Perth/09 HA passage 1 libraries barcoded subamplicon prep ATTEMPT #2

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January 30, 2017 – March 6, 2017

Barcoded subamplicon primers

- ***Subamplicon primers should start and end with codons***
- Lower case is sequence that anneals to Perth/2009 HA
- I tried to aim for ~285 bp of sequence between the forward and reverse primers of each subamplicon, and an annealing temp ~58-60°C for each primer
- P09_HA_for and P09_HA_Rev will be used to create the full-length amplicon
- Note that only primers "Amplicon1_F_P09" and "Amplicon6_R_P09" remain the same as before. The rest are new primers that I re-ordered through IDT so that subamplicons start and end with codons

Amplicon1_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNagcaaaagcaggggataattctattaatc
Amplicon1_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNgttcaacaaaaaggtcccatttcttattttg
Amplicon2_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNggagaccctcagtgtgatgacttc
Amplicon2_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNccccccaaatgtacaatttgtcaaa
Amplicon3_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNcgtgactatgccaaacaatgaacaa
Amplicon3_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNgccaatgggtgcatctgatct
Amplicon4_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNacgaagtgggaaaagctcaataatg
Amplicon4_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNgagtgcttttgagatctgctgc
Amplicon5_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNcatcaaaattctgagggaagaggacaa
Amplicon5_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNccattgcccatatcctcagc
Amplicon6_F_P09	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNgaaaaaacaaagaagcaactgagggaaaat
Amplicon6_R_P09	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNagtagaaacaagggtgtttttaattactaatacac
P09_HA_For	agcaaaagcaggggataattctattaatc
P09_HA_Rev	agtagaaacaagggtgtttttaattactaatacac

Rnd1 adaptor sequence + 8 N barcode

Rnd1for	CTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNN
Rnd1rev	GGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNN

Ordered primers from IDT, 4 nmole Ultramer DNA Oligo, machine mixed (NOT hand-mixed)

January 10, 2017

Testing NEW barcoded subamplicon primers on WT Perth/09 HA plasmid template.

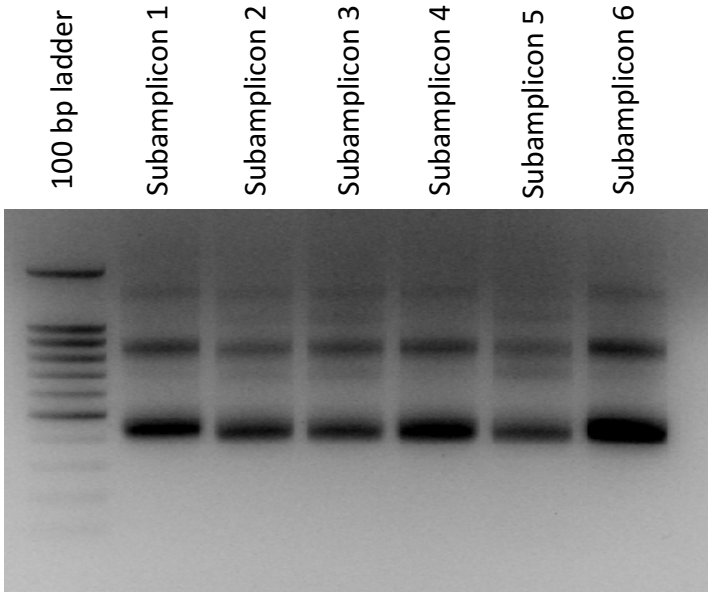
Resuspended 4 nmol round 1 barcoded subamplicon primers (ordered from IDT) in 400 ul water to make **10 uM stocks**.
Tested subamplicon primers to see that they work on 10 ng/ul stock of plasmid #1535 (pICR2-Perth09-HA).

Set up six KOD PCR reactions for each of the six subamplicon primer pairs:
10 ul water
0.75 ul 10 uM F primer
0.75 ul 10 uM R primer
1 ul 10 ng/ul template (#1535)
12.5 ul 2X KOD Hot-Start Master Mix

Ran "jl amplicon" on G-storm thermal cycler:

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 50°C for 30 s cooling to 50°C at 0.5 °C/s
5. 70°C for 40 s
6. Repeat steps 2-5 for 24 additional cycles (25 cycles total)
7. 4°C forever

After PCR, ran a gel of PCR products. Added 5 ul 6X loading dye to each sample, ran 1% agarose gel at 120 V for ~50 min.



Expecting a product size of ~400-500 bp, which I what I saw for each. Subamplicon 5 product band looked lighter than the other bands. There seem to be a lot of secondary bands, especially between 800 and 900 bp. Perhaps some sort of concatemer species? Keep in mind that the PCR conditions I've used here are very different from Round 1 PCR conditions, and I was amplifying off of the plasmid rather than a linear template. At any rate, the ~450 bp product seems to be the main product.

RNA extraction from passaged wild-type and mutant virus libraries – be extra careful not to cross-contaminate between the different WT and library samples!

January 30, 2017

Ultracentrifuged clarified supernatant of passaged wild-type and mutant virus libraries. Used the 25 ml frozen aliquots that I made of each sample (wt, mutvirus lib1, mutvirus lib2, mutvirus lib3 rep. 1, mutvirus lib3 rep. 2) in 50-ml conicals. Thawed the frozen aliquots at 4°C (pulled the frozen aliquots out of the freezer yesterday afternoon and let them sit in the TC room fridge overnight).
Spun down ~12 ml of each sample X 2 ultracentrifuge runs = ~24 ml total. Spun at 22,000 rpm (can use a higher speed since viruses will be lysed anyway – want to more completely spin down viruses) for 1.5 h at 4°C for each spin. Quickly and carefully decanted supernatant as soon as spin was done.

Tube 1: mutvirus lib1 (used 12.8 ml each for both spins)
Tube 2: mutvirus lib2
Tube 3: mutvirus lib3 rep. 1
Tube 4: mutvirus lib3 rep. 2
Tube 5: WT
Tube 6: PBS balance

After decanting off the supernatant after the second spin, proceeded immediately with RNA extraction with the viral pellet.

Notes for working with RNA: cleaned entire bench/pipettes with EtOH, then with RNAzap. RNAzapped everything (bench, pipettes, tube racks, benchtop centrifuge, etc.). Got a fresh box of each of the tips, a fresh box of each of the different types of eppendorf tubes (except 2 ml collection tubes since we were out, but the ones I were using from before should be fine since these are only for collecting flowthrough anyway), and new HyClone water. Used the RNeasy Kit that I used on 20161210

RNA extraction:
Used a new Qiagen RNeasy Mini Kit and followed the protocol from the "Dec2016_Perth09-p1-lib_barcoded-subamplicon-prep" notebook.
During the second spin, prepared reagents from the RNeasy Mini Kit.
Made 2X 1.1 ml buffer RLT + 11 ul beta-mercaptoethanol (need 2000 ul buffer RLT + BME)
Made 2X 1120 ul 100% EtOH + 480 ul RNase-free water (1600 ul total) (need 3000 ul 70% EtOH).
Resuspended pellet in **400 ul Buffer RLT + BME**, and pipetted slowly up and down 30X's. Sterilized pipet between each sample.
Added **600 ul 70% ethanol** to each lysate.
Transferred 700 ul of the lysate to an RNeasy spin column, spun through once, changed collection tubes, and spun an additional 700 ul of the lysate through the column again.
Proceeded with remainder of RNA extraction protocol.
Eluted each sample with 40 ul of RNase free water (included in the kit), and immediately placed samples on ice.
Proceeded with RT of RNA samples. Stored remainder of RNA samples at -80°C.

Accuscript Reverse Transcriptase First-Strand Synthesis:

Used the Accuscript tubes that I had received from Mike and that I had used once previously on 20161210.

*NOTES:

- I used the RNase-free water that the kit provided, since it is not DEPC-treated

I first set up the annealing reaction, with the following samples in order in an 8-strip PCR tube:

1. WT Perth/2009 RNA
2. mutvirus lib1 RNA
3. mutvirus lib2 RNA
4. mutvirus lib3 rep. 1 RNA
5. mutvirus lib3 rep. 2 RNA
6. No RNA template (H2O only)
7. Positive control Perth/2009 RNA – extracted from passage 6 replicate 2 WT Perth/2009 virus (20160105)

Each reaction is:	
3 ul	10X Accuscript RT Buffer
1.2 ul	dNTP mix
3 ul	5 uM P09-HA-For
3 ul	5 uM P09-HA-Rev
2 ul	**RNA template**
6.8 ul	RNase free water
19 ul	Total volume

Made a 10X reaction master mix	
30 ul	10X Accuscript RT Buffer
12 ul	dNTP mix
30 ul	5 uM P09-HA-For
30 ul	5 uM P09-HA-Rev
68 ul	RNase free water

Aliquoted **17 ul** of the 10X master mix into 7 tubes (labeled the 8-strip tube as “RT-”).
Added **2 ul of the RNA**, and mixed by pipetting.
Heated to **65°C for 5 min**, then cooled to 4°C in thermal cycler. Spun down tubes briefly and placed on ice.

While the annealing reaction was going, made the following master mixes:
AccuScript master mix (for 10 reactions): 12.5 ul water + 7.5 ul Accuscript RT (mixed well by pipetting)
RNase block master mix (for 20 reactions): 32.5 ul water + 7.5 ul RNase block

After the annealing reaction was done, added **3 ul DTT** to each reaction.

Split each reaction into two by pipetting 11 ul from each sample into a new PCR tube (labeled the second 8-strip as “RT+”)
To the “RT+” reactions, added: 2 ul Accuscript MM (1.25 ul water + 0.75 ul AccuScript RT) and then 2 ul of the RNase block MM (1.625 ul water + 0.375 ul RNase block).
To the “RT-” reactions, added: 2 ul of RNase-free water and then 2 ul of the RNase block MM.
Proceeded with RT: 42°C for 90 min, 70°C for 15 min, cool to 4°C.
Stored RT reactions at -20°C

Full-length amplicon PCR. Amplifying full-length amplicon from cDNA in addition to plasmid templates and witness band standards.

Templates:

cDNA samples

1. RT+ WT P09 virus
2. RT+ mutvirus lib1
3. RT+ mutvirus lib2
4. RT+ mutvirus lib3 rep. 1
5. RT+ mutvirus lib3 rep. 2
6. RT+ no RNA
7. RT+ positive ctrl P09 RNA
8. RT- WT P09 virus
9. RT- mutvirus lib1
10. RT- mutvirus lib2
11. RT- mutvirus lib3 rep. 1
12. RT- mutvirus lib3 rep. 2
13. RT- no RNA
14. RT- positive ctrl P09 RNA

Plasmid samples (*all diluted to 10 ng/ul)

15. WT plasmid (#1535 pICR2-Perth09-HA)
16. mutDNA plasmid lib1
17. mutDNA plasmid lib2
18. mutDNA plasmid lib3
19. No plasmid ctrl (water only)

Witness standards (2 ul)

20. 1e5 ssDNA molecules (5e4 / ul)
21. 1e6 ssDNA molecules (5e5 / ul)
22. 1e7 ssDNA molecules (5e6 / ul)
23. 1e8 ssDNA molecules (5e7 / ul)
24. 1e9 ssDNA molecules (5e8 / ul)

Used 2 ul of each template for PCR reactions.

Each reaction: 50 ul total	*Made a 25X master mix:*
25 ul 2X KOD Hot-Start Master Mix	*625 ul 2X KOD MM*
3 ul of 5 uM P09-HA-For	*75 ul of 5 uM P09-HA-For*
3 ul of 5 uM P09-HA-Rev	*75 ul of 5 uM P09-HA-Rev*
17 ul water	*425 ul water*
2 ul template	

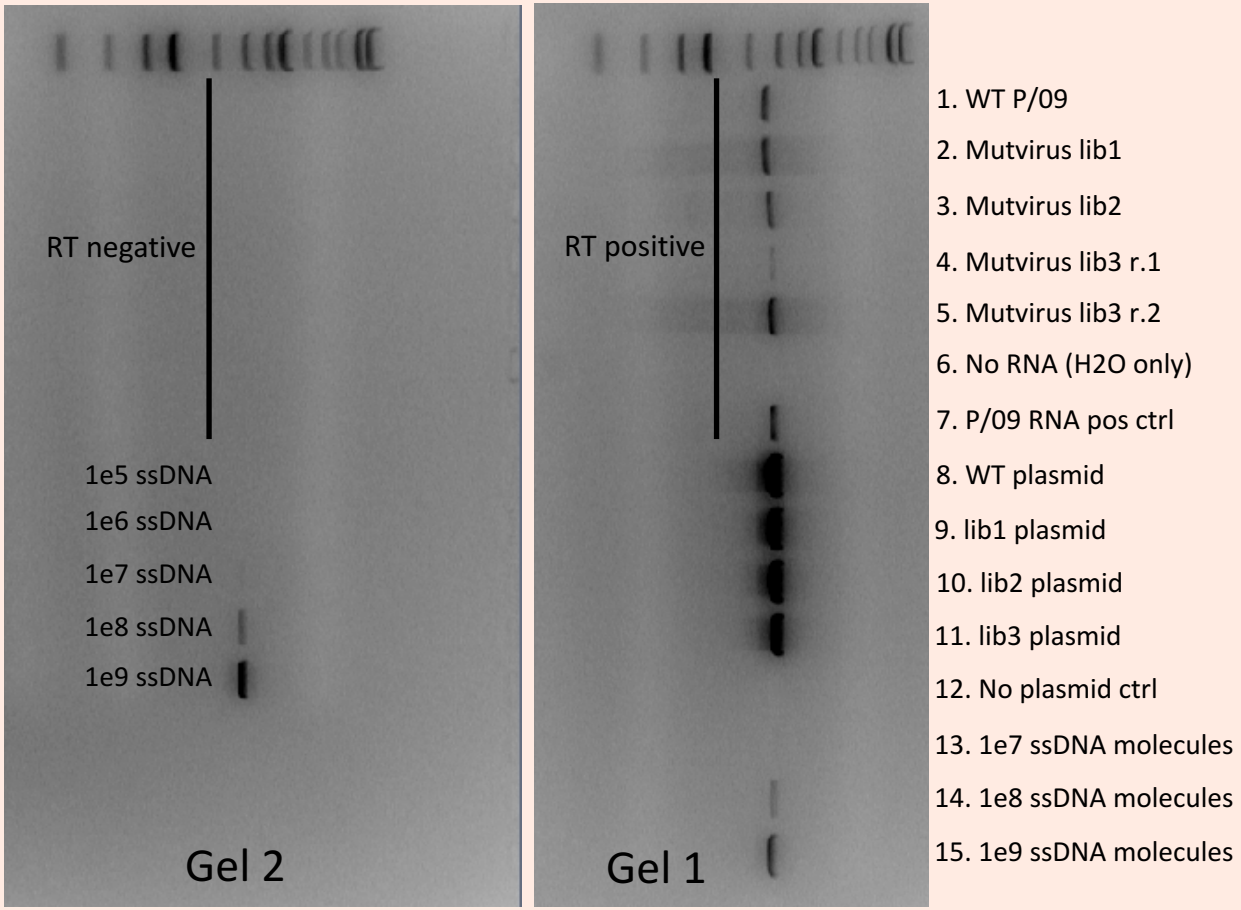
Added **48 ul of the PCR master mix** to specific wells of a 96-well plate, then added **2 ul of template** to each well.
Pipette all wells with multichannel a few times to mix.
Seal with **Microfilm A**, briefly centrifuge.
PCR program ("JLSA" using Mastercycler Gradient machine):

