Operation Tick Hunt: Developing an Experimental Framework to Monitor Lyme Disease in Central Massachusetts

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Abstract

For this project, the student group investigated the presence of Lyme disease in deer ticks in Massachusetts. Tick samples were collected from both urban and rural sites before being sorted by sex and location. Next, ticks were subjected to whole-DNA extraction for use in testing for the presence of the bacteria using PCR. Several individual ticks were positively identified as carrying Lyme disease, and comprehensive protocols were developed to support the study's continuation with future project groups.

Acknowledgements

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4. Background and Introduction

4.1. Lyme disease as a public health concern

In the United States, a notable issue that has been identified over the past two decades is an increase in the infection rates of various tick-borne pathogens. By far the most common among these pathogens is Lyme disease, which has been reported by the Centers for Disease Control and Prevention (CDC) to affect upwards of 30,000 Americans each year (Winny, 2023). This statistic has been rising steadily over the past few decades, and even more concerningly, many thousands more cases are likely to go unreported each year. Additionally, Lyme disease infections are currently increasing in states previously without high historical case rates. The proliferation of Lyme disease in the United States is especially worrisome in the context of several exacerbating factors, such as the rapidly developing threat of antibiotic resistance.

4.2. Ecology and pathogenicity of Lyme disease

The most common vector for Lyme disease in New England is *Ixodes scapularis*, or the deer tick, as a result of its enzootic feeding cycle. The ticks are pathogen-free when they first hatch as larvae, but the causative agent of Lyme disease, *Borellia burgdoferi*, can be contracted from mammalian hosts during the larval ticks' first blood meal (Baer, 2020). *B. burgdorferi* is a spirochete, or spiral-shaped bacteria. Between each of the subsequent nymphal and adult life stages, an infected tick will feed again and can transmit *B. burgdoferi* to multiple other hosts within its ecosystem. To attach to and feed from an animal, ticks will participate in what is often referred to as "questing"; they will climb upwards on vegetation and raise their legs to expose claws and velcro-like bristles at the ends. When a potential host brushes by the vegetation, the tick will latch on readily and bite through the skin to begin feeding on blood. The most

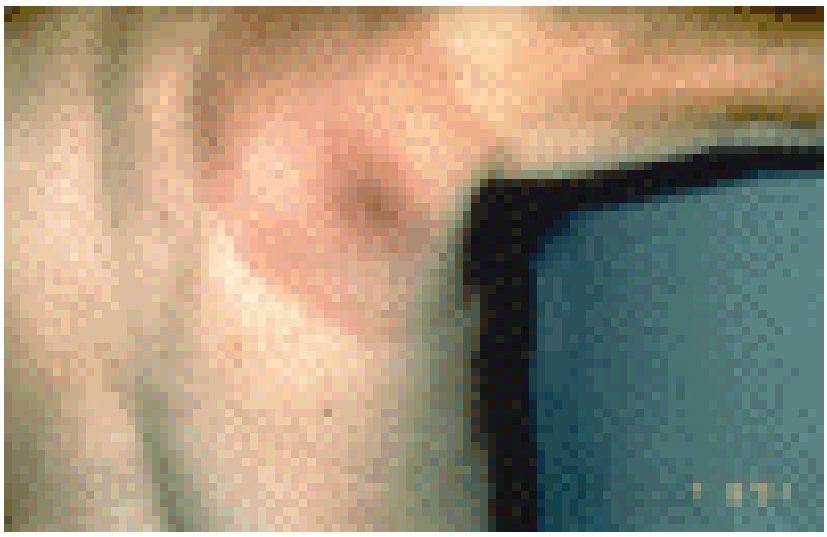
commonly selected hosts for *I. scapularis* are deer, small rodents, and birds. However, humans can easily become incidental hosts when working outdoors, hiking, or even through contact with pets that are carrying ticks (Johns Hopkins Medicine, 2021).

Figure 1: Morphology of *Ixodes scapularis* for both sexes

Note: (University of Rhode Island, 2024)

Once attached, infected ticks will gradually transmit *B. burgdorferi* directly into the bloodstream. *B. burgdoferi* is most likely to be contracted the longer an infected tick remains in blood-contact with a host; an attachment period longer than 48 hours drastically increases the chance of transmission (Radolf, 2012). In humans, the bacterial incubation period can vary widely, with the onset of symptoms occurring anywhere between a few days or a whole month. The most common symptom to appear first is a tell-tale “bull’s-eye rash” which spreads outwards in a banded pattern from the site of the tick bite. This is known formally within the medical community as erythema migrans. This rash is typically followed by flu-like symptoms such as fever, chills, fatigue, and muscle pain. However, erythema migrans does not appear in all infected individuals, which can contribute to incorrect diagnoses of Lyme disease.

Figure 2: *Erythema migrans* rash characteristic of Lyme infection



Note: (Minnesota Department of Health, 2022)

While the clinical manifestation of Lyme disease is unpleasant, the bacteria itself is not known to secrete any toxins or biochemical agents that would cause direct damage to the body; instead, the sickness is caused by the host's immune system working in overdrive, with mediated responses such as inflammation and fever being upregulated to fight the pathogen (Radolf, 2012). As such, many people with underlying autoimmune conditions can be especially affected by Lyme disease, and others may experience chronic, years-long effects such as lasting joint pain or further susceptibility to bacterial infections (Global Lyme Alliance). Additionally, Lyme disease is frequently misdiagnosed because the symptomatic presentation is highly variable. Not all infected individuals will experience all of the negative effects, and symptoms can be inconsistent in terms of severity. The early, acute symptoms of Lyme disease can easily be confused with those commonly observed in people with the annual flu virus (Johns Hopkins Medicine, 2021). Therefore, treatment of this disease can be difficult, and long-term chronic symptoms such as inflammation and autoimmune disorders often develop in patients who are not

properly diagnosed or given correct treatment. As a result, it is particularly important to proactively prevent infections of Lyme disease.

4.3. The discovery of Lyme disease and concerns for public health

Although Lyme disease is now well-known to the general public, it was only relatively recently that it was formally identified as a human pathogen. The first modern reports of the illness came from the small town of Lyme, Connecticut in the early 1970s, when multiple residents reported similar symptoms including inflammation, headaches, and rashes. After several years and dozens of cases reported, people in the town began to take notice of the mysterious illness, and despite the absence of a known cause, the condition became colloquially known by locals and early researchers as Lyme disease (Bay Area Lyme Foundation, 2022).

Lyme Disease and its characteristic symptoms were documented by researchers throughout the late 1970s, but it was not until 1981 that the causative agent of Lyme was finally identified. A Swiss-American tick researcher named Dr. William Burgdorfer was already engaged in studying Rocky Mountain Spotted Fever, another tick-borne pathogen when he became aware of the enigmatic disease afflicting the citizens of Lyme.

