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

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See configuration management system for approval history.

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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	December 2014	ECO-02321	Migration to new protocol template Contingent decisions updated to inform responses to site and plot level delays that are acute (FOPS-1228, FOPS-1582, FOPS-1629, FOPS-1241, FOPS-1171, FOPS-1018) and delays that may be more chronic and require consideration of dropping/replacing one or more sampling plots (FOPS-1568, FOPS-1365, FOPS-1224) SOP A: format for internal sample vial labels changed (locality label format no longer used) SOP B: Text added to clarify what was formerly the “>50% draggable” rule and more clearly define when to use dragging versus flagging (FOPS-1188, FOPS-1183, and FOPS-1170). This text also provides more explanation of the efficacy of dragging vs. flagging in tall grass or where understory vegetation prevents the cloth from touching the ground (in response to FOPS-1167, FOPS-838). The text further explains how to modify sampling when “difficult veg” (including water) is encountered along the sampling path (addresses FOPS-1227). The total distance that can be sampled as each plot has been modified accordingly, and new figures are included here. Ticks of all three life stages can now be stored and shipped in the same sample vial(s) (versus previously, larvae were separate from adults/nymphs). Text has been added to clarify how frequently larvae should be rinsed from the reusable lint rollers during sampling in a plot. Text was added in response to FOPS-1566 (when to use masking

			tape vs. sticky buddy methods to collect larval ticks) indicating that reusable lint rollers should be used to collect larval ticks unless NEON HQ science staff have approved use of masking tape method. VialID format has been modified slightly to create unique identifiers for each sample vial. In response to FOPS-1478, siteID has been added to the datasheet. SOP C: Text was added in response to FOPS-1566 (when to use masking tape vs. sticky buddy methods to collect larval ticks) indicating that reusable lint rollers should be used to collect larval ticks unless NEON HQ science staff have approved use of masking tape method. VialID format has been modified slightly to create unique identifiers for each sample (here, ticks on masking tape attached to cardboard cards) SOP D: Ticks of all three life stages can now be stored and shipped in the same sample vial(s) (versus previously, larvae were separate from adults/nymphs) (addressed FOPS-1574). Information on and format of lab on internal vial label has been changed (was locality label, now vialID) SOP E: format of shipping manifest has been adjusted with the addition of fields and changes to the name and format of some existing fields
F	03/17/2015	ECO-02564	Update of tick TOS protocol based on 2014 field experience and budget analysis. Details of the changes are located in the change record.
G	1/29/2016	ECO-02905	Effective starting 2016 field season: Larval collection uses tape only (NEON-247). Changed storage preservative from 95% ethanol to RNA stabilization solution (NEON-350). Ticks found outside the plot may be discarded instead of released. Low-intensity sampling frequency resumes after a year of no ticks (NEON-354). Added instructions to maintain a narrow sampling path (NEON-554). Removed bout from sampleID format. Internal labels should be printed on all-weather copy paper and inserted inside vials. Reduced sizes of collection tubes to 1.5-2 mL (existing supplies may be used until depleted). Distilled redundant information, restructured for clarity.

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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[05]).

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.050005	Field Operations Job Instruction Training Plan
AD[05]	NEON.DOC.000911	NEON Science Design for Vectors and Pathogens
AD[06]	NEON.DOC.014051	Field Audit 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
RD[07]	NEON.DOC.000793	Tick Drag Cloth Assembly Procedure
RD[08]	NEON.DOC.002163	NEON Algorithm Theoretical Basis Document: TOS Tick Abundance and Diversity - QA/QC of Raw Field and Lab Data and Prevalence Measure Calculations

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 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[06]). Additional quality assurance will be performed on data collected via these procedures according to the NEON Algorithm Theoretical Basis Document: TOS Tick Abundance and Diversity - QA/QC of Raw Field and Lab Data and Prevalence Measure Calculations (RD[08]).

4 SAMPLING SCHEDULE

4.1 Sampling Frequency and Timing

Bouts of tick and tick-borne pathogen sampling will be conducted annually when temperature thresholds are met between March and 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. However, if a site is on a high-intensity sampling plan and no ticks of any life stage are sampled within the plot for a full calendar year, sampling reverts to the low-intensity plan. High intensity sampling will resume when one or more ticks are counted within the site.

4.2 Criteria for Determining Onset and Cessation of Sampling

The annual sampling window is between March and December due to seasonal personnel limitations, but when sampling actually occurs will depend on local temperature within that time period (see Appendix D for estimated dates). The first bout of tick sampling each year will occur as soon as the temperature thresholds are met in March or later. 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. Sampling will continue for the rest of the year as long as temperature thresholds are met, until the end of December or earlier.

For both the high and low intensity sampling plans, a bout of sampling will only be performed if the high temperature two consecutive days 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.

4.3 Timing for Laboratory Processing and Analysis

Tick samples held in vials containing RNA stabilization solution and stored at -20°C (4°C is acceptable for up to one month) will retain their integrity. However, samples should be sent to the identification facility within 3 months of collection to enable publication of the data on the portal prior to the following field season.

4.4 Sampling Timing Contingencies

If temperature thresholds are not met at the time of a scheduled sampling bout, cancel that bout, and resume sampling at the next scheduled bout if temperature thresholds are met.

