Perth/2009 HA mutant library rescue and passage (paired with WSN NA)

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November 10, 2016 – February 4, 2017

Plan for rescue of Perth/2009 HA mutant libraries

- I will transfect recently thawed **MDCK-SIAT1-TMPRSS2** cells with 4X RNP only (negative control), 4X RNP + WT Perth/2009 HA, 4X RNP + 3 plasmid mutant libraries of Perth/2009 HA, or 4X RNP + pICR2-PB1-flank-eGFP (to estimate transfection efficiency) using **lipofectamine** in **6-well plates** (5e5 cells per well)

- 4 hpt, I will change the media of the transfected cells to fresh D10

- 18 hpt, I will infect the transfected cells with Mike's MD WSN helper virus replicate 3 (17782 TCID50/ul) at an MOI = 1.0 (1e6 TCID50 total per well). I will change the media of the infected cells to fresh IGM 3 hpi

- 23 hpi, I will harvest the viral supernatant, spin down to clarify the supernatant, and make aliquots which I will then freeze down at -80°C

- I will titer the rescued viruses by TCID50 in TMPRSS2 cells

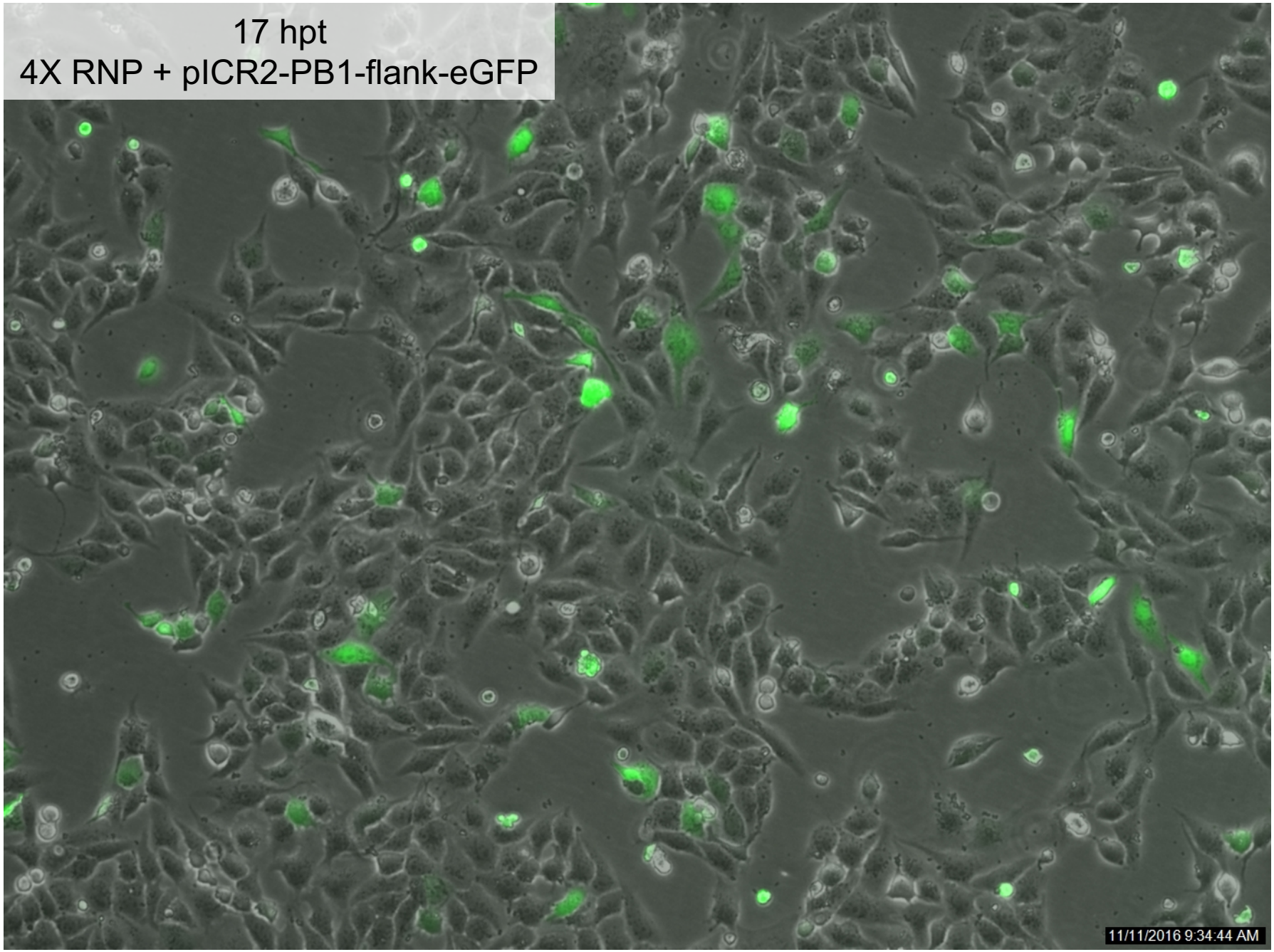
- **When rescued, the Perth/2009 HA mutant virus libraries will be paired with the WSN NA**

November 10, 2016

1. **Transfections:** I will transfect cells to rescue my three replicate Perth/2009 HA libraries. I will also have 4X RNP + WT Perth/2009 HA and 4X RNP only controls. I will also have a 4X RNP + PB1flank-eGFP control to estimate my transfection efficiency based on the number of cells that are green the following morning. All of the HA's and the PB1-flank-eGFP are in the pICR2 plasmid (plasmid #96) under the control of the canine PolI promoter
    - Set up eppendorf tubes with plasmid(s) (see slide 4) – made three master mixes of 4X RNP + each of the Perth/2009 HA libraries. Also made a 4X RNP only master mix, aliquoted this master mix into separate eppendorf tubes, and then added WT Perth/2009 HA or pICR2-PB1-flank-eGFP to its respective tube
    - Added 125 ul Opti-MEM to each transfection mixture
    - Made a master mix of lipofectamine 3000 with Opti-MEM: (79x7.5) 592.5 ul LF + (79x125) 9875 ul Opti-MEM. Vortexed mixtures for 3 s
    - Added 10 ul P3000 reagent to the DNA/Opti-MEM mixture. Mixed by pipetting
    - Added 125 ul of the lipofectamine/Opti-MEM mixture to each tube containing the DNA/Opti-MEM/P3000 mixture. Mixed by pipetting
    - Incubated mixtures at RT for > 80 min
    - While DNA was incubating, split TMPRSS2 cells from confluent 15 cm plates. Spun cells at **100xg for 5 min** and resuspend in fresh D10. Counted cells (want a final concentration of 5 x 10^5 cells/ml). Made two 45 ml master mixes
        - Used TMPRSS2 cells thawed on Nov. 3, 2016
        - Counted 1.8 x 10^6 cells/ml from two confluent 15 cm plates combined => added 13 ml cells to each master mix
    - Added 750 ul D10 to the DNA/lipofectamine mixture (60 ml D10 in two 50-ml conical)
    - Added cells to 6-well plates (14 6-well plates) – added 1 ml cells to each of three wells, then transfected these three wells at a time
    - Added 1 ml of the DNA/lipofectamine/D10 mixture to its corresponding well of cells by slowly pipetting down, then up once, then back down. Rocked each plate gently after addition of DNA mixture. For no transfection wells, added 1 ml D10 only
    - Incubated cells at 37°C (Start first well @ 4:30 PM, finish last well @ 6:20 PM)
        - DNA incubation lasted 1 h 30 min for first transfection tube (4X RNP only), 1 h 45 min for first library transfection (lib1), and 2 h 52 min for last tube (last lib3 transfection tube)
    - ~4 h post-transfection (START: 8:30 PM), I changed the media of all the wells to fresh D10. Aspirated old D10 and gently added 2 ml fresh pre-warmed D10 to each well. Used the P1000 pipet to add 1 ml twice to each well

