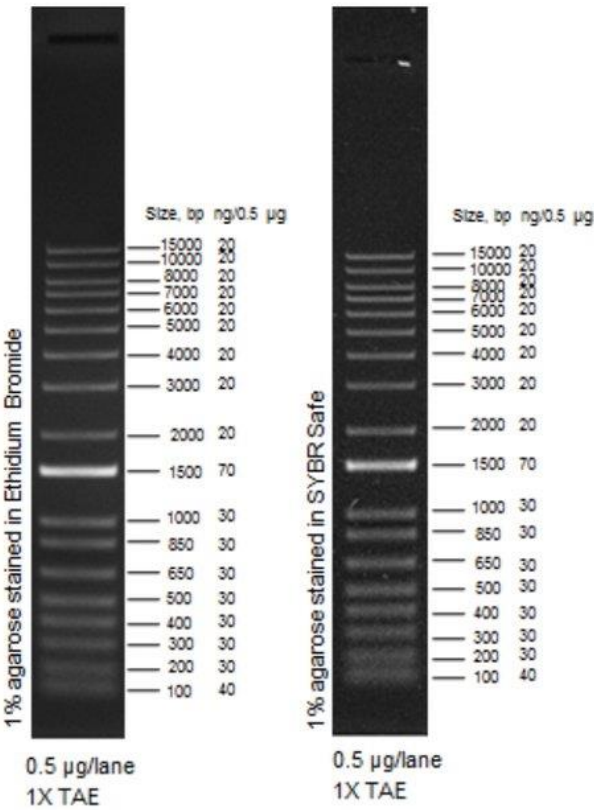


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DNA Ladder used throughout this notebook:



September 2021

Wednesday, September 22, 2021

To Do:

- ~~1. Make MHB~~
- ~~2. PCR Larger Volume~~

Today Hannah and I are doing the PCR that Dan did yesterday on a larger volume scale, plus two more reactions. It worked yesterday, so hopefully it works today! The purpose of the PCR is to amplify the desired inserts, because we start with them in the primers (?) and the PCR adds them to the template DNA.

PCR Protocol New

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

Tube	Product	Template	Primers	Expected size
1	rpsU2 promoter, 5' UTR, and 6 aa	LVS gDNA	KROL472 KROL473	297
2	rpsU2 promoter, with tul4 5' UTR and 6 aa	gBlock PrpsU2_tul4UTR	KROL472 KROL350	252
3	tul4 promoter, with rpsU2 5' UTR and 6 aa	gBlock Ptul4_rpsU2UTR	KROL326 KROL473	271
4	- control	-	KROL472 KROL473	0

Finished at the thermocycler step. On Friday, I will continue onto the next step of DNA purification, and then start digestion.

Reagents

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			62	5
PrimeSTAR GXL Buffer	5x	1x	20	100
dNTPs	2.5 mM	0.2 mM	8	40
oligo F	10 uM	0.3 uM	3	
oligo R	10 uM	0.3 uM	3	
template	100 ng/ul	2 ng/ul	2	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	10
		Total volume	100	460

An oligo is synthetic DNA that is a primer.

Friday, September 24, 2021

To Do:

- ~~1. DNA Purification~~
2. Start Digestion

Only had time to do the DNA Purification. The purpose of the DNA purification is sorta in the name. It purifies the DNA from any remaining contaminants to ensure the best quality of DNA can be used for the remaining part of the protocols.

Monday, September 27, 2021

To Do:

1. ~~DNA Digest~~

The purpose of the digest is to use enzymes to give the inserts and the backbones “stick” ends to do the next step by cleaving specific sequences.

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 1 (rpsU2 promoter, 5' UTR, and 6 aa)	NotI-HF/KpnI-HF	15	-
2	Backbone (pKR70)	NotI-HF/KpnI-HF	5	10
3	Purified PCR 2 (rpsU2 promoter, with tul4 5' UTR and 6 aa)	NotI-HF/KpnI-HF	15	-
4	Purified PCR 3 (tul4 promoter, with rpsU2 5' UTR and 6 aa)	NotI-HF/KpnI-HF	15	-
5	Backbone (pKR89)	NotI-HF/KpnI-HF	5	10

Expected size for Tubes 1,3,4 -250-300 bps

Expected size for Tube 2 -7772 bp fragment and 237 bp fragment; keep larger fragment

Expected size for Tube 5 - should yield 6250 bp fragment and 207 bp fragment; keep larger fragment

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	10.8	64.8
10x Buffer*	3	18
DNA	(15)	
Enzyme 1	0.6	3.6
Enzyme 2	0.6	3.6
Total	30.0 (15.0 actual b/c of DNA)	90

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

3. Add indicated amounts of H₂O and 10x buffer to master mix tube (MM).
4. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
5. Add indicated amount of each enzyme (___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).

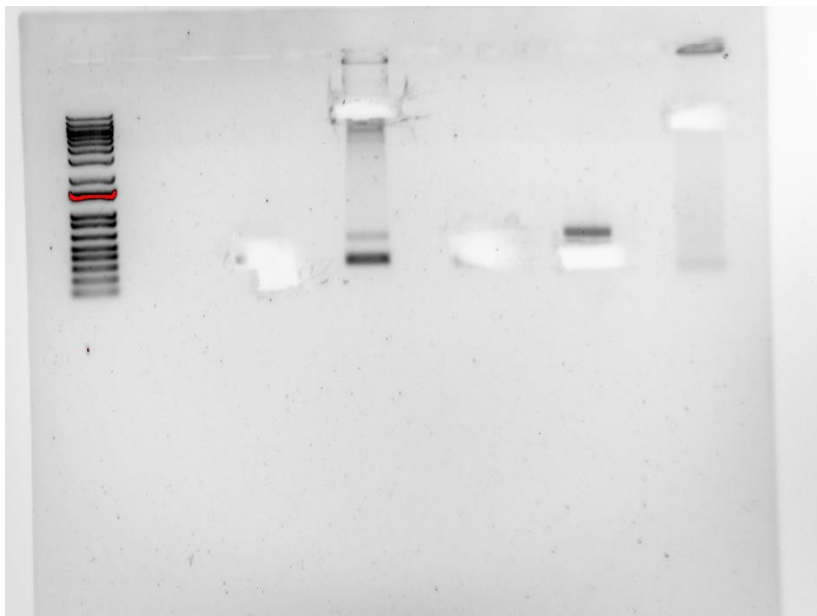
8. Incubate at 37°C for 1 hour or up to overnight.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP

Results and Data:

This shows the bands were where we expected them to be.

Expected size for Tubes 1,3,4 -250-300 bps
Expected size for Tube 2 -7772 bp fragment
and 237 bp fragment; keep larger fragment
Expected size for Tube 5 - should yield
6250 bp fragment and 207 bp fragment;
keep larger fragment

Figure 1



This is after I cut the gel out.

Hannah did the gel purification after I had to leave.

Wednesday, September 29, 2021

To Do:

1. ~~Ligation~~
2. ~~Transformation~~

Results and Data:

Ligation

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	Desired Plasmid
1	Gel purf tube 1	Gel purf tube 2	pKR121
2	-	Gel purf tube 2	Backbone Control
3	Gel purf tube 3	Gel purf tube 5	pKR122
4	Gel purf tube 4	Gel purf tube 5	pKR123
5	-	Gel purf tube 5	Backbone Control

2. Set up master mix table:
3. Master Mix for Tubes 1-2:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.5	34.5
10x ligase buffer	2.0	6
Insert	4.0	
Backbone	2.0	6
Ligase	0.5	1.5
Total	20	48

4. Master Mix for Tubes 3-5:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.5	46
10x ligase buffer	2.0	8
Insert	4.0	
Backbone	2.0	8
Ligase	0.5	2
Total	20	64

5. 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.
6. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.

7. To the individual tubes, add indicated amounts of H₂O (___ uL), 10x buffer (___ uL), insert (___ uL), and backbone (___ uL). Did this in a master mix.
8. Add indicated amount of ligase (___ uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
9. After all of the components have been added, mix each tube with a pipette set to 18 uL.
10. Leave at room temperature for 10 minutes.

Transform Chemically Competent *E. coli* cells

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

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Type of Cells	Final volume to plate	Number of kanamycin-containing plates
1	pKR121	Ligation 1	8 uL	XL1-Blue	100 uL, remaining	2
2	Backbone Control	Ligation 2	8 uL	XL1-Blue	100 uL, remaining	2
3	pKR122	Ligation 3	8 uL	PIR1	100 uL, remaining	2
4	pKR123	Ligation 4	8 uL	PIR1	100 uL, remaining	2
5	Backbone Control	Ligation 5	8 uL	PIR1	100 uL, remaining	2
6	(+) control	pKR117	1 uL	XL1-Blue	20 uL, 100 uL	2
7	(+) control	pKR114	1 uL	PIR1	20 uL, 100 uL	2
8	(-) control	None	0	XL1-Blue	100 uL	1
Total number of plates						15

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.

