Barcoded subamplicon sequencing library prep

June 28 2016

I already prepared full-length amplicons for libraries 2 and 3 selected with H17-L19 and FI6v3
(2016-06-05 notebook file) and all three libraries selected with H17-L10, H17-L7, and H18-S415 (2016-06-14 notebook file).

Here I will begin with purified and picogreen-quantified amplicons as template for Round 1 PCR.

Layout and concentrations for amplicons of Lib2/Lib3 neutralized with H17-L19 and FI6v3 (from 2016-06-05 L2 and L3 selection with H17L19, FI6v3 notebook).
I will prepare sequencing libraries from highlighted samples.

Plate: "2016.06.12 purified full-length amplicons"

	1	2	3	4	5	6	7	8	9
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									

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

Layout and concentrations for amplicons of Lib1/Lib2/Lib3 neutralized with H17-L10, H17-L7, and H18-S415 (from “2016-06-14 H17-L10, H17-L7, and H18-S415 Library Selections” notebook).

I will prepare sequencing libraries from highlighted samples.

selecting mAb	mab conc / Lib	qPCR % infectivity
L10	c3 - Lib1	0.07%
L10	c3 - Lib2	0.06%
L10	c3 - Lib3	0.03%
L10	c2 - Lib1	0.19%
L10	c2 - Lib2	0.17%
L10	c2 - Lib3	0.15%
L7	c3 - Lib1	0.15%
L7	c3 - Lib2	0.12%
L7	c3 - Lib3	0.12%
L7	c2 - Lib1	0.99%
L7	c2 - Lib2	0.75%
L7	c2 - Lib3	1.27%
S415	c3 - Lib1	0.04%
S415	c3 - Lib2	0.02%
S415	c3 - Lib3	0.04%
S415	c2 - Lib1	0.15%
S415	c2 - Lib2	0.09%
S415	c2 - Lib3	0.17%

2016.06.27 purified full-length amplicons L10/L7/S415 (layout for PCR on 6.17 and ampure on 6.27)

	1	2	3	4	5	6	7	8	9	10	11	12
A	L10c3 - Lib1	L10c3 - Lib2	L10c3 - Lib3	L10c2 - Lib1	L10c2 - Lib2	L10c2 - Lib3	L7c3 - Lib1	L7c3 - Lib2	L7c3 - Lib3	L7c2 - Lib1	L7c2 - Lib2	L7c2 - Lib3
B												
C	S415c3 - Lib1	S415c3 - Lib2	S415c3 - Lib3	S415c2 - Lib1	S415c2 - Lib2	S415c2 - Lib3	standard L1 -NV					
D												
E	no template	1e4 (5e3/ul)	1e5 (5e4/ul)	1e6 (5e5/ul)	1e7 (5e6/ul)							

well	sample	ng/ul of undiluted sample
A1	L10c3 - Lib1	1.547
A2	L10c3 - Lib2	3.439
A3	L10c3 - Lib3	0.088
A4	L10c2 - Lib1	2.897
A5	L10c2 - Lib2	8.322
A6	L10c2 - Lib3	1.783
A7	L7c3 - Lib1	2.914
A8	L7c3 - Lib2	5.103
A9	L7c3 - Lib3	1.114
A10	L7c2 - Lib1	7.983
A11	L7c2 - Lib2	10.096
A12	L7c2 - Lib3	10.126
C1	S415c3 - Lib1	0.149
C2	S415c3 - Lib2	1.572
C3	S415c3 - Lib3	0.231
C4	S415c2 - Lib1	2.669
C5	S415c2 - Lib2	1.958
C6	S415c2 - Lib3	1.366
C7	standardL1-NV	0.004
E1	no template	0.002
E2	1e4 (5e3/ul)	0.027
E3	1e5 (5e4/ul)	0.297
E4	1e6 (5e5/ul)	1.567
E5	1e7 (5e6/ul)	21.718

Summary of library selection experiments and sequencing

	library 1			
	replicate A	replicate B	**library 2**	**library 3**
H17-L19 0.5 ug/ml	2.1%	2.5%	4.9%	5.4%
H17-L19 1 ug/ml	0.7%	0.7%	1.9%	1.8%
H17-L19 10 ug/ml	0.3%	0.2%	0.4%	0.4%
H17-L10 3 ug/ml	0.2%		0.2%	0.2%
H17-L10 15 ug/ml	0.1%		0.1%	<0.1%
H17-L7 3 ug/ml	1.0%		0.8%	1.3%
H17-L7 15 ug/ml	0.2%		0.1%	0.1%
H18-S415 3 ug/ml	0.2%		0.1%	0.2%
H18-S415 15 ug/ml	<0.1%		<0.1%	<0.1%
FI6v3 0.1 ug/ml	1.7%	1.4%	2.3%	0.9%
FI6v3 0.2 ug/ml	0.5%	0.3%	0.3%	0.1%
FI6v3 2 ug/ml	<0.01%	<0.01%	<0.01%	<0.01%

Preliminary sequencing run, May 2016

Subsequent sequencing run, July 2016 (hopefully…)

2016.06.28: Round 1 PCR. Before setting up round 1 I will make a plate of 0.5ng/ul dilutions of the full-length HA amplicons. Here I will bring together selected library samples prepared from two different sets of experiments completed on 2016.06.12 and 2016.06.27. From now on I will use a nomenclature referring to these selected samples as experiments 1-24. Experiment 24 is a no-virus control taken through RT/PCR and used as negative control template in round 1 PCR and will not be sequenced. I am placing the samples into columns of 8 in the destination dilution plate to facilitate sample layout for the round 1 PCR plate layouts (next slide).

