LOOP MEDIATED ISOTHERMAL AMPLIFICATION BASED DETECTION OF EQUINE RESPIRATORY PATHOGENS USING A PORTABLE, SMARTPHONE-BASED SETUP

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Bioengineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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Abstract

New tools are needed to enable rapid detection, identification, and reporting of infectious viral and microbial pathogens in a wide variety of point-of-care applications that impact human and animal health. We report the design, construction, and characterization of a multiplexed platform for multiplexed analysis of disease-specific DNA sequences that utilizes a smartphone camera as the sensor in conjunction with a handheld "cradle" that interfaces the phone with a silicon-based microfluidic chip embedded within a credit card-sized cartridge. Utilizing specific nucleic acid sequences for four equine respiratory pathogens as representative examples, we demonstrate the ability of the system to utilize a single 15 μL droplet of test sample to perform selective positive/negative determination of target sequences, including integrated experimental controls, in approximately 30 minutes. Our approach utilizes loop mediated isothermal amplification (LAMP) reagents pre-deposited into distinct lanes of the microfluidic chip, which, when exposed to target nucleic acid sequences from the test sample, generates fluorescent products that, when excited by appropriately selected light emitting diodes (LEDs) are visualized and automatically analyzed by a software application running on the smartphone microprocessor. The system achieves detection limits comparable to those obtained by laboratory-based methods and instruments. Assay information is combined with information from the cartridge and the patient to populate a cloud-based database for epidemiological reporting of test results.

To my friends and especially my family whose support made this work possible. Thank you!

Acknowledgements

I would like to thank my advisor Prof. Rashid Bashir for giving me the opportunity to work on this project and for guiding and supporting me throughout the work. I also want to thank my collaborator Prof. Brian Cunningham who provided me invaluable guidance and support throughout the project. Last, but not least, I want to thank all my colleagues Anurup Ganguli, Tanmay Ghonge, Gregory Damhorst, and Gelson Pagan Diaz, and collaborators Weili Chen, Hojeong Yu, and Fu Sun whose efforts made this work possible.

The following thesis represents a subset of the work "Mobile Platform for Multiplexed Detection and Differentiation of Disease-Specific Nucleic Acid Sequences, Using Microfluidic LoopMediated Isothermal Amplification and Smartphone Detection" published in *Analytical Chemistry*. http://pubs.acs.org/doi/pdf/10.1021/acs.analchem.7b02478

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1. Introduction

1.1. Equine Respiratory Tract infection

1.1.1. Background

Thoroughbred horse racing is an industry that churns out $4 billion per year. Thoroughbred racing stables and breeding farms are professionally run organizations and in many ways are similar to commercial food animal herd producers in terms of their sophisticated record keeping, veterinary care, understanding of genetics, and need to rapidly diagnose an infectious disease that can injure, kill, or render unsafe large numbers of high-value animals though an outbreak. Respiratory disease is common in horses and difficult to diagnose using the current methods available to practicing veterinarians. Currently there is little to no monitoring of the health of horses even though they are the most valuable of livestock. Some respiratory conditions such as Inflammatory Airway Disease (IAD) and Interstitial Lung Disease are poorly understood [1] although animals are predisposed to these conditions by viral and bacterial infections [2], which occur worldwide [3].

1.1.2. Current diagnostic pathway

Respiratory tract infection is a major cause of mortality and morbidity in horse populations. [4] The effect of infectious respiratory tract infection is magnified in race tracks where entire horse population needs to be quarantined due to long diagnostic turnaround times and inadequate on-site intervention strategies. There have been a significant increase in EHV-1 outbreaks in the US in recent years, which have led to millions of dollars in costs. Current methods of to evaluate horse respiratory tract include invasive techniques such as endoscopic examination, or imaging techniques such as ultrasonogram. [5] Other diagnostic approach includes cytology and a bacterial culture of the of the horse respiratory secretion. These techniques are not suitable for a field-tailored application where early diagnosis of sick horses is required for quarantine. Samples for must first be sent to a reference lab, and the turnaround times for test results is usually in days by which time the entire racetrack might have been affected. Furthermore, these tests themselves are not sensitive enough to distinguish between closely related strains of the equine respiratory tract infection such as the Equine Herpesvirus subtypes. Diagnosing the correct Equine Herpesvirus subtype is particularly important since the equine herpesvirus 1 have been associated with neurological

disorders and high rates of mortality among horses. The distinction between the two strains of the Equine Herpesvirus can be determined by highly specific and sensitive nucleic acid amplification tests such as PCR. Infections caused by Equine viral rhinopneumonitis, it is difficult to clinically differentiate from equine influenza, equine viral arteritis, or other equine respiratory infections solely on the basis of clinical signs. Definitive diagnosis is determined by PCR by extracting nucleic acid from the horse respiratory secrection (nasopharyngeal swab). [2] Strangles is another very common equine respiratory tract infection caused by Streptococcus Equi Subsp. Equi (S. Equi) or Streptococcus Equi Subsp. Zooepidemicus (S.Zooep). Nucleic acid amplification tests, such as PCR, has been used to distinguish between these two closely related strains responsible for strangles.

1.2. Nucleic acid amplification tests (NAATs) in point-of-care testing

Polymerase Chain Reaction (PCR) is the most commonly used technique for the detection of nucleic acid target from any bacterial or viral pathogenic source [6-8]. PCR enables sequence-specific concentration amplification of infectious disease DNA through a series of thermally cycled chemical reactions between a set of enzymes, a prepared test sample (of DNA extracted from a pathogen-containing bodily fluid), and disease-specific "primer" molecules. PCR primers for detection of every common human and animal infectious disease have been identified, and may be inexpensively synthesized through readily available commercial sources. Conventional PCR amplification requires expensive laboratory-based instruments that are operated by technicians and housed in central facilities, although there have been strong efforts aimed at miniaturization of PCR for translation closer to the point of care through the engineering of systems integrated into a small chip or cartridge [6, 8-36]. Many of these efforts have been aimed at accelerating the thermal cycling process through reduction in the liquid volume [17, 21, 26, 27, 30, 32, 35, 37-40], while others have explored various modalities for sensing the presence of the amplified product using biosensors [41-45]. Due to the cost and complexity of implementing thermal cycling (generally between 95 °C (denaturation), 72 °C (extension), and 68 °C (annealing) with ~30-40 cycles), various isothermal nucleic acid amplification methods have been proposed and demonstrated with comparable sensitivity to PCR [46-48], but with a requirement to more fully customize and validate the primer sequences. Of these, loop-mediated Isothermal amplification (LAMP) has emerged as a compelling approach for portable applications [49-51], particularly due to

its ability to utilize unprocessed or minimally processed test samples without inhibition of the reaction. [52] As the name suggests, LAMP reactions take place at a single incubation temperature of 65C. This eliminates the use of any complex instruments required for thermal cycling. LAMP reactions can take place on a simple heating block or even a oven maintained at 65C.

1.3. Point-of-care testing for the detection of Equine respiratory pathogens

A key characteristic for a successful system for detection and reporting of infectious disease is speed. Particularly for point-of-care scenarios, where the clinician is testing a patient at a remote clinic, a farm, or a racetrack, the need to send samples to a central laboratory, to wait for the test to be performed, and to wait for the results to be reported, results in an enormous waste of opportunity to determine if aggressive treatment or quarantine is needed before the disease spreads further. The ability to rapidly share the results of positive and negative tests can revolutionize the manner in which infectious diseases are managed. Therefore, it is of paramount importance for the test to be performed at the same location as the patient, so action can be taken within the same day that the sample is gathered. A second key characteristic for a POC test is the ability to simultaneously test for the presence of more than one pathogen with a single test protocol, which lowers cost, saves time/effort, and allows for a panel of pathogens, which may cause similar symptoms, to be identified (Equine influenza vs Equine Arteritis virus).

1.4. Smartphone-based point-of-care setup

A key feature of a POC setup is the use of inexpensive, easily available, and ready to fabricate detection systems. [53-56]. Due to the rapid development of computational, communication, and sensing capabilities of smartphones since the introduction of the iPhone in 2007, these devices have become similar to personal computers with integrated cameras, geolocation capabilities, and access to cloud services. The widespread use of smartphone makes it a very attractive candidate for fluorescence or optical detection modalities. Recent examples include attachments that enable smartphones to serve as stethoscopes [58], ultrasound probes [59], microscopes [60], fluorescent microscopes [61, 62], label-free biosensor detection

instruments [63, 64], fluorimeters [65], and colorimetric assay readers [66]. Portable detection systems for infectious disease are already recognized as a likely extension of mobile technology [49, 67-72], and PCR or LAMP mobile sensing platform that is integrated with a smartphone and a smart service system for reporting and sharing results with a network of users is highly desirable.

1.5. Our technique

In this work, we combine the sensitivity and specificity of nucleic acid amplification assays for the detection of a panel of equine respiratory tract pathogens with a smartphone-based detection system. While our ultimate goal is the diagnosis and mobile reporting of human infectious diseases, we initially focus specifically upon equine respiratory infections due to their economic importance to the horse racing industry, similarity to the tests that would have an impact upon the food animal industry, and the strong need for a service system that can inform networks of field veterinarians. Our work involves the development of a microfluidic approach for identifying the presence of specific nucleic acid sequences from the pathogens of equine respiratory infections using a portable detection system integrated as a disposable cartridge that can be read by a conventional smartphone in conjunction with a custom-designed cradle. Capable of simultaneously performing 10 parallel isothermal amplifications (with two utilized for experimental controls and eight for pathogen-specific DNA detection assays), the system can detect target DNA sequences from more than one pathogen with a single test protocol, and interface with a smartphone app that communicates with a cloud-based service for the immediate reporting of the location, time, identity, and results (positive and negative) of the detection. In this paper, four LAMP-based assays have been developed for the detection of four important equine respiratory infectious pathogens: streptococcus equi (S. Equi), strangles (streptococcus zooepidemicus, abbreviated as S. Zoo), equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4). The smartphone-based detection demonstrates the same detection limits and sensitivity as LAMP reactions performed on commercially available laboratory-based systems, and is capable of identifying the specific nucleic acid sequences of pathogens for both single-infection and co-infection scenarios. To our knowledge, a smartphone-based nucleic acid testing approach for the diagnosis of veterinary infectious diseases has not

been reported in previous literature. This platform can be easily extended for the simultaneous detection of any closely-related human pathogens.

2. Literature review

In this section, we will discuss various competing point-of-care techniques, and details about loop-mediated isothermal amplification and its use in our techniques.

2.1. Current Nucleic Acid Amplification based miniaturized setups

State-of-the-art miniaturized systems that perform DNA amplification reactions can be classified as: small thermocyclers with integrated photodiodes [11, 83], microfluidic systems with customized optics for automated and multiplexed experiments [49, 84, 85], and systems based on electrochemical principles that achieve miniaturization by removing optical elements [86, 87]. Small thermocyclers like 'Palm PCR' [83] let the user perform the reaction in a pocket-size device but all the sample preparation is still performed by the user and there are no multiplexing capabilities. The microfluidic 'FilmArray' [84] system is highly automated and considerably reduces sample preparation and handling. However, the instrument size and complexity results in an expensive bench-top system that is not suitable for portable applications. Electrochemical-based systems like the one demonstrated by DNA Electronics [86] monitors variables like pH change or secondary redox reactions to assess DNA amplification without optical elements. However, these alternatives require the compartmentalization of reactions since the monitoring variables are not specific to the target complicating assay multiplexing.

In general, miniaturized DNA amplification systems seek to minimize footprint, automate processes, and reduce cost without losing sensitivity and specificity of the assay [88]. Additionally, especially for infectious disease detection, it is desirable to have multiplexed assays that enable the screening of multiple agents in a single assay and it is important to create data networks to quickly analyze and share information to contain outbreaks. The devices referenced above fulfill some of these characteristics, but there is no system with all the desired features. In the following sections, we will highlight how our developed device aims to fulfill each of these characteristics.

2.2. Loop mediated isothermal amplification (LAMP)

LAMP is a novel nucleic acid amplification method that was first reported by Notomi et al in 2000.[52] It is a one-step amplification reaction that amplifies target nucleic acid sequence with high specificity and sensitivity under isothermal conditions and has sensitivity comparable to traditional PCR. The LAMP reaction can also be used for detection of viral

RNA or mRNA by adding heat-stable reverse transcriptase to the reaction. This section will discuss the mechanism, primer design and key features of RT-LAMP reaction.

This technique relies on auto-cycling strand displacement DNA synthesis that is performed by a strand displacement DNA polymerase with high activity and a set of two specially designed inner and two outer primers. These sets of primers recognize a total of six distinct sequences on the target DNA. At the LAMP reaction temperature (60 − 65 ◦C) double stranded DNA is in a 'dynamic equilibrium' allowing primers to anneal to the complimentary sequence in the target double stranded DNA and thereby triggering the polymerase to make initial elongations. Initially, all four primers are used, but later during the cycling reaction only the inner primers are used for strand displacement DNA synthesis. The inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), respectively, and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages. During the initial elongation, the polymerase releases a single stranded DNA that is then used for further downstream reactions of making loop structures which serves as the element for downstream amplification.

In the subsequent LAMP cycling one inner primer hybridizes to the loop on the aforementioned product and initiates the displacement DNA synthesis, yielding the original stem–loop DNA in addition to a new stem–loop DNA with a stem twice as long. This cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The reaction will stop when concentration of free deoxyribonucleotides (dNTPs) is too low or the buffer capacity is reached. As a result of this 2 step process, various sized DNA structures (consisting of concatenated inverted repeats of the target sequence) are formed. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. Furthermore, an accelerated LAMP procedure has been developed that employs two additional primers (LoopF and LoopB) for enhanced specificity and reaction efficiency [52].

