

Table of Contents

May 2022	3
Wednesday, May 25, 2022	3
Codon-Optimization Protocol	3
Design T7 NLuc Plasmid	4
Thursday, May 26, 2022	5
Friday, May 27, 2022	6
MHB Protocol	6
LB Carb Plate Protocol	6
Tuesday, May 31, 2022	7
2% Hemoglobin Protocol	7
June 2022	8
Wednesday, June 1, 2022	8
Thursday, June 2, 2022	9
PCR Purification Protocol	12
Friday, June 3, 2022	13
DNA Digest Protocol	13
Gel Purification Protocol	13
Ligation Protocol	15
Transform Chemically Competent <i>E. coli</i> Cells Protocol	15
Tuesday, June 7, 2022	17
Single Use Aliquots Protocol	18
Wednesday, June 8, 2022	19
Miniprep Protocol	19
Thursday, June 9, 2022	22
Miniprep Protocol	24
Sequence Reactions Protocol	25
Wednesday, June 15, 2022	26
Diagnostic Digest Protocol	26
Thursday, June 16, 2022	28
NEB 5-alpha Competent <i>E. coli</i> Transformation Protocol	28
Friday, June 17, 2022	30

Monday, June 20, 2022 31

Tuesday, June 21, 2022 34

Wednesday, June 22, 2022 37

Thursday, June 23, 2022 40

Friday, June 24, 2022 41

Tuesday, June 28, 2022 42

Wednesday, June 23, 2022 43

Thursday, June 30, 2022 44

Phenol:Chloroform DNA Purification and Ethanol Precipitation Protocol 45

May 2022

Wednesday, May 25, 2022

To Do:

1. ~~Start new lab notebook~~
2. ~~Codon optimize nluc~~
3. ~~Design a T7-nluc plasmid off of pKR84~~
4. ~~Order gBlock (send to Kathryn)~~
5. ~~Design and order primers~~

Today I will be starting the cloning process for a luciferase detector for a reporter assay. Luciferases are a class of oxidative enzymes that produce bioluminescence. Since we want high sensitivity, we will try using NanoLuc (NLuc). Nano Luciferase (NanoLuc) is a very small protein (19 kDa) and has approximately 150-fold stronger specific activity compared to conventional luciferases. The overall purpose is to create a highly sensitive system to detect and measure the translational efficacy of different strains of ribosomes. The hypothesis is that ribosomes that contain bs21-2 (encoded by rpsU2) can better recognize the 5' UTR (known as pdpA) compared to ribosomes containing bs21-1 and bs21-3. This will result in bs21-2 producing the same level of fluorescence as wild type ribosomes.

First, I must design and clone a plasmid containing NLuc.

Codon-Optimization Protocol

Use this web-based tool: <http://genomes.urv.es/OPTIMIZER/>

NLuc amino acid sequence: http://parts.igem.org/Part:BBa_K1159001

1. Input amino acid sequence and select protein
2. Select Data from the HEG-DB
3. Select E. coli K12 (MG1655) codon usage table Codon Usage HEG format
4. Select eubacterial genetic code
5. Select One AA – one codon (Most Frequent)

Nluc Amino Acid Sequence:

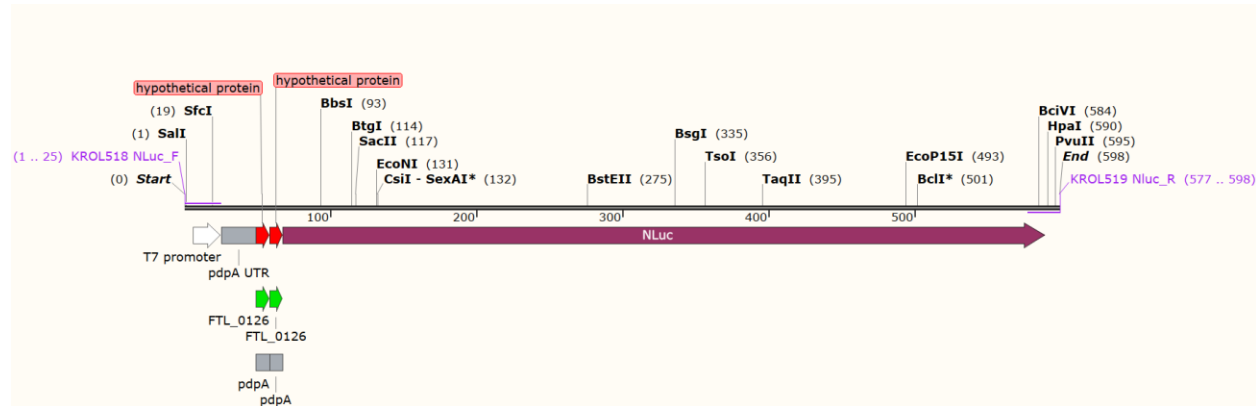
```
MAGVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIIPYEGLSGDQMGQI
EKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLI
FRVTINGVTGWRLCERILATG
```

Codon Optimized Sequence:

```
ATGGCGGGTGTTCACCCCTGGAAGACTTCGTTGGTGACTGGCGTCAGACCGCGGGTTACAACCTGGACC
AGGTTCGGAACAGGGTGGTGTTCCTCTCTGTTCCAGAACCTGGGTGTTTCTGTTACCCCGATCCAGCG
TATCGTTCGTCTGGTGAAAACGGTCTGAAAATCGACATCCACGTTATCATCCCGTACGAAGGTCTGTCT
GGTGACCAGATGGGTGAGATCGAAAAAATCTTCAAAGTTGTTTACCCGGTTGACGACCACCACTTCAAAG
TTATCCTGCACTACGGTACCCTGGTTATCGACGGTGTACCCCGAACATGATCGACTACTTCGGTTCGTCC
GTACGAAGGTATCGCGGTTTTTCGACGGTAAAAAATCACCGTTACCGGTACCCTGTGGAACGGTAACAAA
ATCATCGACGAACGTCTGATCAACCCGGACGGTTCTCTGCTGTTCCGTGTTACCATCAACGGTGTACCG
GTTGGCGTCTGTGCGAACGTATCCTGGCGACCGGT
```

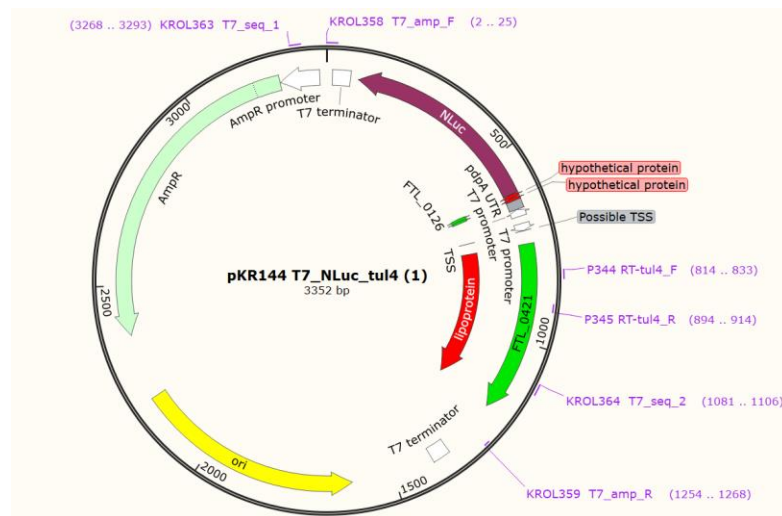
Design T7 NLuc Plasmid

I inserted the codon-optimized NLuc sequence into SnapGene and then added a stop codon (TAA), the restriction enzyme recognition sequences (Sal1 at the 5' end and PvuII at the 3' end), the T7 promoter sequence, and the pdpA UTR sequence with approximately 6 extra bp between its 3' end and the start of the NLuc sequence. I then created a forward and reverse plasmid for this gene called KROL518 and KROL519.



NLuc gene = 606 bp

Using pKR81 as the backbone, I inserted the NLuc fragment above into the plasmid using Sal1 and PvuII restriction enzymes to create pKR144.