Merging samples from 2016.06.12 and 2016.06.27 plates into 0.5ng/ul dilution plate (2016.06.28) for round 1 PCR template:

Experiment	amplicon	ng/ul	origin plate	origin well	ul to 60 ul EB makes 0.5ng/ul	destination well on 0.5ng/ul dilution plate 2016.06.28
1	L2_H17L19_c1	36.3	2016.06.12	A1	0.838	A1
2	L2_H17L19_c2	49.4	2016.06.12	A2	0.613	B1
3	L2_H17L19_c3	19.1	2016.06.12	A3	1.613	C1
4	L2_FI6v3_c1	49.8	2016.06.12	A4	0.609	D1
5	L2_FI6v3_c2	22.9	2016.06.12	A5	1.339	E1
6	L2_FI6v3_c3	0.4	2016.06.12	A6	all sample no EB	F1
7	L3_H17L19_c1	42.8	2016.06.12	A7	0.709	G1
8	L3_H17L19_c2	21.2	2016.06.12	A8	1.449	H1
9	L3_H17L19_c3	10.7	2016.06.12	C1	2.941	A3
10	L3_FI6v3_c1	12.3	2016.06.12	C2	2.542	B3
11	L3_FI6v3_c2	3.1	2016.06.12	C3	11.538	C3
12	L3_FI6v3_c3	0.3	2016.06.12	C4	all sample no EB	D3
13	L2_mock pooled	70.4	2016.06.12	C5	0.429	E3
14	L3_mock pooled	67.6	2016.06.12	C6	0.447	F3
15	L10c2 - Lib1	2.897	2016.06.27	A4	12.516	G3
16	L10c2 - Lib2	8.322	2016.06.27	A5	3.835	H3
17	L10c2 - Lib3	1.783	2016.06.27	A6	23.383	A5
18	L7c3 - Lib1	2.914	2016.06.27	A7	12.428	B5
19	L7c3 - Lib2	5.103	2016.06.27	A8	6.517	C5
20	L7c3 - Lib3	1.114	2016.06.27	A9	48.860	D5
21	S415c2 - Lib1	2.669	2016.06.27	C4	13.831	E5
22	S415c2 - Lib2	1.958	2016.06.27	C5	20.576	F5
23	S415c2 - Lib3	1.366	2016.06.27	C6	34.642	G5
24	standardL1-NV	0	2016.06.27	C7	all sample no EB	H5

2016.06.28: Round 1 PCR plate layouts:

Plate 1:

	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						experiment 9					
B	experiment 2						experiment 10					
C	experiment 3						experiment 11					
D	experiment 4						experiment 12					
E	experiment 5						experiment 13					
F	experiment 6						experiment 14					
G	experiment 7						experiment 15					
H	experiment 8						experiment 16					

Plate 2:

	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 17											
B	experiment 18											
C	experiment 19											
D	experiment 20											
E	experiment 21											
F	experiment 22											
G	experiment 23											
H	experiment 24 (no primers left at this point!)											

2016.06.28 Round 1 PCR set up. Making two plates as shown in slide above.

Round 1 primers are 10uM stocks. For each primer pair (ampX_F/ampX_R), make a mix for 26 reaction's worth (actually only needing 24 reactions for each primer pair). To do this, for each of the 6 subamplicons make a primer mix by adding: **26ul F primer (10uM) + 26ul R primer (10uM) + 52ul water.** Final volume 104ul, each primer is 2.5uM in this final mixture. Each PCR gets 4ul of this combined F/R subamplicon primer pair.

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 in slides above.

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 24 rows of 6) to all rows as indicated. Ran out of primers for plate 2 row H, even though I made a 26x mix for 24. In the future make at least 27x for 24, etc.
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 round1plate1; column 3 of dilution plate is used for columns 7-12 of round1plate1, and column 5 of the dilution plate is used for columns 1-6 of round1plate2. Ran out of template for subamplicon 6 of experiment 6 and experiment 12 (used less than the desired 4ng). In the future, the full-length amplicons should be eluted in > 48ul to be able to use them undiluted in round 1 if necessary. Should have diluted these even though they were less than 0.5ng/ul, to have enough to evenly distribute across all amplicons.

