2017-3-24: I will clone 10 mutations to pHW-WSN-HA (#33) for making PB1flank-GFP viruses to test in neutralization assays. Here are the mutations I'm making and the primer sequences. Rationale for mutant selection and primer design is at:

https://github.com/jbloomlab/WSN-HA_mAb_dms/blob/master/2017analysis/plan_validation_FI6v3_escape.ipynb

K294S_For,gtaacacgTCTtgtcaaacaccccagggag,25nm,STD
K294S_Rev,gtttgacaAGAcgtgttacactcatgcattgacg,25nm,STD
K294T_For,gtaacacgACCtgtcaaacaccccaggg,25nm,STD
K294T_Rev,gtttgacaGGTcgtgttacactcatgcattgacg,25nm,STD
G390R_For,cattaacAGAattacaaacaaggtgaactctgttatcgagaaaatgaac,25nm,STD
G390R_Rev,gtttgtaatTCTgttaatggcattttgtgtgctttttgatccg,25nm,STD
N305S_For,ctataaacagcTCTctccctttccagaatatacacccagtc,25nm,STD
N305S_Rev,gaaagggagAGAgctgtttatagctccctggg,25nm,STD
M360L_For,gactggaCTTatagatggatggtatggttatcatcatcagaatgaac,25nm,STD
M360L_Rev,catctatAAGtccagtccatccccccctcaataaaacc,25nm,STD
K294A_For,gtaacacgGCTtgtcaaacaccccaggg,25nm,STD
K294A_Rev,gtttgacaAGCcgtgttacactcatgcattgac,25nm,STD
G390K_For,cattaacAAAattacaaacaaggtgaactctgttatcgagaaaatgaacac,25nm,STD
G390K_Rev,gtttgtaatTTTgttaatggcattttgtgtgctttttgatccg,25nm,STD
N305D_For,ctataaacagcGATctccctttccagaatatacacccag,25nm,STD
N305D_Rev,gaaagggagATCgctgtttatagctccctggg,25nm,STD
K2T_For,ccaaaatgACAgcaaaactactggtcctg,25nm,STD
K2T_Rev,gttttgcTGTcattttggttgtttttattttcc,25nm,STD
K2Y_For,ccaaaatgTATgcaaaactactggtcctg,25nm,STD
K2Y_Rev,gttttgcATAcattttggttgtttttattttcc,25nm,STD

I resuspend these all to 100uM with EB.

3.24.17 mutagenesis PCR for fi6v3 validation mutants
For each primer pair, mix F+R: 2ul F + 2ul R + 16ul H2O to make 10uM each combo.
Each mutagenesis PCR will be (33.3ul):
15 ul H2O
16.66 ul KOD
1 ul 10 uM combo primers
0.66 ul template (plasmid #33) at 10 ng/ul

Master mix (everything but primers, for 13 reactions):
195 ul H2O
216.6 ul KOD
8.58 ul 10ng/ul template #33

Add 32.3 ul to each well, then add 1 ul of the appropriate set of primers from the 10uM combo dilutions.
Cycling conditions (plasmid is 4736bp):

1. 95 2min
2. 95 20sec
3. 57 10sec
4. 70 3min
5. Go to 2, 34 times
6. 70, 10min
7. 4, hold

Do PCR (run 4ul on a 1% agarose analytical gel (although I would probably run this on 0.7% if I had gel already, this time I used 1% out of convenience) to check products before DpnI), followed by DpnI digest (add 1ul directly into PCR, incubate at 37 for 1hr), then ampure purification (0.6X beads = 18.2ul beads per well = ~220ul beads total), freeze the purified products at -20 overnight, then in-fusion and transformation).

Use this plate layout:

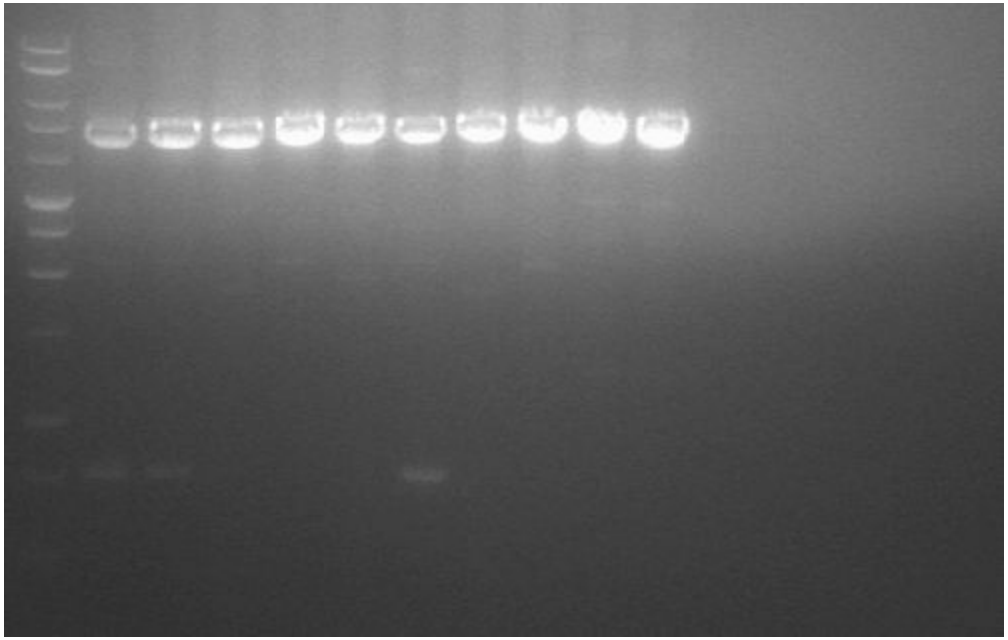
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1:K294S	2:K294T	3:G390R	4:N305S	5:M360L	6:K294A	7:G390K	8:N305D	9:K2T	10:K2Y	11:no primer	12:no primer no DpnI
C												
D												
E												
F												
G												
H												

3.24.17 check gel for mutagenesis pcr products, which should be 4.7kbp.

Run 4ul of the PCR products taken before DpnI digest. Each lane is 4ul PCR + 2ul 6x sample buffer + 6ul water. Make MM of diluted sample buffer (26 ul sample buffer + 78 ul water; add 8 ul to 12 tubes and add 4 ul PCR to load gel)

See expected bands in reactions 1-10, no bands in no-primer reactions 11-12.

