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## Bibliography

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## July 2022

### Friday, July 1, 2022

To Do:

1. ~~Miniprep candidate pKR135-2, pKR135-3, and pKR135-4 colonies~~
2. ~~Look at KRLVS174, KRLVS175, KRLVS177, and KRLVS113 under the microscope with Colby~~

### Results and Data:

N/A.

### Miniprep of pKR135 Mixed Population Candidates from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column with 750uL Buffer PE. Centrifuge 30-60s x3 and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Due to timing, I pelleted the 9 cultures prior to the joint lab meeting and stored the pellets in their tubes in the fridge for later miniprep. Was originally planning on miniprepping them after the meeting, before fluorescent microscopy, however, I wanted to make sure to give myself ample time so that I do not mess anything up, as I would really like to get a single isolated plasmid prep. I think it should be okay in the fridge for the couple more hours. Stored in cloning box '135-2 1 EV' format.

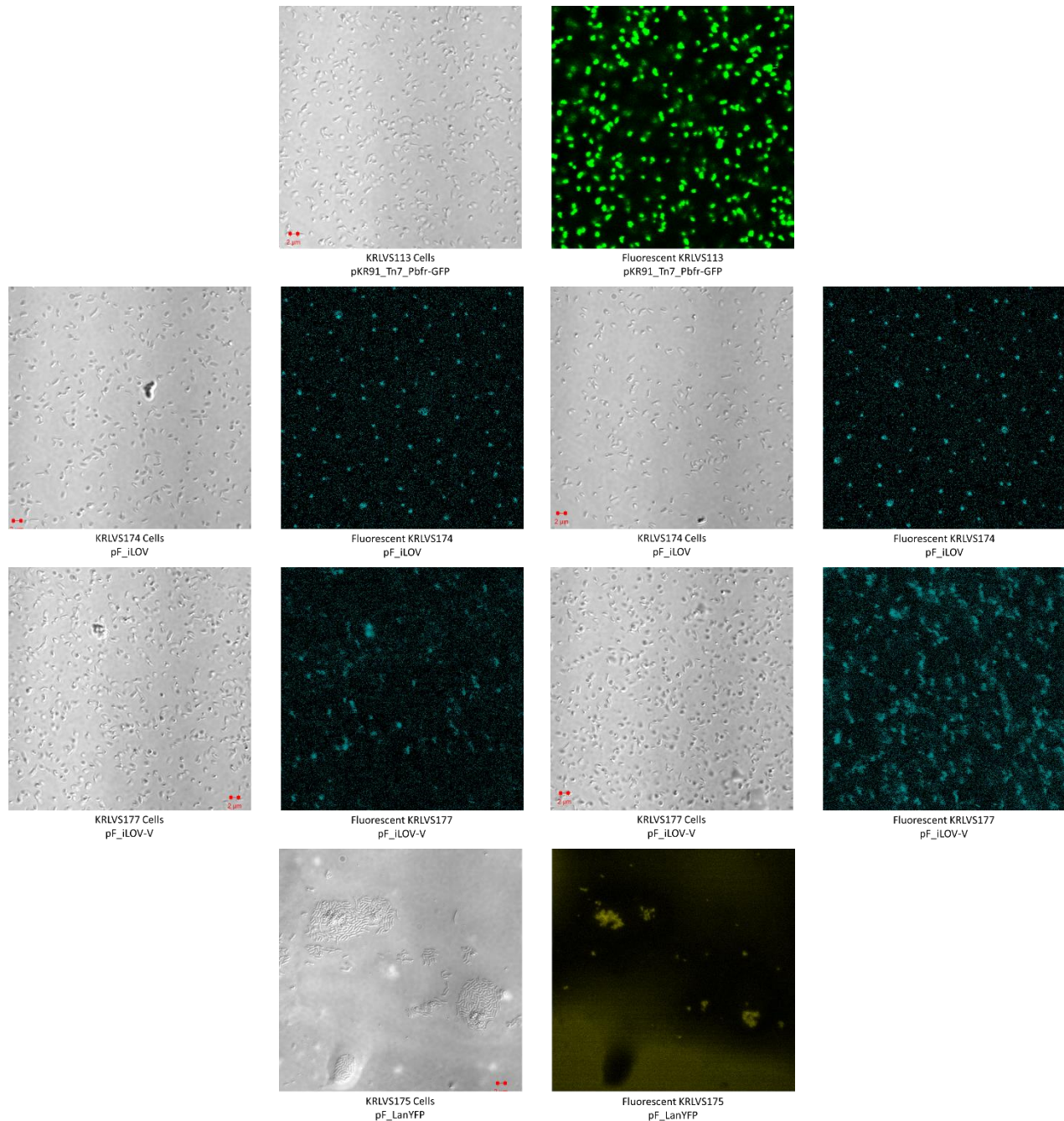
For the fluorescent microscopy, Colby and I will be using poly-L lysine as opposed to an agarose pad as it is a quicker method. The chemical will stick the bacteria directly to the slide, the drawback, and why the Camberg lab doesn't necessarily use, is that the cells will die off quicker, but that does not matter for this application. I grew up plates overnight of each of our strains that we are hoping to look at and we will resuspend the patches in buffer prior to the poly-L lysine treatment.

