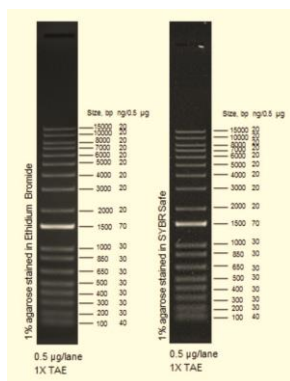


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## September 2022

### Monday, September 12, 2022

1. ~~Update excel tables~~
2. ~~Make hemoglobin~~

### Hemoglobin

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

### Tuesday, September 13, 2022

3. ~~Meeting with Dr. Ramsey~~
4. ~~Make LB agar~~

### Transformation Efficiency

- In patches of component cells
- The DNA isn't limiting
- The limiting factor is how efficient the cells are picking up the DNA
- For my cells
- Presuming it doesn't change over time

Next steps:

- Make mutant library
  - Using EC cells and plasmid
  - Get lotta colonies
- Frozen glycerol is the final product of mutant library
- Quality Checks
  - Southern Blot on mutants
  - Isolate gDNA from aliquot of library and straight LVS
    - Test tn-seq library protocol
    - Product: single band at right size

For Mutant Library

- For making big 150 mm square KAN-CHAH
  - 8 plates (2 electroporation's)
  - 145 vs 56 = 2.5 fold change
  - 65 mL CHAH per plate
    - 520 mL
  - 60 ul of KAN (?)
  - Super dry !!
- Electroporation the same as last time:
  - Recover in 4 mL
  - Separate 1 mL on big plates
  - Take out 30 uL before plating on big plates
    - Plate 10 uL twice
    - 1:10 once
    - Track plate style
- Harvesting

- 15% glycerol
- 50 mL conical
- Add MHB (2.4 mL)
- Add glycerol (200uL 75%)
- Scrape the cells up using a cell scraper and resuspended

#### Southern Blot

- DIG – probe through PCR, instead of radioactivity
- Look Roche PCR dig kit in freezer

### Wednesday, September 14, 2022

1. ~~Figure out schedule for mutants library~~
2. ~~Filled up ddiH<sub>2</sub>O~~

### Friday, September 16, 2022

1. ~~Double check DIG probe has the nucleotide thing~~
2. Work on grant
3. ~~Order primers KROL567/8~~
4. ~~Make MHB~~

### Muller Hinton Broth (500 mL)

- 10.5 g of MHB
- 500 mL of ddiH<sub>2</sub>O
- Spin
- Autoclave for liquid 30'

### Monday, September 19, 2022

1. Make KAN-CHAH plates

For making big 150 mm square KAN-CHAH

- 8 plates (2 electroporation's)
- 145 vs 56 = 2.5 fold change
- 65 mL CHAH per plate
  - 520 mL
- 60 ul of KAN (?)
- Super dry !!

### Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

For 600 mL of CHA (double everything)

1. Take out 15.3g x 2 of cystine heart agar into two 1L flask (non-baffled; 10.2g/100mL)
2. Add 150mL x 2 of ddiH<sub>2</sub>O (type I)
3. Add stirbar to flasks
4. Heat on low, stirring, for about 10 minutes (media should be totally dissolved)

5. Autoclave on 30' liquid cycle, filling the water bin up to the height of the media
6. Cool down (ideally to ~55C)
7. Separately (before), prepare 2 flasks hemoglobin 2% solution
8. Using sterile technique, pour hemoglobin into CHA
9. Add 60 uL of Kanamycin to each flask. Stir.
10. Using a 50mL pipet, aliquot 30mL of CHAH mixture into each 100mm plate (should make approximately 2 plates) Try to avoid bubbles! 65 mL per big plate. (should make 8 plates)

### Tuesday, September 20, 2022

- ~~1. Work on grant~~
- ~~2. Made Hemoglobin~~

### Wednesday, September 21, 2022

1. Work on grant
2. Prep for Friday

### Friday, September 23, 2022

- ~~1. Electroporate~~

*Electroporate plasmid into EC cells\**

Part one of four of the mutant library creation for Tn-Seq.

1. -For each electroporation, aliquot 4 mL MHB (no KAN) into glass test tubes for recovery, warm in shaker at 37°C
  - a. Not cold to the touch , let warm up for 10 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette (NEW and blue cap) (found under hannahs bench), combine:
  - a. 1 µg in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
    - Current concentration is 156.2
    - Adding 6.4 ul of pKR141
  - b. 50 µL electrocompetent cells
    - 1 aliquots (110 uL ) done in duplicate
    - Thaw on bench then on ice
    - Aliquots from July 14, 2022
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
  - a. Sample 1 = 2.6
  - b. Sample 2 = 2.9

5. -Immediately after individual electroporation, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
  - a. Use blue pipette for 1 mL
  - b. Maybe clumpy due to dense cells
  - c. When adding the 1 mL try to break up the clumps as best as possible
6. -Recover cells for 3 hours, shaking at 37°C
7. -Plate the following:
  - a. Separate 1 mL on big plates
    - Used a 1000 ul micropipette
    - For EP1 I poured out the final 2mL into a sterile 2 mL tube to plate
    - For EP2 I used a serological pipettes
    - Lots of bubbles
    - Used the beads and let sit for 45 minutes before getting rid of the beads
  - b. Take out 30 uL before plating on big plates for each electroporation
    - Did this first before plating the 1 mL
    - Plate 10 uL twice
    - 1:10 once
      1. 90 ul PBS to 10 ul of cells
    - Track plate style
8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

Took a little longer than I would like. I think other than that it went ok, but we will see. Also there was much more clumping than the previous times.

### Monday, September 26, 2022

1. ~~Harvest~~
2. ~~Count Track Plates~~
3. ~~Streak out 5 plates of LVS~~
4. ~~Make more CHAH KAN plates for Friday~~

Harvesting in MHB with 15% glycerol

- 50 mL conical
- Add MHB (2.4 mL)
- Add glycerol (200uL 75%)
- Scrape the cells up using a cell scraper and resuspended
- Label and freeze
- Remember in the future that the next ones will go in there as well

### track plate

				Avg CFU	Transformation	
					Efficiency	Avg TE
EP-1	row 1	row 2	row 3			
	3			4	1.60E+03	1.90E+03
	5					
EP-2	6			5.5	2.20E+03	

## 5

The transformation efficiency changed by a log fold. Not good. So I talked to Kathryn and we decided the following:

- Making more EC cells on wed. As many as possible.
- On Friday, do another test electroporation to determine transformation efficiency
  - o Plate 100 ul for each electroporation on a round plate with beads
  - o Plate a track plate like last time (1, 0.1, and 0.01) for each electroporation
  - o And for the next attempt at mutant library, leave in the incubator for another day (Tuesday) before harvesting.

