WSN-HA library selections with monoclonal antibodies (continued)

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i previously selected and sequenced library 1 with three concentrations of each of the first two antibodies (H17-L19 and FI6v3), performing full technical replicates of the experiment. using the differential selection algorithm employed in dms_diffselection, i identified sites with escape mutants selected by H17-L19 and observed strong correlations across technical replicates and antibody concentrations.

here I will repeat the experiment using biological replicate libraries 2 and 3, performing one technical replicate for each of these additional two biological replicate mutant libraries.

2016-06-06 neutralization selection of libraries 2 and 3 with H17-L19 and FI6v3.

2016-06-06 **2pm:** Plated 2.5E5 MDCKSIAT-1 cells per well in **7 6-well plates**. [Not sure now why the cell number is this low. Did I pick this to limit the cellular RNA:viral RNA ratio? In any case it looks like I definitely used this cell number last time, as the RNA yields were ~half of what I got during the initial pilot with 5e5 cells/well plated. So This seems to be the number I plated for L1, and again here for L2 and L3 -- however they looked a little sparse after plating, and as shown later the RNA yields were lower than back in April for L1, so I think the actual cell number really was lower this time than before. Might be worth thinking about for next time -- to plate, perhaps, 2.5e5 and 5e5, and then infect the dish that have ~40-50% confluency.]

I will use the 20ul aliquots of H17-L19 (1mg/ml) from when we first received it. I will use FI6v3 aliquoted 4/4/16 at -20 (0.83mg/ml). Same three concentrations of each library: Neutralize with 0.5, 1, and 10 ug/ml of H17-L19; 0.1, 0.2, and 2 ug/ml FI6v3 (this is the concentration during incubation with virus).

Make antibody and virus dilutions:

H17-L19:

Add 60 ul of 1mg/ml stock to 3mL IGM to make 20ug/ml. (c3)
Transfer 450ul of 20ug/ml to a final volume of 4.5mL IGM to make 2ug/ml. (c2)
Transfer 2mL of 2ug/ml to a final volume of 4mL IGM to make 1ug/ml. (c1)

FI6v3:

Add 14.4ul To 3mL IGM to make 4ug/ml. (c3)
Transfer 450ul of 4ug/ml to final volume of 4.5mL IGM to make 0.4 ug/ml. (c2)
Transfer 2mL of 0.4ug/ml to a final volume of 4mL IGM to make 0.2 ug/ml. (c1)

Bring both Libraries to 1E6 TCID50/ml:

Add 2095 ul of library 2 (3700 tcid50/ul) to final volume 7.75 mL IGM to make 1E6 TCID50/ml.
Add 5962 ul of library 3 (1300 tcid50/ul) to final volume 7.75 mL IGM to make 1E6 TCID50/ml.

Neutralize **5:30-6:30pm:** In 5mL eppendorf tubes, mix 1mL of each diluted antibody with 1 mL library. There are 12 tubes neutralizing libraries (6 per each of the two libraries as shown on plate layout next page, “LibraryX selections” plates.) Also make one mock-neutralization for each library (1 mL library 2 or 3 + 1 mL IGM). **Incubate neutralization reactions at 37C for 1 hr.**
Store remaining 1E6/ml at 4˚ C for ~30 min, until making standard curves immediately prior to infection.

Just before infecting, use the remaining 1E6/ml stocks of each library to make standard infection curve inocula for each library:
Make 50 TCID50/ul (5 ml total), (this is 1E5 TCID50 per 2mL infection, or 10% of library dose for each neutralization):
To make 50 TCID50/ul (“1E5” tcid50 per 2mL infection), add 250ul of the 1E6/ml (1,000T/ul) to final volume 5 ml in IGM.
Then make 1E4, 1E3, 1E2, and 1E1 by serially transferring 0.5 ml to 4.5 mL IGM. (one dilution series per library, technical replicates of infection for the standard curves)

Infect at **6:30pm**: Aspirate D10 from cells and replace with each 2mL neutralization reaction. *For the mock-neutralizations, split across three wells* by bringing the final volume of each of the two “mock-neutralized” virus to 6 mL. (effective MOI for mock neutralization in this case is 1.3).
Change media at **8:30pm** by washing cells **1x with 1 mL PBS**. Replace with 2 mL IGM+5%D10.
Lyse cells & Extract RNA at **9:30AM** (15 hours post-infection). Lyse all wells and purify in two batches, keeping the lysates on ice when waiting to purify.

Library2 selections

H17-L19 c1	H17-L19 c2	H17-L19 c3
Fl6v3 c1	Fl6v3 c2	Fl6v3 c3

Library3 selections

H17-L19 c1	H17-L19 c2	H17-L19 c3
Fl6v3 c1	Fl6v3 c2	Fl6v3 c3

Mock-neutralized

Lib2a	Lib2b	Lib2c
Lib3a	Lib3b	Lib3c

Standard curve Lib2 replicate A

Lib2 - 1E5	Lib2 - 1E4	Lib2 - 1E3
Lib2 - 1E2	Lib2 - 1E1	NoVirus

Standard curve Lib2 replicate B

Lib2 - 1E5	Lib2 - 1E4	Lib2 - 1E3
Lib2 - 1E2	Lib2 - 1E1	NoVirus

Standard curve Lib3 replicate A

Lib3 - 1E5	Lib3 - 1E4	Lib3 - 1E3
Lib3 - 1E2	Lib3 - 1E1	NoVirus

Standard curve Lib3 replicate B

Lib3 - 1E5	Lib3 - 1E4	Lib3 - 1E3
Lib3 - 1E2	Lib3 - 1E1	NoVirus

I will lyse all wells, collect lysate in epi tubes, keep half of the samples on ice while I purify the others, and then finish the second replicate.