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, 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

After running the PCR, freeze at -20 overnight. Then add 26 ul of EB to each well to bring the volume to 50 ul. Then purify with 1x Ampure beads: (one plate at a time)

1. Take bead aliquot (~7.5ml) 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 2 new racks ("**2016.06.29 purif. R1 subamps Plate 1/2**") with the same layout as the PCR plates above.

2016.06.29 picogreen of purified round 1 products. This will require three picogreen assay plates, here are the layouts:

Plate 1: exp 1-8 (round1 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	experiment 1												TE	1.00E+00	1.00E+00
B	experiment 2												TE	5.00E-01	5.00E-01
C	experiment 3												TE	2.50E-01	2.50E-01
D	experiment 4												TE	1.25E-01	1.25E-01
E	experiment 5												TE	6.25E-02	6.25E-02
F	experiment 6												TE	3.13E-02	3.13E-02
G	experiment 7												TE	1.56E-02	1.56E-02
H	experiment 8												TE	7.81E-03	7.81E-03

Plate 2: exp 9-16 (round1 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	experiment 9					
B				5.00E-01	5.00E-01	TE	experiment 10					
C				2.50E-01	2.50E-01	TE	experiment 11					
D				1.25E-01	1.25E-01	TE	experiment 12					
E				6.25E-02	6.25E-02	TE	experiment 13					
F				3.13E-02	3.13E-02	TE	experiment 14					
G				1.56E-02	1.56E-02	TE	experiment 15					
H				7.81E-03	7.81E-03	TE	experiment 16					

Plate 3: exp
17-24

	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	experiment 17						TE	1.00E+00	1.00E+00
B	experiment 18						TE	5.00E-01	5.00E-01
C	experiment 19						TE	2.50E-01	2.50E-01
D	experiment 20						TE	1.25E-01	1.25E-01
E	experiment 21						TE	6.25E-02	6.25E-02
F	experiment 22						TE	3.13E-02	3.13E-02
G	experiment 23						TE	1.56E-02	1.56E-02
H	experiment 24						TE	7.81E-03	7.81E-03

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

1. Make 2 standards at **2ng/ul by adding 4ul of the standard to 196ul TE**.
2. Add 99 ul 1xTE to the wells for sample dilutions.
3. Add 1 ul of ampure-purified products to the 99 ul 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 standard curve wells, 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 (40ul to 8ml TE), add 100 ul to all wells used in assay, cover to protect from light and incubate 5 minutes before reading on plate reader.

Results for picogreen quantification of round 1 subamplicon products:

	subamp 1	subamp 2	subamp 3	subamp 4	subamp 5	subamp 6
experiment 1	3.72	2.41	3.78	2.79	3.18	2.60
experiment 2	2.67	1.67	2.76	2.32	2.98	2.11
experiment 3	2.39	1.57	2.59	2.14	2.66	2.01
experiment 4	3.18	1.93	2.54	2.33	3.07	2.08
experiment 5	3.22	2.22	2.97	2.44	2.84	2.00
experiment 6	2.61	1.93	2.42	2.24	2.77	0.11
experiment 7	4.01	2.30	3.35	2.99	3.39	2.28
experiment 8	4.05	2.66	3.85	3.12	4.30	2.60
	subamp 1	**subamp 2**	**subamp 3**	**subamp 4**	**subamp 5**	**subamp 6**
experiment 9	2.74	1.84	2.62	2.74	3.56	2.60
experiment 10	2.55	1.66	2.30	2.37	3.14	2.13
experiment 11	2.46	1.48	2.84	2.16	2.70	1.84
experiment 12	1.29	0.90	1.37	1.09	1.48	0.65
experiment 13	2.84	2.00	2.73	2.70	3.79	2.73
experiment 14	2.51	1.66	2.45	2.20	3.13	1.98
experiment 15	3.09	2.12	3.22	2.96	3.75	2.64
experiment 16	3.88	2.74	3.43	3.38	5.19	2.85
	subamp 1	**subamp 2**	**subamp 3**	**subamp 4**	**subamp 5**	**subamp 6**
experiment 17	2.61	1.37	2.85	1.89	2.93	2.10
experiment 18	2.54	1.01	2.89	1.88	3.37	2.18
experiment 19	2.65	0.98	3.10	2.02	3.36	2.38
experiment 20	2.43	1.10	2.98	2.04	2.93	1.99
experiment 21	2.70	1.54	3.47	2.14	3.29	2.31
experiment 22	2.50	1.51	3.04	2.01	3.16	2.01
experiment 23	3.04	1.74	3.81	2.26	3.29	1.96
experiment 24	0.08	0.08	0.08	0.08	0.08	0.08

Using these concentrations, make a new set of plates with normalized concentrations of round 1 products.

NORMALIZATION PLATES: 0.4 ng/ul

Add this much EB to new plates labeled "**2016.06.30: 0.4 ng/ul round 1 products**" and then multichannel 15 ul of round 1 products to these dilution wells to make 0.4ng/ul. Wells highlighted in red will be less than 0.4ng/ul.