October 2021

Friday, October 1, 2021

To Do:

1. ~~Miniprep plasmids~~

Before the meeting, I did the first step of miniprepping the plasmids. After, Dan and I finished it together. Later that day, Hannah did the following:

Here's a brief summary of what your tubes are for miniprepping today:

I'm making the following overnight cultures, using LB + kan (50 ug/mL) for all of the tubes except for tube 8 which I used LB-carb (100 ug/mL):

1-4 – pKR121 colonies

5 – pKR122

6 – pKR123

7 – pKR117

8 – pYES2

Put in 37C shaking incubator at 4:15 pm.

Overnight Cultures Protocol

By John Church

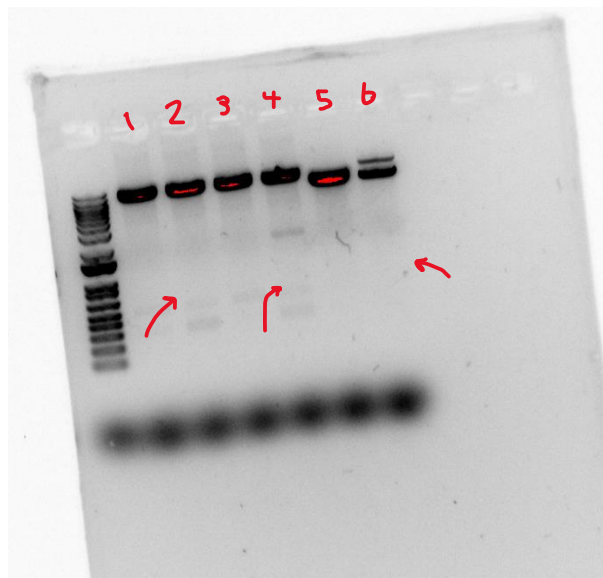
1. Add 45 mL of LB broth to a sterile 50 mL conical (makes enough for 8 tubes: $9 \times 5 = 45$)
2. Add 45 uL of kanamycin (Kan50) to the conical.
3. Label 4 glass sterile test tubes for each new plasmid and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB + Kan media 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

Monday, October 4, 2021

To Do:

1. ~~Diagnostic Digest~~

Today, I prepared the table and some of the other components for Dan to do the diagnostic digest and gel.



The red arrows are pointing at the expected length of the insert for tubes 2, tube 4, and a very faint tube 6. Tube 2 and 4 correlates to pKR121, and tube 6 correlates to pKR123. Unfortunately, pKR123 or tube 5 did not have a band in the appropriate place. As a reminder pKR121 has the rpsU2 promoter and the 5' UTR, and pKR123 has the tul4 promoter and the rpsU2 5' UTR. Since the pKR122 didn't work, we will be trying the double PCR method that Hannah did last week to create the DNA insert to put directly into the *Francisella*.

Wednesday, October 6, 2021

To Do:

1. ~~Prepare sequences~~

Today I prepared pKR121 that Dan miniprep'd for sequencing to see if the insert of tul4 promoter, with rpsU2 5' UTR and 6 aa is actually inserted in the backbone. I did this by adding water, the primer something-257 (?), and insert. After I went to the grant workshop at the Launch Pad and worked on the grant for the rest of my time. I finished everything except for the original ideas part.

Friday, October 8, 2021

To Do:

1. ~~Analyze sequencing results~~
2. ~~Work on grant~~

Our sequencing results came back from Janett for pKR121 that Dan sent, and the pKR123 that I sent in. They both look good! There were some deletions and "unknowns" outside of the area of interest, which is the part between where the two enzymes start sites were. (Hannah told us this) So it is confirmed that we have these two plasmids! Next week we will not be making a plasmid for the rpsU2 promoter and tul4 5' UTR, but instead doing the PCR protocol that Hannah designed.

Tuesday: all 20 uL rxns

Reaction 1: – Template is pKR121, primers are KROL482 and 257 (expected size about 500)

Reaction 2: – Template is pKR89, primers are KROL 483 and 363 (expected size about 5700)

Reaction 3: negative control - no DNA template, Use KROL482 and 257 (no product expected)

Wednesday: all 100 uL rxns

Dilute PCR1 from Tuesday 1:10 in 0.1xEB

Dilute PCR2 from Tuesday 1:10 in 0.1xEB

Reaction 1: Template is half 1:10 PCR1 and half 1:10 PCR2; primers are KROL257 and 363. Expected size is about 6200 bps

Run on a gel with rxn 3 from Tuesday to confirm

PCR purify and elute in 35 ul of 0.1xEB

Wednesday, October 13, 2021

To Do:

- ~~1. PCR of larger fragment for pKR122~~

Today I completed the PCR reaction for combining the PCR1 and PCR2 that Dan completed on Tuesday. On Friday we will run the gel to see if we completed all the reactions right, and if the product of the rpsU2 promoter with the tul4 5' UTR and 6 aa with lacZ and Tn7 components were successfully completed. We will know this if a band around 6200 bp is seen. After I think we will send it to be sequenced (not really sure though)

PCR New Protocol

Before doing PCR I need to dilute the PCR1 and PCR2 to one microliter with nine microliters of 0.1xEB.

Tube	Product	Template	Primers	Expected size
1	rpsU2 promoter, with tul4 5' UTR and 6 aa with lacZ and Tn7 components	Half 1:10 PCR1 and half 1:10 PCR2	KROL257 KROL363	6200 bps

Master Mix:

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			62	2
PrimeSTAR GXL Buffer	5x	1x	20	124
dNTPs	2.5 mM	0.2 mM	8	40
oligo F	10 uM	0.3 uM	3	16
oligo R	10 uM	0.3 uM	3	
template	100 ng/ul	2 ng/ul	2	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	4
		Total volume	100	184

Oligo F = KROL257

Oligo R = KROL363

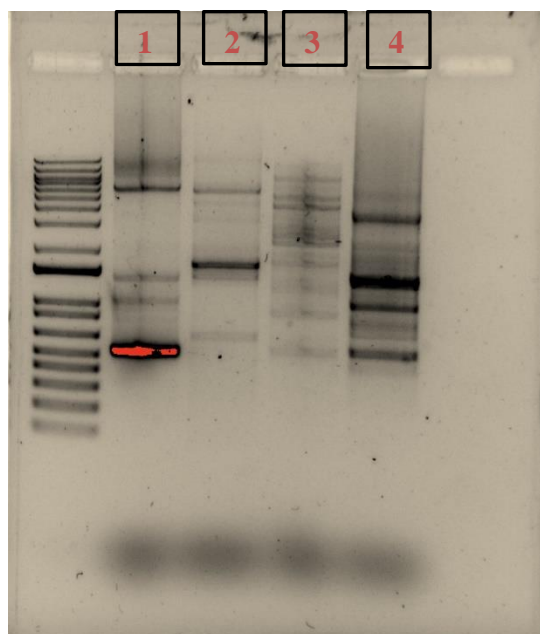
Friday, October 15, 2021

To Do:

- ~~1. Run gel of the PCR1, PCR2, and PCR1 + PCR2 reactions~~
- ~~2. Tell Hannah I won't be there on Monday~~
3. Submit Grant?

Today I completed the PCR reaction for combining the PCR1 and PCR2 that Dan completed on Tuesday. On Friday we will run the gel to see if we completed all the reactions right, and if the product of the rpsU2 promoter with the tul4 5' UTR and 6 aa with lacZ and Tn7 components were successfully completed. We will know this if a band around 6200 bp is seen. After I think we will send it to be sequenced (not really sure though)

Results:



Reaction 1('insert'): expected size about 500

Reaction 2 ('backbone'): expected size about 5700

Reaction 3(negative?): no product expected

Reaction 4(rxn 1+2): Expected size is about 6200 bps

We cannot see the expected band in the reaction 2, which means that the reaction 4 was also not able to be completed correctly. I think the primers were the reason for this? We will be continuing with the electroporation for insertion of pKR121 and pKR123 into *F. tularensis* (?) on Wednesday/Friday.

Monday, October 18, 2021

To Do:

1. ~~Lab chores~~
2. ~~Edit grant~~

Today in lab we did some lab chores (dishwasher, fill the water, and make media) Dan and I made the hemoglobin/cysteine heart agar plates in preparation for the culturing after the electroporation. We also made edits to the grant and I just submitted it!

Wednesday, October 20, 2021

To Do:

1. ~~Day two and six of the Tn7 in *F. tularensis*~~

Today in lab I did day 2 and day 6 of the Tn7 in *F. tularensis* protocol with a lot of help from Hannah. This is done to insert the plasmids into the genomic DNA of *F. tularensis* with the help of the helper plasmid which has a transposon. This transposon creates a protein which assists with the uptake of the plasmid during the Electroporate process. In the afternoon, I plated the cells on CHAH-kanamycin after being on the warm-shaker for 4.5 hours.

I electroporated into LVS cells, because Dan did the delta the day before.

Tn7 in F. tularensis Day 2 and 6

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
 - split into two tubes. Added 600 microL sucrose to each
- 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 80 μ L. It should be about equal amounts of cells as sucrose.
- For any extra EC cells, aliquot $\sim 110 \mu$ L / sterile tube (enough for 2 electroporations) and freeze at -80°C
 - only got one tube of 110 μ L of extra

Day 6:

Take patches of strain with helper plasmid from CHAH-Hyg plates and freeze down as electrocompetent cells (same protocol as above) should get about 50-110 μ L of electrocompetent cells per patch.

