LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR DETECTION OF *PASTEURELLA MULTOCIDA*, *MANNHEIMIA HAEMOLYTICA*, AND *HISTOPHILUS SOMNI* IN BOVINE NASAL SAMPLES

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To my friends and family for their unconditional love and support.

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LIST OF ABBREVIATIONS

LoD:	Limit of Detection
ADDL:	Animal Disease Diagnostic Laboratory
BHI:	Brain Heart Infusion
BLAST:	Basic Local Alignment Search Tool
DNA:	Deoxyribonucleic Acid
dNTPs:	Deoxyribonucleotide Triphosphate
FPR:	False-positive Rate
gDNA:	genomic DNA
NCBI:	National Center for Biotechnology Information
PACUC:	Purdue Animal Care and Use Committee
ROC:	Receiver Operator Characteristic
RPA:	Recombinase Polymerase Amplification
QPCR:	Real Time Polymerase Chain Reaction
TPR:	True Positive Rate
TSB:	Tryptic Soy Broth
BRD:	Bovine Respiratory Disease
PCR:	Polymerase Chain Reaction
LAMP:	Loop-mediated Isothermal Amplification
UDG:	Uracil DNA Glycosylase
UTP:	Uracil Triphosphate

ABSTRACT

This thesis aims to develop loop mediated isothermal amplification (LAMP) assays that can be used with bovine nasal samples to detect the presence of bacterial pathogens *(Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*) that cause bovine respiratory disease. The most common method to diagnose and treat BRD involves a physical examination and follow-up trial-and-error antibiotic therapies. Unfortunately, physical symptoms are often not consistent with the presence of BRD and antibiotic treatments incur a failure rate of 33%. This can lead to a surgency of antibiotic resistant pathogens, posing a significant risk to the beef cattle industry. Nucleic acid-based diagnostics, such as polymerase chain reaction (PCR), offer a robust approach for identifying BRD pathogens by amplifying species-specific genes from genetic material present in nasal samples. However, PCR-based approaches are limited to a lab setting due to expensive equipment required for maintaining assay reactions, and inhibitors that necessitate preprocessing to optimize assay performance. LAMP, on the other hand, offers an accurate, inhibitor resistant approach to detecting BRD causing bacteria in a format more amenable to field use. This assay was developed to have an accuracy of 97% in pure DNA samples, sensitivity and specificity of 99% and 89% respectively in DNA-spiked bovine nasal samples, and has a limit of detection of 10^4 DNA copies/reaction.

CHAPTER 1. INTRODUCTION

1.1 Background and Significance

Bovine respiratory disease complex (BRD) is the costliest disease to affect North American beef cattle feedlots with an approximate incidence rate of 18-21%. [1,2] Current methods of detecting or diagnosing pathogenic causes of BRD range from inspection of physical symptoms in cattle (loss of appetite, elevated temperature, and depression) to laboratory assays (serology, cell culture, immunohistochemistry, and in-situ hybridization on collected biological materials). [3] However, these methods suffer drawbacks that can make effective diagnosis and treatment of BRD a problematic endeavor. Physical indicators do not determine the causative pathogen and thus are not sufficient for guiding appropriate therapy. Laboratory-based tests can: i) require lengthy periods of time to culture sample pathogens for identification, ii) require specialized supplies and expensive equipment to be performed properly with minimal error, and iii) involve complicated procedures that necessitate trained personnel to operate. Here, we address these limitations by developing a molecular diagnostic assay—using loop-mediated isothermal amplification (LAMP) [4]—capable of detecting primary bacterial pathogens for BRD in nasal samples. This work presents novel primers and optimizes them for conducting LAMP in a timely (<45 minutes), sensitive (99%), and specific (89%) for BRD-associated bacterial pathogens.

BRD serves as an umbrella term for a series of respiratory illnesses caused by infections occurring along the respiratory tract [5]. Cattle afflicted with BRD are likely to develop pneumonia with physical symptoms including elevated temperatures, nasal discharge, depression, and reduced appetite [6]. While treatable, unmanaged cases of BRD can lead to expensive diagnosis/treatments, high morbidity rates, and decreases in overall meat quality [7,8]. Multiple bacteria have been cited to cause BRD, but the most common are *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni*, and *Mycoplasma bovis* [9].

Aside from assessing the physical symptoms of cattle to determine BRD disease state, which can often have inconsistent results (Wolfger et al., 2015), there are have been a growing number of diagnostic tests to detect these BRD pathogens. While these assays have had nearly three decades

of development and standardization, polymerase chain reaction (PCR) offers an approach for detecting the presence of BRD in samples through amplification of targeted DNA sequences unique to pathogenic strains [10] [11]. However, PCR assays suffer from requiring expensive lab equipment with multi-temperature cycling which often limits them to use in laboratory settings. Moreover, influxes of livestock samples sent to these labs for testing can cause delays to occur between sample submission and assay diagnosis. This can result in long wait times for feedlot operators or veterinarians, and more seriously, possible losses in livestock before treatment.

LAMP has seen recent development as a promising technology for the detection of infectious agents from multiple biological sources [12]. LAMP defies the restrictions placed on other diagnostic methods by amplifying DNA under a single temperature incubation, specifically targeting sequences through the use of four to six DNA primers, achieving detection limits similar to conventional PCR, and requiring only a simple heating element for assay operation as opposed to complex thermocyclers [4].

1.2 Thesis Objectives

The objective of this work is to:

1. Design and characterize a LAMP assay that can be used to specifically target and detect the presence of *P. multocida*, *M. haemolytica*, and *H. somni* from bovine nasal samples.

1.3 Organization of Thesis

This thesis is organized in a traditional style format. Chapter 2 provides a detailed summary of the background of BRD and approaches to diagnosing it in relevant literature. Chapter 3 details the materials and procedures involved in conducting experiments. Chapter 4 reports experimental results and examines their implication to the following work. Chapter 5 summarizes key findings and provides insights on future directions that can be taken with this work.

CHAPTER 2. LITERATURE REVIEW

2.1 Bovine Respiratory Disease and its effects on the beef cattle industry

Bovine respiratory disease is considered to be one of the costliest diseases to the beef cattle industry with an annual cost of up to 900 million dollars annually for beef cattle farmers. [13–16]. These costs are mainly split between post handling of BRD-related deaths, treatment plans, and lowered sales due to decreased meat quality and quantity [7,8,13,16]. Up to 97% of all feedlots annually have more than 1000 head of cattle can have occurrences of BRD, which can be responsible for up to 57% of cattle mortality in a given feedlot. [2,17]. While cattle at any age are capable of contracting BRD, higher risk groups include newly post-weaned calves, cattle experiencing high levels of transportation stress, and cattle in close proximity with other pathogen carrying cattle within the same pen [6,18,19].

BRD itself is characterized as a bacterial infection in the respiratory tract in cattle which can cause illnesses such as pneumonia, bronchitis, rhinitis, and tracheitis. [20]. These infections can be caused by low-area housing conditions, shipping stresses, and inclement weather that lead to compromised immune systems. Additionally, underlying viral infections that are caused by viruses (such as Bovine Respiratory Syncytial Virus (BRSV), ParaInfluenza 3 Virus (PI3V), Bovine Viral Diarrhea Virus (BVDV), and Infectious Bovine Rhinotracheitis Virus (IBRV)) encourage subsequent bacterial infections to occur. [21–24]. Most bacterial infections are primarily caused by large microbial populations of *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni, and Mycoplasma bovis*. [9]. These bacterial species are often commensal organisms in the respiratory microbiome of cattle and can thus easily propagate to infectious concentrations if any of the previously mentioned causes are met [25]. Due to the sheer number of variables that are involved in causing these bacterial infections, BRD can be incredibly complicated to diagnose and treat promptly

2.2 Current Diagnostic Method and Treatments for BRD

Various methods have been developed to standardize the classification of BRD symptoms and treatment plans often require the combined effort of veterinarians, laboratory scientists, and cattle

owners. The most common method for diagnosing BRD involves looking for physical symptoms such as depression, nasal discharge, coughing, and loss of appetite as well as measuring rectal temperatures. [26–28]. Based on the presence of these symptoms and having temperatures higher than 104 °F, a veterinarian would then suggest an antibiotic therapy to use for treatment [20]. In most feedlots, the breakdown of antibiotics used is as follows: tulathromycin (66%), fluoroquinolones (43%), cephalosporin (35%), florfenicol (35%), tetracylines (28%), and tilmicosin (26%) [2].

Despite being a frequently used approach, the physical examination methods are often inconsistent with a sensitivity and specificity of 61.8% and 62.8% [28]. The symptoms used for examination are not necessarily unique to BRD and can be hard to spot due to evasive tendencies of afflicted cattle when inspected [29]. Moreover, of the 89% of sick cattle that are treated with these antibiotics, 33% of the treatments fail [2,30]. As a response to failed initial treatments, selection of different antibiotics by trial and error becomes the next best treatment option for farmers. At best, this would result in the farmer incurring increased treatment expenses. At worst, this would lead to animal mortality and the emergence of antibiotic resistant pathogens that can have the potential to spread in and between feedlots.

2.3 Nucleic Acid-Based Approaches for Detecting BRD in Samples

Polymerase Chain Reaction (PCR) is known to be one of the most effective molecular techniques for conducting disease diagnostics on clinical samples [31]. By using a combination of DNA oligonucleotide primer sequences unique for BRD pathogen genetic markers (including discovered antibiotic resistant genes) and DNA polymerases, PCR is capable of amplifying and detecting the presence of pathogenic DNA from clinical samples [10,11]. Through additional use of indicator molecules such as fluorescent DNA intercalating dyes or fluorescent-labeled probes, and the use of a serial dilution of a positive control DNA template (standard curve), quantitative PCR assays (qPCR) are capable of quantitatively determining the presence of pathogenic DNA. Moreover, the addition of primer sequences that target different genes of different pathogens allows for the ability to detect multiple pathogens from a single sample (multiplex PCR). To date, there are qPCR assays designed for BRD bacterial pathogens with a limit of detections (LoD) between 10-250 genomic

copies/reactions, and diagnostic sensitivities and specificities ranging from 71%-96% and 72%-96% respectively [32,33]

While having multiple advantages compared to traditional cell culturing methods, PCR still faces limitations with being easily adopted by direct stakeholders to BRD, such as cattle farmers. The requirement of expensive thermal cyclers to conduct the appropriate temperature cycles for successful reactions limits the operation of PCR assay primarily to laboratory-based settings. Additionally, the presence of inhibitors from transport media or sample matrixes can adversely affect the performance and detection limits of PCR assays [31,34].

A possible solution to the equipment limitation was proposed by using Recombinase Polymerase Amplification (RPA) to detect the presence of BRD pathogens in deep nasopharyngeal swabs [35]. This technique allows for the binding and amplification of target DNA sequences at temperatures between 37-42 °C with recombinase enzymes and DNA binding proteins. Despite having comparable performance to PCR on clinical samples, RPA does not have openly available software for designing primers and probes and may experience interference or premature detection in field settings due to environmental temperatures within the amplification range [36].

2.4 LAMP as an effective solution for diagnosing BRD

Loop mediated isothermal amplification (LAMP) offers a unique approach to detecting BRD pathogens in clinical samples without the limitations inherent to traditional PCR methods. LAMP utilizes 4-6 oligonucleotide primers and a strand displacing DNA polymerase to specifically amplify a target sequence multiple orders of magnitude more than traditional PCR assays [4]. Due to the inherent strand displacement activity of Bst polymerase between 60 °C-65 °C, only a single temperature is required to allow the reaction to proceed. This opens up the use of cheap, simple heat sources, such as water baths or hot plates, for conducting LAMP reactions in a lab or field setting [4]. The increased yield of DNA amplicons additionally allows for the use of visual detection methods such as turbidity, colorimetric indicators, and fluorescent indicators to obtain a visual observation of results after completed reactions [37]. Moreover, LAMP reactions are more tolerant to inhibitors that reduce PCR performance, making it a good candidate for pathogen detection in

crude bovine samples. [38]. These advantages allow LAMP to be amenable to point-of-care based diagnostics and a viable approach for user-based diagnoses of BRD.