PLATE 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	124.64	75.46	126.58	89.68	104.39	82.61	87.85	54.16	83.27	87.68	118.33	82.52
B	85.02	47.76	88.60	72.15	96.61	64.21	80.78	47.11	71.40	74.05	102.72	64.79
C	74.47	43.76	82.11	65.28	84.60	60.33	77.29	40.66	91.69	66.03	86.35	54.08
D	104.14	57.29	80.36	72.48	99.96	62.97	33.50	18.67	36.50	25.82	40.41	9.51
E	105.65	68.42	96.20	76.54	91.60	60.09	91.44	60.00	87.35	86.27	127.17	87.27
F	82.77	57.29	75.71	69.17	88.85	-10.92	79.03	47.19	76.87	67.60	102.30	59.18
G	135.27	71.15	110.60	97.20	112.11	70.33	100.88	64.54	105.65	95.86	125.57	83.85
H	136.71	84.85	129.53	101.97	146.43	82.36	130.37	87.85	113.62	111.61	179.65	91.85

PLATE 2:

	1	2	3	4	5	6
A	82.77	36.50	91.85	55.89	95.03	63.71
B	80.28	22.92	93.52	55.48	111.35	66.69
C	84.35	21.71	101.30	60.91	110.85	74.22
D	76.04	26.14	96.70	61.57	94.86	59.67
E	86.27	42.61	115.05	65.20	108.25	71.57
F	78.62	41.47	99.12	60.25	103.47	60.41
G	99.12	50.30	128.01	69.83	108.33	58.44
H	-11.88	-11.95	-12.03	-11.95	-12.03	-12.03

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 of the first plate, and column 1 of the second plate), 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.

Pool the concentration-normalized (**15 ul of the 0.4ng/ul of each**) six subamplicons for each experimental sample into a single 0.4ng/ul pool for each experimental sample 1-24 as shown below, and 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	experiment 1	2.67E-02	1.78E-03	1.19E-04	2.37E-05		experiment 9	2.67E-02	1.78E-03	1.19E-04	2.37E-05	
B	experiment 2						experiment 10					
C	experiment 3						experiment 11					
D	experiment 4						experiment 12					
E	experiment 5						experiment 13					
F	experiment 6						experiment 14					
G	experiment 7						experiment 15					
H	experiment 8						experiment 16					

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
A	experiment 17	2.67E-02	1.78E-03	1.19E-04	2.37E-05	
B	experiment 18					
C	experiment 19					
D	experiment 20					
E	experiment 21					
F	experiment 22					
G	experiment 23					
H	experiment 24					

2016-06-30 Calculations for round 2 barcode complexity bottlenecking: I will bottleneck barcode complexity to 150,000 as before for the antibody-selected samples. In contrast with the last experiment in which I bottlenecked mock-selected samples to 700,000, this time I will bottleneck them to 500,000, since I know I don't need extensive depth for the mock-selected samples to get good signal for differentially-selected mutants. *The next slide shows how I intend to partition the lane into these 23 samples in order to achieve a target of 4 reads per barcode to build consensus sequences for each barcode.*

bottleneck calculation **for the mock samples (exp 13 and 14)**:
I want *5E5 barcodes for each subamplicon in each sample*, which means 2.5E5 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.

2.5E5 dsDNA molecules per subamplicon per sample, * 6 subamplicons in each sample = 1.5E6 dsDNA molecules per sample in each round 2 PCR, so that within each sample's round 2 PCR there will be 5E5 unique barcodes for each of the 6 subamplicons.

So each of my round 2 PCRs gets 1.5E6 dsDNA molecules of pooled sample as template, which corresponds to **0.000660** ng of dsDNA 407bp long.

6.60e-4 ng of the pooled subamplicons for each sample results in 2.5E5 dsDNA molecules for each subamplicon in the sample, for 5E5 total barcodes for each subamplicon.

bottleneck calculation **for the selected samples (all expt besides 13 and 14)**:
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.

selected bottleneck bc/subamp:	150000
mock bottleneck bc/subamp:	500000
% lane for each mock:	12
Expect this many PE reads:	1.00E+08

