2016-04-04 pilot neutralization of library 1 with H17-L19 and FI6v3.

Monday 2016-04-04: 3pm: Plated 2.5E5 MDCKSIAT-1 cells per well in 5 plates of 6-wells **at 3pm**.
I have 20ul aliquots of H17-L19 (1mg/ml) from when we first received it, I thawed three of them for this experiment. I will thaw a fresh vial of FI6v3 from -80C (0.83mg/ml), make 50ul aliquots and freeze at -20 before performing this experiment (these aliquots are dated 4/4/16).
Will neutralize with 0.5, 1, and 10 ug/ml of H17-L19; 0.1, 0.2, and 2 ug/ml FI6v3. (final concentration during incubation with virus).
Make antibody dilutions and virus dilutions:
H17-L19:
Add 60 ul of 1mg/ml stock to 3mL IGM to make 20ug/ml.
Transfer 450ul of 20ug/ml to a final volume of 4.5mL IGM to make 2ug/ml.
Transfer 2mL of 2ug/ml to a final volume of 4mL IGM to make 1ug/ml.
FI6v3:
Add 14.4ul To 3mL IGM to make 4ug/ml.
Transfer 450ul of 4ug/ml to final volume of 4.5mL IGM to make 0.4 ug/ml.
Transfer 2mL of 0.4ug/ml to a final volume of 4mL IGM to make 0.2 ug/ml.
Library 1:
Add 2 mL of library 1 (7600 tcid50/ul) to final volume 15.2mL IGM to make 1E6 TCID50/ml.

Neutralize **6:40pm-7:40pm**: In 5mL eppendorf tubes, mix 1mL of each diluted antibody with 1 mL library - do duplicates for each antibody dilution (12 tubes of 2 mL) and make two additional no MAb controls by mixing 1 mL library with 1 mL IGM. **Incubate at 37C for 1 hr.**
Store remaining 1E6/ml at 4C until ready to make standard curves (immediately prior to infection).

Before infecting, use the remaining 1E6/ml to make standard infection curve inocula:
Make 50 TCID50/ul (5 ml total), (1E5 TCID50 per 2mL infection, or 10% of library dose for each neutralization).
To make 50T/ul ("1E5 standard"), add 250ul of the 1E6T/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. Only made one dilution series but infected in duplicate.

Infect at **7:40PM**: Aspirate D10 from cells and replace with each 2mL neutralization reaction. *For the no MAb controls, split each duplicate 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). In total there are two plates of standard curve (2 wells per 5 concentrations and 2 negative control wells), one plate of H17L19 (2 wells per 3 concentrations), one plate of FI6v3 (2 wells per 3 concentrations), and one plate of mock neutralizations (2 mock neutralizations spread out over 3 wells each). Plates 1 and 2 are the standard curves. Plates 3 and 4 and the neutralized samples and got to them between 8pm and 8:15. Plate 5 is the mock neutralizations.
Change media at **9:40PM** by washing cells **1x with 1 mL PBS**. Replace with 2 mL IGM+5%D10. Got to Plate 3 and 4 between 10:20 and 10:40. Finished up around 11.
Lyse cells & Extract RNA at **10:40AM** (15 hours post-infection). Lyse all wells and purify in two batches, keeping the lysates on ice when waiting to purify.

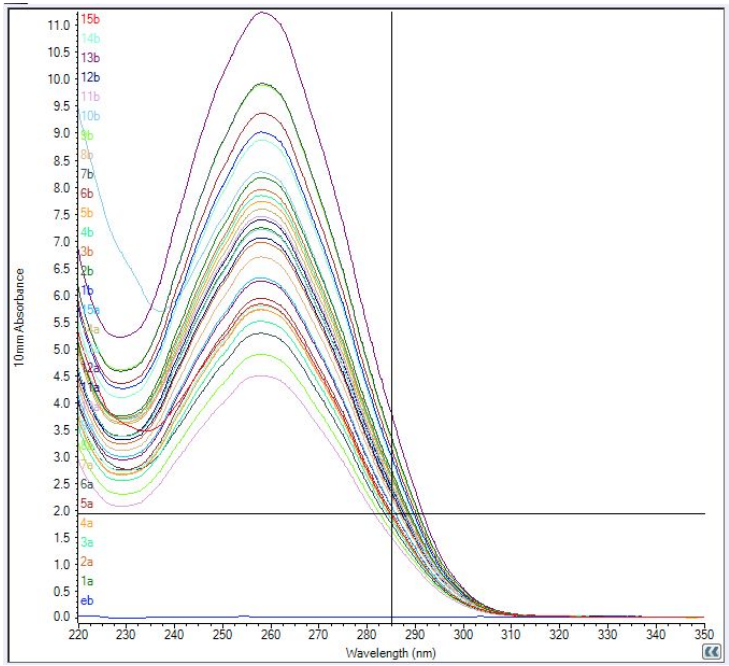
	2 plates:			3 more plates:	
	Standard curve of infection			Neutralizations of Library 1	
	One Dilution and infect in duplicate			Neutralize and infect in duplicate	
MOI for 2.5e5/well	% not neutralized	TCID50 per well		Sample	[MAb] during neutralization
0.4	10.000%	100,000		H17-L19 conc 1	**0.5 ug/ml**
0.04	1.000%	10,000		H17-L19 conc 2	**1 ug/ml**
0.004	0.100%	1,000		H17-L19 conc 3	**10 ug/ml**
0.0004	0.010%	100		FI6v3 conc 1	**0.1 ug/ml**
0.00004	0.001%	10		FI6v3 conc 2	**0.2 ug/ml**
0	no virus control	0		FI6v3 conc 3	**2 ug/ml**
				No neutralization (spread over 3 wells each replicate)	0 ug/ml

2016-04-05 RNA samples: 1-6 are the duplicate infections with standard curves. 7-9 are neutralization with H17L19 at 0.5, 1.0, and 10.0 ug/ml respectively; 10-12 are FI6v3, 13-15 are the three wells of a “mock-neutralized” 1E6 TCID50 spread out over three wells.

(rep A and B for each) RNA TUBE #	Sample
1	1E5
2	1E4
3	1E3
4	1E2
5	1E1
6	0
7	H17-0.5
8	H17-1.0
9	H17-10.0
10	FI6-0.1
11	FI6-0.2
12	FI6-2.0
13	mock
14	mock
15	mock

2016-04-05: RNA purification

I will lyse all wells and keep half of the samples (one entire set of replicates) on ice while I purify the others, and then finish the second replicate. For 30 samples, aliquot RLT and add BME: 12 mL RLT + 120 ul BME. 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. Eluted in 35ul.



Sample ID	Nucleic Acid Conc.	Unit
eb	0.1	ng/µl
1a	324.7	ng/µl
1b	358.4	ng/µl
2a	316.1	ng/µl
2b	287.7	ng/µl
3a	311.5	ng/µl
3b	276.9	ng/µl
4a	307.4	ng/µl
4b	218.5	ng/µl
5a	372.6	ng/µl
5b	227.1	ng/µl
6a	394.7	ng/µl
6b	235.4	ng/µl
7a	266	ng/µl
7b	209.6	ng/µl
8a	393.6	ng/µl
8b	227.2	ng/µl
9a	286.4	ng/µl
9b	193.9	ng/µl
10a	296.2	ng/µl
10b	329	ng/µl
11a	280.3	ng/µl
11b	178.5	ng/µl
12a	447.2	ng/µl
12b	293.5	ng/µl
13a	230.2	ng/µl
13b	248.1	ng/µl
14a	301.5	ng/µl
14b	352.4	ng/µl
15a	250.6	ng/µl
15b	231.1	ng/µl

Dilute all RNA samples to 10 ng/ul.

Sample ID	Nucleic Acid Conc.	ul to Add to 100ul EB to make 10ng/ul dilution:
eb	0.1	
1a	324.7	3.18
1b	358.4	2.87
2a	316.1	3.27
2b	287.7	3.60
3a	311.5	3.32
3b	276.9	3.75
4a	307.4	3.36
4b	218.5	4.80
5a	372.6	2.76
5b	227.1	4.61
6a	394.7	2.60
6b	235.4	4.44
7a	266	3.91
7b	209.6	5.01
8a	393.6	2.61
8b	227.2	4.60
9a	286.4	3.62
9b	193.9	5.44
10a	296.2	3.49
10b	329	3.13
11a	280.3	3.70
11b	178.5	5.93
12a	447.2	2.29
12b	293.5	3.53
13a	230.2	4.54
13b	248.1	4.20
14a	301.5	3.43
14b	352.4	2.92
15a	250.6	4.16
15b	231.1	4.52

2016-04-06 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 20ul.

I made master mixes as so:
54x RT+ MM: 540 ul One-Step MM + 21.6 ul qScript RT + 194.4 ul water
2x RT- MM: 20ul One-STep MM + 8 ul H2O (replaces H2O and RT)

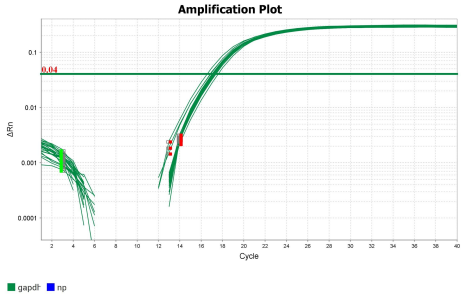
Split RT+ MM into two 27x MM tubes (each tube gets 378ul) and add NP primers or GAPDH primers to respective tubes (each tube gets 27 ul of each primer from 5uM stocks).
Aliquot the two RT+NP and RT+GAPDH master mixes to appropriate rows as labeled below (16 ul per well).
Aliquot the RT- MM to G1 and H1 (14ul per well) and add 1ul each F and R primer accordingly.

