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DNA ladder used throughout this notebook.



## January 2023

### Thursday, January 12, 2023

1. ~~MHB~~
2. ~~Alphabetize chemicals~~
3. ~~Clean flasks~~

### Muller Hinton Broth (250 mL)

- 5.25 g of MHB
- 250 mL of diiH2O
- Spin
- Autoclave for liquid 30'

Made 2 x 250 mL.

### Monday, January 16, 2023

1. ~~Meet with Dr. Ramsey~~

Meeting Notes with Dr. Ramsey:

- Topotia kit
- Validate library with the kit
  - o Finish making the library
  - o PCR product (lib) perform the topo rxn using the topo kit
  - o Take rxn transform into E. coli cells (in kit)
  - o Pick colonies after transformation
  - o Miniprep
  - o Send for sequencing
- Provided it works
  - o Sequence on MiSeq with Janet
- Data analysis
  
- Review the topo kit
- 12 GP1002

### Tuesday, January 17, 2023

1. ~~Make CHA mix~~

### Wednesday, January 18, 2023

2. ~~Make hemoglobin~~
3. ~~Take out trash~~
4. ~~Save fall notebook as pdf~~
5. ~~Submit NIH!~~
6. 50x TAE

### Hemoglobin

- 6 g heme
- 300 mL diiH2O

- Liquid 20 min

### 50x TAE Buffer

- 242 g Tris Base in diH<sub>2</sub>O (Trizma Base)
  - o Added 400 mL to dissolve in
  - o Use heat to dissolve
- 57.1 mL of acetic acid
- 100 mL 0.5 M EDTA (pH 8.0)

Adjust to 1 L volume.

### Tuesday, January 24, 2023

- ~~1. Kanamycin aliquots~~
- ~~2. Ran the gel!~~

Made 20 Kanamycin 50 ug/mL aliquots.

- It is in a small red cap container in the door of the big refrigerator
- Made in a 50 mL conical
- The filters and the syringes are above Hannah's bench.

### 2% Agarose for Big Gel Rig

- Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
- Add stirbar to container.
- Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
- Let the 2% agarose solution cool to approximately 50-55°C.

### Wednesday, January 25, 2023

- ~~1. Gel Purification~~

- From the protocol paper:
- Clean up the sample using QIAquick gel purification columns according to the manufacturer's instructions. Be certain that all of the ethanol-containing wash buffer is removed from the column before elution by pipetting around the inner rim with a 10-uL tip, if necessary. Elute in 32 uL of buffer EB. Gel purification ensures that primer-dimers or other erroneous products do not contribute to the DNA quantification or sequencing in subsequent steps.

### Gel Purification

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.

6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 40uL Buffer 0.1 x EB and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm. Do this twice.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
Input Library	290 mg	870 ul	290 ul

### Thursday, January 25, 2023

1. ~~Meet with Janet and Kathryn~~

### Notes from meeting

- 125 bp
- Gel extraction – not clean
- Illumina linkers
- 1 uL for qPCR
- QuBit = fluorometric binds to double stranded DNA, super sensitive
  - o More accurate
  - o Nanodrop can overestimate
- qPCR – primers match the end
  - o DNA that has the linkers
  - o Functional library
  - o Helps ensure that the adapters and the linkers are there
  - o Doesn't tell us that the DNA isn't just the same exact sequence
  - o Quantity
  - o Based on 399 bp
  - o Have to size adjust the library (normalize)
- TA-Clone
  - o Ensure that the DNA is what we want
  - o Cloning
- Bioanalyzer
  - o Doesn't quantity
  - o Size (estimated)
  - o DNA – can't tell if there is the right product
- MiSeq Questions:
  - o Low diversity?
  - o V2 vs V3 (chemistry)
  - o V2 – under clustered with low diversity 33
  - o V3 -
  - o Nano kit – flow cell, where they black out 80% of the cell
  - o Indexes (barcode) – 6
    - Specific barcodes – so we can sequence together
    - Region where the DNA sequencing (in the adaptor)

- Prada/puritz
  - o Blue pipin (gel extractor automatic)
- Take 5 uL out of gel extraction (for TA-Clone)
- Tuesday: if no kit – start the next step after linear PCR
  - o If kit – do the cloning

#### TA KIT:

- Invitrogen pCR4-TOPO TA Vector
- 5 min rxn with 95% recombinants
- Disrupts the lethal *E. coli* gene, *ccdB*
- Minimal multiple cloning site to shorten the distance
- Both amp and kan resistance
- LacZalpha-*ccdB* gene fusion for positive selection
- The polymerase
  - o Extensive 3' to 5' exonuclease activity (Platinum SuperFi) do not leave 3' A-overhangs

### Tuesday, January 31, 2023

1. ~~2% hemoglobin~~
2. ~~Prep for tomorrow~~

### Hemoglobin

- 6 g heme
- 300 mL diH<sub>2</sub>O
- Mix for 10 minutes minimally
- Liquid 20 min
- Made 4x 300mL flasks

#### Dilute the dNTPs:

- Add 2.5 uL of water with 2.5 uL of dNTPs (2.5 mM). Mix well.
- For the 1:2 dilution remove 2.4 uL into the mix tomorrow.

## February 2023

### Wednesday, February 1, 2023

1. ~~TOPO TA~~

### PCR to add 3' A Overhang

Component	1 rxn volume
DNA Template	3
10X PCR Buffer	1.5
2.5 mM dNTPs (0.2 mM)	2.4
Water	7.9
<i>Taq Polymerase</i>	0.2

Total Volume	15
--------------	----

- Add the above volumes to a tube.
- Incubate in the thermocycler for 20 minutes at 72C
- During this 20 minute, put the plates in the incubator to warm and closer to the end of the 20 minutes, start to thaw the chemically competent cells. Turn on shaking incubator to 37C in the environmental room.

### TOPO Cloning Reaction

Reagent	Volume
Fresh PCR	4 uL
Salt Solution	1 uL
Water	0 uL
TOPO vector	1 uL
<b>Final Volume</b>	<b>6 uL</b>

1. Mix the reaction gently and incubate for **5 minutes** at room temperature
  - a. Can vary from 30 seconds to 30 minutes depending on size of PCR product
  - b. Our product is 125 bp (?) so 5 minutes should be sufficient
2. Place the reaction on ice and proceed to next step

### Chemical Transformation

- Vial with no DNA, vial with plasmid pKR6/7/8 pF with kanamycin w/o lamda pir requirement (ask Hannah)
1. Warm vial of S.O.C. medium to room temperature
  2. Warm selective plates at 37C for 30 minutes
  3. Thaw **on ice** one vial of One Shot cells for each transformation

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of Kanamycin - containing plates
1	(+) control	pKR8	2 uL	20 ul, 200 ul, remaining	3
2	(-) control	None	0	20 ul, 200 ul, remaining	3
3	TOPO-TA	Tn-Seq Lib 1	2 uL	20 uL, 200 uL, remaining	3
<b>Total number of plates</b>					<b>9</b>

4. Add 2 µL of the TOPO® Cloning reaction from Set up the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli and mix gently. Do not mix by pipetting up and down.
5. Incubate on ice for 5-30 minutes.

Note: Longer incubations on ice do not seem to affect transformation efficiency. The length of the incubation is at the user's discretion.

6. Heat-shock the cells for 30 seconds at 42°C without shaking.
7. Immediately transfer the tubes to ice.
8. Add 250 µL of room temperature S.O.C. medium.
9. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
  - a. Use the shaking incubator

10. Spread 10–50  $\mu$ L from each transformation on a pre-warmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20  $\mu$ L of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
  - a. 20  $\mu$ L, 200  $\mu$ L, and remaining
  - b. 9 plates total of LB kanamycin
  - c. Sierra spread these for me, thank you Sierra!!
11. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see Analyze positive clones on page 20).

### Thursday, February 2, 2023

- ~~1. Check transformation plates~~
- ~~2. Make more LB-Kan plates~~
- ~~3. Overnights~~

Got colonies! Yay

	20 $\mu$ L	200 $\mu$ L	R (~30 $\mu$ L)
Tn-Seq Lib 1	89	164	
Positive	Too many to count	Too many to count	Too many to count
Negative	None	None	none

### Analyzing Transformants

1. Pick 12 colonies and culture them overnight in LB containing 50  $\mu$ g/mL kanamycin
  - a. Normal overnight volumes?
  - b. 5 mL of LB ( $12 \times 5 = 60$  mL)
  - c. 5  $\mu$ L of kanamycin (60  $\mu$ L)
  - d. Pipette 5 mL into each test tube
2. Isolate plasmid DNA using the PureLink Quick Plasmid Miniprep
  - a. Use our miniprep kit?
3. Sanger Sequence?

### Friday, February 3, 2023

- ~~1. Pellet~~

1. Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes.
  - a. Removed all liquid since the next step is lysating.

### Tuesday, February 7, 2023

- ~~1. Miniprep~~
- ~~2. Receiving Primer M13~~
- ~~3. Sequencing~~

### MiniPrep

1. Added 250  $\mu$ L Buffer P1 to resuspend pelleted bacterial cells.
2. Added 250  $\mu$ L Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution was more translucent

3. Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
  - a. Makes like a white precipitate
4. Centrifuge for 10 minutes at 13,000 rpm
  - a. Prep primers
5. Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 1 minute. Discard flow-through. (x2 because double the initial volume)
6. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 minute. Discard flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 minute and discard flow-through 3 times (added 750ul of PE three times). Transfer to collection tube.
8. Centrifuge for 3 minutes to remove residual wash buffer.
9. Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 50 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.

**Nanodrop Results (1 ul)**

#	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	247.4	ng/μl	4.947	2.463	2.01	2.58	DNA	50
2	418.1	ng/μl	8.361	4.278	1.95	2.38	DNA	50
3	83.6	ng/μl	1.673	0.772	2.17	3.32	DNA	50
4	362.1	ng/μl	7.241	3.694	1.96	2.48	DNA	50
5	278.3	ng/μl	5.566	2.831	1.97	2.52	DNA	50
6	295.8	ng/μl	5.916	2.98	1.99	2.46	DNA	50
7	307.5	ng/μl	6.15	3.103	1.98	2.51	DNA	50
8	191.7	ng/μl	3.835	1.897	2.02	2.52	DNA	50
9	333.2	ng/μl	6.665	3.377	1.97	2.45	DNA	50
10	299.5	ng/μl	5.989	3.04	1.97	2.45	DNA	50
11	364	ng/μl	7.28	3.716	1.96	2.41	DNA	50
12	199.2	ng/μl	3.984	1.967	2.03	2.49	DNA	50

**Receiving Primers Protocol KROL618**

By: Hannah Trautmann

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
  - a. KROL618 = 40.16 nmoles = 401 uL of 0.1 xEB
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. If a sequencing primer, make a 2.5 uM stock by diluting 5 uL of the 100 uM stock into 195 uL of 0.1x EB. Label with blue sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

Meeting with Kathryn:

- Clone in either direction
- Should be around the Not cut site

- Amplified with BioSamA – onto the genomic DNA
- Cut with MMEI (recognition but not cut) (when in the transposon)
- After cut, very small amount of genome DNA
- Linker ligation
  - o Linker can only add on the non-beaded (3' if the top strand)
  - o Linker is a specific DNA sequence
- Example is in the folder
- Index A (1) should be immediately after the linker
- The question:
  - o Is the gDNA all the same, different, or in between
- Make a file with different clones gDNA sequences
- Check that all the other things (transposon and the linker) are the same as well
- A bit on either end that will be slightly different from the pCR-4 plasmid as well

## Sequencing

Sample number	Well	Template Type	Template Name	Primer Name	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H <sub>2</sub> O needed
							$(A + 100) \times 2.5$	$(C + B) \mu\text{l}$	$2x(-200 + B) \mu\text{l}$	(12 less D or E - 2.56) μl
AM1		plasmid	Tn-SeqLib1-1	KROL618	150	247.4	3.75	0.02	1.62	7.82
AM2		plasmid	Tn-SeqLib1-2	KROL618	150	418.1			0.96	8.48
AM3		plasmid	Tn-SeqLib1-3	KROL618	150	83.6			4.78	4.66
AM4		plasmid	Tn-SeqLib1-4	KROL618	150	362.1			1.10	8.34
AM5		plasmid	Tn-SeqLib1-5	KROL618	150	278.3			1.44	8.00
AM6		plasmid	Tn-SeqLib1-6	KROL618	150	295.8			1.35	8.09
AM7		plasmid	Tn-SeqLib1-7	KROL618	150	307.5			1.30	8.14
AM8		plasmid	Tn-SeqLib1-8	KROL618	150	191.7			2.09	7.35
AM9		plasmid	Tn-SeqLib1-9	KROL618	150	333.2			1.20	9.44
AM10		plasmid	Tn-SeqLib1-10	KROL618	150	299.5			1.34	8.10
AM11		plasmid	Tn-SeqLib1-11	KROL618	150	364			1.10	8.34
AM12		plasmid	Tn-SeqLib1-12	KROL618	150	199.2			2.01	7.43

- Messed up the water volumes for samples 2 and 3. Did 9.44 uL instead of the 8.48 uL and 4.66 uL.

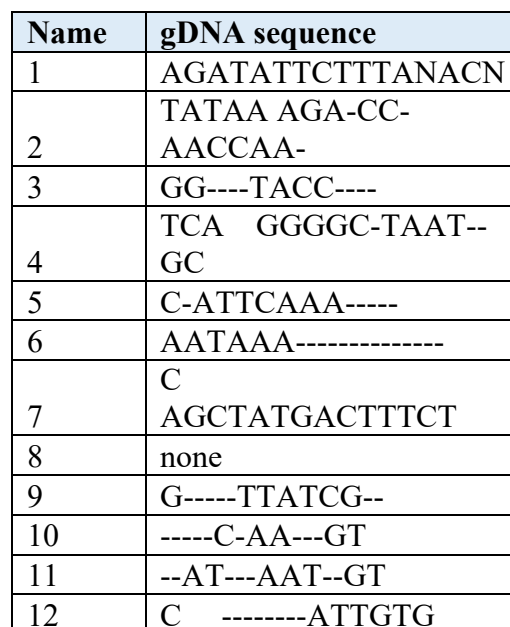
## Wednesday, February 8, 2023

1. ~~Check sequencing results~~
2. ~~Go through notebook~~
3. ~~Plan next week or so according to sequencing results~~

### Sequencing Results:

1. First index A is (N) but looking at the peak it looks like it could be an A. In the linker there is one (N) that looks like it could (maybe) be the G, and then four other errors in the P5 capture site
2. Looks fine?
3. Looks fine?
4. One deletion in the P5 capture site
5. Two of first index A's got deleted
6. One deletion in the MmeI recognition site,
7. Weird big G peak from bases 88 to 92. Under the black peak looks like the correct sequence though?
8. No signal
9. Looks fine?
10. Looks fine?
11. One incorrect A in the linker and a deletion in the P5 capture site
12. Looks fine?





**Thursday, February 9, 2023**

- ~~1. Linear PCR~~
- ~~2. PCR Purification~~

Reaction	Template	Concentration (ng/uL)	Volume to add for 2 ug	0.1x EB to normalize concentration
1	day 0	1883.2	1.06	18.94
2	day 7	312	6.41	13.59
3	day 14	192.2	10.41	9.59
4	LVS gDNA	100	20.00	0.00

### Linear PCR (2 hours) – Starting Point

1. Assemble the linear PCR reactions on ice
  - a. Where everything is?
    - i. Buffer: IN-Seq box in -20C
    - ii. dNTPs: IN-Seq box in -20C
    - iii. BioSAMA: KROL584 – IN-Seq box (1 uM aliquot)
    - iv. Platinum poly: yellow cooler in top left corner with purple cap

Total Reaction Vol	100
Total # of reactions	4

Component	Stock Concentration	Final Concentration	1 rxn volume	Factor
ddiH2O			52	4.5
Platinum SuperFi Buffer	5x	1 x	20	234
dNTPs	10 mM	0.2 mM	2	90
BioSama	1 uM	0.05 uM	5	9
DNA		2 ug	20	22.5
Platinum SuperFi Polymerase	2 U/uL	0.02 U/uL	1	add individually
Total Volume			100	360

2. Split the reaction into 2 x 50 uL in PCR tubes and run them on a thermocycler as follows: 94°C for 2 min, followed by 50 cycles of 94°C for 15s and 68°C for 1 minute. 4°C hold (~1 hour and 40 minutes total)
  - a. Put into 1.5 mL tubes then divided into PCR strip. Next time, I would just go directly into PCR tubes when normalizing the concentration, then moving 10 uL into another tube. Then add 40 uL of the mastermix to the strip tubes for a total of 50 uL.
  - b. Labeled the PCR tubes: 0A, 0B, 7A, 7B, 14A, 14B, +A, +B
3. Pool the tubes containing the same DNA sample, run them over a QIAquick PCR cleanup column according to the instructions, and elute them in 50 uL of buffer EB.

### PCR Purification

- 500 buffer PB in a 1.5 ml tube with 100 ul of PCR reaction
  - Forgot to put into a separate tube and placed right into the column
- Put entirety into a column
- Centrifuge for 60s at 13000 rpm
- Remove flow through and place back into the same 2 ml tube
- Wash add 750 ul of buffer PE to column and centrifuge for 60s at 13000 rpm
- Remove flow through and place into the same 2 ml tube
- Centrifuge one more time for 3 minutes at 13000 rpm

- Place column into a clean 1.5 ml tube
  - a. Elute in 50 uL of buffer EB (**Undiluted**) sit for one minute then spin for 1 minute
  - b. Got distracted and used 0.1xEB instead. Extremely silly of me. Kathryn says to continue on anyways.

Next Steps: Linear PCR for day 0,7,14, and LVS gDNA

### Tuesday, February 14, 2023

1. Sick 😞

### Wednesday, February 15, 2023

1. Bind linear PCR products

#### Bind linear PCR products to beads (1 hour)

##### Prepare Prior: B&W buffer, LoTE buffer

1. Resuspend streptavidin-coated beads (Dynabeads tm M-280) by shaking.
  - a. In box in the fridge (bottom left corner) Parafilm when done!
2. Add beads (32uL per sample) to a new microcentrifuge tube (1mL max, use multiple tubes if necessary)
  - a.  $32 \times 4 = 128 \text{ uL}$
3. Place the tube on the (Magnetic Particle Collector) MPC for 1-2 minutes
4. Carefully remove the supernatant with a pipette.
5. Remove the tube from MPC and add 1,000 uL of 1x B&W buffer; gently resuspended by pipetting.
  - a. B&W buffer  $2\times = 2 \text{ M NaCl}$ , 10 mM Tris, and 1 mM EDTA, pH 7.5
  - b. Dilute with water
6. Repeat steps 3-5 twice for a total of three washes.
7. Remove the final wash and add 2xB&W buffer (52uL per sample). Aliquot into PCR strip tubes (one tube per sample, 50 ul per tube).
  - a.  $52 \text{ uL} \times 4 = 208 \text{ uL}$
8. Add the entire volume of one sample from step 23 to the tube.
9. Mix at room temperature for 30 minutes. Taped to vortex and kept at lowest setting (pretty vigorous!)
10. Place the tube on the MPC for 2 min.
11. Carefully remove the supernatant with a pipette.
  - a. To avoid disturbing beads, set the electronic multichannel pipettor to its slowest setting, place the end of the tip against the opposite side of the tube from the beads, and slowly move the tip downward as the supernatant is removed.
12. Remove the tube from the MPC and add 100uL of 1 x B&W buffer, gently resuspend by pipetting.
13. Repeat steps 10-12 twice, but resuspend beads in 100ul of the LoTE buffer ea. time.
  - a. Can be stored at 4°Covernight.
  - b. LoTE buffer: 3 mM tris and 0.2 mM EDTA, pH 7.5

**Thursday, February 16, 2023**

1. Second Strand Synthesis
2. Check if I need to make more of the M12 oligos and MmeI buffer

**Second strand synthesis (1 hour)**

1. Denature the sample by heating in a thermocycler: 95°C for 2 min, then chill quickly to 4°C.
2. Prepare second strand mix on ice.
  - a. diiH2O – 16uL x4 = 72 uL
  - b. 10x hexanucleotide mix – 2 uL x 4 = 9 uL
  - c. 10 mM dNTPs – 1 uL x 4 = 4 uL
  - d. KLenow (exo-) – 1 uL = 4 uL
3. Collect the beads with the MPC, carefully discard the supernatant, remove the tube from the MPC
4. Gently resuspend the sample in 20 uL of second strand mix.
5. Incubate in a thermocycler at 37°C for 30 min. Mix by gently tapping the tube every 10 min.
6. Add 100 uL of LoTE buffer to the sample, collect the beads in the MPC, and then carefully discard the supernatant.
7. Repeat step 6.
8. Resuspend the beads in 100uL of LoTE buffer
  - a. Pause point 4C

**Meeting with Kathryn:**

- Sequence that plasmid DNA could be because of homologous recomb
- Small gDNA
  - o Could be MmeI degradation
  - o Could be completion over the long period of time
- Include the steps along the way
  - o Made the library
  - o Did the experiment
  - o Did a preliminary checks
    - Topo-ta plasmids
    - Bioanalyzer
    - qPCR
  - o Include the initial gDNA sequences
- Email PCR purtitz and prada
  - o 130 bp
  - o What reagents
  - o Borrow reagents and then replace

**Tuesday, February 21, 2023**

1. MmeI digestion
2. Linker Ligation

**MmeI digestion (2.5 hr)**

1. Prepare 50 uM double stranded M12 oligonucleotide by combining the following in a new PCR tube.

- a. M12\_top (100 uM in EB) – 15 uL
  - b. M12\_bot (100 uM in EB) – 15 uL
  - c. 1 M NaCl – 1.5 uL
    - i. THAW on ICE
2. Anneal oligonucleotides in a thermocycler: 95°C for 5 min, cool to 4°C at a rate of 0.1 °C s<sup>-1</sup>,
3. Store in 5-uL aliquotes at -20°C for future use.
  - a. Did not do steps 1-3 since Kathryn prepared prior
4. Prepare MmeI buffer mix on ice.
  - a. dH<sub>2</sub>O – 15.8 uL x 6 = 94.8 uL
  - b. 10 x NEBuffer 4 – 2 uL (Cutsmart) x 6 = 12 uL
  - c. M12 dsDNA – 0.2 uL x 6 = 1.2
    - i. Make enough for 6 rxns
5. Collect the beads from second strand synthesis with the MPC, carefully discard the supernatant, remove the tube from the MPC
6. Gently resuspend each sample in 19 uL of MmeI buffer mix.
7. Add 1 uL of MmeI to each sample. (lot ? exp ?, shipped from NEB on 2/17/23)
8. Incubate in the thermocycler at 37°C for 1 hour. Gently mix the sample every 10 min.
9. Add 100 uL of LoTE buffer to the sample, collect the beads in the MPC, and then carefully discard the supernatant.
10. Repeat step 9
11. Resuspend the beads in 100 ul of LoTE bbuffer.
  - a. Pause point, 4°C overnight