Round2Rev Index	Expt #	sample	bottleneck bc/subamp	target % clusters	target # PEreads	target PEreads/subamp	target PEreads/subampbarcode	
NF01 Rnd2Rev	1	L2_H17L19_c1	150000	3.6	3.62E+06	6.03E+05	4.0	
NF03 Rnd2Rev	2	L2_H17L19_c2	150000	3.6	3.62E+06	6.03E+05	4.0	
NF08 Rnd2Rev	3	L2_H17L19_c3	150000	3.6	3.62E+06	6.03E+05	4.0	
NF09 Rnd2Rev	4	L2_FI6v3_c1	150000	3.6	3.62E+06	6.03E+05	4.0	
NF10 Rnd2Rev	5	L2_FI6v3_c2	150000	3.6	3.62E+06	6.03E+05	4.0	
NF11 Rnd2Rev	6	L2_FI6v3_c3	150000	3.6	3.62E+06	6.03E+05	4.0	
TS22Rnd2rev	7	L3_H17L19_c1	150000	3.6	3.62E+06	6.03E+05	4.0	
TS25Rnd2rev	8	L3_H17L19_c2	150000	3.6	3.62E+06	6.03E+05	4.0	
TS06Rnd2rev	9	L3_H17L19_c3	150000	3.6	3.62E+06	6.03E+05	4.0	
TS12Rnd2rev	10	L3_FI6v3_c1	150000	3.6	3.62E+06	6.03E+05	4.0	
TS19Rnd2rev	11	L3_FI6v3_c2	150000	3.6	3.62E+06	6.03E+05	4.0	
TS13Rnd2rev	12	L3_FI6v3_c3	150000	3.6	3.62E+06	6.03E+05	4.0	
TS14Rnd2rev	13	L2_mock pooled	500000	12.0	1.20E+07	2.00E+06	4.0	
TS15Rnd2rev	14	L3_mock pooled	500000	12.0	1.20E+07	2.00E+06	4.0	
TS18Rnd2rev	15	L10c2 - Lib1	150000	3.6	3.62E+06	6.03E+05	4.0	
TS20Rnd2rev	16	L10c2 - Lib2	150000	3.6	3.62E+06	6.03E+05	4.0	
TS21Rnd2rev	17	L10c2 - Lib3	150000	3.6	3.62E+06	6.03E+05	4.0	
TS23Rnd2rev	18	L7c3 - Lib1	150000	3.6	3.62E+06	6.03E+05	4.0	
TS27Rnd2rev	19	L7c3 - Lib2	150000	3.6	3.62E+06	6.03E+05	4.0	
NF29Rnd2rev	20	L7c3 - Lib3	150000	3.6	3.62E+06	6.03E+05	4.0	
NF30Rnd2rev	21	S415c2 - Lib1	150000	3.6	3.62E+06	6.03E+05	4.0	
NF31Rnd2rev	22	S415c2 - Lib2	150000	3.6	3.62E+06	6.03E+05	4.0	
NF32Rnd2rev	23	S415c2 - Lib3	150000	3.6	3.62E+06	6.03E+05	4.0	

Round 2 PCR set-up: **This will use the pooled and diluted round 1 products as template for round 2 PCR**.

For samples Exp1-Exp12 and 15-23, I want 1.98e-4 ng of the pooled subamplicons for each experimental sample.
Therefore, for Exp1-12 and 15-23, round 2 PCR will use: **8.354** ul of dilution column 5 or 11 (2.37E-5 ng/ul column).

For mock samples Exp13 and Exp14, I want 6.6e-4 ng of the pooled subamplicons for each sample.
Therefore, For Exp13-14, round 2 PCR will use: **5.546** ul of dilution from COLUMN 10 (1.19E-4 ng/ul column).

I will not use expt 24 (no virus control) anymore, since it clearly did not amplify in round 1. Instead, for round 2, I will perform a negative control by doing PCR on experiment 23 without a round 2 reverse primer (although it still has the universal round 2 Forward primer). Here is the plate layout for the round 2 PCR:

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

Each Round 2 PCR:
20 ul of 2X KOD Master Mix + **4 ul** of 5 uM Rnd2ForUniversal (*make 26x MM: 520ul KOD + 104 ul R2UniF 5uM*)
4 ul of 5 uM of the appropriate Rnd2RevIndex as indicated on following slide.
5.546 ul - 8.354 ul of template as indicated on following slide.
3.65 ul - 6.454 ul water as indicated on following slide, 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

Round 2 PCR set-up checklist

"Md" round2rev primers are 5uM stocks I ordered from IDT. the rest are 5uM dilutions I made from Adam's 100uM stocks.

