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Introduction

Monday, August 31, 2020

To Do:

1. Observe Hannah complete steps 1 and 2 of the 70S Ribosomes Purification Protocol
2. Observe Hannah complete steps 3 and 4 of the 70S Ribosomes Purification Protocol

Method and Results:

Preparation of 70S Ribosomes for Biochemistry

(Updated from original protocol of J. Thompson and S. Gregory from 3.22.03)

1. Scrape up a plate of *Francisella* into 1.5 mL tube of MHB.
2. Inoculate 500 ml of MHB in 1 liter baffled flasks with correct volume of culture. The initial OD₆₀₀ should be around 0.005. Incubate with shake at 37 °C to an OD₆₀₀ = 0.3-0.4.

Take a 1 mL sample for whole cell proteins. Add equal amounts OD of 1x SLB (i.e. 0.3 OD, add 0.3 mL 1x SLB). Store sample at -20C.
3. Chill the cultures on ice for 20 minutes, then harvest the cells by centrifuging at max speed for 30 minutes, at 4°C if using benchtop centrifuge. Using JA-10 rotor in floor centrifuge, use 8,000 rpm for 5 mins (11,295xg).
4. Resuspend the cell pellet in a total of 100 ml H10M10A1000 (optional). Pellet the cells in centrifuge as above. This wash step is intended to remove any ribonucleases that might be sticking to the cell exterior. Resuspend the cells in 100 ml H10M10A50, pellet again. Resuspend the cell pellet in 30 ml H10M10A50 and pellet the cells in a 50 mL screw-cap tube. If using benchtop, do max speed for 30 mins, if using JA-20 rotor, do 8,000 rpm for 15 mins (7,720xg). Freeze the cell pellet at -80C.
5. Thaw the cell pellet and resuspend it in 2.5 ml H¹⁰M¹⁰A⁵⁰ per gram of cells (recommended: add buffer to increase volume for French Press, so it is at least 13 mL per sample). Add 20 µl of 1 U/ul DNase and pass three times through the French Press set on high until about 800 on the gauge. Collect the lysate in a glass beaker then transfer to a 30 ml Oakridge tube (red caps).
6. Pellet the cell debris in a 50.2 Ti rotor at 30,000 rpm, 15 minutes, 4 °C.
7. Transfer the S30 supernatant avoiding the pellet, to a 15 ml conical tube and add Brij 58 to 0.5 % (1/10 vol of 5 % stock; optional). Incubate on ice for 30 minutes or more.
8. Layer the S30 over a 10 ml sucrose cushion, H¹⁰M¹⁰A⁵⁰⁰ + 20 % sucrose being sure not to let the two layers mix. Centrifuge in a 50.2 Ti rotor at 40,000 rpm, 4 hours, 4 °C. An orange layer of carotenoids and membrane should appear at the interface and a glassy 70S ribosome pellet should be at the bottom of the tube. Rinse the ribosome pellet with 10 mL H¹⁰M¹⁰A⁵⁰ twice, without disturbing the pellet, and resuspend it in 1.0 ml of H¹⁰M¹⁰A⁵⁰. This may take a while. Do not pipet or vortex. Microfuge the suspension for 5 minutes and keep the supernatant.

9. Layer 1 ml of ribosomes onto 15 ml $H^{10}M^{10}A^{50}$ + 40 % sucrose. Centrifuge in a 50.2 Ti rotor at 30,000 rpm, 14 hours, 4 °C.
10. Rinse the pellet with 10 mL $H^{10}M^{10}A^{50}$ twice, without disturbing the pellet. Dissolve the ribosome pellet in 250 μ l or less (minimal volume, start with 150 μ L) of $H^{10}M^{10}A^{50}$. Pellet any remaining debris in the microfuge. Dispense into small aliquots (20 μ l) in small Eppendorf tubes and freeze at -80 °C.

 $H^{10}M^{10}A^{1000}$ (if using)

10 mM Hepes KOH pH7.6	2 ml 1M Hepes KOH pH 7.6
10 mM $MgCl_2$	2 ml 1M $MgCl_2$
1M NH_4Cl	40 ml 5M NH_4Cl
	<u>156 ml H_2O</u>
	200 ml

 $H^{10}M^{10}A^{50}$

10 mM Hepes KOH pH7.6	8 ml 1M Hepes KOH pH 7.6
10 mM $MgCl_2$	8 ml 1M $MgCl_2$
50 mM NH_4Cl	8 ml 5M NH_4Cl
	<u>H_2O to 800 mL</u>
	800 ml

 $H^{10}M^{10}A^{500}$ + 20 % sucrose

10 mM Hepes KOH pH7.6	1 ml 1M Hepes KOH pH 7.6	300 μ L 1 M HEPES
10 mM $MgCl_2$	1 ml 1M $MgCl_2$	300 μ L 1M $MgCl_2$
500 mM NH_4Cl	10 ml 5M NH_4Cl	3 ml 5M NH_4Cl
Sucrose	20 g	6 g
	<u>H_2O to 100 ml</u>	<u>H_2O to 30 ml</u>
	100 ml	30 ml

 $H^{10}M^{10}A^{50}$ + 40 % sucrose

10 mM Hepes KOH pH7.6	2 ml 1M Hepes KOH pH 7.6	400 μ L 1M HEPES
10 mM $MgCl_2$	2 ml 1M $MgCl_2$	400 μ L 1M $MgCl_2$
50 mM NH_4Cl	2 ml 5M NH_4Cl	400 μ L 5M NH_4Cl
Sucrose	80 g	16 g
	<u>H_2O to 200 ml</u>	<u>H_2O to 40 ml</u>
	200 ml	40 ml

Stock Solutions

Brij 58 stock	5 % (w/v) Brij 58 in $H^{10}M^{10}A^{50}$
1M $MgCl_2$	203.3 g/mol = 20.33 g/ 100 ml
1M Hepes pH 7.6	238.3 g/mol = 23.83 g/ 100 ml = 47.66 g/ 200 ml pH with 2.14 g KOH/ 100 ml = 4.28 g/ 200 ml
5M NH_4Cl	53.489 g/mol = 8.02 g / 30 mL for 5M

Make all stock solutions using good H_2O and good, clean glassware. Autoclave buffers before adding sucrose. Use disposable plastic pipets, not the glass ones.

The baffled flasks went into the incubator at 1615 on 8/31 to shake for 16 hours.

September 2020

Tuesday, September 1, 2020

To Do:

- ~~1. Observe Hannah complete steps 1 and 2 of the 70S Ribosomes Purification Protocol~~
2. Observe Hannah complete steps 3 and 4 of the 70S Ribosomes Purification Protocol

Methods and Results:

The baffled flasks were taken out of the incubator at 0845 (9/1) and the OD was checked. The OD was not high enough so the flasks were incubated for another 30 minutes. The second reading was 0.302

The cultures were chilled and the floor centrifuge was used for all spins. The pellets were frozen at -80°C. The protocol will be continued in a couple of weeks. On Friday I will begin the Glycerol Stocks Protocol.

Friday, September 4, 2020

To Do:

- ~~1. Observe Hannah complete steps 1 and 2 of the 70S Ribosomes Purification Protocol~~
- ~~2. Observe Hannah complete steps 3 and 4 of the 70S Ribosomes Purification Protocol~~
- ~~3. Perform Glycerol Stocks and Single-Use Aliquots Protocol~~
4. Perform DNA Digest

Methods and Results:

Glycerol Stocks

Making Glycerol Stocks Protocol

1. Make 3 cryovials for each strain (permanent stocks), label!
2. Prepare 2.4mL of MHB in a 50mL conical (adjust if you are also making single use stocks)
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 600ul of 75% glycerol to the 2.4mL mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. For single use stocks follow the same protocol but pipet 50ul of solution to tubes

The plates were scraped and added to 3 mL MHB each. 750 uL glycerol were added to each tube, the tubes were vortexed and given a quick spin, then 50 uL were aliquoted into 5 cryotubes, and 5 uL were aliquoted to 5 centrifuge tubes were single use. Six extra single use tubes of LVS and Δ rpsU2 were aliquoted at Hannah's request for her own use. The aliquots were stored at -80°C.

Note: Purity plates were set up on some of the tubes to ensure I used proper aseptic technique.

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	Ex: enzyme1,enzyme2	15	-
2	Backbone	Ex: enzyme1,enzyme2	5	10

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 (___ uL) and 10x buffer (___ uL) to master mix tube (MM).
4. 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).
5. Add indicated amount of each enzyme (___ 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 (___ uL).
8. Incubate at 37°C for 1 hour or up to overnight.
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)

Originally we were going to use Eco53kl site but Hannah noticed on SnapGene that there were 2 Eco53kl sites. Instead we used SmaI.