For 42 samples, aliquot RLT and add BME: 16.8 mL RLT + 168 ul BME. Also make fresh 70% EToH.

Lyse on plate by adding 350ul RLT and carefully pipetting several times to mix and lyse; transfer lysate to RNAse-free tube. Once all lysates are collected in TC hood, bring to lab and vortex each tube for ~20 sec to homogenize. Then continue with protocol as written on gDNA eliminator columns.

Elute in 35ul.

Lysed cells at 9:30am and kept on ice after lysis, purified RNA for tubes 1-22 first (finished around 1pm) then 23-42 (finished around 3pm). Kept all at -20 overnight before nanodropping.

2016-06-07 RNA

sample #	Identity
1	L2_H17L19_c1
2	L2_H17L19_c2
3	L2_H17L19_c3
4	L2_FI6v3_c1
5	L2_FI6v3_c2
6	L2_FI6v3_c3
7	L3_H17L19_c1
8	L3_H17L19_c2
9	L3_H17L19_c3
10	L3_FI6v3_c1
11	L3_FI6v3_c2
12	L3_FI6v3_c3
13	L2_mockA
14	L2_mockB
15	L2_mockC
16	L3_mockA
17	L3_mockB
18	L3_mockC
19	st_L2_1e5_a
20	st_L2_1e4_a
21	st_L2_1e3_a
22	st_L2_1e2_a
23	st_L2_1e1_a
24	st_L2_NoVirus_a
25	st_L2_1e5_b
26	st_L2_1e4_b
27	st_L2_1e3_b
28	st_L2_1e2_b
29	st_L2_1e1_b
30	st_L2_NoVirus_b
31	st_L3_1e5_a
32	st_L3_1e4_a
33	st_L3_1e3_a
34	st_L3_1e2_a
35	st_L3_1e1_a
36	st_L3_NoVirus_a
37	st_L3_1e5_b
38	st_L3_1e4_b
39	st_L3_1e3_b
40	st_L3_1e2_b
41	st_L3_1e1_b
42	st_L3_NoVirus_b

2016-06-08 RNA yields from yesterday are a little lower than I got last time I did this experiment (2016-04-05). I'm not sure why exactly but expect this is due to several factors -- the seemingly lower cell number (hard to say if this is true, but the cells *did* look less confluent than I was expecting. However, I didn't take good notes about what confluency they looked like the first time I did this back in April, so this is hard to say.), batch effect on RNA purification, and a freeze-thaw before nanodropping… In any case I will use these concentrations to make a plate of 10ng/ul dilutions which I will use as templates for qPCR.

Make 10ng/ul dilutions in a 96-well plate in the following layout for RNA sample numbers. First add 50ul of RNAse-free water to these wells, then add the indicated amount of RNA to make final concentration of 10ng/ul.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	2	3	4	5	6	7	8	9	10	11	12
B												
C	13	14	15	16	17	18	19	20	21	22	23	24
D												
E	25	26	27	28	29	30	31	32	33	34	35	36
F												
G	37	38	39	40	41	42						
H												

2016-06-07

RNA sample #	Identity	RNA yield ng/ul	**ul to 50ul for 10ng/ul**
1	L2_H17L19_c1	142.7	**3.77**
2	L2_H17L19_c2	110.2	**4.99**
3	L2_H17L19_c3	139.2	**3.87**
4	L2_FI6v3_c1	87	**6.49**
5	L2_FI6v3_c2	106.1	**5.20**
6	L2_FI6v3_c3	123.8	**4.39**
7	L3_H17L19_c1	130.9	**4.14**
8	L3_H17L19_c2	106.5	**5.18**
9	L3_H17L19_c3	115.7	**4.73**
10	L3_FI6v3_c1	81.6	**6.98**
11	L3_FI6v3_c2	165.2	**3.22**
12	L3_FI6v3_c3	165.5	**3.22**
13	L2_mockA	93.9	**5.96**
14	L2_mockB	103.2	**5.36**
15	L2_mockC	114.3	**4.79**
16	L3_mockA	133.3	**4.06**
17	L3_mockB	144.1	**3.73**
18	L3_mockC	136.1	**3.97**
19	st_L2_1e5_a	109.4	**5.03**
20	st_L2_1e4_a	94.4	**5.92**
21	st_L2_1e3_a	160.9	**3.31**
22	st_L2_1e2_a	108.4	**5.08**
23	st_L2_1e1_a	141	**3.82**
24	st_L2_NoVirus_a	112.1	**4.90**
25	st_L2_1e5_b	101.3	**5.48**
26	st_L2_1e4_b	126.7	**4.28**
27	st_L2_1e3_b	119.4	**4.57**
28	st_L2_1e2_b	134.8	**4.01**
29	st_L2_1e1_b	145.5	**3.69**
30	st_L2_NoVirus_b	152.5	**3.51**
31	st_L3_1e5_a	59.9	**10.02**
32	st_L3_1e4_a	166.1	**3.20**
33	st_L3_1e3_a	132	**4.10**
34	st_L3_1e2_a	122.9	**4.43**
35	st_L3_1e1_a	95.2	**5.87**
36	st_L3_NoVirus_a	134.7	**4.01**
37	st_L3_1e5_b	140.2	**3.84**
38	st_L3_1e4_b	151.8	**3.53**
39	st_L3_1e3_b	84.6	**6.70**
40	st_L3_1e2_b	114.3	**4.79**
41	st_L3_1e1_b	146	**3.68**
42	st_L3_NoVirus_b	131.6	**4.11**

2016-06-08 qRT-PCR

Each qRT-PCR reaction (**20ul** total volume) will contain:

10 ul One-Step SYBR Green Master Mix
0.4 ul RT (or water for RT- controls)
1 ul of 5 uM forward primer (250nM final)
1 ul of 5 uM reverse primer (250nM final)
4 ul RNA (40 ng total)
3.6 ul water to bring volume to 25ul.

