Laboratory of Medical Zoology
Mosquito Testing
Standard Operating Protocol

Ver 2.0 Modified Date: 7/12/2018

Receipt and Custody of specimens

Samples sent by courier (UPS or FedEx) may arrive throughout the business day.

Incoming samples:

- Samples submitted to LMZ by contractor agencies 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). Each sample (tube) must have a unique ID number at this point. Notify the Contract Officer immediately of any problems with the shipment within 48 hours of receipt.
- Samples submitted from NEON were placed into Fisher Cat. No. 05-408-145 tubes. One tube contains one mosquito pool. One pool has <=50 mosquitoes. DO NOT open any tubes in this step. Store samples at proper temperature (rm, 4C, -20C, -80C) according to the sample store condition.
- After reconciling with contractor manifest, samples are then moved forward for In-Processing. Samples from contract agencies should be accompanied with Internal Routing Sheet (Appendix A) indicating service required (photos, tests, etc.)

In-Processing of specimens

1. Each pool (tube) should be individually processed.
2. Carefully remove the tube from the bag/container it was sent in and place them in the hood.
3. Carefully check the label on each sample tube. Each tube should have a unique label.
4. Prepare a 2.0 ml safe-lock tubes (Qiagen Cat. No. 990381) containing one 5mm metal bead (VXB, 3/16" inch Diameter Stainless Steel 440C G16

Bearing Balls, Code: Kit12748). (Must use Qiagen or Eppendorf 2.0 ml Safe-Lock Tubes, Cheap tubes will crack after shaking). Place the same ID number that it was assigned on the lid of the sample tube.

Total Nucleic Acid Extraction

Things to do before starting:

- This protocol was modified from Zymo Research Quick-DNA/RNA Pathogen Miniprep kit (See Appendix C).
- 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 centrifuge at room temperature.
- Add 260 µl or 1,040 µl Proteinase K Storage Buffer to reconstitute per 5 mg or 20 mg lyophilized Proteinase K, respectively. Vortex to dissolve and store frozen aliquots (-20C freezer).
- Add beta-mercaptoethanol to 0.5% (v/v) i.e., add 250 µl or 500 µl per 50 ml or 100 ml Pathogen DNA/RNA Buffer,respectively.
- Add 24 ml of 100% ethanol to the 6 ml Pathogen DNA/RNA Wash Buffer concentrate (R1042) or 192 ml of 100% ethanol to the 48 ml Pathogen DNA/RNA Wash Buffer concentrate (R1043) before use.

1. Transfer all mosquitoes (maximum 50 mosquitoes) from the original tube into a 2.0 ml safe-lock tubes containing a stainless steel bead. This must be done in the hood.
2. Add 800 ul DNA/RNA Shield to the above sample in the tube. This must be done in the hood.
3. Place the safe-lock tube into a TissueLyser reaction-tube holder (24 samples/holder).
4. Assemble the TissueLyser Adapter Set 2 x 24 using a bottom plate, the reaction- tube holder (with 2.0 ml safe-lock tubes containing samples, one 5mm bead and TNA mastermix), and a top plate with sealing strips.
5. Ensure that the top and bottom plate fit securely, and make sure that the reaction tubes are sealed securely.
6. 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.

7. 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.
8. Homogenize the samples at 30Hz, 5 minutes. A larger sample may need a second homogenization.
9. Centrifuge the tube for 20 min at maximum speed and transfer 200 ul of supernatant to a new tube.
10. Add 2 μl Proteinase K, 400 μl Pathogen DNA/RNA Buffer with beta-mercaptoethanol to each 200 μl sample mix well and incubate at room temperature for 10 minutes.
11. Transfer the mixture into a Zymo-Spin IIC Column in a Collection Tube and centrifuge (10000rpm). Discard the flow-through.
12. Add 500 μl Pathogen DNA/RNA Wash Buffer (make sure ethanol was added) to the column and centrifuge. Discard the flow-through and repeat this step.
13. Add 500 μl ethanol (95-100%) to the column and centrifuge for 1 minute to ensure removal of any residual ethanol. Discard the collection tube and carefully transfer the column into a new nuclease-free tube.
14. Add 100 μl DNase/RNase-Free Water directly to the matrix of the column and centrifuge to elute the DNA/RNA.
15. Store samples (29 samples and 1 extraction control in general) at -20C in the freezer box and proceed to the next section for real-time PCR setup.