Sample	vol template	source template (pooled/diluted round 1 products)	r2plate destination	vol H2O	reverse primer
Exp1	8.354	plate1-A5	A2	3.65	md NF01 Rnd2Rev
Exp2	8.354	plate1-B5	B2	3.65	md NF03 Rnd2Rev
Exp3	8.354	plate1-C5	C2	3.65	md NF08 Rnd2Rev
Exp4	8.354	plate1-D5	D2	3.65	md NF09 Rnd2Rev
Exp5	8.354	plate1-E5	E2	3.65	md NF10 Rnd2Rev
Exp6	8.354	plate1-F5	F2	3.65	md NF11 Rnd2Rev
Exp7	8.354	plate1-G5	G2	3.65	md TS22Rnd2rev
Exp8	8.354	plate1-H5	H2	3.65	md TS25Rnd2rev
Exp9	8.354	plate1-A11	A4	3.65	TS06Rnd2rev
Exp10	8.354	plate1-B11	B4	3.65	TS12Rnd2rev
Exp11	8.354	plate1-C11	C4	3.65	TS19Rnd2rev
Exp12	8.354	plate1-D11	D4	3.65	TS13Rnd2rev
Exp13	**5.546**	**plate1-E10**	E4	6.454	TS14Rnd2rev
Exp14	**5.546**	**plate1-F10**	F4	6.454	TS15Rnd2rev
Exp15	8.354	plate1-G11	G4	3.65	TS18Rnd2rev
Exp16	8.354	plate1-H11	H4	3.65	TS20Rnd2rev
Exp17	8.354	plate2-A5	A6	3.65	TS21Rnd2rev
Exp18	8.354	plate2-B5	B6	3.65	TS23Rnd2rev
Exp19	8.354	plate2-C5	C6	3.65	TS27Rnd2rev
Exp20	8.354	plate2-D5	D6	3.65	NF29Rnd2rev
Exp21	8.354	plate2-E5	E6	3.65	NF30Rnd2rev
Exp22	8.354	plate2-F5	F6	3.65	NF31Rnd2rev
Exp23	8.354	plate2-G5	G6	3.65	NF32Rnd2rev
Exp23noprimer	8.354	plate2-G5	H6	3.65	water

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.

Then picogreen in triplicate:

1. Make 2 standards at **2ng/ul by adding 4ul of the standard to 196ul TE**.
2. Add 99 ul 1xTE to the wells for sample dilutions.
3. Add 1 ul of ampure-purified products to the 99 ul 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 standard curve wells, 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	12
A	1.00E+00	1.00E+00	TE	experiment 1	experiment 1	experiment 1	experiment 9	experiment 9	experiment 9	experiment 17	experiment 17	experiment 17
B	5.00E-01	5.00E-01	TE	experiment 2	experiment 2	experiment 2	experiment 10	experiment 10	experiment 10	experiment 18	experiment 18	experiment 18
C	2.50E-01	2.50E-01	TE	experiment 3	experiment 3	experiment 3	experiment 11	experiment 11	experiment 11	experiment 19	experiment 19	experiment 19
D	1.25E-01	1.25E-01	TE	experiment 4	experiment 4	experiment 4	experiment 12	experiment 12	experiment 12	experiment 20	experiment 20	experiment 20
E	6.25E-02	6.25E-02	TE	experiment 5	experiment 5	experiment 5	experiment 13	experiment 13	experiment 13	experiment 21	experiment 21	experiment 21
F	3.13E-02	3.13E-02	TE	experiment 6	experiment 6	experiment 6	experiment 14	experiment 14	experiment 14	experiment 22	experiment 22	experiment 22
G	1.56E-02	1.56E-02	TE	experiment 7	experiment 7	experiment 7	experiment 15	experiment 15	experiment 15	experiment 23	experiment 23	experiment 23
H	7.81E-03	7.81E-03	TE	experiment 8	experiment 8	experiment 8	experiment 16	experiment 16	experiment 16	exp 23 no primers	exp 23 no primers	exp 23 no primers

Here are the concentrations of the purified round 2 products.

Based on these concentrations, I will mix experimental samples proportionally to the desired % of reads. Here I chose to mix 2.5ug total, in approx 163 ul.

I will run some portion of this on a 2% agarose gel, excise the band (~470bp), purify with qiagen gel extraction kit, and quantify once more with picogreen in order to make a dilution for the sequencing core of approx 4 nM.