If the sampling conditions below are not met, delay the bout according to Table 1 (high-intensity plan) or Table 2 (low-intensity plan):

- 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 (mid to late afternoon) on days for which the high temperature is at or near the annual high temperature for the site.
- Sampling may be delayed in high wind conditions (in excess of 20 mph) where winds disrupt appropriate execution of tick sample protocols.

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, and the issue(s) causing the delay affects all plots at the site, reattempt the entire bout at the conclusion of the delay. If the issue(s) causing the delay does not affect all plots at the site, submit a problem ticket in JIRA for guidance. If the delay occurs during the sampling bout, and the issue(s) causing the delay	Increases potential for temporal variability/inconsistency in time series data.

	affects all plots at the site, resume and complete the bout at the conclusion of the delay. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and resume and complete sampling at the affected plots 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.	
2 days < delay ≤ 10 days	If the delay occurs prior to the start of or during the sampling bout, and the issue(s) causing the delay affects all plots at the site, reattempt the entire bout at the conclusion of the delay. Submit a problem ticket for delays exceeding one week. Do not push back dates for subsequent sampling bouts. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and submit a problem ticket in JIRA for guidance about sampling at affected plots. In either case, note the duration and cause of the delay in the notes section of the datasheet.	
Delay > 10 days	If the delay occurs prior to the start of or during the sampling bout, and the issue(s) causing the delay affects all plots at the site, cancel the sampling bout and submit a problem ticket. Do not push back dates for subsequent sampling bouts. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and submit a problem ticket in JIRA for guidance about sampling at affected plots. In either case, note the duration and cause of the delay in the notes section of the datasheet.	

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, and the issue(s) causing the delay affects all plots at the site, reattempt the entire bout at the conclusion of the delay. If the issue(s) causing the delay does not affect all plots at the site, submit a problem ticket in JIRA for guidance. If the delay occurs during the sampling bout, and the issue(s) causing the delay affects all plots at the site, resume and complete the bout at the conclusion of the delay. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and resume and complete sampling at the affected plots 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 time series data.
7 days < delay ≤ 21 days	If the delay occurs prior to the start of or during the sampling bout, and the issue(s) causing the delay affects all plots at the site, reattempt the complete bout at the conclusion of the delay. Submit a problem ticket for delays exceeding one week. Do not push back dates for subsequent sampling bouts. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and submit a problem ticket in JIRA for guidance about sampling at affected plots.	
Delay > 21 days	If the delay occurs prior to the start of or during the sampling bout, and the issue(s) causing the delay affects all plots at the site, cancel the sampling bout and submit a problem ticket. Do not push back dates for subsequent sampling bouts. If the issue(s) causing the delay does not affect all plots at the site, conduct sampling at the plots that are not affected by the delay and submit a problem ticket in JIRA for guidance about sampling at affected plots.	

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 arthropods, but there is no increased risk of infection by zoonotic pathogens during implementation of this protocol than in general fieldwork. We recommend that field personnel wear light-colored clothing when implementing this protocol to improve visibility of ticks on clothing prior to and following sampling. 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]. Generally, the incidence of tick-borne diseases in humans is extremely rare and is typically associated with working outside in vegetated areas.

IMPORTANT: Use of insect repellent will reduce tick sampling success and data quality, but application is left as a personal safety choice. 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-free hand wipes) before handling any sampling equipment. Both permethrin (0.5%) and DEET (up to 30%) are excellent repellents and can be used to treat field clothes well in advance of field sampling (two to four hours prior).

This protocol does require the use of chemicals (Ethanol, RNA stabilization solution). Safety Data Sheets (SDS) shall be readily available for review whenever chemicals are being transported or used during this activity.

6 PERSONNEL RESOURCES

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 freezers, 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. Preparation for field sampling

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
Durable Items					
MX105087	R	Ice pack, 0°C	Pre-freeze for field preservation of samples	Variable	N
Consumable items					
MX103942	R	All weather copy paper	Print datasheets, prepare internal vial labels (pre-print or use pencil)	5	N
	S	Waterproof adhesive label or label tape	Prepare external vial labels (pre-print or use permanent marker)	Variable	N
MX100213	R	Ethanol, 190 proof (95%)	Weaken packing tape adhesive, to remove larval ticks	Variable	Y
MX100372	R	pH meter	Prepare RNA stabilization solution	1	N

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
	R	RNA stabilization solution	Preserve collected ticks	Variable	N

R/S=Required/Suggested

Table 4. Equipment list – Field sampling a single bout, team of two

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
Durable Items					
MX104723	R	Cooler, 16 qt	Chill perishable samples in field	1	N
MX100659	R	Forceps (with flagging or lanyard)	Collect ticks	2	N
MX100319	S	Clipboard	Hold and write on datasheets	1	N
	R	Pencils	Write on datasheets and internal labels	3	N
MX100703	S	GPS receiver, recreational accuracy	Navigate to sampling location	1	N
MX105087	R	Ice pack, 0°C	Chill perishable samples in field	3	N