2.2.1. Key features

The key features of LAMP reaction are as follows:

- Isothermal: LAMP does not require complicated thermal cyclers as the ones used for PCR for carrying out reactions.
- Simple visual assessment of amplification: using turbidity or by addition of fluorescent or chromogenic reagents (like SYBR green intercalating dye),
- Low detection limit: As few as 1 copy per reaction have been detected using LAMP assays. This is similar to PCR
- High specificity: 4-6 primers target 6-8 regions in the target nucleic acid, reducing the chances for non-specific amplification.
- Robustness: It requires minimal sample preparation such as purification steps. LAMP reactions have been shown to be specific and perform efficiently in the presence of PCR inhibitors like cellular debris, urine, stool etc.
- Assay time: Lamp assays can be give detection results in as little as 10min - 15min. It depends on the initial template concentration and using LF / LB loop primers is possible to decrease a long detection time.
- Amplification yield: While PCR yields 10ug/mL of DNA copies, LAMP produces 500 ug/mL allowing detection through turbidity measurements. Overall LAMP has been proposed as an ideal technique for nucleic acid amplification for point-of-care devices since it has less demanding equipment and has better sensitivity and specificity over PCR and other methods.

3. Materials and Methods

In this section, we present the materials and methods used in the characterization of LAMP assays and development of the portable smartphone-based infectious disease platform. The setup composed of three main components:

1. LAMP assays for the specific detection of nucleic acid targets
2. A microfluidic chip that served as the reaction chambers for our parallel, multiplexed detection of the nucleic acid targets
3. Smartphone-based reader for the fluorescence-based detection of LAMP amplification.

3.1. LAMP assay development

For this work, we developed four LAMP assays for the specific detection of four equine respiratory pathogen targets. The assays were first developed and characterized off-chip in pcr tubes in a commercial thermocycler. The assay development can be categorized in three broad categories:

1. Primer design – novel primer sets were designed for each of the four targets
2. Characterization of LAMP primers – the developed LAMP primers were tested for specificity with the target of interest. Cross- reactivity tests were carried out to perform the most specific primer sets
3. Limit of detection tests – lower limits of detection for each of the primer sets were determined so as to ensure that our assays could detect targets in the clinically relevant concentration of these pathogens.
4. Translation of assays to on-chip – the steps 2 and 3 were directly translated and tested on-chip to validate our smartphone-based detection system for the multiplexed detection of 4 of the targets.

Gold standard qPCR assays were also designed for each of the four targets and compared with the LAMP assay. Figure 3.1 shows a flowchart outlining the assay development starting from the design of primer to on-chip assay translation.

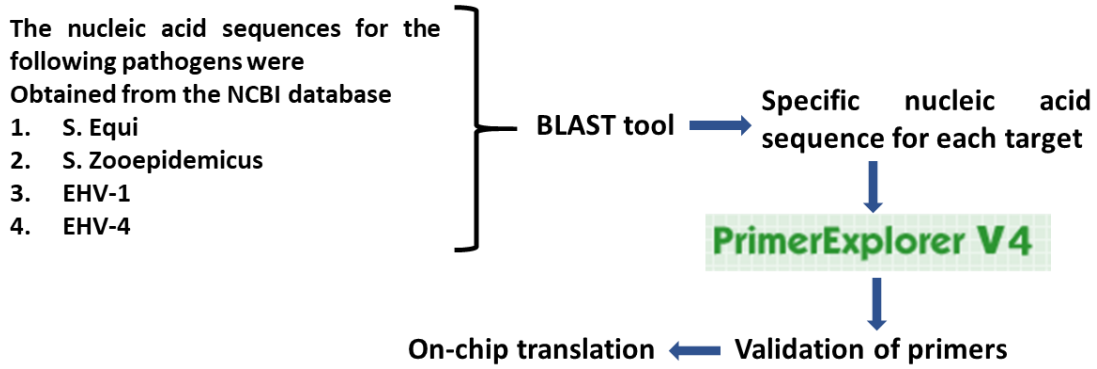

Figure 3.1. Flowchart for primer design and assay

We chose Streptococcus Equi (ATCC® 9528™), Streptococcus Zooepidemicus (ATCC® 39920™), Equine Herpesvirus-1 (USDA 040-EDV), and Equine Herpesvirus-4 (USDA 044-EDV) as the pathogens of interest due to their widespread virulence among horse populations. The S. Equi and S. Zoo bacteria were received in lyophilized form and were propagated in bovine-brain heart infusion medium (Sigma Aldrich) for a period of 16 hours to obtain a carrying concentration of 1×10^8 colony forming units (CFU)/mL. 500uL of glycerol stocks were prepared from this concentration and are stored at -80 °C until needed for experiments. The Equine Herpesvirus-1 and Equine Herpesvirus-4 stocks are obtained suspended in culture medium and were aliquoted into smaller volumes and also stored at -80 °C until needed. DNA from all the pathogens were extracted via a standard heat-lysing protocol. Heat lysis was chosen as the method of DNA extraction for this work due to the simple protocol, short processing time, and low cost. Briefly, 1 mL of culture-grown equine pathogens is centrifuged at 12,000 rpm for 1 minute. The supernatant is discarded and the cells are suspended in 200uL of nuclease free water. The cells are then heat-lysed at 95 ˚C for 5 minutes, followed by 3 minutes of centrifugation at 12,000 rpm. The supernatant containing the extracted DNA was retrieved and ready for immediate use or is stored at -80 ˚C until use. This protocol can be easily adapted for use in field testing with a simple heating block. LAMP assays using unfiltered sample material can be performed without loss of specificity. The extracted DNA is quantified using PCR standard curves that were established using synthetic

targets. The PCR standard curves are shown in Figure 4.1..PCR reactions were carried out using the Promega GoTaq Green MasterMix according to the manufacturer's instructions.

Novel LAMP primers were designed for S. Equi and S. Zoo, targeting the seM and sorD genes respectively. The target sequence for S. Equi was obtained from the NCBI database using GenBank AJ249868.1 and U73162.1. The target sequences for S. Zoo were also obtained from the NCBI database using GenBank CP002904.1, NC_011134.1, and FM204884.1. LAMP primers were designed using Primerexplorerv4 (https://primerexplorer.jp/e/). LAMP primers for EHV-1 and EHV-4 were utilized from the sequences reported from literature. All LAMP primers were synthesized by Integrated DNA Technologies (IDT DNA) and are listed in Table S2. Briefly, each primer mix consists of 0.2 µM of F3 and B3, 1.6 µM of FIP and BIP, and 0.8 µM of Loop F and Loop B primers.

A 25 µL LAMP reaction mix were prepared containing the following: 3.5 µL of 10mM dNTPs (New England Biolabs), 2.5 µL of 10X Isothermal Amplification Buffer (New England Biolabs), 2 µL of 5M Betaine (Sigma Aldrich), 1.5uL of 100mM Magnesium Sulfate Solution (New England Biolabs), 2 µL of primer mix, 2 µL of 8000units/mL Bst 2.0 Warmstart DNA Polymerase (New England Biolabs), 1.25 µL of 20X Evagreen Dye (Biotium), 2.25 µL of DEPC-treated water (Invitrogen), and 8 µL of the template DNA. The LAMP reactions are validated on a benchtop thermocycler (Eppendorf Realplex Thermocycler) at 65 °C for 40 minutes and terminated by heating at 85 °C for 5 minutes. For the off-chip experiments performed on thermocycler, the 25 µL reaction mix is divided into three equal parts (~8 µL) for triplicate repetition. Ten-fold serial dilutions of the extracted DNA are carried out in DEPC-treated water to determine the working range of our LAMP assays. For on-chip experiments, 15 µL of the reaction mix without primers is used for injection.

The specificity of the LAMP primers were validated by testing each of the 4 targets each of the 4 primers. The results of the specificity test are shown and summarized in Figure 4.4 and Table 4.1. respectively. Both the specificity and limits of detection tests were performed with both plasmid targets and nucleic acid extracted directly from the pathogen.

The primer sequences (PCR and LAMP) designed for these assays are listed below:

Table 3.1. Primer sets used in this study

Pathogens	**LAMP assay primer sequences**
Streptococcus Equi	**F3**: AAA ACT AAG TGC CGG TGC2
	B3: AAA ACT AAG TGC CGG TGC2
	FIP: TAC GAC TAA CCT CAG AGT TCG CTA TCA GTA TTA GTT GCA ACA AGT G
	BIP: CGA CTC CAA GAT TAT CGC GTG ATT GAA CTT TTT GGG CTG ATG A
	Loop F: ACA GTT GTC CCT CCC AAC A
	Loop B: GCG ATA TAG CCA TAA GTG GAG ATG
	F: CGG ATA CGG TGA TGT TAA AGA
	R: TTC CTT CCT CAA AGC CAG A
Streptococcus Zooepidemicus	**F3:** AAA GAC CCT CAT GGG AAA T
	B3: CCT TAG TTG CCG CAT AGG
	FIP: CCT GAC TAA CCA AAT ATA AGC CCT TGA GCT GGA CGA TAA GAC CT
	BIP: TGT TGG ACG TAT TTT GGT TGC TCT TCT GAG CCT TCT AAA CCT G
	Loop B: GGT GTC ATT ATT AAC ATG GCC TCT
	F: CAG CAT TCC TGC TGA CAT TCG TCA GG
	R: CTG ACC AGC CTT ATT CAC AAC CAG CC
Herpesvirus 4	**F3:** CAA GAC GTA ACA ACG GGA GT

Table 3.1. continued

	B3: CGC AAG TAA CGG CGA TGA
	FIP: CGC TCT CCG TTT TCT TCC GAC AAG CCA CCC AGG ATT AGT CAA
	BIP: TTA CCC GGA CGG CCT TCC AAC GGG CAT GTC CTC AAC AA
	Loop F: GCC TGC TAC TCC GCA TG
	Loop B: AGC GTT GTA TAT GAT GCA TCC CCT
	F: GAC CTC TCC GTT CAC CCA AG
	R: TCC GTT TTC TTC CGA CAG GG
Herpesvirus 1	**F3:** GGC ATT TAC GTG TGG TCC TT
	B3: TCG CGG GCA TTT TTG TAC C
	FIP: GTC CAG CAA CGG TGC GTT GTG GCA CGC TCG TTA ACA GT
	BIP: CGA GCC TGA AGG GGG AAA ACT GGA GCT GTG TGG AAA GTA GC
	Loop F: AGG TTG AGA CGG TAA CGC TG
	Loop B: CAC GTG CGT CGT CGC AA
	F: GCG CCA GCT GTT TAA CCT TC
	R: CGG GCA TTT TTG TAC CAC CG

3.2. Chip fabrication

The fabrication for chip fabrication was developed by Weili Chen from Professor Brian Cunningham's group. The protocol is illustrated in Figure 3.2. Briefly, an oxidized silicon microfluidic chip contained ten parallel lanes with a common sample injection inlet. Primers specific to the targets of interest was pre-dried in each of the lanes, and LAMP reaction mix (prepared off-chip) without the primers was manually injected into the sample inlet. The chip size was the same as a standard SIM card (25mm*15mm). Each of the lanes were 1mm in length, 500um in width, and 200um in depth, thereby allowing a total of 1uL of reaction mix per well. The fabrication procedure for the chip is illustrated in Figure 3.2. A single-side polish 4" Silicon wafer was used as the substrate. The wafer was cleaned using standard degreasing protocol and a positive tone photoresist was spin-coated to form a 4.5um thick covering layer. A soft bake of 2 mins at 60C followed by 1min at 110C was performed. The photoresist was then exposed with an i-line (365nm) mask aligner (EVG 620) at a exposure dose of 180 J/cm^2. The exposed regions of the positive tone photoresists were weakened upon exposure and were subsequently removed by immersing the wafer in a 1:4 ratio of AZ developer in deionized water. 4 minutes incubation was sufficient to remove all the photoresist in the exposed regions. The developer activity was quenched by holding wafer against running deionized water. A hard-bake at 110C for 1 minute was then performed to harden the remaining unexposed photoresist. Anisotropic etching of the exposed silicon was carried out using the Bosch process. In the Bosch process, alternating steps of SF_6/O_2 etching and C_4F_8 passivation was used to create 200 µm deep trenches in an inductively coupled plasma reactive ion etcher (ICP-RIE). The SEM image in Figure 3.2c shows vertical wells of 200um. The remaining photoresist after the Bosch process was stripped by using a combination of acetone degrease followed by O_2 descumming. The etched silicon wafer was then thermally oxidized at 1150C for 2 hours to form a 200nm film of SiO_2. The wafer was then diced to form individual devices which were later used for downstream testing.