Streaking out for EC cells

- Use 50 ul single use aliquots
- Pipette full amount onto regular circular CHAH plate
- Use a sterile stick to make a patch
- When I did this, I forgot that the beads make it take forever, and Hannah reminded me. So I did two with beads and three with stick.

CHAH-KAN plates

- 15.3 g of CHAH
- 150 mL dH<sub>2</sub>O
- Instant pot
- Pour 150 mL of hemoglobin into CHAH flask
- Add 30 uL of KAN
- Mix on stir plate
- Make 4 track plates and 4 circular plates ( $4 \times 30 + 4 \times 24 = 216$  mL)

**Tuesday, September 27, 2022**

- ~~1. Work on grant~~
- ~~2. Prep primers~~

### Receiving Primers Protocol

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. KROL567 – 27.69 nm – 22 uL of 0.1xEB
  - b. KROL568 – 30.28 nm – 303 uL of 0.1xEB
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. For southern blot add 1.2 uL of 100 uM primer and 398.8 uL of 0.1xEB for a final concentration of 0.3 uM.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.



### Wednesday, September 28, 2022

#### ~~1. Make EC cells~~

#### *Prepare electrocompetent (EC) cells*

Trying to make more cells for mutant library part 2. Overall, I think it went ok. It took around 3.5 hours start to finish.

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)
  1. Pretty difficult to resuspend without bubbles
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 5x in 10% sucrose
6. -After final spin, remove most of supernatant. Resuspended with remaining supernatant. Transfer all tubes to one tube (2mL tubes)
  1. There was about 1.5 mL in one tube so I divided 700 uL into another tube.
7. Pellet one final time at 10,000 rpm for 3 minutes.
  1. Both tubes.
8. -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.
  1. Ask Hannah or Kathryn to look
  2. Added 200uL of 10% of sucrose to each tube
  3. Then aliquoted 110 ul into nine different 1.5 mL sterile tubes
  4. With the extra 25 ul I plated on a kan plate to see if there was any contamination.
9. -For any extra EC cells, aliquot  $\sim 110 \mu\text{L}$  / sterile tube (enough for 2 electroporations) and freeze at  $-80^\circ\text{C}$

### Friday, September 29, 2022

#### ~~1. Electroporate (test)~~

#### *Electroporate plasmid into EC cells\**

Looking for transformation efficiency, check recovery time, and how to plate. This is testing the cells that I made on Wednesday, September 28<sup>th</sup>.

1. -For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^\circ\text{C}$
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a.  $1 \mu\text{g}$  in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least  $100 \text{ ng}/\mu\text{L}$ )
    - Current concentration is  $156.2 \text{ ug}/\mu\text{L}$
    - Adding 6.4 ul of pKR141
    - $1 \text{ ug}$

- b. 50  $\mu$ L electrocompetent cells
    - 1 aliquots (110  $\mu$ L ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25  $\mu$ F, and 600  $\Omega$ 
  - a. Sample 1 = 3.5 (super clumpy when resuspending)
  - b. Sample 2 = 0.8 (not clumpy at all)
5. -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
  - a. Maybe clumpy due to dense cells
  - b. When adding the 1 mL try to break up the clumps as best as possible
6. -Recover cells for 3 hours, shaking at 37°C
  - a. Had the Fulbright meeting, so I was a bit late to get back, and I asked Hannah to pull them out at exactly 3 hours. This may have been the wrong decision, but I plated them regardless after them sitting on my bench for 8 minutes.
7. -Plate the following (on CHAH-KAN):
  - 4 round plates per electroporation = 4 total
    1. 100  $\mu$ L
  - 2 track plates per electroporation = 4 total
    1. Only 3 dilutions 1, 0.1 and 0.01
    2. From recovery tube, take out 500  $\mu$ L into 1.5 mL
    3. 2 more 1.5 mL put 900  $\mu$ L of PBS
    4. 100  $\mu$ L of undiluted into 1<sup>st</sup> 1.5 mL
      - a. Vortex!!
    5. Take 100  $\mu$ L from previous to next tube
    6. After 10  $\mu$ L from each tube onto track plates
  - Transformants per microgram of DNA = transform efficiency
  - Before with pKL97:  $1 \times 10^4$  to  $7 \times 10^4$  transformation efficiency
  - Library of 400,000 mutants is the goal
  - 2 million sequence
8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

\*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.

## October 2022

Monday, October 3, 2022

- ~~1. Count transformants~~
- ~~2. Make more CHAH-KAN plates~~

**100  $\mu$ L      0.01      0.1      1**

EP1-A	1	0	0	0
EP1-B	0	0	0	0
EP2-A	49	0	0	2
EP2-B	40	0	0	4

Troubleshooting:

1. Increasing the DNA concentration, but NOT the volume
2. Making more dense EC cells
3. Left plates in for two days instead of one

Made \_10\_ square CHAH-KAN plates. 300 mL of CHAH and 30 uL of KAN.

### Tuesday, October 4, 2022

- ~~1. Make more LB media~~
- ~~2. Patch out 5 plates for EC cells~~
- ~~3. Throw out trash~~

Patched out 5 plates using 50 uL single use aliquots of LVS to use tomorrow for EC.

### Wednesday, October 5, 2022

- ~~1. Make more EC cells pt 3~~

Trying to make more cells for mutant library part 3. Scraped up from five plates.

10. -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)
11. -Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
12. -Spin for 3 minutes at 10,000 rpm
  1. One tube was very clear supernatant, never seen that before.
13. -remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
14. -Repeat 5x in 10% sucrose
15. -After final spin, remove most of supernatant. Resuspended with remaining supernatant. Transfer all tubes to one tube (2mL tubes)
16. -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.
  1. Did not add any extra sucrose. Very dense.
  2. Ask Hannah or Kathryn to look
  3. Added 200uL of 10% of sucrose to each tube
  4. Then aliquoted 110 ul into nine different 1.5 mL sterile tubes and then one 55 uL tube.
  5. With the extra 10 ul I plated on a kan plate to see if there was any contamination.
17. -For any extra EC cells, aliquot  $\sim 110 \mu\text{L}$  / sterile tube (enough for 2 electroporations) and freeze at  $-80^\circ\text{C}$

### Friday, October 7, 2022

- ~~1. Electroporate~~

No contamination on the 10 uL of the extra EC on the CHAH-KAN plate!

Looking for transformation efficiency, check recovery time, and how to plate. This is testing the cells that I made on Wednesday, October 5<sup>th</sup> with Dr. Ramsey.