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 50°C for 30 s, cooling to 50°C at 0.5°C/sec
5. 70°C for 40 s
6. Repeat steps 2-5 for 21 additional cycles (22 cycles total)
7. 4°C forever

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

Made dilutions of witness band standards:
Concentration of witness band amplicon = 160.2 ng/ul
10 ng of FL amplicon (1765 bp) ~ 1e10 ssDNA molecules
Made a 5 ng/ul dilution (5e9 ssDNA/ul) =
3.12 ul witness amplicon + 96.88 ul H2O
Made serial 10-fold dilutions of the 5 ng/ul dilution, down to 5e3 ssDNA/ul

Ran **two** 1% agarose gels of full-length amplicon PCR products. Ran 4 ul of each sample + 6 ul water + 2 ul 6X loading dye.
Made a 30X mix of loading dye + water: 180 ul water + 60 ul 6X loading dye.
Added 8 ul of the 30X master mix to wells of a new plate corresponding to wells of the PCR plate. Transferred 4 ul from PCR plate to buffer plate.
Ran gels at 120 V for 45 min.



I saw bands for witness band standards 1e8 and 1e9 ssDNA molecules. Again, not seeing much of a band for 1e7 ssDNA molecules and below.
I am amplifying HA from > 1e8 ssDNA molecules (compare bands for RT positive to 1e8 ssDNA standard), although mutvirus lib3 replicate 1 seems to have the faintest band of all RT positive samples.
I will store this 96-well plate as “P/09 HA FL amplicon” at -20°C for bead purification and picogreen tomorrow.

January 31, 2017

Ampure bead purification of full-length amplicons. Purified remaining full-length amplicon PCR products from yesterday using **0.9x beads**. I will Ampure bead purify the following:

cDNA samples

1. RT+ WT P09 virus
2. RT+ mutvirus lib1
3. RT+ mutvirus lib2
4. RT+ mutvirus lib3 rep. 1
5. RT+ mutvirus lib3 rep. 2
6. RT+ no RNA
7. RT+ positive ctrl P09 RNA

Plasmid samples (*all diluted to 10 ng/ul)

15. WT plasmid (#1535 pICR2-Perth09-HA)
16. mutDNA plasmid lib1
17. mutDNA plasmid lib2
18. mutDNA plasmid lib3
19. No plasmid ctrl (water only)

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

There are 46 ul of PCR product remaining, so I added **42 ul beads**.

1. Added 42 ul beads to each highlighted well and mixed by pipetting 20X's
2. Incubated at RT for 10 min to bind
3. Placed on magnet for 5 min
4. Aspirated
5. Washed twice with 190 ul freshly made 80% ethanol
6. Air dried 10 min
7. Removed from rack, and dispersed beads in 60 ul EB
8. Incubated for 5 min to resuspend DNA
9. Incubated on magnet for 5 min
10. Transferred supernatant to a new 96-well plate in the following setup:

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

Nanodropped Ampure-purified products to get a rough idea of what the DNA concentration of my PCR products are:
These concentrations generally correspond with band intensities I saw on the gel, with mutvirus lib3 rep.1 having the least amount of product. I will picogreen these samples and use a 1:100 dilution

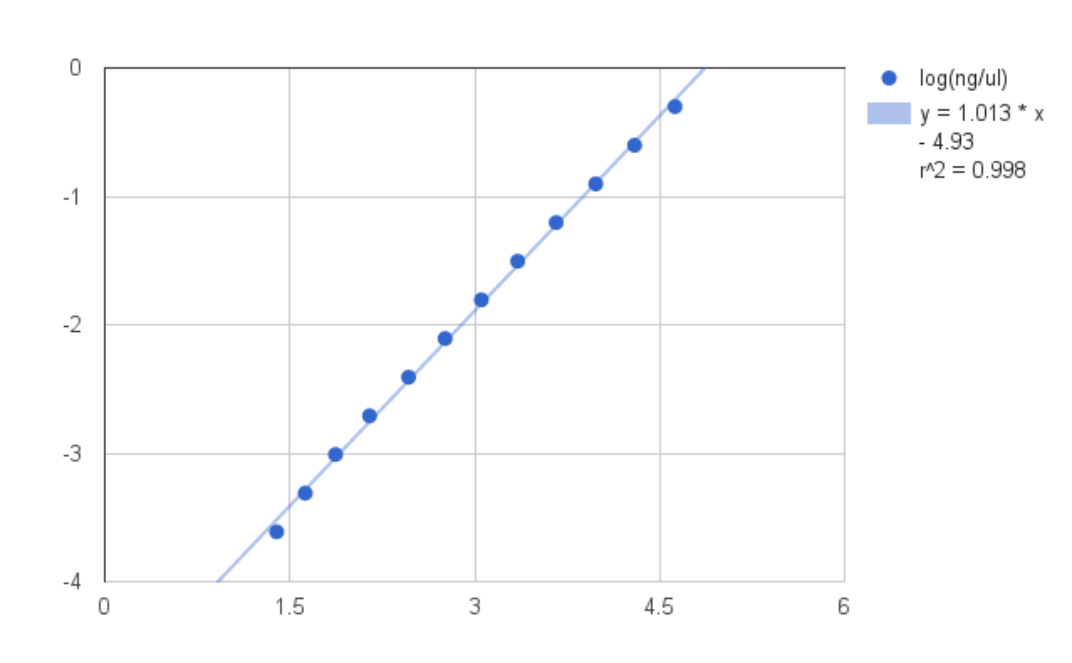
Sample	DNA concentration (ng/ul)
WT virus	4.7
Mutvirus lib1	7.9
Mutvirus lib2	5.4
Mutvirus lib3 rep. 1	1.7
Mutvirus lib3 rep.2	10.9
No RNA	1.0
Perth/09 RNA positive ctrl	4.2
WT plasmid	16.8
mutDNA plasmid lib1	12.8
mutDNA plasmid lib2	12.5
mutDNA plasmid lib3	12.1
No plasmid ctrl	1.0

Picogreen of purified full-length amplicons.

1. Made two standards at 1 ng/ul by adding 2 ul of the standard to 198 ul 1X TE. *Remember to vortex the stock solution of lambda DNA before adding to buffer TE!*
2. Added 99 ul 1X TE to rows A and C (for replicate measurements of DNA), and wells D1-D4 as negative control wells.
3. Added 1 ul of the Ampure-purified products to the 99 ul TE rows A and C to measure 1:100 dilutions of each sample, in duplicate. Added from column 1 of the Ampure-purified plate to the first 8 wells of each column, and the four wells in column 7 of the Ampure plate to the last 4 wells of each column (12 samples total for each replicate)
4. Made standard rows by adding 100 ul TE to rows F and G, and added 100 ul of the independent standards to F1 and G1. Made serial dilutions of the standard by transferring 100 ul across columns, then removing 100 ul from the last column to equalize volumes (no need to change pipet tips here).
5. Made 1:200 picogreen dilution (30 ul to 5970 ul TE), and added 100 ul to all wells containing liquid. Covered plate to protect from light, and incubated for 5 min before reading on the plate reader.

Plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	WTvirus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	P09 RNA pos	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3	no plasmid
B												
C	WTvirus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	P09 RNA pos	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3	no plasmid
D	TE only	TE only	TE only	TE only								
E												
F	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	2.44E-04
G	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	2.44E-04
H												



Sample	ng/ul
WT virus	2.77
mutvirus lib1	5.32
mutvirus lib2	3.92
mutvirus lib3-1	0.76
mutvirus lib3-2	9.28
no RNA	0.027
P09 RNA pos	3
no plasmid	0.014
WT plasmid	17.30
plasmid lib1	12.32
plasmid lib2	11.74
plasmid lib3	11.21

Picogreen concentrations have a good correlation with nanodrop readings.
As expected, the no RNA and no plasmid controls have very low picogreen readings.
However, I am concerned that overall, my yields after FL amplicon PCR are lower for what I was getting before (see Dec2016 notebook). In December, I was getting much higher yields overall, especially for amplification off of the plasmid, and I was getting in the ~20-45 ng/ul range, which is consistent with what Mike was getting. Perhaps because I was using a different machine? Although I am concerned about this, I will move forward with Round 1 since I still have enough product to work with.
I will store the Ampure-purified full-length amplicon products at -20°C to use for round 1 PCR.

February 1, 2017

Round 1 PCR. Diluted Ampure-purified full-length PCR products to 0.5 ng/ul. Added the following volumes of each sample to 70 ul water in row A of the corresponding column of a new 96-well PCR plate (note that mutvirus lib3-1 has low concentration, so I added 45.83 ul of the PCR product to 24.17 ul H2O)

Column	1	2	3	4	5	6	7	8	9	10
Sample	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Well on Ampure-purified amplicons plate taken from	A1	A2	A3	A4	A5	A6	A8	A9	A10	A11
Volume added to 70 ul water (ul)	15.42	7.28	10.24	45.83 + 24.17 H_2O	4	40	2.09	2.98	3.12	3.27

For each subamplicon primer pair, made a mix in a strip tube, from #1-6, for each subamplicon primer pair #1-6, respectively. I added 11 ul F primer (10 uM) + 11 ul R primer (10 uM) + 22 ul water. This final mixture is 44 ul and each primer will be at 2.5 uM final concentration. I then added 4 ul of each primer mix to each PCR reaction.

Each round 1 PCR reaction is set up as follows (total volume = 24 ul)
12 ul 2X KOD Hot-Start MM
2 ul of 5 uM forward primer
2 ul of 5 uM reverse primer
8 ul of 0.5 ng/ul template (4 ng total)

Added 130 ul of the 2X KOD MM to the first 7 tubes of an 8-strip. Using a multichannel, added 12 ul of the KOD MM column-wise.
Then added primers one column at a time from the primer mix strip tube (4 ul added column-wise).
Then added 0.5 ng/ul templates one row at a time (8 ul added row-rise).
Seal with microfilm A, spin briefly, and run “HAsubampR1” under “^^ike” to run 9 total PCR cycles:

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 54°C for 20 s, cooling at 0.5°C/s
5. 70°C for 20 s
6. Go to 2, 8 times
7. 95°C for **1 min** (this step to ensure identical pairs are not annealed at the end)
8. 4°C forever

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:	1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1 A												
Subamplicon2 B												
Subamplicon3 C												
Subamplicon4 D												
Subamplicon5 E												
Subamplicon6 F												
no primers G												
H												

Ampure purification of Round 1 products.

After PCR was finished, added 26 ul water to each well to bring the volume to 50 ul.

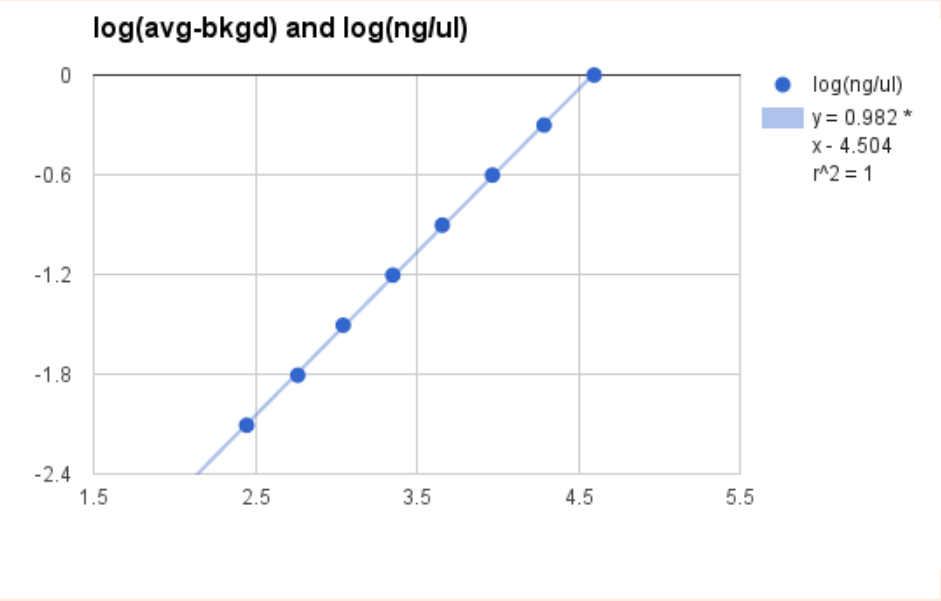
Purified PCR products with 1X Ampure beads.

