

**DEVELOPMENT OF MULTIPLEX REAL-TIME
PCR ASSAYS FOR THE SURVEILLANCE OF TICK-BORNE
PATHOGENS IN DELAWARE**

by

Michael H. Buoni

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences

Fall 2023

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ACKNOWLEDGMENTS

I would first like to thank Dr. Esther Biswas-Fiss and Dr. Subhasis Biswas for giving me the amazing opportunity to pursue this Ph.D. program. Their support throughout this process is very much appreciated. I am also thankful for the support of the other amazing people on my committee, Dr. Virginia Hughes and Dr. Ashley Kennedy, who have given me advice and direction throughout this process. I would also like to thank Dr. Lauren Maestas, Dr. Rosh Roy and Dr. Scott Gardner for their support with my research. I would also like to thank the Delaware Department of Natural Resources and Environmental Control for funding this project. In addition, I would like to thank Dr. Mark Brainard and all of Delaware Tech for their continued support of this project, especially Christina Evans, for helping me with review and formatting.

On a personal level, I would like to thank my parents, Bo and Eileen Buoni, as well as my sister Andrea Kelly, for instilling in me at a young age the notion of always finishing what you start. One never really realizes the impact of a great family until later in life.

Finally, I dedicate this dissertation to my wife Charlotte and my daughters Sarah and Julia. Thank you for understanding the times that I had to stay home from family events to finish writing or researching. I am forever grateful for the continued love and support that you all have given me. I would have never even attempted this program without the unconditional support from you. Mew.

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ABSTRACT

Tick-borne pathogens are responsible for most vector-borne human diseases in the United States. In the United States, close to 650,000 cases of vector-borne diseases were reported from 2004–2016¹. Of the 650,000 cases, tick-borne diseases accounted for >75% of these cases¹. From 2016-2019, there were 206,568 reported cases of tick-borne diseases¹. This demonstrates increasing numbers of tick-borne pathogen cases reported in the United States. Given the increasing recognition of tick-borne diseases, as well as the increase in the range and distribution of ticks, it is imperative to understand which pathogens, and in what prevalence, are carried by tick species in areas populated by humans. Few studies exist surveying the presence and distribution of tick-borne pathogens in the state of Delaware. The goal of this study was to create multiplex real-time PCR assays to identify *Borrelia burgdorferi sensu stricto*, *Babesia microti*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* from their respective reservoir tick species.

Three multiplex, real-time PCR assays were developed and tested on 1527 ticks comprising *Ixodes scapularis*, *Dermacentor variabilis*, and *Amblyomma americanum*, three species of ticks relevant to Delaware. The results showed that of a sample of 500 *Ixodes scapularis* ticks from Delaware, 30.20% were positive for *Borrelia burgdorferi*, 2.60% were positive for *Babesia microti*, and 1% were positive

for *Anaplasma phagocytophilum*. Testing of 500 *D. variabilis* ticks revealed that 0.00% were positive for *E. chaffeensis* and 0.20% were positive for *A. phagocytophilum*. Finally, of the 527 *A. americanum* ticks tested, 4.74% were positive for *E. chaffeensis* and 1.14% were positive for *E. ewingii*. These findings support the notion that real-time PCR assays can be used to successfully identify and monitor tick-borne pathogen activity in Delaware.

Chapter 1

INTRODUCTION

1.1 Ticks and Tick-Borne Pathogens of Interest

Worldwide, ticks are the second most important arthropod vector of human pathogens.² In the last few decades, changes in climate, vegetative communities, and wildlife density have contributed to distributional shifts of many ticks and their associated pathogens, and the recognition of previously known, as well as novel tick-borne disease agents has become more frequent³⁻⁷. In the United States, greater than 95% of reported cases of vector-borne diseases are caused by tick-borne pathogens². The increase in reporting of vector-borne disease may be associated with human expansion into wild areas and increases in forays into nature, associated with healthier living styles, which may lead to an increase in potential interactions with infected ticks².

Ticks are mites, included in the taxonomical class Arachnida, along with spiders⁸. There are four stages of tick development: egg, larva, nymph, and adult⁸. Ticks are obligate sanguineous parasites, feeding on the blood of mammals, birds, amphibians, and reptiles⁸. They are characterized by the presence of chelicera, non-segmented body, simple eyes, a Haller's organ, and have a hemimetabolous lifestyle with the immature larvae having six legs and nymphs and adults having eight⁹. Ticks have a near-worldwide distribution, though the highest diversity of tick species, like other plant and

animal life, is near the equator¹⁰. Ticks are limited by their tolerance to desiccation and environmental susceptibility¹⁰. The combination of a long life, multiple feeding sessions, and warming global temperatures contribute to the efficiency of ticks as a vector of pathogens, including bacteria, viruses, protozoans, as well as sources of toxins¹¹.

Ticks are categorized into three taxonomic families, the Nuttalliellidae, Argasidae, and Ixodidae. Ticks of the Nuttalliellidae family are found in Africa and are represented by only one species, *Nuttalliella namaqua* Bedford¹². Argasidae ticks are referred to as soft-bodied ticks because they lack the hard shield, or scutum, present in the hard ticks. There are about 193 species of Argasidae ticks¹³. The family Ixodidae, or the hard ticks, is found in large numbers in the eastern United States and is significant as vector of tick-borne disease. For this reason, the Ixodidae family of ticks was chosen as the focus of this study. There are around 700 species of Ixodidae ticks, and many of them are known to carry human pathogens¹⁴. Three of the 17 genera of Ixodid ticks most commonly encountered in the Northeastern United States, using common surveillance techniques, are *Amblyomma*, *Dermacentor*, and *Ixodes*¹⁴.

Tick-borne pathogens pose a health risk to the United States public. With increased reports of the numbers and diversity of tick-borne pathogens, it is critical to identify the distribution of tick species, as well as the inherent pathogens they transmit¹⁵. The National Institute for Occupational Safety and Health (NIOSH) reported that of the many tick-borne diseases, some of the most common tick-associated human

diseases in the United States are anaplasmosis, babesiosis, ehrlichiosis, Lyme disease, and Rocky Mountain spotted fever¹⁶.

Tick-associated disease data from the Office of Infectious Disease Epidemiology at the Delaware Department of Health and Social Services Division of Public Health was obtained through a public records request. Data was obtained for 2017-2021 in reference to human cases of disease in relation to the following tick-borne disease pathogens. The five human disease-causing tick-borne pathogens noted below in Table 1 are representative of the pathogens relevant to this study.

Tick-Borne Disease Pathogens	# of confirmed cases	# of probable cases	Total
<i>Anaplasma phagocytophilum</i>	7	37	44
<i>Borrelia burgdorferi</i>	1966	312	2278
<i>Babesia microti</i>	17	3	20
<i>Ehrlichia chaffeensis</i>	86	61	147
<i>Ehrlichia ewingii</i>	3	0	3

Table 1. Incidence of relevant tick-borne pathogen disease cases in Delaware from 2017-2021¹⁷.

There is substantial evidence that the northeastern United States not only has high numbers of reported cases of tick-borne pathogen infections per capita, but may also have high numbers of unreported cases¹⁸. For a 3-year average ending in 2018, Delaware had the 6th highest incidence of Lyme disease in the United States with 45.7 cases per 100,000 persons¹⁸. In addition to Lyme disease, an increased number of cases of ehrlichiosis caused by *Ehrlichia* pathogens have been observed in south-central and the southeastern United States with the highest incidence occurring in Missouri,

Arkansas, Delaware, Tennessee, Virginia, and Oklahoma¹⁹. For example, in the year 2000, only 200 cases of ehrlichiosis were reported across the nation²⁰. By the year 2018, the number of reported cases rose to 1,799 cases²¹.

1.2 Tick Species Relevant to this Study

Most of the ticks used in this study were collected across the state of Delaware by the Delaware Department of Natural Resources and Environmental Control (DNREC). DNREC employs a full-time tick biologist who collects, identifies, and preserves ticks from across the state. DNREC collected 59,786 ticks in from Delaware from 2019-2020. Of the ticks collected and identified to species, 94.7% of ticks collected were *Amblyomma americanum*, 1.9% were *Ixodes scapularis*, and 0.5% were *Dermacentor variabilis*. These three species represented 97% of all the ticks collected in Delaware from 2019-2020. A subset of ticks from this effort was used for this study. DNREC maintains a database of information for every tick that is collected. Each time a tick was collected, information about the tick was recorded including the date, location, sex, life stage, and species. Ticks were stored in 95% ethanol at -20°C until processing.

Amblyomma americanum Linnaeus, more commonly referred to as the lone star tick, is a common tick of the Eastern, Midwestern, and Southeastern United States. *Amblyomma* ticks are large, variegated ticks with long, strong mouthparts. The hypostome and palps are long and the second palp segment is twice as long as it is wide. The eyes are present and the festoons are well developed¹. They are known or suspected to cause diseases or vector several organisms that are implicated in human diseases such as alpha-gal syndrome, ehrlichiosis, tularemia, and southern tick-associated rash illness

(STARI). In addition, these ticks can also carry and transmit emerging viral human pathogens such as Bourbon virus and Heartland virus²².



Figure 1. Larva, nymph, adult male and adult female lone star ticks *Amblyomma americanum*. Image retrieved from <https://web.uri.edu/tickencounter/species/lone-star-tick/>

Ixodes scapularis Say ticks are commonly referred to as the deer tick or black-legged tick. The males and females have black legs and dorsal shield, and the female has a dark red abdomen. The males are entirely black or dark brown and have a larger scutum than females. This tick also has a characteristic suture around the anal opening on the lower edge of the abdomen of the ventral side which resembles a horseshoe-shaped ridge. Black legged ticks, unlike other hard-bodied ticks, do not have festoons²³. *Ixodes scapularis* ticks are an established vector of *Borrelia burgdorferi*, the causative agent of Lyme disease, but can also carry and transmit pathogens that cause anaplasmosis, babesiosis, *Borrelia miyamotoi* disease and Powassan virus disease¹⁶. Over the past few decades, expansion in both the geographic range of *I. scapularis* and

the geographic range an incidence of Lyme disease, as well as other *I. scapularis*-associated diseases, has been apparent²⁴.



Figure 2. Larva, nymph, adult male and adult female black legged ticks (deer ticks) *Ixodes scapularis*. Image retrieved from <https://web.uri.edu/tickencounter/species/blacklegged-tick/>

Dermacentor variabilis Say, also referred to as the American dog tick, are characterized by decorated, light-colored dorsal patterns that include many geometric shapes against an otherwise reddish-brown to brown body. *Dermacentor* ticks also have a square shaped head, condensed palps and mouth parts. The American dog tick can carry and transmit pathogens and toxins that cause such human conditions as Rocky Mountain spotted fever, tick paralysis, and tularemia. In the United States, *D. variabilis* has also been shown to also carry *Anaplasma phagocytophilum*, the causative agent of anaplasmosis, though vector competency has not yet been established²⁵.



Figure 3. Larva, nymph, adult male and adult female American dog ticks *Dermacentor variabilis*. Image retrieved from <https://web.uri.edu/tickencounter/species/dog-tick/>

1.3 Human Tick-Borne Pathogens of Interest

1.3.1 *Borrelia burgdorferi*

Borrelia burgdorferi sensu stricto (s.s.) is the causative agent of Lyme disease in North America, the most common vector-borne infectious disease in the United States²⁶. They are gram-negative, microaerophilic, intracellular spirochetes²⁷. Phylogenetic analysis has led to the division of *Borrelia burgdorferi* into 21 species, collectively referred to as the *B. burgdorferi sensu lato* species complex²⁷. The *B. burgdorferi sensu stricto* genospecies is the only one currently known to cause human cases of Lyme disease in the Northeastern United States^{28,29}. *Borrelia mayonii* and *Borrelia bissetti* can also cause Lyme disease. However, present data shows that within

the United States, *B. mayonii* is only found in the Upper Midwest and West Coast of the United States^{30,31}. *Borrelia bissetti* is mainly found mainly in the Upper Midwest and West Coast of the United States. Therefore, the primer and probe sequences for this study were developed specifically for the genospecies *B. burgdorferi s.s.* (henceforth referred to as *B. burgdorferi*). The *I. scapularis* tick spreads the *B. burgdorferi* pathogen in the northeastern, mid-Atlantic, south, and north-central United States³².

In the northeastern United States, the principal reservoir host of this pathogen is the white-footed mouse (*Peromyscus leucopus* Rafinesque), but chipmunks (*Tamias striatus* Linnaeus), short-tailed and masked shrews (*Blarina brevicauda* Say and *Sorex cinereus* Kerr), and eastern gray squirrels (*Sciurus carolinensis* Gmelon) can serve as infectious hosts³³. Because *B. burgdorferi* is not transmitted transovarially, ticks acquire the bacteria by feeding on infected hosts during their larval and/or nymphal stages of development³⁴⁻³⁸. Other potential pathogen hosts must acquire *B. burgdorferi* by being fed upon by one or more infected nymphs or adults³⁹.

The Centers for Disease Control and Prevention (CDC) reports approximately 30,000 cases of Lyme disease through data collected from state health departments and the District of Columbia³⁰. At least 24 hours of tick attachment are generally required to transmit this pathogen to humans.⁴⁰ The spirochete spreads through lymph or blood and it may be weeks or months for the human immune system to control the spread of the pathogen, despite the presence of antibiotics, and if not treated, *B. burgdorferi* can cause a characteristic rash, and in later stages, potentially arthritis, carditis, and in late stages, encephalopathy⁴¹.

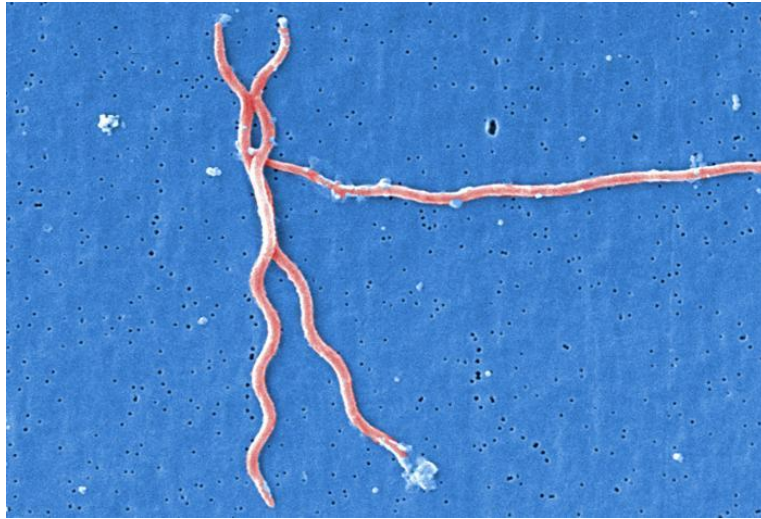


Figure 4. Image of *B. burgdorferi* spirochetes. Image retrieved from <https://phil.cdc.gov/Details.aspx?pid=13177>

1.3.2 *Babesia microti*

Human babesiosis is caused by a protozoan parasite that infects red blood cells⁴². There are more than 100 species of *Babesia*, and in the United States, most human infections are caused by *Babesia microti*, *Babesia duncani* and *Babesia divergens*⁵. *Babesia duncani* is primarily identified on the West Coast of the United States, and *B. divergens* is associated with babesiosis in Europe^{43,44}. In the Northeastern United States, most cases of human babesiosis are caused by *Ba. microti*⁴⁵. An established reservoir for *Ba. microti* is white-footed mice (*Peromyscus leucopus* Rafinesque). However, other small mammals such as northern short-tailed shrews (*Blarina brevicauda* Say), eastern chipmunks (*Tamias striatus* Linnaeus), and raccoons

(*Procyon lotor* Linnaeus) have been shown to be competent reservoirs^{46,47}. The *Ixodes scapularis* tick is the main vector host for *Ba. microti*⁴⁴.

Babesiosis infection is caused by a *Babesia*-infected tick introducing sporozoites into the human host through the tick bite⁴⁷. In most patients, symptoms begin one to six weeks after infection and the symptoms are non-specific. Typical earlier presentation of symptoms includes intermittent fevers accompanied by fatigue, chills headache, and myalgias. In worse cases, reduced appetite, nausea, vomiting, and depression may occur. The usual course of the disease can last from several weeks to several months, but some patients take even longer to recover fully⁴⁸. More specifically, the *Babesia* parasites infect and destroy human erythrocytes. This can cause hemolytic anemia that can cause jaundice, dark urine, thrombocytopenia and systemic anemia⁴⁹.

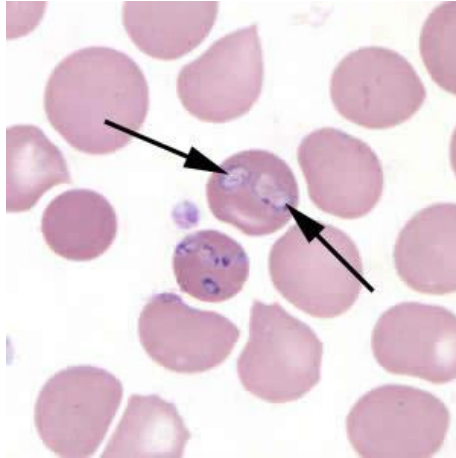


Figure 5. *Babesia microti* MO-1 in a thin blood smear stained with Giemsa. Note the vacuolated parasites (black arrows) in the image. Image retrieved from <https://www.cdc.gov/dpdx/babesiosis/index.html>

1.3.3 Anaplasma phagocytophilum

Anaplasmosis is a bacterial infection caused by the gram-negative, intracellular bacterium *Anaplasma phagocytophilum*⁴⁵. It has an unusual affinity for growth in neutrophils of its hosts⁴⁵. The growth of this organism in the neutrophils can cause tissue injury, explaining the clinical manifestations associated with anaplasmosis⁵⁰. *Anaplasma phagocytophilum* has been known to coinfect with *Borrelia* spp^{51,52}.

The strain of *A. phagocytophilum* relevant to human disease is *A. phagocytophilum-human active (ha)*. This strain is known to infect humans and can be carried by myriad small rodents and wildlife, including the white-footed mouse (*Peromyscus leucopus*)⁵³. The *A. phagocytophilum-ha* pathogen has been found in the *I. scapularis* tick and the *D. variabilis* tick, however vector transmission has only been

clearly established in *I. scoularis*⁵⁴. Another strain, *A. phagocytophilum-V1*, carried by wildlife, especially by white tailed deer (*Odocoileus virginianus* Zimmermann), is not associated with human infection⁵⁵. Any positive *A. phagocytophilum* samples will need to be further sequenced to distinguish the V1 strain from the human disease-causing strains.