1. -For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
  - a. Warmed for 15 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a. 1 µg in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
    - Current concentration is 156.2 ug/uL
    - Adding 6.4 ul of pKR141
    - 1 ug
  - b. 50 µL electrocompetent cells
    - 1 aliquots (110 uL ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
  - a. Sample 1 = 2.5 (barely clumpy)
  - b. Sample 2 = 2.5 (a bit more clumpy at)
  - c. No pop was heard
5. -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
  - a. Maybe clumpy due to dense cells
  - b. When adding the 1 mL try to break up the clumps as best as possible
6. -Recover cells for 3 hours, shaking at 37°C
7. -Plate the following (on CHAH-KAN):
  - 4 round plates per electroporation = 4 total
    1. 100 ul
  - 2 track plates per electroporation = 4 total
    1. Only 3 dilutions 1, 0.1 and 0.01
    2. From recovery tube, take out 500 ul into 1.5 mL
    3. 2 more 1.5 mL put 900 ul of PBS
    4. 100 ul of undiluted into 1<sup>st</sup> 1.5 mL
      - a. Vortex!!
    5. Take 100 ul from previous to next tube
    6. After 10 ul from each tube onto track plates
  - Transformants per microgram of DNA = transform efficiency
  - Before with pKL97:  $1 \times 10^4$  to  $7 \times 10^4$  transformation efficiency
  - Library of 400,000 mutants is the goal
  - 2 million sequence
8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

\*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.

### Monday, October 10, 2022

- ~~1. Count electroporation plates to calculate TE~~
- ~~2. Patch out LVS to make more aliquots~~
  - ~~a. Patch out from glycerol stock~~
- ~~3. Make more CHAH KAN plates~~

	100 uL	0.01	0.1	1		
EP1-A	31	0	0	5		
					Average of 100	TE of 100
EP1-B	39	0	1	7	uL	uL
EP2-A	20	0	0	3		28.5 1.14E+03
EP2-B	24	0	0	2		

Still not the TE that we are looking for :/. But after some discussion, we are going ahead with the project with a less than perfect library just so we can perform the double checks. We are aiming for 10,000 mutants.

### Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

For 600 mL of CHA (double everything)

1. Take out 15.3g of cystine heart agar into two ½ L flask (non-baffled; 10.2g/100mL)
2. Add 150mL of ddiH<sub>2</sub>O (type I)
3. Add stirbar to flasks
4. Heat on low, stirring, for about 10 minutes (media should be totally dissolved)
5. Autoclave on 30' liquid cycle, filling the water bin up to the height of the media
6. Cool down (ideally to ~55C)
7. Separately (before), prepare 2 flasks hemoglobin 2% solution
8. Using sterile technique, pour hemoglobin into CHA
9. Add 60 uL of Kanamycin to each flask. Stir.
10. Using a 50mL pipet, aliquot 30mL of CHAH mixture into each 100mm plate (should make approximately 2 plates) Try to avoid bubbles! 65 mL per big plate. (should make 8 plates)

Made 9 big plates and 5 small plates

### Tuesday, October 11, 2022

- ~~1. Make single use aliquots~~
- ~~2. Make more sucrose~~

~~3. Made LB Agar~~

LB Agar:

1. 6 g Agar
2. 5 g NaCl
3. 5 g Tryptone
4. 2.5 g Yeast extract
5. 500 mL dH<sub>2</sub>O
6. Liquid 30' autoclave
7. Made two flasks today

Sucrose: (10%)

8. 50 mL of 50% sucrose
9. 200 mL of dH<sub>2</sub>O
10. Filter sterilize

### 50 ul single use aliquot of LVS

- day before streak out one lawn from glycerol stock (today was from 1 of 2 2/2020)
  - 800 ul MHB (400 ul + resuspend and then add remaining 400 ul)
  - Take all of lawn
- 200 ul of 75% glycerol
- Vortex
- 50 ul aliquots
- Made 20 aliquots

### Wednesday, October 12, 2022

~~1. CHA mix~~

Meeting with Kathryn

11. Put back at 37C
12. On Friday, pick the smallest that I see (6 colonies if possible)
13. And patch on kan and CHA
  - i. Cross-patch
  - ii. Kan first then CHA
14. Are they really tiny mutants?

### Friday, October 14, 2022

~~1. Electroporation~~

15. On Friday, pick the smallest that I see (6 colonies if possible)
16. And patch on kan and CHA
  - i. Cross-patch
  - ii. Kan first then CHA
  - iii. 1-3 were smaller than 4-6
17. Are they really tiny mutants?

This is the real dealio. For the mutant library for the next steps in the experiment. Dr. Ramsey is coming to help because we will be doing a total of 17 electroporations. A change is recovering in 3 mLs instead of 4 like previously.

1. -For each electroporation, aliquot 3 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
  - a. Warmed for 15 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a. 1 µg in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
    - Did not have enough, so we diluted the concentrated amount of pKR141
    - $C1V1=C2V2$
    - $C1= 4.035 \text{ ug/mL}$
    - $V1= 5 \text{ uL}$
    - $V2= 17.5 \times 5 = 87.5 \text{ uL}$
    - $C2= 200 \text{ ng/uL}$
    - Adding 5 ul of pKR141
    - 1 ug
    - $17.5 \text{ ug}/4.035 \text{ ug/mL} = 4.34 \text{ uL of stock}$
    - 83.16 uL 0.1xEB
    - $4.34 \text{ uL} + 83.16 \text{ uL} = 87.5 \text{ uL}$
  - b. 50 µL electrocompetent cells
    - 1 aliquots (110 uL ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
  - a. Sample 1 = 4.00
  - b. Sample 2 = 4.00
  - c. Sample 3 = Arc
  - d. Sample 4 = Arc
  - e. Sample 5 = Arc
  - f. Sample 6 = 4.00
  - g. Sample 7 = 2.30
  - h. Sample 8 = 3.90
  - i. Sample 9 = Arc
  - j. Sample 10 = 4.1
  - k. Sample 11 = 4.1
  - l. Sample 12 = Arc
  - m. Sample 13 = 3.9
  - n. Sample 14 = 4.1
  - o. Sample 15 = 4.0
  - p. Sample 16 = Arc
  - q. Sample 17 = 1.10

5. -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
  - a. Maybe clumpy due to dense cells
  - b. When adding the 1 mL try to break up the clumps as best as possible
  - c. Generally, Arcs were not clumpy
  - d. But the number ones were pretty clumpy
6. -Recover cells for 3 hours, shaking at 37°C
7. -Plate the following (on CHAH-KAN):
  - a. Two EP per 1 big plate (7 big plates)
  - b. 3 EP on two small plates
  - c. Using tubes 1 and 8 for dilution plates
  - d. Take out 30 uL before plating on big plates for each electroporation
    - Did this first before plating the 1 mL
    - Plate 10 uL twice
    - 1:10 once
      1. 90 ul PBS to 10 ul of cells
    - Track plate style
    -
  - e. Spinning down the samples into 2 mL sterile tubes for 3 min at 10,000 rpm
    - Did this 3 times for tubes 1-14
    - Got ride of supernatant each time except last
      1. Did this by dumping into the waste container.
    - Tubes 15,16,19 I did 2 cause less volume and going on smaller plate.
    - Resuspended with ~1 mL of supernatant at end before plating
      1. Even with the three smaller plates, which in hindsight was dumb, but I shook them for a while and they were very dry when taking off the beads.
    - Plated with beads and then shook for about 2-3 minutes until the plates looked dry
    - Will check on Tuesday!

