King, Jenny. Validation of Droplet Digital PCR (ddPCR) for the Detection and Absolute Quantification of *Borrelia* DNA in *Ixodes* Ticks. Master of Science (Biomedical Sciences, Forensic Genetics). May 2015. 48 pages, 8 tables, 12 figures, 34 references.

In this research, QX200 Droplet Digital PCR (ddPCR™) system protocols for the detection of bacterial (*Borrelia burgdorferi* and *Borrelia miyamotoi*) DNA were developed and tested. Existing *Ixodes scapularis* samples collected from Cape Cod, Massachusetts and previously determined to be 60% positive for *B. burgdorferi* were utilized to investigate absolute bacterial genome carriage per tick using the ddPCR assays optimized here. The ddPCR technology proved to be a reliable means for detection and absolute quantification of control bacterial DNA with sensitivity as low as 10 spirochetes per µl input DNA. Application of ddPCR revealed an average *B. burgdorferi* carriage level of 27,239 copies in infected ticks (range: 231-118,407 copies), 2,197 copies in infected nymphs (range: 231- 4,983 copies), and 45,620 copies in infected adults (range: 5,647- 118,407 copies). This is the first known and validated application of ddPCR for the detection of *Borrelia* DNA in *Ixodes* ticks.

VALIDATION OF DROPLET DIGITAL PCR (DDPCR) FOR THE DETECTION AND ABSOLUTE QUANTIFICATION OF *BORRELIA* DNA IN *IXODES* TICKS

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Jenny King, B.S.

Fort Worth, TX

April 2015

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Michael Allen, for all of the assistance and guidance throughout my educational career at the University of North Texas Health Science Center. I would like to thank Dr. Martin Schriefer with the Division of Vector-Borne Disease at the Centers for Disease Control and Prevention for supplying bacterial specimens for testing in this project. A special thanks to my committee members: Dr. Joseph Warren, Dr. Raghu Krishnamoorthy, and Dr. Anuja Ghorpade for their support and guidance over the course of my graduate career. I am extremely grateful for the suggestions, encouragement, and guidance I received from Elizabeth Mitchell and Ashley Smith during the entirety of this research project. I would also like to give a special thanks to my classmates in the Forensic Genetics program, especially Lindsey Thompson, for their friendship and support throughout our schooling. Most importantly, I want to thank my amazing family for their unwavering love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Background on Tickborne Illnesses and* Borrelia *Pathogens

Borrelia burgdorferi and *Borrelia miyamotoi* are spirochetes and tickborne obligate parasites. These pathogenic bacteria are commonly found in the hard bodied tick *Ixodes scapularis*, more commonly known as the deer tick (*1, 2*). *B. burgdorferi* is the causative agent of Lyme disease and *B. miyamotoi* is in the relapsing fever *Borrelia* group (*3, 4*). The prevalence of these tickborne illnesses and the pathogenic agents which cause them are of growing concern in several areas nationwide and around the world (Fig. 1) (*3-9*).

Lyme disease is transmitted to humans via infected tick bites (*6*). Signs and symptoms of this illness vary between infected individuals and over time (*10*). However, this disease is typically associated with a characteristic rash which resembles a "bull's eye". Later onset symptoms can include fever, headache, and fatigue. If left untreated, Lyme disease can lead to more serious complications including nerve, joint, and heart damage. Lyme disease is diagnosed based on symptoms, physical rashes, the possibility of exposure to infected ticks, and correct and validated laboratory testing methods (*4*). According to the Centers for Disease Control and Prevention (CDC), Lyme disease is contracted by more than 20,000 individuals each year in the United States (*4, 6*).

Relapsing fever is an illness which is known to be caused by the tickborne pathogen *Borrelia miyamotoi (3)*. Symptoms of relapsing fever include alternating episodes of fever, several forms of body pains, as well as, vomiting and a rash (which could present similarly to the characteristic bull's eye rash of Lyme disease), among numerous other symptoms. Interestingly,

most relapsing fever-causing agents are transmitted among vertebrates via soft tick vectors; whereas, *B. miyamotoi* use *Ixodes*, or hard ticks, as vectors (*11*).

Lyme disease is typically associated with co-infection with other diseases because *Ixodes* ticks often carry and transmit other pathogens in addition to *B. burgdorferi (12)*. Additionally, *B. miyamotoi* is known to be present in all tick species that transmit Lyme disease (i.e. hard ticks). In recent years, several cases of *B. miyamotoi* infection have been found in areas where Lyme disease is endemic in the United States and other parts of the world (*3, 5, 8, 13-15*). Therefore, it stands to reason that *Ixodes* tick screenings should test for *B. burgdorferi* as well as *B. miyamotoi* infections. However, current detection and diagnostic approaches for Lyme disease- and relapsing fever-causing pathogens, such as qPCR, have significant room for improvement (*16, 17*). Droplet Digital™ PCR (ddPCR™) (Bio-Rad Laboratories, Inc., Hercules, California) is a promising technology for this task because it allows for sensitive, specific detection of single template molecules as well as precise quantification of target DNA (*17-20*).

Confirmed cases of Lyme disease by state or locality, 2001-2013													
Year	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
U.S. Total	17,029	23,763	21,273	19,804	23,305	19,931	27,444	28,921	29,959	22,561	24,364	22,014	27,203

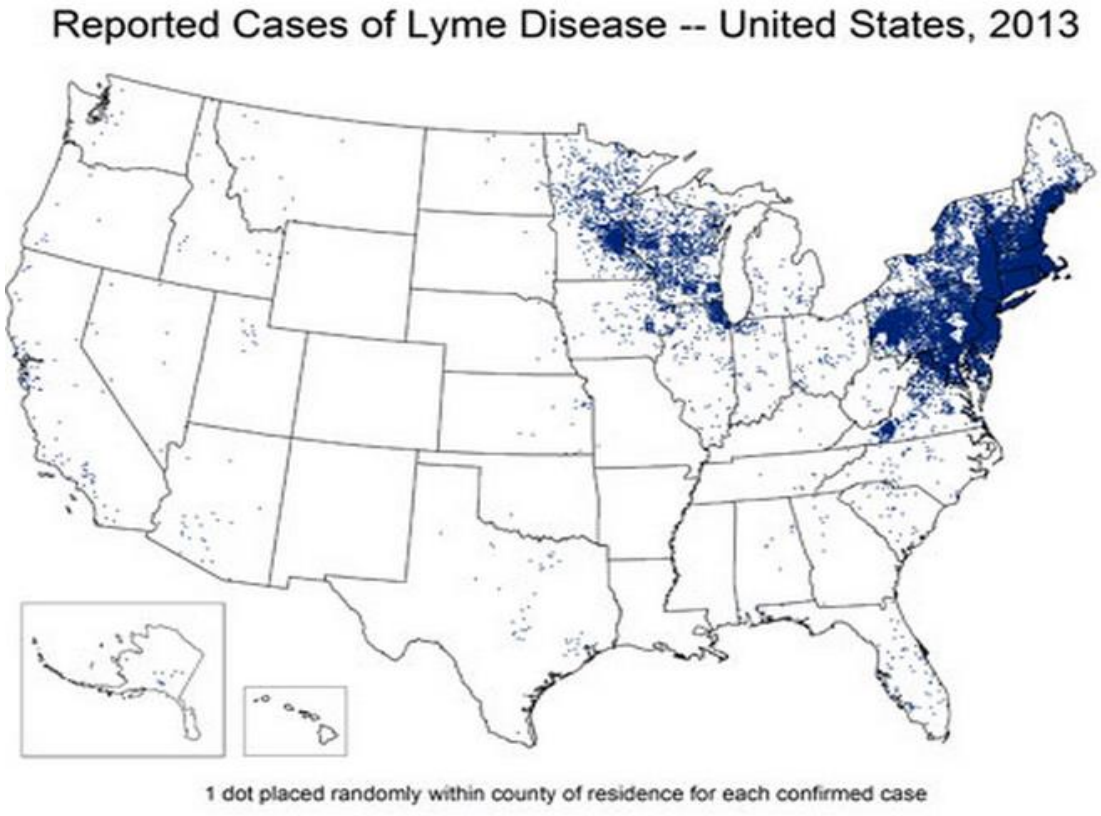

Figure 1. Lyme disease is endemic and of growing concern in areas of the nation. This map and information were obtained from the CDC website (CDC, in *Lyme Disease*. (Centers for Disease Control and Prevention, http://www.cdc.gov/lyme/stats/maps/interactivemaps.html, 2014), vol. 2014, pp. Map of Reported Cases of Lyme Disease in the United States in 2013). Each blue dot represents a confirmed case of Lyme disease in the US during 2013. The table shows that the number of confirmed cases of Lyme disease has seen an almost 60% increase over the past decade in the US.

Background on PCR Methods of Detection

The molecular method currently employed in diagnostics for the detection of pathogens in ticks is the polymerase chain reaction (PCR) (*3, 14, 16*). The first generation of PCR methods yielded qualitative detection results obtained through end-point analysis by gel electrophoresis (*18*). The second generation of PCR platforms is known as real-time or quantitative PCR (qPCR) (*18, 21*). In one iteration, real-time detection of PCR products is achieved in each qPCR reaction through the attachment of a fluorescent reporter molecule to specific TaqMan® (Life Technologies, Carlsbad, California) probes which, upon amplification of target DNA products, becomes cleaved and fluoresces (Fig. 2). This fluorescence can be measured and a DNA quantity interpolated from a constructed standard curve. In essence, an increased amount of fluorescence corresponds to an increased amount of target DNA product (*21*). Though the quantitative approach has proven to be a relatively successful diagnostic tool, the process of conventional PCR methods can be time-consuming, costly, and imprecise compared to more novel PCR methods (*18, 22*).

It has been suggested that ddPCR is a more reliable, sensitive, and robust technique compared to qPCR. This is due in part to the fact that the accuracy of qPCR is limited by the fact that amplification efficiencies and cycle threshold (C_T) values generated can vary greatly between runs (*18*). Unlike the relative measurements of DNA concentration obtained through qPCR methodologies, ddPCR enables the absolute quantification of target DNA molecules (*18, 20, 23*). This capability is a defining characteristic of ddPCR and is advantageous to a wide variety of applications, including large bodies of research and a growing number of molecular

diagnostic tests (*18*, *20*, *22*). For instance, ddPCR has proven to be an effective diagnostic technology suitable for research and clinical use in diagnosing ocular *Chlamydia trachomatis* infections (*24*).

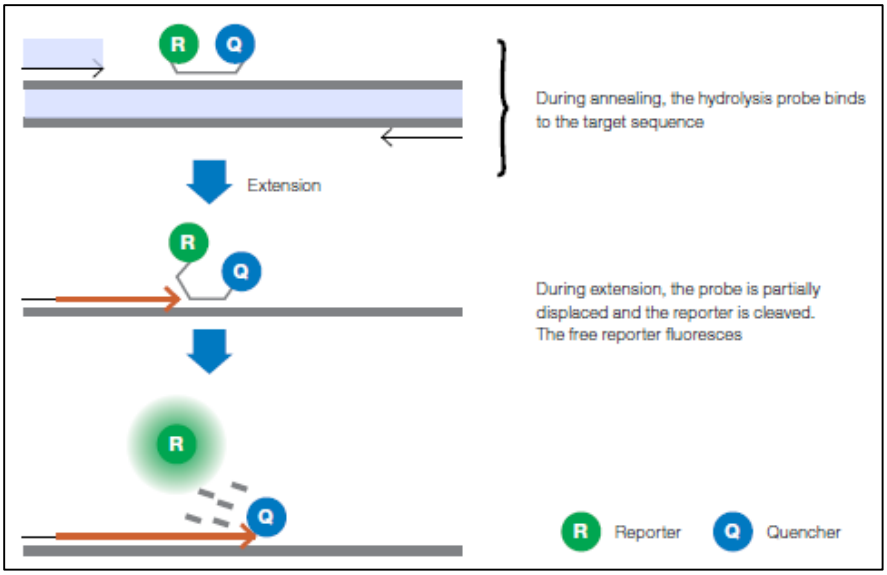

Figure 2. Hydrolysis of TaqMan probes during qPCR amplification process *(*I. Bio-Rad Laboratories. (Life Science Group), pp. 1-100.**)**(*19*). When a target sequence bond by a TaqMan probe is amplified during PCR, the reporter dye will emit a fluorescent color which can then be detected and measured. An increase in amplified template yields an increase in fluorescence. This probe application is compatible for both qPCR and ddPCR assay chemistries.