Specifically for today's protocol:

Tube	DNA	Enzyme(s)	DNA Volume (uL)
1	pKR7	NotI, EcoRV	2
2	pKR33	NotI, SmaI	2

1. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H ₂ O	23.8	32.4
10x Buffer*	3.0	9
DNA	(2.0)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (28.0 actual b/c of DNA)	45.0

Digest pKR7 pF-rpsU2-V with NotI / EcoRV

76 bp = DNA specifying VSV-G tag)

7,390 bp = plasmid backbone

Digest pKR33 pEX-rpoC-TAP with NotI / Eco53kl

682 bp = TAP tag

7,142 bp = plasmid backbone

Gel-purify

Ligate together purified pF backbone with TAP tag

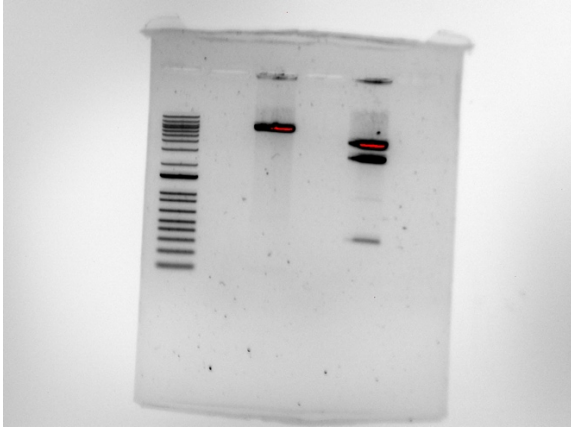
For SmaI substitute:

Notes:

1. Digest in CutSmart Buffer (or NEBuffer 4 + BSA) at 25 °C with SmaI, then add NotI-HF and raise temperature to 37 °C.

The 75 bp band did not show on the gel:

Ladder pKR7 pKR33



plasmid backbone

Furthermore, the TAP tag is showing up at around 400 bp rather than 700 bp. Kathryn thinks it may be because the SmaI is acting up. She suggests maybe sequential digests.

Tuesday, September 8, 2020

To Do:

1. Continue 70S Ribosome Purification Protocol
2. Order primers for PCR for the TAP-tag purification

Methods and Results:

See Protocol from pg 2. Continuing from Step 5, I observed Hannah perform the Ribosome Purification Protocol. The cell pellets were thawed and resuspended in 2.5 ml $H^{10}M^{10}A^{50}$. We brought the samples over to the Camberg Lab to use their French Press. 20 μ L of 1 U/ μ L DNase were added to the samples and they were passed through the French Press 3 times. The lysate was collected in a glass beaker and transferred to Oakridge Tubes. The samples were spun on the high speed centrifuge in the INBRE lab at 30,000 rpm for 15 minutes. The supernatant was transferred to a 15 mL conical tube and Brij 58 was added. The samples were incubated on ice for 30 minutes. The samples were then layered over $H^{10}M^{10}A^{500}$ + 20 % sucrose and spun on the high speed centrifuge for 4 hours at 40,000 rpm. The pellets were twice rinsed with 10 mL $H^{10}M^{10}A^{50}$, and resuspended in 1 mL $H^{10}M^{10}A^{50}$. The suspension was microfuged for 5 minutes and then layered onto 15 ml $H^{10}M^{10}A^{50}$ + 40 % sucrose.

Hannah and I went over SnapGene software to see how I would determine what primers were needed. Kathryn ordered the primers for me.

Wednesday, September 9 2020

To Do:

1. ~~Continue 70S Ribosome Purification Protocol~~
2. ~~Order primers for PCR~~
3. Observe Hannah perform PCR
4. Autoclave

Methods and Results:

PCR Protocol (updated 7/1/19)

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/primestar buffer
 - dNTPs
 - oligo F (10uM)
 - oligo R (10uM)
 - template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
 - The file is located in the Protocols folder
 - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi 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:
 - Add ddi H₂O
 - Add dNTPs
 - Add KOD buffer
 - Add KOD enzyme
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

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	rpsU1	LVS gDNA	P742 and P743	1165
2	+ control	LVS gDNA	KROL15, KROL16	441
3	- control	-	KROL15, KROL16	-

Today Hannah did a PCR with 100 uL with only one sample and the negative control

After 3 attempt, the autoclave cycle was complete

Thursday, September 10, 2020

To Do:

1. ~~Order primers for PCR~~

- ~~2. Observe Hannah perform PCR~~
3. Pour LB-Kan plates
4. Read over Ligation and Transformation Protocols
5. Observe Hannah perform Transformation Protocol
6. Look over TAP Purification Protocol to prepare for rotation talk

Methods and Results:

Ligations

Protocol by John Church

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

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

Tube	Insert	Backbone
1	BamHI, KpnI digested, purified PCR	BamHI, KpnI digested, purified pKL80
2	-	BamHI, KpnI digested, purified pKL80

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)
H ₂ O	11.5	15.5
10x ligase buffer	2.0	2.0
Insert	4.0	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

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 in order to avoid degradation.
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 (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
6. Add indicated amount of ligase (___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. Place in the thermocycler overnight at 16°C.

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 kanamycin-containing plates	Number of carbenicillin-containing plates
1	(+) control	pKL02	1 uL	20 uL, 100 uL	2	
2	(-) control	None	0	20 uL, 100 uL	2	
3	Backbone Ligation	pEX (digested)	8 uL	100 uL, remaining	2	
4	Ligation 1	pKR2	8 uL	100 uL, remaining	2	
5	(+) control	pUC19	1 uL	20 uL, 100 uL		2
6	(-) control	None	0	20 uL, 100 uL		2
7	Backbone Ligation	pEX-Carb (digested)	8 uL	100 uL, remaining		2
8	Ligation 2	pKR4	8 uL	100 uL, remaining		2
Total number of plates					8	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)
- 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.
- 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-Kan), 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.

Pour LB Plates:

500 mL of LB was melted and stirred and then cooled down in the water bath. 500 uL kanamycin were added to the LB agar and the flask was stirred again. The plates were poured, marked with blue sharpie and left on the bench to solidify. Final yield was 23 plates because I was probably a little heavy on my pour.

Transformation:

I observed Hannah do the Transformation protocol on a ligation. Because I had class and didn't get into the lab until 11, I only was able to observe beginning from Step 8. Since Hannah was performing this on a ligation, she used 8 uL of her sample. The samples were placed on the 42°C heat block for 30 seconds, then put on ice while 1 mL LB was added to microfuge tubes. The tubes were then fixed to the 37°C shaking incubator and left for an hour. After shaking, the tubes were moved to a tube rack to plate. Beads were added to the plates. Each control was inoculated to 2 plates: 20 uL and 100 uL respectively, and ligations were plated 100 uL and remaining volume respectively. For the remaining, the tubes were spun down at max speed for about a minute and then 800 uL of the supernatant were removed. The cells were resuspended with a 200 uL pipette and inoculated on the plate. The beads were removed and the plates were incubated at 37°C.

Friday, September 11, 2020

To Do:

1. ~~Pour LB Kan plates~~
2. Make MHB
3. Make LB agar

Methods and Results:

Mueller-Hinton broth protocol- by Jamie Wandzilak

For 500 mL of MHB

1. Weigh out 10.5g of Mueller-Hinton broth into 1L square-bottle
2. Add 500mL of ddiH₂O (type I)
3. Autoclave on 30' liquid cycle, filling the water bin up
4. Cool down to 37 °C or cooler
5. Can keep this sterile media indefinitely without supplements
6. Add 5mL of 10% glucose
7. Add 5mL of 2.5% iron pyrophosphate
8. Add isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)
9. Can keep this supplemented media for 2 weeks, storing at 4 °C

Reagents

Mueller-Hinton Broth media dry

10% Glucose (filter-sterilized)

2.5% Iron pyrophosphate (1.25g iron pyrophosphate in 50mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks (filter-sterilized)

Isovitalex (commercially available)

Making LB-agar plates

Protocol written by KMR

Preparing LB-agar

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

If pouring plates later:

Let LB-agar solidify and keep in lab. When you want to pour plates, heat up LB-agar on hotplate, stirring, until entirely melted (**watch carefully** so it doesn't boil over! Don't walk away). Let agar cool down or place in warm over (50°C) until ready to pour plates.

If pouring plates the same day:

After autoclaved, keep in warm oven (50°C) until ready to pour plates

Pouring plates

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
Carbenicillin	100 mg/mL	100 µg/mL

Antibiotic	Stock Concentration	Working Concentration (Concentration in plates)
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.
- 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.

The MHB was stored without supplements, the LB agar will be poured at a later date. Since my primers have not arrived yet, my project will have to continue next week.