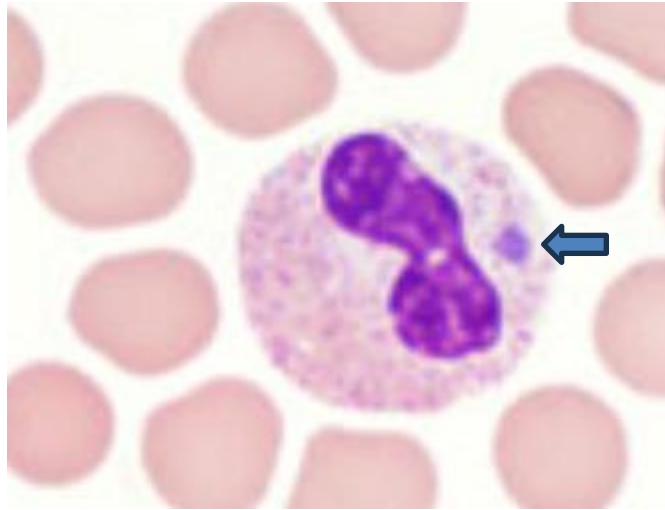


Figure 6. Image of *Anaplasma phagocytophilum* infecting a white blood cell. Image retrieved from: <https://www.cdc.gov/ticks/tickbornediseases/anaplasmosis.html>

1.3.4 Ehrlichia chaffeensis and Ehrlichia ewingii

Ehrlichia chaffeensis and *E. ewingii* have been identified as two of the most common emerging tick-borne pathogen agents of ehrlichiosis¹⁵. Ehrlichiosis is primarily caused by *Ehrlichia chaffeensis* but can also be caused by *Ehrlichia ewingii*²¹, *Ehrlichia muris euclairensis* and more rarely by the Panola Mountain *Ehrlichia* species

pathogen⁵⁶. In 2008, the CDC split the ehrlichiosis case definition into four categories: *Ehrlichia chaffeensis* infection, *Ehrlichia ewingii* infection, *Anaplasma phagocytophilum* infection, and undetermined ehrlichiosis/anaplasmosis²¹. The reason that *A. phagocytophilum* was included in the ehrlichiosis reporting was that some clinical and laboratory signs were consistent with either an ehrlichiosis or anaplasmosis infection, but the distinction between the specific organism of causation could not be made due to the limitations of diagnostic test results²¹. *Ehrlichia ewingii* was chosen for this study because of the inclusion of this species on Class B2 reporting on human identified cases of ehrlichiosis and anaplasmosis by the Delaware Public Health of patient ehrlichiosis infections⁵⁷. *Ehrlichia ewingii* was indicated to be reported as a separate case ID, different than other *Ehrlichia* species, making it important to know the causative agent of the ehrlichiosis cases. The main tick vector for this disease is the lone star tick, or *A. americanum*²¹. However, *D. variabilis* and *I. scapularis* have also been found to be potential vectors, although vector competency in these species of tick have yet to be established²¹.

Ehrlichiosis and anaplasmosis are similar tick-borne illnesses in several ways. They can both cause flu-like symptoms, including muscle aches, headache, and fever⁵³. The signs and symptoms of ehrlichiosis and anaplasmosis are generally the same, however they can be more severe in ehrlichiosis⁵⁸. *Ehrlichia* primarily infect peripheral blood leukocytes, with *E. chaffeensis* affecting human monocytic cells, and *E. ewingii* affecting neutrophils⁶.

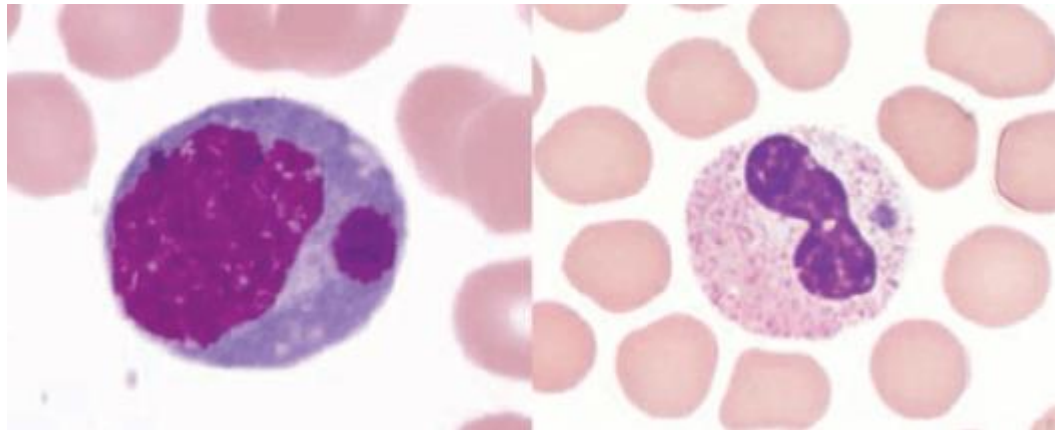


Figure 7. Wright stain of peripheral blood smears showing an intramonocytic morula associated with *Ehrlichia chaffeensis* infection (left) and an intragranulocytic morula (right), associated with *Ehrlichia ewingii*. Image retrieved from: https://www.cdc.gov/mmwr/volumes/65/rr/rr6502a1.htm#F1_down

1.4 Current Tick Pathogen Testing

Given the high prevalence of Lyme disease and other tick-borne diseases in Delaware, it is necessary to survey the distribution of tick species, as well as the inherent pathogens they may carry. Molecular tests for identifying the presence of tick-borne pathogens are not new. Molecular tests based on Polymerase Chain Reaction (PCR) have been around for decades and have provided valuable information that has been critical to identifying the prevalence of tick-borne pathogens as well as identifying reservoirs and hosts^{34,59–63}. For example, one of the first PCR tests that was published to identify *B. burgdorferi* was in 1996⁶⁴. Since then, studies have also used nested PCR to search for *B. burgdorferi*, *E. chaffeensis*, and *A. phagocytophilum*⁵⁴. Real-time PCR

probe-based assays intended to identify *B. burgdorferi*, *A. phagocytophilum* and *Ba. microti*³⁴ have also been developed, as well as assays to test and differentiate *Ehrlichia* species^{36,59,65}.

1.5 Discussion

Although some methods exist for tick pathogen testing, there exists a gap not only in the knowledge of the prevalence of pathogen-infected ticks, but also in current, high-throughput, affordable applications for the genetic detection of tick-borne pathogens posing the biggest threat to Delaware. There have been few and limited studies in Delaware involving tick pathogen testing. Although Delaware is one of a few states with a dedicated, tick biologist, much of that work involves the collecting and surveillance of tick species, which generally does not involve tick-borne pathogen testing. Residents of Delaware have submitted ticks for human pathogen testing in the past, but the number of ticks tested before this study was relatively insignificant. Between 1998 and 2019, only 430 ticks from Delaware were submitted to the Army Defense Center for Public Health's Tick-Borne Disease Laboratory (TBDL) in Aberdeen Proving Grounds, Maryland for pathogen testing²². In 2018, 258 *Ixodes scapularis* nymph ticks from New Castle County, Delaware were tested for *Borrelia burgdorferi*, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, and *Babesia microti*⁶⁶. In 2019, 119 *Amblyomma maculatum* Koch ticks collected from Delaware were tested for *Rickettsia parkeri*⁶³. However, as of the date of this study, there are no published,

holistic studies of the prevalence of *B. burgdorferi*, *Ba. microti*, *A. phagocytophilum*, *E. chaffeensis* and *E. ewingii* across Delaware.

In addition, there is currently a lack of current, efficient, and affordable methods for high throughput screening of these five tick-borne pathogens in Delaware. Four out of the top five tick-borne disease by incidence in Delaware are: anaplasmosis, babesiosis, ehrlichiosis, and Lyme disease^{6,18,67}. Although PCR targets do exist for each of these diseases, there are not readily available, current, affordable, real-time multiplex PCR assays to test for these pathogens. In addition, this study aimed to create a suite of tests for each species of tick using the most up to date genetic information available, as well as see if the genetic loci of the respective tick-borne pathogens are compatible with QSY™ probes. Some technologies do exist for multi-target pathogen testing, but are generally proprietary, expensive, outdated, and/or not designed for high-throughput sampling. Success of this study could provide the tools to affordably test large numbers of ticks for select pathogen infections.

Chapter 2

DESIGN AND TESTING OF NOVEL TAQMAN™ QSY PROBES FOR THE DETECTION OF PATHOGEN TARGETS

2.1 Introduction

The use of conventional PCR with a single target has dominated the molecular field since the 1990s⁶⁸. Conventional PCR testing typically involves about a two-hour PCR protocol, followed by an agarose gel verification. However, conventional PCR and gel verification do not provide specific evidence that the target in question is the actual amplicon apparent on a gel. Advances in molecular technologies such as quantitative PCR (qPCR) have led to decreases in cost per sample and time efficiency by eliminating the need for running gels. With the ever-increasing number of genetic sequence data available worldwide, development of qPCR methods, as well as efficient multiplex Taqman™ PCR probe-based assays designed for screening of tick-borne pathogens of specific tick species (multiplex – a multi target assay vs singleplex – a single target assay) is of the utmost importance. Design of this tick species-specific pathogen suite, using the latest genetic information, and Taqman™ probe-based technology, will be useful for efficient tick-borne pathogen screening efforts in Delaware. Design of a tick species-specific pathogen suite is especially important for

use by stakeholders with limited resources/technical abilities, such as DNREC. There are advantages to designing multiplex Taqman[®] PCR assays in lieu of the many singleplex assays testing for these pathogens. One major advantage over conventional PCR testing is specificity and efficiency.

Real-time PCR applications, such as qPCR and Taqman[™] technology, differ from conventional PCR in that they both use a fluorescent dye. A common fluorescent dye for real-time applications is SybrGreen[®] technology. SybrGreen[®] is a cyanine dye that has a high affinity for binding to double stranded DNA⁶⁹. This makes it a good tool to reflect the direct amplification DNA in a sample by the production of a sigmoidal curve, relevant to the amount of new double-stranded DNA being produced in a conventional PCR reaction. In addition, real-time cyanine dye technology is much faster than conventional PCR protocols and does not require the use of an agarose gel for verification, as the amplification signal is being produced in “real-time”. However, a major drawback of Sybr dyes is that they can create false positive signals. This is because the Sybr dyes bind to any double-stranded DNA, including any nonspecific double-stranded DNA sequences that may not be the intended target sequence⁷⁰.

Real-time probe-based multiplex assays such as those that use Taqman[®] probes provide speed, specificity, and sensitivity beyond that of conventional PCR technology^{71,72}. TaqMan[™] probes rely on the action of hydrolysis and are designed to increase the specificity of real-time PCR. This is the reason many TaqMan[™] probe-based assays are widely used in quantitative real-time PCR (qPCR) in research and medicine⁷³. Quantitative real-time PCR (qPCR) can not only detect specific nucleic acid

sequences in a sample, but more importantly it can measure the quantity of nucleic acid copies by comparing the sample to a reference gene⁷⁴. This study does not involve the use of qPCR, as the methods for this study were developed to detect only the presence or absence of the specific nucleic acid sequences of the tick-borne pathogens.

Taqman™ probes are oligonucleotide probes designed to anneal to target DNA within the area of standard primers, but not overlapping with any primer sequence nucleotides⁷². Along with target-complementary single stranded DNA, they consist of a 5' fluorophore and a 3' quencher molecule. The quencher “quenches” the fluorescent emission of the fluorophore via Förster resonance energy transfer (FRET)⁷⁵. As the Taq DNA polymerase extends the new strand of DNA during the PCR extension phase, the exonuclease activity of the DNA polymerase breaks the probe that has annealed to the target DNA. The destruction of the probe separates the fluorophore from the quencher and allows fluorescence of the fluorophore.

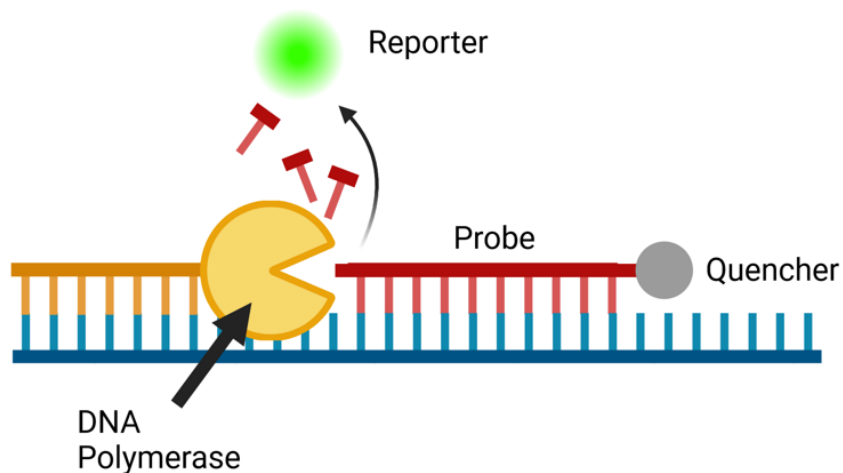


Figure 8. Image of the hydrolysis process of Taqman probes by exonuclease activity of DNA polymerase. Image created using BioRender™ software.

This fluorescence is directly proportional to the number of probes being degraded by polymerases and hence, is directly proportional to the exponential rate of DNA replication in the PCR process. Simply put, the final PCR sigmoidal curve of a positive real-time Taqman Probe assay reflects the increased fluorescence of cleaved probes- a valid sign of DNA polymerase activity at the exact location of the target of interest.

There are a few different types of quenchers available. Some quenchers can increase the melting temperature of your probe, where some do not. Others can have high fluorescence, weak fluorescence, or be nonfluorescent. The choice of which one to use with a particular assay depends on the type of assay being designed. A common type of quencher to use for flexibility and specificity are Minor Groove Binder (MGB) quenchers. They have the advantage of using shorter probes and therefore increased

specificity. However, they are not ideal (nor recommended) in multiplex assays, especially with more than two targets in one sample test⁷⁶. A recommended quencher for Taqman™ probes used with multiplexing are QSY™ quenchers. QSY quenchers are recommended for multiplexing because they come in a variety of dyes that support multiplexing. Because they are also nonfluorescent quenchers, real-time PCR instruments can measure the reported dyes more precisely⁷⁶.

One drawback of QSY probes is that they normally require a longer sequence than other probe types such as MGB. This is due in part to the general requirement of QSY probe melting temperature of 70°C. In addition, the primers used in Taqman QSY real-time PCR require primer sets to have a melting temperature, at, or close to 60°C. These relatively stringent parameters sometimes make primer and probe development difficult. However, if sequences are found that are compatible, then specificity and sensitivity of these assays has been proven to be very effective for identification of intended target sequences⁷⁶.

To begin the process of designing multiplex real-time PCR assays to identify tick-borne pathogens relevant to the ticks of Delaware, it was necessary to look at existing multiplex assays in the literature, as well as in practice³⁴⁻³⁸. There are many published, single target real-time PCR tests available from various sources. However, to test for more than one target, the notion of testing each target in singleplex would be time consuming and significantly more expensive than multiplexing, as separate assays would have to be run independently on each target. In addition, multiplexing is more efficient and cheaper than a singleplex in that multiplexing allows higher throughput by

allowing more targets to be tested per singular sample which uses less reagents than multiple singleplexes. Multiplexing also is much quicker than performing multiple singleplex reactions.

There are also published multiplex tests available for some of the same pathogens relevant to this study^{34,77-79}. Some of the tests are outdated, expensive, and/or do not disclose their target primers and probes. For example, one commonly used Multiplex real-time probe-based assay to test for *Borrelia burgdorferi*, *Babesia microti*, *Anaplasma phagocytophilum* in the *Ixodes scapularis* tick was created in 2014, when less genetic information was known about these pathogens than today³⁴. Analysis of current bioinformatic data through National Center for Biotechnology Information (NCBI) shows more robust genetic data available that could improve on the performance and specificity of these older tests. In terms of cost and transparency of other tick pathogen testing methods, the company TickReport® charges consumers anywhere from \$50 to \$200 to test one tick for myriad pathogens⁸⁰. Furthermore, they do not offer high throughput testing at a less expensive rate. Like many companies of this type, they also do not release target sequences for any of their assays to the public.

In summary, multiplex Taqman QSY assays were developed for each relevant tick pathogen to be tested in Delaware. These assays were designed for pathogens relevant to Delaware ticks and are not only suited for small scale testing, but also can be applied to high throughput testing as well. The notion of creating singular multiplex assays for *I. scapularis*, *D. variabilis*, and *A. americanum* to test for the presence of relevant pathogens has the advantage of efficiency, lower cost, and high throughput

capabilities. This is important to help identify the prevalence of *B. burgdorferi*, *Ba. microti*, *A. phagocytophilum*, *E. chaffeensis*, and *E. ewingii* in ticks of Delaware.

2.2 Materials and Methods

2.2.1 *In Silico* Design of Prospective Primer and Probe Sequences

The goal of this chapter was to develop primer and probe sequences and to test whether the primer sequences correctly amplified only the intended target DNA for which they were designed. Therefore, *in silico* testing was first performed to design potential suitable primers and probe sequences for the multiplex targets. *In silico* design testing was performed using the NCBI BLAST database, Primer Express 3.0.1 (ThermoFisher, Waltham, MA), and Snapgene[®] (GSL Biotech LLC, Chicago, IL). The gene sequences for each target as well as relevant homologs were uploaded to Snapgene[®] software and submitted to a multiple DNA sequence MUSCLE alignment. The results of the alignment were used to verify unique and specific regions of the target sequences. After verification of such, the target DNA sequences were uploaded to Primer Express 3.0.1, a software designed to test sequences for use with QSY probes⁸¹.

The design of the prospective *in silico* primer and probe combinations for each individual pathogen focused on identifying current or novel genetic loci to employ as potential targets for real-time PCR multiplexing using QSY probes. The targets for primer and probe sequences applied to the following tick-borne pathogens: *Anaplasma phagocytophilum*, *Babesia microti*, and *Borrelia burgdorferi*, to be tested on *I.*

scapularis ticks, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* to be tested on *D. variabilis* ticks, and *Ehrlichia chaffeensis* and *Ehrlichia ewingii* to be tested on *A. americanum* ticks.

The target loci for *B. burgdorferi* was the Tryptophan-tRNA Ligase protein (GenBank: ARS32776.1) of the *B. burgdorferi* strain B31_NRZ genome (CP019767.1). This gene is of the Amino acyl tRNA synthetase family, also identified as gene BB005 of *B. burgdorferi*. It was identified as a potential target not only because of its specificity to *B. burgdorferi*, but also because of its unique region with little similarity to other species in terms of primer and probe loci based on sequence alignment. To our knowledge, this is the first study to use the location of the tryptophan--tRNA ligase protein of *B. burgdorferi* for genetic identification.

The target for *Ba. microti* was based on many literature sources showing the *I8s* rRNA loci (Genbank ID: M93660.1) as a plausible location for species identification of this organism^{34,62,82}. The *I8s* rRNA variable region has shown to contain several conserved sequences specific to *Ba. microti*. The conserved regions, as well as the commonality of this gene loci for identification of the species conveyed its potential to be a useful as a target for real-time PCR with Taqman QSY probes.

The target for *A. phagocytophilum* was the major surface protein 4 gene (*mSP4*) (GenBank ID: JQ522935.1). This gene has been shown to be an important part of the interactions with host proteins during infection⁸³. Among the unique genes used to detect and identify *A. phagocytophilum*, *mSP4* is one of the most common belonging to the OMP-1/MSP-2/P44 superfamily, characteristic of Anaplasmataceae^{60,84} and the

sequence of *msp4* has also been shown to be compatible with probed-based, real-time PCR⁸³.

Because of the emergence and increase of ehrlichiosis cases in the United States, as well as in Delaware⁶, new multiplex targets for *Ehrlichia chaffeensis* and *Ehrlichia ewingii* were also designed for this study. The target for *Ehrlichia chaffeensis* is the 120 kDa outer membrane protein (*TRP120*) gene (Genbank ID:AF474899.1). This protein of *Ehrlichia chaffeensis* is exposed on the surface of whole ehrlichial cells⁸⁵. The gene for this protein has been used as a target in several previous studies using standard and real-time PCR assays⁸⁶⁻⁸⁸.