Once you have electrocompetent cells that contain the helper plasmid you can electroporate these cells with the Tn7 plasmid that has been designed with your gene (should be in the background of pKR55). The Tn7 plasmid is Kanamycin resistant, so these electroporation's should be plated onto CHA-Kan plates.

*Electroporate Tn7 transposon plasmid into helper plasmid containing EC cells**

- For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
- **tube 1 = pKR123 , tube 2 = pKR121, tube 3 = negative control**
- For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
 - 5 μ L of Tn7 transposon plasmid
 - 50 μ L electrocompetent cells containing helper plasmid
- Have recovery media ready
- Electroporate using the following settings: 2.5 kV, 25 μ F, and 600 Ω (setting Ec2)
- 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 4.5 hours, shaking at 37°C
- Plate 20 μ L, 200 μ L, and remaining on CHAH-Kan plates
- Incubate plates at 37°C for 3 days (or until single colonies appear)
 - It took 2 days for single colonies to appear

Friday, October 21, 2021

To Do:

- ~~1. Look to see if there is any colony growth~~
- ~~2. Make more sucrose~~
- ~~3. Talk about Honors Project~~

Today in lab I looked at the colonies. Lots and lots of growth. We got our primers in to try the 3 PCR method again, and we prepped the primers.

Receiving Primers Protocol

By: Hannah Trautmann

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 μ M. Calculate this by multiplying the reported nm by 10 and adding that volume in μ L (i.e. 12.7 nmol = add 127 μ L of 0.1xEB).
 - a. KROL486 era_PCR_f = 15.88 nmol = add 158.8 μ L of 0.1xEB
 - b. KROL487 = 14.96 nmol = add 149.6 μ L of 0.1xEB
 - c. KROL488 = 15.73 nmol = add 157.3 μ L of 0.1xEB
3. Put on 42°C heat block for 5 minutes to help primers dissolve

After this step Hannah took over, so Dan and I could go talk to Dr. Ramsey.

4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
 - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
 - b. If a sequencing primer, make a 2.5 uM stock by diluting 5 uL of the 100 uM stock into 195 uL of 0.1x EB. Label with blue sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

Hannah also did this for us:

“Hi here's a brief summary of what I did today for you. The plates have sooooo many colonies that I think it will be too many to count:

I patched out the electroporations for Dan and Aisling. They had no colonies on the negative controls but tons and tons on the actual Eps. I took from the 20 ul plate for all of them, and only patched out 2 for each strain (8 patches total) to CHAH-kan. Put the kan plates with patches at room temp over the weekend.”

Thanks Hannah!!

Monday, October 25, 2021

To Do:

- ~~1. Complete Tn7 in *F. tularensis* Day 10 (PCR)~~

Today I completed the PCR reactions and left it in the thermocycler overnight. Tomorrow, Dan will run the gel. The purpose of this PCR is to confirm that the inserts and backbone were successfully inserted into the *Francisella* spp. If this is completed successfully, then Dan and I have created eight new strains!

***Tn7 in F. tularensis* Day 10**

Day 10:

-At this point you want to “cure” the primary integrant of the hygromycin resistance. To do this you can streak out the primary integrants (from the patches on CHAH-Kan) to single colony on regular CHAH plates (no antibiotic). Plates should be incubated at 37°C for 3 days (or until single colonies appear).

- You can freeze down the rest of the patch from the CHAH-Kan plate to go back to later if needed.

* At this point you should have the transposon and therefore your gene of interest incorporated, so you can check by colony PCR to see if your gene of interest has been incorporated into the Tn7 site.

* Primers KROL252 and KROL253 amplify the insertion region of the Tn7 transposon (~350bp)

*Colony PCR from patches **

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

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
LVS pKR121	LVS pKR121	LVS pKR123	LVS pKR123	ΔrpsU2 pKR121	Δ rpsU2 pKR121	ΔrpsU2 pKR123	ΔrpsU2 pKR123

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

-Dilute lysates 1:10

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

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

Example colony PCR using PrimeSTAR GXL DNA Polymerase (use a master mix):

Number of samples		12
Master mix factor		13
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.8	153.4
5X Primestar GXL buffer	4	52
2.5 mM dNTPS	1.6	20.8
Forward primer (10 uM)	0.6	7.8
Reverse primer (10 uM)	0.6	7.8
Primestar DNA polymerase	0.4	5.2
Cell lysate	1	
Total	20	247

Putting 1 uL of cell lysate with 19 uL of Master Mix into PCR tube. Dilute the two plasmids (pKR121 and pKR123) but not the LVS gDNA.

Primers KROL252 and KROL253

Reactions:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
LVS pKR121	LVS pKR121	LVS pKR123	LVS pKR123	ΔrpsU2 pKR121	ΔrpsU2 pKR121	ΔrpsU2 pKR123	ΔrpsU2 pKR123

Controls:

Tube 9	Tube 10	Tube 11	Tube 12
LVS gDNA Low band at 350	pKR121 high band at 8000	pKR123 high band at 8000	Water No band

-Use PCR program:

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

- This is the PCR program SNT1 which is already programed. The extension time is 5 minutes because the DNA should be ~5,000 bp. The amount in the tubes is 20 uL.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

Friday, October 29, 2021

To Do:

1. ~~Run gel for PCR that I did on Monday~~
2. ~~Dishes~~



Tube	Reaction	Expected size	y/n/?
1	LVS pKR121	5000	n
2	LVS pKR121	5000	Y
3	LVS pKR123	5000	N
4	LVS pKR123	5000	Y
5	Δ rpsU2 pKR121	5000	Y
6	Δ rpsU2 pKR121	5000	Y?
7	Δ rpsU2 pKR123	5000	Y
8	Δ rpsU2 pKR123	5000	N
9	LVS gDNA	350	Y
10	pKR121	8000	?
11	pKR123	8000	?
12	water	0	y

Steps:

- Tubes 3 and 12 looked to have less than 20 uL, was found to have ~15 uL
- Added cyberspace and gel to the gel rig and let cool for 30 minutes
- Added 4 uL of cyber space to all reactions except for tubes 3 and 12, which 3 uL of cyberspace was added
- Loaded the gels and let run for 45 minutes (double check with Dan)

November 2021

Monday, November 1, 2021

To Do:

- ~~PCR of the lanes with no bands from Fridays Gel~~

Today in I redid the PCR for lanes 1,3, and 8 since there were no bands for each of these reactions. Tube 3 still looks weird, but it all looks more even than previously.

Tn7 in *F. tularensis* Day 10 (just PCR)

Colony PCR from patches

Dilute lysates 1:10

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

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

Reactions:

Tube 1	Tube 3	Tube 8
LVS pKR121	LVS pKR123	ΔrpsU2 pKR123

Controls:

Tube 9	Tube 10	Tube 11	Tube 12
LVS gDNA Low band at 350	pKR121 high band at 8000	pKR123 high band at 8000	Water No band

Number of samples

7

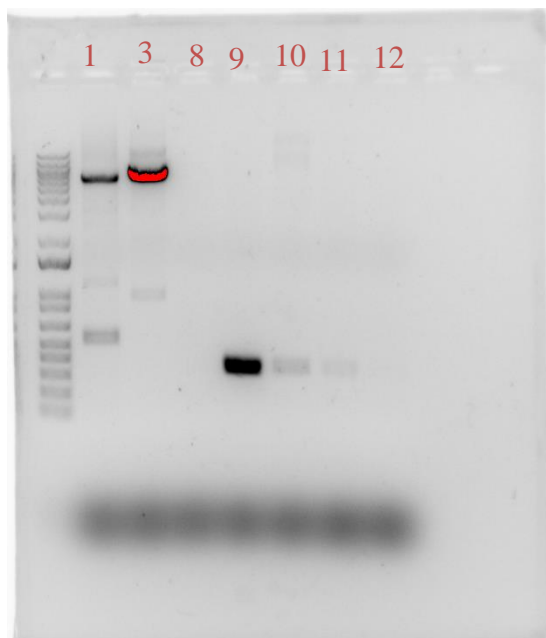
Master mix factor

8

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.8	94.4
5X PrimeStar GXL buffer	4	32
2.5 mM dNTPS	1.6	12.8
Forward primer (10 uM)	0.6	4.8
Reverse primer (10 uM)	0.6	4.8
PrimeStar DNA polymerase	0.4	3.2
Cell lysate	1	
Total	20	152

1 uL of cell lysate and 19 uL of Mastermix

Results: (Dan ran the gel)



Tube	Reaction	Expected size	y/n/?
1	LVS pKR121	5000	y
3	LVS pKR123	5000	y
8	Δ rpsU2 pKR123	5000	N
9	LVS gDNA	350	Y
10	pKR121	8000	y
11	pKR123	8000	Y (faint)
12	water	0	n

Wednesday, November 3, 2021

To Do:

- Check gDNA conc.
- Dilute to 100 uL/uC (10 uL)
- Set up 50 uL PCR
 - Control and 8 samples
 - KROL252 and KROL253

The purpose of the PCR for today is to get a higher concentration of the PCR reactions, so it can be prepared and sent for sequencing later in the week.

To check the gDNA, use the 0.1 x EB which is the dilutant, as the control.