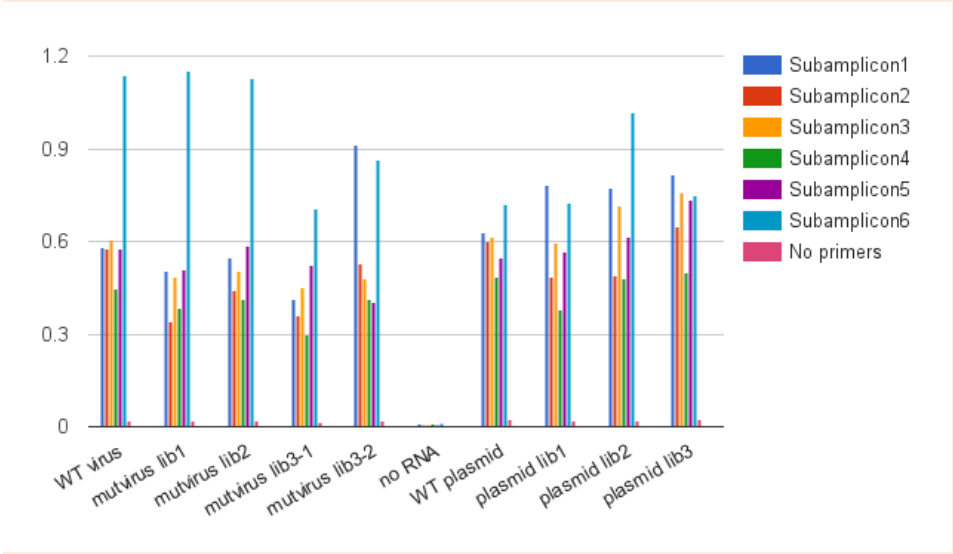
1. Added 50 ul beads from 3.7 ml aliquot to each well and mixed by pipetting 20X's
2. Incubated at RT for 10 min to bind
3. Placed on magnet for 5 min
4. Aspirated
5. Washed twice with 190 ul freshly made 80% ethanol
6. Air dried 7 min
7. Removed from rack, and dispersed beads in 75 ul EB
8. Incubated for 5 min to resuspend DNA
9. Incubated on magnet for 5 min
10. Transferred supernatant to a new 96-well plate in the same set-up as the round 1 PCR plate (*I noticed some liquid at the bottom of the tube after transferring supernatant – perhaps residual ethanol, which is not good?*). Used 10 ul for picogreen, and stored the remainder at -20°C

Picogreen of Ampure-purified Round 1 products.

Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 100 ul TE to row H (H1-H10) and columns 11 & 12. Added 90 ul TE to remaining wells (wells that will receive PCR products for a final 1:10 dilution).
Made standard curve by serially transferring 100 ul of the 2 ng/ul into A11 and A12 and then down rows.
Transferred 10 ul of the purified Round 1 PCR products to the picogreen plate for a final dilution of 1:10.
Made a 1:200 dilution of picogreen (55 ul to 11 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

Template:		*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:		1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1	A	10 ul of purified rnd1 products to 90 ul TE (to measure 1:10 dilution)										1.00E+00	1.00E+00
Subamplicon2	B											5.00E-01	5.00E-01
Subamplicon3	C											2.50E-01	2.50E-01
Subamplicon4	D											1.25E-01	1.25E-01
Subamplicon5	E											6.25E-02	6.25E-02
Subamplicon6	F											3.13E-02	3.13E-02
no primers	G											1.56E-02	1.56E-02
	H	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	7.81E-03	7.81E-03





ng/ul of purified Round 1 product

	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Subamplicon1	0.582	0.502	0.545	0.412	0.912	0.008	0.630	0.780	0.774	0.818
Subamplicon2	0.574	0.340	0.442	0.362	0.529	0.005	0.602	0.483	0.490	0.647
Subamplicon3	0.603	0.483	0.506	0.452	0.480	0.006	0.615	0.594	0.717	0.758
Subamplicon4	0.448	0.383	0.413	0.296	0.412	0.009	0.483	0.379	0.479	0.500
Subamplicon5	0.575	0.506	0.584	0.522	0.405	0.005	0.545	0.568	0.615	0.734
Subamplicon6	1.138	1.154	1.128	0.704	0.862	0.011	0.719	0.727	1.019	0.750
No primers	0.021	0.019	0.019	0.015	0.019	0.002	0.024	0.021	0.021	0.025

These concentrations are very low, which concerns me greatly. The next step requires a dilution to 0.5 ng/ul, and many of these concentrations are **below** 0.5 ng/ul, with the lowest being 0.296 ng/ul. Although I can probably make a lower dilution (say, 0.2 ng/ul) which will be eventually be diluted further to bottleneck for Round 2, I am concerned that I am already bottlenecking the library after round 1. Especially since I was seeing secondary bands from a pilot of my subamplicon primers (see 20170110), I'm worried that a considerable proportion of the round 1 PCR products are comprised of these non-specific products, which will affect my bottleneck calculation.

I have a suspicion that these low yields may mostly be due to residual ethanol left from the second wash during ampure bead purification. As I was adding EB, I noticed a small amount of liquid still left in the bottom of the tube. This can negatively impact the DNA's ability to elute off of the beads. I will reattempt to make full-length amplicon, keeping in mind to remove all residual ethanol and drying for 10 min. Hopefully this should help improve yields.

February 2, 2017

Full-length amplicon PCR. Amplifying full-length amplicon from cDNA in addition to plasmid templates and witness band standards.

Templates:

cDNA samples

1. RT+ WT P09 virus
2. RT+ mutvirus lib1
3. RT+ mutvirus lib2
4. RT+ mutvirus lib3 rep. 1
5. RT+ mutvirus lib3 rep. 2
6. RT+ no RNA
7. RT+ positive ctrl P09 RNA
8. RT- WT P09 virus
9. RT- mutvirus lib1
10. RT- mutvirus lib2
11. RT- mutvirus lib3 rep. 1
12. RT- mutvirus lib3 rep. 2
13. RT- no RNA
14. RT- positive ctrl P09 RNA

Plasmid samples (*all diluted to 10 ng/ul)

(made new dilutions of plasmid libraries)

15. WT plasmid (#1535 pICR2-Perth09-HA)
16. mutDNA plasmid lib1
17. mutDNA plasmid lib2
18. mutDNA plasmid lib3
19. No plasmid ctrl (water only)

Witness standards (2 ul)

20. 1e7 ssDNA molecules (5e6 / ul)
21. 1e8 ssDNA molecules (5e7 / ul)
22. 1e9 ssDNA molecules (5e8 / ul)

Used 2 ul of each template for PCR reactions.

Each reaction: 50 ul total	*Made a 25X master mix:*
25 ul 2X KOD Hot-Start Master Mix	*625 ul 2X KOD MM*
3 ul of 5 uM P09-HA-For	*75 ul of 5 uM P09-HA-For*
3 ul of 5 uM P09-HA-Rev	*75 ul of 5 uM P09-HA-Rev*
17 ul water	*425 ul water*
2 ul template	

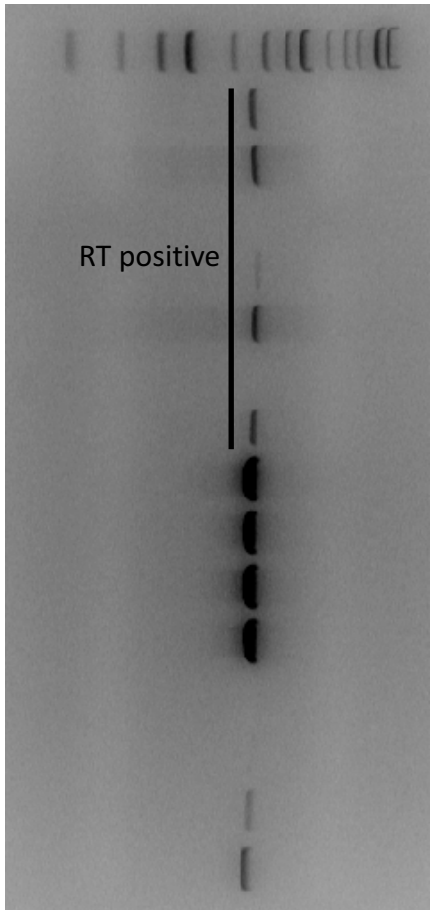
Added **48 ul of the PCR master mix** to specific wells of a 96-well plate, then added **2 ul of template** to each well.
Pipette all wells with multichannel a few times to mix.
Seal with **Microfilm A**, briefly centrifuge.
PCR program (Under "^^ike" use "mike kod HA"):

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 50°C for 30 s, cooling to 50°C at 0.5°C/sec
5. 70°C for 40 s
6. Repeat steps 2-5 for 21 additional cycles (22 cycles total)
7. 4°C forever

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

Made dilutions of witness band standards:
Concentration of witness band amplicon = 160.2 ng/ul
10 ng of FL amplicon (1765 bp) ~ 1e10 ssDNA molecules
Made a 5 ng/ul dilution (5e9 ssDNA/ul) =
3.12 ul witness amplicon + 96.88 ul H2O
Made serial 10-fold dilutions of the 5 ng/ul dilution, down to 5e5 ssDNA/ul

Ran **two** 1% agarose gels of full-length amplicon PCR products. Ran 4 ul of each sample + 6 ul water + 2 ul 6X loading dye.
Made a 30X mix of loading dye + water: 180 ul water + 60 ul 6X loading dye.
Added 8 ul of the 30X master mix to wells of a new plate corresponding to wells of the PCR plate. Transferred 4 ul from PCR plate to buffer plate.
Ran gels at 120 V for 50 min.



1. WT P/09
2. Mutvirus lib1
3. Mutvirus lib2
4. Mutvirus lib3 r.1
5. Mutvirus lib3 r.2
6. No RNA (H2O only)
7. P/09 RNA pos ctrl
8. WT plasmid
9. lib1 plasmid
10. lib2 plasmid
11. lib3 plasmid
12. No plasmid ctrl
13. 1e7 ssDNA molecules
14. 1e8 ssDNA molecules
15. 1e9 ssDNA molecules

This lane was acting weird, and whatever I loaded into this lane all ended up in the mutvirus lib1 lane for whatever reason, so it looks like there is no sample for mutvirus lib2

Did not take a picture of the RT negative gel, but I did not see any bands, as before.
I saw bands for witness band standards 1e8 and 1e9 ssDNA molecules. Again, not seeing much of a band for 1e7 ssDNA molecules and below.
I am amplifying HA from > 1e8 ssDNA molecules (compare bands for RT positive to 1e8 ssDNA standard). Again, mutvirus lib3 replicate 1 seems to have the faintest band of all RT positive samples.
I will proceed with Ampure bead purification of FL amplicon products and subsequent Picogreen.

Ampure bead purification of full-length amplicons. Purified remaining full-length amplicon PCR products using **0.9x beads**. I will Ampure bead purify the following:

cDNA samples

1. RT+ WT P09 virus
2. RT+ mutvirus lib1
3. RT+ mutvirus lib2
4. RT+ mutvirus lib3 rep. 1
5. RT+ mutvirus lib3 rep. 2
6. RT+ no RNA
7. RT+ positive ctrl P09 RNA

Plasmid samples (*all diluted to 10 ng/ul)

15. WT plasmid (#1535 pICR2-Perth09-HA)
16. mutDNA plasmid lib1
17. mutDNA plasmid lib2
18. mutDNA plasmid lib3
19. No plasmid ctrl (water only)

Witness standards

20. 1e7 ssDNA molecules (5e6 / ul)
21. 1e8 ssDNA molecules (5e7 / ul)
22. 1e9 ssDNA molecules (5e8 / ul)

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

There are 46 ul of PCR product remaining, so I added **42 ul beads**.

1. Added 42 ul beads to each highlighted well and mixed by pipetting 20X's
2. Incubated at RT for 10 min to bind
3. Placed on magnet for 5 min
4. Aspirated
5. Washed twice with 190 ul freshly made 80% ethanol
6. Air dried 10 min
7. Removed from rack, and dispersed beads in 60 ul EB
8. Incubated for 5 min to resuspend DNA
9. Incubated on magnet for 5 min
10. Transferred supernatant to a new 96-well plate in the following setup:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C			1	2	3	4	5	6	7			
D												
E			15	16	17	18	19					
F												
G			20	21	22							
H												

Nanodropped Ampure-purified products to get a rough idea of what the DNA concentration of my PCR products are:
These concentrations generally correspond with band intensities I saw on the gel, with mutvirus lib3 rep.1 having the least amount of product. I will picogreen these samples and use a 1:100 dilution

Sample	DNA concentration (ng/ul)
WT virus	21.6
Mutvirus lib1	41.4
Mutvirus lib2	17.9
Mutvirus lib3 rep. 1	4.3
Mutvirus lib3 rep.2	35.1
No RNA	0.1
Perth/09 RNA positive ctrl	13.4
WT plasmid	40.7
mutDNA plasmid lib1	35.1
mutDNA plasmid lib2	30.9
mutDNA plasmid lib3	31.7
No plasmid ctrl	0.4
1e7 ssDNA WB	0.7
1e8 ssDNA WB	5.5
1e9 ssDNA WB	15.6

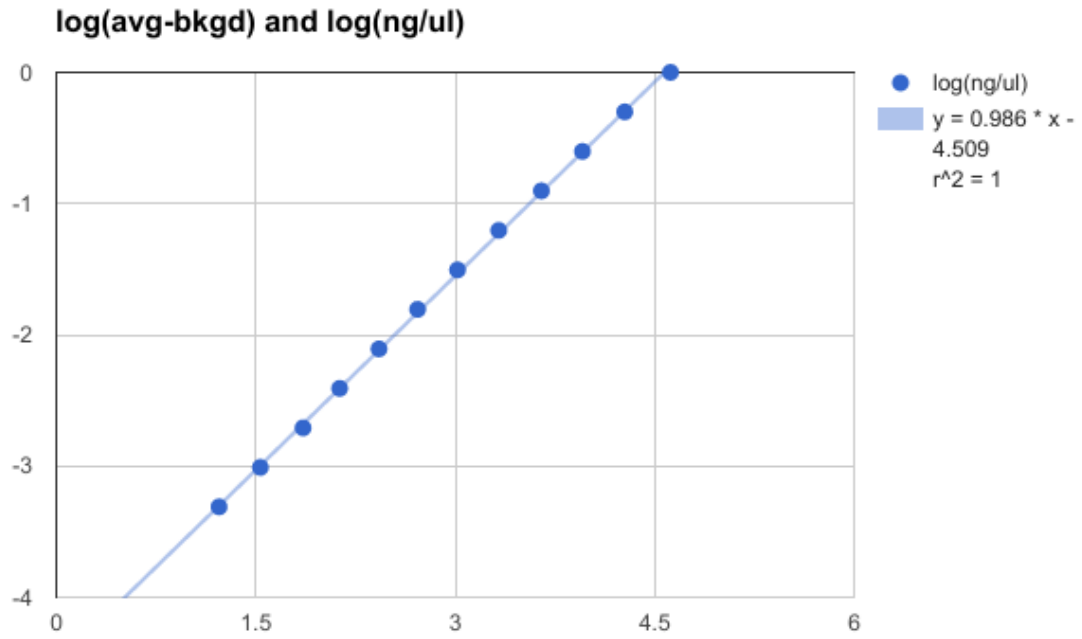
Picogreen of purified full-length amplicons.

