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Influenza GFP Neutralization Assay Protocol

Goal

This approach allows us to measure neutralizing titers against influenza virus encoding an HA of interest and a PB1flank_GFP. Serial dilutions of serum or antibodies are incubated with a given virus for 1 hr, and then transferred to MDCK/SIAT1+PB1 cells. Infectivity is measured by GFP fluorescence at 18-24 hrs (generally aim for ~20 hpi). By measuring infectivity, we can quantify the potency of serum or antibodies against the virus of interest.

Supplies needed for neutralization assay of influenza GFP viruses:

Cells

We use MDCK-SIAT1-CMV-PB1 cells for the neutralization assays. 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.**

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 confirm that 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-fold dilutions of antibody (achieved by transferring 50 ul of antibody to 50 ul of media, mixing, and then transferring 50 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 12.5 ul (instead of 50ul) into 50 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 50 ul NAM + 50 ul antibody stock (for 2-fold dilutions), so the concentration of antibody BEFORE addition of virus = 2x final concentration. Then, after serial dilutions there is only 50 ul left. After adding 50 ul of virus, the effective concentration of

antibody during incubation of virus is half the concentration you initially started with in this well. This is the ug/ml we plot on the neutralization curve: the concentration of antibody during incubation with virus for 1-1.5hr.

Protocol for neutralization assay of influenza GFP viruses:

Day 1: Make serum dilutions, incubate with virus, and transfer to cells infect

- Thaw serum or antibodies and viral aliquots needed for running assay. For these assays we are using PB1flank_GFP encoding influenza (all mutants that we will measure effect of were introduced into this background). Bring NAM and D10 to 37°C, and bring trypsin-EDTA to room temp. Have one nearly confluent 15-cm dish of MDCK-SIAT1-PB1 cells per 4-5 plates of neutralization assay to set up.

- First, set up the plate by adding the appropriate amount of NAM to all of the wells. I typically use one 96-well plate for four antibodies (or sera) with one virus. If using this plate design, my final plate setup design looks like this:

Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells
Virus only	Cells+Virus	Sera1	Sera1	Sera2	Sera2	Sera3	Sera3	Sera4	Sera4	Cells+Virus	Cells

Alternatively, you can rotate the plate to run one virus and antibody pair per plate and obtain triplicate measurements with 12 concentrations.

- To start, I add NAM to the whole plate first, for all wells that are getting virus and sera, we need 50 uL per well final volume for wells with serum. All wells that are not getting virus (column 12) or cells (column 1) will get 100 uL. The first dilution will be done into row A of the plate, so leave this row empty for now, as we may need to add different amounts of media depending on the initial dilution for each serum sample:

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	50									50	100
B	100	50	50	50	50	50	50	50	50	50	50	100
C	100	50	50	50	50	50	50	50	50	50	50	100
D	100	50	50	50	50	50	50	50	50	50	50	100
E	100	50	50	50	50	50	50	50	50	50	50	100
F	100	50	50	50	50	50	50	50	50	50	50	100
G	100	50	50	50	50	50	50	50	50	50	50	100
H	100	50	50	50	50	50	50	50	50	50	50	100

- Now, we will fill row A with media according to the initial dilution that we want to perform. Depending on the desired initial dilution (chosen based on serum potency), I vary how much media and how much sera I added to the first dilution well. We are going to add 50 uL of virus, so know that initial dilution into that 100 uL is going to be diluted 2-fold. Also, prior to running neutralization assays, I RDE treat my serum which results in a 1:4 dilution. Keep this in mind when setting up dilutions for serum, if you want a starting dilution of 1:20, you will need to use 40 uL of RDE treated sera into 60 uL NAM.

- After performing the initial dilution into 100 uL, I then perform serial dilutions down the plate. If doing 2-fold dilutions, the total volume in row A prior to starting dilutions was 100 uL, and then 50 uL was transferred down the plate, discarding the extra 50 uL from the bottom row. This will leave 50 uL in each well.

- Prepare virus. 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 so that you are working within the linear range for GFP signal. Typically, this is in the range of 200-1000 IP/uL.

- Add 50 uL virus to columns 1-11 of the plate using multichannel pipette.

- Incubate plate with serum and virus at 37C for 1-1.5 hr

- At about 35-40 min into incubation, begin trypsin-treating MDCK/SIAT1/CMV-PB1 cells, harvest cells from plate, wash once in PBS, and resuspend in NAM at a concentration of 0.8×10^5 cells/mL.

- After 1 hr incubation is complete add 50 uL cells to each well in columns 2-12. Return plate to 37C incubator with 5% CO2.

Day 2: Read fluorescence measurements to calculate infectivity

- At about 18-20 hpi, first check plates under the microscope. There should be infected cells in columns 2 and 11 rows A – H, additionally, by checking infectivity at highest concentrations for the serum samples (rows B and C, columns 3-10) you can get an idea of how well your samples neutralized.

- Read GFP fluorescence on the plate – I typically use a gain normalization.

- Examine curves. If you started at a particularly high concentration of sera, in the wells containing high concentrations of sera, there may be some high background fluorescence in this top well, to ablate this signal and get more reasonable reads, you can carefully remove 100 uL per well for each plate and discard this volume into wescodyne. You can then re-read the plate following the removal of this excess media.

- Transfer data to a .csv file which includes concentration of sera used for each dilution and the calculated fraction infectivity – use this file as your input for *neutcurve* to calculate IC50s.