Monday, September 14, 2020

To Do:

- ~~Make MHB~~
- ~~Make LB agar~~
- Test PCR plasmid construction and run gel
- Larger volume PCR for pKR72
- PCR purify and digest overnight

Methods and Results:

Primers arrived at around 11 today

Both the TAP and the sequencing primers arrived. They were reconstituted by being dry spun in the bench top centrifuge at top speed for 3 minutes, then the 0.1 EX buffer was added in the following volumes to make 100 µMol solution:

Primer	Concentration	Buffer added
KROL333	11.9 nMol	119 µL
KROL334	31.2 nMol	312 µL
KROL335	13.4 nMol	134 µL
KROL336	19.2 nMol	192 µL

The tubes were then put on the 42°C hot plate for 5 minutes. For the TAP primers, 90 uL of EX buffer were added to 10 uL primer to make a 10 nMol working concentration. For the sequencing primers, 07.5 uL of EX buffer were added to 2.5 uL primer to make a 2.5 nMol working concentration.

PCR test run:

ç	Plasmid/Region	Source DNA	Primers	Length (bps)
1	TAP tag	pKR33	KROL333, KROL334	570
2	+ control	LVS gDNA	KROL15, KROL16	441
3	- control	-	KROL15, KROL16	-

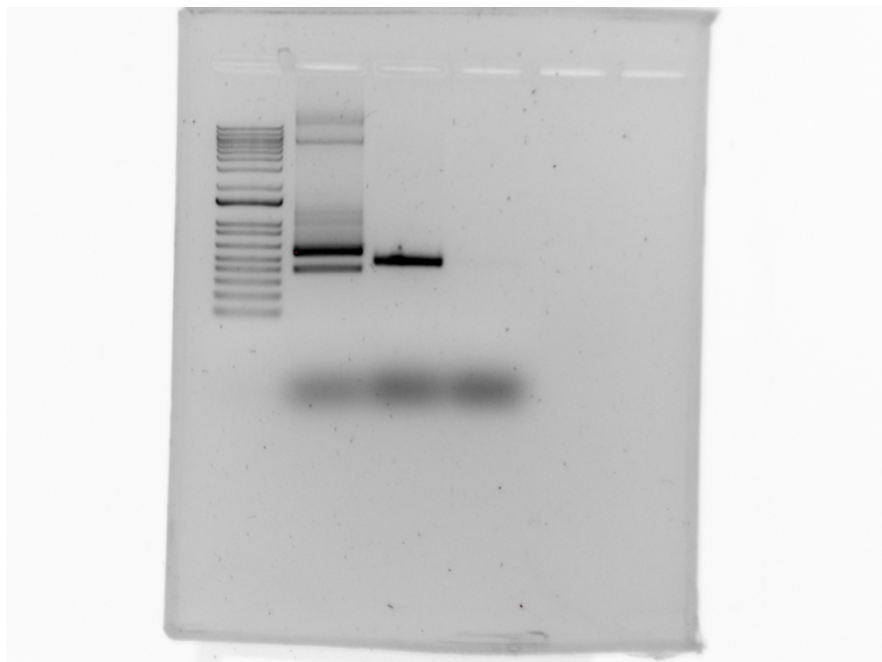
Only TAP tag is being amplified, backbone pKR7 digest with NotI and BamHI

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	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	1.6
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
Total volume			20	80

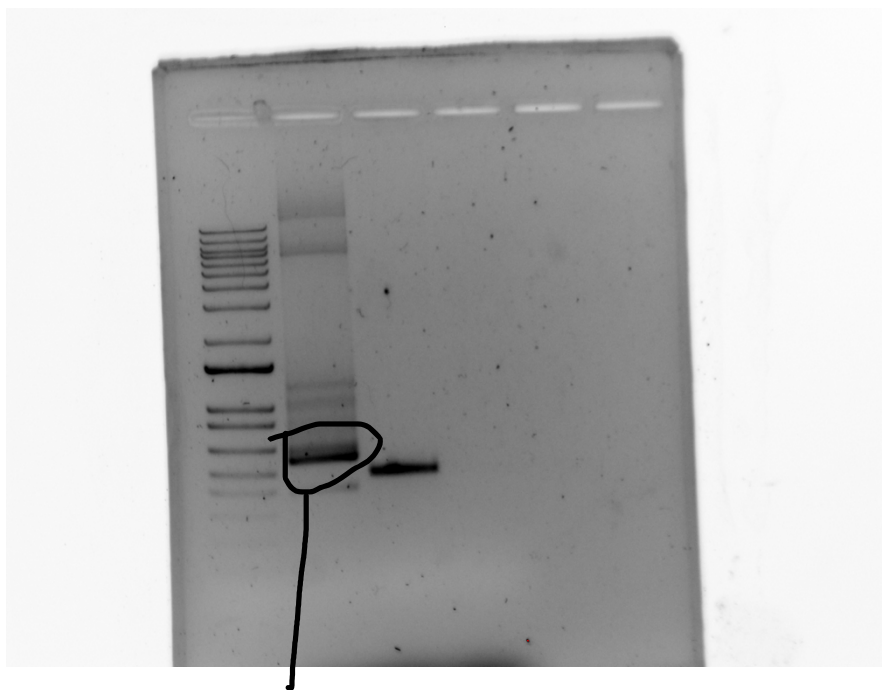
The volumes of plasmids and DNA noted on the table above were added to the PCR tubes. In a 1.5mL centrifuge tube the master mix was made, with the enzyme added last, and pipetted to mix. The PCR tubes were quickly spun to bring the material to the bottom of the tube and 18.4 uL of master mix was added to each tube.

Ladder pKR33 LVSgDNA Neg Control



The first picture image isn't very clear and there appears to be an extra band. I thought the brighter band was the TAP, but because the bands were close together I ran the gel a little longer to get a clearer image:

Ladder pKR33. LVS gDNA Neg control



This image is much better. The test PCR worked. I proceeded to set up the larger volume PCR

	Plasmid/Region	Source DNA	Primers	Length (bps)
1	TAP tag	pKR33	KROL333, KROL334	570
2	- control	-	KROL333, KROL334	-

Total reaction volume	100
Total number of reactions	2

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	2.5
ddiH2O			62	155
PrimeSTAR GXL Buffer	5x	1x	20	50
dNTPs	2.5 mM	0.2 mM	8	20
oligo F	10 uM	0.3 uM	3	7.5
oligo R	10 uM	0.3 uM	3	7.5
template	100 ng/ul	2 ng/ul	2	5
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	5
Total volume			100	250

Because I did not have to set up a positive control for this run, I used the same primers for both the negative control and the TAP. I set up the master mix by a factor of 2.5 to ensure I would have enough product. I put the PCR tubes on the Thermocycler on the STN-1 setting for the night.

Tuesday, September 15, 2020

To Do:

1. ~~Test PCR plasmid construction and run gel~~
2. ~~Larger volume PCR for pKR72~~
3. PCR purify and digest
4. Gel purify
5. Overnight ligation

The tubes were taken out of the thermocycler and the PCR was purified using the QIAquick PCR Purification Kit. I set up the digest:

Digest Table:

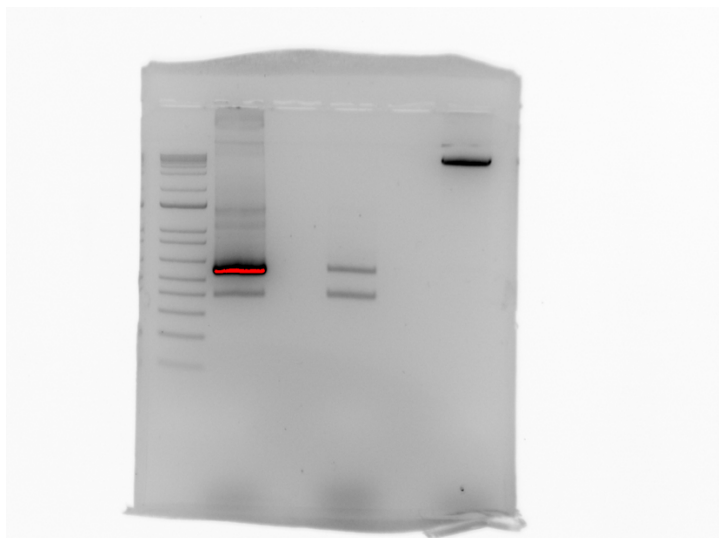
Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	NotI, BamHI	15	-
2	pKR7	NotI, BamHI	5	10

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)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

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).
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.
7. 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)

The gel was run (Hannah loaded the gel for me while I was in class):

Neg Control. Pure PCR pKR7



The negative control is contaminated. I spoke to Kathryn about this; this could have resulted from perhaps the water being contaminated in the negative control or else the pipette tip wasn't changed in between pipetting master-mix in the TAP tube and the negative tube. I threw my aliquot of water away and got a new one. It was determined that the experiment could continue. I cut out the TAP and the pKR7 from the gel.