The other species of *Ehrlichia* selected for the real-time multiplex assays was *Ehrlichia ewingii*. The target gene chosen for this assay development was the 28-kDa major antigenic protein, *p28* (Genbank ID: AF287961.1). The *p28* gene family represents a series of 21 homologous genes, also found in the *E. chaffeensis* genome⁸⁹. The *p28* gene is homologous to the major antigenic proteins of *E. chaffeensis* (*p28*) and the *E. canis* immunodominant major outer membrane protein *p30*. However, there are unique regions of the *E. ewingii p28* homologue were identified to design primers and probes for this study⁹⁰.

The Primer Express 3.0.1 software was used in part for QSY primer and probe development. This software uses submitted DNA sequences to determine if any loci fit the criteria required for the creation of possible primer and probe sequences. For this assay development, Primer Express 3.0.1 software criteria were used that satisfy the conditions for QSY probe development. These include, but are not limited to, primer

melting temperatures between 58°C-60°C, probe melting temperatures between 68°C-70°C, proper GC content, analysis of secondary structure such as dimerization, and correct primer, probe, and amplicon length. The software accounted for penalty scores that include, but are not limited to, cross-dimerization, hairpins, length abnormalities, and less than ideal GC percentages of the proposed oligos. Primer and probe sequences were chosen for downstream use in this study were based in part, on the penalty score of the primer and probe sets.

2.2.2 Conventional PCR of Prospective Primer and Probe Sequences

The exogenous controls were genomic DNA (gDNA) for each target organism (ATCC, BEI Resources, CDC) and cloned plasmids. The endogenous controls consisted of direct gDNA from pathogen-negative ticks spiked with gDNA (ATCC, BEI Resources, CDC) from each pathogen. Primers and probes for each target were purchased and conventional PCR was performed using exogenous and endogenous positive controls with the primer pairs for each target. The purpose of testing the proposed oligos on the pathogen gDNA as well as gDNA from pathogen-negative ticks spiked with gDNA from each pathogen was to ensure that the oligos did not amplify any unintended targets in either the tick gDNA or the inherent microbiome of the ticks. Conventional PCR was performed on all endogenous and exogenous controls in 50µl reactions using 25µL of Promega GoTaq Green Master Mix[®], .2µM final concentration of each forward and reverse primer, 1µL of pathogen or tick gDNA controls,

normalized to 2ng/ μ L, and sterile nuclease-free water to 50 μ L. In addition, each target pathogen gDNA was tested not only with its intended primer sequences, but also with the primer sequences of the other four targets to test for cross reactivity. The Applied Biosystems MiniAmp Thermal Cycler (Thermo Scientific, Waltham, MA) was used for all standard PCR experiments. The Promega GoTaq Green Master Mix[®] thermal cycler protocol for these reactions was an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30seconds, 60°C for 30 seconds, and 72°C for 1 minute. Finally, a concluding extension of 72°C for 5 minutes was performed. The 60°C annealing temperature was used based on the stringent development of the primers to function best with this temperature. After PCR, 10 μ L of each 50 μ L sample was run on a 1% agarose gel with 1% sodium borate buffer and SybrSafe DNA stain (Thermofisher, Waltham, MA) for 13 minutes at 290V.

2.2.3 Confirmation of Amplification Via Sanger Sequencing

Upon verification of a target amplicon via gel electrophoresis, the remaining 40 μ L of the conventional PCR sample was cleaned the using the ReliaPrep[™] DNA Clean-Up and Concentration System (Promega, Madison, WI) to prepare for sequencing. The cleaned PCR amplicons were analyzed for concentration and purity using the Nanodrop One UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA).

The Sanger sequencing PCR procedure was followed using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Thermo Scientific, Waltham, MA). Cleaned DNA amplicons were normalized to 1ng/μL per the recommendations of the BigDye™ Terminator v1.1 Cycle Sequencing Kit. Cycle reactions were prepared using the Diluted reaction (0.5X) protocol in the BigDye™ Terminator v1.1 Cycle Sequencing Kit manual. Each 20μL Reaction was prepared with 4μL of BigDye™ Terminator v1.1 Ready Reaction Mix, 2μL of BigDye™ Terminator v1.1 5X Sequencing Buffer, 3.2pmol (final concentration) of the respective primer, 2μL of 1ng/μL of DNA template and the remaining volume of nuclease-free sterile deionized water to 20μL. Forward primer reactions and reverse primer reactions were set up individually in triplicate for each DNA template. The cycle sequencing protocol for each sample was an initial incubation of 96°C, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Once completed, the samples were purified using the BigDye XTerminator™ Purification Kit (Thermo Scientific, Waltham, MA). To each sample well, 90μL of SAM solution and 20μL of XTerminator™ Solution are added, and the samples were subjected to 30 minutes of shaking at 1800 rotations per minute (RPM).

All Sanger sequencing was performed on the SeqStudio Genetic Analyzer (Thermofisher, Waltham, MA). The plate manager setting for the SeqStudio Genetic Analyzer required specifying the use of the BigDye™ Terminator v1.1 Cycle Sequencing Kit (versus the 3.1 kit) as well as selecting Short BDX, which informs the genetic analyzer that the DNA template is relatively short. After genetic analysis, the SeqStudio Genetic Analyzer software displays the results of each sample as a raw .ab1

file. The .ab1 files were uploaded to Snapgene[®] software to assess the quality of the electropherogram data and to assemble contigs when necessary. Subsequent sequence data was uploaded to NCBI BLAST to verify the sequence of the conventional PCR amplicon.

2.2.4 Creation of Positive Control Plasmids

After all PCR amplicon sequences were verified via Sanger sequencing, positive control plasmids were created for downstream determination the limit of detection (LOD). Conventional PCR amplicons for each target were cloned into pGEM-T Easy Vector System I (Promega, Madison, WI) using the manufacturer's instructions for PCR ligation. After ligation, the plasmids were transformed into JM109 competent *E. coli* cells plated on LB/amp/IPTG/X-gal plates and incubated at 37°C overnight. Since the multiple cloning site for the pGEM-T plasmids is located in the LacZ gene of the plasmid, blue-white screening of colonies was used to identify potentially successful ligations by choosing white colonies for processing. Prospective cloned, white colonies were grown up in LB/AMP broth cultures overnight at 37°C. The following day, plasmids were isolated using the PureYield[™] Plasmid miniprep System (Promega, Madison, WI). Quantity and quality of plasmids were determined using the Nanodrop One UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). The plasmids were then sent to the Plasmidsaurus[™] company for full plasmid sequencing to ensure proper ligation of the target ligations. The plasmids were then subjected to conventional

PCR with corresponding primers and probes to ensure amplification of the target sequences.

2.3 Results

Figure 9 below shows the conventional PCR agarose gel of all 5 pathogen targets with the novel primers used in this study. All amplicons were visible and located at expected positions in the gel. The following were the expected amplicon lengths of each of the five pathogen targets: *B. burgdorferi*- 99 base pairs, *Ba. microti*- 75 base pairs, *A. phagocytophilum*- 71 base pairs, *E. chaffeensis*- 125 base pairs and *E. ewingii*- 85 base pairs. In addition, there was no unexpected amplification of any other DNA from the pathogen gDNA sources.

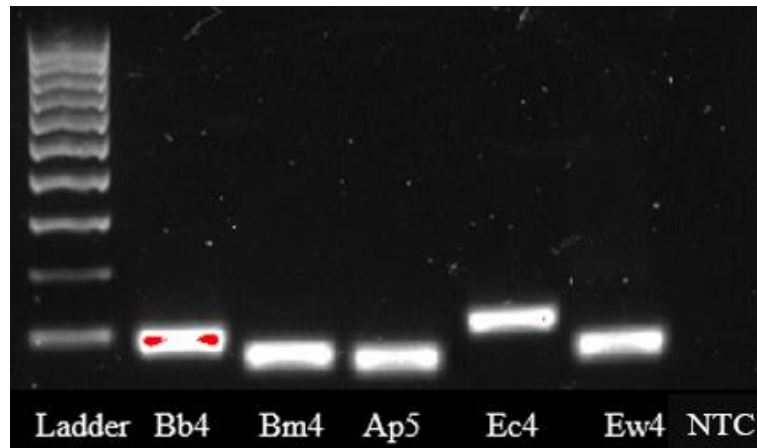


Figure 9. This image represents the conventional PCR results of the successful pathogen targets using pathogen gDNA as the template. Ladder: 100-1000 base pairs. Bb4= *Borrelia burgdorferi* Bm4=*Babesia microti* Ap5=*Anaplasma phagocytophilum* Ec4=*Ehrlichia chaffeensis* Ew4=*Ehrlichia ewingii* NTC= No Template Control

Table 2 below displays the following results of the final primer and probe sequences for the five targets. The Organism column of Table 2 provides the name of the pathogen as well as the length of the real-time target DNA sequence. The Loci column is the strain or gene name of the target for each pathogen. The Accession number column refers to the unique identifier that each GenBank submission is given in such databases as those in NCBI. Next, the forward and reverse primer sequence column are indicated in the standard 5'→3' orientation. Finally, the 5'→3' probe sequence is indicated, along with the assigned 5'dye and 3'QSY quencher.

Organism	Loci	Accession #	5'→3' Forward primer Sequence	5'→3' Reverse primer Sequence	5'→3' Probe Sequence
<i>B. burgdorferi</i> (99 bp)	TRP-tRNA Ligase	CP019767.1	CCCAACGGGAACTAAATTTGC	GCAGCTGGACTTAAAGAGATTCCT	FAM-CTCATAAGAACAGGATACCCAAAAGGCCA-QSY
<i>Ba. microti</i> (75 bp)	18s rRNA	M93660.1	CGCGTGGCGTTTATTAGACTT	GCCATGCGATTTCGCTAATTT	ABY-CCAACCCTTCGGGTAATCGGTGATTC-QSY
<i>A. phagocytophilum</i> (71 bp)	msp4	JQ522935.1	CAAGAATTGAGCACGTTGAATG	CGAACTTGAATGAGGGATCATG	VIC-TTCAGATCCTGCCAGCTTCACGCA-QSY
<i>E. chaffeensis</i> (125 bp)	120 omp	AF474899.1	CGGAGTAATAC TGATGGCTTGATAGA	ACTGTGTCATCTTCTTGCTCTTGC	FAM-TTGTGCAACCACAGGATCTGGGTCTG-QSY
<i>E. ewingii</i> (85 b)	omp/p28	AF287961.1	GATGCCCATATG TATTACCTTTTG	TGAGCAAGACAGATTGTTAACTTGA	JUN-TGTTGGCGAAGAACTATCAACTTCTCGTGC-QSY

Table 2. List of primer and probe sequences used in this study for tick pathogen targets.

2.4 Discussion

The target primer and probes in Table 2 met all of the required standards of optimal multiplex real-time performance. The sequences were developed to be specific to their intended target. This was tested by comparing all primer and probe sequences

in NCBI Blast for specificity. Any subject sequences that were not 100% aligned and 100% query covered to the primer or probe sequences were not chosen for further development. When uploaded to NCBI Blast, the combined primers and probe sequence regions were in 100% subject alignment and 100% query cover with only the intended pathogen sequences.

Twenty-eight primer and probe combinations for all targets were tested over a 16-month period for this assay development. Appendix B shows the list of the primer sequences that did not pass all of the criteria for further assay development. There were a few reasons why some of the primers failed to be used for further development. One reason was a lack of strong bonding to intended targets, as indicated by repeated conventional PCR and subsequent electrophoresis agarose gel verification. For this type of assay development, it is critical that the primers convey a strong bond to their target DNA at, or very close to 60°C. Therefore, any primers that did not show strong amplification were discontinued. Another reason for discontinuing sequences was the amplification of unintended targets, again indicated by repeated conventional PCR and subsequent electrophoresis agarose gel verification. The goal for this chapter of developing primer and probe sequences that correctly amplified only the intended target DNA for which they were designed was successfully completed.

The remainder of this dissertation will focus on the development, testing, and deployment of the primer and probe sets in Table 2. At this point in the study, the primers from Table 2 were shown to pass all requirements for downstream Taqman™ QSY real-time PCR development. Finally, it is important to note that this is the first

real-time assay to use the specific target sequences from Table 2 to identify *B.*

burgdorferi, *Ba. microti*, *A. phagocytophilum*, *E. chaffeensis*, and *E. ewingii* in ticks.

Chapter 3

DESIGN AND TESTING OF MULTIPLEX REAL-TIME PCR ASSAYS FOR THE DETECTION OF *BORRELIA BURGDORFERI*, *BABESIA MICROTI*, *ANAPLASMA PHAGOCYTOPHILUM*, *EHRlichia CHAFFEENSIS*, AND *EHRlichia EWINGII* IN TICKS

3.1 Introduction

Real-time PCR assays require many tedious steps of optimization and efficiency testing to ensure a stringent and effective assay. The goal of this chapter was to successfully design and test the primers and probes from Table 2 in Taqman, multiplex real-time assays. To begin the design of these assays, it is critical to define the LOD and how it will be tested. LOD was defined as the lowest concentration of target in a sample that can be detected in repeated trials⁷⁴. LOD testing with plasmid positive controls allow the indication of a theoretical limit of detection value as the minimum concentration of nucleic acid that identifies a positive PCR result in all replicates tested⁹¹. Furthermore, multiplex assays require the testing of each target in singleplex and in multiplex to ensure consistent and reliable amplification. Finally, negative samples are usually spiked with endogenous positive target samples in both singleplex and multiplex assays to determine validity and reliability. Many of the steps in the

optimization and efficiency testing involve serial dilutions, usually in triplicate, to ensure enough data points and consistency in the results.

Before beginning testing such as LOD, the dyes for each sample were assigned to each target pathogen probe. The assignment of each dye to sequence was determined using parameters specified by ThermoFisher™, the proprietor of the QSY probes used in this study⁹². Each target was assigned a specific reporter dye. The reporter dyes have different emission wavelengths that allow the instrument to detect each dye independent of the other dyes (see Table 3). The FAM dye was used for both *B. burgdorferi* and *E. chaffeensis* because these pathogens are not found in the same tick species and will therefore never be deployed in the same multiplex assay on ticks.

Target Organism	Reporter Dye	Emission Max
<i>B. burgdorferi</i> (<i>Bb</i>)	FAM	517
<i>Ba. microti</i> (<i>Bm</i>)	ABY	580
<i>A. phagocytophilum</i> (<i>Ap</i>)	VIC	551
<i>E. chaffeensis</i> (<i>Ec</i>)	FAM	517
<i>E. ewingii</i> (<i>Ee or Ew</i>)	JUN	617
Passive Reference	Mustang Purple	654

Table 3. List of Reporter dyes, wavelength emission maximums and associated targets.

3.2 Materials and Methods

3.2.1 Determination of Primer and Probe Concentrations

For this study, Taqman™ Multiplex Master Mix (Applied Biosystems, Waltham, MA) was recommended, given its optimization for multiplexing and the use of the QSY probe chemistry in this assay development⁹². All real-time assays were performed on the 96-well Applied Biosystems QuantStudio 5™ (ThermoFisher Scientific, Waltham, MA). Before any operations were performed, the instrument was calibrated and certified by a ThermoFisher® technician. The recommended probe concentration for Taqman QSY probes is 250nM, as indicated by ThermoFisher⁹². Therefore, no probe concentration studies were necessary. To determine which primer concentrations were optimal for each assay, serial dilutions of all 5 plasmids were prepared with concentrations from 10^0 to 10^{-10} in triplicate. Each triplicate set was tested with primer concentrations of 900nM, 500nM, 250nM and 150nM. Each sample well of a 0.2ml, 96-well plate was prepared in triplicate with 10μL of Taqman Multiplex Master Mix™ (ThermoFisher, Waltham, MA), primer concentrations of 900nM, 500nM, 250nM and 150nM respectively, 1μL of plasmid DNA, and nuclease-free water to 20μL. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. This test also ensured that the probes would fluoresce correctly when in the presence of their respective targets and would elucidate the best potential primer concentrations. The lowest cycle threshold (C_T) values of any one sample dilution are considered the best, as

it represents the earliest detection⁹³. The C_T value is defined as the number of cycles of amplification that are needed for the fluorescence of a PCR product target to be detected while crossing a C_T , which is above the normal background signal (a low-level signal that is present in the assay regardless of whether target is present)⁹⁴.

3.2.2 Results

The results of the serial diluted triplicate plasmid tests of various primer concentrations in Table 4 showed that a primer concentration of 250nM appeared to amplify the normalized targets with the earliest C_T values.

[Primer]	<i>B. burgdorferi</i>	<i>Ba. microti</i>	<i>A. phagocytophilum</i>	<i>E. chaffeensis</i>	<i>E. ewingii</i>
AVG 900nM	7.71	7.85	6.66	6.78	7.96
SD 900nM	0.239	0.313	0.344	0.225	0.244
CV 900nM	3.100	3.987	5.165	3.319	3.065
AVG 500nM	7.07	7.39	5.91	6.70	7.19
SD 500nM	0.222	0.247	0.313	0.303	0.344
CV 500nM	3.140	3.342	5.296	4.522	4.784
AVG 250nM	6.97	7.13	5.58	6.73	7.12
SD 250nM	0.241	0.277	0.266	0.247	0.229
CV 250nM	3.458	3.885	4.767	3.670	3.216
AVG 150nM	7.15	7.17	6.59	6.86	7.10
SD 150nM	0.334	0.345	0.298	0.317	0.282
CV 150nM	4.671	4.812	4.522	4.621	3.972

Table 4. Chart of the average lowest C_T values (i.e. earliest amplification) for each tested primer concentration. All probe concentrations were 250nM. AVG= Average SD=Standard Deviation CV= Coefficient of Variation

The standard deviation of all samples was very low, which represented satisfactory reproducibility and showed the reactions to be accurately prepared. The primer

concentration of 500nM for *E. chaffeensis* did elicit a lower C_T value than 250nM, however, the difference was only 0.03, and the standard deviation of the 500nM primer concentration was 0.05 higher than 250nM. Also, the coefficient of variation (CV) for 250nM was 0.852 less than CV for 500nM. For *E. ewingii*, the primer concentration of 150nM also had a lower C_T than 250nM by a difference of 0.02, however, the standard deviation of 150nM was 0.053 higher than 250nM and the CV of 250nM was lower by a difference of 0.756. Therefore, the 250nM primer concentration was used for the remainder of this assay development for all targets.