November 11, 2016

1. Checked transfected cells (see above) ~17 h post-transfection (9:30 AM). Saw some cell death for all cells that were transfected. Generally, ~60-75% confluent throughout the well, which is higher confluency than I have typically observed from other transfections. Untransfected cells were ~90-100% confluent throughout the entire well. Looking at the 4X RNP + PB1-flank-eGFP transfection, approximately 25-30% of the cells were green. Picture on slide 3. Transfection efficiency looks reasonable judging from the three 4X RNP + PB1-flank-eGFP transfections, so I will go ahead and infect the cells with MD WSN helper virus rep. 3


17 hpt
4X RNP + pICR2-PB1-flank-eGFP
11/11/2016 9:34:44 AM

#	Transfection	HA plasmid	Helper virus infection
1-21	4X RNP + P09 HA lib1	Perth/2009 HA lib1	MD WSN hv Rep. 3
22-42	4X RNP + P09 HA lib2	Perth/2009 HA lib2	MD WSN hv Rep. 3
43-63	4X RNP + P09 HA lib3	Perth/2009 HA lib3	MD WSN hv Rep. 3
64-72	4X RNP + Perth/09 HA	WT Perth/2009 HA	MD WSN hv Rep. 3
73-75	4X RNP only	none	MD WSN hv Rep. 3
76-78	4X RNP + PB1-flank-eGFP	PB1-flank-eGFP	none

Library master mixes 5 ug total *Prepare 3x 4X RNP MM*

4X RNP	Plasmid	Plasmid name	Conc (ng/ul)	Amount (ng)	Vol for amt (ul)	# Transfections	Vol in 22X master mix	Total vol MM	Vol per tube
	1053	HDM-Nan95-PB2	1012.3	937.5	0.93	21	20.46		
	1054	HDM-Nan95-PB1	879.9	937.5	1.07		23.54		
	1055	HDM-Nan95-PA	830	937.5	1.13		24.86		
	793	HDM-Aichi68-NP	789.9	937.5	1.19		26.18		
		Perth/09 HA lib1	699.3	1250	1.79	X 22	39.38	134.42	6.11
		Perth/09 HA lib2	799.2	1250	1.57	X 22	34.54	129.58	5.89
		Perth/09 HA lib3	942.8	1250	1.33	X 22	29.26	124.3	5.65

Control master mixes

4X RNP	Plasmid	Plasmid name	Conc (ng/ul)	Amount (ng)	Vol for amt (ul)	# Transfections	Vol in 16X master mix	Total vol MM
	1053	HDM-Nan95-PB2	1012.3	937.5	0.93	15	14.88	69.12
	1054	HDM-Nan95-PB1	879.9	937.5	1.07		17.12	Vol MM/TF
	1055	HDM-Nan95-PA	830	937.5	1.13		18.08	4.32
	793	HDM-Aichi68-NP	789.9	937.5	1.19		19.04	
WT Perth/09 HA	1535	pICR2-Perth09-HA	531.3	1250	2.36			
PB1-flank-eGFP	1405	pICR2-PB1flank-eGFP	749.3	1250	1.67			

2. **Infected transfected cells:** Infected transfected cells from yesterday ~18h post-transfection with various helper viruses
    - Thawed 5 aliquots of MD WSN hv replicate 3 and made master mixes so that the final concentration would be 500 TCID50/ul. Made four separate master mixes in 50-ml conicals – one for each library and one for the 4X RNP controls (4X RNP only and 4X RNP + WT Perth/09 HA). 3 aliquots of Mike's MD WSN hv rep. 3 remain
    - Aspirated D10 from each well and gently added 2 ml of the inoculum to each well (used P1000 pipet to add 1 ml twice)
    - Rocked plates gently and incubated at 37°C
        - Infected first well at 10:40 AM, finished at 1 PM
    - ~3h post-infection (start first well at 2:30 PM, finish at 4:15 PM), aspirated the inoculum and gently added 2 ml fresh, pre-warmed IGM to each well. Incubated at 37°C

Need 500 TCID50/ul in MM						
Helper virus	**Titer (TCID50/ul)**	**Number of infections**	**Vol virus (ul)**	**Vol IGM (ul)**	**Total vol (ml)**	**# aliquots needed**
MD WSN hv (rep. 3)	17782	75				5
		4X controls	703	24297	25	
		Lib1	1270	43730	45	
		Lib2	1270	43730	45	
		Lib3	1270	43730	45	

November 12, 2016

1. Checked infected cells at 9 AM
    - Saw lots of CPE (~60%) for 4X RNP transfections infected with each of the helper viruses
    - Saw 50-60% CPE for 4X RNP + HA transfections infected with each of the helper viruses
2. **Harvested viral supernatant** from transfected cells infected with helper virus. Harvested viral supernatant ~23.5 hpi (start at 10AM, finish at 12:30 PM)
    - For each library, harvested ~40 ml of viral supernatant in separate 50-ml conical tubes. Harvested ~18 ml for WT Perth/2009 HA control, and ~6 ml for 4X RNP only controls. For each, made 1 ml aliquots in labeled Cryotubes
        - Made 40 aliquots of LIB1, 38 aliquots of LIB2, 39 aliquots of LIB3, 8 aliquots of WT Perth/2009 HA, and 4 aliquots of 4X RNP only control
3. **TCID50 assay**: set up TCID50 of the rescued libraries
    - Added 50 ul of the undiluted viruses above to row A. Had 8 replicates of each virus. Did serial 10-fold dilutions down each plate
    - Used TMPRSS2 cell lines to titer
    - Incubated plates at 37°C (START: 6PM)

November 15, 2016

1. **TCID50 titer:** scored TCID50 at 11 AM (65 hpi). Saw only CPE in row A of 4X RNP only control for some replicates
    - WT Perth/2009 HA: 734.932 TCID50/ul
    - Perth/2009 LIB1: 92.832 TCID50/ul
    - Perth/2009 LIB2: 536.539 TCID50/ul
    - Perth/2009 LIB3: 536.539 TCID50/ul
    - These titers look reasonable, and I will go ahead with piloting passage of one of my libraries

