution for each of the elution buffer conditions (n = 5 per condition) and quantified using ImageJ. After establishing the feasibility of using chitosan to capture and elute DNA, a simple device design was created to perform on-chip sample pretreatment directly on the lateral flow strip.

On-chip sample pretreatment using chitosan-modified paper

Wax-patterned filter paper was modified with 2 μL of 1% w/v chitosan oligosaccharide lactate prepared in MES buffer pH 5.0, this layer is referred to as the 'chitosan layer'. The chitosan layer was cut and affixed to a laser cut polymethyl methacrylate (PMMA) support layer. Upon completion of the P1 nuclease digestion step, 10 μL each of the sample was applied to the chitosan layer while in contact with an absorbent pad. The chitosan layer was washed in this position with 40 μL of 100 mM MES buffer pH 5.0 to remove the interfering compounds from the sample while retaining DNA from the sample. The membrane was then moved to the lateral flow strip and the DNA retained in the membrane was eluted on the lateral flow strip using three 10 μL volumes of Tris buffer pH 8.6. The sample pretreatment setup was then removed from the lateral flow strip and 10 μL of Tris buffer + 0.05% Tween® 20 was applied to the inlet followed by the remaining chromogenic components (Strep-HRP, Tris buffer + 0.05% Tween® 20, and the TMB blotting solution).

Image analysis

After color development, the strips were allowed to dry briefly to enhance the color contrast. These devices were then scanned using a V600 Epson scanner. The resulting image was inverted, and the detection region mean intensity was quantified using ImageJ 1.05i image processing software (open-source software, National Institutes of Health).

Results and Discussion

Nucleic acid detection

Colorimetric detection in the lateral flow devices was based on enzymatic conversion of TMB by HRP in the presence of H_2O_2. The use of reporter enzyme allowed for signal amplification to improve detection sensitivity. The binding of digoxigenin/biotin-labeled oligo probe to the capture antibody allowed for accumulation of streptavidin-HRP on the binding sites and subsequent color formation. To assess detection limit of this detection scheme, a series of solutions with varying probe quantities were tested on the flow devices. Colorimetric responses obtained from 64 amol to 5.0 pmol oligo probe are shown in Figure 2.2. The colorimetric signal reached a plateau at around 200 fmol of probe and the detection limit (i.e., mean intensity of blank sample + 3x standard deviation) was approximately 0.57 fmol (5.7×10^{-16} mol).

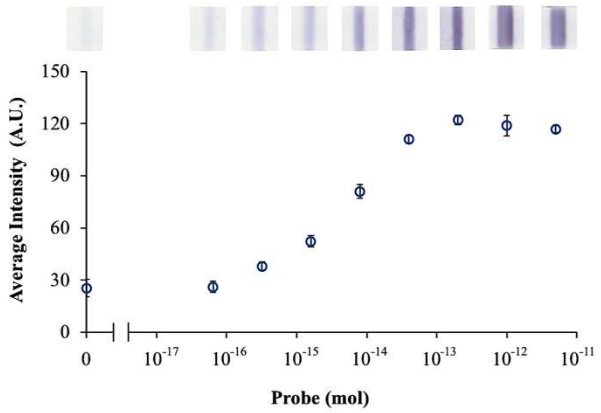

Figure 2.2. Dose response of varying amounts of digoxigenin/biotin-labeled oligo probes. Error bars indicate standard deviations (n = 4 devices)

Nuclease protection assay (NPA)

Hybridization between the probes and the target oligos and nuclease digestion of the unhybridized oligos are the two major steps in NPA. For nucleic acid hybridization, stringency/specificity can be manipulated by controlling three parameters: temperature, salt

concentration and formamide concentration.[26,27] Although cross reaction among related based sequences can be reduced at higher temperature in aqueous solution, conditions required for specificity are much more easily adjusted by varying ionic strength and formamide concentration.[28] Thus, to optimize hybridization condition for the NPA, formamide concentrations were varied in the presence of 400 mM sodium salt.

Prior to performing NPA in solutions containing formamide, we assessed the effect of formamide on the binding of labeled probes to the detection elements in the lateral flow devices. This step is important to help distinguish the influence of formamide in different aspects of the PB-NPA including hybridization, nuclease digestion and detection in the flow devices. Formamide concentrations above 20% reduced binding between the probes and the capture antibodies as shown by linear decrease in colorimetric signal at formamide concentrations ranging from 20-50% (Figure 2.3A). While antigen-antibody binding activity was reported to increase in the presence of several organic-water miscible solvents such as ethanol and methanol,[29] formamide and similar solvents have been shown to disrupt antigen-antibody binding potentially by inducing conformation change of the antibodies or lowering the binding affinity by solvating either or both antigen-labeled oligo probes and capture antibodies.[29,30] Therefore, dilution of test solution to formamide concentrations equal to 20% or lower was required prior to testing using the lateral flow devices.

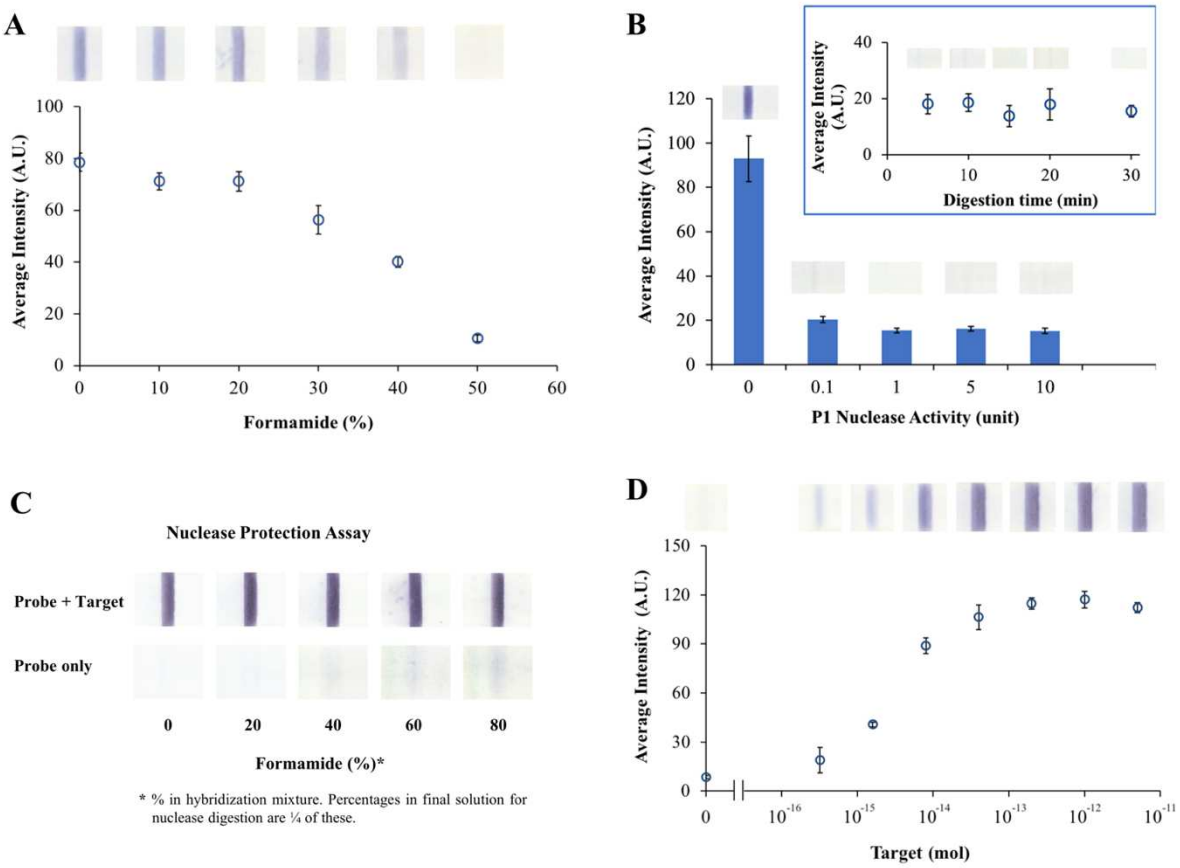

Figure 2.3. Optimization of NPA protocol and assay sensitivity: **(A)** Effect of formamide on the detection of 8 fmol oligo probe; **(B)** Effect of P1 nuclease quantity/activity and (digestion time-*right top inset*) on degradation of single-strand oligo probe. Digestion was carried out for 30 min in experiment shown in the main graph; **(C)** Effect of formamide concentration on NPA; and **(D)** Dose response of varying amounts of target complementary oligos. Error bars indicate standard deviations (n = 4 devices)

The use of a single-strand-specific nuclease is crucial to remove unhybridized labeled probes from the test solution such that any detected signal is only dependent on the quantity of the protected target-probe duplexes. Although more than 30 single-strand-specific nucleases have been identified from various sources, only a few enzymes including S1 nuclease and P1 nuclease have been characterized to a significant extent.[31] S1 nuclease is often employed in NPA [14,32,33] due to the well-characterized nature of the enzyme and high selectivity toward single-stranded DNA in the presence of double-stranded DNA.[31] However, we found that the catalytic activity of P1

nuclease in digesting the labeled oligo probes is higher than S1 nuclease, and thus selected P1 nuclease for our NPA protocol. To optimize digestion conditions, the amount of P1 nuclease and digestion times were varied in the presence of 10 pmol probes and incubation at 37°C. As shown in Figure 2.3B, significant depletion of colorimetric signal was obtained by using 0.1 U P1 nuclease in a 10 μL solution as compared to control experiment in the absence of nuclease. The background signal was further reduced by increasing nuclease activity to 1 U/10 μL, however, no more discernable reduction of signal was observed at higher nuclease activities. The probes were then digested using 1 U P1 nuclease with digestion time varied from 5 to 30 min. No significant difference was observed in measured colorimetric signals from probes digested for 5 to 30 min (Figure 3B inset) and thus, digestion with 1 U P1 nuclease for 5 min at 37°C was selected for the NPA.

Formamide is commonly used during hybridization in NPA to increase hybridization stringency. The effect of formamide concentrations on the NPA is shown in Figure 2.3C. No visible difference in colorimetric signals was observed at 0-80% formamide used in hybridization mixtures, suggesting that formamide neither interferes nor improves duplex formation within the experimental conditions described in the method section. However, increased concentration of formamide did affect the P1 nuclease activity in degrading unhybridized probes. Background signals were observed at 10-20% formamide concentrations in digestion mixtures (equivalent to 40-80% formamide concentration in hybridization mixtures due to 1:4 dilution of solution prior to nuclease digestion). Since no signal improvement was obtained by incorporating formamide into the hybridization mixture and the organic solvent has shown some detrimental effects to nuclease digestion and probe binding to the capture antibody, formamide was omitted from the PB-NPA protocol.

Figure 2.3D shows the intensities of colorimetric signal as a function of target oligo quantities in PB-NPA using the optimized protocol. Similar to the probe dose-response shown in Figure 2.2, NPA with target sequence complementary to the probe also reached saturation at approximately 200 fmol of target oligo, suggesting that the hybridization between target oligo and the probe is highly efficient. The background signal in NPA with target oligo was lower than that in probe only detection, potentially due to the change in pH and ionic strength in assay buffer from addition of P1 buffer to the hybridization buffer that was used solely in probe detection. This lower background signal gave slightly improved detection limit for PB-NPA, which was approximately 0.24 fmol (2.4×10^{-16} mol).

Assay specificity

To investigate the specificity of PB-NPA, target oligos with 1, 2, 3, or 4 base mismatches were designed and tested at 1:1 ratio and 125:1 ratio to the quantity of the probes. A completely random sequence was also tested at similar ratios. The results are shown in Figure 2.4A. Significant reduction of signal was observed for the 1 base mismatch compared to that in complementary oligo, indicating sequence selectivity within the assay. However, the considerably high colorimetric responses obtained in various mismatch sequences (in comparison to the baseline level signal obtained from the random sequence) suggested the formation of duplexes or secondary structures between the probe and the mismatch oligos that P1 nuclease was not able to degrade. Increasing quantity of either mismatch or random oligo relative to probe quantity did not affect the observed signals. Single-base mismatches are the least accessible to the single-strand specific nucleases because they present the smallest region for enzyme binding.[34] This difficulty in accessing single-strand binding sites is possibly the reason why considerable amounts of signal

were obtained in all mismatch oligo tested by PB-NPA. Although 4 bases were non-complementary in the 4 bases mismatch oligo, the location of mismatched bases was designed to be single-base mismatch at 4 different locations within the sequence, posing the same difficulty for the nuclease to bind.