Plasmid (3352 bp)

Thursday, May 26, 2022

To Do:

- ~~1. Organize / do calculations for upcoming protocols~~

Friday, May 27, 2022

To Do:

- ~~1. Make mueller-hinton broth~~
- ~~2. Make LB-Carb plates~~
- ~~3. Make aliquots for PCR reactions~~
- ~~4. Reconstitute primers~~

MHB Protocol

1. Add 10.5 g MHB and 500 mL ddiH₂O to 1 L orange-topped jar
2. Shake to mix
3. Autoclave on 30 min. liquid cycle

LB Carb Plate Protocol

All steps below should be performed aseptically

1. Mark sleeve of plates with black marker
2. Heat up 500 mL LB agar on hot plate
3. Add 500 uL carbenicillin to agar - mix
4. Pour agar into plates
5. Leave plates at room temp. until cool
6. Store in standing incubator

Today I prepared aliquots for my future PCR reactions. I already had PrimeSTAR GXL Buffer and dNTPs in my box in the freezer from last summer so I will use those.

1. Micropipette 50 uL PrimeSTAR GXL DNA Polymerase into non sterile 1.5 mL tube
2. Store buffer and dNTPs in box in -20°C fridge
3. Store enzyme in yellow cooler in -20°C fridge

I will also reconstitute primers KROL518/519.

1. Centrifuge primers at highest speed (150,60 rpm) for 2 mins
2. Add nmol x 10 uL of 0.1X elution buffer to primer tubes to make 100 uM
3. Vortex
4. Put on 42°C heat block for 5 mins
5. Dilute primers 1:10 in purple tagged tubes to a volume of 300 uL – store in -20°C (30 uL primer; 270 uL buffer)

Tuesday, May 31, 2022

To Do:

1. ~~Prepare hemoglobin~~

2% Hemoglobin Protocol

1. Add 6 g freeze-dried hemoglobin to a 1 L flask
2. Add stir bar
3. Add 300 mL ddiH₂O
4. Mix on stir plate until fully dissolved
5. Autoclave using 20 min. liquid cycle

June 2022

Wednesday, June 1, 2022

To Do:

- ~~1. Patch out LVS~~
- ~~2. Read research papers~~

Thursday, June 2, 2022

To Do:

1. ~~Reconstitute gBlock~~
2. ~~PCR~~
3. ~~Run gel~~
4. ~~PCR Purify~~

Today I will reconstitute the NLuc gBlock.

1. Centrifuge gBlock using benchtop centrifuge for 1 min
2. Add ng / 10 uL of 0.1X elution buffer to tube to make 10 ng/uL
3. Vortex briefly
4. Incubate at 42°C for 20 mins
5. Vortex briefly
6. Centrifuge

Since the gBlock was 500 ng, I added 50 uL of elution buffer.

I then amplified this gBlock, along with a positive and negative control, and ran a gel to confirm it was correct.

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
 - Primestar buffer
 - dNTPs
 - oligo F (10uM)
 - oligo R (10uM)
 - template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Add appropriate volume (based on PCR worksheet below) of each experiment specific primer (forward and reverse) to PCR tubes
7. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
8. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTP

- Add Primestar buffer
 - Add Primestar enzyme
9. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
 10. Add appropriate volume of master-mix to negative control PCR tube
 11. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
 12. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
 13. Close PCR Tubes until the caps are tight
 14. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
 - Heat at 94°C for 2 min.
 - 94°C for 20 seconds
 - 50°C for 30 seconds
 - 68°C for 1 minute/kb (adjust based on expected size of product)
 - Go back to step 2
 - Repeat 32x
 - 68°C for 5 minutes
 - 12°C for infinity

Prepare PCR to begin amplifying the NLuc gene.

Reaction Numbers	Primers	Template	Expected Size
1	KROL518/519	NLuc gBlock	606
2	KROL15/16	water	-
3	KROL15/16	LVS gDNA	400

Total reaction volume		100		
Total number of reactions		3		
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			62	248
PrimeSTAR GXL Buffer	5x	1x	20	80
dNTPs	2.5 mM	0.2 mM	8	32
oligo F	10 uM	0.3 uM	3	12
oligo R	10 uM	0.3 uM	3	12
template	100 ng/ul	2 ng/ul	2	8
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	8
		Total volume	100	400

I added 92 uL of master mix to each tube, which contained its respective template and primers. Since the largest product is approximately 606 bp, I set step 4 for 45 seconds.

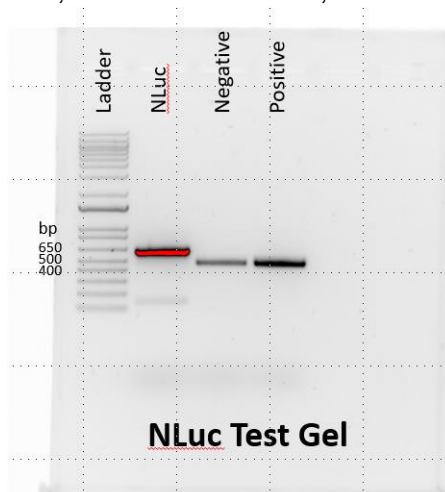
PCR Gel Protocol

1. Prepare gel consisting of 1% agarose in TAE
 - a. melt and let cool down

2. Micropipette 7 uL SYBR safe into the gel rig
3. Pour liquid gel into plate until level reaches just under the two notches
4. Use comb to gently mix together
5. Let gel solidify (takes ~1 hour)
6. Pull gel out of rig and then put back in (with comb facing longwise)
7. Submerge gel in 1X TAE buffer; fill wells on either side of gel
8. Remove comb
9. Micropipette 10 uL ladder into first well
10. Micropipette three, 4 uL drops of dye on parafilm (volume of dye used is 1/5 volume of PCR product)
11. Micropipette 20 uL of each PCR product onto its respective dot of dye
12. Mix by pipetting up and down
13. Insert each PCR product + dye into its respective well
14. Connect wires to gel (run to red)
15. Turn device on and set to 112 V (bubbles running up sides of well means its working)
 - a. Run until dye travels ~1/2 down the gel (takes ~35 min.)
16. Turn off device and unplug wires
17. Remove gel from rig and place in image lab - center it
18. Take image of gel and save
19. Cut part of gel not used during run and return to gel flask
20. Pour buffer from gel rig into the used buffer jug
21. Rinse gel rig with water

Used 2 uL of dye and 10 uL of template for each reaction.

Lane 1: ladder; Lane 2: Reaction 1; Lane 3: Reaction 2; Lane 4: Reaction 3



Both the NLuc and positive control bands were at their expected locations of 606 and 400 bp, respectively. There was a strong band in the negative control column at the same location as the positive control, which indicates some LVS gDNA must have gotten cross contaminated. I ran the gel further to ensure the NLuc product was not contaminated. No band became present around 400 bp for the NLuc column. The presence of the second, faint band in the NLuc column is most likely from a secondary product and should not effect the cloning process.

Next, I PCR purified the NLuc product and stored it in the -20°C fridge overnight.

PCR Purification Protocol

1. Add five volumes of Buffer PB (500 μ L) to one volume of the PCR reaction (100 μ L) and mix by pipetting up and down
2. Place a QIAquick column in a 2 mL collection tube
3. To bind DNA, transfer sample to QIAquick column and centrifuge for 60s at 13,000 rpm
4. Drain filtered contents from 2 mL collection tube
5. To wash, add 750 μ L of Buffer PE to QIAquick column, and centrifuge for another 60s at 13,000 rpm
6. Drain filtered contents from 2 mL collection tube
7. Centrifuge QIAquick column for 3 min at 13,000 rpm to remove any residual wash buffer
8. Place each QIAquick column in a clean 1.5 mL microfuge tube
9. To elute DNA, add 35 μ L of 0.1x elution buffer to the center of the QIAquick membrane, and centrifuge for 1 min at 13,000 rpm
10. Discard top filter and save 1.5 mL tube in -20°C fridge

Friday, June 3, 2022**To Do:**

1. ~~DNA Digest~~
2. ~~Run Gel~~
3. ~~Gel Extract / Purify~~

Today I will digest the purified NLuc and the backbone from pKR81 using Sal1 and PvuII.

DNA Digest Protocol

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR (NLuc)	Sal1 ; PvuII	15	-
2	Backbone (pKR81)	Sal1 ; PvuII	5	10

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)	-
Sal1	0.6	1.8
PvuII	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.

1. Add indicated amounts of H₂O (___uL) and 10x buffer (____uL) to master mix tube (MM).
2. Add indicated amount of DNA (___uL) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler.
 - a. For backbone, add 5 uL and 10 uL water
3. Add indicated amount of each enzyme (___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 (____uL).
6. Incubate at 37°C for 1 hour or up to overnight.