1. Made two standards at 2 ng/ul by adding 4 ul of the standard to 196 ul 1X TE. *Remember to vortex the stock solution of lambda DNA before adding to buffer TE!*
2. Added 99 ul 1X TE to rows A and C (for replicate measurements of DNA), and wells D1-D4 as negative control wells.
3. Added 1 ul of the Ampure-purified products to the 99 ul TE rows A and C to measure 1:100 dilutions of each sample, in duplicate. Added from column 1 of the Ampure-purified plate to the first 8 wells of each column, and the four wells in column 7 of the Ampure plate to the last 4 wells of each column (12 samples total for each replicate)
4. Made standard rows by adding 100 ul TE to rows F and G, and added 100 ul of the independent standards to F1 and G1. Made serial dilutions of the standard by transferring 100 ul across columns, then removing 100 ul from the last column to equalize volumes (no need to change pipet tips here).
5. Made 1:200 picogreen dilution (30 ul to 5970 ul TE), and added 100 ul to all wells containing liquid. Covered plate to protect from light, and incubated for 5 min before reading on the plate reader.

Plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	WTvirus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	P09 RNA pos	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3	no plasmid
B	1e7 WB	1e8 WB	1e9 WB	1e7 WB	1e8 WB	1e9 WB						
C	WTvirus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	P09 RNA pos	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3	no plasmid
D	TE only	TE only	TE only	TE only								
E												
F	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
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												

Sample	ng/ul
WT virus	19.852
mutvirus lib1	35.525
mutvirus lib2	15.334
mutvirus lib3-1	3.756
mutvirus lib3-2	27.825
no RNA	0.023
P09 RNA pos	11.936
WT plasmid	37.583
plasmid lib1	33.651
plasmid lib2	28.582
plasmid lib3	28.366
No plasmid	0.007
1e7 ssDNA	0.771
1e8 ssDNA	5.529
1e9 ssDNA	14.554



Picogreen concentrations have pretty good correlation with nanodrop readings. Again, mutvirus lib3-1 has the lowest picogreen reading
As expected, the no RNA and no plasmid controls have very low picogreen readings.
The yields this time around are much better ☺
The Picogreen readings also indicate that I am definitely amplifying from at least 1e7 ssDNA molecules.
I will store this plate as “P/09 LIB FL Amplicon Ampure purified” at -20°C and use tomorrow to make dilutions for Round 1 PCR.

February 3, 2017

Round 1 PCR. Diluted Ampure-purified full-length PCR products to 0.5 ng/ul. Added the following volumes of each sample to 70 ul water in row A of the corresponding column of a new 96-well PCR plate

Column	1	2	3	4	5	6	7	8	9	10
Sample	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Well on Ampure-purified amplicons plate taken from	C3	C4	C5	C6	C7	C8	E3	E4	E5	E6
Volume added to 70 ul water (ul)	1.81	1	2.36	10.75	1.29	40	0.95	1.06	1.25	1.26

For each subamplicon primer pair, made a mix in a strip tube, from #1-6, for each subamplicon primer pair #1-6, respectively. I added 11 ul F primer (10 uM) + 11 ul R primer (10 uM) + 22 ul water. This final mixture is 44 ul and each primer will be at 2.5 uM final concentration. I then added 4 ul of each primer mix to each PCR reaction.

Each round 1 PCR reaction is set up as follows (total volume = 24 ul)
12 ul 2X KOD Hot-Start MM
2 ul of 5 uM forward primer
2 ul of 5 uM reverse primer
8 ul of 0.5 ng/ul template (4 ng total)

Added 130 ul of the 2X KOD MM to the first 7 tubes of an 8-strip. Using a multichannel, added 12 ul of the KOD MM column-wise.
Then added primers one column at a time from the primer mix strip tube (4 ul added column-wise).
Then added 0.5 ng/ul templates one row at a time (8 ul added row-rise).
Seal with microfilm A, spin briefly, and run “HAsubampR1” under “^^ike” to run 9 total PCR cycles:

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 54°C for 20 s, cooling at 0.5°C/s
5. 70°C for 20 s
6. Go to 2, 8 times
7. 95°C for **1 min** (this step to ensure identical pairs are not annealed at the end)
8. 4°C forever

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:	1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1 A												
Subamplicon2 B												
Subamplicon3 C												
Subamplicon4 D												
Subamplicon5 E												
Subamplicon6 F												
no primers G												
H												

Ampure purification of Round 1 products.

After PCR was finished, added 26 ul water to each well to bring the volume to 50 ul.

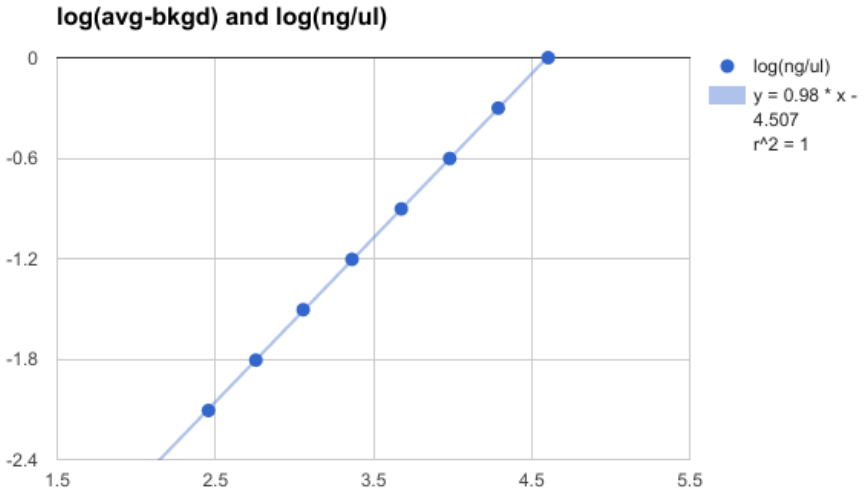
Purified PCR products with 1X Ampure beads.

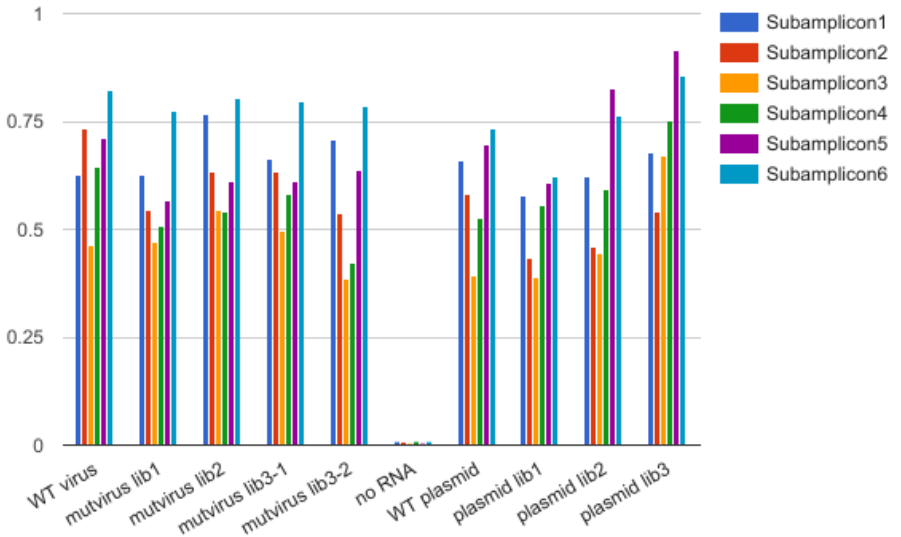
1. Added 50 ul beads from 3.7 ml aliquot to each well and mixed by pipetting 20X's
2. Incubated at RT for 10 min to bind
3. Placed on magnet for 5 min
4. Aspirated
5. Washed twice with 190 ul freshly made 80% ethanol
6. Air dried 10 min
7. Removed from rack, and dispersed beads in 75 ul EB
8. Incubated for 5 min to resuspend DNA
9. Incubated on magnet for 5 min
10. Transferred supernatant to a new 96-well plate in the same set-up as the round 1 PCR plate. Used 10 ul for picogreen, and stored the remainder at -20°C

Picogreen of Ampure-purified Round 1 products.

Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 100 ul TE to row H (H1-H10) and columns 11 & 12. Added 90 ul TE to remaining wells (wells that will receive PCR products for a final 1:10 dilution).
Made standard curve by serially transferring 100 ul of the 2 ng/ul into A11 and A12 and then down rows.
Transferred 10 ul of the purified Round 1 PCR products to the picogreen plate for a final dilution of 1:10.
Made a 1:200 dilution of picogreen (55 ul to 11 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

Template:		*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:		1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1	A	10 ul of purified rnd1 products to 90 ul TE (to measure 1:10 dilution)										1.00E+00	1.00E+00
Subamplicon2	B											5.00E-01	5.00E-01
Subamplicon3	C											2.50E-01	2.50E-01
Subamplicon4	D											1.25E-01	1.25E-01
Subamplicon5	E											6.25E-02	6.25E-02
Subamplicon6	F											3.13E-02	3.13E-02
no primers	G											1.56E-02	1.56E-02
	H	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	7.81E-03	7.81E-03





ng/ul of purified Round 1 product

	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Subamplicon1	0.626	0.624	0.766	0.664	0.706	0.009	0.661	0.579	0.621	0.679
Subamplicon2	0.735	0.546	0.633	0.634	0.535	0.007	0.582	0.434	0.459	0.541
Subamplicon3	0.464	0.471	0.545	0.496	0.387	0.004	0.392	0.391	0.445	0.672
Subamplicon4	0.643	0.507	0.542	0.581	0.421	0.009	0.526	0.556	0.594	0.752
Subamplicon5	0.712	0.565	0.610	0.611	0.637	0.004	0.697	0.609	0.827	0.914
Subamplicon6	0.824	0.772	0.806	0.796	0.785	0.009	0.733	0.622	0.764	0.855
No primers	0.010	0.010	0.006	0.005	0.009	0.000	0.010	0.007	0.008	0.010

These concentrations are still very low. Perhaps the round 1 primers don't work very well? One thing I noticed from my initial pilot of these new round 1 primers (20170110) is that there is a substantial amount of secondary bands present at ~800-900 bp, and this band is present for all subamplicon primer pairs. The adapter sequences are all shared for the primers, and since the pilot conditions are different than the conditions used for round 1 (used a plasmid template, 25 cycles, 50°C annealing temp, more starting template), perhaps these are factors that led to the formation of these secondary bands. I am also suspicious that formation of these secondary bands may be affecting my calculation of how many barcodes to bottleneck to for round 2, as I was seeing half as many barcodes as I should have from my initial sequencing run (see December notebook).

I will run a gradient PCR to see if raising the annealing temp gets rid of these secondary bands, and run TapeStation of my Round 1 products.

Gradient PCR of full-length amplicons and plasmid template.

I will run a gradient PCR of ampure-purified WT full-length amplicon (from 20170131) and WT plasmid template, using the subamplicon primers. I will try to follow round 1 PCR conditions as closely as possible.