Increasing the amount of P1 nuclease reduced colorimetric responses from 1 base mismatch oligo (Figure 2.4B). However, the increased nuclease activity also decreased colorimetric signals in complementary oligo, suggesting that P1 nuclease also degrades double-strand DNA at high concentrations. Nevertheless, the signal-to-noise ratio (i.e., ratio of colorimetric signal from complementary oligo to that of 1-base mismatch) was improved by increasing the amount of P1 nuclease from 1 U (S/N = 1.4) to 50 U (S/N = 2.2) despite the lower sensitivity.

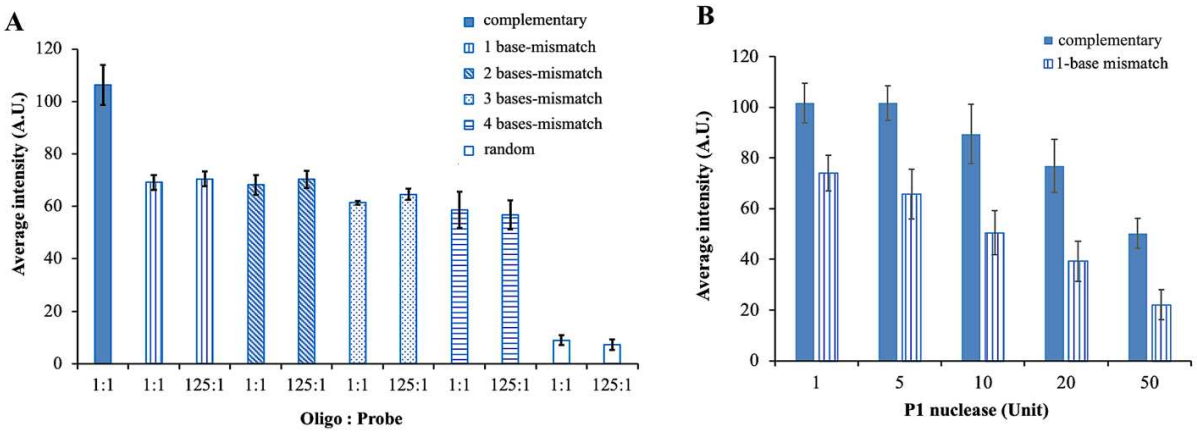

Figure 2.4. (A) Colorimetric signals for NPA with complementary, mismatch and random sequences. 1 U P1 nuclease was used for the digestion. Error bars indicate standard deviations (n = 4 devices). **(B)** NPA signal for complementary and 1-base mismatch at different activities of P1 nuclease. Error bars indicate standard deviations (n = 3 devices)

On-chip sample pretreatment with chitosan-modified paper

In conventional NPA, extensive sample preparation steps are needed in order to perform detection using colorimetric or radiological methods. These steps include inactivation of nuclease enzymes, centrifugation to remove free-label resulting from nuclease digestion and precipitation of protected probe-target hybrids for detection. In PB-NPA we perform on-chip sample pretreatment utilizing chitosan modified filter paper. Chitosan (pKa ~6.4) is a polycationic polymer that exhibits a pH dependent affinity for the negatively charged backbone of nucleic acids.[35] This pH-dependent nucleic acid affinity exhibited by chitosan has been exploited to create nanoparticle carriers in gene therapy delivery systems [36,37], but there are limited reports on the use of chitosan as a means to perform sample pretreatment in lab-on-a-chip or point-of-care nucleic acid sensors.[38,39] Yager et al. described the use of chitosan to perform preconcentration in a lateral flow format by capturing DNA at a low pH of ~5 and eluting the captured DNA into a secondary channel for detection by increasing the pH to ~8.5.[40] Chitosan-modified paper can also be used to preconcentrate DNA from the sample which could further improve the LOD of the PB-NPA device.[40,41] Schlappi et al. showed that a preconcentration factor of 5000× can be achieved using chitosan-modified porous membranes integrated with *in situ* PCR.[41] Moreover, chitosan complexed with DNA has been shown to provide protection against nuclease digestion and potentially eliminating the need for nuclease enzyme inactivation in the PB-NPA.[35-37]

DNA interaction with chitosan-modified paper

Free digoxigenin generated from the nuclease digestion of unhybridized probe can compete with protected probe-target hybrids for binding sites on the anti-digoxigenin antibody. It is therefore important to remove digoxigenin from the reaction in order to improve the limit of detection of the

PB-NPA. We first studied the interaction between chitosan and DNA to determine optimal conditions for the capture and elution of DNA using chitosan-modified paper. Fluorescently labeled oligo DNA and a fluorescent microscope was used to perform these experiments. A two-layered wax patterned paper device was prepared using Whatman grade 1 qualitative filter paper, the top layer was treated with 1% w/v chitosan while the bottom layer served as an absorbent pad. DNA solution prepared in MES buffer pH 5.0 was flowed through the paper stack and a fluorescence image of each layer was recorded (n = 9 per layer). Figure 2.5A shows the mean intensity of each layer including the intensity measured from untreated paper wetted with the same volume of MES buffer to use as background signal. The ratio of background subtracted relative fluorescence intensity in the chitosan layer and the total fluorescence was used to estimate the capture efficiency in chitosan-modified paper. It was determined that the capture efficiency of filter paper modified with 1% (w/v) chitosan solution was 88.9%, which is comparable to previous reports using lateral flow to capture DNA.[40] Next, we studied the effect of elution buffer volume and buffer pH on the elution of DNA from the chitosan-modified filter paper using fluorescently labeled oligos. DNA captured using the method described above was then eluted using TBS buffer with varying volumes (3 μL – 6 μL) and pH (ranging from 7.5-9.0). Fluorescence intensity of the chitosan layer post-elution under different elution buffer conditions was plotted (Figure 2.5B). The data shows that DNA was retained in the chitosan layer when using elution buffer pH 8.0 or less while a significant improvement in elution was observed when using buffer pH ≥ 8.5. Increasing buffer volume above the saturation volume of the chitosan layer (3 μL) improved elution performance, with 6 μL removing nearly all DNA from the chitosan layer and returning the fluorescence to background levels.

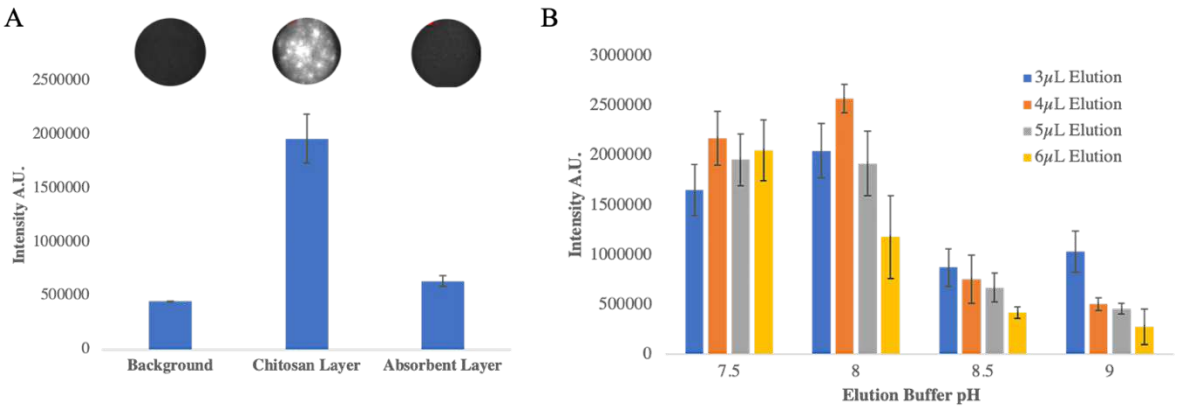

Figure 2.5. (A) The capture efficiency of 1% w/v chitosan-modified filter paper in the vertical flow format (n = 9 per layer). **(B)** The captured DNA was eluted using different elution buffer volume and pH. The fluorescence intensity in the chitosan layer pre- and post-elution was measured using a fluorescence microscope.

On-chip sample pretreatment

We created a simple vertical flow device using 1% chitosan-modified filter paper affixed to a PMMA support layer (Figure 2.6).

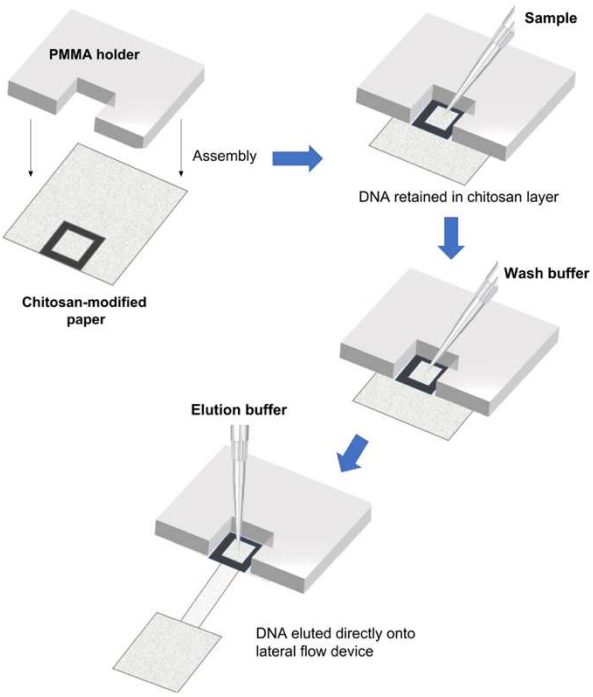

Figure 2.6. On-chip sample pretreatment device made of chitosan-modified filter paper affixed to a PMMA template: DNA from nuclease digested samples is captured in the chitosan layer at an acidic pH (pH ~5). The chitosan layer is then washed with a wash buffer (MES pH 5.0), followed by elution of the DNA directly onto the lateral flow device using an alkaline pH buffer (Tris pH 8.6).

We have integrated this sample pretreatment device with the PB-NPA to pretreat nuclease-digested samples and remove digoxigenin and other potential interferents from the reaction. Nuclease digested samples were flowed through the chitosan-modified filter paper, the nucleic acids from the sample are retained in the chitosan layer through electrostatic interaction while interfering compounds are washed to the absorbent waste pad. The captured DNA in the chitosan layer can then be eluted directly on to the lateral flow strip for detection using an alkaline pH elution buffer (pH ~8.6). Wax printing provides a hydrophobic barrier and ensures that all sample flows through the chitosan deposition zone.

Nuclease protection assays were run with varying target oligo amounts and 200 fmol of the NPA probe and then the samples were pretreated using the on-chip sample pretreatment method described above (n = 3 devices). Controls were run in triplicates without sample pretreatment, i.e., samples were applied directly to the inlet of the lateral flow strip (Figure 2.7). Limit of detection was calculated using mean blank + 3.3 × standard deviation. The limit of detection obtained for the control condition was 5.34 x 10^{-15} mol of target oligo DNA whereas with chitosan sample pretreatment, the LOD improved to 1.16 x 10^{-15} mol. The slope of the linear region (sensitivity) of the assay also improved using chitosan sample pretreatment (Avg. Intensity = 23.6 × ln(Target) + 858.25) compared to the control condition (Avg. Intensity = 21.5 × ln(Target) + 737.43). This on-chip pretreatment allowed for removal of free-label and other potential interferents from the reaction, improved the sensitivity and limit of detection of the PB-NPA, and eliminated the need for a nuclease enzyme inactivation step.

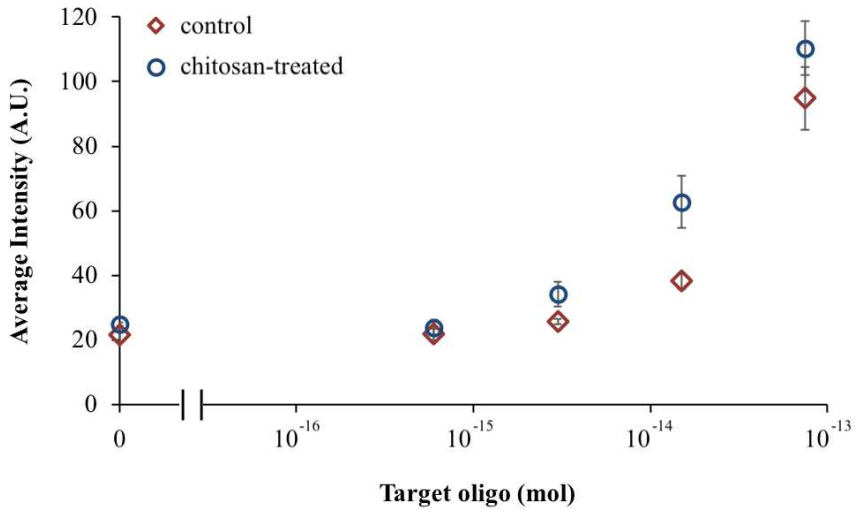

Figure 2.7. Colorimetric signals obtained for samples run with chitosan sample pretreatment (blue circle) and untreated controls (red diamond).