Incubated for 1 hour in the standing incubator.

Gel Purification Protocol

If using digest for plasmid construction then after incubation at 37°C, follow these steps:

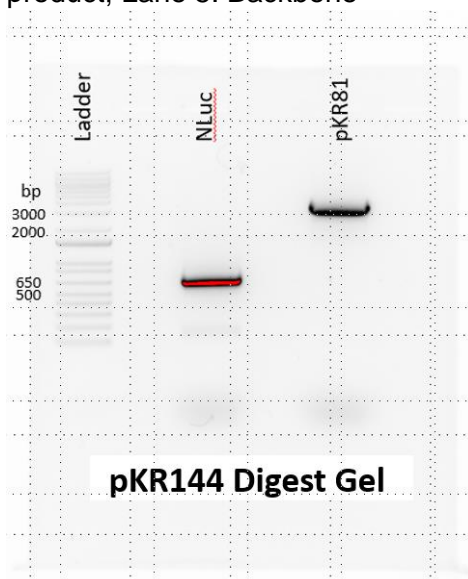
1. Add 1 uL of Quick CIP to backbone
2. Mix by pipetting and put in incubator for 10 mins.
3. Put in 80°C heat block for 2 mins.

(this step removes the phosphates from the ends of the plasmid to prevent re-ligation)

1. Prepare and run gel
2. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel
3. Weigh the gel slice in a colorless tube
4. Add 3 volumes buffer QG to 1 volume gel (100 mg gel ~ 100 uL) The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes buffer QG
5. Incubate at 50°C for 10 min. Vortex the tube every 2-3 min. to help dissolve the gel
 - a. After the gel slice has dissolved completely the mixture should be yellow; if it is orange or violet add 10 uL 3 M sodium acetate, pH 5.0, and mix
6. Add 1 gel volume isopropanol to the sample and mix
7. Place a QIAquick spin column in a provided 2 mL collection tube. Apply the sample to the column and centrifuge for 1 min. Discard flow-through and place column back in same tube
8. Add 500 uL buffer QG to column and centrifuge for 1 min. Discard flow-through
9. Add 750 uL buffer PE to column and centrifuge for 1 min. Discard flow-through
10. Centrifuge column for 5 min
11. Place column in a clean 1.5 mL micro centrifuge tube
12. Add 35 uL 0.1X buffer EB to center of membrane and centrifuge for 1 min.
13. Discard top filter and store tubes in -20°C fridge

Used 6 uL of dye for 30 uL of template.

Lane 1: Ladder; Lane 3: PCR product; Lane 5: Backbone



The gel looked good, with both bands at their expected locations. (PCR product: 606 bp; Backbone: 2762 bp).

I then cut out the bands for both the insert and backbone.

Insert: 0.14 g (140 mg) ; added 420 uL buffer QG

Backbone: 0.18 g (180 mg) ; added 540 uL buffer QG

I did not heat the gel slice to dissolve it, I only vortexed it.

Monday, June 6, 2022**To Do:**

1. ~~Ligate~~
2. ~~Transform~~

Ligation Protocol

Tube	Insert	Backbone
1	Sal1, PvuII digested, purified PCR (NLuc)	Sal1, PvuII digested, purified pKR81
2	-	Sal1, PvuII digested, purified pKR81

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

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 in order 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. Create a master mix of water, 10x buffer, backbone, and ligase (keep ligase in mini cooler)
4. Add 16 uL master mix to each tube
5. Add 4 uL insert to tube 1
6. Add 4 uL water to tube 2
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Place in the thermocycler overnight at 16°C or room temperature for 10 min.

Incubated for 10 mins at room temperature. The tubes must have gotten bumped at some point during the incubation since after the 10 mins I noticed that the contents of both tubes were splashed up the sides. I centrifuged them and then incubated for another 10 mins at room temperature to ensure there was a proper reaction.

Transform Chemically Competent *E. coli* Cells Protocol

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	pKR81	1 uL	20 ul, 100 ul	2
2	(-) control	None	0	20 ul, 100 ul	2
3	Backbone Ligation	pKR81 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR114 (digested)	8 uL	100 uL, remaining	2
Total number of plates					8

1. Check to be sure you have enough LB-Carb plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed.
2. Obtain DNA and thaw on ice if necessary.
3. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
4. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
5. 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.
6. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
7. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
8. After heat shock, place tubes back on ice until next step (don't keep them here too long).
9. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
10. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
11. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
12. 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.
13. Put plates in standing incubator

Side Project

Today I will also start a side project which will compare the growth rates of wild-type and Δ rpsU2 *Francisella tularensis*.

Today I streaked out LVS and Δ rpsU2 on no antibiotic CHAH plates and stored them in the standing incubator overnight.

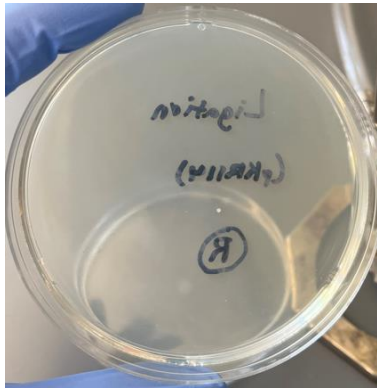
Tuesday, June 7, 2022

To Do:

1. ~~Prepare overnight cultures~~
2. ~~Make single use aliquots of LVS~~

Transformation Plate Analysis

The transformation plates looked good, with no growth on either of the negative control or backbone plates. The two positive control plates had a lot of growth, both being completely covered in colonies (too many to count). The 100 uL ligation plate had no colonies, however, the remaining ligation plate had one colony. The low transformation efficiency is most likely due to the use of a blunt-end restriction enzyme. I will make one overnight culture using this single colony and continue the experiment.



Overnight Cultures Protocol

Today I will prepare an overnight culture using my pKR144 insert plate, which I will then use tomorrow to mini prep and prepare for sequencing.

1. Add 5 mL of LB broth to a sterile glass culture tube
2. Add 5 uL of carbenicillin (Carb) to the tube
3. Vortex briefly to mix
4. Warm in shaking incubator
5. Using a stick, pick the isolated colony from the plate and inoculate the tube
6. Place in the 37°C shaking incubator overnight. ~16-22 hours

Put in the incubator at 2:21 pm.

Side Project

I will prepare a 50 mL overnight culture using the LVS plate I streaked out yesterday.

1. Add 50 mL of MHB to a 125 mL sterile glass baffled flask
2. Warm in shaking incubator

I want a starting OD600 of 0.05. I followed the steps below:

1. Add 400 uL MHB to 1.5 mL microcentrifuge tube
2. Use a loop to scrape a chunk of cells into tube – resuspend using a pipette
3. Add 1 mL MHB to a cuvette (blank)

4. Make a 1:20 dilution in a cuvette (950 uL MHB; 50 uL cells) – mix by turning upside down using parafilm
5. Check OD600

OD600 = 0.422

Added 296 uL of the non-diluted cells to the flask – swirled to mix.

Starting OD600 = 0.058

I then placed it in the 37°C shaking incubator overnight starting at 2:16 pm.

I left the Δ rpsU2 plate in the incubator to continue to grow and streaked out another LVS plate and left it in the standing incubator overnight.

Single Use Aliquots Protocol

After making the overnight culture of LVS, I used the remaining cells on the plate to make single use aliquots. I stored them in my box in the -80°C freezer.

1. Add 150 uL of MHB to a 1.5 mL microfuge tube
2. Heat up a loop over the flame
3. Use loop to gently scrape the cells off the gel plate
4. Swirl the loop in the microfuge tube to release the cells
5. Once the majority of the cells are scraped off the plate, discard the plate
6. Add 250 uL MHB to tube and pipette up and down to mix
7. Add 100 uL of 75% glycerol – mix by pipetting
7. Pipette 100 uL of cell mixture to 4 1.5 mL microfuge tubes
 1. Open/close container of microfuge tubes near flame

Wednesday, June 8, 2022

To Do:

- ~~1. Miniprep~~
- ~~2. Transform~~
- ~~3. Ligate~~
- ~~4. Grow cultures~~
- ~~5. Design / order primer~~

Side Project

Today I will start the side experiment. I will grow 8 cultures using different media and different cells and compare their growth:

- 2 cultures of LVS in fresh MHB
- 2 cultures of LVS in conditioned media
- 2 cultures of Δ rpsU2 in fresh MHB
- 2 cultures of Δ rpsU2 in conditioned media

To make the conditioned media I followed the steps below, starting with the 50 mL overnight culture I made yesterday.