Tube	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Plate	1	2	1	2	3	3	4	4	5	5	6	6	7	7	8	9	10

### Monday, October 17, 2022

- ~~2. Check serological bin~~
- ~~3. Check mutant cross patches~~
- ~~4. Transformation to make more pKR141~~
- ~~5. 5 plates of LVS for EC~~
- ~~6. Half flask of CHAH and CHAH-KAN~~

So I checked the plates and there is some pretty bad contamination. On Friday when I was pouring off the supernatant on the third step I had the gas on and forgot to light the flame for a few of them, and was hoping it was going to be ok. (a very silly mistake that I now will hopefully never make again). The plates are really contaminated though, so I think its best to make more EC cells on Wednesday and try again on



Friday. So I am transforming today to make more pKR141 and then making more EC to test on Wed. **big bummer.**

### 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 carbenicillin-containing plates
1	(-) control	None	0	20 ul, 100 ul	2
2	Make more pKR141	pKR141	1 uL	20 ul, 100 ul	2
Total number of plates					4

2. Check to be sure you have enough LB-Carb 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 PIR!!!
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.
  - 250 rpm for the shaking
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

I made a ½ flask of CHAH and ½ flask of CHAH-KAN for electroporation's on Friday

### Tuesday, October 18, 2022

1. ~~Overnights~~
2. ~~EC cells~~

Trying to make more cells for mutant library part 4. Scraped up from five plates.

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 5x in 10% sucrose
6. -After final spin, remove most of supernatant. Resuspended with remaining supernatant. Transfer all tubes to one tube (2mL tubes)
  1. Added 200 mL to tube 1 to resuspend
  2. Added the volume of tube 1 to tube 2 to resuspend.
  3. Ask Hannah or Kathryn to look
  4. Then aliquoted 110 ul into 3 different 1.5 mL sterile tubes and then one 55 uL tube.
  5. With the extra 10 ul I plated on a kan plate to see if there was any contamination.
7. -For any extra EC cells, aliquot ~110  $\mu$ L / sterile tube (enough for 2 electroporations) and freeze at -80°C

### Overnight for E. coli @ 5 pm

- 2 tubes of 5 mL of LB-Carb
  - 5 mL of LB
  - 5 uL of Carbenicillin
- Place in shaking incubator overnight at 37C
- 2 colonies from transformation plates = 2 tubes

### Wednesday, October 18, 2022

- ~~1. MaxiPrep pKR141~~
- ~~2. Meeting with Dr. Greene~~

### Maxi-Prep Protocol (CompactPrep)

Did this to maximize the amount of DNA since my plasmid is low copy number.

For back dilution:

1. Making a culture, calculate OD then put in shaking incubator = very annoying
2. For E. coli, it grows consistently well, so no scrapping from patch or OD, know if you back dilute then it will grow to proper OD in 5 hours
  - a. At 2 hours one mL of sample B = 0.063
3. 625 uL in 250 mL of LB
  - i. 250 ul carb
1. Harvest cells by spinning down at 6000 x g for 15 minutes at 4°C
  - a. When doing this, transfer the 250 ml daytime cultures (which should be grown to an OD600 of 0.4-0.5) to 250 ml centrifuge canisters
  - b. OD of culture 1 = 0.47
  - c. OD of culture 2 = 0.549

- d. So mine are definitely above the 0.5 but Hannah said to just go for it.
2. Remove supernatant and resuspend cells in 5 ml of Buffer P1; after this step, transfer cell solution to 50 ml tubes
  - a. Did each one at a time. So took out the supernatant from one then immediately resuspended in buffer P1 then transferred to a new conical tube directly after.
3. Add 5 ml of Buffer P2 to each tube and mix by rigorously inverting 4-6 times and incubate at room temp for 3 minutes
  - a. Also did this one at a time.
4. Add 5 ml Buffer S3 to each tube, immediately mixing by inverting 4-6 times
5. Centrifuge the tubes for 45 minutes at 14365 x g at 4°C and then transfer the supernatant containing the plasmid to a new 50 ml tube
  - a. Got to 8C during this spin.
  - b. Transferred about 15 mL into the new tubes.
6. Centrifuge again for 25 minutes at 14365 x g at 4°C, transferring the supernatant to another new tube
  - a. During this step, set up vacuum manifold, making sure all holes are plugged besides the ones to be used
  - b. Before starting this spin I set the centrifuge and waited for it to cool down to 4C again. It got up to 7C this time.
  - c. Roughly mL to the new tubes.
7. Add 5 ml Buffer BB to each tube, mixing by inverting 4-6 times and then transfer to the tube extenders, which are to be attached to the CompactPrep columns on the vacuum manifold
8. Use the vacuum manifold to draw the lysate through the column, making sure all the lysate is through
  - a. Once all lysate has been pulled through the column, immediately shut off the vacuum and transfer CompactPrep column to a 2 ml collection tube
9. Wash DNA via microcentrifuge
  - a. Add 700 ul of Buffer PE to the column, spinning for 1 minute at 13000 rpm , then removing the flowthrough and spinning again for 3 minutes to remove residual buffer
10. Elute DNA
  - a. Add 50 ul 0.1x EB to the columns, letting stand for 1 minute before spinning for 1 minute
  - b. Then, pipetting the flowthrough and putting on the column again, letting stand 1 minute before spinning for 1 minute
  - c. I feel like there was a little less than 50 ul when I pipetted it through for the second time. Maybe this was because there was some in the column or I pipetted too fast initially.

### Nanodrop from MaxiPrep

#	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	2283.8	ng/μl	45.675	23.884	1.91	2.33
2	1980.1	ng/μl	39.603	20.724	1.91	2.36

Friday, October 21, 2022

1. ~~Electroporate~~
2. ~~LB media~~

- ~~3. MHB~~
- ~~4. Serological bin~~

Looking for transformation efficiency, check recovery time, and how to plate. This is testing the cells that I made on Tuesday, October 18<sup>th</sup>. Test with 55 uL EC.

1. -For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
  - a. Warmed for 15 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a. 1 µg in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
    - Current concentration is 156.2 ug/uL
    - Adding 6.4 ul of pKR141
    - 1 ug
  - b. 50 µL electrocompetent cells
    - 1 aliquots (110 uL ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
  - a. Sample 1 = 2.49
5. -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
  - a. Maybe clumpy due to dense cells
  - b. When adding the 1 mL try to break up the clumps as best as possible
6. -Recover cells for 3 hours, shaking at 37°C
7. -Plate the following (on CHAH-KAN):
  - 4 round plates per electroporation = 2 total
    1. 100 ul
  - 2 track plates per electroporation = 2 total
    1. Only 3 dilutions 1 x 2, 0.1
    2. From recovery tube, take out 300 ul into 1.5 mL
    3. 2 more 1.5 mL put 90 ul of PBS
    4. 10 ul of undiluted into 1<sup>st</sup> 1.5 mL
      - a. Vortex!!
    5. After 10 ul from each tube onto track plates
  - Transformants per microgram of DNA = transform efficiency
  - Before with pKL97:  $1 \times 10^4$  to  $7 \times 10^4$  transformation efficiency
  - Library of 400,000 mutants is the goal
  - 2 million sequence
8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

\*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.

