TOS PROTOCOL AND PROCEDURE:
TICK AND TICK-BORNE PATHOGEN SAMPLING

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See configuration management system for approval history.

Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
-	05/16/2011	ECO-00151	Draft protocol
A_DRAFT	10/03/2011	ECO-00280	Updated draft after 2011 field season
B_DRAFT	07/12/2012	ECO-00497	Updated draft for 2012 field season
C_DRAFT	01/10/2014	ECO-01139	Updated draft for 2013 field season
D	03/19/2014	ECO-01669	Production release, template change, and other changes as detailed in Appendix C (only in rev D)
E	10/01/2014	ECO-02321	Migration to new protocol template

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1 OVERVIEW

1.1 Background

Ticks transmit numerous pathogens of wildlife, livestock, and humans, including the etiological agent of Lyme disease (*Borrelia burgdorferi*), the most frequently reported vector-borne disease of humans in the United States. Among arthropod vectors, ticks are particularly sensitive to meteorological conditions and associated physiological constraints, making it highly likely that the demography and biogeography of many tick species, and the pathogens they transmit, will be affected by climate change.

Further, the multi-host lifecycles of most tick species increase their ecological connectivity and sensitivity to community-level perturbations that may arise from changes in human land- and resource-use practices. Based on these epidemiological and ecological characteristic ticks and tick-borne pathogens will be sampled within the National Ecological Observatory Network (NEON). The objectives of sampling are to quantify spatio-temporal changes in the abundance of ticks at NEON sites and in the prevalence of infection by associated tick-borne pathogens. Rationale for the sampling protocol provided in this document can be found in the NEON Science Design for Vectors and Pathogens (AD[06]).

1.2 Scope

This document provides a change-controlled version of Observatory protocols and procedures. Documentation of content changes (i.e. changes in particular tasks or safety practices) will occur via this change-controlled document, not through field manuals or training materials.

1.2.1 NEON Science Requirements and Data Products

This protocol fulfills Observatory science requirements that reside in NEON's Dynamic Object-Oriented Requirements System (DOORS). Copies of approved science requirements have been exported from DOORS and are available in NEON's document repository, or upon request.

Execution of this protocol procures samples and/or generates raw data satisfying NEON Observatory scientific requirements. These data and samples are used to create NEON data products, and are documented in the NEON Scientific Data Products Catalog (RD[03]).

1.3 Acknowledgments

N/A

2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain higher-level information that is implemented in the current document. Examples include designs, plans, or standards.

AD[01]	NEON.DOC.004300	EHS Safety Policy and Program Manual
AD[02]	NEON.DOC.004316	Operations Field Safety and Security Plan
AD[03]	NEON.DOC.000724	Domain Chemical Hygiene Plan and Biosafety Manual
AD[04]	NEON.DOC.001155	NEON Training Plan
AD[05]	NEON.DOC.050005	Field Operations Job Instruction Training Plan
AD[06]	NEON.DOC.000911	NEON Science Design for Vectors and Pathogens
AD[07]	NEON.DOC.014051	Field Audit Plan
AD[08]	NEON.DOC.000824	Data and Data Product Quality Assurance and Control Plan

2.2 Reference Documents

Reference documents contain information that supports or complements the current document. Examples include related protocols, datasheets, or general-information references.

RD[01]	NEON.DOC.000008	NEON Acronym List
RD[02]	NEON.DOC.000243	NEON Glossary of Terms
RD[03]	NEON.DOC.005003	NEON Scientific Data Products Catalog
RD[04]	NEON.DOC.001271	NEON Protocol and Procedure: Manual Data Transcription
RD[05]	NEON.DOC.001100	TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing
RD[06]	NEON.DOC.001583	Datasheets for TOS Protocol and Procedure: Tick and Tick-borne Pathogen Sampling

2.3 Acronyms

All acronyms used in this document are defined in RD[01].

2.4 Definitions

N/A

3 METHOD

Tick and tick-borne pathogen sampling involves the collection of ticks using drag and/or flag sampling. Following minimal in-house processing, samples will be sent to one or more external facilities where ticks will be identified to lowest taxonomic rank (preferably species). A subset of identified ticks will be tested to quantify the prevalence of infection by various prokaryotic pathogens. Some ticks will be set aside for long-term archiving.

Standard Operating Procedures (SOPs), in Section 7 of this document, provide detailed step-by-step directions, contingency plans, sampling tips, and best practices for implementing this sampling procedure. To properly collect and process samples, field technicians **must** follow the protocol and associated SOPs. Use NEON's problem reporting system to resolve any field issues associated with implementing this protocol.

The value of NEON data hinges on consistent implementation of this protocol across all NEON domains, for the life of the project. It is therefore essential that field personnel carry out this protocol as outlined in this document. In the event that local conditions create uncertainty about carrying out these steps, it is critical that technicians document the problem and enter it in NEON's problem tracking system.

The procedures described in this protocol will be audited according to the Field Audit Plan (AD[07]). Additional quality assurance will be performed on data collected via these procedures according to the NEON Data and Data Product Quality Assurance and Control Plan (AD[08]).

4 SAMPLING SCHEDULE

4.1 Sampling Frequency and Timing

Bouts of tick and tick-borne pathogen sampling will be conducted annually from March through December. At each site, sampling occurs at six distributed plots that are iteratively resampled for every sampling bout. Two sampling plans, each with a different sampling bout (aka event) frequency, are possible.

At each site, sampling begins with the **low intensity plan**, which involves one bout every six weeks. Collection of one or more ticks triggers a switch to the **high intensity plan**, which involves one bout every three weeks. Once high intensity sampling is initiated at a site it continues for the remainder of the season and all subsequent seasons irrespective of per-bout sampling success

4.2 Criteria for Determining Onset and Cessation of Sampling

The first bout of tick and tick-borne sampling each year will occur as soon as the temperature thresholds are met during the March-December sampling window. Subsequent events will be scheduled according to the fixed sampling frequency: every three weeks for high intensity sampling, every six weeks for low intensity sampling. Site-specific dates appear in Appendix E.

4.3 Timing for Laboratory Processing and Analysis

From a technical/scientific perspective, tick samples held in vials containing ethanol and stored at -20C (or 4C) will retain their integrity for many months.

4.4 Sampling Timing Contingencies

For both the high and low intensity sampling plans, a bout of sampling will only be performed if the high temperature on the day prior to planned sampling was >0°C and the mean high temperature in the five days prior to planned sampling was >7°C. Obtain this information from a publically-available source of meteorological data based on sensors located as close as possible to the sampling site. Eventually this information will be available as data streaming from the NEON tower.