I set up the gel purification, but the melting of the gel during gel purification was interrupted by a lab meeting, however Kathryn says that the gel will melt at room temperature. It turns out I didn't initially add enough buffer; after adding the appropriate amount of buffer, the gels melted. I continued with the gel purification and after the elution step I stored them in the -20 to continue to the ligation tomorrow.

Wednesday, September 16, 2020

To Do:

1. ~~PCR purify and digest~~
2. ~~Gel purify~~
3. Ligation
4. Transformation
5. Make CHA-kan plates

Ligation:

Tube	Insert	Backbone
1	TAP 3X	pKR7
2	TAP 5X	pKR7
3	-	pKR7

4	TAP	-
---	-----	---

Master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)
H ₂ O	11.5	15.5
10x ligase buffer	2.0	2.0
Insert	4.0	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

Instead of the overnight ligation I did a ten minute room temp ligation this morning. Before I began the ligation I checked the DNA concentration of the insert and backbone using the NanoDrop.

The concentrations were 4.2 and 6.2 respectively, so we didn't think I had enough to continue with the ligation, but I put it in the ligation calculator that Hannah made for me and it turned out I had enough.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	Tap	RI-INBRE	9/16/2020 10:04:59 AM	4.2	ng/μl	0.085	0.015	5.8	0.01	DNA	50
2	Backbone pKR7	RI-INBRE	9/16/2020 10:07:10 AM	6.2	ng/μl	0.124	0.036	3.4	0.01	DNA	50

Ligation Calculator

Ligation Calculator				
ng vector	ratio of insert/bb bps	molar ratio	ng of insert	
50	0.074932615	3	11.23989218	
50	0.074932615	5	18.73315364	
	concentration	uL needed		
backbone	6.2	8.06		
insert (3x)	4.2	2.68		
insert (5x)	4.2	4.46		
	3X	5X	No Insert	No backbone
Water	6.76	4.98	9.44	14.82
Ligation Buffer	2	2	2	2
Backbone	8.06	8.06	8.06	0
Insert	2.68	4.46	0	2.68
Ligase	0.5	0.5	0.5	0.5

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.
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 (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
4. Add indicated amount of ligase (___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. Place in the thermocycler overnight at 16°C.

I have 4 ligation reactions: 3X, 5X, no insert, and no backbone. I added the appropriate DNA to the reaction tubes with the water, and for the “master mix” I added 10 uL of ligation buffer and 2.5 uL of the ligase. I added 2.5 uL of the master mix to the reaction tubes and pipetted to mix. Rather than put them on the thermocycler, I let them sit at room temperature for ten minutes before proceeding to the transformation.

Transformation:

Transform chemically competent *E. coli* cells

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	3X	Ligation 1	8 uL	100 uL, remaining	2	
2	5X	Ligation 2	8 uL	100 uL, remaining	2	
3	No insert	Ligation 3	8 uL	100 uL, remaining	2	
4	No backbone	Ligation 4	8 uL	100 uL, remaining	2	
5	(+) control	pKR68	1 uL	20 uL, 100 uL	2	
6	(-) control	None	0	20 uL, 100 uL	2	
Total number of plates					12	

- 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.
- 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)
- 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.
- 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-Kan), 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.

The transformation proceeded as written in the protocol above. I set up 6 tubes: one positive control, one negative control, and each ligation sample. I used beads to spread the inoculum on the plates, and after removing the beads I left the plates in the 37°C incubator to sit overnight.

CHA-kan plates:

Cystine heart agar with hemoglobin plates protocol- by Jamie Wandzilak

For 600 mL of CHA

1. out 30.6g of cystine heart agar into 1L flask (non-baffled; 10.2g/100mL)
2. Add 300mL 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 6g freeze-dried hemoglobin to 300mL 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 24mL of CHAH mixture into each 100mm plate (should make approximately 25 plates) Try to avoid bubbles!

Reagents

Cystine heart agar
2% hemoglobin
100mm plates

Modifications

Plates with kanamycin

For 5 ug/ml, add 60 ul of 50 mg/mL kanamycin to 600 mL of media

Plates with X-gal

For 100 ug/mL X-gal, add 1.2 mL of 50 mg/mL X-gal (dissolved in DMF, stored protected from light at -20°C). Keep plates out of light.

Hannah actually made the medium and the hemoglobin because she said she was making regular CHA for John and it was easier to make both, so I only did Steps 11 and 12. I aseptically added the hemoglobin to the CHA, stirred it, aseptically added 60 uL of kanamycin, stirred it, and aliquoted 24 mL to each plate, avoiding bubbles. The yield was 24 plates.

Thursday, September 17, 2020

To Do:

1. — Ligation
2. — Transformation

3. ~~Make CHA-kan plates~~
4. Repeat digest
5. Gel purification
6. Overnight ligation

There were colonies on the positive plates, and no colonies on the negative control plates. There was only one colony on the #3 Remaining plate and no other plates had growth. This indicates that the transformation worked but the ligation did not. After meeting with Kathryn and Hannah to diagnose what could have gone wrong, it was suggested that adding the ligase to the buffer directly may have been a bad idea. I will repeat the ligation and transformation, but first I have to set up a new digest on the backbone because I don't have enough material leftover.

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR7	NotI, BamHI	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)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

Since I was only preparing one tube, there was no need to set up a master mix separately; everything was prepared in the same tube. The tube was then incubated for 33 minutes while the gel was set up. After the gel was poured, 0.5uL of CIP were added to the sample tube and the tube was incubated for an additional ten minutes.

I set up the gel but I realized that I never put the comb in the gel after I poured it. I tried to use the comb to make wells after the fact but it didn't work; the sample ran across the top of the gel. I had enough agar to start over so I did so. This next time the sample incubated for 26 minutes while the gel was prepared, then additional 10 after the CIP was added.

Ladder

pKR7



The gel looks good; onto purification. The nanodrop says the DNA concentration is 8.0

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	pKR7	RI-INBRE	9/17/2020 5:04:48 PM	8	ng/ μl	0.15	0.06	2.48	0.01	DNA	50

Ligation:

Tube	Insert	Backbone
1	TAP 3X	pKR7
2	-	pKR7
3	TAP	-
4	HT insert	HT backbone
5	-	HT backbone

Ligation Calculator

ng vector	ratio of insert/bb bps	molar ratio	ng of insert	
50	0.074932615	3	11.23989218	
50	0.074932615	5	18.73315364	

	concentration	uL needed		
backbone	8	6.25		
insert (3x)	4.2	2.68		
	3X	5X	No Insert	No backbone
Water	8.57	4.98	11.25	14.82
Ligation Buffer	2	2	2	2
Backbone	6.25	8.06	6.25	0
Insert	2.68	4.46	0	2.68
Ligase	0.5	0.5	0.5	0.5

Master Mix Table

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.
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 (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
4. Add indicated amount of ligase (___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. Place in the thermocycler overnight at 16°C.

Friday, September 18, 2020

To Do:

1. ~~Repeat digest~~
2. ~~Gel purification~~
3. ~~Overnight ligation~~
4. Transformation

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	3X	Ligation 1	8 uL	100 uL, remaining	2	
2	No insert	Ligation 2	8 uL	100 uL, remaining	2	
3	No backbone	Ligation 3	8 uL	100 uL, remaining	2	
4	HT's insert	Ligation 4	8 uL	100 uL, remaining	2	
5	HT's backbone	Ligation 5	8uL	100 uL, remaining	2	
6	(+) control	pKR54	1 uL	20 ul, 100 ul		2
7	(-) control	None	0	20 ul, 100 ul		2
Total number of plates						6

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.
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)
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.

6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), 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.

This morning I took the ligation tubes off the thermocycler and set up the transformations. After I plated them I put them in the 30°C incubator. I will come in on Sunday to check the plates and if they are good I will set up overnight cultures.

Sunday, September 20, 2020

To Do:

1. ~~Transformation~~
2. Set up overnight cultures
3. Streak out LVS from frozen

Transformation Results:

Reaction	Contents	20 uL plate	100 uL plate	Remaining plate
1	3X	-	1 colonies	5 colonies
2	Backbone Only	-	2 colonies	3 colonies
3	Insert Only	-	0 colonies	0 colonies
4	Hannah's Insert + Backbone	-	1 colony	19 colonies
5	Hannah's Backbone Only	-	0 colonies	1 colony
6	Positive Control	0 colonies	0 colonies.	-
7	Negative Control	0 colonies	0 colonies.	-

For some reason the positive control didn't work but the transformation did! And so did the ligation as demonstrated in the comparison between Reactions 4 and 5.

