Rescue of Perth/2009 GFP viruses *I am going to rescue wild-type Perth/2009 GFP viruses using reverse genetics in a coculture of 293T-CMV-PB1::MDCK-SIAT1-CMV-PB1-TMPRSS2 cells. These viruses will express the Perth/2009 HA and the WSN NA, similar to the HA and NA components of my Perth/2009 HA libraries. I will use this stock of GFP viruses for GFP neutralization assays.*

September 9, 2017

1. **Seeded a coculture** of 293T-CMV-PB1:MDCK-SIAT1-CMV-PB1-TMPRSS2 cells
    - Seeded 4 x 10^5 293T-CMV-PB1 cells and 0.5 x 10^5 MDCK-SIAT1-PB1-TMPRSS2 cells per well, 2 ml per well, into two 6-well plates
    - Made a 40 ml master mix of cells
    - Added 2 ml of the master mix of cells to its corresponding well. Incubated at 37°C (Start: 4:30 PM)

September 10, 2017

1. **Transfections:** transfected cocultures seeded yesterday with influenza reverse genetics plasmids to rescue.
    - Checked cocultures ~18 h after seeding – they all were ~50% confluent
    - Prepared 18 test tubes containing their corresponding transfection mix as described in tables below and on the next page
    - Made DMEM + BioT master mix: 2000 ul DMEM + 60 ul BioT
    - Added 103 ul of the DMEM + BioT master mix to each transfection tube and mixed by pipetting up and down. Incubated at RT for ~20 min. Added the plasmid-DMEM-BioT mix dropwise to its corresponding well. Mixed by tilting the plate
    - Incubated the plates at 37°C (Start: 10 AM)

TRANSFECTIONS	Master Mix (ul)	HA plasmid (ul)	NA plasmid (ul)	BioT (ul)	DMEM (ul)	Transfection (ul)/well
Perth09 HA + WSN NA GFP	5	1.67	1.67	3	100	111.34
Perth09 HA-NA GFP	5	1.67	1.67	3	100	111.34
no HA WSN NA GFP	5	0	1.67	3	100	109.67

2. **Changed media** of transfected cells to **IGM**
    - Aspirated D10 and gently added 2 ml IGM with P1000 pipet (10:30 PM)

September 12, 2017

1. **Harvested viral transfection supernatant**: I'm seeing substantial spread of GFP to MDCK cells in IGM across all transfections. I saw nearly 100% spread of green to neighboring MDCK cells even 48 hpt. I collected various timepoints for some of the viruses
    - Perth/09 HA + WSN NA GFP virus and no HA + WSN NA GFP virus: collected one plate at 48 hpt, and the other plate at 52 hpt. For each timepoint, collected 18 x 500 ul aliquots of the P09 HA + WSN NA viruses and 2 x 800 ul aliquots of the no HA ctrls
    - Perth/09 HA + NA GFP virus and no HA + P09 NA GFP virus: collected one well of P09 GFP virus at 48 hpt and the rest at 54.5 hpt

Master Mix (all WSN reverse genetics plasmids)

Plasmid	Name	Conc (ng/ul)	Plasmid vol (ul)	Amount (ng)	# Transfections	Master Mix Vol (ul)
30	pHW181-PB2	363.5	13.76	250	18	100
208	pHH-PB1flank-eGFP	609.7	8.22			
32	pHW183-PA	863.6	5.8			
34	pHW185-NP	448.5	11.16			
36	pHW187-M	537.6	9.32			
37	pHW188-NS	438.7	11.4			
1422	pHAGE2-TMPRSS2	769.3	6.5			
	Plasmid total vol		66.16			
	H2O		33.84			
	Total volume		100			

HA plasmid to add to Master Mix for each transfection

Plasmid	Name	Conc (ng/ul)	Vol to make 150 ng/ul dil (ul)	Vol H2O (ul)	Vol (ul)	Amount (ng)
1561	pHW-P09-HA-G78D-T212I	215.5	20.9	9.1	1.666666667	250

NA plasmid to add to Master Mix for each transfection

Plasmid	Name	Conc (ng/ul)	Vol to make 150 ng/ul dil (ul)	Vol H2O (ul)	Vol (ul)	Amount (ng)
1443	pHWPerth09-NA	452.4	16.58	33.42	1.666666667	250
35	pHW186-NA	541.3	13.86	36.14	1.666666667	250