Sample ID	Nucleic Acid	Unit	260/280	260/230	Sample Type	Factor
148.1	539.7	ng/ μ l	1.88	1.58	DNA	50
148.2	943.7	ng/ μ l	1.88	1.33	DNA	50
149.1	1080.9	ng/ μ l	1.92	1.93	DNA	50
149.2	731.2	ng/ μ l	1.84	1.61	DNA	50
150.1	630.7	ng/ μ l	1.9	1.82	DNA	50
150.2	768.3	ng/ μ l	1.93	1.69	DNA	50
151.1	391.2	ng/ μ l	1.88	1.66	DNA	50
151.2	1043.9	ng/ μ l	1.9	1.57	DNA	50

Dilute using $C1(V1)=C2(V2)$ in order to get a concentration of 100 uL/uC and volume of 10 uL.

Sample ID	Amount of DNA	Amount of 0.1 x EB
148.1	1.85	8.15
148.2	1.06	8.94

149.1	0.93	9.07
149.2	1.37	8.63
150.1	1.59	8.41
150.2	1.30	8.70
151.1	2.56	7.44
151.2	0.96	9.04

PCR New Protocol

Total reaction volume	50
Total number of reactions	9

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			31.0	310
PrimeSTAR GXL Buffer	5x	1x	10.0	100
dNTPs	2.5 mM	0.2 mM	4.0	40
oligo F	10 uM	0.3 uM	1.5	15
oligo R	10 uM	0.3 uM	1.5	15
template	100 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1.0	10
		Total volume	50	490

1.0 uL of template and 49 uL of Master mix. Primers are KROL252 and KROL253. The expected size for all is 5000 bp. This is amplifying the promoter and UTR and lacZ region.

Reactions:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
KRLVS 148.1	KRLVS 148.2	KRLVS 149.1	KRLVS 149.2	KRLVS 150.1	KRLVS 150.2	KRLVS 151.1	KRLVS 151.2	water

Strains:

Tube	Name	Genotype	Background	Integrated Plasmid
1	KRLVS148.1	LVS $\Delta rpsU2$ Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1	KRLVS9.1	pKR121
2	KRLVS148.2	LVS $\Delta rpsU2$ Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-2	KRLVS9.1	pKR121
3	KRLVS149.1	LVS Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1	LVS	pKR121
4	KRLVS149.2	LVS Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-2	LVS	pKR121
5	KRLVS150.1	LVS $\Delta rpsU2$ Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1	KRLVS9.1	pKR123
6	KRLVS150.2	LVS $\Delta rpsU2$ Tn7_Ptul4_rpsU2UTR_lacZ aphA E-2	KRLVS9.1	pKR123
7	KRLVS151.1	LVS Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1	LVS	pKR123
8	KRLVS151.2	LVS Tn7_Ptul4_rpsU2UTR_lacZ aphA E-2	LVS	pKR123

Friday, November 5, 2021

To Do:

- PCR purify (don't do negative control)
- Elute in 50 uL 0.1 x EB

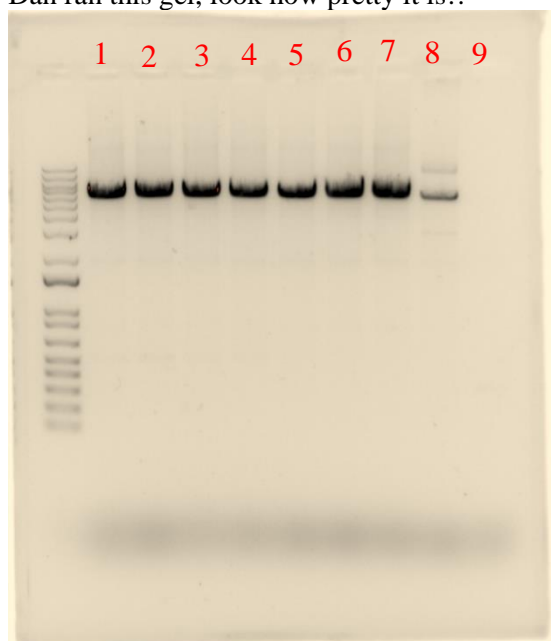
~~— Run 5 uL on gel~~

- Set up sequencing reactions (will due Monday)

For the PCR purification I used 250 uL of buffer to dilute 50 uL of the PCR reaction. Used 5 uL of cybersafe for the gel, used the smaller of gel rigs. Followed the PCR purification for all the reactions including water.

Results

Dan ran this gel, look how pretty it is!!



Tube	Reaction	Expected size	y/n/?
1	KRLVS148.1	5000	Y
2	KRLVS148.2	5000	Y
3	KRLVS149.1	5000	Y
4	KRLVS149.2	5000	Y
5	KRLVS150.1	5000	Y
6	KRLVS150.2	5000	Y
7	KRLVS151.1	5000	Y
8	KRLVS151.2	5000	?
9	water		Y

These bands came out well due to their darkness. I think they are around 5000 bp which is what was expected. After looking at the ladder, which can be found after the table of contents, the bands are slightly above the 5000 bp marker.

Monday, November 8, 2021

To Do:

- ~~— Sequencing Reactions~~
- ~~— Large batch of CHA~~
- ~~— Made MHB~~

Dan and I prepared the KRLVS 148.1, KRLVS 149.1, KRLVS 150.1 and KRLVS 151.1 for sequencing. I forgot to pull out KROL181 when pulling out the sequencing primers and had to redo the strip for LVS 148.1.

#NAME?	Well	Template Type	Template Name	Primer Name ^a	A.		B.	C.	D.	E.	F.
	(GSC use <u>only</u>)	(plasmid or PCR)			Template Size (bases)		Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMI D template: Volume =	Volume H ₂ O needed (12 less D or E - 2.56)μl
								$(A + 100) \times 2.5$	$(C + B)\mu l$	$2x(\sim 200 \div B)\mu l$	
AM1		PCR	148.1	KROL472	5000		116.3	125.00	1.07		8.37
AM2		PCR	148.1	KROL177	5000		116.3	125.00	1.07		8.37
AM3		PCR	148.1	KROL178	5000		116.3	125.00	1.07		8.37
AM4		PCR	148.1	KROL179	5000		116.3	125.00	1.07		8.37
AM5		PCR	148.1	KROL180	5000		116.3	125.00	1.07		8.37
AM6		PCR	148.1	KROL181	5000		116.3	125.00	1.07		8.37
AM7		PCR	148.1	KROL182	5000		116.3	125.00	1.07		8.37
AM8		PCR	148.1	KROL253	5000		116.3	125.00	1.07		8.37
AM9		PCR	149.1	KROL472	5000		116.9	125.00	1.07		8.37
AM10		PCR	149.1	KROL177	5000		116.9	125.00	1.07		8.37
AM11		PCR	149.1	KROL178	5000		116.9	125.00	1.07		8.37
AM12		PCR	149.1	KROL179	5000		116.9	125.00	1.07		8.37
AM13		PCR	149.1	KROL180	5000		116.9	125.00	1.07		8.37
AM14		PCR	149.1	KROL181	5000		116.9	125.00	1.07		8.37
AM15		PCR	149.1	KROL182	5000		116.9	125.00	1.07		8.37
AM16		PCR	149.1	KROL253	5000		116.9	125.00	1.07		8.37
AM17		PCR	150.1	KROL326	5000		94.6	125.00	1.32		8.12
AM18		PCR	150.1	KROL177	5000		94.6	125.00	1.32		8.12
AM19		PCR	150.1	KROL178	5000		94.6	125.00	1.32		8.12
AM20		PCR	150.1	KROL179	5000		94.6	125.00	1.32		8.12
AM21		PCR	150.1	KROL180	5000		94.6	125.00	1.32		8.12
AM22		PCR	150.1	KROL182	5000		94.6	125.00	1.32		8.12
AM23		PCR	150.1	KROL253	5000		94.6	125.00	1.32		8.12
AM24		PCR	150.1	KROL181	5000		94.6	125.00	1.32		8.12
AM25		PCR	151.1	KROL326	5000		117.6	125.00	1.06		8.38
AM26		PCR	151.1	KROL177	5000		117.6	125.00	1.06		8.38
AM27		PCR	151.1	KROL178	5000		117.6	125.00	1.06		8.38
AM28		PCR	151.1	KROL179	5000		117.6	125.00	1.06		8.38
AM29		PCR	151.1	KROL180	5000		117.6	125.00	1.06		8.38
AM30		PCR	151.1	KROL182	5000		117.6	125.00	1.06		8.38
AM31		PCR	151.1	KROL253	5000		117.6	125.00	1.06		8.38
AM32		PCR	151.1	KROL181	5000		117.6	125.00	1.06		8.38
a. Add 2.56 μl of 2.5 μM stock to each reaction											

Excel Spread sheet: 211108_AM_SequencingSubmission

We made master mixes for each strain of the template and water to make it easier to pipette.

	templ	water	Total
148.1	9.67	75.29	9.44
149.1	9.62	75.34	9.44
150.1	11.89	73.07	9.44
151.1	9.57	75.39	9.44

Added 9.44 uL of the master mix and 2.56 of the appropriate primer to each tube.

After, Dr. Ramsey, Dan, and I tag teamed making the CHA powder and mixed in the appropriate amount of water. After we also made the MHB media.