While the current format of the assay still relies on multiple manual steps that take a few hours to complete, further optimization by lyophilizing the NPA components for hybridization and

nuclease digestion in tubes and storing the detection reagents on the lateral flow strip can provide a rapid, simple, and easy-to-use assay with minimal user interaction. An inexpensive, portable resistive heater can be supplemented to the assay kits to allow for an easy and controlled assay at the point-of-care. To make the PB-NPA suitable for nucleic acid detection in real samples, sample preparation steps need to be incorporated to the test platform. A lysis/extraction buffer can be provided and combined with the chitosan-based sample pretreatment for the extraction and purification of nucleic acid targets from the sample matrices. Depending on the characteristics of the nucleic acid targets (e.g., the lengths, types, origins, and required specificity), the assay components/steps can be adjusted to meet the detection requirement. For example, a longer probe can be used to improve the assay specificity while targeting a class of pathogens with similar, shared traits may benefit from using a shorter probe sequence. Simultaneous detection of multiple pathogens is also possible with the PB-NPA as probes with different labeling molecules and antibodies to the labels can be obtained from commercial vendors. Despite the higher detection limit compared to other conventional techniques including PCR, PB-NPA can serve a good screening tool for infections in which a high viral load is present in the biological samples such as nasopharyngeal fluids in patients with acute adenovirus infection and patients with chronic infection of hepatitis B virus.[42,43]

Conclusions

These results demonstrate the proof-of-concept for the on-chip sample pretreatment capable lateral flow platform for end-point detection in nuclease protection assays. The PB-NPA can detect sub-femtomole of target DNA with extremely high specificity. In addition, our vertical flow on-chip sample pretreatment using chitosan-modified paper eliminated the need for a

nuclease inactivation step in the assay and further improved the detection limit by ~5 fold. The paper-based format allows for simplification of the assay compared to the traditional NPA including: (1) easy readout for minimally trained users based on the intensity of the colored lines, (2) lower reagent consumption and less waste generated, and (3) potentially improved assay time for point-of-care applications. The proposed assay only takes around 2-3 h to complete, which is substantially faster than the conventional culture-based method. The assay is also simpler to perform than conventional amplification-based nucleic acid tests, which often require amplification steps, multiple probes, enzymes, and expensive external readers. While the LOD is much higher than the amplification-based techniques, PB-NPA can serve a good screening tool for infections in which a high viral load is present in the samples.

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CHAPTER 3: PADLOCK PROBE-BASED ROLLING CIRCLE AMPLIFICATION LATERAL FLOW ASSAY FOR POINT-OF-NEED NUCLEIC ACID DETECTION

Chapter Overview:

Sensitive, reliable, and cost-effective detection of pathogens has wide ranging applications in clinical diagnostics and therapeutics, water and food safety, environmental monitoring, biosafety, and epidemiology. Nucleic acid amplification tests (NAATs) such as PCR and isothermal amplification methods provide excellent analytical performance and significantly faster turnaround times than conventional culture-based methods. However, the inherent cost and complexity of NAATs limit their application in resource-limited settings and the developing world. To help address this urgent need, we have developed a sensitive method for nucleic acid analysis based on padlock probe rolling circle amplification (PLRCA), nuclease protection (NP) and lateral flow detection (LFA), referred to as PLAN-LFA, that can be used in resource-limited settings. The assay involves solution-phase hybridization of a padlock probe to target, sequence-specific ligation of the probe to form a circular template that undergoes isothermal rolling circle amplification in the presence of a polymerase and a labeled probe DNA. The RCA product is a long, linear concatenated single-stranded DNA that contains binding sites for the labeled probe. The sample is then exposed to a nuclease which selectively cleaves single-stranded DNA, the double-stranded labeled probe is protected from nuclease digestion and detected in a lateral flow immunoassay format to provide a visual, colorimetric readout of results. We have developed specific assays targeting beta-lactamase resistance gene for monitoring of antimicrobial resistance and Severe

Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2, the novel coronavirus discovered in 2019) using the PLAN-LFA platform. The assay provides a limit of detection of 1.08 pM target DNA (or 1.3×10^6 copies/reaction). We also demonstrate the versatility and robustness of the method by performing analysis on DNA and RNA targets, and perform analysis in complex sample matrices like saliva, plant tissue extracts and bacterial culture without any sample pretreatment steps. This work was published in the Analyst journal.

Introduction:

Accurate, reliable and efficient pathogen detection methods are critical in lowering the global burden of communicable diseases.[1, 2] Conventional culture-based methods of pathogen detection have long turnaround times.[2] Increasingly nucleic acid amplification tests (NAATs) such as polymerase chain reaction (PCR) and isothermal amplification techniques have been employed for sensitive, sequence-specific pathogen detection. NAATs amplify the amount of target nucleic acids in the sample enabling detection using fluorescent or colorimetric methods. PCR, the most utilized NAAT, involves three main steps, i.e., denaturation, annealing and extension performed at three different temperatures requiring a thermocycler for precise temperature control and cycling.[3] PCR is also prone to inhibition from compounds present in unprocessed biological, environmental and food samples, requiring significant sample matrix-specific pre-processing steps.[4-7] Isothermal amplification techniques such as recombinase polymerase amplification (RPA), template-mediated amplification (TMA), helicase-dependent amplification (HAD), loop-mediated isothermal amplification (LAMP) and rolling circle amplification (RCA) perform nucleic acid amplification at a fixed lower temperature, therefore do not require a thermocycler.[3] Furthermore, isothermal techniques can be more robust and resilient than PCR to unprocessed samples, though they still require significant infrastructure, equipment and highly trained personnel to perform.[8, 9] Inexpensive, portable, accurate and reliable nucleic acid sensor platforms that can be used by minimally trained personnel are needed to lower the global burden of communicable diseases and improve global health.[3, 10]

Rolling circle amplification offers a simple and versatile method for nucleic acid amplification frequently employed in biosensing, sequencing, and cloning applications.[11, 12] RCA

uses a small primer sequence to amplify a circular DNA template in the presence of specialty polymerases (like phi29 or Bst DNA polymerase) and deoxynucleotides (dNTPs).[13] RCA can also be used to detect a short strand of nucleic acid using a circular primer in the presence of dNTPs and DNA polymerase. The DNA polymerases used in the RCA reactions possess both polymerase and strand displacement activity, and therefore the RCA product is a long, concatenated single stranded DNA with 100s to 1000s of tandem repeats.[14-17] The RCA product has a high molecular weight (100-1000× of the primers) and can be detected using gel electrophoresis or fluorescence or colorimetric methods.[11-13] Lateral flow assays offer a simple and cost-effective method for the visualization of results.[18] RCA has also been combined with lateral flow assays for equipment-free detection.[11, 13, 19, 20] Another popular variation of RCA is padlock rolling circle amplification, wherein, a linear DNA probe is specifically ligated in the presence of the target sequence forming a primed-circular template that can undergo rolling circle amplification in the presence of DNA polymerase.[21-27] We previously reported a nucleic acid sensor platform based on nuclease protection and lateral flow detection.[28] Nuclease protection or hybridization protection assay is a simple technique wherein post-denaturation and annealing of a labeled probe, the sample is exposed to a single-strand specific nuclease. The nuclease cleaves single-stranded DNA while perfectly hybridized dsDNA is protected from nuclease digestion. These protected probes can be detected using a lateral flow immunoassay format.[28] This assay lacked the sensitivity needed for clinical diagnosis of communicable diseases, though the platform offered a simple, and inexpensive method for nucleic acid analysis.[28]

To improve the sensitivity of the assay we have developed a padlock rolling circle amplification, nuclease protection and lateral flow detection (PLAN-LFA) (Figure 3.1).

Combining padlock rolling circle amplification with our paper-based nuclease protection assay gave a 1000-fold improvement in the limit of detection of the assay.[28] The PLAN-LFA assay provides 1.08 pM target DNA limit of detection in 3 hours of analysis time and can be used to develop point-of-need nucleic acid sensor platforms. PLAN-LFA can be used for the detection of pathogens (human, animal, or plant pathogens) and infectious disease diagnosis, to monitor the spread of antimicrobial resistance, and monitoring of nucleic acid biomarkers like miRNA. We have developed specific assays for beta-lactamase resistance gene detection to enable environmental monitoring of antimicrobial resistance, and for the detection of SARS-CoV-2.

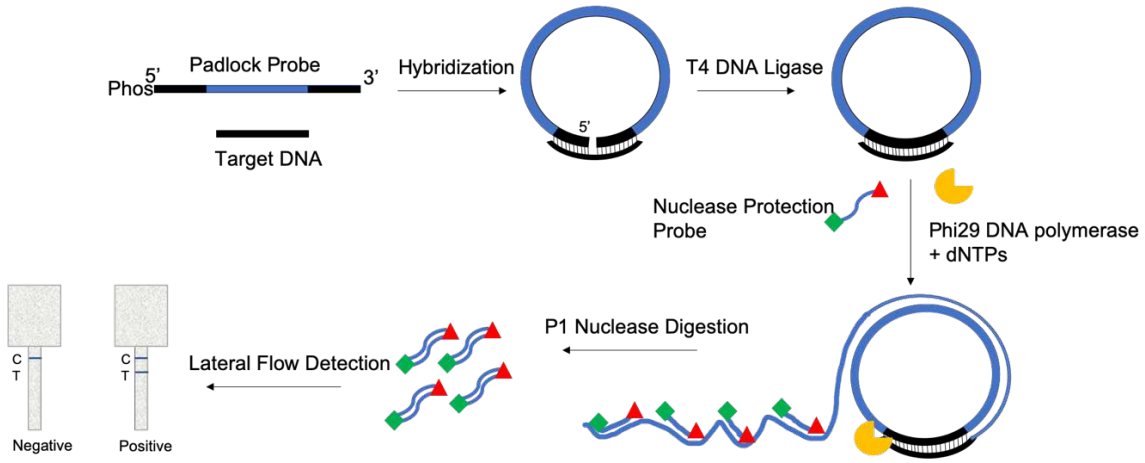

Figure 3.1 Padlock Rolling Circle Amplification Nuclease Protection Lateral Flow Assay (PLAN-LFA) Schematic Padlock probe hybridization with target DNA oligo enables sequence specific ligation of the 5' and 3' end of the padlock probe by T4 DNA ligase. The target-primed circular template undergoes rolling circle amplification in the presence of Phi29 DNA polymerase. The resulting ssDNA product binds the labeled nuclease protection probe. Upon P1 nuclease digestion, the protected labeled NPA probes are detected in an immunoassay format.

The primer design for padlock rolling circle amplification is simpler than other isothermal amplification methods like LAMP.[12] The assay can be performed in a variety of sample matrices, such as serum, urine, saliva, plant extract, without the need for sample pretreatment making it an attractive platform for development of point-of-need nucleic acid sensors.

Materials and Methods:

All DNA sequences used in the development of the PLAN-LFA assay shown in Table 3.1 were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). T4 DNA ligase, adenosine triphosphate (ATP), P1 nuclease (from P. citrinum), Phi29 DNA polymerase and deoxynucleotides were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). The supplied buffers were used for each of the ligation, amplification, and nuclease protection steps of the assay. Nitrocellulose (FF120HP) and Whatman Grade 4 filter paper were purchased from GE Lifesciences (Pittsburgh, PA, USA). Streptavidin conjugated to Horseradish Peroxidase (Strep-HRP) was purchased from R&D Systems, Inc. (Minneapolis, MN). Pierce™ 1-Step Ultra TMB-Blotting Solution was purchased from Thermo Scientific (Waltham, MA). Anti-digoxigenin monoclonal antibody was purchased from Abcam, Inc (Cambridge, MA). StabilGuard® Immunoassay Stabilizer (BSA-free) was purchased from Surmodics, Inc. (Eden Prairie, MN). Trehalose dihydrate was purchased from EMD Millipore (Burlington, MA). Glycerol was purchased from Mallinkrodt (Staines-upon-Thames, United Kingdom).

