2016-12-19: Reverse transcription of WSN-HA library 2 selected by mixtures of L7+L19

Each RT annealing 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
8.8 ul RNA diluted to 1000 ng total in RNase-free water.
====== 19 ul total volume

First, thaw RNA on ice, flick tubes gently to mix, and spin down. Keep on ice.

Make annealing master mix for 30 reactions' worth (for the 26 reactions I will do) on ice:
90 ul 10X AccuScript RT Buffer
36 ul of dNTP mix
90 ul of 5 uM WSN-For
90 ul of 5 uM WSN-Rev

Keep RNA and reaction plates on ice while setting up.
Use HyClone molecular biology-grade water.

Check heather's protocol for how much RNA to put in what volume of accuscript RT reaction, and then how much of that RT reaction to add to the subsequent PCR.

Primers for RT/PCR:

WSN-For: AGCAAAAGCAGGGGAAAATAAAAACAAC; Length: 28 bp; Tm= 60.9°C

WSN-Rev: AGTAGAAACAAGGGTGTTTTTCCTTATATTTCTG; Length: 34 bp; Tm= 60.1°C

The stocks should be at 100uM.

Makes 1775 bp product.

Make RNA dilutions in the plate (on ice) layout and pipetting calculations as shown in next slide. Each well has 1000ng RNA diluted to 8.8ul in water. Once all RNA dilutions are made in plate, add 10.2 ul of annealing master mix to each well and mix by pipetting.

Heat to 65 C for 5 minutes, then cool to 4 C. Make the following master mixes while heating and cooling:

AccuScript master mix (for 29 rxn):
72.5 ul of water and 43.5 ul of AccuScript RT
RNAse block master mix (for 29 rxn):
94.25 ul of water and 21.75 ul of Rnase block

Bring the plate to ice. 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.
T**otal RT reaction volume is 30ul.**
I goofed and skipped the DTT step until after I already started the block for the RT. I don't think the block actually started the 42C step - it was probably still heading the lid when I realized and stopped it, put the plate back on ice and added 3ul DTT to each.

2016-12-19: RT plate layout and pipetting calculations.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
C												
D	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
E												
F	**25**	**26**										
G												

Sample ID	ng/ul	ul for 1000ng	ul water to 8.8
1	337.5	2.96	5.84
2	443.5	2.25	6.55
3	220.6	4.53	4.27
4	428.8	2.33	6.47
5	435	2.30	6.50
6	184.8	5.41	3.39
7	495.6	2.02	6.78
8	435.4	2.30	6.50
9	288.6	3.47	5.33
10	388.8	2.57	6.23
11	394.6	2.53	6.27
12	205.2	4.87	3.93

Sample ID	ng/ul	ul for 1000ng	ul water to 8.8
13	489.5	2.04	6.76
14	370.9	2.70	6.10
15	412.4	2.42	6.38
16	418.2	2.39	6.41
17	267.6	3.74	5.06
18	162.4	6.16	2.64
19	473.7	2.11	6.69
20	483.9	2.07	6.73
21	270.3	3.70	5.10
22	403.9	2.48	6.32
23	212.9	4.70	4.10
24	222.5	4.49	4.31
25	384.3	2.60	6.20
26	355.1	2.82	5.98

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
C												
D	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
E												
F	**25**	**26**	**27: no PCR template**	**28: 1e4**	**29: 1e5**	**30: 1e6**	**31: 1e7**	**32: 1e8**				
G												

2016-12-19: PCR on cDNA to make full-length HA amplicons.

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 am making a fresh 5ng/ul dilution, and making 10-fold dilutions by transferring 20ul to 180 ul to make **5e3, 5e4, 5e5, 5e6** per ul to use as witness band standards.

Do in **50ul reactions: (make 33x master mix)**
Each reaction:
25 ul of 2X KOD Master Mix (825 ul)
3 ul of 5 uM WSN-for (99 ul)
3 ul of 5 uM WSN-rev (99 ul)
17 ul of water (561 ul)
2 ul template

Add 48 ul of PCR mastermix to each well, then add 2ul of template.
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).