3.2.3 Limit of Detection and Efficiency Using Positive Control Plasmids

Once the optimum primer concentration was determined for the assays, the positive control plasmids were serially diluted from concentrations of 10^0 to 10^{-10} in triplicate. The samples were prepared with 10 μ L of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probe, 1 μ L of plasmid DNA, and nuclease-free water to 20 μ L. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

From the results, standard curves were created for each singleplex positive control plasmid to determine the LOD and the efficiency for each target reaction. These standard curves of known template concentrations were used to establish the initial starting amount of the target template in experimental samples⁷⁶. The auto baseline and

auto threshold settings were used by the QuantStudio 5™ to determine the C_T value for the amplification curves. The log of each concentration of the dilutions is plotted against their C_T values. The highest C_T value for a serial diluted plasmid represents the lowest concentration of amplified positive plasmid. To determine the LOD of the target, the concentration in ng/ μ L of the sample dilution with the lowest concentration of positive plasmid is used with the known mass of the plasmid to convert to number of molecules. This provides a low-end limit of template detection for each target⁹⁵. The efficiency was determined by taking the standard curve and applying it to two mathematical formulas. The first is the standard formula $E = -1 + 10^{(-1/\text{slope})}$ for efficiency³⁵. For example, a slope of -3.322 means that the reaction has an efficiency of 100% and that the PCR product is doubling every cycle as expected⁹⁶. Acceptable efficiency percentages for real-time PCR assays are between 90% and 110% in relation to the slope⁹⁷.

The second test of efficiency is determining the R^2 value of each standard curve for each of the three triplicate serial dilutions. In PCR and qPCR efficiency, R^2 refers to the strength of the linear fit of serial diluted samples in a series of reactions⁹⁸. The R^2 value of the curve is influenced by the precision of replicate standard material C_T measurements. All R^2 values for acceptable efficiency of standard curves are from 0.98 to 1.00⁹³. Lower R^2 values indicate possible pipetting errors, standard dilution preparation errors, or instrument variation⁹⁹. These values are shown in Table 5 below.

Plasmid Target Pathogen	Average Highest Positive C _T Value	SD of Highest Positive C _T Value	LOD in # of molecules	Efficiency	Average R ² of triplicates
<i>B. burgdorferi</i>	35.95	0.219	4.73	104.16%	0.9947
<i>Ba. microti</i>	35.519	0.198	7.46	98.03%	0.9938
<i>A. phagocytophilum</i>	35.515	0.498	5.88	105.07%	0.9957
<i>E. chaffeensis</i>	35.337	0.378	2.41	109.82%	0.9923
<i>E. ewingii</i>	36.47	0.045	6.09	101.40%	0.9994

Table 5. Positive control plasmid data from 10-fold serial dilutions in triplicate using 250nM primer and probe concentrations. SD= Standard Deviation

3.2.4 Efficiency Testing of Singleplex Versus Multiplex Assays

The real-time PCR protocol required testing singleplex reactions versus the multiplex reactions of all targets to ensure their consistency, specificity, sensitivity, and efficiency. Table 6 displays the multiplex assays that were designed for each of the three species of species of tick relevant to this study. Multiplex ISC1 was developed for *I. scapularis*, DVA1 was developed for *D. variabilis*, and AAM1 was developed for *A. americanum*.

Tick species	Multiplex Assignment	Pathogen Organism
<i>I. scapularis</i>	ISC1	<i>B. burgdorferi</i> <i>Ba. microti</i> <i>A. phagocytophilum</i>
<i>D. variabilis</i>	DVA1	<i>A. phagocytophilum</i> <i>E. chaffeensis</i>
<i>A. americanum</i>	AAM1	<i>E. chaffeensis</i> <i>E. ewingii</i>

Table 6. Pathogen assignments for each of the three multiplexes in relation to tick species.

To evaluate the results of the three multiplex assays, the reaction efficiency of each multiplex assay was determined by testing each target in singleplex, then also in multiplex against all four other pathogen primers and probes. The reason for testing each singleplex against all five-target primer and probe sets was to ensure efficiency of all primers and probes in the presence of each other. If each multiplex reaction for all five targets works the same as the singleplex, then any combination of mixing any singleplex targets to create a new multiplex from the five targets would be equally effective. The singleplex testing involved creating serial diluted pathogen gDNA from concentrations of 10^0 to 10^{-10} in triplicate. The samples were prepared with 10 μ L of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probe for only the intended singleplex pathogen target, 1 μ L of pathogen gDNA, and nuclease-free water to 20 μ L. The real-time PCR protocol was set to an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

Unlike the singleplex efficiency testing, the multiplex efficiency testing involved testing the target gDNA under several conditions. The first condition was to test the pathogen gDNA of a given target in the presence of not only its intended primers and probe, but also the other primers and probes relevant to each multiplex assay. For example, to test for the efficiency of the *B. burgdorferi* target in ISC1, *B. burgdorferi* target gDNA would be tested in the presence of not only *B. burgdorferi* primers and probe, but also in the same sample well with the primers and probes for *Ba. microti* and *A. phagocytophilum*. These samples were prepared with 10 μ L of Taqman

Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 1μL of pathogen gDNA specific to only one pathogen, and nuclease-free water to 20μL. Ten-fold dilutions were made in triplicate. This ensured that the range of the standard curve was broad enough to cover most of the experimental samples¹⁰⁰. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

The second condition was to test for each pathogen gDNA in the presence of tick gDNA. A sample of pathogen-negative gDNA from each species of tick was spiked with 1μL normalized to 2ng/μL of relevant pathogen gDNA and tested in each multiplex assay. The purpose of this was to ensure that the target primers and probes were not affected by, or bound to, any unintentional DNA loci. This is a critical step to perform, as ticks can have many thousands of different species in their microbial biomes¹⁰¹.

The spiked samples were prepared with 10μL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 1μL of pathogen-negative gDNA from a 100μL sample of gDNA spiked with 2μL of pathogen gDNA, and nuclease-free water to 20μL. Ten-fold dilutions were again made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

The relative standard curve method and the comparative C_T method were then used to analyze data for the efficiency of the multiplex assay by comparing the singleplex assay C_T values for each pathogen to the multiplex assay C_T values for the same pathogen. Ideally, there should not be a significant difference between the resulting C_T values from the singleplex and the multiplex reactions³⁵. Once all the dilutions have been plotted on a standard curve, the R^2 values were determined for each sample. Finally, an independent t-test, with a significance level of 0.05 was used to compare the mean C_T values of each singleplex and multiplex trial. For example, if the singleplex and multiplex reactions do not result in similar C_T values, then further optimization of the primer and probe concentrations would need to be performed. Upon correct amplification, a further parameter of an amplification score above 1.2 must also be met for verified positive result, as required by the ThermoFisher Connect™ software, utilized by the Quantstudio™ 5 Real-Time PCR system¹⁰².

3.3 Results

The results of the singleplex versus multiplex testing showed that the primers and probes that were designed specifically for each target only amplified the intended targets from intended gDNA sources, and non-source primer and probes did not amplify any gDNA sequences or affect the efficiency of the reactions. Tables 7-11 showed that the specific primer and probe sequences for each target were not only successful, but also that no loss of efficiency occurred when in the presence of the other four primer

and probe targets. Probes were purchased for the *E. chaffeensis* targets with versions using both FAM and JUN dyes. The JUN dye version of *E. chaffeensis* was used to test the entire 5 series assay, ensuring that *B. burgdorferi* was the only FAM dye in the initial test. *E. chaffeensis* was returned to FAM dye for all other testing.

All statistical formulas and tools for this study were performed using IBM® SPSS Statistics, version 29.0 (Chicago, IL). The R² values for all 5 singleplex versus multiplex trials fell well within the acceptable range of 0.98 to 1.0, with most well above 0.990. This indicates very strong positive linear correlation. In terms of the results of the independent t-test, all samples showed very high p values, which indicated that the singleplex trials and the multiplex trials for each target were not statistically significant from one other (table 7-11). This is designed to show that there is no efficiency loss or interference from non-target primer and probe oligos in the reaction. Simply put, the results should be very similar regardless of whether the reaction for one target occurs in singleplex or in multiplex with the other primers and probes.

<i>B. burgdorferi</i> Singleplex with primers and probes for <i>B. burgdorferi</i>			<i>B. burgdorferi</i> Multiplex with primers and probes for <i>B. burgdorferi</i>, <i>Ba. microti</i>, <i>A. phagocytophilum</i>, <i>E. chaffeensis</i>, and <i>E. ewingii</i>		
Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
13.060	14.162	15.128	14.400	14.592	15.125
17.948	17.740	17.860	17.843	17.828	17.813
21.283	21.174	21.359	21.844	21.488	21.454
25.230	24.937	24.959	25.334	24.934	25.062
28.810	28.507	28.330	28.906	28.827	28.638
31.938	32.330	31.858	32.209	32.618	32.717
R ² = 0.9959	R ² = 0.9998	R ² = 0.9986	R ² = 0.9994	R ² = 0.9992	R ² = 0.9971
The t-value is -0.13269. The p-value is .895225. The result is not significant at p < .05. Degrees of freedom:34					

Table 7. Table of *B. burgdorferi* singleplex versus multiplex results tested in triplicate.

<i>Ba. microti</i> Singleplex with primers and probes for <i>Ba. microti</i>			<i>Ba. microti</i> Multiplex with primers and probes for <i>B. burgdorferi</i>, <i>Ba. microti</i>, <i>A. phagocytophilum</i>, <i>E. chaffeensis</i>, and <i>E. ewingii</i>		
Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
18.235	18.041	18.181	18.439	18.807	18.887
20.681	20.323	20.234	20.605	20.775	20.701
24.093	23.774	23.928	23.965	24.555	24.306
27.579	27.333	27.226	27.725	28.165	27.745
30.983	30.631	30.661	31.067	31.202	31.304
33.459	33.544	34.864	34.452	35.347	35.070
R ² = 0.9968	R ² = 0.997	R ² = 0.9928	R ² = 0.9954	R ² = 0.9932	R ² = 0.9921
The t-value is -0.26898. The p-value is .789574. The result is not significant at p < .05. Degrees of freedom:34					

Table 8. Table of *Ba. microti* singleplex versus multiplex results tested in triplicate.

<i>A. phagocytophilum</i> Singleplex with primers and probes for <i>A. phagocytophilum</i>			<i>A. phagocytophilum</i> Multiplex with primers and probes for <i>B. burgdorferi</i> , <i>Ba. microti</i> , <i>A. phagocytophilum</i> , <i>E. chaffeensis</i> , and <i>E. ewingii</i>		
Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
19.102	19.729	20.108	20.297	20.249	20.297
25.724	25.084	24.874	25.338	24.974	25.338
29.084	29.647	29.041	29.490	29.360	29.490
32.773	32.735	32.872	32.441	32.636	32.441
36.163	37.372	35.547	36.239	37.653	36.239
R ² = 0.981	R ² = 0.9927	R ² = 0.9901	R ² = 0.9912	R ² = 0.9965	R ² = 0.9912

The t-value is -0.08015. The p-value is .936686. The result is not significant at p < .05.
Degrees of freedom:28

Table 9. Table of *A. phagocytophilum* singleplex versus multiplex results tested in triplicate.

<i>E. chaffeensis</i> Singleplex with primers and probes for <i>E. chaffeensis</i>			<i>E. chaffeensis</i> Multiplex with primers and probes for <i>B. burgdorferi</i> , <i>Ba. microti</i> , <i>A. phagocytophilum</i> , <i>E. chaffeensis</i> , and <i>E. ewingii</i>		
Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
25.901	25.967	25.226	26.480	26.199	25.872
30.457	29.870	30.104	30.538	30.655	30.444
34.007	33.801	33.574	33.863	33.513	34.373
R ² = 0.9949	R ² = 1	R ² = 0.9906	R ² = 0.9967	R ² = 0.9843	R ² = 0.9981

The t-value is -0.20721. The p-value is .838463. The result is not significant at p < .05.
Degrees of freedom:16

Table 10. Table of *E. chaffeensis* singleplex versus multiplex results tested in triplicate.

<i>E. ewingii</i> Singleplex with primers and probes for <i>E. ewingii</i>			<i>E. ewingii</i> Multiplex with primers and probes for <i>B. burgdorferi</i> , <i>Ba. microti</i> , <i>A. phagocytophilum</i> , <i>E. chaffeensis</i> , and <i>E. ewingii</i>		
Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
24.805	24.711	24.803	25.292	25.329	25.742
28.124	27.902	27.856	28.162	28.535	28.407
31.518	31.654	31.366	31.749	31.624	31.880
34.953	35.673	34.539	36.924	36.941	36.441
R ² = 0.9999	R ² = 0.9974	R ² = 0.9993	R ² = 0.9819	R ² = 0.981	R ² = 0.9859
The t-value is -0.44147. The p-value is .663182. The result is not significant at p < .05. Degrees of freedom:22					

Table 11. Table of *E. ewingii* singleplex versus multiplex results tested in triplicate.

Table 12 below summarizes the relationship between each multiplex assay and their reliability to identify the intended pathogen targets. The primer and probe combinations in this study were shown to only amplify the intended pathogen target without any interference or cross-reactivity with unintended targets. This is critical to the development and optimization of any multiplex assay.

Multiplex	Pathogen Target	Primer and Probe Combination				
		<i>B. burgdorferi</i>	<i>Ba. microti</i>	<i>A. phagocytophilum</i>	<i>E. chaffeensis</i>	<i>E. ewingii</i>
ISC1	<i>B. burgdorferi</i>	+	-	-	-	-
	<i>Ba. microti</i>	-	+	-	-	-
	<i>A. phagocytophilum</i>	-	-	+	-	-
DVA1	<i>A. phagocytophilum</i>	-	-	+	-	-
AAM1	<i>E. chaffeensis</i>	-	-	-	+	-
	<i>E. chaffeensis</i>	-	-	-	+	-
	<i>E. ewingii</i>	-	-	-	-	+

Table 12. Results of singleplex versus multiplex testing of pathogen gDNA with all primers and probes per each multiplex assay. (+ means positive sample and – means negative sample)

Tables 13-23 display the real-time Multiplex PCR data. On each table, the column named SAMPLE indicated the type of pathogen gDNA that was present in the corresponding well. The column named TARGET indicates the primer/probe added to the well. The column named DYES represents the specific dye used for each probe. The column named Cq is representative of the C_T, or the cycle where the signal crosses the threshold as a positive signal. The column named Cq CONF indicates the reliability of the Cq/C_T value in the context of the curve itself and not in the relationship to other curves¹⁰³. The column called AMP SCORE is the amplification score value, where an amp score above 1.2 is one indication of a positive amplification. Amp scores below 1.2 generally indicate inconclusive, or late-stage partial fluorescence. It is normal for probes to sometimes degrade a small amount, even with a negative sample, which can be recorded as inconclusive. This is caused by the repeated heating a cooling of dozens of cycles. The probes can sometimes indicate initial fluorescence, but they are apparent very late in the number of cycles, and not in the standard sigmoidal curve. The instrument sees the fluorescence and can differentiate it from noise, as well as a positive signal. This is indicated by the final computation of the instrument as: Amp, No Amp, or Inconclusive on the last column called AMP STATUS. Amp means a positive sample, no amp means a negative sample and inconclusive means the machine cannot tell and other factors must be considered for verification, such as C_T score, Amp Score, and the actual linear curve. In addition, Tables 13-23, utilize the following sample and target symbols: Bb=*Borrelia burgdorferi*, Bm=*Babesia microti*, Ap=*Anaplasma phagocytophilum*, Ec=*Ehrlichia chaffeensis*, and Ee or Ew=*Ehrlichia ewingii*.

Tables 13-15 display the results from three trials of pathogen-negative *I. scapularis* tick gDNA that was spiked with 1µL of pathogen DNA. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of tick gDNA spiked with indicated pathogen gDNA, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single pathogen gDNA sample from each indicated tick pathogen was amplified, and no unintended amplification of any other target was present.

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Bb	Bb	FAM	24.125	0.978	1.531	Amp
A1	Bb	Ec	FAM	Undetermined	0.000		No Amp
A1	Bb	Ee	JUN	Undetermined	0.000		No Amp
A1	Bb	Bm	ABY	Undetermined	0.000	0.585	No Amp
A1	Bb	Ap	VIC	Undetermined	0.000		No Amp
A2	Bm	Bb	FAM	Undetermined	0.000		No Amp
A2	Bm	Ec	FAM	Undetermined	0.000		No Amp
A2	Bm	Ee	JUN	Undetermined	0.000		No Amp
A2	Bm	Bm	ABY	23.027	0.981	1.335	Amp
A2	Bm	Ap	VIC	Undetermined	0.000		No Amp
A3	Ap	Bb	FAM	Undetermined	0.000		No Amp
A3	Ap	Ec	FAM	Undetermined	0.000		No Amp
A3	Ap	Ee	JUN	Undetermined	0.000		No Amp
A3	Ap	Bm	ABY	Undetermined	0.000		No Amp
A3	Ap	Ap	VIC	26.397	0.980	1.457	Amp
A4	Ap	Bb	FAM	Undetermined	0.000		No Amp
A4	Ap	Ec	FAM	Undetermined	0.000		No Amp
A4	Ap	Ee	JUN	Undetermined	0.000		No Amp

A4	Ap	Bm	ABY	Undetermined	0.000		No Amp
A4	Ap	Ap	VIC	25.221	0.976	1.450	Amp
A5	Ec	Bb	FAM	Undetermined	0.000		No Amp
A5	Ec	Ec	FAM	31.247	0.943	1.523	Amp
A5	Ec	Ee	JUN	Undetermined	0.000		No Amp
A5	Ec	Bm	ABY	Undetermined	0.000		No Amp
A5	Ec	Ap	VIC	Undetermined	0.000	0.471	No Amp
A6	Ec	Bb	FAM	Undetermined	0.000	0.913	No Amp
A6	Ec	Ec	FAM	31.417	0.969	1.530	Amp
A6	Ec	Ee	JUN	Undetermined	0.000		No Amp
A6	Ec	Bm	ABY	Undetermined	0.000		No Amp
A6	Ec	Ap	VIC	Undetermined	0.000		No Amp
A7	Ew	Bb	FAM	Undetermined	0.000		No Amp
A7	Ew	Ec	FAM	Undetermined	0.000		No Amp
A7	Ew	Ee	JUN	31.059	0.968	1.500	Amp
A7	Ew	Bm	ABY	Undetermined	0.000		No Amp
A7	Ew	Ap	VIC	Undetermined	0.000		No Amp
A9	NCT	Bb	FAM	Undetermined	0.000		No Amp
A9	NCT	Ec	FAM	Undetermined	0.000		No Amp
A9	NCT	Ee	JUN	Undetermined	0.000		No Amp
A9	NCT	Bm	ABY	Undetermined	0.000		No Amp
A9	NCT	Ap	VIC	Undetermined	0.000		No Amp
A10	NCT	Bb	FAM	Undetermined	0.000	0.528	No Amp
A10	NCT	Ec	FAM	Undetermined	0.000		No Amp
A10	NCT	Ee	JUN	Undetermined	0.000		No Amp
A10	NCT	Bm	ABY	Undetermined	0.000		No Amp
A10	NCT	Ap	VIC	Undetermined	0.000	0.485	No Amp
A11	NCT	Bb	FAM	Undetermined	0.000		No Amp
A11	NCT	Ec	FAM	Undetermined	0.000		No Amp
A11	NCT	Ee	JUN	Undetermined	0.000		No Amp
A11	NCT	Bm	ABY	Undetermined	0.000		No Amp
A11	NCT	Ap	VIC	Undetermined	0.000		No Amp

Table 13. Results from pathogen-negative tick gDNA spiked with 1 μ L of pathogen DNA (Trial 1).