CHAPTER 3. MATERIALS AND METHODS

3.1 Design of LAMP Primers for BRD Pathogens

Published literature was investigated for highly conserved genes present in individual BRD pathogens and candidate gene sequences were run through the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Any species-specific genes that had <= 50% similarity with other pathogen genomes were considered unique gene targets and used as template sequences for LAMP primer design (Table 1). Additionally, all available genomes of a single BRD pathogen in the BLAST database were aligned, and gene sequences that were conserved amongst these alignments were manually determined (>99% similarity). Chosen genes were then compared to genomes of other tested BRD pathogens to ensure species-specificity by using the <=50% similarity criteria.

Table 1: List of candidate gene targets for BRD bacterial pathogens

Bacteria Name	Candidate Gene Name	Sequence/Genbank ID
Pasteurella multocida	kmt1	AF016259.1
	ompP1	QGV32322.1
	omp16	AJ271673
Mannheimia haemolytica	rsmL	QEC27547.1
	rsmC	QEC27614.1
	lktA	QEC25656.1
Histophilus somni	lolA	ACA31013.1
	lolB	ACA31225.1
	lppB	ACA32113.1

Three different gene targets were chosen for each BRD pathogen to verify target pathogen identification for the final assay. Three unique primer sets were designed for each gene target to conduct preliminary screening and optimize reaction performance.

All LAMP primer sets were generated using Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.html). Primer sets that spanned <= 200 bp of a target gene sequence, had loop primers with a length of18-21 bp, and had dG values <= -4.0 kcal/mol for i) 3’

end of F2, ii) 5’ of F1c, iii) 3’ of B2, and iv) 5’ of B1c were selected for initial screening. For each gene target, a total of 3 different LAMP primer sets were designed.

3.2 Bacterial Isolates and Complex Sample Collection

Pure isolates of *P. multocida*, *M. haemolytica*, and *H. somni* were acquired in the form of glycerol stocks from the Indiana Animal Disease Diagnostic Laboratory (ADDL) at Purdue University. Nasal swabs were collected from 45 healthy heifers at the Purdue Animal Sciences Research and Education Center Beef Unit (Purdue Animal Care and Use Committee Approval # 1906001911) using rayon tipped polyester swabs with liquid Amies transport media (BD 220146). All nasal samples were then pooled, vortexed until homogenous, and aliquoted for use as a complex substrate for cross-reactivity studies.

3.3 Bacterial DNA Extraction

P. multocida and *M. haemolytica* isolates were streaked on tryptic soy agar plates supplemented with defibrinated sheep blood (blood agar) and incubated for 16-18 hours aerobically at 37 °C. Single, isolated colonies of *P. multocida* and *M. haemolytica* were picked from plates, inoculated into brain-heart infusion (BHI) broth, and incubated aerobically at 37 °C for 16-18 hours. *H. somni* isolates were similarly streaked on blood agar plates, stored in BD GasPak™ EZ container systems (BD 260672) with BD BBL™ CO_2 gas generators (BD 260679), and incubated in a 5% CO_2 atmosphere at 37 °C for 2-3 days or until sufficient colony growth was present. *H. somni* colonies were inoculated into tryptic soy broth (TSB), stored in the previously mentioned BD GasPak™ EZ container system with the CO_2 gas generators and incubated with 5% CO_2 at 37 °C for 2-3 days.

Genomic DNA of all bacterial isolates was extracted from 1-2 mL of saturated liquid culture using the PureLink™ Genomic DNA Mini Kit (Invitrogen K182002) with a final eluted volume of 30 µL. Final DNA concentrations (ng/µL) of eluted extracts were measured using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen P11496).

3.4 Bacterial DNA Verification

PCR reactions were conducted on extracts using gene-specific target primers and run on 2% agarose gels to confirm bacterial genome identity. Gels were 2% w/v agarose and were run in a horizontal electrophoresis chamber in Tris Borate EDTA for 60 min at a voltage of 80 V. The bands were seen at expected locations as confirmed by a 1kb ladder (gel images not shown).

3.5 Quantitative LAMP Assay (qLAMP)

LAMP reactions were conducted by following manufacturer instructions of the Warmstart® LAMP Kit (DNA & RNA) (New England Biolabs E1700L). 25 µL reactions comprised 12.5 µL of Warmstart LAMP 2x Master Mix (40 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 100 mM KCl, 16 mM $MgSO_4$, 2.8 mM dNTPs,0.28 µM dUTP, 0.64 U/µL Warmstart Bst 2.0 DNA polymerase, 0.6 U/µL Warmstart Reverse Transcriptase [RTx], $4x10^{-4}$ U/µL Antarctic Thermolabile UDG, 0.2% Tween 20, pH 8.8@25°C), 2.5 µL of a 10x LAMP primer mixture (2 µM F3, 2 µM B3, 4 µM LF, 4 µM LB, 16 µM FIP, 16 µM BIP) 5 µL of a 1:101 dilution of the included LAMP fluorescent dye, and 5 µL of the template DNA containing solution. Antarctic Thermolabile UDG and dUTP were added to the LAMP reaction mixture for limit of detection and complex cross-reactivity studies to minimize carryover contamination during assay preparation. In-house validation experiments have confirmed that UDG/UTP does not affect reaction performance at the concentration used. Unless specified, the final concentration of template DNA for qLAMP reactions was 1 ng/reaction. Reactions were pipetted into wells of white 96-well full-skirted PCR plates (Thermofisher Scientific AB-0800W). Wells were sealed with VersiCap Mat Cap Strips (Thermo Fisher AB1820) and were inserted into either a CFX96 Touch Real-Time PCR Detection System (Bio Rad) or a qTOWER^3 G (Analytik Jena) for real-time fluorescence measurement. Reaction plates were incubated at 65 °C for 1 hour with fluorescence measurements taken using the FAM/SYBR Green I filter every minute. A ramp rate of 6 °C/s and 8 °C/s was used on the CFX96 and qTOWER^3 G respectively. A ramp rate of 0.1 °C/s was used on the qTOWER^3 G for limit of detection and complex reactivity experiments to improve the overall limit of detection of the LAMP reactions.

To minimize false-positives due to amplicon aerosol contamination, LAMP reaction mixtures preparation, template DNA loading, and reaction incubation/measurement was conducted in

separate lab spaces. RNase AWAY® Surface Decontaminant (Thermo Fisher 14-754-34) was thoroughly applied to all working surfaces. Reagent containers, pipettes, and lab gloves before and after each lab space operation and wiped completely with Kimwipes to prevent residue formation. Care was taken in the following three ways: i) minimize plate agitation during reaction preparation and DNA loading, ii) securely depress cap strips to wells before and after assay steps, and iii) wrap plates with aluminum foil (cleaned with RNase AWAY®) for transport between lab spaces.

3.6 Data Analysis and Figure Generation

Collected fluorescent data from real-time thermal cyclers were exported as excel worksheets (.xlsx) and manipulated in Microsoft Excel or custom MathWorks MATLAB® scripts (A1-A3).

3.6.1 Primer Screening

We used five metrics to characterize the primer performance based on their amplification curves: i) response time (min), ii) response time spread (min), iii) maximum fluorescent intensity (RFU), iv) maximum intensity spread (RFU), and v) total false-positives. Response time was based on the time point at which 90% of the maximum reaction intensity occurs. False-positive reactions were defined as reactions with negative/non-target controls that had fluorescent intensities higher than 20% of the maximum reaction intensity. Each of the five previously mentioned metric data for all screened primer sets were normalized and multiplied by predefined numerical weights (whole numbers) to generate individual characteristic scores for each primer set. All individual metric scores for a single primer set were then summed to generate a total performance score. Any primer sets that produced non-target amplification in < 30 min were automatically rejected and given a total performance score of 0.

3.6.2 Multi-Isolate/Cross-reactivity Data

Multi-isolate data refers to qLAMP assays run on gDNA from different strains (isolates) of *P. multocida*, *M. haemolytica,* and *H.somni*. Cross-reactivity data refers to qLAMP assays run on different gDNA combinations of single isolates of *P. multocida, M. haemolytica,* and *H. somni*. For multi-isolate data, fluorescent intensities were extracted for the 30-minute time-point for all

primer set reaction replicates and arranged in a table ordered by isolate. Table values were converted to a heat map and formatted using OriginLab® OriginPro, to display amplification differences between primer sets for all isolates visually. For cross-reactivity data, fluorescent intensities were normalized and used to find reaction Tt values (time required for intensity to reach/exceed defined reaction threshold) for each reaction replicate. These Tt values were then compared to Tt thresholds determined from limit of detection studies to classify reaction replicates as positive and negative reactions. These classifications were classified as a table ordered by spike-in combination.

3.6.3 Receiver Operator Characteristic Curve

Receiver-operator characteristic (ROC) curves were generated by comparing formatted multi-isolate data and cross-reactivity to a predefined threshold via binary classifications to assess positive vs. negative reactions. Thresholds were defined as a percentage of the maximum fluorescent intensity of the data set. Various thresholds (0%-100%) at an increment of 1% were tested and used to calculate the true positive rate and false-positive rate for each threshold classification. Diagnostic sensitivity and specificity of the LAMP assay to multi-isolate data were defined as the true positive rate (Equation 1) and 1-false-positive rate (Equation 2) for the lowest threshold value that created the most significant difference in sensitivity between the ROC curve and the random chance line. Accuracy was determined by taking the area under the ROC curve.

$$True\ Positive\ Rate\ (TPR) = \frac{\#\ of\ true\ positives}{\#\ of\ true\ positives + \#\ of\ false\ negatives} \quad (1)$$

$$False\ Positive\ Rate\ (FPR) = \frac{\#\ of\ false\ positives}{\#\ of\ false\ positives + \#\ of\ true\ negatives} \quad (2)$$

3.6.4 Limit of Detection

Fluorescent intensities were extracted for the 45-minute time-point for all primer set reactions. Intensities were normalized and multiplied by 100 to represent a % amplification value. Any amplification values that were greater than the previously determined ROC threshold (% amplification) were highlighted light blue and considered successful amplifications. The lowest DNA concentrations that had successful amplification for all three replicates of a given primer set were classified as the Limit of Detection (LoD) for the primer set. Tt value thresholds for each

primer set were determined as the time when all amplification values at the complex sample LoD were greater than or equal to the ROC threshold.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Evaluation of Primer Performance through qLAMP

We selected three genes for targeting each bacterial target. While some of these genes were published as PCR targets (Kumar et al., 2015), others were discovered by comparing BLAST available genomes as explained in the Materials and Methods section (Table 1). We determined which LAMP primer sets are optimal for our target genes by first designing multiple primer sets per gene and then characterized their performance. Initial screening of primer sets was carried out to identify sets that could amplify genomic DNA from a target pathogen, while maintaining little/no amplification on other pathogens or negative samples. All designed primers (Table S2) were run with genomic DNA in water to test for primer dimerization (early amplification of negative controls) or cross-reactivity with off-target DNA. Moreover, primer sets with faster reaction speeds and more consistent amplification trends (smaller standard deviation) were given higher priority for selection. The results of the screening (Table 2 and Figures A5, A6, and A7) indicate that the following primer sets were considered optimal (Table 3 for sequences): i) kmt1.2, ompP1.2, and omp16.1 for *P. multocida*, ii) rsmC.3, rsmL.2 and lktA.3 for *M. haemolytica*, and iii) lolA.2, lolB.3, and lppB.3 for *H. somni*.

Table 2: Performance characteristics of all designed primer sets for screening. Highlighted rows symbolize primer sets with optimal reaction features that were chosen for further assay development.