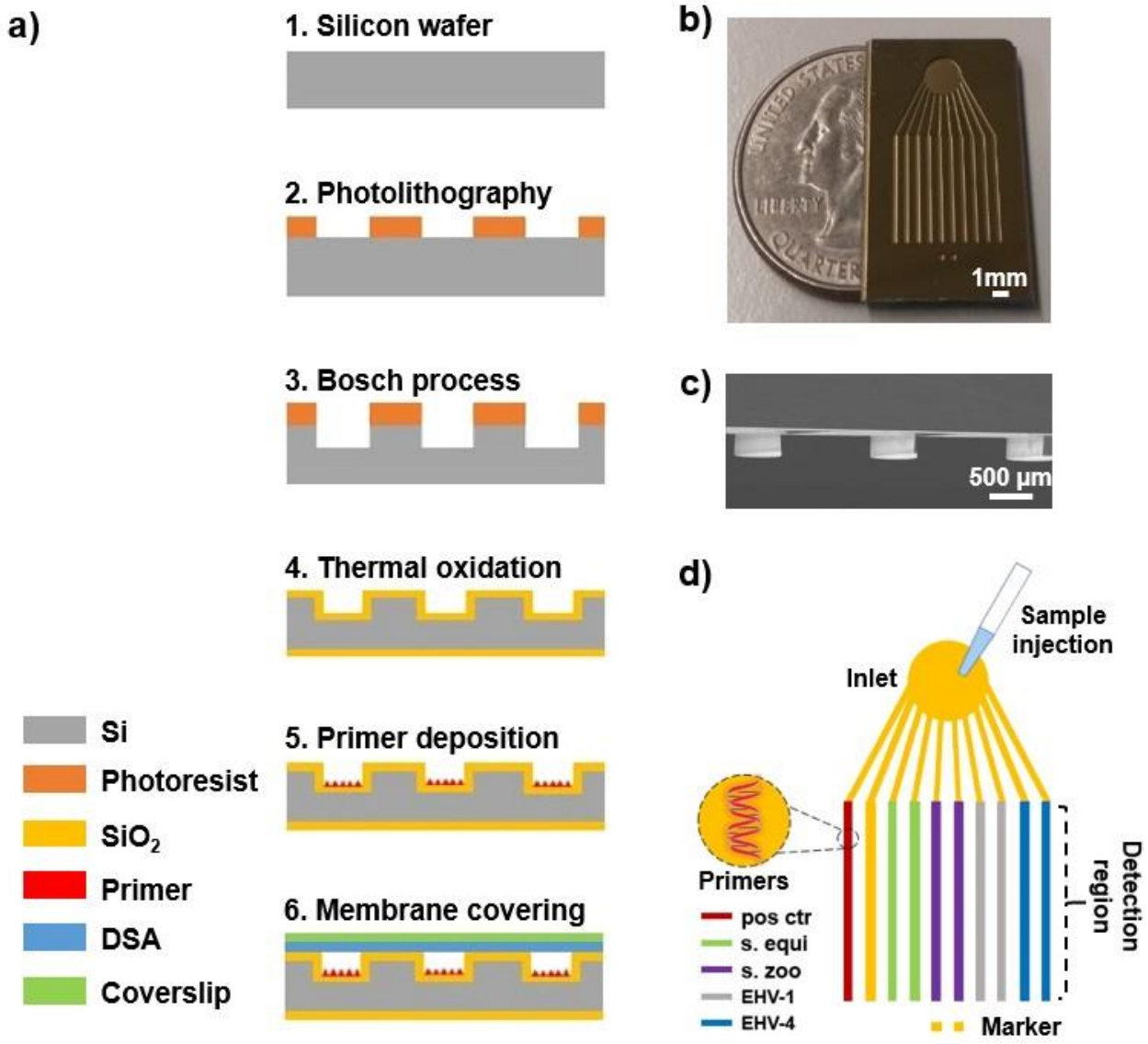

Figure 3.2: Ten-flow channel microfluidic chip for multiplexed LAMP detection. a) Schematic diagram of the fabrication process for the microfluidic chip. b) Photo of a fabricated chip taken with a US quarter. c) Scanning electron microscope (SEM) image of the cross-section of the microfluidic channels. d) Primer deposition plan for the on-chip reactions.

3.3. Chip preparation

Before being used for the assays, the chips are treated with Piranha solution (a 3:1 mixture of 98% sulfuric acid (H_2SO_4) and 30% hydrogen peroxide (H_2O_2)), and rinsed with deionized water and blow-dried using nitrogen gas. The chips were stored at room temperature in a desiccator until use. The chip preparation can be divided into two categories:

1. Primer deposition
2. Chip sealing

1. Chip sealing

   To be compatible with our workflow, our microfluidic chip must be able to

   a. Prevent evaporation of LAMP reaction mix when incubated with 65C
   b. Prevent cross-contamination between adjacent wells

   Mineral oil has been previously reported as the medium to prevent evaporation [55], but cannot be used in point-of-care systems. Towards this goal, we developed a simple protocol to seal the chips before incubating them at 65C for the LAMP reaction. The chips are covered with a transparent double-sided adhesive (DSA) (ARseal™ 90880, Adhesives Research). The sample inlet and the ten outlets are laser-cut so that the reaction mix can be flowed in. For reactions testing the limits of detection of primers on chip, 15uL of the complete reaction (with the primers) was flowed in. For experiments with dried primers (see section below), 15uL of the reaction mix without the primers were flowed in. The chip was degassed for 5 minutes in a desiccator to remove any air bubbles. The upper protective layer of the DSA was peeled off, and the chip was completely sealed with a glass coverslip. The binding is strong enough to prevent any leakage or cross-contamination during the reaction. This resulted in a amplification ready and completely sealed chip with ten independent reaction chambers.

2. Primer deposition:

   For on-chip assays testing the limits of detection (Figure 4.5-4.8)reaction mix with the primer and target of interest was directly injected into the sample inlet. For reactions testing the primer specificity and co-infections [Figure 4.9] specific primers were deposited into each of the 10 lanes. Figure 3.2d illustrates the primer printing schematic. Lane #1 served

as a universal positive control. In this lane, 1uL of a high concentration of S.Zooep DNA (5e6 CFU/mL) was mixed in 1:1 ratio with 1uL of S.Zooep primer (diluted 1:20 ratio in nuclease-free water). 1uL of this solution was then pipetted pipetted at the outlet of lane 1. Since this lane contained both the target and primer sequence for S.Zooep, we expected this lane to amplify regardless of the sample present in sample solution. Hence, this lane served as a validation of the reaction components on-chip. Lane#2 served as the no-primer negative control. No primer sequences were printed on this lane, and no amplification reaction was expected from this lane. The primer printing configuration of the other lanes were as follows: Lanes 3,4 = S.Equi. Lane 5,6 = S.Zooep Lanes 7,8 – Equine Herpesvirus 1 Lanes 9,10 = Equine Herpesvirus 4. Since the maximum reaction capacity of each lane was only 1uL, the primer mix was diluted in 1:20 ratio in nuclease-free water. 1 uL of the 1:20 dilution of the appropriate primer mix was loaded at the outlets of each of the lanes. The liquid, upon pipetting, moved up the lane, but did not reach sample inlet. This allowed us to achieve uniform distribution of primers while preventing any primer cross-talk between the lanes. Due to the small volumes, the primers dry within a few minutes. The chips prepared with the dried primers can either be used immediately for testing or stored in a desiccator to be used for later experiments. The primers are hydrated when the reaction mix (without the primers) are injected into the sample inlet, and can then participate in the LAMP reaction. Amplification and a corresponding exponential increase in fluorescence is observed when the primer finds the specific template.

Once the chip is ready for amplification, it is inserted into our custom-made cartridge (~ the size of a credit card). The cartridge allows the chip to inserted into our smartphone-based setup (Figure 3.2b), and allows easy handling of the chip during transition. The cartridge was made from Polyoxymethylene (POM) due to the material's low auto-fluorescence, low cost, ease of machining, and excellent dimensional stability. The chip was attached inside the card with adhesive, so the chip's location within the imaging field of view was fixed inside the reader. Information about the chip and the assays were encoded in the quick response code (QR code).

3.4. Chip Imaging

3.4.1. Fluorescence microscopy

We used fluorescence microscopy to verify the on-chip assays on-chip before moving to our smartphone-based reader. Nikon Eclipse FN1 fluorescence microscope with a 2× objective and a Nikon 96311 B-2E/C FITC fluorescence filter was used to capture real-time images of the LAMP reaction. For fluorescence microscope-based real time LAMP reactions, a commercial heating stage (INSTECT mK1000) was used to incubate the chip at 65C. At a 2X objective, the field of view of the microscope allowed 6 out of the 10 wells to be imaged. The fluorescent image was used to construct amplification curves for the reactions tested. For limit-of-detection experiments, threshold times were determined for each of amplification curves, and a standard curve of threshold time vs input DNA concentration was constructed.

3.4.2. Smartphone-based instrument

The smartphone-based reader system was designed by Dr. Hojeong Yu from Professor Brian Cunningham's group. The details about the instrumentation can be found in the manuscript http://pubs.acs.org/doi/pdf/10.1021/acs.analchem.7b02478The schematic of the reader is shown in Figure 3.3.

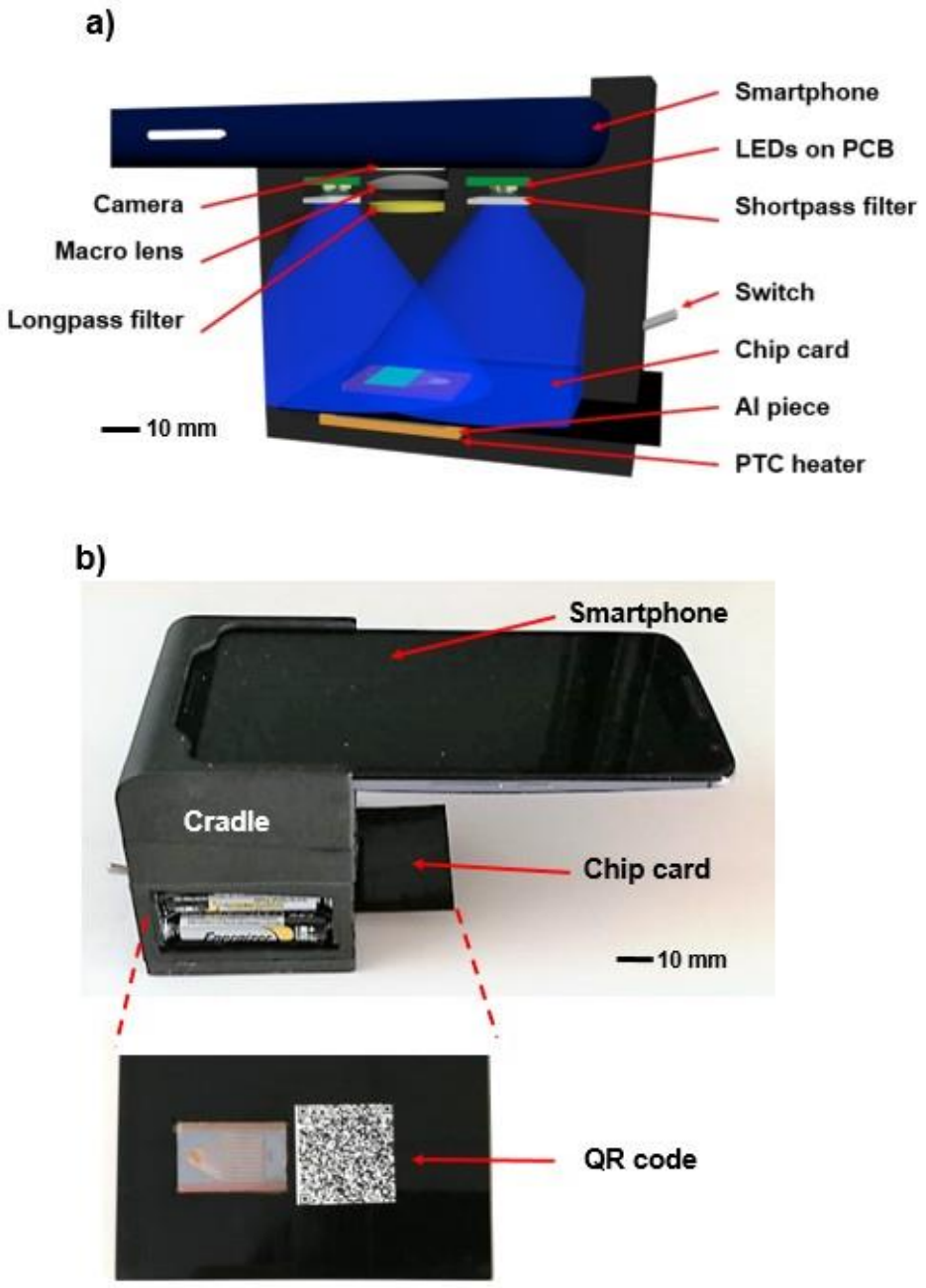

Figure 3.3: Smartphone-based instrument. a) Schematic diagram to show the internal structure of the cradle that integrates optical and electrical components used for smartphone fluorescence microscopy. The microfluidic chip, integrated in its card, is inserted into the cradle that incorporates a PTC heater to maintain a constant ~ 65 °C temperature. b) Photo of the smartphone-based instrument taken with the smartphone and chip card. A QR code label is printed on the chip card to provide information about the on-chip detection.

