Laboratory of Medical Zoology (LMZ) NEON Tick Pathogen Testing SOP

Version: 4.01

Date Modified: 3/22/2023

Log of SOP Changes:

Version: 4.01; Date Modified: 3/22/2023

Major differences between SOP 3.0 and 4.01: 1) the real-time PCR system was changed from Agilent MX3000P instrument (Agilent no longer provides supports) to Bio-Rad CFX instrument. CFX instrument has 6 filtered LEDs; It can detect up to 5 multiplex targets per well, no need to use ROX as a reference dye; 2) Merged NEON archival sample transferring and shipping protocol.

Version: 3.10; Date Modified: 8/29/2016

Equipment and materials list

Equipments:

- TissueLyser
- -20°C and -80°C Freezers
- Hybridization Oven
- Centrifuge
- Pipettes
- Bio-Rad CFX96 qPCR Machine
- PCR hood
- Brady Label printer

Materials	Manufacturer #
PCR Plate (Bio-Rad)	Bio-Rad #HSP9601
PCR Plate Sealing Film	Bio-Rad #MSB-1001
2.0 ml Microcentrifuge Tubes	Eppendorf #022363352
1.5 ml Microcentrifuge Tubes - Fisherbrand	Fisher Scientific 14-666-319
Fisherbrand™ SureOne™ Aerosol Barrier Pipette 1000 uL Tips	Fisher Scientific #02-707-404
Fisherbrand™ SureOne™ Aerosol Barrier Pipette 200 uL Tips	Fisher Scientific #02-707-430

Fisherbrand™ SureOne™ Aerosol Barrier Pipette 10 uL Tips	Fisher Scientific #02-707-442
Fisherbrand™ SureOne™ Aerosol Barrier Pipette 20 uL Tips	Fisher Scientific #02-707-432
VWR Wide bore pipette tips 1000ul	VWR 76635-652
Integra Multi-Channel adjustable Tips	Integra #3403
epMotion Reservoir 30 mL	Eppendorf #960051009
Brady Printer Ribbon	Brady IP-R6406
Brady Printer Label	Brady THT-59-492-10
Externally Threaded Cryogenic Storage Vials (NEON)	Fisher Catalog No.10-500-26
Lysis Buffer	Lucigen/Epicentre #MTC096H
Proteinase K	Lucigen/Epicentre #MPRK092
MPC Protein Precipitation Reagent	Lucigen/Epicentre #MMP095H
Red Cell Lysis Solution	Lucigen/Epicentre #MRC0912H
Isopropanol, Molecular Biology Grade	Fisher #BP26184
Ethanol, Molecular Biology Grade 200 Proof	Fisher #BP28184
Water, Molecular Biology Grade	Fisher #BP2819-10
qPCR Master Mix	Agilent #600881
RTqPCR Master Mix	Agilent #600885
12.5% Bleach	VWR BDH7038-4L

Receipt and Custody of Specimens

Ticks sent by US Mail arrive on weekdays in the morning. Ticks sent by courier (UPS or FedEx) may arrive throughout the business day. Ticks may also be dropped off at the Reception Office (Rm 101).

Incoming ticks:

- Ticks submitted by NEON will come in large batches. Open the container(s) and ensure all samples listed on the manifest have been received and are in good condition (e.g., not broken or missing; labels are legible). Notify the NEON Contract Officer immediately of any problems with the shipment within 48 hours of receipt.
- The electronic receipt form included in the NEON shipping email must also be completed to document condition of samples upon receipt. Each completed receipt form will be uploaded to the NEON Data Portal per instructions provided by NEON.
- After reconciling with contractor manifest, ticks are then moved forward for In-Processing. Ticks from contract agencies should be accompanied with Internal Routing Sheet (**Appendix A**) indicating service required (photos, tests, etc.)

In-Processing of specimens

1. Each tick should be individually processed.
2. Carefully remove the tick from the bag/container it was sent in and place in a plastic weigh boat.
3. Place the weigh boat with the tick under a microscope and identify the species, life stage, and feeding state (fed, no signs of feeding) using James Keirans Identification key (Keirans and Litwak 1989)
4. For specimens requiring photo-micrograph, take a picture of both the dorsal and ventral sides of the tick. Tick images are labeled according to the filename template 20150202_01_v_1.5x (filename template YYYYMMDD_01_v_1.5x, where YYYYMMDD is the year, month and day of the Monday of that week, 01 is the tick number, v=ventral, 1.5x being the magnification on microscope.). Save pictures as JPEG data type in a file for the week by selecting "Browse." (For details, please see Appendix Leica LAS X Imaging Protocol). (**Appendix B1 and B2**)
5. Contract ticks should be labeled with an internal sample number (LMZ 000001). The FT tick data should be input into FT database (see **Appendix C** for curating rules of FT Database).
6. Place one tick into a 2.0 ml safe-lock tubes (Qiagen Cat. No. 990381) containing one 5 mm metal bead (VXB, 3/16" inch Diameter Stainless Steel 440C G16 Bearing Balls, Code: Kit 12748). (Must use Qiagen or Eppendorf 2.0 ml Safe-Lock Tubes, Cheap tubes will crack after shaking). Place the tick ID number that it was assigned on the lid of the tube.
7. The samples will be stored at -80°C and can be placed in line to be extracted.

Total Nucleic Acid Extraction

Things to do before starting:

- This protocol was modified from Epicentre MasterPure Complete DNA and RNA Purification Kit Bulk Reagents (https://www.lucigen.com/MasterPure-Complete-DNA-and-RNA-Purification-Kit/#subcat-tabs2). (see **Appendix D**)
- Total Nucleic Acid Extractions are performed in the "dirty" room, Room B1.
- Total Nucleic Acid extractions are carried out on a bench that is cleaned daily with 10% Bleach.
- All waste from the DNA extraction is placed in a plastic "trash" container on the bench and then emptied in to the biohazard trash.
- There should be 1 extraction control for each set of extractions. This is typically the 30th tube in the set. This no template control allows us to monitor for DNA

contamination in the extraction reagents as well as DNA contamination between samples due to human error or a problem with the protocol.