Drag and/or flag sampling should only be conducted when conditions are dry (e.g., not during or immediately after a rain event or on a morning with heavy dew). Additionally, while sampling can be conducted during any time of day, the hottest period of the day (mid to late afternoon) should be avoided if possible.

When unexpected conditions require deviations from the field protocols outlined in this document, implementation guidelines outlined in the tables below should be followed.

General contingent decisions that apply broadly to tick sampling include:

- Sampling must be conducted when the ground is dry. Do not sample if the ground is moist (e.g., heavy morning dew or following a rain event).
- If possible, avoid sampling during the hottest part of the day on days for which the high temperature is at or near the annual high temperature for the site.

Table 1. Contingent decisions for high-intensity sampling

Delay/Situation	Action	Outcome for Data Products
Delay ≤ 2 days	If the delay occurs prior to the start of the sampling bout, reattempt the complete bout at the conclusion of the delay. If the delay occurs during the sampling bout, resume and complete the bout at the conclusion of the delay. In either case, note the duration and cause of the delay in the notes section of the datasheet. Do not push back dates for subsequent sampling events	Increases potential for temporal variability/inconsistency in timeseries data.
2 days < delay ≤ 10 days	If the delay occurs prior to the start of or during the sampling bout, reattempt the complete bout at the conclusion of the delay. Submit a problem ticket. Do not push back dates for subsequent sampling bouts.	
Delay > 10 days	If the delay occurs prior to the start of or during the sampling bout, cancel the sampling bout and submit a problem ticket. Do not push back dates for subsequent sampling bouts.	

Table 2. Contingent decisions for low intensity sampling

Delay/Situation	Action	Outcome for Data Products
Delay ≤ 7 days	If the delay occurs prior to the start of the sampling bout, reattempt the complete bout at the conclusion of the delay. If the delay occurs during the sampling bout, resume and complete the bout at the conclusion of the delay. In either case, note the duration and cause of the delay in the notes section of the datasheet. Do not push back dates for subsequent sampling events	Increases potential for temporal variability/inconsistency in timeseries data.
7 days < delay ≤ 21 days	If the delay occurs prior to the start of or during the sampling bout, reattempt the complete bout at the conclusion of the delay. Submit a problem ticket. Do not push back dates for subsequent sampling bouts.	
Delay > 21 days	If the delay occurs prior to the start of or during the sampling bout, cancel the sampling bout and submit a problem ticket. Do not push back dates for subsequent sampling bouts.	

5 SAFETY

This document identifies procedure-specific safety hazards and associated safety requirements. It does not describe general safety practices or site-specific safety practices.

Personnel working at a NEON site must be compliant with safe field work practices as outlined in the Operations Field Safety and Security Plan (AD[02]) and EHS Safety Policy and Program Manual (AD[01]). Additional safety issues associated with this field procedure are outlined below. The Field Operations Manager and the Lead Field Technician have primary authority to stop work activities based on unsafe field conditions; however, all employees have the responsibility and right to stop their work in unsafe conditions.

Field personnel are collecting biting insects, but there is no increased risk of infection by zoonotic pathogens during implementation of this protocol than in general fieldwork. Follow guidelines provided the Operations Field Safety and Security Plan (AD [02]) to prevent tick bites and take appropriate action if an embedded tick is found. Personnel working with ticks should familiarize themselves with the Zoonotic Diseases section of AD [02]. The incidence of these diseases in humans is extremely rare, with the exception of Lyme disease in certain regions of the country, and is typically associated with working outside in vegetated areas.

IMPORTANT: If used, insect repellent must be applied at least 30 minutes prior to arriving in the field. If applying insect repellent in spray form DO NOT apply in the vicinity of sampling equipment. After applying insect repellent, clean the palms of hands (e.g., with soap/water or alcohol swabs) before handling any sampling equipment.

6 PERSONNEL AND EQUIPMENT

6.1 Equipment

The following equipment is needed to implement the procedures in this document. Equipment lists are organized by task. They do not include standard field and laboratory supplies such as charging stations, first aid kits, drying ovens, ultra-low refrigerators, etc. Quantities specified represent ideal scenarios for a team of two conducting a sampling bout (sampling at 6 plots at a site). Staff may wish to bring extra equipment to account for contingencies.

Table 3. Equipment list – Field sampling a single bout, team of two

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	GPS receiver, handheld, recreational accuracy	For locating sampling plots	1	N
	R	Hand towel, cloth	For drying reusable lint roller	1	N
	R	Funnel or coffee cup filter	For collecting larval ticks from reusable lint roller	1	N
	R	Wash Bottle, LDPE, Unitary or equivalent, 500 mL	For transporting water and ethanol used during collection of larval ticks	1	N
	R	Beverage Cooler, 2.5 gallon capacity, w/spigot, Plastic; IGLOO or Equivalent	For transporting water to be used in larval tick collection	1	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
EB03180000	R	39.5x29.5 inch drag cloth with weights (bringing spare is recommended in the event that equipment becomes damaged (e.g., torn) or wet during sampling)	For collecting nymphal and adult ticks	1	N
	R	Forceps, Dissecting Microforceps, Jeweler Style Straight or Curved, Stainless Steel	For collecting nymphal and adult ticks	2	N
	R	Tube, Transport, 10 mL, Rounded Bottoms with Freestanding Skirts, w/o Caps, Polypropylene; Capital Vial or Equivalent.	Sample vials	6	N
	R	Cap, For 10mL Transport Tubes, Leak-Resistant (e.g. with gasket), Blue; Capital Vial or Equivalent.	Sample vials	6	N
	R	Cooler, 16qt, Material HDPE, Insulation Ultratherm Foam	For transporting samples from field to lab	1	N
	R	Ice Pack, 0 degree C Refrigerant Gel Pack, Wet Ice Alternative, 16 Ounce; Tegrant ThermoSafe PolarPack or Equivalent	For transporting samples from field to lab	3	N
	R	Marker, Permanent, Archival, Pigma Micron Brand, Point size 01, 0.25mm, Black	For filling out locality labels	1	N
	R	Ethanol, 190 proof (95%), Reagent Grade, 55 gallon drum, tax exempt	For preserving samples		Y
	S	Survey Marking Flags, Vinyl Flag with Wire Stake, 18 inch wire, 2.5 inch x 3.5 inch flag, orange	For marking plot perimeter	4	N
	S	Tape measure, 50m	For measuring deviations from the drag path	1	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
	S	Magnifier Hand Lens, 20X, 1/2 Inch Focus, 5/8 Inch Lens Diameter; Bausch and Lomb or Equivalent.	For identifying ticks	1	N
	S	Permanent Markers, Fine, Black; Sharpie or Equivalent	For labeling sample vials	1	N
Consumable items					
	R	Label Paper, Sheet, Acid Free Archival Quality, Withstands Ethanol	For locality labels	1	N
	R	Paper, Copy, All Weather, 8-1/2 inches W x 11 inches L, White; Rite in the Rain or Equivalent.	For field datasheets	1	N
	R	Reusable lint roller (Sticky Buddy)	For collecting larval ticks	1	N
	S	Label Paper, Adhesive-backed, Sheet, Withstands Ethanol	For labeling sample vials, optional	1	N
	S	Mosquito Repellent, 30-50% DEET, in Spray Pump Dispenser		1	N
	S	Wipe, Alcohol Pad, Individually Packaged, to refill First Aid Kit	For cleaning off repellent residue	2	N
	R	Paper coffee filters	For collecting larval ticks from reusable lint roller	12	N
	R	Duct Tape, Vinyl, Gray, 2 in x 50yds	For removing larval ticks from clothing	1	N
	R	Freezer Bag, Reclosable, 10" x 12" or 1 gal, 4 mil thickness	For holding larval ticks in coffee filters	6	N

