Generating amplicons from the viral RNA and plasmids.
November-19-2014:
I ordered primers to amplify HA amplicons from the RNA or plasmids. These are the same primers that Bargavi used. They anneal at the termini of the vRNA and do **not** extend into the coding sequence. They should amplify the entire vRNA.
1) WSN-For: AGCAAAAGCAGGGGAAAATAAAAACAAC; Length: 28 bp; Tm= 60.9°C
2) WSN-Rev: AGTAGAAACAAGGGTGTTTTTCCTTATATTTCTG; Length: 34 bp; Tm= 60.1°C

January-6-2015:
I will do RT-PCR to make the amplicons from my RNA from the passage 1 (November-10-2014) and passage 2 (November-14-2014) extractions.
I will number the RNA samples as follows:
1) wildtype #1 passage 1
2) Wildtype #2 passage 1
3) Wildtype #3 passage 1
4) Mutvirus #1 passage 1
5) Mutvirus #2 passage 1
6) Mutvirus #3 passage 1
7) No-HA control passage 1
8) No-template control
9) wildtype #1 passage 2
10) Wildtype #2 passage 2
11) Wildtype #3 passage 2
12) Mutvirus #1 passage 2
13) Mutvirus #2 passage 2
14) Mutvirus #3 passage 2

I will set up the AccuScript (Agilent, 200820-12) reactions as follows (I have slightly modified the protocol to avoid pipetting small volumes). Each reaction 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
2 ul of RNA template
6.8 ul of water for 19 ul total volume.

To make these mixes for 16 reactions (I need enough for 14 reactions, but made a bit extra), prepared a master mix of:
48 ul 10X AccuScript RT Buffer
19.2 ul of dNTP mix
48 ul of 5 uM 5'-BsmBI-Aichi68-NP
48 ul of 5 uM 3'-BsmBI-Aichi68-NP
108.8 ul of water
Aliquoted 17 ul of this master mix into PCR tubes, added the 2 ul of the RNA to each tube and mixed by pipetting. Heated to 65 C for 5 minutes, cooled to 4 C in the PCR machine. Added 3 ul of DTT to each reaction, then split the mixes into two by pipetting 11 ul out of each reaction into a new PCR tube so that I have both an +RT and no RT control reaction.

Generating amplicons from the viral RNA and plasmids, continued
January-6-2015, continued
To the RT reactions, I then added:
2 ul of a mix of 1.25 ul of water and 0.75 ul of AccuScript RT, followed by 2 ul of a mix of 1.625 ul of water and 0.375 ul RNase Block
To the no RT reactions, I then added:
2 ul of water, followed by 2 ul of a mix of 1.625 ul of water and 0.375 ul RNase Block

To do this, first made the following master mixes:
AccuScript master mix: 20 ul of water and 12 ul of AccuScript RT
RNAse block master mix: 52 ul of water 12 ul of Rnase block
Then added 2 ul of these master mixes to give the volumes indicated above.

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

I then set up PCRs used my cDNA and also 10 ng/ul plasmids (either wildtype or the plasmid DNA libraries) from Bargavi. The reactions all included 2 ul of template, so this is 2 ul of cDNA or 20 ng/ul of plasmid. The reactions also each contained 17.5 ul of 2X KOD Master Mix, 2.1 ul of 5 uM WSN-for, 2.1 ul of 5 uM WSN-rev, and 13.3 ul of water.
Used the following PCR program (note use of **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
Note that I also used a standard for quantification of DNA based on using linear WSN HA PCR product. The conversion is that 10 ng of WSN gene corresponds to 1e10 ssDNA molecules. So 1e4 ssDNA molecules corresponds to 1 ul of a 1:1e6 dilution of 10 ng of HA DNA, 1e5 corresponds to a 1:1e5 dilution, 1e6 corresponds to 10 ng of a 1:1e4 dilution, and 1e7 corresponds to 10 ng of a 1:1e3 dilution. The PCRs were in a 96-well plate with the following layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	wildtype plasmid #1	wildtype plasmid #2	wildtype plasmid #3	mutDNA plasmid #1	mutDNA plasmid #2	mutDNA plasmid #3	no template					
B												
C	wildtype virus p1 #1	wildtype virus p1 #2	wildtype virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3	no-HA control p1	no template control RT				
D	wildtype virus p2 #1	wildtype virus p2 #2	wildtype virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3						
E												
F	no RT, wildtype virus p1 #1	no RT, wildtype virus p1 #2	no RT, wildtype virus p1 #3	no RT, mutvirus p1 #1	no RT, mutvirus p1 #2	no RT, mutvirus p1 #3	no RT, no-HA control p1	no RT, no template control RT				
G	no RT, wildtype virus p2 #1	no RT, wildtype virus p2 #2	no RT, wildtype virus p2 #3	no RT, mutvirus p2 #1	no RT, mutvirus p2 #2	no RT, mutvirus p2 #3						
H	standard 1e4 molecules	standard 1e5 molecules	standard 1e6 molecules	standard 1e7 molecules								

Checking amplicons, January-7-2015.

I ran 4 ul of each of my PCR reactions out on a 1.0% analytical gel. Very frustratingly, the comb appeared to have punctured the bottom of four wells, and the sample leaked out of those. But I will go ahead and run the gel and decide what to do at that point. Fortunately, all of the punctured are samples that should have DNA, not negative controls. So if everything else looks good, I could take the risk of assuming these are OK and then just seeing what things look like after the AmPure beads…

Bottom row of gel: 5 ul of 1 kb ladder, standard 1e4 to 1e7, wells A1-A7 of PCR, wells C1 to C8 of PCR, wells D1 to D5 of PCR.

Top row of gel: 5 ul of 1 kb ladder, standard 1e4 to 1e7, well D6 of PCR, rows F1 to F8 of PCR, rows G1 go G6 of PCR.

The punctured samples are in the bottom row in lanes 16, 18, 20, and 23.

So overall, the gel below looks great. None of the no-RT controls or no template controls have detectable HA bands. All of the samples that should have bands do (except for the ones that unfortunately leaked during loading), and in all cases the intensity is such that it suggests >1e7 unique template molecules. So despite not being positive about the leaked samples, I am going to go ahead and do the purification. After that I will do a second check for DNA, so I guess I'll find out then if these actually lacked amplicons…

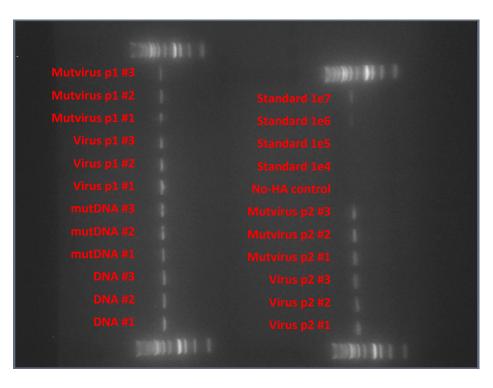**Bead purification of amplicons, January-7-2015.**

I will bead purify all of the amplicons with AmPure XP beads except for the no-RT controls.

The wells in the PCR plates should have 31 ul left, and I will use 0.9X beads. So I vortexed the bead bottle and immediately removed a 1 ml aliquot. I then let this aliquot come to room temperature for 10 minutes, and then added 28 ul to each well, mixing the 1 ml master stock by vortexing before withdrawing each 28 ul aliquot, and mixing the bead-DNA mix by pipetting 10X after each addition. Then let sit at room temperature for 10 minutes. Then put on magnet for 5 minutes. Then removed as much liquid as could be done cleanly, and washed twice with 180 ul freshly made 80% ethanol, adding the ethanol-mix gently so as not to disturb the beads (used multichannel). After the last ethanol wash, let the tubes air-dry for 10 minutes. Then took off the rack and dispersed beads in 75 ul of EB. Let the DNA resuspend for 5 minutes, then put back on the magnetic rack for 5 minutes. Finally, transferred the bead-free supernatants to a new plate in the following orientation:

	1	2	3	4	5	6	7	8	9	10	11	12
A	wildtype plasmid #1	wildtype plasmid #2	wildtype plasmid #3	mutDNA plasmid #1	mutDNA plasmid #2	mutDNA plasmid #3	wildtype virus p1 #1	wildtype virus p1 #2	wildtype virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
B	wildtype virus p2 #1	wildtype virus p2 #2	wildtype virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control p1					
C												
D												
E												
F												
G	standard 1e4 molecules	standard 1e5 molecules	standard 1e6 molecules	standard 1e7 molecules								
H												

Analytical gel of amplicons, January-7-2015.

