Influenza GFP Neutralization Assay Protocol

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Cells

We use MDCK-SIAT1-TMPRSS2-PB1 cells. For trypsin-independent viruses (i.e., WSN), use MDCK-SIAT1-CMV-PB1 cells. I grow these cells in D10 as usual and split 1:5 for a one-day growth to confluency, or 1:10 for a two-day growth to confluency, and try to avoid splitting lower/growing longer.

Media

We use NAM, or Neutralization Assay Media (Medium 199 supplemented with 0.01% heat-inactivated FBS, 0.3% BSA, 100 U of penicillin/ml, 100 µg of streptomycin/ml, 100 µg of calcium chloride/ml, and 25 mM HEPES). For WSN virus, we use WSN Neutralization Media, or WNM. This is a variant of NAM (neutralization assay media) where the FBS concentration is brought up to 0.5%.

I make sure to use the same batch of NAM for an entire neutralization experiment to avoid any batch effects on the fluorescence signal coming from the media.

Plate setup

I typically use one 96-well flat-bottom plate per antibody/virus pair to test; one plate gives triplicate measurements.

For the 96-well plate setup, I orient the plate such that A-H are the "columns" and 1-12 are the "rows". When I make the antibody dilutions I have row 12 at the top (away from me) with the highest concentration of antibody, making serial dilutions towards me so that row 1 has the lowest amount of antibody. Using 8 columns x 12 rows allows for 12 dilutions of antibody to be tested. The plate can also be oriented with 12 columns x 8 rows, but this is a matter of personal preference.

For the 8 columns, I ignore the edge columns A and H to avoid edge artifacts with the plate reader. I still fill them with some media - however, I don't think this is important to do.

I set up the remaining columns like so:

- Column B: virus-only control to measure background signal. Mike Doud previously found that the GFP viruses contribute more background signal than cells do, likely because some GFP is released into the viral supernatant from lysing cells during virus rescue, but since the “background signal” from the MDCK-SIAT1-TMPRSS2-PB1 cells has never been tested before, you may want to try a column with cells only to see which contributes more background, cells-only or viruses-only

- Columns C and G: cells + virus to measure the GFP signal for "100% infection". Since there can be a bit of variance, it's useful to have two measurements of this for each row and average them
- Columns D, E, and F: cells + virus + antibody. I make antibody dilutions to have triplicate measurements of % infection at each antibody concentration

Example plate setup:

- `E` = empty well (NAM only)
- `VC` = virus only control (or cells only, depending on which contributes greater background signal)
- `C+V` = cells + virus control
- `Dil#` = cells + virus + antibody dilutions

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	E	VC	C+V	Dil1	Dil1	Dil1	C+V	E
2	E	VC	C+V	Dil2	Dil2	Dil2	C+V	E
3	E	VC	C+V	Dil3	Dil3	Dil3	C+V	E
4	E	VC	C+V	Dil4	Dil4	Dil4	C+V	E
5	E	VC	C+V	Dil5	Dil5	Dil5	C+V	E
6	E	VC	C+V	Dil6	Dil6	Dil6	C+V	E
7	E	VC	C+V	Dil7	Dil7	Dil7	C+V	E
8	E	VC	C+V	Dil8	Dil8	Dil8	C+V	E
9	E	VC	C+V	Dil9	Dil9	Dil9	C+V	E
10	E	VC	C+V	Dil10	Dil10	Dil10	C+V	E
11	E	VC	C+V	Dil11	Dil11	Dil11	C+V	E
12	E	VC	C+V	Dil12	Dil12	Dil12	C+V	E

Virus

Titering the GFP viruses by flow cytometry (or on a plate reader) is important to normalize the IP/ul used across different viruses when comparing neutralization curves between mutants or strains. I recommend trying to use the same volume of inoculum across all viruses when titering to have the most consistent results across viruses.