2. **Seeded MDCK cells in 12-well plates to titer GFP viruses**:
    - Split a plate of MDCK-SIAT1-PB1-TMPRSS2 cells. Resuspended pellet in 20 ml IGM
    - Made a master mix of each of these cells, at a density of 1e5 cells/ml. Made a 50 ml master mix in IGM of cells
        - Counted 1.3e6 PB1-TMPRSS2 cells => added 3.9 ml cells to 46.1 ml master mix
    - Added 1 ml of cells to all wells of four 12-well plates
    - Incubated cells at 37°C to allow them to adhere (START: 2 PM)
3. **Infected MDCK cells with GFP viruses:**
    - 4 h after seeding, checked 12-well plates to see that cells were adherent. Thawed virus supernatant
    - Made virus dilutions in a 96-well plate
        - Added 180 ul IGM to rows B/C/D/E of columns 2-10
        - Added 100 ul of undiluted virus supernatant to row A of its corresponding column
        - Transferred 20 ul to each row to make serial 10-fold dilutions
    - Infected cells with 100 ul of virus dilutions in rows B/C/D/E to achieve effective volumes of 10, 1, 0.1, and 0.01 ul, respectively
    - Added dilutions column-wise to the 12-well plate (START infections: 6 PM)

1	Perth/09 HA + WSN NA, 51 hpt, NAM	4	Perth/09 HA + WSN NA, 48 hpt	7	No HA + Perth/09 NA, 54.5 hpt
2	No HA + WSN NA, 51 hpt, NAM	5	No HA + WSN NA, 48 hpt	8	Perth/09 HA + NA, 48 hpt
3	Perth/09 HA + NA, 51 hpt, NAM	6	Perth/09 HA + NA, 54.5 hpt	9	Perth/09 HA + WSN NA, 52 hpt

All in IGM unless otherwise specified

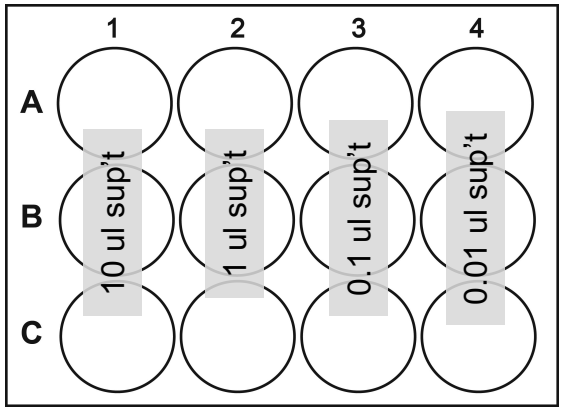
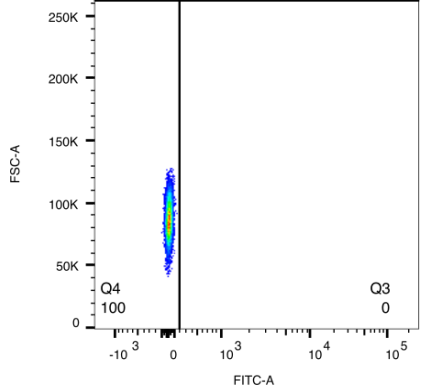


Plate #	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**
Row A	1	4	6	8
Row B	2	5	7	9
Row C	3	Cells only	Cells only	Cells only

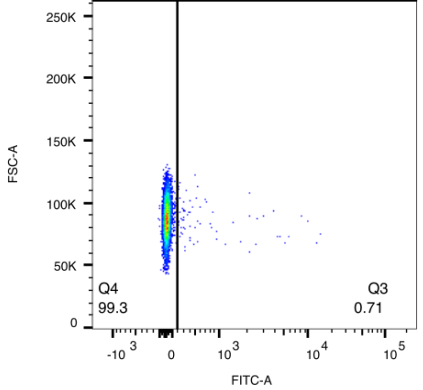
September 13, 2017

1. ~15 hpi, checked plates and picked wells that by eye looked like there were between ~1-10% green cells
    - Collected samples for FACS analysis by aspirating supernatant, adding 350 ul trypsin, neutralizing with 750 ul D10, and pelleting cells at 1200 rpm for 5 min. I did not collect any of the viral samples rescued in NAM because I saw little no GFP in these infections, even at the highest volume
    - Resuspended cells in 400 ul PBS + 1% PFA (made a master mix of 1.5 ml 4% PFA + 4.5 ml PBS). Waited 10 min for PFA to inactivate virus
    - Spun down cells again, and resuspended in FACS buffer (PBS + 1% BSA)
    - Transferred samples from TC room to lab bench. Vortexed briefly, then filtered cells through cell strainer. Titered using Canto1