Primer Set	Response Time (min)	Response Time Spread (min)	Max Intensity (RFU)	Max Intensity Spread (RFU)	Total False Positives	Total Score
kmt1.1	13.5	0.6	19867.7	3140.6	4	0
kmt1.2	**12.3**	**0.5**	**23011.1**	**2308.1**	**0**	**91.4**
kmt1.3	15.8	0.5	18443.5	3842.2	0	85.1
ompP1.1	18.3	0.5	24363.8	3058.9	4	77.8
ompP1.2	**18**	**0**	**26217**	**2191.9**	**2**	**86.9**
ompP1.3	32.5	7	23301.9	2170.7	1	49.6
omp16.1	**18.8**	**0.5**	**21989.6**	**2149.6**	**0**	**86.8**
omp16.2	22.3	0.5	25324	2847	2	81
omp16.3	43	1.6	16808.9	1152.8	0	64.6
rsmL.1	26.3	2.2	27059.9	382.5	7	62.2
rsmL.2	**18.3**	**0.5**	**25720.1**	**4300.4**	**1**	**84.9**
rsmL.3	21.3	1.5	30923.3	5814.4	1	80.6
rsmC.1	19.3	1.5	30716.5	6274.2	10	58.9
rsmC.2	31.5	7.3	14235.6	6248.4	0	42.4
rsmC.3	**30.5**	**1**	**19127.2**	**1916.2**	**0**	**75.8**
lktA.1	25.3	1.3	18785.5	2121	10	52.7
lktA.2	20	0.8	24106.6	2765.7	7	0
lktA.3	**19**	**0**	**18906.3**	**1479.9**	**12**	**57.5**
lolA.1	22	1.4	22003.7	1838.9	0	81.3
lolA.2	**16**	**0**	**25501.2**	**1824.5**	**0**	**93**
lolA.3	13.8	1	24870.2	1616	2	85.4
lolB.1	35.5	0.6	23504.3	802	11	50.5
lolB.2	28	1.2	23133.7	1060.1	0	80
lolB.3	**16.5**	**0.6**	**22503**	**1461.9**	**0**	**88.9**
lppB.1	19.8	0.5	19569.2	1501.7	2	80.4
lppB.2	43.8	5.1	25406.9	11254.2	2	40.7
lppB.3	**23**	**0**	**22834.9**	**1485.6**	**0**	**87.3**

Table 3: Screen-selected primer set sequences used in assay development.

Primer Set	**Sequence (5' to 3')**
kmt1.2 F3	GAATCAAGCGGTCACAG
kmt1.2 B3	CACTCACAACGAGCCATA
kmt1.2 FIP	AGAGCAGTAATGTCAGCACAATATTAAAGACAGCAATTTCGAGCA
kmt1.2 BIP	CGCTATTTACCCAGTGGGGCGCCATTTCCCATTTCAAGTG
kmt1.2 LF	CGTAAAGCCCCACCATTGTT
kmt1.2 LB	ACCGATTGCCGCGAAATTGAGT
ompP1.2 F3	GCAATTTATGTGGACCCAAAT
ompP1.2 B3	AATCGGTTTTACCGCCTA
ompP1.2 FIP	TCGGAACTAACGCATTCGGCTTAACTTCACCAATGCCAGG
ompP1.2 BIP	ATCCAATTAACGAAAAATTCGCTGTGCATATTTGTCATCAAACTCGG
ompP1.2 LF	CAATATTTTTATAGGCGAA
ompP1.2 LB	GGCGGTGGATTGAATGTCAAC
omp16.1 F3	GGCGGTTATTCAGTACAAGA
omp16.1 B3	CATCTGCACGACGTTGAC
omp16.1 FIP	CGCATGTGCATCTAAAATTTGTACAGTTATAATACCGTGTATTTCGGC
omp16.1 BIP	AATGCAACACCTGCAACGAACTAATGCGATGTTATATTCTGGT
omp16.1 LF	CGATATTGTATTTATCGA
omp16.1 LB	CGTTGTTGAAGGTAACACCGA
lktA.3 F3	GTAACGACGGCAATGACC
lktA.3 B3	ATCTTTTAAGTTCGAATCAGAGA
lktA.3 FIP	TTGCCTTTACCGCCATCGATAAAGTAAAGGCGATGATATTCTCG
lktA.3 BIP	GGTGGCAAGGGCGATGATATCATTGCCGTCAGAATCGG
lktA.3 LF	TCATCACCATTTCCACCA
lktA.3 LB	TCGTTCACCGTAAAGGCGAT
rsmL.2 F3	CGAAGACACTCGCCACAG
rsmL.2 B3	AACTTTTACCCCGGCTTGG
rsmL.2 FIP	AACGACCGCTTTCTGCTGTTCATTATTGCTGAGCCACTACGG
rsmL.2 BIP	TGCGTTAATTTCCGATGCCGGACGGCAATGACGGACAAGA
rsmL.2 LF	GTGCAAGGCGAAAAACGGTTTTTTA
rsmL.2 LB	GCCACTGATTAGCGACCCG
rsmC.3 F3	CGGCAGACGTACTTTGGC
rsmC.3 B3	ATGCGTTTGCGACAATTCG
rsmC.3 FIP	GTGGAATGGTGGGTTGGAGACA-AGAGGGGGAAGTGGTAGC
rsmC.3 BIP	ACGGGGTCGATACCGCCTAC-GCTCACCGCCTTTGGTTA

Table 3: continued

rsmC.3 LF	CCGTTCATTAATGTGAGAGAACACA
rsmC.3 LB	TGGAGGAGTTGATTTTCCAAGCT
lppB.3 F3	AGCACAAAAAATACTGAGCA
lppB.3 B3	AGAGAAGGAGATTATTTGGAATG
lppB.3 FIP	TGTTGCCCATACTTCTAAGGTTAAATTTTGTAGCCTCAGTTTTCAAGC
lppB.3 BIP	ACCGAATAAACAAAGCTATCCGATTTTTGCTGATTTTGCTAATGCGG
lppB.3 LF	TTTCTCTGCTTCATAACC
lppB.3 LB	CGCACTTTCTTTGATAACTCTCGT
lolA.2 F3	AGTAATGTAACTTGGGCAAAT
lolA.2 B3	GCAATAATTTGACTTTCTTGAGG
lolA.2 FIP	CACTTGTTGTGTATAGTCAGCACTTCGGTTAATGAGTTACAAAATCG
lolA.2 BIP	ATGCACAGGGAAAAAAAATACAGCTGTTTCATTGTCCATACGAAAT
lolA.2 LF	ACACATCAATTTTATTTAA
lolA.2 LB	GGAAAAATACAACTCAAACGT
lolB.3 F3	GCTACGTGAAATGATTGGTATC
lolB.3 B3	CTTTTCAGAAGAATATCTTTGGGTA
lolB.3 FIP	GCCGACCTGATAATCTGAATTTTCATATTCCATTACAACAAATAGGGAAC
lolB.3 BIP	GCAAGCTTTACTTATTCAGTTGAGGATGCTTTGATCTGTTCGATAG
lolB.3 LF	CTGGTTGACCTTTTAGCC
lolB.3 LB	GAAGTTTGGAGTGCTGAC

4.2 Multi-Isolate Cross-reactivity Studies

We tested the specificity of our optimal primer sets by comparing the amplification results of LAMP primers with purified off-target DNA. Based on the multi isolate data as shown in Figure 1, most of the primer sets amplify the target pathogenic DNA and do not amplify off-target pathogenic DNA. By generating ROC curves (Figure 2) for each pathogen based on the multi isolate data, we determined the diagnostic sensitivities and specificities for each pathogen assay (Table 4). For the ROC curve of the overall BRD LAMP assay (Figure 3), we determined that the primers had 97% accuracy (96 % sensitivity 98% specificity) when using a fluorescent threshold of 28% of the maximum reported intensity. Diagnostic abilities of BRD LAMP were either comparable (sensitivity) or higher (specificity) than reported qPCR values [32,33]. However, samples

used for qPCR assays were derived from collected animal samples and subjected to DNA extractions procedures before use. Either mentioned condition can cause variability on total DNA load of target and off-target gDNA per sample used, which may contribute to lowered diagnostic performance. LAMP samples, in contrast, represented ideal sample conditions by being concentration-controlled additions of target gDNA in nuclease-free water, which would allow for optimal performance of the LAMP assay.

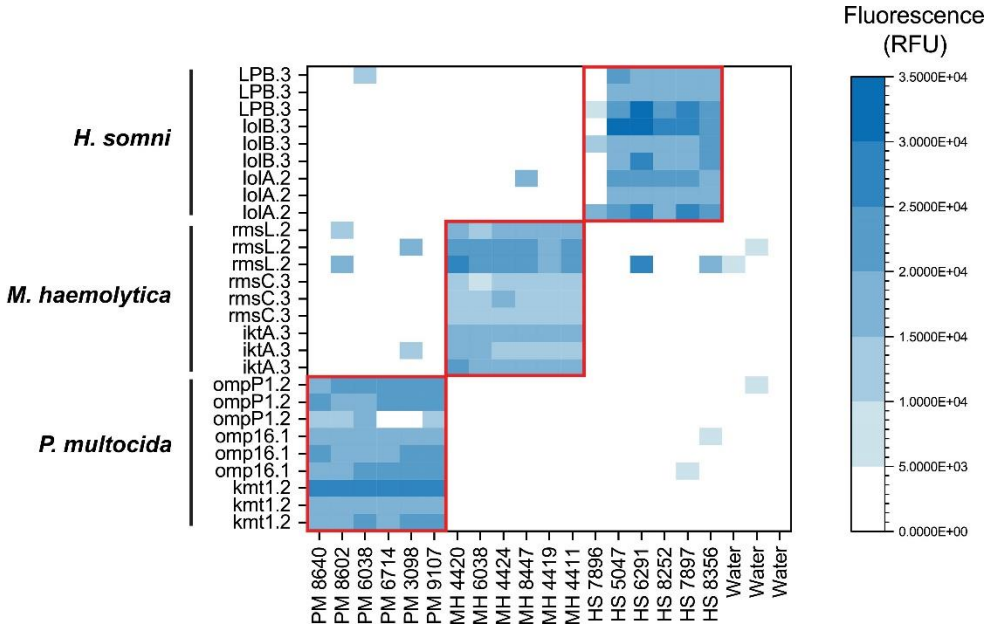

Figure 1: Heat map of selected primer sets tested in triplicate (y-axis) against different isolates (6) of BRD pathogens (multi-isolate data). Isolates of *P. multocida*, *M. haemolytica*, and *H. somni* were used (the initials on the x-axis refer to the bacteria genus and species, the numbers refer to a different strain as labeled by Indiana Animal Disease Diagnostic Lab). LAMP reactions were run in real-time with a real-time thermal cycler at 65 °C and fluorescence intensities were selected at 30 minutes (longest reaction time of 9 selected primer sets) to be plotted on the heat map. Three replicates of each reaction were run and displayed individually on the map. Water was used as a negative control. Outlined red regions on the table represent expected regions of positive reactions.

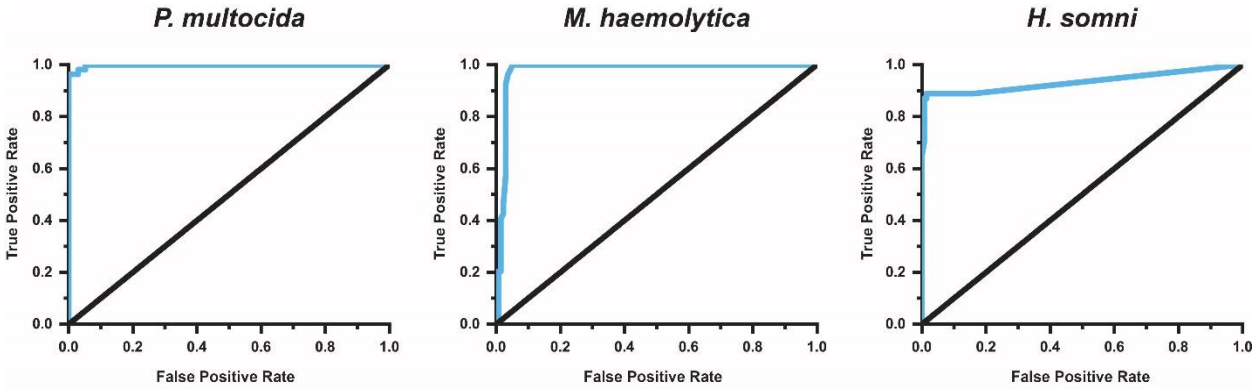

Figure 2: Receiver Operator Characteristic (ROC) curves illustrating true positive rate (TPR) and false-positive rate (FPR) of BRD LAMP assay for each pathogen using multi-isolate data presented in Figure 1.

Table 4: Diagnostic sensitivities and specificities of LAMP and qPCR assays against listed BRD pathogens.