- Turn on and warm Hybridization Oven to 65°C.
- Turn on and cool centrifuge to 4°C.

Process

1. Place the tube into a TissueLyser reaction-tube holder (24 samples/holder).
2. Place the tube holder and tubes into a -80°C freezer overnight (or at least 1 hour). **DO NOT** freeze top and bottom plate of the adaptor, only freeze tube holder.
3. Assemble the TissueLyser Adapter Set 2 x 24 using a bottom plate, the reaction-tube holder (with 2.0 ml safe-lock tubes containing one tick and one 5mm bead), and a top plate with sealing strips.
4. Ensure that the top and bottom plate fit securely, and make sure that the reaction tubes are sealed securely.
5. Make sure that two TissueLyser adapter sets are balanced. Distribute the reaction tubes evenly across the TissueLyser. Balance the lid by ensuring 2.0 ml safe-lock tubes are in all 4 corners of both 24 sample holders.
6. Place the assembled TissueLyser Adapter Sets into the TissueLyser holders (arms), and close the handles tightly. **DO NOT** use the TissueLyser with only one TissueLyser Adapter Set, since this will cause machine imbalance and decrease homogenization efficiency.
7. Homogenize the ticks: 23Hz, 1.5 minutes.
8. Take out tubes from step 7 and centrifuge at 14000rpm for 1 minute at 4oC.
9. Dilute 1 ul of 50 ug/ul Proteinase K (http://www.epibio.com; Proteinase K; MPRK09250 µg/µl) into 300 ul of 1X Tissue and Cell Lysis Solution (http://www.epibio.com; 2X T&C Lysis Buffer should be diluted to 1X using molecular grade water. 2X T&C Lysis Buffer MTC085H) for each sample. For 30 ticks, prepare master mixture: 1X Tissue and Cell Lysis Solution: 300 ul X 31 = 9300 ul; 50 ug/ul Proteinase K : 1 ul X 31 = 31 ul;
10. Take 301ul master mixture for one homogenized tick (602 ul for fully engorged ticks).
11. Mix thoroughly and incubate at 65°C for 15 minutes; vortex every 5 minutes.
12. Place the samples on ice or in 4°C fridge for 10 min.
13. Add 150 ul (200 ul for fully engorged ticks) ice cold MPC Protein Precipitation Reagent (MPC Protein Precipitation; MMP03750 Bulk order) to 301 ul of lysed sample and vortex vigorously for 10 seconds.
14. Pellet the debris by centrifugation at 4°C for 10 minutes at 14,000 RPM in a microcentrifuge.

15. Transfer 350 ul of supernatant to a new tube containing 500 ul of ice cold isopropanol. Invert the tube several times. Store the protein pellets in the 4C fridge until all testing is complete.
16. Pellet the DNA/RNA by centrifugation at 4°C for 10 minutes at 14,000 RPM.
17. Carefully pour off the isopropanol without dislodging the nucleic acid pellet. You can remove all of the residual isopropanol with a pipette.
18. Rinse with 1000 ul 75% ice-cold ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipette.
19. Air-dry your pellets in the 65°C incubator, using 3D printed comb to separate tubes to prevent cross contamination. Resuspend the DNA/RNA in 70 ul of molecular grade water (BP 2819-10 Fischer Scientific).
20. Store samples (29 ticks and 1 extraction control in general) at -20°C and proceed to the next section for real-time PCR setup.

Design of Assays and Master Mix

- We use multiplex Taqman real-time PCR to detect tick pathogens: 3 multiplex assays for Ixodes DNA package and 3 multiplex assay for Non-Ixodes DNA package and 1 multiplex assay for tick virus.
- The multiplexing master mixes are using Agilent Brilliant III Ultra-Fast QPCR (Agilent Cat# 600880) and Agilent Brilliant III Ultra-Fast QRT-PCR (Agilent Cat# 600885). The primers and dual-labeled probes are ordered from IDTDNA. Double-Quenched Probes contain a 5' fluorophore (FAM, HEX or Cy5); 3' IBFQ quencher; and proprietary, internal ZEN or TAO quencher. The dual-labeled probes are also ordered from Biosearch with report dyes: CAL Fluor Red 610, Quasar 670 and Quasar 705 with black hole quencher.
- Hard tick (Family: Ixodidae) 18S gene was used as an internal control for tick DNA quality control.
- Gene fragments (gBlocks) are ordered from IDTDNA as Taqman PCR positive QC controls. These gene fragments (gBlocks) are double-stranded, sequence-verified genomic blocks.
- Multiplex master mix are made each in 50 ml conical tube. Four 15ul reactions from the master mix are tested QC at gBlock concentration 5 pg- 0.005 pg for positive controls (2 reactions) and plain master mix for negative controls (2 reactions). If the master mixes pass the QC, they are aliquoted into 1.5 mL tubes and stored at -80°C (About 70 reactions per tube, 15 ul per reaction). The number of master mix need to be thawed and frozen should be limited (as this