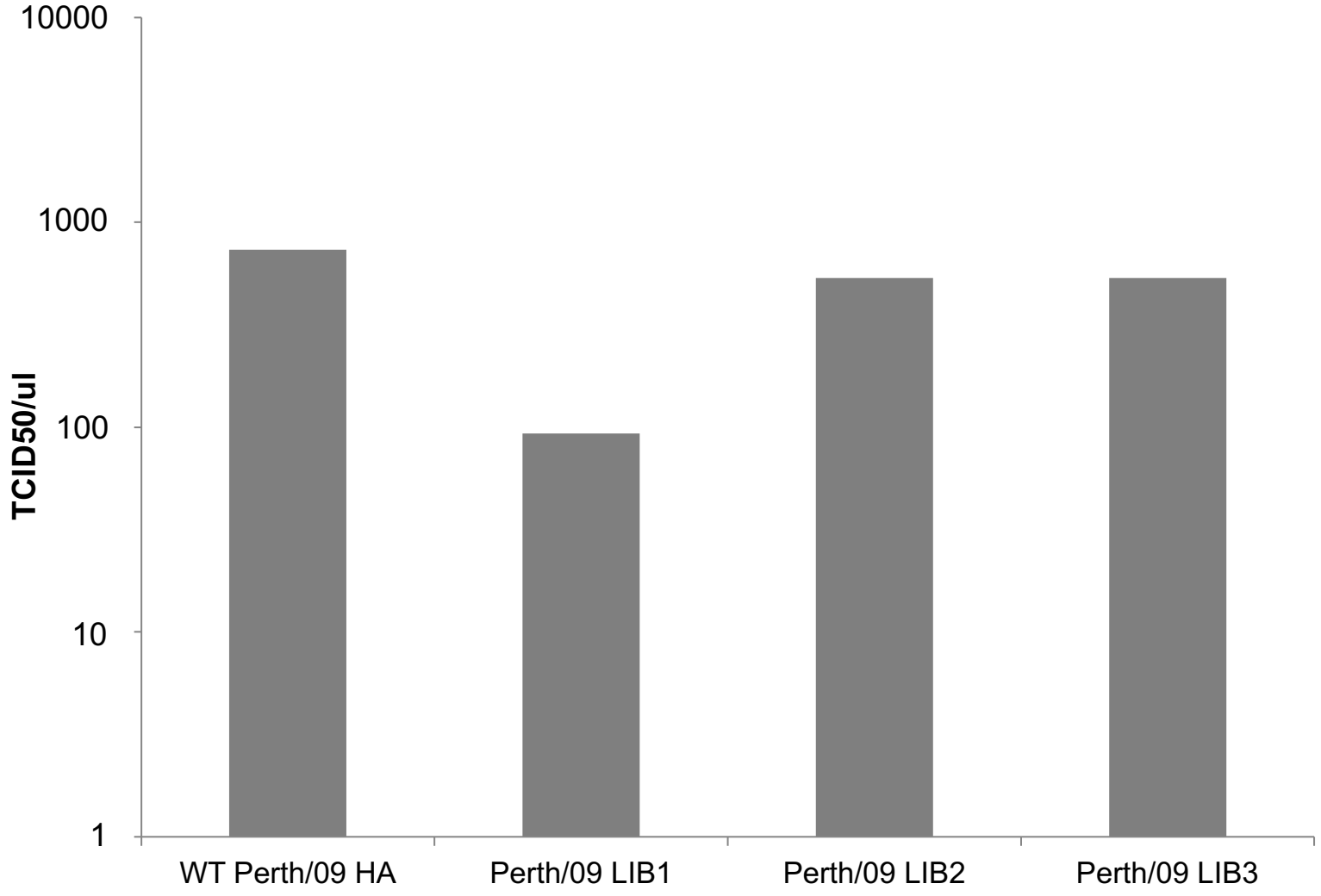
VOLUME 50
DILUTION 10
NREPLICATES 8

SAMPLE WT-Perth09
A, B, C, D
A, B, C, D, E
A, B, C, D
A, B, C, D
A, B, C, D, E, F, G
A, B, C, D, E, F, G
A, B, C, D, E
A, B, C, D, E

SAMPLE Perth09 LIB1
A, B, C, D
A, B, C, D, E
A, B, C, D
A, B, C, D
A, B, C, D, E
A, B, C, D
A, B, C, D
A, B, C, D

SAMPLE Perth09 LIB2
A, B, C, D
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E

SAMPLE WILDTYPE
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D, E
A, B, C, D



PILOT PASSAGE OF LIBRARY 3 – I will pilot a passage of library 3 in TMPRSS2 cells, generally following Mike's passage protocol, and collect at various time points (0, 24, 30, 40 hpi) and titer to see what the best time point to collect my passaged libraries is

November 15, 2016

1. **Seeded TMPRSS2 cells:** split a plate of confluent TMPRSS2 cells (thawed Nov 8, 2016). Seeded 6e6 cells per plate into two 15-cm plates (seeded at 3:35 PM)