4. Results

4.1. Off-chip characterization of the LAMP assay

DNA from the culture-grown pathogenic source was extracted using the heat lysis protocol as described in the Materials and Methods section. The concentration of the extracted DNA was estimated using a PCR standard curve (Figure 4.1) constructed by using known amounts of target plasmid DNA in reaction. The limit of detection of the PCR assays was 1e5 copies/mL for all the targets. The LAMP limit of detection for the four assays were determined first in a tube-based reaction using a commercial thermocycler (Eppendorf MasterCycler RealPlex4). LAMP reactions were performed in tubes with ten-fold serial dilution of the DNA of interest. Figure 4.2(a-d) shows the baseline-subtracted amplification curves for the off-chip LAMP assay of S. Equi, S. Zoo, EHV-1, and EHV-4 using purified DNA templates. Triplicate testing was done for each concentration by dividing a 25uL reaction into three 8uL final reactions. Threshold times from the amplification curves were calculated by determining the time taken for the curve to reach 20% of its maximum intensity. A good log-linear fit was obtained between the threshold times and the concentration of input DNA. The lower limit-of-detection was determined to be 5×10^4 copies/mL for S. Equi, 5×10^3 copies/mL for S. Zoo and EHV-1, and 1×10^3 copies/mL for EHV-4. The limits of detection of our tube-based LAMP assays was superior compared to the PCR assays. Figure 4.3 shows the same assay performed using plasmid DNA targets. A lower limit of detection of 1e5 copies/mL (starting) was obtained for each of the assays. To demonstrate the specificity of our LAMP assays, which is critical to the multiplexed on-chip detection, each of the four target DNA sequences were tested against non-specific primer sets (Figure 4.4). Specific template-primer amplification was observed for all four assays. The results are summarized in Table 4.1.

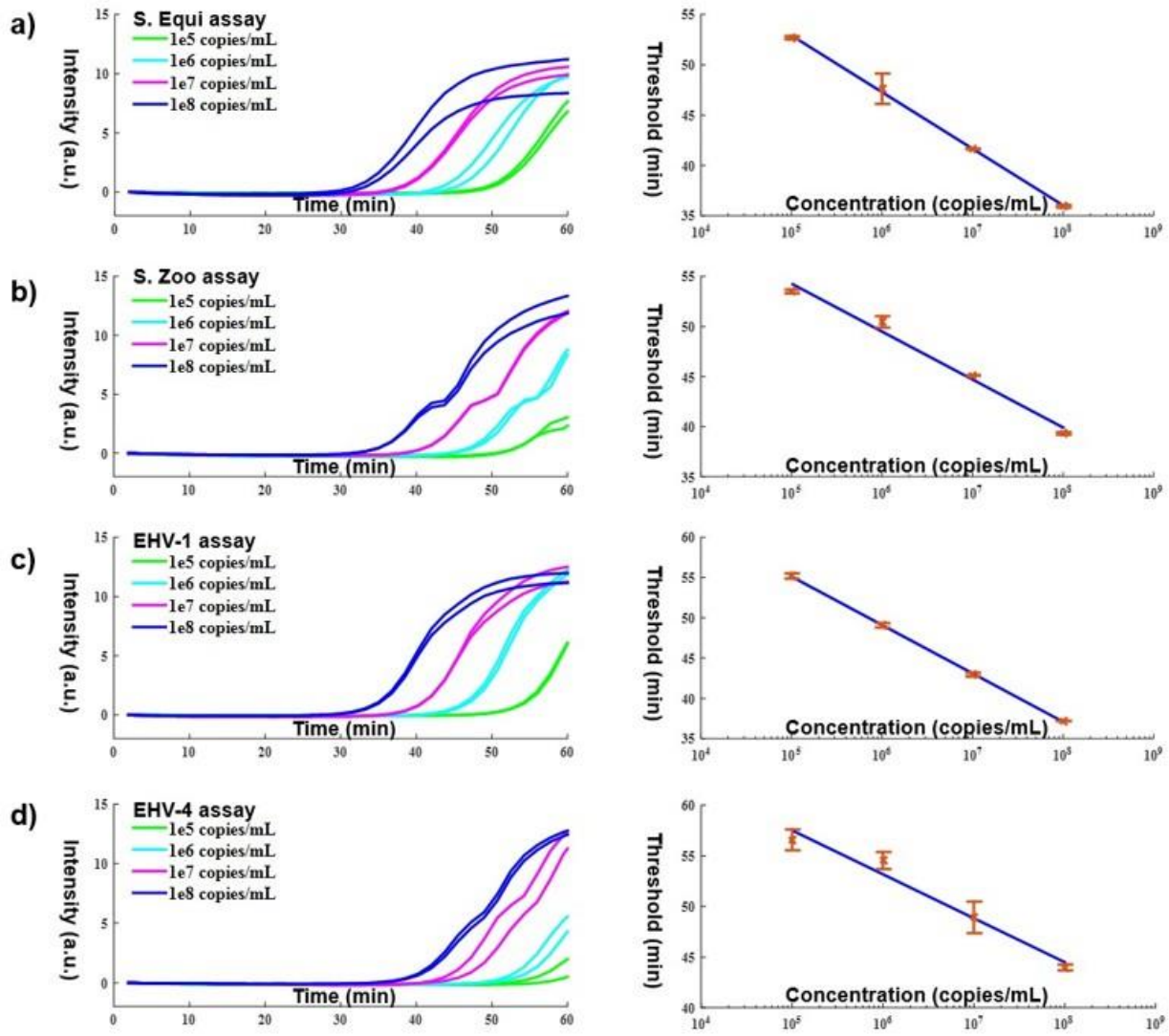

Figure 4.1: Off-chip PCR standard curves for the detection and quantification of equine respiratory infection pathogen DNA. The amplification curves and threshold time versus concentration relationship are plotted for a) S. Equi, b) S. Zoo, c) EHV-1, and d) EHV-4.

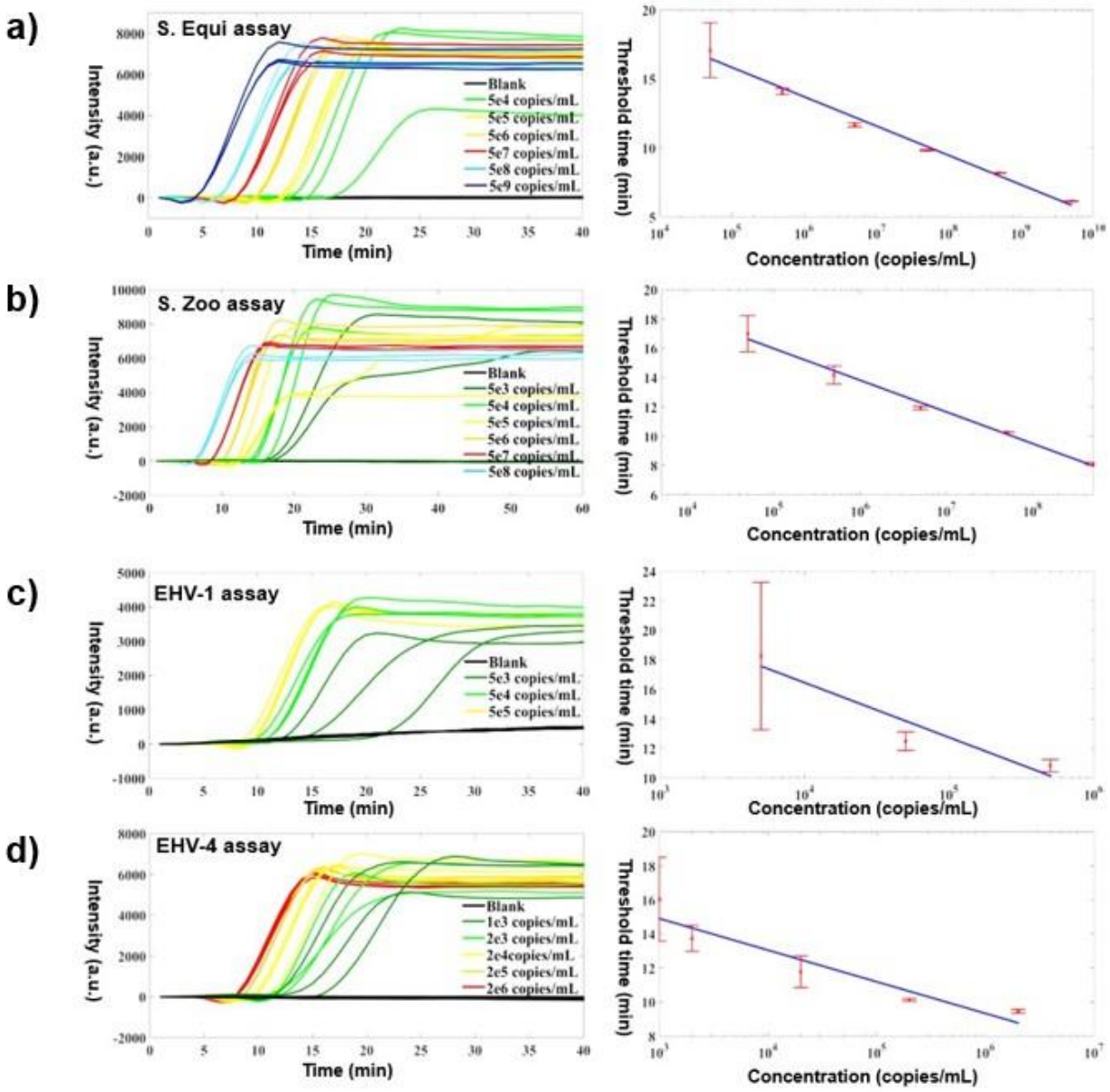

Figure 4.2: Off-chip characterization of the four LAMP assays developed for the detection of equine respiratory infection pathogen DNA. The amplification curves and threshold time versus concentration relationship are plotted for a) S. Equi, b) S. Zoo, c) EHV-1, and d) EHV-4.

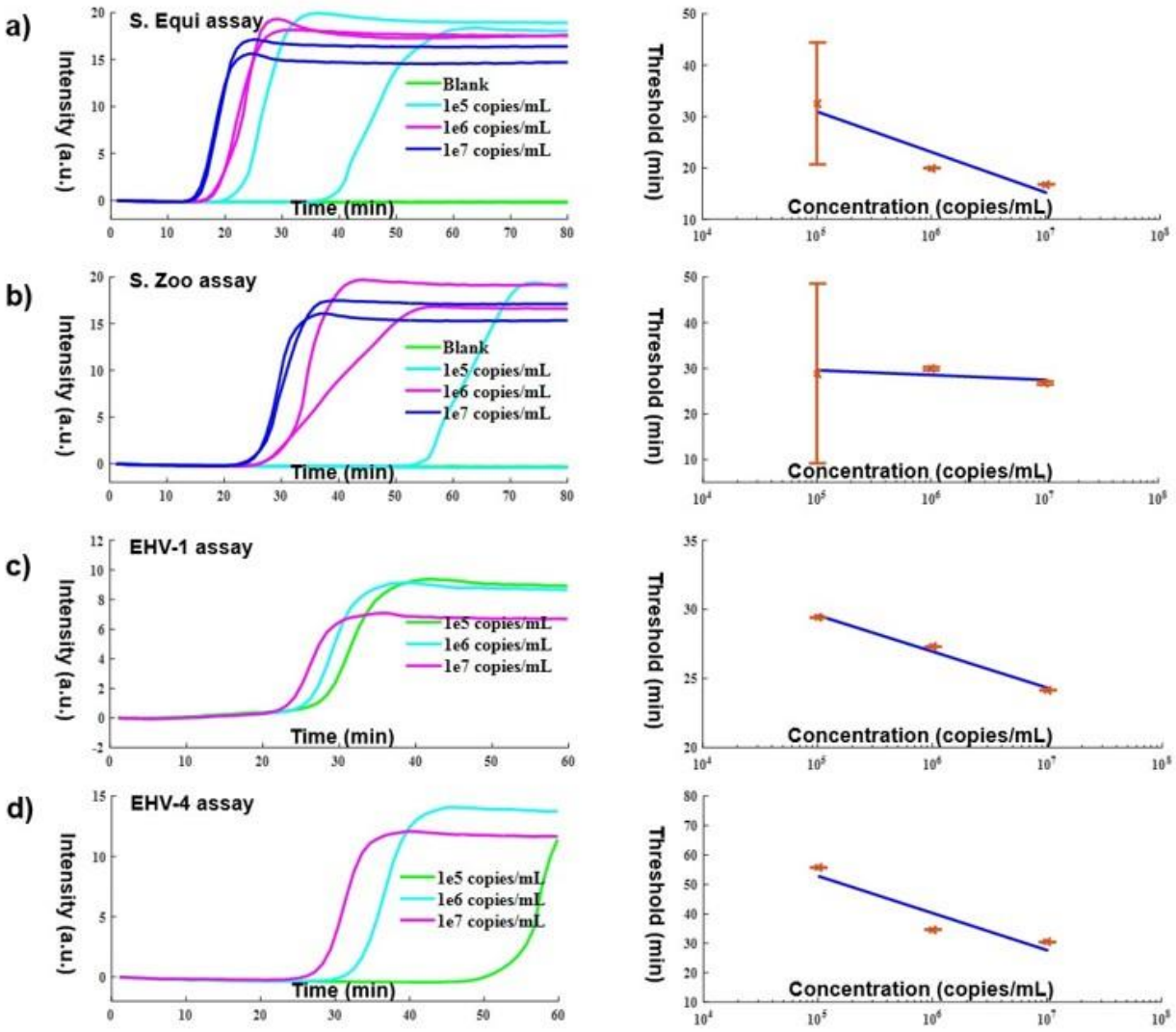

Figure 4.3: Off-chip characterization of the four LAMP assays developed for the detection of equine respiratory infection pathogen using plasmid DNA templates. The amplification curves and threshold time versus concentration relationship are plotted for a) S. Equi, b) S. Zoo, c) EHV-1, and d) EHV-4.