Large batch of pre-mixed CHA powder

Combine the following components, making sure to break up any clumps prior to adding to the container:

75 g Beef Heart Infusion

75 g Protease Peptone

75 g Glucose

7.5 g L-Cystine

112.5 g Agar

37.5 g NaCl

Put in recycled BD container, shake well to mix, and update tape with date made.

Measure out 30.6 g of premade mix for each flask of 300 mL CHA.

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)

Hannah brought down to Autoclave, not sure who finished the rest of the steps.

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

Friday, November 12, 2021**To Do:**

—Sequence KRLVS148.1 with primers KROL178 and 179

—Prepare PCR for pKR122

Today I did a redo of the sequencing since the results from Mondays did not come out well. There should be 600 bp for each primer, and we got around 300-350 bp. So, I redid KRLVS 148.1 with primers KROL178 and KROL179 again the same way and then another way with doubled the concentration. This was done to see if the increased concentration will help with the sequencing.

#NAME?	Well	Template Type	Template Name	Primer Name ^a	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Templat e Stock Conc. (n g/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMI D template: Volume =	Volume H ₂ O needed (12 less D or E - 2.56)μl
							(A + 100) × 2.5	(C + B)μl	2x(~200 + B)μl	
AM1		PCR	148.1	KROL178	5000	116.3	125.00	1.07		8.37
AM2		PCR	148.1	KROL179	5000	116.3	125.00	1.07		8.37
AM3		PCR	148.1	KROL178	5000	116.3	125.00	2.14		7.30
AM4		PCR	148.1	KROL179	5000	116.3	125.00	2.14		7.30
a. Add 2.56 μl of 2.5 μM stock to each reaction										
3130xl Plate Record		Date	11/12/2021	Name	Aisling Macaraeg					
PI	Kathryn Ramsey	Dept	CMB	Email	amacaraeg@uri.edu	PO No.	0000143904			

The PCR completed was the same for the pKR121 from September 22, 2021, but with an increased in amount of template. 4uL instead of 2 uL. This was done to see if it helps with the our ability to increase the concentration on the gel when the digest is completed.

PCR New Protocol

Tube	Product	Template	Primers	Expected size
1	rpsU2 promoter, with tul4 5' UTR and 6 aa	gBlock PrpsU2_tul4UTR	KROL472 KROL350	252
2	- control	-	KROL472 KROL350	0

Total reaction volume	100
Total number of reactions	2.5

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			60.0	3.5
PrimeSTAR GXL Buffer	5x	1x	20.0	154
dNTPs	2.5 mM	0.2 mM	8.0	70
oligo F	10 uM	0.3 uM	3.0	28
oligo R	10 uM	0.3 uM	3.0	10.5
template	100 ng/ul	4 ng/ul	4.0	10.5
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	7
		Total volume	100	280

The primers used were KROL472 and KROL350. The region of amplification is rpsU2 promoter, tul4 5' UTR, and 6 aa. Since 4 uL of the template is being used, 96 uL of the master mix will be added to each. The elongation step was set to 15 seconds due to the expected base pairs being 252. The volume of the PCR reactions was set to 100 uL.

Monday, November 15, 2021

To Do:

- PCR purify the reactions from Friday
- DNA digest
- Gel purify
- Ligation

Today I purified the PCR reactions from Friday. There was 100 uL of PCR reaction, so I added 500 uL of buffer PB to it. I did one minute for all of the steps except for step 5, which requires 3 minutes. For step 7, I eluted the DNA with 35 uL Buffer 0.1 x EB and let stand for 2 minutes before centrifuging for one last time. Then a gel was completed for the digest.

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 (rpsU2 promoter, with tul4 5' UTR and 6 aa)	NotI-HF/KpnI-HF	15	-
---	--	-----------------	----	---

Expected size for Tubes 1 250-300 bps

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	0	
10x Buffer*	3	
DNA	(25.8)	
Enzyme 1	0.6	
Enzyme 2	0.6	
Total	30.0 (15.0 actual b/c of DNA)	

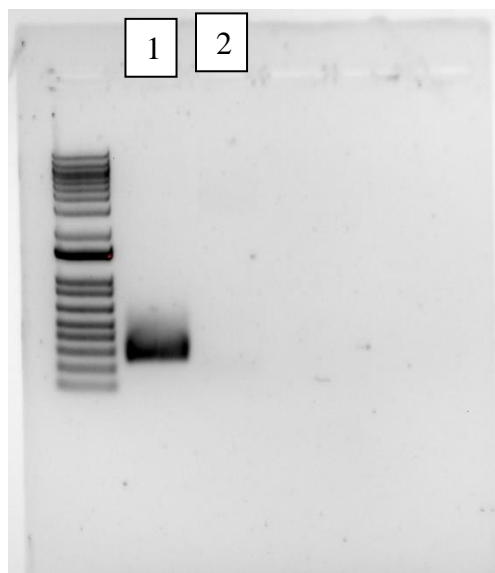
*Cutsmart Buffer is used for all the “HF” enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

3. Add indicated amounts of H₂O and 10x buffer to individual tube
4. Add indicated amount of each enzyme (_0.6_uL) to individual tube
5. Mix the tube by pipetting up and down.
6. Incubate at 37°C for 30 minutes.

Since there is only one reaction, a master mix is not necessary. We are only using DNA and no water, which is why the 10.8 uL of water, the volume was added to the DNA. So 25.8 uL of DNA was used. This was done in order to increase the chances of the amount of DNA being able to be digested and ligated.

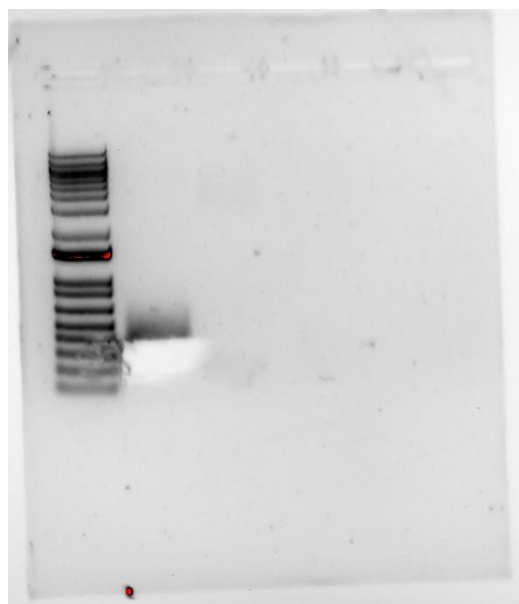
For the gel: 36uL of the first reaction and negative control was added. (30 uL of PCR + 6 uL of dye) 15 uL of the ladder was added. Forgot to skip a lane, so it went right next to one another. Hannah did the cutting for us. (thanks Hannah)

Before:



Tube	Reaction	Expected size	y/n/?
1	Insert (rpsU2 promoter, with tul4 5' UTR and 6 aa)	250-300	Y
2	Negative control	0	Y

After:



Gel extraction:

- Full 2uL , so dividing into two 1.5 uL, with 1 uL in each. Adding 255 uL of isopropyl to each tube.

I had to leave but Dan did a nanodrop and ligation.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	insert	20.4	ng/μl	0.407	0.186	2.19	0.07

2 backbone 9.3 ng/μl 0.186 0.064 2.92 0.02

Ligation

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	Desired Plasmid
1	Gel purf 1 Purified PCR 2 (rpsU2 promoter, with tul4 5' UTR and 6 aa)	Gel purf tube 2	pKR122
2	-	Gel purf tube 2 Backbone (pKR89)	Backbone Control

2. Set up master mix table:
3. Master Mix for Tubes 1 and 2 (insert and backbone):

Number of samples

2

Master mix factor

3

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H ₂ O	11.5	34.5
10x ligase buffer	2.0	6
Insert	4.0	
Backbone	2.0	6
Ligase	0.5	1.5
Total	20	48

4. 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.
5. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
6. To the individual tubes, add indicated amounts of H₂O (11.5_uL), 10x buffer (_2.0_uL), insert (__4.0__uL), and backbone (__2.0__uL). Did this in a master mix.
7. Add indicated amount of ligase (__0.5_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
8. After all of the components have been added, mix each tube with a pipette set to 18 uL.
9. Leave at room temperature for 10 minutes.