Table 3.1: Oligonucleotide padlock probe and primers used for PLAN-LFA

Sequence Name	DNA sequence (5' – 3')
Padlock Probe (Beta-lactamase resistance)	5'Phosphate/CAACGATCAAGGCGAGAGATTTAGGTGACACTATAGCGCCCCTATAGTGAGTCGTATTAGCGTTCAGCTCCGGTTCC
Beta-lactamase resistance target	5'/CATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATAC/3'
Nuclease Protection Probe 1	5'biotin/GATTTAGGTGACACTATAG/3'Digoxigenin
Padlock probe (SARS-CoV-2)	/5'Phosphate/GGGCAAATTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTGAAGCGCTGG
SARS-CoV-2 Target	5'/TTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGC/3'
Nuclease Protection Probe 2	/5'Biotin/AAAAAAAAAAAAAAAAAAAA/3'Digoxigenin

PLAN-LFA Assay

In a 200 µL centrifuge tube, 2 µL of 10X ligation buffer, 5 µL of 100 nM Padlock Probe, 2 µL of varying concentration of target DNA and 11 µL of nuclease-free water was added. The sample was denatured at 95°C for 2 min and hybridization was performed at 37°C for 10 min. After hybridization, 2 µL of 10 mM adenosine triphosphate (ATP) and 20U of T4 DNA ligase were added to the reaction. Ligation was performed at 37°C for 30 min. After ligation, 3 µL of 10X Phi29 buffer, 0.5 µL of 10 mM dNTP mix, 0.5 µL of 10 mg/mL bovine serum albumin (BSA) and 20U of Phi29 DNA polymerase were added to the reaction. Rolling circle amplification was

performed at 30°C for 2 hours. 2 μL of 100 nM nuclease protection probe was added to the reaction post amplification, and the sample was denatured at 95°C for 2 min and annealed at 37°C for 10 min. Finally, 4 μL of 10X P1 nuclease buffer, and 20U of P1 nuclease were added to the reaction and the reaction was incubated at 37°C for 20 min. 10 μL of the reaction were pipetted on to the lateral flow strip inlet, followed by wash buffer (100 mM Tris buffer saline + 0.05% Tween20), 10 μL of 1:200 diluted strep-HRP enzyme, 10 μL of wash buffer, and 20 μL of 1-step Ultra TMB blotting solution. After the development of the colorimetric signal, the lateral flow strips were scanned using an Epson Perfection V600 scanner. ImageJ (open-source image processing software, National Institute of Health) was used to analyze the images and quantitate the colorimetric signals.

Assay Feasibility

The PLAN-LFA assay was run in the presence of all the necessary components, and without either the padlock probe or target DNA or T4 DNA ligase or the Phi29 DNA polymerase to show that the assay does not produce non-specific signal in the absence of one of the constituents. 5 μL of the undigested samples were run on a 1% agarose gel in duplicates to analyze the results of the amplification reaction. Gel electrophoresis was run in 0.5X Tris Acetate EDTA buffer, at 120V for 30 minutes. Nuclease digested and undigested samples were also run on the lateral flow strips along with a negative control (no Target DNA).

Analytical Performance

Different concentrations (100 nM, 1 nM, 10 pM, 100 fM, 1 fM) of target DNA solutions were prepared in DI water along with negative controls (no target and non-target (random) DNA of

equal length as the target). 2 μL of each of these solutions were added to the PLAN-LFA reactions. Assay limit of detection was calculated using mean blank + 3.3*standard deviation. All samples were tested in triplicate. The lateral flow strips were scanned after completion of the test. The color intensity of the developed signal was quantified using ImageJ.

Matrix Effects

Human saliva was collected from a healthy volunteer into a sterile tube and spiked with the target oligonucleotide. PLAN-LFA were run with positive (10 pM SARS-CoV-2 target) and negative (no target) samples in undiluted human saliva as per the procedure outlined above. Target and no target samples prepared in 1X PBS were used as controls for the experiment. The colored signal obtained in the paired saliva and buffer positive and negative samples were compared to determine the effect of sample type on assay performance. To prepare cacao plant extract, five-0.5 cm cacao leaf punches were added to 1 mL PBS and heated at 98°C for 5 min. The extract was cooled to room temperature, aliquoted and stored at –20°C until use. Positive (10pM beta-lactamase resistance target oligo) and negative (no target) samples were prepared in leaf extract and buffer; and tested using the assay protocol described above. The color signal obtained from leaf extract and buffer samples was compared to determine the effect of plant extract on assay performance.

RNA detection using PLAN-LFA

The beta-lactamase padlock probe is designed to bind a sequence in the beta-lactamase resistance gene, and therefore also binds the mRNA expressed by ampicillin resistant cells in the presence of the antibiotics. To demonstrate that the PLAN-LFA assay can be used to detect a specific target RNA, we performed the assay on ampicillin-resistant cells and chloramphenicol resistant cells

grown in the presence of ampicillin and chloramphenicol respectively. Briefly, *E. coli* (XL10-Gold) cells were transformed with pUC19 plasmid DNA (carries ampicillin resistance gene) by heat shocking the cells at 42°C. 20 μL of transformed cells were added to 5 mL of LB broth along with 5 μL of 50 mg/mL Ampicillin. The cells were grown overnight in a shaker incubator set to 37°C. *E. coli* (BL-21 (DE3) pLysS) cells with chloramphenicol resistance grown overnight in the presence of chloramphenicol was used as a negative control. Buffer samples spiked with ssDNA target oligo and no target controls were used as controls for the PLAN-LFA assay performance. Chloramphenicol resistant culture was also spiked with ssDNA target oligo was used to control for sample matrix effects arising from the presence of bacterial culture. All samples were tested in triplicates. No changes were made to the PLAN-LFA assay protocol for mRNA detection. To further support our claim that the PLAN-LFA assay can be utilized for RNA targets, we used the SARS-CoV-2 padlock probe to analyze SARS-CoV-2 genomic RNA. SARS-CoV-2 RNA (extracted from plaque cultures using Trizol extraction method) was obtained from Brian Geiss lab (stock concentration: $2.6*10^{10}$ copies/mL). Different concentrations of the SARS-CoV-2 RNA along with appropriate controls were tested using the PLAN-LFA assay protocol. The mean intensity of blank + 3.3*sigma was used to calculate the limit of detection of the assay.

Results and Discussion

Beta-lactamase resistance genes and SARS-CoV-2 were used as model sequences to demonstrate PLAN-LFA assay feasibility and characterize assay performance. Padlock probe was designed such that the 5’ and 3’ ends of the probe are complementary to the target sequence; these ends of the probe are referred to as hybridization arms. The middle region of the padlock probe is referred to as the padlock probe backbone is designed such that the RCA product that is generated contains

a region complementary to a biotin-and-digoxigenin-labeled nuclease protection probe, referred to as nuclease protection probe or NPA probe. A second padlock probe was designed based on a poly-adenine backbone sequence and targeting the novel coronavirus discovered in 2019 (SARS-CoV-2).[29] Spacer sequences were added between the padlock probe hybridization arms and padlock probe backbone regions to minimize probe secondary structure formation, which is known to interfere with efficient ligation and rolling circle amplification.[30, 31]

Assay Feasibility

After determining the melting temperature and appropriate annealing temperature for the padlock probe and target sequences, assay feasibility was determined by performing the PLAN-LFA reactions in the presence of all assay components. Control reactions were run in the absence of one of the assay components. The samples were tested on a 1% agarose gel (0.5X Tris Acetate EDTA running buffer, 120V for 35 min) in duplicate wells (Figure 3.2A). The RCA product is a large, single-stranded concatenated DNA which tends to remain in the well of the gel. RCA product is only observed in the Test condition (T) which includes all assay components. No RCA product was observed in the absence of the padlock probe (C1) or target DNA oligonucleotide (C2) or T4 DNA ligase (C3) or Phi29 DNA polymerase (C4). Nuclease protection probe was added to each of the target (T) and no target control conditions followed by P1 nuclease digestion of the samples. These samples were tested on lateral flow strips as per previously published protocol.[28]

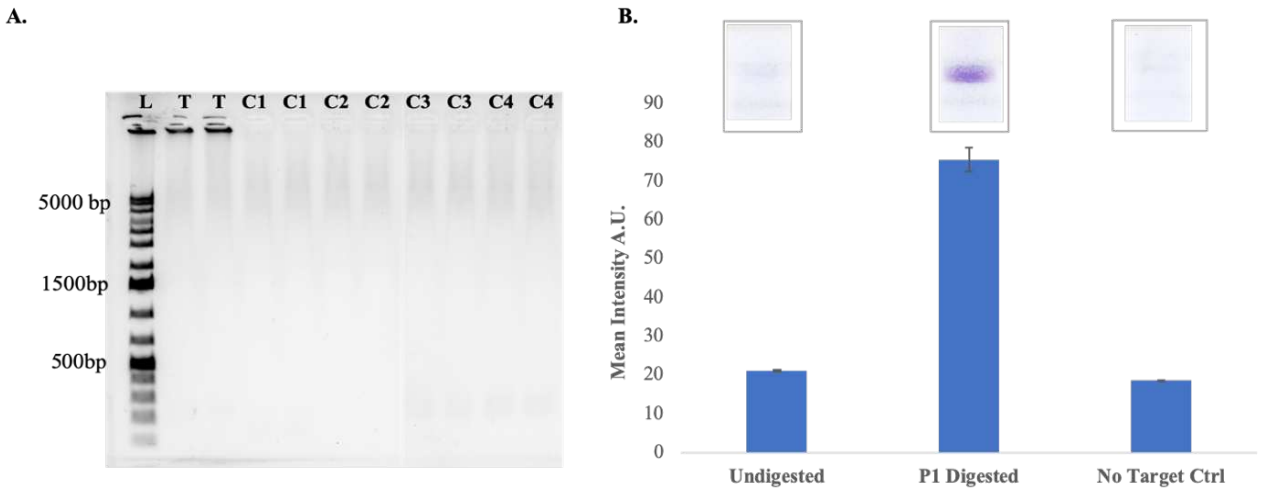

Figure 3.2 PLAN-LFA Assay Feasibility A. Gel electrophoresis data showing large molecular weight RCA product is generated in the presence of all assay components (T) which remains in the well of the gel. No product is observed in the absence of the padlock probe (C1) or Target oligo (C2) or T4 DNA ligase (C3) or Phi29 DNA polymerase (C4). **B.** P1 nuclease digested, and undigested samples were run on lateral flow strips (n =3).

The lateral flow strips (Figure 3.2B, upper) show that strong signal is produced when the samples are P1 nuclease digested while the undigested sample or no target control samples do not produce a signal. Given that the RCA product is a large, concatenated DNA, it is likely that the nuclease protection probe bound to its target site on the RCA product is unable to flow through the pores of the nitrocellulose membrane or the digoxigenin label on the nuclease protection probe is unavailable to the anti-digoxigenin antibody on the detection region of the strip. Upon P1 nuclease digestion, the protected NPA probe-hybrid becomes available for binding on the detection region. No signal is observed in the absence of the target DNA oligonucleotide indicating that the assay is specific to the target sequence.

Analytical Performance

After showing that the PLAN-LFA was feasible, we studied the analytical performance of the assay. Sensitivity analysis was performed by running the assay in the presence of different amounts

of target oligo (Figure 3.3A). Assay specificity was studied by performing reactions in the presence and absence of the target oligo and in the presence of a non-target oligonucleotide of similar length as the target.

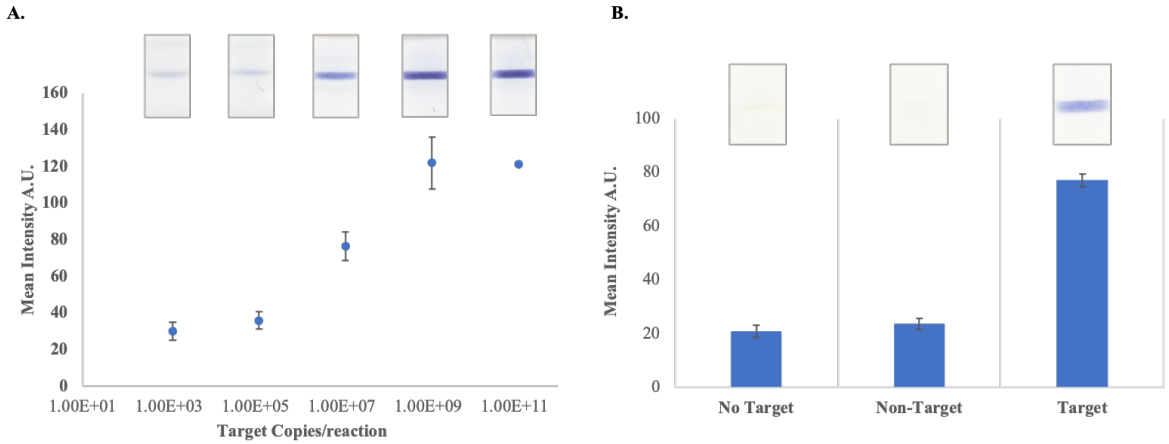

Figure 3.3 PLAN-LFA Assay Performance A. Assay sensitivity was studied in the presence of increasing amount of target oligonucleotides. The color response observed for each sample is shown in the images above each data point. (n=3 per sample) **B.** Specificity analysis was performed by running the assay in the absence of target oligo and in the presence of non-target and target oligonucleotides. (n=3 per sample)

The limit of detection of the assay was 1.08 pM (2.16×10^{-18} moles or 1.3×10^{6} copies) of target oligo, which is 20-40 fold improvement over other reported methods using linear rolling circle amplification and lateral flow detection.[32] The assay also provides excellent specificity as no target and non-target conditions do not produce a signal, while strong colorimetric response is observed in the presence of target oligo (Figure 3.3B). These results indicate that the assay provides reasonable assay performance and can be applied for the detection of pathogenic nucleic acids in a simple, cost-effective assay that can be used by minimally trained personnel as it only requires a few reagent additions and visual colorimetric detection.