1. Transferred overnight culture to a 50 mL conical tube (sterile)
2. Centrifuged in the big centrifuge at 10,000xg for 10 mins
3. Filter sterilized supernatant using a 50 mL centrifuge tube filter

I then prepared the cultures following the steps below:

1. Added 600 uL fresh MHB to two 1.5 mL tubes
2. Scraped LVS from the fresh plate (made yesterday) into one tube - resuspended
3. Scraped Δ rpsU2 from the plate made two days ago into the other tube – resuspended
4. Diluted 1:20 in a cuvette

LVS: OD600 = 0.455 (added 70.2 uL to each 8 mL culture tube)

Δ rpsU2: OD600 = 0.544 (added 58.8 uL to each 8 mL culture tube)

1. Checked starting OD600s of each culture (600 uL)
2. Checked OD600s every 1.5 hours for 4.5 hours total

Left in the shaking incubator to grow overnight starting around 4 pm.

Miniprep Protocol

Today I will miniprep the overnight culture of my plasmid.

1. Pellet bacterial overnight culture by centrifugation (max speed for 3 min.) at room temp.
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (in fridge) and transfer to micro centrifuge tube
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times

5. Centrifuge for 10 min. at 13000 rpm
6. Apply 800 uL supernatant from previous step to QIAprep 2.0 spin column by pipetting
7. Centrifuge for 1 min and discard flow through
8. Wash column with 0.5 mL Buffer PB
9. Centrifuge for 1 min and discard flow through
10. Wash column with 0.75 mL Buffer PE
11. Centrifuge for 1 min and discard flow through (wash with PE 3x)
12. Centrifuge for 3 min.
13. Place column in 1.5 mL microcentrifuge tube; add 50 uL 0.1X Buffer EB to center and let stand 1 min.
14. Centrifuge for 1 min.
15. Save in -20 fridge

Re-Do Experiments

Concentration of pKR144 miniprep ~ 5 ng/uL

Since the concentration was so low, I will re-do several steps.

First, I will transform my saved ligations.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR81	1 uL	20 ul, 100 ul	2
2	(-) control	None	0	20 ul, 100 ul	2
3	Backbone Ligation	pKR81 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR114 (digested)	8 uL	100 uL, remaining	2
Total number of plates					8

I incubated the plates in the standing incubator overnight.

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.

1. Put plates in standing incubator

I will also set up a new overnight ligation with a positive control.

Tube	Insert	Backbone
1	Sal1, PvuII digested, purified PCR (NLuc)	Sal1, PvuII digested, purified pKR81
2	-	Sal1, PvuII digested, purified pKR81
3	Pac1, Kpn1 digested, purified PCR (LnSeqA)	Pac1, Kpn1 digested, purified pKL97
4	-	Pac1, Kpn1 digested, purified pKL97

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (5x)
-----------	-----------------	-----------------	-----------------

H ₂ O	11.5	11.5	57.4
10x ligase buffer	2.0	2.0	10
Insert	4.0	-	-
Backbone	2.0	2.0	-
Ligase	0.5	0.5	2.5
TOTAL	20.0	20.0	70

Used a brand new, big tube of ligase buffer and thawed completely on ice then mixed well. As a positive control I used Aisling's gel purified insert and backbone (tubes 3 + 4).

Incubated at 16°C in the thermocycler overnight.

Thursday, June 9, 2022

To Do:

1. ~~Overnight cultures~~
2. ~~Transform cells~~
3. ~~Make single use aliquots of Δ rpsU2~~
4. ~~Analyze side project data~~

Transformation Results

The transformation went well. I got the same results as last time. There was no growth on the negative control and backbone plates, lots of growth on both positive control plates, and only one colony on the remaining ligation plate.

Today I will prepare two overnight cultures using the colony from my pKR144 insert plate and one from my pKR81 positive control plate, which I will then use tomorrow to mini prep.

1. Using a stick, pick the isolated colony from the plate and inoculate the tube
2. Place in the 37°C shaking incubator overnight. ~16-22 hours

Put in the incubator at 3:30 pm.

Single Use Aliquots

Today I also created single use aliquots for Δ rpsU2 from the plate I streaked out on Monday. I stored them in my box in the -80°C freezer.

Transformation

I will also perform another transformation using the overnight ligation from yesterday.

Reaction table

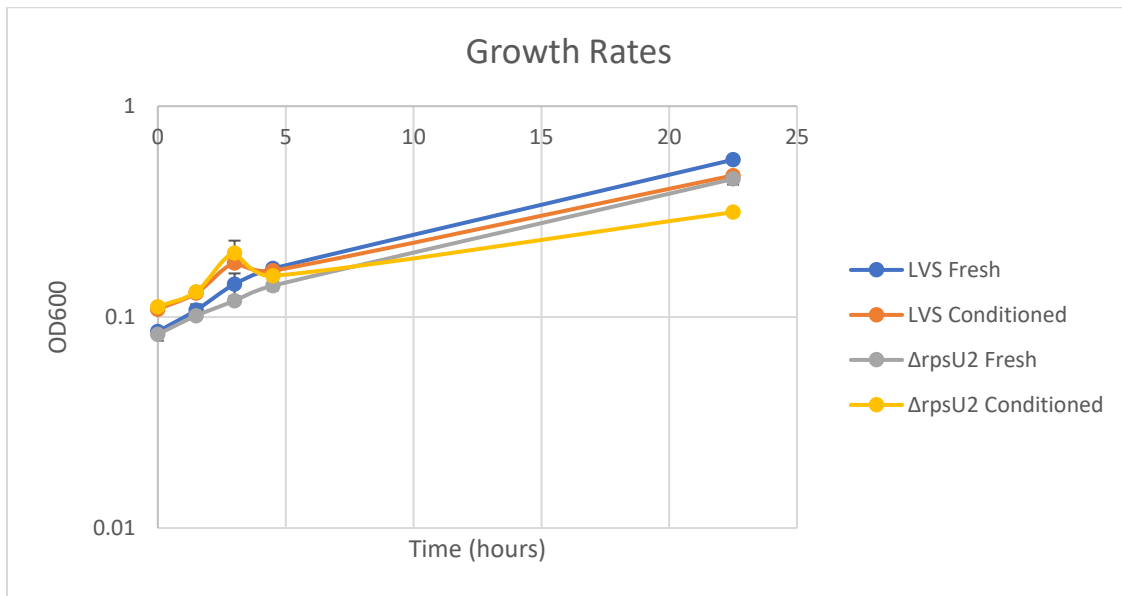
Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR81	1 uL	20 ul, 100 ul	2
2	(-) control	None	0	20 ul, 100 ul	2
3	Backbone Ligation	pKR81 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR114 (digested)	8 uL	100 uL, remaining	2
5	Ligation Positive Control	pKR140 (digested)	8 uL	100 uL, remaining	2
Total number of plates					10

Put in standing incubator overnight.

Side Project Analysis

I took out the overnight cultures around 10 am and checked their OD600s. The OD600 averages of the two duplicates for each sample can be seen below with a graphical representation of the growth curves for each condition.

Sample	0 (hours)	1.5 (hours)	3 (hours)	4.5 (hours)	22.5 (hours)
LVS Fresh	0.0855	0.108	0.1435	0.17	0.5575
LVS Conditioned	0.109	0.13	0.1805	0.166	0.4695
Δ rpsU2 Fresh	0.083	0.1015	0.1195	0.141	0.452
Δ rpsU2 Conditioned	0.112	0.132	0.2015	0.157	0.3145



Overall, LVS in both conditions grew better than Δ rpsU2 and both strains grew better in fresh media compared to conditioned.

Generation Times	1.5-3 (hours)
LVS Fresh	227.9443936
LVS Conditioned	191.3579718
Δ rpsU2 Fresh	385.6540485
Δ rpsU2 Conditioned	158.4067221

As seen by the generation times, all the cells grew very poorly. When compared to Hannah’s previous growth experiment, LVS should have a generation time around 110. My LVS’s generation time was over double that. Therefore, I should re-do the entire experiment for more accurate data analysis.