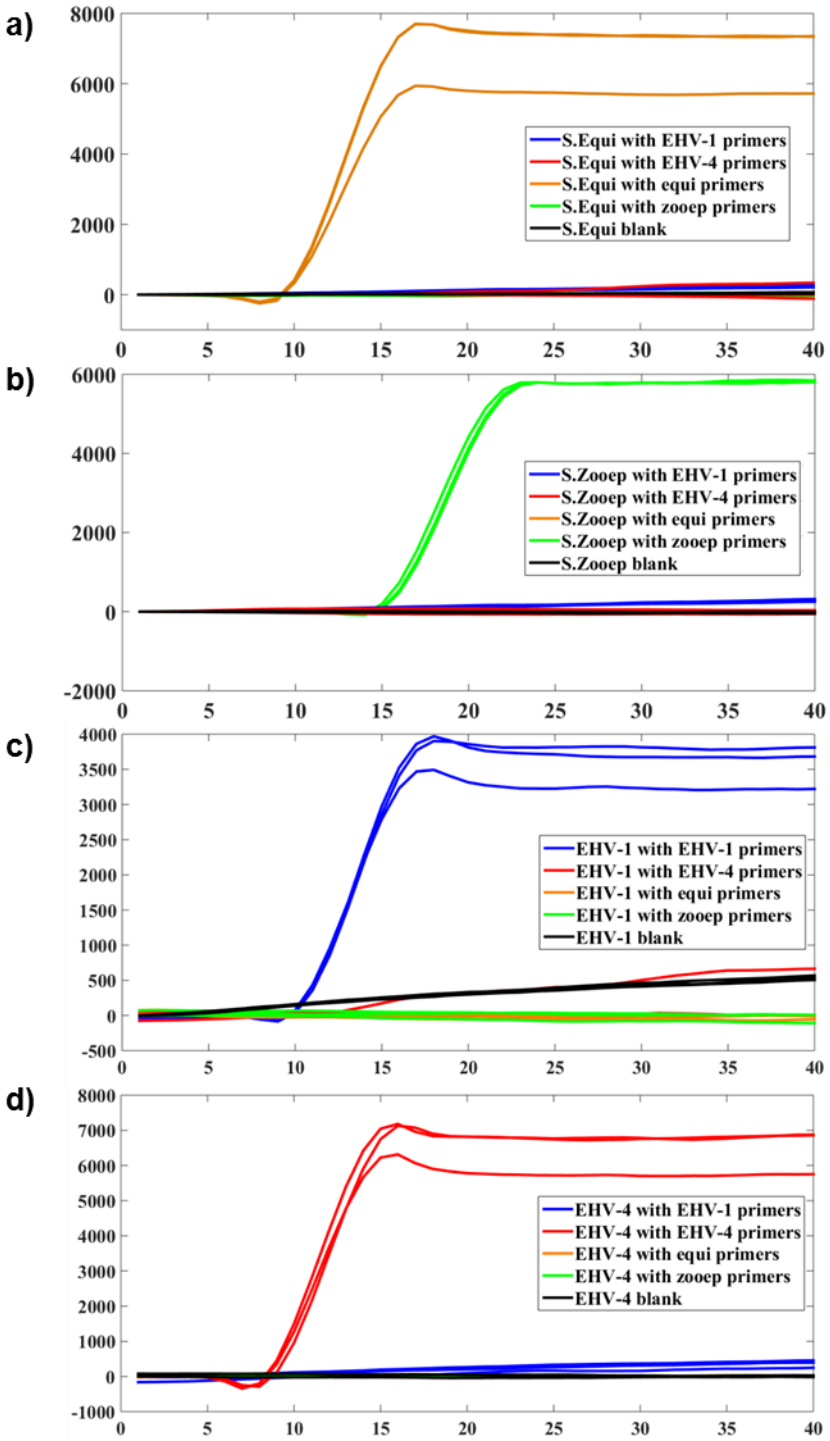

Figure 4.4: Off-chip verification of assay specificity. The real-time reaction curves of a) the S. Equi DNA, b) S.Zoo DNA, c) EHV-1 DNA and d) EHV-4 DNA with all four primers and negative control

		Primer			
		S.Zooep	S.Equi	EHV-1	EHV-4
Template	S.Zooep	✓	✗	✗	✗
	S.Equi	✗	✓	✗	✗
	EHV-1	✗	✗	✓	✗
	EHV-4	✗	✗	✗	✓

Table 4.1: Summary of the specificity of the four LAMP assays.

4.2. On-chip characterization of the LAMP assay

The experiments in this section were done in collaboration with Dr. Weili Chen and Ms. Fu Sun from Professor Cunningham's group.

4.2.1. On-chip limits of detection test

Figures 4.5 – 4.8 summarizes the on-chip LOD tests for S. Zooep, S.Equi, EHV-1 and EHV-4 respectively. We observed an on-chip limit of detection of 5e4 copies/mL for S.Equi and S.Zooep, and 5e3 copies/mL for EHV-1 and EHV-4. No non-specific amplification (no template control) was observed in any of the assays for a period of 60 minutes reaction time.The concentration of the purified DNAs are within the clinically relevant ranges for the four pathogens

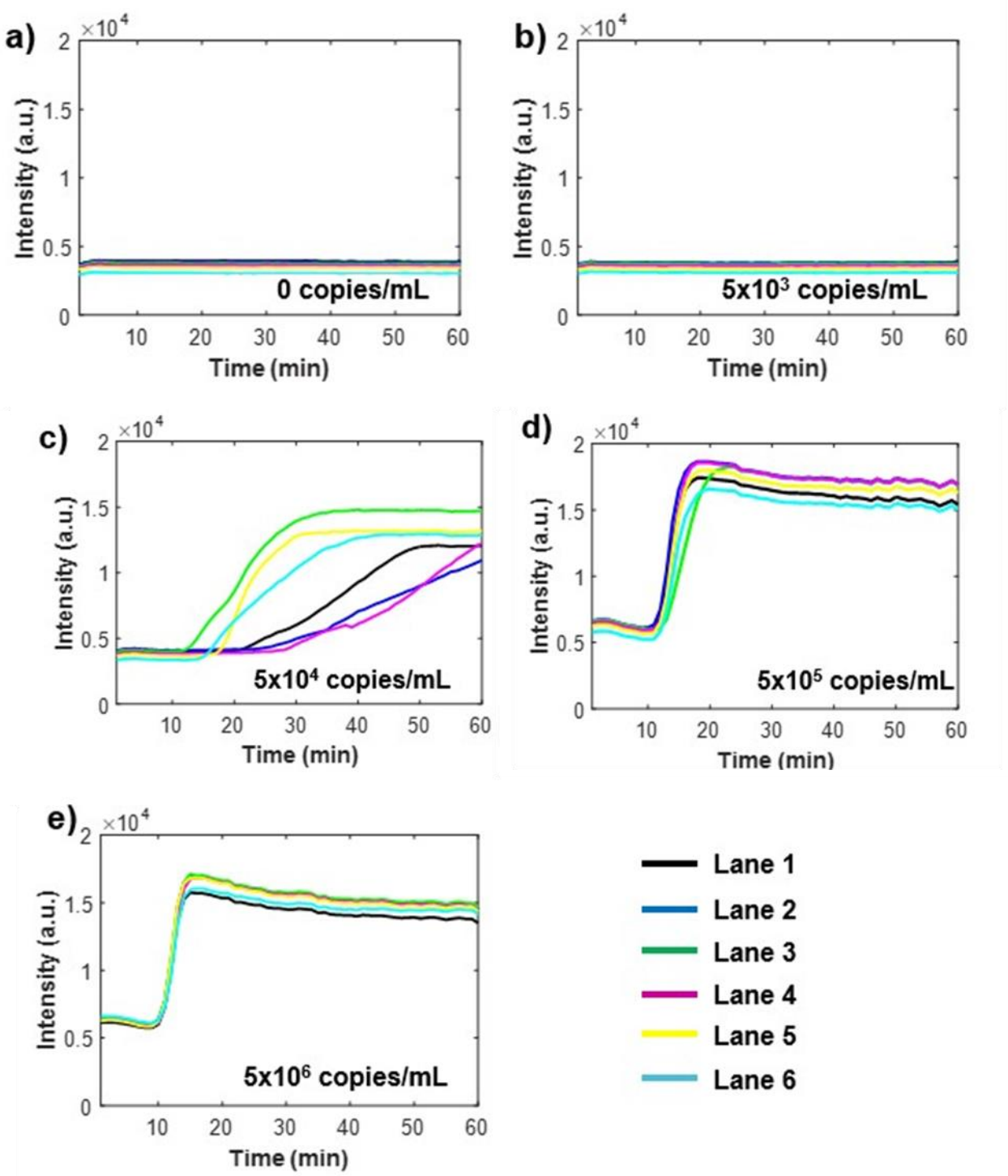

Figure 4.5: On-chip characterization of the S. Zoo assay. The negative control a) and low DNA concentration test b) show no amplification within 60 minutes c) The limit of detection for the on-chip reaction is 5×10^4 copies/mL. The amplification reactions begin within 15 minutes for the high concentration tests at d) 5×10^5 copies/mL and e) 5×10^6 copies/mL.

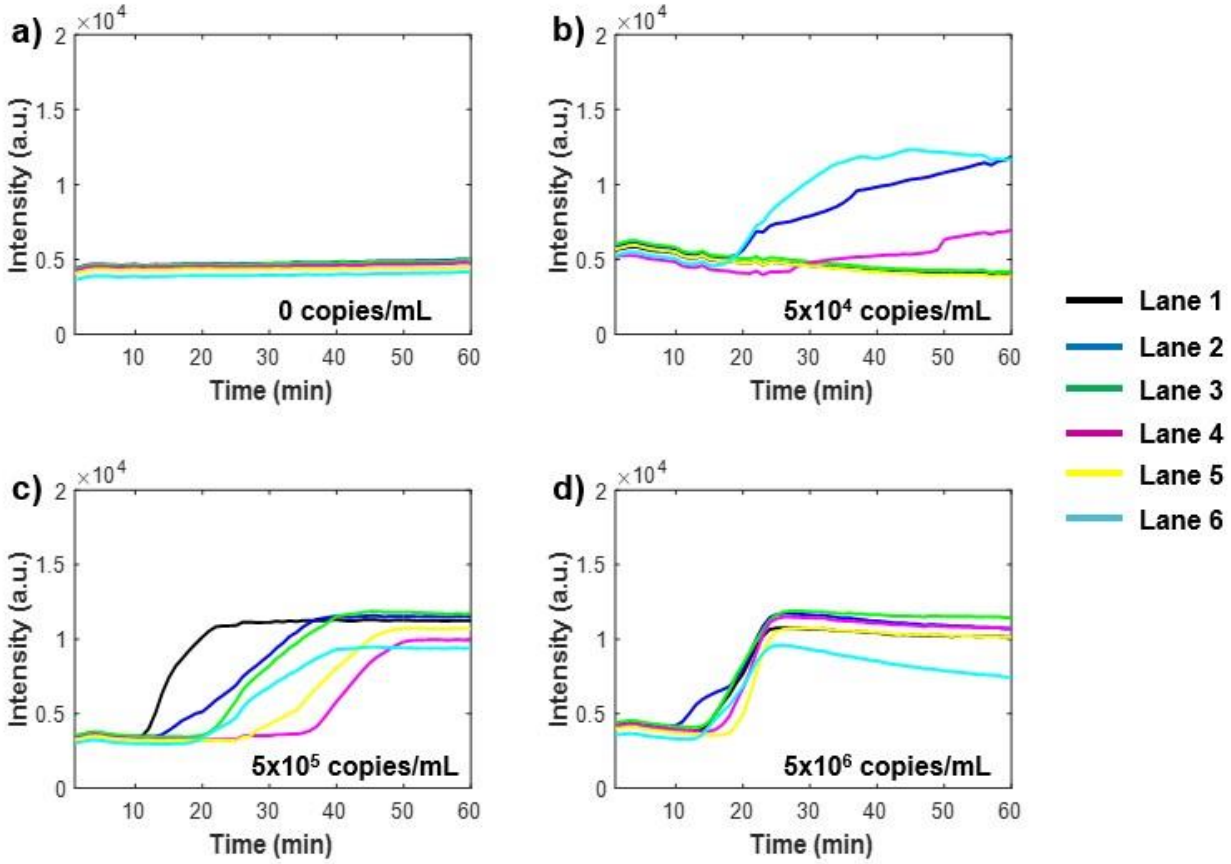

Figure 4.6: On-chip characterization of the S. Equi assay detection limit. The limit of detection for the on-chip reaction is 5×10^4 copies/mL