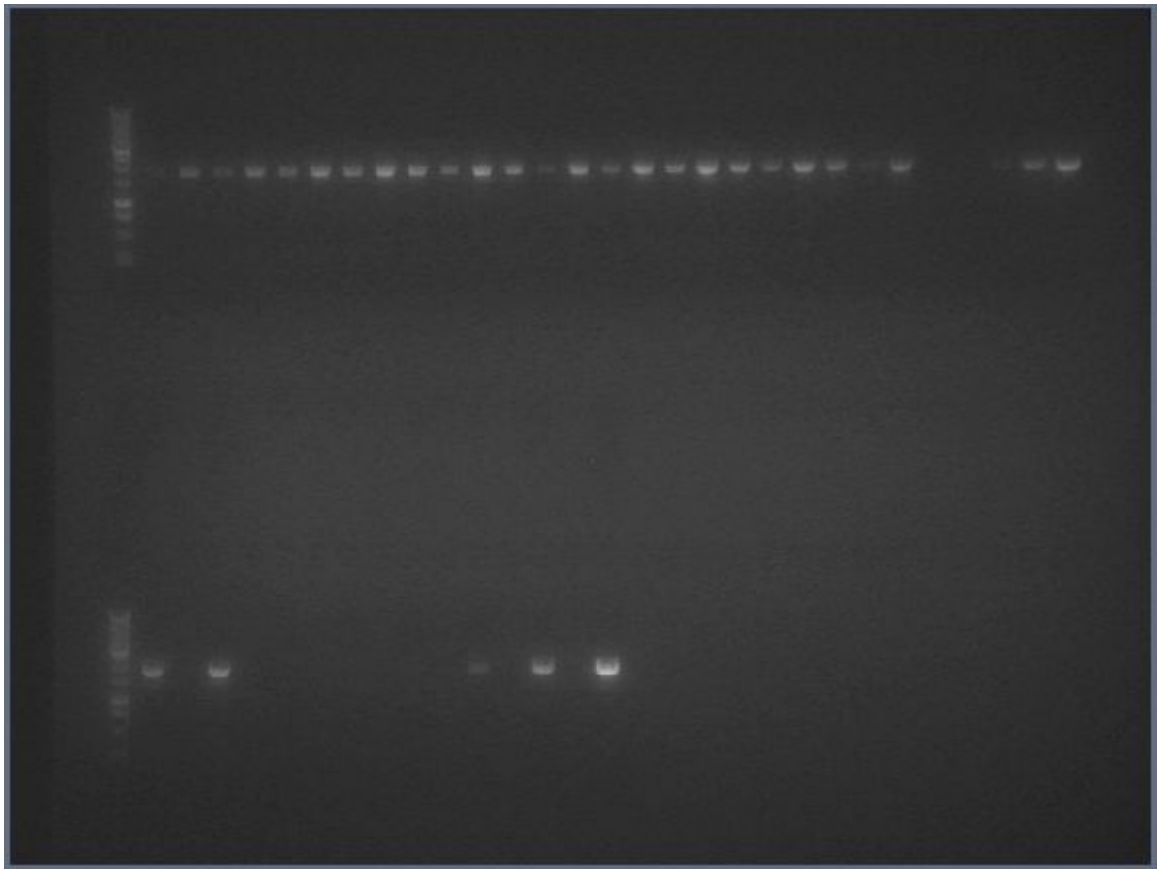
Samples 28-32 use a known number of linear WSN HA amplicon molecules as template in each reaction, so that you can use band intensities on the gel to approximate how much cDNA was used as template for each PCR.

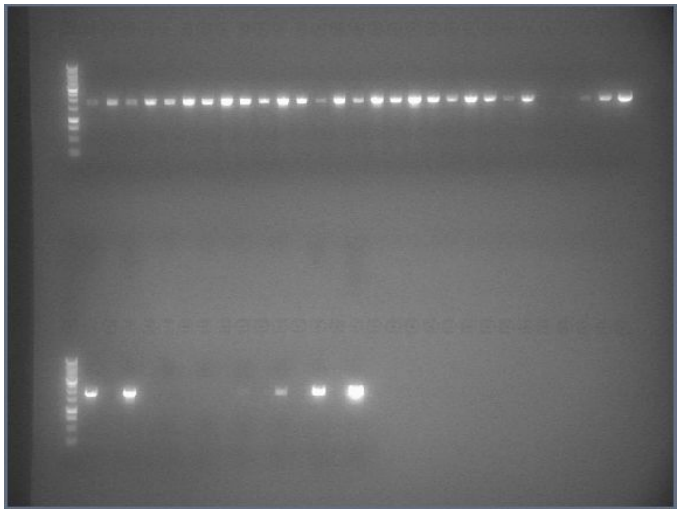
Bring a wt DNA sample amplified from plasmid into the workflow at this point. Plasmid #33 in the plasmid log should be pHW-WSN-HA. I will give you some of this.

For all these PCR: Use your own, fresh thaw tube of KOD (stays good at 4 C for a while, but start out with a fresh tube and keep it to yourself.)

Top: 1, 13, 2, 14… 11, 23, 12, 24, 28.29.30.31.32. For 12, only half was loaded to gel.

Bottom: 25-32, every other.





2016-12-19: Ampure XP cleanup of full-length amplicons.

I will purify all 24 PCR amplicons (some of these are negative controls and standard curves for witness band amplification, and some I don't plan to sequence, but will purify regardless). I will use 0.9X ampure beads. Each PCR was 50 ul and I used 5ul to run on the gel (and 10 ul for witness samples 28-32), so there remains **45ul of PCR to which I will add 40.5ul beads.**

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. (During the second wash) Aspirate any remaining ethanol with multichannel, Air dry 10 min. I usually cover for these incubations with the lid of a tip box. When dried you'll see "caking" and slight cracking?
8. **Add 60 ul of EB over the dried beads while on the rack; then** remove from rack, mix beads by pipetting the 60 ul EB. Make sure beads from all wells are well resuspended. You might need to touch-up on individual wells with a single channel pipetter to resuspend.
9. Incubate 5 min to resuspend DNA
10. Put on magnet for 5 minutes
11. Transfer bead-free DNA solutions to a new plate ("2016.12.19 purified full-length amplicons")

Protocol is actually for 70% ethanol for wash steps

It's ok to elute with 70 ul of EB and then transfer 65 ul of bead-free solution should be OK, to avoid carrying over stray beads.