	Sensitivity (%)			Specificity (%)		
Pathogen	**LAMP** (current work)	**qPCR** [32]	**qPCR** [33]	**LAMP** (current work)	**qPCR** [32]	**qPCR** [33]
P. multocida	96	85	84	100	69	70
M. haemolytica	100	72	92	95	91	73
H. somni	89	85	100	99	84	76

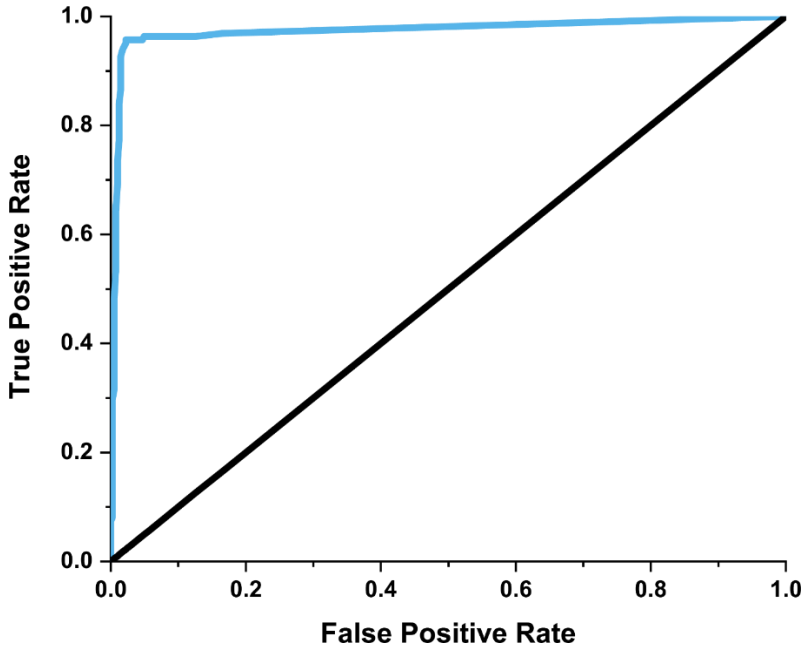

Figure 3: Receiver Operator Characteristic (ROC) curve illustrating true positive rate (TPR) and false-positive rate (FPR) of BRD LAMP assay with multi-isolate data presented in Figure 1.

Using different isolates of the same species helps check for cross-reactivity in case there were strain-specific genetic differences that could influence reaction performance. Since most isolates show consistent amplification results with their own species, our LAMP primer sets are functioning as expected. One exception was the *H. somni* isolate 7896 which did not amplify reliably with any of our LAMP primers. Further sequencing and genome annotation using RAST revealed that this isolate was putatively identified as *Staphylococcus hominis*, which has no significant similarity with *H. somni* and whose genomic DNA would not be expected to amplify with our *H. somni* primer sets. It is likely that when handling this isolate, it might have been mislabeled before isolate collection from the ADDL or contaminated during culturing for DNA isolation.

4.3 Determination of Assay Detection Limits in Water and Liquid Amies Media

Optimal primer sets were characterized using LAMP reactions of decreasing concentrations of target gDNA template to assess limits of detection (LoD)—defined as the lowest concentration at which 3/3 replicates show amplification. LoD experiments were conducted on target gDNA

suspended in water and Liquid Amies separately to determine inhibitory effects on reaction performance. The performance of our optimal primer sets is highlighted in Table 5. LoDs were predominately i) 10^3 copies/reaction in water samples and ii) 10^4 copies/reaction in Liquid Amies samples. This order of magnitude difference in LoD between the two media types was likely due to the LAMP reaction composition being altered by the increased salt concentrations present in Liquid Amies, negatively impacting reaction sensitivity. As DNA concentration decreases, there was an associative increase in response time of all primer sets (Figures A2-A4). In general, the overall LoDs of the LAMP reactions are higher than what is reported for PCR [32]. However, the difference between the maximum reported PCR LoD (250 copies/reaction) and the LAMP LoD of water samples indicates that conducting a finer LoD study between 10^2 and 10^3 copies/reaction may reveal a closer alignment.

Table 5: Limit of detection characterization of assay primer sets. 5 µL of gDNA ($1x10^0$ to $1x10^5$ copies/reactions) were added to reactions (20 µL reagents) in triplicate and incubated for 60 minutes at 65˚C. Fluorescent intensities of primer set replicates at 45 minutes were extracted, and compared to a threshold fluorescent intensity (28%) determined from ROC analysis. Light blue highlights represent reactions crossing the threshold considered positive. The lowest concentration at which all three replicates amplify is the limit of detection. lktA.3 seems to form dimers in water and lead to false amplification, which is inhibited in Liquid Amies media.

DNA Concentration (copies/reaction)	*P. multocida*																	
	kmt1.2						ompP1.2						omp16.1					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	60.56	57.55	54.79	61.89	67.05	70.77	81.57	79.60	81.86	74.33	75.14	73.42	81.11	79.50	77.84	66.15	66.35	59.27
1.00E+04	71.64	77.09	76.37	75.53	69.27	76.16	93.37	100.00	87.69	69.60	76.94	73.00	86.53	79.42	84.01	66.49	65.44	61.41
1.00E+03	80.67	77.15	81.19	0.80	87.19	0.06	84.01	84.12	94.94	0.40	0.30	69.59	68.76	77.72	78.27	0.15	0.42	47.03
1.00E+02	-0.36	83.90	89.71	0.73	0.12	1.25	76.05	89.93	-0.66	0.29	0.52	0.64	-0.44	-0.56	-0.43	0.40	25.03	0.43
1.00E+01	-0.47	-0.21	-0.32	0.77	0.64	0.16	-0.39	-0.26	-0.47	0.61	0.57	0.66	-0.12	0.02	-0.06	0.50	0.68	0.37
1.00E+00	0.02	-0.63	-0.38	0.73	0.55	0.48	0.76	-0.54	-0.70	0.26	0.29	0.32	-0.69	-0.65	10.77	0.53	0.47	0.34

DNA Concentration (copies/reaction)	*M. haemolytica*																	
	rsmL.2						rsmC.3						lktA.3					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	34.38	32.16	33.32	31.33	33.12	36.57	42.34	39.50	38.01	32.71	31.23	31.63	39.14	38.14	38.15	32.60	35.98	37.67
1.00E+04	38.96	36.95	37.71	33.92	33.06	34.06	39.21	40.98	39.84	32.33	33.49	33.91	36.68	42.45	38.52	33.89	34.02	36.32
1.00E+03	41.42	42.22	43.63	32.46	34.08	32.12	39.36	40.56	37.43	11.11	0.30	24.30	40.79	42.45	41.09	34.47	35.38	36.14
1.00E+02	36.69	36.77	40.81	0.38	0.38	26.32	25.93	0.10	-0.31	0.54	0.48	0.54	30.16	42.10	27.60	0.03	0.46	31.77
1.00E+01	-0.53	0.04	-0.42	0.35	0.35	0.24	-0.30	-0.08	-0.17	0.69	0.54	0.84	32.04	30.51	31.12	1.80	0.03	0.05
1.00E+00	-0.49	-0.23	-0.54	0.20	0.22	0.13	-0.29	-0.54	-0.46	0.62	0.53	0.38	28.80	29.54	31.28	0.15	9.15	0.66

DNA Concentration (copies/reaction)	*H. somni*																	
	lolA.2						lolB.3						lppB.3					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	75.54	71.39	76.26	61.24	62.34	64.67	85.20	79.31	78.40	70.94	58.13	65.37	75.47	70.00	73.40	55.88	57.32	54.26
1.00E+04	73.00	70.00	73.87	62.91	64.24	63.94	92.57	89.26	93.93	70.29	65.47	73.80	69.57	71.08	68.48	60.75	60.01	54.66
1.00E+03	73.35	62.74	76.67	66.82	67.12	63.03	76.72	83.23	86.00	0.67	0.99	-0.80	67.04	64.70	63.40	12.43	0.06	0.06
1.00E+02	0.04	-0.44	5.46	12.81	0.37	16.33	-0.54	-0.35	-0.39	-1.06	-0.86	-0.83	0.28	0.11	0.00	-0.13	0.23	1.63
1.00E+01	-0.20	-0.51	-0.27	0.70	0.41	0.20	-0.46	-0.33	-0.34	-0.97	-0.68	-0.58	0.28	0.26	0.30	0.48	0.06	0.14
1.00E+00	-0.31	-0.35	-0.47	0.69	0.51	0.46	-0.73	0.64	-0.55	-1.00	-0.63	-0.72	0.53	0.19	0.03	0.42	0.18	0.00

4.4 Assessing Primer Performance in Complex Bovine Nasal Samples

LAMP reactions with optimal primer sets were conducted on pooled bovine nasal swabs in Liquid Amies to determine assay performance on complex samples. Due to the lack of literature on quantifiable concentrations of BRD bacterial pathogens in sick bovine nasal swabs, pathogen gDNA was spiked into complex samples at equivalent to 10^4 copies/reaction to simulate elevated levels of bacteria for BRD related infection. Different combinations of bacterial pathogen gDNA were spiked into complex samples to reflect possible bacterial communities present in collected samples. Most primer sets could amplify spiked target DNA regardless of combination (Figure 4). Diagnostic sensitivity and specificity for the assay based on ROC analysis of the cross-reactivity data were 99% and 89% respectively.

Off-target amplification was observed for of *P. multocida* ompP1.2 primer set. PCR conducted on base complex samples (Figure A1) revealed DNA sequences coding for the kmt1 gene in *P. multocida*. This suggests the presence of naturally occurring *P. multocida* in the original swab samples that could contribute to high background amplification in non-*P. multocida* target LAMP assays. However, the significantly higher false-positive rate for ompP1.2 could indicate a higher DNA load of the ompP1 gene compared to the other *P. multocida* conserved genes. Verification can be done via a quantitative comparison of DNA load between the three *P. multocida* genes using qPCR. Alternatively, primer-specific cross-reactivity with off-target gDNA is possible as well. To date, there have been no reported PCR assays that can detect the presence of BRD bacterial pathogens in crude bovine nasal samples. While one false negative reaction occurs for kmt1.2 in the "PM+MH" column, the calculated Tt value for this reaction was larger than the threshold by merely 2 minutes. Further replicates of these complex sample reactions would provide better tolerance values for Tt when determining positive vs. negative reactions.

PCR assays experience significant inhibition under the presence of transport media, therefore requiring some form of DNA purification step before detection can be done [31,34]. Serology tests, capable of being run with crude samples, predominantly screen for BRD viral pathogens. As such, this assay represents a novel approach to screen for BRD bacterial pathogens in crude bovine nasal samples.

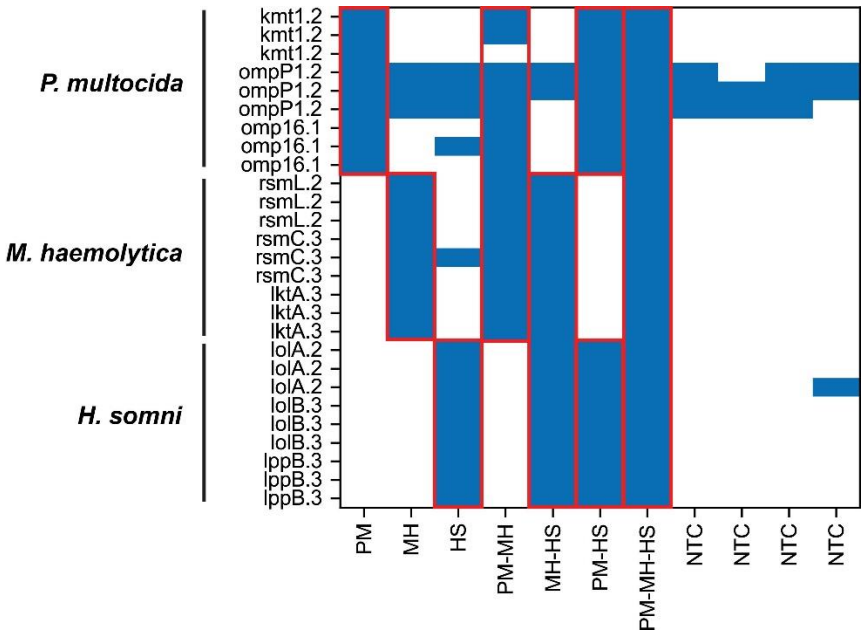

Figure 4: Classification table of selected primers tested against pooled bovine nasal samples with combinations of spiked in bacterial gDNA (PM - *P. multocida*, MH - *M. haemolytica*, HS - *Histophilus somni*). The final concentrations of spiked DNA in all reactions were 10^4 copies/reaction. LAMP reactions were conducted in a real-time thermal cycler at 65 °C. Calculated Tt values of reactions were compared to primer set-specific Tt thresholds established from LoD studies to determine positive vs. negative reactions. Reactions that had Tt values less than or equal to Tt threshold were considered positive reactions and plotted blue on the classification table. All primer reactions were run in triplicate and base bovine nasal sample spiked with water was used as a negative control (NTC). Outlined red regions on the table represent expected regions of positive reactions.