### Muller Hinton Broth (500 mL)

- 10.5 g of MHB
- 500 mL of ddiH2O
- Spin
- Autoclave for liquid 30'

Made 1 500 mL.

### LB Media

Protocol written by KMR

#### Preparing LB media

1. For 500 mL of LB, weigh out the following components and add to a 500 mL bottle:
  - a. 5 g NaCl
  - b. 5 g Tryptone
  - c. 2.5 g Yeast extract
2. Add 500 mL type I ddiH2O
3. Close tightly and shake to mix
4. Loosen cap and add a small piece of autoclave tape with the date
5. Autoclave on 30 minute liquid cycle
6. Sterile media can be stored indefinitely

Made 1 x 500ml and 2 x 250 mL

I didn't remember I had pKR141 still in the plasmids box, so now there's A LOT of pKR141. Here's the first dilution I did to make it 100 ul/ug.

### Monday, October 24, 2022

- ~~1. Check EP plates~~
- ~~2. Dilute pKR141~~
3. More EC

	100 uL	0.1	1	1	Average of 100 uL	TE of 100 uL
EP1-A	182	3	59	42		
EP1-B	205	10	25	40	193.5	7.74E+03

For diluting pKR141:

$$V1 = (200 \times 100) / 2283 = 8.76 \text{ uL of combined maxi prep}$$

91.24 uL of 0.1 x EB which should have been 191.24 lol, I again, wasn't thinking.

Nanodrop:

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	194.4	ng/ul	3.888	2.017	1.93	2.39

After adding the additional 100  $\mu$ L of 0.1 x EB:

90.2 ng/ $\mu$ L

Trying to make more cells for mutant library part 5. Scraped up from four plates.

8. -Scrape up entire plate of cells into 400  $\mu$ L of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
9. -Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
10. -Spin for 3 minutes at 10,000 rpm
11. -remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
12. -Repeat 5x in 10% sucrose
13. -After final spin, remove most of supernatant. Resuspended with remaining supernatant. Transfer all tubes to one tube (2mL tubes)
  1. Added 200  $\mu$ L to tube 1 to resuspend
  2. Added the volume of tube 1 to tube 2 to resuspend.
  3. Ask Hannah or Kathryn to look
  4. Then aliquoted 105  $\mu$ L into 6 different 1.5 mL sterile tubes.
  5. With the extra 10  $\mu$ L I plated on a kan plate to see if there was any contamination.
14. -For any extra EC cells, aliquot  $\sim$ 110  $\mu$ L / sterile tube (enough for 2 electroporations) and freeze at -80°C

## Tuesday, October 25, 2022

1. ~~Electroporate~~
2. ~~LB agar~~
3. ~~CHAH KAN big plates~~

No contamination on test plate.

Looking for transformation efficiency, check recovery time, and how to plate. This is testing the cells that I made on Monday, October 24<sup>th</sup> with Kathryn.

1. -For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
  - a. Warmed for 15 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a. 1  $\mu$ g in 10  $\mu$ L or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/ $\mu$ L)
    - Current concentration is 156.2  $\mu$ g/ $\mu$ L
    - Adding 6.4  $\mu$ L of pKR141
    - 1  $\mu$ g
  - b. 50  $\mu$ L electrocompetent cells
    - 1 aliquots (110  $\mu$ L ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25  $\mu$ F, and 600  $\Omega$

- a. Sample 1 = 3.3 (loud pop)
  - b. Sample 2 = arc
5. -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
  - a. Maybe clumpy due to dense cells
  - b. When adding the 1 mL try to break up the clumps as best as possible
6. -Recover cells for 3 hours, shaking at 37°C (went in at 7:54 am and took out at 11:05 am)
7. -Plate the following (on CHAH-KAN):
  - 2 round plates per electroporation = 4 total
    1. 100 ul
  - 2 track plates per electroporation = 2 total
    1. Only 3 dilutions 1 x 2, 0.1
    2. From recovery tube, take out 300 ul into 1.5 mL
    3. 2 more 1.5 mL put 90 ul of PBS
    4. 10 ul of undiluted into 1<sup>st</sup> 1.5 mL
      - a. Vortex!!
    5. After 10 ul from each tube onto track plates
  - Transformants per microgram of DNA = transform efficiency
  - Before with pKL97:  $1 \times 10^4$  to  $7 \times 10^4$  transformation efficiency
  - Library of 400,000 mutants is the goal
  - 2 million sequence

My plates were a tad wetter than I would have liked. I didn't dry the 100 ul but I did dry the track plates for 5 minutes which helped.

8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

\*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.

Made two flasks of LB agar as well as 10 big CHAH-KAN plates for the electroporation on Friday (fingers crossed). Made two ½ flasks of CHAH and added 30 uL of kanamycin.

### Thursday, October 26, 2022

1. ~~check ep plates~~
2. ~~clean cuvettes~~

	100 uL	0.1	1	Average of 100 uL	TE of 100 uL
EP1-A	8	0?	0?		
EP1-B	4	n/a	0?	6	2.40E+02
EP2-A	122	4	30	116	4.64E+03

EP2-B      110      n/a      26

### Friday, October 28, 2022

#### ~~1. mutant ep!~~

Prior to EP, needed to make more dilute pKR141. Did a dilution of 1 to 5 to accurately measure the super concentrated maxi prep from July. It as 787.9 ng/uL. Here's is some math that should make sense:

$$787.9 \text{ ng/uL} \times 5 = 3939.5 \text{ ng/ul} = C1$$

$$V2 = 100 \text{ uL}$$

$$C2 = 1000 \text{ ng/5ul} = 200 \text{ ng/ul}$$

$$(100 \times 200) / 3939.5 = 5 \text{ uL}$$

So I used 5 uL of concentrated pKR141 and 95 uL of WATER. (Molecular grade) not exactly sure why, I should ask.

Making the mutant library! Whoot whoot! Fingers crossed it all goes well. Putting them in at 8 am and plating at 11 am.