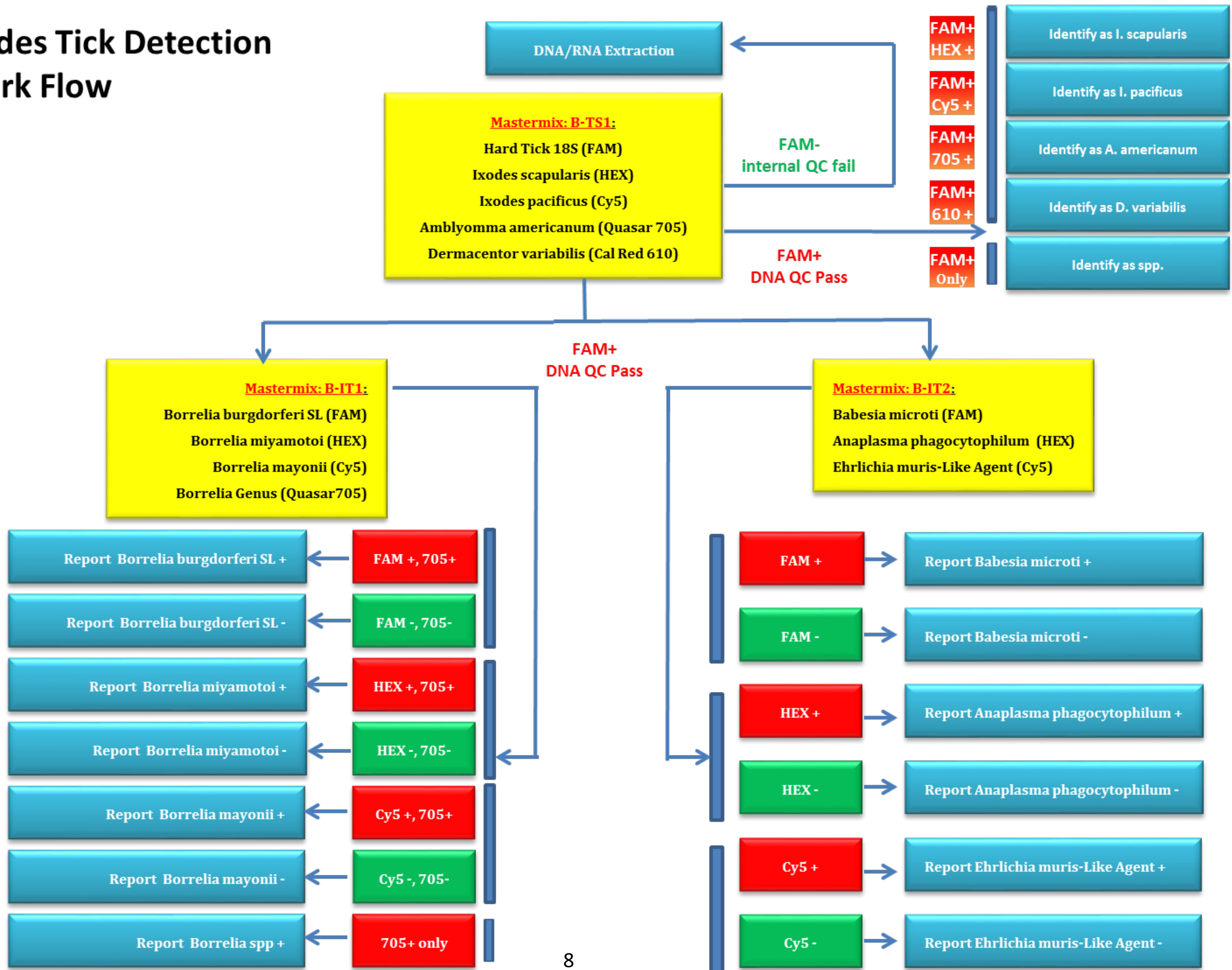
may reduce the efficacy of the master mix). Exposure to light can also damage the master mix.

- Tick pathogens for Ixodes and Non-Ixodes tick species, target genes and probe label dyes for each multiplexing are also listed below: (See **Appendix E**)