Friday, June 10, 2022

To Do:

- ~~1. Miniprep~~
- ~~2. Sequence Reactions~~

Transformation Results

The positive controls had good growth, both pKR81 and pKR140 (Aisling's plasmid), which means both the ligation and transformation were successful. However, there were no colonies on either of the ligation plates. I talked to Sierra and we checked the concentrations of my gel purifications of the backbone and insert and they were good (approximately 30 and 50 ng/uL respectively). In the future I will try another overnight ligation using the ligation calculator to try and improve my ligation efficiency.

Miniprep Protocol

Today I will miniprep the two overnight cultures I prepared yesterday. I took the overnight cultures out of the incubator at 11:00 am.

1. Pellet bacterial overnight culture by centrifugation (max speed for 3 min.) at room temp.
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (in fridge) and transfer to micro centrifuge tube
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
5. Centrifuge for 10 min. at 13000 rpm
6. Apply 800 uL supernatant from previous step to QIAprep 2.0 spin column by pipetting
7. Centrifuge (13,000 rpm) for 1 min and discard flow through
8. Wash column with 0.5 mL Buffer PB
9. Centrifuge (13,000 rpm) for 1 min and discard flow through
10. Wash column with 0.75 mL Buffer PE
11. Centrifuge (13,000 rpm) for 1 min and discard flow through (wash with PE 3x)
12. Centrifuge (13,000 rpm) for 3 min.
13. Place column in 1.5 mL microcentrifuge tube; add 50 uL 0.1X Buffer EB to center and let stand 1 min.
14. Centrifuge (13,000 rpm) for 1 min.
15. Save in -20 fridge

Right out of the incubator both cultures had a good amount of precipitate at the bottom. I vortexed to mix prior to centrifuging them.

I realized that the last time I miniprep I used buffer PE with no ethanol added. This could have been what caused the very low DNA concentration.

pKR81 miniprep concentration: 184.2 ng/uL

pKR144 miniprep concentration: 183.3 ng/uL

Since the miniprep concentration was good, I used it to set up sequence reactions and left them in the freezer for Janet to sequence next week.

Sequence Reactions Protocol

1. Use nanodrop to check concentrations of mini preps (dilute if necessary)
2. Dilute primers (2.5% - dilute 1:4 from working primer stock)
3. Set up sequence reaction worksheet
4. Add appropriate primer to each tube (2.56 uL for each)
5. Add appropriate miniprep to each tube
6. Add appropriate volume of water to each tube
7. Centrifuge tubes until all components are at bottom
8. Pipette up and down to mix
9. Bring PCR tubes and worksheet to genome sequencing lab

Sample number	Well	Template Type	Template Name	Primer Name ^a	A.	B.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PLASMID template:	Volume H₂O needed
							Volume =	
							2x(~200 ÷ B)μl	(12 less D or E - 2.56)μl
MC1		Plasmid	pKR144	KROL358	3352	183.3	2.18	7.26
MC2		Plasmid	pKR144	KROL64	3352	183.3	2.18	7.26

Wednesday, June 15, 2022

To Do:

1. ~~Diagnostic Digest~~
2. ~~Overnight Ligation~~
3. ~~Streak out LVS~~

Diagnostic Digest Protocol

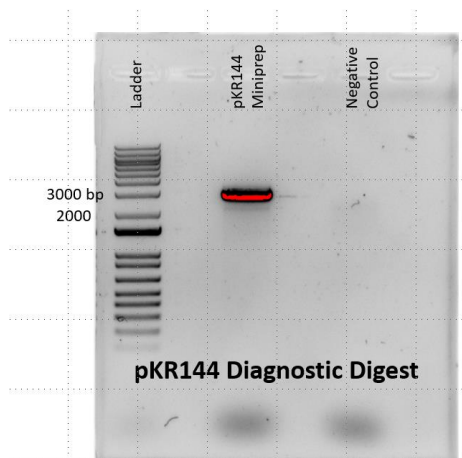
Today I will diagnostically digest the miniprep of my plasmid to see if NLuc was properly inserted.

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Miniprep pKR144	SalI, PvuII	4	-
2	Water	SalI, PvuII	-	4

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H ₂ O	13	39
10x Buffer*	2	6
DNA	(4)	-
SalI	0.5	1.5
PvuII	0.5	1.5
Total	20.0 (18.0 actual b/c of DNA)	

1. Add indicated amounts of H₂O (___ uL) and 10x buffer (____ uL) to master mix tube (MM)
2. Add 2 uL of DNA to individual tubes for digest
3. Add indicated amount of each enzyme (___ uL) to the master mix tube (MM)
4. Mix the master mix by pipetting up and down
5. Add 18 uL master mix to individual tubes
6. Incubate at 37°C for 1 hour or up to overnight
7. Run PCR gel

Incubated in standing incubator for 1 hour.



Used 4 uL of dye for each 20 uL sample

Lane 1: ladder; Lane 3: pKR144; Lane 5: negative control

Backbone: 2762 bp

Insert: 606 bp

As seen in the gel, there is no band where the insert should be, which either means it is too faint to see or not there. There are two bands between 2000-3000 bp, which could be an incomplete digest of the plasmid. The lower, fainter band represents the digested backbone, while the darker one is the full plasmid.

Due to these inconclusive results, I will prepare an overnight ligation using my insert and backbone with adjusted backbone:ligation ratios to try and improve my transformation efficacy.

Overnight Ligation

Diluted gel purified NLuc 1:5 (4 uL NLuc; 16 uL water)

Tube	Insert	Backbone
1	Sal1, PvuII digested, purified PCR (NLuc)	Sal1, PvuII digested, purified pKR81
2	Sal1, PvuII digested, purified PCR (NLuc)	Sal1, PvuII digested, purified pKR81
3	-	Sal1, PvuII digested, purified pKR81

	Tube 1 (3X)	Tube 2 (5X)	Tube 3 (BB only)
Water	15.44	15.08	15.98
Ligation Buffer	2	2	2
Backbone	1.52	1.52	1.52
Insert	0.55	0.91	-
Ligase	0.5	0.5	0.5
	20.00	20.00	20.00

Incubated at 16°C overnight in the thermocycler (starting around 4 pm)

Side Project

Today I streaked out LVS on a no antibiotic CHAH plate and stored it in the standing incubator overnight. This will be used to prepare more conditioned media to repeat the experiment.

Thursday, June 16, 2022**To Do:**

1. ~~Overnight culture~~
2. ~~Make LB-Carb plates~~
3. ~~Transformation~~

Transformation

Today I will prepare more LB-Carb plates and transform the overnight ligation.

I dried the plates by the flame for 30 mins then in the fume hood for another 30 mins.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR81	1 uL	20 uL	1
2	(-) control	None	0	20 uL	1
3	Backbone Ligation	pKR81 (digested)	8 uL	100 uL, remaining	2
4	3X Ligation	pKR144 (digested)	8 uL	20 uL, 100 uL, remaining	3
5	5X Ligation	pKR144 (digested)	8 uL	20 uL, 100 uL, remaining	3
Total number of plates					10

NEB 5-alpha Competent *E. coli* Transformation Protocol

For my transformation I will use NEB 5-alpha competent cells since they have much higher efficiency. The protocol is below:

1. Check to be sure you have enough LB plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed
2. Obtain DNA and thaw on ice if necessary
3. Thaw appropriate number of competent cell tubes (1 per reaction) on ice for 10 mins.
4. Add appropriate amount of DNA to each tube. Flick 4-5 times to mix. Do not vortex
5. Incubate cells on ice for 30 minutes. During incubation, find or set heat block to 42°C. Do not mix
6. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step). Do not mix
7. After heat shock, place tubes back on ice for 5 mins. Do not mix
8. Using aseptic technique, add 950 uL of room temperature SOC media to each microfuge tube
9. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
10. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
11. 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.

12. Put plates in standing incubator overnight

Side Project

Today I will prepare a 50 mL overnight culture using the LVS plate I streaked out yesterday and fresh MHB.

I diluted the cells 1:40 in the cuvette

OD600 = 0.366

Added 171 uL of the non-diluted cells to the flask – swirled to mix

Starting OD600 = 0.048

I then placed it in the 37°C shaking incubator overnight starting at 3:54 pm.