### iLOV Excitation and Emission Maximums Used

State	Ex $\lambda$	Em $\lambda$	EC ( $M^{-1} cm^{-1}$ )	QY	Brightness	pKa	Maturation (min)	Lifetime (ns)
default	447	497		0.44				

### LanYFP Excitation and Emission Maximums Used

Ex $\lambda$	Em $\lambda$	EC ( $M^{-1} cm^{-1}$ )	QY	Brightness	pKa	Maturation (min)	Lifetime (ns)
513	524	150,000	0.95	142.5	3.5		



Resuspended some of each patch into 20uL 1xPBS. Meanwhile, left cover slides in poly-L lysine for 5-10 minutes. Let cover slides air dry in fume hood. Applied 5uL of resuspended LVS to microscope slide, followed by cover slip, then imaged using GFP protocol adjusted to corrected excitation. Had to re-dilute both iLOV cultures as they were too thick, cells were moving a lot as it seemed the pol-L lysine did not stick as well, so images were a bit blurry but fluorescence was observed. LanYFP was odd throughout the slide, with patches of yellow fluorescence in weird places, potentially agar, however, clearly was not being expressed in each cell of the population. GFP seemed to have the most 'coverage' of fluorescence, but it seemed like iLOV is good too. Will need to be tested quantitatively. LanYFP-V was not ready yet.

## Monday, July 11, 2022

### To Do:

1. ~~Nanodrop candidate pKR143 minipreps~~
2. ~~Run diagnostic restriction digest on candidate pKR143 minipreps~~
3. ~~Run diagnostic gel on candidate pKR143 minipreps~~
4. ~~Nanodrop candidate pKR135 minipreps~~
5. ~~Run diagnostic restriction digest on candidate pKR135 minipreps~~
6. ~~Run diagnostic gel on candidate pKR135 minipreps~~
7. ~~Set up sequencing reactions for candidate pKR143 minipreps~~
8. ~~Set up sequencing reactions for candidate pKR135 minipreps~~

### Results and Data:

Sample	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pkR143-1	276.7	5.535	2.921	1.89	2.41
pkR143-2	423.9	8.479	4.513	1.88	2.37
pkR143-5	353.9	7.079	3.765	1.88	2.38
pKR135-3.1	765.1	15.303	8.328	1.84	2.37
pKR135-3.2	694.4	13.889	7.505	1.85	2.37
pKR135-3.3	637.9	12.757	6.921	1.84	2.35

### Diagnostic Digest of Candidate pKR143 Miniprep w/EcoRI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	pKR143-1	EcoRI, BamHI	2.0	-
2	pKR143-2	EcoRI, BamHI	2.0	-
3	pKR143-3	EcoRI, BamHI	2.0	-
4	pKR143-4	EcoRI, BamHI	2.0	-
5	pKR143-5	EcoRI, BamHI	2.0	-
6	pKR143-6	EcoRI, BamHI	2.0	-
7	Water	EcoRI, BamHI	-	2.0

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H <sub>2</sub> O	15	105
10x Buffer (Cutsmart)	2	14
DNA	(2)	
EcoRI	0.5	3.5
BamHI	0.5	3.5
Total	20.0 (18.0 actual b/c of DNA)	126