I make master mixes as so:

Need 84, make 88 RT+ MM: 880 ul One-Step MM +35.2 ul qScript RT + 316.8 ul water
Need 12, make 12x RT- MM: 120 ul One-Step MM + 48 ul H2O

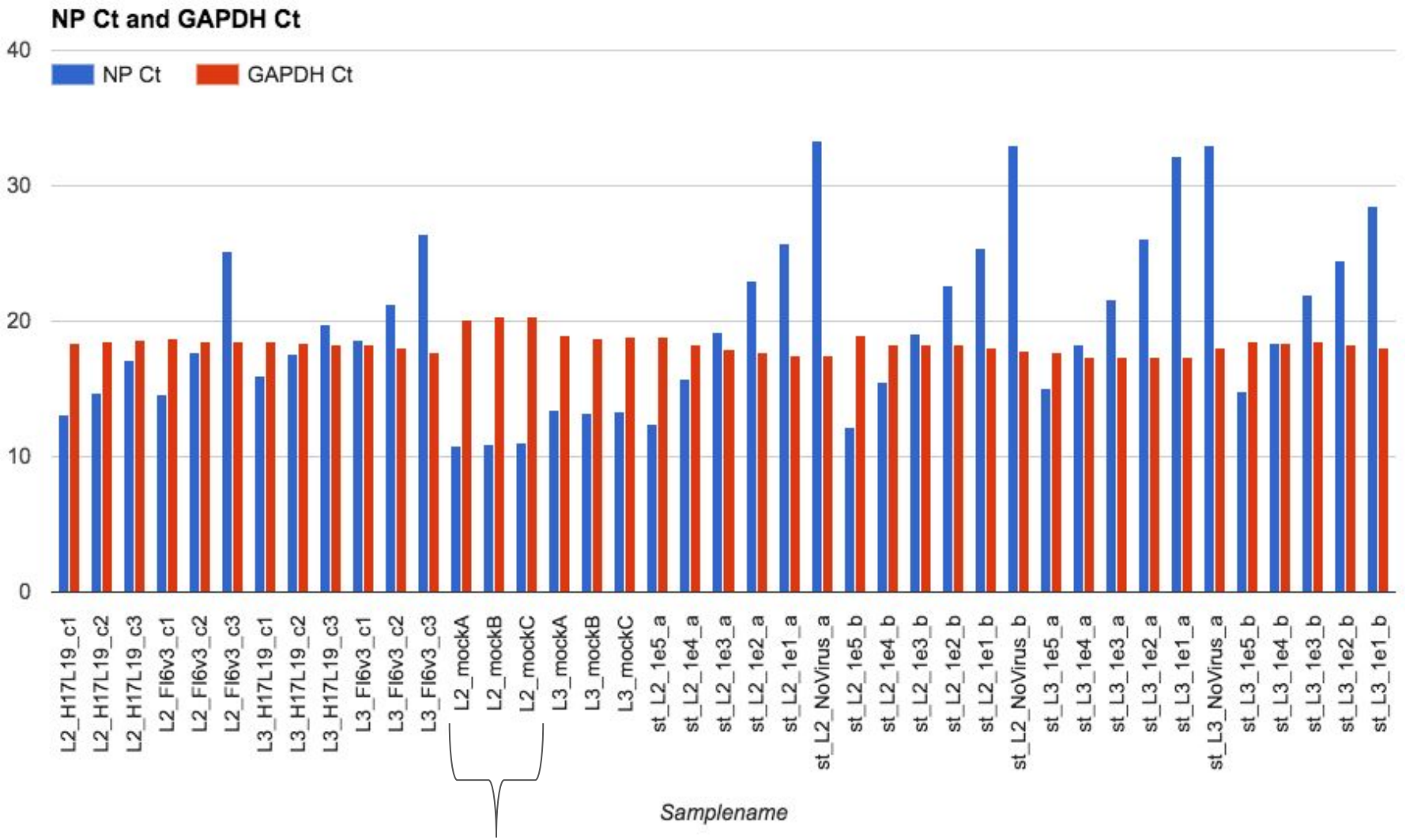
Split RT+ MM into two 44x MM tubes (each tube gets 616 ul) and add NP primers or GAPDH primers to respective tubes (each tube gets 44 ul of each primer from 5uM stocks).
Aliquot the two **RT+ NP** and **RT+ GAPDH** master mixes to appropriate rows as labeled below (16 ul per well).
Split RT- MM into two 6x MM tubes (each tube gets 84 ul) and add NP primers or GAPDH primers to respective tubes (each tube gets 6 ul of each primer for 5uM stocks).
Aliquot the **RT- NP** and **RT- GAPDH** master mixes to the appropriate noRT wells (16ul per well).

Lastly, Add 4 ul RNA (from 10ng/ul dilutions) to each as indicated. Ran out of master mix for the wells highlighted in red, so ignore those.