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
MX104829	S	Magnifier hand lens, 2X/5X	Aid in tick identification	1	N
MX100474	S	Counting device	Count ticks	2	N
MX104369	S	Measuring tape, minimum 50 m	Measure deviations from the drag path	1	N
323580000	S	Sinker weights for tick drag cloth assembly	Weigh drag cloth to maintain contact with ground	5	N
EB03180000	R	Tick drag cloth assembly	Collect ticks	2	N
Consumable items					
MX100714	S	Alcohol-free hand wipes	Remove repellent residue	2	N
MX102000	R	Duct tape	Remove and discard ticks not being archived	1	N
MX103942	R	All-weather copy paper	Internal labels for sample vials (pre-print or use pencil)	1	N
	S	Waterproof adhesive label or label tape	External labels for sample vials (pre-print or use permanent marker)	Variable	N
MX104461	S	Scissors	Cut labels	1	N
MX105587 MX104522	R	Clear packing or masking tape	Collect larval ticks from cloth	1 roll	N

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
MX100710	S	Mosquito repellent, up to 30% DEET or 0.5% permethrin	Protect personnel from insect bites	1	N
MX104422	R	Permanent marker, archival	Write external labels for sample vials	1	N
MX100592	R	Resealable plastic bag, 1 gal, 4 mil	Organize sample tubes	6	N
	S	Rubber band	Organize sample tubes	Variable	N
	S	Survey marking flag, PVC or fiberglass stake	Delineate sampling area	4	N
	S	Resealable plastic bag, 1 gal, 4 mil	Store and transport drag cloths	Variable	N
MX108277	S	Tubes with caps, 50 mL, or larger as needed	Prepare pre-filled tubes with 95% ethanol for soaking tape with larval ticks	Variable	N
MX110350 MX110351	R	Tubes, 1.5 to 2 mL with screw-top cap and O ring	Prepare pre-filled sample vials with RNA stabilization solution	6	N
Resources					
RD[06]	R	Field datasheet	Record data		N

R/S=Required/Suggested

Table 5. Equipment list – Laboratory processing and analyses

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
Durable Items					
MX104751	S	Artist paintbrush	Count and transfer ticks to tubes	2	N
MX100659	S	Forceps	Count and transfer ticks to tubes	2	N
Consumable items					
	S	Copy paper, white	Aid in visibility of ticks	1	N
MX103249	R	All weather copy paper	Internal vial labels (pre-print or use pencil)	1	N
	S	Waterproof adhesive label or label tape	External vial labels (pre-print or use permanent marker)	Variable	N
MX100635	R	Liquid laundry detergent, fragrance free	Wash tick drag cloth	1	N
	S	Resealable plastic bag, 1 gal, 4 mil	Organize sample tubes	Variable	N
	S	Rubber band	Organize sample tubes	Variable	N
MX110350 MX110351	R	Tubes, 1.5-2 mL with screw-top cap and O ring	Store ticks in RNA stabilization solution for shipping	12	N

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
Resources					
RD[06]	R	Completed field datasheet	Record data		N

R/S=Required/Suggested

Table 6. Equipment list – Shipping

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
Durable Items					
MX100358	R	Ice pack	Chill specimens during shipment	Variable	N
Consumable items					
	R	Cardboard box, UN packing group III	Package specimens with dry ice for shipment	Variable	N
	S	Plastic bag, 2 mil	Spill containment	2	N
	S	Plastic liner, 2 mil	Spill containment	1	N
	S	Styrofoam sheet	Insulation for samples in shipment boxes	6	N

Item No.	R/S	Description	Purpose	Quantity*	Special Handling
	R	Up arrow shipping label	Label shipments containing liquids	2	N
MX104220	R	Absorbent pad	Absorb liquid spills during shipment	Variable	N
MX109205	R	Vermiculite, grade 2	Absorb liquid spills during shipment	Variable	N
Resources					
RD[06]	R	Shipping manifest	Inventory of specimens being shipped	1	N

R/S=Required/Suggested

6.2 Training Requirements

All technicians must complete Field Safety Training as defined in Operations Field Safety and Security Plan (AD[02]) and NEON EHS Safety Policy and Program Manual (AD[01]) and protocol-specific training for safety and implementation of this protocol as required in Field Operations Job Instruction Training Plan (AD[04]).

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 can complete sampling of ticks at a single plot in approximately 30 to 120 minutes. This entails dragging/flagging around the perimeter of the plot and transferring ticks into one or more sample vials.