I then ran an analytical 1.0% gel of the amplicons. Loaded the equivalent of 4 ul of purified amplicon (4 ul diluted into 6 ul of water + 2 ul 6X loading buffer). The samples are simply in the left-to-right, top-to-bottom orientation above, with the first well 10 ul of Promega 1 kb ladder (this corresponds to 1 ug of total ladder). The analytical gel looks great. I got good recoveries of all of the amplicons. They all are still at least as bright as the 1e7 template molecules band. Given that the total amount of ladder loaded in each lane is 1000 ng, I would guess that each amplicon band is about 50 ng. Given that I loaded 4 ul per well, that would give a concentration of about 10 ng/ul for the amplicon. This is certainly plenty. I will do more accurate quantification by TapeStation going forward...

January-7-2015, design of primers:

Primers that anneal at 5' end of gene. The overlap between the two primers should have a Tm of 59 C. The second primer has 8 N nucleotides that serve as part of the read-specific barcode. The *x*'s indicate where the second primer overlaps with the WSN HA in the sense direction.

The primers shown here are named *Rnd2forUniversal* and *Rnd1for???* where *???* indicates the first nucleotide in the HA coding sequence downstream from where the primer anneals.

5′–AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC–3′

5′–CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNxxxxxxxx…–3′

Primers that anneal at 3' end of the gene. The 6 n nucleotides indicate the primer specific index. The overlap between the two primers should have a Tm of 59 C. The second primer has 8 N nucleotides that serve as part of the read-specific barcode. The x's indicate where the second primer overlaps with the WSN HA in the anti-sense direction.

The primers shown here are named *Rnd2revIndex???* where *???* indicates the index number, and *Rnd1rev???* where *???* Indicates the last nucleotide in the HA coding sequence upstream from where the primer anneals. The index numbers are the TruSeq indices.

5′–CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGTGCTCTTCC–3′

5′–GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNxxxxxxxx…–3′

The Rnd1 primers all have melting temperatures of at least 57 C. Note that the round 1 primers are ***designed to span full codons!***

Rnd1for1, CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNaagcaggggaaaataaaaacaaccaaa
Rnd1rev426, GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNcatgatactgttactccgttgaatgtgtg
Rnd1for427, CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNccaaggaaagttcatggcccaac
Rnd1rev849, GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNcactcatgcattgacgcgtttga
Rnd1for850, CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNgtttgagtccggcatcatcacc
Rnd1rev1275, GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNaaatgtccagaaacccatcatcaacttt
Rnd1for1276, CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNcaacaacttagaaaaaaggatggaaaatttaaataaa
Rnd1rev1698, GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNaagggtgtttttccttatatttctgaaatcctaatc
Rnd2forUniversal, AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC
Rnd2revIndex1, CAAGCAGAAGACGGCATACGAGATatcacgGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex2, CAAGCAGAAGACGGCATACGAGATcgatgtGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex3, CAAGCAGAAGACGGCATACGAGATttaggcGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex4, CAAGCAGAAGACGGCATACGAGATtgaccaGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex5, CAAGCAGAAGACGGCATACGAGATacagtgGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex6, CAAGCAGAAGACGGCATACGAGATgccaatGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex7, CAAGCAGAAGACGGCATACGAGATcagatcGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex8, CAAGCAGAAGACGGCATACGAGATacttgaGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex9, CAAGCAGAAGACGGCATACGAGATgatcagGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex10, CAAGCAGAAGACGGCATACGAGATtagcttGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex11, CAAGCAGAAGACGGCATACGAGATggctacGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex12, CAAGCAGAAGACGGCATACGAGATcttgtaGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex13, CAAGCAGAAGACGGCATACGAGATagtcaaGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex14, CAAGCAGAAGACGGCATACGAGATagttccGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex15, CAAGCAGAAGACGGCATACGAGATatgtcaGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex16, CAAGCAGAAGACGGCATACGAGATccgtccGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex17, CAAGCAGAAGACGGCATACGAGATgtagagGTGACTGGAGTTCAGACGTGTGCTCTTCC
Rnd2revIndex18, CAAGCAGAAGACGGCATACGAGATgtccgcGTGACTGGAGTTCAGACGTGTGCTCTTCC
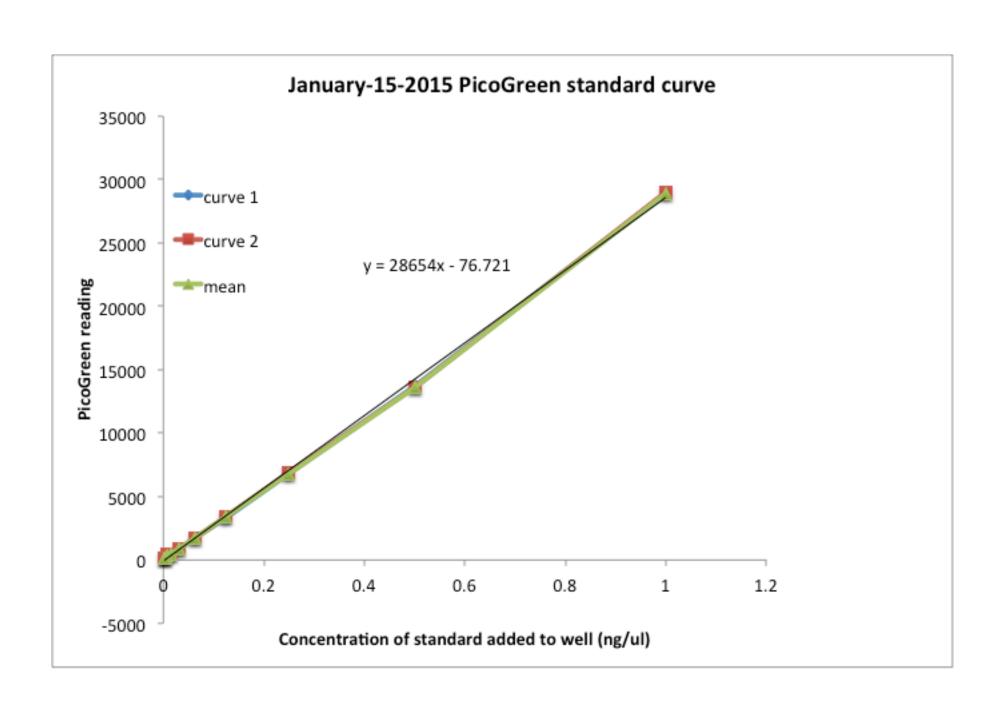
January-15-2015, quantification of amplicons.
I will quantify my bead purified amplicons from January-7-2015. From my gel, I estimated that the amplicons are about 10 ng/ul. The PicoGreen assay is linear up to 1 ng/ul (and the measured DNA is diluted 1:2). I will therefore dilute the amplicons 1:5 in EB, which will give an estimated concentration of 2 ng/ul for the PicoGreen assay. Made my dilution plate layout as follows, by multichannel pipetting 20 ul from my January-7-2015 amplicon plate into 80 ul of EB. Note that I have made a duplicate entry of **wildtype plasmid #1** in well B8 for my "no-primer" controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	wildtype plasmid #1	wildtype plasmid #2	wildtype plasmid #3	mutDNA plasmid #1	mutDNA plasmid #2	mutDNA plasmid #3	wildtype virus p1 #1	wildtype virus p1 #2	wildtype virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
B	wildtype virus p2 #1	wildtype virus p2 #2	wildtype virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control p1	wildtype plasmid #1 (for no-primer control)				

