**Evaluating ZIKV binding and entry**

Day 1 – seeding cells:

1. Seed cells in 12-well plates at density of 3x105 cells/well (1ml/well)
   1. Have samples seeded in duplicate

Day 2 – infecting and harvesting cells:

1. Pre-cool cells at 4C
2. Prepare virus inoculum in DMEM as shown in calculations
3. Aspirate media from wells and wash cells once in **ice-cold** 1xPBS and remove PBS wash (this is to remove serum from cells)
4. Add 200ul DMEM or virus inoculum to appropriate wells
5. Incubate plates in 4C fridge in TC for 1 hour to allow the virus to bind to cells
   1. During incubation, serially dilute 500ul of remaining virus stocks down to 10^0 and store at -80C (for extracellular vRNA extraction to generate standard curve for qPCR)
6. Remove inoculum and wash **three times** with **ice-cold** 1X PBS to remove unbound virus and minimize viral entry
7. Add 1ml fresh **pre-warmed** cDMEM to wells and return plates to incubator at 37C and allow infection to go on until appropriate time points (0h, 1h, 2h, 4h, 6h, 8h, 10h, and 24h)
   1. Have 1 plate for RNA lysates after adsorption to assess differences in viral binding
8. For duplicate 1 of 2:
   1. Collect supernatant from all samples (to assess differences in **extracellular** vRNA levels and infectious particles)
      1. Supernatants were centrifuged at 2000rpm for 10min. at 4C
      2. Aliquot 2x 450ul of cleared supernatant into clean Eppendorf tubes and store at -80C
   2. Wash cells with **ice-cold** stringent wash buffer to remove bound/extracellular virus for 3min.
   3. Wash cells two times with 1X PBS to wash off stringent wash buffer
   4. Harvest RNA lysates from cells (to assess differences in **intracellular** vRNA levels)
      1. See detailed protocol on p.14 of Notebook #1 for isolating RNA
9. For duplicate 2 of 2:
   1. Collect cell pellets from all samples (to assess differences in **intracellular** infectious particles)
      1. Wash cells with **ice-cold** stringent wash buffer to remove bound/extracellular virus for 3min.
      2. Wash cells two times with 1X PBS to wash off stringent wash buffer
      3. Trypsinize cells and resuspend in 1ml cDMEM
      4. Centrifuge cells at 300xg for 5 min. and resuspend in 500ul cDMEM
      5. Store resuspended cells at -80C
      6. Prior to plaque assays, thaw resuspended cells at 37C to lyse cells
      7. Remove cellular debris by centrifuging at 3200xg for 5 min. at 4C
      8. Use cleared supernatant for plaque assays