Sample Matrix Effects

PCR can often produce false-negative results due to PCR inhibition caused by complex biomolecules present in samples. For example, polyphenolic compounds present in plant tissues are known inhibitors of PCR, and therefore detection of plant pathogens by PCR often requires laborious and costly sample pretreatment steps.[33] To understand the effect of sample matrices on the performance of the PLAN-LFA assay, we performed analysis in saliva and cacao leaf extract. SARS-CoV-2 positive (10pM) and negative samples were prepared by spiking saliva collected from a healthy individual, while positive beta-lactam resistance (10pM) and negative samples were prepared by spiking cacao healthy leaf extract. The color intensity of the positive samples was compared to the positive sample in buffer to determine if PLAN-LFA was affected by the presence of the complex sample matrix. Figure 3.4A shows that the assay performance in saliva was nearly identical to the performance in 1X PBS buffer samples with less than 10% decrease in signal intensity compared to the buffer control.

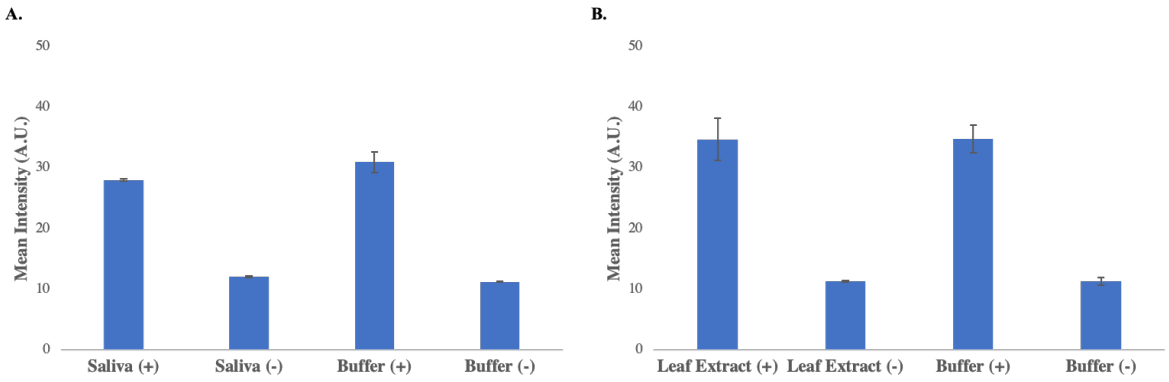

Figure 3.4 Sample Matrix Effects: A. Positive (10pM) and negative samples prepared in saliva and buffer were tested using the PLAN-LFA assay protocol (n = 3 per sample). **B.** Positive (10pM) and Negative samples prepared in cacao leaf extract and buffer were tested using the PLAN-LFA assay protocol (n=3 per sample).

Figure 3.4B shows that the assay performance was unaffected by the presence of cacao leaf extract. The negative samples were free of any background signal indicating that the presence of

these complex sample matrices does not affect produce a false-positive response in the PLAN-LFA assay.

RNA Detection using PLAN-LFA

To demonstrate the versatility of the PLAN-LFA assay, we performed analysis on SARS-CoV-2 genomic RNA using the SARS-CoV-2 padlock probe. T4 DNA ligase can ligate DNA-RNA hybrids, and therefore no changes to the protocol or components were necessary to detect RNA targets. Different concentrations of purified SARS-CoV-2 RNA were prepared in water and tested using the protocol described in the methods section. A limit of detection of approximately $1.78*10^5$ copies/mL of SARS-CoV-2 RNA was obtained using the mean intensity of the blank + 3.3* sigma (Figure 3.5A). We also tested bacterial cultures of ampicillin resistant cells and chloramphenicol resistant cells were tested using the beta-lactam padlock probe. Figure 3.5B shows the mean intensity obtained from the lateral flow strips run with different samples. The ampicillin resistant cells produced a positive signal while chloramphenicol resistant culture did not produce a positive signal. The ssDNA oligo spiked (10 pM) in chloramphenicol resistant culture gave a strong color response indicating that the PLAN-LFA assay is unaffected by the presence of bacterial culture.

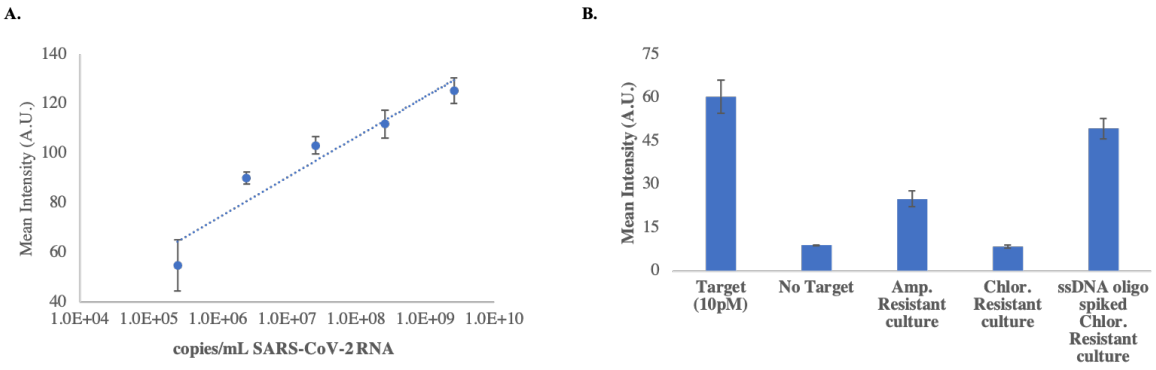

Figure 3.5 RNA detection using PLAN-LFA: A. Dose-response with purified SARS-CoV-2 RNA. Using blank + 3.3* sigma a limit of detection of approximately $1.78*10^5$ copies/mL was obtained. **B.** PLAN-LFA performed on bacterial cultures of *E. coli* transformed with ampicillin resistance (test condition) and chloramphenicol resistance (negative control). ssDNA oligo target positive (10 pM) and negative samples were used to control for assay performance. The ssDNA oligo spiked in chloramphenicol resistant culture was used to control for sample matrix effects.

Though the signal intensity obtained with the ampicillin resistant culture was fainter than expected, the clear difference between positive and negative cultures indicates that the PLAN-LFA assay can be used to detect RNA of interest. It should also be noted that no changes were made to the assay protocol to perform RNA detection. Assay performance for RNA detection can be improved by altering the assay protocol specifically for RNA targets by optimizing denaturation and hybridization conditions, along with the use of other ligases (T2 and T4 RNA ligase) that may improve ligation efficiency for RNA targets.[25, 34]

Conclusions

We describe a nucleic acid sensor platform based on padlock probe rolling circle amplification, nuclease protection and lateral flow detection. The assay provides a visual colorimetric readout of results with the appearance of a test line if the sequence of interest is present in the sample. Semi-quantitation of the copy number is possible by performing image analysis on a photograph or scan of the lateral flow test strip further improving the utility of the test. The assay provides picomolar sensitivity ($1.3*10^6$ copies/reaction) and can be used to detect both DNA and RNA targets with

excellent specificity. We demonstrated assay feasibility by developing specific assays for SARS-CoV-2 and beta-lactamase resistance genes. The PLAN-LFA assay can be completed within 3 hours, making it an attractive platform for point-of-need nucleic acid sensing applications. We also demonstrate assay robustness by performing nucleic acid analysis in complex sample matrices like saliva, plant extract and bacterial cultures without the need for any sample pretreatment steps. The probe design is simplified in PLAN-LFA compared to other isothermal amplification methods such as LAMP, as the padlock probe hybridization arms are complementary to the target sequence. Alternative targets can be assayed by simply changing the hybridization arm sequences, while a common padlock probe backbone region such as the poly-adenine sequence can be used to design probes. The assay sensitivity, turnaround time and ease-of-use would need to be further improved to make it more applicable for point-of-need nucleic acid sensing applications. Using exponential mode RCA or hyperbranched RCA can significantly improve both assay sensitivity and turnaround times.[35] The lateral flow strip can be made into a sequential delivery microfluidic device to improve the sensitivity and ease of use of the assay.

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CHAPTER 4: EXPONENTIAL PADLOCK ROLLING CIRCLE AMPLIFICATION

Chapter overview:

As discussed in the previous chapters, rolling circle amplification (RCA) is one of the most versatile isothermal amplification techniques with several known modes of operation, i.e., linear, exponential and hyperbranched RCA. In continuation of our work on rolling circle amplification, this chapter discusses the use of exponential rolling circle amplification to improve the limit of detection of the Padlock probe-based rolling circle amplification lateral flow assay (PLAN-LFA). The linear PLAN-LFA assay gave a limit of detection of 1.08pM ($1.3*10^6$ copies/reaction) and therefore is not sensitive enough for most point-of-need infectious disease diagnosis. To perform exponential amplification, a nickase enzyme recognition site was added to the padlock probe. Exponential amplification reaction starts with hybridization of the padlock probe to the target, followed by sequence-specific ligation using T4 ligase to generate the circular template like the linear RCA reaction. The circular template is amplified using Phi29 DNA polymerase in the presence of Nb.BbvCI nickase enzyme, which cleaves the newly synthesized amplicon strand to free up a target sequence region and a region containing a polyT segment complementary to the polyA nuclease protection probe. The target sequence region can bind additional padlock probes and initiate exponential amplification. Following nuclease protection with P1 nuclease, the nuclease protected probes can be detected in a lateral flow format similar to the linear PLAN-LFA assay.

In this work, end-point fluorescence was measured using SyBr Gold intercalating dye in a well plate reader to demonstrate the feasibility of the nickase-assisted exponential rolling circle

amplification. We have developed nickase assisted exponential rolling circle amplification assay for the detection of (severe acute respiratory syndrome coronavirus 2, the novel coronavirus discovered in 2019 (SARS-CoV-2)). The assay provides a limit of detection of approximately 0.01 pM (or $1.2*10^4$ copies/reaction) which is an 100X improvement over the linear RCA method.

Introduction:

Ultrasensitive nucleic acid detection methods that can be utilized at the point-of-need, especially in resource-limited settings and low-and-middle income countries (LMICs), could significantly improve global health and help lower the global burden of disease.[1] PCR is one of the most utilized nucleic acid amplification technique though it requires extensive sample preparation, thermocycler for precise temperature control and cycling, and complex detection equipment which limit its application in infectious disease diagnostics in the developing world.[1-3] Isothermal amplification methods like LAMP, RPA, RCA, SDA and HDA have been proposed as alternatives to PCR, though very few platforms have reached the market.[4, 5] Rolling circle amplification is one of the simpler and more versatile amplification techniques as it only requires one primer and a strand-displacing DNA polymerase to amplify a circular template.[6, 7] Linear sequences can be detected using padlock RCA wherein a linear probe is specifically ligated in the presence of the target sequence to form the circular template that subsequently undergoes rolling circle amplification.[8-11] In the previous chapter, we reported a nucleic acid sensor platform based on padlock probe rolling circle amplification, nuclease protection and lateral flow detection. This method employs linear rolling circle amplification mode and provides picomolar detection limits (or $1.2*10^6$ copies/reaction) which is insufficient for POC infectious disease diagnostic applications. In order to improve the PLAN-LFA assay sensitivity, we used a restriction enzyme (Nb.BbvCI nickase) to perform exponential mode rolling circle amplification.[12] The padlock probe was altered to include the complement of the recognition site for Nb.BbvCI nickase. Nb.BbvCI is a nicking endonuclease which cleaves only one strand of a double stranded DNA substrate. The exponential padlock RCA reaction proceeds as the linear PLAN-LFA assay with hybridization to target followed by ligation and linear rolling circle amplification. The amplification reaction contains the nickase enzyme

which cleaves the newly synthesized amplicon strand to produce a polyT segment that binds the polyA nuclease protection probe, while the target segment binds additional padlock probes initiating exponential amplification of target (Figure 4.1). Following nuclease protection with P1 nuclease, the double-stranded NPA probes can be detected in a lateral flow format similar to the PLAN-LFA assay. Using the nickase-assisted exponential padlock RCA method (ePLRCA), we have developed a SARS-CoV-2 assay which provides 0.01pM (or $1.2x10^4$ copies/reaction) limit of detection, which is a 100X improvement over the linear RCA method.