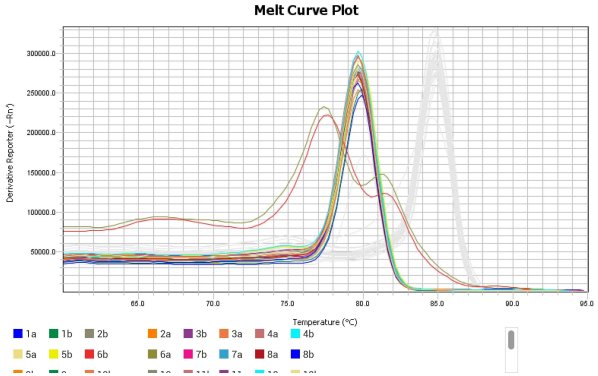
Add 4 ul RNA (from 10ng/ul dilutions) to each as indicated.

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)

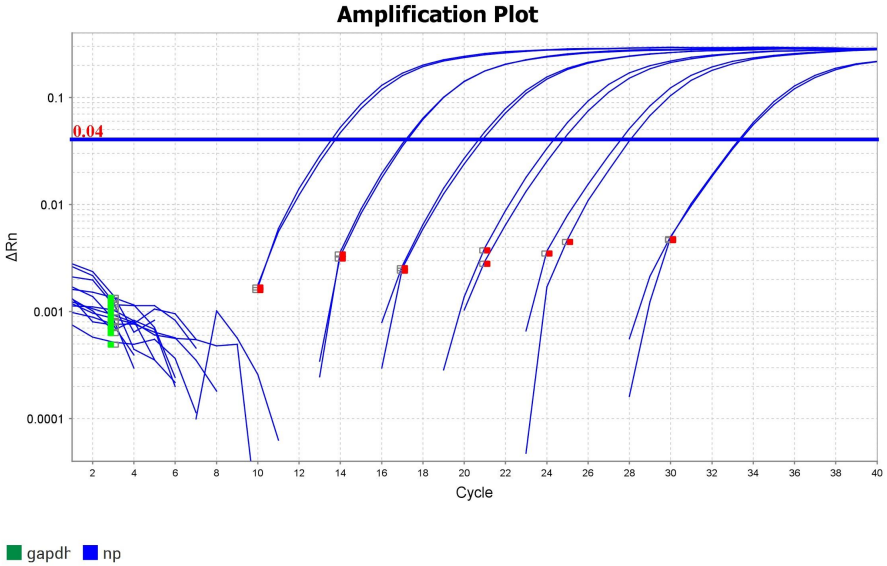
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A - NP	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B
B - GAPDH	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B
C - NP	7A	7B	8A	8B	9A	9B	10A	10B	11A	11B	12A	12B
D - GAPDH	7A	7B	8A	8B	9A	9B	10A	10B	11A	11B	12A	12B
E - NP	13A	13B										
F - GAPDH	13A	13B										
G - NP NO RT	13A											
H - GAPDH NO RT	13A											