Figure 3: Dr. William Burgdorfer photographed in his laboratory examining tick specimens in 1954

Note: (Yardley, 2014)

Given his previous experience with working with tick-borne diseases, Dr. Burgdorfer gathered deer ticks from the forests around Lyme and used surgical methods in his laboratory to investigate the ticks for potential disease-causing agents. From within the ticks' thorax, he isolated a novel bacteria, that was later confirmed to be the causative agent of Lyme Disease. At long last, the mysterious pathogen had been identified, and those who had been infected finally knew what had been making them ill for years (U.S. Department of Health and Human Services, National Institutes of Health).

As Lyme disease poses a direct threat to human health, its spread and incidence rate across the United States must be tracked closely by government and public health agencies. The Massachusetts Department of Public Health (MDPH) maintains an electronic data-collection Syndrome Surveillance program, which tracks many metrics including the incidence of Lyme-related hospital visits by month and location within the state. In 2023, the total number of

emergency room visits in Massachusetts related to tick-borne pathogens was reported at 2,125, with Lyme disease accounting for the majority of cases among young children aged five to fourteen (Massachusetts Bureau of Infectious Disease and Laboratory Sciences, 2023).

Figure 4: Reported cases of Lyme disease in the United States, 1991–2018

Furthermore, the Centers for Disease Control's (CDC) national Lyme disease tracking system has documented incidence rates between 1991 and 2018 and determined that the annual case rate has almost doubled in this period (United States Environmental Protection Agency, 2021). The rising spread of Lyme disease becomes increasingly worrisome when considering that, according to Johns Hopkins University, anywhere between one and fifty percent of deer ticks in the United States can be expected to carry Lyme disease (Johns Hopkins Medicine, 2021). This statistic is highly localized and is contingent on several ecological factors, such as vegetation, host availability, and proximity to human-developed areas.

4.4. Project goal

If Lyme disease prevalence is increasing year over year, then it is important to take action to reduce it. Ideally, Lyme disease prevention strategies such as rodent trapping, deer killing, and vegetation trimming would be performed uniformly to reduce Lyme prevalence everywhere. However, targeting certain at-risk areas can be more efficient and realistic to achieve. The goal of this project was to create a framework for tick collection and testing. Using this framework, future groups can investigate Lyme disease prevalence across an urban-to-rural gradient. With this information, areas of need can be identified.

4.5. Consulting modern research to develop project methodology

To begin establishing the potential scope and direction of our project, it was necessary to read scientific literature to gain an understanding of the methods used by researchers to study *B. burgdorferi* in the laboratory. We identified one such study that was conducted in the Pacific Northwest between July 2006 and August 2017 and involved the analysis of 549 ticks belonging to the *Ixodes* genus (Xu et al., 2019). The researchers compiled these ticks to determine how many carried one or more of three different bacterial pathogens: *Borrelia burgdorferi sensu lato*, *Borrelia miyamotoi*, and *Anaplasma phagocytophilum*. To diagnose the presence of these bacteria, the researchers opted to employ a polymerase chain reaction (PCR) procedure. PCR is a widely used and highly modular method for amplifying small amounts of nucleic acids to high concentrations for quantification or sequence analysis.

Every PCR protocol involves the usage of four basic components: template DNA, primers, free nucleotides, and DNA polymerase. When all components are combined in a PCR tube, the target DNA sequence, called the amplicon, is propagated exponentially through the use

of a thermal cycler machine. This machine allows for the input of a programmed sequence of temperature increases and decreases. First, the temperature is raised high enough to denature the template DNA by breaking the hydrogen bonds that hold the complementary strands together. Next, the temperature is lowered to allow for the template-specific primers to bind to their target sequences. This results in the DNA forming small oligonucleotides of a size dictated by the sequence of the forward and reverse primers. Finally, the temperature is raised again to catalyze the action of the DNA polymerase, which incorporates free nucleotides (adenine, guanine, thymine, and cytosine) onto the overlapping ends of the oligonucleotide fragments. This programmed sequence is repeated for a set number of cycles, producing more and more of the desired amplicon each time (Garibyan et al., 2013).

Because the exact size of the amplicon is known as determined by the primers used, its presence can be visualized using gel electrophoresis. Gel electrophoresis is an extremely common laboratory method that uses electric current to separate DNA sequences, which are negatively charged, based on their relative size. DNA sequences are transferred into wells within a rectangular agar gel, and a negative current is generated from one end to cause the samples to migrate through the gel to the opposite side. Before applying current, dye is added to the DNA samples to allow for them to be viewed readily as colored bands while they travel through the gel. Smaller DNA sequences will migrate further along the gel than larger ones, and their relative sizes are determined by comparing their position to the bands of a gel ladder. Gel ladders are prepared solutions that are added to electrophoresis gels that contain multiple DNA fragments of known lengths; the experimental sequences can thus be compared side-by-side with the ladder to confirm their size.

5. Methodology

5.1. Sampling site selection

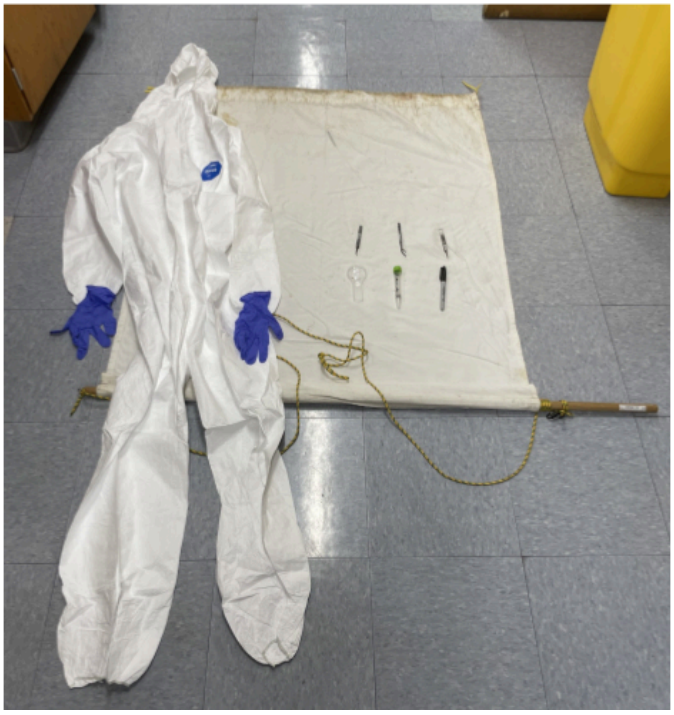
To accomplish the goal of assessing the abundance of Lyme disease in a rural-to-urban gradient, it was necessary to select collection sites that were distinguishable into either of these two categories. Using a map that was accessed through the website of the Massachusetts State Office of Rural Health, all of the cities and towns within Massachusetts were able to be designated as either rural or urban. Furthermore, the regions that were categorized as rural were further split up into groups labeled rural level one and rural level two (Massachusetts State Office of Rural Health). Rural level one sites were identified as being slightly more developed than rural level two sites, which lacked any major human development. Ultimately, four collection sites were selected from both urban and rural level 1 locations. The sites were chosen based on the presence of hiking trails or walking paths that were easily accessible on foot and open to the public. After selecting our sites, a complete tick-sampling protocol was written before beginning the collection process.