Sample	GFP positive percent	GFP positive fraction	number cells	uL used	IP/uL
P09 HA + WSN NA 48 hpt	0.71	0.0071	100000	1	712.5324943
P09 HA + WSN NA 52 hpt	1.31	0.0131	100000	1	1318.65618
P09 HA + P09 NA 48 hpt	0.44	0.0044	100000	1	440.9708489
P09 HA + P09 NA 48 hpt	18.5	0.185	100000	10	2045.671657
P09 HA + P09 NA 54.5 hpt	4.33	0.0433	100000	1	4426.54163
no HA P09 NA 54.5 hpt	0	0	100000	1	0
no HA WSN NA 48 hpt	0	0	100000	1	0



Specimen_001_Cells only.fcs
Live MDCK cells
9972



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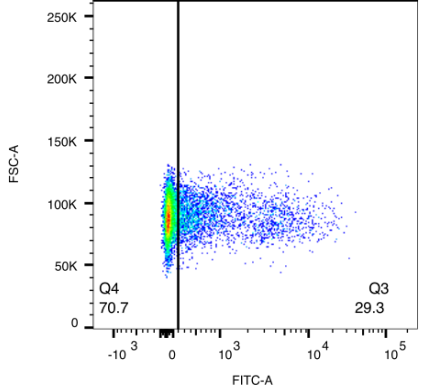
Live MDCK cells
9968



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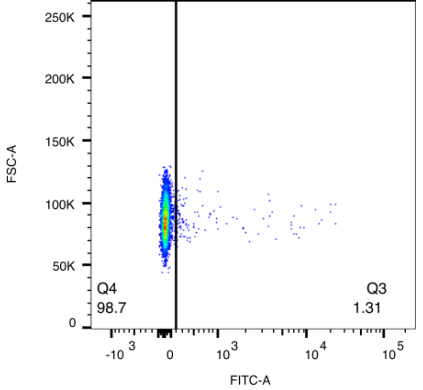

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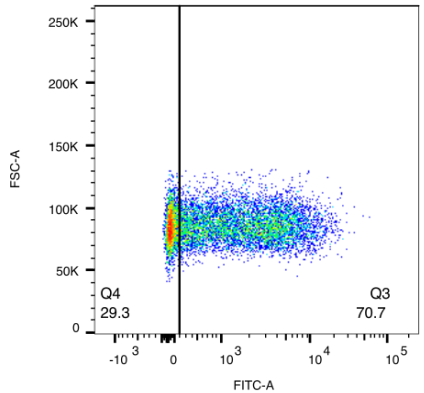
Live MDCK cells
9948



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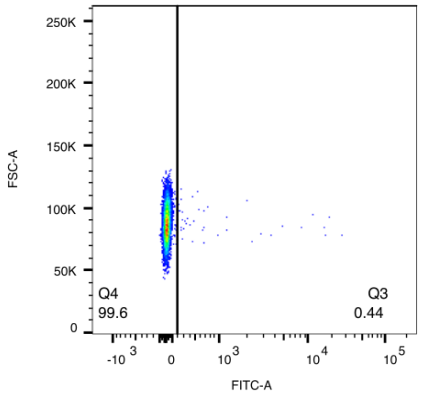
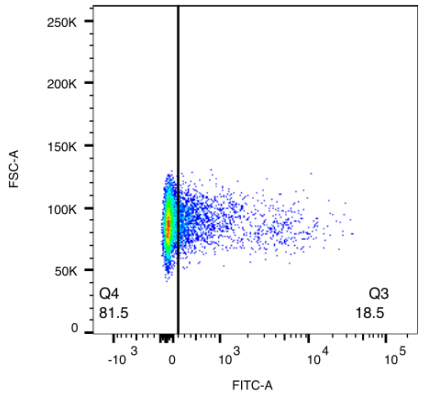
Live MDCK cells
9886