CHAPTER 5. CONCLUSION

In this work, we developed a LAMP assay that can i) specifically detect the presence of BRD causing bacteria (*P. multocida, M. haemolytica, and H. somni*) in less than 45 minutes, ii) detect pathogen DNA in both simple water samples and unprocessed bovine nasal samples, iii) translate more easily to field use due to its ease of incubation and amenability to more visual forms of detection.

A major limitation of LAMP as a mainstream assay for pathogen screening is the occurrence of false-positives either due to poor reagent handling or carryover contamination from previous experiments. However, this concern can be minimized by employing multiple spaces for reaction preparation, pre-aliquoting required reagents to reduce contamination losses, and adding increased concentrations of UDG/UTP to degrade leftover amplicons in incubation environments [39].

Applications of this assay can be extended further by the following four steps i) expanding the list of pathogens to other bacteria (e.g., *Mycoplasma bovis*), viruses, and fungi associated with BRD, ii) selecting antibiotic resistance-related genes as LAMP targets to allow for timely diagnosis of drug-resistant strains before outbreaks, iii) coupling reactions with pH or magnesium-based indicators to allow for more visual inspection of assay results [40–42], and iv) converting assay into a format more amenable for resource-limited, field-use settings to bypass sample shipment and lab processing altogether.

APPENDIX A.

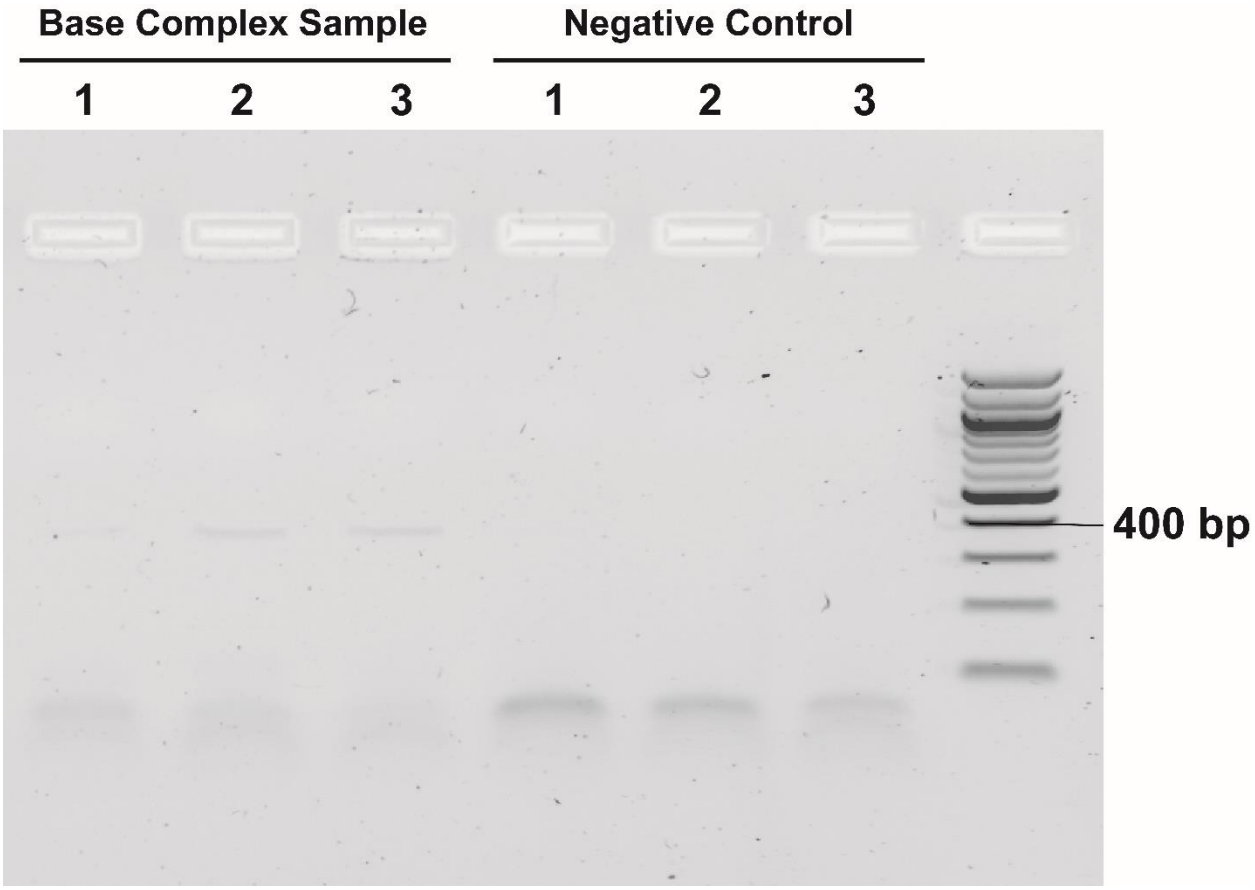

Figure A1: Agarose gel containing amplification results of PCR targeting kmt1 gene of *Pasteurella multocida* conducted on pooled bovine nasal samples.

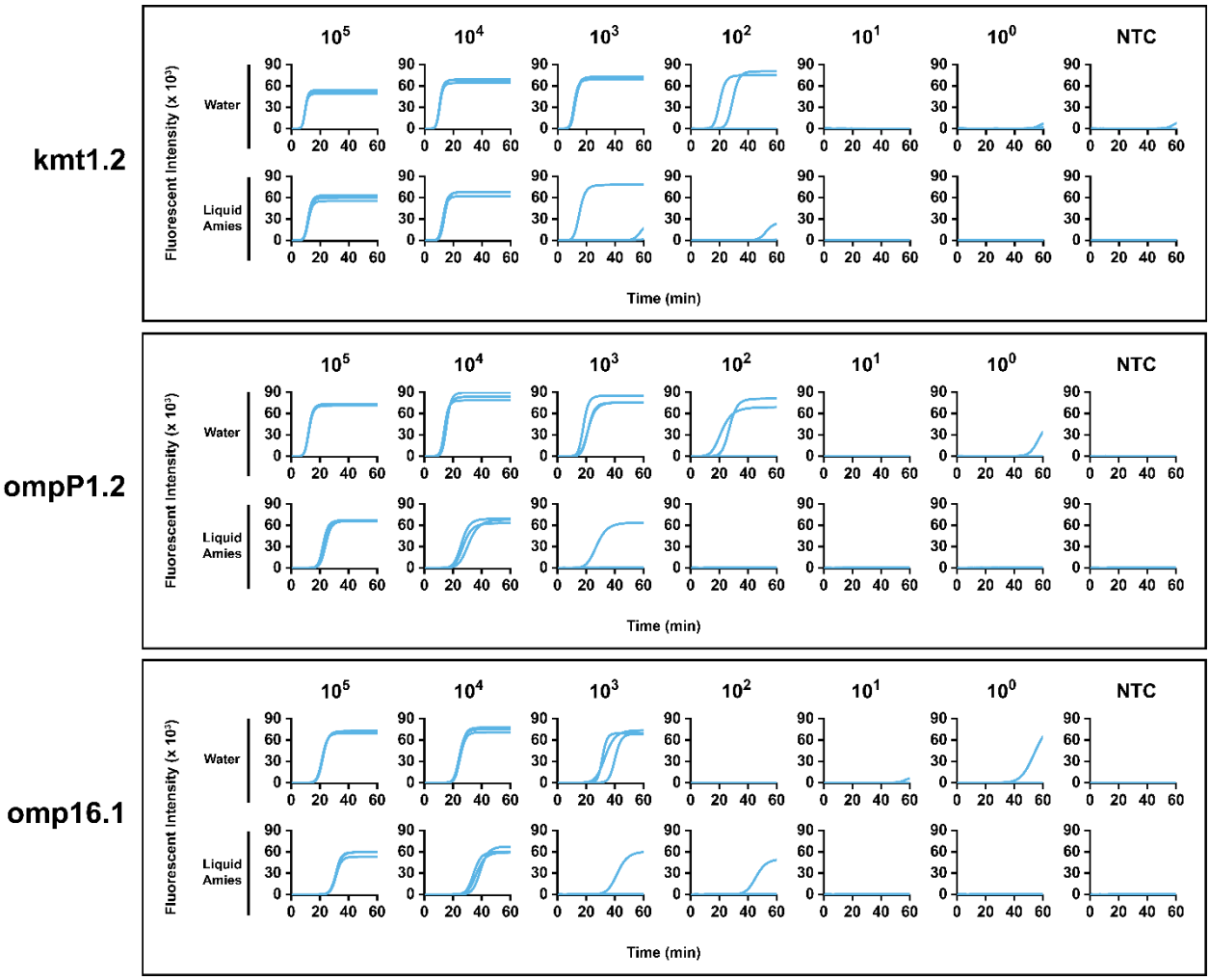

Figure A2: Amplification of *P. multocida* gDNA present in water and DNA-spiked liquid Amies. Water and liquid Amies samples were spiked with various concentrations of water-suspended DNA extracts (*P. multocida*, *M. haemolytica*, and *H. somni*) to generate serial dilutions ($1.0x10^0$ to $1.0x10^5$ copies of DNA/reaction), and were added to qLAMP assays with *P. multocida* specific primer sets for 60 minutes at 65 °C. Water and water spiked liquid Amies were used as negative controls.