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. Plot validation/acceptance:
   a. Tick sampling should only be conducted in plots in which you are able to sample for ticks (using dragging, flagging, or a combination, as described in SOP B) over 50% or more of the plot perimeter. Problems arise when woody vegetation is so tall (i.e., >3-4 ft) and/or dense that a drag cloth cannot easily be pulled over or around the base of plants and flagging in between plants becomes exceedingly time consuming (i.e., >120 min). Other obstacles include standing/flowing water or wet terrain. If obstacles are present over 50% or more of the entire perimeter of the plot, then the plot should be rejected.
   b. When obstacles are temporally variable: In some cases a plot may be acceptable for sampling for a proportion of the sampling season and unacceptable for the remainder of the season. For example, large portions of a plot perimeter may be wet early in the sampling season but dry out later. Alternatively, large portions of the plot perimeter may be associated with supple, low growing vegetation early in the sampling season that becomes tall/dense/woody later in the season. You will need to use local knowledge to estimate these proportions, and one or two field seasons may be required to quantify them with confidence for questionable plots. Over the long term, each accepted plot needs to be amenable to sampling on $\geq$50% of the planned sampling dates.
   c. Representative cover type: When inclined to reject a plot based on obstacles, consider whether the conditions are unique to this plot or are typical of plots within this vegetation type. If the former is likely, then rejecting the plot and evaluating alternative plots in the same vegetation type is advisable. Alternatively, if all of the plots in the vegetation type are likely to be characterized by these features (e.g., all of the woody wetland plots are too wet, or the plant density in all of the shrub plots is too high), then issue a problem ticket in JIRA. Reallocating plots to one or more other vegetation types at the site may be considered.
3. Prepare RNA stabilization solution for tick collection and storage. This solution is non-hazardous and does not have special handling or shipping requirements. Specimens stored in this solution are stable for one week at 25°C, one month at 4°C, or indefinitely at -20°C.
   a. Combine the following (makes 1.5 liters):
      i. 935 mL sterile distilled water
      ii. 700 g ammonium sulfate
      iii. 25 mL 1M sodium citrate
      iv. 40 mL 0.5M EDTA
   b. Adjust to pH 5.2 using concentrated H_2SO_4 (about 20 drops = 1 mL).

c. Store at room temperature (25°C). The solution can be stored indefinitely, however, to reduce risk of contamination, fresh solution should be mixed at the beginning of each field season. If a precipitate forms, warm to 37°C and agitate to redissolve.

4. Prepare sample vials with both external and internal labels. The label format (vialID) consists of the sampleID and a vial number, separated by a period (**Figure 1**). The sampleID is the plotID and the date (YYYYMMDD), separated by a period. The vial number is the two-digit number of the vial relative to the total number of vials containing ticks collected during a single bout at one plot, 01 or higher. As an example, the vialID "OSBS_002.20130802.02" would indicate that the labeled vial is the second vial containing ticks collected in plot 002 at Ordway Swisher Biological Station on August 2, 2013.
   a. External labels: External labels may be legibly written directly on the vial with permanent marker or pre-printed on adhesive labels (preferred). Labels should be oriented with the beginning of the vialID towards the vial opening.
   b. Internal labels: Print internal labels on all-weather copy paper, cut to fit inside the vial. Labels should be oriented with the beginning of the vialID towards the vial opening. Ideally, you should be able to read the vialID without opening the tube. Pre-printed labels are recommended, although hand-writing legibly with pencil is acceptable.
   c. Labeling in the field: If temporary labels are added to vials in the field, be sure that vials have both internal and external labels before shipping samples.

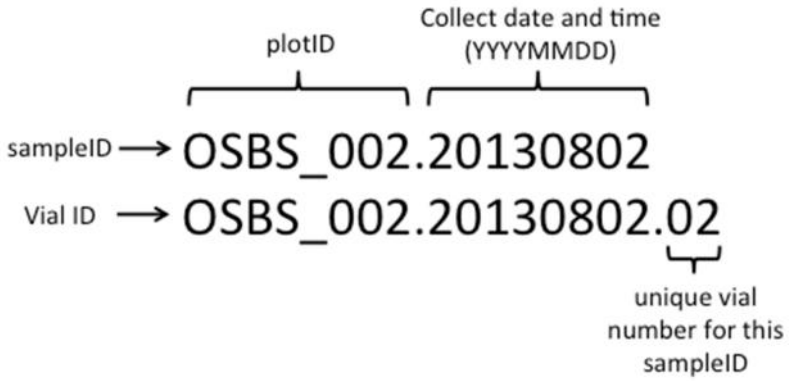


Figure 1. Annotated example of sampleID and vial ID

5. Print out datasheet(s) on waterproof paper.
6. Be sure reusable ice packs (0°C) are frozen.

A.2 Just prior to field sampling

1. Gather all necessary equipment for field sampling.
2. Fill sample vials with RNA stabilization solution.
3. Use of insect repellent reduces sampling success. 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-free wet wipes) before handling any sampling equipment.

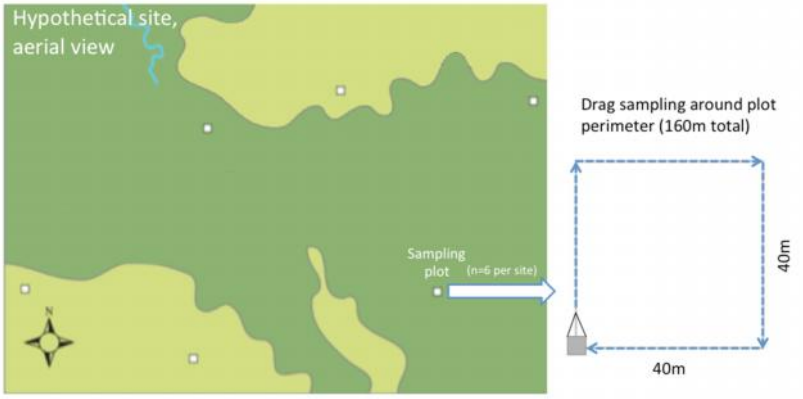
SOP B Field Sampling

Sample using one or both of the two sampling methods. Drag sampling (SOP B.2) is the preferred method used for tick collection. Flagging (SOP B.3) is used as a substitute for dragging when vegetation is too thick to allow the drag cloth to be pulled along the ground.