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
B1	Bb	Bb	FAM	24.050	0.974	1.539	Amp
B1	Bb	Ec	FAM	Undetermined	0.000		No Amp
B1	Bb	Ee	JUN	Undetermined	0.000		No Amp
B1	Bb	Bm	ABY	Undetermined	0.000	0.567	No Amp
B1	Bb	Ap	VIC	Undetermined	0.000		No Amp
B2	Bm	Bb	FAM	36.213	0.919	1.279	Amp
B2	Bm	Ec	FAM	Undetermined	0.000	0.745	No Amp
B2	Bm	Ee	JUN	Undetermined	0.000		No Amp
B2	Bm	Bm	ABY	23.067	0.984	1.340	Amp
B2	Bm	Ap	VIC	Undetermined	0.000		No Amp
B3	Ap	Bb	FAM	Undetermined	0.000	0.557	No Amp
B3	Ap	Ec	FAM	Undetermined	0.000		No Amp
B3	Ap	Ee	JUN	Undetermined	0.000		No Amp
B3	Ap	Bm	ABY	Undetermined	0.000		No Amp
B3	Ap	Ap	VIC	26.668	0.974	1.445	Amp
B4	Ap	Bb	FAM	Undetermined	0.000	0.564	No Amp
B4	Ap	Ec	FAM	Undetermined	0.000	0.709	No Amp
B4	Ap	Ee	JUN	Undetermined	0.000		No Amp
B4	Ap	Bm	ABY	Undetermined	0.000		No Amp
B4	Ap	Ap	VIC	25.576	0.979	1.454	Amp
B5	Ec	Bb	FAM	Undetermined	0.000		No Amp
B5	Ec	Ec	FAM	31.390	0.957	1.533	Amp
B5	Ec	Ee	JUN	Undetermined	0.000		No Amp
B5	Ec	Bm	ABY	Undetermined	0.000		No Amp
B5	Ec	Ap	VIC	Undetermined	0.000	0.519	No Amp
B6	Ec	Bb	FAM	Undetermined	0.000		No Amp
B6	Ec	Ec	FAM	31.171	0.963	1.536	Amp
B6	Ec	Ee	JUN	Undetermined	0.000		No Amp
B6	Ec	Bm	ABY	Undetermined	0.000		No Amp
B6	Ec	Ap	VIC	Undetermined	0.000		No Amp
B7	Ew	Bb	FAM	Undetermined	0.000		No Amp
B7	Ew	Ec	FAM	Undetermined	0.000		No Amp
B7	Ew	Ee	JUN	31.427	0.946	1.499	Amp
B7	Ew	Bm	ABY	Undetermined	0.000		No Amp
B7	Ew	Ap	VIC	Undetermined	0.000		No Amp
B9	NCT	Bb	FAM	37.957	0.761	1.130	Inconclusive
B9	NCT	Ec	FAM	Undetermined	0.000		No Amp
B9	NCT	Ee	JUN	Undetermined	0.000		No Amp
B9	NCT	Bm	ABY	37.958	0.909	1.053	Inconclusive

B9	NCT	Ap	VIC	Undetermined	0.000		No Amp
B10	NCT	Bb	FAM	Undetermined	0.000		No Amp
B10	NCT	Ec	FAM	Undetermined	0.000	0.631	No Amp
B10	NCT	Ee	JUN	Undetermined	0.000		No Amp
B10	NCT	Bm	ABY	37.961	0.973	1.051	Inconclusive
B10	NCT	Ap	VIC	Undetermined	0.000	0.467	No Amp
B11	NCT	Bb	FAM	Undetermined	0.000	0.600	No Amp
B11	NCT	Ec/ Ee-CTA	JUN	Undetermined	0.000		No Amp
B11	NCT	Bm	ABY	Undetermined	0.000		No Amp
B11	NCT	Ap	VIC	Undetermined	0.000		No Amp

Table 14. Results from pathogen-negative tick gDNA spiked with 1 μ L of pathogen DNA (Trial 2).

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
C1	Bb	Bb	FAM	24.123	0.979	1.517	Amp
C1	Bb	Ec	FAM	Undetermined	0.000		No Amp
C1	Bb	Ee	JUN	Undetermined	0.000		No Amp
C1	Bb	Bm	ABY	Undetermined	0.000	0.513	No Amp
C1	Bb	Ap	VIC	Undetermined	0.000		No Amp
C2	Bm	Bb	FAM	Undetermined	0.000		No Amp
C2	Bm	Ec	FAM	Undetermined	0.000	0.762	No Amp
C2	Bm	Ee	JUN	Undetermined	0.000		No Amp
C2	Bm	Bm	ABY	23.111	0.984	1.340	Amp
C2	Bm	Ap	VIC	Undetermined	0.000	0.541	No Amp
C3	Ap	Bb	FAM	Undetermined	0.000		No Amp
C3	Ap	Ec	FAM	Undetermined	0.000		No Amp
C3	Ap	Ee	JUN	Undetermined	0.000		No Amp
C3	Ap	Bm	ABY	Undetermined	0.000		No Amp
C3	Ap	Ap	VIC	26.399	0.974	1.442	Amp
C4	Ap	Bb	FAM	Undetermined	0.000	0.594	No Amp
C4	Ap	Ec	FAM	Undetermined	0.000	0.725	No Amp
C4	Ap	Ee	JUN	Undetermined	0.000		No Amp
C4	Ap	Bm	ABY	Undetermined	0.000		No Amp
C4	Ap	Ap	VIC	25.745	0.974	1.447	Amp
C5	Ec	Bb	FAM	Undetermined	0.000		No Amp
C5	Ec	Ec	FAM	30.995	0.959	1.540	Amp
C5	Ec	Ee	JUN	Undetermined	0.000		No Amp
C5	Ec	Bm	ABY	Undetermined	0.000		No Amp

C5	Ec	Ap	VIC	Undetermined	0.000	0.525	No Amp
C6	Ec	Bb	FAM	Undetermined	0.000		No Amp
C6	Ec	Ec	FAM	31.197	0.958	1.541	Amp
C6	Ec	Ee	JUN	Undetermined	0.000		No Amp
C6	Ec	Bm	ABY	Undetermined	0.000		No Amp
C6	Ec	Ap	VIC	Undetermined	0.000		No Amp
C7	Ew	Bb	FAM	Undetermined	0.000		No Amp
C7	Ew	Ec	FAM	Undetermined	0.000		No Amp
C7	Ew	Ee	JUN	31.508	0.953	1.510	Amp
C7	Ew	Bm	ABY	Undetermined	0.000		No Amp
C7	Ew	Ap	VIC	Undetermined	0.000		No Amp
C9	NCT	Bb	FAM	Undetermined	0.000	0.528	No Amp
C9	NCT	Ec	FAM	Undetermined	0.000		No Amp
C9	NCT	Ee	JUN	Undetermined	0.000		No Amp
C9	NCT	Bm	ABY	Undetermined	0.000		No Amp
C9	NCT	Ap	VIC	Undetermined	0.000		No Amp
C10	NCT	Bb	FAM	Undetermined	0.000		No Amp
C10	NCT	Ec	FAM	Undetermined	0.000		No Amp
C10	NCT	Ee	JUN	Undetermined	0.000		No Amp
C10	NCT	Bm	ABY	Undetermined	0.000		No Amp
C10	NCT	Ap	VIC	Undetermined	0.000	0.591	No Amp
C11	NCT	Bb	FAM	Undetermined	0.000		No Amp
C11	NCT	Ec	FAM	Undetermined	0.000		No Amp
C11	NCT	Ee	JUN	Undetermined	0.000		No Amp
C11	NCT	Bm	ABY	Undetermined	0.000		No Amp
C11	NCT	Ap	VIC	Undetermined	0.000		No Amp

Table 15. Results from pathogen-negative tick gDNA spiked with 1 μ L of pathogen DNA (Trial 3).

Table 16 displays the results from pathogen-negative tick gDNA spiked with 2 μ L pathogen DNA. The experiment was performed twice: once with Bb as FAM and once with Ec as FAM. This separate testing was indicated, as pathogens Bb and Ec do not exist in the same species of tick. Wells A1-C11 tested singular infections, while wells F1-F6 tested confections. Each well of the test included 10 μ L of Taqman

Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of tick gDNA spiked with indicated pathogen gDNA, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single pathogen gDNA sample from each indicated tick pathogen was amplified, and no unintended amplification of any other target was present.

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Bb	Bb	FAM	24.125	0.978	1.531	Amp
A1	Bb	Ec	FAM	Undetermined	0.000		No Amp
A1	Bb	Ee	JUN	Undetermined	0.000		No Amp
A1	Bb	Bm	ABY	Undetermined	0.000	0.585	No Amp
A1	Bb	Ap	VIC	Undetermined	0.000		No Amp
A2	Bm	Bb	FAM	Undetermined	0.000		No Amp
A2	Bm	Ec	FAM	Undetermined	0.981		No Amp
A2	Bm	Ee	JUN	Undetermined	0.000	0.657	No Amp
A2	Bm	Bm	ABY	23.027	0.981	1.335	Amp
A2	Bm	Ap	VIC	Undetermined	0.000		No Amp
A3	Ap	Bb	FAM	Undetermined	0.000		No Amp
A3	Ap	Ec	FAM	Undetermined	0.980		No Amp
A3	Ap	Ee	JUN	Undetermined	0.000		No Amp
A3	Ap	Bm	ABY	Undetermined	0.000		No Amp
A3	Ap	Ap	VIC	26.397	0.982	1.457	Amp
A4	Ap	Bb	FAM	Undetermined	0.000		No Amp
A4	Ap	Ec	FAM	Undetermined	0.000		No Amp
A4	Ap	Ee	JUN	Undetermined	0.000		No Amp
A4	Ap	Bm	ABY	Undetermined	0.000		No Amp
A4	Ap	Ap	VIC	25.221	0.976	1.450	Amp
A5	Ec	Bb	FAM	Undetermined	0.000		No Amp
A5	Ec	Ec	FAM	31.247	0.969	1.523	Amp
A5	Ec	Ee	JUN	Undetermined	0.000		No Amp
A5	Ec	Bm	ABY	Undetermined	0.000		No Amp

A5	Ec	Ap	VIC	Undetermined	0.000	0.471	No Amp
A6	Ec	Bb	FAM	Undetermined	0.000	0.913	No Amp
A6	Ec	Ec	FAM	31.417	0.967	1.530	Amp
A6	Ec	Ee	JUN	Undetermined	0.000		No Amp
A6	Ec	Bm	ABY	Undetermined	0.000		No Amp
A6	Ec	Ap	VIC	Undetermined	0.000		No Amp
A7	Ee	Bb	FAM	Undetermined	0.000		No Amp
A7	Ee	Ec	FAM	Undetermined	0.000		No Amp
A7	Ee	Ee	JUN	31.059	0.979	1.500	Amp
A7	Ee	Bm	ABY	Undetermined	0.000		No Amp
A7	Ee	Ap	VIC	Undetermined	0.000		No Amp
A9	NCT	Bb	FAM	Undetermined	0.000		No Amp
A9	NCT	Ec	FAM	Undetermined	0.000		No Amp
A9	NCT	Ee	JUN	Undetermined	0.000		No Amp
A9	NCT	Bm	ABY	Undetermined	0.000		No Amp
A9	NCT	Ap	VIC	Undetermined	0.000		No Amp
A10	NCT	Bb	FAM	Undetermined	0.974	0.528	No Amp
A10	NCT	Ec	FAM	Undetermined	0.000		No Amp
A10	NCT	Ee	JUN	Undetermined	0.000		No Amp
A10	NCT	Bm	ABY	Undetermined	0.000		No Amp
A10	NCT	Ap	VIC	Undetermined	0.919	0.485	No Amp
A11	NCT	Bb	FAM	Undetermined	0.000		No Amp
A11	NCT	Ec	FAM	Undetermined	0.984		No Amp
A11	NCT	Ee	JUN	Undetermined	0.000		No Amp
A11	NCT	Bm	ABY	Undetermined	0.000		No Amp
A11	NCT	Ap	VIC	Undetermined	0.000		No Amp
B1	Bb	Bb	FAM	24.050	0.974	1.539	Amp
B1	Bb	Ec	FAM	Undetermined	0.000		No Amp
B1	Bb	Ee	JUN	Undetermined	0.000		No Amp
B1	Bb	Bm	ABY	Undetermined	0.000	0.567	No Amp
B1	Bb	Ap	VIC	Undetermined	0.000		No Amp
B2	Bm	Bb	FAM	36.213	0.979	1.279	Inconclusive
B2	Bm	Ec	FAM	Undetermined	0.000		No Amp
B2	Bm	Ee	JUN	Undetermined	0.957	0.745	No Amp
B2	Bm	Bm	ABY	23.067	0.980	1.340	Amp
B2	Bm	Ap	VIC	Undetermined	0.000		No Amp
B3	Ap	Bb	FAM	Undetermined	0.000	0.557	No Amp
B3	Ap	Ec	FAM	Undetermined	0.965		No Amp
B3	Ap	Ee	JUN	Undetermined	0.000		No Amp
B3	Ap	Bm	ABY	Undetermined	0.000		No Amp
B3	Ap	Ap	VIC	26.668	0.946	1.445	Amp
B4	Ap	Bb	FAM	Undetermined	0.000	0.564	No Amp
B4	Ap	Ec	FAM	Undetermined	0.000		No Amp
B4	Ap	Ee	JUN	Undetermined	0.000	0.709	No Amp

B4	Ap	Bm	ABY	Undetermined	0.000		No Amp
B4	Ap	Ap	VIC	25.576	0.936	1.454	Amp
B5	Ec	Bb	FAM	Undetermined	0.000		No Amp
B5	Ec	Ec	FAM	31.390	0.981	1.533	Amp
B5	Ec	Ee	JUN	Undetermined	0.000		No Amp
B5	Ec	Bm	ABY	Undetermined	0.000		No Amp
B5	Ec	Ap	VIC	Undetermined	0.973	0.519	No Amp
B6	Ec	Bb	FAM	Undetermined	0.000		No Amp
B6	Ec	Ec	FAM	31.171	0.982	1.536	Amp
B6	Ec	Ee	JUN	Undetermined	0.000		No Amp
B6	Ec	Bm	ABY	Undetermined	0.000		No Amp
B6	Ec	Ap	VIC	Undetermined	0.000		No Amp
B7	Ee	Bb	FAM	Undetermined	0.000		No Amp
B7	Ee	Ec	FAM	Undetermined	0.000		No Amp
B7	Ee	Ee	JUN	31.427	0.979	1.499	Amp
B7	Ee	Bm	ABY	Undetermined	0.000		No Amp
B7	Ee	Ap	VIC	Undetermined	0.000		No Amp
B9	NCT	Bb	FAM	37.957	0.984	1.130	Inconclusive
B9	NCT	Ec	FAM	Undetermined	0.000		No Amp
B9	NCT	Ee	JUN	Undetermined	0.000		No Amp
B9	NCT	Bm	ABY	37.958	0.979	1.053	Inconclusive
B9	NCT	Ap	VIC	Undetermined	0.000		No Amp
B10	NCT	Bb	FAM	Undetermined	0.000		No Amp
B10	NCT	Ec	FAM	Undetermined	0.000		No Amp
B10	NCT	Ee	JUN	Undetermined	0.974	0.631	No Amp
B10	NCT	Bm	ABY	Undetermined	0.000		No Amp
B10	NCT	Ap	VIC	Undetermined	0.982	0.467	No Amp
B11	NCT	Bb	FAM	Undetermined	0.974	0.600	No Amp
B11	NCT	Ec	FAM	Undetermined	0.000		No Amp
B11	NCT	Ee	JUN	Undetermined	0.959		No Amp
B11	NCT	Bm	ABY	Undetermined	0.963		No Amp
B11	NCT	Ap	VIC	Undetermined	0.000		No Amp
C1	Bb	Bb	FAM	24.123	0.958	1.517	Amp
C1	Bb	Ec	FAM	Undetermined	0.000		No Amp
C1	Bb	Ee	JUN	Undetermined	0.000		No Amp
C1	Bb	Bm	ABY	Undetermined	0.966	0.513	No Amp
C1	Bb	Ap	VIC	Undetermined	0.000		No Amp
C2	Bm	Bb	FAM	Undetermined	0.000		No Amp
C2	Bm	Ec	FAM	Undetermined	0.000		No Amp
C2	Bm	Ee	JUN	Undetermined	0.000	0.762	No Amp
C2	Bm	Bm	ABY	23.111	0.989	1.340	Amp
C2	Bm	Ap	VIC	Undetermined	0.000	0.541	No Amp
C3	Ap	Bb	FAM	Undetermined	0.000		No Amp
C3	Ap	Ec	FAM	Undetermined	0.000		No Amp

C3	Ap	Ee	JUN	Undetermined	0.000		No Amp
C3	Ap	Bm	ABY	Undetermined	0.000		No Amp
C3	Ap	Ap	VIC	26.399	0.979	1.442	Amp
C4	Ap	Bb	FAM	Undetermined	0.000	0.594	No Amp
C4	Ap	Ec	FAM	Undetermined	0.000		No Amp
C4	Ap	Ee	JUN	Undetermined	0.000	0.725	No Amp
C4	Ap	Bm	ABY	32.027	0.991	1.360	Amp
C4	Ap	Ap	VIC	25.745	0.983	1.447	Amp
C5	Ec	Bb	FAM	Undetermined	0.970		No Amp
C5	Ec	Ec	FAM	30.995	0.987	1.540	Amp
C5	Ec	Ee	JUN	Undetermined	0.000		No Amp
C5	Ec	Bm	ABY	Undetermined	0.000		No Amp
C5	Ec	Ap	VIC	Undetermined	0.000	0.525	No Amp
C6	Ec	Bb	FAM	Undetermined	0.000		No Amp
C6	Ec	Ec	FAM	31.197	0.989	1.541	Amp
C6	Ec	Ee	JUN	Undetermined	0.000		No Amp
C6	Ec	Bm	ABY	Undetermined	0.000		No Amp
C6	Ec	Ap	VIC	Undetermined	0.930		No Amp
C7	Ee	Bb	FAM	Undetermined	0.973		No Amp
C7	Ee	Ec	FAM	Undetermined	0.971		No Amp
C7	Ee	Ee	JUN	31.508	0.972	1.510	Amp
C7	Ee	Bm	ABY	Undetermined	0.000		No Amp
C7	Ee	Ap	VIC	Undetermined	0.000		No Amp
C9	NCT	Bb	FAM	Undetermined	0.000	0.528	No Amp
C9	NCT	Ec	FAM	Undetermined	0.000		No Amp
C9	NCT	Ee	JUN	Undetermined	0.000		No Amp
C9	NCT	Bm	ABY	Undetermined	0.000		No Amp
C9	NCT	Ap	VIC	Undetermined	0.000		No Amp
C10	NCT	Bb	FAM	Undetermined	0.000		No Amp
C10	NCT	Ec	FAM	Undetermined	0.000		No Amp
C10	NCT	Ee	JUN	Undetermined	0.981		No Amp
C10	NCT	Bm	ABY	Undetermined	0.000		No Amp
C10	NCT	Ap	VIC	Undetermined	0.000	0.591	No Amp
C11	NCT	Bb	FAM	Undetermined	0.000		No Amp
C11	NCT	Ec	FAM	Undetermined	0.000		No Amp
C11	NCT	Ee	JUN	Undetermined	0.980		No Amp
C11	NCT	Bm	ABY	Undetermined	0.000		No Amp
C11	NCT	Ap	VIC	Undetermined	0.000		No Amp
F1	Bb/Bm	Bb	FAM	25.038	0.982	1.532	Amp
F1	Bb/Bm	Ec	FAM	Undetermined	0.976		No Amp
F1	Bb/Bm	Ee	JUN	Undetermined	0.000		No Amp
F1	Bb/Bm	Bm	ABY	23.912	0.943	1.350	Amp
F1	Bb/Bm	Ap	VIC	Undetermined	0.976		No Amp
F2	Bm/Ap	Bb	FAM	Undetermined	0.000		No Amp

F2	Bm/Ap	Ec	FAM	Undetermined	0.000		No Amp
F2	Bm/Ap	Ee	JUN	Undetermined	0.000	0.685	No Amp
F2	Bm/Ap	Bm	ABY	23.801	0.969	1.354	Amp
F2	Bm/Ap	Ap	VIC	27.553	0.973	1.463	Amp
F4	Ap/Ec	Bb	FAM	Undetermined	0.000		No Amp
F4	Ap/Ec	Ec	FAM	32.311	0.967	1.530	Amp
F4	Ap/Ec	Ee	JUN	Undetermined	0.000		No Amp
F4	Ap/Ec	Bm	ABY	Undetermined	0.000		No Amp
F4	Ap/Ec	Ap	VIC	26.888	0.995	1.451	Amp
F6	Ec/Ee	Bb	FAM	Undetermined	0.000	0.492	No Amp
F6	Ec/Ee	Ec	FAM	32.090	0.993	1.522	Amp
F6	Ec/Ee	Ee	JUN	31.535	0.989	1.654	Amp
F6	Ec/Ee	Bm	ABY	39.835	0.000	0.906	Inconclusive
F6	Ec/Ee	Ap	VIC	Undetermined	0.000		No Amp

Table 16. Results from pathogen-negative tick gDNA spiked with 2 μ L pathogen DNA. The experiment was performed twice- once with *B. burgdorferi* as FAM and once with *E. chaffeensis* as FAM.