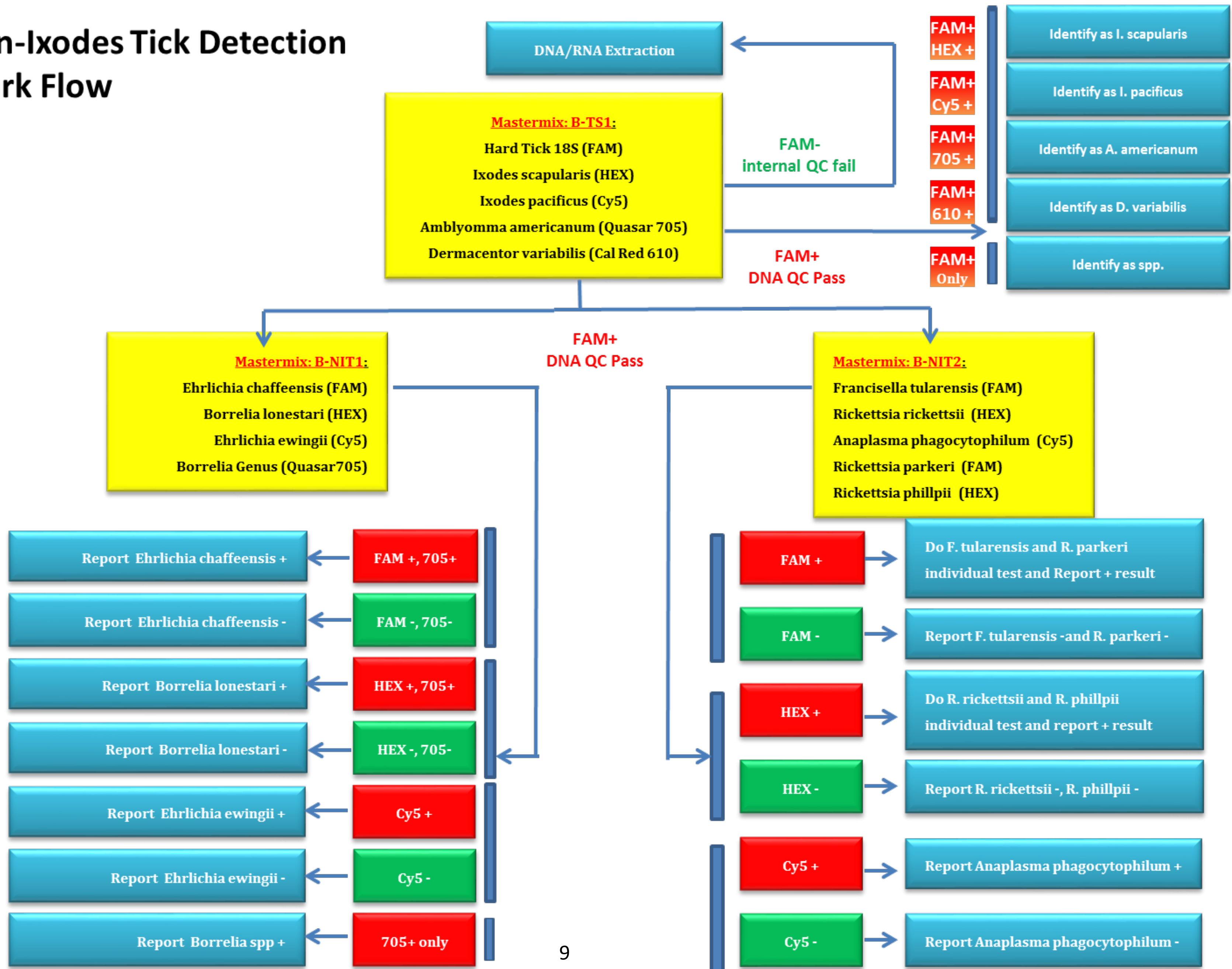
Tick Species and Pathogen Tests	Ixodes Ticks	Non-Ixodes Ticks	Master Mix	Dye	Target Gene	Positive control (SPC)	Thermal Program
Tick DNA Internal quality (Hard tick Ixodidae 18S gene)	Included	Included	B-TS1	FAM	mtDNA 18S	B-SPC	DNA STD program
Ixodes scapularis (molecular identification for *I. scapularis*)	Included	Included	B-TS1	HEX	ITS2	B-SPC	DNA STD program
Ixodes pacificus (molecular identification for *I. pacificus*)	Included	Included	B-TS1	CY5/Quasar 670	ITS2	B-SPC	DNA STD program
Amblyomma americanum (molecular identification for A. americanum)	Included	Included	B-TS1	Quasar705	ITS2	B-SPC	DNA STD program
Dermacentor variabilis (molecular identification for D. variabilis)	Included	Included	B-TS1	Cal Red 610	ITS2	B-SPC	DNA STD program
Borrelia general species (Lyme or relapsing fever)	Included	Included	B-IT1	Quasar705	16S	B-SPC	DNA STD program
Borrelia burgdorferi sensu lato (Lyme borreliosis)	Included		B-IT1	FAM	ospA	B-SPC	DNA STD program
Borrelia miyamotoi	Included		B-IT1	HEX	glpQ	B-SPC	DNA STD program
Borrelia mayonii	Included		B-IT1	CY5	ospC	B-SPC	DNA STD program
Babesia microti (Babesiosis often found in humans)	Included		B-IT2	FAM	Tubulin	B-SPC	DNA STD program
Anaplasma phagocytophilum (Human Granulocytic Anaplasmosis; HGA)	Included	Included	B-IT2	HEX	MSP-2	B-SPC	DNA STD program
Ehrlichia muris-Like Agent	Included		B-IT2	CY5	P13	B-SPC	DNA STD program
Borrelia general species (Lyme or relapsing fever)	Included	Included	B-NIT1	Quasar705	16S	B-SPC	DNA STD program
Ehrlichia chaffeensis (Human Monocytic Ehrlichiosis)		Included	B-NIT1	FAM	dsb	B-SPC	DNA STD program
Borrelia lonestari (Southern Tick-Associated Rash Illness; STARI)		Included	B-NIT1	HEX	glpQ	B-SPC	DNA STD program
Ehrlichia ewingii (Human Ehrlichiosis)		Included	B-NIT1	CY5	dsb	B-SPC	DNA STD program
Francisella tularensis (Tularemia)		Included	B-NIT2	FAM	ISFtu2	B-SPC	DNA STD program
Rickettsia rickettsii (Rocky Mountain Spotted Fever; RMSF)		Included	B-NIT2	HEX	RRi6	B-SPC	DNA STD program
Anaplasma phagocytophilum (Human Granulocytic Anaplasmosis; HGA)		Included	B-NIT2	CY5	MSP-2	B-SPC	DNA STD program
Rickettsia parkeri (Rickettsia parkeri rickettsiosis)		Included	B-NIT2	FAM	sca2	B-SPC	DNA STD program
Rickettsia philipii (Pacific Coast Tick Fever)		Included	B-NIT2	HEX	Fragment from 364D genome	B-SPC	DNA STD program

- Detection work flow are listed below:

Ixodes Tick Detection Work Flow



Non-Ixodes Tick Detection Work Flow



Setup a Sample Loading Map

- All sample are run as a 96-well plate format.
- Use a below sample as a template map. It contains 29 samples, 1 extraction control (EC), 1 super positive control (SPC) and 1 no template control (NTC) for 3 multiplex assays (B-TS1, B-IT1 and B-IT2).
- Use the map to determine which samples and master mixes need to be thawed at 4C in order to set up this plate.
- This printed map should also be labeled with the file name that contains the results and should be filed in the office according to month as well as a copy by contract/project name (if applicable).
  An example of sample loading map (See **Appendix F**):