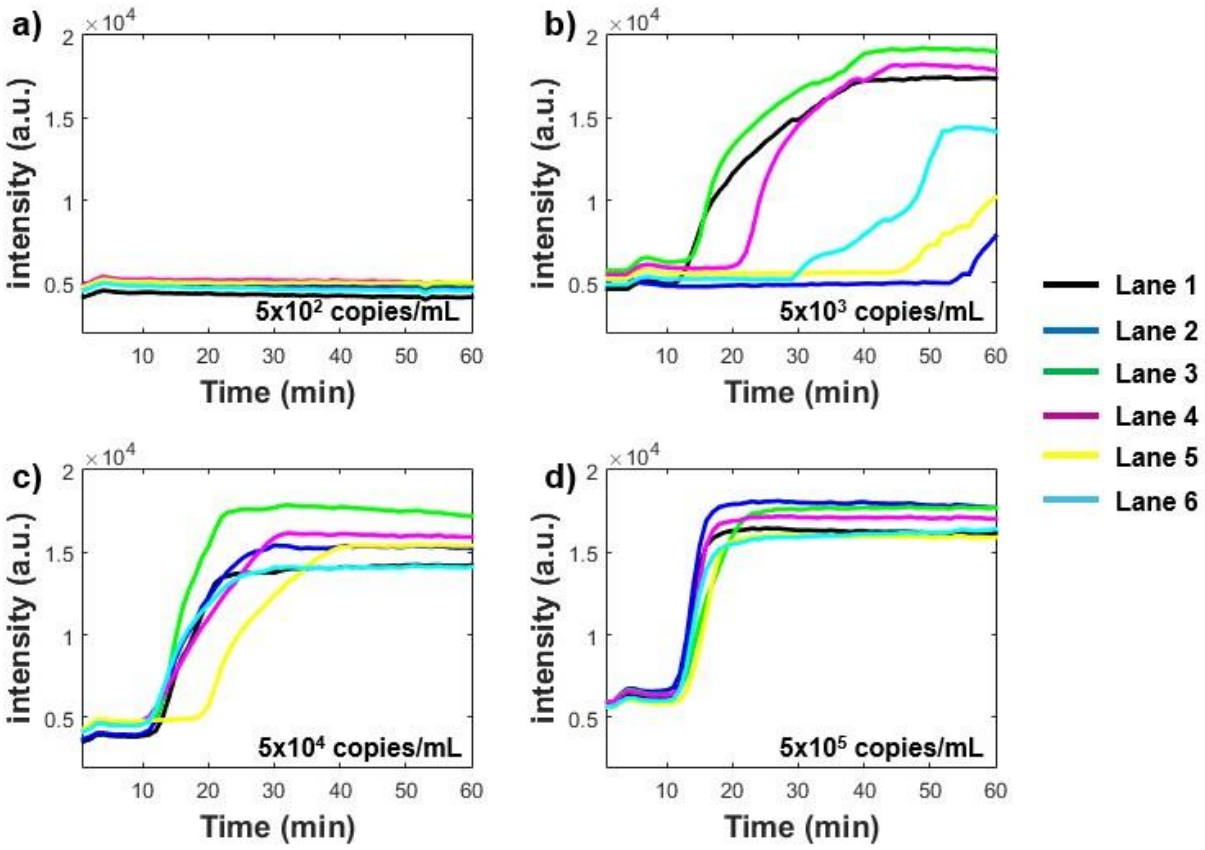

Figure 4.7: On-chip characterization of EHV-1 assay detection limit. The limit of detection for the on-chip reaction is 5×10^3 copies/mL

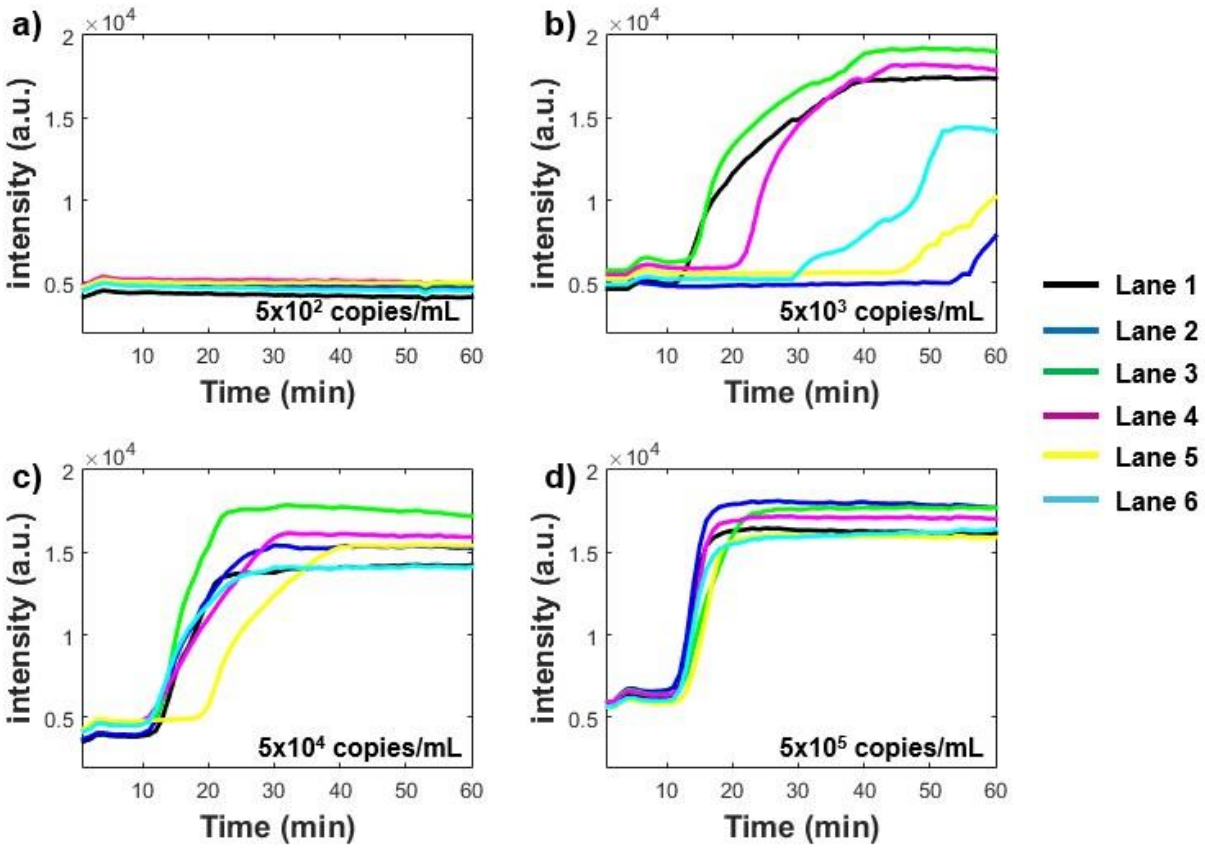

Figure 4.8: On-chip characterization of EHV-4 assay detection limit. The limit of detection for the on-chip reaction is 5×10^3 copies/mL

4.2.2. On-chip simultaneous detection tests

We performed end point LAMP assays to demonstrate the primer specificity on-chip. End-point images of the LAMP reactions were taken by our smartphone reader. (Figure 4.10). We pre-printed the chips with target specific primers as described in the primer deposition section in the Materials and Methods section. Figure 4.10a shows the end-point LAMP fluorescent image as captured by our smartphone reader when the 5e7 copies/mL of S.Equi target DNA was introduced into the reaction. As evident from the figure, only lanes 1 (universal positive control, See Materials and Methods section), and lanes 3 and 4 (containing the S.Equi specific primer sets) showed an increased fluorescence above the pre-set intensity threshold. . The threshold value was designated as half of the intensity of the positive control as the value that is used to differentiate a positive test (target DNA present) from a negative test (target DNA not present). This demonstrated the

specific detection of the S,Equi target DNA in only the S.Equi specific primer printed lanes. Specific detection was observed when 5e6 copies/mL of S.Zooep DNA was used as the template (Figure 4.9b) where only lanes 1 (positive control), and lanes 5 & 6 (S.Zooep primers) showed an increased fluorescence above the threshold. The Figure 4.9c and Figure 4.9d show the results for detection of target DNA sequences from viral DNA targets EHV-1 and EHV-4 respectively. Both experiments showed clear evidence that the presence of target DNA can be specifically identified without inducing amplification in non-target lanes. The concentrations for the injected EHV-1 and EHV-4 DNAs were 5×10^6 copies/mL and 2×10^6 copies/mL respectively. Again, clear distinction between the positive and negative lanes were observed with the fluorescence intensity in the negative lanes being below the threshold.