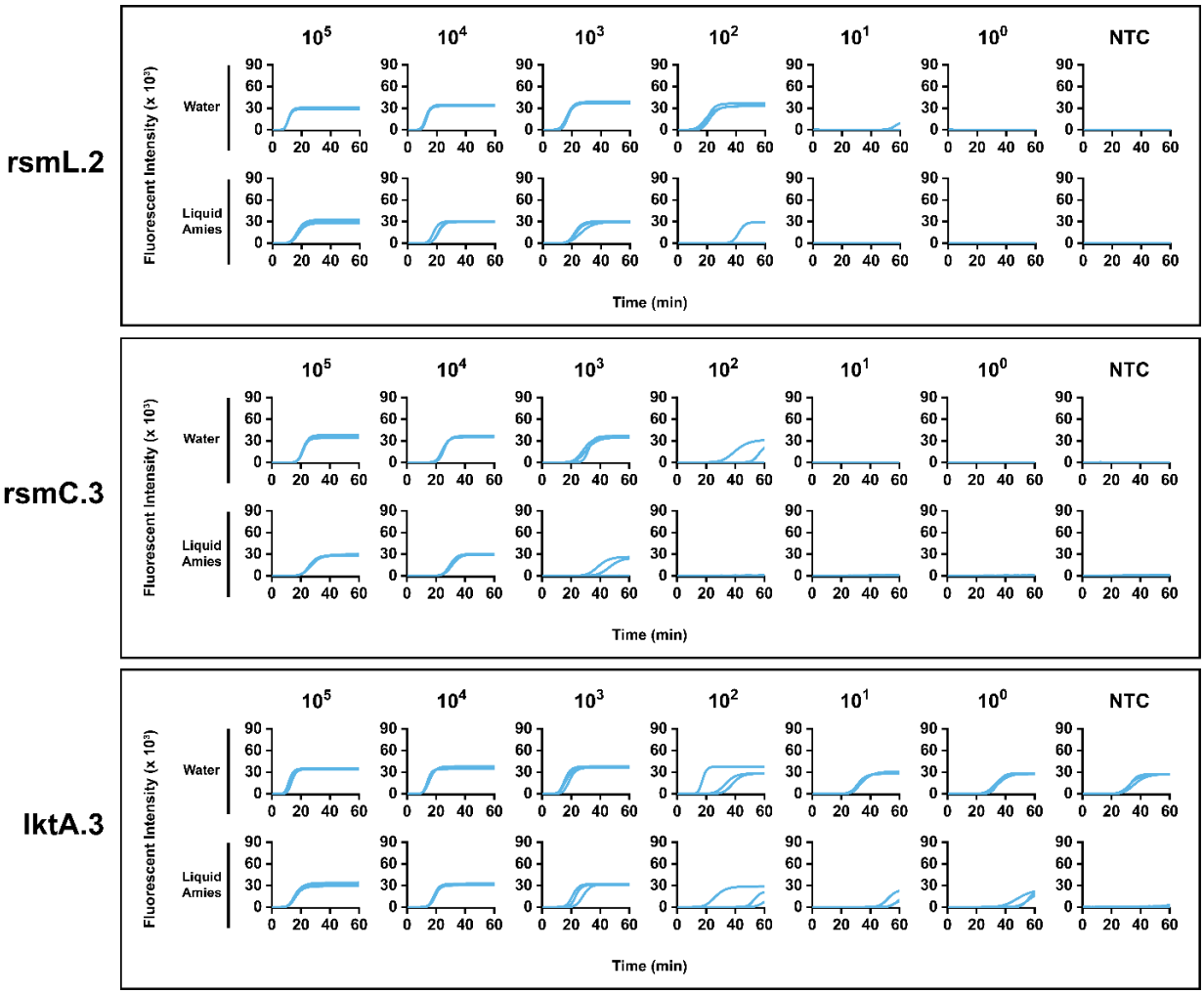

Figure A3: Amplification of *M. haemolytica* gDNA present in water and DNA-spiked liquid Amies. Water and liquid Amies samples were spiked with various concentrations of water-suspended DNA extracts (*P. multocida*, *M. haemolytica*, and *H. somni*) to generate serial dilutions ($1.0x10^0$ to $1.0x10^5$ copies of DNA/reaction), and were added to qLAMP assays with *M. haemolytica* specific primer sets for 60 minutes at 65 °C. Water and water spiked liquid Amies were used as negative controls.

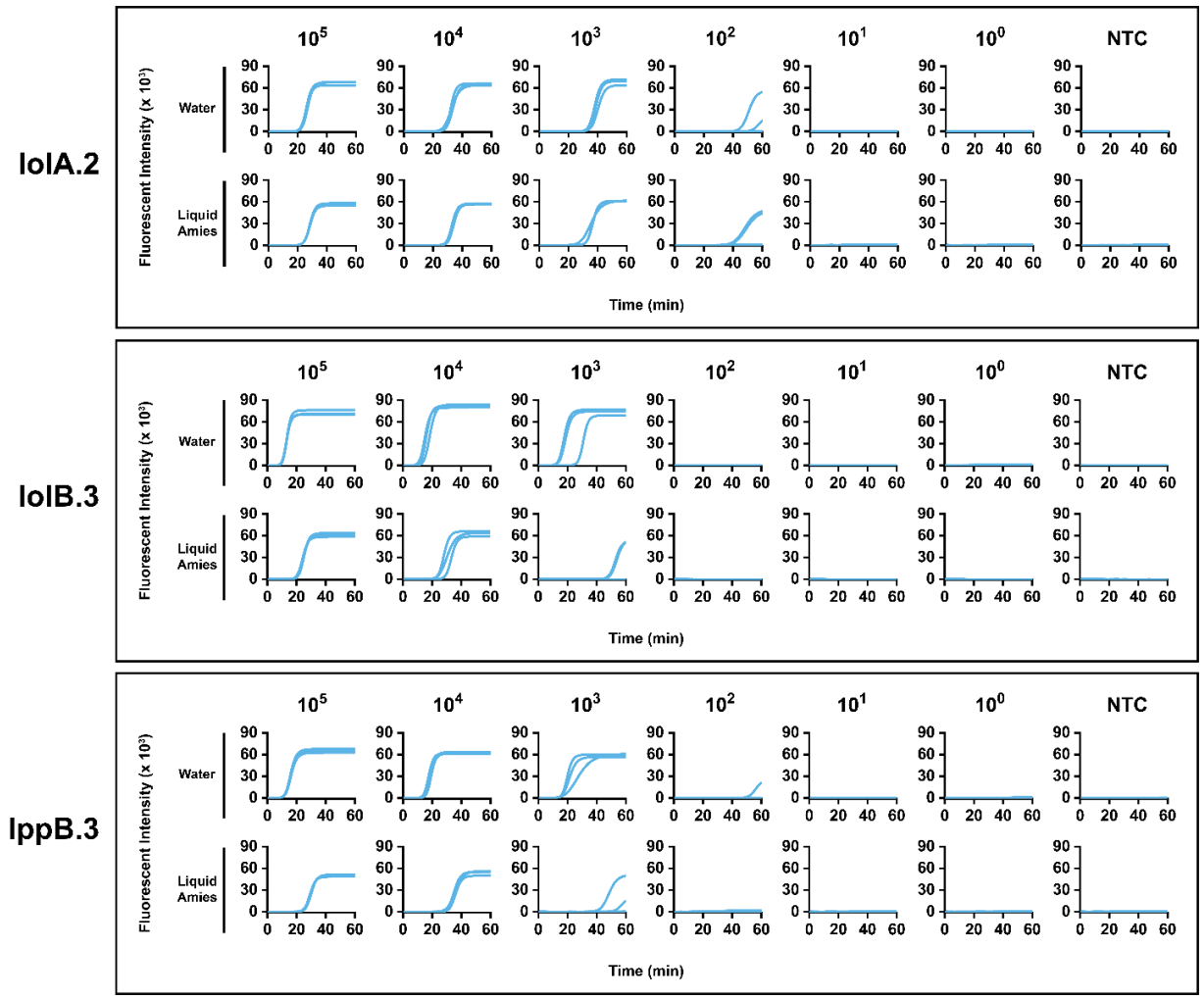

Figure A4: Amplification of *H. somni* gDNA present in water and DNA-spiked liquid Amies. Water and liquid Amies samples were spiked with various concentrations of water-suspended DNA extracts (*P. multocida*, *M. haemolytica*, and *H. somni*) to generate serial dilutions ($1.0x10^0$ to $1.0x10^5$ copies of DNA/reaction), and were added to qLAMP assays with *H. somni* specific primer sets for 60 minutes at 65 °C. Water and water spiked liquid Amies were used as negative controls.

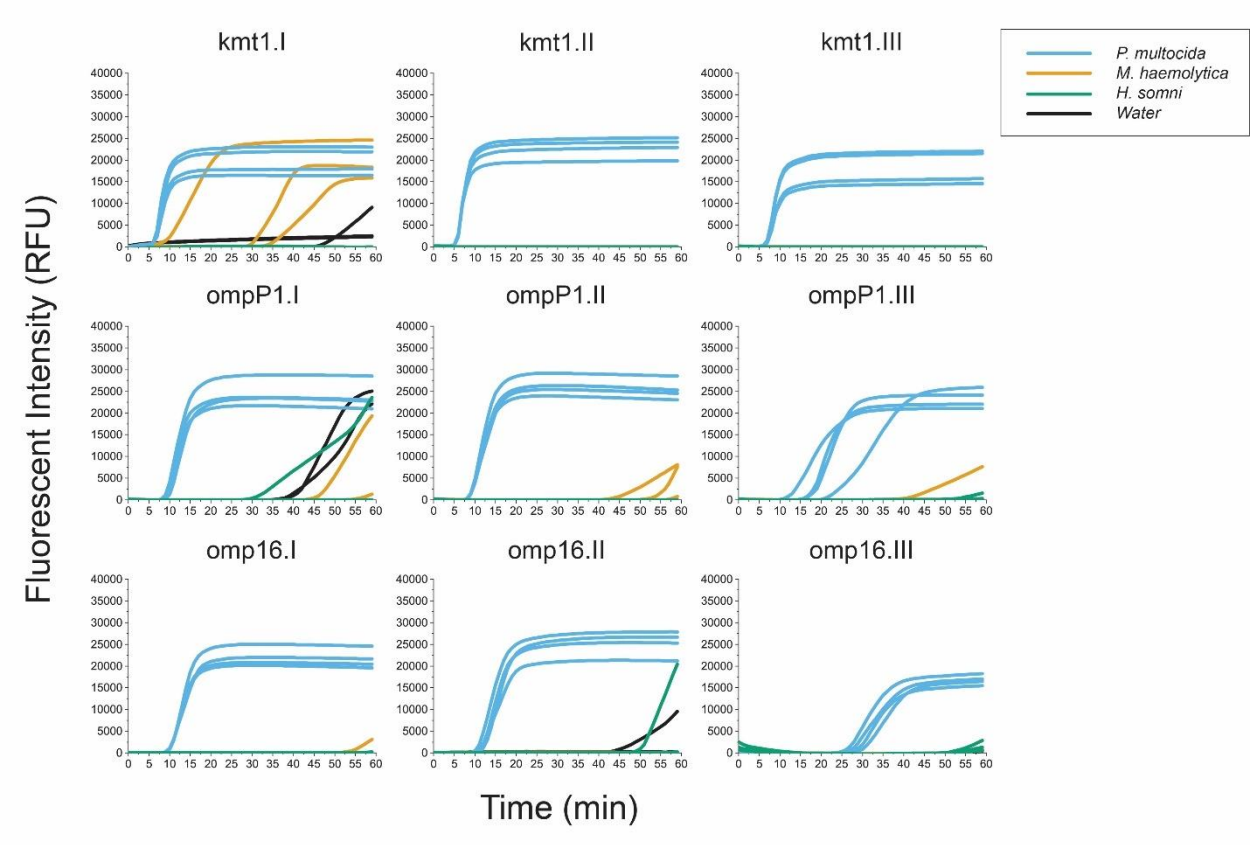

Figure A5: qLAMP amplification curves for *P. multocida* targeting LAMP primer sets. 5 µL of gDNA from *P. multocida*, *M. haemolytica*, and *H. somni* (0.2 ng/uL) was added to separate reactions in quadruplicate and incubated for 60 minutes at 65 °C. Nuclease-free water was used as a negative control. Blue lines: *P. multocida* gDNA reactions; Orange lines: *M. haemolytica* gDNA reactions; Dark Green lines: *H. somni* gDNA reactions; Black lines: Water reactions.

