

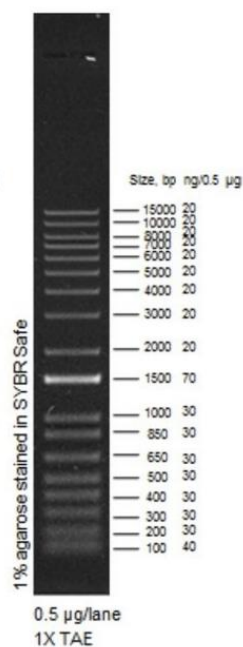
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DNA ladder used throughout this notebook.

May 2022

Monday, May 23, 2022

To Do:

- ~~Count day 21~~

Day 21 Counts:

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	125.3333	1253333	278672.4
	0.0001	0	0	0
Track Plates 4,5,6	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
		0.666667	2.222222	2.545875
Track Plates 7,8,9	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	0	0	0
	0.0001	0	0	0

Talked to Hannah about making pKR140:

- PCR from gblock (InseqA – insert) with primers (KROL516/7)
 - pcr clean up and gel purify
 - Digest PCR and pKL97 with enzymes Kpn1 and Pac1
 - pcr clean up and gel purify
 - Ligation
 - backbone and insert (complete plasmid, and you want more of it)
 - BB only (self ligate)
 - make LB – carb plates
 - Transform with PIR
 - select on carb
 - miniprep 2x with colony
 - 1 colony in 10 mL (incubate for 30 minutes)
 - next day 2x the cells
 - sequence
- RESTART for pKR141
- gblock: LnseqB
 - Primers:
 - digest with Pst1 and Bamh1 (since this insert is wicked small need to use a thicker gel which is 2% agarose) and the backbone is InseqB

Tuesday, May 24, 2022

To Do:

- ~~2. Plate day 28~~
 - ~~a. Track plates for 1-3 and 300 ul for 4-6 and no more 7-9~~
3. Annotate pKL97

Track Plating Protocol

- 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

I did plates 1 and 3 an extra time, because it looked like they converged. Other than that, went well and I am expecting to see countable colonies in the 10^{-2} column.

300 ul Plating Protocol

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.

This was done with samples 4-6, and it went well. I did not plate samples 7-9 as they have had no countable colonies for the past two weeks.

Wednesday, May 25, 2022

To Do:

- ~~1. Make primers for pKR141~~
- ~~2. Take out trash~~

Also the gBlocks Tn_InSeqA/B came in today. I prepared them by centrifuging on the mini-centrifuge for about a minute. Then I added 25 ul of 0.1xEB to each, and briefly vortexed and spun down. After, I placed on the 42°C heat block for 30 minutes. Finally, I briefly vortexed and spun down again, labeled, and placed in the gBlock box in the -20°C.

Thursday, May 26, 2022

To Do:

- ~~1. PCR amplify for pKR140~~
- ~~2. Ask Hannah questions about next week~~
- ~~3. Receiving primers~~

Why do we need a new Tn-Seq vector?

The technology that we use will not be compatible with the previous vectors.

Hannah said that with new primers and new gblock to test them out first. So do 20ul reaction volumes with a positive, negative and insert. Run on a gel and check that size is correct. Then after that is all checked and good, do 2 reactions with negative and insert. Make the insert to be 100 ul and then the negative will be remaining. (1.5 master mix)

Receiving Primers Protocol

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
 - a. KROL516: 27.73 nm x 10 = 277 ul
 - b. KROL517: 36.03 nm x 10 = 360 ul
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
 - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

PCR Protocol

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
 - Molecular grade H₂O in 1.5 mL microfuge tube
 - KOD/primestart buffer
 - dNTPs
 - oligo F (10uM)
 - oligo R (10uM)

- template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
 5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
 6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
 - The file is located in the Protocols folder
 - Also setup table below to specify which primers and source DNA will be used
 7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
 8. Add dszdi H2O to negative control tube
 - Template volume for 1 reaction
 9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:

Total reaction volume	20
Total number of reactions	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	2.4
oligo R	10 uM	0.3 uM	0.6	2.4
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
		Total volume	20	78.4

10. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
 - Heat at 94 degrees for 2 minutes,
 - 94 degrees C for 20 seconds

- 50 degrees C for 30 seconds
- 68 degrees C for 1 minute/kb (adjust based on expected size of product)
- Go back to step 2
- Repeat 32x
- 68 degrees C for 5 minutes
- 12 degrees C for infinity
- 400 bp (always use larger so primase has enough time) so 00:25 or 25 sec for step 4 (elongation?) and ensure that the volume is set for 20 ul

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	InseqA	gblock	KROL516 and 517	168
2	+ control	LVS gDNA	KROL15 and 16	400
3	- control	-	KROL516 and 517	-

Friday, May 27, 2022

To Do:

- ~~1. Make plates for day 28 (2 hours)~~
 - ~~a. 6 square plates for samples 1-3 and 6 circular plates for samples 4-6~~
- ~~2. Make LB agar~~
- ~~3. Move plates to room temperature~~
- ~~4. Run PCR on gel from yesterday to double check that the bands are in the right spot (30 minutes)~~
- ~~5. PCR with 100 ul of reaction volume~~
- ~~6. PCR purify~~
- ~~7. Work on CITI training~~
- ~~8. Make document on how to make primers in snap gene~~
- ~~9. Received primers KROL520 and 521~~
- ~~10. Made and purchased primers KROL522 and KROL523~~
- ~~11. Annotate pKL97~~

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!

I made 8 square plates and 3 circular plates for next Tuesdays plating with one ½ flask.

Preparing LB-agar

I made 2 flasks.

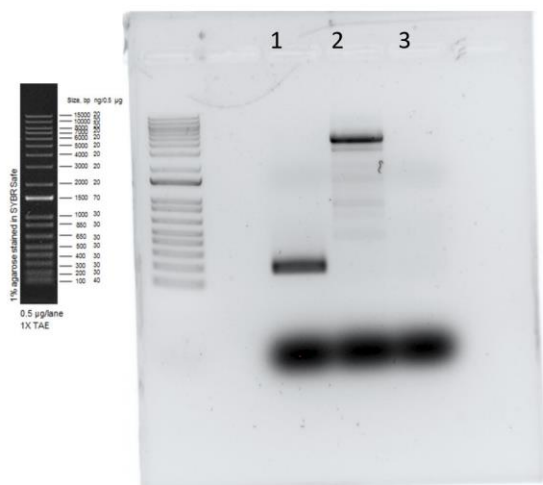
1. For 500 mL of LB-agar, weigh out the following components and add to a 1 L (non-baffled) flask:
 - a. 6 g agar
 - b. 5 g NaCl
 - c. 5 g Tryptone
 - d. 2.5 g Yeast extract
2. Add stirbar to flask
3. Add 500 mL ddiH₂O
4. Mix on stirplate until components are dissolved
5. Cover top of flask with foil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

Run on a gel to confirm that the bands are in the expected length.

For the small rig PCR, used 5 ul of cybersafe, 10 ul of ladder, and 24 ul of samples (20 ul of sample + 4 ul of loading dye).

Results:

Purpose:	To check new primers and gBlock
Results	Well, I messed up a bit, but overall it went as expected
Future directions	100 ul PCR reaction and PCR purification



Lane	Expected bp	Expected: Yes or No
1 - InseqA	168	Yes
2 – positive control	400	No, but I used the wrong primers (KROL516/517 instead of KROL15/16) so it is ok because it is just a positive control
3 – negative control	0	Yes

5/27/22

PCR again with 1.5x master mix, with a negative control and InseqA. The reaction volume for InseqA should be a 100 and the negative control is remaining. PCR purify after. To elute in 35 ul of 0.1xEB.

PCR Protocol

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	InseqA	gblock	KROL516 and 517	168
2	- control	-	KROL516 and 517	-

Total reaction volume	100
Total number of reactions	1.5

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			62.0	2.5
PrimeSTAR GXL Buffer	5x	1x	20.0	155
dNTPs	2.5 mM	0.2 mM	8.0	50
oligo F	10 uM	0.3 uM	3.0	20
oligo R	10 uM	0.3 uM	3.0	7.5
template	100 ng/ul	2 ng/ul	2.0	7.5
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	
		Total volume	100	5
				245

Thermocycler settings: 168 bp $\{(168/1000)*60\}$ so 15 seconds and 100 ul reaction volume.

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 35 ul of Buffer 0.1xEB and let sit for 1 minute then centrifuge for 1 minute

Receiving Primers KROL520 and 521

- Spin at max speed for 3 minutes
- Add 0.1xEB for final concentration of 100 uM
 - KROL520: $33.41 \text{ nm} \times 10 = 334 \text{ uL}$
 - KROL521: $33.91 \text{ nm} \times 10 = 339 \text{ uL}$
- Heat at 42C for 5 min
- Vortex and spin
- Label with KROL #
- Since this is for PCR primer
 - At 20 ul from 100 uM stock
 - 180 ul of 0.1xEB
 - Add purple sticker

Tuesday, May 31, 2022

To Do:

- ~~1. Plate day 35~~
- ~~2. Count Hannah's Plates~~
- ~~3. Image Hannah's plates~~
- ~~4. Count day 28 plates~~
5. Make aliquots of 1kb ladder

Imaging Hannah's Plates

- No filter on the top right side of imager
- Application > select > custom > zone inhibition assay > position > no filter > center plate > run > export into Hannah's folder > data > spot plate assays > 220527 sigma 32 folder
- Double check that t= 0 is at the top!!
- Label with the strain names

Track Plating Protocol

The plates were very wet, and some were strangely bumpy. I decided to also plate on 300 ul as well just so if there is something wrong with the square plates I can get some data for this week for samples 1-3.

300 ul Plating Protocol

I plated samples 1-6 on circular plates. These plates looked normal and all went smoothly.

I wanted to do the digestion, ligation, and transformation today, but I wasn't able to find any Pac1, so I had to be patient.

Day 28 Counts

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	24.66667	246666.7	151767.4
	0.1	67.5	67500	58845.14
Track Plates 4,5,6	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
		0	0	0
Track Plates 7,8,9	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	0	0	0
	0.0001	0	0	0

Making ready-to-load 1 Kb Plus DNA ladder stock

Combine:

- 60 uL 1 Kb Plus DNA ladder
- 100 uL 6x Orange-G dye
- 140 uL water (molecular biology grade)
- 300 uL total volume

Making 6 aliquots

Jk we couldn't find the DNA ladder, so paused until its found.