Ampure-purified products in 60ul are ~50 ng/ul (average from four samples; no-primer sample 12 looked like blank as expected.)
Froze -20 overnight.



2017-3-25 In-Fusion and transformation of HA mutant constructs

Each in-fusion reaction:
1.5 ul purified linear mutagenesis product (~75ng)
2 ul 5X In-Fusion enzyme mix
6.5 ul H2O

Make an in-fusion master mix for 15 reactions' worth and dispense 8.5ul to 14 wells in a fresh plate using the same layout as the PCR products:
30 ul 5x infusion enzyme mix
97.5 ul H2O

Then add 1.5ul of purified DNA to each well and mix the reaction with multichannel pipette(s).
Incubate 15 min at 50C using thermal cycler, then place on ice and begin transformations (do in batches).

Transformation protocol (perform in batches):
Pre-warm 7ml SOC media and 14 LB-AMP plates.
Thaw stellar cells on ice. Take 30ul stellar cells into a fresh 1.5ml tube and add 1 ul of the infusion reaction.
Incubate on ice for 30 minutes.
Heat shock at 42C for 45 seconds, then return to ice for 2 minutes.
Add 500 ul warm SOC to each transformation; plate 50 ul and store the remainder ~400ul at 4C.

InFusion plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1:K294S	2:K294T	3:G390R	4:N305S	5:M360L	6:K294A	7:G390K	8:N305D	9:K2T	10:K2Y	11:no primer	12:no primer no DpnI
C												
D	In-fusion control backbone only (1ul)	In-fusion control backbone (1ul)+insert (2ul)										
E												
F												
G												
H												

2017-3-26: Got good colonies on all expected plates (maybe around 100), NO colonies on the no primer control, and only 5 colonies on the no primer no DpnI control; this is consistent with my previous experiments in that the DpnI digest does reduce background from what begins at a very low level to essentially zero. In general I'd probably be comfortable doing this without DpnI each time, but it's not too much extra work to ensure the parental plasmid doesn't get through.

I will inoculate LB-amp cultures for one or two clones per construct.

For all constructs I made two glycerol stocks for each of the two clones.

I will prioritize these mutants for miniprep and sequencing:

1:K294S
2:K294T
3:G390R
4:N305S
5:M360L
6:K294A
9:K2T

The other mutants I will just freeze bacterial pellet and prep and sequence later:

7:G390K
8:N305D
10:K2Y

For clones .1 and .2 for constructs 1-6+9, Miniprepped and normalized concentrations as shown on next slide

Sample ID	Nucleic Acid Conc. (in 42ul)	Add EB to normalize to 95ng/ul
1.1	147.2	23.1
1.2	123	12.4
2.1	180.4	37.8
2.2	165.5	31.2
3.1	161.9	29.6
3.2	146.8	22.9
4.1	170.9	33.6
4.2	129.1	15.1
5.1	134.9	17.6
5.2	159.6	28.6
6.1	109.6	6.5
6.2	95.6	0.3
9.1	123.8	12.7
9.2	107	5.3

All are normed to 95ng/ul

Genewiz: wants 500 ng of plasmid + 2.5ul of 10uM primer for each sequencing reaction, in a total volume of 15 ul with water.
So each well:

5.2 ul plasmid + 2.5 ul primer + 7.3ul water.

Make 15x primer/water mix for each primer: 37.5 ul primer + 109.5 ul water. Aliquot 9.8 ul per well, then add plasmids.

Row a: WSN-F,
row b: seq373, (Samples 1.1 - 6.2)
row c: WSN-R.

Row d: 9.1F 9.1Int 9.1R 9.2F 9.2Int 9.2R

Clone 1: 1-1 is good. 1-2 has a deletion near the mutation site.
K294S_For,gtaacacgTCTtgtcaaacaccccagggag,25nm,STD
K294S_Rev,gtttgacaAGAcgtgttacactcatgcattgacg,25nm,STD

Clone 2: Both are good.
K294T_For,gtaacacgACCtgtcaaacaccccaggg,25nm,STD
K294T_Rev,gtttgacaGGTcgtgttacactcatgcattgacg,25nm,STD

Using clone 1 for all of these.

Clone 3: Both are good.
G390R_For,cattaacAGAattacaaacaaggtgaactctgttatcgagaaaatgaac,25nm,STD
G390R_Rev,gtttgtaatTCTgttaatggcattttgtgtgctttttgatccg,25nm,STD

Clone 4: Both are good,
N305S_For,ctataaacagcTCTctccctttccagaatatacacccagtc,25nm,STD
N305S_Rev,gaaagggagAGAgctgtttatagctccctggg,25nm,STD

Clone 5: Both are good,
M360L_For,gactggaCTTatagatggatggtatggttatcatcatcagaatgaac,25nm,STD
M360L_Rev,catctatAAGtccagtccatcccccctcaataaaacc,25nm,STD

Clone 6: Both are good,
K294A_For,gtaacacgGCTtgtcaaacaccccaggg,25nm,STD
K294A_Rev,gtttgacaAGCcgtgttacactcatgcattgac,25nm,STD

Clone 9: Both are good,
K2T_For,ccaaaatgACAgcaaaactactggtcctg,25nm,STD
K2T_Rev,gttttgcTGTcattttggttgtttttattttcc,25nm,STD

Clones 7, 8, and 10: Not miniprepped or sequenced. Bacterial pellets in NEWEST -20 in rack on top of hybridoma supts.
7: G390K_For,cattaacAAAattacaaacaaggtgaactctgttatcgagaaaatgaacac,25nm,STD
G390K_Rev,gtttgtaatTTTgttaatggcattttgtgtgctttttgatccg,25nm,STD
8: N305D_For,ctataaacagcGATctccctttccagaatatacacccag,25nm,STD
N305D_Rev,gaaagggagATCgctgtttatagctccctggg,25nm,STD
10: K2Y_For,ccaaaatgTATgcaaaactactggtcctg,25nm,STD
K2Y_Rev,gttttgcATAcattttggttgtttttattttcc,25nm,STD

2017-3-30: Starting tissue culture. Making fresh D10 and fresh WNM (NAM+0.5% FBS, or 2.5 ml FBS per 500 ml NAM), and thawing MDCK-SIAT1-CMV-PB1 from my stock and 293T-CMV-PB1 from katherine's stock. For each cell thaw, just adding directly to a plate of 9 ml pre-warmed media today and will change media tomorrow.