The ddPCR technique is centered on the partitioning of a single DNA sample into an emulsion containing tens of thousands of smaller individual reaction liposomes, known as "droplets" (Fig. 3) (*19, 20, 23*). Polymerase chain reaction is subsequently carried out to amplify the target DNA template within each droplet. The droplets are then counted as either positive or negative based on the presence or absence of amplicons which is measured by target-dependent fluorescence signals (*23*). The "digital" aspect of the system refers to the simple readout of droplet partitions as a binary code of ones (positive) and zeroes (negative) and, because the presence of a target molecule in a given droplet is a random event, the associated data fits a Poisson distribution (*19, 23*). This allows for direct and simple calculation of DNA copy number in a given sample without the obligation of a standard curve, an improvement upon qPCR requirements (*17, 18, 20, 22, 23*). Droplet partitioning during ddPCR reduces bias from PCR amplification efficiency and inhibitors, as well, which in turn reduces error rates and enables accurate quantification of DNA template (*19, 20*). Droplet partitioning also reduces competitive amplification effects, allowing template detection an order of magnitude more sensitive than that obtainable through qPCR (*18*). ddPCR has also proven to be a more robust technique than qPCR for the amplification of DNA in the presence of known PCR inhibitors (*25*).

Although the quality of the results yielded by ddPCR are distinct from those obtained through qPCR, this technology uses assay chemistries homologous to those widely used for qPCR applications (i.e. TaqMan) (*16, 18, 26, 27*). This allows for a relatively seamless transition between second and third generation PCR platforms in laboratories world-wide. While ddPCR detection of certain pathogens (e.g. *Borrelia spp*.) has clear and notable advantages over existing technologies; by way of contrast, the use of this next generation PCR technology for this endeavor has yet to be validated in a controlled laboratory setting.

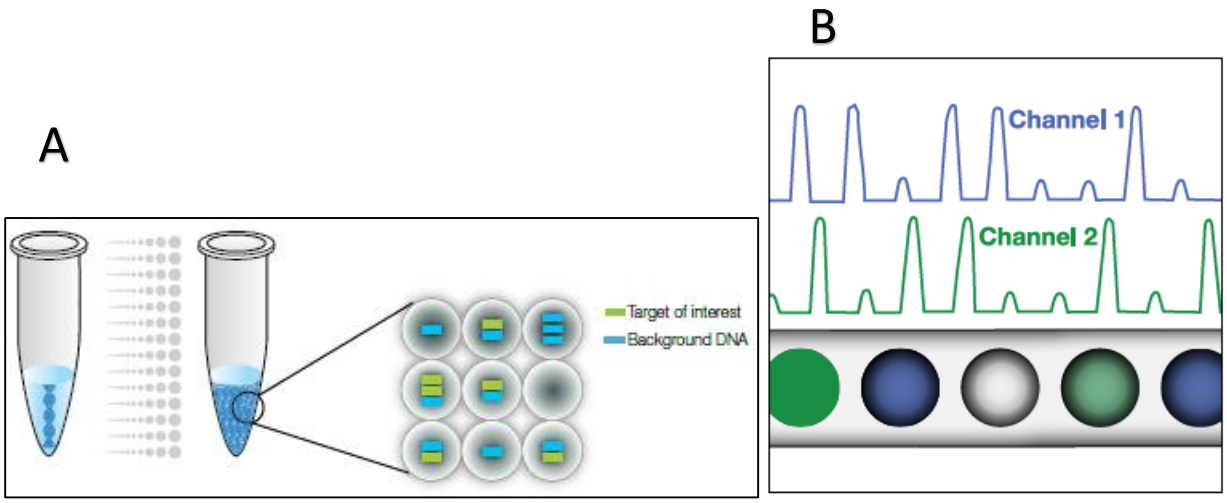

Figure 3. ddPCR droplet partitioning and reading uses a two-color fluorescence system (I.Bio-Rad Laboratories. (Life Science Group), pp. 1-100.)(*19*). **A.** In ddPCR, a single PCR sample is partitioned into ~20,000 droplets. Target and background DNA are distributed at random into the droplets during the partitioning process. **B.** After PCR, fluorescent readings are measured by the QX200 Droplet Reader for each droplet in two channels based on color. "Positive" droplets will contain at least one copy of the target DNA molecule and will exhibit a higher intensity of fluorescence. "Negative" droplets will contain zero copies of the target DNA molecule and will exhibit little or no fluorescence.

Research Significance

It is hypothesized that ddPCR can be used to reliably detect the presence of *B. burgdorferi* and *B. miyamotoi* DNA using optimized assays with specifically designed primers and probes, and that accurate quantification of *B. burgdorferi* template will reveal the actual pathogen carriage-level of infected *I. scapularis* ticks. Since these pathogens are known associates in the transmission of Lyme disease and other tickborne illnesses via tick vectors,

precise detection of *Borrelia spp*. is important from the following standpoints: public health, emerging infectious disease monitoring, and biosecurity. Extensive evidence of the prevalence of *Borrelia spp*. infection among people in the United States and other parts of the world lends additional support to the present need for monitoring of tickborne diseases (*8, 13*). This is the first known and validated application of ddPCR for the detection and quantification of *Borrelia* DNA in infected *Ixodes* ticks.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

As previously stated, the hypothesis associated with this research focused on the ability of ddPCR to reliably detect the presence of *Borrelia* DNA through optimized assays with species-specific primers and probes, and that accurate quantification of *B. burgdorferi* or *B. miyamotoi* will reveal the actual pathogen carriage-level in infected ticks. This hypothesis was tested through three different experimental phases. The first phase was to determine the absolute quantification capabilities and sensitivity of each ddPCR assay targeting *Borrelia spp*. During the second phase of this research, these optimized assays were tested on ddPCR reactions containing *I. scapularis* extracts, previously determined to be negative for *Borrelia* infection, and spiked with serial-diluted amounts of control *Borrelia* DNA. This was done to evaluate inhibition of *Borrelia* quantification in the presence of host DNA. In the third and final phase, the developed ddPCR assay specific to *B. burgdorferi* was applied to existing DNA extracts of *B. burgdorferi*-infected deer ticks. The results of this phase were used to estimate the number of genomic copies of *B. burgdorferi* possessed by each positively-infected tick.

DNA Samples

Known *Borrelia burgdorferi* DNA used as a positive control in this study was *B. burgdorferi* strain B31 genomic DNA (35210^{TM}) (ATCC®, Manassas, VA).

Known *Borrelia miyamotoi* DNA used as a positive control in this study was *B. miyamotoi* strain US178 (Rhode Island) provided by the Center for Disease Control and Prevention (CDC).

Tick samples analyzed during the course of this study were available from the tick laboratory at the UNT Health Science Center. These *Ixodes scapularis,* i.e. deer ticks, were originally obtained and tested for *B. burgdorferi* infection prior to the start of this thesis research (*28*). This sampling of *I. scapularis* was collected from the Lyme disease-endemic region of Cape Cod. This collection, consisting of nymph (sex not determined) and adult female ticks, was previously tested and determined to be 60% positive for the presence of *B. burgdorferi.* This testing process was accomplished through the following steps in a total of ten, female deer ticks: pulverization and DNA extraction of entire tick specimens (using the Omega Biotek E.N.Z.A. ® Mollusc DNA Kit according to the manufacturer's protocol) with elution in 140 µl of DNA Elution Buffer, followed by a control-PCR analysis (using primers targeting the mitochondrial 16S rRNA gene found in tick DNA), and a nested PCR analysis (amplifying a portion of the *flaB* gene via *Borrelia* genus-specific primers). Of these tested samples, two negative and six positive ticks were utilized in the second and third phases of this research project, respectively. *I. scapularis* extracts were quantified prior to ddPCR analysis using the Qubit® dsDNA HS Assay Kit (Life Technologies) to determine total concentration of double-stranded DNA present in each sample (Table 1).

Table 1. Deer tick DNA samples. *I. scapularis* DNA extracts previously tested for *B. burgdorferi* infection and analyzed via ddPCR in experimental Phases 2 or 3. Adult-stage deer ticks are in bold.

Tick Sample #	Stage	Results of *B. burgdorferi* Testing	ddPCR Phase Analyzed
1	Nymph	Positive	Phase 3
2	Nymph	Negative	Phase 2
3	Nymph	Negative	Phase 2
4	Nymph	Positive	Phase 3
6	**Adult**	**Positive**	**Phase 3**
7	**Adult**	**Positive**	**Phase 3**
9	**Adult**	**Positive**	**Phase 3**
10	Nymph	Positive	Phase 3

Genospecies-Specific PCR Primers and Probes

Widely accepted qPCR primer design guidelines also apply to the design of ddPCR primers and probes (*19*); therefore, *Borrelia* species-specific primer and probe sets used during this research were selected according to those previously described in Ullmann et al. 2005 and are detailed in Table 2 (*16*).

Oligonucleotide primers and probe sequences specific for the *B. burgdorferi ospA* gene and the *B. miyamotoi glpQ* gene were used. Primer sequences specific for *the B. burgdorferi ospA* gene were identified as MOspA-F (5'-GYAAAGTAAAATTAACART) and MOspA-R (5'-TGTTTTRCCATCTTCTTT) to generate a 74-bp fragment. TaqMan probe was identified as MBurg-P (5'- 6-FAM-GACGATCTAGGTCAAACC-MGBNFQ) and labeled with a 5' 6-FAM

(blue) dye and a 3' MGB/nonfluorescent quencher (MGBNFQ). Primer sequences for the hard tick relapsing fever group *Borrelia glpQ* gene were identified as MglpQ-F (5'-GATAATATTCCTGTTATAATGC) and MglpQ-R (5'-CACTGAGATTTAGTGATTTAAGTTC) to generate a 100-bp fragment. TaqMan probe was identified as MglpQ-P (5'-VIC-CCCAGAAATTGACAACCAC-MGBNFQ) and labeled with a 5' VIC (green) dye. Sequences were evaluated for specificity, length of amplicon, secondary structuring from internal primer binding, G-C content, and the melting temperature of primers and probes.

Table 2. Primers and probes. Oligonucleotide primers and probes used in ddPCR assays targeting *Borrelia* DNA. Coloring of FAM/ VIC probe represents the color of fluorescent dye, and therefore the color of generated and detected PCR-positive droplets, associated with the particular *Borrelia spp*. which it targets (i.e. *B. burgdorferi* template will fluoresce blue and *B. miyamotoi* template will fluoresce green).

Pathogen	Target gene	Name	Primers and Probe Sequence (5' →3')	Size (bp)	Reference
Borrelia burgdorferi	*ospA*	MOspA-F	GYA AAG TAA AAT TAA CAR T *(forward)*	74	Ullmann et al. 2005
		MOspA-R	TGT TTT RCC ATC TTC TTT *(reverse)*		
		MBurg-P	**6-FAM**-GAC GAT CTA GGT CAA ACC-**MGBNFQ**		
Borrelia miyamotoi	*glpQ*	MglpQ-F	GAT AAT ATT CCT GTT ATA ATG C *(forward)*	100	Ullmann et al. 2005
		MglpQ-R	CAC TGA GAT TTA GTG ATT TAA GTT C *(reverse)*		
		MglpQ-P	**VIC**-CCC AGA AAT TGA CAA CCA C-**MGBNFQ**		

Droplet Digital PCR

Droplet Digital PCR for both *ospA* and *glpQ* assay formats was performed, with a PCR reaction volume of 20 µl, using the ddPCR™ Supermix for Probes (no dUTP) master mix (Bio-Rad, Hercules, CA). Reaction conditions included 10 µl of ddPCR Probe Supermix, forward and reverse primers at 900nM each, probes at 250nM, and template DNA. The PCR reaction mixture was loaded into an 8-well DG8™ Cartridge (Bio-Rad) and droplets were formed with the emulsion device, Bio-Rad QX100™ Droplet Generator, following the manufacturer's instructions. During emulsion, the QX100 droplet generator partitions the samples into 20,000 nanoliter-sized droplets. The droplet contents were then transferred to a 96-well plate and sealed with a Bio-Rad PX1™ PCR Plate Sealer, as per Bio-Rad recommendations.