November 16, 2016

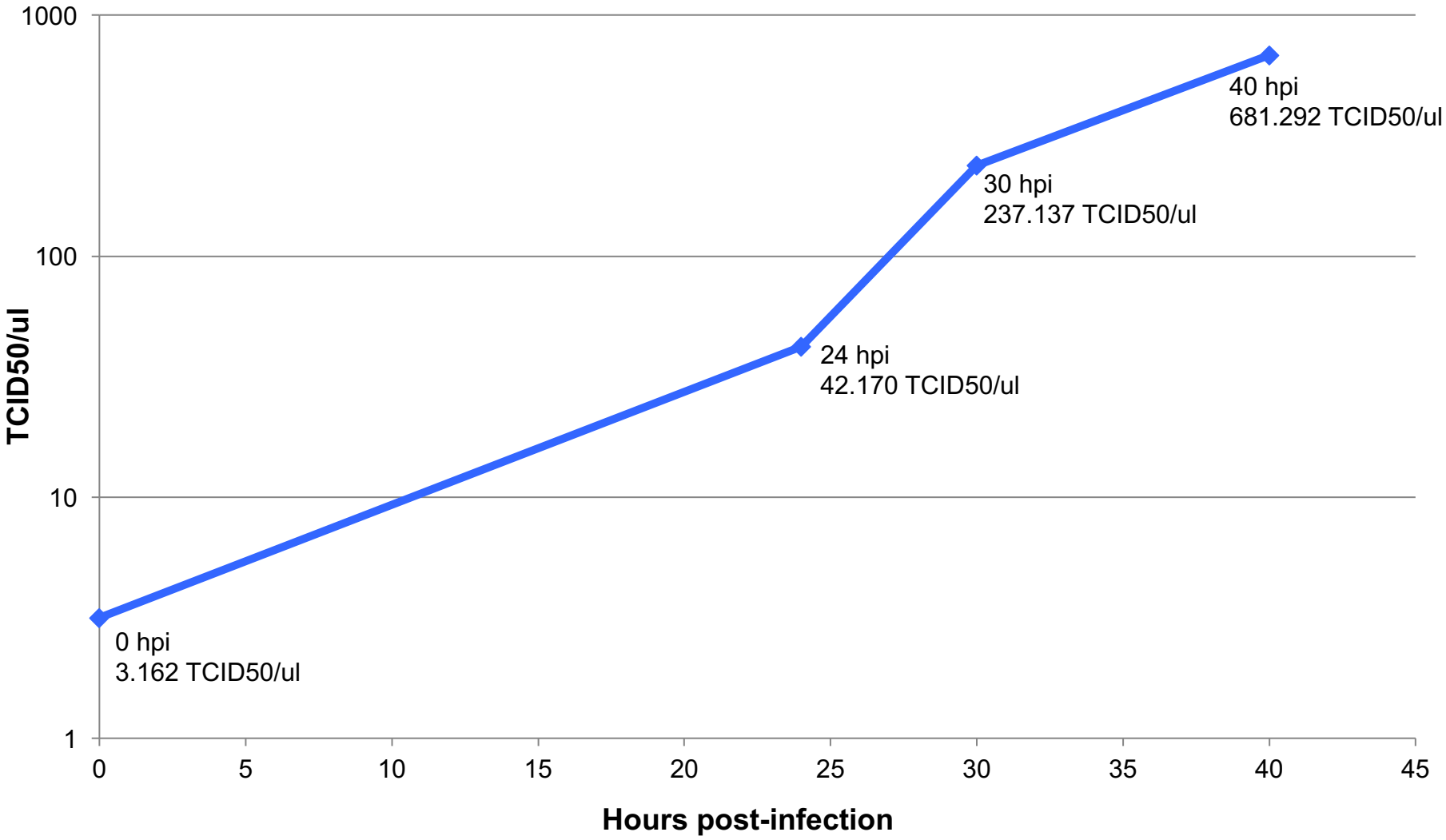
1. **Infected TMPRSS2 cells**: at 4 PM, split the extra 15 cm plate of TMPRSS2 cells and counted cells using hemacytometer. Counted 0.9 x 10^6 cells/ml, for a total of 22.5e6 cells total. I will infect the other 15 cm plate of TMPRSS2 cells at an MOI of 0.0075 = 168,750 TCID50 total
    - Perth09 lib3 rescue titer: 536.539 TCID50/ul => need 315 ul to infect the plate of cells
    - Made an inoculum of 25 ml IGM + 315 ul Perth/09 HA lib3 rescued and harvested 20161112, and gently mixed 3X's with serological pipet
    - Aspirated D10 from plate of cells. Added the viral inoculum to the plate by pipetting down the side of the plate. Rocked gently back and forth a couple of times. Removed a 400 ul aliquot from the plate, which is a 0 hpi time point collection, and froze this aliquot at -80°C. Incubated the plate at 37°C (START: 5:15 PM)

November 17, 2016

1. At **24 hpi**, removed a 400 ul aliquot of the viral supernatant from the 15 cm plate. Froze down aliquot at -80°C
2. At **30 hpi**, removed another 400 ul aliquot of the viral supernatant from the 15 cm plate. Froze down aliquot at -80°C
3. At both of these time points, I noticed very little CPE. The cells were getting quite confluent and were small in size

November 18, 2016

1. At **40 hpi**, removed a 400 ul aliquot of the viral supernatant from the 15 cm plate. Froze down aliquot at -80°C. Noticed small islands of slightly sick-looking cells and some floaties, but not very many
2. **TCID50 assay**: set up a TCID50 of the library 3 pilot expansion time points that I collected (0, 24, 30, and 40 hpi). Added 10 ul of viral supernatant to 90 ul IGM to row A, and titered 5 replicates of each sample
    - From the pilot expansion, it looks like 40 hpi is the best time to collect the viral supernatant. I probably don't want to collect past this point to minimize formation of defective particles


1000
100
10
1
TCID50/ul
0
5
10
15
20
25
30
35
40
45
Hours post-infection
0 hpi
3.162 TCID50/ul
24 hpi
42.170 TCID50/ul
30 hpi
237.137 TCID50/ul
40 hpi
681.292 TCID50/ul

Plan for passage of Perth/2009 HA mutant libraries

- I will stagger passage of each of my libraries + WT control + no HA control
- I will passage my libraries + ctrls by seeding MDCK-SIAT1-TMPRSS2 cells at 6e6 cells per dish, infecting ~24 h after seeding at an MOI of 0.0075, then harvesting the viral supernatant 40 hpi
- I will use 15x15 cm plates for each library (~375 ml viral supernatant), 10x15 cm plates for WT Perth/2009 HA control, and 2x15 cm plates for the no HA 4X RNP control

PASSAGE OF WT Perth/2009 HA and no HA controls

November 26, 2016

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells into a 10 cm plate

November 27, 2016

Split the thawed plate of cells into a 15 cm dish by adding all 10 ml of cells to 15 ml D10

November 29, 2016

Split the confluent 15 cm plate of cells into 4x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

December 1, 2016

Split each of the four confluent 15 cm plates into 12x15 cm plates (10 for WT passage and 2 for no HA passage). Added 6e6 cells per plate. Counted ~1.6e6 cells/ml from two of the plates, so added 3.75 ml of cells to each plate. Plated 5:35-5:45 PM

December 2, 2016

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 90-100% confluent on all plates, with some areas where cells were big and still had some room to grow, and some areas where the cells were starting to get compressed.

- Made two inoculums – made the WT Perth/09 HA inoculum in a T75 flask and the no HA inoculum in a 50 ml conical. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 10x15 cm plates with WT Perth/2009 HA virus and 2x15 cm plates with no HA control. Infected 6:15-6:30 PM:

WT Perth/2009 HA ctrl	
10x15 cm plates	250 ml supt
Titer from rescue (TCID50/ul)	734.932
Total cells	225000000
MOI 0.0075	1687500
Vol virus (ul)	2297
Aliquots needed	3

no HA ctrl	
2x15 cm plates	50 ml supt
Vol virus (ul)	2000

December 4, 2016

Checked infected cells 40 hpi: saw isolated foci of rounded/sick cells scattered across the plate, but little overall CPE (hard to say, but I estimate ~5-10%). At 11 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T75 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1 h. After 1 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 200 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 81 1-ml aliquots (1 freezer box) of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 7 12.5 ml aliquots in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 3, replicate 1 (because of the possibility of snow on Monday and a chance I may not be able to make it into lab early if there is snow, I will passage library 3 first since I have enough aliquots to repeat lib3 passage)

November 26, 2016

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells into a 10 cm plate

November 27, 2016

Split the thawed plate of cells into a 15 cm dish by adding 5 ml of cells to 15 ml D10

November 30, 2016

Split the confluent 15 cm plate of cells into 4x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

December 2, 2016

Split each of the four confluent 15 cm plates into 15x15 cm plates. Added 6e6 cells per plate. Counted ~2.15e6 cells/ml from two of the plates, so added 2.8 ml of cells to each plate. Plated 8:15 PM

December 3, 2016

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 90-100% confluent on all plates, with some areas where cells were big and still had some room to grow, and some areas where the cells were starting to get compressed. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 8:15-8:30 PM