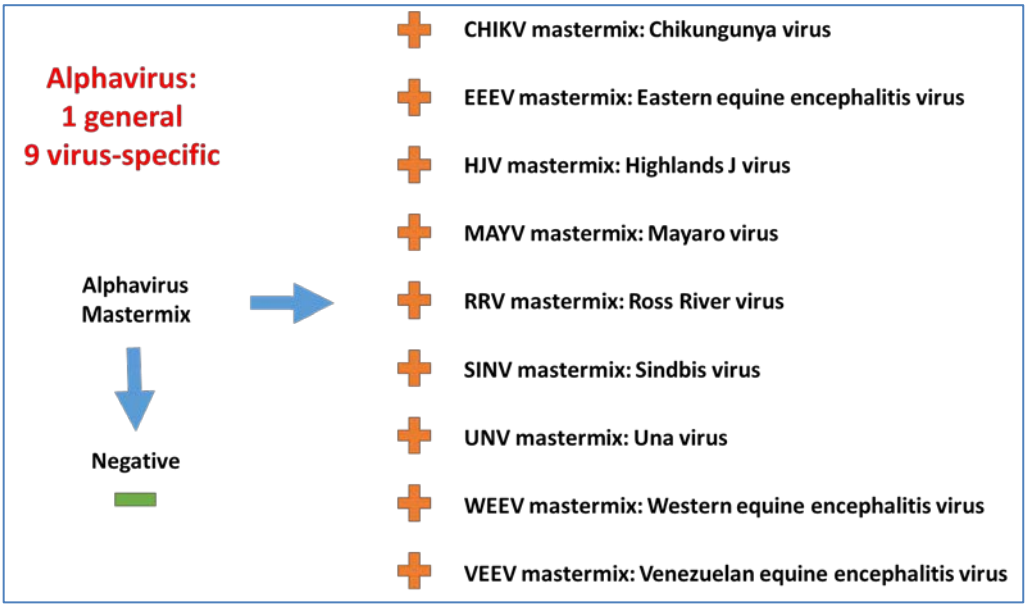
Design of Assays and Master Mix

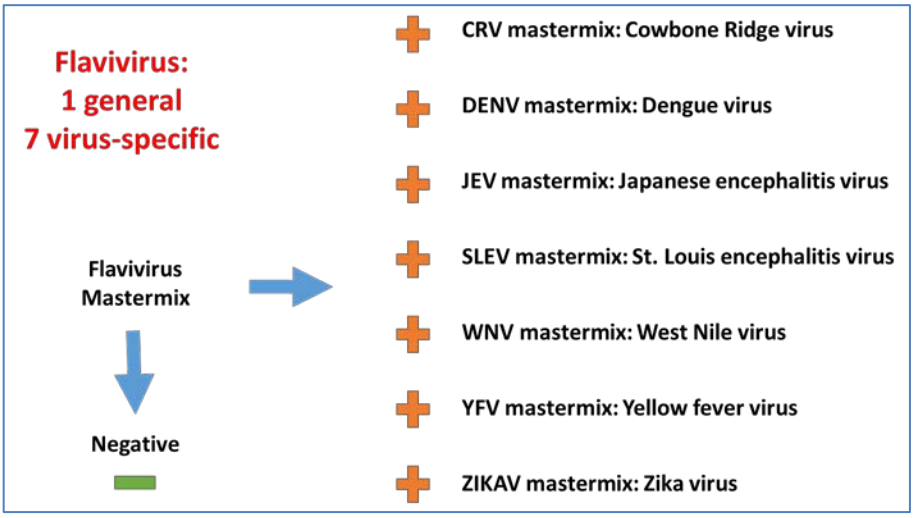
- We use Taqman real-time PCR to detect mosquito Alphavirus, Flavivirus and Orthobunyavirus.
- Alphavirus: 1 general detection and 9 virus-specific detections (CHIKV mastermix: Chikungunya virus; EEEV mastermix: Eastern equine encephalitis virus; HJV mastermix: Highlands J virus; MAYV mastermix: Mayaro virus; RRV mastermix: Ross River virus; SINV mastermix: Sindbis virus; UNV mastermix: Una virus; WEEV mastermix: Western equine encephalitis virus; VEEV mastermix: Venezuelan equine encephalitis virus).
- Flavivirus: 1 general detection and 7 virus-specific detections (CRV mastermix: Cowbone Ridge virus; DENV mastermix: Dengue virus; JEV mastermix: Japanese encephalitis virus; SLEV mastermix: St. Louis encephalitis virus; WNV mastermix: West Nile virus; YFV mastermix: Yellow fever virus; ZIKAV mastermix: Zika virus).
- Orthobunyavirus: 1 general detection and 4 virus-specific detections (CEV