2016-12-19 Picogreen of full-length amplicons

Thaw the picogreen pouch ahead of time. Use the 20x TE to make a stock of 1x TE to use for the whole assay.

1. VORTEX and SPIN the DNA standard tube well to resuspend DNA. Then Make 2 standards at **2ng/ul by adding 4ul of the standard to 196ul TE**. Vortex to mix.
2. Add 99 ul (or 100 ul) 1xTE to all wells.
3. Add duplicates of 1 ul of ampure-purified products to the 99 ul TE wells to measure 1:101 dilutions of the samples in replicates as shown in the plate layout below. (**sometimes multichanneling 1 ul is sketchy. Pipette with the same technique consistently. Try to watch all channels when you pipette to make sure you actually draw solution into each tip. Also helpful to watch as you eject to make sure there weren't any empty tips.**)
4. Add 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. In the dark, make picogreen working solution (I think this is 1:200 dilution?) (55ul to 11ml TE), and from a reservoir of this add 100 ul to all wells used in assay, **mixing 4 times by pipetting**, cover with aluminum foil 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	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
C	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
D	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
E	**25**	**26**	**27: no PCR template**	**28: 1e4**	**29: 1e5**	**30: 1e6**	**31: 1e7**	**32: 1e8**	**TE**	**TE**	**TE**	**TE**
F	**25**	**26**	**27: no PCR template**	**28: 1e4**	**29: 1e5**	**30: 1e6**	**31: 1e7**	**32: 1e8**	**TE**	**TE**	**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

Rows G and H are picogreen standard so that you can relate your samples to an absolute concentration of ds DNA. The standards in orange are PCR products that began with a certain number of molecules - it is not imperative to purify and quantify them but might be nice to see (?) --

2016.12.21 Round 1 PCR set up. Making two plates as shown below. Round 1 primers are 10uM stocks. For each primer pair (ampX_F/ampX_R), make a mix for 30 reaction's worth (actually only needing 26 reactions for each primer pair). To do this, for each of the 6 subamplicons make a primer mix by adding: **30ul F primer (10uM) + 30ul R primer (10uM) + 60ul water.** Final volume 120ul, each primer is 2.5uM in this final mixture. Each PCR gets 4ul of this combined F/R subamplicon primer pair. I make these 6 primer mixes in a labeled strip of tubes to facilitate multichanneling.

First make the 0.5 ng/ul dilution of the full-length amplicon plate as described below, then set up round 1 as follows.

Each round 1 PCR 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) taken from the dilution plate which is laid out to facilitate transferring 8 samples at a time, 6 times, to the 6 subamplicon reactions for those samples.

Set these reactions up with a multichannel with the format shown in slides below. You may want to only set up one plate at a time since you are making two plates. Set up on ice :-).

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). Be careful not to dispense air bubbles (stop at the first stop like you're supposed to).
2. Add 4ul primers from a striptube of primer pairs 1-6 (6 transfers at a time to 27+ rows of 6) to all rows as indicated. I like to use a fresh row of 6 tips for each dispensing so that I can look at the box of tips and know which row I'm on.
3. Add templates to each column from the 0.5ng/ul plate, which is arranged to facilitate easy transfer using the multichannel 8 at a time (8ul). Basically, column 1 of the dilution plate is used for columns 1-6 of plate1; column 3 of dilution plate is used for columns 7-12 of plate1, column 5 of the dilution plate is used for columns 1-6 of plate 2, etc.

Seal with microfilm A, spin, and thermocycle as described here. I 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**" on the Mastercycler (be careful becuase there is some other program called something like "ORRROUND1"), or if using C1000 I will save this program as "**HA subamplicons R1**".

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

Make 0.5ng/ul dilutions of full-length amplicons **in a new 96-well plate** by first adding 100ul EB to the dilution plate columns 1, 3, 5, and 7, and then transfer these volumes of each sample from the purified full-length amplicons plate to the dilution plate. The dilution plate sample layout will facilitate setting up round 1 pcr.

Source well	Selection #	ng/ul	ul of sample to dilution plate	Destination well (0.5ng/ul dilution plate)
B1	**1**	5.6	**9.838**	A1
B2	**2**	10.5	**4.977**	B1
B3	**3**	19.1	**2.691**	C1
B4	**4**	26.0	**1.960**	D1
B5	**5**	35.5	**1.429**	E1
B6	**6**	52.0	**0.971**	F1
B7	**7**	5.7	**9.642**	G1
B8	**8**	16.2	**3.177**	H1
B9	**9**	29.2	**1.744**	A3
B10	**10**	34.3	**1.478**	B3
B11	**11**	43.4	**1.165**	C3
B12	**12**	10.6	**4.929**	D3
D1	**13**	20.2	**2.535**	E3
D2	**14**	27.0	**1.886**	F3
D3	**15**	37.3	**1.358**	G3
D4	**16**	44.4	**1.139**	H3
D5	**17**	21.2	**2.419**	A5
D6	**18**	31.1	**1.633**	B5
D7	**19**	43.2	**1.171**	C5
D8	**20**	44.2	**1.145**	D5
D9	**21**	51.3	**0.984**	E5
D10	**22**	18.9	**2.721**	F5
D11	**23**	29.0	**1.756**	G5
D12	**24**	40.6	**1.248**	H5
F1	**25**	44.8	**1.128**	A7
F2	**26**	54.4	**0.928**	B7
	negative control		just EB	C7

Layouts for round 1 PCR.

