

Contents

July 2022.....	3
Friday, July 1, 2022	3
Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak.....	3
Tuesday, July 5, 2022	4
300 ul Plating Protocol.....	4
Supplemented MHB.....	5
Wednesday, July 6, 2022	5
Diagnostic Digest.....	6
Track Plating Protocol	7
Sequencing.....	7
Thursday, July 7, 2022.....	8
Preparing LB-agar.....	8
Friday, July 8, 2022	9
Monday, July 11, 2022.....	9
Receiving Primers Protocol KROL532 and KROL533	10
Tuesday, July 12, 2022	10
Wednesday, July 13, 2022	11
300 ul Plating Protocol.....	12
Track Plating Protocol	12
Transform chemically competent <i>E. coli</i> cells.....	12
Thursday, July 14, 2022.....	13
Overnight for <i>E. coli</i>	14
Friday, July 15, 2022	14
Maxi-Prep Protocol (CompactPrep).....	14
Monday, July 18, 2022.....	16
Tuesday, July 19, 2022	17
Wednesday, July 20, 2022	19
300 ul Plating Protocol.....	19
Thursday, July 21, 2022.....	19
Friday, July 22, 2022	19
Monday, July 25, 2022.....	20
Isolate gDNA	20
Freeze down strain	22

Tuesday, July 26, 2022 23

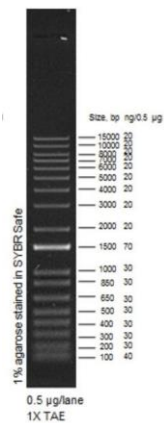
300 ul Plating Protocol..... 23

Wednesday, July 27, 2022 23

Thursday, July 28, 2022..... 24

Friday, July 29, 2022 24

Pay Period Information 24



DNA ladder used throughout this notebook.

July 2022

Friday, July 1, 2022

To Do:

1. ~~Make plates for day 69? And day 14~~
 - a. ~~8 square and 6 circular~~
2. ~~Pull out day 63 and day 7~~
3. ~~Sequencing~~

These are sequencing reactions using KROL523 and 2 colonies from 3x and 2 colonies from 5x from my transformation plates from 6/28/22.

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 ₂ O needed
							$(A \div 100) \times 2.5$	$(C \div B) \mu l$	$2x(-200 \div B) \mu l$	(12 less D or E - 2.56) μl
AM1		Plasmid	pKR141-1	KROL523	4564	457.2			0.87	8.57
AM2		Plasmid	pKR141-2	KROL523	4564	373.1			1.07	8.37
AM3		Plasmid	pKR141-3	KROL523	4564	355.3			1.13	8.31
AM4		Plasmid	pKR141-4	KROL523	4564	249.7			1.60	7.84
a. Add 2.56 μl of 2.5 μM stock to each reaction										
3130xl Plate Record										
PI	Kathryn Ramsey	Date	7/1/2022	Name	Aisling Macaraeg					
		Dept	CMB	Email	amacaraeg@uri.edu	PO No.				

Went well, no issues that I can think of. Will get results on Wednesday hopefully.

Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

For 300 mL of CHA

1. Take out 15.3g of cystine heart agar into 1L flask (non-baffled; 10.2g/100mL)
2. Add 150mL of ddiH₂O (type I)
3. Add stirbar to flask
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 hemoglobin 2% solution
8. Add 3g freeze-dried hemoglobin to 150mL of ddiH₂O (type I)
9. Autoclave on 20' liquid cycle with water in the bin
10. Cool down (ideally to ~55C)
11. Using sterile technique, pour hemoglobin into CHA
12. Using a 50mL pipet, aliquot 30mL of CHAH mixture into each 100mm plate (should make approximately 20 plates) Try to avoid bubbles! 24 mL per circular plate.

Made 15 CHAH circular plates and 8 square plates.

Tuesday, July 5, 2022

To Do:

1. ~~Plate day 69~~
2. ~~Plan electroporation's~~
3. ~~Write rough drafts~~
4. ~~Supplemented MHB~~

300 ul Plating Protocol

Plated day 69 of experiment 2.