This is what Dan did:

Ligation

Used a ligation calculator Hannah made

Ligation Calculator			
ng vector	ratio of insert/bb bps	molar ratio	ng of insert

50	0.02997941328	3	4.496911992
50	0.02997941328	5	7.49485332
	concentration	uL needed	
backbone	9.3	5.38	
3X	2.04	2.20	
5X	2.04	3.67	
	3X	5X	BB only
Water	9.92	8.45	12.12
Ligation Buffer	2	2	2
Backbone	5.38	5.38	5.38
Insert	2.20	3.67	-
Ligase	0.5	0.5	0.5
	20.00	20.00	20.00

- Set up the three ligation reaction tubes according to this table
 - Tube 1 was the 3:1 BB/Insert ratio
 - Tube 2 was the 5:1 BB/Insert ratio
 - Tube 3 was just the backbone
- Made a diluted solution of the insert since the initial concentration from the extraction was 20.4 ug/ml
 - The volumes for the reaction tubes would have been too small to pipette, so I made a 1:10 reaction (1 ul purified DNA, 9 ul .1X EB)
- Once the reactions were prepared, they went in the thermal cycler at 16°C for transformation tomorrow

Wednesday, November 17, 2021

To Do:

- ~~Count transformation plates~~
- ~~Redo PCR for sequencing~~
- ~~Make new primers~~

Yesterday Dan finished up *E. coli* transformation. So this are the colonies on each of the plates:

Backbone Ligation (pKR89) 100 uL	3x Ligation (pKR122) 100 uL	5x Ligation (pKR122) 100 uL	Positive control 20 uL	Negative control 20uL
0	3	0	lots	none

Backbone Ligation (pKR89) remaining	3x Ligation (pKR122) remaining	5x Ligation (pKR122) remaining	Positive control 100 uL	Negative control 100uL
3	14	3	Lots (lawn)	none

Redoing the PCR that was completed on Wednesday, November 3rd. This is being done in hopes of fixing the sequencing issues.

Dilute using $C1(V1)=C2(V2)$ in order to get a concentration of 100 uL/uC and volume of 10 uL.

Sample ID	Amount of DNA	Amount of 0.1 x EB
148.1	1.85	8.15
148.2	1.06	8.94
149.1	0.93	9.07
149.2	1.37	8.63
150.1	1.59	8.41
150.2	1.30	8.70
151.1	2.56	7.44
151.2	0.96	9.04

PCR New Protocol

Total reaction volume	30
Total number of reactions	9

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			18.6	186
PrimeSTAR GXL Buffer	5x	1x	6.0	60
dNTPs	2.5 mM	0.2 mM	2.4	24
oligo F	10 uM	0.3 uM	0.9	9
oligo R	10 uM	0.3 uM	0.9	9
template	100 ng/ul	2 ng/ul	0.6	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.6	6
		Total volume	30	294

0.6 uL of template and 29.4 uL of Master mix. Primers are KROL252 and KROL253. The expected size for all is 5000 bp. This is amplifying the promoter and UTR and lacZ region. 5 minutes for the elongation.

Reactions:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
--------	--------	--------	--------	--------	--------	--------	--------	--------

KRLVS 148.1	KRLVS 148.2	KRLVS 149.1	KRLVS 149.2	KRLVS 150.1	KRLVS 150.2	KRLVS 151.1	KRLVS 151.2	water
----------------	----------------	----------------	----------------	----------------	----------------	----------------	----------------	-------

Strains:

Tube	Name	Genotype	Background	Integrated Plasmid
1	KRLVS148.1	LVS $\Delta rpsU2$ Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1	KRLVS9.1	pKR121
2	KRLVS148.2	LVS $\Delta rpsU2$ Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-2	KRLVS9.1	pKR121
3	KRLVS149.1	LVS Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1	LVS	pKR121
4	KRLVS149.2	LVS Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-2	LVS	pKR121
5	KRLVS150.1	LVS $\Delta rpsU2$ Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1	KRLVS9.1	pKR123
6	KRLVS150.2	LVS $\Delta rpsU2$ Tn7_Ptul4_rpsU2UTR_lacZ aphA E-2	KRLVS9.1	pKR123
7	KRLVS151.1	LVS Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1	LVS	pKR123
8	KRLVS151.2	LVS Tn7_Ptul4_rpsU2UTR_lacZ aphA E-2	LVS	pKR123

Remade primers for KROL 253, KROL177-182, and KROL472.

- take 100 uM stock and place 5 uL into new 1.5 tubes
- add 195 uL of 0.1 x EB
- add blue sticker with label

Friday, November 19, 2021

To Do:

- miniprep
- PCR purify

Before the lab meeting I miniprepped the overnight cultures of the 3x ligation that Hannah made for us last night.

MiniPrep

1. Added the broth to 2mL tubes to pellet at maximum rpm (13000 rpm) for 3 minutes. Did this 3 times.
2. Added 250uL Buffer P1 to resuspend pelleted bacterial cells.
3. Added 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution was more translucent
4. Added 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
5. Centrifuge for 10 minutes at 13,000 rpm
6. Apply 800 uL of supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 1 minute. Discard flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 minute. Discard flow-through.
8. Wash the QIAprep 2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 minute and discard flow-through 3 times. Transfer to collection tube.
9. Centrifuge for 3 minutes to remove residual wash buffer.
10. Place the QIAprep 2.0 column to a clean 1.5 ml microcentrifuge tube. To elute DNA add 50 uL buffer 0.1xEB. Let stand for 1 minute. Centrifuge for 1 minute.

PCR purify

This is being done for the re-do of the sequencing.

Added 150 μ L of the buffer PB to the 30 μ L of the PCR reaction. I did one minute for all of the steps except for step 5, which requires 3 minutes. For step 7, I eluted the DNA with 35 μ L Buffer 0.1 x EB and let stand for 1 minute before centrifuging for one last time.

Monday, November 22, 2021

To Do:

- Nanodrop
- Gel
- Sequencing reactions

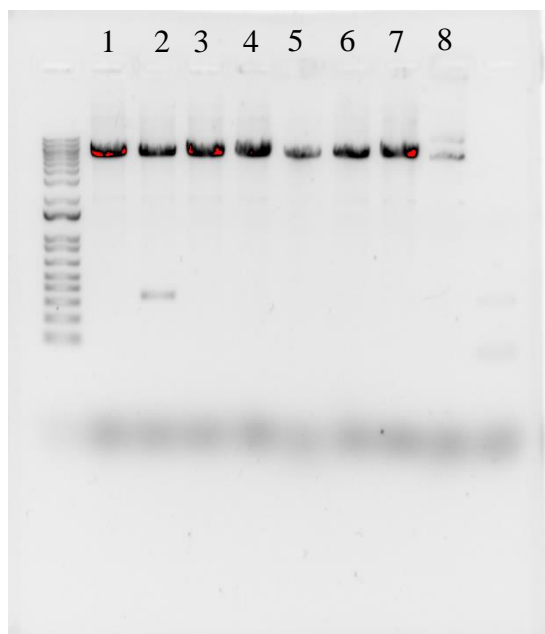
Before the lab meeting I minipreped the overnight cultures of the 3x ligation that Hannah made for us last night.

Nano-dropped to check of the concentrations of the miniprep and the pcr purification.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
MP1	289.8	ng/ μ L	5.797	2.417	2.4	4.52
MP2	400.5	ng/ μ L	8.011	4.238	1.89	2.29
MP3	395.9	ng/ μ L	7.918	4.173	1.9	2.21
MP4	439.7	ng/ μ L	8.794	4.655	1.89	2.28
PCR1	62.4	ng/ μ L	1.248	0.677	1.84	2.79
PCR2	86.8	ng/ μ L	1.736	0.921	1.88	2.31
PCR3	103	ng/ μ L	2.06	1.124	1.83	2.44
PCR4	108	ng/ μ L	2.16	1.197	1.8	2.16
PCR5	93.8	ng/ μ L	1.877	1.045	1.8	2.35
PCR6	98.5	ng/ μ L	1.97	1.092	1.8	2.26
PCR7	94.1	ng/ μ L	1.881	1.018	1.85	2.33
PCR8	40.1	ng/ μ L	0.802	0.459	1.75	2.43

PCR gel: 5 μ L of the PCR reaction, 1 μ L of the dye, 5 μ L of cyberspace, and small gel rig

Tube	Reaction	Expected size	y/n/?
1	KRLVS148.1	5000	Y
2	KRLVS148.2	5000	Y but 2 bands
3	KRLVS149.1	5000	Y
4	KRLVS149.2	5000	Y



5	KRLVS150.1	5000	Y
6	KRLVS150.2	5000	Y
7	KRLVS151.1	5000	Y
8	KRLVS151.2	5000	Same as 11/5/21
9	water		Y

Sequencing Submission:

#NAME?	Well	Template	Template Size	Primer	Template Size (bases)	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)				Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H ₂ O needed
							$(A + 100) \times 2.$	$(C + B)\mu l$	$2x(\sim 200 + B)\mu l$	(12 less D or E - 2.56)μl
AM1		PCR	148.1	KROL178	5000	62.4	125.00	2.00		7.44
AM2		PCR	150.1	KROL178	5000	93.8	125.00	1.33		8.11
AM3		plasmid	pKR122	KROL257	8000	289.8	144.9		2.76	6.68
AM4		plasmid	pKR122	KROL257	8000	400.5	200.25		2.00	7.44
AM5		plasmid	pKR122	KROL257	8000	395.9	197.95		2.02	7.42
AM6		plasmid	pKR122	KROL257	8000	439.7	219.85		1.82	7.62

a. Add 2.56 μl of 2.5 μM stock to each

The sequencing came back, and the PCR (samples 1 and 2) looked a lot better! The plasmid looks not super great.

Contamination:

Sample 3 FTL0295

Sample 4 FTL0295

Sample 5 PDPA

Sample 6 pKR123

Monday, November 29, 2021

To Do:

— Sequencing reactions

Dan and I tag teamed the re-doing of the sequencing of strains 148.1, 149.1, 149.2, 150.1, 150.3. Did not do 148.2 because of the double band in the PCR completed on 11/22/2021 and 150.2 because of the faint band.