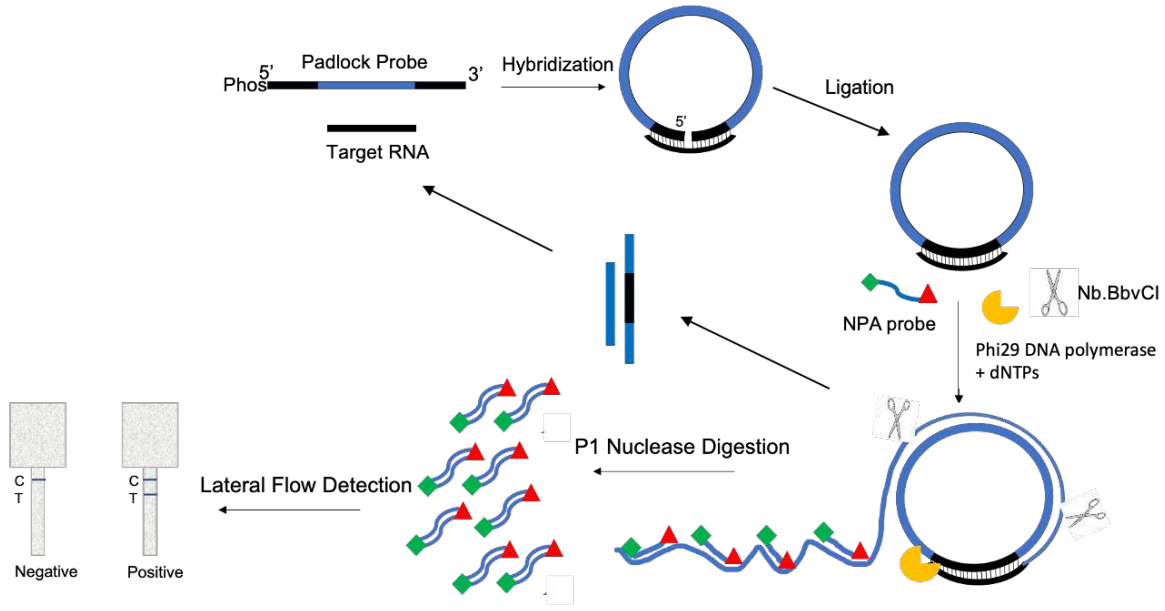

Figure 4.1: Nickase-assisted exponential padlock rolling circle amplification lateral flow assay schematic. The reaction proceeds as the linear PLAN-LFA assay with hybridization to target followed by ligation and linear rolling circle amplification. The amplification reaction contains the nickase enzyme which cleaves the newly synthesized amplicon strand to produce a polyT segment that binds the polyA nuclease protection probe, while the target segment binds additional padlock probes initiating exponential amplification of target. Following nuclease protection with P1 nuclease, the double-stranded NPA probes can be detected in a lateral flow format similar to the PLAN-LFA assay.

Materials and Methods:

All DNA sequences used in the development of the PLAN-LFA assay, shown in Table 4.1, were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). T4 DNA ligase, adenosine triphosphate (ATP), P1 nuclease (from *P. citrinum*), Phi29 DNA polymerase, Nb.BbvCI nickase enzyme (*Bacillus brevis*), SyBr® Gold and deoxynucleotides were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). The supplied buffers were used for each of the ligation, amplification, and nuclease protection steps of the assay.

Table 4.1: Padlock probes and primers used in the development of ePLRCA

Sequence Name	DNA sequence (5' – 3')
Padlock Probe*	5'Phosphate/GTAATGCGGGGCCTCAGCAAAAAAAAAAAA AAAAAAAAAAAAAAAAAACCTCAGCGTCCACCAAAC
Target	5'/CCCCGCATTACGTTTGGTGGAC/3'
Nuclease Protection Probe	/5'Biotin/AAAAAAAAAAAAAAAAAAA/3'Digoxigenin

***The nickase recognition site is shown in red.**

Assay Feasibility and Amplification Time

To determine assay feasibility, amplification reactions were run in the presence and absence of nickase enzyme. In a 200 µL centrifuge tube, 2 µL of 10X ligation buffer, 5 µL of 100 nM Padlock Probe, 2 µL of varying concentration of target DNA and 11 µL of nuclease-free water were added. The sample was denatured at 95°C for 2 min and hybridization was performed at 37°C for 10 min.

After hybridization, 2 µL of 10 mM adenosine triphosphate (ATP) and 20U of T4 DNA ligase were added to the reaction. Ligation was performed at 37°C for 30 min. After ligation, 3 µL of 10X Phi29 buffer, 0.5 µL of 10 mM dNTP mix, 0.5 µL of 10 mg/mL bovine serum albumin (BSA) and 20U of Phi29 DNA polymerase and 5U Nb.BbvCI nickase were added to the reaction. Reactions were in the presence and absence of nickase to determine the contribution of nickase to the amplification reaction. Control reactions were run with no target oligo and in the presence of a non-target oligo of similar length as the target. Rolling circle amplification was performed at 30°C for 24 hours. End-point fluorescence was measured in each reaction condition using SyBr Gold® nucleic acid stain (excitation: 495nm emission: 537nm) in a well plate reader. To determine amplification time, 10µL of the reaction volume was mixed with SyBr® gold at 2 hours, 4 hours, and 24 hours. All reactions were run in triplicates.

Analytical Performance:

To determine assay sensitivity and specificity, varying concentrations of the target DNA were tested with the ePLRCA well plate assay along with no target and non-target controls. All reactions were run in triplicates.

SARS-CoV-2 RNA Detection:

SARS-CoV-2 RNA was tested using the ePLRCA protocol by our collaborators at Edoceo Inc. (Fort Collins, CO). No target and non-target (Kunjin virus) controls were run. All reactions were run in triplicates.

Lateral Flow Detection:

Following assay feasibility studies performed on well plate analyzers, we tested the ePLRCA on the lateral flow platform to determine if lateral flow detection of exponential RCA amplicons was feasible. 2 µL of 100 nM nuclease protection probe was added to the reaction post amplification, and the sample was denatured at 95°C for 2 min and annealed at 37°C for 10 min. Finally, 4 µL of 10X P1 nuclease buffer, and 20U of P1 nuclease were added to the reaction and the reaction was incubated at 37°C for 20 min. 10 µL of the reaction were pipetted on to the lateral flow strip inlet, followed by wash buffer (100 mM Tris buffer saline + 0.05% Tween20), 10 µL of 1:200 diluted strep-HRP enzyme, 10 µL of wash buffer, and 20 µL of 1-step Ultra TMB blotting solution. After the development of the colorimetric signal, the lateral flow strips were scanned using an Epson Perfection V600 scanner. ImageJ (open-source image processing software, National Institute of Health) was used to analyze the images and quantitate the colorimetric signals.

Results and Discussion:

After determining the optimal annealing temperature and annealing times for the padlock probe and selecting a probe sequence that does not produce significant background signal (data not shown), we performed a feasibility study to determine if the ePLRCA reaction performs as described above. Reactions with target, no target and non-target sequences were run in the presence and absence of nickase to determine the contribution of nickase to the amplification reaction. Figure 4.2A shows that significantly higher yield of DNA amplicon is achieved in the presence of nickase. The high yield in the negative control shows that the amplification reaction at 24 hours produced non-specific results. The linear RCA reaction (no nickase) does not produce signal even with the target condition. This experiment showed that even though the response was non-specific,

the use of nickase was feasible in achieving exponential amplification. After determining feasibility, amplification time was determined by taking an aliquot of the reactions and measuring the SyBr® gold counts at 2, 4, and 24 hours. Figure 4.2B shows that specific amplification is achieved at 4 hours, while the assay specificity is lost when allowed to incubate for 24 hours. From this data set, it was determined that the highest signal is achieved at 4 hour incubation time, and therefore was selected as the incubation time in subsequent experiments.

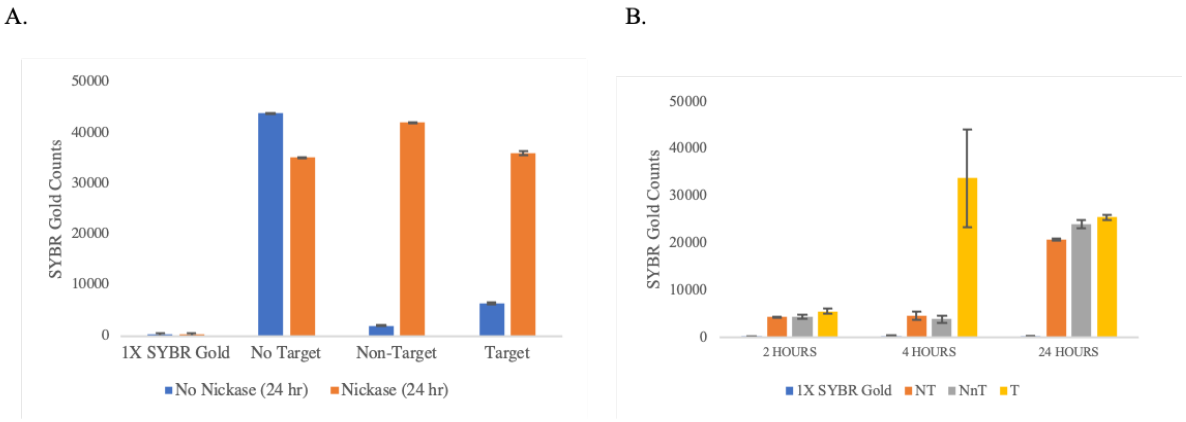

Figure 4.2: ePLRCA assay feasibility and amplification time A. Assay feasibility No Target, non-target and Target sequences were tested with the ePLRCA protocol in the presence and absence of nickase. **B. Amplification time** was determined by running ePLRCA reactions with the target, no target and non-target sequences and measuring SyBr gold counts at 2 hours, 4 hours, and 24 hours. (n = 3 per condition)

The ePLRCA assay analytical sensitivity and specificity were evaluated using varying concentrations (0, 0.001, 0.01 and 1pM) of the target (T) sequence along with no target (NT) and non-target (NnST) controls. The data shows that the assay provides approximately 0.01pM detection limits (or 1.2 x 10^4 copies/reaction), with 4 hours incubation time (Figure 4.3). The data also shows that the assay can at times give non-specific amplification as was observed in the case of 0.01pM and 0.001pM non-target DNA conditions.

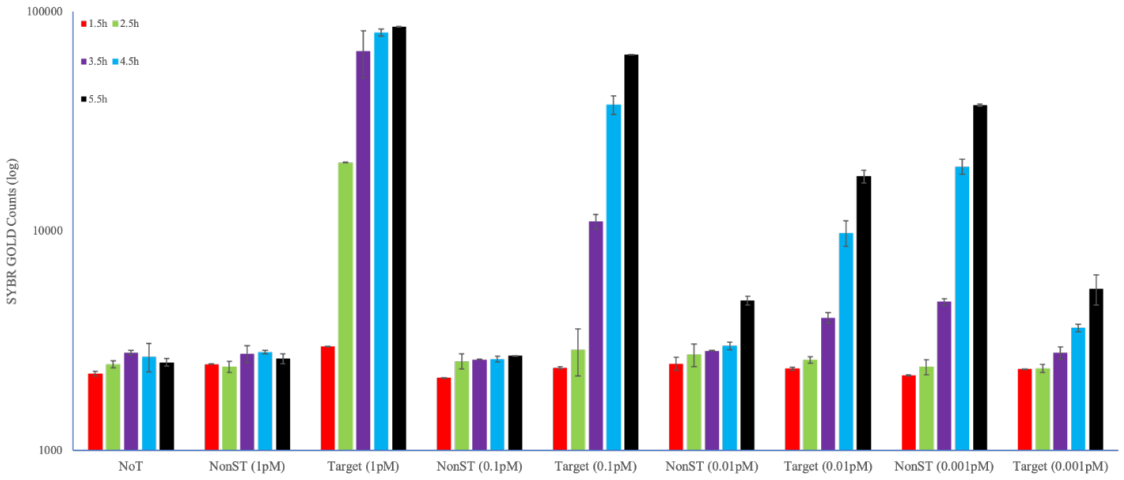

Figure 4.3: ePLRCA assay analytical performance The assay provides approximately 0.01 pM target DNA detection limit.