3. Add indicated amounts of H<sub>2</sub>O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

### Diagnostic Digest of Candidate pKR135 Miniprep w/KpnI and BamHI

1. Make a reaction table with desired digests:



Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	pKR135-1	KpnI, BamHI	2.0	-
2	pKR135-2	KpnI, BamHI	2.0	-
3	pKR135-3	KpnI, BamHI	2.0	-
4	pKR135-4	KpnI, BamHI	2.0	-
5	pKR135-5	KpnI, BamHI	2.0	-
6	pKR135-6	KpnI, BamHI	2.0	-
7	pKR135-7	KpnI, BamHI	2.0	-
8	pKR135-8	KpnI, BamHI	2.0	-
9	pKR135-9	KpnI, BamHI	2.0	-
10	Water	KpnI, BamHI	-	2.0

2. Set up master mix table:

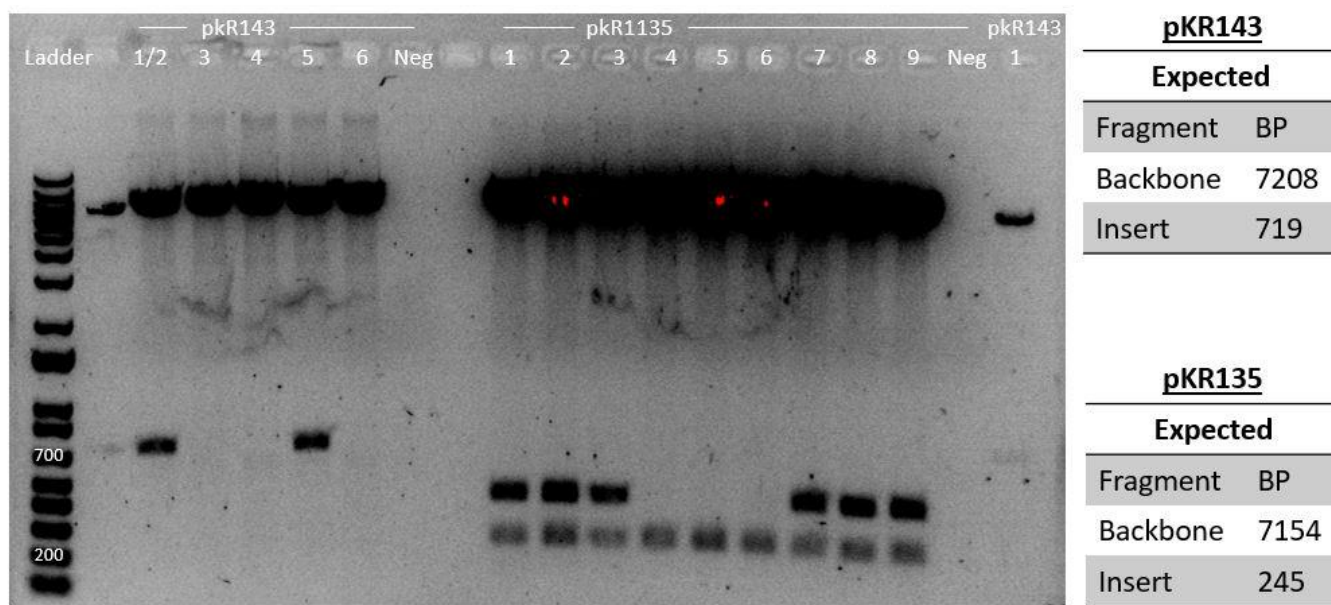
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 11x (uL)
H <sub>2</sub> O	15	165
10x Buffer (Cutsmart)	2	22
DNA	(2)	
KpnI	0.5	5.5
BamHI	0.5	5.5
Total	20.0 (18.0 actual b/c of DNA)	198

3. Add indicated amounts of H<sub>2</sub>O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

### Gel of Diagnostic Digest of Candidate pKR143 and pKR135 Minipreps

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up large gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe dye to gel rig, pour ~120uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 15 uL ladder, and 20 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Diagnostic Digest of Candidate pKR143 and pKR135									
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Ladder		MP <sub>143</sub> -1/2	MP <sub>143</sub> -3	MP <sub>143</sub> -4	MP <sub>143</sub> -5	MP <sub>143</sub> -6	Neg <sub>143</sub>		MP <sub>135</sub> -1
Lane 11	Lane 12	Lane 13	Lane 14	Lane 15	Lane 16	Lane 17	Lane 18	Lane 19	Lane 20
MP <sub>135</sub> -2	MP <sub>135</sub> -3	MP <sub>135</sub> -4	MP <sub>135</sub> -5	MP <sub>135</sub> -6	MP <sub>135</sub> -7	MP <sub>135</sub> -8	MP <sub>135</sub> -9	Neg <sub>135</sub>	MP <sub>143</sub> -1



Accidentally loaded candidate pKR143 1 and 2 on top of each other, due to dimpling in the ladder region. Loaded the last 4uL in the resultant last empty lane, lane 20. Since it was such a small volume compared to the rest, might not be able to see it. If I can, I'll be able to compare to Lane 3 in order to compare and determine what each has.