B.1 Sampling transects

1. Sample along a fixed path that follows the shortest straight-line distance between plot corners and thus covers the full perimeter of the plot (**Figure 2**). You can sample in either a clockwise or counterclockwise direction. Ideally, you will sample 160 meters (four 40 meter transects). Always record the total horizontal distance sampled.
2. Try to maintain as narrow and consistent a path as possible to minimize trampling surrounding vegetation. Should a path become worn, you may need to drag the cloth beside the path, just inside the plot perimeter.
3. If straight-line transects are not possible, choose an alternative path that minimizes detours from the original perimeter, while staying in range of acceptable sampling distance (minimum 80 meters, maximum 180 meters). Always record the total horizontal distance sampled, with a target accuracy of +/-2 meters. If the distance covered falls out of the range of accepted sampling distance, issue a problem ticket in JIRA. Flagging landmarks may help maintain consistency across bouts in transect detours.
    a. If a large obstacle (e.g., rock, tree, cluster of shrubs) is present along the transect, divert the sampling path as little as possible into the plot.
    b. If the obstacle is too large to divert around, you can sample up to the obstacle, pick up the cloth, make your way through or over the obstacle, and begin sampling again on the other side. For example, if a narrow creek runs down the middle of the plot, you can simply step over or cross the creek where it crosses the sampling transect.
    c. Be sure that all diversions are directed into the plot, rather than outside the plot boundaries.

Figure 2. Schematic of sampling plot and transects.



B.2 Overview of drag sampling method and frequency of checks

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

2. 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 15 meters at the proper pace.
3. 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.
4. 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 to keep the cloth in contact with the ground.
5. 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.

6. Stop to count/collect ticks every 5-10 meters as described in SOP B.5.5.

B.3 Overview of flag sampling method and frequency of checks

1. The flagging cloth is a modified drag cloth: unclip the drag cloth pull cord and any attached weights from the drag cloth.

2. 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.
3. 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 sweep the flag underneath vegetation. Note that when flagging, especially underneath vegetation, the cloth will generally wrinkle. This will require estimating the total distance sampled with less precision than when dragging.
4. Do not attempt to flag spiny/thorny vegetation (e.g., brambles, cacti) as this will damage the cloth. Instead, drag underneath this vegetation if you can avoid catching the cloth on spines/thorns. Alternatively, if the vegetation is low growing and you cannot get the cloth underneath, consider the vegetation an obstacle (see SOP B.1.2).
5. Stop to count/collect ticks (SOP B.5.5) 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 3-5 square meters.

B.4 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.
2. During sampling, it is important to try and keep the cloth in direct physical contact with the ground, vegetation, or overlying leaf litter. When dragging, attempt to make a qualitative assessment of whether the cloth is on or close to these surfaces: is it touching the surface most or all of the time, is it "surfing" up 2-3 inches above the surface as it passes over flexible-stem grasses/forbes, or is it "stilting" 4 or more inches above the surface 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 surface. In particular, flagging is an effective means of getting the sampling cloth underneath shrubs and taller/more dense vegetation.
3. A combination of dragging and flagging may be used if necessary to sample over vegetation types or obstacles. Here are some examples of scenarios that may be encountered:
    - Low stature grass, herbs, or leaf litter: you can sample using dragging or flagging, but the former is preferred since it allows for more accurate quantification of total distance covered during sampling.
    - Medium to tall grass or herbs, supple vegetation (not woody/rigid): the cloth might not be in physical contact with the ground, but it can be easily pulled (dragging) or waved/passed (flagging) over the top of the vegetation. Because the vegetation is supple, the weight of the cloth will allow it to be pulled down into the vegetation and closer to the ground by gravity. This can be further accentuated with flagging as the cloth can be pushing down into the

vegetation by holding the dowel lower to the ground. In this scenario, you can sample by dragging or flagging, but the latter may be preferred when the vegetation is tall because the cloth can be pushed down to a greater degree than by gravity alone.

- Medium to tall shrubs, woody vegetation (non-supple) and patchy: if the vegetation (e.g., woody shrubs) is present at low density such that the drag cloth can be pulled between plants, then use dragging to sample the ground underneath the shrubs. If vegetation density is higher and the drag cloth cannot be pulled between plants, use flagging to sample this interstitial area. If the vegetation is not tall (i.e., ≤3 ft) you can additionally sample the sides and tops of shrubs using flagging.
- Tall woody (non-supple) vegetation: As with low/medium stature woody vegetation, drag or flag the ground between plants if density is low enough to allow space. If plants are >4 ft tall, just sample the ground between and underneath plants (i.e., do not sample trunks or woody stems).

B.5 Tick Collection in the Field

1. Use maps and/or a handheld GPS as necessary to navigate to one corner of the plot. 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 ticks using duct tape and discard.