LIBRARY 3	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	337500000
MOI 0.0075	2531250
Vol virus (ul)	4718
Aliquots needed	5

December 5, 2016

Checked infected cells 40 hpi: saw isolated foci of rounded/sick cells scattered across the plate, but little overall CPE. At 12:30 PM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T150 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1 h. After 1 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 18 250 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 81 1-ml aliquots (1 freezer box) of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 7 12.5 ml aliquots in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 1

November 26, 2016

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells into a 10 cm plate

November 27, 2016

Split the thawed plate of cells into a 15 cm dish by adding 5 ml of cells to 15 ml D10

November 30, 2016

Split the confluent 15 cm plate of cells 1:2 into a new 15 cm plate to split tomorrow

December 1, 2016

Split the confluent 15 cm plate of cells into 4x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

December 3, 2016

Split each of the four confluent 15 cm plates into 15x15 cm plates. Added 6e6 cells per plate. Counted ~2.1e6 cells/ml from two of the plates, so added 2.8 ml of cells to each plate. Plated 7 PM

December 4, 2016

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 90-100% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 7:15 PM

LIBRARY 1	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	92.832
Total cells	337500000
MOI 0.0075	2531250
Vol virus (ul)	27267
Aliquots needed	29

December 6, 2016

Checked infected cells 40 hpi: saw isolated foci of rounded/sick cells scattered across the plate, but little overall CPE. At 11:15AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a 500 ml media bottle, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1 h. After 1 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 200 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 81 1-ml aliquots (1 freezer box) of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 7 12.5 ml aliquots in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 2

November 26, 2016

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells into a 10 cm plate

November 27, 2016

Split the thawed plate of cells into a 15 cm dish by adding 5 ml of cells to 15 ml D10

November 30, 2016

Split the confluent 15 cm plate of cells 1:10 into a new 15 cm plate to split Friday

December 2, 2016

Split the confluent 15 cm plate of cells into 4x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

December 4, 2016

Split each of the four confluent 15 cm plates into 15x15 cm plates. Added 6e6 cells per plate. Counted ~2.11e6 cells/ml from two of the plates, so added 2.8 ml of cells to each plate. Plated 5:45 PM

December 5, 2016

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 90-100% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 6:15 PM

LIBRARY 2	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	337500000
MOI 0.0075	2531250
Vol virus (ul)	4718
Aliquots needed	5

December 7, 2016

Checked infected cells 40 hpi: saw isolated foci of rounded/sick cells scattered across the plate, but little overall CPE. At 10:15AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a 500 ml media bottle, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 250 ul aliquots and a couple of 400 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 81 1-ml aliquots (1 freezer box) of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 7 12.5 ml aliquots in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 3, replicate 2

December 4, 2016

Split a confluent 15 cm plate of cells into 4x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

December 6, 2016

Split each of the four confluent 15 cm plates into 15x15 cm plates. Added 6e6 cells per plate. Counted ~2.1e6 cells/ml from two of the plates, so added 2.8 ml of cells to each plate. Plated 7 PM

December 7, 2016

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 90-100% confluent on all plates, with some areas where cells were big and still had some room to grow, and some areas where the cells were starting to get compressed. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 7:15-7:30 PM

LIBRARY 3	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	337500000
MOI 0.0075	2531250
Vol virus (ul)	4718
Aliquots needed	5

December 9, 2016

Checked infected cells 40 hpi: saw isolated foci of rounded/sick cells scattered across the plate, but little overall CPE. At 11:30AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a 500 ml media bottle, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 5 1 ml aliquots in Cryotubes of the concentrated virus (we were on a limited supply of Crytotubes, so I made fewer aliquots in larger volumes).

During the first spin, made 81 1-ml aliquots (1 freezer box) of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 7 12.5 ml aliquots in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

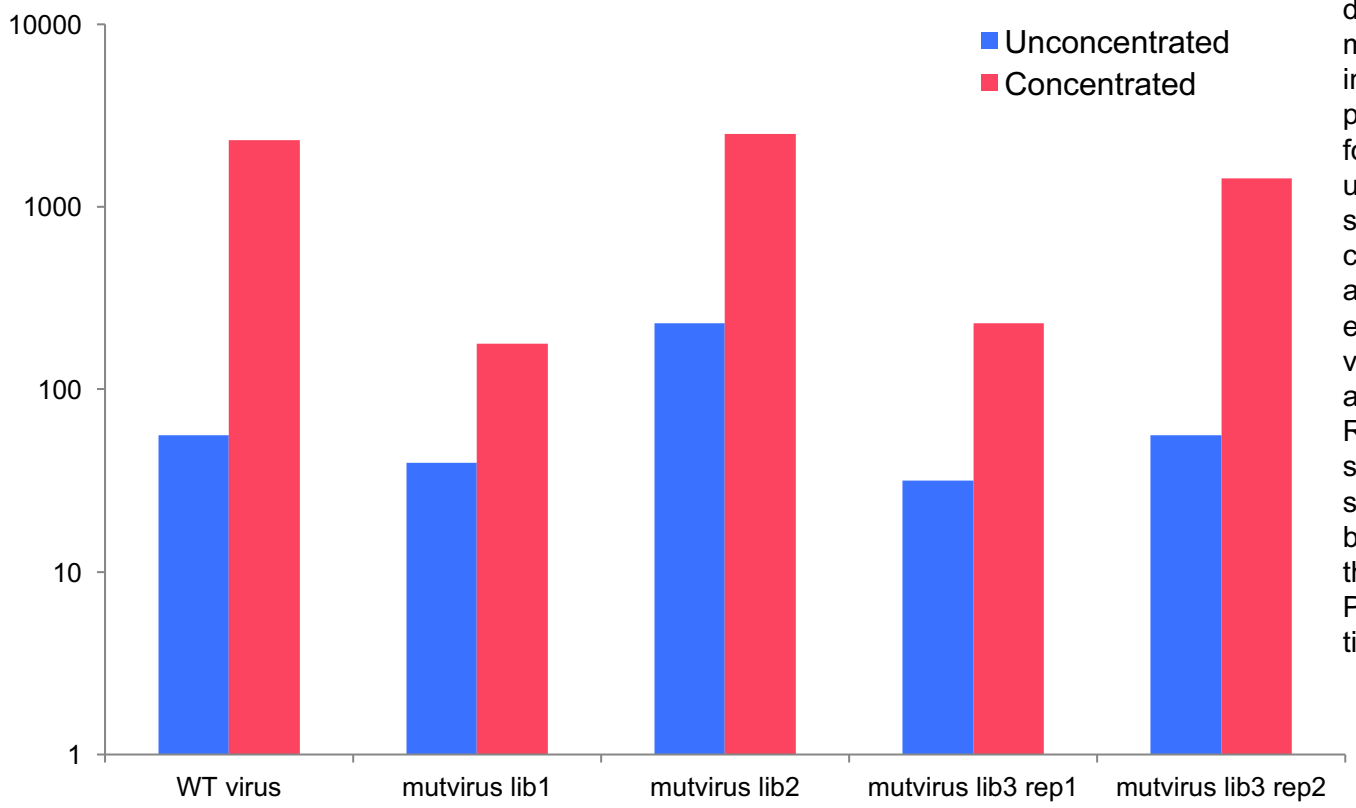
December 12, 2016

TCID50 of passaged WT and mutant virus libraries: set up TCID50 of passaged WT virus, lib1, lib2, lib3 rep. 1, lib3 rep. 2, all unconcentrated and concentrated. Also titered passaged no HA control virus. Thawed an aliquot of each virus. Used 10 ul undiluted virus in row A for unconcentrated viruses. Made a 1:10 dilution of concentrated virus and used 10 ul of the dilution for row A of the TCID50. Had 6 replicates of each virus, and made 10-fold dilutions down. Used TMPRSS2 cells to titer.