Amplicon PCR (24 ul total):

1 ul 10 uM F primer
1 ul 10 uM R primer
8 ul 0.5 ng/ul WT plasmid full-length amplicon template (ampure-purified from 20170131, made a 0.5 ng/ul dilution by adding 5.78 ul DNA to 194.22 ul H2O)
12 ul 2X KOD MM
2 ul H_2O

In six tubes of an 8-strip PCR tubes, made a 5X master mix in each of the six tubes, each containing one pair of subamplicon primers. Added 16 ul of each master mix to 4 tubes of 4 separate strip tubes (four strips for each annealing temp to try)

5 ul 10 uM F primer
5 ul 10 uM R primer
60 ul 2X KOD MM
10 ul H2O

Then added 8 ul template to each reaction. 6 subamplicons x 4 annealing temps = 24 PCR reactions total

Plasmid template PCR (24 ul total):

1 ul 10 uM F primer
1 ul 10 uM R primer
1 ul 10 ng/ul template (plasmid #1535)
12 ul 2X KOD MM
9 ul H2O

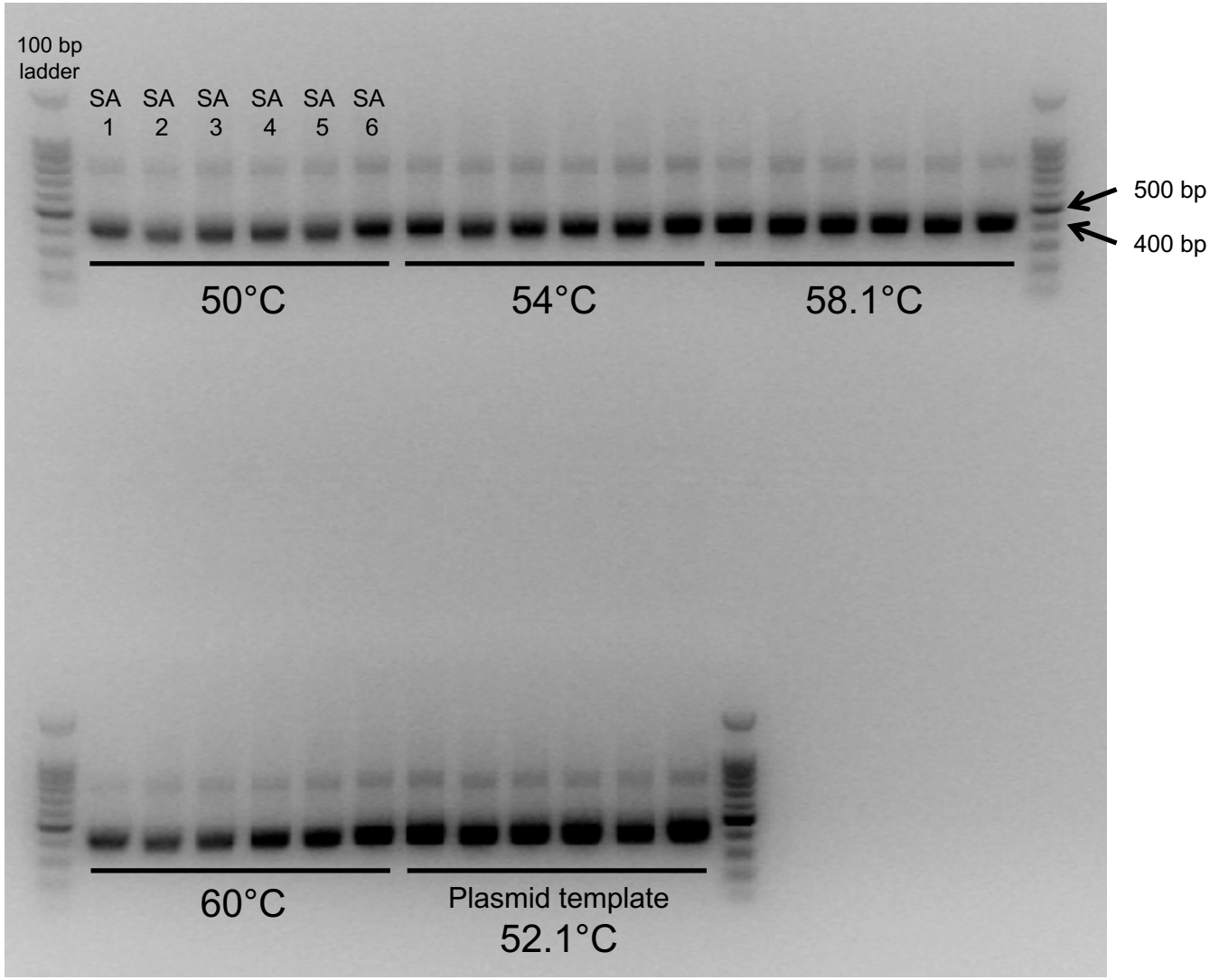
For plasmid template PCR, used annealing temp of 52.1°C

Ran “jl gradient” for 20 cycles total:

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 50°C (row A), 54°C (row E), 58.1°C (row C), 60°C (row A), 52.1°C for plasmid template PCR, for 20 s – note: no ramp option for gradient PCR
5. 70°C for 20 s
6. Go to 2, 19 times
7. 4°C forever

February 4, 2017

After PCR was done, added 1 ul H2O + 5 ul 6X loading dye to each sample, and ran on 1% agarose gel for 1 h 25 min at 120 V



From the gel, it looks like the secondary band between 800 and 900 bp is still present for all annealing temps and for linear and plasmid templates. Although it is hard to tell, it looks like there might be a bit more product at 54°C and 58.1°C than at 50°C and 60°C, but overall, the yield of the desired specific product at ~450 bp does not improve much at these different annealing temps. Since it looks like these secondary bands are still present for linear template, I will submit a couple of my samples for TapeStation at the Hutch Genomics core to see how abundant these secondary products are relative to the desired product.

February 7, 2017

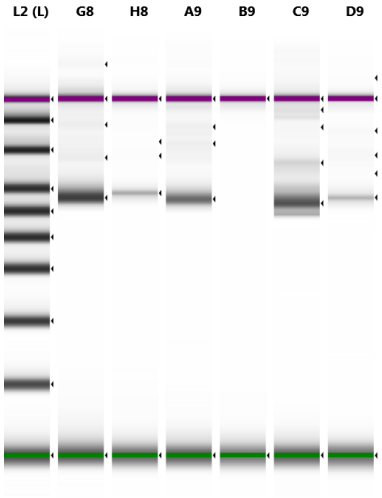
TapeStation Sample Submission.

Prepared samples to submit for TapeStation analysis at the Fred Hutch Genomics core. Submitted online order form at Hutch base: 6 samples, each at 0.5 ng/ul concentration, 5 ul total. I am using the HiSens D1K assay (35-1000 bp), ≥ 3 ul at 0.075-1 ng/ul. In addition to my own samples and water only control, I am also submitting two of Mike's previously Ampure-purified Round 1 products to see if these additional PCR products are present in his samples as well as mine (from Mike 20160628).

Submitted samples with each sample in a tube of an 8-strip tube. Put a piece of tape on the top of the strip tubes labelled with name, Hutch ID, date, # samples, and assay. Placed the tubes in the -20°C freezer of the genomics core and put a copy of the order sheet on the clipboard.

Labelled samples "JL0#" with # being from 1 to 6 for each sample. Although one sample was diluted and one sample had only water, I completed the online form with all samples having a concentration of 0.5 ng/ul.

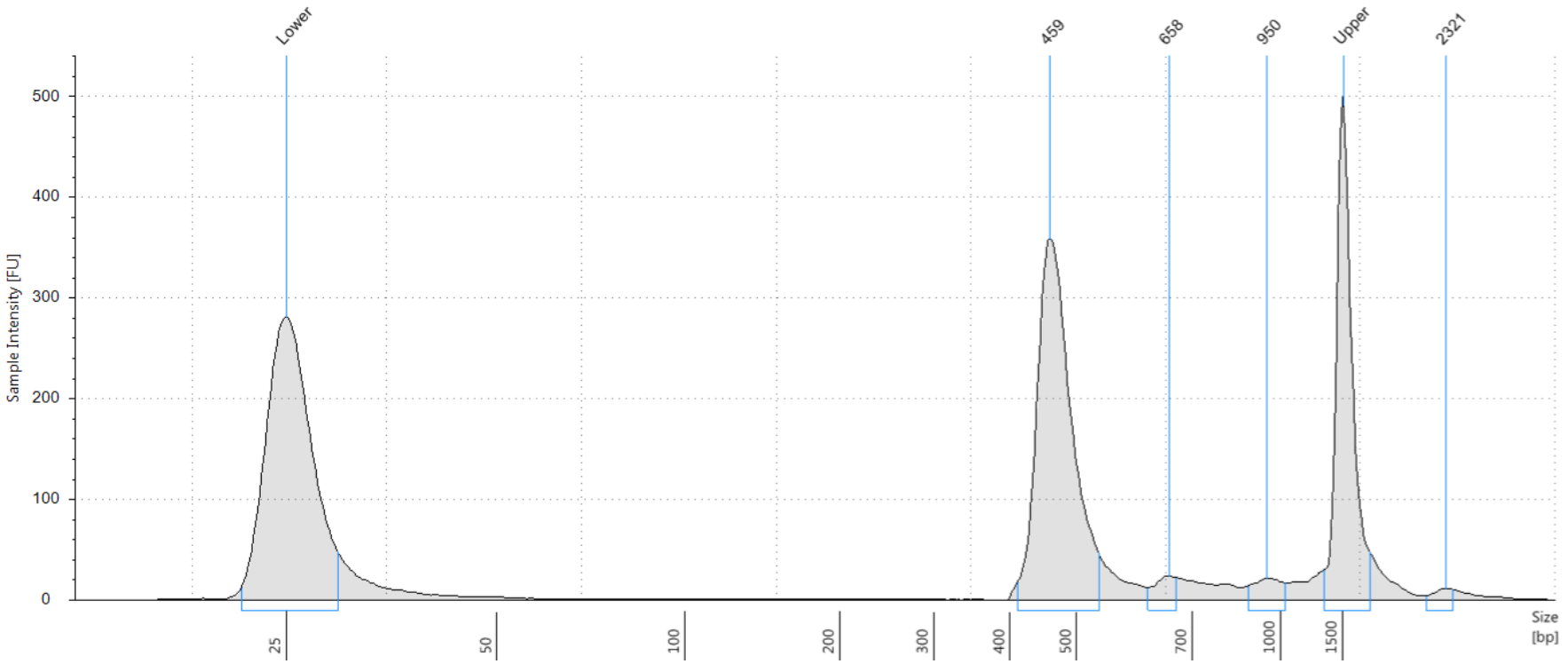
Sample ID	Sample name	Vol sample (ul)	Vol H2O (ul)	Concentration
JL01	WT virus, subamplicon 1	4	1	0.5 ng/ul
JL02	WT virus, subamplicon 1, dil.	0.8	4.2	0.1 ng/ul
JL03	Mutvirus lib3-2, subamplicon 6	3.2	1.8	0.5 ng/ul
JL04	Water only neg ctrl	-	5	-
JL05	MD experiment 1, subamplicon 1	0.68	4.32	0.5 ng/ul
JL06	MD experiment 1, subamplicon 6	0.97	4.03	0.5 ng/ul



Sample Info

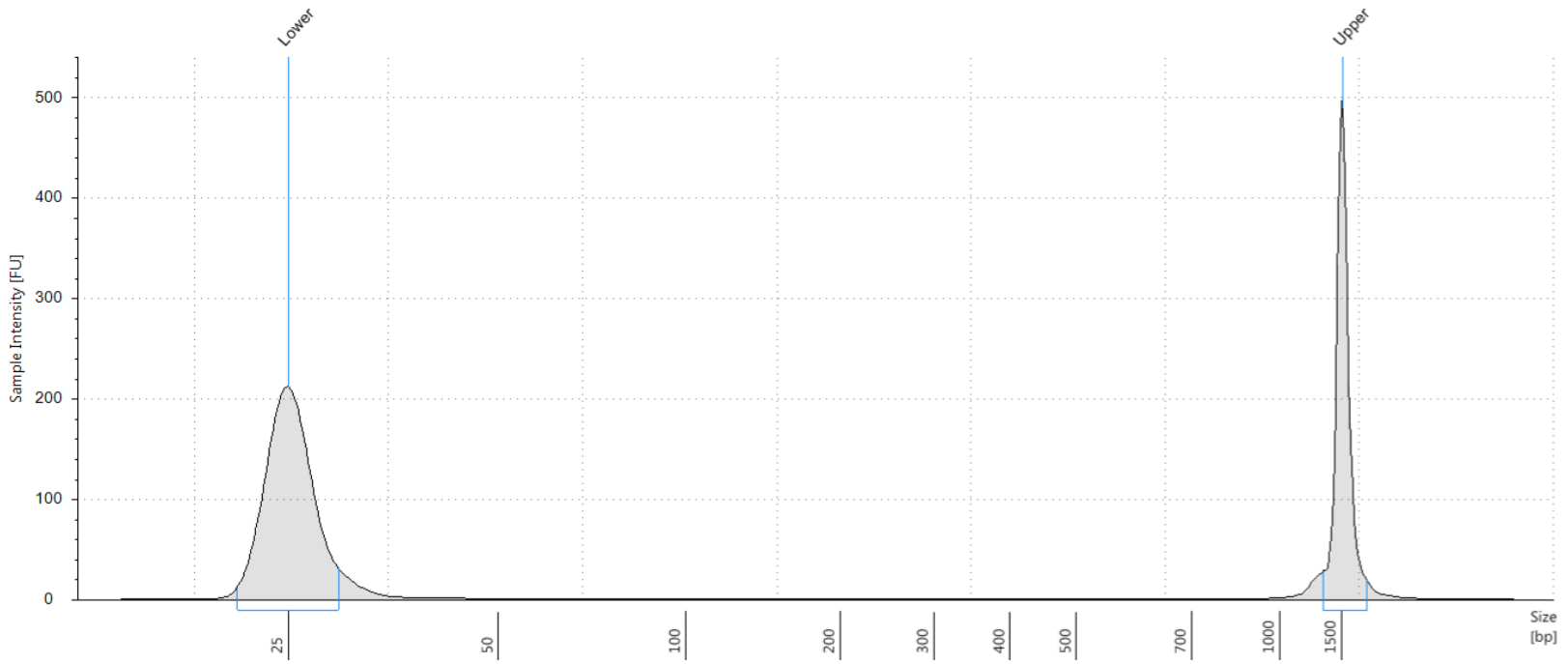
Well	Conc. [pg/µl]	Sample Description	Alert	Observations
L2	2420	Ladder		Ladder
G8	426	JL01		
H8	87.6	JL02		
A9	296	JL03		
B9		JL04		
C9	533	JL05		
D9	105	JL06		