For analyses focused on *B. burgdorferi* detection, reactions involving the *ospA* assay format were amplified in a Bio-Rad C1000 Touch™ thermal cycler with the following cycling conditions: initial activation of the *Taq* polymerase at 95ºC for 10 min, followed by 50 cycles of 30-s denaturation at 94 ºC and a 1 min annealing-extension step at 49 ºC, followed by a final 10 min enzyme deactivation step at 98 ºC, with an overall ramp rate of 2 ºC/s.

For analyses focused on *B. miyamotoi* detection, reactions involving the *glpQ* assay were amplified in a Bio-Rad C1000 Touch™ thermal cycler with the following cycling conditions: an initial activation of the *Taq* polymerase at 95ºC for 10 min, followed by 50 cycles of 30-s denaturation at 94 ºC and a 1 min annealing-extension step at 52 ºC, followed by a final 10 min enzyme deactivation step at 98 ºC, with an overall ramp rate of 2 ºC/s.

After cycling, droplets were immediately analyzed or stored at 4ºC overnight and until analysis in the QX200™ Droplet Reader.

Data Acquisition and Analysis

The QX200 Droplet Reader analyzes each droplet individually, in a single-file fashion, using a two-color, two-channel detection system (Fig. 3). Products amplified via the *ospA* assay were read under the Channel 1, "blue dye" system while products amplified via the *glpQ* assay were read as "green dye" under Channel 2. Droplets were classified as PCR-positive or PCR-negative according to a threshold manually set across all wells within a single run based upon results of the no template or negative control sample. Positive droplets contain at least one copy of the target DNA molecule and display increased fluorescence compared to its negative counterparts.

The number of positive and negative droplets read in each channel is used by the QuantaSoft™ v.1.7.4.0917 (Bio-Rad) software to calculate the concentration of the target DNA sequences, along with their Poisson-based 95% confidence intervals (*18*). The number of template copies per unit volume μ was estimated from the number of positive events *n* detected by the droplet reader in the corresponding channel (channels 1 and 2 for FAM and VIC dyes, respectively), and the number of total droplets *N* by maximum likelihood (*27*). The distribution of templates within a drop was assumed to follow a Poisson distribution, and the number of positive droplets was assumed to follow a binomial distribution. 95% confidence intervals were estimated under the same assumptions. The droplet size was assumed to be 0.91 nl, consistent with the instrument manufacturer's software. (*27*) The concentration reported by QuantaSoft is copies of template per μl of the final 1x ddPCR reaction. This value was multiplied by the reaction volume to generate results in terms of template copies detected per sample. This data was generated for each well and used for analysis.

Phase 1: Limit of Detection and Absolute Quantification

A 1: 100 dilution of each control *Borrelia spp*. DNA sample was made and total double-stranded DNA (dsDNA) concentration quantified via Qubit® dsDNA HS Assay Kit (Life Technologies). From this, a 7-sample standard dilution series was created for testing via ddPCR during Phases 1 and 2 of this study. Serial dilutions of control DNA were prepared separately for each *Borrelia spp*. in order to determine the sensitivity of the ddPCR instrument in the case of each pathogen. Serial dilutions ranged from 10 to 100,000 copies per reaction of control *B. burgdorferi* DNA, and 6 to 165,000 copies per reaction of control *B. miyamotoi* DNA. PCR reactions were prepared according to the ddPCR reaction conditions previously detailed, with an added template volume of 1 µl diluted *Borrelia* DNA. Expected genomic copies were calculated at each sample concentration according to the equation found in Figure 4. Expected copies were then compared with measured copies generated by the QuantaSoft (Bio-Rad) software in order to evaluate the ddPCR system's ability to provide absolute quantification of known samples of *Borrelia* DNA.

Figure 4. Equation for expected copy numbers in control DNA samples. The theoretical bacterial genome copies expected at a concentration of control *Borrelia* DNA was found during Phase 1 analyses. *Note: Linear* Borrelia *chromosomes are ~910 Kb and ~907 Kb in length for* B. burgdorferi *and* B. miyamotoi*, respectively (29, 30); Avogadro's constant represents the amount of molecules per mole; molecular weight of a double-stranded DNA molecule is the number of basepairs multiplied by the average weight of a basepair (650 daltons) (31); 1 x 10^9 is the unit conversion between nanograms and grams.*

$$\text{\# genome copies} = \frac{(\text{ng template}) \times (6.022 \times 10^{23})}{(\text{genome size in bp}) \times 650\ \text{Da/bp}) \times (1 \times 10^{9})}$$

Phase 2: PCR Inhibition by Background Host DNA

To evaluate potential inhibitory effects on the performance of ddPCR detection and absolute quantification, serial diluted amounts of known *Borrelia* DNA were spiked into pre-determined amounts of host background (*I. scapularis* tick) DNA prior to droplet formation. PCR volumes were created to include 10 µl of ddPCR Probe Supermix, forward and reverse primers at 900nM each, probes at 250nM, and 4.9 µl of uninfected deer tick DNA (tick #2 or tick #3 sample extracts) spiked with 1 µl of varying amounts of control *Borrelia* template. The *I. scapularis* samples, previously determined as negative for *B. burgdorferi,* which were used in Phase 2 were tick #2 and tick #3 for the *ospA* and *glpQ* assays, respectively (Table 1). The spiked-in concentrations consisted of the same template amounts generated from the serial dilutions performed during Phase 1 of this study. Once ddPCR was completed for Phase 2 samples, the results were evaluated for concordance with the results obtained for correlating samples run in Phase 1 and background tick DNA inhibition evaluated.

Phase 3: Validation and Estimation of Pathogen Carriage Levels in Ticks

Six *Ixodes* ticks (3 adults, 3 nymphs) collected from endemic areas of Cape Cod, available from the tick laboratory at the UNT Health Science Center, and previously determined as being positive for *B. burgdorferi* infection, were selected for ddPCR testing in order to validate the *ospA* assay (Table 1). Validation of developed ddPCR protocols was carried out only for the *ospA* assay because the presence of *B. miyamotoi* had not previously been determined in available tick samples. The previously described ddPCR *ospA* assay format was applied to each positive tick sample in replicate. PCR reactions were created to include 10 µl of ddPCR Probe Supermix, *ospA* forward and reverse primers at 900nM each, *ospA* probe at 250nM, and 5.9 µl of

B. burgdorferi-infected deer tick DNA extract at various concentrations. The results generated by the QuantaSoft (Bio-Rad) software regarding these samples were used for analysis. The carriage level in infected *I. scapularis* hosts was estimated by calculating the number of spirochete detected per infected tick. This value was found by multiplying the elution volume of tick sample extract (140 µl) by the number of template copies detected per µl template (a product of input template amount and generated copy number).

CHAPTER III

RESULTS AND DISCUSSION

Optimal Annealing Temperatures for **Borrelia** *DNA Amplification*

The annealing temperatures which produced optimal ddPCR results for the *ospA* and *glpQ* assays created for the purposes of this research were determined by incorporating a temperature gradient into thermal cycling conditions for the annealing-extension step of amplification. PCR reactions were made following the ddPCR conditions previously described for eight samples containing the same concentrations of template of respective *Borrelia spp*. DNA. The resulting droplet digital data from the investigation of optimal annealing temperature for the *B. burgdorferi*- targeting assay, *ospA*, can be found in Figure 5A. No amplification of bacterial DNA was achieved with the *ospA* assay above an annealing temperature of 54.8°C. The optimal ddPCR result had a clustering of positive droplets at a fluorescent amplitude above 6,000 rfu (relative fluorescent units) and minimal "rain"-down of positive droplets from this line of positive-clustering, and is seen in the sample annealed at 48.8°C. Due to this result, 49°C was selected as the optimal annealing temperature at which to amplify *B. burgdorferi* DNA when using the *ospA* assay for ddPCR technology.

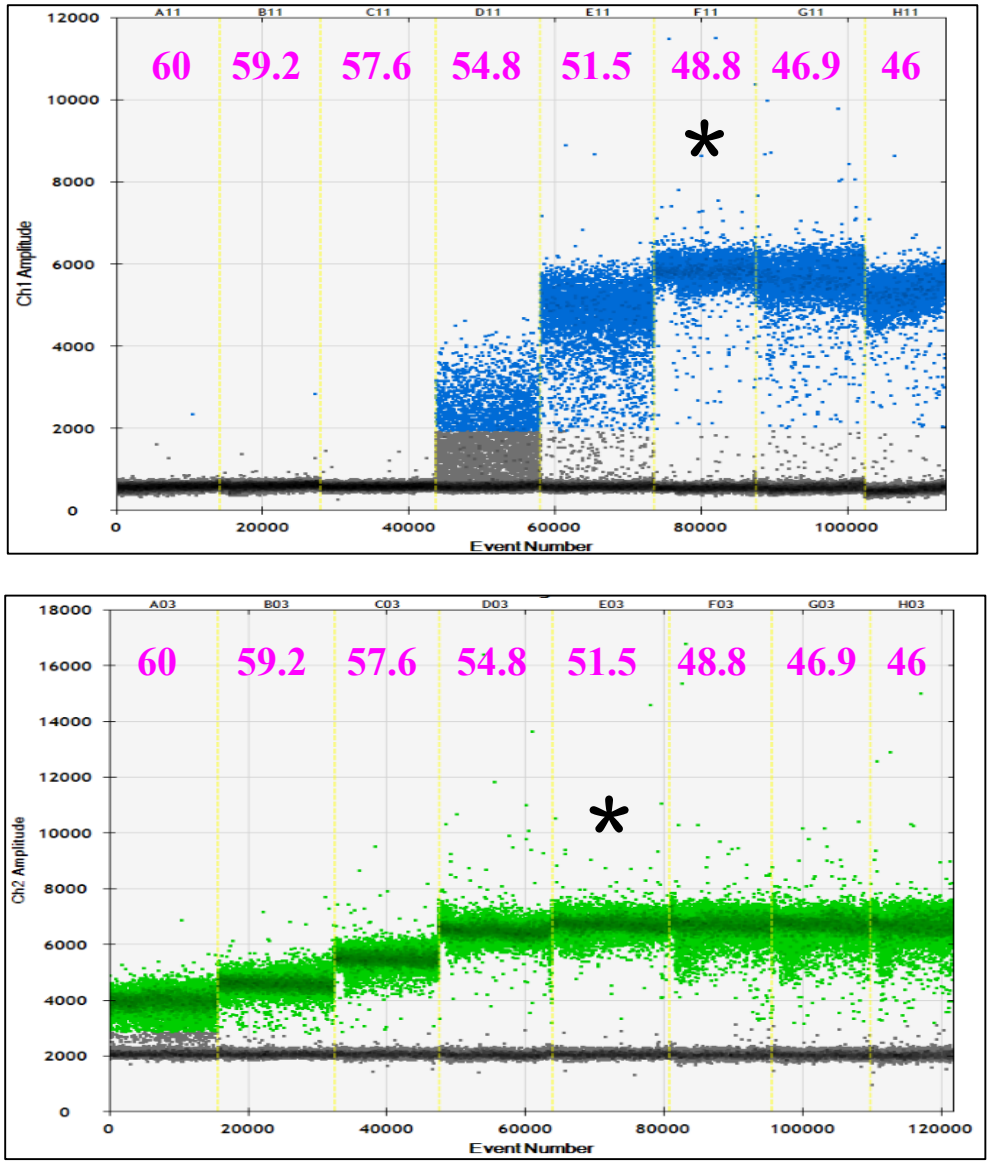

Figure 5. Optimal annealing temperatures for *Borrelia* DNA assays. A temperature gradient was used to determine optimal annealing temperatures for assays. The annealing temperature tested per sample is in pink and indicated in each ddPCR well in degrees Celsius (°C). Optimal ddPCR sample results are indicated by an asterisk. **A.** ddPCR results of optimal annealing temperature for *ospA* assay amplification of *B. burgdorferi* target. **B.** ddPCR results of optimal annealing temperature for *glpQ* assay amplification of *B. miyamotoi* target.