Sample#	Type	Template	Primer Name	A.	B.	E.	F.
				Template (bp)	Conc. (ng/μl)	<u>DNA Add</u>	<u>H<sub>2</sub>O Add</u>
SS1	Plasmid	pKR135-3.1	KROL44	7399	765.1	0.52	8.92
SS2	Plasmid	pKR135-3.2	KROL44	7399	694.4	0.58	8.86
SS3	Plasmid	pKR135-3.3	KROL44	7399	637.9	0.63	8.81
SS4	Plasmid	pKR143-1	KROL44	7927	276.70	1.45	7.99
SS5	Plasmid	pKR143-2	KROL44	7927	423.90	0.94	8.50
SS6	Plasmid	pKR143-5	KROL44	7927	353.90	1.13	8.31

Tuesday, July 12, 2022

To Do:

1. Drop off sequencing sheet at loading dock (oops!)

### Results and Data:

Kathryn and I agreed that I will go ahead and complete these cloning projects, specifically in regards to pKR143 despite the fact that LanYFP seems to fluoresce oddly (toxic?) in LVS. Simply seems like a good idea to have it since I am already almost done with it. Regardless, with how LanYFP acts, determined that it would not be worth my time to complete any microscopy, this isn't really my project anyways. Otherwise, in meeting, discussed how its *interesting* how all of the third miniprep of the candidate pKR135 prior to retransformation were all correct. Regardless, sent them out for sequencing so we will know (hopefully) tomorrow, and will then transform into LVS on Friday.

Otherwise, discussed the purpose of nanodrop before and after DNase treatment for the RNA samples. After treatment and re-purification, will run on a gel. If I have time tomorrow, then I will do it tomorrow as well. After I have completed this, then I will discuss with Kathryn.

### Wednesday, July 13, 2022

#### To Do:

- ~~1. Analyze sequencing results~~
2. Patch out LVS for electrocompetent cells

#### Results and Data:

pKR135 candidates: All of them were good, mismatches showing in the poor sequencing region, but otherwise all of the insert, tetO sites and such appeared to be quite good. I think the sequence for pKR135-3.3 looked a little worse? Maybe it didn't but I feel it in my heart and pKR135-3.1 looked great so will go ahead with that for the transformation. Need to ask Kathryn about the transformation though, what strain should I be using?

pKR143 candidates: pKR143-1 and -2 both look good. pKR143-5 does not contain the LanYFP-V gene. pKR143-1 does not seem to have the preceding 50 base pairs as I would expect, which is present in the pKR143-2 sample, but the region of bad sequence is literally not in -1, shorter on both sides. As such, I will transform pKR143-2 into LVS rather than -1.

### Thursday, July 14, 2022

#### To Do:

- ~~1. Nanodrop RNA/DNA Samples~~
- ~~2. DNase Treatment and Re-Purify RNA~~

#### Results and Data:

Sample	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
LVS BR-1	935.2	18.704	8.787	2.13	2.47
LVS BR-2	829.4	16.587	8.032	2.07	2.06
LVS BR-3	636.1	12.721	6.301	2.02	2.3
<i>Δrpsu2</i> BR-1	544.1	10.882	5.339	2.04	2.34
<i>Δrpsu2</i> BR-2	510.1	10.203	4.892	2.09	2.45
<i>Δrpsu2</i> BR-3	395.0	7.899	3.873	2.04	2.38

Table 1. Total nucleic acid concentrations.

#### DNase treatment

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube

15. Add 100  $\mu$ L RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

During the last spin, sample 4's,  $\Delta$ rpsu2 BR-1, lid broke off in the centrifuge. Transferred to a new RNase free 1.5mL tube. If there is something wrong with it that may be why. Hopefully it's okay though:(.

Additionally, accidentally used 1.5mL tubes as collection tubes, but given how much liquid was expected to come out, I switched to collection tubes from the kit. As such, next time I do an RNA purification, I will use 2 mL centrifuge tubes for each collection step (so 3x the number of samples).

