**Quantification of WNV by Real Time (probe-based)**

1. This assay is based off of the primer/ probe set reported by Lanciotti et al.

Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, et al. (2000) Rapid

detection of West Nile virus from human clinical specimens, field-collected

mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay.

J Clin Microbiol 38: 4066–4071.

2. A 2 kb fragment from the WNV E gene was previously amplified using the WNV 1031 F and WNV3430 R primers. The resultant amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen) downstream of the T7 promoter. The recombinant vector was linearized with KpnI, purified and used as template for in vitro transcription using the T7 Megascript kit according the manufacturer’s instructions (Ambion, Austin, TX). The resultant RNA was quantified and aliquoted in serial ten-fold dilutions.

3. Using a probe specific for the E gene, the WNV 1160 F and WNV 1229 R primers, and the viral RNA copy numbers can be determined.

4. We previously used the TaqMan H One-Step RT-PCR Master Mix Reagent (Applied Biosystems, Foster City, CA) for this assay. However, we have found that ABI products do not work on the Bio-Rad machines. Therefore, we have purchased the Probe-based assay kit for Bio-Rad.

5. Get an aliquot of the ~1011 genome equivalent/ ml stock from Doug and dilute so that your final concentrations will be between 107-101/ 5ul. For example, if you want a final concentration of 1x107 per reaction you should make your concentration 2x106/ ul because you will be adding 5 ul to the reaction.

6. Make master mix for enough reactions to do all experimental samples in duplicate as well as your standards in duplicate and then add 10% more of each reagent so that you have a little extra.

Recipe

12.5 ul 2x RT-PCR reaction mix for probes

0.5 ul 1160 Fwd primer

0.5 ul 1229c Rev primer

0.5 ul 1160 TAM-FAMRA probe

0.5 ul iScript RT for one-step RT-PCR

5.5 ul ddH2O

5 ul of RNA template

25 ul total volume per reaction

Program

1 20 m at 50˚

2 5 m at 95˚

3 10 s at 95˚

4 1 m at 60˚

5 repeat cycles 3-4 39X