Cycling conditions:

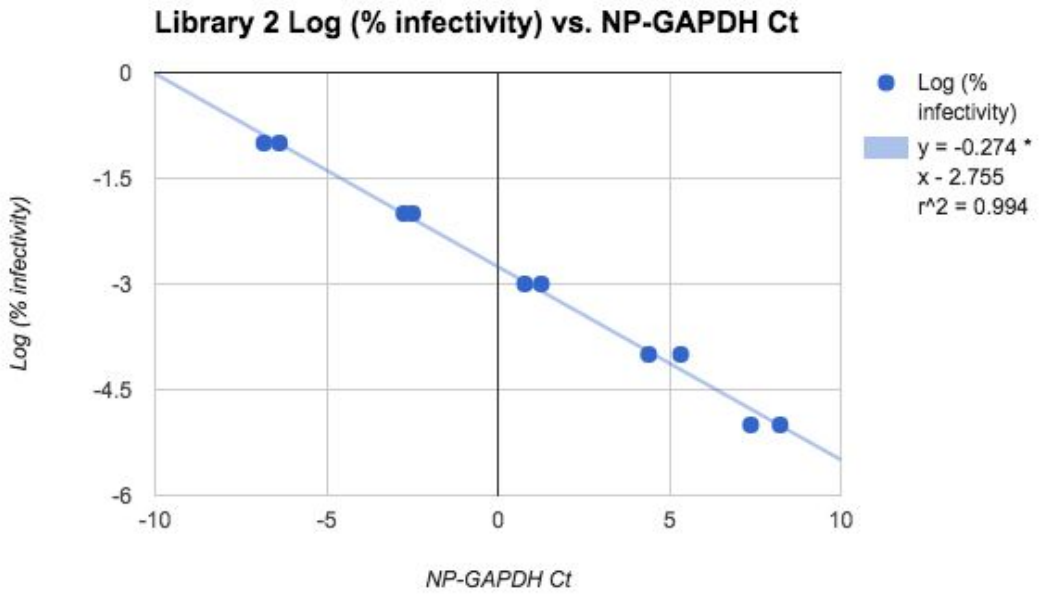
50C for 10 minutes
95C for 5 minutes
40 cycles of: 95C for 15 sec, 58C for 30 sec with data acquisition
(Followed by instrument's default melt curve program)

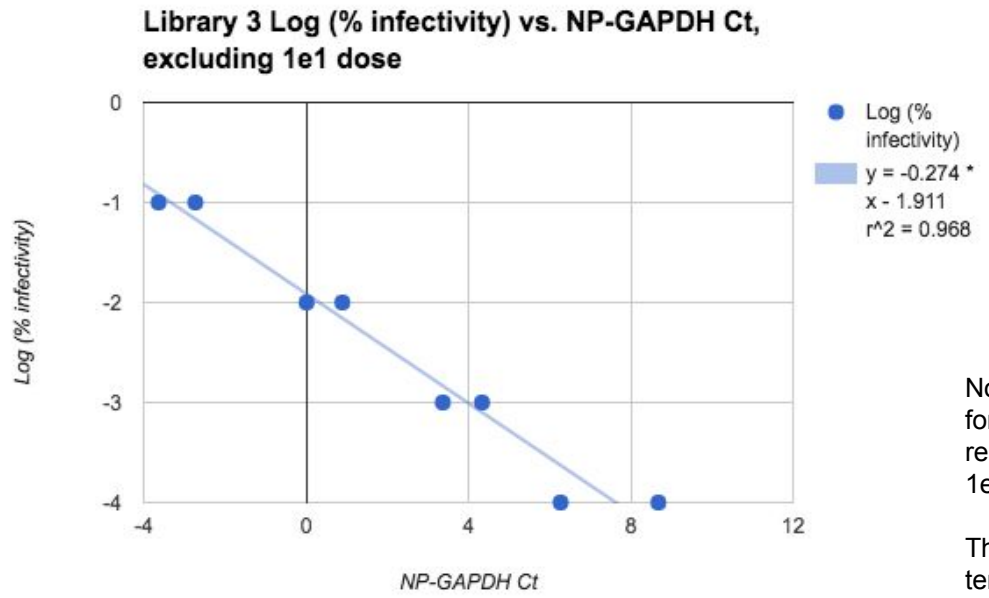
	1	2	3	4	5	6	7	8	9	10	11	12
A - NP	1	2	3	4	5	6	7	8	9	10	11	12
B - GAPDH	1	2	3	4	5	6	7	8	9	10	11	12
C - NP	13	14	15	16	17	18	19	20	21	22	23	24
D - GAPDH	13	14	15	16	17	18	19	20	21	22	23	24
E - NP	25	26	27	28	29	30	31	32	33	34	35	36
F - GAPDH	25	26	27	28	29	30	31	32	33	34	35	36
G - NP	37	38	39	40	41	42	37 noRT	38 noRT	39 noRT	40 noRT	41 noRT	42 noRT
H - GAPDH	37	38	39	40	41	42	37 noRT	38 noRT	39 noRT	40 noRT	41 noRT	42 noRT



Something strange with the three samples for the mock-neutralized Library 2 infection. The GAPDH Cts are a few higher than all the other samples. The fact that this showed up in three independent infections with the same viral inoculum (three independent wells receiving the same pool of library 2 mock-neutralized, processed independently as three independent RNA preps) suggests it is something biological and not technical. Is there really that much less GAPDH per ng RNA in those samples? The high GAPDH Ct here may be inflating the NP-GAPDH quantification for % infectivity of mock-neutralized library 2 (which appeared as ~%60 when extrapolating from the standard curve, not the expected 33% -- mock samples not shown on next slide). Mock-neut library 3 showed the expected ~33% infectivity (since it was one infection split across three wells), similarly to library 1 back in april. In any case, this doesn't really affect the interpretation of the infectivities of library 2 neutralized with antibody, since the standard curve and antibody-neutralized samples all look as expected.