Labelled	Contents
1 RNA	LVS BR-1
2 RNA	LVS BR-2
3 RNA	LVS BR-3
4 RNA	$\Delta$ rpsu2 BR-1
5 RNA	$\Delta$ rpsu2 BR-2
6 RNA	$\Delta$ rpsu2 BR-3

Spoke with Hannah and Kathryn regarding the pKR135 plasmid and where to move forward with it. It is not clear if this empty vector will be able to go into the triple deletion strain, as this would leave LVS without any bS21 at all. As such, I will be doing a number of electroporations with different strains and a few different plasmids:

Experiment 1: Will put pKR135 into the double deletion strain, LVS  $\Delta$ rpsU1  $\Delta$ rpsU3, KRLVS24. Given that induction was seen with this strain, this will allow us to sus out if there is toxicity from ATc or something to do with the production of bS21 (overproduction causing toxicity).

Experiment 2: Will electroporate pKR135 and pKR30 (pf-nat) into electrocompetent KRLVS153. Trying to get KRLVS153 to lose kanamycin resistance, so will grow up on regular CHAH plates for preparation of the cells. After electroporation will let transformants grow to single colony on Nat plates, then cross-patch onto Kan and Nat plates to confirm if the Kan plasmid has been lost.

Experiment 3: Will electroporate pF (kanamycin resistant) into pKR157 and allow to grow to single colony then cross-patch on Nat and Kan to try to lose the nat resistant plasmid. Will grow the cells on regular CHAH to encourage loss of the plasmid prior to preparation of the electrocompetent cells.

Friday, July 15, 2022

To Do:

1. — Run dishwasher

## Monday, July 18, 2022

### To Do:

1. ~~Put away dishes~~

### Results and Data:

N/A. Worked on Program of Study and other graduate school forms. Also looked for proposal templates or guidelines but couldn't find any specific to the program.

## Tuesday, July 19, 2022

### To Do:

1. ~~Nanodrop RNA~~
2. ~~Run gel of RNA~~
3. ~~cDNA Synthesis~~
4. ~~Start making iron pyrophosphate~~
5. ~~Run dishwasher~~

### Results and Data:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
LVS BR-1	552.4	13.811	6.439	2.14	2.31	RNA
LVS BR-2	333.7	8.343	3.950	2.11	2.31	RNA
LVS BR-3	369.1	9.227	4.348	2.12	2.30	RNA
$\Delta$ rpsu2 BR-1	280.8	7.020	3.319	2.11	2.24	RNA
$\Delta$ rpsu2 BR-2	297.7	7.442	3.313	2.25	2.54	RNA
$\Delta$ rpsu2 BR-3	232.6	5.815	2.717	2.14	2.41	RNA

Sample	ng/uL	RNA	Water
LVS BR-1	552.4	2.72	7.28
LVS BR-2	333.7	4.50	5.50
LVS BR-3	369.1	4.06	5.94
$\Delta$ rpsu2 BR-1	280.8	5.34	4.66
$\Delta$ rpsu2 BR-2	297.7	5.04	4.96
$\Delta$ rpsu2 BR-3	232.6	6.45	3.55

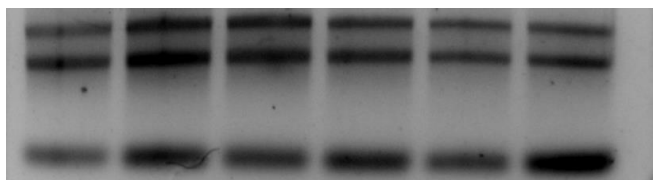
## Gel of LVS and $\Delta$ rpsu RNA Samples

1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x and run on gel.
2. Rinse comb and gel rig with DI water, then ethanol both as well
3. Make fresh agarose gel by adding 1.0g to 100mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
6. Make fresh 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 12 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA), rather than smears.

There seemed to be less loading dye in the last two samples, meant to add an additional 0.5uL but forgot before loading the samples. Oops.

Loading Order of RNA Samples Gel					
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
LVS-1	LVS-2	LVS-3	$\Delta$ rpsu2-1	$\Delta$ rpsu2-2	$\Delta$ rpsu2-3

LVS LVS LVS  $\Delta$ rpsu2  $\Delta$ rpsu2  $\Delta$ rpsu2  
BR-1 BR-2 BR-3 BR-1 BR-2 BR-3



Properly shows 26S, 16S, and tRNA/5SRNA. Omg yay! Look at it! It's beautiful. Kathryn also looked at my concentrations and purity during the lab meeting and demonstrated how they are quite pure and high concentrations, and the gel confirms that there's no degradation much soooo yay I did it!