June 2022

Wednesday, June 1, 2022

To Do:

1. ~~DNA digest~~
2. ~~Ligation~~
3. ~~Transformation~~
4. ~~Received primers KROL522 and 523~~

DNA digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	Kpn1 and Pac1	15	-
2	Backbone – pKL97	Kpn1 and Pac1	5	10

Expected size of tube 1: 168 bp

Expected size of tube 2: 4472 bp keep larger fragment

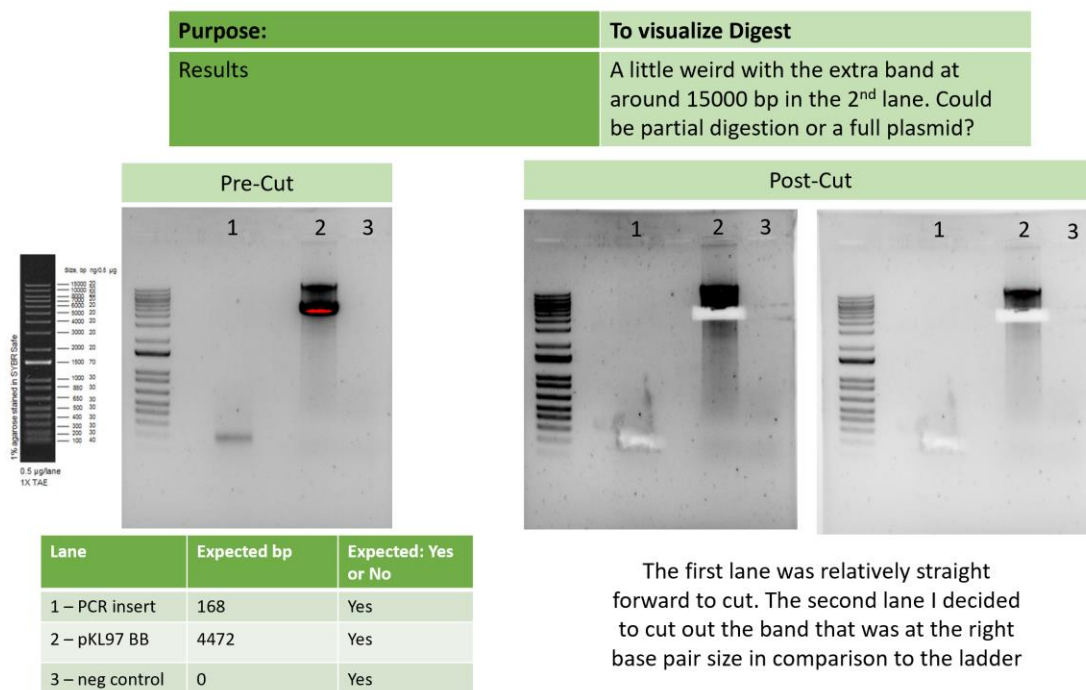
2. Set up master mix table:

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

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

3. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
4. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
5. Add indicated amount of each enzyme (_1.8_uL) to the master mix tube (MM).
6. Mix the master mix by pipetting up and down.
7. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_15_ul).
8. Incubate at 37°C for 1 hour.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
10. Run on gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
 - a. 5 ul of cybersafe
 - b. 10 ul of ladder
 - c. 6 ul of loading dye + 30 ul of sample (for tubes 1 and 2)
 - d. Negative control

Results



6/1/22

Receiving Primers KROL522/523

- Spin at max speed for 3 minutes
- Add 0.1xEB for final concentration of 100 uM
 - KROL522: $25.88 \text{ nm} \times 10 = 258.8 \text{ uL}$
 - KROL523: $23.64 \text{ nm} \times 10 = 236.4 \text{ uL}$
- Heat at 42C for 5 min
- Vortex and spin
- Label with KROL #
 - 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.

Gel Purification

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
 - a. This took about 8 minutes
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.

8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer 0.1x EB and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
Backbone pKL97	410 mg	1230uL	410uL
PCR – InSeqA	290 mg	870uL	290uL

Ligations

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

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

Tube	Insert	Backbone
1	Kpn1 and Pac1 digested, purified PCR	Kpn1 and Pac1 digested, purified pKL97
2	-	Kpn1 and Pac1 digested, purified pKL97

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H ₂ O	11.5	15.5	34.5
10x ligase buffer	2.0	2.0	6
Insert	4.0	-	-
Backbone	2.0	2.0	6
Ligase – enzyme box	0.5	0.5	1.5
TOTAL	20.0	20.0	48

3. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H₂O (11.5_uL), 10x buffer (2.0_uL), insert (4.0 or 0.00_uL), and backbone (2.0_uL).
6. Add indicated amount of ligase (0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. room temp for 10 minutes

Transform chemically competent *E. coli* cells

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL and 100 uL. If transforming a ligation, plate 100 uL and remaining culture.
 - Note: The plasmid used for the positive control plates should be the regular circular plasmid, not the digest/purified one.

- There should always be a backbone control if testing a ligation. This is the digested and ligated backbone with NO INSERT.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKL97	0.5 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Ligation 1	pKR140	8 uL	100 uL, remaining	2
4	Backbone Ligation	pKL97(digested)	8 uL	100 uL, remaining	2
Total number of plates					8

2. Check to be sure you have enough LB-Kan plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of **competent cell tubes** on ice (5 reactions per tube of competent cells)
 - Double check 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.
 - I read my notebook wrong and put them in the 37C incubator for probably 30 seconds
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-carb), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.
 - Used the beads

Thursday, June 2, 2022

To Do:

- ~~1. Submit Timesheet~~
- ~~2. Check plates~~
 1. Make table with plates and each count
 2. Look for 2:1 ratio
- ~~3. Make more LB agar~~
- ~~4. Make more 1xTAE~~
- ~~5. Make more agarose Gel~~
- ~~6. Make more CHAH plates~~
- ~~7. Look up Tn Seq~~
- ~~8. Work on Marshall/Mitchell~~
- ~~9. Make overnights~~

I think that I labeled by plates and tubes wrong with my transformation. I fixed it in the parts above, but if sequencing comes back wrong, I know that it was the ligation that went wrong.

DNA	Volume	Colony Count
pKL97	20 ul	Too many to count
pKL97	100 ul	Too many to count
Negative control	20 ul	None
Negative control	100 ul	None
Ligation	100 ul	197
Ligation	R	Too many to count
BB Ligation	100 ul	5
BB Ligation	R	80

Preparing LB-agar

Made 2 1L flasks

Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

Made a ½ flasks so _12_ plates

1xTAE

We couldn't find the 25xTAE, so used 50xTAE instead

- 20 mL of 50 x TAE into a 1 L graduated cylinder
- Fill to 1L mark with water
- Added to the new 1xTAE bottle

1% agarose gel

- 5.80 g of agarose
- 580 mL of 1xTAE
- Added both to the existing flask of gel
- Spin and brought to a boil
- Put in water bath so Marissa can use for her gel later

Do this after 3pm

Overnight for E. coli: (for double miniprep)

I am doing the double miniprep because this plasmid is a transposon so it will integrate into the E. coli chromosome on some level. This will allow me to hopefully get the same amount of plasmid as if I did only 1.

- 5 tubes of 10 mL of LB-Carb
 - 10 mL of LB
 - 10 uL of Carbenicillin
- 1 colony into 10 ml of LB-Carb for 30 min in shaking incubator
- Split each tube into 2 tubes of 0.5 ml.

- Place in shaking incubator overnight at 37C
- 4 colonies from ligation plates = 8 tubes
- 1 colony from positive control = 2 tubes
- 10 tubes total for the overnight

1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
pKR140-1A	pKR140-1B	pKR140-2A	pKR140-2B	pKR140-3A	pKR140-3B	pKR140-4A	pKR140-4B	pKL97	pKL97

Friday, June 3, 2022

To Do:

- ~~1. Mini prep x2~~
- ~~10. Nanodrop~~
- ~~11. Make plates for day 42~~
 - ~~1. Make 12 circular and 6 square~~
- ~~12. Pull out day 35 plates~~
- ~~13. Work on marshal/Mitchell~~

MiniPrep

- Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes.
 - Added about 1.7 mL to each tube for the first and second times, then added the remaining
- Added 250uL Buffer P1 to resuspend pelleted bacterial cells.
- Added 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution was more translucent
- Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
 - Makes like a white precipitate
- Centrifuge for 10 minutes at 13,000 rpm
 - Combine into 5 columns
- Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 1 minute. Discard flow-through. (x2 because double the initial volume)
- Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 minute. Discard flow-through.
- Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 minute and discard flow-through 3 times (added 750ul of PE three times). Transfer to collection tube.
- Centrifuge for 3 minutes to remove residual wash buffer.
- Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 50 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.

Nanodrop Results (1 ul)

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR140-1	201.4	ng/μl	4.028	2.097	1.92	2.53
pKR141-2	148.7	ng/μl	2.974	1.5	1.98	2.82
pKR141-3	48.3	ng/μl	0.966	0.455	2.12	3.37
pKR140-4c	94	ng/μl	1.879	0.983	1.91	1.74
pKL97	123.3	ng/μl	2.466	1.3	1.9	2.02

Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

Made a 1 L flask so 8 square plates and 14? Circular plates

Monday, June 6, 2022

To Do:

1. ~~Label plates and leave out on bench~~
2. ~~Count day 35~~
3. ~~PPT slides for meeting~~
4. ~~Sequencing~~
5. ~~Took out trash~~

My plates for day 42 were bumpy (which Dr. Ramsey says that they are very wet) so I dried them on my bench for an hour or so.

Day 35 Counts

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	#DIV/0!	#DIV/0!	#DIV/0!
	0.1	40.25	40250	#VALUE!
Track Plates 4,5,6	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
		0	0	0
Track Plates 7,8,9	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	0	0	0
	0.0001	0	0	0

Sequencing

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:	PCR template:	PLASMID template:	Volume H₂O needed	
							ng needed =	Volume =	Volume =	
							$(A \div 100) \times 2.5$	$(C \div B)\mu\text{l}$	$2x(\sim 200 \div B)\mu\text{l}$	(12 less D or E - 2.56)μl
1		Plasmid	pKR140-1	KROL522	4556	201.4	0.00	#DIV/0!	1.99	10.01
2		Plasmid	pKR140-2	KROL522	4556	148.7			2.69	9.31
3		Plasmid	pKR140-3	KROL522	4556	48.3			8.28	3.72
4		Plasmid	pKR140-4	KROL522	4556	94			4.26	7.74

Primer: KROL522

Tuesday, June 7, 2022

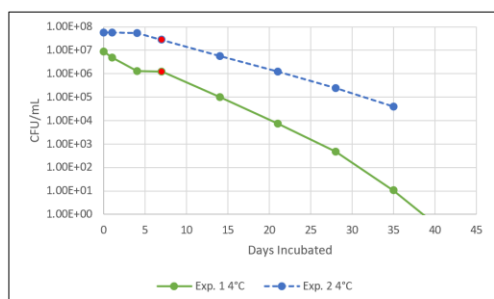
To Do:

1. ~~Lab meeting~~

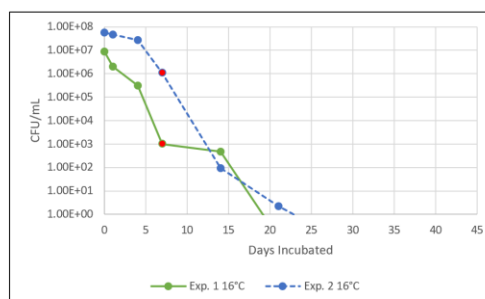
2. ~~Plan next plasmid in separate doc~~
3. ~~Plate day 42~~
4. ~~Make a chart for 4C~~
5. ~~LB-Carb plates~~

Comparisons of Experiments 1 and 2 of the viability of *F. tularensis* from day 0 to day 35 in 4C, 16C, and 25C

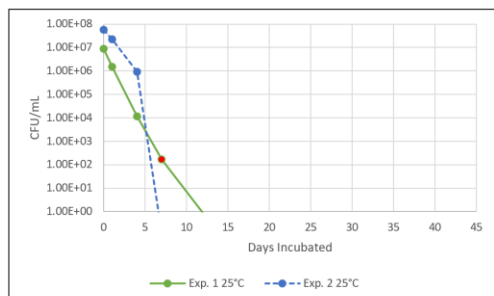
4°C



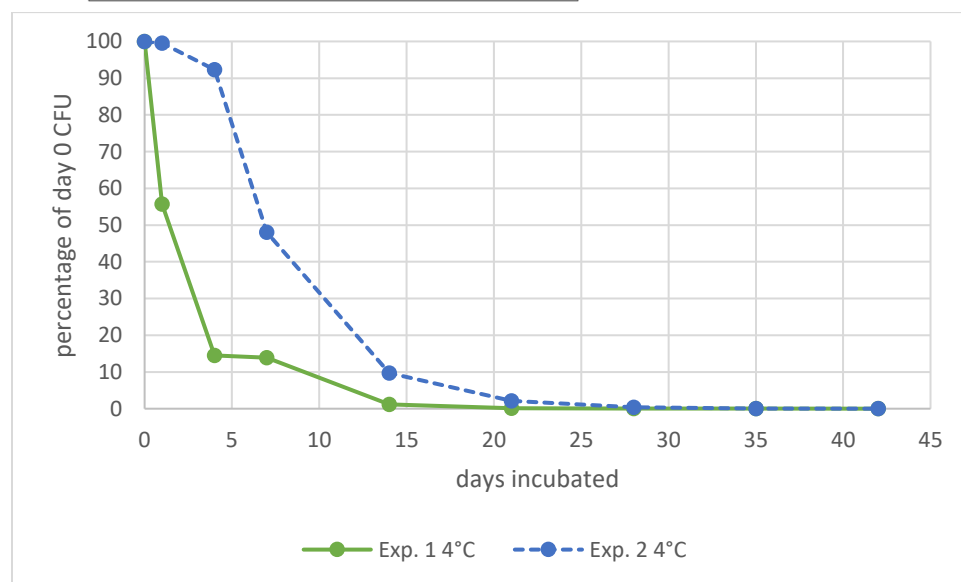
16°C



25°C

**Notes:**

On the day before plating day 7 the incubators broke so my samples were moved for a day and that have caused some disturbances. (marked with red markers)

**Pouring plates LB-Carb**

1. Add antibiotic using aseptic technique if necessary. Typical antibiotic concentrations:

Antibiotic	Stock Concentration	Working Concentration (Concentration in plates)
Ampicillin	100 mg/mL	100 µg/mL

Antibiotic	Stock Concentration	Working Concentration (Concentration in plates)
Carbenicillin	100 mg/mL	100 µg/mL
Kanamycin	50 mg/mL	50 µg/mL

- Keep antibiotic stocks on ice. For kanamycin, add 500 µL to 500 mL of LB-agar media. Stir on stirplate to mix media.
 - 500 µL of carb = 500 mL of media
- Open a new sleeve of sterile plates. Don't open plates unless next to flame.
- Next to flame, open foil top of flask. Pass flask opening through flame.
- Open a plate next to flame and pour ~20-25 mL LB-agar into plate; the bottom of the plate should be covered in media.
- Continue to pour media into plates until media is gone.
- Immediately rinse out flask
- Stack plates and mark the side to indicate which antibiotic is used. (blue = kanamycin, black = carbenicillin / ampicillin).
- Let plates sit at room temperature for one night, then store at 4°C with tape indicating date poured. Don't use antibiotic plates older than 3 months.

Track Plating Protocol

I did 6 track plates for samples 1-3. Last week the CFU 40250, and looking at the previous experiment it should be 10^{-3} serial dilution next week, so I decided to not do circular 300 µL.

Wednesday, June 8, 2022

To Do:

- ~~Look at sequencing results~~
- ~~PCR 20µL and gel~~
- ~~PCR 100µL and purification~~
- ~~DNA digestion~~
 - ~~Check at 30 minutes and 40 minutes~~
 - ~~Use a big gel rig~~
- Ligation
- Transformation

Sequencing results came back... successfully made pKR140!! Whoot!

PCR Protocol

- Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
- Get a container of ice to keep the components on
- Acquire the following components and put them on ice, labeling tubes if necessary:
 - Molecular grade H₂O in 1.5 mL microfuge tube
 - KOD/primestar buffer
 - dNTPs
 - oligo F (10µM)
 - oligo R (10µM)

- template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
19. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
 20. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
 21. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
 - The file is located in the Protocols folder
 - Also setup table below to specify which primers and source DNA will be used
 22. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
 23. Add dszdi H2O to negative control tube
 - Template volume for 1 reaction
 24. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:

Total reaction volume	20
Total number of reactions	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	2.4
oligo R	10 uM	0.3 uM	0.6	2.4
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
		Total volume	20	78.4

25. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
26. Add appropriate volume of master-mix to negative control PCR tube
27. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
28. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)

29. Close PCR Tubes until the caps are tight

30. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:

- Heat at 94 degrees for 2 minutes,
- 94 degrees C for 20 seconds
- 50 degrees C for 30 seconds
- 68 degrees C for 1 minute/kb (adjust based on expected size of product)
- Go back to step 2
- Repeat 32x
- 68 degrees C for 5 minutes
- 12 degrees C for infinity
- 400 bp (always use larger so primase has enough time) so 00:25 or 25 sec for step 4 (elongation?) and ensure that the volume is set for 20 ul

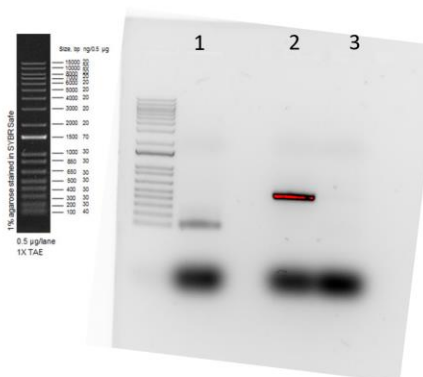
Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	InseqB	gblock	KROL520/521	127
2	+ control	LVS gDNA	KROL15 and 16	400
3	- control	-	KROL16 and 17	-

Forgot that I was added the primers (0.6 ul and 0.6 ul = 1.2 ul) to each individual tube. Added 19.4ul of MasterMix instead of 18.4ul. This means that I may see some primer dimers or extra PCR artifacts.

Gel Rig: small, 5ul of cyber safe, 10 ul of ladder, 20ul rxn + 4ul of loading dye, ran for 30 minutes.

Results:

Purpose:	To check new primers and gBlock
Results	Well, I messed up a bit, but overall it went as expected
Future directions	100 ul PCR reaction and PCR purification



Lane	Expected bp	Expected: Yes or No
1 - InseqB	127	Yes
2 - positive control	400	yes
3 - negative control	0	yes

PCR Protocol

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	InseqB	gblock	KROL520/1	127
2	- control	-	KROL520/1	-

Total reaction volume	100
Total number of reactions	1.5

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	2.5
ddiH2O			62.0	155
PrimeSTAR GXL Buffer	5x	1x	20.0	50
dNTPs	2.5 mM	0.2 mM	8.0	20
oligo F	10 uM	0.3 uM	3.0	7.5
oligo R	10 uM	0.3 uM	3.0	7.5
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	5
		Total volume	100	245

Thermocycler settings: _127_ bp :{(127/1000)*60 }so _15_ seconds and 100 ul reaction volume.

2% Agarose for Big Gel Rig

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

PCR Purification

- 500ul buffer PB in a 1.5 ml tube with 100 ul of PCR reaction
- 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 35 ul of Buffer 0.1xEB and let sit for 1 minute then centrifuge for 1 minute

DNA digest

11. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	PstI and BamHI	15	-
2	Backbone – pKR140	PstI and BamHI	5	10

Expected size of tube 1: _67_ bp

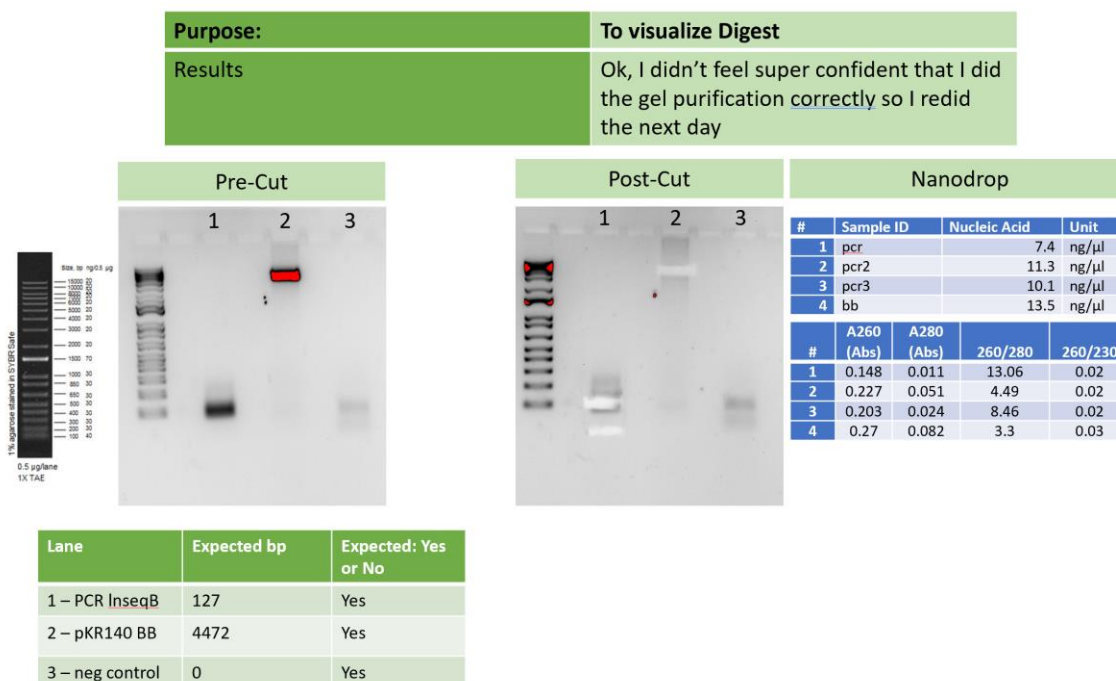
Expected size of tube 2: 4497 bp keep larger fragment

12. Set up master mix table:

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

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

13. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
14. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
15. Add indicated amount of each enzyme (_1.8_uL) to the master mix tube (MM).
16. Mix the master mix by pipetting up and down.
17. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_15_ul).
18. Incubate at 37°C for 1 hour.
19. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate at 37C for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
20. Run on **2% Agarose** gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
 - a. Ran for _60_ minutes before cutting
 - b. 12 ul of cybersafe
 - c. 10 ul of ladder
 - d. 6 ul of loading dye + 30 ul of sample (for tubes 1 and 2)
 - e. Negative control



6/8/22

Gel Purification

10. Excise the DNA fragment from the agarose gel with a clean, sharp razor
11. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
12. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
13. Add 1 gel volume isopropanol to the sample and mix.
14. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
 - a. Forgot to do this multiple times with extra sample. Missed probably around 200 ul of sample.
15. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
16. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
17. Place columns in a fresh 1.5mL microcentrifuge tube.
18. Elute: Add 30uL Buffer 0.1 x EB and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
Backbone pKR140	191.2 mg	573.6 ul	191.2 ul
PCR – InSeqB	218.5 mg	655.5 ul	218.5 ul

Thursday, June 9, 2022

To Do:

1. ~~Re-do of the digestion~~
2. ~~Add gel pictures to slides~~
3. ~~LB Agar~~
4. ~~1xTAE buffer~~

Preparing LB-agar

Made 2 1L flasks

I had a bad feeling about how the digestion went yesterday so I nano dropped the gel purifications, and it didn't look good. So, I am going to re-do the digestion and gel purification and be extra cautious.

Nanodrop Results

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	pcr	7.4	ng/μl	0.148	0.011	13.06	0.02
2	pcr2	11.3	ng/μl	0.227	0.051	4.49	0.02
3	pcr3	10.1	ng/μl	0.203	0.024	8.46	0.02
4	bb	13.5	ng/μl	0.27	0.082	3.3	0.03

2% Agarose for Big Gel Rig

- Add 1.2 g agarose powder to 60 mL 1x TAE buffer in 250 mL.
- Add stirbar to container.
- Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
- Let the 2% agarose solution cool to approximately 50-55°C.

DNA digest

21. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	PstI and BamHI	15	-
2	Backbone – pKR140	PstI and BamHI	5	10

Expected size of tube 1: ~127 bp

Expected size of tube 2: 4497 bp and 67 bp keep larger fragment

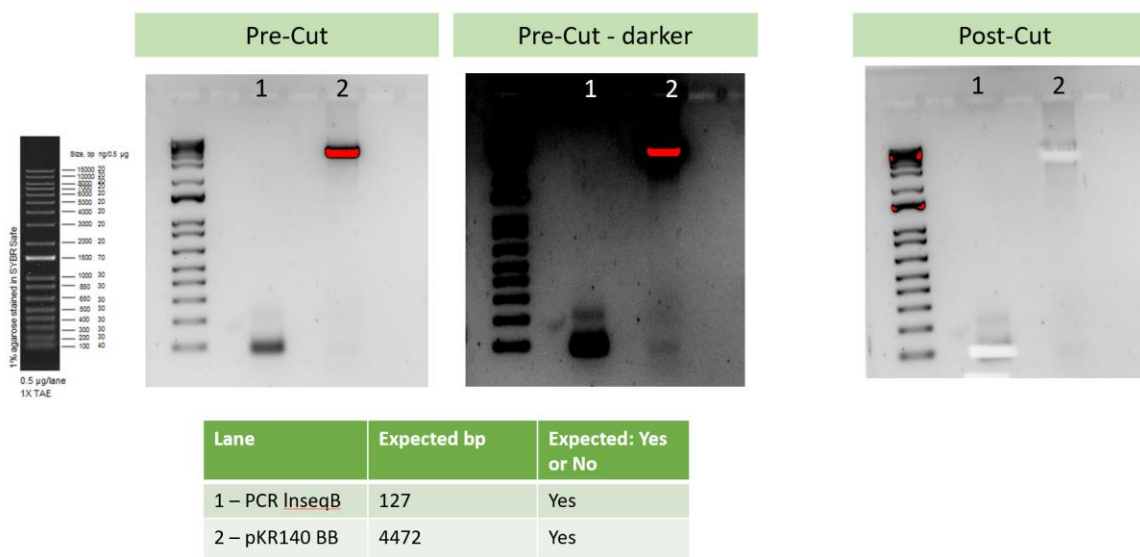
22. Set up master mix table:

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

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

Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).

23. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
24. Add indicated amount of each enzyme (_1.8_uL) to the master mix tube (MM).
25. Mix the master mix by pipetting up and down.
26. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_15_ul).
27. Incubate at 37°C for 1 hour or up to overnight.
28. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate at 37C for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
29. Run on **2% Agarose** gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
 - a. Ran for _1 hour and ten minutes_ before cutting
 - b. 12 ul of cybersafe
 - c. 10 ul of ladder
 - d. 6 ul of loading dye + 30 ul of sample (for tubes 1 and 2)
 - e. Negative control



Gel Purification

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

- a. Forgot to do this multiple times with extra sample. Missed probably around 200 ul of sample.
24. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
 25. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
 26. Place columns in a fresh 1.5mL microcentrifuge tube.
 27. Elute: Add 30uL Buffer 0.1 x EB and let stand for 3 minutes and 42 seconds. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
Backbone pKR140	185.2 mg	555.6	185.2
PCR – InSeqB	222.8	668.4	223

1xTAE

We couldn't find the 25xTAE, so used 50xTAE instead

- 20 mL of 50 x TAE into a 1 L graduated cylinder
- Fill to 1L mark with water
- Added to the new 1xTAE bottle

Friday, June 10, 2022

To Do:

1. ~~Make plates for day 49~~
 - a. Already have a lot of extra circular plates, just gonna make a half flask for square plates
2. ~~Pull out day 42 plates~~
3. Lab meeting at 10 am

[Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak](#)

Made a 1/2 flask so _8_ square plates

Sunday, June 12, 2022

To Do:

1. Take pictures of plates to count

Monday, June 13, 2022

To Do:

2. BBM

Tuesday, June 14, 2022

To Do:

1. BBM

Wednesday, June 15, 2022

To Do:

- ~~1. Plate day 50~~
- ~~2. Ligation~~
- ~~3. Transform~~
- ~~4. Count plates from Sunday~~

Stocks of pKR140 and pKL97: permanent stocks were made with 5ul from the regular stock. Add green stickers and write on the side as well and put in KMR permanent box in -80C.

Ligations

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

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

Tube	Insert	Backbone
1	PstI and BamHI digested, purified PCR (lnSeqB)	PstI and BamHI digested, purified pKR140
2	-	PstI and BamHI digested, purified pKR140

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H ₂ O	11.5	15.5	34.5
10x ligase buffer	2.0	2.0	6
Insert	4.0	-	-
Backbone	2.0	2.0	6
Ligase – enzyme box	0.5	0.5	1.5
TOTAL	20.0	20.0	48

3. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H₂O (11.5_uL), 10x buffer (2.0_uL), insert (4.0 or 0.00_uL), and backbone (2.0_uL). ON ICE!!
6. Add indicated amount of ligase (0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Rm temp for 10 min

Transform chemically competent *E. coli* cells

- 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	pKR140	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Ligation 1	pKR141	8 uL	100 uL, remaining	2
4	Backbone Ligation	pKR140(digested)	8 uL	100 uL, remaining	2
Total number of plates					8

- 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.
- Obtain DNA and thaw on ice if necessary.
- Thaw appropriate number of **competent cell tubes** on ice (5 reactions per tube of competent cells)
 - Double check PIR!!!
- Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
- 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.
- Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
- Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
- After heat shock, place tubes back on ice until next step (don't keep them here too long).
 - Put the first two in the tube rack for about 30 secs to a minute because I forgot to put them on ice directly afterwards
- Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
- Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
 - 250 rpm for the shaking
- Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
- 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

Track Plating Protocol

The plates were really wet again, just the square ones though. I used the hood this time, because last week I used the flame, and had a LOT of contamination. The plates were pretty good, and I had no convergence. Only did samples 1-3.

300 ul Plating Protocol

Did samples 1-3 and it all went smoothly.

Day 42 Counts

Track Plates 1,2,3	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
	0.01	#DIV/0!	#DIV/0!	#DIV/0!
	1	15	1500	1909.188

Thursday, June 16, 2022

To Do:

- ~~1. Look at transformation plates~~
- ~~2. Plan third viability experiment~~
- ~~3. Make LB agar~~
- ~~4. Clean flasks?~~
5. Running a gel with purified gel from 6/9 to get an estimate of DNA concentration
6. Redo ligation with 3x and 5x

Transformation Plates

DNA	Volume	Colony Count
pKR140	20 ul	Too many to count
pKR140	100 ul	Too many to count
Negative control	20 ul	none
Negative control	100 ul	none
Ligation	100 ul	0
Ligation	R	9
BB Ligation	100 ul	2
BB Ligation	R	9

Determine DNA concentration from band brightness

10 uL – what we add for 1kb ladder

500 ng/ul is concentration of 1ul

We make a dilution of 60ul/300ul = 0.2 therefore

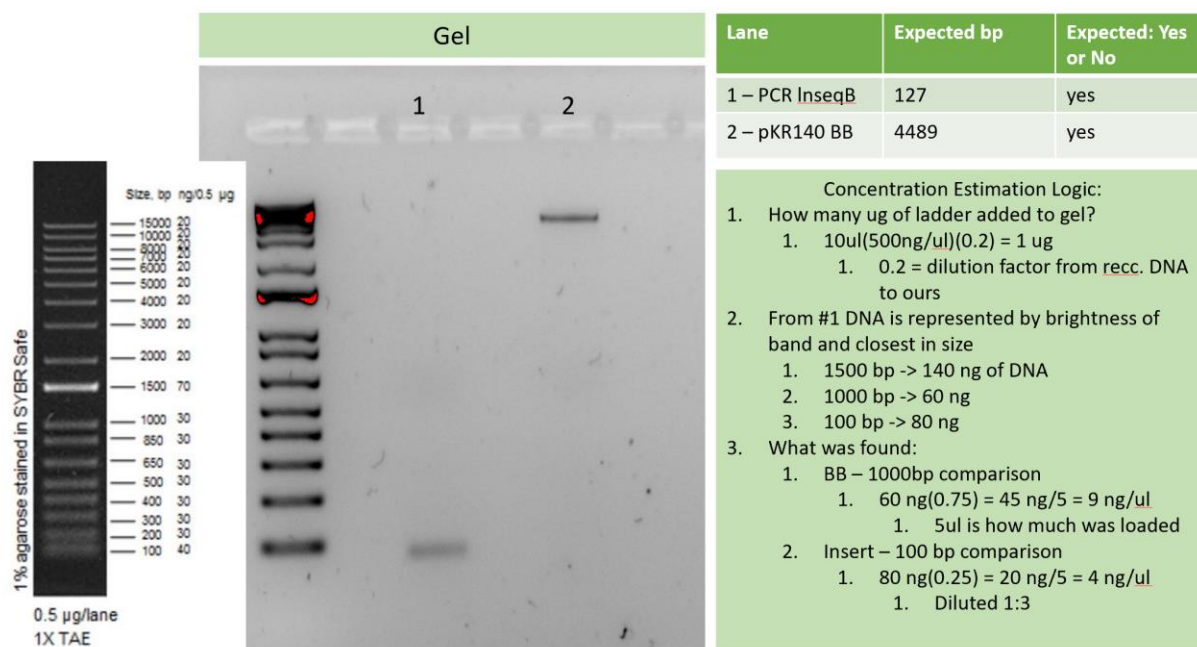
10ul (500 ng/ul) (0.2) = 1000 ng = 1 ul

And then the 1500 bp band is 70 ng on theirs (1ng/0.5 ul) so on mine is 140 ng of DNA and the 100 bp is 80 ng

Loaded 5ul of sample + 1ul of dye, 10 ul of ladder, and 12ul of cybersafe

Results

Determined that the concentration of the backbone was 9ng and the insert was 4ng based on the comparison of the 100bp (80ng) and assuming its $\frac{1}{4}$ as bright. And then the comparison of the 1000bp (60ng) and assuming it is $\frac{3}{4}$ as bright.



4ng makes the amount of insert not pipettable, so I did a 1:3 ratio so 2ul of DNA added to 4ul of water.

Ligation attempt #2

Ligation Calculator

ng vector	ratio of insert/bb bps	molar ratio	ng of insert
50	0.014925373	3	2.23880597
50	0.014925373	5	3.731343284

	concentration	uL needed	
backbone	9	5.56	
3X	1.33	1.68	
5X	1.33	2.81	
	3X	5X	BB only
Water	10.26	9.14	11.94
Ligation Buffer	2	2	2
Backbone	5.56	5.56	5.56
Insert	1.68	2.81	-
Ligase	0.5	0.5	0.5
	20.00	20.00	20.00

1. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H₂O , 10x buffer , insert and backbone. ON ICE!!
4. Add indicated amount of ligase (0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Overnight in thermocycler at 16C

Friday, June 17, 2022

To Do:

1. ~~Make plates for day 56 — 1/2 flask of circular~~
2. ~~Pull out day 50 plates~~
3. ~~Freeze ligation~~
4. ~~Submit time sheet~~

Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

Made a 0.5 L flask so _10_ square plates

Bad contamination on 1B circular plate, so I threw it out. The rest have no visible colonies right now, but I feel confident that there will be some on Monday. Or maybe not I guess we will see.

Monday, June 20, 2022

To Do:

1. ~~Pull out plates for day 56~~
2. ~~Transformation — 10 plates~~
3. ~~Count day 50 plates~~
4. ~~Label all plates for day 0, day 56~~
5. ~~Work on project outline~~
6. ~~Struck out plate for LVS aliquot~~

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 digested/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	pKR140	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Ligation 3x	pKR141 - not made	8 uL	100 uL, remaining	2
4	Ligation 5x	pKR141 – not made	8 uL	100 uL, remaining	2
5	Backbone Ligation	pKR140(di gested)	8 uL	100 uL, remaining	2
Total number of plates					10

- 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.
- Obtain DNA and thaw on ice if necessary.
- Thaw appropriate number of **competent cell tubes** on ice (5 reactions per tube of competent cells)
 - Double check PIR!!!
- Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
- 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.
- Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
- Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
- After heat shock, place tubes back on ice until next step (don't keep them here too long).
- Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
- Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
 - 250 rpm for the shaking
- Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
 - Didn't prewarm the shaking incubator, so didn't start the timer until it hit 37C
- 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
 - Plates were quite wet, so I let them sit with the beads for 23 minutes.

Day 50 counts: no growth

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

Tuesday, June 21, 2022

To Do:

- Make ½ flask of CHAH square plates for day 1
 - 4 more squares
 - Offered to make circular plates
- ~~Check ligation plates~~
- Plate day 56
- ~~Make a lawn of LVS on two CHAH plates (square plates)~~
- ~~Prepare 40 mL of sterile freshwater into a 50mL conical for OD~~

6. ~~Prepare 50 mL of sterile freshwater into a conical~~
7. ~~Make LVS aliquots~~
8. Re-do PCR

Transformation Plates

DNA	Volume	Colony Count
pKR140	20 ul	Too many to count
pKR140	100 ul	Too many to count
Negative control	20 ul	None
Negative control	100 ul	none
Ligation 3x	100 ul	3
Ligation 3x	R	24
Ligation 5x	100 ul	8
Ligation 5x	R	48
BB Ligation	100 ul	11
BB Ligation	R	70

50 ul single use aliquot of LVS

- 800 ul MHB (400 ul + resuspend and then add remaining 400 ul)
- 200 ul of 75% glycerol
- Vortex
- 50 ul aliquots
- Made 20 aliquots

Made two plates of LVS lawns using 50 ul aliquot and beads.

300 ul Plating Protocol

Made square plates instead of circular plates, which was silly of me. So I used the square plates instead of circular plates. Shouldn't make much of a difference as I believe that all of the cells are dead.

PCR Protocol

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	InseqB	gblock	KROL520/1	127
2	- control	-	KROL520/1	-

Total reaction volume	100
Total number of reactions	1.5

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	2.5
ddiH2O			62.0	155

PrimeSTAR GXL Buffer	5x	1x	20.0	50
dNTPs	2.5 mM	0.2 mM	8.0	20
oligo F	10 uM	0.3 uM	3.0	7.5
oligo R	10 uM	0.3 uM	3.0	7.5
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	5
		Total volume	100	245

Thermocycler settings: _127_ bp : $\{(127/1000)*60\}$ so _15_ seconds and 100 ul reaction volume.

Took 1 hour and 22 minutes (I think)

2% Agarose for Big Gel Rig

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

PCR Purification

- 500ul buffer PB in a 1.5 ml tube with 100 ul of PCR reaction
- 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 35 ul of Buffer 0.1xEB and let sit for 1 minute then centrifuge for 1 minute

DNA digest

30. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	PstI and BamHI	15	-
2	Backbone – pKR140	none	5	10
3	Backbone – pKR140	PstI	5	10
4	Backbone – pKR140	BamHI	5	10
5	Backbone – pKR140	PstI and BamHI	5	10

Expected size of tube 1: _67_ bp

Expected size of tube 2: 4497 bp keep larger fragment

31. Set up master mix table:

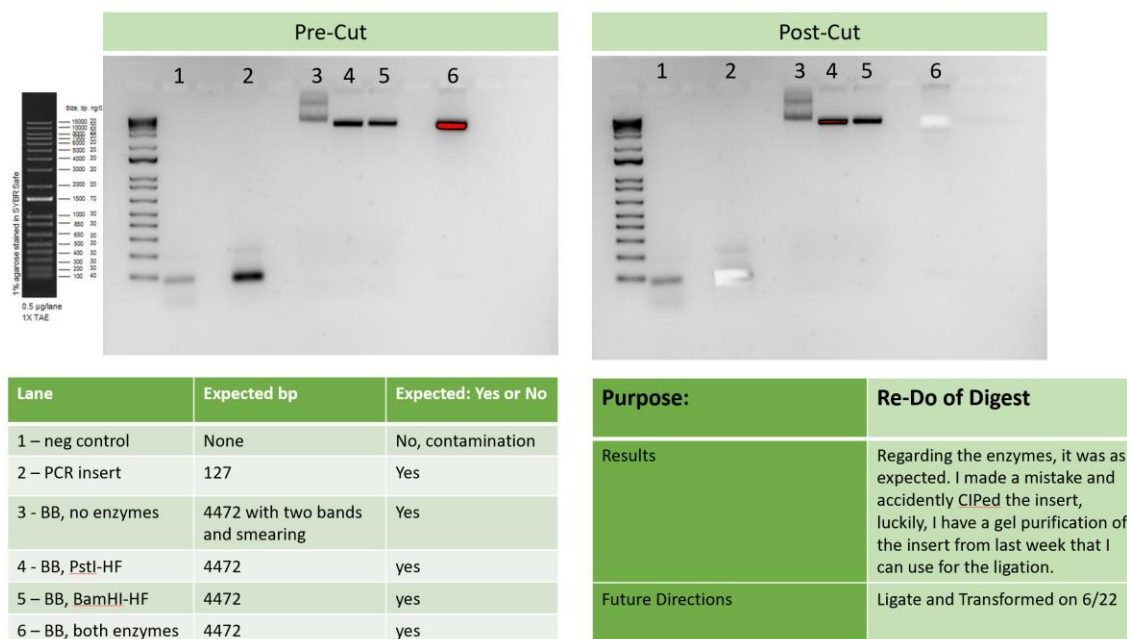
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 6x (uL)
H ₂ O	10.8	64.8
10x Buffer*	3.0	18.0
Total	30.0 (15.0 actual b/c of DNA)	

Tube	PstI (uL)	BamHI (uL)	DNA (uL)	H2O (uL)	MM (uL)	Total
1	0.6	0.6	15	-	13.8	30
2	-	-	2	14.2	13.8	30
3	0.6	-	2	13.6	13.8	30
4	-	0.6	2	13.6	13.8	30
5	0.6	0.6	5	10	13.8	30

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

32. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
33. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
34. Add indicated amount of each enzyme to each tube.
35. Mix the master mix by pipetting up and down.
36. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_13.8_ul).
37. Incubate at 37°C for 1 hour.
38. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate at 37C for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
39. Run on **2% Agarose** gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
 - a. Ran for _70_ minutes before cutting
 - b. 12 ul of cybersafe
 - c. 10 ul of ladder
 - d. 6 ul of loading dye + 30 ul of sample (for tubes 1-5)
 - e. Negative control

Results:



6/21/22

Gel Purification

28. Excise the DNA fragment from the agarose gel with a clean, sharp razor
29. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
30. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
31. Add 1 gel volume isopropanol to the sample and mix.
32. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
33. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
34. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
35. Place columns in a fresh 1.5mL microcentrifuge tube.
36. Elute: Add 30uL Buffer 0.1 x EB and let stand for 1 minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
PCR- insert	214.6 mg	643.8 uL	214.6
BB - backbone	161.3	483.9	161.3

Wednesday, June 22, 2022

To Do:

1. ~~Plate day 0~~
2. ~~Ligate~~
3. ~~Transform~~

~~4. Add gel image to ppt~~

~~5. Add pKR140 to spreadsheet~~

Inoculation

- Get a sterile 1.5 mL tube and pipette 500 uL of freshwater into it.
- Scrap up all of cells that were prepared the previous day into the 1.5mL tube with freshwater. Resuspend the cells.
 - Only scraped from one plate
- Add 500 mL more freshwater to the 1.5 tube
 - Started with 1000 ul pipette and switched to 200 ul to resuspend.
- 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.
- Use spectrometer to measure OD. Obtain an OD₆₀₀ of 0.03. Don't forget the blank!
 - Needs to be between 0.05-0.60 (dynamic range of the spec), if more than 0.6 need to dilute
 - $C1V1=C2V2$
 - $V1 = 100.4 \text{ ul}$
 - Amount of resuspended cells to add
 - $C1 = \text{the OD measured} \times 50 \text{ (amount diluted)}$
 - 0.239
 - $C2 = 0.03$
 - $V2 = 40 \text{ mL}$
- Take prepared 40 mL freshwater in 50 mL conical tube and add V1 of resuspended cells to it.
- Put the cells into three technical replicates. 10 mL in 3 different flasks for the one condition. 3 flasks total.
- Put flasks in respective locations.
 - 4°C – cold room

Flask 1B	Flask 2B	Flask 3B
4°C	4°C	4°C

Plating Protocol

- 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.
- 7. Pipette 180 uL of 1 x PBS into wells row B1/2-G1/2.
 - a. Use multichannel and a reservoir.
- 8. Pipette 200 uL from 40mL (which is now 10ml) of resuspended cells and freshwater into A1 and A2
 - a. Use a 200 uL pipette.

9. Using the multichannel pipette, pipette 20 uL from A into B. Mix 150 uL of the volume three times.
10. 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!
11. Use the less volume multichannel pipette set to 10 uL and change the orientation of the plate to vertical before starting the dripping process.
12. 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

Plating is done to get the starting point for all of the samples.

Nanodrop Results: from gel extraction 6/21/22

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	pcr	11.4	ng/μl	0.228	0.054	4.21	0.02
2	bb	9.9	ng/μl	0.199	0.063	3.17	0.12

Diluted insert 1:10 for a concentration of 1.14.

Ligation #3

To preface this, I messed up and added 5.50 ul of BB into the first three tubes instead of 5.05 ul. Hannah suggested that I scale up the reactions by 1.09 to account for this. Not ideal at all, but maybe it will work.

	3x (uL)	5x (uL)	BB (uL)	Insert + BB (from 6/1) (uL)	BB only (from 6/1) (uL)
Water	11.43	10.00	13.57	11.5	15.5
Ligation Buffer	2.18	2.18	2.18	2	2
Backbone	5.50	5.50	5.50	2	2
Insert	2.14	3.57	-	4	-
Ligase	0.55	0.55	0.55	0.50	0.50

1. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H₂O, 10x buffer, insert and backbone. ON ICE!!
4. Add indicated amount of ligase (0.55 or 0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Room temperature for 10 minutes

Transform chemically competent *E. coli* cells

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

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR140	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Ligation 3x	pKR141 – not made	8 uL	100 uL, remaining	2
4	Ligation 5x	pKR141 – not made	8 uL	100 uL, remaining	2
5	Backbone Ligation	pKR140(digested)	8 uL	100 uL, remaining	2
6	Pos control lig	pKR140 (6/1)	8 uL	100 uL, remaining	2
7	Pos control bb	pKL97(digested) (6/1)	8 uL	100 uL, remaining	2
Total number of plates					14

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).
 - Didn't prewarm the shaking incubator, so didn't start the timer until it hit 37°C
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
 - Plates were quite wet, so I let them sit with the beads for 23 minutes.

Thursday, June 23, 2022**To Do:**

1. ~~Plate day 1~~
2. ~~Check day 50 plates~~
3. ~~Check transformation plates~~
4. ~~Overnights~~
5. ~~DNA digest~~
6. ~~Meeting with Dr. Ramsey~~

Transformation Plates from 6/22/22

DNA	Volume	Colony Count
pKR140	20 ul	Too many to count
pKR140	100 ul	Too many to count
Negative control	20 ul	None
Negative control	100 ul	none
Ligation 3x	100 ul	None
Ligation 3x	R	20 – 5 for overnight
Ligation 5x	100 ul	1
Ligation 5x	R	8 – 5 for overnight
BB Ligation	100 ul	1
BB Ligation	R	32 – 2 for overnight
Positive control ligation	100 ul	86
Positive control ligation	R	
Positive control BB	100 ul	463
Positive control BB	R	

For my positive controls, the 100 ul seemed to be flipped. This is because the R plates looked to be as expected (more on the ligation and less on the BB). With these plates I took overnights and will miniprep to run a diagnostic digest to see if maybe some of them worked.

Track Plating Protocol

Day 1: went well the plates were very nice, the perfect wet/dry to be honest.

Looked at my day 50, and there are 27 colonies! I will count them later or tomorrow.

Overnight for E. coli: (for double miniprep)

I am doing the double miniprep because this plasmid is a transposon so it will integrate into the E. coli chromosome on some level. This will allow me to hopefully get the same amount of plasmid as if I did only 1.

- 12 tubes of 10 mL of LB-Carb
 - 10 mL of LB
 - 10 uL of Carbenicillin
- 1 colony into 10 ml of LB-Carb for 30 min in shaking incubator
- Split each tube into 2 tubes of 5 ml.
- Place in shaking incubator overnight at 37C
- 5 colonies from ligation plate 3x = 10 tubes
- 5 colonies from ligation plate 5x = 10 tubes

- 2 colonies from backbone = 4 tubes
- 1 colony from pos control = 2 tubes
- 24 tubes total for the overnight

1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Lig 3x-1A	Lig 3x-1B	Lig 3x - 2A	Lig 3x - 2B	Lig 3x - 3A	Lig 3x - 3B	Lig 3x - 4A	Lig 3x - 4B	Lig 3x-5A	Lig 3x - 5B
6A	6B	7A	7B	8A	8B	9A	9B	10A	10B
Lig 5x-6A	Lig 5x-6B	Lig 5x - 7A	Lig 5x - 7B	Lig 5x - 8A	Lig 5x - 8B	Lig 5x - 9A	Lig 5x - 9B	Lig 5x-10A	Lig 5x - 10B
11A	11B	12A	12B	13A	13B	14A	14B		
BB - 11A	BB - 11A	BB - 12A	BB - 12B	BB - 13A	BB - 13B	pKR140	pKR140		

Accidentally did 3 of the backbone instead of 2

2% Agarose for Big Gel Rig

Did this in a 250 mL beaker instead of flask.

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

DNA digest

This is attempt #3? For making pKR141.

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	PstI-HF and BamHI-HF	15	-
2	Backbone – pKR140	PstI-HF and BamHI-HF	5	10

Expected size of tube 1: ~67_ bp

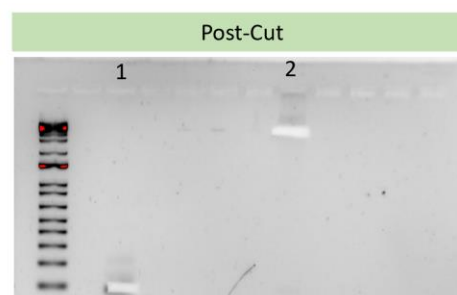
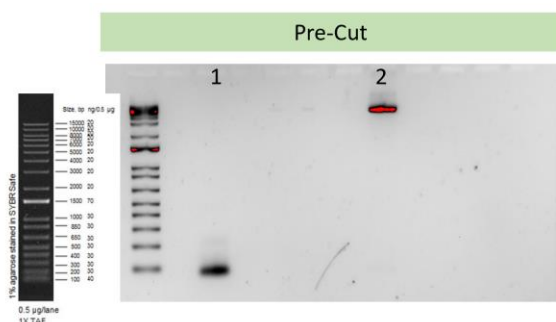
Expected size of tube 2: 4497 bp keep larger fragment

2. Set up master mix table:

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

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

3. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
4. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
5. Add indicated amount of each enzyme (_1.8_uL) to the master mix tube (MM).
6. Mix the master mix by pipetting up and down.
7. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_15_ul).
8. Incubate at 37°C on hour.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate at 37C for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**
10. Run on **2% Agarose** gel : **REMEMBER TO SKIP LANES** and take pictures before and after cutting out.
 - a. Ran for __60_ minutes before cutting
 - b. 12 ul of cybersafe
 - c. 10 ul of ladder
 - d. 6 ul of loading dye + 30 ul of sample (for tubes 1 and 2)
 - e. Negative control



Lane	Expected bp	Expected: Yes or No
1 – PCR insert	127	yes
2 – BB	4472	Yes

Purpose:	Re-Do of Digest – prep for next round!
Results	The pcr is the darkest I think I have gotten, due to the new pcr I did previously. I think it went well and I have the gel in the fridge.
Future Directions	Ligate and Transform

6/23/22

Friday, June 24, 2022**To Do:**

1. ~~Pull out day 56 and day 0~~

- ~~2. LB Media~~
- ~~3. Miniprep~~
- ~~4. nanodrop~~
- ~~5. Draft of project outline~~
- ~~6. Diagnostic Digest~~

2% Agarose for Big Gel Rig

Did this in a 250 mL beaker instead of flask.

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

MiniPrep

These samples are from transformation plates from 6/22/22. Not confident in the outcome of these samples, but what the hecc why not try. This time I used the vacuum for the first time. The overall goal of this is to sequence and see if the plasmid is pKR141 or not.

1. Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes.
 - a. Did this three times using the pouring method. Since all 24 samples didn't fit I did this in two parts for centrifuging.
2. Added 250uL Buffer P1 to resuspend pelleted bacterial cells.
3. Added 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution was more translucent
4. Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
 - a. Makes like a white precipitate
5. Centrifuge for 10 minutes at 13,000 rpm
 - a. Combine into 14 columns
 - b. Move to vacuum after
6. Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Use vacuum. (x2 because double the initial volume)
7. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Use vacuum.
8. Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Vacuum 3 times (added 750ul of PE three times). Transfer to collection tube.
9. BACK!! Centrifuge for 3 minutes to remove residual wash buffer.
10. Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 50 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.
 - a. Next time, Hannah suggests eluting in 30 uL.

Nanodrop Results from the miniprep above.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	137.5	ng/μl	2.75	1.354	2.03	2.46
2	106.6	ng/μl	2.133	1.017	2.1	2.48

	3	84.4	ng/μl	1.688	0.968	1.74	1.68
	4	115.5	ng/μl	2.31	1.159	1.99	2.18
	5	69	ng/μl	1.379	0.644	2.14	3.19
	6	82	ng/μl	1.639	0.769	2.13	2.86
	7	130.2	ng/μl	2.604	1.912	1.36	1.68
7b		122	ng/μl	2.44	1.337	1.82	1.66
	8	61.3	ng/μl	1.225	0.542	2.26	2.83
	9	85.7	ng/μl	1.714	0.785	2.18	2.97
	10	101.6	ng/μl	2.032	0.969	2.1	2.13
	11	64.2	ng/μl	1.283	0.575	2.23	3.17
	12	89.6	ng/μl	1.793	0.829	2.16	2.54
	13	84.5	ng/μl	1.69	0.785	2.15	2.84
	14	83.4	ng/μl	1.668	0.783	2.13	2.99

LB Media

Protocol written by KMR

Preparing LB media

- For 250 mL of LB, weigh out the following components and add to a 500 mL bottle:
 - 2.5 g NaCl
 - 2.5 g Tryptone
 - 1.25 g Yeast extract
- Add 250 mL type I ddiH₂O
- Close tightly and shake to mix
- Loosen cap and add a small piece of autoclave tape with the date
- Autoclave on 30 minute liquid cycle
- Don't screw the tops on tight until fully cooled
- Sterile media can be stored indefinitely

Made two 250 mL bottles of LB Media.

Saturday, June 25, 2022

To Do:

- ~~Pull out day 1~~
- ~~Plate day 3~~

Track Plating Protocol

Day 3: went swimmingly. No issue, no convergence.

Monday, June 27, 2022

To Do:

- ~~Pull out day 3~~
- ~~Make 1/2 CHAH plates for day 7~~
- ~~Diagnostic digest with minipreps~~
- ~~Sequencing~~
- ~~Gel Purification~~
- ~~Ligation~~
- ~~Make LB carb~~
- ~~Nanodrop gel purification~~

Diagnostic Digest

This diagnostic digest is to use the samples from the minipreps done on 6/24/22 and see if the insert can be seen to be more confident before selecting which should go to sequencing. Once again this is for pKR141.

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Lig 1	PstI-HF and BamHI-HF	5	-
2	Lig 2	PstI-HF and BamHI-HF	5	-
3	Lig 3	PstI-HF and BamHI-HF	5	-
4	Lig 4	PstI-HF and BamHI-HF	5	-
5	Lig 5	PstI-HF and BamHI-HF	5	-
6	Lig 6	PstI-HF and BamHI-HF	5	-
7	Lig 7	PstI-HF and BamHI-HF	5	-
8	Lig 8	PstI-HF and BamHI-HF	5	-
9	Lig 9	PstI-HF and BamHI-HF	5	-
10	Lig 10	PstI-HF and BamHI-HF	5	-
11	BB 11	PstI-HF and BamHI-HF	5	-
12	BB 12	PstI-HF and BamHI-HF	5	-
13	BB 13	PstI-HF and BamHI-HF	5	-

For diagnostic digest:

Number of samples

13

Master mix factor

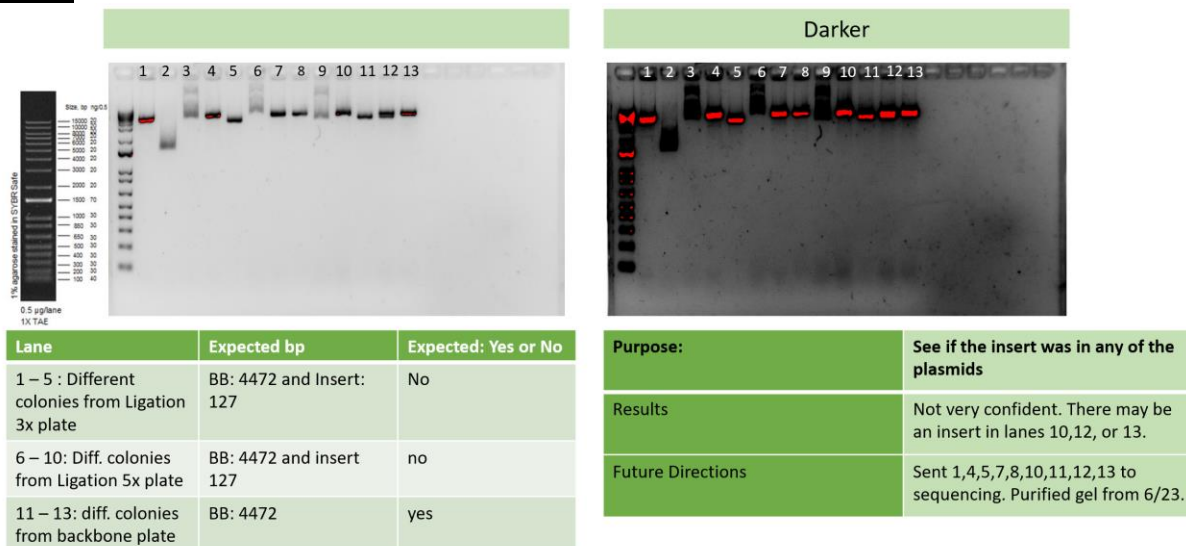
14

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	15	210
10x Buffer*	2	28
DNA	(5)	
Enzyme 1	0.5	7
Enzyme 2	0.5	7
Total	20.0 (15.0 actual b/c of DNA)	252

1. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
2. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
3. Add indicated amount of each enzyme (_7_uL) to the master mix tube (MM).
4. Mix the master mix by pipetting up and down.
5. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (_15.0_ul)
6. Incubate at 37°C for 1 hour
7. Run on a large gel rig with the smaller comb (with 24 loading spaces)
 - a. 12 ul of cyber safe
 - b. 10 ul of lader
 - c. 20 ul of sample + 4 ul of dye

I didn't really consider the change of the Master mix before starting, since I am using 5ul of DNA instead of 2. Not really sure how this will impact this. Additionally the amount of backbone (weight) if you do some math to find the molecules, we would have about 4 ng of insert, which is not visible on a gel.

Results:



Day 50 Counts: (exp 2)

There are more than when I counted last week. Maybe this means I should save all my plates for this third experiment and wait to count them a week or so later after to confirm no more further growth?

Sequencing

This is from the miniprep 6/24/22 and selected from the gel above.

Sample number	Template Type	Template Name	Primer Name ^a	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	<u>PLASMID</u> template:	<u>Volume H₂O</u> needed
						Volume =	
						2x(~200 ÷ B)μl	(12 less D or E - 2.56)μl
1	Plasmid	pKR141-1	KROL523	4564	137.5	2.91	9.09
2	Plasmid	pKR141-2	KROL523	4564	115.5	3.46	8.54
3	Plasmid	pKR141-3	KROL523	4564	69	5.80	6.20
4	Plasmid	pKR141-4	KROL523	4564	122	3.28	8.72
5	Plasmid	pKR141-5	KROL523	4564	61.3	6.53	5.47

6	Plasmid	pKR141-6	KROL523	4564	101.6	3.94	8.06
7	Plasmid	pKR141-7	KROL523	4564	64.2	6.23	5.77
8	Plasmid	pKR141-8	KROL523	4564	89.6	4.46	7.54
9	Plasmid	pKR141-9	KROL523	4564	84.5	4.73	7.27

Gel Purification

Used gel purifications from the fridge from 6/23.

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer 0.1 x EB and let stand for 4 minutes. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
PCR- insert	160.9	482.7	160.9
BB - backbone	191.2	573.6	191.2

Nanodrop Results – 2 uL of the gel purification.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	pcr	1.2	ng/ul	0.025	-0.061	-0.41	0.01
2	bb	4.3	ng/ul	0.086	-0.047	-1.85	0.03
3	pcr	2	ng/ul	0.04	-0.059	-0.68	0.02

Ligation pt 4

Not super confident above the sequencing coming back correctly, so being proactive and starting the next set of ligations. Changed the vector ratios because the concentration of the backbone was so low.

ng vector	ratio of insert/bb bps	molar ratio	ng of insert
40	0.014898821	3	1.787858572
40	0.014898821	5	2.979764287

	concentration	uL needed	
backbone	4.3	9.30	
3X	1	1.79	
5X	1	2.98	

Diluted the insert 1:2.

	3X	5X	BB only	+BB and insert (6/1)	+BB only (6/1)
Water	6.41	5.22	8.20	11.5	15.5
Ligation Buffer	2	2	2	2	2
Backbone	9.30	9.30	9.30	2	2
Insert	1.79	2.98	-	4	-
Ligase	0.5	0.5	0.5	0.50	0.50
	20.00	20.00	20.00		

1. **Obtain ice to assemble and keep the reactions on.** This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H₂O, 10x buffer, insert and backbone. ON ICE!!
4. Add indicated amount of ligase (0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Leave overnight at 16C in thermocycler.

Pouring plates LB-Carb

500 ul of carb into 500 mL of LB agar

Made _20_ circular plates.

Tuesday, June 28, 2022

To Do:

1. ~~Plate day 63~~
2. ~~Transformation~~
3. ~~Count plates day 0 and 1~~
4. ~~LB Agar~~
5. ~~Work on project outline~~

Day 0 Counts:

Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
0.0001	28.5	28500000	707106.8
0.001	#DIV/0!	#DIV/0!	#DIV/0!

	Initial Inoculum							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A					26			0.0001	29	29000000
1-B					32			0.001	0	0
2-A					converged			0.0001	28	28000000
2-B					28			0.001	#DIV/0!	#DIV/0!
Dilution Factor	1	0.1	0.01	0.001	0.0001	0.00001	0.000001			

Day 1 Counts:

	Dilution Factor Counted	Avg. Cells	CFU per mL				
	4	5	6	7			
1-A	94	13			0.001	97	9700000
1-B	100	11			0.0001	12	12000000
2-A	107	16			0.001	110.5	11050000
2-B	114	14			0.0001	15	15000000
3-A	127	21			0.001	107.5	10750000
3-B	88	16			0.0001	18.5	18500000
Dilution Factor	0.001	0.0001	0.00001	0.000001			

300 ul Plating Protocol

Plated day 63. When moving plate 1B, the top came off so there may be contamination. Other than that went smoothly.

Transform chemically competent *E. coli* cells

Transforming ligations from 6/27/22 trying to make pKR141.

- 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	Ligation 3x	pKR141 – not made	8 uL	100 uL, remaining	2
2	Ligation 5x	pKR141 – not made	8 uL	100 uL, remaining	2
3	Backbone Ligation	pKR140(digested)	8 uL	100 uL, remaining	2

4	Pos control lig	pKR140 (6/1)	8 uL	100 uL, remaining	2
5	Pos control bb	pKL97(digested) (6/1)	8 uL	100 uL, remaining	2
6	(+) control	pKR140	0.5 uL	20 ul, 100 ul	2
7	(-) control	None	0	20 ul, 100 ul	2
Total number of plates					14

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
 - Used the bigger centrifuge at max speed this time for the remaining. Hopefully this will help.

Wednesday, June 29, 2022

To Do:

1. ~~Plate day 7~~
2. ~~Colony PCR from day 50 plates~~
3. ~~Count day 3 plates~~
4. ~~Check transformation plates~~
5. ~~Check sequencing results~~
6. ~~Overnights~~

Sequencing Results: no signal, did not use the correct amount of primers. Used 4 ul of KROL523 instead of 2.56 ul.

Transformation Plates from 6/28/22

DNA	Volume	Colony Count
pKR140	20 ul	TMTC
pKR140	100 ul	TMTC
Negative control	20 ul	None
Negative control	100 ul	None

Ligation 3x	100 ul	34
Ligation 3x	R	184
Ligation 5x	100 ul	51
Ligation 5x	R	~302
BB Ligation	100 ul	2
BB Ligation	R	34
Positive control ligation	100 ul	TMTC
Positive control ligation	R	TMTC
Positive control BB	100 ul	4
Positive control BB	R	86

Yayay! The ratios look great!

Colony PCR from patches

I did this PCR because we wanted to double check if the newer colonies on the day 50 plates are actually *F. tularensis*. This lead to the use of KROL15 and KROL16 as they amplify regions on the chromosome.

-Take small amounts of each patch and resuspend in 50 µL molecular water using a sterile toothpick.

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
Big colony from 3B	Small colony from 1B	Small colony from 2A	Small colony from 2B	Small colony from 3B	Small colony from 3B	LVS gDNA	water

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

- only for tubes 1 through 6 NOT LVS gDNA!

-Dilute lysates 1:10

-18 µL of molecular grade water to 2 µL of cells

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use LVS cells, LVS gDNA, the Tn7 plasmid, and water only.

Number of samples

8

Master mix factor

9

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.8	106.2
5X PrimeStar GXL buffer	4	36
2.5 mM dNTPS	1.6	14.4
Forward primer (10 uM)	0.6	5.4
Reverse primer (10 uM)	0.6	5.4
PrimeStar DNA polymerase	0.4	3.6
Cell lysate	1	

Total	20	171
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Primers used KROL15 and KROL16.

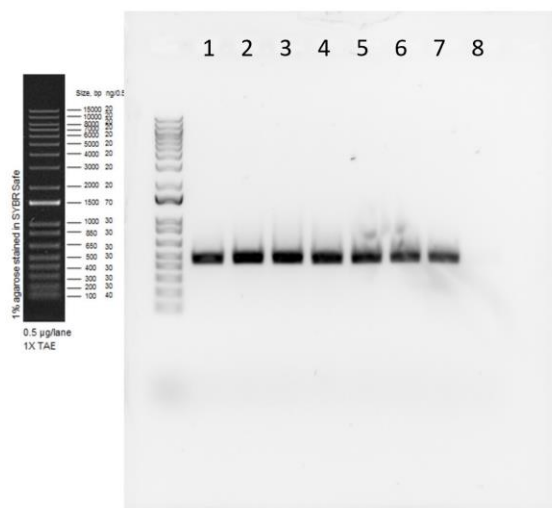
-Use PCR program:

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

- This is the PCR program SNT1 which is already programed. The extension time is _25 seconds_ because the DNA should be ~400 bp. The amount in the tubes is 20 uL.

Results:

Purpose:	Colony PCR from day 50 plates
Results	All <i>F. tularensis</i>
Notes	KROL15 and KROL16



Lane	Expected bp	Expected: Yes or No
1 – Big colony from 3B	400	yes
2 – small colony from 1A	400	yes
3 – small colony from 2A	400	yes
4 – Small colony from 2B	400	yes
5 – small colony from 3B	400	yes
6 – small colony from 3B	400	yes
7 – LVS gDNA	400	yes
8 – negative control	400	yes

6/29/22

Track Plating Protocol

Day 7: my plates were wetter than I would have liked. I think this is due to the humidity. There were convergences for samples 1 and 2, so I did extra plates for both.

Day 3 Counts:

Track Plates	Dilution Factor Counted	Avg. Cells	CFU per mL	Stdev
1,2,3	0.001	53.5	5350000	522015.3
	0.0001	0	0	0

	Samples 1,2,3 - 4°C							Dilution Factor Counted	Avg. Cells	CFU per mL
	1	2	3	4	5	6	7			
1-A				70	11			0.001	75.5	7550000
1-B				81	8			0.0001	9.5	9500000
2-A				68	11			0.001	71	7100000
2-B				74	9			0.0001	10	10000000
3-A				73	7			0.001	65.5	6550000
3-B				58	7			0.0001	7	7000000

Overnight for E. coli: (for double miniprep)

I am doing the double miniprep because this plasmid is a transposon so it will integrate into the E. coli chromosome on some level. This will allow me to hopefully get the same amount of plasmid as if I did only 1. Additionally, this is a low copy plasmid, so there will be less DNA in comparison to a high copy plasmid.

- 4 tubes of 10 mL of LB-Carb
 - 10 mL of LB
 - 10 uL of Carbenicillin
 - MM: 50 mL LB with 50 ul of carb
- 1 colony into 10 ml of LB-Carb for 30 min in shaking incubator
- Split each tube into 2 tubes of 5 ml.
- Place in shaking incubator overnight at 37C
- 2 colonies from ligation plate 3x = 4 tubes
- 2 colonies from ligation plate 5x = 4 tubes
- 8 tubes total for the overnight

Tube 1A	Tube 1B	Tube 2A	Tube 2B	Tube 3A	Tube 3B	Tube 4A	Tube 4B
pKR141-1A	pKR141-1B	pKR141-2A	pKR141-2B	pKR141-3A	pKR141-3B	pKR141-4A	pKR141-4B

Thursday, June 30, 2022

To Do:

- ~~1. Meeting with Dr. Ramsey~~
- ~~2. miniprep~~
- ~~3. Fix notebook and save as a pdf~~
- ~~4. Submit timesheet~~
- ~~5. Submit outline~~
- ~~6. Make LB agar~~
- ~~7. Make LB Media~~

Preparing LB-agar

Made two 500 mL flasks.

LB Media

Made one 500 mL bottle.

MiniPrep

Samples were in incubator for 15 hours. These samples were from the transformation plates from 6/28/22, and are for hopefully *fingers crossed* pKR141, which is the final Tn-Seq vector.

1. Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes.
 - a. Did this three times using the pouring method.
 - b. After removing the last of the supernatant, so just the pellet was left, I put in the freezer to continue after my meetings.
2. Added 250uL Buffer P1 to resuspend pelleted bacterial cells.
3. Added 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution was more translucent
4. Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
 - a. Makes like a white precipitate
5. Centrifuge for 10 minutes at 13,000 rpm
 - a. Combine into 4 columns
6. Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 1 minute. Discard flow-through. (x2 because double the initial volume)
7. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 minute. Discard flow-through.
8. Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 minute and discard flow-through 3 times (added 750ul of PE three times). Transfer to collection tube.
9. Centrifuge for 3 minutes to remove residual wash buffer.
10. Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 30 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.

Nanodrop Results from the minipreps above. Much better than last time!!

#	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

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