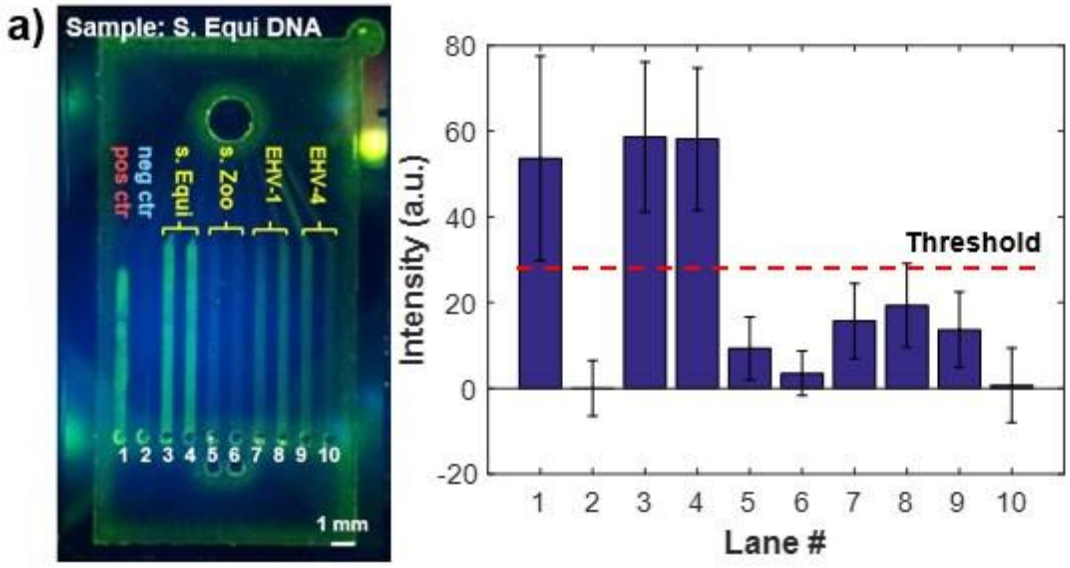

Figure 4.9 Continued

Figure 4.9 Continued

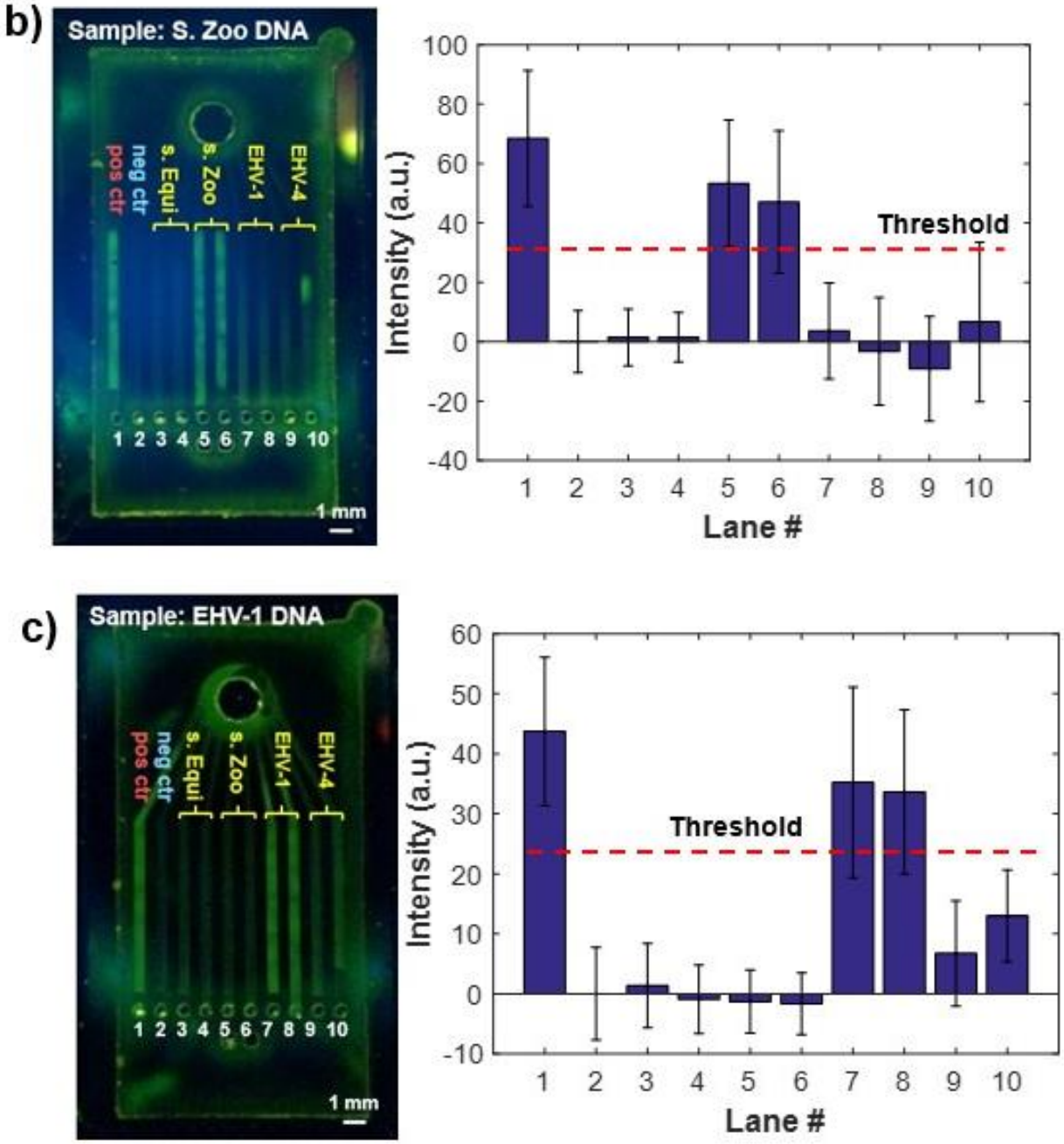

Figure 4.9 Continued

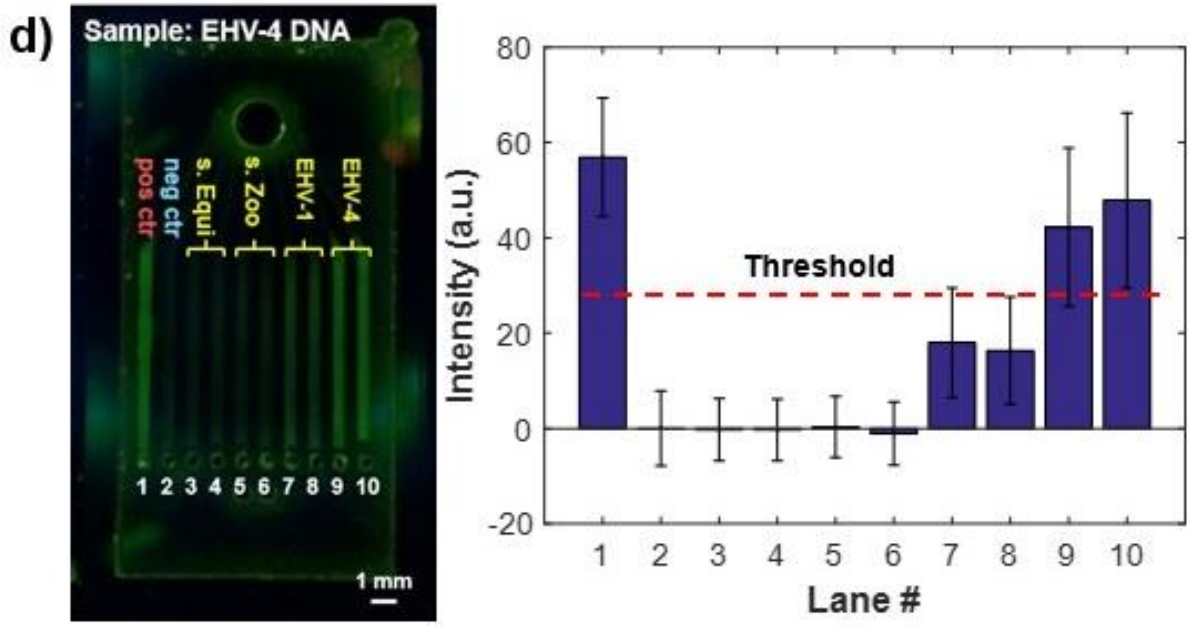

Figure 4.9 a-d: Experiments demonstrating one-at-a-time detection of target DNA sequences. Smartphone-captured images and intensities from each of the ten lanes on the fluorescence images are shown in a) for S. Equi detection, b) S. Zoo detection, c) EHV-1 detection and, d) EHV-4 detection. The LAMP reactions generate fluorescent output only in the positive control channel and the channels prepared with the specific primers. The presence of specific DNA sequences can be identified by the brightness of the lanes on the fluorescence images.

5. Conclusion

We demonstrate a compact, rapid, multiplexed, and inexpensive system for smartphone-based detection and identification of disease-specific nucleic acid sequences within a single-droplet test sample. The system utilizes a microfluidic approach for performing LAMP-based isothermal amplification of a multiplexed array of 1 to 10 pathogen-specific nucleic acid sequences, and uses a hand-held cradle that interfaces with the rear-facing camera of a conventional smartphone to capture the fluorescence images. The captured images are analyzed by a smartphone app and shared to a cloud-based database for the rapid reporting of the detection results. Four LAMP assays have been developed for the detection of the specific genes of four major pathogens that cause equine respiratory infectious diseases, including streptococcus equi, streptococcus zooepidemicus, equine herpesvirus types 1 and 4. As compared with the assays performed on a conventional laboratory thermocycler apparatus the detection sensitivity is not compromised using the microfluidic approach and the smartphone-based instrument. Importantly, the system is capable of detecting multiple nucleic acid targets at the same time, and thus is capable of identifying co-infections of multiple pathogen strains. By generating a positive/negative determination of the presence of specific pathogens with integrated experimental controls and replicates, the mobile system can assist physicians in rapid point-of-care decision-making for treatment and quarantine response that is currently not possible with tests performed at central laboratory facilities. We believe this approach provides a mobile, simple and inexpensive capability for clinicians to perform infectious disease diagnostics, and it represents a significant stride towards a practical solution to the infectious disease diagnostics at resource-limited-settings.

6. References

[1] B. Rush and T. Mair, *Equine Respir. Dis.*, 2007, 1–322.

[2] Overview of Respiratory Diseases of Horses - Respiratory System - Merck Veterinary Manual: "www.merckvetmanual.com/respiratory-system/respiratory-diseases-of-horses/overview-of-respiratory-diseases-of-horses"

[3] 1 H. Cartei, L. E. Shepard, M. Whitney, L. E. Shepard, M. D. Whitney, K. Blackman, R. K. Craft and P. Poe, An Economic Analysis of the California Thoroughbred Racing Industry, *Giannini Research Report Number 342*, 1991

[4] C. van Maanen, *Vet. Q.*, 2002, **24**, 57–78.

[5] Strangles in Horses - Respiratory System - Merck Veterinary Manual: "www.merckvetmanual.com/respiratory-system/respiratory-diseases-of-horses/strangles-in-horses"

[6] S. H. Lee, S. W. Kim, J. Y. Kang, and C. H. Ahn, "A polymer lab-on-a-chip for reverse transcription (RT)-PCR based point-of-care clinical diagnostics," *Lab on a Chip*, vol. 8, pp. 2121-2127, 2008.

[7] J. Wang, Z. Y. Chen, P. L. A. M. Corstjens, M. G. Mauk, and H. H. Bau, "A disposable microfluidic cassette for DNA amplification and detection," *Lab on a Chip*, vol. 6, pp. 46-53, 2006.

[8] C. S. Zhang and D. Xing, "Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends," *Nucleic Acids Research*, vol. 35, pp. 4223-4237, 2007.

[9] Z. Y. Chen, M. G. Mauk, J. Wang, W. R. Abrams, P. L. A. M. Corstjens, R. S. Niedbala, *et al.*, "A microfluidic system for saliva-based detection of infectious diseases," *Oral-Based Diagnostics*, vol. 1098, pp. 429-436, 2007.

[10] D. F. Chen, M. Mauk, X. B. Qiu, C. C. Liu, J. T. Kim, S. Ramprasad, *et al.*, "An integrated, self-contained microfluidic cassette for isolation, amplification, and detection of nucleic acids," *Biomedical Microdevices*, vol. 12, pp. 705-719, Aug 2010.

[11] F. Ahmad and S. A. Hashsham, "Miniaturized nucleic acid amplification systems for rapid and point-of-care diagnostics: A review," *Analytica Chimica Acta*, vol. 733, pp. 1-15, Jul 6 2012.

[12] S. L. Angione, A. Chauhan, and A. Tripathi, "Real-Time Droplet DNA Amplification with a New Tablet Platform," *Analytical Chemistry*, vol. 84, pp. 2654-2661, Mar 20 2012.

[13] J. M. Bienvenue, L. A. Legendre, J. P. Ferrance, and J. P. Landers, "An integrated microfluidic device for DNA purification and PCR amplification of STR fragments," *Forensic Science International-Genetics*, vol. 4, pp. 178-186, Apr 2010.

[14] M. C. Breadmore, K. A. Wolfe, I. G. Arcibal, W. K. Leung, D. Dickson, B. C. Giordano, *et al.*, "Microchip-based purification of DNA from biological samples," *Analytical Chemistry,* vol. 75, pp. 1880-1886, Apr 15 2003.

[15] N. C. Cady, S. Stelick, M. V. Kunnavakkam, and C. A. Batt, "Real-time PCR detection of Listeria monocytogenes using an integrated microfluidics platform," *Sensors and Actuators B-Chemical,* vol. 107, pp. 332-341, May 27 2005.

[16] L. Chen, A. Manz, and P. J. R. Day, "Total nucleic acid analysis integrated on microfluidic devices," *Lab on a Chip,* vol. 7, pp. 1413-1423, 2007.

[17] B. C. Giordano, J. Ferrance, S. Swedberg, A. F. R. Huhmer, and J. P. Landers, "Polymerase chain reaction in polymeric microchips: DNA amplification in less than 240 seconds," *Analytical Biochemistry,* vol. 291, pp. 124-132, Apr 1 2001.

[18] J. A. Higgins, S. Nasarabadi, J. S. Karns, D. R. Shelton, M. Cooper, A. Gbakima, *et al.*, "A handheld real time thermal cycler for bacterial pathogen detection," *Biosensors & Bioelectronics,* vol. 18, pp. 1115-1123, Aug 15 2003.

[19] C. S. J. Hou, M. Godin, K. Payer, R. Chakrabarti, and S. R. Manalis, "Integrated microelectronic device for label-free nucleic acid amplification and detection," *Lab on a Chip,* vol. 7, pp. 347-354, 2007.

[20] E. Kai, S. Sawata, K. Ikebukuro, T. Iida, T. Honda, and I. Karube, "Detection of PCR products in solution using surface plasmon resonance," *Analytical Chemistry,* vol. 71, pp. 796-800, Feb 15 1999.

[21] J. Khandurina, T. E. McKnight, S. C. Jacobson, L. C. Waters, R. S. Foote, and J. M. Ramsey, "Integrated system for rapid PCR-based DNA analysis in microfluidic devices," *Analytical Chemistry,* vol. 72, pp. 2995-3000, Jul 1 2000.

[22] M. U. Kopp, A. J. de Mello, and A. Manz, "Chemical amplification: Continuous-flow PCR on a chip," *Science,* vol. 280, pp. 1046-1048, May 15 1998.

[23] E. T. Lagally, I. Medintz, and R. A. Mathies, "Single-molecule DNA amplification and analysis in an integrated microfluidic device," *Analytical Chemistry,* vol. 73, pp. 565-570, Feb 1 2001.

[24] J. H. Leamon, W. L. Lee, K. R. Tartaro, J. R. Lanza, G. J. Sarkis, A. D. deWinter, *et al.*, "A massively parallel PicoTiterPlate (TM) based platform for discrete picoliter-scale polymerase chain reactions," *Electrophoresis,* vol. 24, pp. 3769-3777, Nov 2003.

[25] P. G. R. Liu, J. Yang, and R. Lenigk, "Self-Contained, Fully Integrated Biochips for Sample Preparation, PCR Amplification and DNA Microarray Analysis," in *Integrated Biochips for DNA Analysis*, R. L. a. A. Lee, Ed., ed: Springer New York, 2007, pp. 46-67.

[26] A. L. Markey, S. Mohr, and P. J. R. Day, "High-throughput droplet PCR," *Methods*, vol. 50, pp. 277-281, Apr 2010.

[27] S. Mohr, Y. H. Zhang, A. Macaskill, P. J. R. Day, R. W. Barber, N. J. Goddard, *et al.*, "Numerical and experimental study of a droplet-based PCR chip," *Microfluidics and Nanofluidics*, vol. 3, pp. 611-621, Oct 2007.

[28] A. F. Sauer-Budge, P. Mirer, A. Chatterjee, C. M. Klapperich, D. Chargin, and A. Sharon, "Low cost and manufacturable complete microTAS for detecting bacteria," *Lab on a Chip*, vol. 9, pp. 2803-2810, 2009.

[29] F. Shen, W. B. Du, J. E. Kreutz, A. Fok, and R. F. Ismagilov, "Digital PCR on a SlipChip," *Lab on a Chip*, vol. 10, pp. 2666-2672, 2010.

[30] R. Tewhey, J. B. Warner, M. Nakano, B. Libby, M. Medkova, P. H. David, *et al.*, "Microdroplet-based PCR enrichment for large-scale targeted sequencing," *Nature Biotechnology*, vol. 27, pp. 1025-U94, Nov 2009.

[31] F. Wang and M. A. Burns, "Performance of nanoliter-sized droplet-based microfluidic PCR," *Biomedical Microdevices*, vol. 11, pp. 1071-1080, Oct 2009.

[32] W. Wang, Z. X. Li, R. Luo, S. H. Lu, A. D. Xu, and Y. J. Yang, "Droplet-based micro oscillating-flow PCR chip," *Journal of Micromechanics and Microengineering*, vol. 15, pp. 1369-1377, Aug 2005.

[33] L. C. Waters, S. C. Jacobson, N. Kroutchinina, J. Khandurina, R. S. Foote, and J. M. Ramsey, "Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing," *Analytical Chemistry*, vol. 70, pp. 158-162, Jan 1 1998.

[34] S. W. Yeung, T. M. H. Lee, H. Cai, and I. M. Hsing, "A DNA biochip for on-the-spot multiplexed pathogen identification," *Nucleic Acids Research*, vol. 34, Oct 2006.

[35] C. S. Zhang, J. L. Xu, W. L. Ma, and W. L. Zheng, "PCR microfluidic devices for DNA amplification," *Biotechnology Advances*, vol. 24, pp. 243-284, May-Jun 2006.

[36] Y. H. Zhang and P. Ozdemir, "Microfluidic DNA amplification-A review," *Analytica Chimica Acta*, vol. 638, pp. 115-125, Apr 13 2009.

[37] N. R. Beer, B. J. Hindson, E. K. Wheeler, S. B. Hall, K. A. Rose, I. M. Kennedy, *et al.*, "On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets," *Analytical Chemistry*, vol. 79, pp. 8471-8475, Nov 15 2007.