R/S=Required/Suggested

Table 4. Equipment list – Laboratory processing and analyses

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Ethanol, 190 proof (95%), Reagent Grade, 55 gallon drum, tax exempt	For sample preservation		Y
	R	Forcep, Dissecting Microforceps, Jeweler Style Straight or Curved, Stainless Steel	For handling ticks	2	N
	R	Paintbrush, artist, PK 5	For handling ticks	2	N
Consumable items					
	R	Laundry Detergent, Liquid, Fragrance Free	For laundering drag cloth	1	N
	S	Label Paper, Adhesive-backed, Sheet, Withstands Ethanol	For labeling sample vials	1	N
	R	Label Paper, Sheet, Acid Free Archival Quality, Withstands Ethanol	For locality labels	1	N
	R	Tube, Transport, 10 mL, Rounded Bottoms with Freestanding Skirts, w/o Caps, Polypropylene; Capital Vial or Equivalent	Sample vials	12	N
	R	Cap, For 10mL Transport Tubes, Leak-Resistant (e.g. with gasket), Blue; Capital Vial or Equivalent	Sample vials	12	N

R/S=Required/Suggested

6.2 Training Requirements

All technicians must complete required safety training as defined in the NEON Training Plan (AD[04]). Additionally, technicians must complete protocol-specific training for safety and implementation of this protocol as required in Field Operations Job Instruction Training Plan (AD[05]).

6.3 Specialized Skills

Prior experience collecting ticks or conducting entomological fieldwork is desirable but not required. Personnel should have good fine manual coordination for handling individual specimens.

6.4 Estimated Time

The time required to implement a protocol will vary depending on a number of factors, such as skill level, system diversity, environmental conditions, and distance between sample plots. The timeframe provided below is an estimate based on completion of a task by a skilled two-person team (i.e., not the time it takes at the beginning of the field season). Use this estimate as framework for assessing progress. If a task is taking significantly longer than the estimated time, a problem ticket should be submitted.

An experienced two-person team will require approximately 45-60 minutes to complete sampling of ticks at a single plot. This entails dragging/flagging around the perimeter of the plot and transferring all adult and nymphal ticks into one or more sample vials, and transferring all larval ticks into coffee filters (filter paper).

7 STANDARD OPERATING PROCEDURES

SOP A Preparing for Sampling

A.1 At least one week prior to a sampling bout

1. Identify the locations of sampling plots and determine how to access them.
2. As an optional step, visit field plots and mark the perimeter path using pin flags.
3. If using pre-printed labels for sample vials, prepare labels on adhesive-backed, ethanol-safe paper. The format for the vialID labels is described in SOP B. Note that while labeling can also be done by writing directly on vial with an ethanol-safe marker, use of pre-printed labels is recommended.
4. Prepare locality labels for sample vials. The general format for and process of generating these labels is described in the lab protocol for beetles and mosquitoes (RD[05]). These labels should be printed on ethanol resistant paper. An example of a locality label for tick sampling is illustrated in Figure 1.
5. Print out datasheet(s) on waterproof paper.
6. Be sure reusable ice packs (0°C) are frozen.

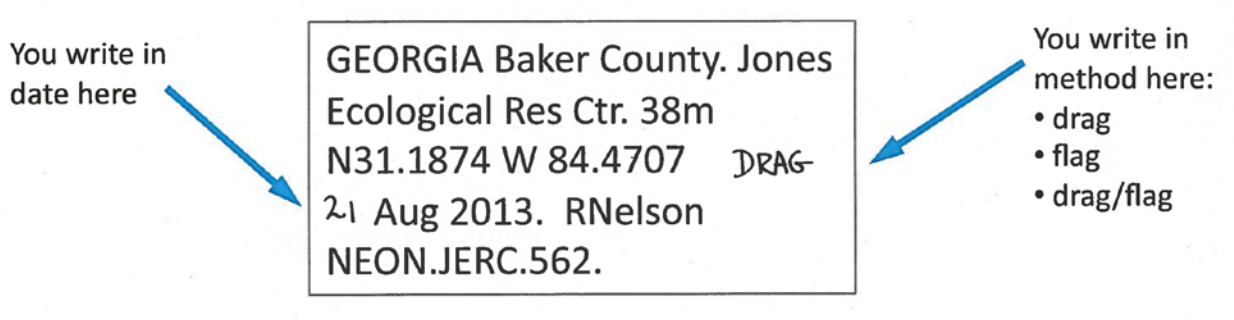


Figure 1. Example of a locality label for tick sampling

A.2 Just prior to heading to the field for sampling

1. Gather all necessary equipment for field sampling.
2. Fill sample vial with 95% ethanol.
3. If used, insect repellent must be applied at least 30 minutes prior to arriving in the field. If using insect repellent in spray form do not apply in the vicinity of sampling equipment. After applying insect repellent clean the palms of hands (e.g., with soap/water or alcohol swabs) before handling any sampling equipment.

SOP B Field Sampling

This SOP begins with a brief overview of the basic steps involved in two sampling methods that can be used to collect ticks. Following this are specific instructions on how to carry out sampling during a bout of tick and tick-borne pathogen sampling.