For each sampling interval, conduct steps 3 through 5:

3. Begin drag or flag sampling. If necessary, use a compass to orient yourself along the plot perimeter. If the plot corners are not easily visible between intervals, the person following may remain at the plot corner and provide direction until the next plot corner is located. Flagging landmarks may help. Stop at intervals appropriate to the sampling method and vegetation density, to examine the cloth and your person(s) for ticks.
4. Avoid contact with the cloth. Be aware that ticks may attempt to crawl onto your hands, arms, or body while you inspect the drag cloth.
5. Perform a quick scan over the cloth and your person(s) for adult ticks first, as they tend to drop off more quickly than the other life stages. Scan the cloth 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 ticks from other arthropods and debris.
    a. Collect ticks and transfer them into a labeled sample vial containing RNA stabilization solution. When handling a tick, use the forceps to pick it up by the leg rather than pinching the body. Ticks of all life stages collected during a sampling plot/bout combination should be placed together 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.

Record the number of ticks of each life stage (

b. **Error! Reference source not found.**) observed. This number **should include** ticks that were dropped or otherwise lost during the collection process. If lost samples result in no vial for that bout, but field counts for the bout are greater than zero, note "tick(s) lost" in the remarks field, otherwise no additional recording of lost ticks is required. It is preferred that these counts are recorded during sampling in the field. If time does not allow for this, however, counting may be done in the lab.
c. If counting and collecting **larvae** with forceps is excessively time-consuming, collect **larvae** with tape for counting in the lab. A lint roller may also be used if preferred, follow the same following procedures as for tape. Ticks collected with tape will be stored in 95% ethanol until counted in the lab, at which point they will be transferred to vials with RNA stabilization solution for shipping. Collection and storage in 95% ethanol does not preserve viral genetic material, therefore, this method should only be used for larvae (for which viral detection is not a priority).
    i. Remove larval ticks from the drag cloth and your person with packing tape or masking tape. Do not use duct tape for sampling. Collect as many larvae as possible using as little tape as possible. You should strive to find and remove every larval tick from the cloth.
    ii. Completely submerge the tape with ticks in a labeled 50 mL vial (or larger if needed) with 95% ethanol to weaken the tape adhesive. Leave in ethanol for transit back to the lab.
d. Spend no more than 10-20 seconds checking your person(s) for ticks between intervals. Examine areas around the lower legs and feet especially closely. This inspection may be more thorough if done reciprocally (i.e., each team member inspects the other).

6. Continue sampling at appropriate intervals until the sampling transect is complete. Inspect your person(s) for ticks, add them to the sampling vial, and include them on the datasheet.
7. Fill in the Tick_QC_datasheet (RD[06]), with one record for every sampling interval inspected. Record end time and meters sampled. Be sure to also record any notes regarding unusual field conditions that may have affected sampling results. (e.g., cows walking through plot during sampling).
8. Place all labeled samples in an insulated cooler with frozen ice packs for transit back to the lab.
9. After leaving the plot, any ticks found on your person(s) should be removed with duct tape and discarded.

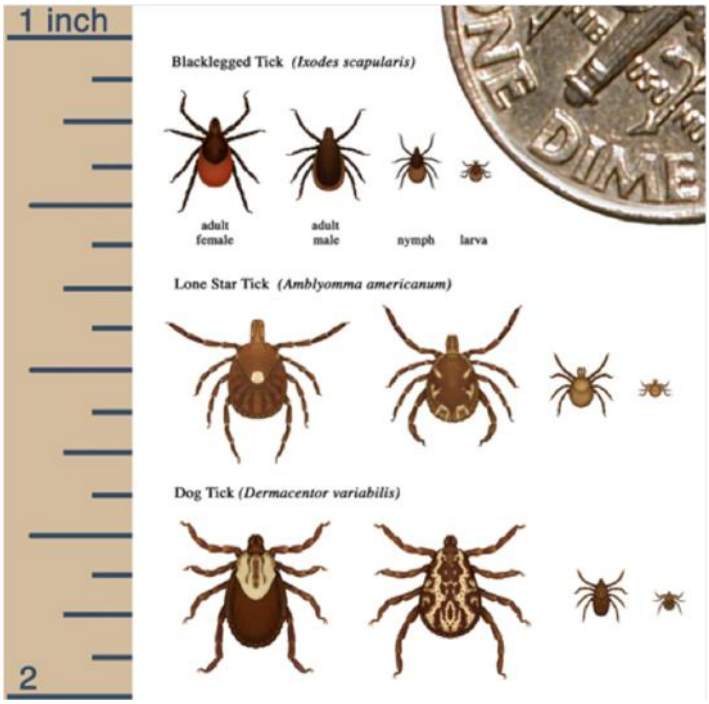


Figure 3. Relative sizes of life stages for selected species (courtesy of the Centers for Disease Control and Prevention)

B.6 Sample storage

Upon returning to the lab, immediately store ticks that need processing (counting, labeling) in a refrigerator (4°C) until specimens can be processed, and process within one month. Otherwise, if the ticks are ready for shipping, store them in a -20°C freezer. Store samples from the same site/bout combination together (e.g., using a rubber band and/or placing within a resealable bag).