2016-04-06 qRT-PCR

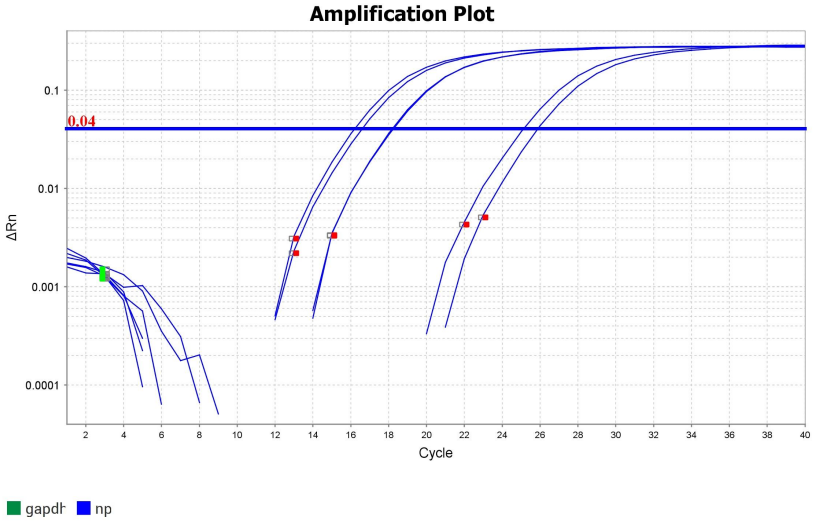




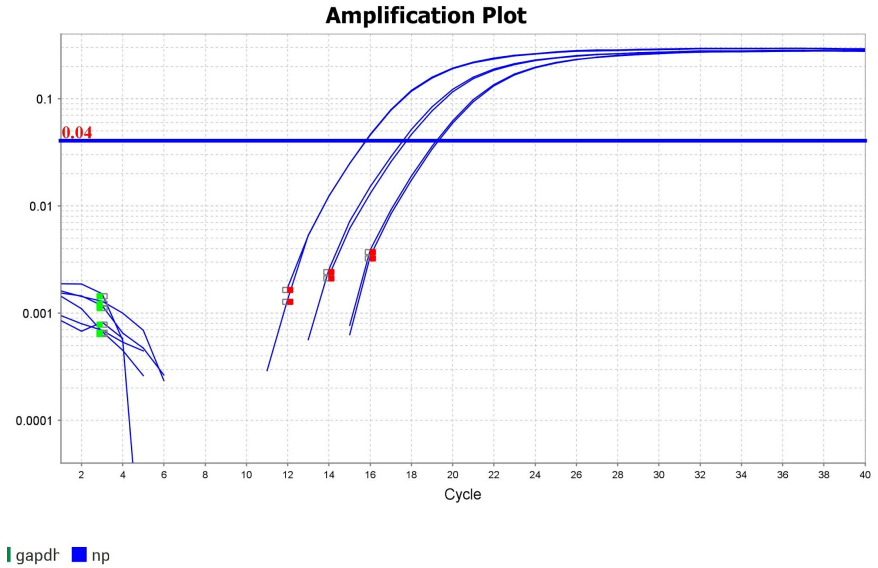
Infectious dose standard curve

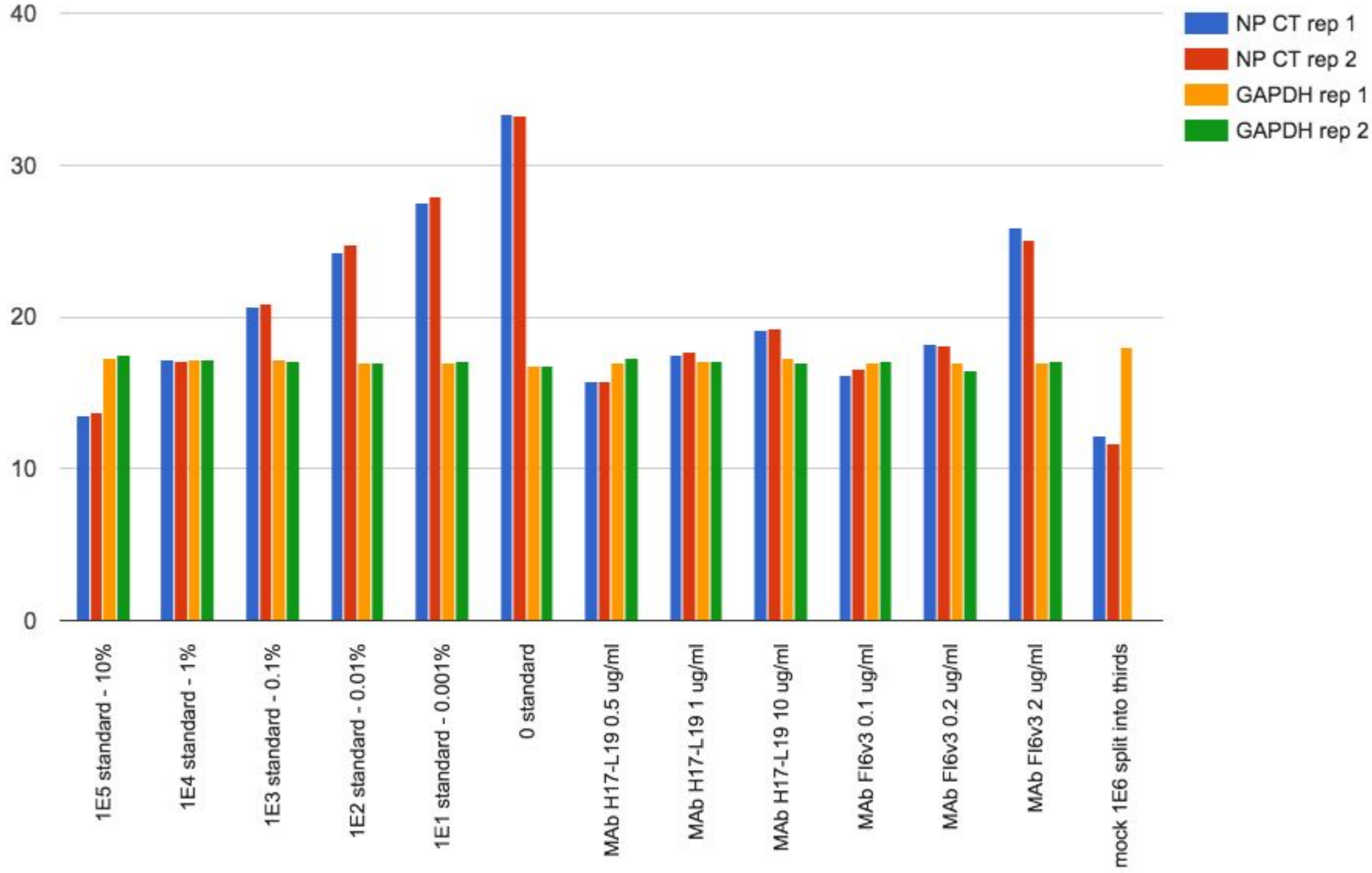


FI6v3 neutralized samples

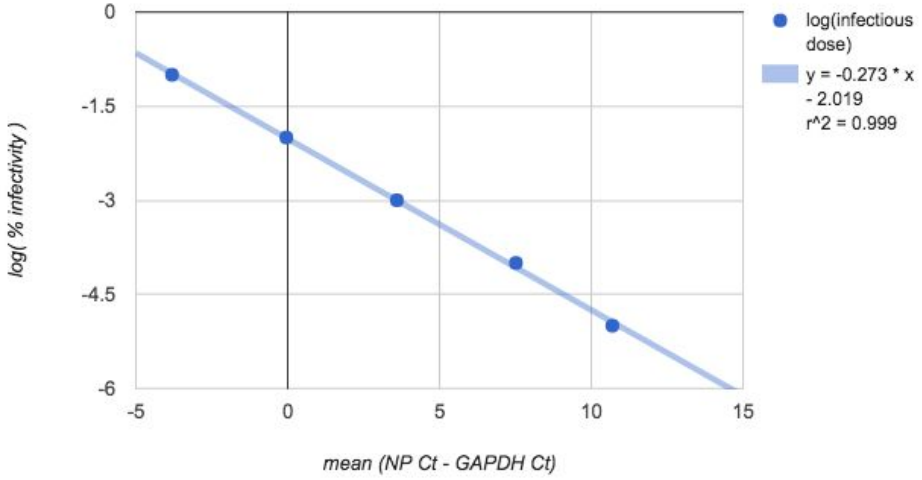


H17-L19 neutralized samples




40
30
20
10
0
NP CT rep 1
NP CT rep 2
GAPDH rep 1
GAPDH rep 2
1E5 standard - 10%
1E4 standard - 1%
1E3 standard - 0.1%
1E2 standard - 0.01%
1E1 standard - 0.001%
0 standard
MAb H17-L19 0.5 ug/ml
MAb H17-L19 1 ug/ml
MAb H17-L19 10 ug/ml
MAb FI6v3 0.1 ug/ml
MAb FI6v3 0.2 ug/ml
MAb FI6v3 2 ug/ml
mock 1E6 split into thirds

Standard curve of % infectivity from dilution series (no neutralization)

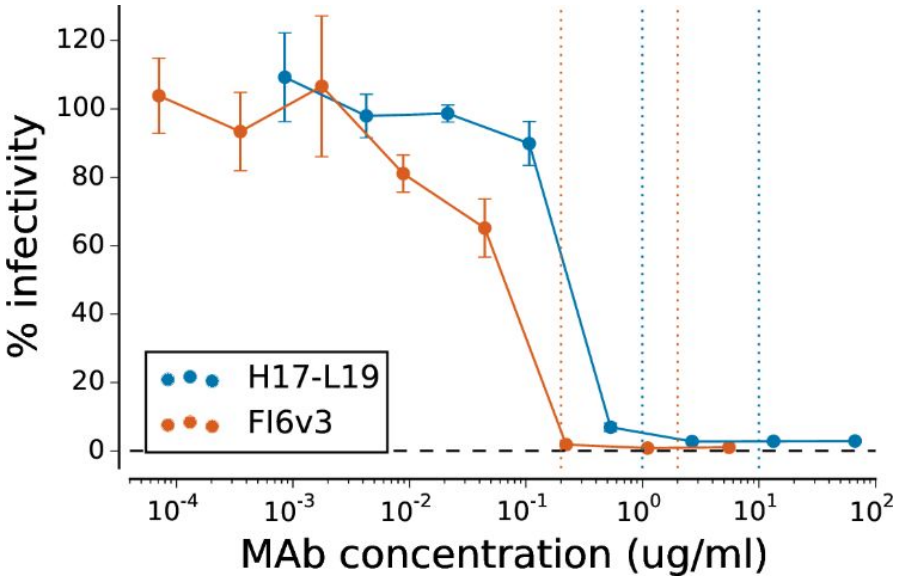


Interpolated % infectivity of neutralized samples

	% infectivity rep 1	% infectivity rep 2
MAb H17-L19 0.5 ug/ml	2.11%	2.54%
MAb H17-L19 1 ug/ml	0.74%	0.65%
MAb H17-L19 10 ug/ml	0.29%	0.23%
MAb FI6v3 0.1 ug/ml	1.66%	1.39%
MAb FI6v3 0.2 ug/ml	0.47%	0.34%
MAb FI6v3 2 ug/ml	0.004%	0.006%
mock 1E6 split into thirds	37.8%	

Maybe bottleneck 100,000 per selected sample instead of 700,000 like last time. But bottleneck same way 700,000 as last time for the mock selected samples.

Old data: WSN HA/NA pseudotyped PB1flankGFP neutralization curves



2016-04-10 Making new witness band amplicon

KOD PCR: (made 4x)
25ul KOD MM
1ul 10ng/ul template [plasmid #1310, pHH-WSN-HA]
3ul 5uM F primer [WSN-for]
3ul 5uM R primer [WSN-rev]
18 ul water to 50ul

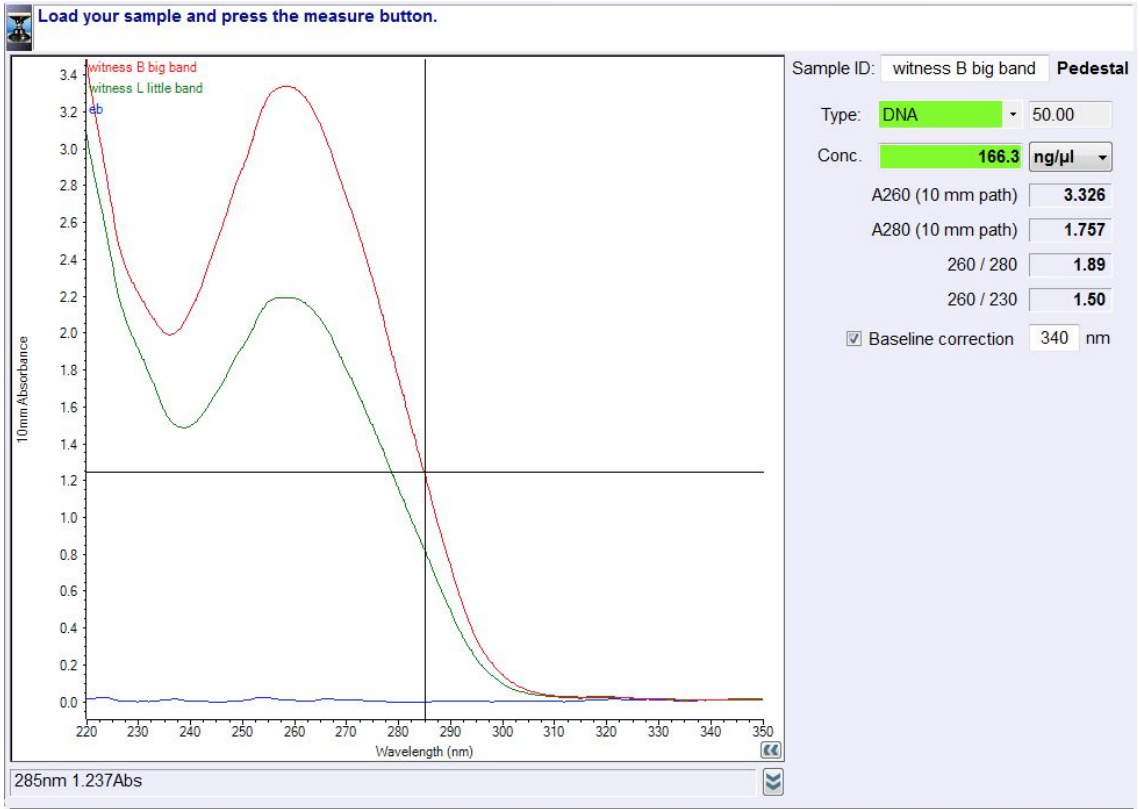
1. 95 C 2:00
2. 95 C :20
3. 50 C :30, cooling to 50 C and 0.5 C/second
4. 70 C :40
5. Goto 2, 34 times
7. 4C forever

4x MM:

100 ul KOD
4 ul 10ng/ul template #1310
12 ul each primer
72 ul water

Split into 4 50-ul reaction tubes.