Specimen_001_P09_48h_1ul.fcs
Live MDCK cells
9970



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Live MDCK cells
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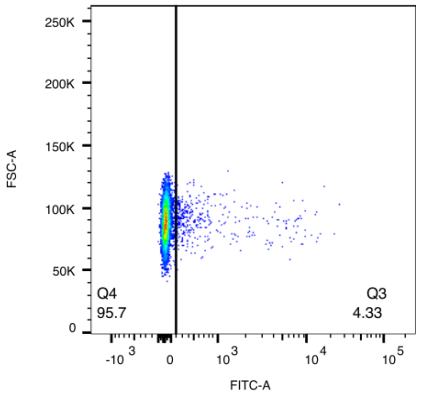
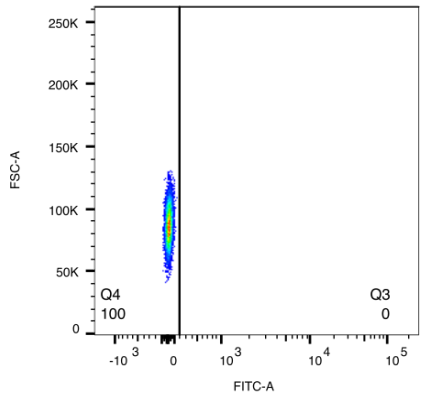


Specimen_001_P09_54h_1ul.fcs
Live MDCK cells
9967



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Live MDCK cells
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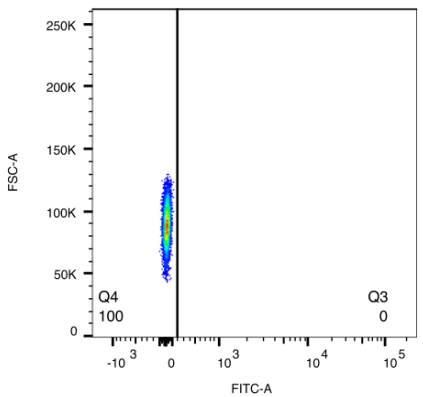


Specimen_001_noHA_WSN-NA_48h_1ul.fcs
Live MDCK cells
9985

September 14, 2017

1. **MOI test:** I am setting up an MOI test to find the appropriate MOI for future neutralization assays. I am going to make two-fold serial dilutions of virus to test an MOI of 1 to 0.0078

IP/well	MOI	Row
40000	1	A
20000	0.5	B
10000	0.25	C
5000	0.125	D
2500	0.0625	E
1250	0.03125	F
625	0.015625	G
312.5	0.0078125	H

Virus 1	Perth/2009 HA + WSN NA 48 hpt
Virus 2	Perth/2009 HA + WSN NA 52 hpt
Virus 3	Perth/2009 HA + NA 54.5 hpt

Plate setup: (doing 2 replicates of virus 2)