2017-04-03:
12 pm: seed co-cultures of 293T-PB1 + MDCKSIAT1-PB1 in 6-well plates. After trypsinizing I spun and resuspended before counting. I plated co-cultures of these mixes at (2e5 293 + 2e4 MDCK) per ml, 2 ml per well.

Reverse genetics master mix (12.5x)

plasmid	ng/ul	ul for 3125ng
30	130	24.04
32	415	7.53
34	208	15.02
35	260	12.02
36	194	16.11
37	188	16.62
208	200	15.63

Make master mix, distribute to 12 tubes (8.56 ul each).
Add 250 of indicated HA plasmid.

Mix 39 ul BioT in 1300ul DMEM.
Add 103ul of BioT/DMEM mixture to each tube.
Incubate 25 min.
Add to cells at 4:30pm

TF well	HA variant	plasmid log #	ng/ul	ul for 250 ng
1	WT	33	200	1.250
2	WT	33	200	1.250
3	K294S	1748	95	2.632
4	K294T	1749	95	2.632
5	G390R	1750	95	2.632
6	N305S	1751	95	2.632
7	M360L	1752	95	2.632
8	K294A	1753	95	2.632
9	K2T	1754	95	2.632
10	P89D	1590	97	2.577
11	V148T	1579	200	1.250
12	No HA			

2017-04-04: Change media to WNM at 11am by aspirating one well at a time and gently adding 2 ml of WNM to side of wall with P1000.

2017-04-06: Harvest supernatants at 5 pm by collecting clarified supernatant (2,000xg 5 min), transferring to a new tube, and storing 6x300ul aliquots at -80.

2017-04-06 Titering fi6v3 escape mutant viruses:

Plate SIAT1/PB1 cells in WNM at 1e5/ml in 12-well plates at 2:15 pm. Make at least 4 plates to titer 12 viruses.

To make dilutions in WNM before infecting, I will use a 96-well plate.

First add 180ul to all of row B/C/D/E.
Then In Row A I will add >20ul transfection supernatant for each virus.
I will then make serial 10-fold dilutions by transferring 20ul to 180 ul to make 1:10, 1:100, 1:1000, 1:10,000.
Then I will use 100ul of Rows C, D, and E to infect cells for titering to achieve the following effective inoculum volumes:

Row A: undiluted (unused)
Row B: 1:10 (unused)
Row C: 100ul of 1:100 is = 1ul effective volume.
Row D: 100ul of 1:1000 is = 0.1ul effective volume.
Row E: 100ul of 1:10,000 is = 0.01ul effective volume.

The effective volume 1ul, 0.1ul, and 0.01ul infections are labeled C, D, E on the titer plates, which corresponds to the row of dilution plate they came from.

Add 100ul of the dilutions from C, D, and E to MDCKSIAT1-PB1 cells as indicated at **8:30 pm.**

Collect at ~17 hrs, which is **1:30pm**

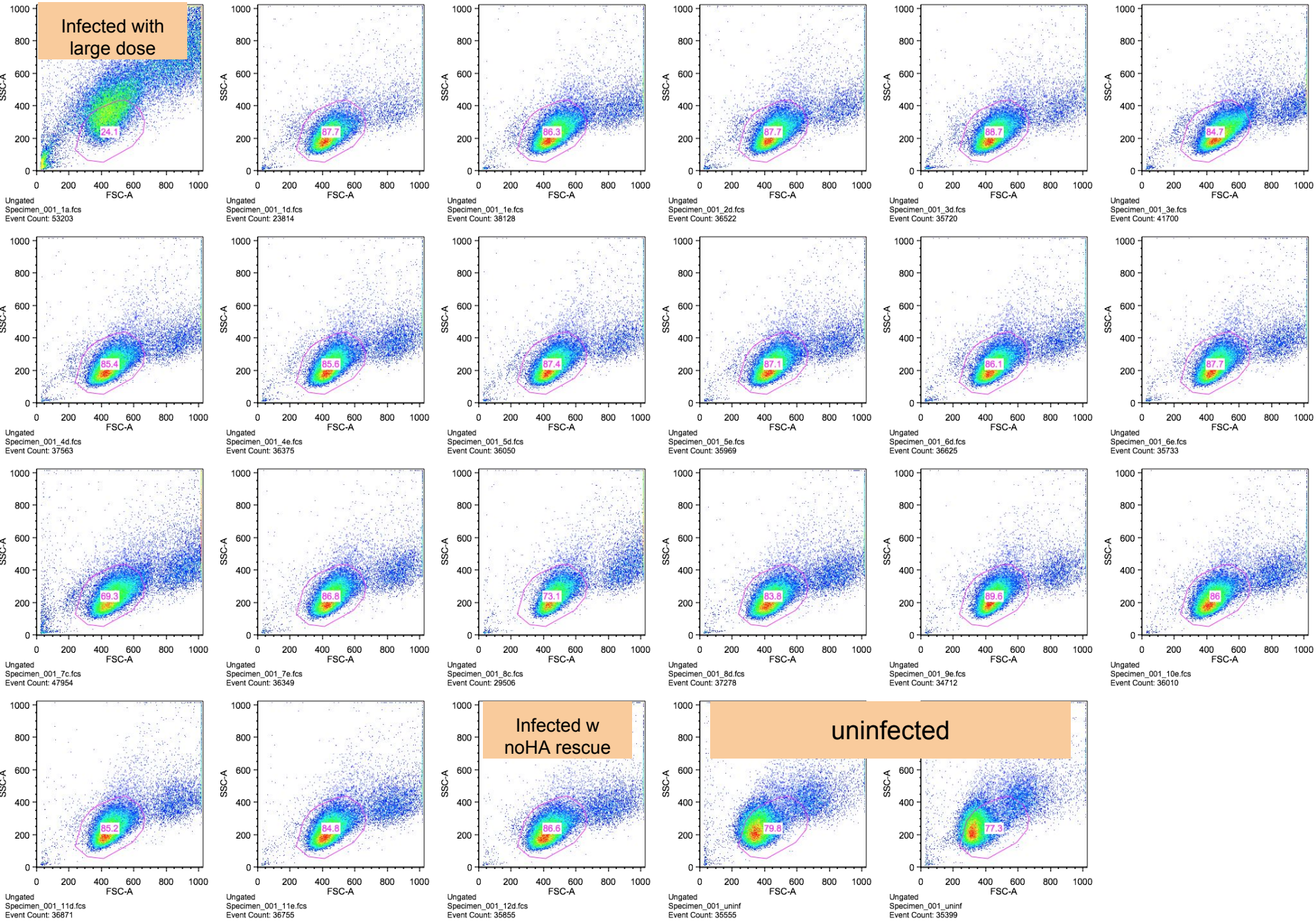
trypsinize with 350ul, quench and collect with 750ul D10, spin 1200rpm 5 min, and resuspend in 400ul PBS+1%PFA.
Allow 10 minutes incubation in resuspension to inactivate virus, then spin again and resuspend in FACS buffer (PBS+1% BSA).
Strain into FACS tubes on ice.