B.1 Overview of drag sampling

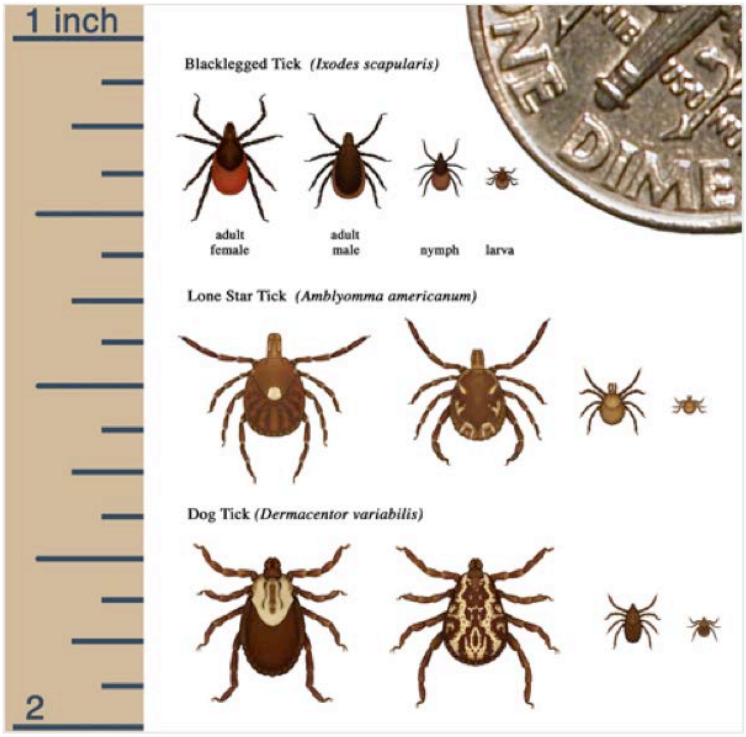
1. Place the drag cloth on the ground
2. One member of the two-person team should pull the cloth while the other walks behind the cloth.

    - The team member pulling the cloth must ensure that the pace of forward progress is slow and steady. Qualitatively, this pace is equivalent to a leisurely stroll (think wedding procession). Slowly counting "1 Mississippi" for each step forward is a good approximation of appropriate cadence. When measured on a grass soccer field it took ~50 seconds to drag 15m at the proper pace.
    - The team member pulling the cloth must also ensure that the entire cloth stays in contact with the ground or vegetation. When pulling the cloth, make sure there is enough pull cord between your person (the individual pulling the cloth) and the cloth so that the leading edge of the cloth stays as flat as possible on the ground. Too little pull cord between your person and the cloth will cause the leading edge of the drag cloth to rise up and not contact the ground.
    - Weights may be attached to the edges of the cloth as necessary if conditions are windy. Note that the weights are not intended to hold the cloth down in the absence of wind. Under calm conditions, the downward pull of gravity on the cloth is acceptable in terms of keeping the cloth in contact with the ground.
    - In instances where the pull of gravity is not sufficient to overcome tall (e.g., >4-6 inches) and rigid-stemmed plants, flagging should be considered. The flagging method is described in more detail below. Flagging allows more direct downward pressure to be applied on the cloth to keep it in contact with the ground or leaf litter.
    - The team member walking behind the cloth must ensure that the cloth does not flip over, get bunched up, or become caught on plants or rocks while being pulled along the ground.

3. After dragging for 5-10m, stop to count and collect ticks.
    a. Inspect the drag cloth. Hold the cloth vertically and scan it in a systematic manner such that you examine the entire cloth on both upper and lower surfaces. Use a hand lens as necessary to distinguish larval and nymphal ticks from dirt.
    b. Inspect your person(s). Examine your body, especially areas around the lower legs and feet. This inspection may be more thorough if done reciprocally (i.e., each team member inspects the other).

c. Remove any nymphal and adult ticks attached to the drag cloth or your persons using forceps (Figure 2). Use the forceps to grab a tick by a leg rather than by its body. Remove larval ticks with a reusable lint roller. More details on how to handle samples are provided below.
d. Be mindful that ticks may attempt to crawl onto your hands, arms, or body while you inspect the drag cloth.

4. Place the drag cloth back on the ground and resume sampling, checking the cloth and your persons and counting/collecting ticks every 5-10m.
5. Continue this iterative process of sampling and collecting ticks until the designated sampling distance has been covered.



Relative sizes of different life stages for three tick species
(Centers for Disease Control and Prevention)

Figure 2. Relative sizes of different tick life stages

B.2 Overview of flag sampling

1. Flagging is used as a substitute for dragging when vegetation is too thick to allow the drag cloth to be pulled along the ground
2. The flag used in flagging is essentially a modified drag cloth. To make a flag, unclip the drag cloth pull cord and any attached weights from the drag cloth.
3. To sample, hold the drag cloth by one end of the wooden dowel. Gently "wave" the flag, guiding it over a sampled area. This movement and manner of holding the cloth allow greater precision to move it over/around/beneath vegetation.
4. While the cloth can be passed over and around vegetation, sampling the ground underneath vegetation will ensure that flagging is most comparable to dragging. To accomplish this, periodically crouch down and insert the flag underneath vegetation.
5. Periodically remove ticks from the cloth and your persons as described for dragging. This should be done with greater frequency than with dragging as sampling in dense vegetation is more likely to dislodge ticks attached to the cloth. It is recommended that you check the cloth every 3-4 sweeps, which should be the equivalent of sampling 3-5m^2.
6. Note that when flagging, especially underneath vegetation, the cloth will generally not remain flat and completely in contact with the sampled surface over its entire area (i.e., it will get wrinkled). This will require estimating the total distance sampled with less precision that when dragging.

B.3 When to use drag sampling versus flagging

1. Drag sampling is the preferred sampling method since it allows the area sampled to be more accurately quantified. This is important for estimating tick density. As such, attempt to sample using the drag method whenever possible.
2. During sampling, it is important to try and keep the cloth in direct physical contact with the ground or overlying leaf litter. When dragging, attempt to make a qualitative assessment of whether the cloth is on or close to the ground/leaf litter: is it touching the ground most or all of the time, is it "surfing" up 2-3 inches above the ground as it passes over flexible-stem grasses/forbes, or is it "stilting" 4 or more inches above the ground as it passes over rigid-stemmed shrubs? The first scenario is ideal for dragging, the second is acceptable for dragging, and the third scenario is one in which flagging should be used to keep the cloth closer to the ground. In particular, flagging is an effective means of getting the sampling cloth underneath shrubs and taller/more dense vegetation.
3. At least 50% of the perimeter of a sampling plot should be amenable to drag sampling. Use flagging to sample any portions of the perimeter of the sampling plot that cannot be sampled using the drag method. If more than 50% of the perimeter of a sampling plot is not amendable to drag sampling, the plot should not have been accepted. Submit a problem ticked for rejected plots.