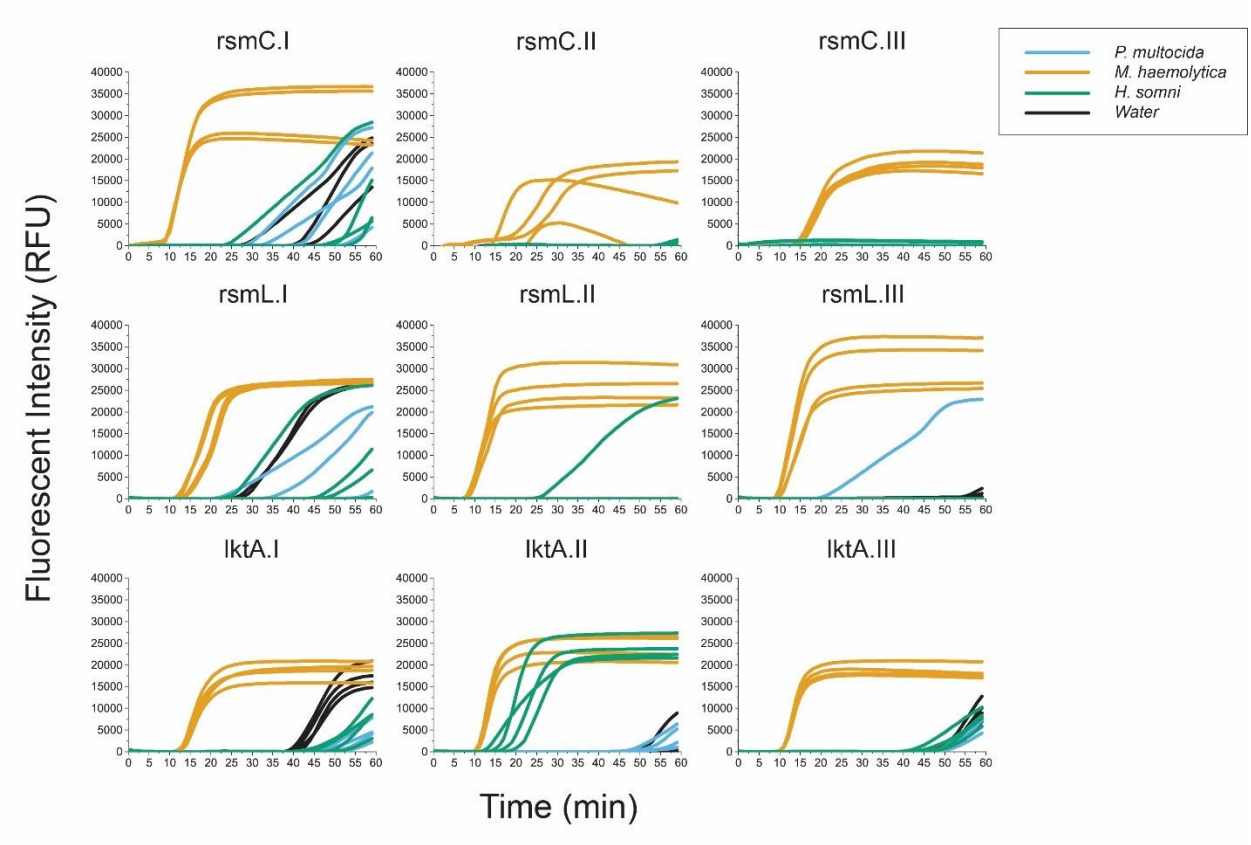

Figure A6: qLAMP amplification curves for *M. haemolytica* targeting LAMP primer sets. 5 µL of gDNA from *P. multocida*, *M. haemolytica*, and *H. somni* (0.2 ng/uL) was added to separate reactions in quadruplicate and incubated for 60 minutes at 65 °C. Nuclease-free water was used as a negative control. Blue lines: *P. multocida* gDNA reactions; Orange lines: *M. haemolytica* gDNA reactions; Dark Green lines: *H. somni* gDNA reactions; Black lines: Water reactions.

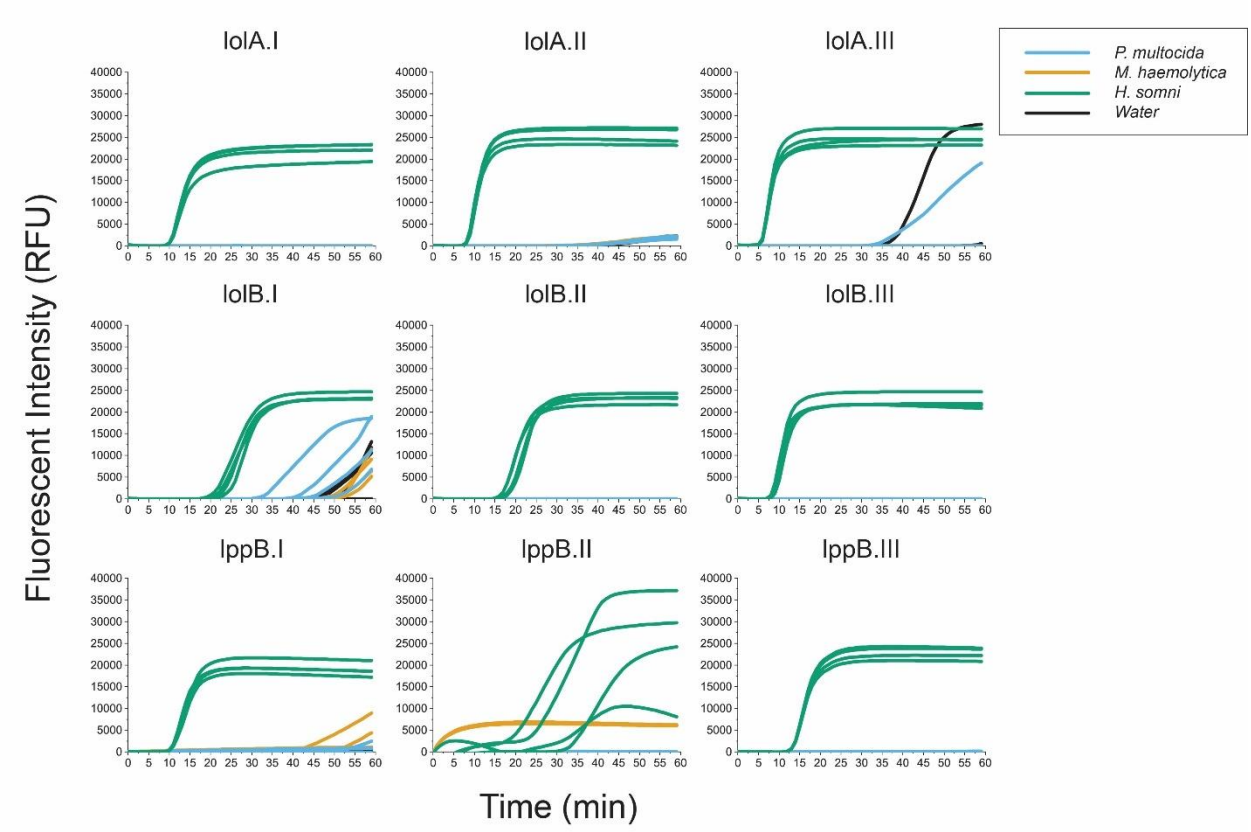

Figure A7: qLAMP amplification curves for *H. somni* targeting LAMP primer sets. 5 µL of gDNA from *P. multocida*, *M. haemolytica*, and *H. somni* (0.2 ng/uL) was added to separate reactions in quadruplicate and incubated for 60 minutes at 65 ºC. Nuclease-free water was used as a negative control. Blue lines: *P. multocida* gDNA reactions; Orange lines: *M. haemolytica* gDNA reactions; Dark Green lines: *H. somni* gDNA reactions; Black lines: Water reactions.

Table A1: Generated primer sets for targeting respective BRD pathogens to be screened

Primer	Sequence (5' to 3')
kmt1.1 F3	GGTGCCTATCTTGCTTCG
kmt1.1 B3	TCGATCGCTAGCACCACA
kmt1.1 FIP	TGCCGTAGCAGAAACTGGACAGGTGAGCCATGTGAGTT
kmt1.1 BIP	CACACCGAAGCCAGGACTTCACCAAATGTACTGGTTGCTTC
kmt1.1 LF	CGACCATCGGTTGCATTTC
kmt1.1 LB	TGGCATTGCATGGCTATCA
kmt1.3 F3	CGCTGATTAATATTGTGCTGA
kmt1.3 B3	CCCAACAAAACTGTGCTT
kmt1.3 FIP	TTCGCGGCAATCGGTTCATTCATTACTGCTCTATCCGCTAT
kmt1.3 BIP	TATGCCACTTGAAATGGGAAATGGCCAAATAAAAGACTACCGACAA
kmt1.3 LF	ACCGCCCCACTGGGTAA
kmt1.3 LB	TTTTATGGCTCGTTGTGAGTGG
omp1.1 F3	TTCCAACTAGCAGAAGTGT
omp1.1 B3	CCTGGCATTGGTGAAGTT
omp1.1 FIP	ACTGCCGCATTATCTGCAATCCAACATCCGGTTTAGGGC
omp1.1 BIP	GCCACGAACCCAGCATTAATACATAAATTGCCCCAACAG
omp1.1 LF	GGCTTCCCCCGCATAAGCA
omp1.1 LB	CTTATTGAAACAACCTGAA
omp1.3 F3	GCTCTTGCACCTGCATTA
omp1.3 B3	GGTCTTACCGTCCGTACC
omp1.3 FIP	CGGTAGCGGTAAATGTAAAGTACCAGTCCTCAAGGTATTACTGC
omp1.3 BIP	TTCTGGCTATCATAAAATGACCGATAACTCTTTAAATTTGCTCCATTG
omp1.3 LF	GGGATTTCTTTGCCACCTGT
omp1.3 LB	TTTTGCAATGCACTATAGCT
omp16.2 F3	CAGTACAAGATTTACAACAACG
omp16.2 B3	AAATAATGTTTAACTGCATCTGC
omp16.2 FIP	CGCATGTGCATCTAAAATTTGTACATTATAATACCGTGTATTTCGGC
omp16.2 BIP	CGTTCTTAAATGCAACACCTGCGATGTTATATTCTGGTGTACCG
omp16.2 LF	CCTTCGATATTGTATTTATCG
omp16.2 LB	TGTTGAAGGTAACACCGATGAAC
omp16.3 F3	GCGTTCTTAAATGCAACACC
omp16.3 B3	TATGCTAACACAGCACGA
omp16.3 FIP	GACGTTGACCTAATGCGATGTTATGTTGTTGAAGGTAACACCG

Table A1: continued

omp16.3 BIP	CAAGCTGGTCAAGTATCAACAGTAGTAAGCTGCTTCATCGTG
omp16.3 LF	TTCTGGTGTACCGCGTTCA
omp16.3 LB	CTTACGGTGAAGAGAAACCTGC
lktA.1 F3	TTCTTTAAAAACTGGGGCAA
lktA.1 B3	CAATAGCGGTTTGAATCGT
lktA.1 FIP	CGCTTTGACTAAATCCTGTAAACCACTCTATATTCCCCAAAATTACCAAT
lktA.1 BIP	CCGAAGAGTTGGGGATTGAGGGCCTAAACTGGTTTGAGCT
lktA.1 LF	TACCTTGTTCAGTATCA
lktA.1 LB	AAGAACGCAATAATATTGCAA
lktA.2 F3	CCGAAGAGTTGGGGATTG
lktA.2 B3	GCATTTTGTACAATGCTTTCG
lktA.2 FIP	CGGTTTGAATCGTGCCTAAACTGGTACAAAGAGAAGAACGCAAT
lktA.2 BIP	CTATTGGCTTAACTGAGCGTGGTGGCCTGCTTTAGTTTTCTG
lktA.2 LF	TTTGAGCTGTTGCAATAT
lktA.2 LB	TTGTGTTATCCGCTCCACAAAT
rsmL.1 F3	ATTTAGTCGCCGCCGAAG
rsmL.1 B3	CCCGGGTCGCTAATCAGT
rsmL.1 FIP	TGGTCGTGCAAGGCGAAAAACACAGCGGTTTATTGCTGAGC
rsmL.1 BIP	ATGAACAGCAGAAAGCGGTCGTCGTTCCGGCATCGGAAAT
rsmL.1 LF	GTTTTTTAATGCCGTAGT
rsmL.1 LB	TTAGCAAAAGGGGAAAACATTGCGT
rsmL.3 F3	TTTAGTCGCCGCCGAAGA
rsmL.3 B3	TGACGGACAAGATGAAAGCC
rsmL.3 FIP	ATGGTCGTGCAAGGCGAAAAACACAGCGGTTTATTGCTGAGC
rsmL.3 BIP	TGAACAGCAGAAAGCGGTCGTTCGTTCCGGCATCGGAAAT
rsmL.3 LF	GTTTTTTAATGCCGTAGT
rsmL.3 LB	ATTAGCAAAAGGGGAAAACATTGCG
rsmC.1 F3	TGCGAGATGATTTTGCCCG
rsmC.1 B3	ACATTTCTTGCCCTGCTTGG
rsmC.1 FIP	CTCACTATGGTGGCGTGCGTATCGTGCGGAAAAGGTTGC
rsmC.1 BIP	TTGAGTGCCGAGTTAGCGGTGACAGCCATTGTAGCAGTTGG
rsmC.1 LF	TCAAAATAGCTACTGAATAC
rsmC.1 LB	AAGAACAAGCAGGAATGCC
rsmC.2 F3	GGAATGCCAATTCCAACTGC
rsmC.2 B3	GCAAGCGGTAGGATTTCCAG