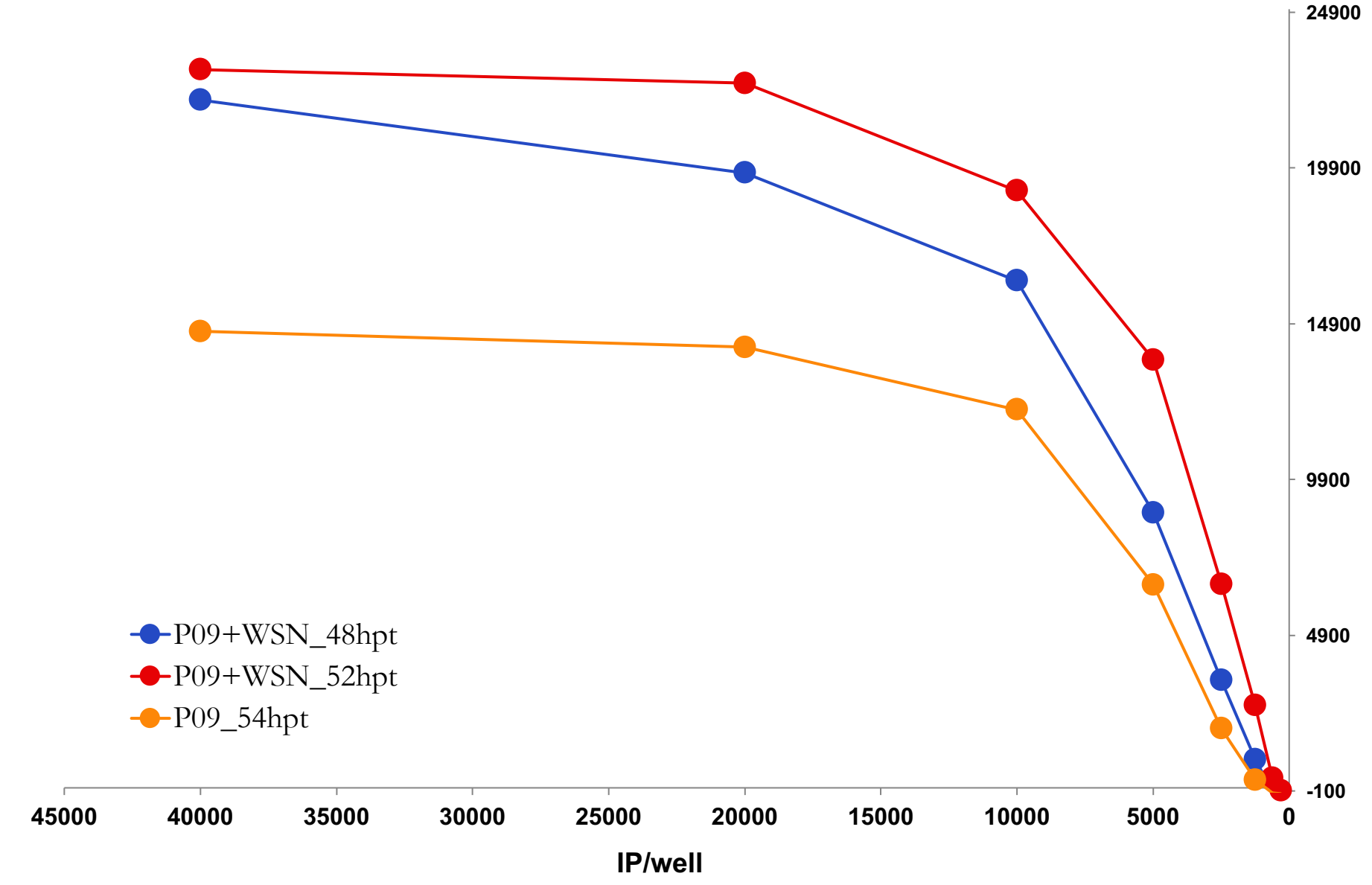
1	2	3	4	5	6	7	8	9	10	11	12
empty	Virus 1 + cells	Virus 1 only	Virus 2 + cells	Virus 2 only	Virus 2 + cells	Virus 2 only	Virus 3 + cells	Virus 3 only	Cells only	empty	

Protocol:

1. Added 80 ul NAM to all wells, and 160 ul NAM to empty wells
2. Added 80000 IP of GFP virus in row A of columns 2-7 (diluted virus to 1000 IP/ul by adding the volumes specified to the right to NAM, then added 80 ul of each to row A). Each virus was added to two columns (virus + cells and virus only)
3. Made serial two-fold dilutions down rows by transferring 80 ul down to each row, removing 80 ul from the final row. Changed tips between each row
4. Incubated plate for ~1 h (actually 50 min) while splitting MDCK-SIAT1-PB1-TMPRSS2 cells. Resuspended cells in NAM
5. Made a 10 ml master mix of cells at 5e5 cells/ml in NAM
6. Added 80 ul of cells to columns 2/4/6/8/10, and 80 ul NAM to columns 3/5/7/9
7. Incubated plate at 37°C (START: 3:45PM)
8. Read plate on plate reader at 9:15 AM (~17.5 hpi)

VIRUS	IP/ul	ul virus	ul NAM
Virus 1	712.53	200	-
Virus 2 (x 2)	1318.6	304	96
Virus 3	4426.5	46	154

From the MOI test, it looks like the best MOI to use for the Perth/2009 HA + WSN NA GFP virus collected at 52 hpt is an MOI = 0.125 (125 IP/ul, or 5000 IP/well). At this point, there is a linear decrease in GFP signal that corresponds with decrease in input viral particles. The MOI test also showed that there was higher background signal coming from the viruses only control compared to the background signal coming from the cells only control, likely due to IGM in the viral supernatant.