Through our collaboration with Edoceo Inc, we have shown that the assay can be used to specifically detect SARS-CoV-2 RNA. These experiments were performed by Dr. Devon Osbourne at Edoceo Inc, though they are included here to show that the assay can be used for real viral genomic RNA (Figure 4.4).

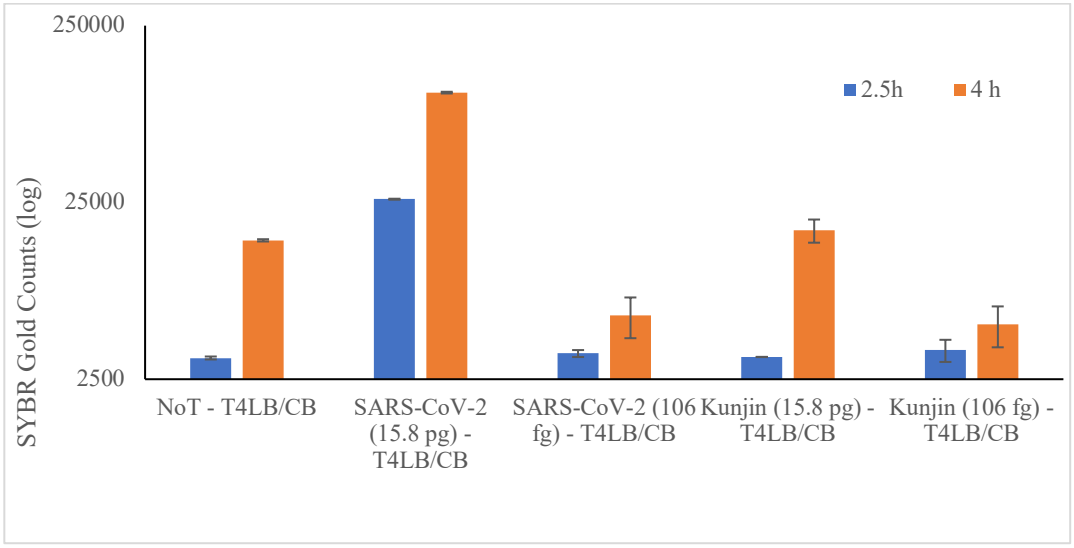

Figure 4.4: SARS-CoV-2 RNA detection using exponential padlock RCA

Several attempts were made at performing lateral flow detection of the ePLRCA reactions (data not shown). However, no signal developed on the positive samples indicating that there was not sufficient protection of the NPA probe. After running a series of controls ensuring that the lateral flow strips performed as expected, it was concluded that either the amplification reaction does not produce the polyT sequence as expected or the segment binds to the padlock probe instead of the NPA probe. Further experiments would be needed to investigate the root cause of the lack of signal on the lateral flow strips. Given the long incubation time (~4 hours) needed to achieve exponential amplification, we have concluded that the nickase-assisted exponential reaction is not conducive to the development of point-of-need NA sensor platforms. Hyperbranched RCA, which utilizes an extra primer to initiate exponential amplification has been shown to provide detection limits on par or exceeding that of PCR within 90 minutes of total analysis time.[13-15] Future work could involve the use of hyper-branched RCA followed by lateral flow colorimetric detection methods.

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CHAPTER 5: MICROFLUIDIC DEVICE FOR ROLLING CIRCLE AMPLICON DETECTION

Chapter Overview:

In this chapter, we discuss the early-phase development of a microfluidic device for the rapid detection of rolling circle amplification (RCA) reaction products. The device is constructed out of low-cost substrates like thin polymer films and double-sided adhesive to create channels that connect to a nitrocellulose strip and an absorbent pad. The flow is driven by capillary forces and enables programmable sequential delivery of reagents to the detection region on the nitrocellulose. The device henceforth referred to as Capillary action driven immunoassay device or CaDI device, reduces the number of user addition steps needed to perform the PLAN-LFA assay from eight to two, improving the ease-of-use and applicability in resource-limited settings. We have shown device feasibility by performing an abbreviated assay using the nuclease protection probe alone to simulate the RCA amplicon. The assay involves user addition of the colorimetric substrate to the substrate inlet and the RCA amplicon to the sample inlet. Glass fiber pads treated with streptavidin-conjugated horseradish peroxidase (Strep-HRP) are placed in the device channels. Upon addition of the two reagents, the RCA amplicon flows through the nitrocellulose, followed by Strep-HRP released from the glass fiber pads, and the colorimetric substrate developing the colored signal for qualitative visual detection of the RCA reaction. The device provides 5 pM detection limit which is comparable to the manual PLAN-LFA method within 20 min and requires only two user steps which is a significant improvement over the manual method.

Introduction:

Ever since the introduction of lateral flow assays (LFA) in the 1970s, they have been one of the most successful and utilized diagnostic platforms. LFAs are easy-to-use, rapid, equipment-free, portable and inexpensive and therefore have been widely applied in diagnostics, pregnancy tests, immunity status, environmental monitoring and drugs of abuse detection.[1, 2] LFAs have also been paired with nucleic acid amplification tests to develop cost-effective methods of amplicon detection.[3-10] However, LFAs have some fundamental limitations in performing multi-step complex bioassays which require sequential delivery or separation of reactions or components.[2] Paper microfluidics have attracted considerable attention from researchers developing low-cost, point-of-need diagnostics.[11] Paper-based devices or microfluidic paper-based analytical devices (μPADs) offer advantages of simplicity, cost-effectiveness, passive pumping through capillary action, dry reagent storage, high surface area to volume ratio, and portability which enable the creation of 'ASSURED' diagnostic devices.[11-15] μPADs constructed out of porous materials like paper however have limitations of non-uniform fluid flow, low flow rate, and poor tolerance to variations in testing conditions. Lamination-based devices have recently been reported which utilize stacks of patterned thin polymers films bonded together using adhesive, plasma treatment or lamination to create microfluidic chips capable of performing complex bioassays. Lamination based devices provide higher fluid flow and better flow control than paper-based devices and could help improve the ease-of-use, analysis time, and analytical performance of complex bioassays. Paper-plastic hybrid devices have also been reported which typically use paper zones to store dry reagents, and plastic capillary channels to direct and control fluid flow. In this chapter, we report a capillary-flown driven immunoassay (CaDI) device for the rapid detection of rolling circle amplification assays. A similar device was first reported by Jang and Carrell et al. for SARS-CoV-

2 antigen and antibodies detection.[16] The device is constructed out of stacked, laser-cut polyethylene terephthalate (PET) and double-sided adhesive (DSA) films to define the channels that connect to a nitrocellulose strip and an absorbent pad (Figure 5.1).

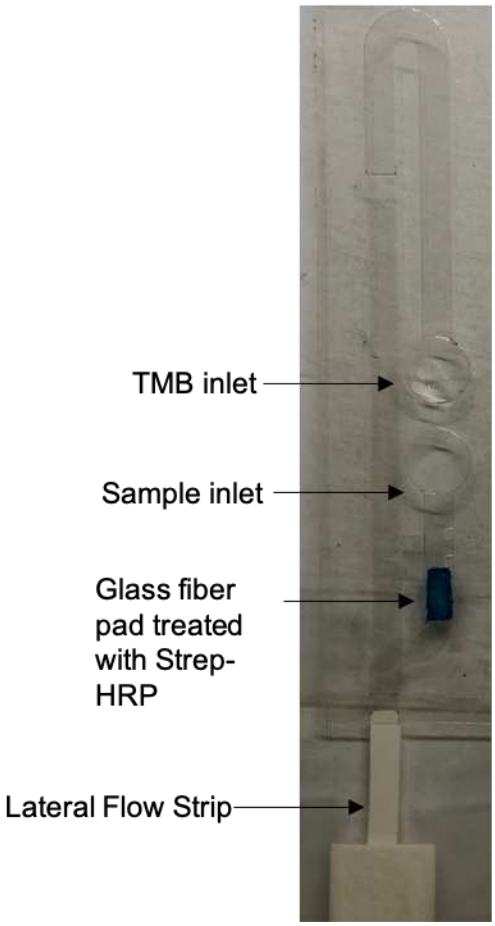

Figure 5.1: Capillary action driven immunoassay (CaDI) device for rapid colorimetric detection of RCA amplicons.

The device contains two inlets, one for the rolling circle amplicon (sample inlet), and the other for the colorimetric substrate (3,3,5,5, tetramethyl benzidine, TMB inlet) to develop the colored band on the nitrocellulose. The plastic channels have two glass fiber pads treated with streptavidin-conjugated horseradish peroxidase (Strep-HRP) at designated locations which release Strep-HRP in the channels when wetted with the RCA amplicon and TMB. The channel geometry is designed such that the RCA amplicon flows through a shorter path to nitrocellulose, while TMB has a much

longer path enabling the sequential delivery of the rolling circle amplicon, Strep-HRP and TMB. Furthermore, an innovative surface pressure valve, made by altering the depth of laser cut materials is placed at the junction where the two channels connect to the nitrocellulose.[17] TMB is added first to its designated inlet, the valve stops and holds the fluid from the TMB channel until the rolling circle amplicon is added to its designated inlet. Once the amplicon is added to its inlet, it flows through the nitrocellulose first, followed by the Strep-HRP released from the glass fiber pads, and finally the TMB flows through the nitrocellulose performing sequential delivery of the reagents needed to develop the colored band. LFAs perform the reactions in the sample matrix, while the CaDI device enables programmable sequential delivery of ELISA or other bioassay reagents, washing buffers and colorimetric substrates which could help improve assay performance at the cost of just one additional step.

The device enables programmable sequential delivery of ELISA or other bioassay reagents through a detection region and therefore can be paired with other isothermal amplification methods for rapid, point-of-need detection. Using an abbreviated assay, we have shown that the nuclease protection probe, which contains a 5' biotin and 3' digoxigenin could be detected using the CaDI device within 20 mins with only two user steps. The assay performance (LOD = 5 pM DNA) which was comparable to a manually run lateral flow assay which requires 8 different user pipetting steps and gives an LOD of 1 pM.

Methods and Materials:

Device Construction:

The CaDI device is constructed out of stacked, laser cut sheets of PET and double-sided adhesive (3M^{TM} MP467, thickness 50 μm). Three layers of PET and two-layers of DSA were cut with the

design shown in Figure 1 using an CO_2 laser cutter (Epilog, Zing1000). Glass fiber pads (dice-cut, 3mm x 5mm) treated with streptavidin-conjugated HRP were placed at the designated locations. Device assembly is completed by connecting a nitrocellulose strip (dice-cut to 20mm x 3mm) CaDI device along with a filter paper (GE Whatman grade 1 filter paper) absorbent waste pad to the main channel of the CaDI device as shown in Figure 5.1.

Sequential Delivery of Reagents:

To demonstrate that sequential delivery of reagents, glass fiber pad treated with a blue food coloring dye were placed in the channel. 1X PBS was added to the 'sample' inlet or the RCA amplicon inlet, while a yellow food coloring dye was added to the 'TMB' or 'substrate' inlet. A video of the device was recorded as the solutions flowed through the channels and into the nitrocellulose and the waste pad. Images at different elapsed time were extracted from the video.

Detection of Nuclease Protection probe:

To demonstrate feasibility of using the CaDI device for the rapid detection of RCA amplicons, we ran an abbreviated assay wherein 60 µL of different concentrations of the Nuclease Protection probe (NPA probe), which contains a 5'biotin and 3' digoxigenin was added to the 'sample' inlet. 70 µL of 1-step™ Ultra TMB blotting solution was added (Thermo Fisher Scientific) to the 'substrate' inlet. An image of the nitrocellulose was recorded and analyzed using ImageJ image processing software (open-access software, NIH). Limit of detection was calculated using the mean blank + 3.3 sigma. All devices were run in triplicates.