The droplet digital data resulting from the investigation of optimal annealing temperature for the *B. miyamotoi*- targeting assay, *glpQ*, can be found in Figure 5B. Positive amplification droplets were generated at each annealing temperature investigated for the *glpQ* assay. However, the sample which generated the optimal ddPCR data (with a positive-droplet line around 7,000 rfu and little droplet rain) was annealed at 51.8°C. Therefore, 52°C was selected as the optimal annealing temperature at which to amplify *B. miyamotoi* DNA when using the *glpQ* assay for ddPCR technology.

Phase 1: Limit of Detection and Absolute Quantification

The limit of detection with the ddPCR assays, *ospA* and *glpQ*, was evaluated. To investigate the intrinsic detection limit for the *ospA* ddPCR assay, a serial dilution of known *B. burgdorferi* DNA was tested in two separate trials. The dilution samples included in the first trial were not quantified prior to droplet generation and ddPCR analysis; therefore, exact template concentrations remain unknown and results obtained were not used to draw final conclusions regarding absolute quantification nor limit of detection. The ddPCR data output from Phase 1, trial 1 analysis of *B. burgdorferi* DNA can be found in Figure 6A. In trial 2, when dilution samples were quantified via Qubit prior to droplet generation, an absolute quantification of *B. burgdorferi* spirochetes was achieved in known samples down to the lowest dilution sample concentration of 10 fg/µl (approximately 10 copies of spirochete DNA per µl) (Table 3).

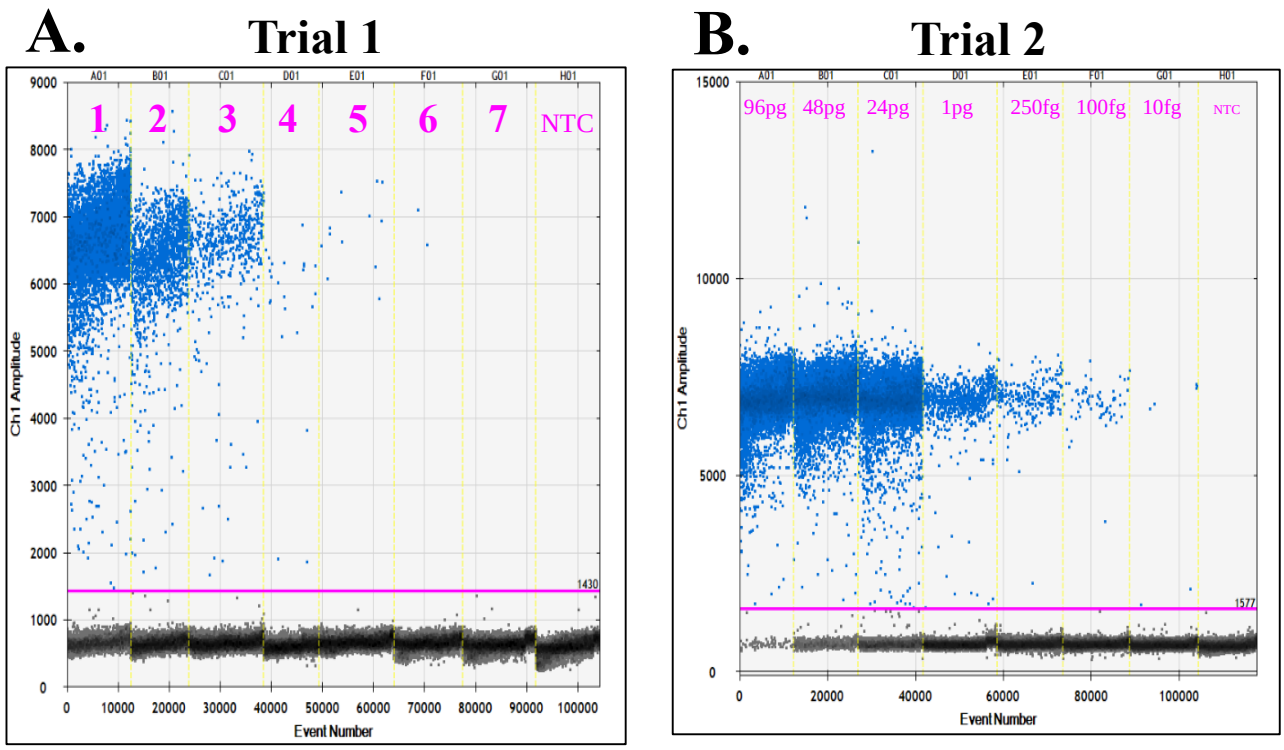

Figure 6. Droplet digital data for Phase 1 analyses with *ospA* assay. Each ddPCR sample-well is numbered in pink according to its sample dilution contents (actual template amounts were quantified and are indicated for trial 2). The pink lines represent the manually-assigned threshold based upon NTC samples (NTC = no template control). **A.** Droplet Digital PCR data generated for samples analyzed in Phase 1, trial 1. Detected number of copies corresponding to each numbered dilution sample can be found in Table 3. **B.** Droplet Digital PCR data generated for samples analyzed in Phase 1, trial 2. Template amount and detected number of copies in each sample can be found in Table 3.

Trial 1 resulted in template detection down to 3 genomic copies per sample (Table 3). However, DNA sample concentration was not determined immediately prior to droplet generation; therefore, exact template input for trial 1 dilution samples remains unknown. Since quantification values were obtained for samples immediately prior to trial 2 droplet formation, the data generated during this trial run of Phase 1 were used to evaluate the ddPCR system's precision at providing absolute quantification of *B. burgdorferi* DNA with the *ospA* assay (Table 3, Fig. 6B). The ddPCR data output from trial 2 of Phase 1 can be found in Figure 6B. DNA template was detectable at every sample concentration, ranging from 95.6 – 0.01 pg/µl input DNA (approximately 100,000 – 10 genomic copies, respectively) (Table 3). A linear relationship was found between the measured copy numbers and expected copy numbers present in control *B. burgdorferi* DNA, diluted in series ($R^2 > 0.999$, slope = 1.25) (Figure 7). In regards to the ddPCR instrument's precision, it is important to note at the lowest dilution sample (which contained an expected copy number of 10.17 spirochetes) ddPCR analysis provided a measurement of 10.6 copies of *B. burgdorferi* genome (Table 3).

To investigate the detection limits associated with the *glpQ* ddPCR assay, a serial dilution of known *B. miyamotoi* DNA was tested in a single trial, with execution similar to that which was carried out for Phase 1, trial 2 analysis of the *ospA* assay format. Prior to droplet formation, dsDNA in dilution sample 1 was quantified via Qubit technology and a serial dilution was performed to create samples of control *B. miyamotoi* DNA with concentrations ranging from 190 – 0.01 pg/µl (approximately 165,000 – 8 genomic copies, respectively). The ddPCR data output from Phase 1 analysis of *B. miyamotoi* DNA can be found in Figure 8. Template was detectable at every concentration of DNA dilution sample and the *glpQ* assay was successful at detection as low as 5 copies of *B. miyamotoi* template (Table 4).

Table 3. Phase 1 limit of detection and quantification with *ospA* assay. Information pertaining to a 7-sample dilution series of known *B. burgdorferi* DNA is detailed below, including: the amount of DNA template added to the PCR reaction, the expected genome copy number (Fig. 4), and the value generated from ddPCR analysis by the QuantaSoft software for total number of copies detected per PCR reaction volume (20 µl). The conduction of trial 2 involved quantification of serial dilution samples immediately prior to droplet generation. Since exact input template amount was not determined via quantification for dilution samples immediately prior to droplet generation in trial 1, the only data reported for this trial is the total number of copies detected and reported by the ddPCR software.

Phase 1: *B. burgdorferi ospA* Assay						
Trial 2				**Trial 1**		
Dilution Sample #	Template Amount	Expected Copies	Total Copies Measured	Dilution Sample #	Template Amount	Total Copies Measured
1	95.6 pg	97,251.99	122,000.00	1	unknown	20,060
2	48.4 pg	49,236.36	60,200.00	2	unknown	3440
3	23.6 pg	24,007.81	31,840.00	3	unknown	906
4	1 pg	1,017.28	1,206.00	4	unknown	50
5	250 fg	254.32	382	5	unknown	19.2
6	100 fg	101.73	110	6	unknown	3.4
7	10 fg	10.17	10.6	7	unknown	0

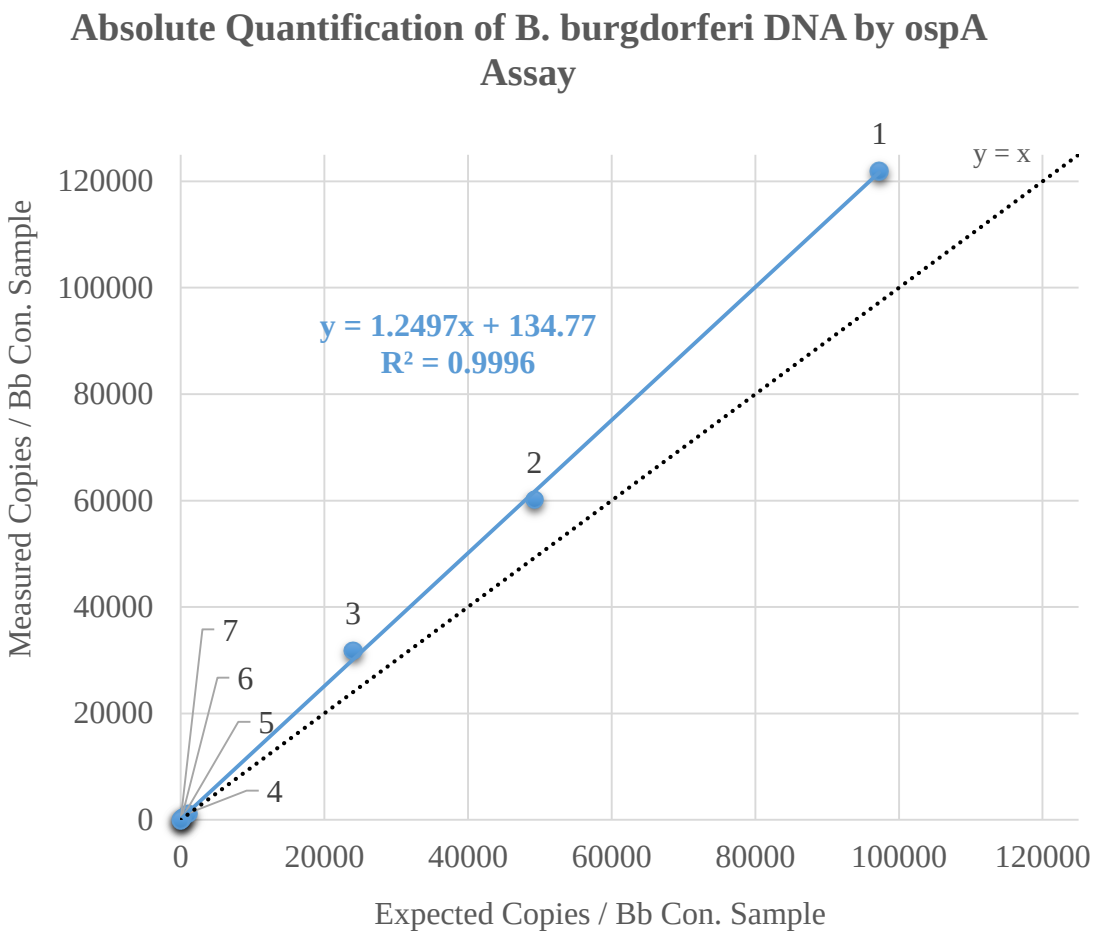

Figure 7. Absolute quantification of *B. burgdorferi* DNA via *ospA* assay. Results from the second trial of research-phase 1, employing the *ospA* assay for targeted amplification of *B. burgdorferi* DNA, are plotted above. The numbers labeled above the blue data points correspond to the dilution sample number analyzed during trial 2. The blue line represents the linear relationship between results of the dilution series. Black dotted line represents the idealistic direct relationship between expected and measured genome copies (y = x). Trend line equations correspond to their line by color. Y-axis values were determined from Fig. 4 equation Copies are reported in template copies per PCR reaction for *B. burgdorferi* control (*Bb* Con.) sample template.

Compared to *ospA* assay, however, the *glpQ* assay appears to be significantly less reliable at providing absolute quantification of copy numbers for targeted *B. miyamotoi* DNA. This can be seen by comparison of originally expected and measured genomic copies at every dilution sample (Table 4, Fig. 9A). Originally assuming the theoretical 907 Kb of purified linear *B. miyamotoi* chromosome, the amount of template measured by the ddPCR system is, on average, approximately 50% less than the expected amount (Table 4). This discrepancy could be due in part to the presence of additional plasmids left behind by the providers of this genetic material during extraction and purification steps of the bacterial DNA isolation process. This would lead to an overestimation in expected *B. miyamotoi* genome copies based on the false assumption that DNA quantifications of "known" samples prior to ddPCR analysis were a measurement of exclusively *B. miyamotoi* DNA, when in fact this was most likely was not the case. To test this theory and account for potentially co-purified large plasmids in addition to genomic *B. miyamotoi* DNA, adjusted expected copy numbers were determined for a genome size of 1,708 Kb (an average of total genome sizes (including megaplasmids) estimated for each dilution sample based on the number of copies measured by ddPCR) (Table 4) (*32*). These expected copy numbers (adjusted) where then compared back to the measured number of template copies amplified by the *glpQ* assay during Phase 1 (Table 4, Fig. 9B). The trend line observed in Figure 9B is a closer fit to the idealistic direct relationship versus the trend line observed in Figure 9A, indicating that measured and expected number of gene copies are more similar when a total genome size plus megaplasmids is considered for control *B. miyamotoi* DNA samples. Adjusting the expected copy numbers to account for the presence of megaplasmids also seems to remedy much of the discrepancies previously seen between expected and measured genome copy quantities (Table 4).

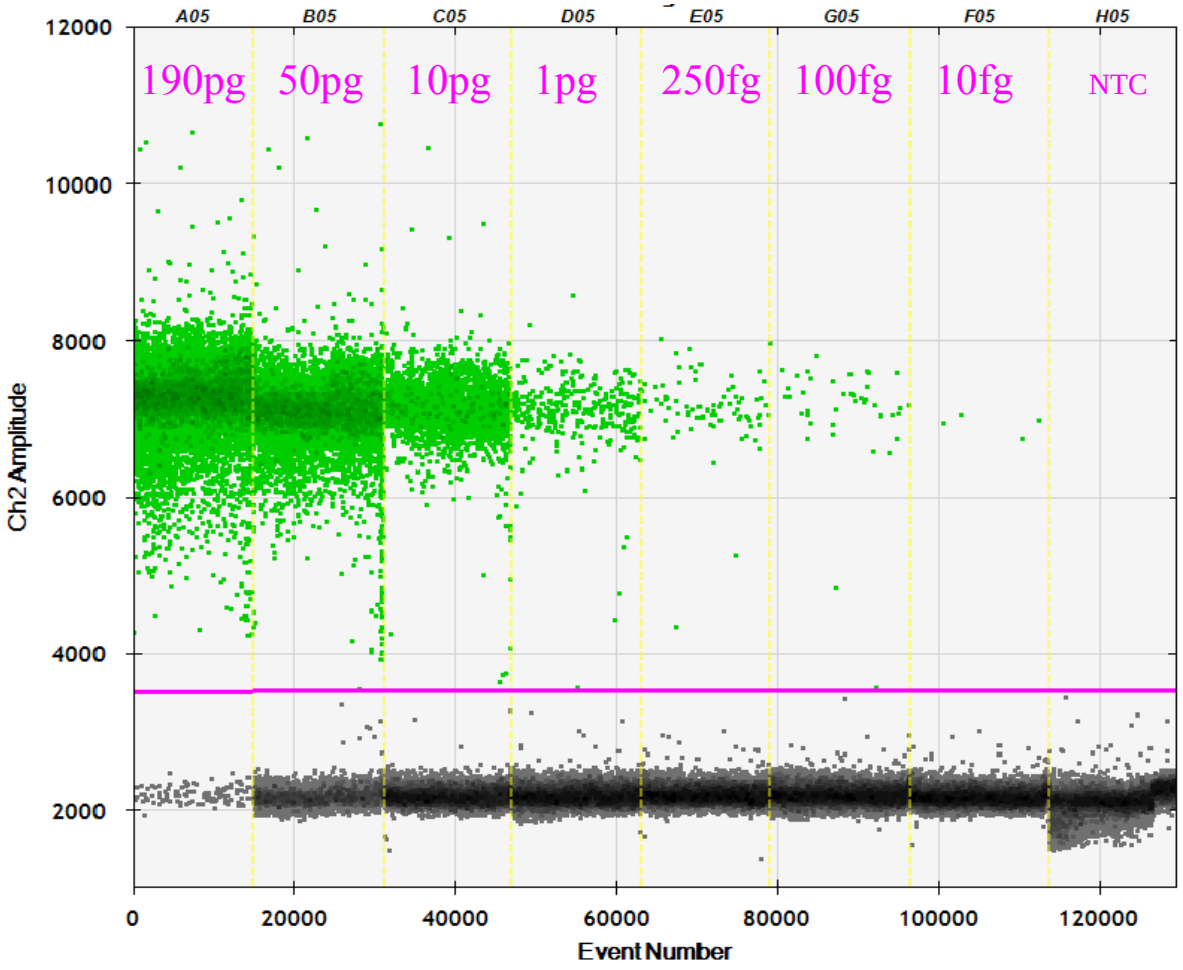

Figure 8. Droplet digital data generated for Phase 1 analysis with *glpQ* assay. Each ddPCR sample-well is numbered in pink according to its serial dilution template contents. The pink lines represent the manually-assigned threshold based upon NTC sample (NTC = no template control). *B. miyamotoi* template amounts and detected number of copies corresponding to each numbered dilution sample can be found in Table 4.

Table 4. Phase 1 limit of detection and quantification with *glpQ* assay.

Information pertaining to a 7-sample dilution series of known *B. miyamotoi* DNA is detailed below, including: the amount of DNA template added to the PCR reaction, the originally calculated expected genome copies using Fig. 4 equation (purple), the total number of copies detected through ddPCR analysis (green), the calculated expected genome copies adjusted to account for anticipated megaplasmids in control sample (purple), and the mean and individually calculated *B. burgdorferi* genome size solved for using the Fig. 4 equation and the ddPCR-measured copies at each sample template concentration.

Phase 1: *B. miyamotoi glpQ* Assay					
Dilution Sample #	Temp. Conc. (ng)	Expected Copies (Original)	Measured copies	Expected Copies (Adjusted)	Calc. Genome size (bp)
1	0.19	194,013.96	115,000.00	103,041.71	1,530,676
2	0.05	51,056.30	29,500.00	27,116.24	1,570,274
3	0.01	10,211.26	5,360.00	5,423.25	1,728,473
4	0.001	1,021.13	500.00	542.32	1,852,923
5	0.00025	255.28	118.00	135.58	1,962,842
6	0.0001	102.11	58.00	54.23	1,597,347
7	0.00001	10.21	5.40	5.42	1,715,670
			Av. Calc. Genome size		**1,708,315**

Due to the fact that the same primer and probe sets used in Ullmann et al. 2005 were also used in this study for detection and quantification of *Borrelia spp*. DNA, limit of spirochete detection results can be directly compared to evaluate the performance of qPCR vs. ddPCR methods. In the study conducted by Ullmann et al. 2005, the qPCR limit of detection consistently observed for the *glpQ* and *ospA* assays was a minimum of 30 spirochetes per 3 µl of template

DNA (*16*). In this study, the ddPCR limit of detection consistently observed for the same assays was a minimum of 10 spirochetes per 1 µl of template DNA. While the same determination was made at similar concentrations between the current and previous studies, the Droplet Digital PCR method was able to make the same determination with one-third of the required volume as previous qPCR methods.

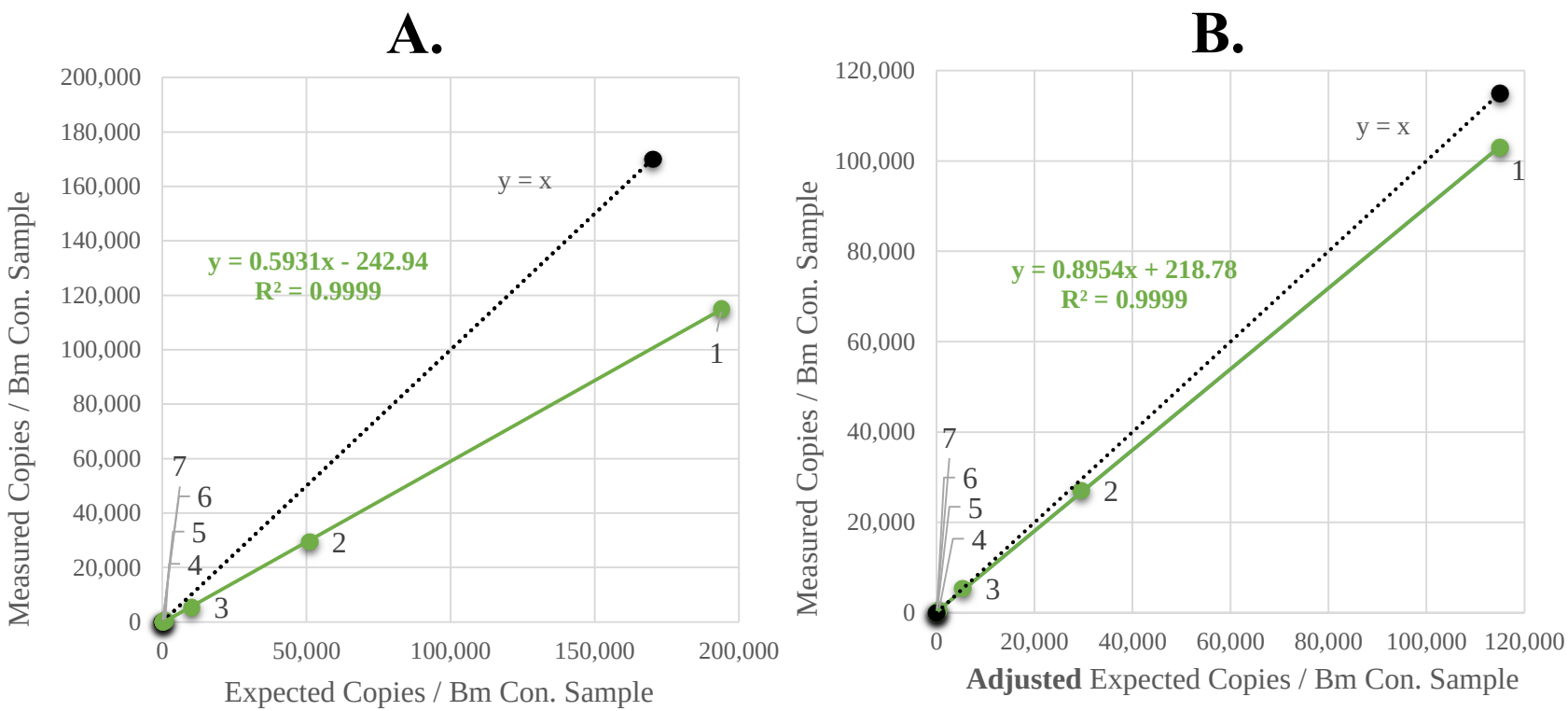

Figure 9. Absolute quantification of *B. miyamotoi* DNA via *glpQ* assay. Genome copies measured, in relation to the expected number of copies, of *B. miyamotoi* control (*Bm* Con.) DNA samples analyzed in Phase 1 are plotted above. Data point labels correspond to the dilution sample number. Green trend lines represent the linear relationship between results of the dilution series. Black dotted line represents the idealistic direct relationship between expected and measured genome copies (y = x). Trend line equations correspond to their line by color. Values for X- and Y- axes can be found in Table 4. **A.** "Expected Copies" were determined for a genome size of 1,067 Kb. **B.** "Adjusted Expected Copies" were determined for a genome size of 1,708 Kb.