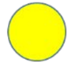
Non-Ixodes			Hard tick	I. scapularis	D. variabilis	A. americanum	I. pacificus			Borrelia16S	E.chaffeensis	B.lonestari	E.ewingii				F.tularensis/R.parkeri	Anaplasma	R.rickettsia/R.philipii		
Sample	Well	Mastermix	**FAM**	**HEX**	**CalRed610**	**Quasar705**	**Quasar670**	Well	asterm	**Quasar705**	**FAM**	**HEX**	**Quasar670**	*CalRed610*	Well	Mastermix	**FAM**	**Quasar670**	**HEX**	*CalRed610*	*Quasar705*
1	A1	B-TS1						A5	B-NIT1						A9	B-NIT2					
2	B1	B-TS1						B5	B-NIT1						B9	B-NIT2					
3	C1	B-TS1						C5	B-NIT1						C9	B-NIT2					
4	D1	B-TS1						D5	B-NIT1						D9	B-NIT2					
5	E1	B-TS1						E5	B-NIT1						E9	B-NIT2					
6	F1	B-TS1						F5	B-NIT1						F9	B-NIT2					
7	G1	B-TS1						G5	B-NIT1						G9	B-NIT2					
8	H1	B-TS1						H5	B-NIT1						H9	B-NIT2					
9	A2	B-TS1						A6	B-NIT1						A10	B-NIT2					
10	B2	B-TS1						B6	B-NIT1						B10	B-NIT2					
11	C2	B-TS1						C6	B-NIT1						C10	B-NIT2					
12	D2	B-TS1						D6	B-NIT1						D10	B-NIT2					
13	E2	B-TS1						E6	B-NIT1						E10	B-NIT2					
14	F2	B-TS1						F6	B-NIT1						F10	B-NIT2					
15	G2	B-TS1						G6	B-NIT1						G10	B-NIT2					
16	H2	B-TS1						H6	B-NIT1						H10	B-NIT2					
17	A3	B-TS1						A7	B-NIT1						A11	B-NIT2					
18	B3	B-TS1						B7	B-NIT1						B11	B-NIT2					
19	C3	B-TS1						C7	B-NIT1						C11	B-NIT2					
20	D3	B-TS1						D7	B-NIT1						D11	B-NIT2					
21	E3	B-TS1						E7	B-NIT1						E11	B-NIT2					
22	F3	B-TS1						F7	B-NIT1						F11	B-NIT2					
23	G3	B-TS1						G7	B-NIT1						G11	B-NIT2					
24	H3	B-TS1						H7	B-NIT1						H11	B-NIT2					
25	A4	B-TS1						A8	B-NIT1						A12	B-NIT2					
26	B4	B-TS1						B8	B-NIT1						B12	B-NIT2					
27	C4	B-TS1						C8	B-NIT1						C12	B-NIT2					
28	D4	B-TS1						D8	B-NIT1						D12	B-NIT2					
29	E4	B-TS1						E8	B-NIT1						E12	B-NIT2					
EC	F4	B-TS1						F8	B-NIT1						F12	B-NIT2					
SPC	G4	B-TS1						G8	B-NIT1						G12	B-NIT2					
NTC	H4	B-TS1						H8	B-NIT1						H12	B-NIT2					
Quasar670=Cy5																					
Date:					Machine #				Operator: GX												
QPCR File Name:									Note:												

Real Time PCR Plate Setup

- This protocol was modified from Brilliant III Ultra-Fast QRT-PCR Master Mix (See **Appendix G**).
- Total volume of each qPCR reaction is 16 ul: 15 ul of master mix and 1ul of template.
- The master mix stocks are made in batches. Each batch is tested to pass QC. If the master mixes pass the QC, they are aliquoted into 1.5 mL tubes and stored at -80°C.
- For master mix transfer, only one master mix can be transferred in the hood each time. For example, when a technician transfers B-TS1 into reservoir in the hood, only B-TS1 master mix and reservoir labeled as B-TS1 can be put into the hood.
- Each technician should have their own freezer box and reservoir in the -20°C freezer. The master mixes that aren't used as frequently should be stored as smaller aliquots to limit the number of times each aliquot needs to be thawed and frozen (as this may reduce the efficacy of the master mix).
- If it takes >60 minutes to prepare a qPCR plate, the master mix and plate should be set on ice.

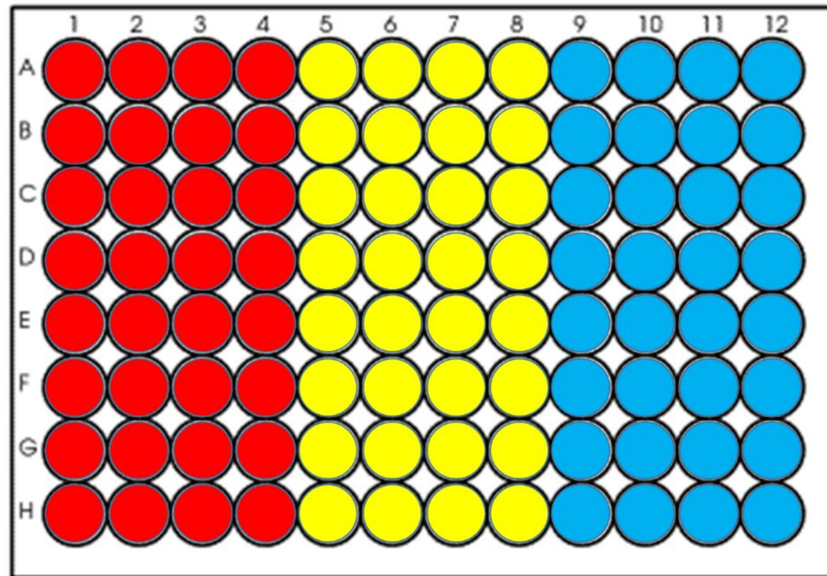
1. Thaw the master mix. Vortex briefly and spin down master mix.
2. Prepare and label a 96 well Bio-Rad qPCR plate. Well A1 is always on the top left corner.
3. Add 15 µl of the master mix solutions, for example: B-TS1, B-IT1 and B-IT2 into the appropriate wells based on the sample loading map, then bring the tube/reservoir back to their storage box at -20°C.