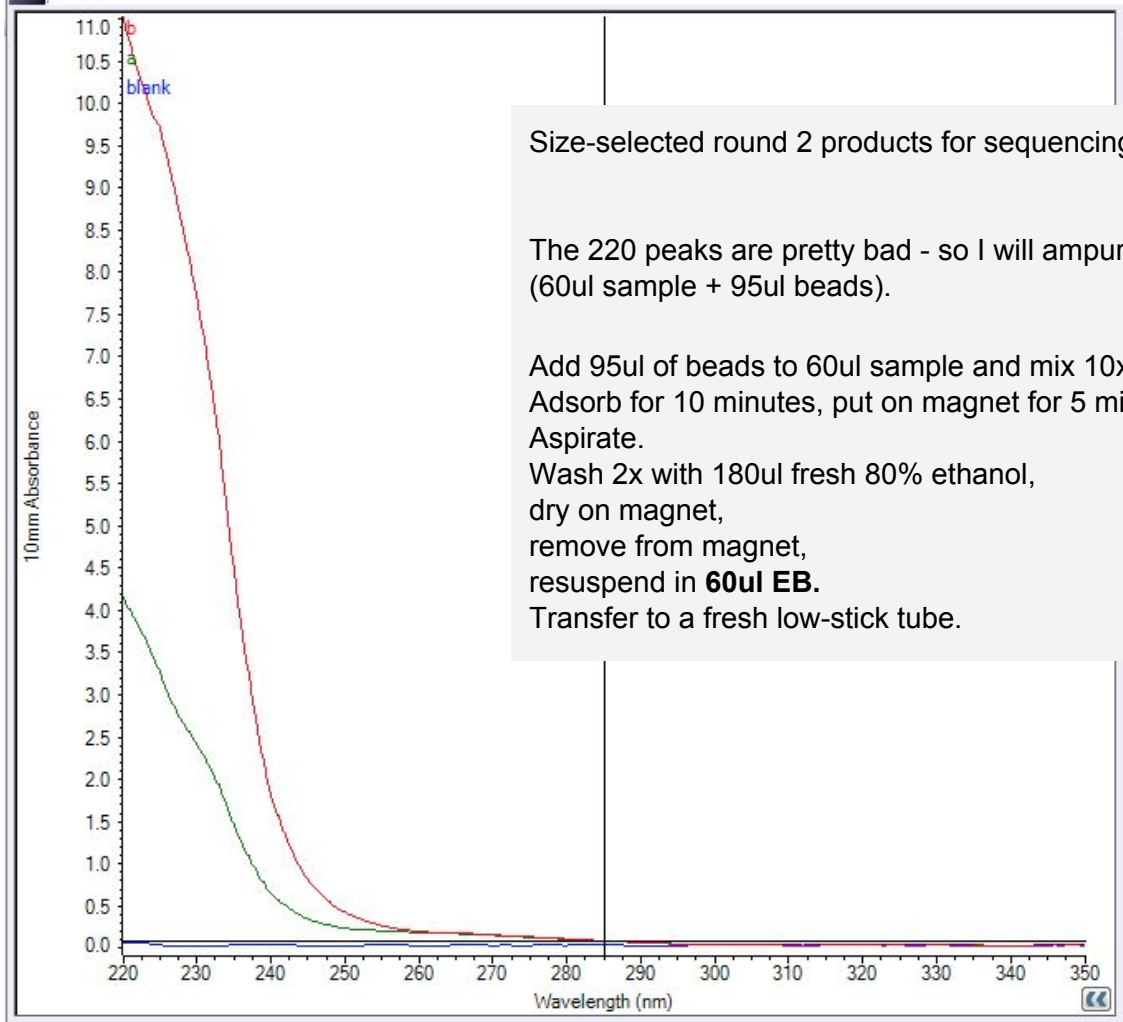
I poured a 2% agarose gel with the small gel tray and used a comb with three slots taped together. I used 83.4 ul of the pool (~1.25 ug) plus 16.6 ul 6x sample buffer and loaded this 100ul to the triple-taped comb. There was probably room for more volume if I wanted to load even more DNA, but I don't think that's necessary.

Eluted from Qiagen gel extraction kit (including the extra QG wash) from two parallel columns in 40ul + 40ul. But only got a total of about 60 ul of elutate when both tubes were combined after nanodrop spec.

The spec is on the next slide - it's pretty bad - so I will ampure purify (60ul sample + 95ul beads).

	ng/ul R2 product	target % clusters	mix volume	check mix ng	check mix %
experiment 1	18.27	3.6	4.953	90	3.6
experiment 2	14.90	3.6	6.072	90	3.6
experiment 3	7.47	3.6	12.120	90	3.6
experiment 4	8.15	3.6	11.102	90	3.6
experiment 5	15.34	3.6	5.898	90	3.6
experiment 6	19.75	3.6	4.582	90	3.6
experiment 7	14.40	3.6	6.281	90	3.6
experiment 8	9.58	3.6	9.447	90	3.6
experiment 9	14.30	3.6	6.326	90	3.6
experiment 10	12.05	3.6	7.506	90	3.6
experiment 11	13.17	3.6	6.872	90	3.6
experiment 12	10.91	3.6	8.295	90	3.6
experiment 13	28.59	12.0	10.495	300	12.0
experiment 14	31.05	12.0	9.660	300	12.0
experiment 15	12.83	3.6	7.050	90	3.6
experiment 16	6.49	3.6	13.946	90	3.6
experiment 17	17.51	3.6	5.166	90	3.6
experiment 18	25.22	3.6	3.588	90	3.6
experiment 19	24.21	3.6	3.736	90	3.6
experiment 20	21.99	3.6	4.114	90	3.6
experiment 21	11.28	3.6	8.019	90	3.6
experiment 22	21.87	3.6	4.137	90	3.6
experiment 23	22.64	3.6	3.996	90	3.6
exp 23 no primers	n.d.				
			163.359	2500	

Load your sample and press the measure button.