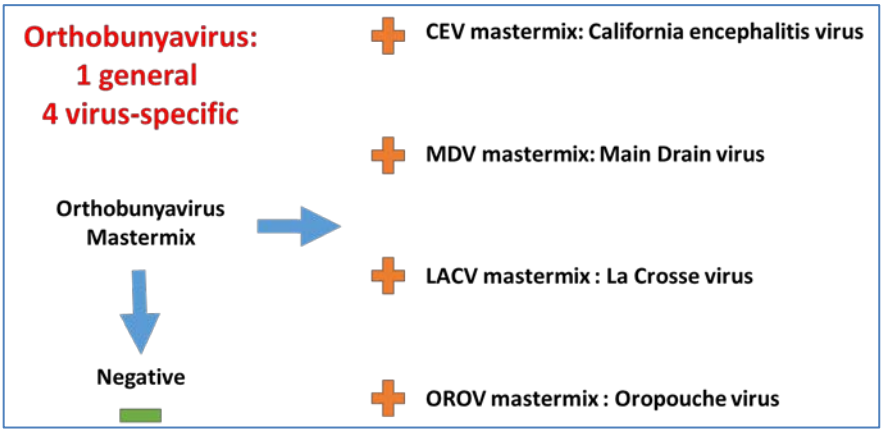
mastermix: California encephalitis virus; MDV mastermix: Main Drain virus; LACV mastermix: La Crosse virus; OROV mastermix: Oropouche virus).

- The 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.
- The master mix is 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 -20C 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.

- The detection flow are listed below:







Setup a Sample Loading Map

- All sample are run as a 96-well plate format.
- Use a below sample as a template map. Each plate has needed master mixes and 3 sets of 29 samples (total 87 samples, make sure the sample ID# matches the sample ID# on the TNA tube); 1 extraction control (EC), 1 super positive control (SPC) and 1 no template control (NTC) for each set of 29 samples.
- 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:

Sample ID	Well	Mastermix	FAM	Sample ID	Well	Mastermix	FAM	Sample ID	Well	Mastermix	FAM
1	A1	Alphavirus		30	A5	Alphavirus		59	A9	Alphavirus	
2	B1	Alphavirus		31	B5	Alphavirus		60	B9	Alphavirus	
3	C1	Alphavirus		32	C5	Alphavirus		61	C9	Alphavirus	
4	D1	Alphavirus		33	D5	Alphavirus		62	D9	Alphavirus	
5	E1	Alphavirus		34	E5	Alphavirus		63	E9	Alphavirus	
6	F1	Alphavirus		35	F5	Alphavirus		64	F9	Alphavirus	
7	G1	Alphavirus		36	G5	Alphavirus		65	G9	Alphavirus	
8	H1	Alphavirus		37	H5	Alphavirus		66	H9	Alphavirus	
9	A2	Alphavirus		38	A6	Alphavirus		67	A10	Alphavirus	
10	B2	Alphavirus		39	B6	Alphavirus		68	B10	Alphavirus	
11	C2	Alphavirus		40	C6	Alphavirus		69	C10	Alphavirus	
12	D2	Alphavirus		41	D6	Alphavirus		70	D10	Alphavirus	
13	E2	Alphavirus		42	E6	Alphavirus		71	E10	Alphavirus	
14	F2	Alphavirus		43	F6	Alphavirus		72	F10	Alphavirus	
15	G2	Alphavirus		44	G6	Alphavirus		73	G10	Alphavirus	
16	H2	Alphavirus		45	H6	Alphavirus		74	H10	Alphavirus	
17	A3	Alphavirus		46	A7	Alphavirus		75	A11	Alphavirus	
18	B3	Alphavirus		47	B7	Alphavirus		76	B11	Alphavirus	
19	C3	Alphavirus		48	C7	Alphavirus		77	C11	Alphavirus	
20	D3	Alphavirus		49	D7	Alphavirus		78	D11	Alphavirus	
21	E3	Alphavirus		50	E7	Alphavirus		79	E11	Alphavirus	
22	F3	Alphavirus		51	F7	Alphavirus		80	F11	Alphavirus	
23	G3	Alphavirus		52	G7	Alphavirus		81	G11	Alphavirus	
24	H3	Alphavirus		53	H7	Alphavirus		82	H11	Alphavirus	
25	A4	Alphavirus		54	A8	Alphavirus		83	A12	Alphavirus	
26	B4	Alphavirus		55	B8	Alphavirus		84	B12	Alphavirus	
27	C4	Alphavirus		56	C8	Alphavirus		85	C12	Alphavirus	
28	D4	Alphavirus		57	D8	Alphavirus		86	D12	Alphavirus	
29	E4	Alphavirus		58	E8	Alphavirus		87	E12	Alphavirus	
EC	F4	Alphavirus		EC	F8	Alphavirus		EC	F12	Alphavirus	
PC	G4	Alphavirus		PC	G8	Alphavirus		PC	G12	Alphavirus	
NTC	H4	Alphavirus		NTC	H8	Alphavirus		NTC	H12	Alphavirus	

Date:		Operator:	
QPCR File Name:		Note:	

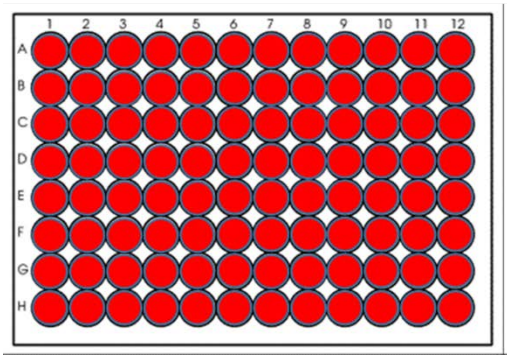
Real Time PCR Plate Setup

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

1. Thaw the master mix.
2. Vortex briefly and spin down master mix. Put the master mix on ice.
3. Prepare a 96 well non-skirt PCR plate.
4. Add 15 µl of the master mix solutions, for example: add 15 ul Alphavirus mastermix into the appropriate wells based on the sample loading map, then bring the tubes back to their storage box at -20°C.
5. 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 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.
6. Add 1ul extraction control (EC), 1 ul positive control (PC) and 1 ul water as NTC (No Template Control).
7. 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.
8. Centrifuge the plate using a plate centrifuge, 800 g, 3 min. Proceed to MX3000P instrument instructions.