B.4 Overview of sampling bouts

1. Use maps and/or a handheld GPS as necessary to travel to one corner of the plot to be sampled. Be sure not to transit through any portion of the plot, especially the plot perimeter.
2. After arriving at the plot corner and before you begin sampling, perform an inspection of your and your partner's person. Remove any attached nymphal and adult ticks and place them into an empty sample vial labeled "release" for release following completion of the sampling. Remove any larval ticks using duct tape.
3. During sampling, you must travel a fixed path that follows the shortest straight-line distance between plot corners and thus describing the full perimeter of the plot (Figure 3). You can sample in either a clockwise or counterclockwise direction.
4. If a large obstacle (e.g., rock, tree, cluster of shrubs) is present along the path, sample around the obstacle by diverting the sampling path into the plot. Use the shortest distance possible to get around the obstacle. If the total increase in the length of the drag path in a plot caused by such diversions exceeds ~20m, submit a problem ticket. Keep track of the distance so that it can be recorded on the datasheet as 160m + 0-20m with a target accuracy of +/-2m.

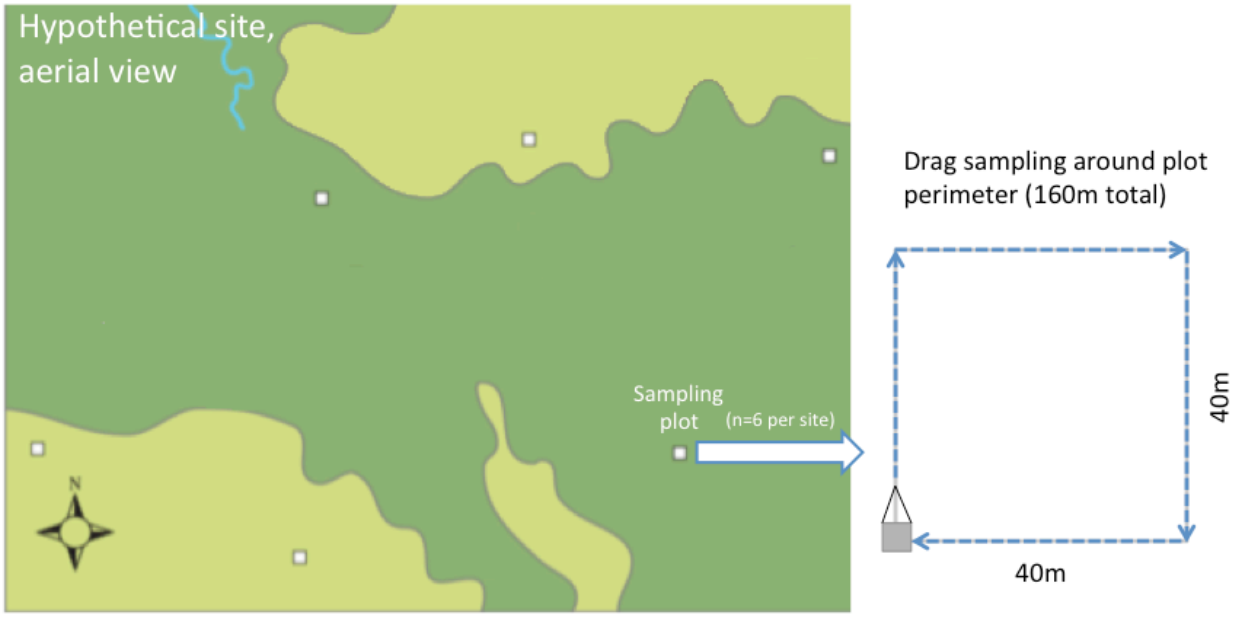


Figure 3. Schematic of high- and low-intensity sampling

5. Begin drag or flag sampling. Stop every 5-10m to examine the drag or flag cloth and your persons for ticks. You may want to check more frequently when dragging/flagging over dense vegetation that could dislodge ticks from the cloth.
    a. Count and collect all nymphal and adult ticks
        1) Collect nymphal and adult ticks using forceps and transfer them into a sample vial containing 95% ethanol. All nymphs and adults collected during a sampling plot/bout combination can be collected into the same sample vial. Additional sample vials can be used if a single vial cannot hold all of the ticks collected during a sampling plot/bout combination.
        2) Record the number of nymphs and the number of adult ticks on the Datasheet for Field and Lab Protocol: Tick and Tick-borne Pathogen Sampling (RD[06]). The total number of nymphs and the total number of adults should be reported for each sampling plot/bout combination.
    b. Collect larval ticks
        1) Gently roll a reusable lint roller over larval ticks on the drag/flag cloth or your persons.
        2) To remove larval ticks from the reusable lint roller, hold the roller vertically over a double paper coffee filter (one filter nested within another) set within a plastic coffee filter cup or funnel, and rinse the roller with water from a wash bottle. This should wash larval ticks off of the roller and down into the coffee filter.
        3) Once larval ticks have rinsed into the filter, spray the larvae with 95% ethanol from a wash bottle to kill them.
        4) Fold the doubled coffee filters flat and roll/fold the upper lip down to seal in the larvae.
        5) Place the doubled coffee filters into a resealable plastic bag.
        6) Larval ticks can be counted upon return to the lab.
        7) Dry the reusable lint roller with a hand towel to reuse.
        8) Two related notes about this larval tick collection method: a) it is not absolutely necessary to wash larval ticks off of the lint roller and into filter paper every time you collect larval ticks from the cloth. As long as the larval ticks are stuck to the lint roller, the roller can be used to collect larval ticks on multiple/successive examinations of the drag/flag cloth before being washed into filter paper. b) If nymphal and/or adult ticks are accidentally collected with the lint roller you can either remove these individuals from the roller using forceps and place them in the adult/nymph sample vial or wash them into the filter paper with the larvae and transfer them to the adult/nymph sample vial when larvae are counted in the lab (just be sure to record counts of these adults and nymphs on the datasheet).
        9) Note: if collection of larval ticks using reusable lint rollers does not work effectively, see SOP C: Collection of Larval Ticks Using Masking Tape.
6. Once all ticks have been collected, continue sampling along the perimeter of the plot. Stop again at 5-10m intervals to examine the drag or flag cloth and your persons and collect ticks. Place any

nymphal and adult ticks into the same sample vial used above. Wash any larval ticks into the same doubled coffee filter used above, or use a new doubled filter as necessary. Store all doubled filters in the same resealable plastic bag.