I will now use PicoGreen (Invitrogen P7859) to quantify the DNA. Thawed the PicoGreen 100 ng/ul standard, vortexed to mix, and then verified that concentration on the NanoDrop (got a concentration of 98 ng/ul, which seems sufficiently close to the declared concentration of 100 ng/ul). I then made entirely independent preparations of the standard curve (doing both the initial dilution from the stock and the subsequent dilutions separately). The dilutions started at 4 ng/ul (made by diluting 8 ul of 200 ng/ul standard into 192 ul of EB), and then serial 2-fold dilutions from there. I pipetted 100 ul of DNA of the standard curve dilutions into a Costar 96-well black plate (3915), and added 10 ul of my 1:5 dilutions (putatively about 2 ng/ul) of amplicons to 90 ul of EB. This makes all wells in the layout below have 100 ul of DNA in EB. I then made a 1:200 dilution of the Quant-IT reagent by adding 42.5 ul of this reagent to 8.1 ml of EB. Then used a multichannel pipette to add 100 ul of this per well. Then incubated for 5 minutes covered with aluminum foil. Then read on the Tecan Infinite M100 with excitation at 485 nm (9 nm bandwidth) and emission at 535 nm (20 nm bandwidth) performing 50 reads at 400 Hz.

	1	2	3	4	5	6	7	8	9	10	11	12
A	wildtype plasmid #1	wildtype plasmid #2	wildtype plasmid #3	mutDNA plasmid #1	mutDNA plasmid #2	mutDNA plasmid #3	wildtype virus p1 #1	wildtype virus p1 #2	wildtype virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
B	wildtype virus p2 #1	wildtype virus p2 #2	wildtype virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control p1	wildtype plasmid #1 (for no-primer control)				
C	wildtype plasmid #1	wildtype plasmid #2	wildtype plasmid #3	mutDNA plasmid #1	mutDNA plasmid #2	mutDNA plasmid #3	wildtype virus p1 #1	wildtype virus p1 #2	wildtype virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
D	wildtype virus p2 #1	wildtype virus p2 #2	wildtype virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control p1	wildtype plasmid #1 (for forward-primer-only control)	wildtype plasmid #1 (for reverse-primer-only control)	wildtype plasmid #1 (for no-primer control)		
E	4 ng/ul	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313
F	4 ng/ul	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313
G												
H												

January-15-2015 PicoGreen standard curve

$y = 28654x - 76.721$

PicoGreen reading

Concentration of standard added to well (ng/ul)

curve 1
curve 2
mean

January-15-2015, creation of plate containing 0.5 ng/ul of amplicons
Used the PicoGreen results to quantify the amounts in my dilution plate. The typical well did have about the expected 2 ng/ul, although there was definite variation. Used the dilution plate to create a new plate with the same layout as the dilution plate where each well had 0.5 ng/ul of amplicon.

well	sample	reading 1	reading 2	reading 1 concentration (ng/ul, accounting for 1:5 dilution)	reading 1 concentration (ng/ul, accounting for 1:5 dilution)	average concentration (ng/ul, after accounting for 1:5 dilution)	volume EB added to 20 ul of sample to give 0.5 ng/ul	
A1	DNA #1	16680	14977	2.90	2.60	2.75	89.9	
A2	DNA #2	12641	13379	2.19	2.32	2.26	70.3	
A3	DNA #3	20387	17794	3.54	3.09	3.32	112.7	
A4	mutDNA #1	6721	7164	1.16	1.24	1.20	55.8	*this well received 40 ul of sample*
A5	mutDNA #2	19510	19759	3.39	3.43	3.41	116.5	
A6	mutDNA #3	19154	20185	3.33	3.51	3.42	116.8	
A7	virus p1 #1	6666	8537	1.15	1.48	1.31	65.0	*this well received 40 ul of sample*
A8	virus p1 #2	13144	15280	2.28	2.65	2.47	78.7	
A9	virus p1 #3	14268	14279	2.48	2.48	2.48	79.1	
A10	mutvirus p1 #1	2901	3999	0.49	0.68	0.59	10.6	*this well received 60 ul of sample*
A11	mutvirus p1 #2	11268	10991	1.95	1.90	1.93	57.1	
A12	mutvirus p1 #3	11781	11206	2.04	1.94	1.99	59.7	
B1	virus p2 #1	15687	16523	2.72	2.87	2.80	91.9	
B2	virus p2 #2	15537	15579	2.70	2.71	2.70	88.1	
B3	virus p2 #3	16083	15585	2.79	2.71	2.75	90.0	
B4	mutvirus p2 #1	14960	15264	2.60	2.65	2.62	84.9	
B5	mutvirus p2 #2	16200	15066	2.81	2.62	2.71	88.6	
B6	mutvirus p2 #3	8667	9454	1.50	1.64	1.57	42.7	
B7	no-HA control virus	32	33	-0.01	-0.01	-0.01	80.0	
B8	DNA #2 for no-primer control	12376	13193	2.15	2.29	2.22	68.7	

January-15-2015, round 1 PCRs
Used my 0.5 ng/ul amplicon plate to set up round 1 PCR reactions. Each reaction had (total volume of 24 ul):
12 ul 2X KOD Master Mix
2 ul of 5 uM forward primer (0.42 uM final concentration)
2 ul of 5 uM reverse primer (0.42 uM final concentration)
8 ul of 0.5 ng/ul template (4 ng total)

Set these up with a multichannel pipette in a 96-well plate with the format shown below.

I decided to run **9 PCR cycles**. With perfect efficiency, this gives a theoretical amplification of 512-fold, which would give about 500 ng of DNA per well (accounting for the fact that the created amplicons are only about ¼ the length of the template). The reaction:
1. 95 C for 2:00
2. 95 C for :20
3. 54 C for :20
4. 70 C for :20
5. Goto 2, 8 times
6. 95 C for 1:00. ***This step ensures that identical pairs are not annealed at the end. This step dissociates the identical pairs and re-anneals them to something else.***
7. 4 C forever

After running the PCR, added 26 ul of EB to each well to bring the volume to 50 ul. Then stored overnight at 4 C.