[38] O. J. Dressler, R. M. Maceiczyk, S. I. Chang, and A. J. deMello, "Droplet-Based Microfluidics Enabling Impact on Drug Discovery," *Journal of Biomolecular Screening*, vol. 19, pp. 483-496, Apr 2014.

[39] J. Liu, M. Enzelberger, and S. Quake, "A nanoliter rotary device for polymerase chain reaction," *Electrophoresis,* vol. 23, pp. 1531-1536, May 2002.

[40] Y. Schaerli and F. Hollfelder, "The potential of microfluidic water-in-oil droplets in experimental biology," *Molecular Biosystems,* vol. 5, pp. 1392-1404, 2009.

[41] L. G. Carrascosa, A. Calle, and L. M. Lechuga, "Label-free detection of DNA mutations by SPR: application to the early detection of inherited breast cancer," *Analytical and Bioanalytical Chemistry,* vol. 393, pp. 1173-1182, Feb 2009.

[42] T. Endo, K. Kerman, N. Nagatani, Y. Takamura, and E. Tamiya, "Label-free detection of peptide nucleic acid-DNA hybridization using localized surface plasmon resonance based optical biosensor," *Analytical Chemistry,* vol. 77, pp. 6976-6984, Nov 1 2005.

[43] H. X. Li and L. J. Rothberg, "Label-free colorimetric detection of specific sequences in genomic DNA amplified by the polymerase chain reaction," *Journal of the American Chemical Society,* vol. 126, pp. 10958-10961, Sep 8 2004.

[44] A. T. Woolley, D. Hadley, P. Landre, A. J. deMello, R. A. Mathies, and M. A. Northrup, "Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device," *Analytical Chemistry,* vol. 68, pp. 4081-4086, Dec 1 1996.

[45] D. C. Zhang, Y. R. Yan, Q. Li, T. X. Yu, W. Cheng, L. Wang, *et al.*, "Label-free and high-sensitive detection of Salmonella using a surface plasmon resonance DNA-based biosensor," *Journal of Biotechnology,* vol. 160, pp. 123-128, Aug 31 2012.

[46] J. Compton, "Nucleic-Acid Sequence-Based Amplification," *Nature,* vol. 350, pp. 91-92, Mar 7 1991.

[47] G. T. Walker, M. S. Fraiser, J. L. Schram, M. C. Little, J. G. Nadeau, and D. P. Malinowski, "Strand Displacement Amplification - an Isothermal, Invitro DNA Amplification Technique," *Nucleic Acids Research,* vol. 20, pp. 1691-1696, Apr 11 1992.

[48] O. Piepenburg, C. H. Williams, D. L. Stemple, and N. A. Armes, "DNA detection using recombination proteins," *Plos Biology,* vol. 4, pp. 1115-1121, Jul 2006.

[49] R. D. Stedtfeld, D. M. Tourlousse, G. Seyrig, T. M. Stedtfeld, M. Kronlein, S. Price, *et al.*, "Gene-Z: a device for point of care genetic testing using a smartphone," *Lab on a Chip,* vol. 12, pp. 1454-1462, 2012.

[50] C. Duarte, E. Salm, B. Dorvel, B. Reddy, and R. Bashir, "On-chip parallel detection of foodborne pathogens using loop-mediated isothermal amplification," *Biomedical Microdevices,* vol. 15, pp. 821-830, Oct 2013.

[51] P. Craw and W. Balachandran, "Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review," *Lab on a Chip,* vol. 12, pp. 2469-2486, 2012.

[52] M. Parida, S. Sannarangaiah, P. K. Dash, P. V. L. Rao, and K. Morita, "Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases," *Reviews in Medical Virology*, vol. 18, pp. 407-421, Nov-Dec 2008.

[53] W. L. Chen, K. D. Long, J. Kurniawan, M. Hung, H. J. Yu, B. A. Harley, *et al.*, "Planar Photonic Crystal Biosensor for Quantitative Label-Free Cell Attachment Microscopy," *Advanced Optical Materials*, vol. 3, pp. 1623-1632, Nov 2015.

[54] W. L. Chen, K. D. Long, H. J. Yu, Y. F. Tan, J. S. Choi, B. A. Harley, *et al.*, "Enhanced live cell imaging via photonic crystal enhanced fluorescence microscopy," *Analyst*, vol. 139, pp. 5954-5963, Nov 21 2014.

[55] G. L. Damhorst, C. Duarte-Guevara, W. Chen, T. Ghonge, B. T. Cunningham, and R. Bashir, "Smartphone-Imaged HIV-1 Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) on a Chip from Whole Blood," *Engineering (Beijing)*, vol. 1, pp. 324-335, Sep 2015.

[56] R. D. Peterson, W. L. Chen, B. T. Cunningham, and J. E. Andrade, "Enhanced sandwich immunoassay using antibody-functionalized magnetic iron-oxide nanoparticles for extraction and detection of soluble transferrin receptor on a photonic crystal biosensor," *Biosensors & Bioelectronics*, vol. 74, pp. 815-822, Dec 15 2015.

[57] T. I. Day and Brent, "Global Analysis of the Smartphones Market: Who's Winning the Necessary Regional Battles," 2013.

[58] G. Comtois, J. I. Salisbury, and Y. Sun, "A Smartphone-Based Platform for Analyzing Physiological Audio Signals," *2012 38th Annual Northeast Bioengineering Conference (Nebec)*, pp. 69-70, 2012.

[59] C. C. Huang, P. Y. Lee, P. Y. Chen, and T. Y. Liu, "Design and Implementation of a Smartphone-Based Portable Ultrasound Pulsed-Wave Doppler Device for Blood Flow Measurement," *Ieee Transactions on Ultrasonics Ferroelectrics and Frequency Control*, vol. 59, pp. 182-188, Jan 2012.

[60] D. N. Breslauer, R. N. Maamari, N. A. Switz, W. A. Lam, and D. A. Fletcher, "Mobile Phone Based Clinical Microscopy for Global Health Applications," *Plos One*, vol. 4, Jul 22 2009.

[61] Q. S. Wei, H. F. Qi, W. Luo, D. Tseng, S. J. Ki, Z. Wan, *et al.*, "Fluorescent Imaging of Single Nanoparticles and Viruses on a Smart Phone," *Acs Nano*, vol. 7, pp. 9147-9155, Oct 2013.

[62] G. L. Damhorst, C. Duarte-Guevara, W. Chen, T. Ghonge, B. T. Cunningham, and R. Bashir, "Smartphone-Imaged HIV-1 Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) on a Chip from Whole Blood," *Engineering*, vol. 1, pp. 324 -335, 2015.

[63] D. Gallegos, K. D. Long, H. J. Yu, P. P. Clark, Y. X. Lin, S. George, *et al.*, "Label-free biodetection using a smartphone," *Lab on a Chip*, vol. 13, pp. 2124-2132, 2013.

[64] J. Jiang, X. H. Wang, R. Chao, Y. K. Ren, C. P. Hu, Z. D. Xu, *et al.*, "Smartphone based portable bacteria pre-concentrating microfluidic sensor and impedance sensing system," *Sensors and Actuators B-Chemical,* vol. 193, pp. 653-659, Mar 2014.

[65] H. J. Yu, Y. F. Tan, and B. T. Cunningham, "Smartphone Fluorescence Spectroscopy," *Analytical Chemistry,* vol. 86, pp. 8805-8813, Sep 2 2014.

[66] K. D. Long, H. Yu, and B. T. Cunningham, "Smartphone instrument for portable enzyme-linked immunosorbent assays," *Biomedical Optics Express,* vol. 5, pp. 3792-3806, Nov 1 2014.

[67] D. Erickson, D. O'Dell, L. Jiang, V. Oncescu, A. Gumus, S. Lee, *et al.*, "Smartphone technology can be transformative to the deployment of lab-on-chip diagnostics," *Lab on a Chip,* vol. 14, pp. 3159-3164, 2014.

[68] C. F. Fronczek, T. S. Park, D. K. Harshman, A. M. Nicolini, and J. Y. Yoon, "Paper microfluidic extraction and direct smartphone-based identification of pathogenic nucleic acids from field and clinical samples," *Rsc Advances,* vol. 4, pp. 11103-11110, 2014.

[69] S. C. B. Gopinath, T. H. Tang, Y. Chen, M. Citartan, and T. Lakshmipriya, "Bacterial detection: From microscope to smartphone," *Biosensors & Bioelectronics,* vol. 60, pp. 332-342, Oct 15 2014.

[70] L. Jiang, M. Mancuso, Z. D. Lu, G. Akar, E. Cesarman, and D. Erickson, "Solar thermal polymerase chain reaction for smartphone-assisted molecular diagnostics," *Scientific Reports,* vol. 4, Feb 20 2014.

[71] J.-D. Kim, Y.-S. Kim, H.-J. Song, and C. Y. Park, "Development of PCR Control Software for Smartphone Using both Wired and Wireless Communications," *International Journal of Multimedia and Ubiquitous Engineering,* vol. 8, pp. 223-230, 2013.

[72] X. Y. Liu, T. Y. Lin, and P. B. Lillehoj, "Smartphones for Cell and Biomolecular Detection," *Annals of Biomedical Engineering,* vol. 42, pp. 2205-2217, Nov 2014.

[73] P. Duarte-Guevara, C. Duarte-Guevara, A. Ornob, and R. Bashir, "On-chip PMA labeling of foodborne pathogenic bacteria for viable qPCR and qLAMP detection," *Microfluidics and Nanofluidics,* vol. 20, Aug 2016.

[74] M. Nemoto, K. Tsujimura, T. Yamanaka, T. Kondo, and T. Matsumura, "Loop-mediated isothermal amplification assays for detection of Equid herpesvirus 1 and 4 and differentiating a gene-deleted candidate vaccine strain from wild-type Equid herpesvirus 1 strains," *Journal of Veterinary Diagnostic Investigation,* vol. 22, pp. 30-36, Jan 2010.

[75] J. Canny, "A Computational Approach to Edge-Detection," *Ieee Transactions on Pattern Analysis and Machine Intelligence,* vol. 8, pp. 679-698, Nov 1986.

[76] M. J. Fu, B. Zhao, C. Carignan, R. K. Shosted, J. L. Perry, D. P. Kuehn, *et al.*, "High-Resolution Dynamic Speech Imaging with Joint Low-Rank and Sparsity Constraints," *Magnetic Resonance in Medicine*, vol. 73, pp. 1820-1832, May 2015.

[77] Y. Kinoshita, H. Niwa, and Y. Katayama, "Development of a Loop-Mediated Isothermal Amplification Method for Detecting Streptococcus equi subsp zooepidemicus and Analysis of Its Use with Three Simple Methods of Extracting DNA from Equine Respiratory Tract Specimens," *Journal of Veterinary Medical Science*, vol. 76, pp. 1271-1275, Sep 2014.

[78] U. B. R. Balasuriya, B. M. Crossley, and P. J. Timoney, "A review of traditional and contemporary assays for direct and indirect detection of Equid herpesvirus 1 in clinical samples," *Journal of Veterinary Diagnostic Investigation*, vol. 27, pp. 673-687, Nov 2015.

[79] S. Park, Y. Zhang, S. Lin, T. H. Wang, and S. Yang, "Advances in microfluidic PCR for point-of-care infectious disease diagnostics," *Biotechnology Advances*, vol. 29, pp. 830-839, Nov-Dec 2011.

[80] B. Rush and T. Mair, *Equine Respiratory Diseases: Blackwell Publishing*: Blackwell Publishing, 2004.

[81] L. Viel and D. Jean, "Inflammatory Airway Disease " in *Blackwell's Five-Minute Veterinary Consult: Equine*, J.-P. Lavoie and K. W. Hinchcliff, Eds., ed: Wiley-Blackwell Publishing, 2008.

[82] L. Couetil and J. Hawkins, *Respiratory Diseases of the Horse: A Problem-Oriented Approach to Diagnosis and Management*

Mason Publishing, 2013.

[83] J. M. Love, R. Marquis-Nicholson, R. C. Love, and D. R. Love, "Portable Battery-Operated Rapid PCR Amplification of the CAG Repeat Region of the Huntington Disease Locus " *Research Journal of Biology*, vol. 02, pp. 191-196 2012.

[84] M. A. Poritz, A. J. Blaschke, C. L. Byington, L. Meyers, K. Nilsson, D. E. Jones, *et al.*, "FilmArray, an Automated Nested Multiplex PCR System for Multi-Pathogen Detection: Development and Application to Respiratory Tract Infection," *Plos One*, vol. 6, Oct 19 2011.

[85] T. Abe, Y. Segawa, H. Watanabe, T. Yotoriyama, S. Kai, A. Yasuda, *et al.*, "Point-of-care testing system enabling 30 min detection of influenza genes," *Lab on a Chip*, vol. 11, pp. 1166-1167, 2011.

[86] C. Toumazou, L. M. Shepherd, S. C. Reed, G. I. Chen, A. Patel, D. M. Garner, *et al.*, "Simultaneous DNA amplification and detection using a pH-sensing semiconductor system," *Nature Methods*, vol. 10, pp. 641-+, Jul 2013.

[87] A. S. Patterson, K. Hsieh, H. T. Soh, and K. W. Plaxco, "Electrochemical real-time nucleic acid amplification: towards point-of-care quantification of pathogens," *Trends in Biotechnology*, vol. 31, pp. 704-712, Dec 2013.

[88] F. L. Kiechle and C. A. Holland, "Point-of-Care Testing and Molecular Diagnostics: Miniaturization Required," *Clinics in Laboratory Medicine*, vol. 29, pp. 555-+, Sep 2009.