**AD-6 peptide neutralisation assay**

**Reagents:**

* AD-6 peptide 5mg/mL stock
* Cells: HFF (in DMEM 1X, 10%FBS, 5mL P/S)
* 2 96w standard plates (one for dilution, one for cells)
* HSV-1 VP-26 GFP virus of known titre. We have 1:100 stock (plenty) and 1:1000 stock (limited).

**Method:**

Day before:

* Design your 96w plate layout. Include several virus-only and media-only wells for normalisation. Include a positive control plate for CMV.
* Calculate the needed molarity for your peptide first. Molar weight of ad-6 is around 6kDa. In order to achieve 50uM molarity, you will need to dilute the stock in 1:(15.9) ratio. You need to account for the fact that 75uL of media will come from the virus dilutions.  
  For one top-row 150uL well you will need 17.8uL of the stock peptide mixed with 132.2ul of media.  
  Normally we run assays in triplicates, let’s calculate enough for 4.4 wells to account for the 10% pipetting error:  
  **For 4.4 top-row 150uL wells altogether you will need:  
  P: 78.3 uL  
  M: 581.7 uL**
* Calculate the quantity of HSV virus needed for MOI 50% (20000 VP per 40000 cells). For the NEW 1:1000 stock we have you will need 1ul per well to achieve MOI 50%.  
    
  **For 10 wells of MOI 50% in 75uL volume you will need:  
  V: 10 uL (1:1000)**   
  **M: 740 uL**
* Seed cells in 2 96-well plates. From fully confluent T175, trypsinise the cells and spin 500g 5min. Discard the supernatant and resuspend the pellet in 30ml media. Use 100uL of cell suspension per well for seeding (counting not necessary). Leave to grow overnight at 37C.

Day of assay:

Make peptide dilution plates:

* Plate layouts will need to be identical to final plates of assay as will be transferred over to virus plates post-dilution and incubation.
* Take fresh 96-well plates, do NOT use the plates with cells.
* Add 150uL media in *media only* wells.
* Add 75uL media in *virus only* wells.
* Add 75uL media to all other wells *apart from* those with the highest concentration of the peptide (top row)
* Add 150uL of 50uM peptide dilution to the wells with the highest concentration of the peptide (top row)
* Dilution series factor 2: transfer 75uL from top row wells to the next row etc., until last and highest dilution well. Pipette up and down to mix every well, change tips between dilutions. Discard last 75ul.
* All wells apart from *media only* should have 75uL total Volume at the end.
* Leave plates in the hood during the next step.

Prepare virus stocks:

* Always keep virus on ice. If possible do not use a previously defrosted virus vial.
* We need to achieve MOI 50% and a volume of 75uL/well.

Make up to the needed number of wells accounting for a 10-12% pipette error. Let’s make enough for 35 wells.

**For 35 wells of MOI 50% in 75uL /well you will need:  
V: 35 uL (1:1000)**   
**M: 2590 uL**

Make up in a 15mL eppendorf

* Mix by inverting gently.

Add virus to serum dilution plates:

* Add 75uL of virus stock to peptide dilution plate to all wells **except media only**. Pipette straight into the liquid already in the well. First pipette *virus only* wells then change tips after every row of peptide dilutions.
* Leave to incubate serum-virus plates for 1 hour at 37C in TC incubator.

Transfer of serum-virus to cell plates:

* Carefully remove all media from cell plates (aspirator with p10 tips would be ok to use here). Be careful and do not damage the cells on the bottom of the well
* Transfer 150uL from peptide-virus plates across to cell plates in the same layout using a multi-channel pipette. Change pipette tips between the plate sections.
* Leave in 37C incubator for 2-3 hours

Change the media 2-3hpi:

* Fully aspirate the liquid from the wells (do NOT use the aspirator with tips, use a multichannel pipette). Be careful and do not damage the cells on the bottom of the well
* Replace with 150ul of fresh media

Grow for 24 hours post-infection in the 37C incubator

**Fix plates 24hpi**

* Carefully remove all media from assay plates (ideally with a multichannel)
* Wash with PBS. Add 100uL/well of PBS, then remove it with a multichannel.
* Add 100uL ice-cold 100% EtOH to fix cells and store at -20C until ready to stain. Store only for a few weeks.

**Immunostaining using standard Reeves protocol (from fixed plate):**

* Work done at molecular lab bench post-fixation.
* Remove EtOH, wash with 100ul sterile PBS.
* Make up primary Antibody.

HSV: Anti-HSV-ICP4 (ab6514 – mAb to HSV-1 ICP4 protein. Lot: GR3400140-1).

* For 1x 96-well plate make up 10mL: 10mL PBS + 5uL primary Antibody.
* Remove PBS and add 100uL primary Antibody dilution to plate.
* Incubate at RT on shaker for 1 hour (does not need to be in the dark as not conjugated antibody).
* Remove primary Antibody and wash with 100ul PBS.
* Make up secondary Antibody (AlexaFluor 568 goat anti-mouse IgG, Invitrogen TF A11004) in 1:2000 dilution + DAPI in 1:500 dilution for 100ul/well:
* For 1x 96-well plate make up 10mL: 10mL PBS + 5uL secondary Antibody + 20uL DAPI
* Remove PBS and add 100uL secondary Antibody/DAPI dilution to plate.
* Incubate at RT on shaker for 1 hour (in the dark in tin foil).
* Remove secondary Antibody/DAPI and wash with 100ul PBS.
* Remove PBS and add exactly 100uL PBS (the more exact the pipetting the better the subsequent images).

Store in fridge in foil after immunostaining is complete, and image on Hermes (separate protocol) within 1-2 weeks.