218586

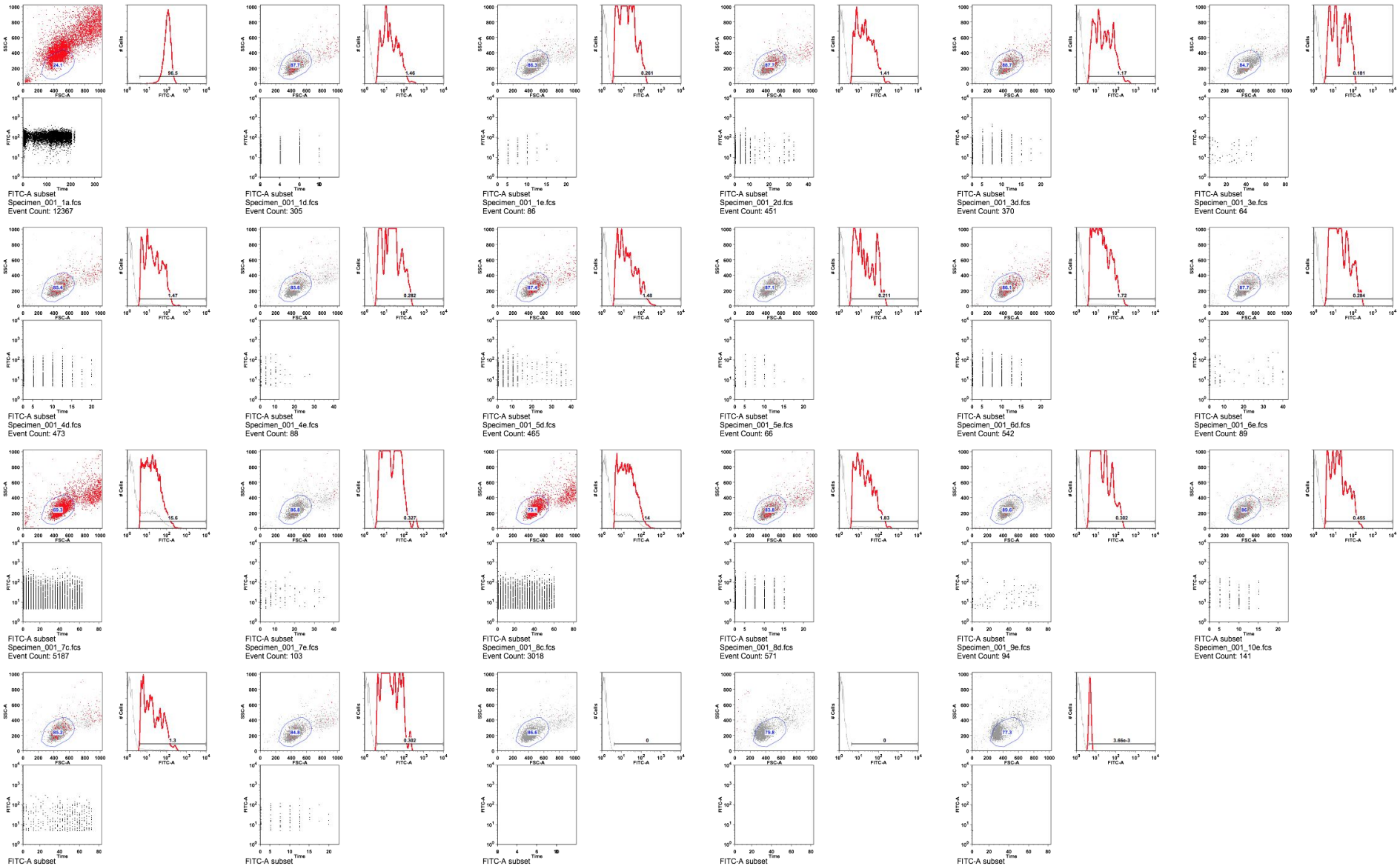
Previous Voltages:
FSC: 10
SSC: 275
FITC: 242

Appointment for 3:15

Here are the FSC/SSC and gate for analysis of GFP+ %.

