**WNV Surveillance RNA Extraction and qRT-PCR Protocol**

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Verify pools and data correlate ( “Samples provided” ) “# of samples” total

**Mosquito Homogenizing:**

1. Prepare 48x samples at a time.
   1. Add 1x steel BB to each mosquito pool in 2ml tube.
   2. Add 1.0ml of mosquito diluent to each tube.
   3. Place in Tissue homogenizer @ 24 Hz for 60 sec.
   4. Centrifuge on desktop centrifuge at max speed for 5 minutes.
   5. Refrigerate at 4 C until ready to extract.

**King Fisher RNA Extraction: (all volumes are per well)**

**Using Omega Mag-Bind Viral DNA/RNA extraction Kit**

1. Sample Plate Master mix : 190ul (191ul if LPA used) total volume. In 2.0ml deep 96 well plates.
   1. Proteinase K…………………………5ul
   2. Mag-Bind Particle beads………5ul
   3. TNA Lysis buffer…………………..60ul
   4. Isopropanol (2-Propanol)…… 70ul
   5. LPA(If needed)…………………….1ul
   6. Sample Homogenate……………50ul
2. VBH Plate Preparation: In 2.0ml deep 96 well plates.
   1. VBH Buffer…………………………..200ul
3. Spr-1 & SPR-2 Plate Preparation: In 2.0ml deep 96 well plates.
   1. SPR Buffer……………………………200ul
4. Elution Plate Preparation: In short 96 well elution plates
   1. H2O……………………………………...50ul
   2. No LPA needed due to high concentration of RNA, If need as 1ul to 49ul H2O.
5. Tip Plate Preparation: In 2.0ml deep 96 well plates.
   1. Add tip comb to tip plate.

Start Fisher Protocol on Ebel Lab PC (protocol omega 50ul adjusted)

**WNV qRT-PCR probe based assay**

**Using BioRad iTaq Universal Probes One Step Kit**

1. Make WNV qRT-PCR Master Mix
   1. 2x iTaq Buffer…………………………………….10ul
   2. WNENV-Forward Primer(10uM)………..1ul
   3. WNENV-Reverse Primer (10uM)………..1ul
   4. WNENV-Probe(10mM)……………………….0.8ul
   5. H2O……………………………………………………1.7
   6. Reverse Transcriptase….……………....…..0.5ul
2. Add 15ul of WNV qRT-PCR Master mix per well
3. Add 5ul of extracted RNA per well
4. On Bio-Rad CFX96, Run WNV Surveillance probe q-RT PCR program:
   1. 50°C for 20 minutes
   2. 95°C for 5 minutes
   3. 95°C for 10 seconds
   4. 60°C for 1 minute
   5. Capture using FAM fluorophore
   6. Repeat steps c-e 35-40 times, depending on amount of WNV

**Materials:**

1. Mosquito Diluent (1L)
   1. PBS……………………….….785ml
   2. FBS……………………….….200ml
   3. Pen/Strep (100x)….….10ml
   4. Fungizome………………..2ml
   5. Gentamycin.……………..2ml
2. WNV Probe qRT-PCR Primers (<http://jcm.asm.org/content/38/11/4066/T1.expansion.html>)
   1. WNENV-forward 1160–1180 TCAGCGATCTCTCCACCAAAG
   2. WNENV-reverse 1209–1229 GGGTCAGCACGTTTGTCATTG
   3. WNENV-probe 1186–1207 TGCCCGACCATGGGAGAAGCTC
      1. Probe included 5’ Fam and 3’ Zen Iowa Black quencher