Make 0.5 ng/ul dilution plates using math on previous slide in 4 columns 1-8, 9-16, 17-24, 25-26 so that each column of 0.5ng/ul in >60ul EB can be multichanneled 6 times to the round 1 PCR plate.

PLATE 1

	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6
	1	2	3	4	5	6	7	8	9	10	11	12
A	**selection 1**						**selection 9**					
B	**selection 2**						**selection 10**					
C	**selection 3**						**selection 11**					
D	**selection 4**						**selection 12**					
E	**selection 5**						**selection 13**					
F	**selection 6**						**selection 14**					
G	**selection 7**						**selection 15**					
H	**selection 8**						**selection 16**					

Note that you can drop the “witness band standards” at this step. Only keep samples that you will be sequencing: all your 26 mice, plus the wt DNA sample, plus a negative control PCR without template for each PCR.

PLATE 2

	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6	subamp1	subamp2	subamp3	subamp4	subamp5	subamp6
	1	2	3	4	5	6	7	8	9	10	11	12
A	**selection 17**						**selection 25**					
B	**selection 18**						**selection 26**					
C	**selection 19**						**water template**					
D	**selection 20**											
E	**selection 21**											
F	**selection 22**											
G	**selection 23**											
H	**selection 24**											

Ampure purification of round 1 PCR products:

FIRST take the PCR product plate that has finished round 1 PCR, and Add 26 ul of EB to each well of the round 1 PCR products to bring the volume to 50 ul - this is important because it is hard to do the ampure bead purification with a small starting volume.

Then purify with 1x Ampure beads: (one plate at a time)

1. Take bead aliquot (~8.5ml (check how much you actually need?) from vortexed stock and allow to come to room temperature
2. Add 50 ul beads to each well and mix 10x (since you have a full plate for at least one of your round 1 plates this might be nice to do with a multichannel by aliquoting beads to a strip of tubes first).
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. disperse beads in 75 ul EB and THEN remove from rack, resuspend well by pipetting.
9. Incubate 5 min to resuspend DNA
10. Put on magnet for 5 minutes
11. Transfer bead-free DNA solutions to 2 new racks ("**purified. R1 subamplicons, Plate 1 of 2 and Plate 2 of 2**) with the same layout as the PCR plates above.

I will use four plates to quantify by picogreen.

Picogreen1:
plate 1, left half

	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6			
	1	2	3	4	5	6	7	8	9
A	selection 1						TE	1.00E+00	1.00E+00
B	Selection 2						TE	5.00E-01	5.00E-01
C	Selection 3						TE	2.50E-01	2.50E-01
D	Selection 4						TE	1.25E-01	1.25E-01
E	Selection 5						TE	6.25E-02	6.25E-02
F	Selection 6						TE	3.13E-02	3.13E-02
G	selection 7						TE	1.56E-02	1.56E-02
H	selection 8						TE	7.81E-03	7.81E-03

Picogreen2
: plate1,
right half

							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	selection 9					
B				5.00E-01	5.00E-01	TE	selection 10					
C				2.50E-01	2.50E-01	TE	selection 11					
D				1.25E-01	1.25E-01	TE	selection 12					
E				6.25E-02	6.25E-02	TE	selection 13					
F				3.13E-02	3.13E-02	TE	selection 14					
G				1.56E-02	1.56E-02	TE	selection 15					
H				7.81E-03	7.81E-03	TE	selection 16					

picogreen3: plate #2, left half

	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6	subamp 1	subamp 2	subamp 3
	1	2	3	4	5	6	7	8	9
A	Selection 17						TE	1.00E+00	1.00E+00
B	Selection 18						TE	5.00E-01	5.00E-01
C	Selection 19						TE	2.50E-01	2.50E-01
D	Selection 20						TE	1.25E-01	1.25E-01
E	Selection 21						TE	6.25E-02	6.25E-02
F	Selection 22						TE	3.13E-02	3.13E-02
G	Selection 23						TE	1.56E-02	1.56E-02
H	Selection 24						TE	7.81E-03	7.81E-03

picogreen4 : plate #2, right half

	1	2	3	4	5	6	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6
							7	8	9	10	11	12
A				1.00E+00	1.00E+00	TE	selection 25					
B				5.00E-01	5.00E-01	TE	selection 26					
C				2.50E-01	2.50E-01	TE	Water only					
D				1.25E-01	1.25E-01	TE						
E				6.25E-02	6.25E-02	TE						
F				3.13E-02	3.13E-02	TE						
G				1.56E-02	1.56E-02	TE						
H				7.81E-03	7.81E-03	TE						

For each of the picogreen plates, here is the protocol:

Make two standards that can be used for all four plates: Make 2 standards at **2ng/ul by adding 10.2 ul of the standard to 500 ul TE**.