Run on 1% gel and extract band. Split sample into a smaller 2-lane combo (little band) and a larger 3-lane combo (big band). Both bands are the same size, obviously.



#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/
1	eb	bloomuser, Service Account	4/10/2016 7:02 PM	-0.1	ng/µl	-0.001	-0.012	0.11	0.15
2	witness L little band	bloomuser, Service Account	4/10/2016 7:03 PM	109.3	ng/µl	2.185	1.154	1.89	1.14
3	witness B big band	bloomuser, Service Account	4/10/2016 7:04 PM	166.3	ng/µl	3.326	1.757	1.89	1.50

2016-04-10 Testing RT with varying amounts of RNA input. Previously I did RT using ~1000ng of total RNA per 30ul RT reaction. (see results on 3.30.2016). Today I will use a few of those samples (representing 2% of library (20,000 TCID50 infection) and 0.02% of library (200 TCID50) escaping neutralization; sample numbers 1 and 5, respectively) to test RT using the following amounts of RNA:

1. 250ng RNA (0.5ul + 8.3 ul H2O)
2. 500ng RNA (1ul + 7.8 ul H2O)
3. 1000ng RNA (2ul + 6.8 ul H2O)
4. 2500ng RNA (5ul + 3.8 ul H2O)

	Nucleic Acid Conc.	Make 500ng/ul stock
1: 20,000 TCID50	511.9	19.5 ul + 0.5 ul H2O
5: 200 TCID50	528.7	18.9 ul + 1.1 ul H2O

Each RT:
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
0.5-5 ul of RNA template
3.8-8.3 ul of water for 19 ul total volume.

Make a master mix for 10 reactions' worth (for the 8 reactions I will do):
30 ul 10X AccuScript RT Buffer
12 ul of dNTP mix
30 ul of 5 uM WSN-For
30 ul of 5 uM WSN-Rev

Number tubes 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4: first number is RNA template tube number from 3.30, second number is RNA concentration as shown above.

Aliquoted 10.2 ul of this master mix into 8 PCR tubes, added the 8.8 ul of the RNA/water combo as shown above to each tube, and mixed by pipetting. Heated to 65 C for 5 minutes, cooled to 4 C in the PCR machine. Make the following master mixes while heating:

AccuScript master mix (for 10 rxn): 25 ul of water and 15 ul of AccuScript RT
RNAse block master mix (for 10 rxn): 32.5 ul of water and 7.5 ul of Rnase block

Added 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. Freeze at -20 overnight before PCR.

2016-04-11 continuation of RT/PCR pilot to optimize amount of RNA used in RT:

I have cDNA samples derived from two different RNA samples (1 and 5, from 3.30) used at four different amounts of RNA in each RT (1: 250ng, 2: 500ng, 3: 1000ng, 4:2500ng) - these are cDNA samples 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4 as previously described. Here I will amplify these cDNA samples alongside fresh witness template and assess overall yield by band intensities on an agarose gel. I typically use 2ul of cDNA in each 50ul PCR; here I will test **both 2ul and 10ul.**

PCR tubes 1-8: **2ul** template of 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4
PCR tubes 9-16: **10ul** template of 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4
PCR tubes 17-21: no template control, dilutions of **fresh witness** 1e4, 1e5, 1e6, 1e7 per reaction.
PCR tubes 22-25: no template control, dilutions of **old witness** 1e5, 1e6, 1e7 per reaction.

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.
The "old witness" concentrated stock is 94.6ng/ul, so make an "old" 5e9/ul solution (5ng/ul) by adding (10.56 ul of 94.7ng/ul) to 189.44 ul EB, and making 10-fold dilutions by transferring 20 ul to 180 ul EB to make "old stock" 5e4, 5e5, and 5e6 per ul.

The "*fresh witness" concentrated stock* is 166 ng/ul ("big band") so make a "fresh" 5e9/ul (5ng/ul) by adding (6 ul to 194 ul EB) and making 10-fold dilutions by transferring 20ul to 180 ul to make fresh stock 5e3, 5e4, 5e5, 5e6 per ul.

Do in **50ul reactions: (make 27x master mix)**
Each reaction:
25 ul of 2X KOD Master Mix (675 ul)
3 ul of 5 uM WSN-for (81 ul)
3 ul of 5 uM WSN-rev (81 ul)
9 ul of water (243 ul)
2 or 10 ul template, plus 8 ul water if only using 2ul template
Add 40 ul of PCR mastermix to tubes, then add 2ul or 10ul of template to each well, plus 8 ul water if only using 2 ul template (tubes 1-8, 17-25.).
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

Add 10ul 6x sample buffer to every sample, run 12ul on 1% agarose.

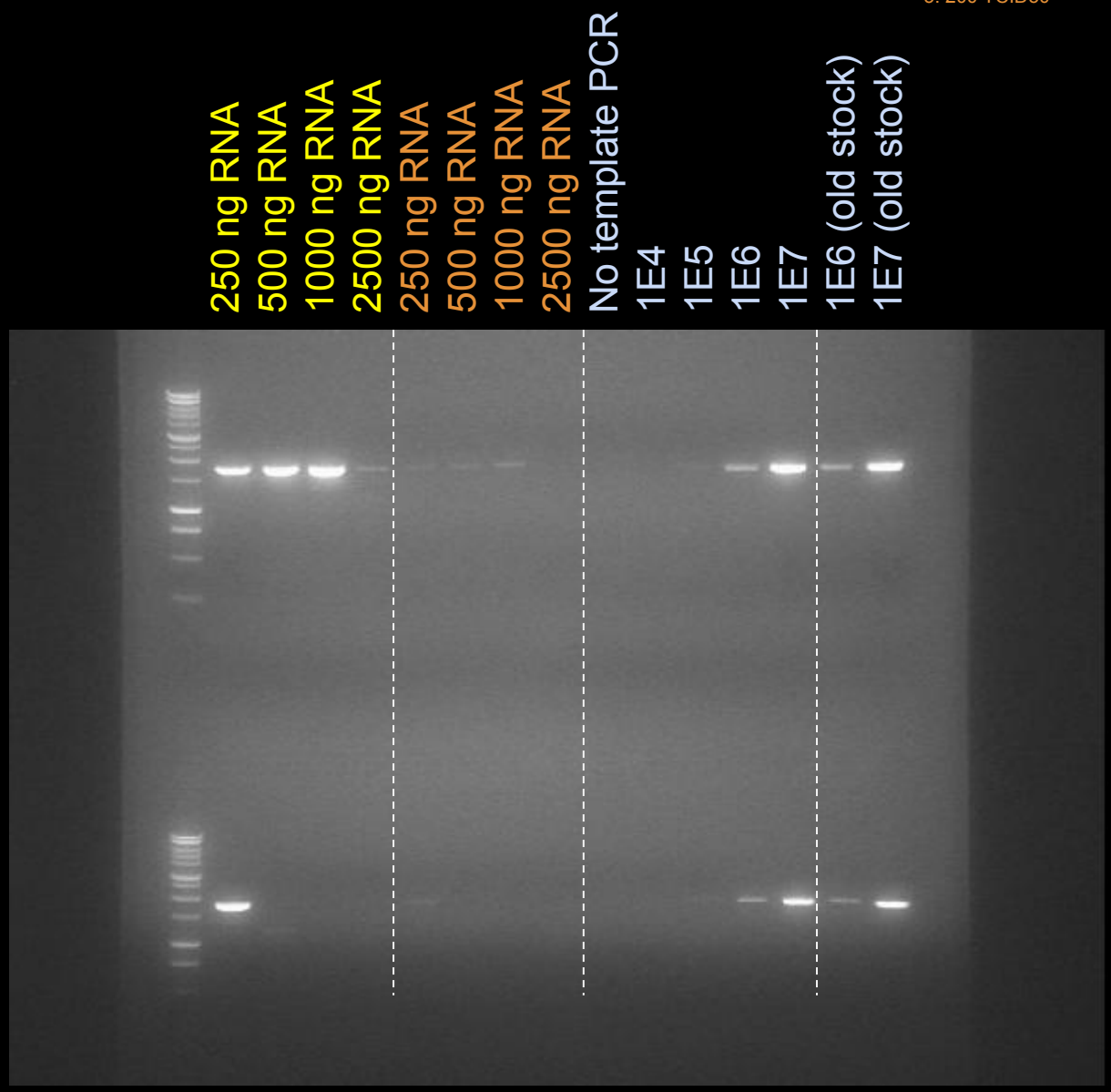
2016-04-11: gel layout
Top row: Ladder, PCR 1-8, 17-21, 24, 25
Bottom row: Ladder, PCR 9-16, 17-21, 24, 25
PCR tubes 1-8: **2ul** template of 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4
PCR tubes 9-16: **10ul** template of 1.1, 1.2, 1.3, 1.4, 5.1, 5.2, 5.3, 5.4
PCR tubes 17-21: no template control, dilutions of **fresh witness** 1e4, 1e5, 1e6, 1e7 per reaction.
PCR tubes 22-25: no template control, dilutions of **old witness** 1e5, 1e6, 1e7 per reaction.