1. -For each electroporation, aliquot 3 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
  - a. Warmed for 15 minutes
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - a. 1 µg in 10 uL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
    - Current concentration is 200 ug/uL
    - Adding 5 ul of pKR141
    - 1 ug
  - b. 50 µL electrocompetent cells
    - 1 aliquots (110 uL ) done in duplicate
3. -Have recovery media ready
4. -Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
  - a. Sample 1 = pop!
  - b. Sample 2 =
  - c. Sample 3 = arc
  - d. Sample 4 = pop
  - e. Sample 5 = arc
  - f. Sample 6 = pop
  - g. Sample 7 = pop
  - h. Sample 8 = 3.6
  - i. Sample 9 = 3.00
  - j. Sample 10 = 3.5
  - k. Samples 1-7 had the wrong settings so we did not capture the correct time. Also the first half were put into the shaking incubator 9 minutes early.
5. -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
  - a. Maybe clumpy due to dense cells



- b. When adding the 1 mL try to break up the clumps as best as possible
- 6. -Recover cells for 3 hours, shaking at 37°C
- 7. -Plate the following (on CHAH-KAN):
  - a. Got distracted in lab meeting so we plated about 3.5 hours later.
  - b. Plate 3 mL of each EP onto individual big plate with beads = 10 plates total
    - Pellet for 3 min at 10000 rpm and then throw away most of supernatant then resuspend with least amount possible
  - c. For TE
    - 100 ul onto smaller plates = 2 plates
- 8. -Incubate plates at 37°C for 3 days (or until single colonies appear)

\*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.

### Monday, October 31, 2022 – Happy Halloween 🎃

1. ~~MHB~~
2. ~~Struck out LVS~~

Made 3 flasks of MHB. Struck out 5 plates of LVS to make more electrocompetent cells tomorrow. I did this because I looked at the representative 100 ul plates and there were only 4 colonies from the electroporation from Friday. (not what we are looking for) I messaged Dr. Ramsey if I should continue to plan on harvesting on Wednesday.

## November 2022

### Tuesday, November 1, 2022

1. CHAH-Kan

Plate	Quad est	Full Plate Est
1	103	412
2	126	504
3	276	1104
4	137	548
5	82	328
7	292	1168
8	63	252
9	147	588
10	167	668
	Total	5572

Above is the very rough estimation from my plates electroporated on Friday 10/28. Even though unideal, I am moving forward with the project and will harvest tomorrow. Leaving all the plates at room temperature until tomorrow except plate \_\_\_\_ which has tiny colonies so it is going back in the incubator.

Making CHAH-KAN square plates for track plating on 11/7 and 11/14.

### Wednesday, November 2, 2022

- ~~2. Make mutant library~~
  - ~~a. Single use aliquots~~

### Harvesting in MHB with 15% glycerol

- 50 mL conical
- Add MHB (2.4 mL)
- Add glycerol (200uL 75%)
- Scrape the cells up using a cell scraper and resuspended
- Label and freeze

### 50 ul single use aliquot of LVS

- day before streak out one lawn from glycerol stock
  - I struck out 5 plates of LVS for the potential for having to make more electrocompetent cells, but then I didn't need to, but I feel bad throwing them all away. So I am going to use one and make back up 50 ul single use aliquots of LVS
  - 800 ul MHB (400 ul + resuspend and then add remaining 400 ul)
  - Take all of lawn
- 200 ul of 75% glycerol
- Vortex
- 50 ul aliquots
- Made 20 aliquots

### Friday, November 4, 2022

- ~~1. Work on grant~~
- ~~2. Prep for Monday~~

Things to prepare:

- Flasks
- Label plates
- 96 well plate

### Sunday, November 6, 2022

- ~~1. Patch out for water~~

### Monday, November 7, 2022

- ~~1. Put into water system~~
- ~~2. Scrape up some and pellet for isolation of gDNA~~

- put 175 mL of sterile freshwater into the 250 mL baffled flask for inoculation.

**Take OD first!**

3. Get a sterile 1.5 mL tube and pipette 500 uL of freshwater into it.
4. Scrape up all of cells that were prepared the previous day into the 1.5mL tube with freshwater. Resuspend the cells.
5. Add 500 uL more freshwater to the 1.5 tube
6. Pipette 980 uL of freshwater and 20 uL of resuspended cells into the cuvette. Place parafilm on top and invert 3 to 5 times, slowly.
7. Use spectrometer to measure OD. Obtain an OD<sub>600</sub> of 0.03. Don't forget the blank!
8. Needs to be between 0.05-0.60 (dynamic range of the spec), if more than 0.6 need to dilute
9.  $C1V1=C2V2$
10.  $V1 = 260 \text{ uL}$ 
  - a. Amount of resuspended cells to add
11.  $C1 = 0.403$ 
  - a. the OD measured x 50 (amount diluted)
12.  $C2 = 0.03$
13.  $V2 = 175 \text{ mL}$
14. Take prepared 175 mL freshwater in 250mL baffled flask and add V1 of resuspended cells to it. Put the cells into three technical replicates. 50 mL into three different flasks.

**Plating protocol**

This is for the initial inoculum so we have an idea of the starting CFU/mL. Using four CHAH-KAN square track plates.

- Prepare 96 well plate, cuvette, parafilm, freshwater samples, square CHAH plate, and 1xPBS
  - Test plate to see if it is dry enough by pipetting 10 uL of freshwater and watching it drip.
1. Pipette 180 uL of 1 x PBS into wells row B1/2-G1/2.
    - a. Use multichannel and a reservoir.
  2. Pipette 200 uL from 100mL (which is now 10ml) of resuspended cells and freshwater into A1 and A2
    - a. Use a 200 uL pipette.
  3. Using the multichannel pipette, pipette 20 uL from A into B. Mix 150 uL of the volume three times.
  4. Repeat step 3 for B to C, C to D.
    - a. hint = say it out loud so you don't lose your place.
    - b. Change the tip each time!
  5. Use the less volume multichannel pipette set to 10 uL and change the orientation of the plate to vertical before starting the dripping process.
  6. Pipette 10 uL from A1-G1 onto the plate, letting each drip down the plate.
    - a. Remember to stay within the 6 inches of the flame, don't put the handle of the multichannel pipette to the flame, have the plates at roughly a 45-degree angle, and place to dry in between two pipette boxes at the same angle.
    - b. Two plates for each column. So should be 4 total plates
    - c. Place in incubator for 2 days and then pull out and count

The first two plates were fine, but the second two were wet and had some convergences unfortunately.

## Pelleting

1. Took what was leftover from the V1 tube, and pelleted it down for 5 min at max speed.
2. This led to a huge pellet, so I pipetted out 500 ul of a resuspended. Pelleted again and again had too much. So I resuspended and divided into two tubes. Pelleted once again. This led to a slightly too big but fine pellet for gDNA isolation.
3. Put into my -80C box
4. Isolate gDNA on Wednesday

## Tuesday, November 8, 2022 – Election Day

1. Turn in grant to Hannah/Kathryn
2. Election day

## Wednesday, November 9, 2022

- ~~1. gDNA isolation on the mutant library~~  
~~i. 100 uL~~
- ~~2. Make Hemoglobin~~
- ~~3. MHB~~
- ~~4. LB Agar~~
- ~~5. 75% glycerol~~
- ~~6. Count plates~~

## Isolate gDNA

I am isolating the gDNA from the mutant library that was patched out on Sunday, November 6<sup>th</sup>.