7. Sample along the perimeter of the plot until you return to the corner of the plot where you began sampling. In total you should have covered 160m+ 0-20m (essentially four 40m transects). If more than 180m was covered due to diversions around obstructions in the drag path, note this on the datasheet in the notes section and submit a problem ticket.
8. Label samples
    a. Fill out locality labels, writing in the date and sampling method(s) used with an ethanol-safe permanent marker. Make sure pre-printed information on the locality label (e.g., site, month/year, plot number) matches the details of the plot and sampling bout. Place one filled out label into each sample vial (containing nymphal and adult ticks) and resealable bag (containing larval ticks).
    b. Externally label each sample vial. External labels can be pre-printed on ethanol-safe label tape or written directly onto sample vials using an ethanol-safe permanent marker. Label the body rather than the lid of the vial.
    c. The external label format (vialID) consists of the plotID (4-character siteID and 3-digit plot number, separated by an underscore), the date (YYYYMMDD), the boutNumber (integer beginning at 1 for each site in each calendar year and increases by 1 for each bout at that site in that calendar year) and the numVialsPlotBout (the number of vials containing ticks collected during the same site/plot/bout combination) (Figure 4). With the exception of siteID and plot number in the plotID, all variables are separated by periods. As an example, “OSBS_002.20130802.3.2” would indicate that the labeled vial is one of two containing ticks collected in plot 002 during the third sampling bout of the year at Ordway Swisher Biological Station on August 2, 2013.

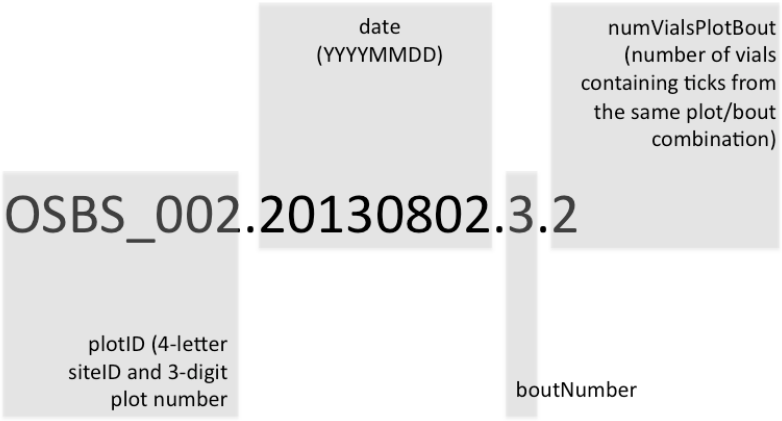


Figure 4. Structure of vialID

9. Place all labeled sample vials generated during sampling in a plot inside the resealable plastic bag containing larval ticks collected in that plot. Seal the bag.
10. Place the sealed resealable plastic bag into an insulated cooler containing frozen reusable ice packs for transit back to the lab.
11. Fill in the following fields on the field datasheet (RD[06]): measuredBy, plotID, date, boutNumber, startTime, endTime, samplingMethod, distanceSampled, and numVialsPlotBout. You may enter information for numAdults and numNymphs in the field or in the lab. Information for numLarvae will be recorded in the lab once larvae are counted. Also record any notes regarding unusual field conditions that could have affected sampling results. (e.g., cows walking through plot during sampling).
12. As you depart from the plot release ticks in the vial labeled “release” at least three meters outside the perimeter of the plot.

B.5 Sample preservation

Upon returning to the lab, immediately transfer sealed resealable bags containing ticks into a refrigerator (4°C) until specimens can be processed. When storing samples, take steps to keep samples from the same site/bout combination together (e.g., using a rubber band and/or placing within a resealable bag).

B.6 Refreshing the sampling kit

1. Print out new adhesive-backed sample labels, locality labels and datasheets as necessary.
2. Obtain new supplies of consumable equipment (e.g., sample vials, ethanol)

B.7 Equipment maintenance, cleaning, and storage

1. Place the drag cloth into an ultralow freezer at the lab to kill any larval ticks attached to the cloth. The duration of exposure to these conditions that is required to kill ticks will likely vary by species but 30 minutes should be sufficient.
2. If the drag cloth is dirty, wash it using fragrance-free laundry detergent and hang it to dry. If a laundry drier is used select a medium heat setting to prevent the drag cloth from shrinking. Always make sure the drag cloth is completely dry before placing in storage.
3. Clean any other equipment as necessary using dilute fragrance-free laundry detergent and a dish sponge and store in a cool, dry place once dry.

SOP C Collection of Larval Ticks Using Masking Tape

This SOP can be implemented if collection of larval ticks using the reusable lint roller method described in SOP B Field Sampling does not work effectively.

C.1 Before going to the field

Prepare cardboard cards by cutting them to a size that is as large as possible to still fit within 1 gal. resealable plastic bags.

C.2 Overview of sampling

1. Collect larval ticks using masking tape:
2. While larval ticks should be removed from the drag or flag cloth and your persons, most attention should be focused on the cloth. Do not spend more than 10-20 seconds examining your persons for larval ticks as they will likely be difficult to see on clothing.
3. Remove a strip of tape with length that does not exceed the width of the cardboard cards.
4. Touch the tape to the drag cloth to remove larval ticks. Do not collect any larval ticks on either of the two ends (1-2 inches) of the strip.
5. When the strip of tape begins to lose its adhesive properties or has many attached ticks, attach it to a cardboard card. Fold the two ends of the strip (which should still be sticky) under so that they touch/stick to the cardboard card. The less sticky side of the strip (with attached larval ticks) should be facing away from the card (Figure 5). Multiple strips of tape can be attached to a single card, but each card should only be associated with larval ticks from a single sampling plot/bout combination.

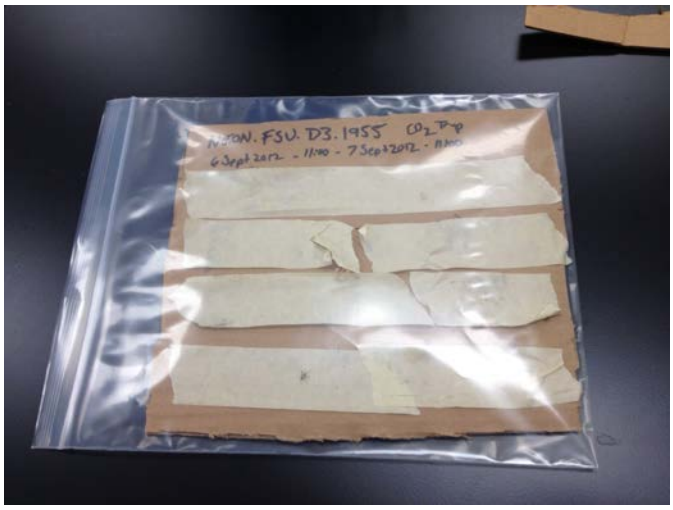


Figure 5. Cardboard card with larval ticks attached to masking tape

6. Use a permanent marker to label the cardboard card(s). Use the same vialID format as for sample vials containing adult and nymphal ticks and count each cardboard card as the equivalent of a vial. The external label format (vialID) consists of the plotID (4-character siteID and 3-digit plot number, separated by an underscore), the date (YYYYMMDD), the boutNumber (integer beginning at 1 for each site in each calendar year and increases by 1 for each bout at that site in that calendar year) and the numVialsPlotBout (the number of vials containing ticks collected during the same site/plot/bout combination) (Figure 6). With the exception of siteID and plot number in the plotID, all variables are separated by periods. As an example, "OSBS_002.20130802.3.2" would indicate that the labeled vial is one of two containing ticks collected in plot 002 during the third sampling bout of the year at Ordway Swisher Biological Station on August 2, 2013. In the notes section on the datasheet record the number of vials and cards generated during sampling for that sampling plot/bout combination.