5.2. Tick sampling

The procedure for locating and collecting *Ixodes scapularis* was developed by consulting online literature to learn how similar have succeeded in obtaining large quantities of environmental tick samples. Among the sources that were used as references, the most common and effective method for collecting tick samples was by dragging a large cloth sheet over vegetation and leaf litter in forested areas (Salomon, 2020). Ticks operate as opportunistic parasites, and as such, they will readily attach themselves to any passing surface. Any ticks that are questing on grasses or shrubs will cling to the cloth sheet and can be removed with a pair of tweezers.

To make use of this approach, the students were provided with two separate 1-meter by 1-meter canvas banners with the ends looped over wooden dowels. These dowels in turn were affixed with short lengths of bungee cord to be held, allowing for the sheets to easily be dragged while walking. These sheets were the main tools in the tick collection kit, which also included tweezers for handling the ticks, conical tubes for sample storage, nitrile gloves, and full-body protective attire to negate the possibility of being infected by any ticks ourselves. Through the WPI Biology and Biotechnology Department, a large quantity of white painters' jumpsuits were provided, which were sufficient to fully cover the body and prevent skin contact with vegetation. For an extra layer of protection, these jumpsuits into the socks and gloves and sealed the opening with duct tape; from the neck down, the students were completely impervious to any skin contact with questing ticks. Additionally, our shoes were treated with an insecticide called Permethrin to further reduce the risk of any ticks becoming attached during collection.

Figure 5: Full complement of tick collection equipment for an individual.



Once our collection equipment had been acquired, the students were prepared to begin visiting sites to collect environmental samples. The first site, the Cascade Falls Loop Trail located in western Worcester, was categorized as urban due to its proximity to residential neighborhoods. In the field, *Ixodes* samples were accumulated through a combination of two techniques involving the canvas banners, both "dragging" and "flagging". Dragging is, as implied, simply pulling the banner across the surface of the ground by the bungee cord. Alternatively, flagging is performed by lifting the banner and passing it over vegetation that is higher than ground level, such as shrubs and small trees. While flagging allows for tick samples to be obtained from larger plants, dragging is typically preferable due to its comparable consistency and overall surface area that is contacted. As the banners were pulled over vegetation, they were checked approximately every ten to fifteen paces to see if any ticks were attached. When a tick was spotted, it was removed from the canvas surface using tweezers and

placed immediately into a conical tube filled with 70-95% ethanol to both neutralize and store the sample.

Figure 6: Dragging method of collecting environmental tick samples

At the end of every sampling session, safety measures were taken by all participating group members to minimize the risk of contracting Lyme disease or any other tick-borne pathogens. Before removing the bodysuits, comprehensive visual checks were performed to ensure that no ticks were attached. Once the absence of ticks was confirmed, the bodysuits and nitrile gloves were removed and placed into trash bags to isolate them; this was to prevent the possibility of any ticks that we missed during our body checks becoming mobile in our vehicles. Upon returning to WPI campus, the ticks that were obtained were placed in the project lab and the equipment was returned to proper storage for the next session. Finally, as a final protective measure, participating group members took hot showers to wash away any ticks that may have been on the skin but had not yet bit and become attached.

Table 1: Categorization of collection sites

Site Name	Development Type
Site 7: Cascade Falls Loop Trail	Urban
Site 8: Pyramids Disc Golf Course	Urban
Site 9: Lake Park	Urban
Site 10: Pakachoag Hiking Trail	Urban
Site 11: Rutland State Park	Rural level one
Site 12: Moore State Park	Rural level one
Site 13: North Brookfield Rail Trail	Rural level one
Site 14: Oakham Wildlife Management Area	Rural level one

To effectively keep track of each tick that was obtained from the collection sites, it was necessary to catalog them according to a standardized system. First, all of the ticks collected from each site were examined under a microscope to determine whether they were male, female, or nymph. A running tally was taken throughout this process, and the total number of ticks from each sex was recorded in a spreadsheet. Additionally, the proportion of ticks from each sex from all of the collection sites combined was measured and visualized in a pie chart.

After organization by sex was completed, a code was developed by which to demarcate and identify a given tick by its origin, sex, and number within its specific cohort. For example, a tick assigned a code of “7F1” is a female tick from site 7 that was identified as the first female; a tick’s number within its location cohort was assigned arbitrarily as the ticks were individually

counted. After a tick was assigned a code, they were placed in correspondingly labeled microcentrifuge tubes for storage.

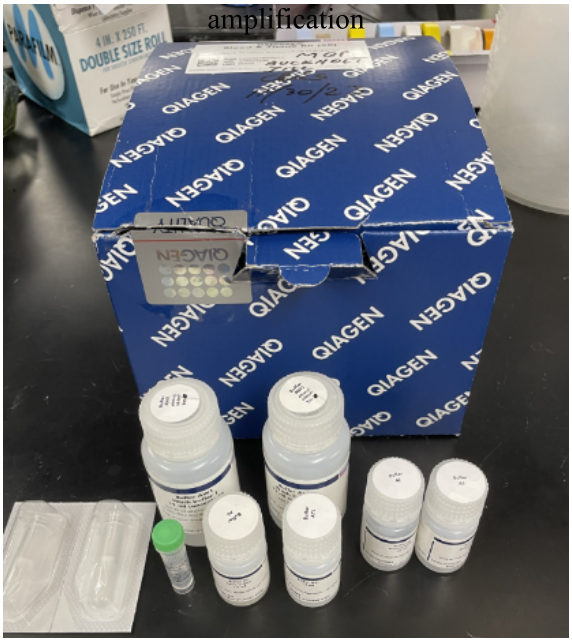
5.3. DNA Extraction

The first DNA extraction kit used was the MasterPure™ DNA Purification Kit. When testing it, it was discovered that the extraction process yielded low amounts of DNA for our tick samples. Additionally, through an interview with Sam Telford, a professor at Tufts Veterinary School, it was determined that it is better to freeze dry ticks without ever storing them in ethanol. The ticks had been previously stored by being frozen in tubes while suspended in ethanol. Ethanol dries out the ticks and makes the DNA extraction protocol more difficult. Therefore, we had to reverse the drying out that the ethanol caused. The ticks were removed from the ethanol and left to dry overnight. Then, the ticks were rehydrated by being suspended in water for 48 hours. Finally, they were dried in water and transferred into individually labeled tubes in the freezer (-20 ℃).

As the ticks were in the process of being rehydrated, it was found that various studies had used the Qiagen DNeasy Blood and Tissue Kit when extracting DNA from ticks. Additionally, an edited protocol of the DNA extraction kit was found which was specifically for extracting DNA from ticks. Therefore, it was decided that the Qiagen DNeasy Blood and Tissue Kit would be used going forward. The edited protocol was followed with ticks that were rehydrated and frozen (Appendix). One of the special instructions for the tick protocol was slicing the ticks in half when using proteinase K as the DNA of pathogens is most commonly found in the gut microbiome of ticks. Additionally, the amount of elution buffer used in the final step was dependent on the size of the tick. The new DNA extraction protocol was tested via NanoDrop.

Knowing the approximate amount of DNA present for each tick would help know the amount of DNA needed for PCR.