1. Place 20 beads on each plate aseptically
2. Pipette 300 ul of each sample onto each plate
3. Shake in each direction
4. Let each plate sit for at least 2-3 minutes so all of the liquid is absorbed
5. Remove the beads within the flame into the 50 mL tube and add ethanol if it is not covering all the beads.

Day 3 Counts (rep 3):

	Track Plate 1 - 4°C							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A			85					0.01	89	890000
1-B			93					0.0001	0	0
2-A			84					0.01	89.5	895000
2-B			95					0.0001	0	0
3-A			111					0.01	102.5	1025000
3-B			94					0.0001	0	0
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			
Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells		CFU per mL		Stdev				
	0.01	93.66667		936666.7		76539.75				
	0.0001	0		0		0				

Day 7 (rep 3):

	Track Plate 1 - 4°C							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A			85					0.01	89	890000
1-B			93					0.0001	0	0
2-A			84					0.01	89.5	895000
2-B			95					0.0001	0	0
3-A			111					0.01	102.5	1025000
3-B			94					0.0001	0	0
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			
Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells		CFU per mL		Stdev				
	0.01	93.66667		936666.7		76539.75				
	0.0001	0		0		0				

Day 56 Counts (exp. 2) :

300 ul circular plates				
Plate	Cells	Avg. Cells	CFU/mL	
1A	1	0.5	1.666667	
1B	0			
2A	1	1	3.333333	
2B	1			
3A	0	0	0	
Track Plates 1, 2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	
				Stdev
		0.5	1.666667	1.666667

Supplemented MHB

- Mixed 0.75 g of iron pyrophosphate with 30 mL of ddiH₂O for 3 hours
- Used a syringe to filter sterilize (above Hannah's bench) into a sterile 50 mL conical that was pre-wrapped in tin foil
- Added 5mL of the iron to MHB
- Added 5mL of 10% glucose
- Added isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)
- Can keep this supplemented media for 2 weeks, storing at 4 °C
- Label with date made and expiration date

Wednesday, July 6, 2022**To Do:**

- ~~1. Plate day 14~~
- ~~2. Look at sequencing results~~
- ~~3. Diagnostic digest~~
- ~~4. Order primers KROL532 and 533~~

Sequencing results came back with no signal for a second time. I feel like there might be something wrong with the primer. Or maybe I should dilute because I was pipetting very small amounts of the DNA. Possible Errors:

- Wrong DNA template
- Wrong primer
 - o Double checked what was ordered binds to the right site and the right temperature
- Too low of DNA concentration
- DNA contamination
 - o From some other source??
- Missing primer annealing site

As a reminder, here are the concentrations from the minipreps from 6/30/22.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	mp1	457.2	ng/μl	9.144	4.725	1.94	2.36
2	mp2	373.1	ng/μl	7.461	3.832	1.95	1.88
3	mp3	355.3	ng/μl	7.106	3.651	1.95	2.43

4 mp4 249.7 ng/μl 4.993 2.546 1.96 2.42

Possible Solutions:

Sequence MP1 with KROL522: checking that there is an backbone

Design a new primer for upstream of KROL520

Design a new primer for downstream of KROL523 (2560)

Diagnostic Digest of kpn1 and BamHI – insert of 1476 and a backbone of 3088 (no insert is 1410)

1% gel for an hour and a half

All four of MP – 2 ul

Positive control pKR140 – cut with kpn1/bamHI should insert – 1468 and 3088

Diagnostic Digest

I am doing this digest to try to see if this is the right backbone and possibly insert by comparing to pKR140.