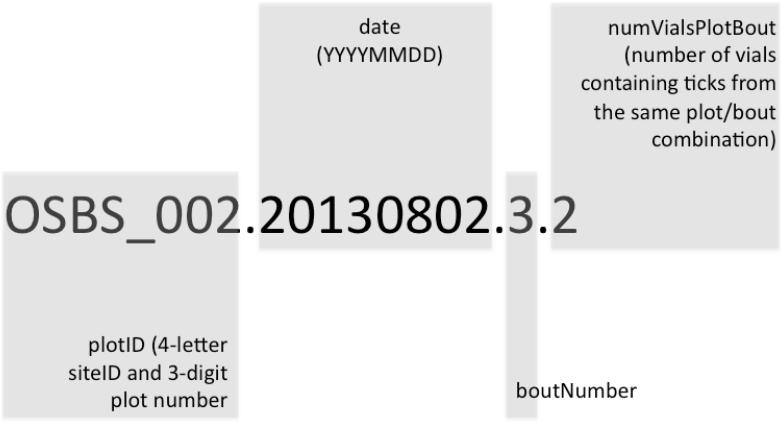


Figure 6. Structure of vialID

7. Place each labeled cardboard card with attached larval ticks into a resealable plastic bag.
8. Place the sealed resealable plastic bag(s) into an insulated cooler containing frozen reusable ice packs for transit back to the lab.

C.3 Sample processing in the lab

1. Count larval ticks attached to masking tape and record on the datasheet. Do not attempt to remove larval ticks from the tape.
2. Send resealable plastic bags containing cardboard cards with attached larval ticks to the external ID facility.

SOP D Laboratory Processing and Analyses

D.1 Preparation

1. Clear space on a lab bench where tick samples can be sorted. It may be helpful to cover the benchtop space where you are working with white paper so that any ticks that may be accidentally lost during sample processing and transfer can be easily located.
2. Gather all necessary equipment for laboratory processing and analyses.

D.2 Sample processing in the lab

1. Remove a resealable bag from the refrigerator and place it on the lab bench.
2. Open the bag and remove any labeled sample vials containing nymphal and adult ticks.
3. Apply 95% ethanol from a wash bottle to the doubled coffee filter(s) as necessary to kill any surviving larval ticks.
4. Count larval ticks enclosed in filter paper and transfer them into one or more sample vials containing 95% ethanol. Larval ticks should be stored in their own vial(s) and not added to the vial(s) containing nymphal and adult ticks collected during the same sampling bout/plot combination. If using forceps, handle larvae gently and try to avoid crushing them during transfer. A paintbrush may be more suitable. Label the vial using the same labeling instructions as provided above for the vial(s) containing nymphal and adult ticks.
5. Fill in any remaining blank fields on the datasheet.

D.3 Sample preservation

1. After counting and transferring larvae into one or more sample vials, transfer all sample vials containing ticks into a freezer (-20°C) or refrigerator (4°C, if a freezer is not available).
2. To facilitate sample sorting and tracking it is recommended that all sample vials associated with a single sampling bout/site combination be grouped (e.g., bind them together with a rubber band or place them in a resealable plastic bag.

D.4 Equipment maintenance, cleaning, and storage

1. Clean all laboratory equipment following use as necessary and according to the manufacturer's instructions.
2. Wash drag/flag cloth(s) as necessary using fragrance free laundry detergent. Air/hang dry cloths or use a clothes dryer on a low setting to be sure that fabric shrinking does not occur.
3. Store all laboratory equipment in a cool, dry location when not in use.

SOP E Data Entry and Verification

As a best practice, field data collected on paper datasheets should be digitally transcribed within 7 days of collection or the end of a sampling bout (where applicable). However, given logistical constraints, the maximum timeline for entering data is within 14 days of collection or the end of a sampling bout (where applicable). See RD[04] for complete instructions regarding manual data transcription.

1. Scan datasheets and save in PDF file format (file location TBD as of Rev E of this document)
2. Save paper copy of datasheets (location TBD as of Rev E of this document)

SOP F Sample Shipment

Note: Shipping details are TBD and will be specified once the taxonomic ID facility has been selected.

CLA or FSU will submit a problem ticket for any required deviation from this shipping protocol.

F.1 Handling Hazardous Material

Ethanol is classified as a hazardous material, and should be handled according to the guidelines in the EHS Safety Policy and Program Manual (AD[01]) and the Domain Chemical Hygiene Plan and Biosafety Manual (AD[03]).

F.2 Supplies/Containers

Whenever a batch of samples is shipped, the batch must be accompanied by a hard-copy shipping manifest enclosed within the shipping container AND a corresponding electronic version of the manifest (excel file) emailed to the taxonomic ID facility.

The hard-copy manifest lists every sample vial in the shipped batch. Include the following fields: vialID, senderID (domain manager, domain number), sentDate (YYYYMMDD), and receiverID (name of lab contact/PI, name of lab). An example of a populated hard-copy manifest is provided in Figure 7.

vialID	senderID	sentDate	receiverID
OSBS_001.20130712.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_001.20130712.1.2	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_002.20130712.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_003.20130712.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_004.20130712.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_006.20130712.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_001.20130724.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_002.20130724.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_003.20130724.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_003.20130724.1.2	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_005.20130725.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_006.20130725.1.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_001.20130804.2.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_002.20130804.2.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_002.20130804.2.2	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_002.20130804.2.3	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_003.20130805.2.1	R. Nelson, D03	20131101	L. Beati, USNTC
OSBS_006.20130805.2.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_001.20130818.2.1	R. Nelson, D03	20131101	L. Beati, USNTC
DSNY_002.20130818.2.1	R. Nelson, D03	20131101	L. Beati, USNTC

Figure 7: Example of a hard-copy shipping manifest for ticks

The electronic manifest is an excel file that should be emailed to the taxonomic ID facility as soon as possible after a batch of samples has been shipped. It is an electronic version of the corresponding hard-copy manifest that additionally contains data columns from the tick and tick-borne pathogen data ingest form to be filled in with taxonomy data and metadata by the taxonomic ID facility facility. The order of samples in the electronic manifest should be the same as the order in the corresponding hard-copy shipping manifest.