I set up overnight cultures with the 6 colonies from Reaction 1. I aseptically added 40 mL of LB broth to a sterile 50 mL conical tube. I aseptically added 40 uL of Kan to the LB. I set up 7 sterile glass tubes. The first 6 were tubes contained 5mL medium with one colony in each. The seventh tube was set up with medium only as a sterility check.

The tubes went in the shaker at 6:30.

I also streaked out LVS from one of my single-use aliquots and put it in the 37°C incubator.

Monday, September 21, 2020

To Do:

1. ~~Set up overnight cultures~~
2. ~~Streak out LVS from frozen~~
3. Miniprep
4. Diagnostic Digest
5. Prepare electrocompetent (EC) cells
6. Set up overnight cultures

The overnight cultures look good, although Tube 1 is not quite as turbid as the others. Tube 7 remained not turbid indicating the sterility check passed.

MiniPrep:

I emptied 2 mL of supernatant of each sample at a time after centrifugation. Once there was a pellet I resuspended it with Buffer P1, added Buffer P2, and washed with PE 3 times. I eluted the samples with 50 mL of 0.1X EB.

Diagnostic Digest:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1-6	TAP tag	BamH1, Not1	2	-

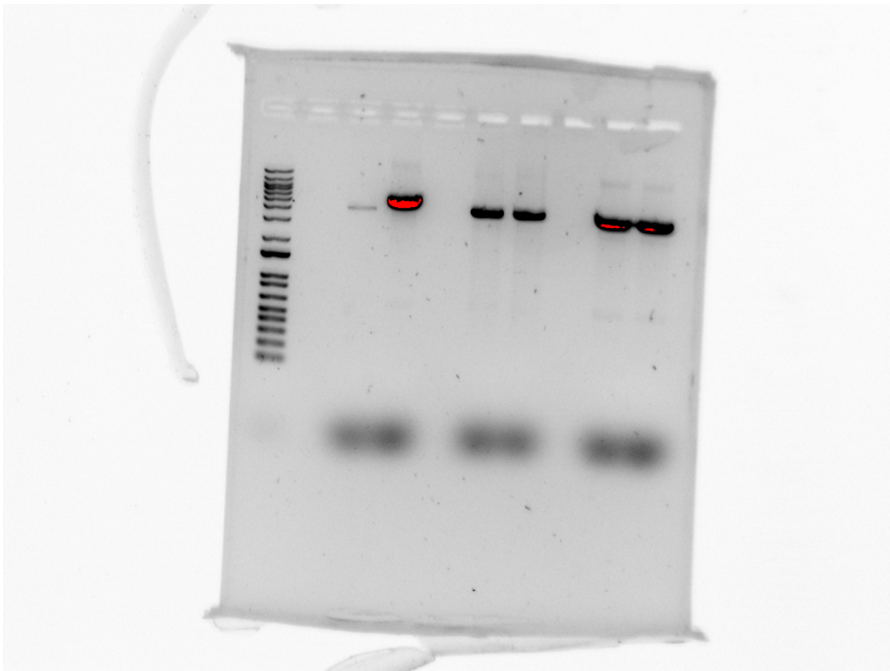
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 7x (uL)
H ₂ O	15.0	105.0
10x Buffer*	2.0	14.0
DNA	(2.0)	-
Enzyme 1	0.5	3.5
Enzyme 2	0.5	3.5
Total	20.0 (18.0 actual b/c of DNA)	

Since this is only a diagnostic digest, there is no need to add CIP.

I set up a master-mix tube and inoculated each sample tube with 2 uL of DNA. I added 18 uL of master-mix to each tube, and incubated the tubes at 37°C while my gel was melting.

Once the gel was set, I put 4 uL of loading dye into each sample tube. I put 10 uL of ladder in the Well 1, skipped Well 2, put samples 1 and 2 in Wells 3 and 4 respectively, skipped Well 5, put samples 3 and 4 in Wells 6 and 7 respectively, skipped Well 8, and put samples 5 and 6 into Wells 9 and 10 respectively.

TAP tag



Looks like there is no insert in any of my samples. The faded band in Well 3 corresponds to the low turbidity relative to the other tubes in Tube 1.

Spoke to Kathryn, I will set up an overnight Ligation today with the 5X, my backbone only, and Hannah's 2 controls that I used the other day with the 3X ligation.

Prepare EC Protocol:Day 1:

-Patch entire plate with recipient LVS strain on CHAH

Day 2:*Prepare electrocompetent (EC) cells*

-Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)

-Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL

-Spin for 3 minutes at 10,000 rpm

-remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose

-Repeat 3x-5x in 10% sucrose

-After final spin, remove all supernatant.

-Resuspend cells in 10% sucrose at high density (corresponding to $\sim 1 \times 10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.

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

I yielded 3 tubes of 110uL and 1 tube of 55 uL. They are in the -80°C .

Ligation:

Tube	Insert	Backbone
1	TAP 5X	pKR7
2	-	pKR7
3	-	HT backbone
4	HT insert	HT backbone

	5X (Reaction 1)	Backbone Only (Reaction 2)
Water	9.47	9.44
Ligation Buffer	2.0	2.0
Backbone	3.57	8.06
Insert	4.46	0
Ligase	0.5	0.5

Master Mix Table (Only for Hannah's samples)

Component	Reaction 3 (uL)	Reaction 4 (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
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Ligation was set up and incubated in thermocycler overnight.

Tuesday, September 22, 2020

To Do:

1. ~~Miniprep~~
2. ~~Diagnostic Digest~~
3. ~~Prepare electrocompetent (EC) cells~~
4. ~~Set up overnight ligation~~
5. Sequencing reaction
6. Transformation
7. Electroporate cells

Sample number	Well	Template Type	Template Name	Primer Name ^a	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H ₂ O needed
							$(A \div 100) \times 2.5$	$(C \div B) \mu\text{l}$	$2 \times (\sim 200 \div B) \mu\text{l}$	$(12 \text{ less D or E} - 2.56) \mu\text{l}$
KB1		PCR	TAP	KROL333	570	3.08	14.25	4.63		4.81
KB2		PCR	TAP	KROL334	570	3.08	14.25	4.63		4.81
a. Add 2.56 μl of 2.5 μM stock to each reaction										
3130xl Plate Record		Date	9/22/20	Name	Kira Bernabe					
PI	Kathryn Ramsey	Dept	CMB	Email	htrautmann@uri.edu	PO No.	0000143904			

The concentration of my PCR product was 30.8 ug/uL. According to the table above the PCR template volume was <.8 uL (0.46 uL), so I made a 1:10 dilution of the PCR product. That brought the PCR concentration to 3.08 ug/uL, and the PCR template volume to 4.63 uL. I made a 2.5 uM dilution of each primer. I added the primers to the respective tubes of template and water and brought the tubes over to GSC.

I only had 11 plates for my transformation and I needed 12, so I melted the LB agar and added 500 uL Kanamycin to 500 mL of the agar, then poured the plates. Due to some spilling and over pouring, the yield is only 14 plates.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	5X	TAP	8 uL	100 uL, remaining	2	
2	Backbone Only	pKR7	8 uL	100 uL, remaining	2	
3	Hannah's Backbone Only		8 uL	100 uL, remaining	2	
4	Hannah's Insert		8 uL	100 uL, remaining	2	
5	(+) control	pKR7	1 uL	20 ul, 100 ul	2	
6	(-) control	None	0	20 ul, 100 ul	2	
Total number of plates					12	

Since pK54 didn't work last time I used pKR7 for the positive control. Unfortunately, at the very last step I forgot to resuspend the pellet and only inoculated the supernatant. I realized this before I removed the beads and so attempted to resuspend with what little fluid was left and added it to the plates. I hope it worked.