1. 250ng RNA (0.5ul + 8.3 ul H2O)
2. 500ng RNA (1ul + 7.8 ul H2O)
3. 1000ng RNA (2ul + 6.8 ul H2O)
4. 2500ng RNA (5ul + 3.8 ul H2O)

RNA templates from 3.30 prep

1: 20,000 TCID50

5: 200 TCID50



RT with RNA from infection with:

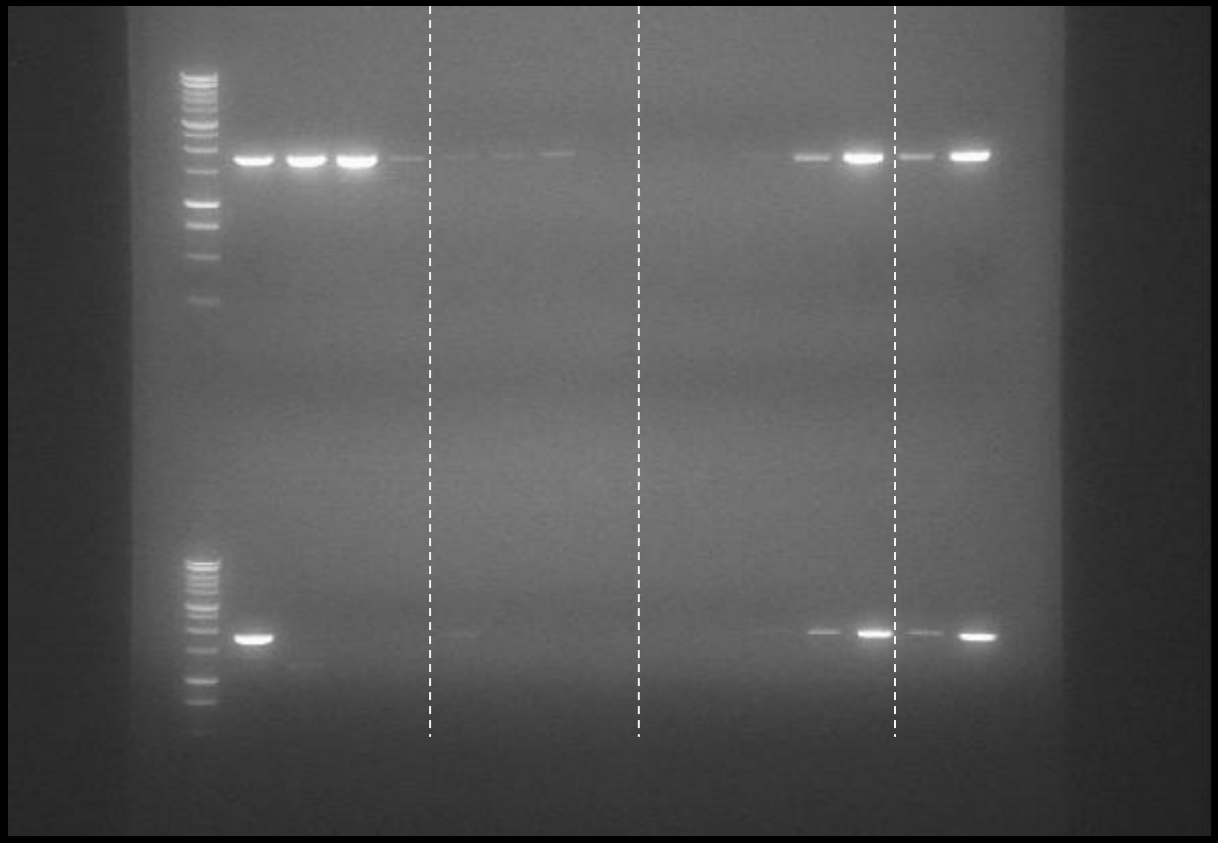
20,000 TCID50

200 TCID50

250 ng RNA
500 ng RNA
1000 ng RNA
2500 ng RNA
250 ng RNA
500 ng RNA
1000 ng RNA
2500 ng RNA
No template PCR
1E4
1E5
1E6
1E7
1E6 (old stock)
1E7 (old stock)

2 ul cDNA to PCR:

10 ul cDNA to PCR:



2016-04-14 Making amplicons for selected samples to sequence.
So far I've found that yield of cDNA is maximized for these types of samples when using 1000ng total RNA as template in a 30ul AccuScript RT reaction. I will use my 2016.04.05 RNA preps from neutralized and mock-neutralized infections with WSN HA Library 1. Here are the tube numbers and sample types; there is an "A" and "B" replicate for each sample (these are replicates at the stage of neutralizing 1E6 TCID50 and infecting cells). Tubes 1 through 5 are a standard curve of unneutralized virus and tube 6 is a no-virus negative control.

I will make amplicons for 7-12 A and B (duplicate neutralizations at three concentrations of two MAbs). I will also make amplicons for "mock-neutralized" infections - these infections were split over three wells each to keep the MOI from getting too high during infection - I will combine RNA from 13A/14A/15A and 13B/14B/15B when making amplicons for the mock neutralized samples.

(rep A and B for each)

2016.04.05 RNA TUBE #	**Sample**
1	1E5
2	1E4
3	1E3
4	1E2
5	1E1
6	0
7	H17-0.5
8	H17-1.0
9	H17-10.0
10	FI6-0.1
11	FI6-0.2
12	FI6-2.0
13	mock
14	mock
15	mock

RT tube	RNA template	RNA ng/ul	ul RNA for 1000ng	water balance to 8.8ul
1	7A	266	**3.76**	**5.04**
2	7B	209.6	**4.77**	**4.03**
3	8A	393.6	**2.54**	**6.26**
4	8B	227.2	**4.40**	**4.40**
5	9A	286.4	**3.49**	**5.31**
6	9B	193.9	**5.16**	**3.64**
7	10A	296.2	**3.38**	**5.42**
8	10B	329	**3.04**	**5.76**
9	11A	280.3	**3.57**	**5.23**
10	11B	178.5	**5.60**	**3.20**
11	12A	447.2	**2.24**	**6.56**
12	12B	293.5	**3.41**	**5.39**
13	**13/14/15A**		**ul RNA for 333ng**	
	13A	230.2	**1.45**	**4.92**
	14A	301.5	**1.10**	
	15A	250.6	**1.33**	
14	**13/14/15B**			
	13B	248.1	**1.34**	**5.07**
	14B	352.4	**0.94**	
	15B	231.1	**1.44**	
			ul for 1000ng	
15	6A (no virus)	394.7	**2.53**	**6.27**

Each RT:
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 1000ng total in RNase-free water.
---
19 ul total volume

Make a 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.

2016-04-14 continued: PCR on cDNA. PCR tube numbers for cDNA are the same numbers as the RT listed on the last page.

PCR tube	cDNA	standard dna
1	7A (H17-0.5)	
2	7B (H17-0.5)	
3	8A (H17-1.0)	
4	8B (H17-1.0)	
5	9A (H17-10.0)	
6	9B (H17-10.0)	
7	10A (FI6-0.1)	
8	10B (FI6-0.1)	
9	11A (FI6-0.2)	
10	11B (FI6-0.2)	
11	12A (FI6-2.0)	
12	12B (FI6-2.0)	
13	13/14/15A (mock)	
14	13/14/15B (mock)	
15	6A (no virus)	
16		no template
17		1e4
18		1e5
19		1e6
20		1e7

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 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.

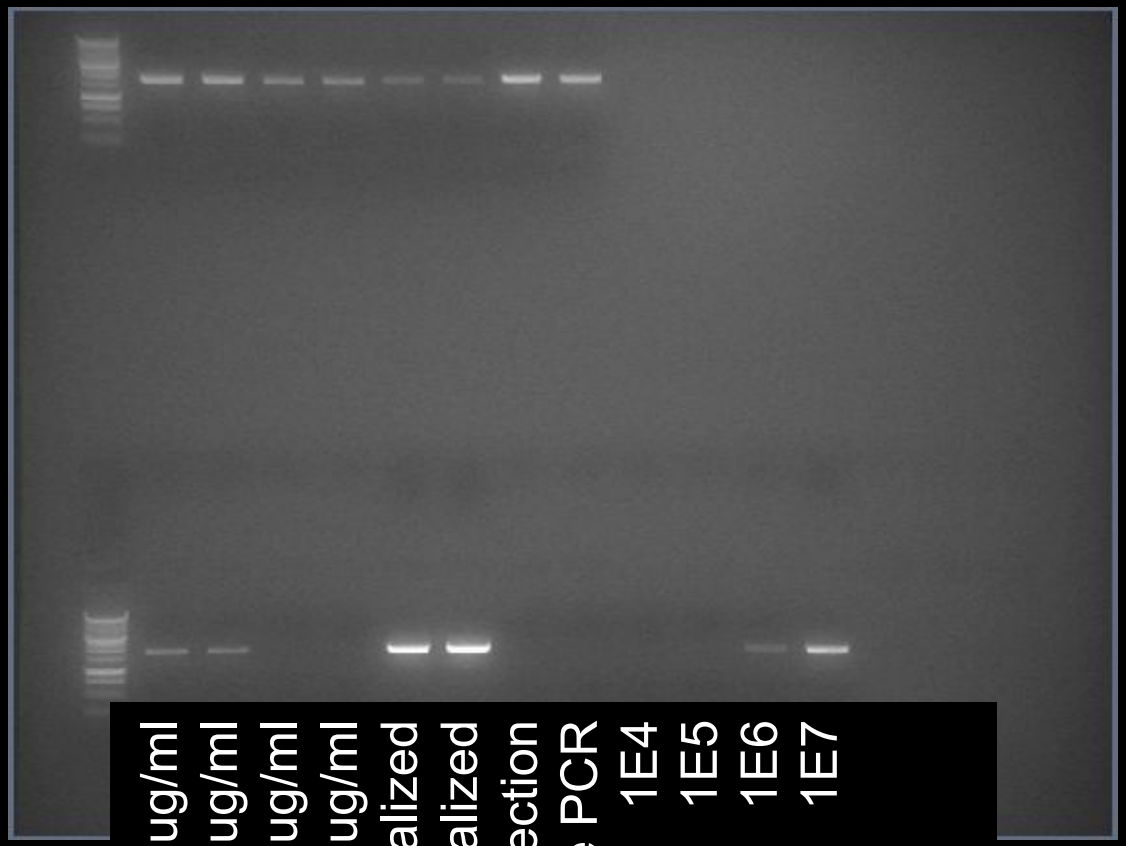
Use the following PCR program (**22 total PCR cycles**):
1.95 C for 2:00
2.95 C for :20
3.70 C for :01
4.50 C for :30, cooling to 50 C at 0.5 C/second
5.70 C for :40
6.Goto 2, 21 times
7.4 C forever