F.3 Timelines

From a technical/scientific perspective, tick samples held in vials containing ethanol and stored at -20C (or 4C) will retain their integrity for many months. Samples should be shipped overnight to external facilities.

F.4 Conditions

Samples should be stored in vials containing 95% ethanol. Sample vials should be stored at -20C (ideally, 4C acceptable) until shipped to an external facility. Samples should be shipped in insulated shipping containers containing frozen, reusable ice packs.

F.5 Grouping/Splitting Samples

Whenever possible, all samples collected during any given site/bout combination should be shipped together. Sample vials containing samples collected as part of the same site/bout combination could be taped or rubber-banded together to allow them to be easily inventoried/sorted at the external facility.

F.6 Return of Materials or Containers

Be sure to include instructions to external facilities on how to return reusable materials (e.g., ice packs). CLA can provide details.

F.7 Laboratory Contact Information and Shipping/Receipt Days

See the CLA shipping document on CLA's NEON intranet site.

8 REFERENCES

N/A

APPENDIX A DATASHEETS

The following datasheets are associated with this protocol:

Table 5. Datasheets associated with this protocol

NEON Doc. #	Title
NEON.DOC.001583	Datasheets for TOS Protocol and Procedure: Tick and Tick-Borne Pathogen Sampling

These datasheets can be found in Agile or the NEON Document Warehouse.

APPENDIX B QUICK REFERENCES

Quick Reference: Collecting Tick Specimens

STEP 1 – Check yourself for ticks and place any ticks in "release" sample vial.

STEP 2 – Start sampling at one corner of the plot.

WALK SLOWLY!

STEP 3 – Walk with drag cloth for 5-10m.

STEP 4 – Stop and inspect drag cloth. Collect and count adult and nymphal ticks and store in vial with 95% ethanol

STEP 5 – Collect larval ticks using a reusable lint roller. Store inside coffee filters in resealable freezer bags.

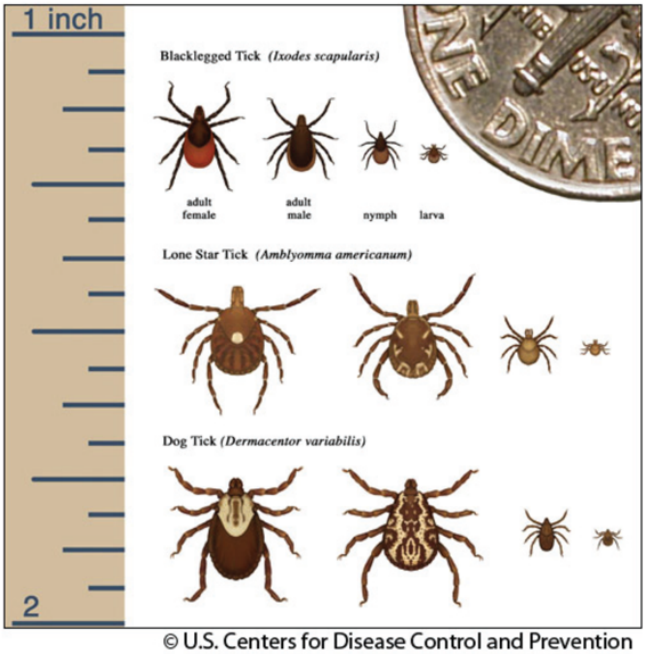
STEP 6 – Repeat 5-10 m drag and collect cycle until you have sampled the entire perimeter of the plot (i.e., returned to the plot corner where you began your sampling).

STEP 7 – Label specimen vial.

STEP 8 – Place tick specimens in cooler with ice packs.

STEP 9 – Release ticks in vial labeled "release" once 3 m outside plot boundary.

Tick Life Stages



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APPENDIX C REMINDERS

Getting Ready for Sampling

Equipment: Be sure to…

- ☑ Inspect drag cloth for tears and ticks.
- ☑ Check that binder clips are attached to dowel.
- ☑ Print Tick and Tick-Borne Pathogen Sampling Data Sheet.
- ☑ Upload sample coordinates to GPS and obtain maps.
- ☑ Bring all supplies and extras.
- ☑ Check your pace. Can you accurately pace 5-10 m?

Personal safety: Protect yourself by…

- ☑ Wearing appropriate clothing.
- ☑ Tucking pant legs into socks.
- ☑ Using tape to seal gaps.
- ☑ Applying insect repellent ½ hour before going into field and away from sampling equipment.

You are collecting live ticks.

If you use insect repellent, apply it at least 30 minutes PRIOR to heading to field site.

Wash hands thoroughly with soap and water after applying insect repellent to avoid transferring repellent to sampling equipment.

Collecting Quality Tick Data

Dragging: Remember to…

- ☑ Check yourself for ticks BEFORE you start dragging.
- ☑ Sample only under dry conditions.
- ☑ Keep drag cloth relatively flat on ground.
- ☑ SLOW DOWN! Your pace is probably too fast.
- ☑ Remain on a path that traces the shortest (straight line) distance between plot corners.
- ☑ Include ticks attached to your clothes (from the waist down) in your count/specimen vial.
- ☑ Label vials and store in cooler with ice packs.

Before leaving drag site: Check that…

- ☑ Data Sheet is complete.
- ☑ All ticks have been removed from drag cloth and your person(s).
- ☑ Drag cloth is stowed in plastic bag for transport to next site.

When you are 3 m outside plot perimeter: Remember to…

- ☑ Release ticks from the "release" vial.

At the end of the day: Limit your exposure to ticks by…

- ☑ Putting your field clothes and the drag cloth in a dryer to kill ticks or, if not possible stowing them in a plastic bag to contain movement of ticks.
- ☑ Check yourself for ticks.

APPENDIX D ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING

The dates in the table below are based on historic records and are estimates for the start and stop dates of sampling. It is essential that domain staff monitor real-time conditions to determine when to start and stop sampling, as described in Section 4 of this protocol. This information will be updated as field data come in.

Table 6. Estimated sampling dates based on historical temperature thresholds

Domain	Site	Approx. Start Date	Approx. End Date
01	HARV	March 15	December 5
	BART	March 20	November 19
02	SCBI	March 1 (Feb 9)	December 25
03	OSBS	March 1	December 31
	DSNY	March 1	December 31
	JERC	March 1	December 31
05	UNDE	April 4	November 3
07	ORNL	March 1	December 31
08	TALL	March 1	December 31
09	WOOD	March 31	November 4
10	CPER	March 1 (Feb 18)	December 6
10	STER	March 1 (Feb 18)	December 6
15	ONAQ	March 1 (Feb 20)	November 27

APPENDIX E SITE-SPECIFIC INFORMATION

N/A