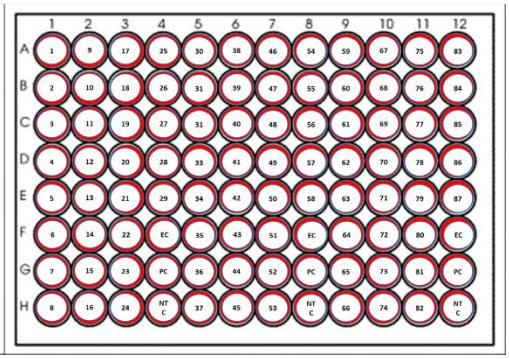
Alphavirus
Mastermix Loading Map

Alphavirus mastermix



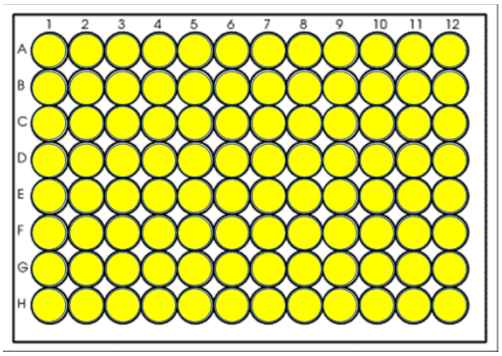
Alphavirus
Sample Loading Map

TNA sample #



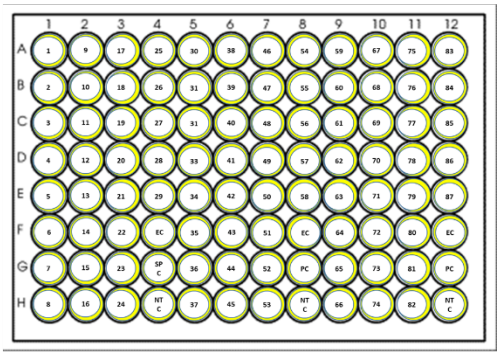
Flavivirus
Mastermix Loading Map

Flavivirus mastermix



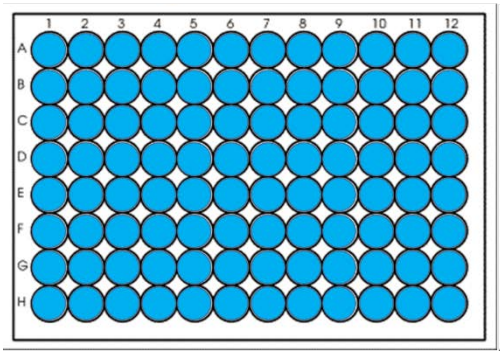
Flavivirus
Sample Loading Map

TNA sample #



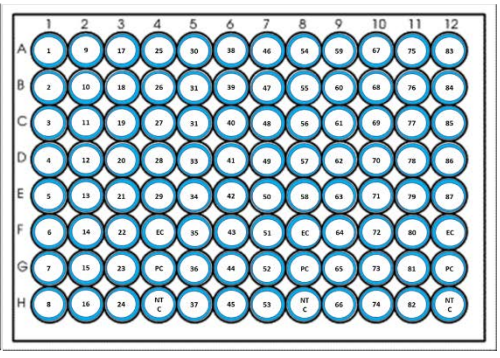
Orthobunyavirus
Mastermix Loading Map

Orthobunyavirus mastermix



Orthobunyavirus
Sample Loading Map

TNA sample #



Instrument Settings and PCR run

Important notes before starting:

- The taqman detection is set up using the Agilent Mx3000P QPCR System. Standard filter sets include FAM/SYBR® Green I (492nm-516nm), HEX/JOE/VIC (535nm-555nm), ROX/Texas Red (585nm-610nm), Cy5 (635nm-665nm).
- ROX is used as a reference dye in all tests.