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

Top row: ladder, 1-8
Bottom row: ladder, 9-16, 17-20

H17-L19 (head binding)
0.5, 1.0, 10.0 ug/ml MAb
2.3%, 0.7%, 0.25%

FI6v3 (stem binding)
0.1, 0.2, 2.0 ug/ml MAb
1.5%, 0.4%, 0.005%



2016-04-15: 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
6. Wash twice with 180ul fresh 80% ethanol
7. 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 ("2016.04.15 purified full-length amplicons") with the following layout:

"2016.04.15 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												

2016-04-15: 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.

	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

2016-04-15: Picogreen of full-length amplicons

Results averaged from two independent readings for each PCR product:

Sample	cDNA or standard	ng/ul full length amplicon	volume added to 110ul H2O to make 0.5ng/ul
PCR 1	7A(H17-0.5)	19.0717	2.9615
PCR 2	7B (H17-0.5)	15.0124	3.7899
PCR 3	8A (H17-1.0)	8.4844	6.8884
PCR 4	8B (H17-1.0)	8.7775	6.6445
PCR 5	9A (H17-10.0)	4.4945	13.7688
PCR 6	9B (H17-10.0)	3.5613	17.9660
PCR 7	10A (FI6-0.1)	16.3451	3.4711
PCR 8	10B(FI6-0.1)	12.6105	4.5415
PCR 9	11A(FI6-0.2)	7.8677	7.4650
PCR 10	11B(FI6-0.2)	6.7677	8.7751
PCR 11	12A (FI6-2.0)	0.1383	use undiluted
PCR 12	12B (FI6-2.0)	0.1538	use undiluted
PCR 13	13/14/15A (mock)	40.2015	1.3853
PCR 14	13/14/15B (mock)	33.0829	1.6880
PCR 15	6A (no virus)	0.0557	use undiluted
PCR 16	no template	0.0476	
PCR 17	1e4	0.0891	
PCR 18	1e5	0.4757	
PCR 19	1e6	4.2802	
PCR 20	1e7	21.6352	

I will use a new plate to make dilutions to 0.5ng/ul for these samples to use as templates in round 1 PCR.

The layout of the 0.5ng/ul full length amplicons plate is on the following slide.

Samples PCR11 and PCR12, which had very strong selection with FI6v3, have very low concentrations, although these are above the background signal from no-template PCR and no-virus RNA used for RT-PCR. Since I expect that the diversity in these samples is already very very low after selection, I will proceed with those samples at the existing concentrations, which will provide ~1ng as template for round 1 PCR instead of the 4ng provided for all other samples.

“2016.04.15 purified full-length amplicons” -- transferring variable volume of most of these samples to the next plate containing 110ul EB in all wells (except for PCR11, PCR12, and PCR15) to make 0.5ng/ul samples.

	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												

“2016.04.17 0.5ng/ul full-length amplicons”: First add 110ul EB to each of these wells EXCEPT E3, E4, E7, and E8. Transfer the indicated amount of each product from the plate above to the plate below (volumes listed on previous slide in order to make final concentration 0.5ng/ul). For E3, E4, and E7, transfer the entire product to the plate below (using undiluted, since these samples are <0.5ng/ul). For E8, transfer 48ul of E6 to have a duplicate well of PCR 14 at 0.5ng/ul, this will be used as template for no-primer control reactions in round 1 pcr.

	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												
D												
E	PCR 9	PCR 10	PCR 11	PCR 12	PCR 13	PCR 14	PCR 15	PCR 14 -48ul*				
F												
G												
H												

2016-04-17 Round 1 PCR. Round 1 primers are 10uM stocks. For each primer pair (ampX_F/ampX_R), make a mix for 16 reaction's worth (actually only needing 15 reactions for each primer pair). To do this, add: **16ul F primer (10uM) + 16ul R primer (10uM) + 32ul water.** Final volume 64ul, each primer is 2.5uM in this final mixture. Each PCR gets 4ul of this.

Using the 0.5 ng/ul amplicon plate to set up round 1 PCR reactions. Each reaction will get (total volume of 24 ul):

12 ul 2X KOD Master Mix

4 ul of mixture of 2.5 uM forward primer + 2.5 uM reverse primer (0.42 uM final concentration each primer)

8 ul of 0.5 ng/ul template (4 ng total)

Set these reactions up with a multichannel with the format shown below.

1. Dispense 12ul KOD to all wells (dispense from strip tubes; 170ul KOD in each of 8 tubes should be enough for 12 dispensings of 12 ul)
2. Add 4ul primers from a striptube of primer pairs 1-6 (6 transfers at a time to 15 rows of 6) to all rows except the no-primer control (H7-H12).
3. Add templates to each column from the 0.5ng/ul plate, which is arranged to facilitate easy transfer using the multichannel (8ul).

Seal with microfilm A, spin, and thermocycle as described here. will run **9 PCR cycles.**. With perfect efficiency, this gives a theoretical amplification of 512-fold, which would give about 340 ng of DNA per well (accounting for the fact that the created amplicons are only about 1/6 the length of the template). Using program "ORRSUBR1"

1. 95 C for 2:00
2. 95 C for :20
3. 70 C for :01
4. 54 C for :20
5. 70 C for :20
6. Goto 2, 8 times
7. 95 C for 1:00. ***This step ensures that identical pairs are not annealed at the end.***
8. 4 C forever

After running the PCR, add 26 ul of EB to each well to bring the volume to 50 ul. Then purify with 1x Ampure beads (see following slides).

round 1 PCR plate layout:

	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6
	1	2	3	4	5	6	7	8	9	10	11	12
A	experiment 1 - (PCR1, RNA 7A(H17-0.5))						experiment 9 - (PCR9, RNA 11A(FI6-0.2))					
B	experiment 2 - (PCR2, RNA 7B (H17-0.5))						experiment 10 - (PCR10, RNA 11B(FI6-0.2))					
C	experiment 3 - (PCR3, RNA 8A (H17-1.0))						experiment 11 - (PCR11, RNA 12A (FI6-2.0))					
D	experiment 4 - (PCR4, RNA 8B (H17-1.0))						experiment 12 - (PCR12, RNA 12B (FI6-2.0))					
E	experiment 5 - (PCR5, RNA 9A (H17-10.0))						experiment 13 - (PCR13, RNA 13/14/15A (mock))					
F	experiment 6 - (PCR6, RNA 9B (H17-10.0))						experiment 14 - (PCR14, RNA 13/14/15B (mock))					
G	experiment 7 - (PCR7, RNA 10A (FI6-0.1))						experiment 15 - (PCR15, RNA 6A (no virus)) - negative template control					
H	experiment 8 - (PCR8, RNA 10B(FI6-0.1))						experiment 14 (NO subamplicon primers) - (PCR14, RNA 13/14/15B (mock))					

2016-04-17: Ampure purification of round 1 PCR products

Add 26 ul EB to all wells of the round 1 PCR plate to bring volume to 50 ul, then purify with 1x ampure beads:

1. Take bead aliquot (~5mL) from vortexed stock and allow to come to room temperature
2. Add 50 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
6. Wash twice with 180ul fresh 80% ethanol
7. Air dry 10 min
8. Remove from rack, disperse beads in 75 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 rack ("2**016.04.17 purified R1 subamplicons**") with the same layout as the PCR plate above.

2016-04-17: Picogreen of purified round 1 products

Make two standard solutions at 2ng/ul by diluting 4.4 ul of the standard to 215.6 ul 1xTE.

Add 90ul TE to columns 1-6 for plate 1 and 7-12 for plate 2 to make dilutions of round 1 products.

Add 100ul TE to Columns 7-9 of plate 1 and 4-6 for plate 2.

Make the standard curve by serially transferring 100ul of the 2ng/ul standard into A8/A9 (plate 1) and A4/A5 (plate w) and down the columns.

Transfer 10ul of purified round 1 products to the picogreen plate in corresponding wells (taking a reading of 1:10 dilutions.).