1. Add 100 ul 1xTE to all wells used in assay
2. Make standard dilutions by adding 100 ul of the independent standards to the 1ng/ul standard wells, and serially transferring 100ul to make 1:2 dilutions.
3. Add 1 ul of ampure-purified products (**sometimes multichanneling 1 ul is sketchy. Pipette with the same technique consistently. Try to watch all channels when you pipette to make sure you actually draw solution into each tip. Also helpful to watch as you eject to make sure there weren't any empty tips.**) to the sample wells to measure (1:101) dilutions of the samples in replicates as shown in the plate layout below.
4. Make picogreen working solution 1:200 dilution, (40ul to 8ml TE; per plate), add 100 ul to all wells used in assay (mix 3 times when adding), cover to protect from light and incubate 5 minutes before reading on plate reader.

For both picogreen and ampure, you'll probably want to refer back to the detailed protocols earlier on, but note any differences.

Round 1 product concentrations:

	1	2	3	4	5	6
selection 1	2.399475411	1.547690499	2.330535669	2.142301696	3.43992651	1.741672134
selection 2	2.406371477	1.664027397	2.266226888	1.839963991	3.331181048	1.883427457
selection 3	1.929199098	1.238308242	1.77137741	1.381461674	2.259338704	1.261010878
selection 4	1.81480983	1.376912616	1.972701317	1.753096108	2.5144583	1.326892054
selection 5	1.913176647	1.379187109	1.890291866	1.536293678	2.553575523	1.222421101
selection 6	1.89715675	1.45656164	1.961251578	1.869700069	2.721677116	1.791948166
selection 7	1.926910021	1.538572914	2.149182922	2.112487928	2.650266635	1.240578152
selection 8	2.385684411	1.575049364	2.282300866	2.165240744	3.261804224	1.340530523

	1	2	3	4	5	6
selection 9	2.487581197	1.905342222	2.718054707	2.482625096	3.734420313	2.43306485
selection 10	2.685836282	2.234838279	2.948555574	2.718054707	3.573260219	2.29678154
selection 11	1.511539771	1.650222813	2.304214889	1.744338535	2.398373516	2.276959439
selection 12	1.759199572	1.717093763	2.460322813	1.682419473	2.50740574	2.269526213
selection 13	1.632886512	1.724524096	2.51484	1.729477676	2.239793652	2.274481693
selection 14	1.734431276	1.818645458	2.301737102	2.006908288	2.980778179	2.162987108
selection 15	1.86323166	1.974703617	2.884111846	2.264570748	3.21378658	2.44545478
selection 16	1.972226365	2.115914005	3.12206759	2.400851445	2.955991516	2.54705548

	1	2	3	4	5	6
selection 17	1.703112345	1.30415928	1.622336079	1.570078207	2.076310597	1.285173442
selection 18	1.676977014	1.299412709	1.638965107	1.91700917	1.928895435	1.418098587
selection 19	1.767269933	1.327893239	1.665097893	1.924140891	1.864713378	1.354002692
selection 20	1.886106362	1.422846931	1.729249394	2.392687451	1.824307285	1.437092371
selection 21	1.966933582	1.268561819	1.453712815	1.836191036	1.795787639	1.401479918
selection 22	2.173819651	1.247205387	1.584329626	2.045397142	1.95504632	1.318399436
selection 23	2.221391894	1.406228024	1.422846931	1.864713378	1.641340741	1.36587134
selection 24	2.397446429	1.479832296	1.764893546	2.2689685	2.114360627	1.337387334

	1	2	3	4	5	6
selection 25	2.525480934	1.741046481	2.433981163	2.656524518	2.755407641	1.963881371
selection 26	2.129701228	1.691512163	2.888876776	2.819673803	2.874048147	2.386989323

Now we want to normalize the concentrations of all the round 1 products so that we can: A) mix together the 6 subamplicons for each experimental sample in equal amounts, and B) dilute those mixtures so that a known number of molecular barcodes for each subamplicon are allowed through.
Make **0.4 ng/ul dilution plates** for round 1 products by **first adding this much EB to each well**, and then **transferring 15 ul of round 1 products with the multichannel. The plate layouts of these 0.4ng/ul dilutions are identical to the plate layouts of the round 1 products.**

Plate 1 diluent volumes:

	1	2	3	4	5	6	7	8	9	10	11	12
A	74.98	43.04	72.40	65.34	114.00	50.31	78.28	56.45	86.93	78.10	125.04	76.24
B	75.24	47.40	69.98	54.00	109.92	55.63	85.72	68.81	95.57	86.93	119.00	71.13
C	57.34	31.44	51.43	36.80	69.73	32.29	41.68	46.88	71.41	50.41	74.94	70.39
D	53.06	36.63	58.98	50.74	79.29	34.76	50.97	49.39	77.26	48.09	79.03	70.11
E	56.74	36.72	55.89	42.61	80.76	30.84	46.23	49.67	79.31	49.86	68.99	70.29
F	56.14	39.62	58.55	55.11	87.06	52.20	50.04	53.20	71.32	60.26	96.78	66.11
G	57.26	42.70	65.59	64.22	84.38	31.52	54.87	59.05	93.15	69.92	105.52	76.70
H	74.46	44.06	70.59	66.20	107.32	35.27	58.96	64.35	102.08	75.03	95.85	80.51

Plate 2 diluent volumes:

	1	2	3	4	5	6	7	8	9	10	11	12
A	48.87	33.91	45.84	43.88	62.86	33.19	79.71	50.29	76.27	84.62	88.33	58.65
B	47.89	33.73	46.46	56.89	57.33	38.18	64.86	48.43	93.33	90.74	92.78	74.51
C	51.27	34.80	47.44	57.16	54.93	35.78						
D	55.73	38.36	49.85	74.73	53.41	38.89						
E	58.76	32.57	39.51	53.86	52.34	37.56						
F	66.52	31.77	44.41	61.70	58.31	34.44						
G	68.30	37.73	38.36	54.93	46.55	36.22						
H	74.90	40.49	51.18	70.09	64.29	35.15						

Pooled/Diluted Round 1 subamplicon products plates: For each experimental sample, the six subamplicon products from round 1 PCR are now mixed together in equal amounts since they can all be used in a single round 2 PCR to append the same experiment-specific index barcode. I will use the following plate setups to first pool together equal amounts of each of the 6 subamplicons for each experimental sample (using columns 1 and 7 to make the initial pools), and then dilute out these pools to use an appropriate dilution for round 2 PCR to control the barcode complexity going forward from this point.

1. Pool the 0.4 ng/ul concentration-normalized products that you just painstakingly made for each experimental samples (**15 ul of the 0.4ng/ul of each subamplicon**) into a single 0.4ng/ul pool for each experimental sample 1-26 as shown below
2. then make the appropriate 1:15 dilutions (columns 2,3,4 and 8,9,10) and finally a 1:5 dilution (into columns 5 and 11) as indicated, to achieve the following ng/ul.

For the 1:15 dilutions, transfer **10 ul to 140 ul of EB**; for the 1:5 dilution transfer **10 ul to 40 ul EB.**

PLATE 1:

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

PLATE 2:

	1: 0.4ng/ul pool	**2:1:15 of col 1**	**3: 1:15 of col 2**	**4:1:15 of col 3**	**5:1:5 of col 4**	6: empty	**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: empty
A	selection 17						selection 25					
B	selection 18						selection 26					
C	selection 19											
D	selection 20											
E	selection 21											
F	selection 22											
G	selection 23											
H	selection 24	2.67E-02	1.78E-03	1.19E-04	2.37E-05			2.67E-02	1.78E-03	1.19E-04	2.37E-05	

Each of the two lanes on the HiSeq will provide at least 100 million paired reads.

For this particular experiment, I want **1.5E5 barcodes (this is *lower* than what I used in the Viruses paper to characterize the plasmid mutant libraries and mutant virus libraries -- because in antibody selection i don't need such great depth to see the escape mutations which are at relatively high frequency --- so what this number is for you will probably be higher?)** 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.

In other words, 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.

Therefore, round 2 PCR will use: **8.354** ul of dilution column 5 or 11 (the column corresponding to 2.37E-5 ng/ul).

Set up a plate in this layout for round 2 pcr: (but first, look at next slide, need to assign an illumina index to each sample)

	1	2	3	4	5	6	7	8
A	selection 1		selection 9		selection 17		selection 25	
B	selection 2		selection 10		selection 18		selection 26	
C	selection 3		selection 11		selection 19		26, no R primer	
D	selection 4		selection 12		selection 20			
E	selection 5		selection 13		selection 21			
F	selection 6		selection 14		selection 22			
G	selection 7		selection 15		selection 23			
H	selection 8		selection 16		selection 24			

Each Round 2 PCR:
20 ul of 2X KOD Master Mix + **4 ul** of 5 uM Rnd2ForUniversal + **3.65 ul** water (*make 29x MM: 580ul KOD + 116 ul R2UniF 5uM + 105.85 water*)
4 ul of 5 uM of the appropriate Rnd2RevIndex as indicated on following slide.
8.354 ul template taken from the appropriate column 5 or 11 from the appropriate dilution plate
(then mix all wells by pipetting, seal with microfilm A, spin, thermalcycler)
======
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

Freeze at -20 overnight (I did but you don't have to) before purifying with ampure:
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 **75ul EB**. Transfer to a fresh plate with the same layout.

Selection	Round2Rev Index	dilution plate source	round 2 plate destination
1	NF01 Rnd2Rev	plate 1, column 5	column 1
2	NF03 Rnd2Rev	plate 1, column 5	column 1
3	NF08 Rnd2Rev	plate 1, column 5	column 1
4	NF09 Rnd2Rev	plate 1, column 5	column 1
5	NF10 Rnd2Rev	plate 1, column 5	column 1
6	NF11 Rnd2Rev	plate 1, column 5	column 1
7	TS22Rnd2rev	plate 1, column 5	column 1
8	TS25Rnd2rev	plate 1, column 5	column 1
9	TS06Rnd2rev	plate 1, column 11	column 3
10	TS12Rnd2rev	plate 1, column 11	column 3
11	TS19Rnd2rev	plate 1, column 11	column 3
12	TS13Rnd2rev	plate 1, column 11	column 3
13	TS14Rnd2rev	plate 1, column 11	column 3
14	TS15Rnd2rev	plate 1, column 11	column 3
15	TS18Rnd2rev	plate 1, column 11	column 3
16	TS20Rnd2rev	plate 1, column 11	column 3
17	TS21Rnd2rev	plate 2, column 5	column 5
18	TS23Rnd2rev	plate 2, column 5	column 5
19	TS27Rnd2rev	plate 2, column 5	column 5
20	NF29Rnd2rev	plate 2, column 5	column 5
21	NF30Rnd2rev	plate 2, column 5	column 5
22	NF31Rnd2rev	plate 2, column 5	column 5
23	NF32Rnd2rev	plate 2, column 5	column 5
24	NF33Rnd2rev	plate 2, column 5	column 5
25	NF34Rnd2rev	plate 2, column 11	column 7
26	NF35Rnd2rev	plate 2, column 11	column 7
26 no primer	*no primer*	*plate 2, B11*	*C7*

I recommend that you use these 26 primers, plus ONE more: NF36 to cover your 27th sample.