1. Open the “MxPro” program.
2. Select “Quantitative PCR (Multiple Standards)” as experiment/project type and select “Turn lamp on for warm up?” (warm-up takes about 20 minutes, so do this just before setting up your plate)
3. SETUP plate and thermal profile **(Attention: RNA Thermal profile is not for the DNA testing)**::
    a. Hit “All” to select all wells
    b. Well type- Unknown
    c. Select HEX, FAM, Cy5 and ROX under “Collect fluorescence data”
    d. Reference Dye: ROX
    e. Thermal profile setup tab: Import from the file of the last plate run
    f. RNA test: 50 ◦C for 10 min (1 cycle); 95 ◦C for 3 min (1 cycle); 95 ◦C for 15 seconds, 60 ◦C for 60 seconds, END* (40 cycles).
    g. DNA test: 95 ◦C for 10 min (1 cycle); 95 ◦C for 15 seconds, 60 ◦C for 60 seconds, END* (40 cycles). *Need endpoint to tell computer to read dye
4. File → Save As → C → Laboratory of Medical Zoology →
5. Name as: Date_Assay_Plate_# for day (20180231_Alph_Plate1)
6. You MUST write this plate name on the qPCR map.
7. Carefully place the plate in the Agilent Mx3000P machine qPCR machine with A1 in the top left-hand corner.
8. Be sure to lock the metal cover down on top of the plate.
9. Verify correct settings in the template file.
10. Select RUN tab, be sure to specify if the light should turn off after the run. Select START. If the lamp is not warmed up yet, click warm-up then run.

Interpretation of results

Checklist for inspecting results:

- Extraction control.
- Negative control.
- Positive control.
- Amplification curve of each sample.

1. Once the program has finished, save the file and open to the Analysis tab.
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 mosquitos.
    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 using 3 replicates.
    d. Extraction control: it will result in an amplification curve if mosquito pathogen 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. Positive control: it consists of a control DNA/RNA 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 database along with other information.

Statement of Work
qPCR Testing for v*iral pathogens in mosquito pools*

University of Massachusetts – Amherst and National Ecological Observatory Network

The National Ecological Observatory Network (NEON) and Laboratory of Medical Zoology, University of Massachusetts-Amherst (UMass), shall furnish the necessary personnel and otherwise do all things for or incidental to the performance of the work set forth in sections (a) through (c).

Tasks

(a) NEON shall coordinate the collection and submission of mosquito pools from environmental surveillance to UMass. NEON shall supply all necessary collection and shipping materials as well as covering the cost of sample shipment to UMass.

(b) NEON shall provide instruction on proper collection, packaging and shipping protocols to staff who conduct surveillance for mosquito pools for testing at UMass. UMass shall notify NEON of discrepancies noted in packaging or shipping that impact the suitability of mosquito pools for testing.

(c) UMass shall analyze all mosquito pools. Testing is performed in two stages. The first stage is for generic testing of alphaviruses, flavivirues, and orthobunyaviruses. Pools positive in first stage are tested secondarily for specific viruses (listed in SOP). UMass will send test results of up to 3,000 mosquito pools to NEON within 35 business days of receiving them.

Deliverable

UMass will report all findings to NEON within 35 business days of receiving pools. Positive findings shall be reported to one of the following people, listed preferentially:

TBA

Period of Performance

The period of performance under this statement of work shall be from April 1, 2018 through June 30, 2018 unless sooner terminated by either party upon 30 days written notice.

Payment

NEON will pay UMass the following rates per mosquito sample:

Mosquito pool	**Price per Sample**
Stage 1 (genus-level) viral testing	$34.00
Stage 2 (specific) viral testing	$14.00

NEON will pay UMass within 30 days after receipt of an invoice voucher and verification of the number of pools tested from review of testing reports.

Dr. Stephen M. Rich
Director
Laboratory of Medical Zoology

Contract Administrator
National Ecological Observatory Network

Work Order Checklist

Sample arriving day/time:

Sample received by:

Sample condition when arriving at LMZ

☐ **Live** ☐ **Dead**

Additional solvents/liquids (water. EtOH)? _____

Identified by species? ☐ **Yes** ☐ **No**

Electronic data sheet from contractor? ☐ **Yes** ☐ **No**

Photomicrograph documentation of sample requested?

☐ **No** ☐ **Yes(** ☐ **Dorsal/** ☐ **Ventral)**

Tests to be applied:

☐ **Stage 1 (genus-level) viral testing**

☐ **Stage 2 (specific) viral testing**

☐

☐

☐

☐

☐

☐

☐

☐

☐ **Other _____**

Results on Contractor Form? ☐ **Yes** ☐ **No**

Report Due Date _____