Friday, June 17, 2022

To Do:

- ~~1. Prepare conditioned media~~
- ~~2. Check transformation plates~~

Transformation Results

The only plate with colonies present was the positive control. There might be one very small colony on the 5X remaining plate, so I left all the plates on the benchtop at room temperature over the weekend.

Since the ligation step seems to be the problem, I will re-do it next week with several adjustments made as follows:

1. Do a positive control during ligation
2. Run a small gel for a better calculation of concentration
3. Make a master mix during ligation

Side Project

Today I finished preparing the conditioned media by centrifuging the overnight culture and then filter sterilizing the supernatant. I stored the media in the 4°C fridge.

Monday, June 20, 2022

To Do:

1. ~~Run gel~~
2. ~~Overnight ligation~~
3. ~~Streak out LVS and rpsU2~~
4. ~~Overnight culture~~
5. ~~Make LB-Carb plates~~
6. ~~Make hemoglobin~~

Side Project

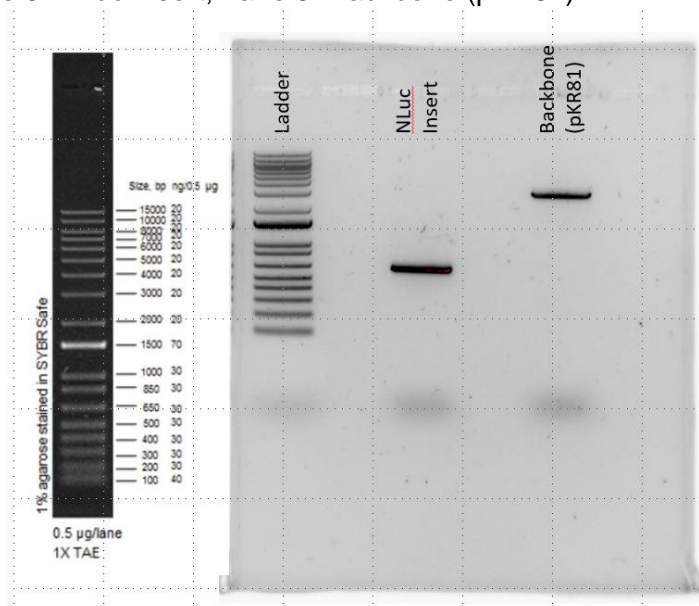
Today I streaked out one plate of LVS and two plates of Δ rpsU2 on CHA to use in my growth experiment tomorrow. I put them in the incubator overnight.

Re-Do of Ligation

Today I will re-do the overnight ligation. First, I will try to get more accurate concentrations for the backbone and insert by running a gel.

Added 10 uL of ladder and 5 uL of each sample (used 1 uL of dye for each sample)

Lane 1: Ladder; Lane 3: NLuc insert; Lane 5: Backbone (pKR81)



NLuc insert: 594 bp

Backbone: 2762 bp

The insert and backbone bands appear at their expected locations. To determine their concentrations, I compared their approximate intensity to the ladder. The backbone band is approximately the same intensity as the 1500 bp band, while the insert band is approximately 3X the intensity.

1500 band = 140 ng (the concentration of the ladder we use is double)

Insert: $420 \text{ ng} / 5 \text{ uL} = 84 \text{ ng/uL}$

Backbone: 140 ng / 5 uL = 28 ng/uL

These concentrations are pretty similar to what was calculated by the nanodrop. I will use the ligation calculator to determine the volume of insert I should use for the ligation using these concentrations. I diluted the insert 1:5 (1 uL NLuc + 4 uL water).

Tube	Insert	Backbone		
1	Sall-HF, PvuII-HF digested, purified PCR (NLuc)	Sall-HF, PvuII-HF digested, purified pKR81		
2	Sall-HF, PvuII-HF digested, purified PCR (NLuc)	Sall-HF, PvuII-HF digested, purified pKR81		
3	-	Sall-HF, PvuII-HF digested, purified pKR81		
	3X	5X	BB only	Master Mix
Water	1.27	0.00	3.18	50.16
Ligation Buffer	2	2	2	8
Backbone	1.79	1.79	1.79	7.16
Insert	1.91	3.18	-	-
Ligase	0.5	0.5	0.5	2
Master Mix	16.82	16.82	16.82	
Total	20.00	20.00	20.00	

1. Make master mix using water, ligation buffer, backbone, and ligase – pipet to mix
2. Add appropriate volume of insert to each tube
3. Add appropriate volume of water to each tube
4. Add 16.82 uL master mix to each tube
5. Pipet to mix using a pipet set to 18 uL
6. Incubate overnight at 16°C in the thermocycler

I will also perform a positive control ligation using Aisling's insert and backbone.

Tube	Insert	Backbone
4	PacI-HF, KpnI-HF digested, purified PCR (LnSeqA)	PacI-HF, KpnI-HF digested, purified pKL97
5	-	PacI-HF, KpnI-HF digested, purified pKL97

Component	Reaction 4 (uL)	Reaction 5 (uL)	Master Mix (3x)
H ₂ O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6.0
Insert	4.0	-	-
Backbone	2.0	2.0	6.0
Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48

I put all the reactions in the thermocycler at 2:45 pm and let them incubate overnight. I also made more LB-Carb plates for the transformation tomorrow.

Transformation Plates

One colony had grown on the 5X remaining ligation plate over the weekend. I made an overnight culture and left it in the shaking incubator at 3:20 pm. I will miniprep it tomorrow.

Tuesday, June 21, 2022**To Do:**

1. ~~Growth experiment~~
2. ~~Transformation~~
3. ~~Miniprep~~
4. ~~Diagnostic digest~~

Side Project

Today I will re-do the growth experiment. I will grow 8 cultures using different media and different cells and compare their growth:

- 2 cultures of LVS in fresh MHB
- 2 cultures of LVS in conditioned media
- 2 cultures of Δ rpsU2 in fresh MHB
- 2 cultures of Δ rpsU2 in conditioned media
- 1 culture of sterile conditioned media (no cells)

I prepared the cultures by first scraping the cells into their own tubes and diluting 1:20 in a cuvette using fresh MHB.

LVS: OD600 = 0.510 (added 62.8 uL to each 8 mL culture tube)

Δ rpsU2: OD600 = 0.578 (added 55.4 uL to each 8 mL culture tube)

I then checked the starting OD600s of each culture (600 uL) and put them in the incubator at 9:26 am.

I then checked the OD600s every 2 hours for 6 hours total

Left in the shaking incubator to grow overnight starting around 3:42 pm.

Transformation

Today I will transform my overnight ligations using NEB 5-alpha cells again.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR81	1 uL	20 ul	1
2	(-) control	None	0	20 ul	1
3	Backbone Ligation	pKR81 (digested)	8 uL	100 uL, remaining	2
4	3X Ligation	pKR144 (digested)	8 uL	100 uL, remaining	2
5	5X Ligation	pKR144 (digested)	8 uL	100 uL, remaining	2
6	(+) Ligation Control	pKR140 (digested)	8 uL	100 uL, remaining	2
Total number of plates					10

Since I think the plasmid may be toxic to *E. coli*, I decided to leave the plates on the bench to grow at room temperature.

Miniprep / Diagnostic Digest

I will also miniprep the overnight culture of my plasmid and check its concentration using the nanodrop.

pKR144 concentration: 161.5 ng/uL

I then performed a diagnostic digest to determine if the NLuc gene was properly inserted into the plasmid.

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Miniprep pKR144	SalI, PvuII	4	-
2	Water	-	-	4
3	pKR82	SalI, PvuII	4	-

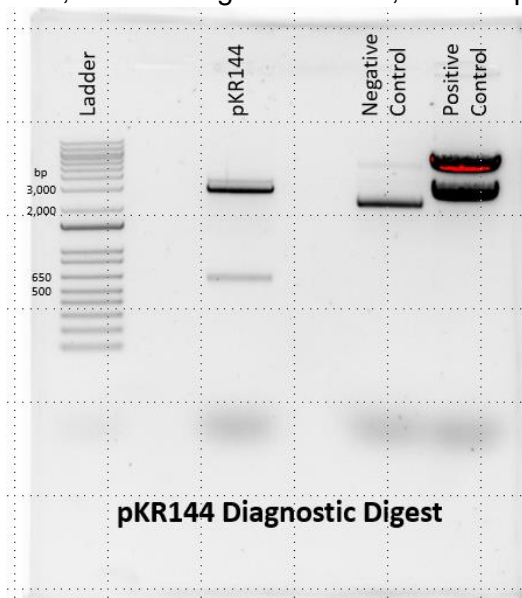
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H ₂ O	13	39
10x Buffer*	2	6
DNA	(4)	-
SalI	0.5	1.5
PvuII	0.5	1.5
Total	20.0 (18.0 actual b/c of DNA)	

I used a master mix for tubes 1 and 3 and added the individual components to tube 2 (no enzymes so added 1 uL of extra water).