September 15, 2017

Neutralization assays against WT Perth/2009 + WSN NA GFP virus with 5A01, 3C06, & 3C04

I am going to set up neutralization assays for three monoclonal antibodies to see the neutralization profiles for these three antibodies.
I will do this for each antibody by making 12 serial dilutions of antibody in triplicate in a 96-well plate, and testing neutralization against WT Perth/2009 + WSN NA GFP virus that I rescued.
I will use three 96-well plates, one for each antibody, and set up the dilutions listed below.
I made the following starting dilutions of each antibody such that when I add the appropriate volume, I will end up with the target concentration in row 1:

5A01:
Stock = 551.8 ug/ml
Need 165 ug/ml working concentration
30 ul stock + 70 ul NAM = 100 ul total

3C06:
Stock = 614.3 ug/ml
Need 330 ug/ml working concentration
53.8 ul stock + 46.2 ul NAM = 100 ul total

3C04:
Stock = 698.9 ug/ml
Need 495 ug/ml working concentration
71 ul stock + 29 ul NAM = 100 ul total

PLATE 1	5A01
Row	3.67-fold dil.
1	30
2	8
3	2.23
4	0.61
5	0.1653701
6	0.04506
7	0.0122779
8	0.003345483
9	0.000911576
10	0.000248386
11	0.00006768
12	1.84414E-05

PLATE 2	3C06
Row	3.67-fold dil.
1	60
2	16
3	4.45
4	1.21
5	0.3307402
6	0.0901199
7	0.0245558
8	0.006690966
9	0.001823151
10	0.000496772
11	0.00013536
12	3.68829E-05

PLATE 3	3C04
Row	3.67-fold dil.
1	90
2	25
3	6.682060153
4	1.820724837
5	0.49611031
6	0.135179921
7	0.036833766
8	0.010036448
9	0.002734727
10	0.000745157
11	0.00020304
12	5.53243E-05

Plate layout:
"A-H" will be referred to as columns, and "1-12" as rows. I had the plate with column A-H going left to right, and rows 12-1 from top to bottom.

	COLUMN							
ROW	A	B	C	D	E	F	G	H
1	NAM only	virus only	virus + cells only	Ab dilution	Ab dilution	Ab dilution	virus + cells only	NAM+IGM

I used the "Perth/2009 HA + WSN NA GFP virus, 52 hpt" batch I rescued on 20170912. Based on the MOI test I conducted, I will use an MOI of 0.125 for these neutralization assays

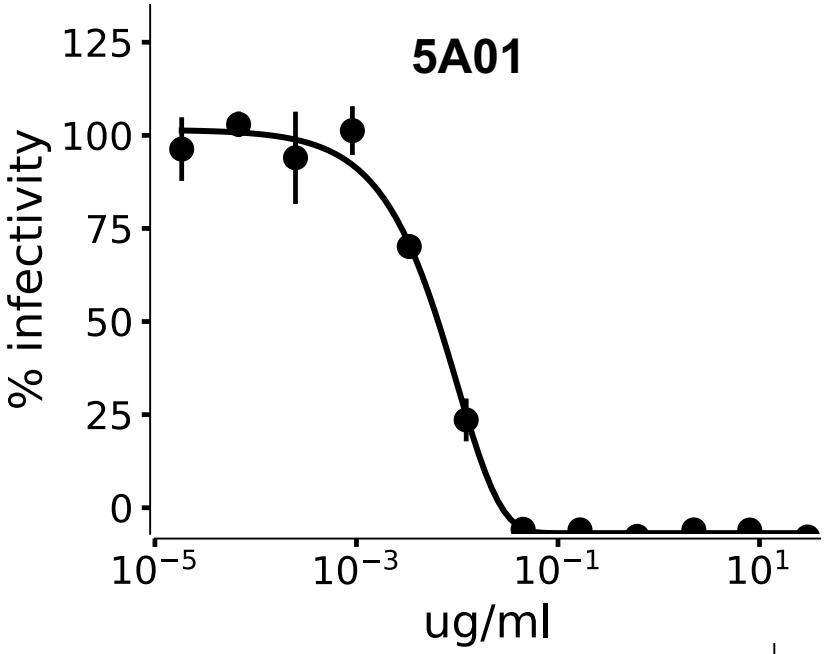
Antibody	Vol of Ab for serial dil	Time start virus + Ab	Time start virus+Ab+cells
5A01	30 ul (3.67-fold dilutions)	3:15	4:15
3C06	30 ul (3.67-fold dilutions)	3:20	4:22
3C04	30 ul (3.67-fold dilutions)	3:26	4:28