Table A1: continued

rsmC.2 FIP	CACCAGCTCGGTTTTCGCCGCAATGGCTGTCGCAATGC
rsmC.2 BIP	CGAACCGTTCGGCAATATTGCC-TGTCGGCACGGTCTGTAG
rsmC.2 LF	TCAACATTTCTTGCCCTGCTT
rsmC.2 LB	AATCGACTCTGCACGCCGTTG
LPB.1 F3	CACAAAAAATACTGAGCAGGTA
LPB.1 B3	AGAGAAGGAGATTATTTGGAATG
LPB.1 FIP	CTGTTGCCCATACTTCTAAGGTTA-GCCTCAGTTTTCAAGCGA
LPB.1 BIP	TACCGAATAAACAAAGCTATCCGAT-GCTGATTTTGCTAATGCGG
LPB.1 LF	TTATGAAGCAGAGAAAAT
LPB.1 LB	CGCACTTTCTTTGATAACTCTCGT
LPB.2 F3	ACATTGGTACTGGAAAGCAA
LPB.2 B3	GCAGAGAAAATTTTAACCTTAGAAG
LPB.2 FIP	ATCAACGATACTCACATTGTTAGCTTTTTCTCACAGTAAAATGTCAATTGC
LPB.2 BIP	GTCACTGTTTTGCGTCTAAATAGTTTTCCTCGCTTGAAAACTGAGG
LPB.2 LF	GTAATTGACGACGTAG
LPB.2 LB	AAAAAATACTGAGCAGGT
lolA.1 F3	AAGTGACTGATGCACAGG
lolA.1 B3	CAAAACAAAGGGGGTATCG
lolA.1 FIP	AGGTGTTTCATTGTCCATACGAAATGAAAAAAAATACAGCAAGGTAGT
lolA.1 BIP	TTGCAGATGGAAAAACATTATGGTTCTTAAGGTATCTTCAACCCAATT
lolA.1 LF	GGACGTTTGAGTTGTATTT
lolA.1 LB	CGATCCTTTTGTTGAGCAAG
lolA.3 F3	TCAAACGTCCTAATTTATTTCGT
lolA.3 B3	CGTATCGGATTTTTGCTCAA
lolA.3 FIP	ACTTGCTCAACAAAAGGATCGTAGAATGGACAATGAAACACCTCA
lolA.3 BIP	AACAGCAAATTGGGTTGAAGATACCTGTTGCCAATGACTTGGAT
lolA.3 LF	TCCATCTGCAATAATTTGACT
lolA.3 LB	GCGATACCCCCTTTGTTTTGC
lolB.1 F3	CATCAGCTTTCAGCAGTTC
lolB.1 B3	AAATGATTTATGCCGACCTG
lolB.1 FIP	GTTGATATCTTTTGTTGAACGCTGATTTCACCTTTCGGTATTACTGT
lolB.1 BIP	AACTTGCTACGTGAAATGATTGGTTGAATTTTCATCTGGTTGACCT
lolB.1 LF	TTCCTTTATGGTCTGA

Table A1: continued

lolB.1 LB	CTCTATTCCATTACAACAAAT
lolB.2 F3	AACGTTTTTCCACTCGCT
lolB.2 B3	CATTTCACGTAGCAAGTTGT
lolB.2 FIP	TGAACTGCTGAAAGCTGATGAAAACTTGGCAATATAACAATCCG
lolB.2 BIP	AGTTTCACCTTTCGGTATTACTGTTTGATATCTTTTGTTGAACGCTG
lolB.2 LF	TAATAAAGAATAGGATT
lolB.2 LB	CAGACCATAAAGGAAA

A1. Primer Screening Code (main)

```
clc
clear
close all
[data, text, alldata] = xlsread('Primer Set Screening 1 Raw Data');
%% Primer Set names
% Creates vector with all primer set names and removes empty spaces
primer_set_names = string(text);
for i=1:length(primer_set_names)
    if primer_set_names(i) == ""
        primer_set_index(i) = 1;
    end
end
primer_set_names(find(primer_set_index == 1)) = [];
%% Graph Primer Data
%Makes GUI with all primer set graph information present
h = tfigure('qLAMP Plots');
j = 1;
%creates and adds individual primer graphs to GUI
for i = 1:16:length(data)
    addPlot(h,convertStringsToChars(primer_set_names(j)));
    primer_set = data(:,i:(i+15));
    primer_plot(primer_set, primer_set_names(j));
    j = j+1;
end


%% Primer Information Extraction
% Extracts primer decision criteria from each primer set screening data
time_avg = [];
time_stdev = [];
max_avg = [];
max_stdev = [];
false_pos_total = [];
true_neg_false_pos = [];
isValid = [];
primer_completion = ones(1,27);
%specifies what the target bacteria for each bacteria in primer set
screening is
primer_bacteria_target = [ ones(1,9)*3 ones(1,9)*2 ones(1,9)*4];
```

```
j = 1;
for i =1:16:length(data)
    %if a primer set has been completed, extract performance
    %characteristics of primer set and add information to master
performance arrays
    if primer_completion(j) == 1

        primer_set = data(:,i:(i+15));
        [time_avg_temp,max_avg_temp,  time_stdev_temp,  max_stdev_temp,
false_pos_total_temp,                          true_neg_false_pos_temp,
isValid_temp]=primer_info(primer_set,primer_bacteria_target(j));
        time_avg = [time_avg, time_avg_temp];
        time_stdev = [time_stdev, time_stdev_temp];
        max_avg = [max_avg, max_avg_temp];
        max_stdev = [max_stdev, max_stdev_temp];
        false_pos_total = [false_pos_total, false_pos_total_temp];
        true_neg_false_pos            =            [true_neg_false_pos,
true_neg_false_pos_temp];
        isValid = [isValid, isValid_temp];
        j = j+1;
    %else mark all performance characteristics as not completed in master
    %performance arrays
    else
        time_avg = [time_avg, -1];
        time_stdev = [time_stdev, -1];
        max_avg = [max_avg, -1];
        max_stdev = [max_stdev, -1];
        false_pos_total = [false_pos_total, -1];
        true_neg_false_pos = [true_neg_false_pos, -1];
        isValid = [isValid, -1];
        j = j+1;
    end
end
%% Primer Set Selection
%Generates weighted scores for each primer set criteria and picks best
%primer set for each gene
time_avg_score = [];
time_stdev_score = [];
max_avg_score = [];
max_stdev_score = [];
false_pos_score = [];
total_score = [];

[time_avg_score,  time_stdev_score,  max_avg_score,  max_stdev_score,
false_pos_score,total_score]  =  score2(time_avg,  max_avg,  time_stdev,
max_stdev, false_pos_total, isValid);

decision_temp = string();
decision = string();

%Determines which primer set to use based off of total primer set scores
for i = 1:3:27
    temp = [total_score(i) total_score(i+1) total_score(i+2)];
    true = find(temp == max(temp));
    false = find(temp ~= max(temp));
    decision_temp(true) = 'Use';
```

```
    decision_temp(false) = 'X';
    decision = [decision, decision_temp];
end
decision(1) = [];

%indicates failed primer sets and generates table with all relavent
%performance characteristics, scores, and decisions
yes = find(isValid == 1);
no = find(isValid == 0);
isValidtext = strings([1,27]);
isValidtext(yes) = 'Yes';
isValidtext(no) = 'No';
%isValidtext(primers_not_completed) = 'N/A';
table_names      =      {'Reaction_Time_min',      'Reaction_Time_stdev',
'Max_Intensity_RFU',   'Max_Intensity_stdev',   'Total_False_Positives',
'True_Neg_False_Positives','Any_Negative_Reactions_pre_30_minutes'
'Total_Score', 'Decision'};
T = table( time_avg', time_stdev', max_avg', max_stdev', false_pos_total',
true_neg_false_pos',isValidtext', total_score',decision');
T.Properties.VariableNames = table_names;
T.Properties.RowNames = primer_set_names;
```

A2. Primer Info Function

```
function [time_avg, max_avg, time_stdev, max_stdev, false_pos_total,
true_neg_false_pos, isValid] = primer_info(primer_set,positive_bacteria)
%primer_info.m: extracts performance information from a given primer set.
% determines which bacteria of inputted primer set is the target pathogen
if positive_bacteria == 1
    num = 1;
elseif positive_bacteria == 2
    num = 5;
elseif positive_bacteria == 3
    num = 9;
elseif positive_bacteria == 4
    num = 13;
end
%references start of fluorescent data for different bacterias in screen
num_series = [ 1 5 9 13];
time = [];
max_intensity = [];
%determines max intensity, 90% max, and response time for taret pathogen
%replicates
for i = 1:4
    max_intensity(i) = max(primer_set(:,num+i-1));
    ninety_percent_max = 0.9*max(primer_set(:,num+i-1));
    time(i) = min(find(primer_set(:,num+i-1) >= ninety_percent_max));

end
%averages target replicate performance criteria and determines spread of
%replicate data
max_avg = mean(max_intensity);
time_avg = mean(time);
max_stdev = std(max_intensity);
```

```
time_stdev = std(time);

%sets fluorescent intensity threshold for determining false postive
%reactions
false_pos_threshold = 0.2*max_avg;

false_pos_total = 0;
true_neg_false_pos = 0;
%removes target bacteria from bacteria reference array for false-positive
%screening
num_series(find(num_series == num))=[];
isValid = 0;
for i = num_series
    for j = 1:4
        %if response time of non target reactions is less than 30 minutes,
        %mark the set for rejection
        if min(find(primer_set(:,i+j-1) >= ninety_percent_max)) <=30
            isValid = 1;
        end
           %if non target reactions have amplified past false-positive
           %threshold, count them as false-positives
        if isempty(find(primer_set(:,i+j-1) > false_pos_threshold)) == 0
            false_pos_total = false_pos_total +1;
        end
        % if Water reactions amplify past false-positive
        % threshold, count them as negative control false-positives
        if i == 1
            if isempty(find(primer_set(:,i+j-1) > false_pos_threshold))
== 0
            true_neg_false_pos = true_neg_false_pos + 1;
            end
        end
    end
end




end
```

A3. Primer Score Function

```
function [time_avg_score, time_stdev_score, max_avg_score,
max_stdev_score, false_pos_score,total_score] = score2(time_avg, max_avg,
time_stdev, max_stdev, false_pos_total, isValid)
%score2.m: Scores performance characteristics of primer sets based off
%normalized characteristic data and assigned weights for desired reaction
%features
%Weights on performance characteristics
time_avg_weight = 20;
time_stdev_weight = 30;
max_avg_weight = 10;
```

```
max_stdev_weight = 10;
false_pos_weight = 30;

A = time_avg;
B = time_stdev;
C = max_avg;
D = max_stdev;
E = false_pos_total;


%leaves unfinished primer sets out of score calculation
index = find(A == -1);
A(index) = [];
B(index) = [];
C(index) = [];
D(index) = [];
E(index) = [];



time_avg_range = range(A);
time_stdev_range = range(B);
max_avg_range = range(C);
max_stdev_range = range(D);
false_pos_range = range(E);

%calculates score of response time for each primer set
for i=1:length(time_avg)
    if time_avg(i) ~= -1
        time_avg_score(i)=(1-((time_avg(i)-
min(A))/time_avg_range))*time_avg_weight;
    else
        time_avg_score(i) = -1;
    end
end
%calculates score for response time spread for each primer set
for i=1:length(time_stdev)
    if time_stdev(i) ~= -1
        time_stdev_score(i)=(1-((time_stdev(i)-
min(B))/time_stdev_range))*time_stdev_weight;
    else
        time_stdev_score(i) = -1;
    end
end
%calculates score for maximum intensity for each primer set
for i=1:length(max_avg)
    if max_avg(i) ~= -1
        max_avg_score(i)=((max_avg(i)-
min(C))/max_avg_range)*max_avg_weight;
    else
        max_avg_score(i) = -1;
    end
end
%calcuates score for max intensity spread for each primer set
for i=1:length(max_stdev)
```

```
    if max_stdev(i) ~= -1
        max_stdev_score(i)=(1-((max_stdev(i)-
min(D))/max_stdev_range))*max_stdev_weight;
    else
        max_stdev_score(i) = -1;
    end
end
%calculates score for false-positive totals for each primer set
for i=1:length(false_pos_total)
    if false_pos_total(i) ~= -1
        false_pos_score(i)=(1-((false_pos_total(i)-
min(E))/false_pos_range))*false_pos_weight;
    else
        false_pos_score(i) = -1;
    end
end


%calculates total score for every primer set
total_score = time_avg_score + time_stdev_score + max_avg_score +
max_stdev_score + false_pos_score;

%if any primer set had any non target amplification before 30 minutes
%(indicative of cross-reactivity), automatically give a failing score to
it
for i = 1:length(isValid)
    if isValid(i) == 1
        total_score(i) = 0;
    end
end


End
```

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