SOP C Laboratory Processing and Analyses

C.1 Preparation

1. Clear space on a lab bench where tick samples can be sorted. It may be helpful to cover the work surface 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.

C.2 Sample processing in the lab

1. Ticks collected on tape will be in 95% ethanol, but after counting should be transferred to RNA stabilization solution for shipping. Ticks of all life stages from the same plot and bout may be combined in the same vial.
2. Count the total number of ticks and add to the Tick_QC_datasheet started in the field. If necessary, use forceps to carefully remove ticks remaining on the tape.
3. Transfer ticks into the appropriate sampling vial(s). If collected on tape and stored in ethanol, try to minimize the amount of excess ethanol transferred into the vial of RNA stabilization solution. A paintbrush may be useful for blotting, and handling larvae gently to avoid crushing them. If more than 500 larvae are collected, only the first 500 need be transferred to the sample vial. The remaining larvae may be discarded. However, ALL collected larvae should be counted and recorded prior to discarding any.
4. Complete Tick_QC_datasheet and fill in Tick_entry_datasheet (RD[06]) for the appropriate bout.
5. Make sure all vials are properly labeled externally and internally. See SOP A for label format instructions.
6. Send labeled vials of ticks (do not send tape) to the external ID facility.

C.3 Equipment maintenance, cleaning, and storage

1. Place the drag cloth in an ultralow (-20°C) freezer for at least 30 minutes to kill any larval ticks attached to the cloth.
2. Remove seeds stuck to the drag cloth to prevent introduction to other plots and sites, either by hand or with duct tape.
3. If the drag cloth is dirty, wash it using fragrance-free laundry detergent, using bleach if necessary, 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 and in good condition (i.e., same size as at the beginning of the season, free of holes) before placing in storage.
4. Clean any other equipment as necessary using dilute fragrance-free laundry detergent, dry and store in a cool, dry place.

SOP D Data Entry and Verification

The importance of thorough, accurate data transcription cannot be overstated; the value of the efforts in the field is only manifested once the data are properly entered for publishing to NEON's data portal. 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). Field data collected on paper datasheets should be digitally transcribed within 14 days of collection or the

end of a sampling bout (where applicable). See RD[04] for complete instructions regarding manual data transcription.

The procedure is as follows:

1. Scan datasheets and save in PDF file format (save scans to the folder designated by the Domain manager; folders found within the DEPT/FOPS/ folders for each domain)
2. Save paper copy of datasheets. At the end of the season, send the paper copies to headquarters for archiving.

Before entering data, all personnel must read RD[04] for complete instructions regarding manual data transcription. Prior to entering data via a web user interface (webUI), each technician shall enter a plot (or subplot) of data from one bout into the protocol-specific webUI housed on the Training portal, as described in RD[04].

Protocol-specific instructions and the associated data ingest workbook for entering tick data can be found on the NEON intranet in the FSU-FOPs folder. Be sure to enter data for all plots within a bout even if collected on a different schedule than originally planned. If an entire bout is missed, no data need to be entered, but issue a problem ticket in JIRA.

SOP E Sample Shipment

Information included in this SOP conveys science-based packaging, shipping, and handling requirements, not lab-specific or logistical demands. For that information, reference the CLA shipping document on CLA's NEON intranet site (https://neoninc.sharepoint.com/sites/cla/SitePages/Home.aspx) and the Domain Chemical Hygiene Plan and Biosafety Manual (AD[03]).

E.1 Supplies/Containers

Dry ice is a Class 9 regulated material and must be shipped according to CFR 49 Subchapter C, Hazardous Materials Regulations.

Dry ice releases carbon dioxide gas which can build up pressure and rupture packaging. Ensure the packaging used allows the release of this pressure to prevent rupturing the package. Dry ice must be packaged using **UN packing group III** compliant materials. The maximum amount of dry ice per package is **200 kg**.

Use corrugated cardboard boxes that meet UN packing group III requirements. Add Styrofoam along the walls of the box as insulation. Alternatively, a pre-insulated container (i.e. polystyrene) may be used (request return from the recipient if cost-reasonable). Precool the shipping container, if possible.

Double-bag the tubes containing samples using minimum 2-mil watertight plastic bags with absorbent liner inserted into the outer bag, and close securely. Pack in insulated container, surrounded by frozen ice packs (as backup in case dry ice sublimates) and dry ice. Fill all void space with grade 2 vermiculite to absorb any spills and prevent movement.

E.2 Conditions

Samples should be stored in vials containing RNA stabilization solution, ideally at -20°C (4°C acceptable for up to one month) until shipped to an external facility. Samples should be shipped 1-day freight with dry ice to maintain temperatures below 4°C during shipping. Avoid shipment on days that will require transit on a weekend or over a holiday period.

E.3 Grouping/Splitting Samples

All samples collected during each bout must be shipped together. Sample vials containing samples collected as part of the same bout can be taped or rubber-banded together, or placed in a separate bag, to allow them to be easily inventoried and sorted at the external facility.

E.4 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.

E.5 Shipping Inventory

Each shipment must be accompanied by a hard-copy shipping manifest AND a corresponding electronic version of the manifest (excel file) emailed to the taxonomic ID facility. The shipping manifest is the Tick_shipping_datasheet tab of RD[06]. Place the hard copy shipping manifest in resealable plastic bag on top of Styrofoam, and send electronic copy to the CLA contact **and** the receiving laboratory.