After titering virus and before doing the neutralization assay, it is crucial to do an “MOI test”. In this test, you use the same number of cells you would use in the neutralization assay, and start with a large dose of virus and make serial dilutions before adding cells to see how many infectious particles of virus should be used per well to ensure reductions in amount of virus lead to proportional reductions in GFP signal. For example, you may find that diluting the virus to 500 IP/ul rather than the standard 1000 IP/ul puts you in the linear range where decrease in GFP intensity corresponds with decrease in infectious virus.

If your virus grows to low titers and does not need to be diluted much, you may want to grow the virus in NAM to limit background fluorescence from the growth media.

Antibody concentrations and serial dilutions

The example protocol below is for 2.3-fold dilutions of antibody (achieved by transferring 60 ul of antibody to 80 ul of media, mixing, and then transferring 60 ul out to the next row, etc.) The dilutions can be modified by changing how much antibody is added and serially transferred. For instance, you might want to do 5-fold dilutions by serially transferring 20ul (instead of 60ul) into 80 ul NAM to cover a wider range of antibody concentrations. Alternatively, you can also make smaller dilutions to test a smaller range of concentrations.

To calculate the antibody concentrations used in the neutralization assay:
In the top row with the highest antibody concentration, you will have 80 ul NAM + 60 ul antibody stock (for 2.3-fold dilutions), so the concentration of antibody BEFORE addition of virus = 60 x stock concentration/(80+60). Then, after serial dilutions there is only 80 ul left. Then, after adding 40 ul of virus, the effective concentration of antibody during incubation of virus = 80 x (antibody concentration BEFORE virus)/(80+40). This is the ug/ml we plot on the neutralization curve: the concentration of antibody during incubation with virus for 1-1.5hr.

Specific protocol for neutralization assay of influenza GFP viruses

Bring NAM and D10 to 37°C, and bring trypsin-EDTA to room temp. Have one nearly confluent 15-cm dish of MDCK-SIAT1-TMPRSS2-PB1 cells per 4-5 plates of neutralization assay to set up.

1. Thaw antibody and make a dilution to X ug/ml stock using NAM
2. Add 80 ul NAM to all wells of all plates. Begin thawing virus near the end of this
3. Add 60 ul (for 2.3-fold dilutions) of the antibody stock at X ug/ml to the top row for columns D, E, and F. I typically do this for each plate (fill the top row of columns D/E/F for each plate from this antibody stock solution) before moving on
4. Using the multichannel pipette with three tips attached, mix the top row of columns D/E/F wells and transfer 60 ul (for 2.3-fold dilutions) to the second row, mix, etc., making serial dilutions all the way down the plate, and removing the 60 ul from the last row. Finish dilutions for all plates before going on
5. Add 40 ul of NAM to column B for each plate to make up for the fact that there will be no cells added to this column
6. Prepare virus inocula. The standard protocol is to dilute virus to 1e3 IP/ul so that the MOI is ~1.0. However, you want to dilute the virus based on the results of the MOI test
7. Add 40 ul of the virus dilution to columns B/C/D/E/F/G. I add one row at a time (starting with the "top row" 12) with 6 tips on the multichannel. Use a new set of 6 tips for each row. When each plate has all rows with virus added, I write down the time and put the plate at 37°C for at least 1 hour (typically 1-1.5 h) to allow virus + antibody to mix and for neutralization to occur
8. While virus/antibody mixtures in plates are incubating, prepare the cells. Trypsinize MDCK-SIAT1-TMPRSS2-PB1 cells, quench with D10, spin down to pellet, decant

D10, and resuspend in NAM (I typically resuspend in ~20 ml of NAM per confluent 15-cm dish). Count cells and make a master mix of cells in NAM at 1e6/ml, making at least 2.5 ml per plate

9. Once it has been 1-1.5 hours incubation of virus + antibody, take each plate out of the incubator in order and add 40 ul of cells to columns C/D/E/F/G, using a fresh set of tips for each row. Write down the time that cells are added
10. Incubate the plates for 16-20 hours at 37°C and then read the fluorescence with the plate reader. Use a spreadsheet to calculate the % infectivity