You will have to get an aliquot of the NF36 primer, possibly from Orr as he might be the only one who has used it in the past.

Keep in mind the concentrations if you get an aliquot from Orr.

Mike's tubes are all at 5uM; Orr's might be at 10uM?

Then picogreen in duplicate:

1. Make 2 standards at **2ng/ul by adding 4ul of the standard to 196ul TE**.
2. Add 100 ul 1xTE to all wells.
3. Add 1 ul of ampure-purified products to measure 1:101 dilutions of the samples in replicates as shown in the plate layout below.
4. Make standard dilutions by adding 100ul of the independent standards to the 1ng/ul standard wells, and serially transferring 100ul to make 1:2 dilutions.
5. Make picogreen working solution (50ul to 10ml 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
A	selection 1	selection 1	selection 9	selection 9	selection 17	selection 17	selection 25	selection 25	**TE**	1.00E+00	1.00E+00
B	selection 2	selection 2	selection 10	selection 10	selection 18	selection 18	selection 26	selection 26	**TE**	5.00E-01	5.00E-01
C	selection 3	selection 3	selection 11	selection 11	selection 19	selection 19	26, no R primer	26, no R primer	**TE**	2.50E-01	2.50E-01
D	selection 4	selection 4	selection 12	selection 12	selection 20	selection 20	**TE**	**TE**	**TE**	1.25E-01	1.25E-01
E	selection 5	selection 5	selection 13	selection 13	selection 21	selection 21	**TE**	**TE**	**TE**	6.25E-02	6.25E-02
F	selection 6	selection 6	selection 14	selection 14	selection 22	selection 22	**TE**	**TE**	**TE**	3.13E-02	3.13E-02
G	selection 7	selection 7	selection 15	selection 15	selection 23	selection 23	**TE**	**TE**	**TE**	1.56E-02	1.56E-02
H	selection 8	selection 8	selection 16	selection 16	selection 24	selection 24	**TE**	**TE**	**TE**	7.81E-03	7.81E-03

	ng/ul	mix vol	SOURCE
Selection 1	11.18	**5.37**	**A1**
Selection 2	11.74	**5.11**	**B1**
Selection 3	11.66	**5.15**	**C1**
Selection 4	12.00	**5.00**	**D1**
Selection 5	11.43	**5.25**	**E1**
Selection 6	11.98	**5.01**	**F1**
Selection 7	11.95	**5.02**	**G1**
Selection 8	12.88	**4.66**	**H1**
Selection 9	5.01	**11.98**	**A3**
Selection 10	5.54	**10.83**	**B3**
Selection 11	5.67	**10.59**	**C3**
Selection 12	6.13	**9.79**	**D3**

	ng/ul	mix vol	SOURCE
Selection 13	6.96	**8.62**	**E3**
Selection 14	7.52	**7.98**	**F3**
Selection 15	7.33	**8.19**	**G3**
Selection 16	8.02	**7.48**	**H3**
Selection 17	5.75	**10.43**	**A5**
Selection 18	8.86	**6.77**	**B5**
Selection 19	9.68	**6.20**	**C5**
Selection 20	8.28	**7.25**	**D5**
Selection 21	4.43	**13.55**	**E5**
Selection 22	6.68	**8.99**	**F5**
Selection 23	7.41	**8.09**	**G5**
Selection 24	6.09	**9.85**	**H5**
Selection 25	6.58	**9.12**	**A7**
Selection 26	6.94	**8.64**	**B7**

You might want to use a NEW gel extraction kit, since we have some idea that the older kits might damage DNA more?

Mix 60ng of each, final volume ~200ul.
Add 100ul of the pool to 20ul of 6x sample buffer and run on 2% agarose.
Got the expected single band somewhere below 500bp.
Gel extracted using two columns for the 500mg slice, stored DNA overnight at -20 before ampure/picogreen.

12-25

Purify gel extraction DNA with 1.58x beads:

Add 139ul of beads to 88ul sample 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 low-stick tube. ("12.25 size-sel lib")

Picogreen 1:100 dilutions in triplicate.

Nanodrop is clean; 2.9ng/ul.
Picogreen is 2.68ng/ul.

I will make a dilution at 2 nM to give to the sequencing core. **For 472bp dsDNA, this is approximately 18.5 ng in 30ul.**

So I will add 6.9ul to 23.1ul EB to make 2nM in 30 ul to give the core.

Notes:

Email Andy Marty (<jamarty@fredhutch.org>) to tell him that you're going to submit sequencing samples soon. Email him ahead of time to ask him to purchase the reagent kit for paired-end 250bp reads on the hiseq; give him the appropriate hutch budget number: 218586

Submit order form on : http://hutchbase.fhcrc.org/base2/

Rapid run mode, 2x250

Now you're done!