Look for isopropanol individual aliquot!!

## Cell Samples

1. Dilute 1  $\mu$ l of Proteinase K into 300  $\mu$ l of Tissue and Cell Lysis Solution for each sample (can use 310 uL to account for pipetting error).
2. Pellet cells by centrifugation (0.5-1 x 10<sup>6</sup> mammalian cells; 0.1-0.5 ml of an overnight culture of E. coli) and discard the supernatant, leaving approximately 25  $\mu$ l of liquid.

This step was completed on Monday, and I put my pellets in the -80C overnight. I left them with the liquid on top and did not resuspend.

1. Vortex for 10 seconds to resuspend the cell pellet.
  - a. Pipetted with 300 uL to resuspend
4. Add 300 ul of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Cool the samples to 37°C and add 1  $\mu$ l of 20 mg/ml RNase A to the sample; mix thoroughly.

Took me 10 minutes to do this

7. Incubate at 37°C for 30 minutes.

8. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation\ (below).

### **Precipitation of Total DNA (for all biological samples)**

1. Add 150  $\mu$ l of MPC Protein Precipitation Reagent to 300  $\mu$ l of lysed sample and vortex vigorously for 10 seconds.

2. Pellet the debris by centrifugation at 4°C for 10 minutes at  $\geq 10,000 \times g$  in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25  $\mu$ l of MPC Protein Precipitation Reagent, mix, and pellet the debris again.

Make ethanol during this

3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.

4. Add 500  $\mu$ l of isopropanol to the recovered supernatant. Invert the tube 30-40 times.

5. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.

6. Carefully pour off the isopropanol without dislodging the DNA pellet.

7. Rinse twice with 70% ethanol (1mL), being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet and let dry completely under hood.

**(the fume hood in dr. bertins lab not the BLS hood)** (for 5 min with the caps open)

- 7 mL of ethanol and 3 mL of diiH<sub>2</sub>O

- pipette the ethanol then remove

8. Resuspend the DNA in 35  $\mu$ l of 0.1x EB Buffer. Put on ice to help dissolve, and add 50  $\mu$ l of additional buffer if DNA is very goopy.

9. Check concentration and purity by nanodrop.

### **Nanodrop results**

#	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	1883.2	ng/ $\mu$ l	37.664	20.01	1.88	1.45
2	2108.1	ng/ $\mu$ l	42.162	22.331	1.89	1.74

### **Muller Hinton Broth (500 mL)**

- 10.5 g of MHB
- 500 mL of diiH<sub>2</sub>O
- Spin
- Autoclave for liquid 30'

Made 3 500 mL.

Oli made the LB and I brought to the autoclave. It boiled over 😞

Day 0 Counts:

Initial Inoculum							Dilution Factor Counted	Avg. Cells	CFU p mL
	1	2	3	4	5	6	7		

1-A					103	10		0.0001	103	1.03E+
1-B					103	9		0.001		0
2-A					converge			0.0001	92	920000
2-B					92	18		0.001		0
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			

Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
0.0001	97.5	97500000	7778175
0.001	#DIV/0!	#DIV/0!	0

### Friday, November 11, 2022 – Veteran's day

- ~~1. Work on grant~~

### Monday, November 14, 2022

- ~~1. Track plating day 7~~
  - ~~a. 6 CHAH-KAN plates~~
  - ~~b. Pelleting day 14~~
- ~~2. Fix gDNA~~

### Plating protocol

This is for the initial inoculum so we have an idea of the starting CFU/mL. Using four CHAH-KAN square track plates.

- Prepare 96 well plate, cuvette, parafilm, freshwater samples, square CHAH plate, and 1xPBS
  - Test plate to see if it is dry enough by pipetting 10 uL of freshwater and watching it drip.
- Pipette 180 uL of 1 x PBS into wells row B1/2-G1/2.
    - Use multichannel and a reservoir.
  - Pipette 200 uL from 100mL (which is now 10ml) of resuspended cells and freshwater into A1 and A2
    - Use a 200 uL pipette.
  - Using the multichannel pipette, pipette 20 uL from A into B. Mix 150 uL of the volume three times.
  - Repeat step 3 for B to C, C to D.
    - hint = say it out loud so you don't lose your place.
    - Change the tip each time!
  - Use the less volume multichannel pipette set to 10 uL and change the orientation of the plate to vertical before starting the dripping process.
  - Pipette 10 ul from A1-G1 onto the plate, letting each drip down the plate.
    - Remember to stay within the 6 inches of the flame, don't put the handle of the multichannel pipette to the flame, have the plates at roughly a 45-degree angle, and place to dry in between two pipette boxes at the same angle.

- b. Two plates for each column. So should be 4 total plates
- c. Place in incubator for 2 days and then pull out and count

### Pelleting

1. Take 15 mL from each flask into a 50 mL conical and max spin for 5 minutes
    - a. Should have three 50 mL
    - b. Remove supernatant as best as possible
  2. Freeze
  3. Isolate gDNA together at the end of 2 weeks
- Removing the supernatant was a tad tricky, the pellet was very loose.

The gDNA was fine, it was out of the linear range, but that shouldn't affect PCR.

### Tuesday, November 15, 2022

- ~~1. Submit grant~~

### Wednesday, November 16, 2022

- ~~1. Count plates day 7~~
- ~~2. Make more CHAH-Kan plates~~

Day 7 Counts:

	Initial Inoculum							Dilution Factor Counted	Avg. Cells
	1	2	3	4	5	6	7		
1-A				94	11			0.0001	10.5
1-B				92	10			0.001	93
2-A				81	10			0.0001	10
2-B				87	10			0.001	84
3-A				90	11			0.0001	11
3-B				83	11			0.001	86.5
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001		

Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
0.0001	10.5	10500000	500000
0.001	87.83333	8783333	464578.7

### Friday, November 18, 2022

- ~~1. Last day before Ireland!~~
- ~~2. Ask someone to plate~~

### Monday, November 21, 2022

2. Track plating day 14
  - a. 6 CHAH-KAN plates
3. Pelleting day 14

## Pelleting

4. Take 15 mL from each flask into a 50 mL conical and max spin for 5 minutes
  - c. Should have three 50 mL
5. Freeze
6. Isolate gDNA together at the end of 2 weeks

## Monday, November 28, 2022

1. ~~Back from Ireland~~
2. ~~Remind Kathryn about URI SURE~~
3. ~~Made 4 flasks of 2% Hemoglobin~~

## Hemoglobin

- 6 g hemoglobin
- 300 mL of diiH2O
- 20 min liquid 20

## Day 7 Plating Data:

	Initial Inoculum							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A				94	11			0.0001	10.5	1.05E+07
1-B				92	10			0.001	93	9300000
2-A				81	10			0.0001	10	10000000
2-B				87	10			0.001	84	8400000
3-A				90	11			0.0001	11	1.10E+07
3-B				83	11			0.001	86.5	8650000
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			

Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
0.0001	10.5	10500000	500000
0.001	87.83333	8783333	464578.7

## Day 14 Plating Data:

	Initial Inoculum							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A				68	9			0.0001	7	7.00E+06
1-B				56	5			0.001	62	6200000
2-A				47	5			0.0001	4	4000000
2-B				45	4			0.001	46	4600000
3-A				62	6			0.0001	6	6.00E+06
3-B				41	6			0.001	51.5	5150000
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			



Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
0.0001	5.666667	5666667	1527525
0.001	53.16667	5316667	812916.6

## Tuesday, November 29, 2022

1. Isolate all the gDNA

### Isolate gDNA

I am isolating the gDNA from the mutant library day 7 and 14.