B-TS1

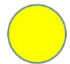


B-IT1

B-IT2

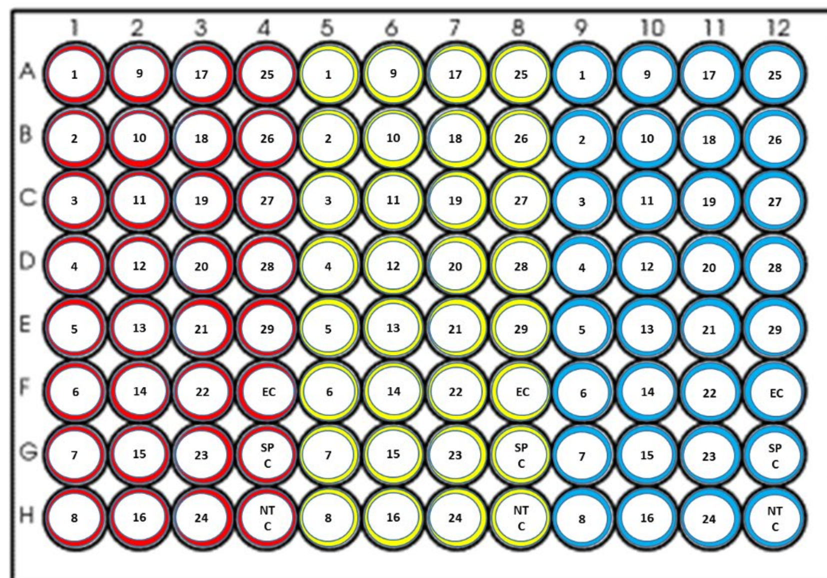


B-TS1



B-IT1

B-IT2



4. Add 1 ul of DNA template to each well, following the layout of the map, for example sample 1-29. When adding template, be sure NOT to open tubes of DNA over the plate. Pipette slowly to avoid contamination of other wells. Always hold pipette straight up and down (not tilted) to get the most accurate volume. Make sure that you are getting a NEW TIP for EVERY well and you are ejecting the tips ABOVE the waste container. It is also helpful to move the template tube to a new tube rack and place in the spot corresponding to the last well it was added to on the 96-well plate. DNA template tubes should be returned to -20°C freezer for short-term and -80°C for long-term storage.
5. Add 1ul extraction control (EC), 1 ul super positive control (SPC) and 1 ul water as NTC (No Template Control).

6. Seal the 96-well PCR plate with optically clear film, Bio-Rad sealer. Make sure to press the film down around all edges and between wells to prevent sample evaporation.
7. Centrifuge the plate using a plate centrifuge for 1 min. Proceed to Bio-Rad CFX96 instrument instructions.

Instrument Settings and PCR Run

- The Taqman detection is set up using Bio-Rad CFX96 Touch Deep Well Real-Time PCR Detection System. (See **Appendix H**) and CFX maestro software (See **Appendix I**).
- CFX instruments are factory calibrated for many fluorescent dye and plate combinations. No need calibration. Compare to Agilent Mx3000P QPCR System, CFX instrument has 6 filtered LEDs; It can detect up to 5 multiplex targets per well, no need to use ROX as a reference dye.
- Standard filter sets for reporter dye detection include Channel 1: FAM (510nm-530nm), Channel 2: HEX (560nm-580nm), Channel 3: Texas Red/Cal Red 610 (610nm-650nm), Channel 4: Cy5/Quasar (675nm-690nm) and Channel 5: Quasar705 (705nm-730nm).

1. Open the “CFX maestro” program.
2. Select run type as “User-defined”.
   For protocol, use below thermal profile for DNA test:
   a. 95°C for 10 min (1 cycle)
   b. 95°C for 15 seconds, 60°C for 60 seconds, END* (40 cycles).
      *Need endpoint to tell computer to read dye.
3. For Plate, set all well type as Unknown, Fluorophores should include all channels, scan mode: all channels.
4. Start Run. Click “Open Lid”, load setup 96-well plate with A1 in the top left-hand corner. Click “Close Lid” and wait the lid to close.
5. File → Save As → C → Bio-Rad_X (X is the QPCR machine number).→
6. Name the file as: Plate#_Date for day (Plate1_20150105).
7. This file name must match the file name on the PCR plate. The plate machine # also need to be recorded on the qPCR map.
8. Verify correct settings in the template file.
9. Select Start Run tab. It takes approximately 90 min to finish a DNA test.

Interpretation of results

Checklist for inspecting results:

- Hard Tick 18S internal control.
- Extraction control.
- Negative control.
- Super positive control.
- Amplification curve of each sample.

1. Once the program has finished, save the file and open it for Data Analysis.
2. Check on each sample:
    a. Software setting is: cq determination mode = Regression; Base line setting= baseline subtracted curve fit and apply fluorescence drift correction; Analysis mode = fluorophore.
    b. Check amplification curve and Ct values. The amplification curves of true positive targets are exponential. The results of the real-time PCR assays are based on cycle threshold (Ct) values obtained from the amplification curves of the targets. The Ct value represents the number of cycles required to reach a particular threshold fluorescence signal level. The fewer cycles it takes to obtain a detectable fluorescence level, the greater the amount of target in ticks.
    c. A positive sample should have an amplification curve and Ct values for up to 5 channels. It is generally accepted that a 3-cycle difference in target Ct compared to the negative control Ct will reliably separate a true positive signal from a contamination result (Bustin, 2004). As the PCR protocol involves 40 cycles, Ct 37 is the appropriate 3 cycle different Ct value.
    d. Ct values above 37, but below 40 may represent cases where target is present at very low levels. In this case, samples should be re-tested.
    e. Hard Tick 18S internal control: it will result in an amplification reaction if tick DNA was successfully extracted and PCR was correctly performed. The purpose of the Hard Tick 18S internal control is to confirm, for each sample and PCR reaction, that the DNA extraction quality and PCR reaction conditions were acceptable for the targets and that the plate setup (pipetting) was correctly performed.
    f. Extraction control: it will result in an amplification curve if tick DNA contamination occurred during extraction step.
    g. Negative control: it will result in no amplification cure. If the Ct value of the target in a negative control is less than 3 cycles apart from the Ct of the same target in a sample, that target in that sample fails to qualify as positive and must be disregarded in the analysis.

h. Super positive control: it consists of a control DNA template that will result in an amplification reaction if the PCR was correctly performed.

3. Record Ct value on the sample loading map.
4. Input the Ct values into the tick database along with tick identification data.

NEON Sample Transferring and Shipping Protocol

Purpose:

- The purpose of this standard operation procedure (SOP) is to clearly list the actions required to transfer and ship samples with varying shipping requirements.