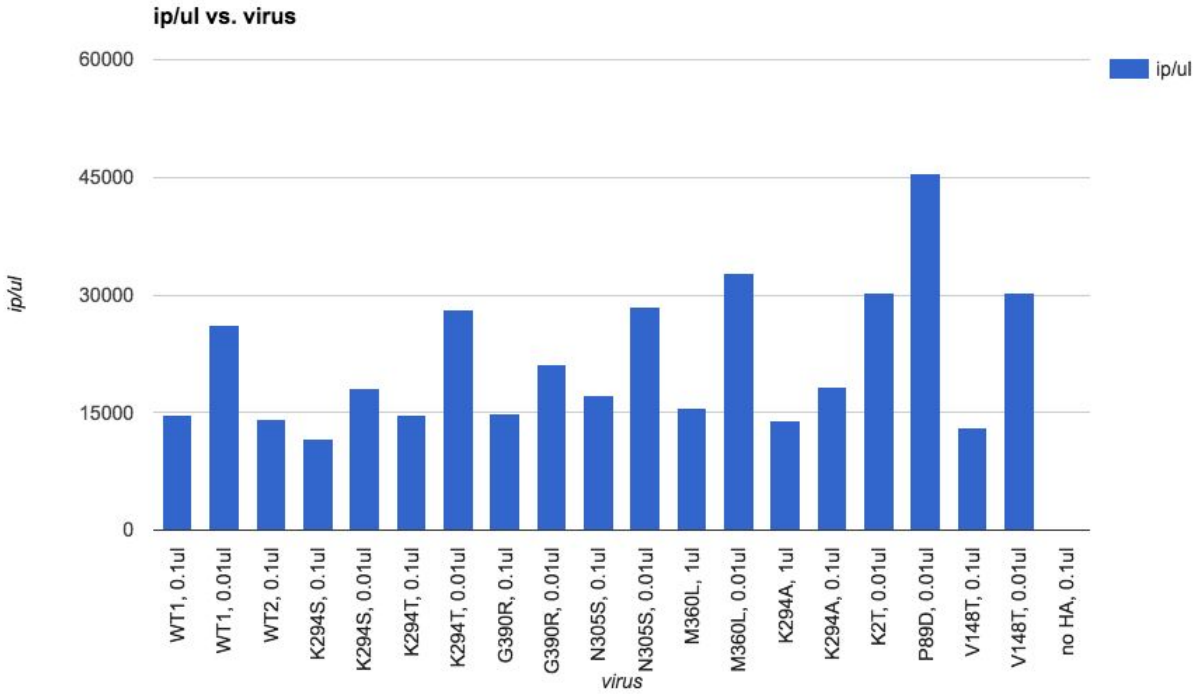


Here is the backgating analysis. I wanted to make sure GFP+ events were evenly distributed throughout time for each sample - this is the case. Interesting how the average GFP intensity among GFP+ cells (red histograms) seems uniformly high for the high-infection dose, but broader among the lower-infection doses actually used for titering.



Here are the titers for each measurement. WT1 and WT2 are biological replicate rescues. For samples titered at both 0.1ul and 0.01ul, it generally seems that the 0.01ul titer divided by two is comparable to the 0.1ul titer.
Thus, to be most consistent in calling titers across all these samples, I will use the titer measured at 0.1ul, OR if this doesn't exist, use the 0.01ul titer / 2 (red text values below). (I am following this convention *only* for the first pilot assay, but after that I will be using the 0.01ul titers to measure saturation with virus to find the linear range of virus to use in the assay.)

virus	ip/ul	0.01ul titer / 2
WT1, 0.1ul	14600	
WT1, 0.01ul	26100	13050
WT2, 0.1ul	14100	
K294S, 0.1ul	11700	
K294S, 0.01ul	18100	9050
K294T, 0.1ul	14700	
K294T, 0.01ul	28200	14100
G390R, 0.1ul	14800	
G390R, 0.01ul	21100	10550
N305S, 0.1ul	17200	
N305S, 0.01ul	28400	14200
M360L, 1ul	15600	
M360L, 0.01ul	32700	16350
K294A, 1ul	14000	
K294A, 0.1ul	18300	
K2T, 0.01ul	30200	15100
P89D, 0.01ul	45500	22750
V148T, 0.1ul	13000	
V148T, 0.01ul	30200	15100
no HA, 0.1ul	0	



2017-04-11 Pilot neutralization assays with FI6v3. This experiment will serve two purposes:

1. Confirm that the range of FI6v3 concentrations tested is optimal
2. Confirm that experimental noise due to the following factors is low:
   a. technical repetition of the neutralization assay with the same virus stock
   b. Neutralization assays with biological replicate reverse genetics rescues of the WT virus
   c. 3-fold variation in the total ip/ul of virus used in the assay

Used the 0.1ul titers in this experiment; in future saturation assay will use 0.01ul titers (which are ~2x)

I will do four assay plates:

1. WT #1
2. WT #1 repeat (technical duplicate of assay)
3. WT #2 (using biological replicate reverse genetics rescue of WT virus)
4. WT #1, lowdose (using 1/3rd the amount of virus as plates 1 and 2)

In contrast to previous neutralization assays, here I will do ~2.3-fold dilutions to try to more finely resolve the curve around the IC50. This will be accomplished by serially transferring 60ul of antibody to 80ul of WNM (before I would usually do 3-fold dilutions by transferring 40ul to 80ul WNM).

only WNM	virus only	cells + virus	cells + virus + ab	cells + virus + ab	cells + virus + ab	cells + virus	only WNM
A	B	C	D	E	F	G	H

WNM (WSN neutralization media) is NAM + 0.5% FBS. Use one batch for entire experiment.
Each well receives 40ul of virus at 1e3 ip/ul (**except for the low-dose plate**), so each full plate as above requires 6columns*12rows = 72wells*40ul = 2880ul @ 1e3ip/ul.
Each well receives 40ul of cells at 1e6/ml, so each full plate as above requires 5 columns x 12 rows = 60 wells * 40ul = 2400ul @ 1e6/ml.
I will use an antibody stock solution diluted to **8ug/ml** and perform serial **1:2.33** dilutions by transferring 60 ul of antibody to 80 ul WNM.
Thus, each plate needs 3*60ul = 180ul of antibody, so need 4*180=720ul of diluted antibody stock. **Add 9.64ul to 990.4ul.**
After serial dilutions and adding 40ul of virus, the final concentration of mAb during incubation with virus is **2.29 ug/ml in row 1**.