#NAME?	Well	Template	Template Size	Primer	A.	B.	C.	D.	E.	F.								
	(GSC use only)	(plasmid or PCR)	(bases)			Template Stock Conc. (ng/μl)	PCR template: ng needed =	PCR template: Volume =	PLASMID template: Volume =	Volume H ₂ O needed								
							$(A + 100) \times 2$	$(C + B) \mu\text{l}$	$2x(-200 + B) \mu\text{l}$	$(12 \text{ less D or E} - 2.56) \mu\text{l}$								
AM1		PCR	148.1	KROL179	5000	116.3	125.00	1.07	8.37	8.37								
AM2		PCR	148.1	KROL180	5000	116.3	125.00	1.07	8.37	8.37								
AM3		PCR	148.1	KROL181	5000	116.3	125.00	1.07	8.37	8.37	148.1	4.30	33.46	37.76				
AM4		PCR	149.1	KROL472	5000	116.9	125.00	1.07	8.37	8.37	149.1	5.35	41.85	47.20				
AM5		PCR	149.1	KROL177	5000	116.9	125.00	1.07	8.37	8.37	149.2	10.42	74.54	84.96				
AM6		PCR	149.1	KROL178	5000	116.9	125.00	1.07	8.37	8.37	150.1	6.61	40.59	47.20				
AM7		PCR	149.1	KROL180	5000	116.9	125.00	1.07	8.37	8.37	150.2	11.42	73.54	84.96				
AM8		PCR	149.2	KROL472	5000	116.9	125.00	1.07	8.37	8.37	151.1	5.31	41.89	47.20				
AM9		PCR	149.2	KROL177	5000	108	125.00	1.16	8.28	8.28								
AM10		PCR	149.2	KROL178	5000	108	125.00	1.16	8.28	8.28								
AM11		PCR	149.2	KROL179	5000	108	125.00	1.16	8.28	8.28								
AM12		PCR	149.2	KROL180	5000	108	125.00	1.16	8.28	8.28								
AM13		PCR	149.2	KROL181	5000	108	125.00	1.16	8.28	8.28								
AM14		PCR	149.2	KROL182	5000	108	125.00	1.16	8.28	8.28								
AM15		PCR	149.2	KROL253	5000	108	125.00	1.16	8.28	8.28								
AM16		PCR	150.1	KROL326	5000	94.6	125.00	1.32	8.12	8.12								
AM17		PCR	150.1	KROL177	5000	94.6	125.00	1.32	8.12	8.12								
AM18		PCR	150.1	KROL178	5000	94.6	125.00	1.32	8.12	8.12								
AM19		PCR	150.1	KROL179	5000	94.6	125.00	1.32	8.12	8.12								
AM20		PCR	150.1	KROL180	5000	94.6	125.00	1.32	8.12	8.12								
AM21		PCR	150.1	KROL181	5000	94.6	125.00	1.32	8.12	8.12								
AM22		PCR	150.2	KROL326	5000	98.5	125.00	1.27	8.17	8.17								
AM23		PCR	150.2	KROL177	5000	98.5	125.00	1.27	8.17	8.17								
AM24		PCR	150.2	KROL178	5000	98.5	125.00	1.27	8.17	8.17								
AM25		PCR	150.2	KROL179	5000	98.5	125.00	1.27	8.17	8.17								
AM26		PCR	150.2	KROL180	5000	98.5	125.00	1.27	8.17	8.17								
AM27		PCR	150.2	KROL181	5000	98.5	125.00	1.27	8.17	8.17								
AM28		PCR	150.2	KROL182	5000	98.5	125.00	1.27	8.17	8.17								
AM29		PCR	150.2	KROL253	5000	98.5	125.00	1.27	8.17	8.17								
AM30		PCR	151.1	KROL179	5000	117.6	125.00	1.06	8.38	8.38								
AM31		PCR	151.1	KROL180	5000	117.6	125.00	1.06	8.38	8.38								
AM32		PCR	151.1	KROL181	5000	117.6	125.00	1.06	8.38	8.38								
AM33		PCR	151.1	KROL253	5000	117.6	125.00	1.06	8.38	8.38								

a. Add 2.56 μl of 2.5 μM stock to each

December 2021

Friday, December 10, 2021

To Do:

— Look at sequencing reactions results

Today I looked at the sequencing reactions results for 148.1, 149.1, 149.2, 150.1, 150.2, and 151.1. They all looked good to me for the most part. My only area of concern is in some of the overlapping regions around the primer there are a few mistakes (Ns) on one sequencing reaction, but it's fine on the other one.

For the gblock sequencing (for 20ul for the pKR122 in Dans notebook December 3rd 2021) Dr. Ramsey said that "Hi folks, sorry our conversation was cut short earlier! With respect to the sequencing we were looking at earlier (the rpsU2 promoter fused to the tul4 UTR), I confirmed that the weird artifact we proposed was what was sequenced BUT that the sequence itself (so the dsDNA PCR product you generated) has the correct sequence. I think you should move forward with that PCR product!" From my understanding this means that the sequencing had a palindrome so it bound together in one section and continued on. This is why we had around 436 bp when we were expecting 250. I am not sure if this means that the pKR122 sequencing is ok, because I think it looked bad last time I remember. So, we may have to re-do the pKR122 from the beginning.

- Dan summarized it well with: Got the sequencing for PrpsU_tul4UTR back, looked weird at first, but Kathryn confirmed that the sequence was correct
 - The two ends have the same sequence, so they bonded together, making the overall size of the fragments almost double the size of what we were expecting (~460 bp vs the original 250 bp)
 - Dr Ramsey said the ends were palindromes, caused unintended binding interactions.

Sequencing Results			
148.1	3 - 211130	150.1	23 - 211109
	1 - 211130	16	211130
	2 - 211130	17	
	1 - 211123	18	
lace region?	4 - 211116	19	
	1 - 211116	20	
	1 - 211109	21	
	2 - 211109		
		150.2	22
148.2	did not do	23	211130
		24	
149.1	7 - 211130	25	
	4 - 211130	26	
around KPOL180	5 - 211130	27	
	6 - 211130	28	211109
	13 - 211109	29	
	14 - 211109		
	15 - 211109	151.1	
	21 - 211109	25	
		26	211130
		27	
149.2	8	28	
	9	29	
around TO region	10	30	
	11	31	211130
very end	12	32	
	13	33	
	14		
	15		

Monday, December 13, 2021

To Do:

- 100 uL PCR of pKR122 re-do
- Streaking for isolation

Today I did a larger concentration of the PCR that Dan did last week. I also isolated colonies for the strains for the beta-galactosidase assay.

PCR New Protocol

Tube	Product	Template	Primers	Expected size
------	---------	----------	---------	---------------

1	rpsU2 promoter, with tul4 5' UTR and 6 aa	gBlock PrpsU2_tul4UTR	KROL472 KROL350	252
2	control	-	KROL472 KROL350	0

Total reaction volume	100
Total number of reactions	1

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			62.0	93
PrimeSTAR GXL Buffer	5x	1x	20.0	30
dNTPs	2.5 mM	0.2 mM	8.0	12
oligo F	10 uM	0.3 uM	3.0	4.5
oligo R	10 uM	0.3 uM	3.0	4.5
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	3
		Total volume	100	147

100uL of PCR: 98 uL of master mix and 2 uL of template

Control: 20 uL of master mix

Streaking for isolation:

Hannah made the Kanamycin plates today, so we let them dry in a circle around the flame for around 15 minutes. I used the KRLVS 112 and 111 from Hannah's personal glycerol stock and then the others from what Dan (?) made.

Strain	Genotype	Genotype
KRLVS 112	LVS	LVS Tn7::Ptul4-tul4 5'UTR-lacZ aphA
KRLVS 149.1	LVS	LVS Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1
KRLVS 151.1	LVS	LVS Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1
KRLVS 111	Δ rpsU2	LVS Δ rpsU2 Tn7::Ptul4-tul4 5'UTR-lacZ aphA
KRLVS 148.1	Δ rpsU2	LVS Δ rpsU2 Tn7_PrpsU2_rpsU2UTR_lacZ aphA E-1
KRLVS 150.1	Δ rpsU2	LVS Δ rpsU2 Tn7_Ptul4_rpsU2UTR_lacZ aphA E-1

Thursday, December 16, 2021

To Do:

- ~~Make culture, media, cuvettes~~
- ~~Patch out single colonies~~

Today I did a

Patching out single colonies: With wooden stick, scraped one colony and made a patch by swiping back and forth on a quarter of the kanamycin plate. Made four patches for each of the strains.

Beta-Galactosidase Assay Prep

Z-Buffer (1L)16.1g Na₂HPO₄•7H₂O5.5g NaH₂PO₄•H₂O

0.75g KCl

0.246g MgSO₄•7H₂O997.3ml ddH₂O

pH 7

ONPG solution

160 mg O-Nitrophenyl-beta-galactoside (ONPG) in 40 mL (4 mg/mL) in Z-buffer.

Store at -20°C.