Figure 7: QIAGEN DNeasy Blood and Tissue Kit used for extracting DNA from *Ixodes scapularis* in preparation for PCR amplification



5.4. Acquisition of primers and controls

To determine whether any of the ticks had *B. burgdorferi*, a PCR primer and a positive control were acquired. The control for *B. burgdorferi* was provided as dried DNA from Dr. Jory Brinkerhoff, a professor at the University of Richmond. The *B. burgdorferi* primer was acquired from one of our advisors, Dr. Chris Collins. Additionally, though the ticks were determined as *I. scapularis* by morphological features, further confirmation was needed due to other *Ixodes* species bearing resemblance to *I. scapularis*. An *I. scapularis* primer could verify the species of the tick and serve as a control for each tick. If the tick primer was negative, then the results would be discarded or indicate that the tick was a different species. Through a literature review, a sequence that has previously been used as a primer for *I. scapularis* was found (Hojgaard et al., 2014). Using this sequence, primers were ordered from Integrated DNA Technologies™. Once

primers and a positive control were collected, PCR could be performed using the tick DNA extracts.

Table 2: PCR primer information

Primer Name	Forward Sequence	Reverse Sequence	Product Length	Source
Ixodes scapularis actin	5' GGC CTG GAC TCC GAG CAG 3'	5' CCG TCG GGA AGC TCG TAG G 3'	77 bp	(Hojgaard et al., 2014)
Borrelia burgdorferi primer	5' GGT ATC AGA AAA TCC ATT CAT ACT TG 3'	5' TAC ATT GCT GAA AAT TCA CCA CTA CTT 3'	135 bp	Dr. Chris Collins

5.5. Polymerase chain reaction (PCR)

The PCR was performed using One*Taq*® 2X Master Mix with Standard Buffer (New England Biolabs) under the following conditions, modified from Hojgaard et al., 2014: 95°C for 3 minutes followed by 40 steps of 30 s at 95°C, 30s at 48°C, 30s at 68°C, with a final hold of 5 minutes at 68°C. To avoid unintended primer interactions, the *I. scapularis* actin and *B. burgdorferi* primers were run separately on each tick sample. Therefore, each tick would undergo PCR twice, one time for each primer set. Where possible, two samples from each site were tested, one male and one female. A *B. burgdorferi* positive control was used alongside 2 negative controls, one for the *I. scapularis* primer and another for the *B. burgdorferi* primer.

5.6. Gel electrophoresis

To assess the results of the PCR, DNA gel electrophoresis was performed. Initially, a 0.8% agar gel was used, however, this provided inadequate separation of bands and poor resolution. Tests were performed on higher agar percentages, such as 2.5%, but they ultimately

were too cloudy for the gel imager to read. An experiment was performed where the same samples were imaged on differing percentages of gels. It was determined that a 1.8% gel was the most ideal for our purposes. All gels were run at 140 V, 300 mA for 30 minutes.

6. Results and discussion

6.1. Outcomes of environmental tick sampling

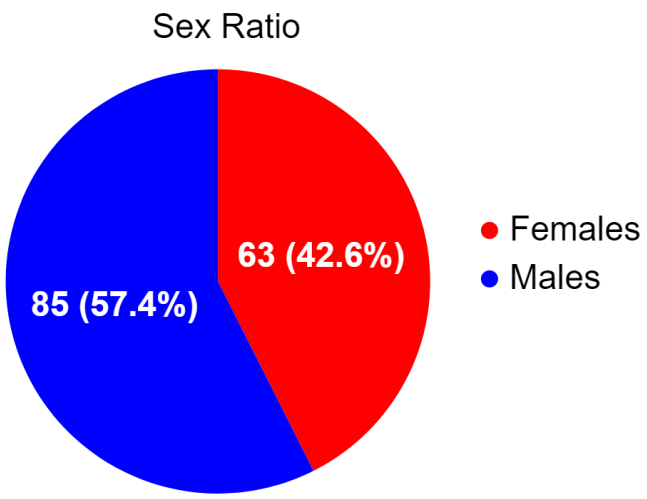
Including those provided by our project advisors at the start of the academic year, a total of 475 ticks were obtained for testing. Each tick was assigned a code as described in the methodology to allow for straightforward counting and organization. For each collection site, the number of ticks belonging to each sex, as well as the number of ticks identified as nymphs, were tabulated in a Google Sheet. Additionally, the overall ratio of female to male ticks was visualized as a pie chart. Sites highlighted in red were provided by our advisor Dr. Collins and were collected in New York. Ticks collected from these sites were used for testing and troubleshooting our methodologies because they did not originate from Massachusetts and were therefore not representative of our intentions to study Lyme disease strictly within Worcester County.

Table 3: Total number of ticks obtained from each chosen collection site

Site name/number	# of female ticks	# of male ticks	# of nymphs
APB-Burned (1)	42	22	0
APB-Unburned (2)	46	39	1
Hartwick (3)	7	2	0
Wiber Lake (4)	30	27	0
APB-Burned (5)	14	27	0
APB-Unburned (6)	21	49	0

Cascade Falls Loop Trail (7)	7	10	0
Woods/pond behind Pyramid Disc Golf Course (8)	4	15	0
Lake Park Trail (9)	0	0	0
Pakachoag Trail (10)	11	13	0
Rutland State Park (11)	9	17	0
Moore State Park (12)	4	2	0
North Brookfield Rail Trail (13)	26	28	0
Oakham Wildlife Management Area (14)	2	0	0

Figure 8: Sex ratio of student collected ticks.

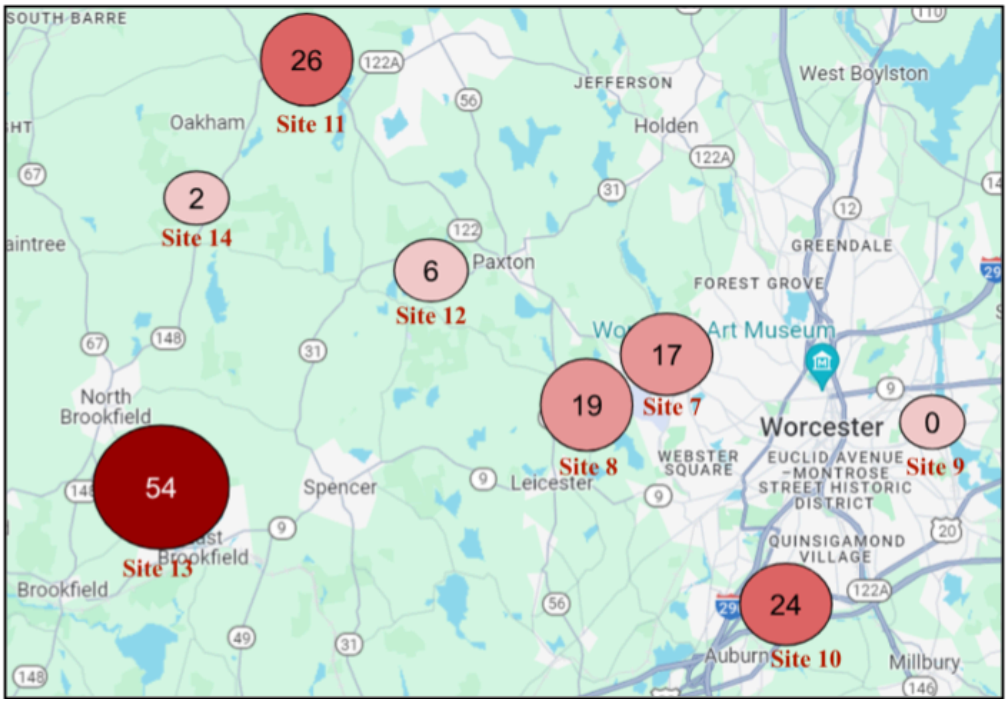


Across all fourteen of the collection sites, there was a high degree of variation in the number of ticks that were gathered. A typical collection session spanned between two and four hours, during which time collection was performed continuously without major pauses. The methodology used to collect tick samples was invariable; apart from minor adjustments to

account for site-specific differences in terrain and vegetation, the protocol for flagging and dragging was performed uniformly across all sites. As such, the observed differences in the number of ticks from each site must be attributable to a range of independent, environmental factors.

While the temperature during collection sessions affected the number of ticks that could be obtained, weather patterns during the tick collection period (October through November) as a whole were often not permissive to sample collection. High rainfall totals were experienced in central Massachusetts, especially during the weekends when group members were available to participate in the collection. In short, the total number of sites that were visited was restricted by environmental factors, which in turn determined the quantity of *I. scapularis* samples that were obtained.

Figure 9: Location of student-collection sites (7-14) within Worcester County, MA



6.2. Issues with DNA extraction and troubleshooting

While tick collection was still in progress, whole-DNA extraction was initially conducted using MasterPure™ DNA Purification Kit. This was done to ensure that the kit was effective in purifying nucleic acids before focusing solely on in-lab activities; essentially, to save time and avoid future delays during the winter months during which the group planned to complete the majority of the in-lab activities relevant to the project. The initial products yielded by the MasterPure™ DNA Purification Kit were deemed inadequate for use in PCR as determined by the resultant NanoDrop readings. The average measured nucleic acid concentration for the twelve samples was calculated at 1.69 nanograms per microliter; given the small size of the tick specimens, this value was expectedly low and not cause for concern. However, the measured A260/A230 ratios indicated issues with the extraction procedure regarding possible contamination.

As measured by a spectrophotometer, a A260/A230 value can detect the presence of undesirable organic compounds within a nucleic acid extraction sample; a low value relative to 1 typically indicates impurity. For the first round of twelve extraction samples, the average A260/A230 value was calculated at approximately 0.39. To investigate potential causes of this result, technical papers were accessed online which listed phenol, guanidine, and glycogen as possible contaminants (Yale School of Medicine). Without a known or readily available method to test whether these contaminants were present in our DNA extracts, we elected to rehydrate the ticks according to Dr. Telford's previous advice and try a different DNA extraction kit, the Qiagen DNeasy Blood and Tissue Kit. After changing these protocols, the extracted DNA from each tick increased 2-3 fold. For 36 tested ticks, the new average DNA concentration was measured at 10.57 nanograms per microliter, and the average A260/A230 ratio at 0.55.

6.3. PCR and gel results

The PCR thermal cycler conditions were adapted from where the *I. scapularis* primer sequence was provided (Hojgaard et al., 2014). This protocol was adjusted to allow both primers to run under the same temperature parameters. To accomplish this, the annealing temperature was lowered to 48°C because the *B. burgdorferi* primer had a lower melting temperature than the *I. scapularis* primer. Additionally, this adjustment helped improve the efficiency of being able to process and analyze samples.

For the analysis of the PCR product through gel electrophoresis, there was some troubleshooting. One adjustment that was made was adjusting the voltage and amperage. It was decided to be 140 V and 300 mA. Another adjustment was the agarose gel percentage. Originally, the samples were being run on a 0.8% gel. The resolution was poor and the separation between the two bands was small. Therefore, we raised the agarose gel percentage to 1.8%. These changes allowed for a gel that had better resolution and better separation between the bands of *B. burgdorferi* and *I. scapularis.*

Figure 10: 0.8% Gel

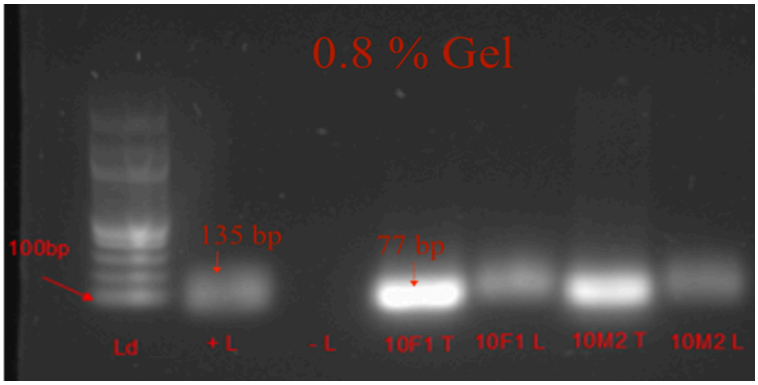
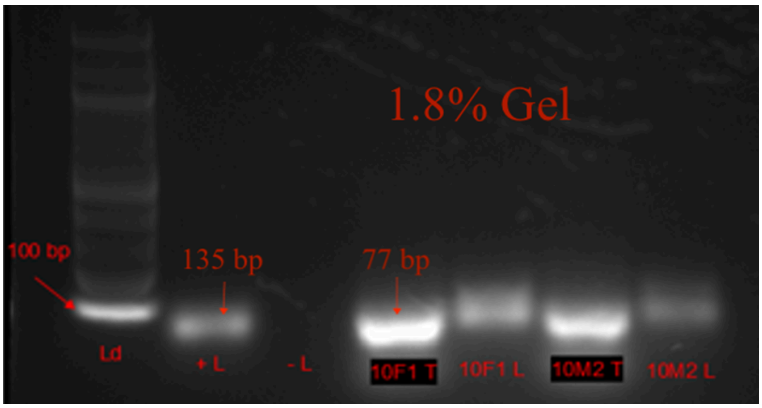


Figure 11: 1.8% Gel



From obtaining that gel the next step was testing 2 samples from each site with one being male and female. This gel was found to be contaminated as both of the negative controls came out positive. The samples were run again and once again the negative controls came back as positive. Through testing, it was found that the primers had been contaminated. This setback was useful in showing how easy it is for contamination to occur. The group recommends that when handling the negative and positive controls they should be done in separate spaces to eliminate chances of contamination. Additionally, the group suggests that further adjustments such as finding primers for *B. burgdorferi* and *I. scapularis* that have more base pairs and differing sizes should be taken to further improve detection and quality of bands.