In dark, Make 1:200 dilution of picogreen (80ul to 16mL 1xTE) and add 100ul to each well, incubate 5 min, read on plate reader.

plate 1:

	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6	TE	standard1	standard2			
	1	2	3	4	5	6	7	8	9	10	11	12
A	experiment 1 - (PCR1, RNA 7A(H17-0.5))						TE	1.00E+00	1.00E+00			
B	experiment 2 - (PCR2, RNA 7B (H17-0.5))						TE	5.00E-01	5.00E-01			
C	experiment 3 - (PCR3, RNA 8A (H17-1.0))						TE	2.50E-01	2.50E-01			
D	experiment 4 - (PCR4, RNA 8B (H17-1.0))						TE	1.25E-01	1.25E-01			
E	experiment 5 - (PCR5, RNA 9A (H17-10.0))						TE	6.25E-02	6.25E-02			
F	experiment 6 - (PCR6, RNA 9B (H17-10.0))						TE	3.13E-02	3.13E-02			
G	experiment 7 - (PCR7, RNA 10A (FI6-0.1))						TE	1.56E-02	1.56E-02			
H	experiment 8 - (PCR8, RNA 10B(FI6-0.1))						TE	7.81E-03	7.81E-03			

Plate 2

				standard 1	standard 2	TE	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6
	1	2	3	4	5	6	7	8	9	10	11	12
A				1.00E+00	1.00E+00	TE	experiment 9 - (PCR9, RNA 11A(FI6-0.2))					
B				5.00E-01	5.00E-01	TE	experiment 10 - (PCR10, RNA 11B(FI6-0.2))					
C				2.50E-01	2.50E-01	TE	experiment 11 - (PCR11, RNA 12A (FI6-2.0))					
D				1.25E-01	1.25E-01	TE	experiment 12 - (PCR12, RNA 12B (FI6-2.0))					
E				6.25E-02	6.25E-02	TE	experiment 13 - (PCR13, RNA 13/14/15A (mock))					
F				3.13E-02	3.13E-02	TE	experiment 14 - (PCR14, RNA 13/14/15B (mock))					
G				1.56E-02	1.56E-02	TE	experiment 15 - (PCR15, RNA 6A (no virus)) - negative template control					
H				7.81E-03	7.81E-03	TE	experiment 14 (NO subamplicon primers) - (PCR14, RNA 13/14/15B (mock))					

2016-04-17 Picogreen concentrations of Round 1 products.

This is pretty much as expected - the samples for exp 11 and exp 12 had less template amplicon to begin with, but they clearly amplified relative to the no virus sample (exp 15) and the no primer sample.

Using these concentrations, I will normalize concentrations across all samples so that I can pool equal amounts of each subamplicon for each experimental sample prior to round 2 PCR. I will normalize to 0.4 ng/ul.

ng/ul

	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6
Exp 1	1.764	1.398	2.661	1.959	2.192	1.337
Exp 2	1.959	1.463	2.736	2.039	2.157	1.453
Exp 3	2.062	1.530	2.926	2.270	2.407	1.411
Exp 4	2.164	1.684	3.181	2.216	2.336	1.476
Exp 5	2.411	1.862	3.411	2.467	2.720	1.598
Exp 6	2.603	1.821	3.448	2.444	3.080	1.716
Exp 7	2.472	1.966	3.066	1.972	2.840	1.663
Exp 8	3.040	2.171	3.292	2.157	3.551	1.798

ng/ul

	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6
Exp 9	1.638	1.858	2.331	1.727	2.959	1.837
Exp 10	1.686	1.754	2.464	1.654	2.763	2.099
Exp 11	0.458	0.462	0.710	0.449	0.846	0.653
Exp 12	0.524	0.524	0.730	0.499	0.876	0.663
Exp 13	1.922	1.576	2.282	1.779	2.639	2.149
Exp 14	1.963	1.535	2.485	1.830	3.094	2.577
Exp 15	0.013	0.013	0.013	0.013	0.011	0.011
Exp 14-noprimer	0.029	0.029	0.030	0.026	0.035	0.028

2016-04-21 Make 0.4 ng/ul plate by first filling a fresh plate with the following volumes of EB, and then transferring 20 ul of samples from the purified round 1 products plate.

Volume of EB to add to	**20**	**ul of sample**
To normalize	**0.4**	**ng/ul**

		1	2	3	4	5	6	7	8	9	10	11	12	
		subamp1	**subamp2**	**subamp3**	**subamp4**	**subamp5**	**subamp6**	**subamp1**	**subamp2**	**subamp3**	**subamp4**	**subamp5**	**subamp6**	
A	**Exp 1:**	68.2	49.9	113.05	77.95	89.6	46.85	61.9	72.9	96.55	66.35	127.95	71.85	**Exp 9:**
B	**Exp 2:**	77.95	53.15	116.8	81.95	87.85	52.65	64.3	67.7	103.2	62.7	118.15	84.95	**Exp 10:**
C	**Exp 3:**	83.1	56.5	126.3	93.5	100.35	50.55	2.9	3.1	15.5	2.45	22.3	12.65	**Exp 11:**
D	**Exp 4:**	88.2	64.2	139.05	90.8	96.8	53.8	6.2	6.2	16.5	4.95	23.8	13.15	**Exp 12:**
E	**Exp 5:**	100.55	73.1	150.55	103.35	116	59.9	76.1	58.8	94.1	68.95	111.95	87.45	**Exp 13:**
F	**Exp 6:**	110.15	71.05	152.4	102.2	134	65.8	78.15	56.75	104.25	71.5	134.7	108.85	**Exp 14:**
G	**Exp 7:**	103.6	78.3	133.3	78.6	122	63.15	none	none	none	none	none	none	**Exp 15:**
H	**Exp 8:**	132	88.55	144.6	87.85	157.55	69.9	none	none	none	none	none	none	**Exp 14-noprimer:**

2016-04-21; Pool the normalized round 1 products for each experimental sample. In other words, mix subamplicons 1 through 6 in equal amounts for each sample. Do this by pipetting all six subamplicons to a single well for pooled samples as shown in Columns 1 and 7 below. Then use these pooled samples to make 1:15 and 1:5 dilutions as noted:

Transfer 20ul of each 0.4ng/ul subamplicon to a pooled plate like this, such that Exp 1-8 0.4ng/ul pooled is in A1-H1; Exp9-16 0.4ng/ul pooled is A7-H7.

Then make 1:15 dilutions of each set of 8 pools by transferring 10ul to 140ul EB to get the following concentrations, with the final dilution being 1:5! (transfer 10ul to 40ul EB)

	1: 0.4ng/ul pool	2: **1:15** of col 1	3: **1:15**	4: **1:15**	5: ***1:5***	6	7: 0.4ng/ul pool	8: **1:15** of col 7	9: **1:15** of col 8	10: **1:15** of col 9	11: ***1:5*** of col 10	12
A	**Exp 1**	2.67E-02	1.78E-03	1.19E-04	2.37E-05		**Exp 9**	2.67E-02	1.78E-03	1.19E-04	2.37E-05	
B	**Exp 2**						**Exp 10**					
C	**Exp 3**						**Exp 11**					
D	**Exp 4**						**Exp 12**					
E	**Exp 5**						**Exp 13**					
F	**Exp 6**						**Exp 14**					
G	**Exp 7**						**Exp 15**					
H	**Exp 8**						**Exp 16**					

2016-04-21:

bottleneck calculation **for the mock samples (exp 13 and 14)**:

I want *7E5 barcodes for each subamplicon in each sample*, which means 3.5E5 dsDNA molecules per subamplicon per sample, since the dissociation step at the end of round 1 PCR results in unique barcodes on each ssDNA molecule in the final duplexes.

3.5E5 dsDNA molecules per subamplicon per sample, * 6 subamplicons in each sample = 2.1E6 dsDNA molecules per sample in each round 2 PCR, so that within each sample's round 2 PCR there will be 7E5 unique barcodes for each of the 6 subamplicons.

So each of my round 2 PCRs gets 2.1E6 dsDNA molecules of pooled sample as template, which corresponds to **0.000924** ng of dsDNA 407bp long.

9.24e-4 ng of the pooled subamplicons for each sample results in 3.5E5 dsDNA molecules for each subamplicon in the sample, for 7E5 total barcodes for each subamplicon.

bottleneck calculation **for the selected samples (exp 1-12)**:

I want ***1.5E5 barcodes*** *for each subamplicon in each sample*, which means **7.5E4 dsDNA** molecules per subamplicon per sample, since the dissociation step at the end of round 1 PCR results in unique barcodes on each ssDNA molecule in the final duplexes.

7.5E4 dsDNA molecules per subamplicon per sample, * 6 subamplicons in each sample = **4.5E5 dsDNA** molecules per sample in each round 2 PCR, so that within each sample's round 2 PCR there will be **1.5E5** unique barcodes for each of the 6 subamplicons.

So each of my round 2 PCRs gets **4.5E5 dsDNA**molecules of pooled sample as template, which corresponds to **0.000198** ng of dsDNA 407bp long.

1.98e-4 ng of the pooled subamplicons for each experimental sample results in 7.5E4 dsDNA molecules for each subamplicon in the sample, for 1.5E5 total barcodes for each subamplicon.