Standard curves using serial dilution infection of L2 and L3

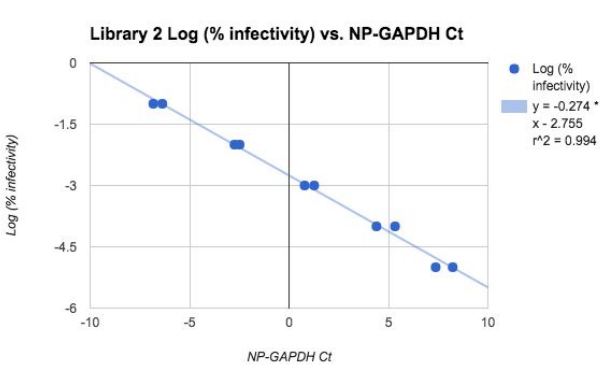


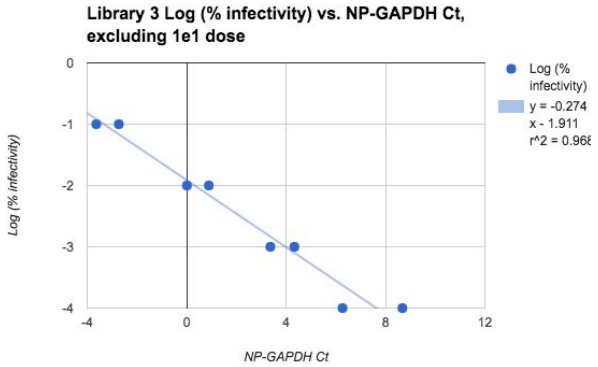


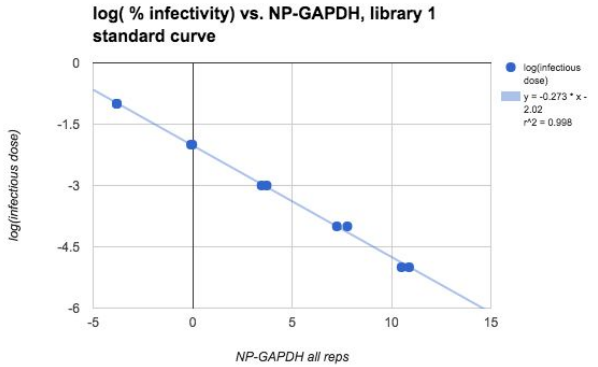
Library 2 and Library 3 neutralization samples:	% infectivity after neutralization
L2_H17L19_c1	4.902%
L2_H17L19_c2	1.849%
L2_H17L19_c3	0.437%
L2_FI6v3_c1	2.322%
L2_FI6v3_c2	0.278%
L2_FI6v3_c3	0.003%
L3_H17L19_c1	5.410%
L3_H17L19_c2	1.809%
L3_H17L19_c3	0.434%
L3_FI6v3_c1	0.903%
L3_FI6v3_c2	0.144%
L3_FI6v3_c3	0.004%

Note: I omit the 1e1 TCID50 dose infection in the standard curve for library 3. This dose was so low for library 3 that one of the two replicates had a Ct value that looked closer to no virus than to 1e1 virus.

These % infectivities should probably be presented only to the tenths of a percent.







Library 2 and Library 3 neutralizations:

	Interpolated % infectivity
L2_H17L19_c1	4.902%
L2_H17L19_c2	1.849%
L2_H17L19_c3	0.437%
L2_FI6v3_c1	2.322%
L2_FI6v3_c2	0.278%
L2_FI6v3_c3	0.003%
L3_H17L19_c1	5.410%
L3_H17L19_c2	1.809%
L3_H17L19_c3	0.434%
L3_FI6v3_c1	0.903%
L3_FI6v3_c2	0.144%
L3_FI6v3_c3	0.004%

(Compare with: Technical replicate neutralizations of Library 1, done in April 2016)

	% infectivity rep 1	% infectivity rep 2
MAb H17-L19 0.5 ug/ml (c1)	2.115%	2.539%
MAb H17-L19 1 ug/ml (c2)	0.740%	0.646%
MAb H17-L19 10 ug/ml (c3)	0.295%	0.231%
MAb FI6v3 0.1 ug/ml (c1)	1.662%	1.390%
MAb FI6v3 0.2 ug/ml (c2)	0.465%	0.345%
MAb FI6v3 2 ug/ml (c3)	0.004%	0.006%

Next, I do RT-PCR to make full-length HA amplicons from mock- and antibody-selected samples of L2 and L3.

The RNA concentrations are a little lower than I have been getting in the past. I will use 1000ng of RNA per RT when possible, and when that's not possible I will use the maximum volume of RNA permissible by the RT reaction specifications -- in the worst cases this reduces the amount of RNA to around 700ng, but I think this is acceptable. calculations on next page

As I did for the L1 prep back in April, I will pool equal amounts of RNA to use as RT template from the mock-neutralized samples that were split across three wells each. Here I will make an RNA pool using 1000ng of each of the three mock samples for each library, and then use these pooled mock RNA samples for each library in RT. As stated above, because some of these samples are kind of low concentration, I will not be able to use a full 1000ng in the RT for mock Library2 (but I will be able to use 900ng - again, I think this is acceptable). calculations on next page.

Some calculations for RNA going into RT.

Maximum vol per RT is 8.8

2016-06-07 RNA sample #	Identity	RNA yield ng/ul	RT tube #	ul for 1000ng RNA	ul RNA in RT	ng RNA in RT
1	L2_H17L19_c1	142.7	**1**	7.01	7.01	1000
2	L2_H17L19_c2	110.2	**2**	9.07	**8.8**	969.76
3	L2_H17L19_c3	139.2	**3**	7.18	7.18	1000
4	L2_FI6v3_c1	87	**4**	11.49	**8.8**	765.6
5	L2_FI6v3_c2	106.1	**5**	9.43	**8.8**	933.68
6	L2_FI6v3_c3	123.8	**6**	8.08	8.08	1000
7	L3_H17L19_c1	130.9	**7**	7.64	7.64	1000
8	L3_H17L19_c2	106.5	**8**	9.39	**8.8**	937.2
9	L3_H17L19_c3	115.7	**9**	8.64	8.64	1000
10	L3_FI6v3_c1	81.6	**10**	12.25	**8.8**	718.08
11	L3_FI6v3_c2	165.2	**11**	6.05	6.05	1000
12	L3_FI6v3_c3	165.5	**12**	6.04	6.04	1000