### Linker ligation (2.5 hours)

1. Prepare a 50 uM stock of barcoded, double-stranded sequencing adapters (one barcode sequence per sample) by combining the following in a new PCR tube.
  - a. LIB\_AdaptT\_(barcode) (100 uM in EB) 15 ul
  - b. LIB\_AdaptB\_(barcode) (100 uM in EB) 15 ul
  - c. 1 M NaCl 1.5 ul
    - i. This step has already been done
2. Anneal oligonucleotides in a thermocycler: 95°C for 5 min, cool to 4°C at a rate of 0.1 C s<sup>-1</sup>
  - a. Pause point, adapters can be stored for months in 5-uL single-use aliquots at -20°C for future use.
3. Dilute adapters for use from 50 uM to 1 uM in 1x T4 DNA ligase buffer. Don't want to pipette less than 1 uL, so combine on ice:
  - a. 44 uL of water
  - b. 5 uL of 10x T4 DNA ligase buffer
  - c. 1 uL of 50 uM adapter
    - i. **When you handle the adapters, and the different samples CRITICAL to have no CROSSOVER!!! Open one tube of adapter at a time, and then between the two adapters, wipe down hands and pipette with ethanol.**
4. Prepare ligation mix on ice
  - a. dH<sub>2</sub>O 14.3 uL x 5 = 71.5 uL
  - b. 10x T4 DNA ligase buffer 1.7 ul x 5 = 8.5 uL
5. Collect the beads from MmeI digestion with the MPC, carefully discard the supernatant, remove the tube from the MPC
6. Gently resuspend the sample in 16 uL of ligation mix.

7. Add 3 uL of 1 uM dsDNA sequencing adapter (from step 3) containing a unique barcode to the sample. Record which barcode is associated with the sample.
  - a. (typically, a different adapter for each sample to be sequenced! Here there is only one sample and two controls, so only use one adapter). Record here which barcode is used for which sample- all barcode A.
  - b. Use one for A, B, C, D
  - c. Day 0 – index A
  - d. Day 7 – index B
  - e. Day 14 – index C
  - f. Positive Control – index D
8. Add 1 uL of T4 DNA ligase to the sample.
  - a. **NEB M0202T, 2,000,000 U/mL**
9. Incubate in a thermocycler at 16°C for 1 h. Gently mix every 10 minutes.
10. Add 100 uL of LoTE buffer to the sample, collect the beads in the MPC and carefully discard the supernatant.
11. Repeat step 11.
12. Resuspend the beads in 100 uL of LoTE buffer.
  - a. Pause point the sample can be stored at 4°C overnight at this point

### Wednesday, February 22, 2023

1. PCR

#### PCR and Final purification (4 hours)

1. Assemble the PCR mix on ice

Total Reaction Vol	50			
Total # of reactions	4			
Factor	4.5			
Component	[Stock]	[Final]	1 rxn vol	Master mix
ddiH2O			23.5	105.75
Platinum SuperFi Buffer	5x	2 x	20	90
dNTPs	10 mM	0.2 mM	2	9
<b>LIB-PCR5</b>	5 uM	0.2 uM	2	9
<b>LIB-PCR3</b>	5 uM	0.2 uM	2	9
Platinum SuperFi Polymerase	2 U/uL	0.02 U/uL	0.5	2.25
		<b>Total Vol</b>	<b>50</b>	

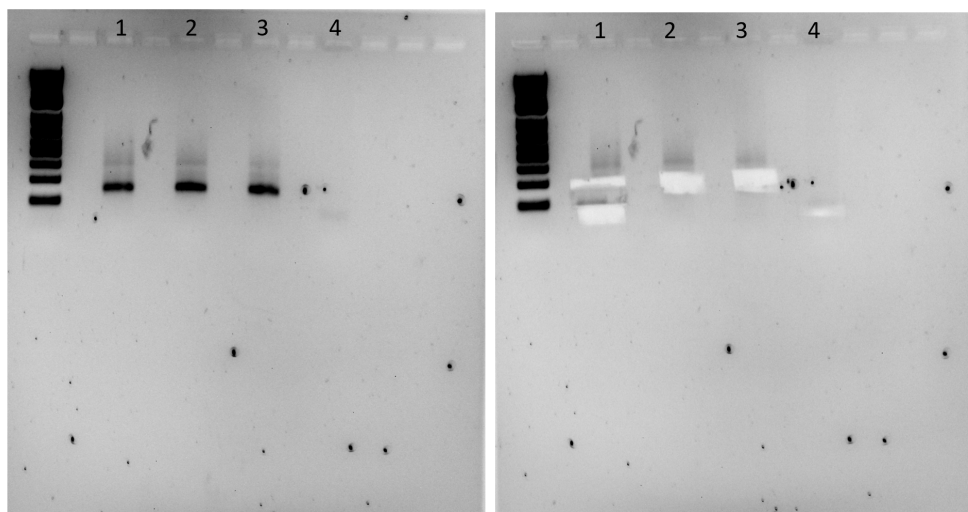
2. Collect the beads from step 63 with the MPC, carefully discard the supernatant, remove the tube from the MPC
3. Gently resuspend the sample in 50 uL of PCR mix on ice.
4. Run on a thermocycler, as follows, and prepare a 2% (wt/vol) agarose gel (1-2 lanes per sample plus 2 ladder lanes per gel; GelGreen dye at 1:10,000 dilution; wide-tooth comb) while PCR is running. Run at 94°C for 2 min followed by 18 cycles of: 94°C for 15 s, 60°C for 1 min, 68°C for 2 min and then 68 °C for 4 min.
  - a. Pause point, can be stored at 4°C overnight

## 2% Agarose for Big Gel Rig

- Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
- Add stirbar to container.
- Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
- Let the 2% agarose solution cool to approximately 50-55°C.

## Thursday, February 23, 2023

1. Run the Gel
2. Gel Purification
  1. Collect the beads on the MPC and transfer the supernatant to a new PCR tube.
  2. Add 10 uL of 6x orange-G dye to each 50 uL volume (final volume of 60 uL)
  3. Load Gel
    - a. 12 uL of ladder (10 ul orange + 2 ul blue 5x dye)
    - b. 12 uL syber safe
  4. Run gel at 200 V for 30 min
  5. Using the blue light screen, visualize bands and take image
  6. Using a clean blade for each band, excise band at ~130 bp. Minimize the amount of agarose per band



Lane	Sample
1	Day 0
2	Day 7
3	Day 14
4	Pos Cont

7.

## Gel Purification

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.

7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 40uL Buffer 0.1 x EB and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm. Do this twice.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
Day 0	290 mg	870 ul	290 ul
Day 7	320 mg	960 uL	320 ul
Day 14	340 mg	1020 ul	340 ul
Positive Control	120 mg	360 ul	120 ul

## Tuesday, February 28, 2023

1. No school

### Kathryn Meeting:

- FastQC results
  - o Expected 12 million reads
  - o Had 4.4 million reads
  - o Very underclustered
- Kathryn's previous Tn-Seq
  - o Aimed to see how many sequences....
  - o Only needed 1million reads
  - o Hit every TA site
  - o This project is less complex
- Quality of the reads were good for most of the middle (Phred score = good)
- Sequence content across all bases
  - o Most bias toward AT cause tularensis
- No N calls = good
- Duplication
  - o Most of the time not useful
  - o For us it is useful and is a good sign since the istful?
- % adapter
  - o Most is our adapter
  - o 0.1% plasmid dna and 0.3% is unknown
- Isftul: insertion sequences, stable, ancient evolution, 100 spots with identical sequences
- DATA ANALYSIS
  - o Cutadapt (program)
    - ID with 5' end with TTTT (moves to a new file to trim further)
  - o Trim the adapter off on the 3' end
  - o Then have a fast QC file that is trimmed
  - o Map to the genome
    - Format that is accessible to humans (SAM)



- Format that is compressed and computer intensive (BAM)
- Analysis of trimming
  - Should be 47 nt left
  - Removed 30 to 31 nt
    - Due to the MmeI cut site and sticky end (one nt longer overhang)
  - Small 38 and 39 nt
  - About 400,000 good reads (10% of initial)
    - Not the most efficient but gets the job done!
  - After trimming looked quality of sequence again
    - Quality score not so good past 16 nt but that is ok
    - Bias GC content in first
    - Bump on the left (from extra nt on the ends)
    - Most sequences are 16 nt what we anticipated
    - Overrep sequences (kinda concerning)
- Mapping
  - 313,680 sequences mapped to the genome
  - Signal on both the minus and plus strand show
    - Key parameter
    - Mapping to spurious sites
    - Real insertion site
  - FTL1753 insertion is present!
    - Lose mutants over time
    - Exciting
- Data analysis
  - Only pull out reads that map to TA site
  - Bonified site should have TA site on the end
  - Pull out sequence with TA site on the end of the genomic DNA
- Wig: # of reads that correlated to the TA site and this file will be used for the essentiality of genes
- Next steps:
  - Meet with Janet
    - Discuss results and how to change for the next run
    - As about the lower limit of detection on bioanalyzer
    - PCR for bubble
- PCR bubble
  - Adapters may have reannealed to itself.
  - Lack of homology in the middle
  - Might occur if not enough primers.
- Presentation
  - Take home message of the temperature stuff
  - Attempts of making the mutant library
    - Include the steps of making the library
  - Show quality control
  - Mapping images???

**Wednesday, February 29, 2023**

1. ~~Hemoglobin~~
2. ~~PCR bubble~~

Made 300mL x 4 2% Hemoglobin.