Wednesday, September 23, 2020

To Do:

1. ~~Sequencing reaction~~
2. ~~Transformation~~
3. Repeat PCR run
4. PCR purification
5. Overnight digest

Transformation Results:

Reaction	Contents	20 uL plate	100 uL plate	Remaining plate
1	5X	-	0 colonies	0 colonies
2	Backbone Only	-	0 colonies	0 colonies
3	Hannah's Backbone Only	-	15 colonies	200 colonies
4	Hannah's Insert + Backbone	-	0 colonies	0 colonies
5	Positive Control	50 colonies	TNTC	-
6	Negative Control	0 colonies	0 colonies.	-

Not good. Looks like transformation worked and ligation worked, but not on my plates. I received my sequencing results and went over them with Kathryn. Both primers have insertions, but specifically pKR334 has a very large insertion towards the beginning. Kathryn and I went over my notebook and it is possible that way in the beginning tubes were mixed up and that's why nothing has worked as expected. So I will start from the beginning with a PCR run on pKR33:

ϕ	Plasmid/Region	Source DNA	Primers	Length (bps
1	TAP tag	pKR33	KROL333, KROL334	570
2	- control	-	KROL333, KROL334	-

Total reaction volume	100
Total number of reactions	2

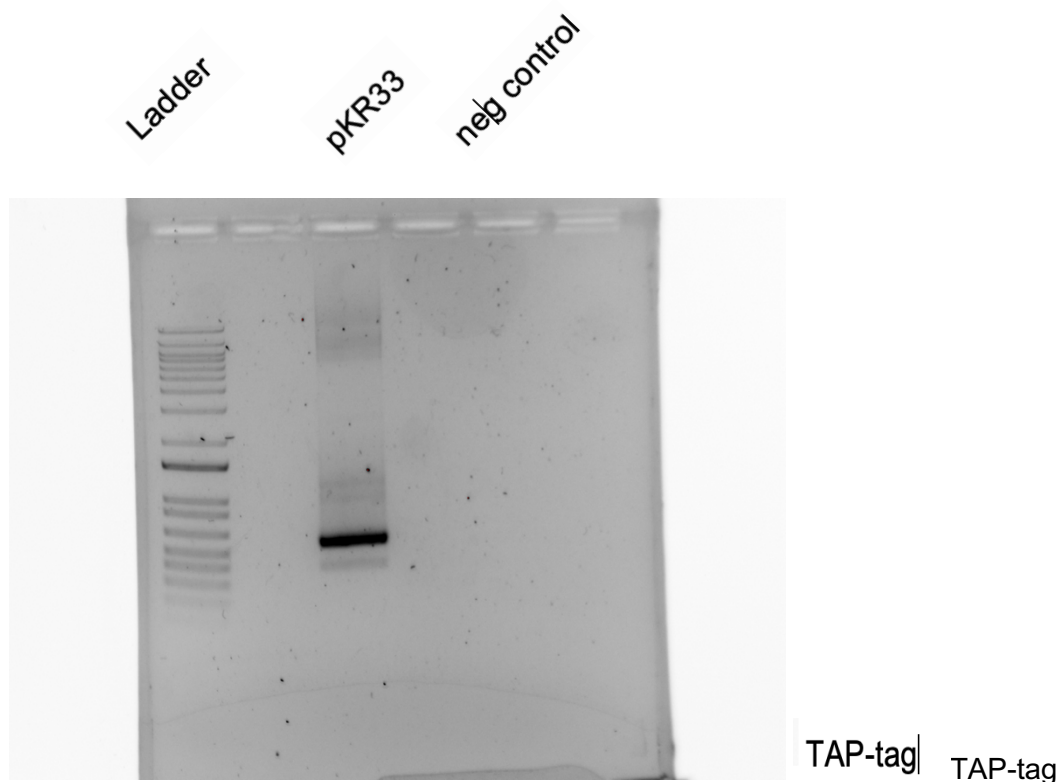
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	2.5
ddiH2O			62	155
PrimeSTAR GXL Buffer	5x	1x	20	50
dNTPs	2.5 mM	0.2 mM	8	20
oligo F	10 uM	0.3 uM	3	7.5
oligo R	10 uM	0.3 uM	3	7.5
template	100 ng/ul	2 ng/ul	2	5
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	5
Total volume			100	250

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/primestar buffer
 - dNTPs
 - oligo F (10uM)
 - oligo R (10uM)
 - template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
 - The file is located in the Protocols folder
 - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi 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:
 - Add ddi H₂O
 - Add dNTPs
 - Add KOD buffer
 - Add KOD enzyme
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

I set up a 100 uL volume PCR run with the pKR33 and a negative control.

Next I ran the PCR products on a gel to make sure they are the target products:



Looks good! Nothing in the negative control and the TAP has a nice band. There's still that second band underneath though.

I immediately threw away the negative control tube. The PCR product was purified using the QIAquick PCR Purification Kit. I eluted it with 35 uL 0.1X EB. Then I set up an overnight digest:

Digest Table:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	Purified PCR	NotI, BamHI	15	-
2	pKR7	NotI, BamHI	5	10

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.

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).
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.
7. 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)

Thursday, September 24, 2020

To Do:

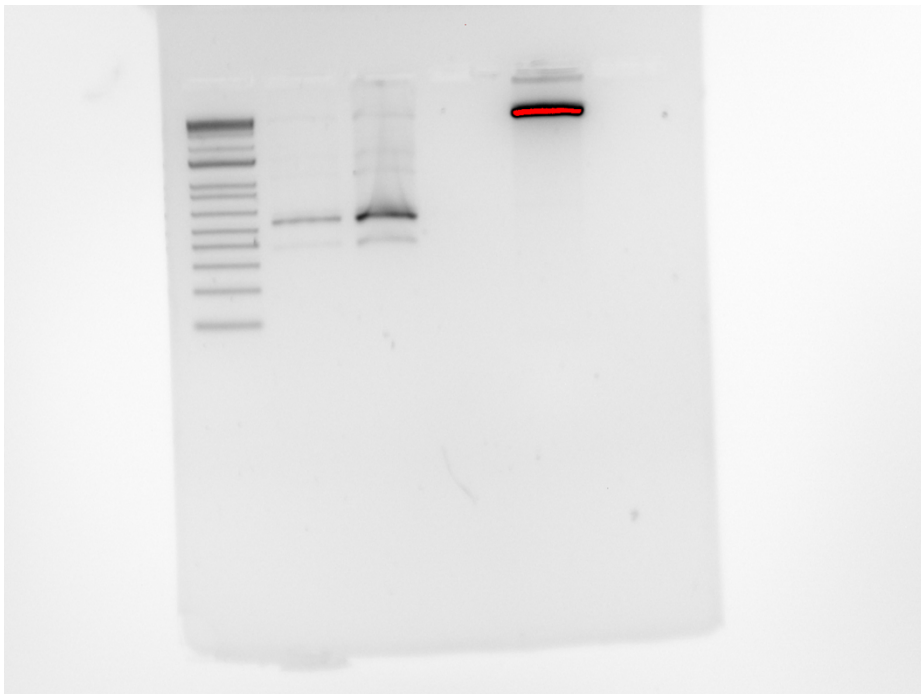
1. ~~Repeat PCR run~~
2. ~~PCR purification~~
3. ~~Overnight digest~~
4. Gel purification
5. Benchtop ligation
6. Transformation
7. Make LB plates

Digest:

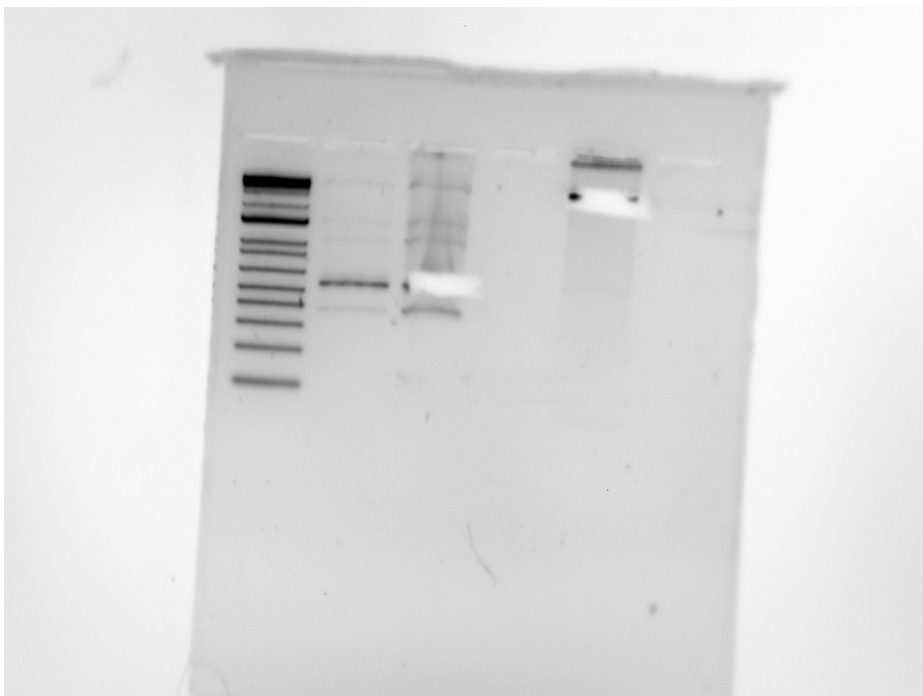
After overnight incubation I added 0.5uL CIP to pKR7 and incubated it for ten more minutes, then I loaded the gel. I used 2% agarose at Hannah's suggestion since that other band is close to the TAP. When I loaded the samples the insert spread out a little in the agarose, I'm concerned that I didn't have the pipette in deep enough. I hope I am able to see good bands.

While I'm in class Hannah ran the gel for me and cut it.

Insert Backbone



Because I didn't get all of the insert into the well, some of it ran in Lane 2, but I think it's ok.



Hannah made the cuts.