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Miniprep 1	Miniprep 2	Miniprep 3	Miniprep 4	pKR140 (positive control)

For diagnostic digest:

Number of samples

5

Master mix factor

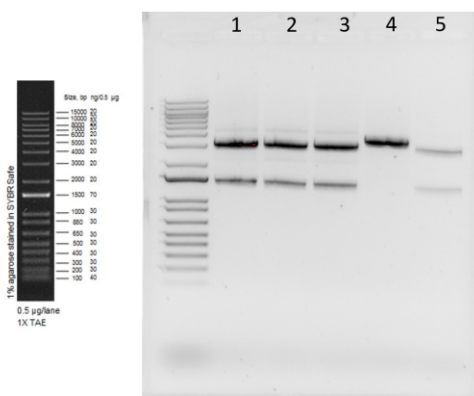
6

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	15	90
10x Buffer*	2	12
DNA	(2)	
Enzyme 1 – Kpn1-HF	0.5	3
Enzyme 2 – BamHI - HF	0.5	3
Total	20.0 (18.0 actual b/c of DNA)	108

- Mix the master mix by pipetting up and down.
 - Used 100ul to mix
- 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)
- Incubate at 37°C for 1 hour
 - 25C room incubator
- Run on a small gel rig with the smaller comb (with 6 loading spaces)
 - 5 ul of cyber safe
 - 10 ul of lader
 - 20 ul of sample + 4 ul of dye

Results – ran for an hour and 15 minutes

Purpose:	Diagnostic Digest of Minipreps
Results	Looks like a plasmid?
Notes	KpnI and BamHI



Lane	Expected bp	Expected: Yes or No
1 – miniprep 1	insert of 1476 backbone of 3088	yes
2 – miniprep 2	insert of 1476 backbone of 3088	yes
3 – miniprep 3	insert of 1476 backbone of 3088	yes
4 – miniprep 4	insert of 1476 backbone of 3088	No – looks like one of the enzymes worked but not both, most likely due to lack of mixing before incubation
5 – pKR140	insert of 1468 and bb of 3088	yes

Track Plating Protocol

Day 14 for experiment 2. Went well, some of the plates were a little wetter than I would have hoped, but no convergences.

- 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

Sequencing

This was done after I remade KROL523. One did one miniprep as a test run to see if remaking the stock changed anything.

To do this I:

- Add 195 ul of 0.1xEB to a new 1.5 mL tube
- Vortexed the 100 uM tube of KROL523
- Add 5 ul of 100 uM stock to new tube
- Vortexed and spun down
- Labeled with blue sticker.

Sample number	Well	Template Type	Template Name	Primer Name ^a	A.	B.	C.	D.	E.	F.
	(GSC use <u>only</u>)	(plasmid or PCR)			Template Size (bases)		Template Stock Conc. (ng/μl)	PCR template; <u>PCR template:</u>	PCR template; <u>PLASMID template:</u>	Volume H ₂ O <u>needed</u>
								ng needed =	Volume =	Volume =
								(A ÷ 100) × 2.	(C ÷ B) μl	2x(-200 ÷ B) μl
AM1		Plasmid	pKR141-1	KROL523	4564	712.7	457.2		0.87	8.57
a. Add 2.56 μl of 2.5 μM stock to each reaction										
3130xl Plate Record		Date	7/6/2022	Name	Aisling Macaraeg					
PI	Kathryn Ramsey	Dept	CMB	Email	amacaraeg@uri.edu	PO No.				

Thursday, July 7, 2022

To Do:

- ~~1. Work on abstract~~
- ~~2. Meet with Dr.~~
- ~~3. LB Agar~~

Preparing LB-agar

I made 2 flasks.

1. For 500 mL of LB-agar, weigh out the following components and add to a 1 L (non-baffled) flask:
 - a. 6 g agar
 - b. 5 g NaCl
 - c. 5 g Tryptone
 - d. 2.5 g Yeast extract
2. Add stirbar to flask
3. Add 500 mL ddiH2O
4. Mix on stirplate until components are dissolved
5. Cover top of flask with foil and add a small piece of autoclave tape
6. Autoclave on 30 minute liquid cycle

pKR141 why is it important?

The ends are different – in the old plasmid pKL97 the transposon and adapter have a two for one sequence, it is fine but adaptors didn't have sequences compatible with new illumina (next gen sequencing) techniques

Biotinylation

Friday, July 8, 2022

To Do:

1. ~~Pull out day 69 and day 14~~
2. ~~Lab meeting presentation~~
3. ~~Add plasmid differences to poster~~

Still no sequencing signal from the redo of primer KROL523. Dr. Ramsey found that the snapgene file for pKR141 was wrong. So I fixed it and KROL523 can't anneal to the actual plasmid. Luckily, the primers ordered yesterday should work!

Notes from talking to Dr. Ramsey:

- pKL97 used to complete the InSeq library protocol
 - o outcome: short pieces of DNA sequences on illumina flow cell
- InSeq product is the SAME for EVERY piece of DNA library sequence (EXCEPT for gDNA)
- plasmid DNA: part of transposon
- gDNA: next to hop
- bitinylated primer: used to amplify -> double stranded -> digest with mmeI
- mmeI recognition sit in plasmid DNA brings in enzyme
 - o TCCAAC
- mmeI restriction sit
 - o TCCRAC(N)₁₈...NN cut
- P7 flow cell attachment
 - o In 2011 added some bp, which lead to pKL97 no longer working
- InSeq product modified is the NEW outcome
 - o using pKR141
 - o adds 8 bp so it will be usable
- read one sequencing primer (illumine)

Monday, July 11, 2022

To Do:

1. ~~Streak out 4 plates of LVS on CHAN (lawn)~~
2. ~~Make CHAH plates for day 21~~
3. ~~Count day 69 and day 14~~
4. ~~Received Primers KROL 532 and 533~~
5. ~~Sequencing of pKR141 pt 4~~

Sequencing with KROL 532 for Minipreps from 6/30 with hopefully pKR141.

Sample number	Well	Template Type	Template Name	Primer Name ^a	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 ₂ O needed (12 less D or E - 2.56) μl
							(A ÷ 100) × 2.5	(C ÷ B) μl	2x(-200 ÷ B) μl	
AM1		Plasmid	pKR141-1	KROL532	4564	457.2			0.87	8.57
AM2		Plasmid	pKR141-2	KROL532	4564	373.1			1.07	8.37
AM3		Plasmid	pKR141-3	KROL532	4564	355.3			1.13	8.31
a. Add 2.56 μl of 2.5 μM stock to each r										
3130xl Plate Record	Date	7/6/2022	Name	Aisling Macaraeg						
PI	Kathryn Ramsey	Dept	CMB	Email	amacaraeg@uri.edu	PO No.				

Day 14 Counts (rep 3):

	Track Plate 1 - 4°C							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A	24							1	33.5	3350
1-B	43							0.0001	0	0
2-A	73							1	75.5	7550
2-B	78							0.0001	0	0
3-A	118							1	96	9600
3-B	74							0.0001	0	0
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			

Track Plates	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
1,2,3	1	68.33333	6833.333	3186.037
	0.0001	0	0	0

Receiving Primers Protocol KROL532 and KROL533

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. KROL532 = 15.83 nm = 158.3 uL of 0.1xEB
 - b. KROL533 = 8.88 nm = 88.8 uL 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. 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.

Tuesday, July 12, 2022**To Do:**

- ~~1. Draft of abstract~~
- ~~2. Outline of poster~~
- ~~3. Make electrocompetent LVS cells~~
 - ~~a. 5 washes of sucrose~~
- ~~4. Check day 56, 62, and 69 plates~~

Prepare electrocompetent (EC) cells

Making these for when electroporating pKR141.

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)
 - 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
 - Still very turbid supernatant., so I spun it twice.
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. Transfer all tubes to one tube (2mL tubes)
7. Pellet one final time at 10,000 rpm
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.
 - Ask Hannah or Kathryn to look
 - Added 200uL of 10% of sucrose
 - Then aliquoted 110 ul into six different 1.5 mL sterile tubes
 - 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°C

Day 62 counts (rep 3):

Plate	Cells	Avg. Cells	CFU/mL
1A	0	0	0
1B	0		
2A	0	0	0
2B	0		
3A	0	0	0
3B	0		

Day 69 Counts (rep 3)

Plate	Cells	Avg. Cells	CFU/mL
1A	0	0	0
1B	0		
2A	0	0	0
2B	0		
3A	0	0	0
3B	0		

Wednesday, July 13, 2022

- ~~1. Check sequencing results~~
- ~~2. Plate day 21~~

- ~~a. Circular and square plates~~
- ~~3. Transform~~
- ~~4. Streak out four plates of LVS~~
- ~~5. 10% Sucrose~~
- ~~6. Work on poster~~

Sequencing results came back! Definitely not the best design of primer, but MP 2 looks good! Going to move forward with this and transform today.

