**Plaque assay protocol**

Seed 6-well plates with Vero WHO cells the day before at 1.85 x 105 cells/well. Seed plates with cDMEM with 10% HI-FBS, 2mL/well. *\*I usually seed cells on Thursdays and infect on Fridays so that the first 2dpi are over the weekend when you won’t likely see plaques\**

Make 2X DMEM (1L)

2 packets of powdered DMEM (Gibco 12100-046)
 (or measure out the appropriate amount of powder from a larger bottle)
900mL ddH2O
*mix
pH to 7.4 (use 5M NaOH)*
*bring volume up to ~920mL
bring into TC*

*add:*
20mL antibiotic/antimicotic
20mL L-glutamine
20mL Sodium pyruvate
20mL NEAA (Non-essential amino acids)
*Filter, store at 4˚*

Make 20X NaHCO3
 37g powder
 500mL H2O
 *\*may have to heat while stirring*

Diluting samples (in 48-well plate)
 -1 = 450uL D-MEM w/1%HI FBS + 50uL Sample
 -2 = 450uL D-MEM w/1%HI FBS + 50uL of -1 Sample
 -3 = 450uL D-MEM w/1%HI FBS + 50uL of -2 Sample
 *Continue to appropriate dilutions for type of sample (typically three dilutions within -1 to -8)
 (Multichannel Pipet can be used for aliquoting media and serial dilutions)*

*Before infecting, remove media from wells.*

(Cells should not be left dry for very long so choose a small number of plates to work with at a time, maybe 4-10 plates)

*Infect with 300uL of diluted sample per well, in duplicate, for one hour.*

*~~Rock plates every 15 minutes during infection~~. \*not necessary if you use at least 250ul to 300ul of diluted sample per well\**

Initial media/agarose overlay

*Add initial overlay one hour after infection*

*2mL of mixture needed per well*

 ½ + 10% final volume 1% Low Melt Point agarose in dH2O

 5% FBS
 20X NaHCO3 (5% of final volume)
 fill up to final volume with 2X DMEM\*

\**warm everything to 37oC prior to mixing. I usually make up agarose overlay before I begin my dilutions so that the agarose overlay can warm up at 37C for at least an hour*

*Overlay must be set at 4oC for at least 20 min prior to incubation of plates at 37oC*

After an appropriate length of time (see below), make Neutral Red overlay (these are approximate)

* WNV-TX = 3 dpi
* WNV-MAD = 5-6 dpi
* WNV-KUN & JEV = 4-5 dpi
* DENV = 7 dpi
* ZIKV = 4-5 dpi

Neutral Red/agarose overlay

*2mL of mixture needed per well*

 ½ + 10% final volume 1% LMP agarose
 10X PBS (10% of final volume)
 50X Neutral Red (3% of final volume)
 up to final volume with dH2O
 *Warm everything to 37oC prior to mixing. I usually make up agarose overlay before I begin my dilutions so that the agarose overlay can warm up at 37C for at least an hour*

*Overlay must be set at 4oC for at least 20 min prior to incubation of plates at 37oC*

Plaques are visible 3-5 hours later (varies depending on the virus and strain). Plates can also be read the next day depending on when the Neutral Red overlay is added.