Appendix B. Real Time Reverse Transcriptase-Polymerase Chain Reaction Protocol

Real time RT-PCR can be performed using a number of commercially available kits. We currently use either the BioRad iScript 1 Step RT-qPCR (#170-8895) or the QIAGEN QuantiTect Probe RT-PCR kit (#204443). The two kits are nearly identical in the reaction setup with the one exception; the volume of enzyme used in the QIAGEN kit is 0.5ul per reaction instead of 1.0ul-the volume of water in the master mix is adjusted by 0.5ul to account for this. The setup shown below is for the QIAGEN kit. Note also that the volume of RNA added per reaction below is 10 ul but can be increased or decreased with the appropriate adjustment of total volume with water.

COMPONENT	VOL PER REACTION	10 REACTIONS
RNase free water	13.2 ul	132 ul
2X Ready mix	25 ul	250 ul
primer 1 (100 uM stock)	0.5 ul	5 ul
primer 2 (100 uM stock)	0.5 ul	5 ul
FAM/ probe (25 uM stock)	0.30 ul	3.0 ul
enzyme	0.5 ul	5 ul

Prepare a reagent "master mix" according to the number of reactions desired. The master mix should be prepared in a “clean room” physically separated from all other laboratory activities with dedicated reagents and equipment (i.e., pipette’s). For 10 samples make a 10X master mix (see above) by multiplying the volumes of all individual reagents by 10. Combine the reagents in the above order in an RNase free centrifuge tube **on ice**. Divide the master mix into 10 portions of 40ul each into either 0.2 ml optical (specifically for TaqMan assays; emission fluorescence is read through the cap) PCR tubes or a 96 well optical PCR plate. Finally add 10 ul of the individual RNA sample to each tube/well. All samples are tested in duplicate wells. **Include several “NO RNA”** negative controls (NTC) by adding water instead of any RNA. Include a positive control or a dilution series of known quantities of positive control RNA if setting up a quantitative assay.

Cycling conditions (QIAGEN conditions for Real Time RT-PCR):

1 cycle :		**45 cycles**:
50° for 30 min	(RT reaction)	95° 15 sec
95° for 15 min	(enzyme activation)	60° 1 min

Interpretation

We use the following algorithm to evaluate the TaqMan results.

Positive:	Ct value 38 in duplicate wells.
Equivocal:	Ct value 38 in one of two wells.
Negative:	Ct values >38 in duplicate wells.

All positive and equivocal samples are repeated with a second set of primer/probes for confirmation. A positive result in any of the negative controls invalidates the entire run. Failure of the positive control to generate a positive result also invalidates the entire run.

I. RNA EXTRACTION

NOTES: Avoiding Contamination & Working with RNA

- Maintain physically separated work areas; one dedicated to **pre-amplification RNA work** (RNA extraction & RNA addition) and the other for **Master mix** production.
- Utilize dedicated/separate equipment within pre and post amplification areas; especially pipets and centrifuges.
- Always wear gloves; even when handling unopened tubes.
- Open & close tubes quickly and avoid touching any inside portion.
- Use RNase free plastic disposable tubes and pipet tips.
- Use aerosol block pipet tips.
- Use RNase free water.
- Prepare all reagents on ice.

1. Solid phase samples (mosquitos or tissues) are first homogenized in an isotonic buffer to produce a liquid homogenate. RNA is extracted from liquid specimens (CSF or serum) without any pre-treatment as described below. Tissue specimens ~(10mm^3) are homogenized in 1 ml of BA-1 diluent using Ten Broeck tissue grinders. Mosquito specimens are homogenized in Ten Broeck tissue grinders or by using the copper clad steel bead (BB) grinding technique. With both techniques the homogenates are clarified by centrifugation in a microcentrifuge (i.e. Eppendorf) at maximum speed for 5 minutes to pellet any particulate material.

2. Extract RNA from 140 ul of the liquid specimen (CSF, serum, or clarified homogenate) using the QiAmp viral RNA kit (QIAGEN part # 52904). Follow the manufacturer's protocol exactly. **NOTE**: For mosquito specimens add 1 additional wash with AW1. Extract at least two negative controls and two positive controls along with the test specimens. The positive controls should differ in the amount of target RNA present (i.e. a pre-determined high positive and a low positive). The volume of sample extracted can be greater or less than the standard volume stated in the QIAGEN protocol (140 ul) with the appropriate adjustments to all other volumes in the protocol.