Table 17 displays the results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *B. burgdorferi*. Each well of the test included 10 μ L of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2 μ L of *B. burgdorferi* gDNA normalized to 1-2ng/ μ L, and nuclease-free water to 20 μ L. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *B. burgdorferi* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Bb	FAM	20.080	0.975	1.546	Amp
A1	Bm	ABY	Undetermined	0.000		No Amp
A1	Ap	VIC	Undetermined	0.000		No Amp
A2	Bb	FAM	23.572	0.980	1.539	Amp
A2	Bm	ABY	Undetermined	0.000		No Amp
A2	Ap	VIC	Undetermined	0.000		No Amp
A3	Bb	FAM	28.045	0.983	1.541	Amp
A3	Bm	ABY	Undetermined	0.000	0.539	No Amp
A3	Ap	VIC	Undetermined	0.000		No Amp
A4	Bb	FAM	31.079	0.968	1.540	Amp
A4	Bm	ABY	37.762	0.934	1.044	Inconclusive
A4	Ap	VIC	Undetermined	0.000		No Amp
A5	Bb	FAM	35.059	0.947	1.431	Amp
A5	Bm	ABY	Undetermined	0.000	0.329	No Amp
A5	Ap	VIC	Undetermined	0.000	0.388	No Amp
A6	Bb	FAM	37.169	0.866	1.216	Inconclusive
B1	Bb	FAM	20.449	0.984	1.545	Amp
B1	Bm	ABY	Undetermined	0.000	0.556	No Amp
B1	Ap	VIC	Undetermined	0.000		No Amp
B2	Bb	FAM	24.340	0.973	1.532	Amp
B2	Bm	ABY	Undetermined	0.000	0.529	No Amp
B2	Ap	VIC	Undetermined	0.000		No Amp
B3	Bb	FAM	27.703	0.972	1.525	Amp
B3	Bm	ABY	Undetermined	0.000		No Amp
B3	Ap	VIC	Undetermined	0.000		No Amp
B4	Bb	FAM	30.681	0.971	1.541	Amp
B4	Bm	ABY	Undetermined	0.000	0.521	No Amp
B4	Ap	VIC	Undetermined	0.000		No Amp
B5	Bb	FAM	34.623	0.966	1.453	Amp
B5	Bm	ABY	Undetermined	0.000	0.427	No Amp
B5	Ap	VIC	Undetermined	0.000		No Amp
B6	Bb	FAM	Undetermined	0.000		No Amp
B6	Bm	ABY	Undetermined	0.000		No Amp
B6	Ap	VIC	Undetermined	0.000		No Amp
B7	Bb	FAM	37.227	0.827	1.228	Inconclusive
B7	Bm	ABY	Undetermined	0.000		No Amp
B7	Ap	VIC	Undetermined	0.000		No Amp
C1	Bb	FAM	20.410	0.977	1.528	Amp
C1	Bm	ABY	Undetermined	0.000		No Amp
C1	Ap	VIC	Undetermined	0.000		No Amp
C2	Bb	FAM	24.443	0.974	1.523	Amp
C2	Bm	ABY	Undetermined	0.000	0.500	No Amp

C2	Ap	VIC	Undetermined	0.000		No Amp
C3	Bb	FAM	27.559	0.975	1.522	Amp
C3	Bm	ABY	Undetermined	0.000	0.479	No Amp
C3	Ap	VIC	Undetermined	0.000		No Amp
C4	Bb	FAM	32.115	0.963	1.529	Amp
C4	Bm	ABY	Undetermined	0.000	0.481	No Amp
C4	Ap	VIC	Undetermined	0.000		No Amp
C5	Bb	FAM	33.628	0.962	1.527	Amp
C5	Bm	ABY	Undetermined	0.000	0.493	No Amp
C5	Ap	VIC	Undetermined	0.000		No Amp
C6	Bb	FAM	36.966	0.902	1.242	Inconclusive
C6	Bm	ABY	Undetermined	0.000		No Amp
C6	Ap	VIC	Undetermined	0.000		No Amp
C12	NCT	FAM	Undetermined	0.000		No Amp
C12	NCT	ABY	Undetermined	0.000		No Amp
C12	NCT	VIC	Undetermined	0.000		No Amp

Table 17. Results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *B. burgdorferi*.

Table 18 displays the results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *Ba. microti*. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of *Ba. microti* gDNA normalized to 1-2ng/µL, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *Ba. microti* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
F1	Bb	FAM	Undetermined	0.000		No Amp
F1	Bm	ABY	18.723	0.984	1.365	Amp
F1	Ap	VIC	Undetermined	0.000		No Amp
F2	Bb	FAM	Undetermined	0.000		No Amp
F2	Bm	ABY	8.454	0.945	1.382	Amp
F2	Ap	VIC	Undetermined	0.000		No Amp
F3	Bb	FAM	Undetermined	0.000		No Amp
F3	Bm	ABY	12.099	0.986	1.380	Amp
F3	Ap	VIC	36.657	0.886	1.084	Inconclusive
F4	Bb	FAM	Undetermined	0.000		No Amp
F4	Bm	ABY	13.600	0.988	1.383	Amp
F4	Ap	VIC	Undetermined	0.000		No Amp
F5	Bb	FAM	Undetermined	0.000	0.573	No Amp
F5	Bm	ABY	15.072	0.989	1.389	Amp
F5	Ap	VIC	Undetermined	0.000		No Amp
F6	Bb	FAM	Undetermined	0.000		No Amp
F6	Bm	ABY	19.919	0.992	1.379	Amp
F6	Ap	VIC	Undetermined	0.000		No Amp
F7	Bb	FAM	Undetermined	0.000	0.532	No Amp
F7	Bm	ABY	23.767	0.992	1.385	Amp
F7	Ap	VIC	Undetermined	0.000	0.500	No Amp
F8	Bb	FAM	Undetermined	0.000	0.585	No Amp
F8	Bm	ABY	28.043	0.989	1.398	Amp
F8	Ap	VIC	Undetermined	0.000		No Amp
F9	Bb	FAM	Undetermined	0.000	0.584	No Amp
F9	Bm	ABY	30.574	0.986	1.379	Amp
F9	Ap	VIC	Undetermined	0.000		No Amp
F10	Bb	FAM	Undetermined	0.000		No Amp
F10	Bm	ABY	34.821	0.977	1.287	Amp
F10	Ap	VIC	Undetermined	0.000		No Amp
F11	Bb	FAM	Undetermined	0.000	0.539	No Amp
F11	Bm	ABY	35.954	0.970	1.237	Amp
F11	Ap	VIC	Undetermined	0.000		No Amp
F12	Bb	FAM	Undetermined	0.000	0.452	No Amp
F12	Bm	ABY	Undetermined	0.000		No Amp
F12	Ap	VIC	Undetermined	0.000		No Amp
G1	Bb	FAM	Undetermined	0.000		No Amp
G1	Bm	ABY	19.244	0.987	1.363	Amp
G1	Ap	VIC	Undetermined	0.000		No Amp
G2	Bb	FAM	37.143	0.887	1.196	Inconclusive
G2	Bm	ABY	8.741	0.949	1.369	Amp
G2	Ap	VIC	Undetermined	0.000		No Amp
G3	Bb	FAM	Undetermined	0.000		No Amp

G3	Bm	ABY	12.142	0.985	1.384	Amp
G3	Ap	VIC	Undetermined	0.000		No Amp
G4	Bb	FAM	Undetermined	0.000	0.553	No Amp
G4	Bm	ABY	13.525	0.986	1.386	Amp
G4	Ap	VIC	Undetermined	0.000		No Amp
G5	Bb	FAM	Undetermined	0.000	0.536	No Amp
G5	Bm	ABY	15.518	0.991	1.384	Amp
G5	Ap	VIC	Undetermined	0.000		No Amp
G6	Bb	FAM	Undetermined	0.000		No Amp
G6	Bm	ABY	19.846	0.990	1.377	Amp
G6	Ap	VIC	Undetermined	0.000		No Amp
G12	NCT	FAM	Undetermined	0.000		No Amp
G12	NCT	ABY	Undetermined	0.000		No Amp
G12	NCT	VIC	Undetermined	0.000		No Amp

Table 18. Results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *Ba. microti*.

Table 19 displays the results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *A. phagocytophilum*. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of *A. phagocytophilum* gDNA normalized to 1-2ng/µL, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *A. phagocytophilum* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Bb	FAM	Undetermined	0		No Amp
A1	Bm	ABY	Undetermined	0		No Amp
A1	Ap	VIC	19.0456	0.978	1.468	Amp
A2	Bb	FAM	Undetermined	0	0.648	No Amp
A2	Bm	ABY	Undetermined	0		No Amp
A2	Ap	VIC	24.86489	0.980	1.472	Amp
A3	Bb	FAM	Undetermined	0		No Amp
A3	Bm	ABY	Undetermined	0		No Amp
A3	Ap	VIC	29.53437	0.976	1.465	Amp
A4	Bb	FAM	Undetermined	0		No Amp
A4	Bm	ABY	Undetermined	0		No Amp
A4	Ap	VIC	31.88608	0.974	1.469	Amp
A5	Bb	FAM	Undetermined	0		No Amp
A5	Bm	ABY	Undetermined	0		No Amp
A5	Ap	VIC	35.91395	0.947	1.321	Inconclusive
B1	Bb	FAM	Undetermined	0		No Amp
B1	Bm	ABY	Undetermined	0		No Amp
B1	Ap	VIC	19.78737	0.985	1.474	Amp
B2	Bb	FAM	Undetermined	0		No Amp
B2	Bm	ABY	Undetermined	0		No Amp
B2	Ap	VIC	24.84941	0.989	1.466	Amp
B3	Bb	FAM	Undetermined	0		No Amp
B3	Bm	ABY	Undetermined	0		No Amp
B3	Ap	VIC	28.79041	0.977	1.449	Amp
B4	Bb	FAM	Undetermined	0		No Amp
B4	Bm	ABY	Undetermined	0		No Amp
B4	Ap	VIC	32.41282	0.973	1.465	Amp
B5	Bb	FAM	Undetermined	0		No Amp
B5	Bm	ABY	Undetermined	0		No Amp
B5	Ap	VIC	36.38597	0.924	1.230	Inconclusive
C1	Bb	FAM	Undetermined	0		No Amp
C1	Bm	ABY	Undetermined	0		No Amp
C1	Ap	VIC	20.46573	0.981	1.457	Amp
C2	Bb	FAM	Undetermined	0		No Amp
C2	Bm	ABY	Undetermined	0		No Amp
C2	Ap	VIC	25.18266	0.981	1.456	Amp
C3	Bb	FAM	Undetermined	0		No Amp
C3	Bm	ABY	Undetermined	0		No Amp
C3	Ap	VIC	28.95838	0.978	1.454	Amp
C4	Bb	FAM	Undetermined	0		No Amp
C4	Bm	ABY	Undetermined	0		No Amp
C4	Ap	VIC	32.68946	0.973	1.452	Amp
C5	Bb	FAM	Undetermined	0		No Amp

C5	Bm	ABY	Undetermined	0		No Amp
C5	Ap	VIC	35.33879	0.947	1.35	Amp
C12	NCT	FAM	Undetermined	0		No Amp
C12	NCT	ABY	Undetermined	0		No Amp
C12	NCT	VIC	Undetermined	0		No Amp

Table 19. Results of the ISC1 Multiplex test with triplicate serial diluted gDNA from *A. phagocytophilum*.

Table 20 displays the results of the DVA1 Multiplex test with triplicate serial diluted gDNA from *A. phagocytophilum*. Since *A. phagocytophilum* was being tested for in two different multiplexes, it was imperative to test this target in the presence of the *E. chaffeensis* primers and probes, as indicated by the DVA1 multiplex assay. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (ThermoFisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of *A. phagocytophilum* gDNA normalized to 1-2ng/µL, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *A. phagocytophilum* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
F1	Ec	JUN	Undetermined	0		No Amp
F1	Ap	VIC	20.52104	0.984981	1.473038	Amp
F2	Ec	JUN	Undetermined	0		No Amp
F2	Ap	VIC	25.04413	0.979864	1.478745	Amp
F3	Ec	JUN	Undetermined	0		No Amp
F3	Ap	VIC	28.78796	0.976837	1.478104	Amp
F4	Ec	JUN	Undetermined	0		No Amp
F4	Ap	VIC	32.23411	0.973495	1.464658	Amp
F5	Ec	JUN	Undetermined	0		Inconclusive
F5	Ap	VIC	35.30633	0.957354	1.353468	Amp
F6	Ec	JUN	Undetermined	0		No Amp
F6	Ap	VIC	38	0.890394	1.085883	Inconclusive
F7	Ec	JUN	Undetermined	0		No Amp
F7	Ap	VIC	Undetermined	0		No Amp
G1	Ec	JUN	Undetermined	0		Inconclusive
G1	Ap	VIC	20.54639	0.980422	1.469285	Amp
G2	Ec	JUN	Undetermined	0		No Amp
G2	Ap	VIC	24.91755	0.977916	1.470894	Amp
G3	Ec	JUN	Undetermined	0		No Amp
G3	Ap	VIC	28.72538	0.976226	1.474333	Amp
G4	Ec	JUN	Undetermined	0		No Amp
G4	Ap	VIC	32.06177	0.967046	1.472525	Amp
G5	Ec	JUN	Undetermined	0		No Amp
G5	Ap	VIC	34.791	0.956588	1.375815	Amp
G6	Ec	JUN	Undetermined	0		No Amp
G6	Ap	VIC	37.84909	0.854292	1.094457	Inconclusive
G7	Ec	JUN	Undetermined	0		No Amp
G7	AP	VIC	Undetermined	0		No Amp
H1	Ec	JUN	Undetermined	0		No Amp
H1	Ap	VIC	20.67731	0.985889	1.461347	Amp
H2	Ec	JUN	Undetermined	0		No Amp
H2	Ap	VIC	24.9929	0.976403	1.457856	Amp
H3	Ec	JUN	Undetermined	0		No Amp
H3	Ap	VIC	28.72203	0.981555	1.463301	Amp
H4	Ec	JUN	Undetermined	0		No Amp
H4	Ap	VIC	32.05211	0.980653	1.476511	Amp
H5	Ec	JUN	Undetermined	0		No Amp
H5	Ap	VIC	36.45718	0.960827	1.226712	Inconclusive
H12	NCT	JUN	Undetermined	0		No Amp
H12	NCT	VIC	Undetermined	0		No Amp

Table 20. Results of the DVA1 Multiplex test with triplicate serial diluted gDNA from *A. phagocytophilum*.

Table 21 displays the results of the DVA1 Multiplex test with triplicate serial diluted gDNA from *E. chaffeensis*. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of *E. chaffeensis* gDNA normalized to 1-2ng/µL, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each *E. chaffeensis* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Ec	Ec	JUN	26.16039	0.972239	1.502359	Amp
A1	Ec	Ap	VIC	Undetermined	0		No Amp
A2	Ec	Ec	JUN	30.38268	0.977915	1.516609	Amp
A2	Ec	Ap	VIC	Undetermined	0		No Amp
A3	Ec	Ec	JUN	32.57182	0.974792	1.525294	Amp
A3	Ec	Ap	VIC	Undetermined	0		No Amp
A4	Ec	Ec	JUN	37.46441	0.912782	1.2734	Inconclusive
A4	Ec	Ap	VIC	Undetermined	0		No Amp
B1	Ec	Ec	JUN	26.1714	0.981795	1.514998	Amp
B1	Ec	Ap	VIC	Undetermined	0		No Amp
B2	Ec	Ec	JUN	30.41747	0.976586	1.521436	Amp
B2	Ec	Ap	VIC	Undetermined	0		No Amp
B3	Ec	Ec	JUN	32.41871	0.975434	1.527773	Amp
B3	Ec	Ap	VIC	Undetermined	0		No Amp
B4	Ec	Ec	JUN	37.95158	0.867562	1.232169	Inconclusive
B4	Ec	Ap	VIC	Undetermined	0		No Amp
C1	Ec	Ec	JUN	26.35249	0.96347	1.51102	Amp
C1	Ec	Ap	VIC	Undetermined	0		No Amp
C2	Ec	Ec	JUN	30.4856	0.971023	1.523008	Amp
C2	Ec	Ap	VIC	Undetermined	0		No Amp
C3	Ec	Ec	JUN	36.12109	0.952091	1.429404	Amp
C3	Ec	Ap	VIC	Undetermined	0		No Amp
C4	Ec	Ec	JUN	37.0079	0.911226	1.312434	Inconclusive

C4	Ec	Ap	VIC	Undetermined	0	No Amp
E1	NCT	Ec	JUN	Undetermined	0	No Amp
E1	NCT	Ap	VIC	Undetermined	0	No Amp
E2	NCT	Ec	JUN	Undetermined	0	No Amp
E2	NCT	Ap	VIC	Undetermined	0	No Amp
E3	NCT	Ec	JUN	Undetermined	0	No Amp
E3	NCT	Ap	VIC	Undetermined	0	No Amp

Table 21. Results of the DVA1 Multiplex test with triplicate serial diluted gDNA from *E. chaffeensis*.

Table 22 displays the Results of the AAM1 Multiplex test with triplicate serial diluted gDNA from *E. chaffeensis*. Since *E. chaffeensis* was being tested for in two different multiplexes, it was imperative to test this target in the presence of the *E. ewingii* primers and probes, as indicated by the AAM1 multiplex assay. Each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2µL of *E. chaffeensis* gDNA normalized to 1-2ng/µL, and nuclease-free water to 20µL. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *E. chaffeensis* gDNA sample was amplified, and no unintended amplification of any other target was present.