2016-06-07 RNA sample #	Identity	RNA yield ng/ul	ul RNA for 1000ng to pool mock RNA before RT
13	L2_mockA	93.9	10.65
14	L2_mockB	103.2	9.69
15	L2_mockC	114.3	8.75
	total pool vol, ul:	29.09	total pool ng/ul: 103.13
16	L3_mockA	133.3	7.50
17	L3_mockB	144.1	6.94
18	L3_mockC	136.1	7.35
	total pool vol, ul:	21.79	total pool ng/ul: 137.68

RT tube #:	ul RNApool to RT	ng RNA in RT
mockpool13	8.8	907.6
mockpool14	7.263	1000

2016-06-11: Reverse transcription of WSN-HA

First, thaw RNA on ice, flick tubes gently to mix, and spin down. Make the pools of the mock-L2 and mock-L3 RNA from the triplicate infection wells of each as shown on right. Then make annealing master mix, add the 8.8ul of RNA plus water as listed on lower right, and follow protocol below.

Each RT will be:

3.0 ul 10X AccuScript RT Buffer

1.2 ul of dNTP mix

3 ul of 5 uM WSN-For

3 ul of 5 uM WSN-Rev

8.8 ul RNA diluted to 1000 ng total in RNase-free water.

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19 ul total volume

2016-06-07 RNA sample #	Identity	RNA yield ng/ul	ul RNA pool mock-L2:
13	L2_mockA	93.9	**10.65**
14	L2_mockB	103.2	**9.69**
15	L2_mockC	114.3	**8.75**
			ul for mock-L3 pool
16	L3_mockA	133.3	**7.50**
17	L3_mockB	144.1	**6.94**
18	L3_mockC	136.1	**7.35**

Make annealing master mix for 17 reactions' worth (for the 15 reactions I will do) on ice:

51 ul 10X AccuScript RT Buffer
20.4 ul of dNTP mix
51 ul of 5 uM WSN-For
51 ul of 5 uM WSN-Rev

Aliquot 10.2 ul of this master mix into 15 PCR tubes on ice, added the 8.8 ul of the RNA/water combo as listed to each tube, and mix by pipetting.

Heat to 65 C for 5 minutes, cooled to 4 C in the PCR machine. Make the following master mixes while heating and cooling:

AccuScript master mix (for 17 rxn):

42.5 ul of water and 25.5 ul of AccuScript RT

RNAse block master mix (for 17 rxn):

55.25 ul of water and 12.75 ul of Rnase block

On ice, add 3 ul of DTT to each reaction.

Then add:

4 ul of a mix of 2.5 ul of water and 1.5 ul of AccuScript RT (**4ul Accuscript mastermix**),

4 ul of a mix of 3.25 ul of water and 0.75 ul RNase Block (**4ul RNAseblock mastermix**)

reverse transcription: 42 C for 90 minutes, followed by 70 C for 15 minutes, then cooled to 4 C.

Total RT reaction volume is 30ul.

2016-06-07 RNA sample #	Identity	RT tube #	ul RNA in RT	ul water to 8.8
1	L2_H17L19_c1	1	7.01	1.79
2	L2_H17L19_c2	2	**8.80**	0.00
3	L2_H17L19_c3	3	7.18	1.62
4	L2_FI6v3_c1	4	**8.80**	0.00
5	L2_FI6v3_c2	5	**8.80**	0.00
6	L2_FI6v3_c3	6	8.08	0.72
7	L3_H17L19_c1	7	7.64	1.16
8	L3_H17L19_c2	8	**8.80**	0.00
9	L3_H17L19_c3	9	8.64	0.16
10	L3_FI6v3_c1	10	**8.80**	0.00
11	L3_FI6v3_c2	11	6.05	2.75
12	L3_FI6v3_c3	12	6.04	2.76
13/14/15 pooled	L2_mock pooled	**13: mock2 pool**	8.8	0.00
16/17/18 pooled	L3_mock pooled	**14: mock3 pool**	7.26	1.54
24	st_L2_NoVirus_a	**15: novirus**	8.8	0

PCR tube	cDNA	standard dna
1	L2_H17L19_c1	
2	L2_H17L19_c2	
3	L2_H17L19_c3	
4	L2_FI6v3_c1	
5	L2_FI6v3_c2	
6	L2_FI6v3_c3	
7	L3_H17L19_c1	
8	L3_H17L19_c2	
9	L3_H17L19_c3	
10	L3_FI6v3_c1	
11	L3_FI6v3_c2	
12	L3_FI6v3_c3	
13	L2_mock pooled	
14	L3_mock pooled	
15	st_L2_NoVirus_a	
16		no template
17		1e4 (5e3/ul)
18		1e5 (5e4/ul)
19		1e6 (5e5/ul)
20		1e7 (5e6/ul)

2016-06-11 PCR on cDNA to make full-length HA amplicons.

PCR tube numbers for cDNA are the same numbers as the RT listed on the last page.

10 ng of WSN amplicon corresponds to 1e10 ssDNA molecules. So a 5 ng/ul dilution is 5e9 ssDNA/ul, or 1e10 ssDNA per 2 ul used in PCR. I used the 5e9/ul dilution from 4/11 (“fresh witness”) and making 10-fold dilutions by transferring 20ul to 180 ul to make 5e3, 5e4, 5e5, 5e6 per ul.

Do in **50ul reactions: (make 22x master mix)**

Each reaction:
25 ul of 2X KOD Master Mix (550 ul)
3 ul of 5 uM WSN-for (66 ul)
3 ul of 5 uM WSN-rev (66 ul)
17 ul of water (374 ul)
2 ul template

Add 48 ul of PCR mastermix to tubes, then add 2ul of template to each well.
Pipette all wells with multichannel a few times to mix.

Use the following PCR program (**22 total PCR cycles**):
1.95 C for 2:00
2.95 C for :20
3.70 C for :01
4.50 C for :30, cooling to 50 C at 0.5 C/second
5.70 C for :40
6.Goto 2, 21 times
7.4 C forever

Then check products on a 1% agarose gel (each lane: 5ul PCR product + 5ul water + 2ul 6x sample buffer).

2016-06-11 Checking amplicon PCR products on 1% agarose: All looks good; seeing the expected variations in band intensity based on % neutralized already measured by qPCR; amplifying from >= 1e6 cDNA molecules in almost all cases. Storing PCR products at -20 overnight before cleaning up.

Sample number	cDNA template
1	L2_H17L19_c1
2	L2_H17L19_c2
3	L2_H17L19_c3
4	L2_FI6v3_c1
5	L2_FI6v3_c2
6	L2_FI6v3_c3
7	L3_H17L19_c1
8	L3_H17L19_c2
9	L3_H17L19_c3
10	L3_FI6v3_c1
11	L3_FI6v3_c2
12	L3_FI6v3_c3
13	L2_mock pooled
14	L3_mock pooled
15	st_L2_NoVirus _a
16	no template
17	1e4 witness
18	1e5 witness
19	1e6 witness
20	1e7 witness

And here is the qPCR infectivity data for reference:

Corresponding lane in *this* PCR experiment:	Sample	qPCR % infectivity
1	L2_H17L19_c1	4.902%
2	L2_H17L19_c2	1.849%
3	L2_H17L19_c3	0.437%
4	L2_FI6v3_c1	2.322%
5	L2_FI6v3_c2	0.278%
6	L2_FI6v3_c3	0.003%
7	L3_H17L19_c1	5.410%
8	L3_H17L19_c2	1.809%
9	L3_H17L19_c3	0.434%
10	L3_FI6v3_c1	0.903%
11	L3_FI6v3_c2	0.144%
12	L3_FI6v3_c3	0.004%

Sample number	PCR template
1	L2_H17L19_c1
2	L2_H17L19_c2
3	L2_H17L19_c3
4	L2_FI6v3_c1
5	L2_FI6v3_c2
6	L2_FI6v3_c3
7	L3_H17L19_c1
8	L3_H17L19_c2
9	L3_H17L19_c3
10	L3_FI6v3_c1
11	L3_FI6v3_c2
12	L3_FI6v3_c3
13	L2_mock pooled
14	L3_mock pooled
15	st_L2_NoVirus _a
16	no template
17	1e4 witness
18	1e5 witness
19	1e6 witness
20	1e7 witness

2016-06-12: Ampure XP cleanup of full-length amplicons.

I will purify all 20 PCR amplicons (some of these are negative controls and standard curves for witness band amplification). I will use 0.9X ampure beads. Each PCR was 50 ul and I used 5ul to run on the gel, so there remains **45ul of PCR to which I will add 40.5ul beads. Transfer PCR products to a plate in the following configuration for ampure and elute in the same configuration.**

1. Take bead aliquot from vortexed stock and allow to come to room temperature
2. Add 40.5 ul beads to each well and mix 10x
3. Incubate at RT for 10 minutes to bind
4. Put on magnet for 5 minutes
5. Aspirate ~5-10ul less than the total volume using multichannel, careful not to disrupt beads
6. Wash twice with 180ul fresh 80% ethanol (gently add and aspirate)
7. Aspirate any remaining ethanol with multichannel, Air dry 10 min
8. Remove from rack, disperse beads in 60 ul EB
9. Incubate 5 min to resuspend DNA
10. Put on magnet for 5 minutes
11. Transfer bead-free DNA solutions to a new plate (“2016.06.12 purified full-length amplicons”) with the following layout:

Plate: “2016.06.12 purified full-length amplicons”

	1	2	3	4	5	6	7	8	9	10	11	12
A	PCR 1	PCR 2	PCR 3	PCR 4	PCR 5	PCR 6	PCR 7	PCR 8				
B												
C	PCR 9	PCR 10	PCR 11	PCR 12	PCR 13	PCR 14						
D												
E	PCR 15	PCR 16	PCR 17	PCR 18	PCR 19	PCR 20						
F												
G												
H												

2016-06-12: Picogreen of full-length amplicons

1. Make 2 standards at **2ng/ul by adding 4ul of the standard to 196ul TE**.
2. Add 99 ul 1xTE to the block between A1 and F8 for making sample dilutions.
3. Add 1 ul of ampure-purified products to the 99ul TE wells to measure 1:100 dilutions of the samples in replicates as shown in the plate layout below.
4. Make standard dilutions by adding 100ul TE to all wells of rows G and H, adding 100ul of the independent standards to G1 and H1 to make the highest concentration 1ng/ul, and serially transferring 100ul across columns to make 1:2 dilutions.
5. Make picogreen working solution (45ul to 9ml TE), add 100 ul to all wells used in assay, cover to protect from light and incubate 5 minutes before reading on plate reader.

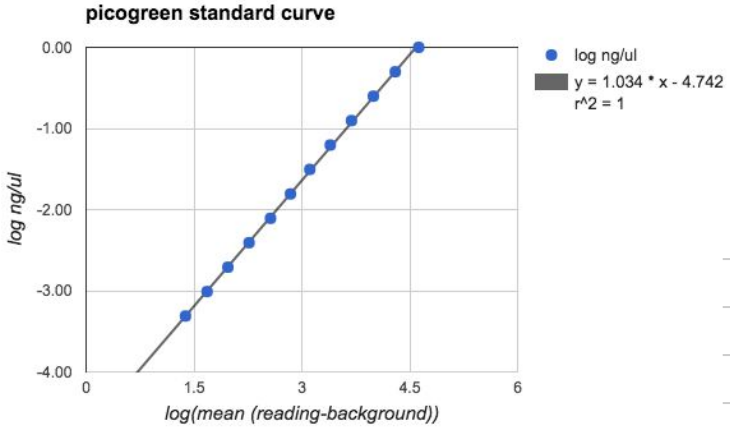
picogreen plate layout:

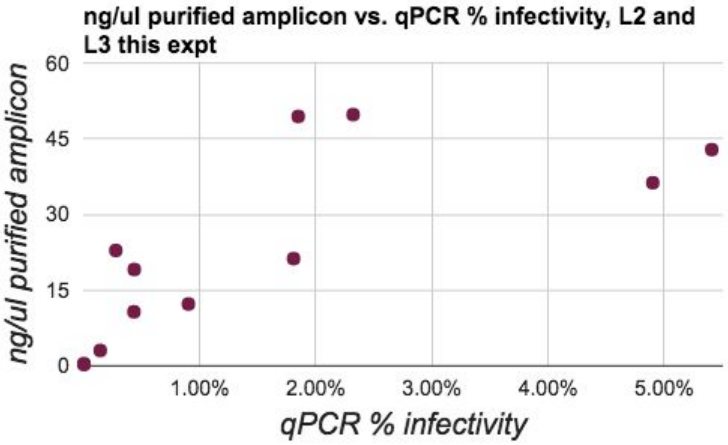
	1	2	3	4	5	6	7	8	9	10	11	12
A	PCR 1	PCR 2	PCR 3	PCR 4	PCR 5	PCR 6	PCR 7	PCR 8				
B	PCR 1	PCR 2	PCR 3	PCR 4	PCR 5	PCR 6	PCR 7	PCR 8				
C	PCR 9	PCR 10	PCR 11	PCR 12	PCR 13	PCR 14	(TE)	(TE)				
D	PCR 9	PCR 10	PCR 11	PCR 12	PCR 13	PCR 14	(TE)	(TE)				
E	PCR 15	PCR 16	PCR 17	PCR 18	PCR 19	PCR 20	(TE)	(TE)				
F	PCR 15	PCR 16	PCR 17	PCR 18	PCR 19	PCR 20	(TE)	(TE)				
G	1.00E+00	5.00E-01	2.50E-01	1.25E-01	6.25E-02	3.13E-02	1.56E-02	7.81E-03	3.91E-03	1.95E-03	9.77E-04	4.88E-04
H	1.00E+00	5.00E-01	2.50E-01	1.25E-01	6.25E-02	3.13E-02	1.56E-02	7.81E-03	3.91E-03	1.95E-03	9.77E-04	4.88E-04

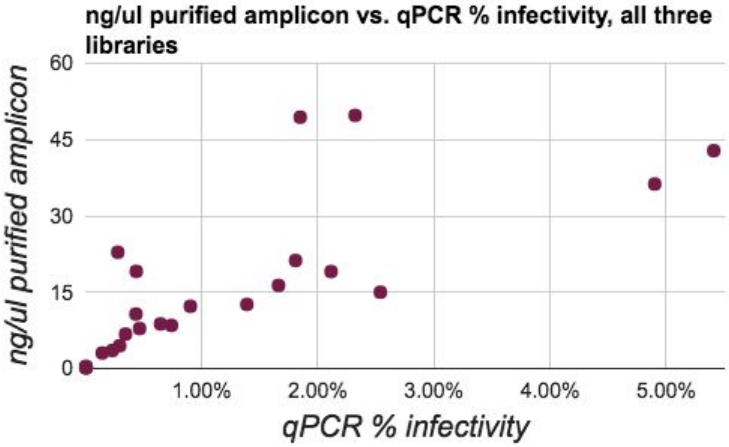
raw readings from plate reader:

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	14577	19289	7752	19424	9290	240	16912	8599				
B	14433	19800	7887	19940	9307	248	17144	8726				
C	4513	5171	1364	173	27492	25999	43	41				
D	4467	5039	1363	171	27509	26851	43	42				
E	50	43	78	537	2824	15163	45	45				
F	82	44	78	537	2775	14872	45	43				
G	43743	20947	10186	5162	2603	1376	768	418	234	141	91	67
H	40669	18963	9626	4715	2456	1291	709	402	222	133	92	68

2016-06-12: Picogreen results: pretty much as expected. Consistent with eyeing band intensities on the gel, most samples used >= 1e6 cDNA molecules as template.







ID	sample	ng/ul purified amplicon	qPCR % infectivity
1	L2_H17L19_c1	36.3	4.902%
2	L2_H17L19_c2	49.4	1.849%
3	L2_H17L19_c3	19.1	0.437%
4	L2_FI6v3_c1	49.8	2.322%
5	L2_FI6v3_c2	22.9	0.278%
6	L2_FI6v3_c3	0.4	0.003%
7	L3_H17L19_c1	42.8	5.410%
8	L3_H17L19_c2	21.2	1.809%
9	L3_H17L19_c3	10.7	0.434%
10	L3_FI6v3_c1	12.3	0.903%
11	L3_FI6v3_c2	3.1	0.144%
12	L3_FI6v3_c3	0.3	0.004%
13	L2_mock pooled	70.4	
14	L3_mock pooled	67.6	
15	st_L2_NoVirus_a	0.0	
16	no template	0.0	
17	1e4 witness	0.1	
18	1e5 witness	1.1	
19	1e6witness	6.5	
20	1e7witness	37.6	