December 15, 2016

Scored TCID50 of passaged viruses.

TCID50/ul	**Unconcentrated**	**Concentrated**
WT virus	56.234	2310.13
mutvirus lib1	39.811	177.828
mutvirus lib2	231.013	2511.886
mutvirus lib3 rep1	31.623	231.013
mutvirus lib3 rep2	56.234	1429.461
no HA ctrl	undetectable	



These titers seem very, very low, compared to titers I was getting at 40 hpi for my pilot expansion of lib3. Not sure why they are so low. Perhaps there was lots of adsorption of viral particles during infection (made the inoculum in a large plastic flask) or during collection (collected in large media bottles), all of these steps involving mixing with serological pipets? These may be too low to use for antibody selection (Mike said he uses 1 ml of 1000 TCID50/ul for Ab selection experiments). The concentration worked fairly well for all the samples, but I doubt I have enough aliquots of the concentrated viruses to do further experiments. At any rate, it seems I have enough RNA from the passaged viruses to sequence, so I will go ahead with sequencing the passaged libraries, but try to think about how to remedy the low titers from passage later. Perhaps try another time course, this time going out past 40 hpi…

PILOT PASSAGE OF WT Perth/09 virus – I will pilot a passage of WT Perth/09 virus in TMPRSS2 cells and collect at various time points (0, 24, 30, 40 hpi) and titer to see if collecting past 40 hpi is feasible for getting higher titers of passaged virus since my titer of passaged virus libraries was so low

December 19, 2016

1. **Seeded TMPRSS2 cells:** split a plate of confluent TMPRSS2 cells (passage 5). Counted 2.7e6 cells/ml. Seeded 6e6 cells per plate (2.2 ml) into three 15-cm plates (seeded at 5:15 PM)

December 20, 2016

1. **Infected TMPRSS2 cells**: at 5:15 PM, infected the 15 cm plates of TMPRSS2 cells at an MOI of 0.0075 = 168,750 TCID50 total. The cells were ~90-100% confluent at this point
    - WT Perth09 rescue titer: 734.932 TCID50/ul => need 230 ul to infect the plate of cells
    - Made an inoculum of 25 ml IGM + 315 ul Perth/09 HA lib3 rescued and harvested 20161112, and gently mixed 3X's with serological pipet
    - Aspirated D10 from plate of cells. Added the viral inoculum to the plate by pipetting down the side of the plate. Rocked gently back and forth a couple of times. Removed a 400 ul aliquot from the plate, which is a 0 hpi time point collection, and froze this aliquot at -80°C. Incubated the plate at 37°C (START: 5:15 PM)

December 21, 2016

1. At **24 hpi**, removed a 300 ul aliquot of the viral supernatant from each of the two the 15 cm plates. Froze down aliquots at -80°C. I noticed very little CPE

December 22, 2016

1. At **40 hpi**, removed a 300 ul aliquot of the viral supernatant from each of the two 15 cm plates. I saw scattered foci of infection.
2. At **48 hpi**, removed a 300 ul aliquot of the viral supernatant from each of the two 15 cm plates. Saw a little bit more spread of infection, but not much

December 23, 2016

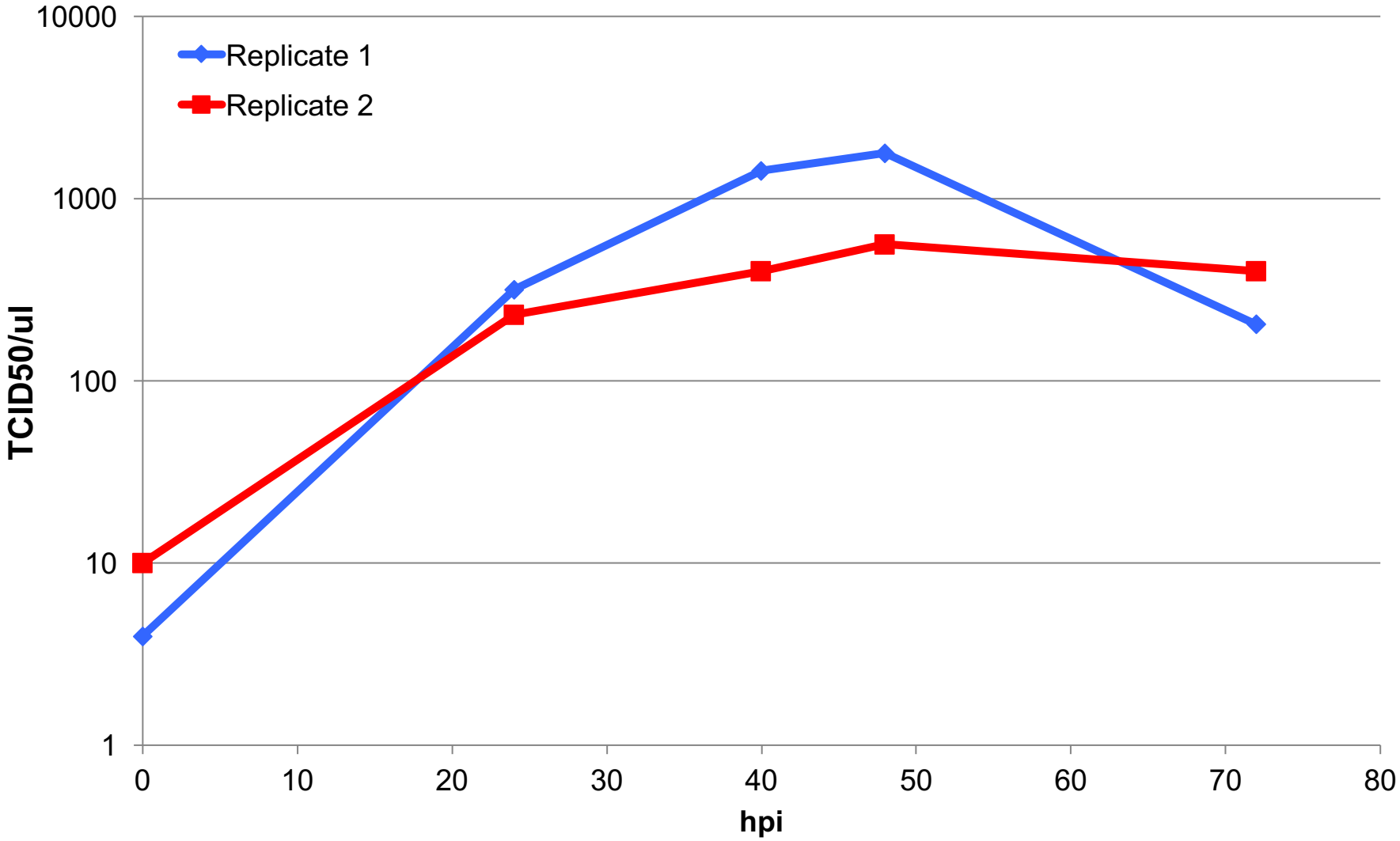
1. At **72 hpi**, removed a 300 ul aliquot of the viral supernatant from each of the two 15 cm plates. At this point I saw extensive CPE, with ~40% of the cell detached from the plate

