**WNV Surveillance Extraction Protocol: Week XX – date xx.xx.xx**

***Sample Coordination***

1. When samples arrive, chill in –20C freezer for about 15 min or more to knock down mosquitoes.
2. Verify pools and data correlate.
   1. Check that the RNA plates you created from the emails correspond to the samples delivered
      1. Check for any missing samples
      2. Check that the first sample given matches the first sample on the first plate
      3. Check that the RNA plate on paper matches the samples set up on the bench for all plates
3. Rack up and color coordinate plates in large racks, 2 racks for ea 85 wells.
4. Get out real pos and real neg samples from the box of pooled controls in –80 freezer.
   1. Thaw and keep on ice
   2. Use ones without a mark on top, the mark indicates it has been thawed once already.
5. Plate layout will look like this, with 85 samples, 3 controls and 6 standards.
   1. You will process 85 samples and use thawed controls and water in wells 86-88
   2. Column 12 is empty for extraction, used only for PCR

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| **RNA Extracts: WNV Surveillance 2023 Week # plate #** | | | | | | |  | **Date: 00.00.00** | |  |  |  |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **A** | 1 | 9 | 17 | 25 | 33 | 41 | 49 | 57 | 65 | 73 | 81 | Std |
| **B** | 2 | 10 | 18 | 26 | 34 | 42 | 50 | 58 | 66 | 74 | 82 | Std |
| **C** | 3 | 11 | 19 | 27 | 35 | 43 | 51 | 59 | 67 | 75 | 83 | Std |
| **D** | 4 | 12 | 20 | 28 | 36 | 44 | 52 | 60 | 68 | 76 | 84 | Std |
| **E** | 5 | 13 | 21 | 29 | 37 | 45 | 53 | 61 | 69 | 77 | 85 | Std |
| **F** | 6 | 14 | 22 | 30 | 38 | 46 | 54 | 62 | 70 | 78 | Real Pos | Std |
| **G** | 7 | 15 | 23 | 31 | 39 | 47 | 55 | 63 | 71 | 79 | Real Neg | Std |
| **H** | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | Extract Neg | empty |

***Mosquito Homogenizing***

1. Process 48x samples at a time.
2. Add 1x steel BB to each tube, clean forceps with 70% EtOH first.
3. Add 1.0ml of mosquito diluent with the repeat pipettor to each tube.

(Make sure to aliquot mosquito diluent into 50 mL conical(s) in BSC).

1. Place in Tissue homogenizer @ 24 Hz for 60 sec.
2. Centrifuge on desktop centrifuge at max speed (13-15K RPM) for 5 minutes at RT or cooler.
3. Refrigerate at 4 C until ready to extract.
   1. This can be a good place to stop and store samples at -80

***King Fisher RNA Extraction***

**King Fisher Start Up:**

1. Turn on machine and let self-test.
2. On the keypad, use right arrow 2X to go to the wrench (service)
3. Use the down arrow 4X to get to Maintenance Protocol
4. Push OK to select maintenance.
5. Use down arrow 3X to get to “Check\_96DW\_tip”
6. Push start button
7. Load the 2.0 ml deep well block with tips in it (block is labeled Maintenance)
8. Push start and let run, push start to finish.

**Reagent Preparation:**

Prior to filling the extraction plate, prepare the following for each extraction.

1. VBH Plate Preparation: In 2.0ml deep 96 well plates.
   1. Add 200ul of VBH Buffer to each well.
2. Spr-1 & SPR-2 Plate Preparation: In 2.0ml deep 96 well plates.
   1. Add 200ul SPR Buffer to each well.
3. Elution Plate Preparation: In short 96 well elution plates

**(Note: remove PCR-standard and master-mix from cold storage to thaw before this step)**

1. Add 50ul nfH2O to each well.
2. No LPA needed due to high concentration of RNA.
3. Tip Plate Preparation: In 2.0ml deep 96 well plates.
   1. Add tip comb to tip plate.

**Start King Fisher Protocol from Lab PC**

1. The file is called “ExportedProtocol071720 50ul adjusted.kfrun”.
2. Start this when you have prepared the sample extraction plate.
3. Load plates as indicated by the machine and be sure to close the door.

**Sample Plate Preparation:**

1. Lysis Master Mix is prepared and aliquoted to plate and samples are added last.
2. 190ul total volume = 140ul Master Mix + 50ul homogenized sample in 2.0ml deep 96 well plates.
3. Make (**X+6)\*1.1** master mix for X samples (X samples + 6/6 controls +10% )
4. Use the table below for automatic calculations.
   1. Add 5ul Proteinase K to each well.
   2. Add 5ul Mag-Bind particle beads to each well. Add these last, vortex very well.
   3. Add 60ul TNA Lysis Buffer to each well.
   4. Add 70ul Isopropanol to each well.
   5. No LPA needed.
   6. **Vortex master mix thoroughly!**
5. Put Master Mix in a reagent boat and use multichannel pipettor to add 140uL of master mix to each well of RNA extraction plate.
6. Add 50ul of the sample supernatant to each well of RNA extraction plate.
   1. You will load 85 samples then 50ul of thawed positive and negative and water in the extraction negative well (see plate layout)
7. Start Kingfisher protocol and load plates as indicated.

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| # Samples |  |  |  |  |  |  |
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**Sample Storage**

1. Once the Kingfisher has finished, proceed directly to q-RTPCR.
2. Place the elution plate on the magnetic plate to pull down residual beads.
3. Using a multichannel pipettor, move the ~50ul of eluent to a 96 well plate
   1. Keep samples in same order as the plate.
   2. You can use 8-well strips for partial plates
   3. Try to avoid picking up the leftover magnetic beads.
4. Seal the plate with optical tape if moving to q-RTPCR and keep on ice.
   1. This is another potential stopping place. You can seal the RNA plate and store at -80
5. Samples need to be stored at –80C after q-RTPCR
   1. For long term storage you can use aluminum plate sealers