2017-1-12: qRT-PCR

First make 10ng/ul dilutions in the following layout by adding the following volumes of RNA to 100ul of water:

	1	2	3	4	5	6	7	8	9	10	11	12
A	sel-1	sel-2	sel-3	sel-4	sel-5	sel-6	sel-7	sel-8	sel-9	sel-10	sel-11	sel-12
B												
C	sel-13	sel-14	sel-15	sel-16	sel-17	sel-18	sel-19	sel-20	sel-21	sel-22	sel-23	sel-24
D												
E	sel-25	sel-26										
F												
G	27. 5e5 (rep A)	28. 5e4 (rep A)	29. 5e3 (rep A)	30. 5e2 (rep A)	31. 5e1 (rep A)	32. No virus (rep A)	33. 5e5 (rep B)	34. 5e4 (rep B)	35. 5e3 (rep B)	36. 5e2 (rep B)	37. 5e1 (rep B)	38. No virus (rep B)
H												

	ng/ul	ul RNA to diluent
sel-1	337.5	3.053
sel-2	443.5	2.307
sel-3	220.6	4.748
sel-4	428.8	2.388
sel-5	435	2.353
sel-6	184.8	5.721
sel-7	495.6	2.059
sel-8	435.4	2.351
sel-9	288.6	3.589
sel-10	388.8	2.640
sel-11	394.6	2.600
sel-12	205.2	5.123
sel-13	489.5	2.086
sel-14	370.9	2.771
sel-15	412.4	2.485
sel-16	418.2	2.450
sel-17	267.6	3.882
sel-18	162.4	6.562
sel-19	473.7	2.157
sel-20	483.9	2.110
sel-21	270.3	3.842
sel-22	403.9	2.539
sel-23	212.9	4.929
sel-24	222.5	4.706
sel-25	384.3	2.672
sel-26	355.1	2.898
27. 5e5 (rep A)	276.7	3.750
28. 5e4 (rep A)	290.2	3.569
29. 5e3 (rep A)	363.1	2.832
30. 5e2 (rep A)	496.4	2.056
31. 5e1 (rep A)	410.2	2.499
32. No virus (rep A)	312.1	3.310
33. 5e5 (rep B)	413.3	2.480
34. 5e4 (rep B)	455.1	2.247
35. 5e3 (rep B)	421.6	2.430
36. 5e2 (rep B)	446.2	2.293
37. 5e1 (rep B)	346.6	2.971
38. No virus (rep B)	215.8	4.859

2017-1-12: 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 76, make 84 RT+ MM: **840 ul One-Step MM + 33.6 ul qScript RT + 302.4 ul water**
Need 12, make 16x RT- MM: **160 ul One-Step MM + 64 ul H2O**
Split RT+ MM into **two 41x MM** tubes (each tube gets 41*14=**574 ul**) and add NP primers or GAPDH primers to respective tubes (each tube gets **41 ul of each** F and R 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 7x MM tubes (each tube gets **98 ul**) and add NP primers or GAPDH primers to respective tubes (each tube gets **7 ul of each** F and R primer).
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 using a multichannel and the dilution plate already made.
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	sel-1	sel-2	sel-3	sel-4	sel-5	sel-6	sel-7	sel-8	sel-9	sel-10	sel-11	sel-12
B - GAPDH	sel-1	sel-2	sel-3	sel-4	sel-5	sel-6	sel-7	sel-8	sel-9	sel-10	sel-11	sel-12
C - NP	sel-13	sel-14	sel-15	sel-16	sel-17	sel-18	sel-19	sel-20	sel-21	sel-22	sel-23	sel-24
D - GAPDH	sel-13	sel-14	sel-15	sel-16	sel-17	sel-18	sel-19	sel-20	sel-21	sel-22	sel-23	sel-24
E - NP	sel-25	sel-26					33. 5e5 (rep B) NORT	34. 5e4 (rep B) NORT	35. 5e3 (rep B) NORT	36. 5e2 (rep B) NORT	37. 5e1 (rep B) NORT	38. No virus (rep B) noRT
F - GAPDH	sel-25	sel-26					33. 5e5 (rep B) NORT	34. 5e4 (rep B) NORT	35. 5e3 (rep B) NORT	36. 5e2 (rep B) NORT	37. 5e1 (rep B) NORT	38. No virus (rep B) noRT
G - NP	27. 5e5 (rep A)	28. 5e4 (rep A)	29. 5e3 (rep A)	30. 5e2 (rep A)	31. 5e1 (rep A)	32. No virus (rep A)	33. 5e5 (rep B)	34. 5e4 (rep B)	35. 5e3 (rep B)	36. 5e2 (rep B)	37. 5e1 (rep B)	38. No virus (rep B)
H - GAPDH	27. 5e5 (rep A)	28. 5e4 (rep A)	29. 5e3 (rep A)	30. 5e2 (rep A)	31. 5e1 (rep A)	32. No virus (rep A)	33. 5e5 (rep B)	34. 5e4 (rep B)	35. 5e3 (rep B)	36. 5e2 (rep B)	37. 5e1 (rep B)	38. No virus (rep B)