300 ul Plating Protocol

Day 21 of replicate 3. Went smoothly.

Track Plating Protocol

Day 21 of replicate 3. The plates were wet even though I made them on Monday, so I dried them in the hood for 5 minutes. 1A had a convergence, but with my extra plates I was able to do a successful 1C.

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-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 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 got contamination on my EC CHAH-KAN plate, so I am going to do the following to try to have electrocompetent cells by Monday:

- Struck out my LVS aliquot from June on a CHAN-KAN to see if sensitive or not
- Used JTC's LVS aliquots to streak out 4 other plates to make EC tomorrow

Used a lot of the sucrose last time so I made some more today.

- 50 mL of 50% Sucrose into a graduate cylinder using a sterile serological pipette
- Add 200 mL of diH₂O
- Filter sterilize
- 250 mL of 10% Sucrose is final product

Thursday, July 14, 2022

1. ~~Overnights~~
2. ~~Make EC cells~~
3. ~~Work on poster~~
4. ~~Submit timesheet~~
5. ~~Submit abstract?~~

Transformation Plate	Count
Neg control 20ul, 100 ul	None
pKR141 20 ul, 100 ul	TMTC

Prepare electrocompetent (EC) cells

Used JTC LVS that I struck out with a wooden stick yesterday.

Making these for when electroporating pKR141.

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)
 - Less bubbles than last time.
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
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. Transfer all tubes to one tube (2mL tubes)
16. Pellet one final time at 10,000 rpm
17. -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.

- Ask Hannah or Kathryn to look
- Added 200uL of 10% of sucrose
- Then aliquoted 110 ul into six different 1.5 mL sterile tubes
- With the extra 25 ul I plated on a kan plate to see if there was any contamination.

18. -For any extra EC cells, aliquot ~110 μ L / sterile tube (enough for 2 electroporations) and freeze at -80°C

Overnight for E. coli

- 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 ligation plates = 2 tubes
- 3 tubes total for the overnight

1	2	3
pKR141-1	pKR141-2	Hannah's

Friday, July 15, 2022

1. ~~Roger Williams Lunch~~
2. ~~Maxiprep~~
3. ~~Pull out day 21 plates~~

My competent cell CHAH-KAN plate had growth ☺. I was super careful last time which makes me worried. I think it could be my sticks or something else on my bench is contaminated. Sierra and I took another one of JTC's 50 ul aliquots (doesn't have the date so I am not sure if it was from the same batch I used last time which was from September) and we both plated 25 ul of it onto CHAH-KAN plates. Sierra went first and I went second using different sticks from the bench with the big centrifuge. Will see if there is growth or not on Monday.

For back dilution:

- Making a culture , calculate OD then put in shaking incubator = very annoying
- 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
- 625 uL in 250 mL of LB
 - i. 250 ul carb

Maxi-Prep Protocol (CompactPrep)

Did this to maximize the amount of DNA since my plasmid is low copy number. Started at 2:11pm and finished at 6:00 pm for future reference.

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 = 0.825
 - c. OD of culture 2 = 0.775
 - d. OD of culture 3 = 0.495

- e. 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.
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 11 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

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	4035.6	ng/ul	80.712	40.522	1.99	2.4
2	5682.9	ng/ul	113.657	59.846	1.9	2.33
3	1245.8	ng/ul	24.915	12.718	1.96	2.37

Dilute: concentration: 200 (C2) and 100 ul (V2) and recheck DNA concentration

Monday, July 18, 2022

1. ~~Make plates for day 28~~
2. ~~Check EC plates.~~
3. ~~Count day 21 plates~~
4. ~~Update lab slides~~
5. ~~Streak out 4 plates~~
6. ~~Dilute concentration of pKR141~~
7. ~~Work on abstract~~

- Combined the two maxi preps into one tube. Diluted using the higher concentration from 7/17/22.
 - i. $V1 = (200 \times 100) / 5682.9 = 3.592$ ul
 - ii. Add 3.52 ul of combined maxi preps
 - iii. Add 96.5 ul of 0.1 x EB
 - iv. Nanodrop – 156.2

Nanodrop for diluted pKR141

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1		156.2 ng/ul	3.123	1.529	2.04	2.81

Day 21 (rep 3):

	300 ul plate 4°C	Dilution Factor Counted	Avg. Cells	CFU per mL
	1			
1-A	19		21	70
1-B	23			
2-A	16		14.5	48.333333
2-B	13			
3-A	5		2.5	8.333333
3-B	0			
Dilution Factor	300			

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	1	12.66667	1266.667	31.28424
	0.0001	#DIV/0!	#DIV/0!	#DIV/0!

Contamination(?) of EC cells update:

- After talking with Dr. Ramsey, she struck out a little bit from the densely grown plate from 20 ul of EC cells from 7/14/22. She thinks that there is a strong possibility that there is a lot of

growth which leads to LVS *F. tularensis* being able to grow even in the presence of Kanamycin. Will check that plate tomorrow. I also struck out four more plates from my LVS June 2022 aliquots to potentially make more EC cells tomorrow. (also the plates that Sierra and I struck out – mine had no growth but Sierra had a little)

Tuesday, July 19, 2022

1. ~~Poster draft due~~
2. ~~Make LB Agar~~
3. ~~Electroporate~~

There was no growth on the KAN plate, so the EC cells are safe!

*Electroporate plasmid into EC cells**

Looking for transformation efficiency, check recovery time, and how to plate.

1. -For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. -For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
 - a. 1 µg in 10 µL or less of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)
 - Current concentration is 156.2
 - Adding 6.4 µL of pKR141
 - b. 50 µL electrocompetent cells
 - 1 aliquots (110 µL) 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 = 1.40
 - b. Sample 2 = 1.30
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:
 - 4 round plates per electroporation = 8 total
 1. 200 µL and 500 µL
 - 2 track plates per electroporation = 4 total
 1. Only 3 dilutions 1, 0.1 and 0.01
 2. From recovery tube, take out 500 µL into 1.5 mL
 3. 2 more 1.5 mL put 900 µL of PBS
 4. 100 µL of undiluted into 1st 1.5 mL
 - a. Vortex!!
 5. Take 100 µL from previous to next tube

6. After 10 ul from each tube onto track plates
 - take some amount of transformation (200uL) plate
 - take 200 uL and serial dilutions
 1. PBS
 - Transformants per microgram of DNA = transform efficiency
 - Before with pKL97: 1×10^4 to 7×10^4 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.

After talking to Kathryn here are the expectations:

			Expected # colonies				
			Round plates		Track plate		
Transformation efficiency	volume MHB (uL)	cells per uL	200 uL	500 uL	Row 1	Row 2	Row 3
1.00E+03	4000	0.25	50	125	2.5	0.25	0.025
1.00E+04	4000	2.5	500	1250	25	2.5	0.25
7.00E+04	4000	17.5	3500	8750	175	17.5	1.75
1.00E+05	4000	25	5000	12500	250	25	2.5
7.00E+05	4000	175	35000	87500	1750	175	17.5

Transformation efficiency	Number of transformations for 400,000 mutants	Max # of mutants
1.00E+03	400	
1.00E+04	40	80000
7.00E+04	5.7	560000
1.00E+05	4.0	800000
7.00E+05	0.6	5600000

Then these are numbers for the expected insertion and more specific details for my experiment. Total number of mutants

400000

Genome size

1,895,994

TA sites	205621	
Mutants per insertion site	1.9453266	fold
Volume of freshwater (mL)	10	coverage of mutants
Starting CFU /mL	5.78E+07	
Total starting CFU	5.78E+08	1.44E+03
7 day CFU/mL	2.78E+07	
Total 7 day CFU	2.78E+08	6.94E+02
14 day CFU/mL	5.58E+06	
Total 14 day CFU	5.58E+07	139.58

Wednesday, July 20, 2022

1. ~~Check electroporation plates~~
2. ~~Work on poster~~
3. ~~Plate day 28 for rep 3~~

No growth on the electroporation plates yet.

[300 ul Plating Protocol](#)

Plated day 28 of replicate 3. Went smoothly, no issues.

Thursday, July 21, 2022

1. ~~Check electroporation plates~~
2. ~~Work on poster~~

Friday, July 22, 2022

1. ~~Pull out day 28 plates~~
2. ~~Check electroporation plates~~
3. ~~Abstracts due~~
4. ~~Poster due~~

Recounted Day 21 Replicate 3

	300 ul plate 4°C	Dilution Factor Counted	Avg. Cells	CFU per mL
	1			
1-A	20		19.5	65
1-B	19			
2-A	15		15.5	51.66667
2-B	16			
3-A	11		5.5	18.33333
3-B	0			
Dilution Factor	300			

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	1	13.5	1350	24.03701
	0.0001	#DIV/0!	#DIV/0!	#DIV/0!

Notes with Dr. Ramsey:

- Make plates 3 days before
 - 8 big plates for 4 transformations
 - Volume per big square plate:
- Day 1 – EP (THU/FRI)
- Day 2 – nothing
- Day 3 – nothing
- Day 4 – some colonies
- Day 5 – harvest Day (MON/TUE)
- Took small amount and determined the transformation efficiency while plating the EP
 - For each EP (4 round CHAN-KAN plates)
 - Three drips
 - 10 ul undiluted
 - 10 ul undiluted
 - 1: 10 dilution
- Taking mutants from plates today
 - Take six colonies and patch out on 2 per plate
 - Leave at RM for the weekend
 - Six freeze down
 - Single aliquots
 - Cryovial
 - Put into strain collections
 - Isolate gDNA (MON/THR)

Monday, July 25, 2022

- ~~1. Count day 28 plates~~
- ~~2. Freeze down mutants~~
- ~~3. Isolate gDNA~~

Isolate gDNA

I am isolating the gDNA from 6 of my mutants from the electroporation plates to see if the insertion is present. Samples 1-3 were from the 1A 200 ul circular plate and samples 4-6 were from the 2A 200 ul circular plate. Sample 7 is LVS gDNA.

Cell Samples

1. Dilute 1 µl of Proteinase K into 300 µl 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 10⁶ mammalian cells; 0.1-0.5 ml of an overnight culture of E. coli) and discard the supernatant, leaving approximately 25 µl of liquid.

200 uL of PBS scrap up small loop-hole, resuspend BEFORE centrifugation

4. 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 µl 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 µl of MPC Protein Precipitation Reagent to 300 µl 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 µl 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 µl 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, 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.

- 7 mL of ethanol and 3 mL of diH₂O

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	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	378.5	ng/µl	7.569	3.984	1.9	2.09
2	427	ng/µl	8.541	4.454	1.92	2.04
3	704.4	ng/µl	14.089	7.568	1.86	1.94
4	390.3	ng/µl	7.806	4.06	1.92	2.16
5	1794.2	ng/µl	35.885	19.011	1.89	2.2
6	398.7	ng/µl	7.973	4.089	1.95	2.16
7	306.3	ng/µl	6.126	3.168	1.93	2.33

Freeze down strain

- This was done for each of my mutants 1-6 from the electroporation of pKR141. Made 1 aliquot of each. Put in freezer 4G -80C.

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

Poster Talk

Francisella tularensis is a Gram – negative pathogenic bacterium. It is the causative agent of a potentially deadly disease tularemia, which is considered a bioweapon. This bacterium can survive in freshwater for long periods of time and can infect humans in this environment. Previously, it has been found that the lower temperatures, but still above freezing, allows for the longest survival. The goal of this project is to determine the gene or genes that are involved in the survival of *F. tularensis* at 4C in freshwater. The first part of this project was finding and validating which temperature in the laboratory allows for the longest survival in sterile freshwater. To figure this out I needed to find the optimal temperature for survival in freshwater, and then use a method to find the specific gene or genes.

Here you can see the method I used which included taking freshwater from the Beaver River, then inoculating it with some LVS *F. tularensis*. I split this initial inoculum into three separate conditions, 4C, 16C, and 25C and did this in triplicate. I then plated for viability on these days.

On this top graph, you can see the on the x-axis is the days incubated, and the y-axis is measure of cell viability . This is showing my first experiment with the three different conditions. The major take-away from this graph is that the cells remained viable for 35 days at 4C.

The second graph shows the second two replicates. As you may or may not know, scientists love to validate results, so these next two replicates are showing the variability of this experiment. The x-axis is the same as the previous, but the y-axis is different. This time, with the different starting inoculums, I plotted the percentage of day 0 CFU to better compare all three replicates. The major take-away from this graph is the cells remained viable from 21 to 56 days at 4C in sterile freshwater. This provides evidence that 4C is the optimal temperature to test for the gene(s) essential for survival of this bacteria in freshwater.

Did this three times, variability, survive at 4C long term (21 days at least)

Tn seq can be used to find genes under any stress condition. Not going to get into the details, I didn't get to do this summer, but will do in the fall. I did modify the vector for this experiment, so we can perform the experiment in a cost effective and easy way. I spent some time cloning, and I changed the edges of the transposon to be more compatible with the new technology. I confirmed that we get transformants at a rate of about 20,000 per electroporation.

The next part of this project will be completed during the fall, but this summer I finished some prep work, by making a new plasmid for the new transposon insertion sequencing. I needed to make a new plasmid, because so the previous plasmid will cost more due to new adaptor sequences. I successfully added these sequences shown in red. I also found the initial transformation efficiency results and completed the math to determine the number of mutants I will be able to make with the electroporation's I have.

I have established the conditions in long term survival in water so I can use this condition for the Tn-Seq experiment. I have created a new plasmid to use for the Tn-Seq experiment, and I will be able to initiate the Tn-Seq experiment in the fall.

Transposon = jumping gene

Tuesday, July 26, 2022

1. ~~Write up a protocol for Sierra/Marisa~~
2. ~~Make more LB agar and media~~
3. ~~Dilute LVS gDNA~~
4. ~~Plate day 34~~

Preparing LB media

1. For 250 mL of LB, weigh out the following components and add to a 500 mL bottle:
 - a. 2.5 g NaCl
 - b. 2.5 g Tryptone
 - c. 1.25 g Yeast extract
2. Add 250 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

For the LB media and LB agar, the autoclave in the basement didn't work, so I brought to CBLS. The tape did not turn black so we figured it probably wasn't going to burn.

Dilute LVS gDNA

- $V1 = (V2 * C2) / C1$
- $= (100 * 45) / 306.3 = 14.69 \text{ ul DNA}$
- Added to 30.3 ul of 0.1xEB

300 ul Plating Protocol

Plated day 34 of replicate 3. Went smoothly, no issues.

Wednesday, July 27, 2022

1. ~~Beach Day~~

Thursday, July 28, 2022

- ~~1. Practice poster talk~~
- ~~2. Submit last time sheet~~

Friday, July 29, 2022

1. RI-SURC!!
2. Pull out day 35 plates

Pay Period Information

Pay Period	Start Date	End Date	Timesheet Due	Payday
25	5/22/2022	6/4/2022	6/3/2022	6/10/2022
26	6/5/2022	6/18/2022	6/17/2022	6/24/2022
1	6/19/2022	7/2/2022	7/1/2022	7/8/2022
2	7/3/2022	7/16/2022	7/15/2022	7/22/2022
3	7/17/2022	7/30/2022	7/29/2022	8/5/2022
4	7/31/2022	8/13/2022	8/12/2022	8/19/2022