Training Requirements:

- Members of LMZ at UMass Amherst involved with shipping samples must complete “Shipping Exempt Biological Samples Category B”, “Shipping with Dry Ice” training modules located in the microbiology department section of SciShield EHS training website yearly. Certificates of completion must be printed and stored in the “LMZ Training Records” binder.

Container and Labeling Requirements for Laboratories Generating NEON Archival Samples, Destined for Cryopreservation via Liquid or Vapor Phase Liquid Nitrogen

- **CryoVial (2ml) approved for use by the NEON Biorepository at Arizona State University (ASU)**
  - *Key features of the vials listed below are external threading, O-ring and self-standing base.*
    1. **Externally Threaded Cryogenic Storage Vials (NEON) Fisher Catalog No.10-500-26**

- **Cryoboxes, Dividers & Securing Bands Approved for use by the NEON Biorepository at ASU**
  - *Key feature of these Cryoboxes is the moisture-repellent coating. Poly boxes are not approved for storage in Liquid or Vapor Phase Liquid Nitrogen.*
    1. **VWR 82007-162 or 97014-322; 2-inch Cryogenic box with grids, 81 cell-divider**
  - *Key feature of these heavy rubber bands is the capability to secure the cryoboxes in transit*
    2. **Alliance Advantage Multi-Purpose Rubber Bands, #84, 1 lb. Box, 150/Box (26845)**
       https://www.staples.com/Alliance-Advantage-26845-Rubber-Band-Crepe/product_388687

- **Cryo-Labels and Printers Approved for use by the NEON Biorepository at ASU**
  - *Key feature of these Cryo-Labels is the capability to withstand long-term storage in liquid nitrogen. Note - Labels needs to be applied to a clean, dry vial before freezing.*

    1. **Brady Printer Ribbon Brady IP-R6406 VWR 490006-116**
    2. **Brady Printer Label Brady THT-59-492-10 VWR 490006-380, new # 89077-356**

Print Barcode Labels:

- Use below format for NEON tick samples: QT00000000001;
- Use below format for NEON rodent samples: QR00000000001;
- Use below format for NEON mosquito samples: QM00000000001.
- Print a sample list with barcode labels (no barcode) with corresponding LMZ internal sample IDs using normal printer (see below sample).
- Print real barcode labels using Brady printer and a subset of (10) these labels be test-scanned with a barcode app.

LMZ internal Label	NEON barcode	containerID	wellCoordinate	
LMZ_M0016032	QM00000000001		A/1	1
LMZ_M0016033	QM00000000002		A/2	2
LMZ_M0016034	QM00000000003		A/3	3
LMZ_M0016035	QM00000000004		A/4	4
LMZ_M0016036	QM00000000005		A/5	5
LMZ_M0016037	QM00000000006		A/6	6
LMZ_M0016038	QM00000000007		A/7	7
LMZ_M0016039	QM00000000008		A/8	8
LMZ_M0016040	QM00000000009		A/9	9
LMZ_M0016041	QM00000000010		B/1	10
LMZ_M0016042	QM00000000011		B/2	11
LMZ_M0016043	QM00000000012		B/3	12
LMZ_M0016044	QM00000000013		B/4	13
LMZ_M0016045	QM00000000014		B/5	14
LMZ_M0016046	QM00000000015		B/6	15
LMZ_M0016047	QM00000000016		B/7	16
LMZ_M0016048	QM00000000017		B/8	17
LMZ_M0016049	QM00000000018		B/9	18
LMZ_M0016050	QM00000000019		C/1	19
LMZ_M0016051	QM00000000020		C/2	20
LMZ_M0016052	QM00000000021		C/3	21
LMZ_M0016053	QM00000000022		C/4	22
LMZ_M0016054	QM00000000023		C/5	23
LMZ_M0016055	QM00000000024		C/6	24
LMZ_M0016056	QM00000000025		C/7	25
LMZ_M0016057	QM00000000026		C/8	26
LMZ_M0016058	QM00000000027		C/9	27
LMZ_M0016059	QM00000000028		D/1	28
LMZ_M0016060	QM00000000029		D/2	29
LMZ_M0016061	QM00000000030		D/3	30
LMZ_M0016062	QM00000000031		D/4	31
LMZ_M0016063	QM00000000032		D/5	32
LMZ_M0016064	QM00000000033		D/6	33
LMZ_M0016065	QM00000000034		D/7	34
LMZ_M0016066	QM00000000035		D/8	35
LMZ_M0016067	QM00000000036		D/9	36
LMZ_M0016068	QM00000000037		E/1	37
LMZ_M0016069	QM00000000038		E/2	38
LMZ_M0016070	QM00000000039		E/3	39
LMZ_M0016071	QM00000000040		E/4	40
LMZ_M0016072	QM00000000041		E/5	41
LMZ_M0016073	QM00000000042		E/6	42
LMZ_M0016074	QM00000000043		E/7	43
LMZ_M0016075	QM00000000044		E/8	44
LMZ_M0016076	QM00000000045		E/9	45
LMZ_M0016077	QM00000000046		F/1	46
LMZ_M0016078	QM00000000047		F/2	47
LMZ_M0016079	QM00000000048		F/3	48
LMZ_M0016080	QM00000000049		F/4	49
LMZ_M0016081	QM00000000050		F/5	50
LMZ_M0016082	QM00000000051		F/6	51
LMZ_M0016083	QM00000000052		F/7	52
LMZ_M0016084	QM00000000053		F/8	53
LMZ_M0016085	QM00000000054		F/9	54
LMZ_M0016086	QM00000000055		G/1	55
LMZ_M0016087	QM00000000056		G/2	56
LMZ_M0016088	QM00000000057		G/3	57
LMZ_M0016089	QM00000000058		G/4	58
LMZ_M0016090	QM00000000059		G/5	59
LMZ_M0016091	QM00000000060		G/6	60
LMZ_M0016092	QM00000000061		G/7	61
LMZ_M0016093	QM00000000062		G/8	62
LMZ_M0016094	QM00000000063		G/9	63
LMZ_M0016095	QM00000000064		H/1	64
LMZ_M0016096	QM00000000065		H/2	65
LMZ_M0016097	QM00000000066		H/3	66
LMZ_M0016098	QM00000000067		H/4	67
LMZ_M0016099	QM00000000068		H/5	68
LMZ_M0016100	QM00000000069		H/6	69
LMZ_M0016101	QM00000000070		H/7	70
LMZ_M0016102	QM00000000071		H/8	71
LMZ_M0016103	QM00000000072		H/9	72
LMZ_M0016104	QM00000000073		I/1	73
LMZ_M0016105	QM00000000074		I/2	74
LMZ_M0016106	QM00000000075		I/3	75
LMZ_M0016107	QM00000000076		I/4	76
LMZ_M0016108	QM00000000077		I/5	77
LMZ_M0016109	QM00000000078		I/6	78
LMZ_M0016110	QM00000000079		I/7	79
LMZ_M0016111	QM00000000080		I/8	80
LMZ_M0016112	QM00000000081		I/9	81

Sample Transferring

1. Place barcode label on the 2ml CryoVial (orientation: bottom up vertical, see below example).

2. Take out TNA extraction samples from freezer and thaw them.
3. Centrifuge briefly.
4. Transfer all liquid from TNA extraction tube (1.5 ml tube) into corresponding CryoVial tube (2 ml external cap tube). Make sure that internal label matches barcode label.
5. Mark the cryobox A/1 position. Place transferred CryoVial tubes with barcode labels into a cryobox from A/1 to I/9. Each cryobox contains 81 CryoVials.
6. Label finished cryobox:
    a. Box Label Template: QR,year,Box #
    b. Example Box Labels: "QR2023 Box 1" (contains samples 1-81), "QR2023 Box 2" (contains samples 82-162), the first sample corresponds to the A/1 position in the box, and the last sample corresponds to the I/9 position.

A/1	A/2	A/3	A/4	A/5	A/6	A/7	A/8	A/9
B/1	B/2	B/3	B/4	B/5	B/6	B/7	B/8	B/9
C/1	C/2	C/3	C/4	C/5	C/6	C/7	C/8	C/9
D/1	D/2	D/3	D/4	D/5	D/6	D/7	D/8	D/9
E/1	E/2	E/3	E/4	E/5	E/6	E/7	E/8	E/9
F/1	F/2	F/3	F/4	F/5	F/6	F/7	F/8	F/9
G/1	G/2	G/3	G/4	G/5	G/6	G/7	G/8	G/9
H/1	H/2	H/3	H/4	H/5	H/6	H/7	H/8	H/9
I/1	I/2	I/3	I/4	I/5	I/6	I/7	I/8	I/9

7. Use #84 multi-purpose rubber bands to secure the cryobox and store them in -80°C freezer.

Packaging and Shipping:

- **Notify receivers (NEON and Biorepository): 1) the manifest, 2) the shipment date and 3) tracking# prior to shipping.**
- **The completed receipt form will be uploaded to the NEON Data Portal by the receiving lab.**
- **Samples shipped on dry ice should be PRIORITY OVERNIGHT only on Monday-Wednesday.**
- **UMass use eShipGlobal's Shipping System: https://umassehs.eshipglobal.com/**

Note: Steps 1 and 2 are to be completed before the day of shipping. **Do not select to ship on a day where shipping delays are expected** (see step 9). When selecting a day to ship, make sure the package is prepared and dropped off at a FedEx location by 2PM. If this is not possible, select a new day to ship.

1. Create a document to be the chain of custody and fill out as much information as possible (what kind of document depends on the destination). Complete this form as you continue through the protocol, or complete before.
2. Determine the temperature requirements for the sample being shipped (RT, 4°C, -20°C, etc.).
   i. For 4°C, make sure ice packs are being frozen overnight the night before the package is assembled.
   ii. For -20°C, go to the fisher store on campus the same morning you plan on shipping the samples. Per the CDC, allow 1 pound of dry ice for every 2 hours of transport.
3. Notify NEON and ASU Biorepository for the coming shipment.
4. When shipping contents that do not require dry ice, package the contents inside a container (Styrofoam cooler for samples requiring cold chain) and secure the contents with packing materials. If multiple sub-containers are needed for a batch of contents, place them in the container such that they are near each other, and in order chronologically. Skip to step 9.
5. When shipping contents that require dry ice, place the samples to be shipped in cryo-boxes. If multiple cryo-boxes are needed for a batch of samples (this will almost always be the case), place them in the container such that they are near each other, and in order chronologically.
6. Secure the cryo-boxes using cardboard, Styrofoam, or other materials that will maintain structural integrity in cold temperature. Do NOT use dry ice to secure the cryo-boxes.
7. Add the dry ice to the container. Spread the dry ice around and throughout the cryo-boxes and packing materials in the container.
8. Cover the Styrofoam container, place it in the cardboard box, and weigh it.
9. Fill out the Dry Ice Label (UN 1845 label), using the shipping information as well as the weight of the package converted to kilograms. Place the dry ice label on the side of the box vertically. Do not place the label on the top or the bottom of the box.
10. Create the FedEx shipping label.
11. Send 1) the manifest, 2) the shipment date and 3) tracking# and the expected arrival time to receivers (NEON and ASU Biorepository).
12. Only ship out temperature sensitive packages on Monday to Wednesday using priority overnight mail to make sure that the package will be received on the next business day.
13. If the chain of custody document has not been completed, complete the document. Seal the document in a 13” x 15” plastic bag and place it in the Styrofoam container.

14. Drop the package off at the FedEx location by 2 PM. Keep the receipt of the package.

Reference:

Keirans, J.E. and Litwak, T.R. 1989. Pictorial key to the adults of hard ticks, family Ixodidae (Ixodidae: Ixodoidea) East of the Mississippi River. Journal of Medical Entomology 26:435-448.