G8: JL01



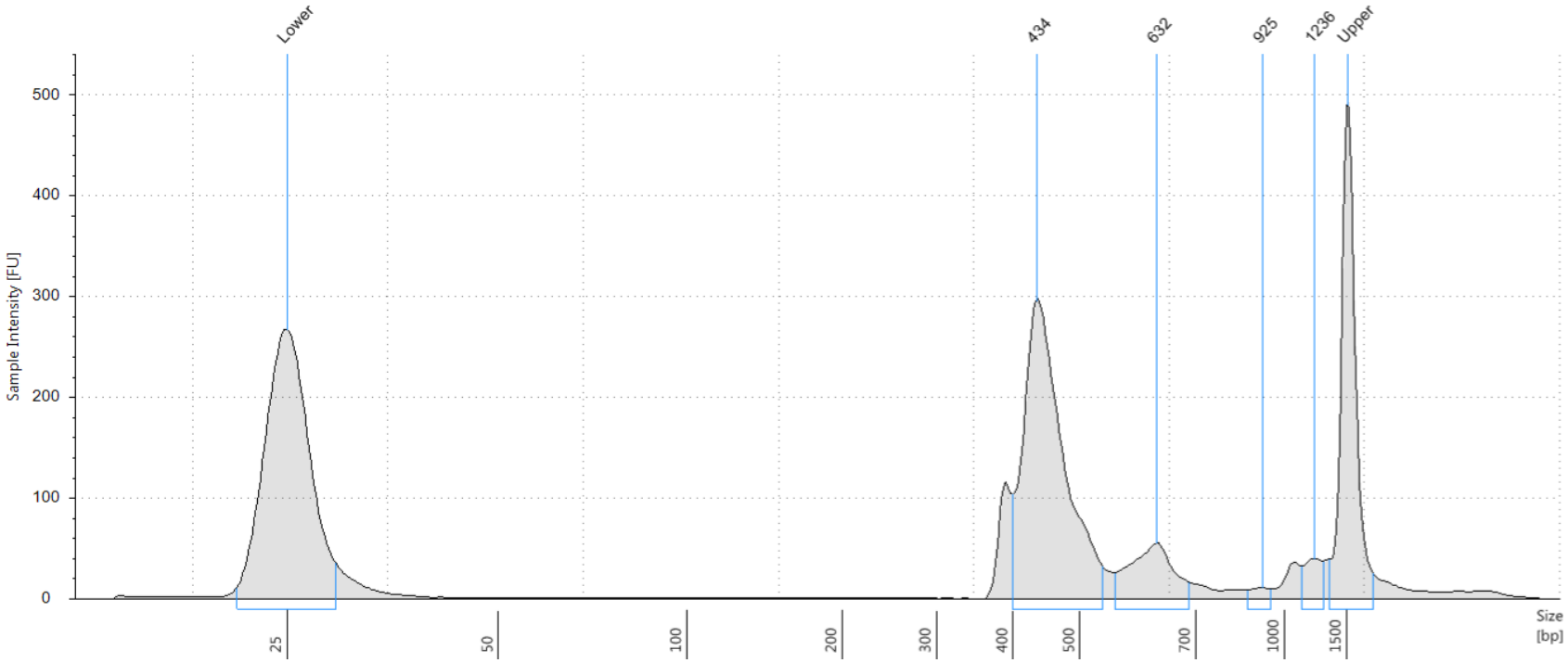
Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	404	-	24900	-		Lower Marker
459	387	-	1300	90.75		
658	14.7	-	34.5	3.46		
950	18.7	-	30.2	4.38		
1500	250	250	256	-		Upper Marker
2321	6.04	-	4.00	1.42		

B9: JL04



Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	401	-	24700	-		Lower Marker
1500	250	250	256	-		Upper Marker

C9: JL05



Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	422	-	26000	-		Lower Marker
434	419	-	1490	78.70		
632	81.1	-	198	15.23		
925	6.99	-	11.6	1.31		
1236	25.4	-	31.6	4.76		
1500	250	250	256	-		Upper Marker

February 8, 2017

Round 1 PCR. Diluted Ampure-purified full-length PCR products to 0.5 ng/ul. Added the following volumes of each sample to 70 ul water in row A of the corresponding column of a new 96-well PCR plate

Column	1	2	3	4	5	6	7	8	9	10
Sample	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Well on Ampure-purified amplicons plate taken from	C3	C4	C5	C6	C7	C8	E3	E4	E5	E6
Volume added to 70 ul water (ul)	1.81	1	2.36	10.75	1.29	~20	0.95	1.06	1.25	1.26

For each subamplicon primer pair, made a mix in a strip tube, from #1-6, for each subamplicon primer pair #1-6, respectively. I added 11 ul F primer (10 uM) + 11 ul R primer (10 uM) + 22 ul water. This final mixture is 44 ul and each primer will be at 2.5 uM final concentration. I then added 4 ul of each primer mix to each PCR reaction.

Each round 1 PCR reaction is set up as follows (total volume = 24 ul)
12 ul 2X KOD Hot-Start MM
2 ul of 5 uM forward primer
2 ul of 5 uM reverse primer
8 ul of 0.5 ng/ul template (4 ng total)

Added 130 ul of a new tube of 2X KOD MM to the first 7 tubes of an 8-strip. Using a multichannel, added 12 ul of the KOD MM column-wise.
Then added primers one column at a time from the primer mix strip tube (4 ul added column-wise).
Then added 0.5 ng/ul templates one row at a time (8 ul added row-rise).
Seal with microfilm A, spin briefly, and run “HAsubampR1” under “^^ike” to run 9 total PCR cycles:

1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 54°C for 20 s, cooling at 0.5°C/s
5. 70°C for 20 s
6. Go to 2, 8 times
7. 95°C for **1 min** (this step to ensure identical pairs are not annealed at the end)
8. 4°C forever

Template:		*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:		1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1	A												
Subamplicon2	B												
Subamplicon3	C												
Subamplicon4	D												
Subamplicon5	E												
Subamplicon6	F												
no primers	G												
	H												

Ampure purification of Round 1 products.

After PCR was finished, added 26 ul water to each well to bring the volume to 50 ul.

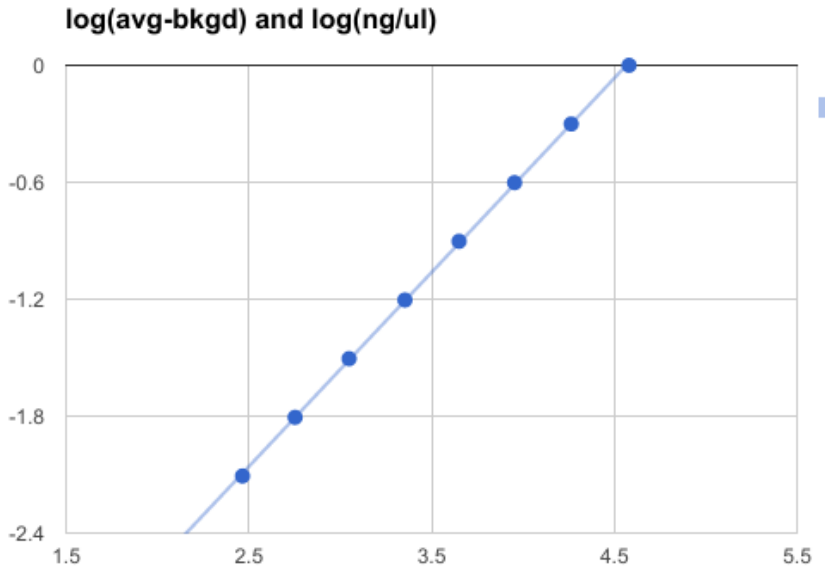
Purified PCR products with 1X Ampure beads.

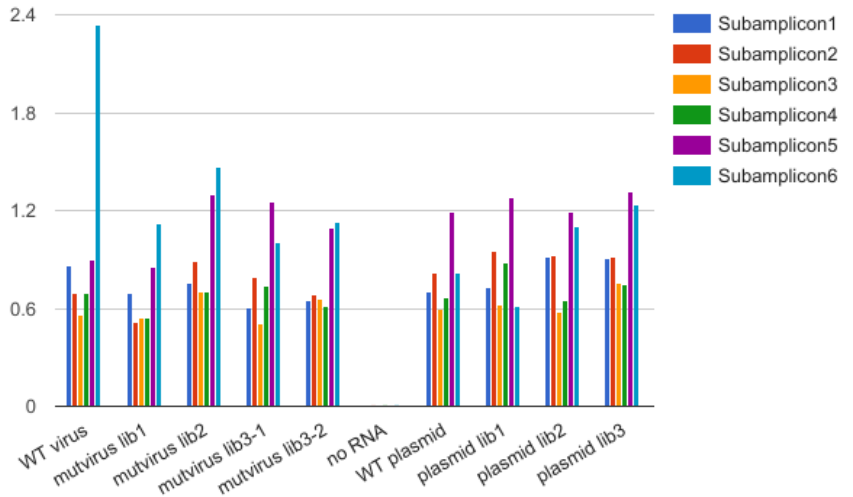
1. Added 50 ul beads from 3.7 ml aliquot to each well and mixed by pipetting 20X's
2. Incubated at RT for 10 min to bind
3. Placed on magnet for 5 min
4. Aspirated
5. Washed twice with 190 ul freshly made 80% ethanol
6. Air dried 10 min
7. Removed from rack, and dispersed beads in 75 ul EB
8. Incubated for 5 min to resuspend DNA
9. Incubated on magnet for 5 min
10. Transferred supernatant to a new 96-well plate in the same set-up as the round 1 PCR plate. Used 10 ul for picogreen, and stored the remainder at -20°C

Picogreen of Ampure-purified Round 1 products.

Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 100 ul TE to row H (H1-H10) and columns 11 & 12. Added 90 ul TE to remaining wells (wells that will receive PCR products for a final 1:10 dilution).
Made standard curve by serially transferring 100 ul of the 2 ng/ul into A11 and A12 and then down rows.
Transferred 10 ul of the purified Round 1 PCR products to the picogreen plate for a final dilution of 1:10.
Made a 1:200 dilution of picogreen (55 ul to 11 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

Template:		*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*		
Primer pair:		1	2	3	4	5	6	7	8	9	10	11	12
Subamplicon1	A	10 ul of purified rnd1 products to 90 ul TE (to measure 1:10 dilution)										1.00E+00	1.00E+00
Subamplicon2	B											5.00E-01	5.00E-01
Subamplicon3	C											2.50E-01	2.50E-01
Subamplicon4	D											1.25E-01	1.25E-01
Subamplicon5	E											6.25E-02	6.25E-02
Subamplicon6	F											3.13E-02	3.13E-02
no primers	G											1.56E-02	1.56E-02
	H	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	7.81E-03	7.81E-03





ng/ul of purified Round 1 product

	WT virus	mutvirus lib1	mutvirus lib2	mutvirus lib3-1	mutvirus lib3-2	no RNA	WT plasmid	plasmid lib1	plasmid lib2	plasmid lib3
Subamplicon1	0.862	0.690	0.757	0.603	0.650	0.005	0.704	0.727	0.914	0.909
Subamplicon2	0.698	0.517	0.886	0.790	0.684	0.006	0.817	0.950	0.926	0.919
Subamplicon3	0.562	0.542	0.701	0.504	0.662	0.005	0.592	0.618	0.579	0.754
Subamplicon4	0.697	0.538	0.698	0.741	0.614	0.007	0.667	0.884	0.646	0.750
Subamplicon5	0.894	0.854	1.297	1.257	1.097	0.004	1.188	1.276	1.191	1.319
Subamplicon6	2.339	1.119	1.466	1.007	1.132	0.008	0.818	0.610	1.106	1.233
No primers	0.007	0.005	0.009	0.006	0.008	-	0.008	0.008	0.008	0.012

The concentrations are now usable – although they are still somewhat low (many below the 1-2 ng/ul range), these are a lot higher than what I was getting the first two times I did round 1.
I will move forward with bottlenecking and Round 2 using these latest round 1 PCR products

February 9, 2017

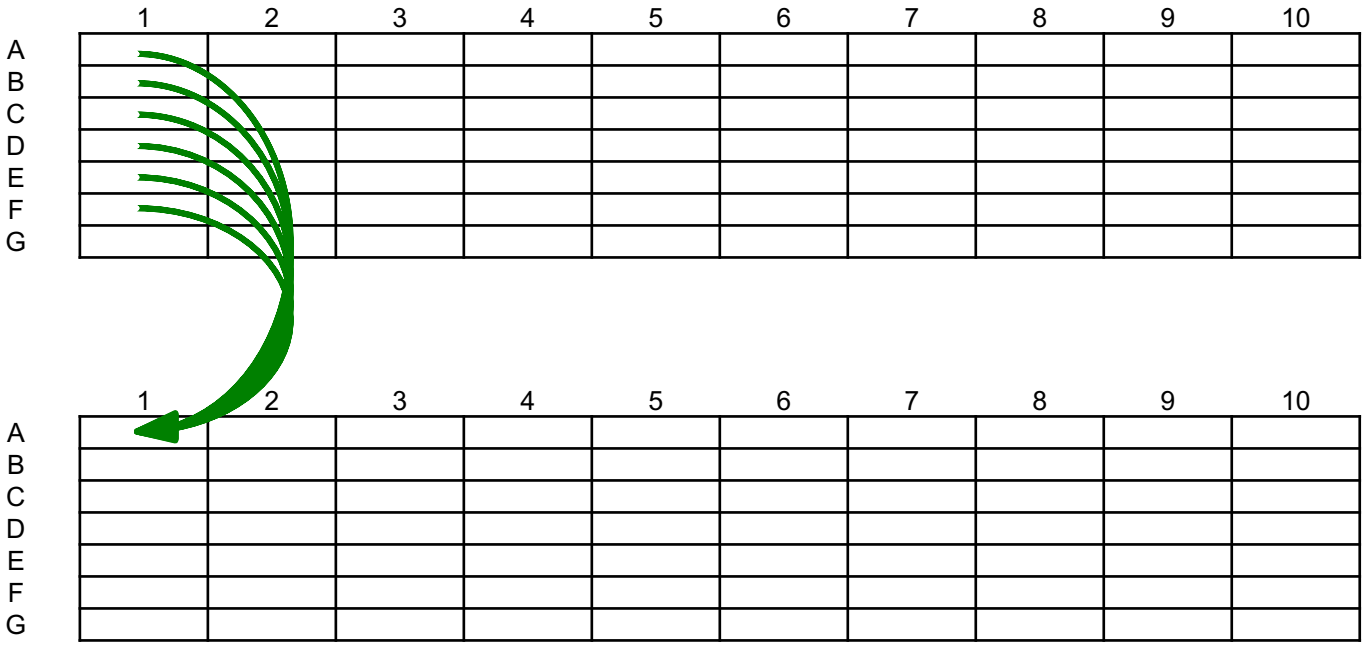
Dilute Round 1 PCR products to 0.5 ng/ul. Diluted Ampure-purified Round 1 PCR products to 0.5 ng/ul. First added the following volumes of water to each well, then **added 20 ul of purified round 1 PCR product** to each well using multichannel. Mixed with multichannel.