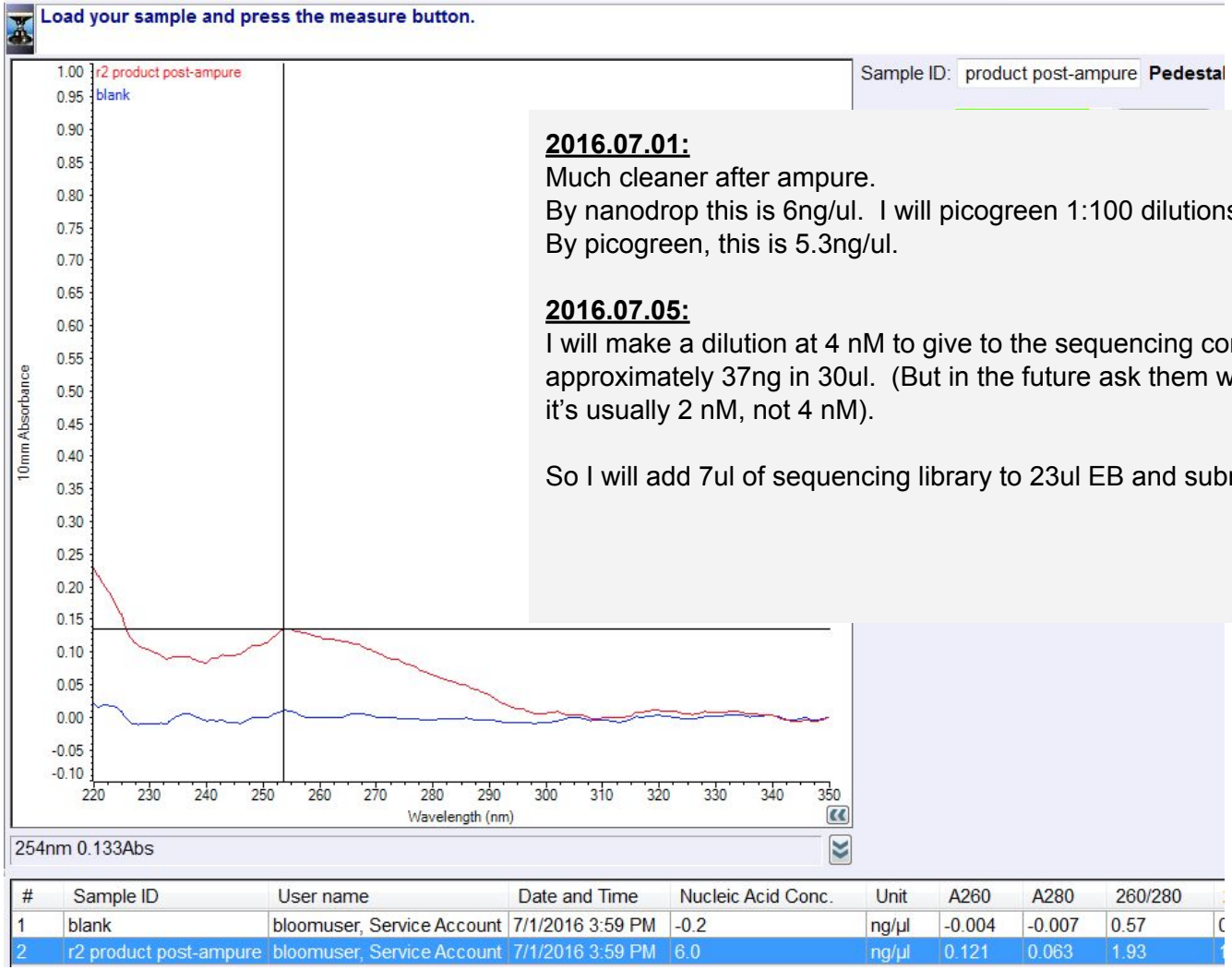


Size-selected round 2 products for sequencing from 2% gel.

The 220 peaks are pretty bad - so I will ampure purify with 1.58x beads (60ul sample + 95ul beads).

Add 95ul of beads to 60ul 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.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230
1	blank	bloomuser, Service Account	7/1/2016 2:28 PM	0.3	ng/µl	0.007	0.007	0.92	-0.84
2	a	bloomuser, Service Account	7/1/2016 2:29 PM	7.9	ng/µl	0.158	0.079	1.98	0.07
3	b	bloomuser, Service Account	7/1/2016 2:29 PM	8.7	ng/µl	0.175	0.084	2.07	0.02



#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280
1	blank	bloomuser, Service Account	7/1/2016 3:59 PM	-0.2	ng/µl	-0.004	-0.007	0.57
2	r2 product post-ampure	bloomuser, Service Account	7/1/2016 3:59 PM	6.0	ng/µl	0.121	0.063	1.93

2016.07.01:
Much cleaner after ampure.
By nanodrop this is 6ng/ul. I will picogreen 1:100 dilutions of this sample now.
By picogreen, this is 5.3ng/ul.

2016.07.05:
I will make a dilution at 4 nM to give to the sequencing core. For 472bp dsDNA, this is approximately 37ng in 30ul. (But in the future ask them what concentration they want it at - it's usually 2 nM, not 4 nM).

So I will add 7ul of sequencing library to 23ul EB and submit to the core facility.