Incubated in standing incubator for 1 hour.

Used 4 uL of dye for each 20 uL sample

Lane 1: ladder; Lane 3: pKR144; Lane 5: negative control, Lane 6: positive control



As a negative control I used the pKR144 miniprep with no enzymes added. I would expect the un-digested plasmid to be supercoiled and therefore run faster on the gel, which was observed. For a positive control I used pKR82, which has cut sites for SalI and PvuII. The expected band sizes for both digested pKR82 and pKR144 are below:

pKR82→

Backbone: 2762 bp

Insert: 2560 bp

pKR144→

Backbone: 2762 bp

Insert: 606 bp

The two bands for the positive control appeared slightly higher than expected, however, the top bands of the ladder were pretty close to each other so the gel should be run longer to get a better estimate of their location. There is a band for the insert, which means the NLuc gene is present in the plasmid. Our theory is that the plasmid is toxic to *E. coli*, which means growing the cells at lower temperatures improves their chances of surviving. However, there is still the chance for mutations so I will sequence the miniprep to ensure there are no base substitutions.

Also, I dug the plate where I took the overnight culture from out of the trash, and made a new overnight culture from it. I will leave it overnight in the shaking incubator at room temperature to try and improve my chances of not getting any mutations in the insert.

Put in overnight culture at 4:07 pm.

Sequencing Results

I got my sequencing results back from my original pKR144 miniprep and there was no insert present, which matches what I saw in the diagnostic digest.

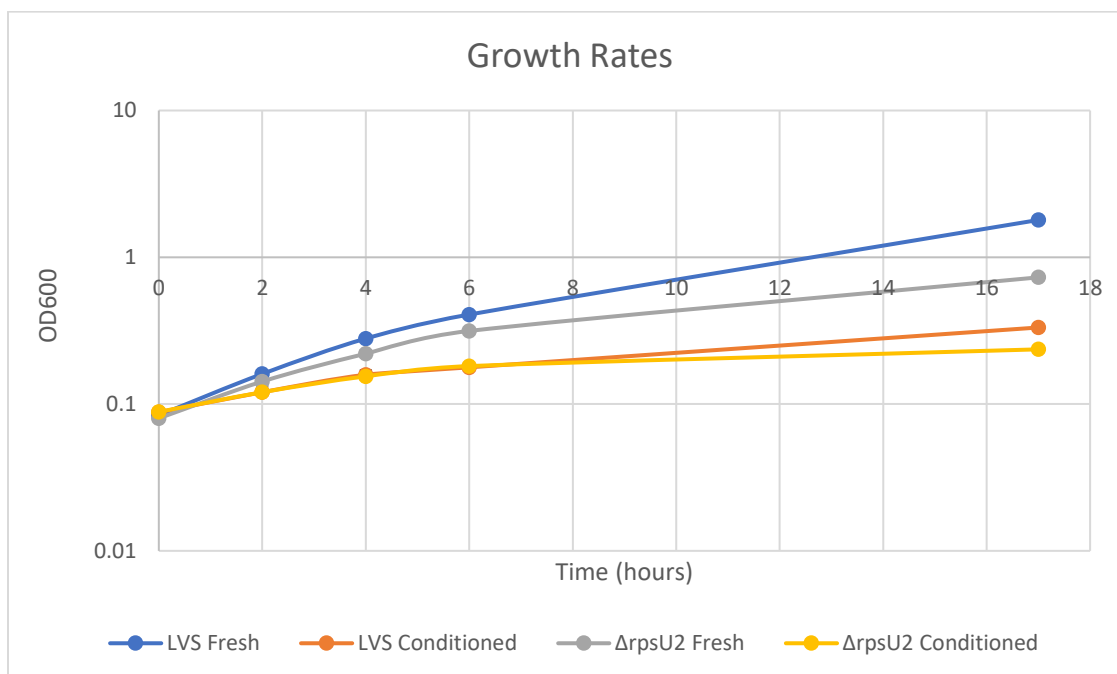
Wednesday, June 22, 2022**To Do:**

1. ~~Analyze side project data~~
2. ~~Miniprep~~
3. ~~Sequence reactions~~

Side Project

I took the growth experiment cultures out of the incubator at 8:37 am and measured their OD600s. I had to dilute all four of the cultures that grew in fresh media 1:4. The average OD600's of the two duplicates for each sample can be seen below:

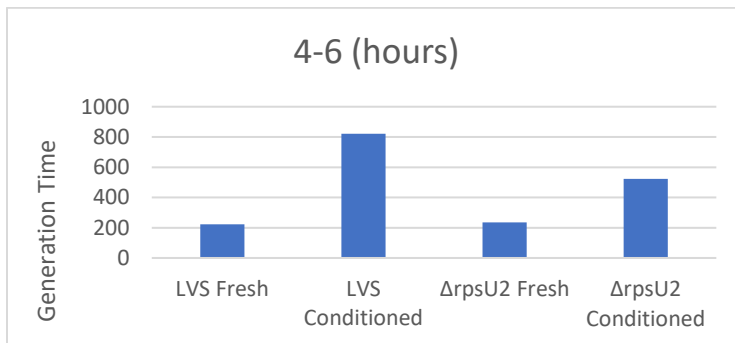
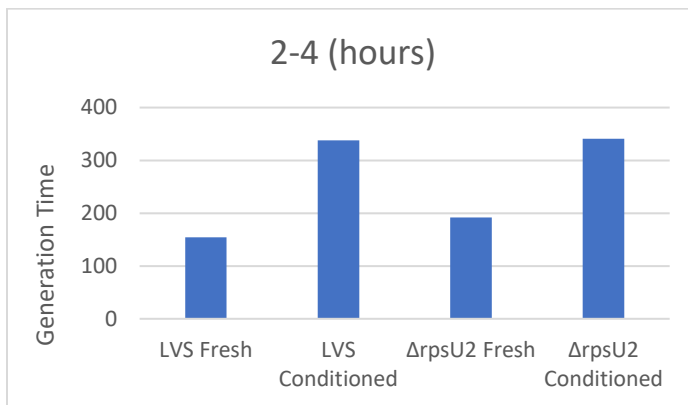
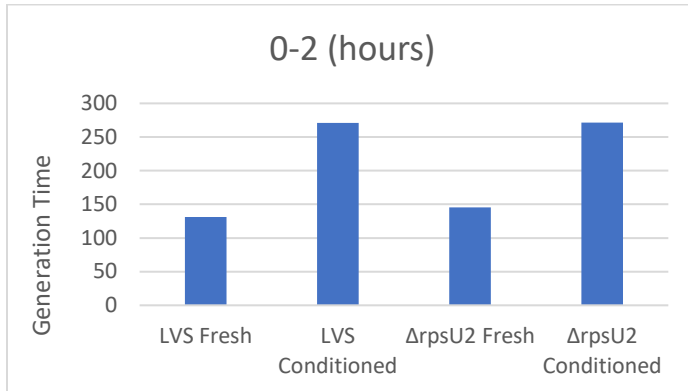
Sample	0 (hours)	2 (hours)	4 (hours)	6 (hours)	17 (hours)
LVS Fresh	0.084	0.1605	0.2795	0.4075	1.796
LVS Conditioned	0.088	0.1205	0.1585	0.178	0.3325
Δ rpsU2 Fresh	0.08	0.1425	0.2205	0.315	0.73
Δ rpsU2 Conditioned	0.0885	0.1205	0.1545	0.1815	0.236



In fresh media, there is a clear growth defect in Δ rpsU2, as seen in the graph by Δ rpsU2's line being below LVS the entire course of the experiment. However, in conditioned media that growth defect is essentially non-existent since in the log phase (0-4 hours) there is no difference between LVS and Δ rpsU2. This can also be seen in the generation times.