6.4. Future directions

With the majority of the exploratory experimentation and troubleshooting complete, the next group to pursue this project can immediately begin collecting and testing ticks. This will allow them to apply the procedure to a higher number of tick specimens and subsequently obtain a larger dataset of the observed pathogen frequency in different collection sites. Although we were not able to investigate the urban-rural hypothesis, further research can be conducted with our provided experimental framework. Additionally, with a more comprehensive understanding

of the prevalence of Lyme disease in central Massachusetts, there will be potential for further investigation into the effects of related environmental factors. Possible examples include urbanization, host animal population dynamics, weather patterns, and any other metrics that may influence the recorded prevalence of Lyme disease in a given site. The conclusions drawn from this future research may be used to increase public awareness of Lyme disease, as well as to model which areas of central Massachusetts pose higher risks for Lyme disease exposure.

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8. Appendix

8.1 Appendix of Tables and Figures

8.2 Appendix of Protocols

Tick Collection Procedure

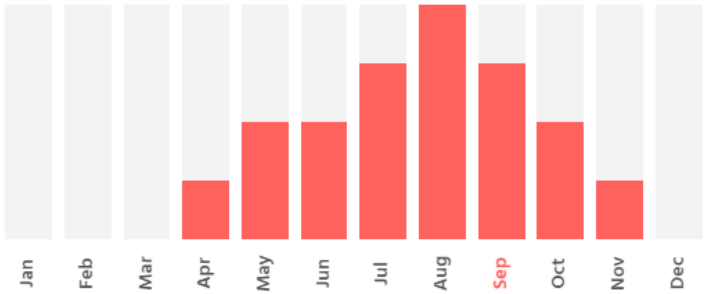
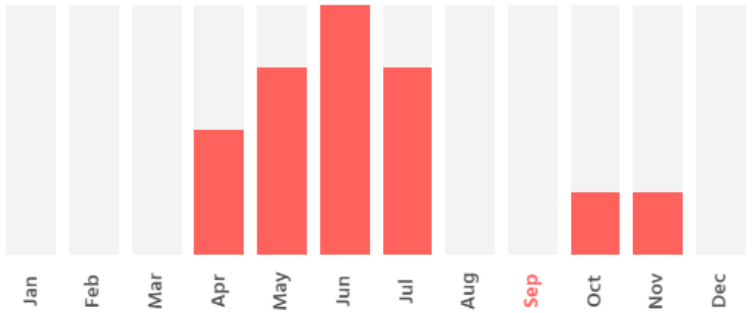
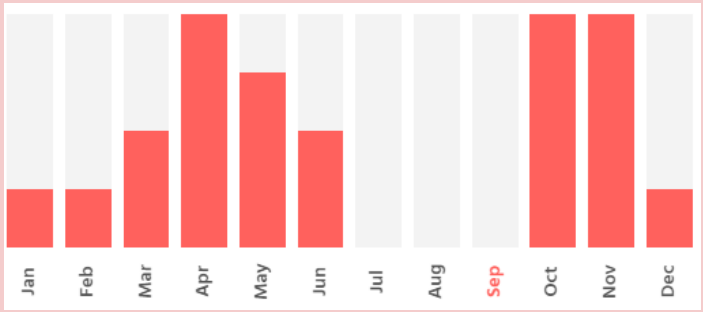
Tick Hunt MQP

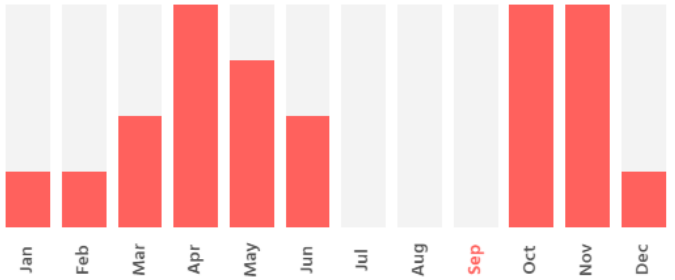
Background

The two main tick species that will be present at the sampling sites are the deer tick (*Ixodes scapularis*), the dog tick (*Dermacentor variabilis*), and the lone star tick (*Amblyomma americanum*). Each tick has a distinct appearance and preferred habitat, meaning that some sampling sites might have different levels of each species than others. In order to correctly identify and sort each species of ticks, it is important to know what each tick looks like at every stage in its life and where they are located.

Species	Preferred Habitat
Deer Tick	Deciduous forest (trees that lose leaves)
Dog Tick	Brushes and open areas where tree cover is limited (trails, grassy fields)
Lone Star Tick	Forests with thick underbrush

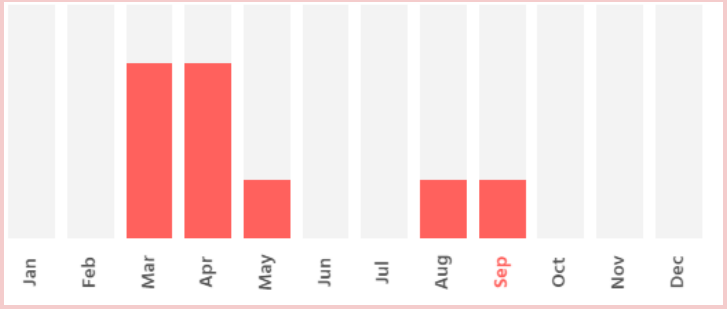
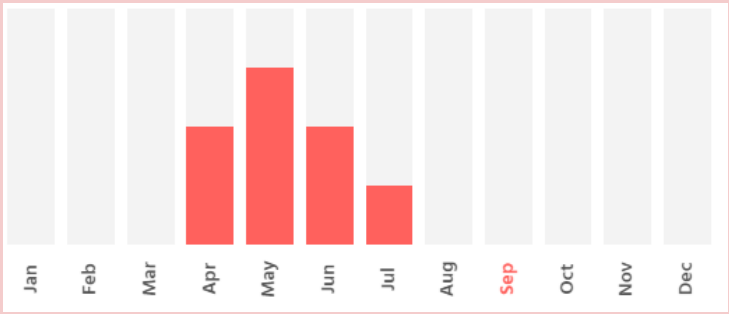
Deer Tick - *Ixodes scapularis*

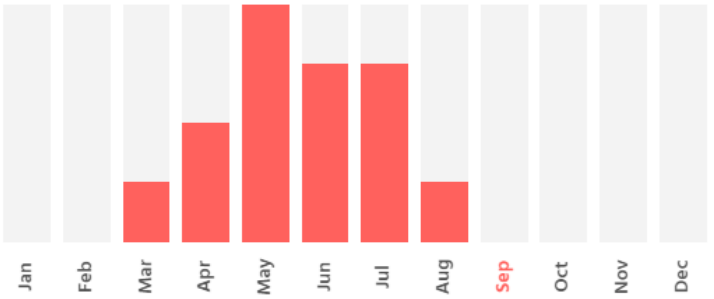
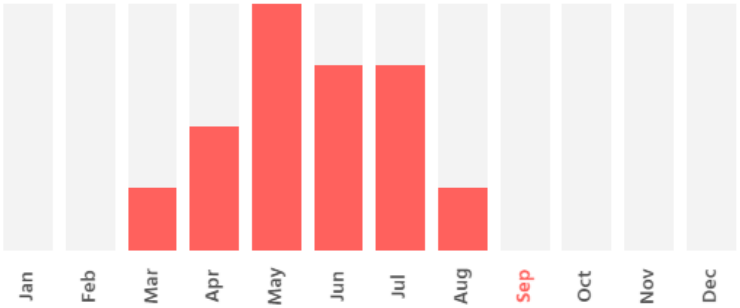
Stage	Picture	Activity
Larva		
Nymph		
Adult Male		

Adult Female		

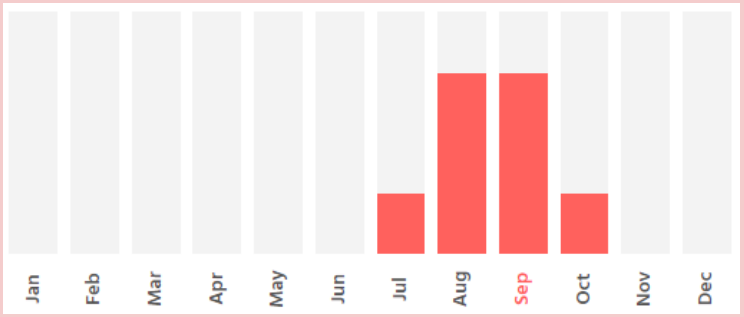
Ticks highlighted in red do not spread Lyme disease to humans

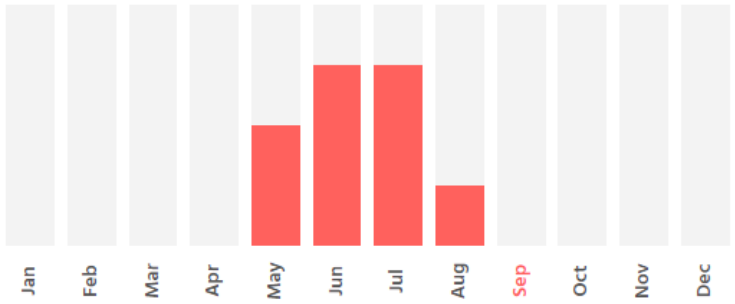
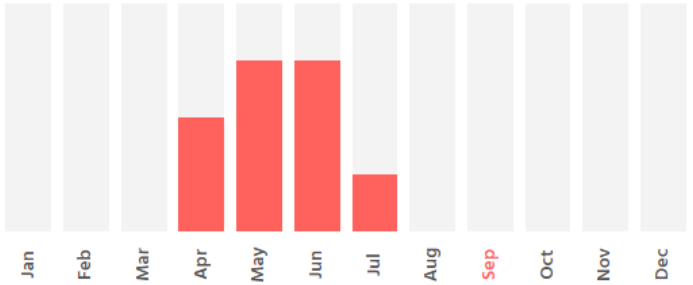
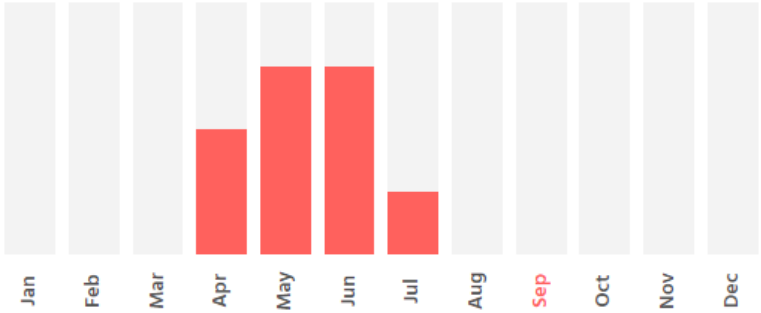
Dog Tick - *Dermacentor variabilis*

Stage	Picture	Activity
Larva		
Nymph		

Adult Male		
Adult Female		

Lone Star Tick - *Amblyomma americanum*

Stage	Picture	Activity
Larva		

Nymph		
Adult Male		
Adult Female		

Procedure

Materials

- Dragging apparatus
- Painter's suit/coveralls
- Nitrile gloves
- Large trash bag
- 3, gallon plastic bags
- Extra pair of shoes
- Microfuge tubes to hold ticks
- Duct tape
- Clear packing tape
- Tweezers

Procedure

1. Arrive at the sampling site and don appropriate PPE
    - Put on painter suits and coveralls
    - Remove day-to-day shoes and put on extra pair of shoes/boots
    - Put on gloves and use duct tape to cover the gap between the gloves and sleeves
    - Tuck pant legs into socks and use duct tape to cover the opening.
2. Take note of weather and atmospheric conditions, vegetation
    - Temperature, humidity, rainfall, and other factors may affect tick activity and subsequently the amount that we are able to collect on any given sampling day.
3. Lay out drag cloth and begin walking at a set pace.
    - Every increment of **15** steps, stop and have a partner examine the drag net, or examine it yourself.
4. Tick collection
    - Remove any ticks on the cloth with tweezers and place in individual tubes. If removing them is difficult, use the clear packing tape tape instead.
5. Tick check

- After every checking of the dragging cloth, also check each other (or yourself) for ticks

6. Once a reasonable amount of ticks or 2 hours have passed, carefully unequip PPE and check thoroughly for ticks on skin.
    - Place painter suits in a trash bag
    - Place second pair of shoes in a plastic bag
    - Place collected test tubes in freezer and label them in bulk with site number
7. At home safety
    - Take a hot shower immediately and perform a tick check
    - Wash all clothes worn in-field using hot water setting or place in the dryer at high heat setting for at least 10 minutes. Cold or warm settings do not kill ticks.
    - Check shoes for ticks or place in dryer with clothes
    - Monitor yourself for any symptoms of Lyme disease for the next couple of days

QIAGEN Supplementary Protocol:

Purification of total DNA from ticks using the DNeasy® Blood & Tissue Kit for detection of *Borrelia* DNA

This protocol provides recommendations for DNA purification from ticks, for use in real-time PCR detection of *Borrelia* spp., using, for example, the QIAGEN® *artus*® Borrelia LC PCR Kit (cat. no. 4551063 or 4551065, not available in the USA).

Introduction

Due to generally low numbers of *Borrelia* in ticks, both complete digestion of tick tissue (except the exoskeleton) and removal of inhibitors are crucial to guarantee the highest possible sensitivity in downstream PCR.

In general, for DNA preparation single, whole ticks are used, except for ticks that are engorged with blood. Preparation of the whole tick is only useful if the body does not exceed 5 mm. Larger ticks most probably have recently sucked blood and therefore may inhibit downstream PCR analysis due to the release of inhibitors from digested blood.

Therefore, use only the head section of large and often dark colored ticks (females). Cut off the abdomen containing most of the digested blood with a sharp scalpel. Frequently, relatively small ticks (males) can be found fixed to the ventral body of a much bigger female. In this case only the females (head) have to be analyzed, because the males did not have contact with the host.

IMPORTANT: Please read the *DNeasy Blood & Tissue Handbook*, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning this procedure.

DNeasy Blood & Tissue Kits: For Research Use Only. Not for use in diagnostics procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease. The *artus* Borrelia LC PCR Kit is not available in the USA. Purification of total DNA from ticks using the DNeasy Blood & Tissue Kit and detection of *Borrelia* DNA using the *artus* Borrelia LC PCR Kit is a research application.