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
A1	Ec	Ew	JUN	Undetermined	0	0.689	No Amp
A1	Ec	Ec-FAM	FAM	23.198	0.986	1.574	Amp
A2	Ec	Ew	JUN	Undetermined	0	0.501	No Amp
A2	Ec	Ec-FAM	FAM	27.922	0.992	1.58	Amp
A3	Ec	Ew	JUN	Undetermined	0		No Amp
A3	Ec	Ec-FAM	FAM	31.14	0.987	1.55	Amp
A4	Ec	Ew	JUN	Undetermined	0	0.47	No Amp
A4	Ec	Ec-FAM	FAM	34.206	0.973	1.449	Amp
A12	NCT	Ew	JUN	Undetermined	0		No Amp
A12	NCT	Ec-FAM	FAM	Undetermined	0		No Amp
B1	Ec	Ew	JUN	Undetermined	0		No Amp
B1	Ec	Ec-FAM	FAM	22.801	0.989	1.58	Amp
B2	Ec	Ew	JUN	Undetermined	0	0.529	No Amp
B2	Ec	Ec-FAM	FAM	27.369	0.987	1.585	Amp
B3	Ec	Ew	JUN	Undetermined	0		No Amp
B3	Ec	Ec-FAM	FAM	30.838	0.987	1.57	Amp
B4	Ec	Ew	JUN	Undetermined	0		No Amp
B4	Ec	Ec-FAM	FAM	34.571	0.968	1.437	Amp
B5	Ec	Ew	JUN	Undetermined	0		No Amp
B5	Ec	Ec-FAM	FAM	Undetermined	0		No Amp
B12	NCT	Ew	JUN	Undetermined	0		No Amp
B12	NCT	Ec-FAM	FAM	Undetermined	0		No Amp
C1	Ec	Ew	JUN	Undetermined	0		No Amp
C1	Ec	Ec-FAM	FAM	22.837	0.988	1.586	Amp
C2	Ec	Ew	JUN	Undetermined	0		No Amp
C2	Ec	Ec-FAM	FAM	27.212	0.988	1.586	Amp
C3	Ec	Ew	JUN	Undetermined	0		No Amp
C3	Ec	Ec-FAM	FAM	31.601	0.992	1.562	Amp
C12	NCT	Ew	JUN	Undetermined	0		No Amp
C12	NCT	Ec-FAM	FAM	Undetermined	0	0.486	No Amp

Table 22. Results of the AAM1 Multiplex test with triplicate serial diluted gDNA from *E. chaffeensis*.

Table 23 displays the results of the AAM1 Multiplex test with triplicate serial diluted gDNA from *E. ewingii* as well as pathogen negative tick gDNA spiked with Ec and Ew (wells H1-H4). In the table, (.5) refers to 1µL of both Ec and Ew template instead of 2µL. Except for wells H1-H4, each well of the test included 10µL of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of

250nM forward and reverse primers and probes for all targets relevant to the species of tick, 2 μ L of *E. ewingii* gDNA normalized to 1-2ng/ μ L, and nuclease-free water to 20 μ L. Ten-fold dilutions were made in triplicate. The real-time PCR standard curve protocol was an initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The results show that each single *E. ewingii* gDNA sample was amplified, and no unintended amplification of any other target was present. For wells H1-H4, the results show that all targets amplified as expected with no unintended results.

Well	Sample	Target	Dyes	Cq	Cq Conf	Amp Score	Amp Status
E1	Ew	Ew	JUN	23.977	0.986	1.466	Amp
E1	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
E2	Ew	Ew	JUN	27.83	0.988	1.478	Amp
E2	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
E3	Ew	Ew	JUN	31.42	0.981	1.459	Amp
E3	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
E4	Ew	Ew	JUN	34.724	0.949	1.237	Amp
E4	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
E12	NCT	Ew	JUN	Undetermined	0		No Amp
E12	NCT	Ec-FAM	FAM	Undetermined	0		No Amp
F1	Ew	Ew	JUN	23.878	0.982	1.466	Amp
F1	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
F2	Ew	Ew	JUN	27.803	0.985	1.476	Amp
F2	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
F3	Ew	Ew	JUN	31.4	0.973	1.465	Amp
F3	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
F4	Ew	Ew	JUN	35.148	0.93	1.226	Inconclusive
F4	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
F12	NCT	Ew	JUN	Undetermined	0		No Amp
F12	NCT	Ec-FAM	FAM	Undetermined	0		No Amp
G1	Ew	Ew	JUN	23.886	0.986	1.46	Amp
G1	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
G2	Ew	Ew	JUN	27.993	0.982	1.47	Amp
G2	Ew	Ec-FAM	FAM	Undetermined	0		No Amp

G3	Ew	Ew	JUN	31.956	0.984	1.44	Amp
G3	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
G4	Ew	Ew	JUN	34.145	0.957	1.352	Inconclusive
G4	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
G12	NCT	Ew	JUN	37.539	0.811	0.932	Inconclusive
G12	NCT	Ec-FAM	FAM	Undetermined	0		No Amp
H1	Ec	Ew	JUN	Undetermined	0		No Amp
H1	Ec	Ec-FAM	FAM	22.768	0.992	1.58	Amp
H2	Ew	Ew	JUN	23.995	0.983	1.466	Amp
H2	Ew	Ec-FAM	FAM	Undetermined	0		No Amp
H3	Ec and Ew (.5)	Ew	JUN	25.417	0.991	1.458	Amp
H3	Ec and Ew (.5)	Ec-FAM	FAM	24.401	0.993	1.583	Amp
H4	NCT	Ew	JUN	Undetermined	0		No Amp
H4	NCT	Ec-FAM	FAM	Undetermined	0		No Amp

Table 23. Results of the AAM1 Multiplex test with triplicate serial diluted gDNA from *E. ewingii* (and spiked *E. chaffeensis* (Ec) and *E. ewingii* (Ew)-Wells H1-H4). (.5) refers to 1 μ L of both Ec and Ew template instead of 2 μ L.

3.4 Discussion

There were a few reasons why some of the 28 prospective primer-probe combinations were not selected from the real-time PCR testing for the final assay. Some of the primer-probe combinations were discontinued from the study because of relatively low fluorescence, later C_T amplification, evidence of primer dimer, self-dimerization, or some other sort of binding anomaly. Some primer-probe combinations showed evidence of binding to unintended target DNA either in the gDNA of the relevant tick, or to DNA from the tick microbiome. This is evidenced by negative control amplification using pathogen-negative tick gDNA. The final primer-probe combinations used in this study (see Table 2) passed all optimization measures and had the earliest and most reliable C_T values.

Chapter 4

DEPLOYMENT OF THE MULTIPLEX REAL-TIME PCR ASSAYS FOR THE DETECTION OF *BORRELIA BURGDORFERI*, *BABESIA MICROTI*, *ANAPLASMA PHAGOCYTOPHILUM*, *EHRlichia CHAFFEENSIS*, AND *EHRlichia EWINGII* ON TICKS ACROSS THE STATE OF DELAWARE

4.1 Introduction

The ISC1, DVA1, and AAM1 real-time PCR multiplex Taqman™ assays were deployed on DNA extracted from a sample of 1527 ticks collected from across the State of Delaware. 500 ticks were tested from *I. scapularis*, 500 ticks were tested from *D. variabilis*, and 527 ticks were tested from *A. americanum*. The ticks were collected by DNREC in 2019 and 2020 from 26 locations across the state of Delaware. At the time of collection, the ticks were collected by dragging a white cloth over areas suspected of harboring ticks. The date, location, species, developmental stage, and sex (if adult), of each tick was recorded. The ticks were placed in 1.5mL microtubes, submerged in 95% ethanol, and stored at -20°C.

4.2 Materials and Methods

4.2.1 DNA Isolation of Ticks

Once the ticks were ready for DNA extraction, each tick was coded, surface-washed in 95% ethanol, and rinsed with sterile water. The washing step can decrease the chance of contamination and false-positive reactions because it helps to remove DNA originating from non-target organisms that are present on the surface of ticks¹⁰⁴. A two-inch square piece of sterilized paper was placed under each tick before dissection and replaced between each tick to limit contamination between ticks. The bench surface was disinfected with a 10% bleach solution between each tick to further mitigate any cross-contamination. Each tick was then bisected sagittally using a sterile scalpel. Half of each tick was placed in a storage tube and frozen at -20°C. The other half of each tick was further dissected transversely and both transverse sections of each tick was placed in a labeled 1.5mL microtube. The only exception to this procedure was to place the entire dissected bodies of smaller nymph ticks in the 1.5mL microtubes to ensure ample DNA was collected. The tick bodies of the select smaller nymphs were stored at -20°C after DNA processing.

DNA was isolated from the sample of 1527 ticks using two different methods. DNA was isolated from 878 ticks using the tick supplementary protocol from the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Frederick, MD). DNA was isolated from the remaining 640 ticks using the Promega Maxwell 48 DNA isolation instrument

(Madison, WI) with the Promega RSC Tissue DNA Isolation Kit (Madison, WI). The reason that two DNA isolation methods were used was because the Promega Maxwell 48 DNA isolation instrument was procured after the assay development was in progress.

4.2.2 DNA Isolation Using the DNeasy® Blood and Tissue Kit

Once the ticks were dissected and placed in a labeled 1.5mL microtube, 180µL of Buffer ATL and 20µL of proteinase K was added to each dissected tick microtube and vortexed. Visual examination of each tube was made to ensure that all tick body parts were submerged. The microtubes were placed in a 56°C water bath overnight, to ensure optimal activity of the buffer and proteinase K. From this step, the Qiagen Supplementary Purification of total DNA from ticks using the DNeasy® Blood and Tissue Kit for detection of *Borrelia* DNA protocol¹⁰⁵ was used for the remainder of the DNA isolation procedures. The purity and concentration of the gDNA was then determined using the Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermofisher, Waltham, MA). The purified tick gDNA was stored in 1.5ml microtubes at -20°C.

4.2.3 DNA Isolation Using the Maxwell 48 Instrument

Once the ticks were dissected and placed in a labeled 1.5mL microtube, 300µL of Tissue Lysis Buffer and 30µL of Proteinase K was added to each dissected tick microtube and vortexed. The tubes were incubated at 56°C in a shaking water bath at 70

RPM overnight. The following day, the entirety of the liquid portion of the microtubes was added to the sample wells of the Maxwell RSC Tissue Kit cartridges. The cartridges were prepared per the instruction manual, and the samples were loaded into the Maxwell 48 DNA Isolation instrument. The RSC Tissue Kit protocol was selected and performed on the machine. The purity and concentration of the gDNA was then determined using the Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermofisher, Waltham, MA). The purified tick gDNA was stored in 0.5ml microtubes at -20°C.

4.2.4 Multiplex Assays Using Tick gDNA

After successful DNA isolation, the DNA samples from each tick were organized by tick species. Master mix assays were prepared for each tick species to be tested. All tick gDNA samples were tested using a 96-well plate. Each master mix was prepared for 105 samples to account for pipette loss. Each master mix contained 10 μ L of Taqman Multiplex Master Mix™ (Thermofisher, Waltham, MA), a final concentration of 250nM forward and reverse primers and probes for all targets relevant to the species of tick, 3 μ L of pathogen gDNA, and nuclease-free water to 20 μ L for each sample. 3 μ L of tick gDNA was considered optimal for all samples based on the results of the Nanodrop Spectrophotometer for the samples. The QuantStudio 5™ Real-time PCR instrument (Thermofisher Scientific, Waltham, MA) was used for all real-time testing of tick pathogen DNA. The real-time standard curve protocol for testing was an

initial 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

4.2.5 Verification of Positive and Negative Multiplex Results

To verify positive samples, each positive sample that resulted from the real-time PCR assay was subject to conventional PCR, followed by Sanger sequencing verification. 100% of all *Ba. microti*, *A. phagocytophilum*, *E. chaffeensis* and *E. ewingii* positives were subjected to conventional PCR and Sanger sequencing. A random sample of the *B. burgdorferi* positive samples were subjected to Sanger sequencing, as testing the large number of positive samples of *B. burgdorferi* was not statistically necessary and was also cost prohibitive. To achieve this, each pathogen-positive tick gDNA sample was first subjected to conventional PCR using the Promega GoTaq® Green Master Mix. 50µl samples contained 25µl of Promega GoTaq® Green Master Mix, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 2µl of sample tick gDNA and 21µl of nuclease-free water. 10µl of the resulting conventional PCR sample was run on a 1% agarose gel in 1X sodium borate buffer and run for 12 minutes at 300V. A DNA ladder was used to compare the appropriate band size of the amplicon.

Upon confirmation of amplification and band size, the remaining 40ul of the post-PCR product was cleaned using the Promega ReliaPrep™ DNA Clean-Up and Concentration System, then Sequenced using the Applied Biosystems SeqStudio™ Genetic Analyzer (Applied Biosystems, Forester City, CA). Cleaned DNA amplicons

were normalized to 1ng/μL per the recommendations of the BigDye™ Terminator v1.1 Cycle Sequencing Kit. Cycle reactions were again prepared using the Diluted reaction (0.5X) protocol in the BigDye™ Terminator v1.1 Cycle Sequencing Kit manual. Each 20μL Reaction was prepared with 4μL of BigDye™ Terminator v1.1 Ready Reaction Mix, 2μL of BigDye™ Terminator v1.1 5X Sequencing Buffer, 3.2pmol (final concentration) of the respective primer, 2μL of 1ng/μL of DNA template and the remaining volume of nuclease-free sterile deionized water to 20μL. Forward primer reactions and reverse primer reactions were set up for each DNA template. The cycle sequencing protocol for each sample was an initial incubation of 96°C, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

Once completed, the samples were purified using the BigDye XTerminator™ Purification Kit (Thermo Scientific, Waltham, MA). To each sample well, 90μL of SAM solution and 20μL of XTerminator™ Solution are added, and the samples were subjected to 30 minutes of shaking at 1800 RPM. The samples were placed in a 96-well reaction plate, then placed in the SeqStudio Genetic Analyzer. The plate manager setting for the SeqStudio Genetic Analyzer required specifying the use of the BigDye™ Terminator v1.1 Cycle Sequencing Kit. Subsequent .ab1 sequence data was uploaded to Snapgene® software, contigs were created, and the sequence was verified via NCBI BLAST.

4.3 Results

In order to interpret the results of the assay deployment, a Google Sheets™ spreadsheet was created to collect and organize all data from all ticks that were tested. The spreadsheet included information about each tick, including the tick ID number, the local location of collection, the county where the tick was collected, the sex of the tick, the developmental stage of each tick, and the real-time PCR results for each tick. Pivot tables were created to tabulate the percentages of infected ticks for each demographic above. In this manner, the data elucidated relative percentages of the tick-borne pathogens present per species, sex, developmental stage, and location in Delaware. In addition, a chi-square test was employed when all chi-square assumptions were met to compare pathogen-infected ticks that were collected in each county of Delaware for distribution significance based on life stage and county.

4.3.1 Collection and Reporting of Tick Pathogen Data

Tables 24 through 26 display the results of the tick pathogen testing of 500 *I. scapularis* ticks used in this study across the state of Delaware. The tables display information related to the species, life stage, sex, county, and chi-square results of the ticks that tested positive for the tick-borne pathogens.

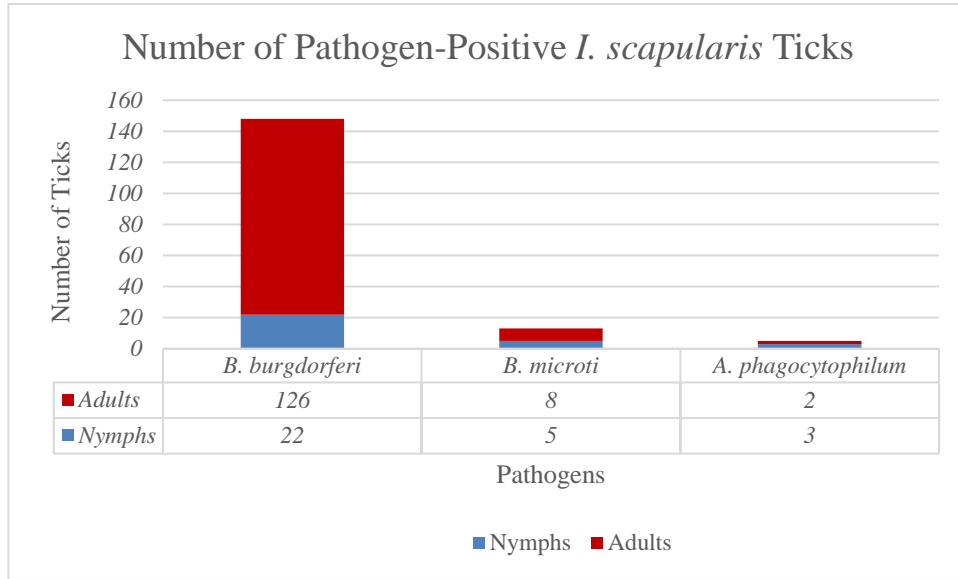


Table 24. The number of pathogen-positive *I. scapularis* ticks of the 500 ticks tested.

	Overall Percent positive	Percent positive by county	Adult percent positive	Nymph percent positive
<i>B. burgdorferi</i>	(151) 30.2%	NCC-(42) 23.73%	(126) 37.84%	(22) 14.38%
		KC-(63) 37.95%		
		SC- (46) 29.30%		
<i>Ba. microti</i>	(13) 2.6%	NCC-(4) 2.26%	(8) 2.4%	(5) 3.27%
		KC-0%		
		SC-(9) 5.73%		
<i>A. phagocytophilum</i>	(5) 1%	NCC-(3) 1.69%	(2) 0.6%	(3) 1.96%
		KC-(1) 0.64%		
		SC-(1)0.64%		

Table 25. Results of percent positive from testing 500 *I. scapularis* ticks by county and life stage.

			NCC	KC	SC	Total
Stage	Nymph	Count	15	9	1	25
		Expected Count	7.0	10.4	7.6	25.0
		% within group	60.0%	36.0%	4.0%	100.0%
Adult	Count	27	54	45	126	
	Expected Count	35.0	52.6	38.4	126.0	
	% within group	21.4%	42.9%	35.7%	100.0%	
Total	Count	42	63	46	151	
	Expected Count	42.0	63.0	46.0	151.0	
	% within group	27.8%	41.7%	30.5%	100.0%	

Table 26. Cross tabulation Chi-square table of life stage by county of Delaware of *B. burgdorferi* positive ticks. NCC= New Castle County KC= Kent County SC= Sussex County

The results of testing 500 *I. scapularis* ticks for the presence of *B. burgdorferi* showed that the overall percentage of the 500 ticks that were positive for *B. burgdorferi* was 30.2% (151/500). Of all the *I. scapularis* ticks infected with *B. burgdorferi*, 37.84% of adults were infected and 14.38% of nymphs were infected. Kent County, Delaware had the highest relative infectivity rate at 37.95%, followed by Sussex County with 29.3% infected and New Castle County with 23.73% infected. The distribution difference across each of the three counties, and including the life stage of positive ticks, was statistically significant using IBM™ SPSS Statistics with a Pearson χ^2 value of 18.281 and $p < .001$. This means that there was a statistically significant difference in the number of positive *B. burgdorferi* samples when comparing each county and life stage of positive *I. scapularis* ticks in Delaware.