Results and Discussion:

As reported by our colleagues, Carrell and Jang et al., the CaDI device allows programmable sequential delivery of bioassay reagents (i.e. sample, wash buffer, substrate) to a detection region, and can be used to disposable ELISA platform for the rapid detection of SARS-CoV-2 antigen and antibodies in a patient sample.[16, 17] Using a CaDI device requires only two user addition steps, the sample and the colorimetric substrate to the designated inlets. We applied a similar device design for the detection of RCA amplicons from the padlock rolling circle amplification assay. We first showed that sequential delivery of reagents was feasible in the CaDI device. Food coloring dye was used to track the flow of solutions through the nitrocellulose detection region. Figure 5.2 shows the time lapsed images of the device. The 'substrate' (yellow food dye) is added first to the 'substrate inlet', followed by the 'sample' solution (1X PBS). Once the 'sample' is added to the device, the blue dye is released from the glass-fiber pad simulating the release of streptavidin-conjugated HRP. This blue dye flows through the nitrocellulose, as the 'sample' inlet is drained out of solution, the TMB (yellow dye) starts to flow through the nitrocellulose. The different colors can be seen on the nitrocellulose as the flow proceeds through the device. The absorbent pad shows a pattern of blue, to green to clear and yellow transition confirming that the different reagents flow sequentially rather than mixing in the channel.

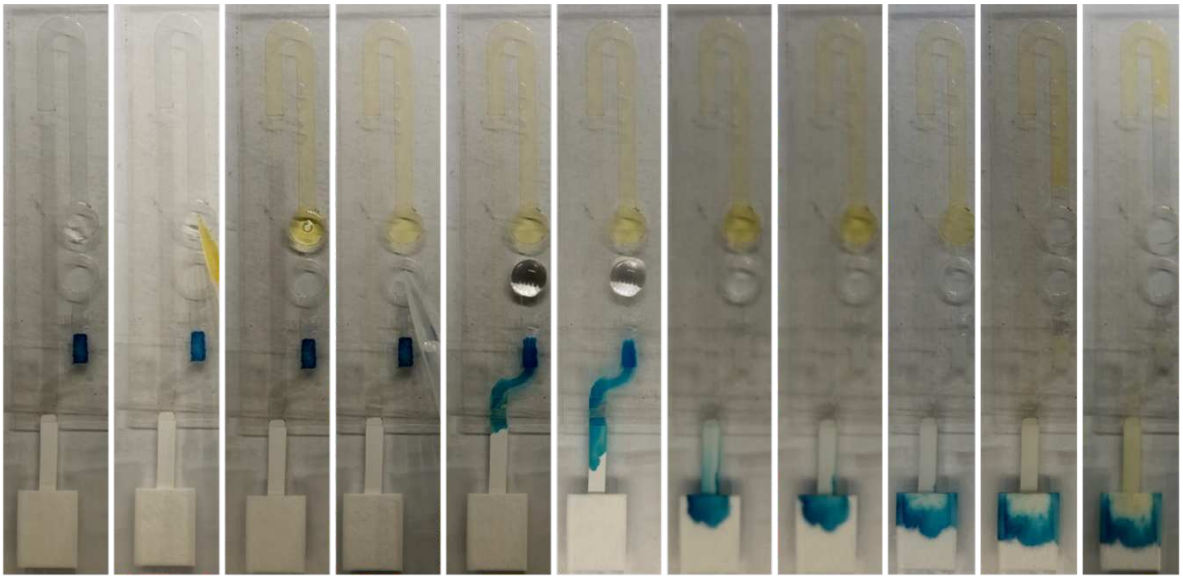

Figure 5.2: Sequential delivery of food coloring dyes to simulate an ELISA reaction. The blue dye stored in the glass fiber pad flows through the nitrocellulose first, followed by the clear 1X PBS solution and the yellow food coloring dye.

The RCA amplicon in the PLAN-LFA reaction is a double-stranded DNA wherein one strand contains a 5'-biotin and a 3' digoxigenin. Padlock rolling circle amplification lateral flow assay (PLAN-LFA) described in an earlier chapter requires eight user addition steps to the nitrocellulose strip to develop the color change. Different concentrations of the nuclease protection probe (ssDNA labeled with biotin and digoxigenin) were run on the CaDI device to simulate the RCA amplicon (Figure 5.3).

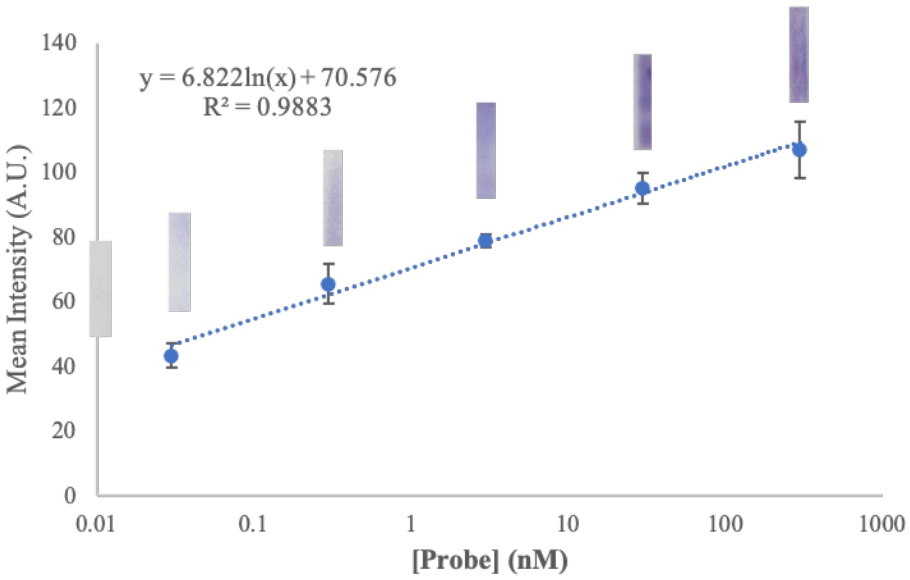

Figure 5.3: CaDI device assay performance using the nuclease protection probe as a surrogate for the RCA amplicon. Different concentrations of nuclease protection probe were run on the CaDI devices in triplicates. LOD was calculated using blank mean + 3.3 sigma.

The CaDI device provided a limit of detection of approximately 5 pM DNA which is comparable to the manual PLAN-LFA assay (1 pM). The number of steps were reduced to only two steps, the addition of the RCA amplicon and the colorimetric substrate which is a significant improvement over the manual method. The use of low-cost substrates makes the device ideal for use in resource-limited settings. As shown by these feasibility experiments, the device can be utilized to detect nucleic acid amplification reactions. With further development, the platform can be utilized to develop a fully integrated sample-to-result nucleic acid sensor platform. Given that the PLAN-LFA assay requires a long incubation time (~2 hours) and multiple steps, it may not be the ideal technique to pair with this platform. LAMP or RPA reactions which have a much shorter amplification time (<30 min), would be ideal for developing such sensor platforms.

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CONCLUSIONS

Nucleic acid testing is rapidly replacing culture-based methods as the gold standard for infectious disease diagnosis in the developed world, however, the associated cost and complexity and the need for trained personnel makes NAT largely inaccessible in the developing world. Although the global infectious disease burden has steadily lowered in the past few decades, emerging infectious diseases and the rapid spread of antimicrobial resistance threaten this recent progress. If left unchecked, antimicrobial resistance has been projected to become the number one cause of death by the year 2050. The recent outbreaks of SARS-CoV, Ebola, H1N1 flu, and SARS-CoV-2 viruses have further highlighted the need to improve the global access to molecular diagnostics to enable rapid diagnosis and quarantine of infected individuals. Inexpensive and portable NAT platforms that can enable rapid, point-of-care diagnosis could significantly improve access to NAT and lower the burden of infectious diseases. Existent molecular diagnostic technologies need to be modified to enable rapid, POC use in the developing world by minimally trained personnel. The few POC NAT platforms that have been proposed thus far are still too expensive for use in level I and II healthcare settings in the developing world.

To briefly summarize, this work discusses the development of a non-amplification based and an isothermal amplification-based nucleic acid sensor platform. We started with the development of a simple and inexpensive non-amplification based nucleic acid sensor based on nuclease protection and lateral flow detection. The nuclease protection lateral flow assay (NPA-LFA) provides an easy-to-use tool for rapid screening of pathogens at the point-of-need as

turnaround time to results is <1 hour. Post-denaturation the entire NPA-LFA reaction can be performed at 37ºC. Various examples of low-cost resistive tube heaters utilizing resistive or chemical heating have been reported. Using a low-cost tube heater, tubes with lyophilized NPA probe and P1 nuclease, and the LF strip, a user could easily perform rapid screening of target nucleic acids. However, the high limit of detection (~10^9 copies/reaction) prevents the application of the platform in point-of-care infectious disease diagnosis. We believe the platform is better suited for sensing applications where a high pathogen load is to be expected. We also showed that paper-modified with chitosan can be used to remove interferents and preconcentrate nucleic acids in samples. We also showed that paper-based sample pretreatment can be performed directly on the lateral flow strip albeit with extra user handling steps. On its own, our paper-based sample pretreatment method provides a simple and inexpensive technique to isolate nucleic acids from a complex sample at the point-of-need and can be used to store nucleic acids for later analysis or in integrated nucleic acid sensor platforms. The NPA-LFA platform can be developed into a dipstick lateral flow strip format in the future to further reduce the number of user-steps and improve usability.

To improve the assay sensitivity and clinical utility, we paired nuclease protection with isothermal padlock rolling circle amplification. We chose rolling circle amplification because it is one of the simplest isothermal amplification techniques requiring only one primer and a strand-displacing polymerase for amplification. Padlock rolling circle amplification utilizes a ligase to specifically ligate the 5' and 3' ends of a DNA probe in the presence of the target. The circularized probe undergoes rolling circle amplification in the presence of Phi29 DNA polymerase. RCA provides a single-stranded concatenated DNA amplicon that can bind the nuclease protection probe and therefore can be detected using a lateral flow strip without an additional denaturation

step. To the best of our knowledge, the combination of padlock rolling circle amplification, nuclease protection and lateral flow detection (PLAN-LFA) has not been previously reported. The PLAN-LFA assay provided a 1000X improvement in the assay sensitivity over the non-amplification based NPA-LFA platform. The detection limit of the assay improved to $1.3*10^6$ copies/mL with a 2-hour amplification time. The assay detection limit is 20-40X better than other similar platforms based on linear rolling circle amplification and lateral flow detection. Using our PLAN-LFA platform, we have developed specific assays for beta-lactamase resistance genes for antimicrobial resistance monitoring, and severe acute respiratory virus coronavirus 2 (SARS-CoV-2).

Primer design is simplified in RCA as compared to LAMP, as the hybridization arms are complementary to the target sequence, and a common backbone region such as a polyA sequence can be used to design probes to assay alternative targets. The only constraint to primer design is the need to minimize the probe secondary structure to reduce the possibility of any non-specific amplification. We also demonstrate the versatility and robustness of the PLAN-LFA platform by designing assays for both DNA and RNA targets and performing analysis in complex sample matrices like saliva and plant tissue without any sample pretreatment steps. As described in chapter 1, nucleic acid sensor platforms that minimize or eliminate the need for sample pretreatment even at the cost of analytical performance would be highly desirable for use in point-of-need and resource-limited settings. While the PLAN-LFA provides a simple, versatile, and robust NAT platform, the turnaround time to results (~3 hours) is still too long for a POC use device. Future efforts should be dedicated to reducing the overall turnaround time to results and development of a fully integrated sample-to-result sensor based on rolling circle amplification, nuclease protection and lateral flow detection.

The WHO recommends that SARS-CoV-2 diagnostics have a limit of detection in the range of 10^4 - 10^6 copies/mL; given the linear PLAN-LFA SARS-CoV-2 assay provides a detection limit just above the WHO recommended range, we decided to explore methods to improve assay LOD. In Chapter 4, we discuss the feasibility of using a nicking endonuclease to initiate exponential-mode rolling circle amplification. The exponential RCA reaction provided a 100X improvement over the linear amplification method for SARS-CoV-2 RNA detection, though we failed to pair lateral flow detection with the exponential RCA reaction. The exponential amplification reaction also has a limitation of long incubation time (~4 hours), therefore is not rapid enough for point-of-care device development. We also discuss the early-phase development of a prototype microfluidic device for sequential delivery of RCA amplicon, and colorimetric substrate to the detection region on the nitrocellulose strip. The device reduces the number of user steps, and allows automated, programmable sequential delivery of reagents. The device can be used to deliver the reagents of an immunoassay reaction, nucleic acid amplicons or components of other bioassays to a detection region. Furthermore, such paper-plastic devices can eventually be used to develop integrated sample-to-result molecular diagnostics platforms. Since RCA has a long incubation time, the device would be better suited for use with LAMP or RPA chemistry to enable the development of truly rapid, point-of-need nucleic acid sensor platforms. The device also has the potential to partially or fully automate all the steps of nucleic acid detection, to improve the usability of the device especially in resource-limited settings.