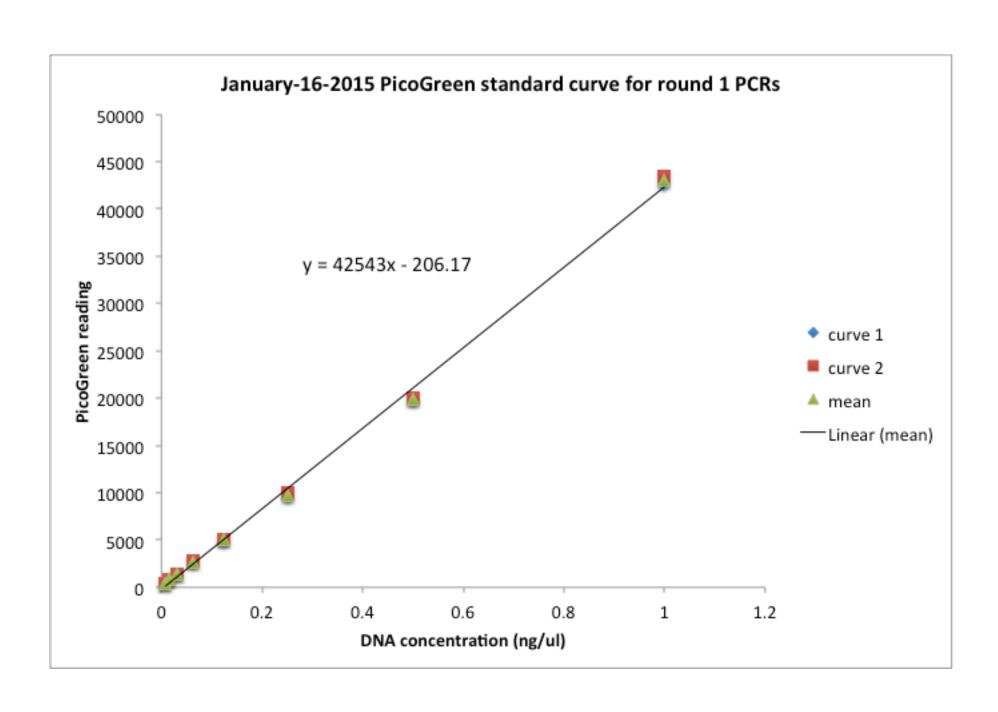|	1	2	3	4	5	6	7	8	9	10	11	12
A	Rnd1for1 / Rnd1rev426, DNA #1	Rnd1for1 / Rnd1rev426, DNA #2	Rnd1for1 / Rnd1rev426, DNA #3	Rnd1for1 / Rnd1rev426, mutDNA #1	Rnd1for1 / Rnd1rev426, mutDNA #2	Rnd1for1 / Rnd1rev426, mutDNA #3	Rnd1for1 / Rnd1rev426, virus p1 #1	Rnd1for1 / Rnd1rev426, virus p1 #2	Rnd1for1 / Rnd1rev426, virus p1 #3	Rnd1for1 / Rnd1rev426, mutvirus p1 #1	Rnd1for1 / Rnd1rev426, mutvirus p1 #2	Rnd1for1 / Rnd1rev426, mutvirus p1 #3
B	Rnd1for1 / Rnd1rev426, virus p2 #1	Rnd1for1 / Rnd1rev426, virus p2 #2	Rnd1for1 / Rnd1rev426, virus p2 #3	Rnd1for1 / Rnd1rev426, mutvirus p2 #1	Rnd1for1 / Rnd1rev426, mutvirus p2 #2	Rnd1for1 / Rnd1rev426, mutvirus p2 #3	Rnd1for1 / Rnd1rev426, no-HA control	DNA #1, no primers				
C	Rnd1for427 / Rnd1rev849, DNA #1	Rnd1for427 / Rnd1rev849, DNA #2	Rnd1for427 / Rnd1rev849, DNA #3	Rnd1for427 / Rnd1rev849, mutDNA #1	Rnd1for427 / Rnd1rev849, mutDNA #2	Rnd1for427 / Rnd1rev849, mutDNA #3	Rnd1for427 / Rnd1rev849, virus p1 #1	Rnd1for427 / Rnd1rev849, virus p1 #2	Rnd1for427 / Rnd1rev849, virus p1 #3	Rnd1for427 / Rnd1rev849, mutvirus p1 #1	Rnd1for427 / Rnd1rev849, mutvirus p1 #2	Rnd1for427 / Rnd1rev849, mutvirus p1 #3
D	Rnd1for427 / Rnd1rev849, virus p2 #1	Rnd1for427 / Rnd1rev849, virus p2 #2	Rnd1for427 / Rnd1rev849, virus p2 #3	Rnd1for427 / Rnd1rev849, mutvirus p2 #1	Rnd1for427 / Rnd1rev849, mutvirus p2 #2	Rnd1for427 / Rnd1rev849, mutvirus p2 #3	Rnd1for427 / Rnd1rev849, no-HA control	DNA #1, no primers				
E	Rnd1for850 / Rnd1rev1275, DNA #1	Rnd1for850 / Rnd1rev1275, DNA #2	Rnd1for850 / Rnd1rev1275, DNA #3	Rnd1for850 / Rnd1rev1275, mutDNA #1	Rnd1for850 / Rnd1rev1275, mutDNA #2	Rnd1for850 / Rnd1rev1275, mutDNA #3	Rnd1for850 / Rnd1rev1275, virus p1 #1	Rnd1for850 / Rnd1rev1275, virus p1 #2	Rnd1for850 / Rnd1rev1275, virus p1 #3	Rnd1for850 / Rnd1rev1275, mutvirus p1 #1	Rnd1for850 / Rnd1rev1275, mutvirus p1 #2	Rnd1for850 / Rnd1rev1275, mutvirus p1 #3
F	Rnd1for850 / Rnd1rev1275, virus p2 #1	Rnd1for850 / Rnd1rev1275, virus p2 #2	Rnd1for850 / Rnd1rev1275, virus p2 #3	Rnd1for850 / Rnd1rev1275, mutvirus p2 #1	Rnd1for850 / Rnd1rev1275, mutvirus p2 #2	Rnd1for850 / Rnd1rev1275, mutvirus p2 #3	Rnd1for850 / Rnd1rev1275, no-HA control	DNA #1, no primers				
G	Rnd1for1276 / Rnd1rev1698, DNA #1	Rnd1for1276 / Rnd1rev1698, DNA #2	Rnd1for1276 / Rnd1rev1698, DNA #3	Rnd1for1276 / Rnd1rev1698, mutDNA #1	Rnd1for1276 / Rnd1rev1698, mutDNA #2	Rnd1for1276 / Rnd1rev1698, mutDNA #3	Rnd1for1276 / Rnd1rev1698, virus p1 #1	Rnd1for1276 / Rnd1rev1698, virus p1 #2	Rnd1for1276 / Rnd1rev1698, virus p1 #3	Rnd1for1276 / Rnd1rev1698, mutvirus p1 #1	Rnd1for1276 / Rnd1rev1698, mutvirus p1 #2	Rnd1for1276 / Rnd1rev1698, mutvirus p1 #3
H	Rnd1for1276 / Rnd1rev1698, virus p2 #1	Rnd1for1276 / Rnd1rev1698, virus p2 #2	Rnd1for1276 / Rnd1rev1698, virus p2 #3	Rnd1for1276 / Rnd1rev1698, mutvirus p2 #1	Rnd1for1276 / Rnd1rev1698, mutvirus p2 #2	Rnd1for1276 / Rnd1rev1698, mutvirus p2 #3	Rnd1for1276 / Rnd1rev1698, no-HA control	DNA #1, no primers				

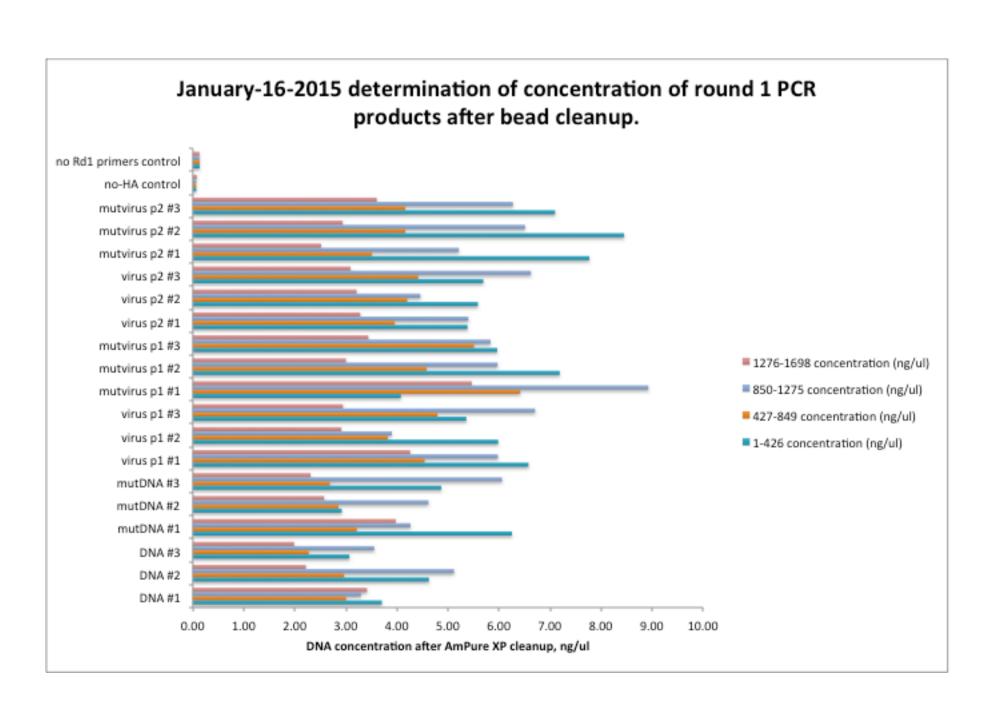**Bead purification and quantification of round 1 PCRs, January-16-2015.**
I will bead purify all of the PCRs with AmPure XP beads. After adding the 26 ul EB, each PCR reaction is a 50 ul volume. I will use a 1:1 ratio of beads. So I vortexed the bead bottle and immediately removed about 4.5 ml into tubes. I then let these tubes come to room temperature for 10 minutes, then vortexed again, put in a reservoir, and added 50 ul per well to the plate with a multichannel, mixing each well by pipetting 10X. Then let sit at room temperature for 10 minutes. Then put on magnet for 5 minutes. Then removed as much liquid as could be done cleanly, and washed twice with 140 ul freshly made 70% ethanol, adding the ethanol-mix gently so as not to disturb the beads (used multichannel). After the last ethanol wash, let the tubes air-dry for 10 minutes. Then took off the rack and dispersed beads in 70 ul of EB. Let the DNA resuspend for 5 minutes, then put back on the magnetic rack for 5 minutes. Finally, transferred the bead-free supernatants to a new plate in the same orientation as before.