Prepare 1M Na₂CO₃**Friday, December 17, 2021****To Do:**

- Move culture tubes to 37°C shaker
- Use loop to scrape patch into microtube with MHB (3 per strain)
- Pipette up and down until no clumps
- Repeat for all 18 patches
- 50 uL from cell suspension and add to cuvette with 950 uL MHB
 - o Invert to mix with parafilm
- Read OD600 in spec: MUST BE BELOW 0.05-0.6
- Calculate volume to add with $C1V1=C2V2$
 - o $V2=6000$ uL
 - o C2 for LVS = 0.1 uL
 - o C2 for rpsu2 = 0.135 uL
 - o $C1 = OD600 * 20$
 - o $V1 = ((6000)(0.1 \text{ or } 0.135)) / (OD600 \times 20)$
- Add appropriate volume to culture tubes
- Put in shaking incubator at 37°C
- Start timer counting up
-

Tubes for culturing:

Tube #	Strain	
1-3	149 (LVS)	Prpsu2-rpsU2UTR
4-6	150 ($\Delta 2$)	Prpsu2-rpsU2UTR
7-9	151 (LVS)	Ptul4-rpsU2UTR
10-12	148 ($\Delta 2$)	Ptul4-rpsU2UTR
13-15	112 (LVS)	Ptul4-tul4UTR
16-18	111 ($\Delta 2$)	Ptul4-tul4UTR

- Switched 150 and 148 when summarized from the previous table

MUST BE BELOW 0.05-0.6

OD600 for initial:

Tube	OD	Tube	OD	Tube	OD
*1	0.396	*7	0.407	13	0.429
*2	0.429	8	0.483	14	0.562
*3	0.373	9	0.540	15	0.513
4	0.368	10	0.295	16	0.163
5	0.374	11	0.481	17	0.125
6	0.426	12	0.590	18	0.151

Originals (before diluting):

The ones with * had to be diluted by taking out 500 uL of the cuvette and then adding 500 uL of the nonsterile MHB broth to the microtube and then reread.

$$1=0.755//2=0.811//3=0.729//7=0.782$$

Volumes: $V1=(6000 \times 0.1 \text{ or } 0.135)/(OD600 \times *20)$

*40 when the OD600 too large

LVS			rpsu2		
Sample	OD600	V1 (uL)	Sample	OD600	V1 (uL)
1	0.396	37.9	4	0.368	110.1
2	0.429	35.0	5	0.374	108.3
3	0.373	40.2	6	0.426	95.1
7	0.407	36.9	10	0.295	137.3
8	0.483	62.1	11	0.481	84.2
9	0.54	55.6	12	0.59	68.6
13	0.429	69.9	16	0.163	248.5
14	0.562	53.4	17	0.125	324.0
15	0.513	58.5	18	0.151	268.2

Added to culture tubes and then took 500 uL from culture tube and added to cuvette to get the time zero OD600. The range of OD600 could be from 0.07 to 0.14.

T=0 OD600

Tube	OD	Tube	OD	Tube	OD
1	0.095	*7	0.094	13	0.101
2	0.099	8	0.093	14	0.092
3	0.096	9	0.100	15	0.095
4	0.127	10	0.113	16	0.120
5	0.129	11	0.114	17	0.114
*6	0.135	12	0.136	18	0.124

Accidentally put both of the cell cultures from the microtubes into tube 7 for microtubes 6 and 7. Originally cuvette 6 was -0.004 and 7 was 0.267. I remade the cell cultures by putting 6mL of sterile MHB broth and 0.6uL of kanamycin into two new sterile culture tubes. They were cold in comparison to the other culture tubes and went into the shaker about 15 minutes after.

Assay

- Grow 6ml cultures until OD600 = 0.3
 - $6 \times 18 = 108 \text{ mL}$

- Turn on 28°C water bath
- Determine amount of Z-buffer needed ($0.8\text{ mL} \times 2 \times \#$ of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer ($2.72 \times \text{X mL Z-buffer} = 95.2\text{ }\mu\text{L}$ of BME).
 - 35 mL of Z-buffer (5 blanks)
 - 41 tubes
- Set up reaction tubes with 800 μL Z-buffer, put on lids
- Turn on spec and gather cuvettes
- Once cultures reach $\text{OD}_{600} = 0.3$, place on ice 30 min and put ONPG in water bath
- After cells have incubated on ice, measure OD_{600} of bacterial cultures
- Add 200 μL culture to each reaction tube (add 200 μL culture media to blank tube)
- Add 30 μL 0.1% SDS to each reaction tube
- Add 60 μL CHCl_3 (chloroform) to each reaction tube
 - 2.46 mL
- Vortex reaction pairs on high for 6 secs (time precisely with timer)
- Put in water bath for 10 min
- Prepare repeater pipette with 1M Na_2CO_3 (stop)
- Add 200 μL ONPG in 5 sec intervals (use timer with hours)
 - $200 \times 41 \text{ tubes} = 8200 \text{ }\mu\text{L}$
- Shake gently and watch for yellow
- Stop with 500 μL 1M Na_2CO_3 , record time, vortex at 4 for 10 sec
 - 20.5 mL
- Give all reaction at least 2 hours
- Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and OD_{550} , using blank reaction as blank in spectrophotometer.

Calculations

$$\text{Miller Units} = 1000 \times [(\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (t \times v \times \text{OD}_{600})]$$

Note that “t” is the time of the reaction in minutes, “v” is the volume of the culture used in the assay in mL (i.e., 0.2) and the OD_{600} is that determined for the culture used in each assay. Use spreadsheet.

Data:

Tube	Sample	OD 420	OD 550	OD 600	Start	Start Time (min)	End Time (clock)	End Time (min)	Miller Units	Average	St Dev
1	1A	0.671	-0.008	0.340	5	0.083	0:21:30	21.500	470.4	466.5	5.5
2	1B	0.679	0.003	0.340	5	0.083	0:21:30	21.500	462.6		
3	2A	0.736	-0.006	0.347	10	0.167	0:21:40	21.667	500.3	500.2	0.1
4	2B	0.741	-0.003	0.347	10	0.167	0:21:40	21.667	500.1		
5	3A	0.812	-0.005	0.348	15	0.250	0:21:45	21.750	548.5	542.3	8.7
6	3B	0.790	-0.007	0.348	15	0.250	0:21:45	21.750	536.1		
7	4A	0.559	0.003	0.376	20	0.333	0:22:30	22.500	332.2	330.5	2.4
8	4B	0.569	0.012	0.376	20	0.333	0:22:30	22.500	328.7		
9	5A	0.564	0.013	0.366	25	0.417	0:22:35	22.583	333.6	329.9	5.1
10	5B	0.554	0.014	0.366	25	0.417	0:22:35	22.583	326.3		
11	6A	0.542	0.004	0.371	30	0.500	0:22:40	22.667	325.3	320.0	7.4
12	6B	0.523	0.003	0.371	30	0.500	0:22:40	22.667	314.8		
13	7A	0.476	0.006	0.216	35	0.583	0:40:05	40.083	272.8	268.7	5.8
14	7B	0.476	0.014	0.216	35	0.583	0:40:05	40.083	264.6		
15	8A	0.661	0.008	0.310	40	0.667	0:40:10	40.167	264.2	258.5	8.1
16	8B	0.626	0.004	0.310	40	0.667	0:40:10	40.167	252.8		
17	9A	0.699	0.005	0.323	45	0.750	0:40:15	40.250	270.5	263.2	10.3
18	9B	0.681	0.016	0.323	45	0.750	0:40:15	40.250	255.9		
19	10A	0.675	0.001	0.327	50	0.833	0:15:05	15.083	722.4	737.3	21.1
20	10B	0.708	0.004	0.327	50	0.833	0:15:05	15.083	752.2		
21	11A	0.698	0.001	0.337	55	0.917	0:15:10	15.167	724.9	735.9	15.5
22	11B	0.719	0.001	0.337	55	0.917	0:15:10	15.167	746.8		
23	12A	0.747	0.003	0.347	60	1.000	0:15:15	15.250	750.0	705.9	62.4
24	12B	0.658	0.002	0.347	60	1.000	0:15:15	15.250	661.8		
25	13A	0.798	0.012	0.336	65	1.083	4:45	4.750	3153.4	3096.1	81.1
26	13B	0.768	0.011	0.336	65	1.083	4:45	4.750	3038.8		
27	14A	0.865	0.017	0.342	70	1.167	4:50	4.833	3330.3	3015.8	444.8
28	14B	0.702	0.014	0.342	70	1.167	4:50	4.833	2701.4		
29	15A	0.865	0.009	0.342	75	1.250	4:55	4.917	3386.164	3301.4	119.8
30	15B	0.833	0.015	0.342	75	1.250	4:55	4.917	3216.707		
31	16A	1.007	0.033	0.378	80	1.333	4:30	4.500	3965.121	3562.0	570.1
32	16B	0.793	0.021	0.378	80	1.333	4:30	4.500	3158.939		
33	17A	0.707	0.006	0.316	85	1.417	4:35	4.583	3480.18	2925.5	784.4
34	17B	0.485	0.006	0.316	85	1.417	4:35	4.583	2370.919		
35	18A	0.84	0.007	0.372	90	1.500	4:40	4.667	3513.37	3470.4	60.8
36	18B	0.818	0.006	0.372	90	1.500	4:40	4.667	3427.419		

Results:

