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

Version: 2.0

Date Modified: 3/22/2023

Log of SOP Changes:

Version: 2.0; ate Modified: 3/22/2023

Major differences between SOP 1.0 and 2.0: 1) the real-time PCR system was changed from Agilent MX3000P instrument (Agilent no longer provides support) 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: 1.0; Date Modified: 11/20/2020

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

Blood and ear samples will be collected in the field by Battelle from rodents that meet taxonomic, size, condition, and bleed/ear sample history criteria (including Peromyscus spp., other cricetid species, and dipodids). The targeted blood volume of each sample collected will range from 10µL to 50µL. Ear samples are collected using a 2000 Micron (2 mm) tissue biopsy punch. Each blood or ear sample will be collected in an individually labeled cryovial rated for storage in vapor phase liquid nitrogen. Samples will be shipped to UMass on dry ice with accompanying shipping manifest.

Incoming rodent samples:

- Rodent samples 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.
- An internal label (LMZ_R00001) will be assigned to each rodent sample at this point. The data should be input into a database.
- After reconciling with internal label and Battelle manifest, samples are then moved forward for Total Nucleic Acid Extraction.

Total Nucleic Acid Extraction

Things to do before starting:

- This protocol was modified from Epicentre MasterPure Complete DNA and RNA Purification Kit Bulk Reagents.
- 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 waste container on the bench and then emptied into 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 65ºC.
- Turn on and cool centrifuge to 4ºC.

For rodent ear samples, part A:

1. Place the tube of rodent ear samples into a TissueLyser reaction-tube holder (24 samples/holder).
2. Place the tube holder and tubes into a -80ºC 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 ear sample 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 rodent ear samples at 23Hz, 1.5 minutes.

For rodent blood samples, part A:

1. Transfer 50 ul of the rodent blood sample to a 2.0ml safelock tube containing 300 ul Red Cell Lysis Solution using VWR wide bore pipette tips (VWR# 76635-652).
2. Invert tubs several times and flick to suspend any remaining material. Incubate tubes at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortexing.
3. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
4. Remove 300-325 ul of the supernatant, leaving approximately 25 ul of liquid. Vortex to resuspend the pellet.

For rodent blood and tissue samples, part B:

1. 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 samples, 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;
2. Take 301ul master mixture for one sample from part A.
3. Mix thoroughly and incubate at 65ºC for 15 minutes; vortex every 5 minutes.
4. Place the samples on ice for 10 min.
5. Add 150 ul of MPC Protein Precipitation Reagent (MPC Protein Precipitation; MMP03750 Bulk order) to 301 ul of lysed sample and vortex vigorously for 10 seconds.
6. Pellet the debris by centrifugation at 4ºC for 10 minutes at 14,000 RPM in a microcentrifuge.
7. Transfer 350 ul of supernatant to a tube containing 500 ul of ice cold isopropanol. Invert the tube several (30-40) times. Store the protein pellets in the 4ºC fridge until all testing is complete.
8. Pellet the DNA/RNA by centrifugation at 4ºC for 10 minutes at 14,000 RPM in microcentrifuge.
9. Carefully pour off the isopropanol without dislodging the nucleic acid pellet. You can remove all of the residual isopropanol with a pipette.
10. 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.
11. Air-dry the pellets in the 65ºC incubator, using 3D printed comb to separate tubes to prevent cross contamination. Resuspend the DNA/RNA in 70 ul of molecular grade water.
12. Store samples (29 samples and 1 extraction control in general) at -20ºC in the freezer box and proceed to the next section for real-time PCR setup.

Design of Assays and Master Mix

- We use multiplexing Taqman real-time PCR to detect rodent pathogen DNAs.
- The multiplexing master mixes are using Agilent Brilliant III Ultra-Fast QPCR (Agilent Cat# 600880). 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.
- Gene fragments (gBlocks) are ordered from IDTDNA as Taqman PCR positive QC controls. These gene fragments (gBlocks) are double-stranded, sequence-verified genomic blocks.
- Multiplexing master mix are made each in 50 ml conical tube. Six 15ul reaction from the master mix are tested QC at gBlock concentration 5 pg- 0.005pg for positive controls (3 reactions) and water for negative controls (3 reactions).
- If the master mixes pass the QC, they are aliquoted into 1.5 mL tubes and stored at -80C (About 100 reactions per tube, 15 ul per reaction). 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). Exposure to light can also damage the master mix. Each technician should have their own freezer box in the -20◦C freezer and they should remove a few tubes of the master mixes that they will need that week and place them at -20C. When preparing to run a plate, the appropriate mastermix tubes should be placed in a DNA-free tube rack at 4C to thaw.
- Rodent pathogen groups are also listed below:

  Base Pathogens:

    Base Pathogens Group B-IT1:

      Borrelia general species

      Borrelia burgdorferi sensu lato

      Borrelia miyamotoi

      Borrelia mayonii

    Base Pathogens Group B-IT2:

      Babesia microti

      Anaplasma phagocytophilum

      Ehrlichia muris-Like Agent

  Option 1 Pathogens:

Option 1 Pathogens Group B-NIT1:

Ehrlichia chaffeensis

Borrelia lonestari

Ehrlichia ewingii

Option 1 Pathogens Group B-NIT2:

Francisella tularensis

Rickettsia rickettsii

Rickettsia parkeri

Rickettsia philipii

Option 2 Pathogens:

Option 2 Pathogens Group Virus:

Powassan virus

Heartland virus

Bourbon virus

Colorado Tick Fever virus

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).
- Print out the map and use this 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:

Rodent_Blood			Borrelia 18S	BBSL	B.miyamotoi	B. mayonii			Babesia	EMLA	Anaplasma			F.tularensis/R.parkeri	A.phagocytophilium	R.rickettsii/R.philipii
Sample	Well	Mastermix	Quasar705	FAM	HEX	Quasar670	Well	Mastermix	FAM	Quasar670	HEX	Well	Mastermix	FAM	Quasar670	HEX
1	A1	B-IT1					A5	B-IT2				A9	B-NIT2			
2	B1	B-IT1					B5	B-IT2				B9	B-NIT2			
3	C1	B-IT1					C5	B-IT2				C9	B-NIT2			
4	D1	B-IT1					D5	B-IT2				D9	B-NIT2			
5	E1	B-IT1					E5	B-IT2				E9	B-NIT2			
6	F1	B-IT1					F5	B-IT2				F9	B-NIT2			
7	G1	B-IT1					G5	B-IT2				G9	B-NIT2			
8	H1	B-IT1					H5	B-IT2				H9	B-NIT2			
9	A2	B-IT1					A6	B-IT2				A10	B-NIT2			
10	B2	B-IT1					B6	B-IT2				B10	B-NIT2			
11	C2	B-IT1					C6	B-IT2				C10	B-NIT2			
12	D2	B-IT1					D6	B-IT2				D10	B-NIT2			
13	E2	B-IT1					E6	B-IT2				E10	B-NIT2			
14	F2	B-IT1					F6	B-IT2				F10	B-NIT2			
15	G2	B-IT1					G6	B-IT2				G10	B-NIT2			
16	H2	B-IT1					H6	B-IT2				H10	B-NIT2			
17	A3	B-IT1					A7	B-IT2				A11	B-NIT2			
18	B3	B-IT1					B7	B-IT2				B11	B-NIT2			
19	C3	B-IT1					C7	B-IT2				C11	B-NIT2			
20	D3	B-IT1					D7	B-IT2				D11	B-NIT2			
21	E3	B-IT1					E7	B-IT2				E11	B-NIT2			
22	F3	B-IT1					F7	B-IT2				F11	B-NIT2			
23	G3	B-IT1					G7	B-IT2				G11	B-NIT2			
24	H3	B-IT1					H7	B-IT2				H11	B-NIT2			
25	A4	B-IT1					A8	B-IT2				A12	B-NIT2			
26	B4	B-IT1					B8	B-IT2				B12	B-NIT2			
27	C4	B-IT1					C8	B-IT2				C12	B-NIT2			
28	D4	B-IT1					D8	B-IT2				D12	B-NIT2			
29	E4	B-IT1					E8	B-IT2				E12	B-NIT2			
EC	F4	B-IT1					F8	B-IT2				F12	B-NIT2			
SPC	G4	B-IT1					G8	B-IT2				G12	B-NIT2			
NTC	H4	B-IT1					H8	B-IT2				H12	B-NIT2			
Quasar670=Cy5																
Date:		Operator: GX														
QPCR File Name:		Note:														

Real Time PCR Plate Setup

- This protocol was modified from Brilliant III Ultra-Fast QRT-PCR Master Mix.

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 into the appropriate wells based on the sample loading map, then bring the tubes back to their storage box at -20°C.
4. Add 1 ul of DNA/RNA 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 in the hood (don't let the pipette move below the edge of the container as this may cause contamination of the pipette with DNA). 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. 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

Important notes before starting:

- The Taqman detection is set up using Bio-Rad CFX96 Touch Deep Well Real-Time PCR Detection System and CFX maestro software.
- 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:

- 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. Click on an individual well and then click Results.
    a. 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 samples.
    b. A positive sample should have an amplification curve and Ct values for FAM, HEX or Cy5. 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.
    c. 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.
    d. Extraction control: it will result in an amplification curve if contamination occurred during extraction step.
    e. 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.
    f. 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 rodent pathogen database along with manifest 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 # 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 and store them into -80C freezer.
    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 per instructions provided by NEON.**
- **Samples shipped on dry ice should be PRIORITY OVERITY 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.

8. 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.
9. 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.
10. Notify NEON and ASU Biorepository for the coming shipment.
11. When shipping contents that does 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.
12. 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.
13. 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.
14. Add the dry ice to the container. Spread the dry ice around and throughout the cryo-boxes and packing materials in the container.
15. Cover the Styrofoam container, place it in the cardboard box, and weigh it.
16. 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.
17. Create the FedEx shipping label.
18. Send 1) the manifest, 2) the shipment date and 3) tracking# and the expected arrival time to receivers (NEON and ASU Biorepository).
19. 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.
20. 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.

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