Equipment and reagents required

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- DNeasy Blood & Tissue Kit (cat. no. 69504 or 69506)
- Pipets and pipet tips
- Scalpel
- Vortexer
- Microcentrifuge tubes (1.5 ml or 2 ml)

- Microcentrifuge with rotor for 1.5 ml and 2 ml tubes
- Thermomixer, shaking water bath, or rocking platform for heating at 56°C and 70°C
- Ethanol (96–100%)*
- Carrier RNA solution, 10 mg/ml (e.g., poly A RNA Homopolymer, Amersham Biosciences, cat. no. 27-4110-01)†
- Optional: Internal control DNA (e.g., as provided in the QIAGEN *artus* Borrelia LC PCR Kit, cat. no. 4551063 or 4551065, not available in the USA)

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" in the *DNeasy Blood & Tissue Handbook*.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 4.
- If using frozen samples, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Place the whole tick or the head in a 1.5 ml microcentrifuge tube with 180 µl Buffer ATL. Vortex thoroughly.**
2. **Draw the moistened body of the tick with the tip of a pipette to the upper rim of the opened microcentrifuge tube and cut it with a scalpel once longitudinally and once diagonally (depending on the sample size, further cuts may be necessary).**

   Cutting the tick is necessary so that the tick tissue is thoroughly lysed and the *Borrelia* contained in it can be quantified.
3. **Close the microcentrifuge tube and centrifuge briefly to collect all tissue pieces at the bottom of the tube.**

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

† This is not a complete list of suppliers and does not include many important vendors of biological supplies.

4. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed (only the exoskeleton remains). Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking shaker.**

   Lysis time varies depending on the size of the tissue pieces. Lysis is usually complete in 30–60 min. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   Heat the thermomixer, shaking water bath, or rocking shaker to 70°C after this step if it will be used for the incubation in step 5.

5. **Vortex for 15 s. Add 200 µl Buffer AL (without added ethanol) to the sample, and mix thoroughly by vortexing. Incubate at 70°C for 10 min.**

   Ensure that ethanol has not been added to Buffer AL (see "Buffer AL" in the *DNeasy Blood & Tissue Handbook*). Buffer AL can be purchased separately.

   It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL. This precipitate does not interfere with the DNeasy procedure.

6. **Add 1 µl of carrier RNA (10 mg/ml), and mix thoroughly by vortexing. Then add 230 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   Optional: Add internal control DNA with the carrier RNA. Adjust the amount of internal control DNA according to the final elution volume. For very small ticks (<5 mm), a final elution volume of 60 µl is recommended. For larger ticks (or part of one), use elution volumes up to 200 µl. When using the *artus* Borrelia LC PCR Kit, add 6 µl or up to 20 µl, respectively, of the Internal Control provided in the kit.

   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. This precipitate does not interfere with the DNeasy procedure.

7. **Pipet the mixture from step 6 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.***

8. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.***

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See *DNeasy Blood & Tissue Handbook* for safety information.

9. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x *g* (14,000 rpm).

10. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 35 µl Buffer AE (for small ticks, <5 mm) or 105 µl Buffer AE (for large ticks) directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**

11. **Pipet another 30 µl Buffer AE (for small ticks, <5 mm) or 100 µl Buffer AE (for large ticks) directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**

    This step leads to increased overall DNA yield.

    Eluting with 35 µl + 30 µl Buffer AE results in an eluate volume of approximately 60 µl. Eluting with 105 µl + 100 µl Buffer AE results in an eluate volume of approximately 200 µl.

    The microcentrifuge tube from step 10 should be reused for this elution step.

    Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Troubleshooting

For general troubleshooting, please consult the Troubleshooting Guide in the *DNeasy Blood & Tissue Handbook*.

Tick MQP PCR Protocol

Materials

- Extracted tick DNA
- OneTaq® 2X Master Mix
- Primers
- Sterile water
- Domed PCR tubes

Procedure

1. Obtain one domed PCR tube for each desired PCR reaction
2. Label each PCR tube with the corresponding tick identification number
3. Pipette 2 µL of extracted tick DNA into its PCR tube
    a. This volume can be adjusted to increase the amount of DNA present for PCR. DNA amount **cannot exceed 1,000 ng**.
4. Pipette into each tube the following:

OneTaq® 2X Master Mix	12.5 µL
100 uM Forward Primer	0.5 µL
100 uM Reverse Primer	0.5 µL
Sterile Water	Amount needed to reach a final volume of 25 µL

5. Place all tubes into the thermal cycler and close the lid. Spin the dial to tighten the lid closed
6. Set thermocycler conditions
    a. Annealing temperature **cannot** be over T_m and is usually a couple of degrees under T_m
7. Wait until the thermal cycler is done, or wait overnight
8. Retrieve sample(s) and proceed to the Gel Protocol
9. Samples can be stored in the freezer if needed

Tick MQP Gel Electrophoresis Protocol

Materials

- PCR Product
- Agar Powder
- 1X TAE Buffer
- Hyperladder
- Loading Dye
- 0.5mL tubes

Procedure

Creating the Gel

1. Choose the appropriate agar percentage for the gel. Higher agarose percentages are better for smaller PCR product sizes.
2. Create the agarose gel using the following formula:
    a. X% gel = X grams of agar powder per 100mL of 1X TAE buffer
        i. Ex: 1.5% gel = 1.5 grams agar powder per 100mL 1X TAE buffer

NOTE: One gel is 50mL of agarose gel mixture

3. Measure out the desired amount of agar powder on a scale and place the powder into an Erlenmeyer flask
4. Measure out the desired amount of 1X TAE buffer and place it into the Erlenmeyer flask
5. Swirl the flask until the powder is mixed with the liquid
6. Ensure that the gel electrophoresis machine is set up and the gel comb is in place
7. Microwave the mixture for 30-second intervals, swirling each time
8. Once the mixture starts bubbling and is completely clear, immediately stop the microwave and remove the mixture. Swirl the mixture until no powder is visible.
9. Add 0.5µL of SYBR Green per 50mL of agarose gel mixture
10. Immediately pour the mixture into the gel cast of the electrophoresis machine
11. Wait until the gel is completely solid (30-45 minutes)
12. Remove the gel comb

Running the Gel

1. Once the gel is solid, pour 1X TAE buffer over the gel until the liquid level is slightly above the gel (both electrode wells must be filled)
2. Obtain the desired number of 0.5mL tubes, one for each well that will be loaded. Label each tube.
3. Place 10µL of PCR product into its corresponding 0.5mL tube
4. Place 2µL of loading dye into each 0.5 mL tube. Gently mix.

5. Using a gel loading tip, load 8µL of hyperladder into the first well
6. Load all 12µL of samples into each well
7. Set the power supply to 140 V, 300 mA
8. Run the machine for 30-45 minutes, until the bottom ladder reaches at least the second comb notch

Imaging the Gel

1. Position the gel onto the gel imaging tray, using the camera to center the gel
2. Run with SYBR Safe setting and ensure the filter is in position 1
3. Run the program