0.5 ng/ul dilutions		1	2	3	4	5	6	7	8	9	10
		WT virus	***mutvirus lib1***	***mutvirus lib2***	***mutvirus lib3-1***	***mutvirus lib3-2***	***no RNA***	***WT plasmid***	***plasmid lib1***	***plasmid lib2***	***plasmid lib3***
Subamplicon1	A	14.47	7.59	10.28	4.14	5.99	none	8.18	9.07	16.54	16.36
Subamplicon2	B	7.91	0.7	15.44	11.61	7.35	none	12.69	18.02	17.03	16.78
Subamplicon3	C	2.47	1.67	8.06	0.16	6.47	none	3.68	4.73	3.17	10.15
Subamplicon4	D	7.87	1.54	7.94	9.66	4.55	none	6.69	15.34	5.86	10.01
Subamplicon5	E	15.76	14.17	31.87	30.27	23.89	none	27.5	31.04	27.65	32.76
Subamplicon6	F	73.56	24.78	38.66	20.27	25.3	none	12.74	4.4	24.22	29.3
No primers	G	none	none	none	none	none	none	none	none	none	none

Pool all 6 subamplicons for each sample into a single well.

Using a new 96-well PCR plate, pooled the six subamplicons for a single sample into one well, using row A for each of the pooled samples. Did NOT add the no primer control row to the pool (therefore, pool only rows A-F)!!! Pooled by transferring 10 ul of each sample from row A using the multichannel into row A of the new 96-well plate. Then transferred 10 ul from row B of the 0.5 ng/ul dilution plate into row A of the pooled plate. Continued this to row F.

Using a P200 pipet set to 60 ul, I made sure each well had 60 ul of volume, to make sure that I pipetted each row and pooled all the samples correctly.



BOTTLENECK CALCULATION

~1.5e8 paired end reads from a lane of a HiSeq x 1/9 samples x 1/6 subamplicons = 2.78E6 PE reads / SA / sample

BOTTLENECK TO **7e5** barcodes per subamplicon, corresponding to 3.9 reads per barcode

7e5 barcodes per SA per sample = 3.5e5 dsDNA molecules per subamplicon per sample

3.5e5 dsDNA molecules per subamplicon per sample * 6 subamplicons = 2.1e6 dsDNA molecules per sample

(product length = primer binding region + informative sequence length + 72 bases of barcode and adaptor sequence after round 1)
Subamp1: 60 + 285 + 72 = 417
Subamp2: 49 + 282 + 72 = 403
Subamp3: 46 + 285 + 72 = 403
Subamp4: 47 + 285 + 72 = 404
Subamp5: 47 + 285 + 72 = 404
Subamp6: 65 + 279 + 72 = 416
Average round 1 product length = 408 bp

So each round 2 PCR gets 2.1e6 dsDNA molecules of pooled sample = 0.000925 ng of dsDNA 408 bp long.

Bottleneck to 7e5 barcodes per subamplicon.
Using the pooled plate of round 1 products for each sample in row A at 0.5 ng/ul, made three consecutive 1:16 dilutions by transferring 10 ul to 150 ul water.
Row D will then be 1.22E-4 ng/ul, so that **7.578 ul of the Row D dilution** can be used in each round 2 PCR reaction to provide 9.25E-4 ng of template DNA ☺

1:16 dilutions		1	2	3	4	5	6	7	8	9	10
		WT virus	***mutvirus lib1***	***mutvirus lib2***	***mutvirus lib3-1***	***mutvirus lib3-2***	***no RNA***	***WT plasmid***	***plasmid lib1***	***plasmid lib2***	***plasmid lib3***
Subamplicon1	A	0.5 ng/ul									
Subamplicon2	B	1:16 dilution of row A									
Subamplicon3	C	1:16 dilution of row B									
Subamplicon4	D	1:16 dilution of row C = 1.22E-4 ng/ul									
Subamplicon5	E										
Subamplicon6	F										
No primers	G										

Use 7.58 ul of row D as template in round 2

Round 2 PCR. Set up round 2 PCR.
Used 5 uM stocks of Rnd2ForUniversal (100 uM stock from Adam) and Round 2 reverse primers from Mike.

*Note WT plasmid is repeated in column 11 as no primer control

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid*
	1	2	3	4	5	6	7	8	9	10	11
A						Use NF01					NO primers
B											
C											
D											
E											
F											
G											
H											

Rnd2 Primers:

Sample	Round 2 reverse primer	Illumina barcode number	NextFlex barcode number	
WT virus	NF01 Rnd2Rev	Illumina 2	nextflex 1	CAAGCAGAAGACGGCATACGAGAT**acatcg**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 1	NF03 Rnd2Rev	Illumina 5	nextflex 3	CAAGCAGAAGACGGCATACGAGAT**cactgt**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 2	NF08 Rnd2Rev	Illumina 3	nextflex 8	CAAGCAGAAGACGGCATACGAGAT**gcctaa**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 3-1	NF09 Rnd2Rev	Illumina 8	nextflex 9	CAAGCAGAAGACGGCATACGAGAT**tcaagt**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 3-2	NF10 Rnd2Rev	Illumina 9	nextflex 10	CAAGCAGAAGACGGCATACGAGAT**ctgatc**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
WT plasmid	NF11 Rnd2Rev	Illumina 10	nextflex 11	CAAGCAGAAGACGGCATACGAGAT**aagcta**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib1	TS12Rnd2rev	Illumina 12	nextflex 6	CAAGCAGAAGACGGCATACGAGAT**tacaag**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib2	NF29Rnd2rev		nextflex 29	CAAGCAGAAGACGGCATACGAGAT**tagttg**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib3	TS06Rnd2rev	Illumina 6	nextflex 4	CAAGCAGAAGACGGCATACGAGAT**attggc**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Each Round 2 PCR: (40 ul total)
20 ul 2X KOD Hot-Start Master Mix
4 ul of 5 uM Rnd2ForUniversal
4 ul of 5 uM of the appropriate Rnd2RevIndex
7.58 ul of 1.22E-4 ng/ul dilutions of purified Round1 products
4.42 ul water

Sealed with Microfilm A, spun briefly, and ran “Round2” under “^^ike” for a total of 24 cycles:
1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 55°C for 20 s
5. 70°C for 20 s
6. Go to 2, 23 times
7. 4°C forever

Analytical gel of Round2 PCR products.

After PCR, ran a 1% EtBr-agarose gel. Used 4 ul PCR products + 6 ul water + 2 ul 6X buffer.
Made a 72 ul water + 24 ul 6X loading dye master mix, aliquoted 8 ul of the master mix to 11 tubes, and added 4 ul PCR products to each tube.
Ran gel at 120 V for 1 h 20 min.
Expect product sizes (round 2 adds 30 bp with F primer and 36 bp with R primer = 66 bp)
Subamp1: 417 + 66 = 483 bp
Subamp2: 403 + 66 = 469 bp
Subamp3: 403 + 66 = 469 bp
Subamp4: 404 + 66 = 470 bp
Subamp5: 404 + 66 = 470 bp
Subamp6: 416 + 66 = 482 bp
Did not see any bands! Perhaps round 2 didn't work? I will do the ampure purification and picogreen to see if any DNA is there.

Ampure purification of Round2 products.

Purified round2 products with 1X beads. Added 36 ul beads to each tube and mixed. Adsorbed for 10 min, placed on magnet for 5 minutes, then aspirated.
Washed twice with 190 ul 80% ethanol, dried for 10 min, removed from magnet, and resuspended in 60 ul EB.
Proceeded with Picogreen of ampure-purified products, and stored remainder at -20°C

Picogreen of Ampure-purified Round 2 products.

Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 98 ul 1X TE to A1-B11 to dilute round2 products. Added 100 ul TE to rows D, F, and G
Made standard curve by serially transferring 100 ul of the 2 ng/ul into F1/G1, and then across columns.
Transferred 2 ul of the purified Round 2 PCR products to the picogreen plate for a final dilution of 1:50.
Made a 1:200 dilution of picogreen (35 ul to 7 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid no primers*	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	
B	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	
C												
D	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE
E												
F	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		
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		
H												

Sample	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid no primers*
ng/ul	18.27	7.80	5.19	7.40	5.67	0.02	17.16	14.96	14.46	15.47	0.00

These concentrations seem rather low compared to what Mike and I had previously gotten from round 2 (usually in the 20-25 ng/ul range). Not sure what happened here, but I will repeat round 2 to see if I can get better yields than these.

Round 2 PCR, ATTEMPT #2. Set up round 2 PCR.
Using pooled round 1 products, made new serial 1:16 dilutions in a new 96-well plate

*Note WT plasmid is repeated in column 11 as no primer control

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid*
	1	2	3	4	5	6	7	8	9	10	11
A						Use NF01					NO primers
B											
C											
D											
E											
F											
G											
H											

Rnd2 Primers:

Sample	Round 2 reverse primer	Illumina barcode number	NextFlex barcode number	
WT virus	NF01 Rnd2Rev	Illumina 2	nextflex 1	CAAGCAGAAGACGGCATACGAGAT**acatcg**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 1	NF03 Rnd2Rev	Illumina 5	nextflex 3	CAAGCAGAAGACGGCATACGAGAT**cactgt**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 2	NF08 Rnd2Rev	Illumina 3	nextflex 8	CAAGCAGAAGACGGCATACGAGAT**gcctaa**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 3-1	NF09 Rnd2Rev	Illumina 8	nextflex 9	CAAGCAGAAGACGGCATACGAGAT**tcaagt**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mutvirus 3-2	NF10 Rnd2Rev	Illumina 9	nextflex 10	CAAGCAGAAGACGGCATACGAGAT**ctgatc**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
WT plasmid	NF11 Rnd2Rev	Illumina 10	nextflex 11	CAAGCAGAAGACGGCATACGAGAT**aagcta**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib1	TS12Rnd2rev	Illumina 12	nextflex 6	CAAGCAGAAGACGGCATACGAGAT**tacaag**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib2	NF29Rnd2rev		nextflex 29	CAAGCAGAAGACGGCATACGAGAT**tagttg**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Plasmid lib3	TS06Rnd2rev	Illumina 6	nextflex 4	CAAGCAGAAGACGGCATACGAGAT**attggc**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Each Round 2 PCR: (40 ul total)
20 ul 2X KOD Hot-Start Master Mix
4 ul of 5 uM Rnd2ForUniversal
4 ul of 5 uM of the appropriate Rnd2RevIndex
7.58 ul of 1.22E-4 ng/ul dilutions of purified Round1 products
4.42 ul water

Sealed with Microfilm A, spun briefly, and ran “Round2” under “^^ike” for a total of 24 cycles:
1. 95°C for 2 min
2. 95°C for 20 s
3. 70°C for 1 s
4. 55°C for 20 s
5. 70°C for 20 s
6. Go to 2, 23 times
7. 4°C forever

Did not run a gel, and just proceeded with ampure-purification and picogreen of Round 2 products

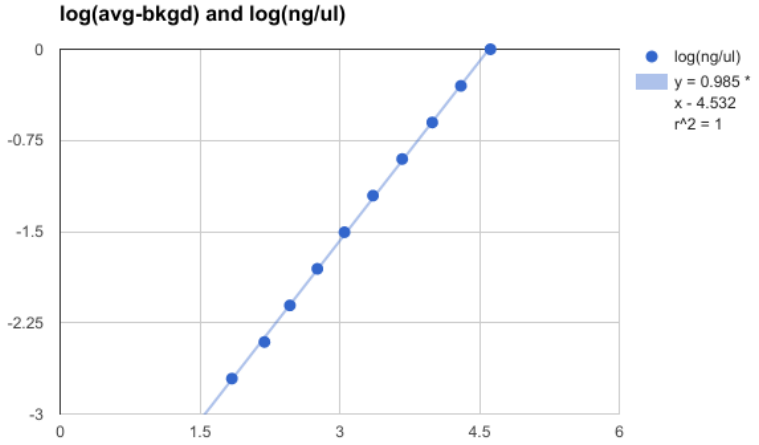
Ampure purification of Round2 products.

Purified round2 products with 1X beads. Added 40 ul beads to each tube and mixed. Adsorbed for 10 min, placed on magnet for 5 minutes, then aspirated.
Washed twice with 190 ul 80% ethanol, dried for 10 min, removed from magnet, and resuspended in 60 ul EB.
Proceeded with Picogreen of ampure-purified products, and stored remainder at -20°C

Picogreen of Ampure-purified Round 2 products.

Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 98 ul 1X TE to A1-B11 to dilute round2 products. Added 100 ul TE to rows D, F, and G
Made standard curve by serially transferring 100 ul of the 2 ng/ul into F1/G1, and then across columns.
Transferred 2 ul of the purified Round 2 PCR products to the picogreen plate for a final dilution of 1:50.
Made a 1:200 dilution of picogreen (35 ul to 7 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

Template:	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid no primers*	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	
B	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50	
C												
D	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE
E												
F	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		
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		
H												



Sample	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid no primers*
ng/ul	33.39	26.69	27.98	29.40	24.40	0.04	25.91	26.58	22.64	23.50	0.00

February 15, 2017

Pool purified Round2 products. Pooled the purified round 2 products using 125 ng of each sample. Did NOT include the no RNA ctrl and WT plasmid no primers ctrl in the pool. Made two separate pools.

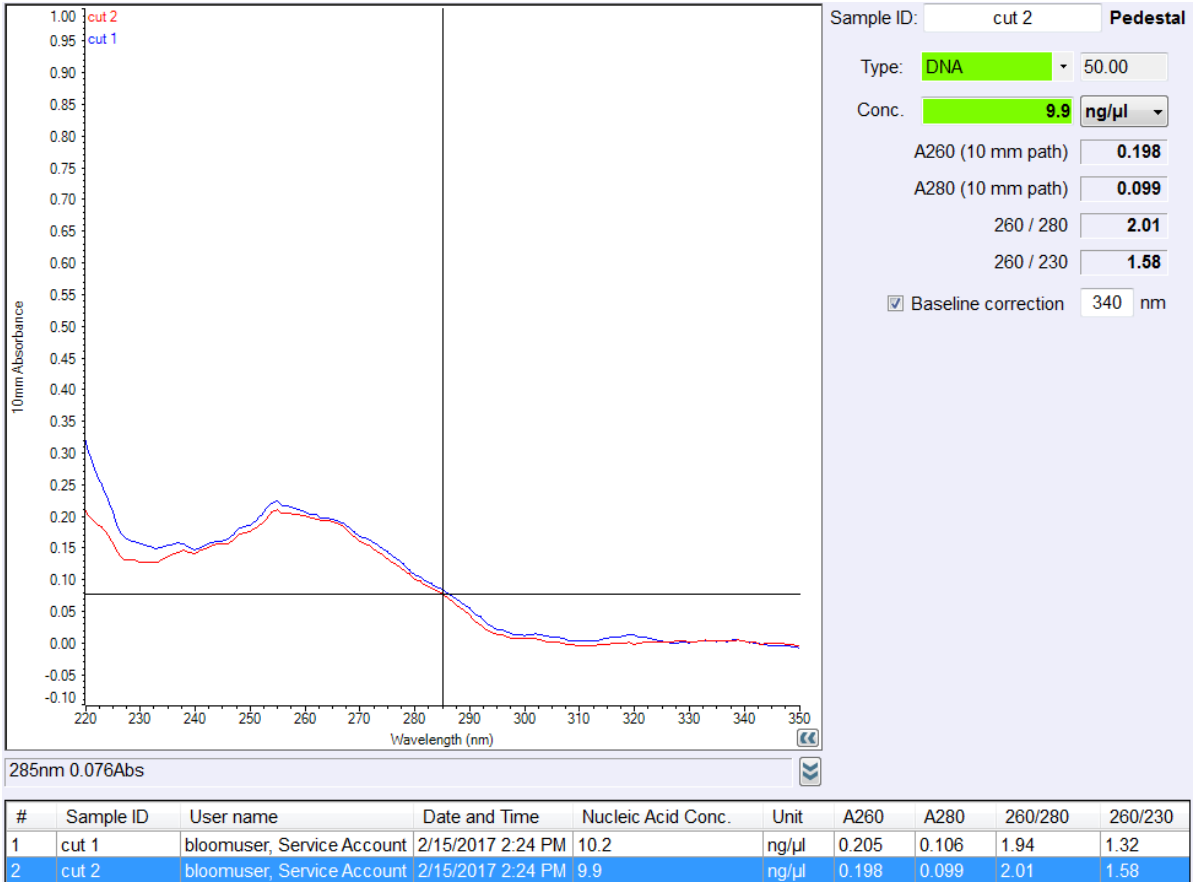
Total volume of pool = 42.64 ul

Sample	*WT virus*	*mutvirus lib1*	*mutvirus lib2*	*mutvirus lib3-1*	*mutvirus lib3-2*	*no RNA*	*WT plasmid*	*plasmid lib1*	*plasmid lib2*	*plasmid lib3*	*WT plasmid no primers*
ng/ul	33.39	26.69	27.98	29.40	24.40	0.04	25.91	26.58	22.64	23.50	0.00
Vol (ul)	3.74	4.68	4.47	4.25	5.13		4.83	4.70	5.52	5.32	

Agarose gel of pooled Round2 products. Ran a 1% SYBR-safe gel of the two pooled Round 2 products.

Added **8.53 ul of 6X loading dye** to each pool, and loaded ~52 ul of the final pool + dye mix in each lane. Skipped a lane between the 100 bp ladder and the two pooled samples since I will be excising bands. Ran gel at 100 V for 1 h 30 min. For both pooled samples, saw a band at ~500 bp, which is the expected product size (did not take a picture of the gel).

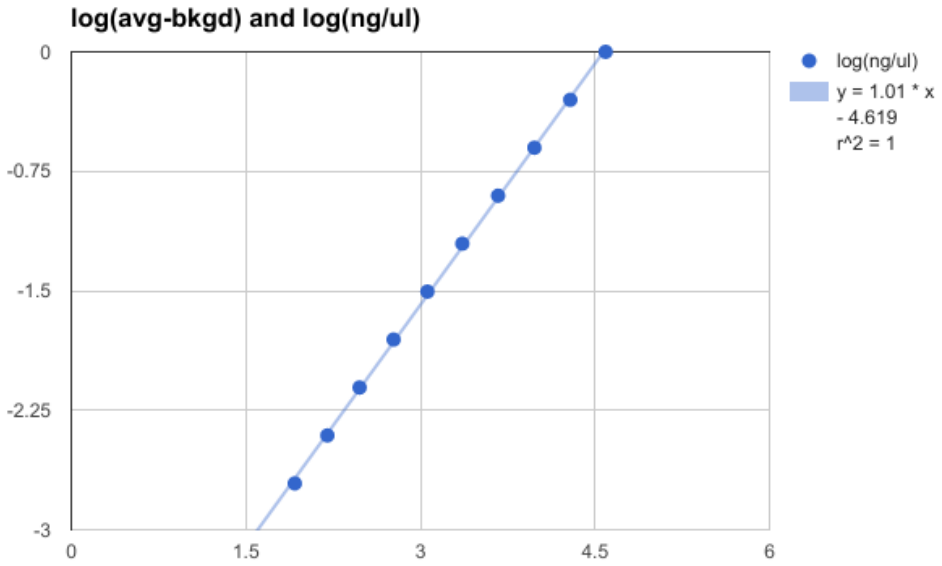
Excised both bands, and did a Zymo gel extraction of both bands using a fresh kit. Added 1 ml ADB buffer to each tube, melted gel slice at 42°C, and **eluted in 50 ul water** for each. Nanodropped. Concentrations look OK, but 260/230 peaks show some contamination, so I will ampure purify the gel-extracted product and picogreen.



Ampure purification of gel-extracted pooled Round2 products.
Purified both gel-extracted pooled products with 1.58X beads. Added 79 ul beads to each tube (containing ~50 ul sample) and mixed. Adsorbed for 10 min, placed on magnet for 5 minutes, then aspirated. Washed twice with 190 ul 80% ethanol, dried for 10 min, removed from magnet, and resuspended in 50 ul EB. Proceeded with Picogreen of ampure-purified products, and stored remainder at -20°C in non-stick 1.5 ml microtubes.

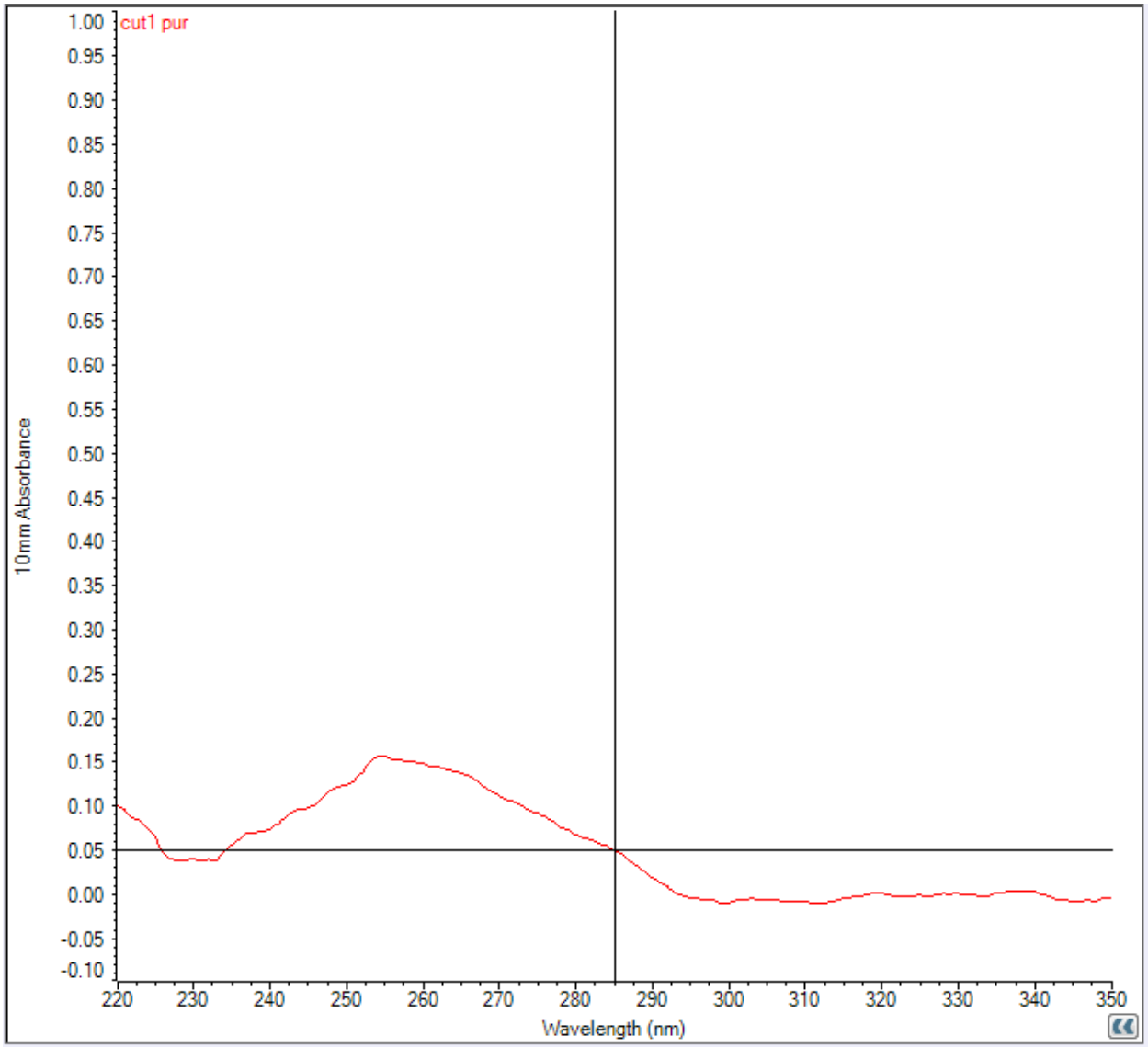
Picogreen of Ampure-purified pooled products.
Made two standard solutions at 2 ng/ul by diluting 4 ul of the standard to 196 ul 1X TE.
Added 98 ul 1X TE to A1-A2 & B1-B2 to dilute pooled products. Added 100 ul TE to rows D, F, and G
Made standard curve by serially transferring 100 ul of the 2 ng/ul into F1/G1, and then across columns.
Transferred 2 ul of the purified pooled products to the picogreen plate for a final dilution of 1:50.
Made a 1:200 dilution of picogreen (20 ul to 4 ml 1X TE) and added 100 ul to each well. Incubated for 5 min, read on plate reader

	1	2	3	4	5	6	7	8	9	10	11	12
A	98 ul TE + 2 ul cut1	98 ul TE + 2 ul cut1										
B	98 ul TE + 2 ul cut2	98 ul TE + 2 ul cut2										
C												
D	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE	100 ul TE
E												
F	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		
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		
H												



Sample	ng/ul
Pooled cut1	6.25
Pooled cut2	5.95

Nanodrop of ampure-purified cut1 product on next slide



285nm 0.048Abs

Sample ID: cut1 pur **Pedestal**

Type: DNA 50.00

Conc. **7.3** **ng/µl**

A260 (10 mm path) **0.146**

A280 (10 mm path) **0.065**

260 / 280 **2.24**

260 / 230 **3.89**

☑ Baseline correction 340 nm

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230
1	cut1 pur	bloomuser, Service Account	2/15/2017 4:17 PM	7.3	ng/µl	0.146	0.065	2.24	3.89

March 6, 2017

Prepare purified pooled library for HiSeq sample submission to Hutch Genomics Core. I will submit purified pooled cut1 at 1.96 nM (Heather and Hugh will spike in each of their samples at 1% for 1 M reads each, while I will get the remainder of reads).

For 2.27 nM in 26 ul, want 18.16 ng of DNA (for 474 bp dsDNA)
Made a mixture of 2.91 ul pooled cut1 + 23.09 ul EB in a 0.5-ml RNase-free low-stick tube.
Attached with label “Juhye pooled-lib 170306 HiSeq”. I added 2 ul of Hugh’s sample and 2 ul of Heather’s sample for a total of 30 ul, and submitted to the genomics core.