Completing the PCR to determine if the bubble is there:

Needed a diluted amount of primers and dNTPs:

Reagents	Stock Conc	Final Conc	Volume
dNTPS	10	4	20 uL
LIBPCR5	100	2	1 uL
LIBPCR3	100	2	1 uL
Water			28 uL
		<b>Total</b>	<b>50 uL</b>

Reagents	Stock Conc.	Final Conc.	Volume
Previous PCR (from Janet)	-	-	5 uL
Buffer	5	2	4 uL
dNTP+Primer solution	10x	1x	1 uL
Polymerase (SuperFi)	2	0.02	0.1 uL
		<b>Total</b>	<b>10 uL</b>

1. Mix the following components together in a PCR tube.
2. Run on a thermocycler, as follows, and prepare a 2% (wt/vol) agarose gel (1-2 lanes per sample plus 2 ladder lanes per gel; GelGreen dye at 1:10,000 dilution; wide-tooth comb) while PCR is running.
  - a. 94°C for 2 min
  - b. 94°C for 15 s
  - c. 60°C for 1 min
  - d. 68°C for 2 min
  - e. 68 °C for 4 min.
  - f. Pause point, can be stored at 4°C overnight

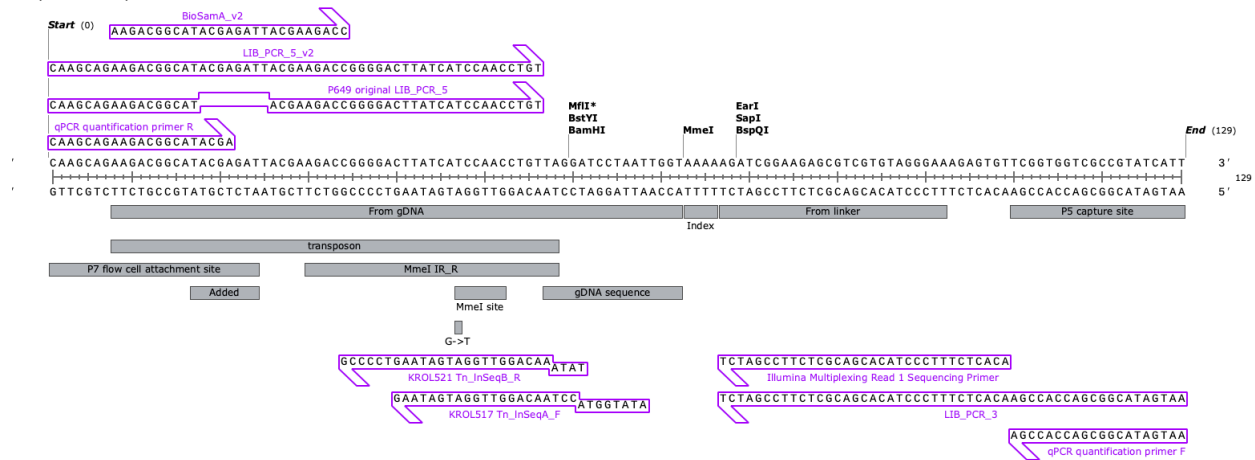
**March 2023****Wednesday, March 8, 2023**

1. ~~PCR-purify~~

NEB library quantification kit

F (Primer 1): 5'-AATGATACGGCGACCACCGA

R (Primer 2): 5'-CAAGCAGAAGACGGCATACGA



## PCR Purification

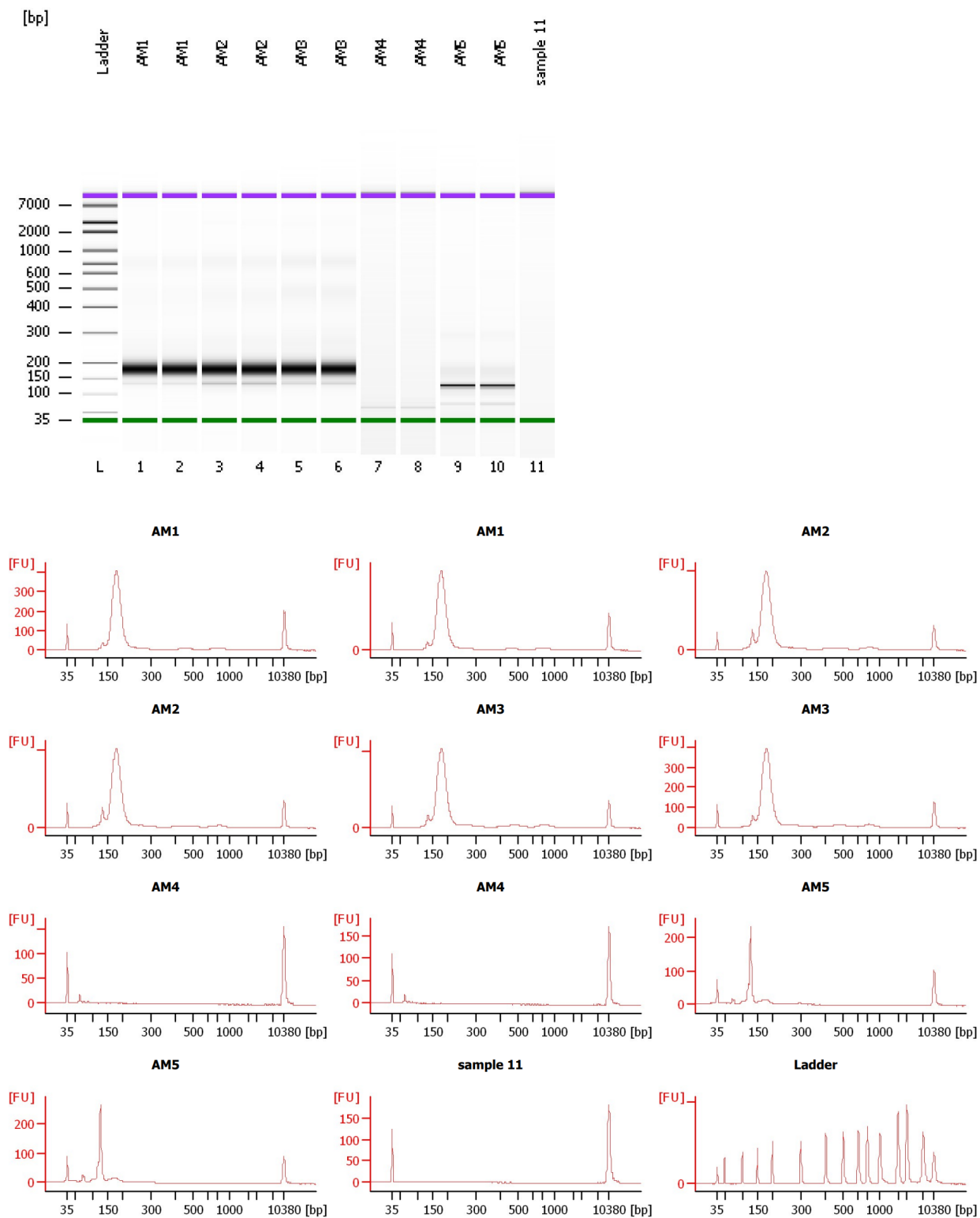
- 50 buffer PB in a 1.5 ml tube with 10 ul of PCR reaction
  - The amount of PB needs to change!!!
- Put entirety into a column
- Centrifuge for 60s at 13000 rpm
- Remove flow through and place back into the same 2 ml tube
- Wash add 750 ul of buffer PE to column and centrifuge for 60s at 13000 rpm
- Remove flow through and place into the same 2 ml tube
- Centrifuge one more time for 3 minutes at 13000 rpm
- Place column into a clean 1.5 ml tube
  - c. Elute in 40 uL of buffer 0.1x EB sit for one minute then spin for 1 minute

Gave Janet samples for qPCR and Bioanalyzer:

- Day 0: AM1
- Day 7: AM2
- Day 14: AM3
- Neg control: AM4
- PCR Bubble: AM5

## Bioanalyzer Results:

It looks like there was in fact a PCR bubble, and my samples have a lot of them! But this shouldn't be an issue as the first step in the illumine sequencing is to denature the strands. Here are some of the highlights of the pdf that janet sent on 3/9/23 and can be found in my folder (Aisling Macaraeg > Tn-Seq > AM1-5BioA-230308)



Tuesday, March 21, 2023

1. Plan FTL\_1753 knockout

~~2. Ordered primers KROL624-7~~

1. Make a plasmid with flanking regions
  - a. Backbone: pK12
  - b. Insert:
    - i. Use primers to make insert from gDNA
  - c. Restriction Enzymes:

Not1 site: GCGGCCGCT

BamHI site: GGATCC

Meeting with Kathryn:

- On the minus strand
- Cut the backbone
- Put in DNA pieces
- BamHI on one side (left)
- Include the codons for the first 3 aa
- Add not1 site GCGGCCGCT
- Kpn1 on the right side
- 3 codons
- Backbone:
  - o Delete a gene successful
  - o sacB can be mutated 😞
  - o need 3 or 4 uL for digest
  - o pKR1/11/12

### Wednesday, March 22, 2023

1. ~~Make a timeline~~
2. ~~Edit planner~~

### Thursday, March 23, 2023

1. ~~Receive Primers~~
2. ~~Make 4 flasks of 2% Hemoglobin~~

### Receiving Primers Protocol

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
  - a. KROL624: 31.13 nm x 10 = 311.1 ul
  - b. KROL625: 20.45 nm x 10 = 204.5 ul
  - c. KROL626: 30.65 nm x 10 = 306.5 ul
  - d. KROL627: 18.24 nm x 10 = 182.4 ul
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.

5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
  - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

## Tuesday, March 28, 2023

1. PCR to amplify FRs
2. PCR purify
3. ~~1xTAE buffer~~
4. ~~More agarose~~
5. Make more LB-Kan plates if needed

## PCR Protocol

Completing two different PCR reactions one for amplifying Flanking Region 1 and one for amplifying Flanking Region 2.

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
  - Molecular grade H<sub>2</sub>O in 1.5 mL microfuge tube
  - KOD/primestar buffer
  - dNTPs
  - oligo F (10uM)
  - oligo R (10uM)
  - template (eg. LVS gDNA, plasmid, colony, etc.)
  - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
  - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR\_worksheet.xlsx to make establish the specifics of what will be added
  - The file is located in the Protocols folder
  - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add dszdi H<sub>2</sub>O to negative control tube
  - Template volume for 1 reaction

9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:

Total reaction volume	100
Total number of reactions	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH <sub>2</sub> O			62.0	248
PrimeSTAR GXL Buffer	5x	1x	20.0	80
dNTPs	2.5 mM	0.2 mM	8.0	32
oligo F	10 uM	0.3 uM	3.0	12
oligo R	10 uM	0.3 uM	3.0	12
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	8
		Total volume	100	392

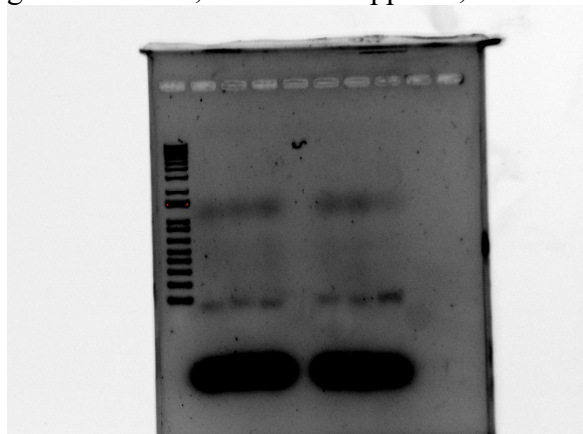
10. Mix the master-mix solution by pipetting up and down
- Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
- Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
- Heat at 94 degrees for 2 minutes,
  - 94 degrees C for 20 seconds
  - 50 degrees C for 30 seconds
  - 68 degrees C for 1 minute/kb (adjust based on expected size of product)
  - Go back to step 2
  - Repeat 32x
  - 68 degrees C for 5 minutes
  - 12 degrees C for infinity
  - 400 bp (always use larger so primase has enough time) so 00:25 or 25 sec for step 4 (elongation?) and ensure that the volume is set for 20 ul

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1A	Flanking Region 1	LVS gDNA	KROL624 and 625	660

2A	+ control	LVS gDNA	KROL15 and 16	400
3A	- control	-	KROL624 and 625	-

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1B	Flanking Region 2	LVS gDNA	KROL626 and 627	663
2B	+ control	LVS gDNA	KROL 15 and 16	400
3B	- control	-	KROL626 and 627	-

Kinda messed up. I made a master mix and added the primers. I also realized I totally over complicated it. The PCR did not work. An image of the gel is below. Also I was unsure of my gDNA content, so I nanodropped it, and it was fine.