2016-04-21: Round 2 PCR specs

For samples Exp1-Exp12, I want **1.98e-4 ng** of the pooled subamplicons for each experimental sample.

Therefore, for Exp1-12, round 2 PCR will use: **8.35** ul of dilution column 5 or 11

For samples Exp13 and Exp14, I want **9.24e-4 ng** of the pooled subamplicons for each sample.

For Exp13-14, round 2 PCR will use: **7.80** ul of dilution from COLUMN 10

Sample	vol template	source template	vol H2O	reverse primer	Index name
Exp1	8.35	A5	3.65	MD-TS01Rnd2rev	Illumina 2 / NextFlex 1
Exp2	8.35	B5	3.65	MD-TS03Rnd2rev	Illumina 5 / NextFlex 3
Exp3	8.35	C5	3.65	MD-TS08Rnd2rev	Illumina 3 / NextFlex 8
Exp4	8.35	D5	3.65	MD-TS09Rnd2rev	Illumina 8 / NextFlex 9
Exp5	8.35	E5	3.65	MD-TS10Rnd2rev	Illumina 9 / NextFlex 10
Exp6	8.35	F5	3.65	MD-TS11Rnd2rev	Illumina 10 / NextFlex 11
Exp7	8.35	G5	3.65	MD-TS22Rnd2rev	Illumina 22 / NextFlex 22
Exp8	8.35	H5	3.65	MD-TS25Rnd2rev	Illumina 25 / NextFlex 25
Exp9	8.35	A11	3.65	ad-ts6rnd2rev	illumina 6
Exp10	8.35	B11	3.65	ad-ts12rnd2rev	illumina 12
Exp11	8.35	C11	3.65	ad-ts19rnd2rev	illumina 19
Exp12	8.35	D11	3.65	ad-ts13rnd2rev	illumina 13
Exp13	**7.80**	**E10**	4.20	ad-ts14rnd2rev	illumina 14
Exp14	**7.80**	**F10**	4.20	ad-ts15rnd2rev	illumina 15
Exp15(negative)	7.80	G10	4.20	ad-ts18rnd2rev	illumina 18 (not sequencing!)

Each Round 2 PCR:
- **20 ul** of 2X KOD Master Mix
- **4 ul** of 5 uM Rnd2ForUniversal
- **4 ul** of 5 uM of the appropriate Rnd2RevIndex as indicated.

7.8 ul - 8.35 of template as indicated.

3.65 ul - 4.2 ul as indicated water for 40 ul of final volume

Here is the PCR reaction:
1.95 C for 2:00
2.95 C for :20
3.70 C for :01
4.55 C for :20
5.70 C for :20
6.Goto 2, 23 times
7.4 C forever

Also did a 16th sample - using pooled exp16 stuff, with no reverse rnd2 primer.
For my MD-TSxx primers, these are already at 5uM in IDT tubes. Note the numering is the nextflex numbering for each of the primer names.
For AD-TSxx primers, I made 5uM dilutions from adam's 100uM stocks.

Round 2 pcr plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A				**Exp 1**				**Exp 9**				
B				**Exp 2**				**Exp 10**				
C				**Exp 3**				**Exp 11**				
D				**Exp 4**				**Exp 12**				
E				**Exp 5**				**Exp 13**				
F				**Exp 6**				**Exp 14**				
G				**Exp 7**				**Exp 15**				
H				**Exp 8**				16-no rev primer				

Ampure the round 2 pcr products with 1x beads to clean up:
Add 40ul of beads to each well and mix 10x. Adsorb for 10 minutes, put on magnet for 5 minutes. Aspirate. Wash 2x with 180ul fresh 80% ethanol, dry on magnet, remove from magnet, resuspend in 60ul EB. Transfer to a fresh plate with the same layout.

picogreen the round 2 PCR products:

Make two standard solutions at 2ng/ul by diluting 4 ul of the standard to 196 ul 1xTE.

Use 1:50 dilutions to measure sample concentrations (2ul + 98ul TE).

	1	2	3	4	5	6	7
A	1.00E+00	1.00E+00	TE	**Exp 1**	**Exp 1**	**Exp 9**	**Exp 9**
B	5.00E-01	5.00E-01	TE	**Exp 2**	**Exp 2**	**Exp 10**	**Exp 10**
C	2.50E-01	2.50E-01	TE	**Exp 3**	**Exp 3**	**Exp 11**	**Exp 11**
D	1.25E-01	1.25E-01	TE	**Exp 4**	**Exp 4**	**Exp 12**	**Exp 12**
E	6.25E-02	6.25E-02	TE	**Exp 5**	**Exp 5**	**Exp 13**	**Exp 13**
F	3.13E-02	3.13E-02	TE	**Exp 6**	**Exp 6**	**Exp 14**	**Exp 14**
G	1.56E-02	1.56E-02	TE	**Exp 7**	**Exp 7**	**Exp 15**	**Exp 15**
H	7.81E-03	7.81E-03	TE	**Exp 8**	**Exp 8**	**16-noprimer**	**16-noprimer**

	ng/ul undiluted
Exp 1	**24.52**
Exp 2	**24.06**
Exp 3	**23.99**
Exp 4	**24.27**
Exp 5	**23.06**
Exp 6	**22.86**
Exp 7	**22.09**
Exp 8	**22.35**
Exp 9	**16.01**
Exp 10	**19.85**
Exp 11	**18.89**
Exp 12	**19.80**
Exp 13	**38.60**
Exp 14	**39.02**
Exp 15	**0.04**
16-noprimer	**0.04**

Bottleneck and pooling plan:

98% fraction of lane for my pool

Total paired-end reads per lane: 1.00E+08

Total PE reads for me: 9.80E+07

# unique barcodes per subamplicon	Experimental sample	cDNA or standard	fraction of my pool	expected PE reads	expected PE reads per subamplicon	expected PE reads per subampliconbarcode
150000	**1**	7A(H17-0.5)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**2**	7B (H17-0.5)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**3**	8A (H17-1.0)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**4**	8B (H17-1.0)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**5**	9A (H17-10.0)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**6**	9B (H17-10.0)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**7**	10A (FI6-0.1)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**8**	10B(FI6-0.1)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**9**	11A(FI6-0.2)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**10**	11B(FI6-0.2)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**11**	12A (FI6-2.0)	**0.0583**	5.72E+06	9.53E+05	6.35
150000	**12**	12B (FI6-2.0)	**0.0583**	5.72E+06	9.53E+05	6.35
700000	**13**	13/14/15A (mock)	**0.1500**	1.47E+07	2.45E+06	3.50
700000	**14**	13/14/15B (mock)	**0.1500**	1.47E+07	2.45E+06	3.50

Mix round 2 products in the following ratios to distribute depth amongst samples as outlined in the previous slide. Storing this pooled product at -20 overnight; tomorrow I will run some of it out on a gel, extract the band, picogreen the purified product alongside adam's sample, mix them 98%-2% at 2nM, and provide to the sequencing core.

	ng/ul R2 product	mix volume
Exp 1	24.52	11.897
Exp 2	24.06	12.121
Exp 3	23.99	12.158
Exp 4	24.27	12.020
Exp 5	23.06	12.645
Exp 6	22.86	12.758
Exp 7	22.09	13.204
Exp 8	22.35	13.048
Exp 9	16.01	18.221
Exp 10	19.85	14.697
Exp 11	18.89	15.443
Exp 12	19.80	14.728
Exp 13	38.60	19.429
Exp 14	39.02	19.220

2016-04-22:

Run 50ul of this mix + 10ul 6X sample buffer on 2% agarose alongside 100bp ladders.

Clean up with Qiagen gel extraction kit, doing the extra QG wash before PE wash. Elute in 50 ul EB.

2016-04-22:

Picogreen alongside Adam's sample.

Make two standard solutions at 2ng/ul by diluting 4 ul of the standard to 196 ul 1xTE.

99ul + 1ul sample for 1:100 dilutions of samples.

picogreen
layout
2016-04-22 for
gel-excised
pool and
adam's pool

	1	2	3	4
A	1.00E+00	1.00E+00	TE	1:100 adam's
B	5.00E-01	5.00E-01	TE	1:100 adam's
C	2.50E-01	2.50E-01	TE	TE
D	1.25E-01	1.25E-01	TE	TE
E	6.25E-02	6.25E-02	TE	1:100 mike's
F	3.13E-02	3.13E-02	TE	1:100 mike's
G	1.56E-02	1.56E-02	TE	TE
H	7.81E-03	7.81E-03	TE	TE

Summary

	tube concentration	averages
Adam's rep 1	2.011362348	
Adam's rep 2	2.17224349	2.091802919
Mike's rep 1	11.02033818	
Mike's rep 2	11.32326315	11.17180067

For 30ul of 472bp dsDNA at 2nM, I want 18.4ng of DNA in the final volume of 30ul.

98% mike's pool:	18.032	ng	1.614063886	ul from the original tube, or	24.21095829 of a 1:15 dilution
2% adam's pool:	0.368	ng	0.1759247951	ul from the original tube, or	2.638871927 of a 1:15 dilution
					3.15016978 ul EB

Mix Mike98%/Adam2%, dilute to 2nM to submit.

Sequencing run perhaps began on 5/6/16 or 5/9/16, delayed due to the core being backed up with a broken sequencer :-(