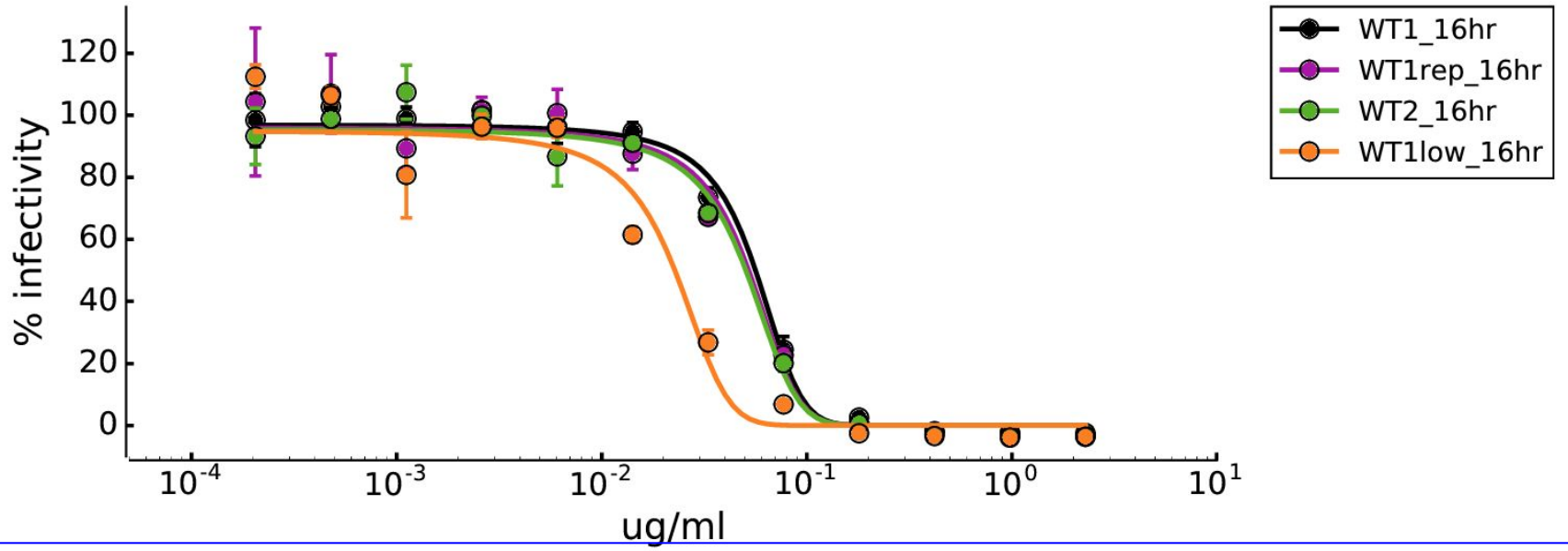
Neutralization assay protocol: started at 4pm, finished at (cells added 6:55-7:10)pm, read plates at 11am=16hpi, ... (try 16, 18, 20, 22 hrs post-infection).

1. Make antibody stock solution by diluting FI6v3 in WNM to **8ug/ml.**
   a. **Add 9.64ul of 830ug/ml FI6v3 storage stock to 990.4ul WNM. Save 830ug/ml stock at 4˚, dated with thaw date.**
2. Add 80 ul WNM to all wells of all plates (<35 mL WNM for 4 plates).
   a. Begin thawing virus aliquots.
3. Add **60 ul of the 8ug/ml FI6v3** to row 1 of mab columns of each plate (Columns D/E/F) and 60ul WNM to the others (A/B/C/G/H). Save the remaining ab at 4˚C.
4. Multichannel **60ul serial** dilutions down rows, without changing tips, and remove 60ul from last row. Finish all plates before going on.
5. Add Extra WNM to wells that don't receive all components so that all volumes will be the same in the end:
   a. Add 80ul WNM to columns A/H to make up for no cells or virus.
   b. Add 40ul WNM to column B to make up for no cells.
6. Prepare virus inocula at 1e3 ip/ul (or 3.3e2 ip/ul) by adding various amounts of TF supt to WNM as shown in worksheet.
7. Add 40 ul virus to columns B/C/D/E/F/G (here I usually gently mix the virus with the antibody by pipetting up and down one time while adding the virus, however, in this experiment i forgot to do this for the first plate and stayed consistent and didn't mix at all while adding… thinking that this probably doesn't matter, given the virus+ab incubates for 1 hr before adding cells...), adding one row of 6 tips at a time. Complete this for each plate, and then place plate in incubator after noting the time and order that plates begin incubation.
8. Incubate plates at 37 C for ~1 hour while preparing cells.
9. Trypsinize MDCK-SIAT1-CMV-PB1 cells, quench with D10, spin 5 min at 200xg, *resuspend in WNM*, and make >>10mL @ 1e6/ml.
10. After the 1 hour incubation of virus with antibody is complete, Add 40 ul cells to all wells in columns C/D/E/F/G (Add one row of 5 tips at a time).
11. Incubate at 37 C for 16-22 hours, and read using plate reader. Only read for columns B through G.

2017-04-11 calculation worksheet for neutralization assays

virus plate	ip/ul	ip/ul diluted virus	ul virus stock	ul WNM
WT1	14600	1.00E+03	239.7	3260.3
WT1repeat	14600	1.00E+03	239.7	3260.3
WT2	14100	1.00E+03	248.2	3251.8
WT1lowdose	14600	3.33E+02	79.9	3420.1

2017-04-12 results of neutralization assay - this is for the 16hr reading; 20hr reading is very similar.



There is **very little experimental noise** involved with either technical repeats of the assay plate for the same stock of WT virus, or between assays on replicate reverse genetics rescues of WT virus. This is pretty good because it should allow for sensitive detection of shifts in the neutralization curve. However, decreasing the amount of virus used in the assay by 3-fold results in a shift in the curve. Why is this?

It's possible that I'm starting with an over-saturating amount of virus. Especially since in contrast with previous assays where I always used titers based on 0.01ul infection, for this assay I used titers based on 0.1ul infection, which are lower, thus I probably added more ip/ul in this assay than in previous assays. IF the MOI is ultimately much higher than it should be, I could imagine that it takes some extra antibody neutralization to get into the range of infectious particles present where changes in % infection can be seen upon further addition of antibody.

Next I will titrate the virus dose used for each variant in the neutralization assay by infecting cells with dilutions of virus (no antibody) to make sure the viral dose used for each variant is in the linear range, where decreases in virus give proportional decreases in GFP signal.

2017-4-14: Testing serial dilutions of each virus to find appropriate dose for neutralization assays

Now I am using **titers measured with 0.01ul infections**, except for K294A, which is estimated based on the 0.1ul infection, and WT2, which is based on the WT 1 titer measured at 0.01ul (the 0.1ul titers for these two viruses were nearly identical). I will make 2-fold virus dilutions to achieve the following MOI:

ip / well	MOI	Row
160000	4	A
80000	2	B
40000	1	C
20000	0.5	D
10000	0.25	E
5000	0.125	F
2500	0.0625	G
1250	0.03125	H

For each virus, I will dilute two columns - one with cells added, and one with no cells to measure background from virus supt. Each plate will test five viruses as shown here:

	virus+cells	virus alone	virus+cells	virus alone	virus+cells	virus alone	virus+cells	virus alone	virus+cells	virus alone	
1	2	3	4	5	6	7	8	9	10	11	12

The two plates will test the viruses in this order:

Plate 1: WT2 (⅔), K294S (⅝), K294T (6/7), G390R (8/9), N305S (10/11)