If there was perfect PCR efficiency and perfect DNA recovery, the concentration in the purified plates should be 7 ng/ul. Presumably the actual concentration will be somewhat lower due to imperfect PCR efficiency and imperfect bead recovery. I will now quantify these by PicoGreen (Invitrogen P7859). Thawed the PicoGreen 100 ng/ul standard, vortexed to mix, and then verified that concentration on the NanoDrop (got a concentration of 98 ng/ul, which seems sufficiently close to the declared concentration of 100 ng/ul). I then made entirely independent preparations of the standard curve (doing both the initial dilution from the stock and the subsequent dilutions separately). The dilutions started at 1 ng/ul (made by diluting 8 ul of 200 ng/ul standard into 792 ul of EB), and then serial 2-fold dilutions from there. The dilutions were set up in a Costar 96-well black plate (3915), which is where I will read the PicoGreen. The dilutions all had final volumes of 100 ul, and had 1 ng/ul in welll B9 and then diluted from B10-B12, D9-D12; the second dilution went F9-F12, H9-H12. I then added 90 ul of EB to all of the other wells, and then pipetted 10 ul of my purified samples into the wells corresponding to the original PCR plate. These wells now putatively have 0.7 ng/ul if the recovery efficiency was perfect, and in reality probably have less. I then made a 1:200 dilution of the Quant-IT reagent by adding 42.5 ul of this reagent to 8.1 ml of EB. Then used a multichannel pipette to add 100 ul of this per well. Then incubated for 5 minutes covered with aluminum foil. Then read on the Tecan Infinite M100 with excitation at 485 nm (9 nm bandwidth) and emission at 535 nm (20 nm bandwidth) performing 50 reads at 400 Hz.

Below are the results; plots are on the next few pages. Note that I am then setting up a new plate that will have 1 ng/ul of these round 1 PCR products.

sample	1-426 reading	427-849 reading	850-1275 reading	1276-1698 reading	1-426 concentration (ng/ul)	427-849 concentration (ng/ul)	850-1275 concentration (ng/ul)	1276-1698 concentration (ng/ul)	volume EB (ul) to which 10 ul of 1-426 DNA is added for a final concentration of 1 ng/ul	volume EB (ul) to which 10 ul of 427-849 DNA is added for a final concentration of 1 ng/ul	volume EB (ul) to which 10 ul of 850-1275 DNA is added for a final concentration of 1 ng/ul	volume EB (ul) to which 10 ul of 1276-1698 DNA is added for a final concentration of 1 ng/ul
DNA #1	15553	12566	13813	14309	3.70	3.00	3.30	3.41	27.0	20.0	23.0	24.1
DNA #2	19478	12394	21566	9216	4.63	2.96	5.12	2.21	36.3	19.6	41.2	12.1
DNA #3	12845	9451	14911	8233	3.07	2.27	3.55	1.98	20.7	12.7	25.5	9.8
mutDNA #1	26394	13454	17943	16706	6.25	3.21	4.27	3.98	52.5	22.1	32.7	29.8
mutDNA #2	12207	11924	19434	10736	2.92	2.85	4.62	2.57	19.2	18.5	36.2	15.7
mutDNA #3	20511	11215	25574	9609	4.87	2.68	6.06	2.31	38.7	16.8	50.6	13.1
virus p1 #1	27781	19105	25227	17912	6.58	4.54	5.98	4.26	55.8	35.4	49.8	32.6
virus p1 #2	25254	16039	16380	12178	5.98	3.82	3.90	2.91	49.8	28.2	29.0	19.1
virus p1 #3	22592	20184	28327	12305	5.36	4.79	6.71	2.94	43.6	37.9	57.1	19.4
mutvirus p1 #1	17135	27087	37774	23048	4.08	6.42	8.93	5.47	30.8	54.2	79.3	44.7
mutvirus p1 #2	30385	19279	25198	12555	7.19	4.58	5.97	3.00	61.9	35.8	49.7	20.0
mutvirus p1 #3	25168	23232	24614	14424	5.96	5.51	5.83	3.44	49.6	45.1	48.3	24.4
virus p2 #1	22694	16615	22761	13746	5.38	3.95	5.40	3.28	43.8	29.5	44.0	22.8
virus p2 #2	23560	17659	18759	13439	5.59	4.20	4.46	3.21	45.9	32.0	34.6	22.1
virus p2 #3	24009	18577	27984	12942	5.69	4.42	6.63	3.09	46.9	34.2	56.3	20.9
mutvirus p2 #1	32852	14718	21969	10490	7.77	3.51	5.21	2.51	67.7	25.1	42.1	15.1
mutvirus p2 #2	35751	17501	27489	12281	8.45	4.16	6.51	2.94	74.5	31.6	55.1	19.4
mutvirus p2 #3	29997	17492	26478	15125	7.10	4.16	6.27	3.60	61.0	31.6	52.7	26.0
no-HA control	69	58	56	122	0.06	0.06	0.06	0.08	30.0	30.0	30.0	30.0
no Rd1 primers control	354	349	330	332	0.13	0.13	0.13	0.13	30.0	30.0	30.0	30.0

January-16-2015 PicoGreen standard curve for round 1 PCRs

$y = 42543x - 206.17$

PicoGreen reading (y-axis)

DNA concentration (ng/ul) (x-axis)

- curve 1
- curve 2
- mean
- Linear (mean)

January-16-2015 determination of concentration of round 1 PCR products after bead cleanup.

X-axis: DNA concentration after AmPure XP cleanup, ng/ul

Legend:
- 1276-1698 concentration (ng/ul)
- 850-1275 concentration (ng/ul)
- 427-849 concentration (ng/ul)
- 1-426 concentration (ng/ul)

Y-axis categories (top to bottom):
- no Rd1 primers control
- no-HA control
- mutvirus p2 #3
- mutvirus p2 #2
- mutvirus p2 #1
- virus p2 #3
- virus p2 #2
- virus p2 #1
- mutvirus p1 #3
- mutvirus p1 #2
- mutvirus p1 #1
- virus p1 #3
- virus p1 #2
- virus p1 #1
- mutDNA #3
- mutDNA #2
- mutDNA #1
- DNA #3
- DNA #2
- DNA #1

Dilution of round 1 PCRs, January-16-2015.
I will now create a new 96-well plate where all the round-1 products are at 1 ng/ul. Did this by adding the indicated volumes of EB to the plate, and the pipetting in 10 ul of the round 1 products. Note that wells B9, D9, F9, and G9 will get the corresponding DNA #1 (A1, C1, E1, G1) for that round 1 PCR – these will be the no-round-2 primer controls.