### 1xTAE

- 20 mL of 50 x TAE
- Fill to 1000 mL of diH<sub>2</sub>O

### Agarose

- 6.08 g of agarose
- 608 mL of 1xTAE new
- Heat to 300 C and melt old agarose
- Check to not boil over
- Put in water bath

### PCR pt 2 – start of the pKR188

For obtaining the inserts of the flanking region for the deltaFTL1753 plasmid. To try to make myself more organized I did the following.

Total reaction volume	100
Total number of reactions	4

<b>Factor</b>
---------------



Component	Stock concentration	Final concentration	1 rxn volume	5
ddiH <sub>2</sub> O			62.0	310
PrimeSTAR GXL Buffer	5x	1x	20.0	100
dNTPs	2.5 mM	0.2 mM	8.0	40
oligo F	10 uM	0.3 uM	3.0	15
oligo R	10 uM	0.3 uM	3.0	15
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	10
		Total volume	100	490

This time I made a master mix with:

- 310 ul of water
- 100 uL of buffer
- 40 uL of dNTPs
- Mixed well

Then I added the individual primers and template DNA to the appropriate tubes:

- 2 uL of LVS gDNA
- 3 uL of forward primer
- 3 uL of reverse primer

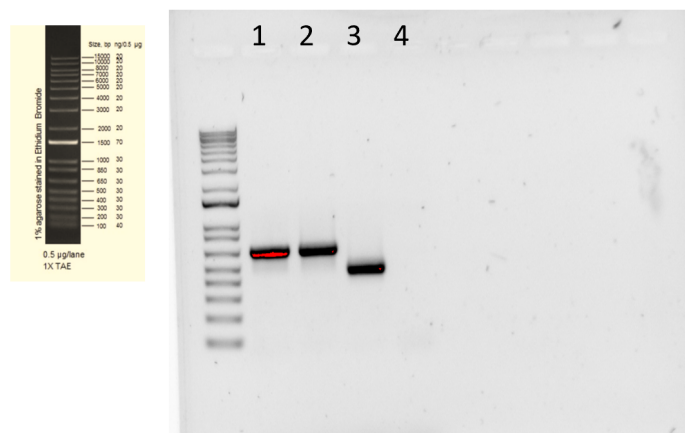
Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	Flanking Region 1	LVS gDNA	KROL624 and 625	660
2	Flanking Region 2	LVS gDNA	KROL626 and 627	663
3	+ control	LVS gDNA	KROL15 and 16	400
4	- control	-	KROL624 and 625	-

Next I added 10 uL of polymerase to the master mix, and mixed by pipetting. This lead to a total of:

Then I pipetted 92 uL of the MM to the pcr tubes, and mixed by pipetting as well. Ran on the STNI program, adjusted for 45 seconds for the 68C repeat step and volume of 100 uL. Overall, went a lot smoother than this morning, so hopefully better results.

Run 5ul on a gel to see if they're the correct band sizes.

Yay! It worked. I ran it for 26 minutes and 21 seconds.



Lane	Sample	Expected	Y/N
1	FR1	660	Y
2	FR2	663	Y
3	Pos cont	400	Y
4	Neg Control	-	Y

Wednesday, March 29, 2023

1. ~~Per purify~~
2. ~~Digest~~
3. ~~Gel PCR~~
4. ~~Nanodrop for ligation calc.~~

### PCR Purification

- 500 buffer PB in a 1.5 ml tube with 100 ul of PCR reaction
  - The amount of PB needs to change!!!
- Put entirety into a column
- Centrifuge for 60s at 13000 rpm
- Remove flow through and place back into the same 2 ml tube
- Wash add 750 ul of buffer PE to column and centrifuge for 60s at 13000 rpm
- Remove flow through and place into the same 2 ml tube
- Centrifuge one more time for 3 minutes at 13000 rpm
- Place column into a clean 1.5 ml tube
- Elute in 40 uL of buffer 0.1x EB sit for one minute then spin for 1 minute

### DNA digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	Purified PCR – FR1	BamHI and NotI	15	-
2	Purified PCR – FR2	Not I and KpnI	15	-
3	Backbone – pKR12	BamHI and KpnI	5	10

Expected size of tube 1: \_660\_ bp

Expected size of tube 2: \_663\_ bp

Expected size of tube 3: \_6826\_ bp (BB) keep larger fragment , 1163 (old insert) the smaller fragment

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H <sub>2</sub> O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

\*Cutsmart Buffer is used for all the “HF” enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

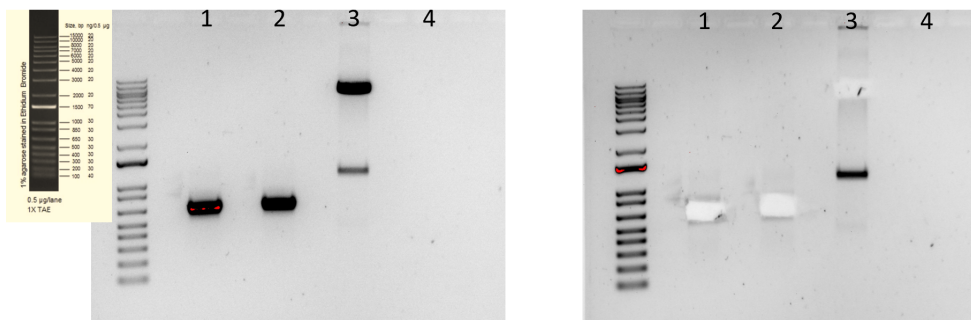
Since these are all separate enzymes. Did not make a master mix.

Tube 1 (FR 1)	Tube 2 (FR2)	Tube 3 (backbone) pKR12
- 10.8 uL water	- 10.8 uL water	- 20.8 uL water
- 3.0 uL 10xBuffer	- 3.0 uL 10xBuffer	- 3.0 uL 10xBuffer
- 15 uL PCR product	- 15 uL PCR product	- 5 uL pKR12
- 0.6 uL BamHI	- 0.6 uL KpnI	- 0.6 uL KpnI
- 0.6 uL NotI	- 0.6 uL NotI	- 0.6 uL BamHI

3. Mix by pipetting up and down.
4. Incubate at 37°C for 1 hour.
5. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
6. Run on gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
  - a. 5 ul of cybersafe
  - b. 10 ul of ladder
  - c. 6 ul of loading dye + 30 ul of sample (for tubes 1, 2,3)

## Results

## Digest for pKR188 - 3/29/23



Lane	Sample	Expected (bp)	Y/N
1	Flanking Region 1	660	Y
2	Flanking Region 2	663	Y
3	Backbone	6826 large, 1163 small	Y

### Gel Purification

- Excise the DNA fragment from the agarose gel with a clean, sharp razor
- Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
- Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
- Add 1 gel volume isopropanol to the sample and mix.
- Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
- Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
- Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
- Place columns in a fresh 1.5mL microcentrifuge tube.
- Elute: Add **40uL Buffer 0.1 x EB** and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
FR1	270 mg	810	270
FR2	290 mg	870	290
BB	290 mg	870	290

### Nanodrop

Had a minor crisis with the nanodrop. Used 8 uL of FR1 and 4 uL of FR2 and BB to get these data.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	FR1		25.2 ng/μl	0.504	0.11	4.59	0.06	DNA	50
2	FR2		20.7 ng/μl	0.414	0.079	5.26	0.06	DNA	50
3	BB		9.6 ng/μl	0.192	-0.035	-5.55	0.03	DNA	50

### LB-Kan plates

- 500 uL of Kan added to 500 mL of LB
- Stirred on stir plate
- Aseptically poured a sleeve of plates
- Marked with blue sharpie and left on benchtop overnight. Will put away tomorrow.

### Thursday, March 30, 2023

- ~~1. Ligation Calculations~~
- ~~2. Ligation~~
- ~~3. Transformation~~

Ligation Calculation:

	Sample Name	Desired Ratio	Sample Concentration (ng/ul)	size (kb)
Backbone	pKR12 BB	1	25.2	1.78
Insert 1	FR1	6	20.7	0.66
Insert 2	FR2	6	9.6	0.663

desired amount of backbone (ng) = 25

ul needed of backbone 0.992063492

ng needed of Insert 1 55.61797753

ul needed of insert 1 2.686858818

ng needed of Insert 2 55.87078652

ul needed of insert 2 5.819873596

### Ligations

The purpose of this is to ligate the cut plasmid backbone to the strand of PCR DNA.

1. Make a reaction table with desired ligations. Always include a backbone only control for each plasmid backbone used.

Tube	Insert	Backbone
1	(BamHI and NotI digested, purified PCR) FR1 + (KpnI and NotI digested, purified PCR) FR2	KpnI and BamHI digested, purified pKR12
2	-	KpnI and BamHI digested, purified pKR12

2. Set up master mix table:

Reaction Mix	Reaction 1	Reaction 2
Backbone	0.99	0.99
Insert 1	2.69	-
Insert 2	5.82	-
T4 Ligase buffer	2.00	2.00
T4 ligase	0.50	0.5
water	8.00	16.51

total	20 ul	20
-------	-------	----

3. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H<sub>2</sub>O, 10x buffer, insert, and backbone.
6. Add indicated amount of ligase (0.5 uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Room temp for 10 minutes

### Transform chemically competent *E. coli* cells

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL and 100 uL. If transforming a ligation, plate 100 uL and remaining culture.
  - Note: The plasmid used for the positive control plates should be the regular circular plasmid, not the digest/purified one.
  - There should always be a backbone control if testing a ligation. This is the digested and ligated backbone with NO INSERT.

### Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of Kanamycin-containing plates
1	(+) control	pKR12	0.5 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Ligation 1	pKR188	8 uL	100 uL, remaining	2
4	Backbone Ligation	pKR12(digested)	8 uL	100 uL, remaining	2
Total number of plates					8

2. Check to be sure you have enough LB-Kan plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of **competent cell tubes** on ice (5 reactions per tube of competent cells)
  - Double check XL1-Blue!!!
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-carb), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in

benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

- Used the beads

*Ask someone to pull out from incubator on Friday and put into the fridge*

## April 2023

### Monday, April 3, 2023

1. Count Transformation Plates
2. Overnights

Transformation Plate	Count
Positive control (20 uL)	18
Positive control (100 uL)	122
Negative control (20 uL)	none
Negative control (100 uL)	none
Ligation (100 uL)	none
Ligation (R)	6
BB Ligation (100 uL)	none
BB Ligation ( R )	none

### Overnight for E. coli

- 6 tubes of 5 mL of LB-Kan
  - 30 mL of LB (total)
  - 30 uL of Kan (double check)
- Place in shaking incubator overnight at 37C
- 6 colonies from ligation plates = 6 tubes (double check)

### Tuesday, April 4, 2023

- ~~3. Miniprep~~
- ~~4. Diagnostic Digest~~
5. Patch out LVS ?