January 9, 2017

1. **TCID50 assay**: set up a TCID50 of the WT pilot expansion time points that I collected for the two replicates. Added 10 ul of viral supernatant to 90 ul IGM to row A, and titered 6 replicates of each sample

hpi	TCID50/ul Replicate 1	Replicate 2
0	3.981	10
24	316.228	231.013
40	1429.461	398.107
48	1778.279	562.341
72	204.336	398.107

From this time course, the titer is still going up at 48 hpi, although only slightly. By 72 hpi, the titers have dropped. This suggests that the furthest time point I should collect is by 48 hpi. For the re-attempt at passaging, I will collect at 48 hpi and *drop the MOI*, both of which should help impose more selection on my libraries, as I am seeing incomplete selection with my initial attempt at passaging (MOI @ 0.0075, collection at 40 hpi). Furthermore, the amount of correlation I'm seeing between the two technical replicates of passaging library 3 suggests that dropping the number of viral particles passaged shouldn't bottleneck my libraries by too much.



PLANS FOR EXPANSION ATTEMPT #2

- Seed MDCK-SIAT1-TMPRSS2 cells onto 15x15 cm plates, at *4.6e6 cells per plate = ~337,500,000 cell total 24 hours after seeding*
- Because lib1 rescue supernatant is most limiting, I will passage my libraries based on how many TCID50's of lib1 I can passage
    - 11 aliquots of lib1 remaining x 950 ul per aliquot x 92.832 TCID50/ul lib1 = 970,000 TCID50 total
- Passage **970,000 TCID50** of each library
- Infect cells 24 h after seeding
- Harvest passaged viral supernatant **48 hpi**

	TCID50/ul	Vol in inoculum (ul)	# aliquots
WT	734.932	1319.85	2
lib1	92.832	10448.98311	11
lib2	536.539	1807.88349	2
lib3	536.539	1807.88349	2

I am going to proceed with OPTION A. This will involve seeding TMPRSS2 cells at a slightly lower density than what I was seeding initially, and the thought behind this is that the lower density may help improve expression of the TMPRSS2 protease.

Option A:

- **Seed 4.6e6 cells per plate onto 15x15 cm plates → 24 h after seeding, assuming these cells grow at the same rate as my initial pilot passage, the cells will have reached a density of ~17,250,000 cells / plate = 258,750,000 cells total**
- **Infecting 970,000 TCID50 for each library gives an MOI = ~0.00375 (half the MOI of what I was passaging the first time)**

Option B:

- Seed 6e6 cells per plate onto 15x15 cm plates → 24 h after seeding, cells will have reached a density of 22,500,000 cells / plate = 337,500,000 cells total
- Infecting 970,000 TCID50 for each library gives an MOI = 0.00287

I will end up with 375 ml of viral supernatant. I would harvest this supernatant precisely as I did for the initial passage (concentrate 144 ml, make ½ a box of 1-ml unconcentrated viral aliquots, make larger aliquots of the rest, save some volume for later concentration and RNA extraction).

PASSAGE OF WT Perth/2009 HA and no HA controls

January 16, 2017

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells and inoculated into a 10 cm plate

January 17, 2017

Split the thawed plate of cells into two 15 cm dishes by adding 5 ml cells to 20 ml D10

January 20, 2017

Split the confluent 15 cm plate of cells into 3x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

January 22, 2017

Split each of the three confluent 15 cm plates into 12x15 cm plates (10 for WT passage and 2 for no HA passage) + 1 plate for counting cells at the time of infection. Added **4.6e6** cells per plate. Counted ~1.85e6 cells/ml, so added 2.45 ml of cells to each plate. Plated 11:15 AM

January 23, 2017

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 75% confluent on all plates.

- Note: counted cells from a separate 15 cm plate – counted 7e5 cells/ml, for a total of 17,500,000 cells per plate. I passaged 970,000 TCID50 of WT virus over 10 plates of cells, so the MOI is higher than what I will passage for libraries
- Made two inoculums – made the WT Perth/09 HA inoculum in a T75 flask and the no HA inoculum in a 50 ml conical. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 10x15 cm plates with WT Perth/2009 HA virus and 2x15 cm plates with no HA control. Infected at 11:30 AM:

WT Perth/2009 HA ctrl	
10x15 cm plates	250 ml supt
Titer from rescue (TCID50/ul)	734.932
Total cells	175000000
MOI 0.0055	970,000
Vol virus (ul)	1320
Aliquots needed	2

no HA ctrl	
2x15 cm plates	50 ml supt
Vol virus (ul)	2000

January 25, 2017

Checked infected cells 48 hpi: saw isolated foci of rounded/sick cells scattered across the plate, and CPE at ~10%. At 11:30 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a 500 ml media bottle, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 5x1 ml aliquots in Cryotubes of the concentrated virus.

During the first spin, made 27x1-ml aliquots of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and one 12.5 ml & one 9 ml aliquot in 15-ml conicals. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 3, replicate 1

January 16, 2017

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells and inoculated into a 10 cm plate

January 17, 2017

Split the thawed plate of cells into two 15 cm dishes by adding 5 ml cells to 20 ml D10

January 20, 2017

Split the confluent 15 cm plate of cells 1:2 into a new 15 cm plate to split tomorrow

January 21, 2017

Split the confluent 15 cm plate of cells into 3x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

January 23, 2017

Split each of the three confluent 15 cm plates into 15x15 cm plates. Added **4.6e6** cells per plate. Counted ~2.1e6 cells/ml, so added 2.2 ml of cells to each plate. Plated 10:15 AM

January 24, 2017

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 75% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 11:30 AM

LIBRARY 3	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	262500000
MOI 0.0037	970000
Vol virus (ul)	1810
Aliquots needed	2

January 26, 2017

Checked infected cells 48 hpi: saw isolated foci of rounded/sick cells scattered across the plate, and overall CPE at around 5-10% (probably a little less CPE than what I saw for WT at 48 hpi). At 11:30 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T75 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 4x1 ml and 1x700 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 36x1-ml aliquots of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 8x12.5 ml aliquots and 1x25 ml aliquot. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 1

January 16, 2017

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells and inoculated into a 10 cm plate

January 17, 2017

Split the thawed plate of cells into two 15 cm dishes by adding 5 ml cells to 20 ml D10

January 22, 2017

Split a confluent 15 cm plate of cells into 3x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

January 24, 2017

Split each of the three confluent 15 cm plates into 15x15 cm plates. Added **4.6e6** cells per plate. Counted ~2.1e6 cells/ml, so added 2.2 ml of cells to each plate. Plated 10:15 AM

January 25, 2017

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 75% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots (NOTE: I had only 10 aliquots remaining, so I was only able to add 10 ml of library 1 rescue supernatant to ~365 ml IGM to make a 375 ml inoculum. Note that this final inoculum has less than the 970,000 TCID50 that I initially decided to passage), added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 10:30-11AM.