D9, F9, G9 that has a duplicate of the #1 sample -- this will be the no round-2 primer control											
	1	2	3	4	5	6	7	8	9	10	11	12
A	27.0	36.3	20.7	52.5	19.2	38.7	55.8	49.8	43.6	30.8	61.9	49.6
B	43.8	45.9	46.9	67.7	74.5	61.0	30.0	30.0	27.0			
C	20.0	19.6	12.7	22.1	18.5	16.8	35.4	28.2	37.9	54.2	35.8	45.1
D	29.5	32.0	34.2	25.1	31.6	31.6	30.0	30.0	20.0			
E	23.0	41.2	25.5	32.7	36.2	50.6	49.8	29.0	57.1	79.3	49.7	48.3
F	44.0	34.6	56.3	42.1	55.1	52.7	30.0	30.0	44.0			
G	24.1	12.1	9.8	29.8	15.7	13.1	32.6	19.1	19.4	44.7	20.0	24.4
H	22.8	22.1	20.9	15.1	19.4	26.0	30.0	30.0	22.8			

I am going to aim to bottleneck each round-1 PCR down to 3e5 ssDNA molecules (or 1.5e5 dsDNA molecules). Since the round-1 PCR had a final dissociation stage, each ssDNA molecule in the dsDNA pairs should be unique. The round-1 PCR products are about 550 nt in length (425 nt insert plus around 60-70 nt added by each primer). This many molecules is 9e-5 ng of DNA.

I will now **demultiplex** my round-1 PCRs. Briefly, pooled in equal volumes (10 ul each) my 1 ng/ul adjusted round-1 PCRs for each primer set. So for instance, this combines equal volumes of A1, C1, E1, and G1. The resulting plate now has the layout below for the first two rows. I will store this plate at -20 C for nw. When I thaw it, I will then make the serial 10-fold dilutions into the next three pairs of rows to get down to 1e-3 ng/ul in the last two rows. Note that when I set up the actual round-2 PCRs, I will want to use 9e-5 * 4 = 3.6e-4 ng of total DNA to get 3e5 molecules of each of the four round-1 products.

January-16-2015												
My "de-multiplexed" round-1 PCR plate. All four round-1 PCRs from each primer pair are pooled in equal volume after adjusting to 1 ng/ul. Serial 10-fold dilutions (10 ul into 90 ul EB) are then made down the plate.												
	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA #1	DNA #2	DNA #3	mutDNA #1	mutDNA #2	mutDNA #3	virus p1 #1	virus p1 #2	virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
B	virus p2 #1	virus p2 #2	virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control	no-round-1 primer control	DNA #1 for no-round-2 primer control	*these two rows are at 1 ng/ul*		
C	DNA #1	DNA #2	DNA #3	mutDNA #1	mutDNA #2	mutDNA #3	virus p1 #1	virus p1 #2	virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
D	virus p2 #1	virus p2 #2	virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control	no-round-1 primer control	DNA #1 for no-round-2 primer control	*these two rows are a 1:10 dilution of previous, so are 1e-1 ng/ul*		
E	DNA #1	DNA #2	DNA #3	mutDNA #1	mutDNA #2	mutDNA #3	virus p1 #1	virus p1 #2	virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
F	virus p2 #1	virus p2 #2	virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control	no-round-1 primer control	DNA #1 for no-round-2 primer control	*these two rows are a 1:10 dilution of previous, so are 1e-2 ng/ul*		
G	DNA #1	DNA #2	DNA #3	mutDNA #1	mutDNA #2	mutDNA #3	virus p1 #1	virus p1 #2	virus p1 #3	mutvirus p1 #1	mutvirus p1 #2	mutvirus p1 #3
H	virus p2 #1	virus p2 #2	virus p2 #3	mutvirus p2 #1	mutvirus p2 #2	mutvirus p2 #3	no-HA control	no-round-1 primer control	DNA #1 for no-round-2 primer control	*these two rows are at 1e-3 ng/ul*		

Round-2 PCRs, January-19-2015.

The dilution plate from January-16-2015 goes down to 1e-3 ng/ul in the bottom two rows. For my round-2 PCRs, I want 3.6e-4 ng of template per reaction (estimated 2.5e5 molecules of each of the four fragments). So in a new 96-well plate, made a 1:15 dilution of 10 ul of the final row of my Jan-16-2015 plate into 140 ul of EB to give 6.7e-5 ng/ul. I then set up the round-2 PCR reactions. Each reaction got:

- 20 ul of 2X KOD Master Mix
- 4 ul of 5 uM Rnd2ForUniversal
- 4 ul of 5 uM of the appropriate Rnd2RevIndex?? as indicated in the plate layout below
- 5.4 ul of my 6.7e-4 ng/ul dilutions of purified round-1 PCRs (for 3.6e-4 ng total).
- 6.6 ul of water for 40 ul of final volume

I then ran the PCR reactions. With a theoretical 2-fold PCR amplification, 24 thermal cycles will give me 6 ug of total product. I think this is a good number of PCR cycles, as this will yield substantial product but should not saturate the reactions. For instance, in my initial PCRs to make the amplicons, with 22 PCR cycles I got clear bands at both 1e6 and 1e7 template molecules, but the band for 1e7 template molecules was noticeably brighter than the 1e6 band but still not as bright as for some of the my samples. Here I am starting with 1.2e6 molecules, so even full amplification for 24 cycles should give a bit less than was the case for my 1e7 amplicon standard which was still below saturation. Here is the PCR reaction:

1. 95 C for 2:00
2. 95 C for :20
3. 55 C for :20
4. 70 C for :20
5. Goto 2, 23 times
6. 4 C forever

Here is the round-2 plate layout:

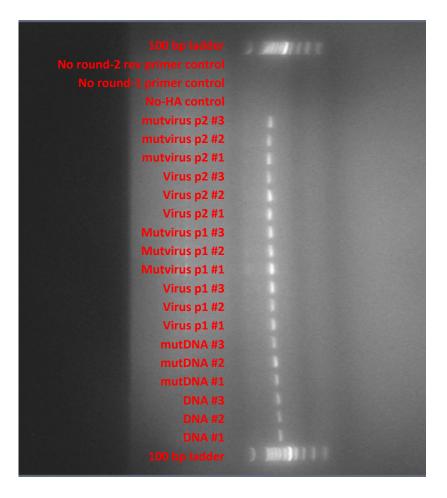|	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA #1 with Rnd2RevIndex1	DNA #2 with Rnd2RevIndex2	DNA #3 with Rnd2RevIndex3	mutDNA #1 with Rnd2RevIndex4	mutDNA #2 with Rnd2RevIndex5	mutDNA #3 with Rnd2RevIndex6	virus p1 #1 with Rnd2RevIndex7	virus p1 #2 with Rnd2RevIndex8	virus p1 #3 with Rnd2RevIndex9	mutvirus p1 #1 with Rnd2RevIndex10	mutvirus p1 #2 with Rnd2RevIndex11	mutvirus p1 #3 with Rnd2RevIndex12
B	virus p2 #1 with Rnd2RevIndex13	virus p2 #2 with Rnd2RevIndex14	virus p2 #3 with Rnd2RevIndex15	mutvirus p2 #1 with Rnd2RevIndex16	mutvirus p2 #2 with Rnd2RevIndex17	mutvirus p2 #3 with Rnd2RevIndex18	no-HA control with Rnd2RevIndex1	no round-1 primer control with Rnd2RevIndex1	DNA #1 with no round-2 rev primer control			