The hard-copy manifest (Figure 4) lists every sample vial in the identifier field (for which the entry will be vialID). The other required information on the shipping manifest, sentTo (name of the facility to which the specimens are being sent), sentDate, and recordedBy, must only be entered once per shipping manifest.

Figure 4. Example shipping manifest for the taxonomic ID facility.

NEON Tick and Tick -borne Pathogen: Shipping

sentTo: _Taxonomic Facility A_____ sentDate: 10/02/2013__ recordedBy: nrobinson@neoninc.org___

identifier *(vialID - for vials of ticks)*	remarks
HARV_001.20130401.01	
HARV_001.20130401.02	
HARV_001.20130401.03	
HARV_002.20130401.01	
HARV_002.20130401.02	

The electronic manifest should be emailed to the taxonomic ID facility as soon as possible after a batch of samples has been shipped.

E.6 Laboratory Contact Information and Shipping/Receipt Days

See the CLA shipping document on CLA's NEON intranet site (https://neoninc.sharepoint.com/sites/cla/SitePages/Home.aspx).

APPENDIX A DATASHEETS

The following datasheets are associated with this protocol:

Table 7. 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, remove with duct tape, and discard.

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

STEP 3 – Drag cloth SLOWLY for 5-10 meters.

WALK SLOWLY!

STEP 4 – Stop and inspect drag cloth. Count ticks and collect into vial containing RNA stabilization solution.

STEP 5 – Label specimen vial.

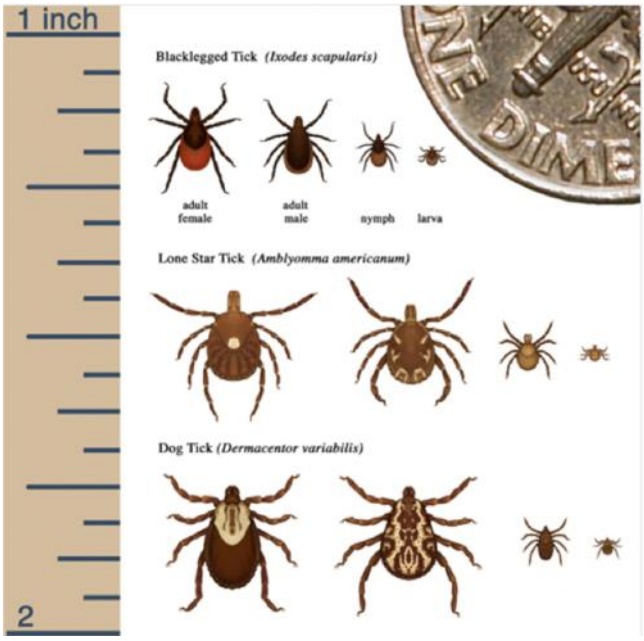
STEP 6 – Collect larval ticks with clear packing tape if necessary. Store in 95% ethanol for transport back to lab (ticks collected on tape only).

STEP 7 – Repeat drag and collection cycle until you have sampled the entire perimeter of the plot (i.e. returned to the plot corner where you began your sampling).

STEP 8 – Store specimen vials in cooler with ice packs.

STEP 9 – After leaving the plot, check yourself for ticks, remove with duct tape, and discard.

Tick Life Stages



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 Datasheet.
- ☑ Upload sample coordinates to GPS and obtain maps.
- ☑ Bring all supplies and extras.
- ☑ Check your pace. Can you accurately pace 5-10 meters?

PERSONAL SAFETY: PROTECT YOURSELF BY…

- ☑ Wearing appropriate clothing.
- ☑ Tucking pant legs into socks.
- ☑ Using tape to seal gaps.
- ☑ Applying insect repellent 30 min or more before going into field and away from sampling equipment.

You are collecting live ticks.

If you choose to 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 on your clothes in your count and specimen vial.
- ☑ Include ticks that were dropped or lost in your count.
- ☑ Label vials and store in cooler with ice packs.

BEFORE LEAVING DRAG SITE, CHECK THAT…

- ☑ Field portion of datasheet 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.

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 ticks.
- ☑ Checking 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.

Table 8. Estimated sampling dates based on historical temperature thresholds

Domain	Site(s)	Approx. Start Date	Approx. End Date
01	HARV	March 15	December 5
	BART	March 20	November 19
02	SCBI, SERC, BLAN	March 1	December 25
03	OSBS, DSNY, JERC	March 1	December 31
04	GUAN	March 1	December 31
05	UNDE, TREE	April 4	November 3
06	KONZ, UKFS	March 1	December 31
07	ORNL, GRSM	March 1	December 31
08	TALL	March 1	December 31
09	WOOD	March 31	November 4
10	CPER, STER	March 1	December 6
11	OAES	March 1	December 31
13	NIWO, MOAB	March 1	December 15
14	SRER, JORN	March 1	December 31
15	ONAQ	March 1	November 27
17	SJER, JORN	March 1	December 31
19	DEJU, HEAL	April 4	October 10

APPENDIX E SITE-SPECIFIC INFORMATION

N/A