### MiniPrep

1. Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes. Did this 3 times.
2. Added 250uL Buffer P1 to resuspend pelleted bacterial cells.
3. Added 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution was more translucent
4. Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
5. Centrifuge for 10 minutes at 13,000 rpm
6. Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 1 minute. Discard flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 minute. Discard flow-through.
8. Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 minute and

discard flow-through 3 times. Transfer to collection tube.

9. Centrifuge for 3 minutes to remove residual wash buffer.

10. Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 40 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.

### Nanodrop results:

#	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	420.1	ng/μl	8.402	4.182	2.01	2.46	DNA	50
2	584.8	ng/μl	11.697	5.952	1.97	2.45	DNA	50
3	514.6	ng/μl	10.293	5.262	1.96	2.42	DNA	50
4	774.3	ng/μl	15.485	7.825	1.98	2.38	DNA	50
5	491.1	ng/μl	9.823	4.849	2.03	2.42	DNA	50
6	681.8	ng/μl	13.636	7.015	1.94	2.44	DNA	50

### Diagnostic Digest

This diagnostic digest is to use

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	Lig 1	KpnI and BamHI	5	-
2	Lig 2	KpnI and BamHI	5	-
3	Lig 3	KpnI and BamHI	5	-
4	Lig 4	KpnI and BamHI	5	-
5	Lig 5	KpnI and BamHI	5	-
6	Lig 6	KpnI and BamHI	5	-
7	pKR12	KpnI and BamHI	5	-
8	-	none	0	5

For diagnostic digest:

Number of samples

8

Master mix factor

9

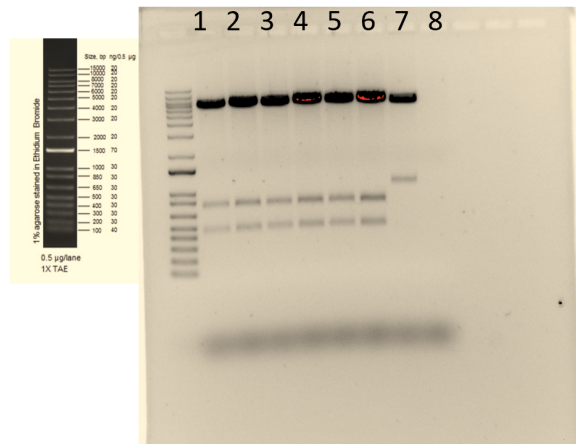
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H <sub>2</sub> O	15	135
10x Buffer*	2	18
DNA	(2)	
Enzyme 1	0.5	4.5
Enzyme 2	0.5	4.5
Total	20.0 (15.0 actual b/c of DNA)	162

1. Add indicated amounts of H<sub>2</sub>O and 10x buffer to master mix tube (MM).



2. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
3. Add indicated amount of each enzyme (\_4.5\_uL) to the master mix tube (MM).
4. Mix the master mix by pipetting up and down.
5. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (\_18.0\_ul)
6. Incubate at 37°C for 1 hour
7. Run on a large gel rig with the smaller comb (with 24 loading spaces)
  - a. 12 ul of cyber safe
  - b. 10 ul of lader
  - c. 20 ul of sample + 4 ul of dye

### Results:



Lane	Sample	Expected (bp)	Y/N
1	Ligation 1	6826 bp, and 1323 bp	sorta
2	Ligation 2	6826 bp, and 1323 bp	sorta
3	Ligation 3	6826 bp, and 1323 bp	sorta
4	Ligation 4	6826 bp, and 1323 bp	sorta
5	Ligation 5	6826 bp, and 1323 bp	sorta
6	Ligation 6	6826 bp, and 1323 bp	sorta
7	pKR12 (pos control)	6826 bp, and 1163 bp	Y
8	Negative control	none	Y

The sorta's were sort of scary, but its alright! There is a BamHI cut site inside flanking region 2, which is why there are bands at about 800 and 400 bp. So, I am going to set up sequencing reactions!

Meeting with Kathryn:

- Try with EC cells
- 8 CHAH-Kan plates

Wednesday, April 5, 2023

1. ~~Sequencing~~
2. ~~Order primer if sequencing doesn't go well~~

Sample number	Well	Template Type	Template Name	Primer Name <sup>a</sup>	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H <sub>2</sub> O needed
							$(A + 100) \times 2.5$	$(C + B)\mu\text{l}$	$2x(\sim 200 \div B)\mu\text{l}$	(12 less D or E - 2.56) $\mu\text{l}$
AM1		plasmid	pKR188	KROL6	660	420.1			0.95	8.49
AM2		plasmid	pKR188	KROL7	663	420.1			0.95	8.49
AM3		plasmid	pKR188	KROL6	660	584.8			0.68	8.76
AM4		plasmid	pKR188	KROL7	663	584.8			0.68	8.76
AM5		plasmid	pKR188	KROL6	660	514.6			0.78	8.66
AM6		plasmid	pKR188	KROL7	663	514.6			0.78	8.66
AM7		plasmid	pKR188	KROL6	660	774.3			0.52	8.92
AM8		plasmid	pKR188	KROL7	663	774.3			0.52	8.92
AM9		plasmid	pKR188	KROL6	660	491.1			0.81	8.63
AM10		plasmid	pKR188	KROL7	663	491.1			0.81	8.63
AM11		plasmid	pKR188	KROL6	660	681.8			0.59	8.85
AM12		plasmid	pKR188	KROL7	663	681.8			0.59	8.85
a. Add 2.56 μl of 2.5 μM stock to each reaction										

### Thursday, April 6, 2023

- Plan next few weeks based on the following

Tuesday – electroporate

Wed – make a lawn of cells, make CHAH-10% sucrose plates

Thur – EC/electroporation

Friday – pick colonies, leave at room temperature over the weekend

Tuesday – glycerol stocks, plate out first integrates

Friday – pick and patch onto CHAH and CHAH-Kan, leave at rm over the weekend

Tuesday – PCR primers

How to make the primer:

- A bp away from flanking region
- Make an additional primer for middle 300 of the flanking region

Colony PCR:

- Pick from CHAH plates with no growth on CHAH-Ka
- Controls
  - o Positive LVS colony
  - o Positive gDNA
  - o Negative – no template

### Tuesday, April 11, 2023

- ~~Electroporation (4 total)~~

### Electroporate plasmid (pKR188) into EC cells\*

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C

-For each electroporation, in a 2 mm sterile electroporation cuvette, combine:

5 μL of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/μL)

50 μL electrocompetent cells

- Have recovery media ready
- Electroporate using the following settings: 2.5 kV, 25  $\mu$ F, and 600  $\Omega$ 
  - 1: 3.5 clumpy
  - 2: 3.7
  - 3: arc
  - 4: 2.9
- Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
- Recover cells for 4 hours, shaking at 37°C
  - put in as close to 10 as possible
- Pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g.
- Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
- Incubate plates at 37°C for 3 days (or until single colonies appear)

- Reaction 1 and 2 = pKR188, Reaction 3 = pKR12 (pos control), Reaction 4 = negative (nothin)

\*Always including a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

Looking at the sequencing results I think MP 3,4,5,6 are all good! Need to double check with Kathryn about MP 4.

### Wednesday, April 12, 2023

- ~~1. 1 lawns of LVS~~
- ~~2. Make CHAH 10% glucose~~

### CHAH plates with 10% sucrose

Mix:

- 5 g Beef Heart Infusion
  - 5 g Protease Peptone
  - 5 g Glucose
  - 0.5 g L-Cystine
  - 7.5 g Agar
1. with **150** mL type I ddiH<sub>2</sub>O. Stir on low heat until completely dissolved, about 10 minutes.
    - a. Next time, use 1L flask
  2. Autoclave 30', being EXTREMELY careful media does not boil over
    - a. It is very viscous
  3. While autoclaving, warm 250 mL of 2% hemoglobin and **sterilized 50% sucrose** (in 50°C oven or in water bath at 55°C)
  4. Place CHA flask in 50°C oven or in water bath at 55°C, let temperature equilibrate
  5. Wipe down flask and bottles with ethanol and using sterile technique, pour hemoglobin into CHA flask
  6. Add 100 mL 50% sucrose to CHA-hemoglobin flask
  7. Mix media
  8. Use sterile pipette, pour ~24 mL media per plate (~20 plates for 500 mL media)

Thursday, April 13, 2023

1. ~~Make EC cells (tears)~~
2. ~~Electroporate~~

### Prepare electrocompetent (EC) cells

1. -Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
2. -Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
3. -Spin for 3 minutes at 10,000 rpm
4. -remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
5. -Repeat 3x-5x in 10% sucrose
6. -After final spin, remove all supernatant.
7. -Resuspend cells in 10% sucrose at high density (corresponding to  $\sim 1 \times 10^{11}$  cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
8. -For any extra EC cells, aliquot  $\sim 110$   $\mu$ L / sterile tube (enough for 2 electroporations) and freeze at  $-80^{\circ}\text{C}$

Made two aliquots of 110 uL. Used the remaining to plate on a CHAH-KAN to see if there is any contamination.

### Electroporate plasmid into EC cells\*

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$

-For each electroporation, in a 2 mm sterile electroporation cuvette, combine:

- 5  $\mu$ L of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/ $\mu$ L)
- 50  $\mu$ L electrocompetent cells

-Have recovery media ready

-Electroporate using the following settings: 2.5 kV, 25  $\mu$ F, and 600  $\Omega$

-1: 5.50

-2: 5.30

- 3: 5.40

-4: 5.60

-Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube

-Recover cells for 4 hours, shaking at  $37^{\circ}\text{C}$

-Pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g.

-Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)

- removed 1.8 mL of the supernatant and resuspended. Plated the resuspension.

-Incubate plates at  $37^{\circ}\text{C}$  for 3 days (or until single colonies appear)

- Reaction 1 and 2 = pKR188, Reaction 3 = pKR12 (pos control), Reaction 4 = negative (nothin)

\*Always including a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

### Friday, April 14, 2023

1. Pick colonies

Pick single colonies from electroporation plates and patch onto CHAH-Kan plates (large patches, maximum 6 per plate, ideally 3-4 per plate). Pick at least 4, if there are 4 colonies (preferably from different electroporations to minimize the chance of picking the same integrant).

Leave at room temperature over the weekend.

Looked at plates from Tuesday's electroporation. Colonies on all plates including the negative control. So I am assuming they are just breakthrough growth. Fingers crossed Thursday's worked!

### Sunday, April 16, 2023

1. Pick colonies

Pick single colonies from electroporation plates and patch onto CHAH-Kan plates (large patches, maximum 6 per plate, ideally 3-4 per plate). Pick at least 4, if there are 4 colonies (preferably from different electroporations to minimize the chance of picking the same integrant).

It didn't work 😞 going to make a lawn on Monday to make more cells on Tuesday.