After running the round-2 PCRs, purified using the Ampure XP beads. Briefly, added 40 ul of beads to the 40 ul reactions, mixed, absorbed for 5 minutes, and then put on magnet. Aspirated liquid and washed twice with 120 ul of fresh 70% ethanol. Then resuspended each reaction in 60 ul of EB.
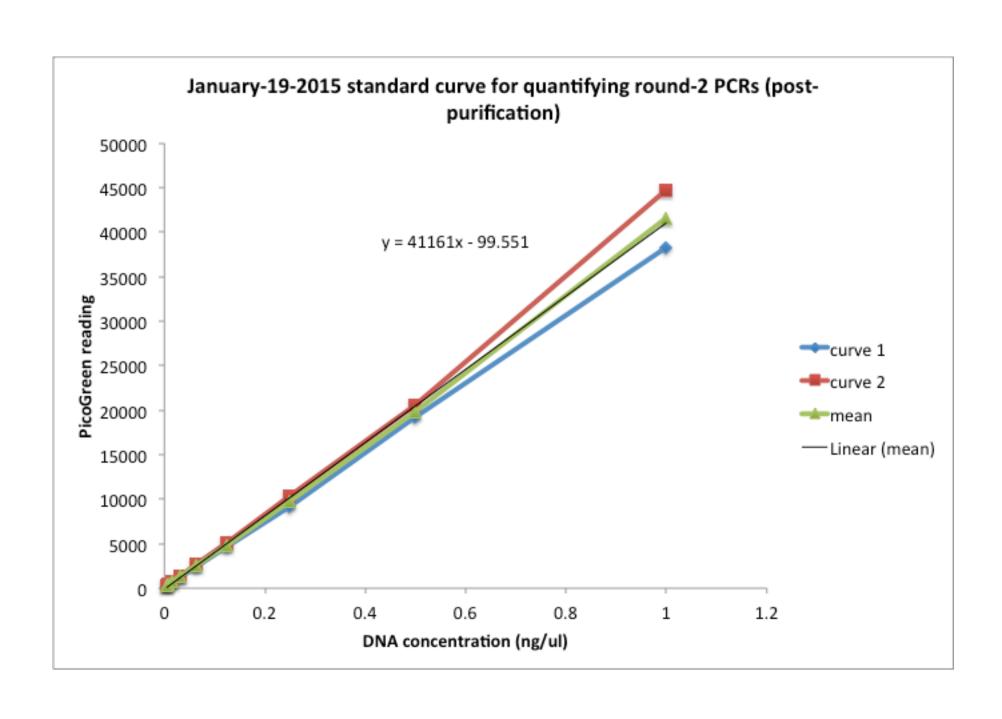
I then ran 4 ul of each reaction out on a 1.5% gel along with 10 ul of Promega 100 bp ladder (which has 1 ug of DNA total). To run out the reactions, I first aliquoted 8 ul of a mix of 2 ul of 6X loading buffer and 6 ul of water into a 96-well plate. I then used a multichannel to transfer 4 ul of my purified round-2 PCRs into this, and then loaded all 12 ul on the gel.

January-19-2015: Quantifying, pooling, and purifying round-2 PCRs.
Here is my gel of the round-2 PCRs. It looks great. All of the samples that are supposed to have bands do, and they are the right size of a bit larger than 600 nt. The three negative controls all lack bands. Given that the ladder overall has about 1 ug of DNA, I would guess that the typical bands are about 100 ng of DNA. Since I loaded 4 ul, this gives a concentration of about 25 ng/ul, which is on the order of what is expected as perfect PCR efficiency and DNA recovery would have yielded about 100 ng/ul. For quantification, used to the next two rows (C and D) of the round-2 PCR plates to make 1:10 dilutions of the round-2 PCRs (5 ul of PCR in 45 ul of EB); these dilutions now putatively have about 2 ng/ul of DNA.

I will now quantify these by PicoGreen (Invitrogen P7859). Took PicoGreen 100 ng/ul standard, vortexed to mix, and then verified that concentration on the NanoDrop (got a concentration of 98 ng/ul, which seems sufficiently close to the declared concentration of 100 ng/ul). I then made entirely independent preparations of the standard curve (doing both the initial dilution from the stock and the subsequent dilutions separately). The dilutions started at 1 ng/ul (made by diluting 8 ul of 200 ng/ul standard into 792 ul of EB), and then serial 2-fold dilutions from there. The dilutions were set up in a Costar 96-well black plate (3915), which is where I will read the PicoGreen. I then added 90 ul of EB to all of the other wells, and then pipetted 10 ul of my purified samples into the wells corresponding to the original PCR plate. These wells now putatively have 0.2 ng/ul. I then made a 1:200 dilution of the Quant-IT reagent by adding 42.5 ul of this reagent to 8.1 ml of EB. Then used a multichannel pipette to add 100 ul of this per well. Then incubated for 5 minutes covered with aluminum foil. Then read on the Tecan Infinite M100 with excitation at 485 nm (9 nm bandwidth) and emission at 535 nm (20 nm bandwidth) performing 50 reads at 400 Hz. The concentrations are on the next page. They come out to right about the 25 ng/ul that I had estimated!

Gel lane labels (top to bottom): 100 bp ladder; No round-2 rev primer control; No round-1 primer control; No-HA control; mutvirus p2 #3; mutvirus p2 #2; mutvirus p2 #1; Virus p2 #3; Virus p2 #2; Virus p2 #1; Mutvirus p1 #3; Mutvirus p1 #2; Mutvirus p1 #1; Virus p1 #3; Virus p1 #2; Virus p1 #1; mutDNA #3; mutDNA #2; mutDNA #1; DNA #3; DNA #2; DNA #1; 100 bp ladder

January-19-2015 standard curve for quantifying round-2 PCRs (post-purification)

y = 41161x - 99.551

January-19-2015: Quantifying, pooling, and purifying round-2 PCRs.
Below are the concentrations from my PicoGreen assays. They look good! I will now make three pools (one for each overall replicate). Did this by mixing equal quantities of DNA from each of the six amplicons for each replicate. Then stored the rest of the 96-well round-2 PCR plate at -20 C.
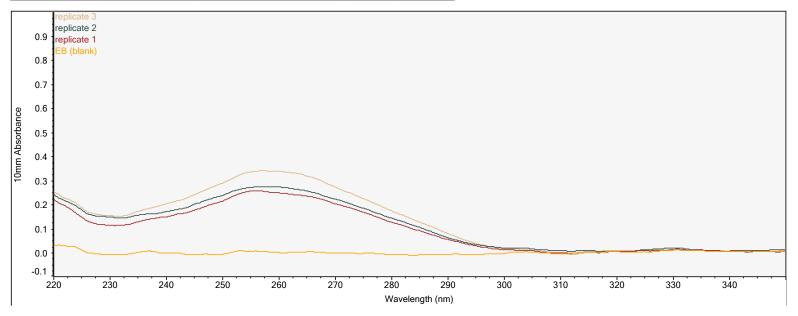
Tomorrow I will gel purify and quantify the three pools before submitting them for sequencing…

sample	reading 1	reading 2	concentration 1 (ng/ul)	concentration 2 (ng/ul)	mean concentration	assigned to this pool:	volume added to pool (ul)
DNA #1	6819	6565	16.8	16.2	16.5	1	12.0
DNA #2	7574	7935	18.6	19.5	19.1	2	12.0
DNA #3	9390	9531	23.1	23.4	23.2	3	12.0
mutDNA #1	10597	10304	26.0	25.3	25.6	1	7.7
mutDNA #2	14716	13886	36.0	34.0	35.0	2	6.5
mutDNA #3	9054	9033	22.2	22.2	22.2	3	12.5
virus p1 #1	10184	8764	25.0	21.5	23.3	1	8.5
virus p1 #2	11244	11374	27.6	27.9	27.7	2	8.3
virus p1 #3	9357	9197	23.0	22.6	22.8	3	12.2
mutvirus p1 #1	9075	8328	22.3	20.5	21.4	1	9.3
mutvirus p1 #2	7066	7036	17.4	17.3	17.4	2	13.2
mutvirus p1 #3	8018	8503	19.7	20.9	20.3	3	13.7
virus p2 #1	9289	7187	22.8	17.7	20.3	1	9.8
virus p2 #2	11183	8560	27.4	21.0	24.2	2	9.5
virus p2 #3	13382	9436	32.8	23.2	28.0	3	10.0
mutvirus p2 #1	9130	9880	22.4	24.2	23.3	1	8.5
mutvrius p2 #2	11493	10712	28.2	26.3	27.2	2	8.4
mutvirus p2 #3	11018	9722	27.0	23.9	25.4	3	11.0
no-HA control	75	67	0.4	0.4	0.4	none	
no round-1 primer control	71	68	0.4	0.4	0.4	none	
no round-2 primer control	60	61	0.4	0.4	0.4	none	

January-20-2015: Preparing pool samples for sequencing.