At 3 hpi (1:45 PM), I changed the media by aspirating the inoculum off each of the plates, and adding 25 ml fresh IGM. I did this to remove any remaining viral particles from the initial inoculum.

LIBRARY 1	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	92.832
Total cells	262500000
MOI 0.0035	928320
Vol virus (ul)	10000
Aliquots needed	10

January 27, 2017

Checked infected cells 48 hpi: I did *not* see isolated foci of infection like I usually see at 48 hpi for the other libraries. Rather, I saw widespread CPE, probably around 30-50%, with 20% detachment, which is much more CPE than I have been seeing for my other infections at 48 hpi. At 11 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T75 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 4x1 ml aliquots and 1x900 ul aliquot in Cryotubes of the concentrated virus.

During the first spin, made 36x1-ml aliquots of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, 8x12.5 ml aliquots, 1x12 ml aliquot, and 1x25 ml aliquot. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 2

January 16, 2017

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells and inoculated into a 10 cm plate

January 17, 2017

Split the thawed plate of cells into two 15 cm dishes by adding 5 ml cells to 20 ml D10

January 23, 2017

Split a confluent 15 cm plate of cells into 3x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

January 25, 2017

Split each of the three confluent 15 cm plates into 15x15 cm plates. Added **4.6e6** cells per plate. Counted ~2e6 cells/ml, so added 2.3 ml of cells to each plate. Plated 9:30 AM

January 26, 2017

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 75% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 9:30 AM
At 3 hpi (12:30 PM), I changed the media by aspirating the inoculum off each of the plates, and adding 25 ml fresh IGM.

LIBRARY 2	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	262500000
MOI 0.0037	970000
Vol virus (ul)	1810
Aliquots needed	2

January 28, 2017

Checked infected cells 48 hpi: saw isolated foci of rounded/sick cells scattered across the plate, and overall CPE at around 10%. At 9:30 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T75 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 5x1 ml and 1x200 ul aliquots in Cryotubes of the concentrated virus.

During the first spin, made 36x1-ml aliquots of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 8x12.5 ml aliquots and 1x25 ml aliquot. Placed the conicals in Ziploc bags to freeze upright.

PASSAGE OF LIBRARY 3, replicate 2

January 16, 2017

Thawed an aliquot of MDCK-SIAT1-TMPRSS2 cells and inoculated into a 10 cm plate

January 17, 2017

Split the thawed plate of cells into two 15 cm dishes by adding 5 ml cells to 20 ml D10

January 24, 2017

Split a confluent 15 cm plate of cells into 3x15 cm plates each at a 1:10 ratio, to grow up enough cells to passage viruses

January 26, 2017

Split each of the three confluent 15 cm plates into 15x15 cm plates. Added **4.6e6** cells per plate. Counted ~2e6 cells/ml, so added 2.3 ml of cells to each plate. Plated 10:30 AM

January 27, 2017

Infected TMPRSS2 cells: ~24 h after seeding, TMPRSS2 cells seeded yesterday were 75% confluent on all plates. Made an inoculum in a T150 flask. Thawed aliquots, added to room temp IGM, mixed with a serological pipet 10X's, and added 25 ml to each plate. Infected 10:15-10:30 AM. At 3 hpi (1:15 PM), I changed the media by aspirating the inoculum off each of the plates, and adding 25 ml fresh IGM.

LIBRARY 3	
15x15 cm plates	375 ml supt
Titer from rescue (TCID50/ul)	536.539
Total cells	262500000
MOI 0.0037	970000
Vol virus (ul)	1810
Aliquots needed	2

January 29, 2017

Checked infected cells 48 hpi: on all the plates, saw part of the plate with isolated foci of infection, with CPE at ~5%, but in other regions of the plates, I saw extensive cell death, up to 20-50% CPE. At 10:30 AM, harvested the viral supernatant from each of the plates, and spun down in 50 ml conicals (with ~40 ml viral supernatant in each conical) at 2000xg for 5 min. Combined clarified viral supernatant in a T75 flask, and mixed with a 50-ml serological pipet up and down gently 10X's.

Concentrated 144 ml of virus: ultracentrifuged 6 tubes with 12 ml of the clarified supernatant in each tube, balanced with PBS, and spun at 20,000 rpm at 4°C for 1.5 h (kept at hold, started my own timer when centrifuged reached 2000 rpm, and stopped after 1.5 h). Poured off supernatant into Wescodyne flask, then added 12 ml more clarified supernatant to each tube and spun again. Poured off supernatant. Added 600 ul cold PBS to each tube, placed tube in 50-ml conical, and put the conicals in the TC fridge to incubate at 4°C for 1.5 h. After 1.5 h, mixed the liquid in each tube by pipetting up and down 20X's, pooled the liquid together, and made 5x1 ml and 1x200 ul aliquot in Cryotubes of the concentrated virus.

During the first spin, made 36x1-ml aliquots of unconcentrated virus in Cryotubes, a 25 ml aliquot in a 50-ml conical for concentration and RNA extraction later, and 8x12.5 ml aliquots, 1x6.5 ml aliquot, and 1x25 ml aliquot. Placed the conicals in Ziploc bags to freeze upright.

February 1, 2017

TCID50 of passaged WT and mutant virus libraries: set up TCID50 of passaged WT virus, lib1, lib2, lib3 rep. 1, lib3 rep. 2, all unconcentrated and concentrated. Also titered passaged no HA control virus. Thawed an aliquot of each virus. Used 10 ul undiluted virus in row A for unconcentrated AND concentrated viruses. Had 6 replicates of each virus, and made 10-fold dilutions down. Used TMPRSS2 cells to titer.

February 4, 2017

Scored TCID50 of passaged viruses.

TCID50/ul	**Unconcentrated**	**Concentrated**
WT	316.228	5623.413
Lib 1	31.623	3162.278
Lib 2	177.828	7551.77
Lib 3-1	31.623	398.107
Lib 3-2	231.013	5623.413
No HA ctrl	undetectable	

Although these titers are still somewhat low, they are somewhat improved over what I was getting from my initial passaging experiments. Especially for the concentrated samples, these will certainly be enough to use for antibody selections (except for Lib 3-1, which is still not above the 1000 TCID50/ul that Mike uses for Ab selections).