### Tuesday, April 18, 2023

- ~~3. Make EC cells (tears)~~
- ~~4. Electroporate~~

#### Prepare electrocompetent (EC) cells (~45 min)

9. -Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
10. -Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
11. -Spin for 3 minutes at 10,000 rpm
12. -remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
13. -Repeat 3x-5x in 10% sucrose
14. -After final spin, remove all supernatant.
15. -Resuspend cells in 10% sucrose at high density (corresponding to  $\sim 1 \times 10^{11}$  cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
16. -For any extra EC cells, aliquot  $\sim 110$   $\mu$ L / sterile tube (enough for 2 electroporations) and freeze at  $-80^{\circ}\text{C}$

Made two aliquots of 110 uL. Used the remaining to plate on a CHAH-KAN to see if there is any contamination.

#### Electroporate plasmid into EC cells\* (~15 min)

- For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$
- For each electroporation, in a 2 mm sterile electroporation cuvette, combine:

- 5  $\mu$ L of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/ $\mu$ L)
  - 50  $\mu$ L electrocompetent cells
  - Have recovery media ready
  - Electroporate using the following settings: 2.5 kV, 25  $\mu$ F, and 600  $\Omega$ 
    - 1: 5.40
    - 2: 5.50
    - 3: 5.60
    - 4: 5.40
  - Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
  - Recover cells for 4 hours, shaking at 37°C
  - Pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g.
  - Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
    - removed 1.8 mL of the supernatant and resuspended. Plated the resuspension.
    - prior to removing the 2ml, I plated 200 uL for reactions 1 and 2.
  - Incubate plates at 37°C for 3 days (or until single colonies appear)
- Reaction 1 and 2 = pKR188, Reaction 3 = pKR12 (pos control), Reaction 4 = negative (nothin)

\*Always including a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

#### Meeting with Kathryn:

- Transposon Insertion Sequencing
- No hyphen
- New transposon delivery plasmid
- Creation
- Survival of *F. tularensis* in river water
  - o Rename the orange diamonds
- In-Seq Data
  - o Kathryn will determine what data
  - o Number of reads and number of high confidence insertions
  - o I
- Conclusion
  - o What FTL\_0508 is?
- Gene found with three insertion sites at day 0 and 7 but not at day 14 suggesting that is important for survival
  - o State what it is involved in
  - o Hypoosmotic environment

Kathryn thought some of the colonies that I previously electroporated might be 1<sup>st</sup> integrants. She very kindly did the following for me as I had to run:

Pick single colonies from electroporation plates and patch onto CHAH-Kan plates (large patches, maximum 6 per plate, ideally 3-4 per plate). Pick at least 4, if there are 4 colonies (preferably from different electroporations to minimize the chance of picking the same integrant).

### Wednesday, April 19, 2023

1. ~~Check on the plates~~

If there is growth on the CHAH-Kan plates, proceed onto glycerol stocks.

\*update: So there was pretty robust growth on the negative control 😞 so we are going to check on the plates tomorrow to see if we can catch any early colonies. I am going to make a lawn of LVS on Thursday to potentially make more EC and electroporate on Friday if it doesn't work out on Friday.

### Thursday, April 20, 2023

1. ~~Check Plates~~
2. ~~2% Hemoglobin~~
3. ~~Lawn of LVS~~

All of my plates have tiny colonies 😞 this means that I will need to do it all over again tomorrow 😞

### Hemoglobin

- 6 g heme
- 300 mL diH<sub>2</sub>O
- Liquid 20 min

### Friday, April 21, 2023

1. Glycerol Stocks
2. Plate out first integrants

Kathryn kindly patched out some of the electroporation colonies from Tuesday. And the negative control did not grow! So I am going to make glycerol stocks and plate out the first integrants.

Just kidding. I don't fully understand the dilution situation for the plating, so I will not be doing it right now.

### Tuesday, April 25, 2023

1. ~~Glycerol stocks~~
2. ~~Plate out first integrants~~
3. ~~Order primers for colony PCR~~
4. ~~Prepare primers KROL653-655 and KROL652~~

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

*To select for cross-outs using sucrose selection*

-Scrape up small loop of cells and resuspend in to 400 uL of 1X PBS

-Dilute culture 1:10 in sterile PBS to  $1 \times 10^{-7}$

Perform first dilution 1:100 so first dilution tube is  $1 \times 10^{-2}$ , adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

- this means that six 1.5 mL tubes will have 225 uL of 1XPBS

- no need to change tips in between, as this is not specific.

- Plate 100  $\mu\text{L}$  of last dilution ( $1 \times 10^{-7}$ ) onto CHAH plates.
- Plate 100  $\mu\text{L}$  of each dilution  $10^{-2} - 10^{-7}$  on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.
  - I plated the  $10^{-3}$  to  $10^{-6}$  dilutions twice since I had extra plates
- Incubate plates at  $37^\circ\text{C}$  for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the  $1 \times 10^{-7}$  dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the  $1^\circ$  integrant may have an inactivating mutation in *sacB* and is not worth keeping.

#### *Make freezer stocks of $1^\circ$ integrants*

- Add 800  $\mu\text{L}$  MHB to cryovial per  $1^\circ$  integrant
- Scrape up  $1^\circ$  integrant cells using loop into cryovial
- Completely resuspend cells in cryovial
- Add 200  $\mu\text{L}$  75% glycerol, close tube, mix by vortexing, and freeze at  $-80^\circ\text{C}$ .

These cells have been growing for awhile, so they were kind of hard to scrape up.

#### Meeting with Kathryn:

- Design plasmids for deletion
- Design plasmid for complementation
  - o pKR7
  - o entire gene with stop codon
  - o ask Kira for how she designed hers
- PCR to confirm the mutant?
  - o KROL286 is the F primer for FTL\_0131
  - o From gDNA extracted
  - o Use 0.5  $\mu\text{L}$  of gDNA in a 30  $\mu\text{L}$  PCR reaction
    - Dilute the input library
    - Make master mix per gDNA
      - Water
      - Buffer
      - gDNA
      - enzyme
      - dNTPs
      - 1  $\mu\text{L}$  of primers
  - o Load 12  $\mu\text{L}$  of sample
  - o Take multiple pictures if necessary

Script: My name is Aisling and I am an undergraduate research in the Ramsey lab, which researches microbial genetics. Our model organism is *Francisella tularensis*, and in the laboratory we use the live vaccine strain as it is a pathogen. The question that I am interested in is “**What are the genetic requirements for survival of *F. tularensis* in freshwater?**”. In order to answer this question we first tested which temperature was most optimal and it was found to be  $4^\circ\text{C}$ .

#### Receiving Primers Protocol

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100  $\mu\text{M}$ . Calculate this by multiplying the reported nm by 10 and adding that volume in  $\mu\text{L}$  (i.e. 12.7 nmol = add 127  $\mu\text{L}$  of 0.1xEB).



- a. KROL652:  $33.42 \text{ nm} \times 10 = 334.2 \text{ ul}$
  - b. KROL653:  $29.47 \text{ nm} \times 10 = 294.7 \text{ ul}$
  - c. KROL654:  $44.40 \text{ nm} \times 10 = 444.0 \text{ ul}$
  - d. KROL655:  $37.36 \text{ nm} \times 10 = 373.6 \text{ ul}$
3. Put on  $42^{\circ}\text{C}$  heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -  $20^{\circ}\text{C}$  freezer.
6. Optional: Make dilution for intended purpose of primer.
  - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
  - b. If a sequencing primer, make a 2.5 uM stock by diluting 5 uL of the 100 uM stock into 195 uL of 0.1x EB. Label with blue sticker and put in appropriate freezer box.
    - i. KROL652 ONLY
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

### Wednesday, April 26 , 2023

#### ~~1. Run PCR validation~~

#### Tn-Seq results PCR validation

Amplify off library gDNA using primers specific to FTL\_0508 and the Tn.

KROL653 TnCh_FTL0508_F	gaaaaaatcctcgcagaaaagttaa
KROL654 TnCh_TnIR	CCGGGGACTTATCATCCAACCTG

If there is an insert in bp 492,464, then this should amplify 259 bp.

If there is an insert in bp 492,820, then this should amplify 614 bp.

If there is an insert in bp 49,018, then this should amplify 813 bp.

#### Controls

Negative: No inserts should be in rpsD (FTL\_0260)

KROL655 TnCh_rpsD_F	atggctagatatctaggacaaaatg
---------------------	---------------------------

With KROL654, there shouldn't be a product smaller than 800bp.

#### Positive:

KROL286 amplifies in FTL\_0131 (133112-133138)

There is a Tn insert in 133444, so should amplify 355 bp

Insert in 133632, so 544 bp

Insert in 133672, so 583 bp

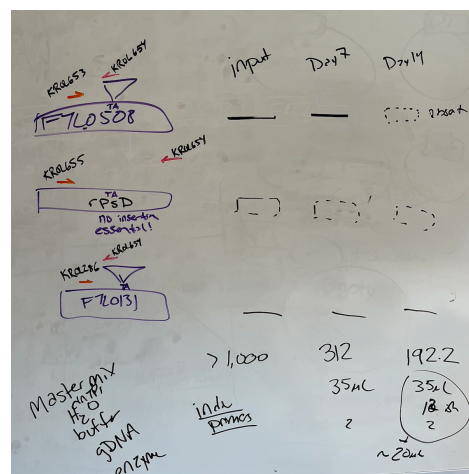
55 second extension time

Add loading dye (6 uL) and load 12 uL.

Run on big rig, load primer sets together. Example:

Lane

Lane	Primer set	gDNA
1	Ladder!	-
2	KROL653 KROL654 (FTL 0508)	Input
3	KROL653 KROL654 (FTL 0508)	Day 7
4	KROL653 KROL654 (FTL 0508)	Day 14
5	KROL655 & KROL654 (rpsD)	Input
6	KROL655 & KROL654 (rpsD)	Day 7
7	KROL655 & KROL654 (rpsD)	Day 14
8	KROL286 & KROL654 (FTL 0131)	Input
9	KROL286 & KROL654 (FTL 0131)	Day 7
10	KROL286 & KROL654 (FTL 0131)	Day 14



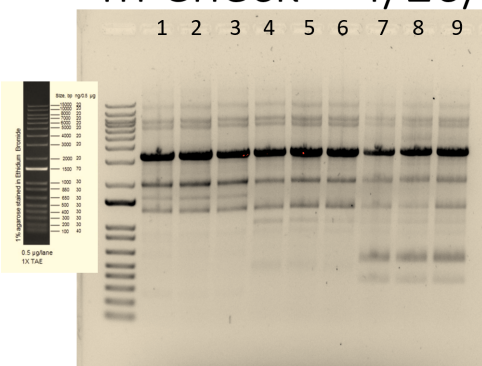
Total reaction volume	30
Total number of reactions	3

1 master  
mix

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			18.5	4
PrimeSTAR GXL Buffer	5x	1x	6.0	74
				24

dNTPs	2.5 mM	0.2 mM	2.4	9.6
oligo F	10 uM	0.3 uM	1.0	
oligo R	10 uM	0.3 uM	1.0	
template	100 ng/ul	2 ng/ul	0.5	2
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.6	2.4
		Total volume	30	112

## Tn Check – 4/26/23



Lane	Sample	Expected (bp)	Y/N
1	Input PS: 653/654	259 bp	N
2	Day 7 PS: 653/654	614 bp	N
3	Day 14 PS: 653/654	813 bp	N
4	Input PS: 655/654	none	N
5	Day 7 PS: 655/654	none	N
6	Day 14 PS: 655/654	none	N
7	Input PS: 286/654	355 bp	Y?
8	Day 7 PS: 286/654	544 bp	Y?
9	Day 14 PS: 286/654	583 bp	Y?