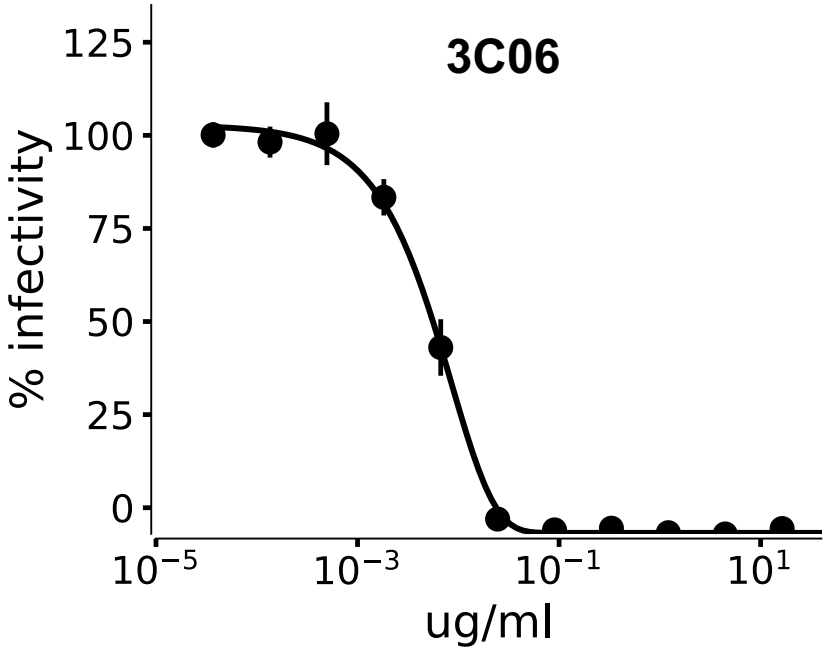
For multichannel, used Fisher brand blue box/green wafer tips, #02-707-431

1. Thawed antibodies and made dilutions to working stock solutions using NAM
2. Added 80 ul NAM to all wells of all three plates. Also began thawing viruses
3. Added 30 ul of the antibody stock to the top row (row 12) of the plate for columns D, E, and F. Did this for each plate
4. Using the multichannel with three tips attached, mixed the top row D/E/F and transferred 30 ul the second row, mixed, etc. Removed 30 ul from the last row. Did not change tips in between rows (not necessary here)
5. Added 40 ul NAM to column B of all plates (virus only column) to make up for no cells being added to these columns. Also added 40 ul NAM to column H of plate 1, as I will add NAM + IGM in this column to see the level of background from Opti-MEM
6. Prepared virus inoculum. Diluted Perth/09 HA + WSN NA GFP 52 hpt virus to 125 IP/ul. Made 15 ml total (5 ml per plate)
    - P09 HA + WSN NA, 52 hpt titer: 1318.6 IP/ul
    - 1422 ul virus + 13,578 ul NAM
7. Using a reservoir and multichannel with six tips attached, added 40 ul of virus to columns B-G. Used new tips for each row, but did not mix virus and Ab (no need to mix). Wrote down time once virus was added to all wells of a plate, and incubated at 37°C
8. While virus + antibody mixtures were incubating, starting preparing cells by trypsinizing MDCK-SIAT1-PB1-TMPRSS2 cells, neutralizing with D10, spinning at 1200 rpm for 4 min, then resuspending in 20 ml NAM
9. Made a master mix of cells at 1e6 cells/ml, making a 15 ml master mix (5 ml per plate)
    - Counted 1.17e6 cells/ml => 12.82 ml cells + 2.18 ml NAM
10. Made a mixture of 47 ul IGM + 453 ul NAM and added 40 ul of this mixture to column H of plate 1. This mixture represents approximately the proportions of viral supernatant diluted in NAM for the virus dilutions
11. After ~1 h of virus + Ab incubation, took each plate out of the incubator in order, and added 40 ul cells to columns C-G, using fresh tips for each row. Used a fresh reservoir for each plate, re-mixed cell suspension before adding to reservoir. Wrote down time cells were added to the wells of a plate
12. At ~17 hpi (9:15 AM), read on plate reader with "Neutralization assay 96 well" program. For 5A01 plate, read columns B-H. For the other two plates, only read columns B-G

September 16, 2017

Results of neutralization assays





The neutralization assays confirm that antibodies 5A01, 3C06, and 3C04 all neutralize the Perth/2009 HA-carrying GFP viruses.
Furthermore, these antibodies seem to neutralize the Perth/2009 HA very potently, with IC50's in the 10^{-2} to 10^{-3} ug/ml range.