Phase 2: PCR Inhibition by Background Host DNA

A study was performed to evaluate susceptibility of the ddPCR technology to inhibition in the presence of background tick-host DNA with regards to each specific assay. This was accomplished by spiking known concentrations of control bacterial template into PCR-mixtures with set volumes of *Borrelia*-negative tick extract and comparing the generated ddPCR results with those obtained during corresponding Phase 1 trials. The deer tick extracts used for analysis during this Phase of the study are described in Table 6

Droplet Digital PCR data output from Phase 2 inhibition studies of *ospA* and *glpQ* assays compared to their Phase 1 counterparts can be seen in Figures 10 and 11, respectively. Generated droplet patterns from both assays were similar between phases, regardless of the presence of tick DNA. Also, the ddPCR system was able to detect the targeted pathogen species down to the same dilution sample number in both phases. These results indicate that, in the case of both assays, limit of detection is not affected by the presence of background DNA (Fig. 10 and 11, Table 5)Upon first glance at the quantitative results generated from this inhibition study, it would appear that the presence of background host DNA was interfering with the amplification of bacterial DNA present within a particular dilution sample due to the fact that the number of copies measured tended to decrease between Phase 1 and Phase 2 analyses of both assays (Table 5). However, after further research, it was observed through consecutive Qubit quantifications that the total DNA concentration of any particular dilution sample would possess decreased amounts of DNA from day-to-day or between freeze/ thaw cycles. This observation suggests that the *Borrelia* DNA present in dilution samples could either be rapidly degrading or binding to the insides of sample tubes, resulting in an unexpectedly low DNA concentration for the samples analyzed during this portion of the research. This explanation could account for why detection

limits remained unaffected in the presence of background host DNA, while *Borrelia* template copy quantities varied from Phase 1 to Phase 2 (which were conducted anywhere from 0 to 5 days subsequent to the creation of particular *Borrelia spp.* dilution series) in both assay studies. In future studies, ddPCR analyses conducted using the same DNA dilution samples should be run in the same day or with freshly diluted *Borrelia* samples.

Table 5. Copies measured by ddPCR during Phase 1 and 2 analyses of assays. Phase 1 reactions tested serial dilutions of control *Borrelia* DNA. Phase 2 reactions tested *Borrelia*-negative tick extracts with serial dilutions of control *Borrelia* DNA spiked-in. Measured copy number results of ddPCR experiments testing the *B. burgdorferi* assay are indicated in blue. Measured copy number results of ddPCR experiments testing the *B. miyamotoi* assay are indicated in green. Results are rounded to the nearest whole copy. The limit of detection observed for both Phases in the case of each assay is highlighted in the table.

	***B. burgdorferi* Assay**		***B. miyamotoi* Assay**	
Dilution Sample #	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**
	Total Copies Measured			
1	20,060	8,680	115,000	99,200
2	3,440	2,412	29,500	19,080
3	906	384	5,360	2,520
4	50	60	500	174
5	19	4	118	68
6	3	3	58	22
7	0	0	5	3

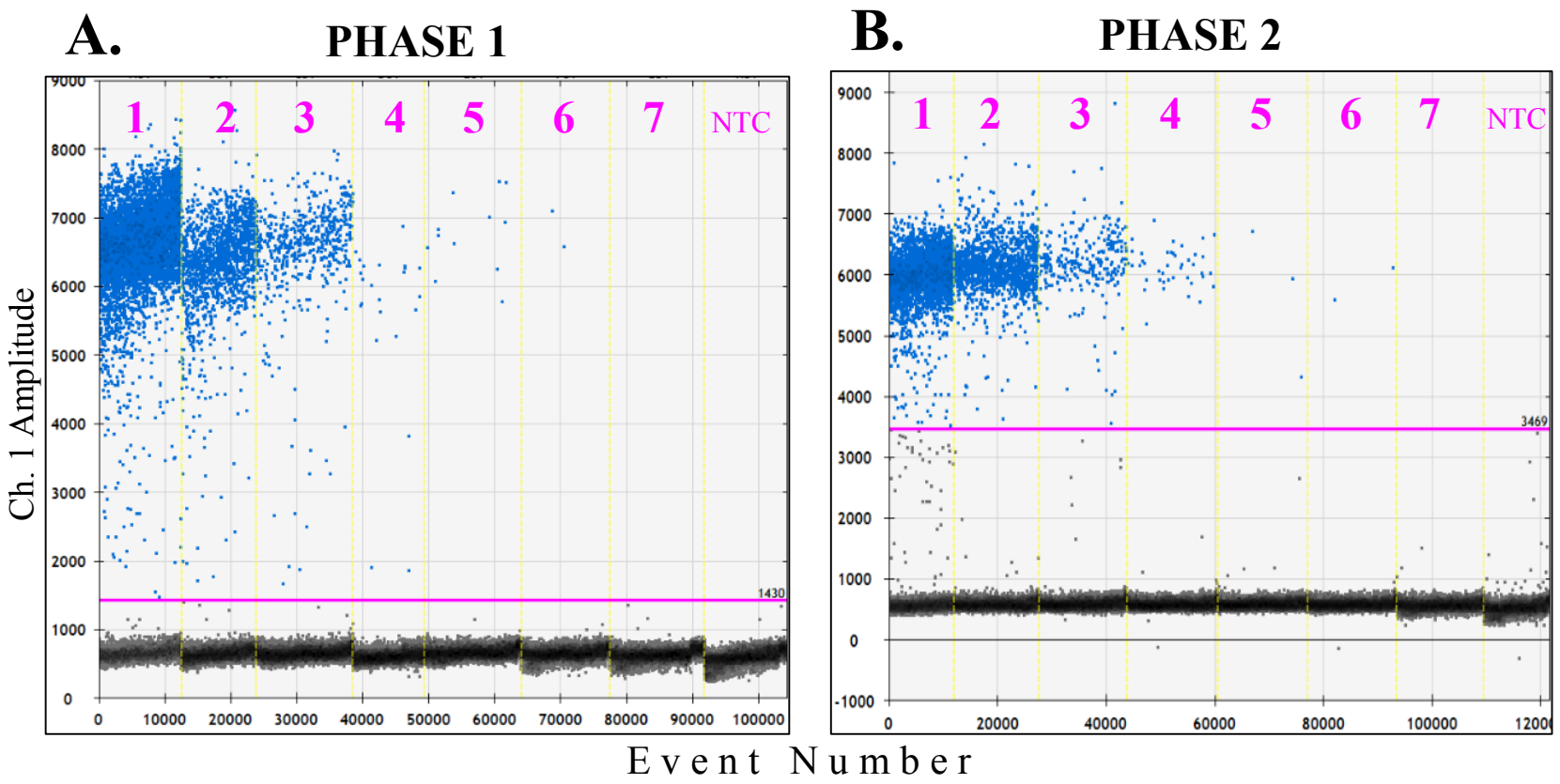

Figure 10. Comparison of ddPCR data generated from Phase 2 (inhibition study) with Phase 1 analyses of *ospA* assay. Each ddPCR well is numbered in pink according to its sample dilution contents. The pink lines represent the manually-assigned threshold based upon NTC samples (NTC = no template control). **A.** Phase 1 results from trial 1 reactions with serial dilutions of control *B. burgdorferi* DNA only. **B.** Phase 2 results from reactions with tick #2 DNA extract (negative for *Borrelia*) spiked with the same dilution series of *Borrelia* created and tested during Phase 1.

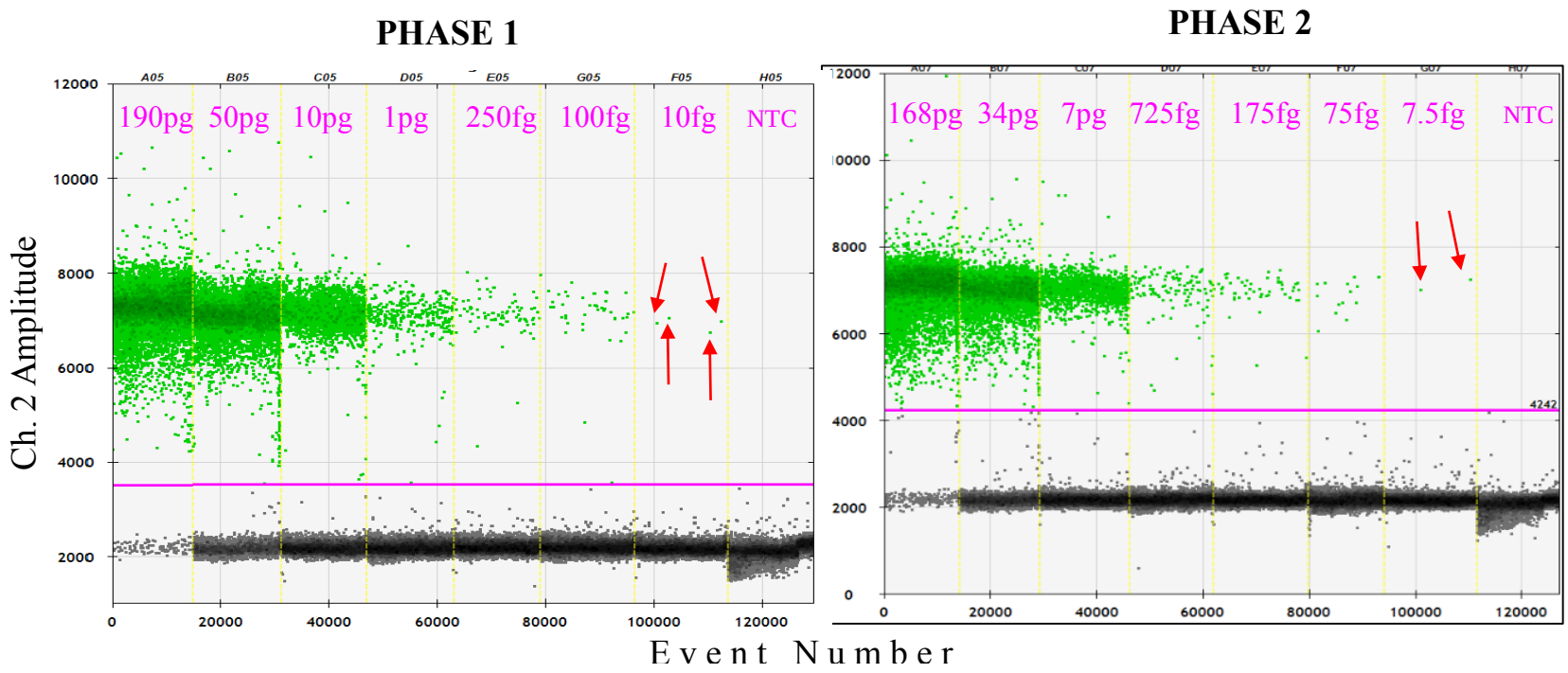

Figure 11. Comparison of ddPCR data generated from Phase 2 (inhibition study) with Phase 1 analyses of *glpQ* assay. Each ddPCR sample-well is named in pink according to its serial dilution template contents. The 7 dilution samples analyzed in Phase 1 are the same as the samples analyzed in Phase 2. The red arrows point out the droplets detected within the most dilute control sample (dilution sample 7), which is the same between both Phases. The pink lines represent the manually-assigned threshold based upon NTC samples (NTC = no template control). **A.** Phase 1 results from reactions with serial dilutions of control *B. miyamotoi* DNA only. **B.** Phase 2 results from reactions with tick #3 DNA extract (negative for *Borrelia*) spiked with the dilution series of *Borrelia* DNA created and tested during Phase 1.