I began the gel purification. The insert weighed 230mg and the backbone weighed 280mg. I added 3X volume buffer BG to each tube (690uL and 840uL respectively). I eluted with 35uL 0.1X EB.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
1	insert	RI-INBRE	9/24/2020 12:07:22 PM	9.1	ng/ μl	0.18	0.11	1.65	0.02	DNA
2	backbone	RI-INBRE	9/24/2020 12:08:39 PM	27	ng/ μl	0.54	0.237	2.28	0.05	DNA

These are better concentrations, although the insert is low because not all of it got in the well of the gel.

Ligation Calculator

ng vector	ratio of insert/bb bps	molar ratio	ng of insert	
50	0.074932615	3	11.23989218	
50	0.074932615	5	18.73315364	

Reactions 1-4

	concentration	uL needed		
backbone	27	1.85		
insert (3x)	9.1	1.24		
insert (5x)	9.1	2.06		
	3X	5X	No Insert	No backbone
Water	6.76	4.98	9.44	14.82
Ligation Buffer	2	2	2	2
Backbone	8.06	8.06	8.06	0
Insert	2.68	4.46	0	2.68
Ligase	0.5	0.5	0.5	0.5

Tube	Insert	Backbone
1	TAP 3X	pKR7
2	TAP 5X	pKR7
3	-	pKR7
4	TAP	-
5	Hannah's Insert	Hannah's Backbone

Reaction 5:

Component	Reaction 1 (uL)
H ₂ O	11.5
10x ligase buffer	2.0
Insert	4.0
Backbone	2.0
Ligase	0.5
TOTAL	20.0

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	3X	Ligation 1	8 uL	100 uL, remaining	2	

2	5X	Ligation 2	8 uL	100 uL, remaining	2	
3	No insert	Ligation 3	8 uL	100 uL, remaining	2	
4	No backbone	Ligation 4	8 uL	100 uL, remaining	2	
5	Hannah's	Ligation 5	8 uL	100 uL, remaining	2	
6	(+) control	pKR7	1 uL	20 ul, 100 ul	2	
7	(-) control	None	0	20 ul, 100 ul	2	
Total number of plates					14	

1. 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.
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-Kan), 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.

During the ligation I poured some more LB w/Kan plates, and I hope they had enough time to solidify when I used them an hour and a half later for the transformation.

Friday, September 25, 2020

To Do:

1. ~~Gel purification~~
2. ~~Benchtop ligation~~
3. ~~Transformation~~
4. ~~Make LB plates~~
5. Make LB agar
6. Make CHA
7. Set up overnight cultures

The transformation worked as expected!

Transformation Results:

Reaction.	Contents	20 uL plate	100 uL plate	Remaining plate
1	3X	-	13 colonies	200 ± colonies
2	5X	-	11 colonies	125 ± colonies
3	Insert Only	-	1 colony	7 colonies
4	Backbone Only	-	0 colonies	0 colonies
5	Hannah's Insert & Backbone	-	28 colonies	240 ± colonies
6	Positive Control	6 colonies	TNTC	-
7	Negative Control	0 colonies	0 colonies.	-

I made 2 500mL batches of LB agar and 1 600mL batch of CHA with hemoglobin and kanamycin.

Overnight Cultures Protocol

By John Church

1. Add 45 mL of LB broth to a sterile 50 mL conical (makes enough for 8 tubes: $9 \times 5 = 45$)
2. Add 45 uL of kanamycin (Kan50) to the conical.
3. Label 4 glass sterile test tubes for each new plasmid and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB + Kan media into each of the tubes.
5. Pick 4 colonies from each reaction and inoculate the tube.
 - Do this by using a stick and picking an isolated colony from plate
6. Place in the 37° shaking incubator overnight. ~16-22 hours

I set up 6 tubes; 1-3 from 3X and 4-6 from 5X, plus a sterility check. I also set up 2 tubes of pKR7 to make more stock.

Saturday, September 26, 2020

To Do:

- ~~1. Make LB agar~~
- ~~2. Make CHA~~
- ~~3. Set up overnight cultures~~
4. Miniprep
5. Diagnostic Digest
6. Electroporation

Miniprep:

All of the tubes looked turbid (except sterility check). I proceeded with the Miniprep by pipetting the samples, resuspending the pellets in Buffer P1, adding Buffer P2 and the Buffer NB. I centrifuged, added Buffer PB and centrifuged again, and then washed with PE 3 times. I eluted the samples with 50 uL of 0.1EB.

Diagnostic Digest:

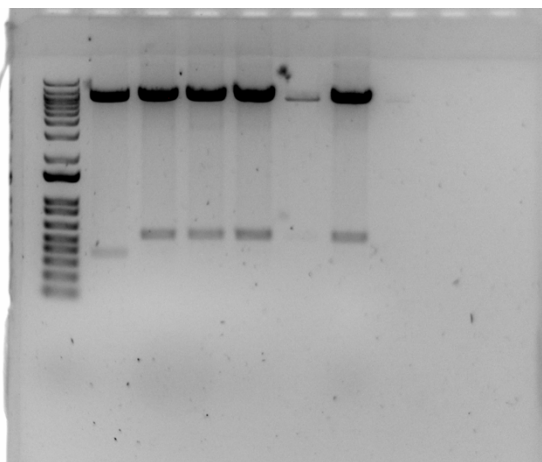
Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1-6	TAP tag	BamH1, Not1	2	-

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 7x (uL)
H ₂ O	15.0	105.0
10x Buffer*	2.0	14.0
DNA	(2.0)	-
Enzyme 1	0.5	3.5
Enzyme 2	0.5	3.5
Total	20.0 (18.0 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.

There is no need to perform the CIP step since this is a diagnostic digest.

When I loaded the gel Sample 5 didn't get all the way into the well so therefore I don't think it will run correctly (I should go slower).



I am not going to use Sample 1 because that doesn't contain the TAP tag, and I'm not going to use Sample 5 because the gel didn't run properly.

Electroporation

Reaction #	Volume DNA	Plasmid	Strain	Time (s)
1	3 uL	pF	LVS	3.8
2	3 uL	pF rpsU2-TAP-2	LVS	Arc
3	3 uL	pF rpsU2-TAP-3	LVS	2.30 + Arc
4	3 uL	pF rpsU2-TAP-4	LVS	1.50 + Pop
5	3 uL	pF rpsU2-TAP-6	LVS	1.20 + Arc
6	3 uL	No plasmid	LVS	0.70

*Electroporate plasmid into EC cells**

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

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

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

50 µL electrocompetent cells

-Have recovery media ready

-Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω

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

-Recover cells for 4-8 hours, shaking at 37°C

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

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

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

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

There are some slight deviations from this method since I am working with replicating plasmids rather than integrating plasmids. For example, I used only 3 uL of DNA rather than 5, and the incubation time was 3 hours rather than 4-8. Lastly, I inoculated one plate with 20 uL of sample and one plate of 100 uL of sample per reaction.

When I set up the EP in the cuvettes, Reaction 3 was not completely thawed and I wasn't sure if I got any sample in the tube. Kathryn advised adding 2 more uL, which I did, but when I took the recovery tube of the shaking incubator 3 hours later, I noticed that that tube was not as turbid as the others.

The actual electroporation didn't give good time for any of the samples. Most of them had arcs, although the display didn't say that.

Worth noting also that when I plated the reactions, I noticed some contamination on some of the CHA plates. 2 plates each had large single colonies towards the edge of the plate. I discarded those, and checked the rest to make sure there was no other growth. The rest looked fine.

Monday, September 28, 2020

To Do:

1. ~~Miniprep~~
2. ~~Diagnostic Digest~~
3. ~~Electroporation~~
4. Sequencing Reaction
5. Make medium for large cultures
6. Make buffers for TAP purification

I checked my EP plates, there is some growth on Rxn #3 20uL and 100 uL, and Rxn #4 100 uL. I am reincubating them until tomorrow for better growth.