If there is an insert in bp 492,464, then this should amplify 259 bp.  
 If there is an insert in bp 492,820, then this should amplify 614 bp.  
 If there is an insert in bp 49,018, then this should amplify 813 bp.

Controls  
 Negative: No inserts should be in rpsD (FTL\_0260)  
 KROL655 TnCK\_rpsD\_F  
 atggctagatatttaggacacaa

atg  
 With KROL654, there shouldn't be a product smaller than 800bp.

Positive:  
 KROL286 amplifies in FTL\_0131 (133112-133138)  
 There is a Tn insert in 133444, so should amplify 355 bp  
 Insert in 133632, so 544 bp  
 Insert in 133672, so 583 bp

Didn't work, there are a lot of bands that are consistent in all of the samples: 3000 ish bp, 2000 bp, and 1200 bp.

### Thursday, April 27, 2023

1. Check plates
2. Make document with boxes content

### Friday, April 28, 2023

1. ~~Cross patch~~
2. ~~Receive primers~~

### Receiving Primers Protocol

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
  - a. KROL658: nm x 10 = ul
  - b. KROL659: nm x 10 = ul
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
  - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.

7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

#### Patching

- Pick single colonies (cross-outs) from sucrose plates and patch onto CHAH, 8 – 16 per 1° integrant.
- Optional: cross-patch to CHAH-Kan. Note that it is common to pick up some background cells so some amount of kanamycin-resistant growth is not yet a concern. However, if there is robust kanamycin-resistant growth from all patches from a single 1° integrant on the next day, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.
- I patched out 12 by using a toothpick and patching onto CHAH then immediately after onto CHAH-Kan

## May 2023

### Monday, May 1, 2023

1. Colony PCR
2. Patch out 12 more just in case

-Take small amounts of each cross-out patch and resuspend in sterile water.

-Heat samples at 95°C for 10' to lyse and kill cells

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use LVS cells, LVS gDNA, the pEX-based allelic exchange vector, and water only.

Example colony PCR using PrimeSTAR GXL DNA Polymerase (use a master mix):

- 14 reactions

Water	12.5 µL	187.5 uL
5X PrimeStar GXL buffer	4.0 µL	60 uL
2.5 mM dNTPs	1.6 µL	24 uL
Forward primer (10 µM) (KROL658)	0.6 µL	9 uL
Reverse primer (10 µM) (KROL659)	0.6 µL	9 uL
Primestar enzyme	0.4 µL	6 uL
Cell lysate	<u>1.00 µL</u>	
Total volume	~20.00 µL	

-Use PCR program:

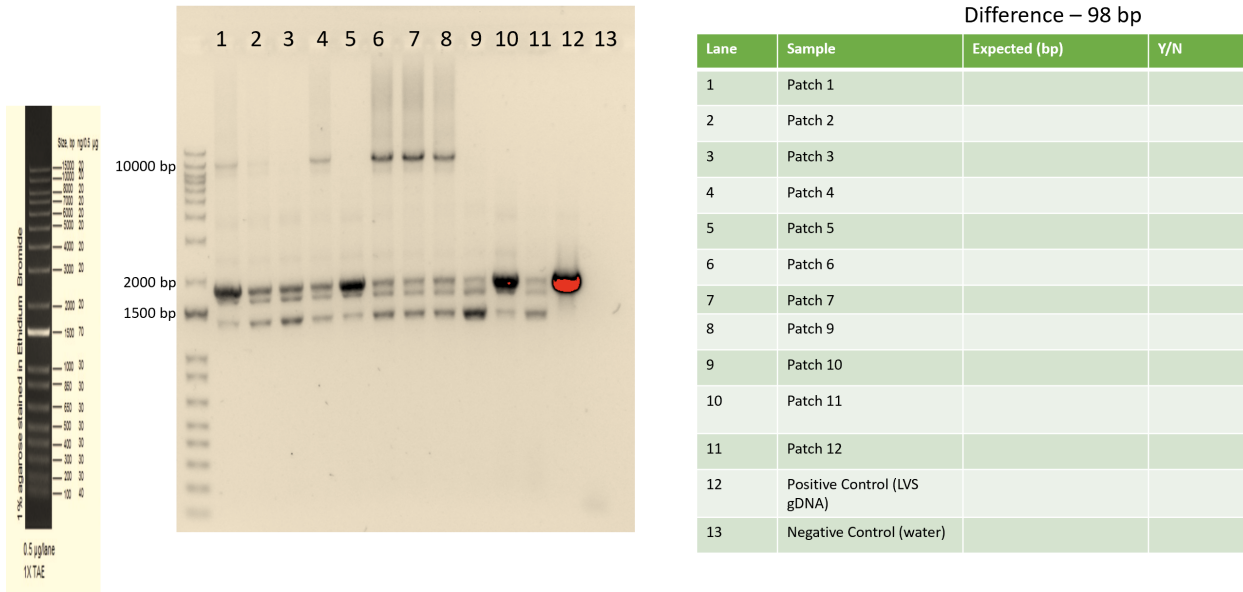
1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C (1' per kb)  
2 minutes
5. Go to step 2, rep 32x
6. 68°C 5'
7. Hold 12°C

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants.  
Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

## Colony PCR for delt\_1753 – 5/1/23

WT – 1851 bp  
Delt – 1753 bp  
Difference – 98 bp



Non conclusive, I think. Sent to Kathryn for her opinion. And I patched out 12 more colonies on CHAH and Kan to test tomorrow.

KROL192 to 195 for ectopic expression.

### Monday, May 8, 2023

1. ~~Label primary integrant for strain LVS 273~~
2. ~~Label deletion mutant for 274~~
3. ~~Prep for gDNA extraction tomorrow~~
4. Fix pKR200
5. Make plasmids pKR202,203

Freeze down strain – KRLVS274

### Use aseptic technique

Per strain, label 2 cryotubes with strain number. Include LVS, strain number, genotype, date.

Add 200 uL sterile 75% glycerol to each tube (2 per strain).

In a sterile 2 mL tube (1 per strain), add 400 uL of MHB

Resuspend patch (all of what you have) in MHB to homogeneity

Correct volume to 1700 uL (add 2x 650 uL MHB)

Transfer 800 uL to each cryotube (final volume should be 1 mL)

Vortex cryotube

Quickly spin (mini-fuge) to get liquid to the bottom of the tube

Freeze at -80°C in appropriate strain box

- Box 10

## Tuesday, May 9, 2023

- ~~1. Fix pKR200~~
- ~~2. Make plasmids pKR202,203~~
- ~~3. gDNA extraction~~

### gDNA extraction for pKR188

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of candidate pKR188 integrant and resuspended in 300uL of MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 70uL of 0.1x EB.

Then I nanodrop'd my gDNA samples:

Meeting with Kathryn:

- Send KRLVS274 for sequencing
  - o Primers: KROL652
  - o LVS gDNA and KRLVS274
    - Colony PCR 658/659
  - o PCR purify
  - o Sequence with KROL652
- pKR202

- move to other side of the promoter
- start from stop codon and BamHI site
  - GGATCC
- forward uses EcoRI
  - GAATTC
- start at Met
- Figures
  - Plating comparisons: transposon mutant library = red
  - Add method graphic
    - Take out the plated days
    - LVS after
    - Delete bacterium
  - Verified what other people have seen with 4C viability
    - It is variable
  - Discussion
    - Different inoculums
    - 21 to 56 days
  - Transposon mutant vector
  - New library
    - Limited library
  - Candidate gene for survival
    - Hopefully validate requirement
  - Talking to author about FTL\_1753
  - Discussion
    - Speculate about cell wall synthesis ligase
- Results
  - Not saying the why
  - Using the figures as a guide
  - Figure 1/2
    - Wanted to validate what temperature *f. tularensis* would survive best at in freshwater
      - Describe figure 1, big picture
      - The results are in figure 2
      - Include the comparison
      - Include day 7 comparisons
      - After 14 days- drop off
  - Figure 3
    - How reproducible is this? (give the logic)
- Development of methods
- Generating a transposon mutant library
  - Modification transposon delivery vector
  - Estimate 6400 mutants
- Assess transposon mutant library for ability to survive in freshwater (fig. 3)
  - Incubation of the library at
- Testing the cells lacking *mpl*
  - Complementation!

### PCR for preparation for sequencing of KRLVS 274

- Reaction 1: KRLVS 274 gDNA
- Reaction 2: LVS gDNA

- Reaction 3: negative control
- Primers: KROL 658/659

Total reaction volume	20
Total number of reactions	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH <sub>2</sub> O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	2.4
oligo R	10 uM	0.3 uM	0.6	2.4
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
		Total volume	20	78.4

- Extension time: 2 minutes

Nano dropped.

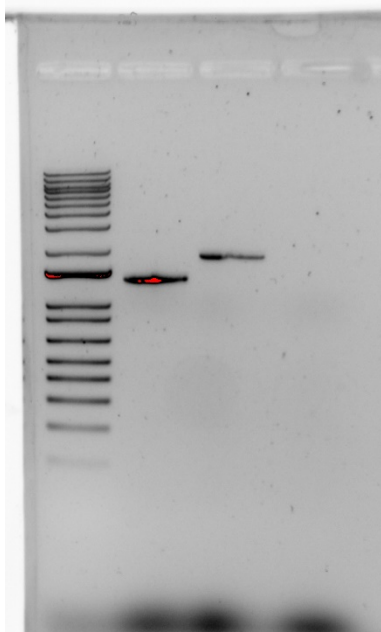
#	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	2477.2	ng/ul	49.544	25.701	1.93	1.71	DNA	50

Monday, May 15, 2023

- ~~1. PCR purification~~
- ~~2. Run on gel~~
- ~~3. Set up sequencing~~
4. Fix plasmid



PCR purified the PCR from the previous day. Ran on the gel. It looks like the following: ]



Lane 1: sample of gDNA from KRLVS274

Lane 2: gDNA from LVS

Lane 3: negative control

Looks good! We expected this to be 1431 bp, which is about what we see here. Sent samples for sequencing!

Sample number	Well	Template Type	Template Name	Primer Name <sup>a</sup>	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H <sub>2</sub> O needed
							$(A \div 100) \times 2.5$	$(C \div B) \mu\text{l}$	$2 \times (\sim 200 \div B) \mu\text{l}$	(12 less D or E - 2.56) μl
AM1		PCR	KRLVS274	KROL652	1493	30	37.33	1.24		8.20
a. Add 2.56 μl of 2.5 μM stock to each reaction										
3130xl Plate Record										
	Date		5/15/2023	Name	Aisling Macaraeg					
PI	Kathryn Ramsey	Dept	CMB	Email	<a href="mailto:amacaraeg@uri.edu">amacaraeg@uri.edu</a>	PO No.				

Things still to do:

- order primers

Meeting with Kathryn:

- add that I sequenced KRLVS274
- fix KROL671
  - o include stop codon