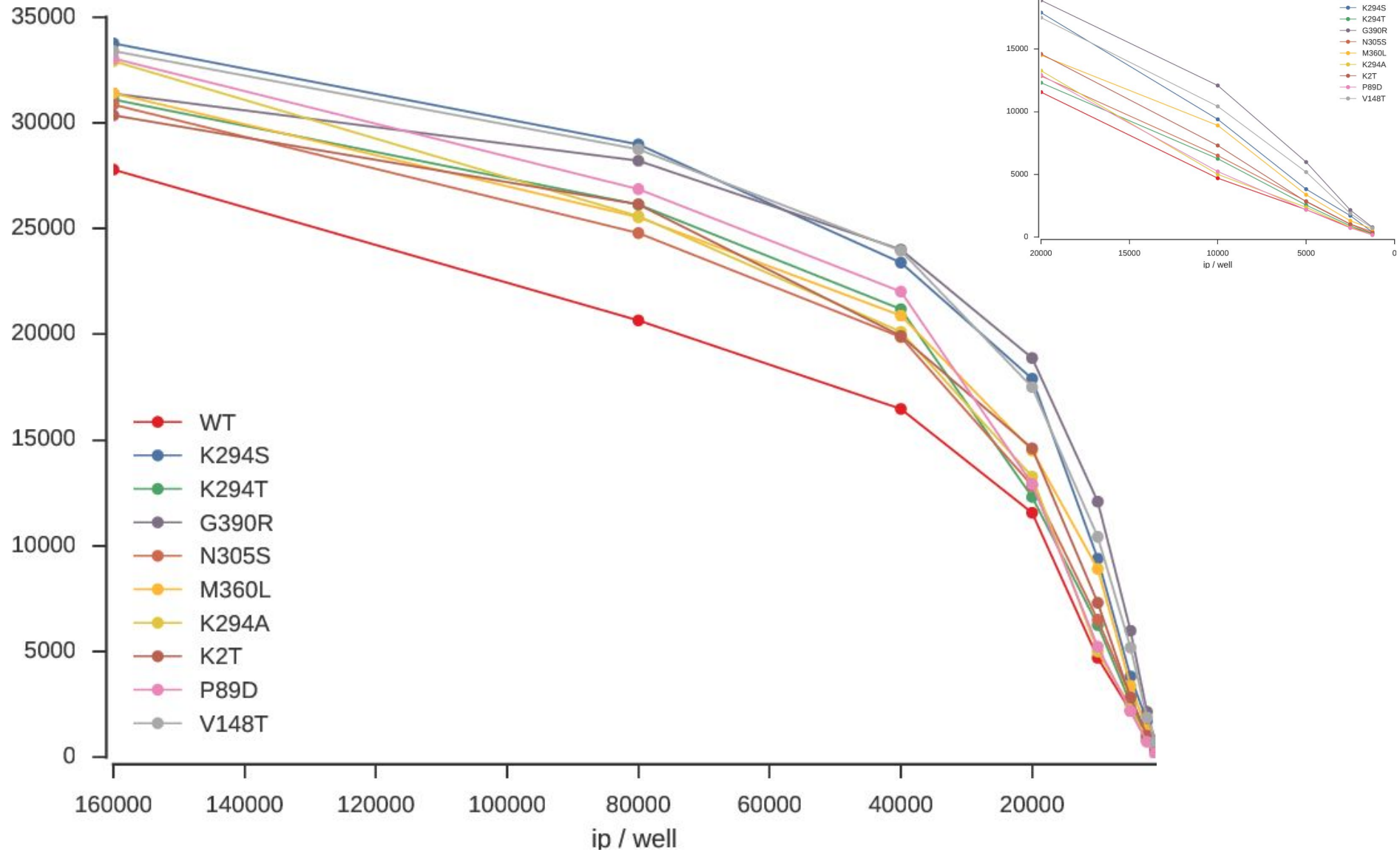
Plate 2: M360L (⅔), K294A (⅝), K2T (6/7), P89D (8/9), V148T (10/11)

Here is the protocol:

1. Add 80 ul WNM all wells
2. Add 3.2e5 ip in 80ul virus to row A (dilute virus to 4e3ip/ul by adding the volumes on the right to 200ul WNM, then add 80ul to each row A well), each virus goes into two cols
3. serial transfer 80ul down rows to make 1:2 dilutions, remove final 80, change tips EACH ROW
4. incubate at 37C for ~1 to 1.5hr while preparing cell suspension
5. prepare SIAT1PB1 cells in WNM at 5e5/ml
6. Add 80ul cells to even columns and 80ul WNM to odd columns. Also add 80ul to unused cols 1 and 12. Added cells at 4:30pm.
7. Read 16hrs later by platereader

virus	ip/ul	ul virus to 200ul WNM
WT2, approx.	26100	36.20
K294S, 0.01ul	18100	56.74
K294T, 0.01ul	28200	33.06
G390R, 0.01ul	21100	46.78
N305S, 0.01ul	28400	32.79
M360L, 0.01ul	32700	27.87
K294A, approx	36600	24.54
K2T, 0.01ul	30200	30.53
P89D, 0.01ul	45500	19.28
V148T, 0.01ul	30200	30.53

Based on these results I will do the neutralization assays with 20,000 ip/well since the signal seems to be linear with decreasing ip doses after that. Insert plot is zoomed in to show the section from 20,000 ip/well and lower.



only WNM	virus only	cells + virus	cells + virus + ab	cells + virus + ab	cells + virus + ab	cells + virus	only WNM
A	B	C	D	E	F	G	H

WNM (WSN neutralization media) is NAM + 0.5% FBS.
Use one batch for entire experiment.
Each well receives 40ul of virus at 5e2 ip/ul, so each full plate as above requires 6columns*12rows = 72wells*40ul = 2880ul @ 5e2ip/ul.
Each well receives 40ul of cells at 1e6/ml, so each full plate as above requires 5 columns x 12 rows = 60 wells * 40ul = 2400ul @ 1e6/ml.
I will use an antibody stock solution diluted to **8ug/ml** and perform serial **1:2.33** dilutions by transferring 60 ul of antibody to 80 ul WNM.
Thus, each plate needs 3*60ul = 180ul of antibody.
After serial dilutions and adding 40ul of virus, the final concentration of mAb during incubation with virus is **2.29 ug/ml in row 1**.