Phase 3: Validation and Estimation of Pathogen Carriage Levels in Ticks

The tick samples which tested positive for *B. burgdorferi* and were used for validation of ddPCR *ospA* assay in this Phase are described in Table 6. Six *Borrelia*-positive, ticks (3 nymphs, 3 adult females) were tested over the course of four trial runs in order to determine via ddPCR information regarding pathogen carriage levels in *Ixodes* ticks (Table 7). These trial runs generated droplet data (Fig.12) and template copy quantification data (Table 7).

The first trial run conducted in Phase 3 consisted of samples with various concentrations of tick #6 extract in order to observe whether tick extracts required dilution prior to optimal ddPCR analysis (Fig. 12A). Based on the droplet data generated from this run, it was determined that dilution was not required in order to obtain satisfactory ddPCR results with which to analyze and compare pathogen quantification results between ticks. Once this was determined, trial 2 was run on duplicated samples with DNA extracts from tick #1, #4, or #6 (Fig. 12B). The total droplet count per ddPCR well from this run, as well as the ddPCR well containing the undiluted extract of tick #6, is shown in Table 7. The third trial run conducted in Phase 3 consisted of duplicated samples with DNA extract from tick #7, #9, or #10. (Fig. 13A) The total droplet count per ddPCR well from this run is shown in Table 7. During this particular trial run, 75% of the ddPCR wells generated a less than desired number of total droplets (the desired amount being greater than or equal to 10,000 total droplets). Due to this fact, a fourth trial run was carried out on the same samples analyzed during trial 3 in order to validate the measured number of droplets produced by the ddPCR system (Fig. 13B). In comparing the measured copy quantities in Table 7 resulting from analysis of duplicates run in trial 3 with those run in trial 4, it was confirmed that droplet counts within each tick replicate were reproducible and ddPCR results from both trial runs may be considered reliable.

Table 6. Deer tick DNA samples with Qubit results. *I. scapularis* tick extracts previously tested for *B. burgdorferi* infection and analyzed in Phase 2 or Phase 3 of this research project. Adult-stage ticks are in bold. Total double-stranded DNA (dsDNA) quantifications were performed using Qubit fluorometer as described in the text.

Tick Sample #	Stage	Results of *B. burgdorferi* Testing	Total dsDNA Conc.	ddPCR Phase Analyzed
2	Nymph	Negative	0.588 ng/ul	Phase 2
3	Nymph	Negative	0.454 ng/ul	
1	Nymph	Positive	0.325 ng/ul	Phase 3
4	Nymph	Positive	0.244 ng/ul	
6	**Adult**	**Positive**	**12.2 ng/ul**	
7	**Adult**	**Positive**	**14.2 ng/ul**	
9	**Adult**	**Positive**	**12.2 ng/ul**	
10	Nymph	Positive	0.783 ng/ul	

The next step in this phase of the experiment was to take the pathogen copy number measured for each ddPCR sample and estimate the total pathogen carriage level in each individual tick. These were conservative estimates due to the fact that they were calculated based on the assumption of 100% extraction efficiency. The details and results of this process can be found in Table 8.It was also assumed that each bacterium contained only one genome copy per cell. The average pathogen carriage level (i.e. infectivity) of individual ticks ranged between 231 and 118,407 copies of *B. burgdorferi* DNA with an overall average of 27,239 copies per tick. Tick #1 proved to be the least infected deer tick (231 copies), while tick #9 had the highest count

of *B. burgdorferi* genome copies in tested ticks (118,407 copies). It is possible that this could be due in part to the developmental stage, and therefore physical size, of the tick. Tick #1 was a nymph, while tick #9 was a larger adult deer tick. To investigate this theory further, the next objective was to determine the mean pathogen carriage level of ticks analyzed at the two different stages of tick development (nymphs vs. adults) and see how they compare (Table 8). The average pathogen carriage level of ticks in the nymph-stage ranged from 231 to 4,983 copies, with an average of 2,197 copies of *B. burgdorferi* genomes per nymph. The average pathogen carriage level ticks in the adult-stage ranged from 5,647 to 118,407 copies, with an average of 45,620 copies of *B. burgdorferi* genomes per adult. So, in the case of this study, while the upper limit of the nymph infectivity range comes close to the lower limit of the adult infectivity range, pathogen carriage levels did not overlap between ticks in different stages of development.

Table 7. Detected copy numbers of pathogen in infected tick samples during Phase 3 of ddPCR analysis. Tick samples were analyzed in duplicate (with the exception of tick #6 which was analyzed in triplicate) and amplified via *ospA* assay. Trial number corresponds with the droplet data visible in Fig. 12 and Fig. 13. Measured copies reported below refer to the number of template copies of *B. burgdorferi* detected within each 20 µl ddPCR well.

Tick Sample #	**1**	**4**	**10**	**6**	**7**	**9**
Stage	Nymph			Adult		
Copies Measured per Tick Replicate	8	62	224	246	1,356	5,040
	11	54	196	230	1,408	4,940
				238		

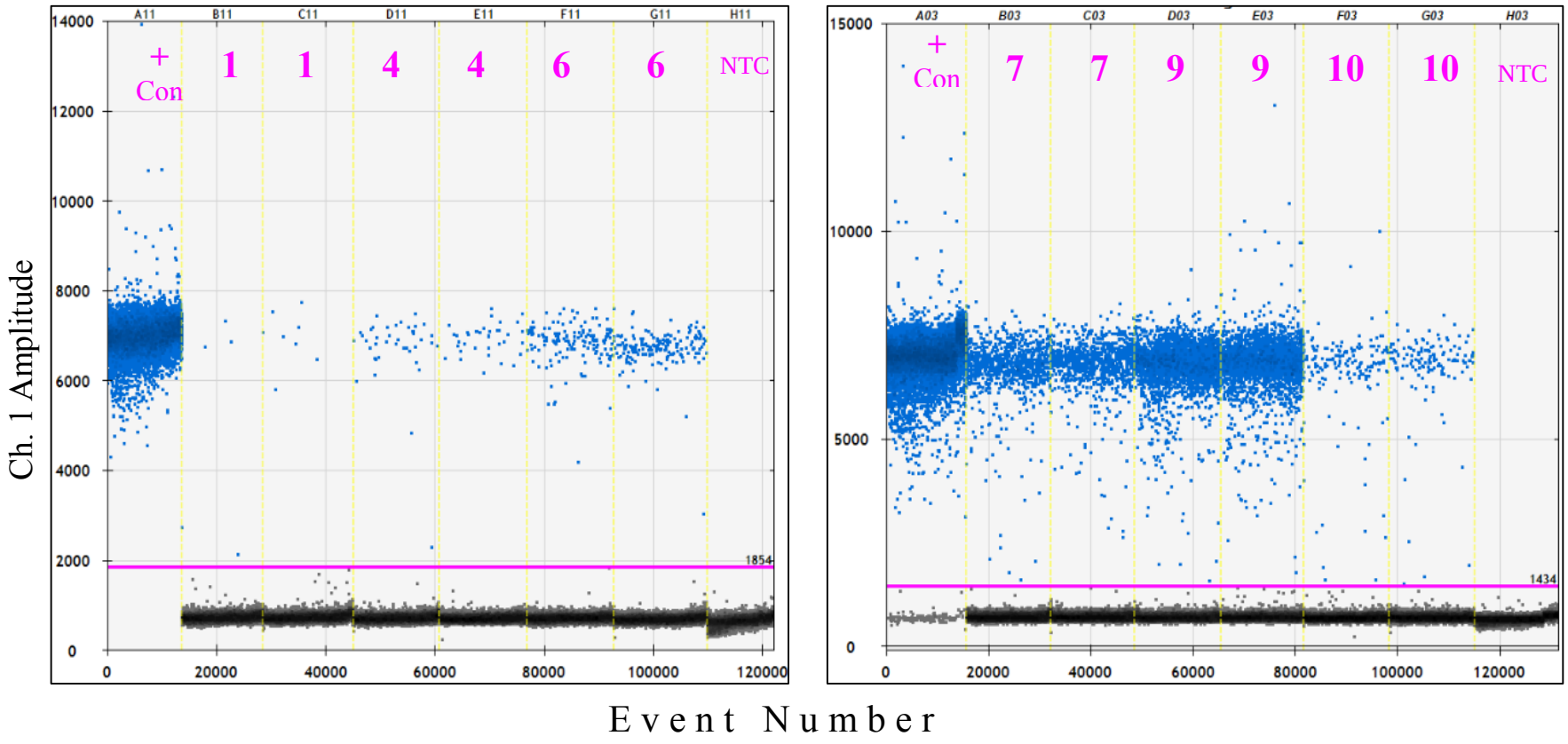

Figure 12. Droplet digital data generated for Phase 3 analysis of *ospA* assay in *B. burgdorferi*-positive ticks. The pink lines represent the manually-assigned threshold based upon NTC samples (NTC = no template control**).** The ddPCR results for tick sample extracts 1, 4, 6, 7, 9 and 10 run in duplicate. The quantities determined through the analysis depicted above were subsequently used to calculate *B. burgdorferi* carriage level in each infected tick.

Table 8. Estimate of *B. burgdorferi* carriage levels in infected *Ixodes* ticks. Assuming 100% extraction and amplification efficiencies, pathogen carriage level values were calculated per individual tick based on template input amount, ddPCR-detected copies per reaction, and original retention volume of tick DNA extract. The mean, range, and standard deviation of these carriage levels was found for each tick sample as well as the two different tick stages of development (nymph vs. adult). The minimum and maximum average pathogen carriage level among ticks analyzed here are in bold.

		PATHOGEN CARRIAGE LEVEL (measured genome copies)						
Tick Sample #	**Stage**	**Per Tick Replicate**			**Per Tick Sample**		**Per Tick Stage**	
					Means	**Std. Error**	**Means**	**Std. Error**
1	**NYMPH**	190	271		**231**	41	2,197	1,432
4		1,471	1,281		1,376	95		
10		5,315	4,651		4,983	332		
6	**ADULT**	5,837	5,458	5,647	5,647	109	45,620	33,978
7		32,176	33,410		32,793	617		
9		119,593	117,220		**118,407**	1,187		

CHAPTER IV

CONCLUSIONS

This project set out to test and optimize genospecies-specific PCR primers and TaqMan probes for the detection and absolute quantification of *Borrelia* DNA by the Bio-Rad Droplet Digital PCR system, in the presence and absence of background host DNA, and then to validate these ddPCR protocols in *Borrelia*-infected deer ticks. The *ospA* and *glpQ* assays tested here (for detection of *B. burgdorferi* and *B. miyamotoi* pathogens, respectively) consistently gave a limit of detection through ddPCR technology of a minimum of 10 spirochetes per µl of template DNA (i.e. per reaction). This is comparable to the study conducted by Ullmann et al. 2005 in which the qPCR limit of detection consistently observed for the *glpQ* and *ospA* assays also analyzed here was a minimum of 30 spirochetes per 3 µl of template DNA (*16*). From this, it can be concluded that the Droplet Digital PCR technology is able to detect and quantify *Borrelia* pathogens with equal or greater accuracy than the qPCR-based technology currently employed in diagnostic testing. In addition to its competitive quantification accuracy, the ddPCR methodology was able to provide the same determination as qPCR but with one-third less volume of template DNA.