Yesterday I made three sample pools, all of which now containg about 1.0 to 1.4 ug of DNA. I will gel purify them to remove anything of the wrong size. To do this, poured three **completely clean (gel rigs, gel trays, combs, etc)** 1.5% agarose gels. I Speed-Vacced the DNA for about 8 minutes to reduce the volume a bit, and then loaded each sample onto its own gel. I ran the gels, and then **cleanly cut (Saran Wrap, new gloves, etc)** the bands, trying to minimize UV damage. To minimize this damage, I did not take photos. But the bands were nice and sharp. I cut around them with a bit of space since each subamplicon is slightly different in size. I then purified the DNA for each pool using Zymo Columns, eluted in 50 ul of EB, and analyzed by NanoDrop. The results are below. The traces look good. The total yield is about 500 ng, which corresponds to about 50% recovery.

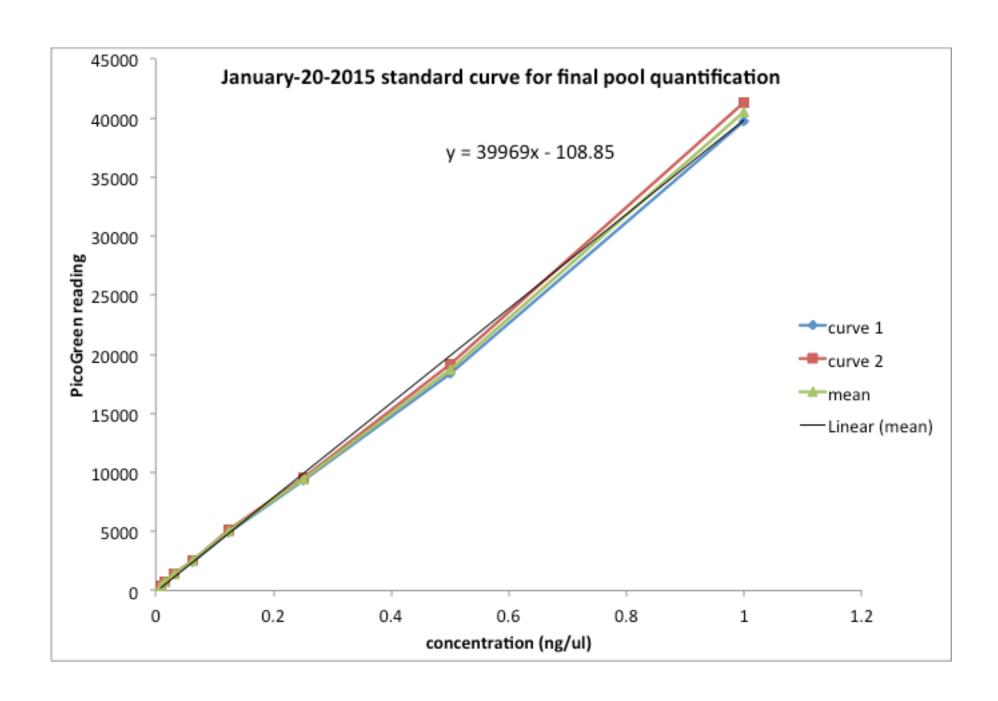| Sample ID	Date and Time	Nucleic Acid Conc.	A260	260/280	260/230
EB (blank)	1/20/2015 3:47:49 PM	-0.3	-0.006	0.42	0.40
replicate 1	1/20/2015 3:48:25 PM	12.1	0.243	2.01	2.24
replicate 2	1/20/2015 3:49:21 PM	13.4	0.268	1.95	1.88
replicate 3	1/20/2015 3:49:57 PM	16.7	0.333	1.98	2.24

January-20-2015: Preparing pool samples for sequencing.
I will now quantify these by PicoGreen (Invitrogen P7859). Took PicoGreen 100 ng/ul standard, vortexed to mix, and then verified that concentration on the NanoDrop (got a concentration of 98 ng/ul, which seems sufficiently close to the declared concentration of 100 ng/ul). I then made entirely independent preparations of the standard curve (doing both the initial dilution from the stock and the subsequent dilutions separately). The dilutions started at 1 ng/ul (made by diluting 8 ul of 200 ng/ul standard into 792 ul of EB), and then serial 2-fold dilutions from there. The dilutions were set up in a Costar 96-well black plate (3915), which is where I will read the PicoGreen. I then added 95 ul of EB to all of the other wells, and then pipetted 5 ul of my purified samples into the 95 ul for a 1:20 dilution. Did this in duplicate for each sample. I then made a 1:200 dilution of the Quant-IT reagent by adding 42.5 ul of this reagent to 8.1 ml of EB. Then used a multichannel pipette to add 100 ul of this per well. Then incubated for 5 minutes covered with aluminum foil. Then read on the Tecan Infinite M100 with excitation at 485 nm (9 nm bandwidth) and emission at 535 nm (20 nm bandwidth) performing 50 reads at 400 Hz. The concentrations are below. They come out to just a bit lower than those estimated by NanoDrop.

The Genomics Core requests the samples at 4 nM. I think they mean dsDNA. For a 625 nt fragment, this corresponds to 1.66 ng/ul. So made dilutions of all of my samples to this concentration as indicated below.

I then submitted *just replicate 1* to the Genomics Core. I will sequence the others if this one looks good. They said that with 2X275 nt reads using v3 reagents on the miSeq, I can expect about 25-million reads. I asked them spike in PhiX at 10%. The cost will be $1800. The submission forms are on the next few slides (after the standard curve).

sample name	reading 1	reading 2	average reading	concentration in dilution (ng/ul)	undiluted concentration (ng/ul)	volume added to 50 ul of EB for 4 nM dsDNA (1.66 ng/ul)
replicate_1	19536	19570	19553	0.49	9.84	10.15
replicate_2	19241	19977	19609	0.49	9.87	10.11
replicate_3	26009	25889	25949	0.65	13.04	7.29

January-20-2015 standard curve for final pool quantification

$y = 39969x - 108.85$

[Header]									
IEMFileVersion	4								
Investigator Name	Jesse Bloom								
Experiment Name	WSN_HA_helper_replicate_1								
Date	January-20-2015								
Workflow	GenerateFASTQ								
Application	FASTQ Only								
Assay	TruSeq								
Description									
Chemistry	Amplicon								
[Reads]									
275									
275									
[Settings]									
ReverseComplement	0								
[Data]									
Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2	Sample_Project	Description
WSN_HA_helper_replicate_1	DNA			TruSeq index 1	atcacg	none	none		
WSN_HA_helper_replicate_2	mutDNA			TruSeq index 2	cgatgt	none	none		
WSN_HA_helper_replicate_3	virus-p1			TruSeq index 3	ttaggc	none	none		
WSN_HA_helper_replicate_4	mutvirus-p1			TruSeq index 4	tgacca	none	none		
WSN_HA_helper_replicate_5	virus-p2			TruSeq index 5	acagtg	none	none		
WSN_HA_helper_replicate_6	mutvirus-p2			TruSeq index 6	gccaat	none	none		