The results of testing 500 *I. scapularis* ticks for the presence of *Ba. microti* showed that the overall percentage of the 500 ticks that were positive for *Ba. microti*

was 2.6% (13/500). Of all the *I. scapularis* ticks infected with *Ba. microti*, 2.4% of adults were infected and 3.27% of nymphs were infected. Sussex county was found to have the largest percentage of *Ba. microti* infected ticks with 5.73%, while New Castle County has 2.26% and Kent County had 0.0%. This difference can be explained in the relatively small sample size. Because of the small sample size, chi-square assumptions have been violated, and therefore no chi-square analysis could be performed.

The results of testing 500 *I. scapularis* ticks for the presence of *A. phagocytophilum* showed that the overall percentage of the 500 ticks that were positive for *A. phagocytophilum* was 1.0% (5/500). Of all the *I. scapularis* ticks infected with *A. phagocytophilum*, 0.6% of adults were infected and 1.96% of nymphs were infected. New Castle County, Delaware had the highest relative infectivity rate at 1.69%, followed by Sussex County with 0.64% infected and Kent County with 0.6% infected. Because of the small sample size, chi-square assumptions have been violated, and therefore no chi-square analysis could be performed.

There was evidence of coinfections of *I. scapularis* ticks with more than one pathogen. Of the 151 positive *B. burgdorferi* ticks 7 ticks (4.6%) were coinfecting with *Ba. microti*. An additional 2 ticks (1.3%) were infected with *A. phagocytophilum*. There was no evidence of *Ba. microti* positive ticks that were coinfecting with *A. phagocytophilum*. Finally, there were no ticks that were coinfecting with *B. burgdorferi*, *Ba. microti*, and *A. phagocytophilum*.

Tables 27 and 28 display the results of the tick pathogen testing of 500

D. variabilis ticks used in this study across the state of Delaware. The tables display information related to the species, life stage, sex and county of the ticks that tested positive for the tick-borne pathogens.

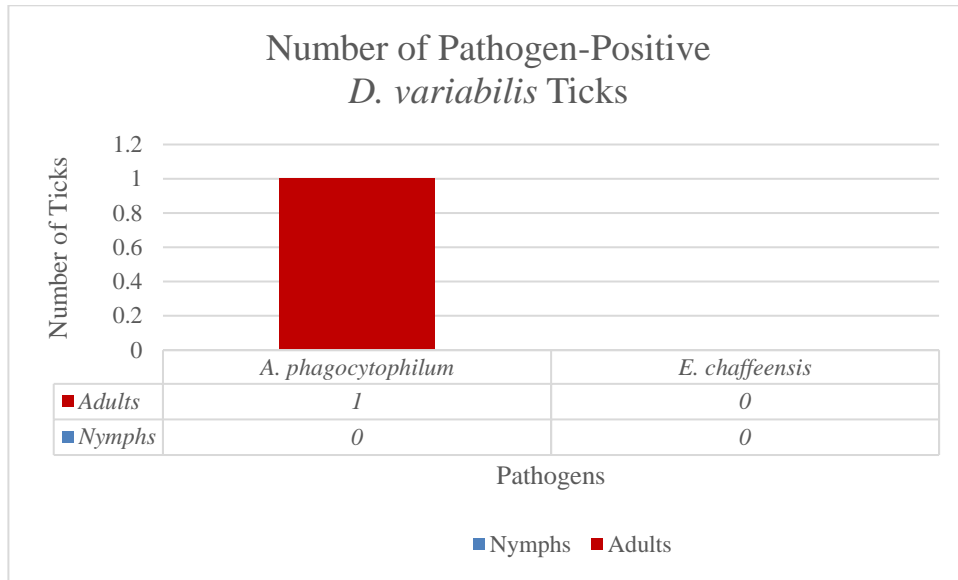


Table 27. The number of pathogen-positive *D. variabilis* ticks of the 500 ticks tested.

	Overall Percent positive	Percent positive by County	Adult percent positive	Nymph percent positive
<i>A. phagocytophilum</i>	(1) 0.2%	NCC-0.0% (1) KC-0.006% SC-0.0%	(1) 0.2%	0.0%
<i>E. chaffeensis</i>	0.0%	NCC-0.0% KC-0.0% SC-0.0%	0.0%	0.0%

Table 28. Results of percent positive from testing 500 *D. variabilis* ticks by county and life stage.

There was no evidence of *E. chaffeensis* in the 500 ticks sampled. However, 1/500 (.2%) of the *D. variabilis* ticks collected from Kent County, Delaware was positive for *A. phagocytophilum*. Because of the small sample size, chi-square assumptions have been violated, and therefore no chi-square analysis could be performed.

Tables 29-30 display the results of the tick pathogen testing of 527 *A. americanum* ticks used in this study across the state of Delaware. The tables display information related to the species, life stage, sex and county of the ticks that tested positive for the tick-borne pathogens.

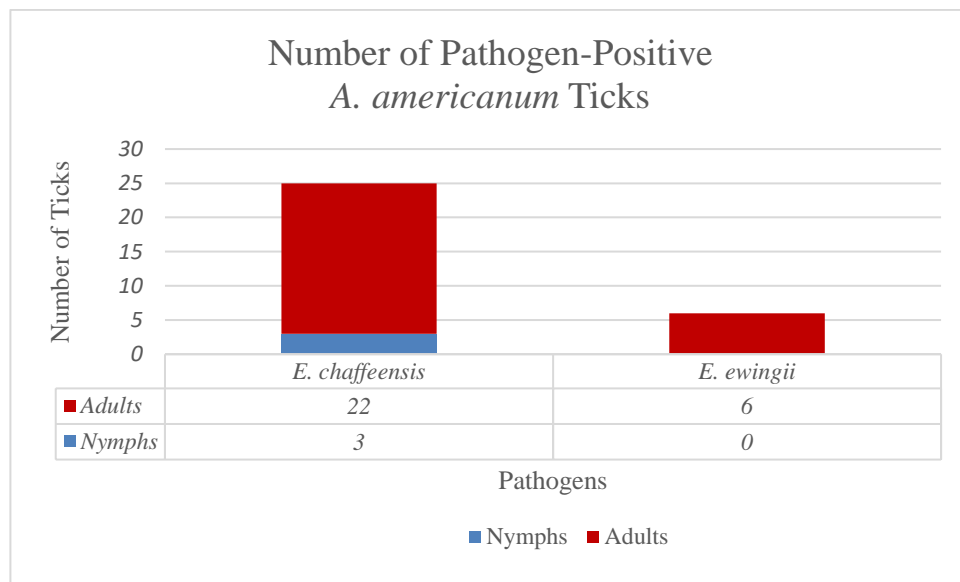


Table 29. The number of pathogen-positive *A. americanum* ticks of the 527 ticks tested.

	Overall Percent positive	Percent positive by county	Adult percent positive	Nymph percent positive
<i>E. chaffeensis</i>	(25) 4.74%	NCC-(2) 1.8% KC-0.0% SC-(23) 6.23%	(22) 13.92%	(3) 0.83%
<i>E. ewingii</i>	(6) 1.14%	NCC-0.0% KC-0.0% SC- (6)1.63%	(6) 3.8%	0.0%

Table 30. Results of percent positive from testing 527 *A. americanum* ticks by county and life stage.

The results of testing 527 *A. americanum* ticks for the presence of *E. chaffeensis* showed that the overall percentage of infection was 4.74% (25/527). Of all the *A. americanum* ticks infected with *E. chaffeensis*, 13.92% of adults were infected and 0.83% of nymphs were infected. Sussex County, Delaware had the highest relative level in infection at 6.23% of ticks infected with *E. chaffeensis*, where New Castle County had 1.8% infection and Kent County found no *A. americanum* ticks infected with *E. chaffeensis*. One of 25 (4%) of the ticks positive for *E. chaffeensis* was coinfecting with *E. ewingii*. Because of the small sample size, chi-square assumptions have been violated, and therefore no chi-square analysis could be performed.

The results of testing 527 *A. americanum* ticks for the presence of *E. ewingii* showed that the overall percentage of the 527 ticks that were positive for *E. ewingii* was 1.14% (6/527). Of all the *A. americanum* ticks infected with *E. ewingii*, 3.8% of adults were infected and 0.0% of nymphs were infected. All positive *A. americanum* ticks infected with *E. ewingii* were found in Sussex County, Delaware. Because of the small

sample size, chi-square assumptions have been violated, and therefore no chi-square analysis could be performed.

4.4 Discussion

The results of the deployment of the ISC1, DVA1, and AAM1 multiplex assays provided evidence of the presence of tick-borne pathogens in ticks collected in Delaware. There are many studies that have assessed the relative percentage of *I. scapularis* ticks infected with *B. burgdorferi*, where some percentages are as low as less than 1% to as high as 10–20 percent of infected nymphs or 50–60 percent infected adults¹⁰⁶. The infectivity rate of 30.2% (151/500) of *I. scapularis* ticks infected with *B. burgdorferi* fell within normal reported percentages. The infectivity rate of 2.6% (13/500) of *I. scapularis* ticks known to be infected with *Ba. microti* fell within reported percentages from 1% in newly endemic areas to 20% in some well-established endemic areas^{11,46,107}. Recent studies showing the relative infection rates of *I. scapularis* ticks with *A. phagocytophilum* range from 0.4% to 9%, however positive percentages can be higher^{53,108}. The infectivity rate of 1.0% (5/500) of the 500 *I. scapularis* ticks that were positive for *A. phagocytophilum* was also within the reported ranges of infection.

There is little data concerning the prevalence of *A. phagocytophilum* and *E. chaffeensis* in *D. variabilis* ticks^{25,109,110}. As expected, the infectivity rates of *D. variabilis* with either *A. phagocytophilum* or *E. chaffeensis* were extremely low at 1/500 (0.2%) and 0.0% respectively. Since these two organisms can be found in the *D. variabilis* tick, this study tested 500 *D. variabilis* ticks for both *A. phagocytophilum* and *E. chaffeensis*. Although there is little evidence of *D. variabilis* infections with *A.*

phagocytophilum or *E. chaffeensis*, testing for these pathogens was performed, given the notion that infections have been reported.

Previous studies concerning the relative infection rates of *A. americanum* with *E. chaffeensis* ranged from 2.6% to 17.4%^{78,79}. The positive *A. americanum* infection rate with *E. chaffeensis* was 4.74% (25/527). This infection rate fell within the normal range of infection found in previous studies. There are few studies identifying *A. americanum* infection rates with *E. ewingii*. However, the few studies concerning the relative infection rates of *A. americanum* with *E. ewingii* ranged from 0.4% to 8.2% in adults¹¹¹⁻¹¹³. The positive *A. americanum* infection rate with *E. ewingii* was 1.14% (6/527). The rate of 1.14% infection of *E. ewingii* found from this study of 527 *A. americanum* ticks fell within the normal range of infection found in other studies. Continued surveillance of these tick-borne pathogens in Delaware can provide a more accurate percentage of their presence in ticks collected in Delaware.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

The creation and implementation of real-time multiplex PCR assays for identifying the presence of tick-borne pathogens in Delaware is critical to providing stakeholders, researchers, and state agencies in Delaware with information relevant to public health. This study designed three multiplex real-time PCR assays to test for five tick-borne pathogens found in Delaware. Multiplex ISC1 successfully identified *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in the *Ixodes scapularis* tick. DVA1 successfully identified *Anaplasma phagocytophilum* in *Dermacentor variabilis* ticks. AAM1 successfully identified *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* in *Amblyomma americanum* ticks.

Given the increased number of interactions with humans^{114,115}, it is imperative for any region to have flexibility in its capability to manipulate testing targets as tick species and inherent pathogens immigrate and emigrate. The use of QSY probes allow for the versatility of adding new pathogen targets to the already created ISC1, DVA1 and AAM1 multiplexes. The results of testing all probes in one sample have shown that the probes using FAM, VIC, ABY and JUN dyes as they applied to this study, did not

show any interference, therefore making it highly possible to add new targets as necessary.

The multiplex assays were designed for both small and large sample testing. This allows the testing of either quantity-limited tick samples, or large-scale testing of thousands of ticks. The versatility of these multiplexes with high throughput testing is conducive for holistic, longitudinal studies of the prevalence of the relevant tick-borne pathogens. The major aspect of the multiplexes that make them useful for multiplexing is not only the versatility of the QSY probes, but also the cost to run each sample. At the time of the study, the average cost of the multiplex chemistry for one target was \$3.31. It is important to note that this cost does not include the cost of DNA isolation, as many methods are available for this purpose with differing costs.

Although the findings of this study will be useful to understanding the distribution and frequency of tick-borne pathogens in Delaware, there are limitations. The first limitation of this study is the relatively small sample size that was tested. The positive infection rates of the species tested was clearly limited by only sampling 500 or 527 ticks per species. Also, the ticks collected were almost exclusively collected in 2019 and 2020. Therefore, the infection rates are only relevant to that time. However, as additional ticks are tested, a more accurate determination of the infection rates will be apparent.

Another limitation was that the efficacy of the real-time assays developed in this study were not tested against more common established assays from the literature. This would include, but not limited to *Detection of Borrelia burgdorferi, Anaplasma*

phagocytophilum and *Babesia microti*, with two different multiplex PCR assays (Hojgaard et al, 2014)³⁴ and A real-time combined polymerase chain reaction assay for the rapid detection and differentiation of *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* (Bell, 2005)¹¹⁶. Funding was not available to test the assays from this study against other current assays using the same samples. Although this study does have limitations, it is important to not only have a better understanding of what ticks and inherent pathogens are present in Delaware, but also to have a tool to elucidate real time data concerning relative frequencies of these tick-borne pathogens in Delaware.

There are immediate plans for the future of this study. Given the success of these assays, an additional project will be developed soon. This project will expand both the DVA1 and the AAM1 Multiplexes with two more bacterial pathogen targets. An additional target for the DVA1 multiplex will be developed to target and potentially test for *Rickettsia rickettsii*. *Rickettsia rickettsii* is the causative agent of Rocky Mountain spotted fever and is the most well-described and deadly of the spotted group *rickettsiae*¹¹⁷. In 2020, there were 20 probable cases of spotted fever rickettsiosis in Delaware¹¹⁸. The pathogen is most often transmitted by the American dog (*Dermacentor variabilis*) tick in the eastern United States¹¹⁹, which is why this target will be added to the DVA1 multiplex if possible.

The second additional target for this first project will be expanding the DVA1 and the AAM1 multiplexes to both include targets for *Francisella tularensis*, the causative agent of tularemia. In the United States, *Dermacentor variabilis* and

Amblyomma americanum can both carry and infect humans with *Francisella tularensis*¹²⁰. The Rocky Mountain wood tick (*Dermacentor andersoni* Stiles) can also carry this pathogen, but this tick is not found in Delaware¹¹⁸. Cases of tularemia have recently been reported in Delaware, making this target of interest to the public¹²⁰. The results of this study, along with the possible addition of more pathogen targets, will provide the State of Delaware with a tool to identify the relative frequencies of tick-borne pathogens that pose a risk to human health.

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APPENDIX A

CENTERS FOR DISEASE CONTROL AND PREVENTION MATERIALS ACKNOWLEDGEMENT

The *Ehrlichia ewingii* DNA sample was obtained through Dr. Alyssa Snellgrove at the Center for Disease Control and Prevention. The sample DNA was extracted from a field-collected *A. americanum* tick (Panola Mountain, GA. The DNA was extracted in July 2019. The C_T was 26.3 using Killmaster, et al (2014) assay “Detection of bacterial agents in *Amblyomma americanum* (Acari: Ixodidae) from Georgia, US, and the use of a multiplex assay to differentiate *Ehrlichia chaffeensis* and *Ehrlichia ewingii*.”

APPENDIX B

LIST OF DISCONTINUED PRIMER AND PROBE SEQUENCES

<i>Borrelia burgdorferi</i>		
FORWARD PRIMER	REVERSE PRIMER	PROBE
GTGGTAGAGCGTCGGGTTG	GGAGTCGAACCTGCAACCTTC	TCCGAATGTCGCGGGTTCAAGCCC
GCCAGGAATATCTGCCCTTATC	CTCGTCCTTTGGTGGGTATTT	TTGCATTATCAAGGCTCTTGCTCA
ACGGTTAAACCTGAGCAAGAT	GGAATGGTCCTCTTGGTGTATT	ATTTGGCAACCTTAGCAGTCCCT
CCCAACGGGAACTAAATTTGC	GCAGCTGGACTTAAAGAGATTC CT	ATGCATTCAACGAGTTTTCTTGG CC
<i>Babesia microti</i>		
FORWARD PRIMER	REVERSE PRIMER	PROBE
CAGGGAGGTAGTGACAAGAAAT AACA	GGTTTAGATTCCCATCATTCCAA T	TACAGGGCTTAAAGTCT
CGACTACGTCCTGCCCTTTG	ACGAAGGACGAATCCACGTTTC	ACACCGCCCGTCGCTCCTACCG
GATCGAGTGATCCGGTGAATTA	CCTTGTACGACTTCTCCTTCC	TCGGACCAAGAAACGTGGATTTCGT
GATCGAGTGATCCGGTGAATTAT	CCTTGTACGACTTCTCCTTCC	TCGGACCAAGAAACGTGGATTTCGT
TGCACGCGCTACT	CCATCACGATGCATACTGTAAG ATT	
<i>Anaplasma phagocytophilum</i>		
FORWARD PRIMER	REVERSE PRIMER	PROBE
TATATCCAACCTCAACTTCCACTC	CATTCAAGTTCGCTAAGAGTTTA C	CTCCGCAATAGCATAGCCAGTTG
TATCCAACCTCAACTTCCACTCTA GC	TGATCCCTCATTCAAGTTCGCTA A	CGCCAATAGCATAGCCAGTTGCACC GT
CAGCGAGCGTTTCAAACCTTTT	CGGTGCAACTGGCTATGCTA	CAACTTCAACTTCCACTCTAGCTCC GCCA
<i>Ehrlichia chaffeensis</i>		
FORWARD PRIMER	REVERSE PRIMER	PROBE
ACTTGGAGAAGCATCACTGAA	CAGCATGGTAGAACTCGATGTA	AGCTGTTCAAGCAGCACTAGCAGT
GAGAGTTTGATCCTGGCTCAGAA	AGCAATTGTCCGTTCCGACTTG	CGCTGGCGGCAAGCCTAACACA
GAGAGTTTGATCCTGGCTCAGAA	AGCAATTGTCCGTTCCGACTTG	CGCTGGCGGCAAGCCTAACACA
<i>Ehrlichia ewingii</i>		
FORWARD PRIMER	REVERSE PRIMER	PROBE
GATGCCATATGTATTACCTTTTG G	TGAGCAAGACAGATTTGTTAAC TTGA	TGGCGAAGAACTATCAACTTCTCGT GC
GCAGGTGCAGCAACTTTA	CCCATTAATCTTATACCTTTGTC TC	TCATGGTGGAGCGAATGAAGCTGT
GGCCATAGGAATCCAGTAAGA	CATTTTCATACCATTGAGCAGACC	CGTTGTTTGCTTTAGCTAGAACATC TGGC
		TGTTGGCGAAGAACTATCAACTTCT CGT

APPENDIX C

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