Sequencing Reactions:

Sample number	Well	Template Type	Template Name	Primer Name ^a	A.	B.	C.	D.	E.	F.	G.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Diluted 1:2	Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H ₂ O needed
								(A ÷ 100) × 2.5	(C ÷ B)μl	2x(~200 ÷ B)μl	(12 less D or E - 2.56)μl
KB1		Plasmid	pKR72 (tube 1)	KROL44	8000	251.4	502.8			1.59	7.85
KB2		Plasmid	pKR72 (tube 1)	KROL335	8000	251.4	502.8			1.59	7.85
KB3		Plasmid	pKR72 (tube 2)	KROL44	8000	278.7	557.4			1.44	8.00
KB4		Plasmid	pKR72 (tube 2)	KROL335	8000	278.7	557.4			1.44	8.00
KB5		Plasmid	pKR72 (tube 3)	KROL44	8000	336.15	672.3			1.19	8.25
KB6		Plasmid	pKR72 (tube 3)	KROL335	8000	336.15	672.3			1.19	8.25
KB7		Plasmid	pKR72 (tube 4)	KROL44	8000	301.45	602.9			1.33	8.11
KB8		Plasmid	pKR72 (tube 4)	KROL335	8000	301.45	602.9			1.33	8.11
KB9		Plasmid	pKR72 (tube 5)	KROL44	8000	263.25	526.5			1.52	7.92
KB10		Plasmid	pKR72 (tube 5)	KROL335	8000	263.25	526.5			1.52	7.92
KB11		Plasmid	pKR7 (tube 6)	KROL44	7500	294.9	589.80			1.36	8.08
KB12		Plasmid	pKR7 (tube 6)	KROL335	7500	294.9	589.80			1.36	8.08
KB13		Plasmid	pKR7 (tube 6)	KROL336	7500	294.9	589.80			1.36	8.08
KB14		Plasmid	pKR7 (tube 7)	KROL44	7500	308.05	616.10			1.30	8.14
KB15		Plasmid	pKR7 (tube 7)	KROL335	7500	308.05	616.10			1.30	8.14
KB16		Plasmid	pKR7 (tube 7)	KROL336	7500	308.05	616.10			1.30	8.14
a. Add 2.56 μl of 2.5 μM stock to each reaction											
3130xl Plate Record		Date	9/28/20	Name	Kira Bernabe						
PI	Kathryn Ramsey	Dept	CMB	Email	kbernabe@uri.edu		PO No.	0000143904			

I made 16 reactions. Each of my Miniprep samples had two preparations: one tube with KROL44 and the other tube with KROL335. I did not use Miniprep 1 since it didn't have the TAP-tag. For the pKR7, I made 3 preparations: in addition to KROL44 and KROL335 I also made a tube of each with KROL336.

Because the concentrations of the samples were $<0.8\text{ng/uL}$, I made a 1:2 dilution on each sample (column B.).

I made 3x400mL of MHB plus 300mL leftover.

I did not make buffers, will make tomorrow

Tuesday, September 29, 2020

To Do:

1. Miniprep
2. Diagnostic Digest
3. Electroporation
4. Sequencing Reaction
5. Make medium for large cultures
6. Make buffers for TAP purification
7. Plate LVS *rpoC*-TAP
8. Patch pF and pF *rpsU2*-TAP from electroporation

Kathryn and I discussed it and I might not actually do the TAP purification this week since my last day of rotation is this Friday and my schedule is very tight.

In the meantime, I plated 50uL of LVS *rpoC*-TAP to a CHA plate.

Electroporation colony count:

Reaction	Plasmid	20 uL	100 uL
1	pF	~35	~75
2	pF <i>rpsU2</i> -TAP-2	NG	NG
3	pF <i>rpsU2</i> -TAP-3	~20	~100
4	pF <i>rpsU2</i> -TAP-4	~200	TNTC
5	pF <i>rpsU2</i> -TAP-6	~150	~300
6	No plasmid	NG	NG

The most efficient reaction is #4.

Wednesday, September 30, 2020

To Do:

1. ~~Make medium for large cultures~~
2. ~~Plate LVS *rpoC*-TAP~~
3. ~~Patch pF and pF *rpsU2*-TAP from electroporation~~
4. Make large cultures (3x400mL)

My sequencing results came back and they look a lot better than the previous results. I set up the large cultures for LVS pF, LVS pF-rpsU2-TAP, and LVS rpoC-TAP. My patches looked good for all, although I only had one plate of pF-rpoC. I put 3 mL each of MHB in 3 sterile 50 mL conical tubes. I scraped the plates and then checked the OD. Initially I diluted all three samples 1:20 by adding 50 uL of sample to 950 uL of MHB. The OD were as follows:

Tube	OD
1	0.547
2	0.317
3	0.667

Because the OD on the 3rd sample was <0.6 I diluted that to 1:40 by removing 500uL of the sample and adding 500 uL of MHB. The resulting OD was 0.274

Multiplied by the factor

$$0.547 \times 20 = 10.94$$

$$0.317 \times 20 = 6.34$$

$$0.274 \times 40 = 10.96$$

To determine the initial volume, I multiplied the final concentration (0.08 OD) by the final volume (400 mL) and divided that by the initial concentration:

$$(0.08)(400000\text{uL})/10.94 = 2925\text{uL}$$

$$(0.08)(400000\text{uL})/6.34 = 5047\text{uL}$$

$$(0.08)(400000\text{uL})/10.96 = 2919\text{uL}$$

These initial volumes are too high.

Next I tried again with 1:40 dilutions by adding 25uL of sample to 975uL of MHB:

Sample	Diluted OD	Multiply by factor	Actual OD	Initial Volume (uL)
1	0.316	X40	12.64	2500
2	0.242	X40	9.68	3305
3	0.338	X40	13.52	2366

These aren't good either, and then I had to get to class. While I was in class, Kathryn and Hannah discussed it and it was decided that I would set up overnight cultures rather than over a few hours. That meant that the final concentration would be 0.002 instead of 0.08. I also needed

to scrape up new plates since the other cells were sitting in very concentrated not truly aerobic conditions. I am concerned that the single plate I have for pF-rpoC isn't sufficient.

After scraping the plates I diluted 1:40 again:

Sample	Diluted OD	Multiply by factor	Actual OD	Initial Volume (uL)
1	0.254	X40	10.16	78
2	0.202	X40	8.08	99
3	0.107	X40	4.28	187

In order to check the OD at time 0, I made sterile 1:10 dilutions of my samples in 1.5mL tubes by adding 100uL of my samples each to 900uL MHB, then took 100uL of that solution and added it to 400 uL of MHB and checked the OD:

Sample	Diluted OD	Multiply by factor	Actual OD	Initial Volume (uL)
1	0.177	X5	0.89	89
2	0.146	X5	0.73	109.6
3	0.068	X5	0.34	235.3

I then added the volumes to the respective baffled flasks and set in the shaking incubator at 37°C overnight.

Thursday, October 1, 2020

To Do:

1. Make large cultures (3x400mL)
2. Check sequencing results for pKR7

When I came in this morning (8:40 am), I immediately checked the OF on my cultures:

Tube	OD
1	0.047
2	0.053
3	0.092

Pretty low. I put them back in the incubator and checked them again after class, around 11 am.

Tube	OD
1	0.065
2	0.083
3	0.155

Still low, but better. It looks like the 3rd sample will be ready before the others. Reincubated for another couple of hours. Just in case I patched all 3 cultures again in case it needs to be repeated tomorrow.

During the downtime while waiting for the incubation, I checked the sequencing results of pKR7. Everything lined up, so I combined my minipreps into the working plasmid tube in the -20.

I also checked the sequencing results of my samples with TAP-tag. All 5 samples lined up except there was one deletion at the very beginning of KB3, which came from Miniprep 2. I did not include that when I combined the Minipreps to freeze.

Next read @ 1 pm:

Tube	OD
1	0.098
2	0.128
3	0.232

It was decided that no matter what my OD was after my class, I was going to pellet the cells and freeze them. After I got out of class at 3, I checked the OD one final time:

Tube	OD
1	0.153
2	0.209
3	0.346*

*a dilution of 1:2 was made to achieve this

I poured the cultures into centrifuge bottles and weighed them to be sure that everything was balanced:

Bottle 1 (KB-1): 472.45g

Bottle 2 (KB-2): 474.51g

Bottle 3 (KB-3): 472.42g

Bottle 4 (H₂O): 474.49g

In the centrifuge I lined up Bottle 1 opposite Bottle 3, and Bottle 2 opposite Bottle 4. Using the INBRE high speed centrifuge with the JA-10 rotor, I spun the samples for at 6000 RMPs for 5 minutes at 22°C. When I took them out there was a pellet but it looked like they could be spun more, so I set it for ten minutes. However, the centrifuge started shaking and stopped spinning. I checked to make sure everything was aligned correctly and that the top was screwed on tight and tried again, but the same thing happened. It was decided that the pellets I had were enough so I unloaded the centrifuge and realized that the Bottle 4 had lost a lot of volume. It was determined that the o-ring on the cap was missing. Fortunately it was water and not culture that spilled. I cleaned up the rotor and the centrifuge, then went back to the lab to pour off the cultures and resuspend the pellets in 10 mL each of PBS. I transferred them to 50 mL conical tubes, weighed the tubes, and spun them again in INBRE this time using the JA-20 rotor at 6000 RMPs for 8 minutes at 22°C. I poured off the supernatant and stored them in the -80°C.

Bibliography

Ramsey, K. M. and Dove, S. L. (2016) ' A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor ', *Molecular Microbiology*. doi: 10.1111/mmi.13418.