### Cell Samples

1. Dilute 1  $\mu$ l of Proteinase K into 300  $\mu$ l of Tissue and Cell Lysis Solution for each sample (can use 310  $\mu$ L to account for pipetting error).
2. Pellet cells by centrifugation (0.5-1 x 10<sup>6</sup> mammalian cells; 0.1-0.5 ml of an overnight culture of E. coli) and discard the supernatant, leaving approximately 25  $\mu$ l of liquid.

This step was completed on Monday, and I put my pellets in the -80C overnight. I left them with the liquid on top and did not resuspend.

2. Vortex for 10 seconds to resuspend the cell pellet.
  - a. Pipetted with 300  $\mu$ L to resuspend
4. Add 300  $\mu$ l of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex every 5 minutes.
6. Cool the samples to 37°C and add 1  $\mu$ l of 20 mg/ml RNase A to the sample; mix thoroughly.

Took me 10 minutes to do this

7. Incubate at 37°C for 30 minutes.
8. Place the samples on ice for 3-5 minutes and then proceed with total DNA precipitation (below).

### Precipitation of Total DNA (for all biological samples)

1. Add 150  $\mu$ l of MPC Protein Precipitation Reagent to 300  $\mu$ l of lysed sample and vortex vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at  $\geq 10,000 \times g$  in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25  $\mu$ l of MPC Protein Precipitation Reagent, mix, and pellet the debris again.

Make ethanol during this

3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500  $\mu$ l of isopropanol to the recovered supernatant. Invert the tube 30-40 times.

5. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the DNA pellet.
7. Rinse twice with 70% ethanol (1mL), being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet and let dry completely under hood.  
(**the fume hood in dr. bertins lab not the BLS hood**) (for 5 min with the caps open)
  - 7 mL of ethanol and 3 mL of diH<sub>2</sub>O
  - pipette the ethanol then remove
8. Resuspend the DNA in 35 µl of 0.1x EB Buffer. Put on ice to help dissolve, and add 50 µl of additional buffer if DNA is very goopy.
9. Check concentration and purity by nanodrop.

### **Nanodrop results**

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	7	Science	11/29/2022 1:22:01 PM	312	ng/µl	6.241	3.18	1.96	2	DNA	50
2	14	Science	11/29/2022 1:22:46 PM	192.2	ng/µl	3.844	1.977	1.94	1.83	DNA	50

### **Wednesday, November 30, 2022**

#### **1. PCR**

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

1. Assemble the linear PCR reactions on ice

Total Reaction Vol	100
Total # of reactions	3

Component	Stock Concentration	Final Concentration	1 rxn volume	Factor
ddiH <sub>2</sub> O			52	3.5
Platinum SuperFi Buffer	5x	1 x	20	182
dNTPs	10 mM	0.2 mM	2	70
BioSamA	1 uM	0.05 uM	5	7
DNA		2 ug	20	17.5
Platinum SuperFi Polymerase	2 U/uL	0.02 U/uL	1	add individually
Total Volume			100	280

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
5	(-) DNA	-	0.00	20.00

Messed up and didn't make enough MasterMix for tubes 4 and 5, so here is the table:

Total Reaction Vol	100
Total # of reactions	2

Component	Stock Concentration	Final Concentration	1 rxn volume	Factor
ddiH2O			52	2.5
Platinum SuperFi Buffer	5x	1 x	20	130
dNTPs	10 mM	0.2 mM	2	50
BioSamA	1 uM	0.05 uM	5	5
DNA		2 ug	20	12.5
Platinum SuperFi Polymerase	2 U/uL	0.02 U/uL	1	add individually
		Total Volume	100	2.5
				200

- 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)
- 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
  - Elute in 50 uL of buffer EB (**Undiluted**) sit for one minute then spin for 1 minute

## December 2022

### Friday, December 2, 2022

- ~~1. Write methods~~

### Monday, December 5, 2022

- ~~1. 1% Agarose~~

### Tuesday, December 6, 2022

- ~~1. Write methods~~

### Wednesday, December 7, 2022

- ~~1. B&W buffer~~
2. LoTE buffer

	Stock Conc (M)	Final Conc(M)	Volume (uL)
NaCl	5	2	8000
Tris	1	0.01	200
EDTA	0.5	0.001	40
Water			9760
		Total	18000

pH vol  
added 20  
water 1980  
End Vol 20000

pH 7.52

added 20 ul of 5N HCl

I hate pHing.

- 8.10 start – added 3 uL of 5N HCl
- 8.01 – 2uL
- 7.959 – 2 uL
- 7.905 – 3 uL
- 7.808 – 2 uL
- 7.756 – 2 uL
- 7.681 – 2 uL
- 7.592 – 2 uL
- 7.552 – 1 uL
- 7.521 – 1 uL

**Friday, December 9, 2022**

1. ~~Meeting~~

**Monday, December 12, 2022**

1. ~~LoTE Buffer~~
2. Filter sterilize the buffers

	Stock Conc (M)	Final Conc(M)	Volume (uL)
Tris	1	0.003	45
EDTA	0.5	0.0002	6
Water			12949
		Total	13000

pH vol  
added 2  
water 1998  
End Vol 15000

Added 2 uL of 1 N NaOH.

- 7.24 starting pH – 2 uL of 1 N NaOH
- 7.45

I did not look at the label of the beads and placed them in the freezer instead of the fridge. Not so smart of me. So, we had to throw them away and Kathryn will continue the experiment to see if it will work.

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

1. Resuspend streptavidin-coated beads by shaking.
2. Add beads (32uL per sample) to a new microcentrifuge tube (1mL max, use multiple tubes if necessary)
3. Place the tube on the MPC for 1-2 minutes
  - a. Magnetic particle collector
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 2x = 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5
  - b. Dilute with water
6. Repeat steps 26-28 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).
8. Add the entire volume of one sample from step 23 to the tube.
9. Incubate at room temperature with gentle mixing for 30 minutes.
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 33-35 twice, but resuspend beads in 100ul of the LoTE buffer ea. Time.
  - a. Can be stored at 4°C overnight.
  - b. LoTE buffer: 3 mM tris and 0.2 mM EDTA, pH 7.5