Some discrepancies were found to exist between calculated-expected and detected copy number of *B. miyamotoi* template produced from analysis of the *glpQ* assay. Upon further investigation, it was revealed that the *B. miyamotoi* genomic DNA supplied by the CDC was

purified by means of a silica-based membrane. The non-size specific nature of this purification method would be expected to co-purify large plasmids in addition to genomic DNA. Reports in the literature indicate that the relapsing fever group *Borrelia* frequently carry an approximately 160 Kb linear plasmid and possibly numerous other plasmids (*32*). Using the detected number of gene copies, a recalculation of the estimated total genome size (including megaplasmids) was made for *B. miyamotoi* of 1,708 Kb, compared with 907 Kb reported for the single chromosome (*30, 32*) . Upon reanalysis (seen in Figure 4B) of expected versus detected number of gene copies, it can be concluded that the *B. miyamotoi* control DNA sample obtained from the CDC most likely contained co-purified large plasmids in addition to genomic DNA. Future ddPCR studies should seek to verify and further remedy this discrepancy encountered between expected and measured genomic copies of *B. miyamotoi* control DNA sample.

By comparison, experiments with *B. burgdorferi* did not suffer this same problem, likely owing to a superior purification method of the genomic DNA (e.g. density ultracentrifugation) performed by ATCC. From a precision standpoint, it is noteworthy that while detected copy number of *B. miyamotoi* did not definitively match up with expected number, the *glpQ* assay format was able to successfully detect the presence of *B. miyamotoi* template through ddPCR in dilution samples with a total DNA concentration as low as 10 fg/µl.

During the process of completing this research, DNA was lost as a function of degradation of template sample or irreversible binding of DNA to the inner walls of the tubes. In future ddPCR studies, when precise template quantifications are a vital component of the research, it is suggested that ddPCR runs involving dilutions be completed in their entirety in the same day and that low-binding tubes be utilized.

This study was able to successfully show that the *ospA* assay can be used in ddPCR protocols to provide absolute quantification *of B. burgdorferi* template copies. This research also provided validation of this ddPCR assay for detection, quantification, and subsequent carriage level estimation of bacterial pathogen in *B. burgdorferi*- infected *Ixodes* ticks. For deer ticks tested in this study, the average pathogen carriage level was determined to be 2,197 copies in nymphs and 45,620 copies in adult females. It was concluded that the stage of tick development might play a role in the level of infectivity, or carriage levels may be simply due to physical capacity with which to possess more or less copies of pathogen (i.e. size). This is an avenue which should be explored further. Due to the unavailability of a 16S assay for total bacterial copy numbers, the number of total bacterial DNA could not be determined per tick. Therefore, the number of target *Borrelia* copies detected relative to the total amount of total bacterial genomic DNA in an individual tick could not be determined during the course of this study. Further studies should be conducted to address this unanswered question upon availability of a suitable 16S assay.

In conclusion, through this research, it was shown that the ddPCR system can be used as an alternative means to the current qPCR methods for bacterial pathogen detection and spirochete quantification in *Ixodes* ticks. Implementation of this modern method into disease diagnostic and prevention facilities could improve testing expenses, time, and production while providing results of equal or better caliber to those obtained through current bacterial DNA-testing methods.

REFERENCES

1. K. Tilly, P. A. Rosa, P. E. Stewart, Biology of Infection with Borrelia burgdorferi. *Infectious Disease Clinics of North America* **22**, 217-234 (2008); published online Epub6// (http://dx.doi.org/10.1016/j.idc.2007.12.013).

2. M. Vayssier-Taussat, S. Moutailler, L. Michelet, E. Devillers, S. Bonnet, J. Cheval, C. Hébert, M. Eloit, Next generation sequencing uncovers unexpected bacterial pathogens in ticks in western Europe. *PloS one* **8**, e81439 (2013).

3. CDC, in *Ticks*. (Center for Disease Control and Prevention, http://www.cdc.gov/ticks/miyamotoi.html, 2013), vol. 2014.

4. CDC. (Center for Disease Control and Prevention, http://www.cdc.gov/lyme/, 2014), vol. 2014.

5. M. S. Dworkin, T. G. Schwan, D. E. Anderson Jr, Tick-borne relapsing fever in North America. *Medical Clinics of North America* **86**, 417-433 (2002); published online Epub3// (http://dx.doi.org/10.1016/S0025-7125(03)00095-6).

6. R. V. A. D. Ticks, Lyme Disease Causes.

7. F. Dietrich, T. Schmidgen, R. G. Maggi, D. Richter, F.-R. Matuschka, R. Vonthein, E. B. Breitschwerdt, V. A. J. Kempf, Prevalence of Bartonella henselae and Borrelia burgdorferi Sensu Lato DNA in Ixodes ricinus Ticks in Europe. *Applied and Environmental Microbiology* **76**, 1395-1398 (2010); published online EpubMarch 1, 2010 (10.1128/aem.02788-09).

8. P. J. Krause, S. Narasimhan, G. P. Wormser, L. Rollend, E. Fikrig, T. Lepore, A. Barbour, D. Fish, Human Borrelia miyamotoi Infection in the United States. *New England Journal of Medicine* **368**, 291-293 (2013)doi:10.1056/NEJMc1215469).

9. S. Varde, J. Beckley, I. Schwartz, Prevalence of tick-borne pathogens in Ixodes scapularis in a rural New Jersey County. *Emerging Infectious Diseases* **4**, 97-99 (1998); published online EpubJan-Mar (

10. P. A. Rosa, T. G. Schwan, A Specific and Sensitive Assay for the Lyme Disease Spirochete Borrelia burgdorferi Using the Polymerase Chain Reaction. *Journal of Infectious Diseases* **160**, 1018-1029 (1989); published online EpubDecember 1, 1989 (10.1093/infdis/160.6.1018).

11. A. G. Barbour, J. Bunikis, B. Travinsky, A. G. Hoen, M. A. Diuk-Wasser, D. Fish, J. I. Tsao, Niche partitioning of Borrelia burgdorferi and Borrelia miyamotoi in the same tick

vector and mammalian reservoir species. *The American journal of tropical medicine and hygiene* **81**, 1120-1131 (2009).

12. N. I. o. Health, in *Health & Research Topics*. (U.S. Department of Health and Human Services, Web, 2014), vol. 2015.

13. J. L. Gugliotta, H. K. Goethert, V. P. Berardi, S. R. Telford, Meningoencephalitis from Borrelia miyamotoi in an Immunocompromised Patient. *New England Journal of Medicine* **368**, 240-245 (2013)doi:10.1056/NEJMoa1209039).

14. K. Padgett, D. Bonilla, A. Kjemtrup, I. M. Vilcins, M. H. Yoshimizu, L. Hui, M. Sola, M. Quintana, V. Kramer, Large Scale Spatial Risk and Comparative Prevalence of Borrelia miyamotoi and Borrelia burgdorferi Sensu Lato in Ixodes pacificus. *PLoS One* **9**, e110853 (2014)10.1371/journal.pone.0110853).

15. CDC, in *Lyme Disease*. (Centers for Disease Control and Prevention, http://www.cdc.gov/lyme/stats/maps/interactivemaps.html, 2014), vol. 2014, pp. Map of Reported Cases of Lyme Disease in the United States in 2013.

16. A. J. Ullmann, E. S. Gabitzsch, T. L. Schulze, N. S. Zeidner, J. Piesman, Three Multiplex Assays for Detection of Borrelia burgdorferi sensu lato and Borrelia miyamotoi sensu lato in Field-Collected Ixodes Nymphs in North America. *Journal of Medical Entomology* **42**, 1057-1062 (2005); published online Epub2005/11/01 (10.1603/0022-2585(2005)042[1057:TMAFDO]2.0.CO;2).

17. R. Hayden, Z. Gu, J. Ingersoll, D. Abdul-Ali, L. Shi, S. Pounds, A. Caliendo, Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. *Journal of clinical microbiology* **51**, 540-546 (2013).

18. B. J. Hindson, K. D. Ness, D. A. Masquelier, P. Belgrader, N. J. Heredia, A. J. Makarewicz, I. J. Bright, M. Y. Lucero, A. L. Hiddessen, T. C. Legler, T. K. Kitano, M. R. Hodel, J. F. Petersen, P. W. Wyatt, E. R. Steenblock, P. H. Shah, L. J. Bousse, C. B. Troup, J. C. Mellen, D. K. Wittmann, N. G. Erndt, T. H. Cauley, R. T. Koehler, A. P. So, S. Dube, K. A. Rose, L. Montesclaros, S. Wang, D. P. Stumbo, S. P. Hodges, S. Romine, F. P. Milanovich, H. E. White, J. F. Regan, G. A. Karlin-Neumann, C. M. Hindson, S. Saxonov, B. W. Colston, High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. *Analytical Chemistry* **83**, 8604-8610 (2011); published online Epub2011/11/15 (10.1021/ac202028g).

19. I. Bio-Rad Laboratories. (Life Science Group), pp. 1-100.

20. I. Bio-Rad Laboratories. (Bio-Rad Laboratories, Inc., http://www.bio-rad.com/en-us/applications-technologies/droplet-digital-pcr-ddpcr-technology#6, 2014), vol. 2014.

21. I. Bio-Rad Laboratories. (Bio-Rad Laboratories, Inc., http://www.bio-rad.com/en-us/applications-technologies/qpcr-real-time-pcr, 2014), vol. 2014.

22. B. Staff. (Bio-Rad Laboratories, Inc., http://www.bioradiations.com/focus-on-technology/783-ddpcr/1340-simpleddpcr, 2012), vol. 2014.

23. M. A. Sze, M. Abbasi, J. C. Hogg, D. D. Sin, A Comparison between Droplet Digital and Quantitative PCR in the Analysis of Bacterial 16S Load in Lung Tissue Samples from Control and COPD GOLD 2. *PLoS ONE* **9**, e110351 (2014)10.1371/journal.pone.0110351).

24. A. Last, S. Molina-Gonzalez, E. Cassama, R. Butcher, M. Nabicassa, E. McCarthy, S. E. Burr, D. C. Mabey, R. L. Bailey, M. J. Holland, Development and evaluation of a next-generation digital PCR diagnostic assay for ocular Chlamydia trachomatis infections. *Journal of clinical microbiology* **51**, 2195-2203 (2013).

25. W. P. Kolar, University of North Texas Health Science Center, (2015).

26. V. Dedkov, M. Markelov, K. Gridneva, M. Bekova, A. Gmyl, L. Kozlovskaya, G. Karganova, L. I. Romanova, V. Pogodina, V. Yakimenko, Prevalence of Kemerovo virus in ixodid ticks from the Russian Federation. *Ticks and tick-borne diseases* **5**, 651-655 (2014).

27. M. C. Strain, S. M. Lada, T. Luong, S. E. Rought, S. Gianella, V. H. Terry, C. A. Spina, C. H. Woelk, D. D. Richman, Highly precise measurement of HIV DNA by droplet digital PCR. *PloS one* **8**, e55943 (2013).

28. D. Rogers, A. Smith, M. Allen, in *14th Annual Biomedical Research Conference for Minority Students*. (University of North Texas Health Science Center, Fort Worth, Texas, 2014).

29. C. M. Fraser, S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J.-F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fujii, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, J. C. Venter, Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. *Nature* **390**, 580-586 (1997); published online Epub12/11/print (

30. F. Hue, A. Ghalyanchi Langeroudi, A. G. Barbour, Chromosome Sequence of Borrelia miyamotoi, an Uncultivable Tick-Borne Agent of Human Infection. *Genome Announcements* **1**, (2013); published online EpubOctober 31, 2013 (10.1128/genomeA.00713-13).

31. I. Integrated DNA Technologies, Molecular Facts and Figures. 2011.

32. S. C. Miller, S. F. Porcella, S. J. Raffel, T. G. Schwan, A. G. Barbour, Large linear plasmids of Borrelia species that cause relapsing fever. *Journal of bacteriology* **195**, 3629-3639 (2013).

33. G. A. Scoles, M. Papero, L. Beati, D. Fish, A relapsing fever group spirochete transmitted by Ixodes scapularis ticks. *Vector Borne and Zoonotic Diseases* **1**, 21-34 (2001).

34. P. J. Krause, S. Narasimhan, G. P. Wormser, A. G. Barbour, A. E. Platonov, J. Brancato, T. Lepore, K. Dardick, M. Mamula, L. Rollend, Borrelia miyamotoi sensu lato seroreactivity and seroprevalence in the northeastern United States. *Emerging infectious diseases* **20**, 1183 (2014).