plate	virus	ip/ul stock	ip/ul diluted	ul stock	ul WNM
1	WT1, 0.01ul	26100	5.00E+02	95.8	4904.2
2	K294S, 0.01ul	18100	5.00E+02	138.1	4861.9
3	K294T, 0.01ul	28200	5.00E+02	88.7	4911.3
4	G390R, 0.01ul	21100	5.00E+02	118.5	4881.5
5	N305S, 0.01ul	28400	5.00E+02	88.0	4912.0
6	M360L, 0.01ul	32700	5.00E+02	76.5	4923.5
7	K294A, approx	36600	5.00E+02	68.3	4931.7
8	K2T, 0.01ul	30200	5.00E+02	82.8	4917.2
9	P89D, 0.01ul	45500	5.00E+02	54.9	4945.1
10	V148T, 0.01ul	30200	5.00E+02	82.8	4917.2

2017-04-17 Neutralization assay protocol: started at 11:15am, finished at 3:40 (plate1)-4:20pm(plate10), read plates at_____

1. Make antibody stock solution by diluting FI6v3 in WNM to **8ug/ml.**
   a. **Add 19.28ul of 830ug/ml FI6v3 storage stock to 1980.8ul WNM. Save 830ug/ml stock at 4˚, dated with thaw date.**
2. Add 80 ul WNM to all wells of all plates (<87.5 mL WNM for 4 plates).
   a. Begin thawing virus aliquots.
3. Add **60 ul of the 8ug/ml FI6v3** to row 1 of mab columns of each plate (Columns D/E/F). Save the remaining ab at 4˚C.
4. Multichannel **60ul serial** dilutions down rows for D/E/F, without changing tips, and remove 60ul from last row. Finish all plates before going on.
5. Add 40ul WNM to column B to make up for no cells.
6. Prepare virus inocula at 5e2 ip/ul by adding various amounts of TF supt to final volume of 5ml WNM as shown in worksheet.
7. Add 40 ul virus to columns B/C/D/E/F/G (no mixing up and down each time, just add), adding one row of 6 tips at a time. Complete this for each plate, and then place plate in incubator after noting the time and order that plates begin incubation.
8. Incubate plates at 37 C for ~1.5 hour while preparing cells.
9. Trypsinize MDCK-SIAT1-CMV-PB1 cells, quench with D10, spin 5 min at 200xg, *resuspend in WNM*, and make ~30+mL @ 1e6/ml. **[two 1:5 15-cm dishes from yesterday (resuspended in ~35ml WNM) were just enough for this]**
10. After the 1 hour incubation of virus with antibody is complete, Add 40 ul cells to all wells in columns C/D/E/F/G (Add one row of 5 tips at a time, >2.4ml per plate, make ~30ml total).
11. Incubate at 37 C for 16-22 hours, and read using plate reader. Only read for columns B through G.

plate	virus	incubate virus/ab	add cells
1	WT1, 0.01ul	1:50	3:40
2	K294S, 0.01ul	1:57	3:45
3	K294T, 0.01ul	2:04	3:50
4	G390R, 0.01ul	2:09	3:54
5	N305S, 0.01ul	2:14	4:00
6	M360L, 0.01ul	2:20	4:04
7	K294A, approx	2:24	4:08
8	K2T, 0.01ul	2:30	4:15
9	P89D, 0.01ul	2:35	4:18
10	V148T, 0.01ul	2:40	4:20

Plate reading times:

Begin at 9:40am (18hr)

Files are called 17 hr but **it is actually 18 hours.**

2017-04-20

H17-L19 neutralization assay on some of these mutants.
Worksheet in “2017-04-17 FI6v3 neut assay mutants setup”.
Using original L19 antibody aliquots from SH.

only WNM	virus only	cells + virus	cells + virus + ab	cells + virus + ab	cells + virus + ab	cells + virus	only WNM
A	B	C	D	E	F	G	H

plate	virus	ip/ul stock	ip/ul diluted	ul stock	ul WNM	incubate virus/ab	add cells
1	WT1, 0.01ul	26100	5.00E+02	95.8	4904.2	1:25	2:50
2	K294S, 0.01ul	18100	5.00E+02	138.1	4861.9	1:30	2:54
3	K294T, 0.01ul	28200	5.00E+02	88.7	4911.3	1:36	3:00
4	G390R, 0.01ul	21100	5.00E+02	118.5	4881.5	1:44	3:04
5	N305S, 0.01ul	28400	5.00E+02	88.0	4912.0	1:50	3:08
6	V148T, 0.01ul	30200	5.00E+02	82.8	4917.2	1:55	3:14

2017-04-20 Neutralization assay protocol (H17-L19 against Fl6v3 muts)

1. Make antibody stock solution by diluting H17-L10 in WNM to **40ug/ml.**
   a. **Add 50 ul of 1000ug/ml H17-L19 storage stock to 1200 ul WNM.**
2. Add 80 ul WNM to all wells of all plates (<87.5 mL WNM for 4 plates).
   a. Begin thawing virus aliquots.
3. Add **60 ul of the 40ug/ml L19** to row 1 of mab columns of each plate (Columns D/E/F). Save the remaining ab at 4˚C.
4. Multichannel **60ul serial** dilutions down rows for D/E/F, without changing tips, and remove 60ul from last row. Finish all plates before going on.
5. Add 40ul WNM to column B to make up for no cells.
6. Prepare virus inocula at 5e2 ip/ul by adding various amounts of TF supt to final volume of 5ml WNM as shown in worksheet.
7. Add 40 ul virus to columns B/C/D/E/F/G (no mixing up and down each time, just add), adding one row of 6 tips at a time. Complete this for each plate, and then place plate in incubator after noting the time and order that plates begin incubation.
8. Incubate plates at 37 C for ~1.5 hour while preparing cells.
9. Trypsinize MDCK-SIAT1-CMV-PB1 cells, quench with D10, spin 5 min at 200xg, *resuspend in WNM*, and make ~15+mL @ 1e6/ml.
10. After the 1 hour incubation of virus with antibody is complete, Add 40 ul cells to all wells in columns C/D/E/F/G (Add one row of 5 tips at a time, >2.4ml per plate, make ~30ml total).
11. Incubate at 37 C for 16-22 hours, and read using plate reader. Only read for columns B through G.