Generation Times	0-2 (hours)	2-4 (hours)	4-6 (hours)
LVS Fresh	131.0987546	154.0235688	223.0153148
LVS Conditioned	271.1895492	337.591975	820.3827353

Δ rpsU2 Fresh	145.3063624	191.7151694	234.795012
Δ rpsU2 Conditioned	271.5490397	341.1966109	523.3159576



Overall, the cells grew much better this time around. The hypothesis is that since the conditioned media has a high concentration of siderophores, the Δ rpsU2 cells are not starved for iron and can grow just as well as LVS. I will repeat this experiment again to validate these results.

Miniprep / Sequence Reactions

Today I will miniprep my second overnight culture and determine its concentration using the nanodrop. I took it out of the shaking incubator at 3:18 pm.

pKR144-2 concentration ~ 52.7 ng/uL

I then prepared sequence reactions for both minipreps.

Sample number	Template Type	Template Name	Primer Name ^a	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PLASMID template:	<u>Volume H₂O</u> <u>needed</u>
						Volume =	
						2x(~200 ÷ B)μl	(12 less D or E - 2.56)μl
MC1	Plasmid	pKR144-1	KROL358	3352	161.5	2.48	6.96
MC2	Plasmid	pKR144-1	KROL64	3352	161.5	2.48	6.96
MC3	Plasmid	pKR144-2	KROL358	3352	52.7	7.59	1.85
MC4	Plasmid	pKR144-2	KROL64	3352	52.7	7.59	1.85

Thursday, June 23, 2022

To Do:

1. ~~Make MHB~~
2. ~~Mentee-Mentor Compact~~
3. ~~Project Outline~~

Today I will make MHB and complete other lab tasks. I will also work on some paperwork.

Friday, June 24, 2022

To Do:

- ~~1. Analyze sequence results~~
- ~~2. Transformation~~

Sequence Results

The sequencing results for my two minipreps came back today. The first miniprep (pKR144-1) has no mutations so I will save it in the plasmids box in the freezer. The second miniprep (pKR144-2) was at a much lower concentration so the sequencing reaction didn't go as far. Therefore, we couldn't 100% validate if there were any mutations or not so I will save it in my personal freezer box but not go further with it.

Transformation

Today I will transform pKR144 using NEB 5-alpha cells. I will use the colonies from this transformation to create more minipreps so I have enough volume of plasmid to use during the phenol-chloroform extraction.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR144	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
Total number of plates					4

I left the plates on the bench over the weekend.

Tuesday, June 28, 2022

To Do:

- ~~1. Overnight cultures~~
- ~~2. Streak out LVS~~
- ~~3. Mentor-Mentee compact~~
- ~~4. Project outline~~

Transformation Results

My transformation from Friday looks good with a very high number of colonies on the pKR144 plates and no colonies on the negative control. In the future, I should plate 10 uL instead of 20 uL, or dilute the miniprep 1:5 prior to transforming, since there was such a high transformation efficacy.

I prepared 8 overnight cultures from the 20 uL plate using LB-Carb media. I put them in the Bertin lab incubator which was set to 30°C at 3:40 pm.

Side Project

I streaked out a plate of LVS on CHA no antibiotic which I will use to create more conditioned media for my growth experiment on Thursday.

Wednesday, June 23, 2022

To Do:

- ~~1. Make hemoglobin~~
- ~~2. Overnight cultures~~
- ~~3. Streak out LVS and Δ rpsU2~~
- ~~4. Make single use aliquots~~

Overnight Cultures Redo

My pKR144 overnight cultures from yesterday did not grow at all. I think I forgot to actually hit the on button on the incubator so when I checked the temperature in the morning it was at 22°C. I prepared 8 more overnight cultures from the 20 uL plate using LB-Carb media. I put them in the Rowley lab incubator which was set to 30°C at 12:13 pm.

Side Project

Today I will prepare a 50 mL overnight culture using the LVS plate I streaked out yesterday and fresh MHB.

I diluted the cells 1:20 in the cuvette
OD600 = 0.658

Added 190 uL of the non-diluted cells to the flask – swirled to mix

Starting OD600 = 0.059

I then placed it in the 37°C shaking incubator overnight starting at 4:00 pm.

I also streaked out another LVS plate and a Δ rpsU2 plate on CHA no antibiotic and left them in the standing incubator overnight.

With the LVS plate from yesterday I prepared more single use aliquots and stored them in my freezer box in the -80°C freezer.

Thursday, June 30, 2022

To Do:

- ~~1. Growth experiment~~
- ~~2. Miniprep~~
- ~~3. Phenol Chloroform extract~~

Side Project

I took out the overnight culture of LVS at 9:20 am and diluted it 1:20 in the cuvette.

OD600 = 0.073

I then finished preparing the conditioned media by centrifuging the overnight culture and filter sterilizing the supernatant.

To re-do the growth experiment I will grow 9 cultures using different media and different cells and compare their growth:

- 2 cultures of LVS in fresh MHB
- 2 cultures of LVS in conditioned media
- 2 cultures of $\Delta rpsU2$ in fresh MHB
- 2 cultures of $\Delta rpsU2$ in conditioned media
- 1 culture of sterile conditioned media (no cells)

I prepared the cultures by first scraping the cells into their own tubes and diluting 1:20 in a cuvette using fresh MHB.

LVS: OD600 = 0.629 (added 50.8 uL to each 8 mL culture tube)

$\Delta rpsU2$: OD600 = 0.472 (added 67.8 uL to each 8 mL culture tube)

I then checked the starting OD600s of each culture (600 uL) and put them in the incubator at 10:05 am.

I then checked the OD600s every 2 hours for 6 hours total

Left in the shaking incubator to grow overnight starting around 4:18 pm.

Miniprep

Today I will miniprep the pKR144 overnight cultures I prepared yesterday. I took the overnight cultures out of the incubator at 10:45 am.

I checked their concentrations using the nanodrop:

- 1: 150.8 ng/uL
- 2: 103 ng/uL
- 3: 105 ng/uL
- 4: 159.3 ng/uL
- 5: 118.9 ng/uL
- 6: 126.3 ng/uL
- 7: 136.5 ng/uL
- 8: 126 ng/uL

Since the concentrations were all very similar, I combined all 8 minipreps into one tube. I then phenol:chloroform purified half of it and stored the remainder in my freezer box.

Phenol:Chloroform DNA Purification and Ethanol Precipitation Protocol

Must be performed in a fume hood!

1. Combine equal amounts of DNA (start with 200 uL) with phenol:chloroform:isoamyl ethanol (25:24:1) in a 1.5 mL polypropylene tube
2. Mix the contents of the tube until an emulsion forms (vortex if DNA <10 kb, gentle shaking if DNA 10-30 kb)
3. Centrifuge the mixture at 80% of the maximum speed for 1 min at room temp. If phases are not well separated, centrifuge again for longer.
4. Use a pipette with the tip trimmed to transfer 125 uL of the aqueous phase (the upper phase and not yellow) to a fresh tube.
 - a. For good recovery, back-extract by adding an equal volume (125 uL) of 0.1x EB to the organic phase (yellow) and interface, mix, and the separate by centrifugation again. Combine the second aqueous phase with the first.
5. Add an equal volume of chloroform to the saved aqueous phase (250 uL) and repeat steps 2-4. Take off 175 uL in each round of centrifugation, so there is a total of 350 uL.

Ethanol precipitation based on PacBio Protocol →

1. Add NH₄OAc to a final concentration of 0.75 M (35 uL of 7.5 M stock) and add 1 uL of glycogen (20 ug/uL). Mix solution well.
2. Add 2.5X volume (963 uL) of 100% ethanol and mix well.
3. Spin for 20 minutes in a 4°C centrifuge at top speed.
4. Decant the supernatant carefully without disturbing the pellet.
5. Wash by adding 300 uL of 80% ethanol and vortex 3 times.
6. Spin for 15 minutes in 4°C centrifuge at top speed.
7. Decant supernatant carefully without disturbing the pellet.
8. Repeat 80% ethanol wash (2x total)
9. Quick spin on a tabletop centrifuge to draw residual ethanol to the bottom, remove with P20 pipette without disturbing pellet.
10. Air dry pellet for 1-2 minutes.
11. Resuspend in appropriate volume of 0.1x EB (35 or 50 uL).

Eluted in 35 uL 0.1X EB and stored in freezer box.

I also checked the concentration using the nanodrop: 313 ng/uL