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
LVS cDNA BR-1	25.2	3.57	56.43
LVS cDNA BR-2	27.4	3.28	56.72
LVS cDNA BR-3	29.8	3.02	56.98
$\Delta$ rpsu2 cDNA BR-1	25.2	3.57	56.43
$\Delta$ rpsu2 cDNA BR-2	26.7	3.37	56.63
$\Delta$ rpsu2 cDNA BR-3	25.1	3.59	56.41

### Generating cDNA (Half Reaction)

- Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	1.5 ul	25 ng/ul
RNase-free water	up to 13.5 ul	

- To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes. Total volume of cDNA and water is 13.5 uL.
- Add the 1.5 uL (NS)<sub>5</sub> oligo to the tubes.
- Incubate using program JSScDNA1 in the thermocycler:

Step	Temp	Time
1	70°C	10'
2	25°C	10'
3	4°C	hold

- Prepare master mix at 1.5x the number of reactions.

Component	Final Concentration	Volume	x7.5
5X 1st strand buffer	1x	6	45
RNase-free water		2.88	21.6
100 mM DTT	10 mM	3	22.5
10 mM dNTPs	0.5 mM	1.5	11.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	12.23

- Aliquot 15 ul of master mix into each PCR tube from the first reaction (total volume now 30ul)
- Incubate using program JSScDNA2

Step	Temp	Time
------	------	------

1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold

Stopped here as I could not locate the Acid/Bases box. Not sure where it's held. Strip tube in my RNA box in the -80C.

### Wednesday, July 20, 2022

#### To Do:

- ~~1. Make patch plate of LVS~~
- ~~2. Make patch plate of KRLVS24~~
- ~~3. Filter sterilize 2.5% iron pyrophosphate~~
- ~~4. Supplement MHB~~
- ~~5. Put away dishes~~

#### Results and Data:

For the patch plates, I made two for KRLVS24, hopefully so that I can make some single use aliquots with the second just in case I have to redo the electroporation at all. However, if I end up needing more cells then I will use that for the electrocompetent prep. I also added two single use aliquots of LVS to the single LVS plate as I was worried about there being enough cells, since there was only enough for two reactions the first time I made electrocompetent cells.

I grew both on no antibiotics plates, LVS for obvious reasons, and KRLVS24 because I saw no indication that it was antibiotic resistant in the strains list.

### Thursday, July 21, 2022

#### To Do:

- ~~1. Make patch plate of KRLVS153~~
- ~~2. Make patch plate of KRLVS157~~
- ~~3. Make electrocompetent LVS~~
- ~~4. Make electrocompetent KRLVS24~~
- ~~5. Electroporate LVS with pKR143~~
- ~~6. Electroporate KRLVS24 with pKR135~~
- ~~7. Continue cDNA generation~~
- ~~8. Purify cDNA~~
- ~~9. Nanodrop cDNA~~
- ~~10. Move plasmid stocks into plasmid boxes in -20 and -80~~

#### Results and Data:

First, I made electrocompetent cells, both LVS and KRLVS24. I decided to make them day of as, it shouldn't particularly matter for the LanYFP-V plasmid, nor really the KRLVS24 since there is no plasmid exchange. However, I am following the protocol that Hannah used for each of the EV electroporation experiments, and she made the electrocompetent cells the day of.

### Preparing Electrocompetent LVS and KRLVS24 Cells

1. Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend
2. Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
3. Spin for 3 minutes at 10,000 rpm
4. Remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
5. Repeat 3x-5x in 10% sucrose



6. After final spin, remove all supernatant.
7. 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  $\mu$ L at a time. It should be about equal amounts of cells as sucrose.
8. For any extra EC cells, aliquot  $\sim 110 \mu$ L / sterile tube (enough for 2 electroporations) and freeze at  $-80^{\circ}\text{C}$

### Electroporating pKR135 into electrocompetent KRLVS24

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - 3  $\mu$ L of pKR135 plasmid DNA
  - 50  $\mu$ L electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program
5. Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
6. Recover cells for 2 hours, shaking at  $37^{\circ}\text{C}$
7. Plate on CHAH-Nat plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
  - a. Plated 10  $\mu$ L and 100  $\mu$ L of each, straight from recovery tube
8. Incubate plates at  $37^{\circ}\text{C}$  for 3 days (or until single colonies appear)

Volume of plasmid used, recovery time, and plating volume based on Hannah's previous experiments. These parameters were specifically used in the plasmid exchange electroporation which I will be completing tomorrow, but I thought that it was potentially good, or else neutral, to keep the parameters the same for the EV plasmid experiments.

Yay, no arcing.

### Electroporating pKR143 into electrocompetent LVS

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - 3  $\mu$ L of pKR143 plasmid DNA
  - 50  $\mu$ L electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program
5. Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
6. Recover cells for 2 hours, shaking at  $37^{\circ}\text{C}$
7. Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
  - a. Plated 20  $\mu$ L and 200  $\mu$ L of each, straight from recovery tube
8. Incubate plates at  $37^{\circ}\text{C}$  for 3 days (or until single colonies appear)

Used these parameters in order to keep with the parameters from the previous fluorescent protein transformation, with pKR142. Also did not arc.

For both electroporations, only did one negative control plate with the higher volume of sample due to number of plates, just in case. Additionally, if something grows, we don't really need to grow two plates to see if they can grow or if there's contamination.

### Generating cDNA (Half Reaction) Cont'd.

8. Remove RNA from sample by degrading with sodium hydroxide:



- a. Add 10 ul of 1N NaOH
- b. Incubate 65°C for 30'
- c. Neutralize with 10 ul of 1N HCl
- d. Final volume is 50 ul
9. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
10. Elute in 60 ul of 0.1x EB
11. Check concentration by Nanodrop
12. Store cDNA at -80°C

### cDNA Purification with PCR Purification Kit

1. Add 300 uL of Buffer PB to each 60 uL cDNA reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 60uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Nanodropped samples with 2 uL. I was initially surprised by the concentration of the cDNA, but I spoke with someone else who does cDNA generation and this is around where they get. Regardless, I will speak to Hannah or Kathryn over whether this is standard.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
LVS cDNA BR-1	25.2	0.503	0.280	1.80	2.14
LVS cDNA BR-2	27.4	0.549	0.305	1.80	2.20
LVS cDNA BR-3	29.8	0.597	0.337	1.77	2.16
$\Delta$ rpsu2 cDNA BR-1	25.2	0.504	0.279	1.81	1.95
$\Delta$ rpsu2 cDNA BR-2	26.7	0.534	0.298	1.79	2.21
$\Delta$ rpsu2 cDNA BR-3	25.1	0.503	0.275	1.83	2.23

Friday, July 22, 2022

#### To Do:

1. ~~Dilute cDNA samples for qRT-PCR~~
2. ~~qRT-PCR of cDNA samples~~

### Results and Data:

Patch plates of KRLVS157 and KRLVS153 have not grown enough to make competent cells. This was an issue with KRLVS157 when I was doing the inducible experiments, however I used a large bit of ice from my glycerol stock (since I'm out of single use aliquots) and thought it would be fine. It was not. Otherwise, I wasn't sure if KRLVS153 had a growth defect, however it makes sense that it would since it seems all the triple mutants have one. I will let them grow another day and do the electroporation tomorrow instead.

Spoke with Kathryn about how to do qRT-PCR specifically in our lab. Discussed what primers to use, which will be KROL343/344 for the 5'UTR region, KROL63/64 for the tul4 region (the control for normalization), and, after speaking with Aisling and looking at her primer efficiencies, KROL504/505 for

downstream of the *rpsu2* gene. Additionally, Hannah showed me how to use the plate spinner and the LightCycler.

Diluted cDNA samples to 1.5 ng/uL as needed for qRT-PCR according to the following table:

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
LVS cDNA BR-1	25.2	3.57	56.43
LVS cDNA BR-2	27.4	3.28	56.72
LVS cDNA BR-3	29.8	3.02	56.98
$\Delta$ rpsu2 cDNA BR-1	25.2	3.57	56.43
$\Delta$ rpsu2 cDNA BR-2	26.7	3.37	56.63
$\Delta$ rpsu2 cDNA BR-3	25.1	3.59	56.41

### qRT-PCR of LVS and $\Delta$ rpsu2 cDNA

- Each experiment will need at least one test primer and one control primer for each sample
  - KROL343/344 and KROL504/505 as test primers, specifically to amplify the 5'UTR and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying *tlu4*.
- Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*3=18 reactions)
- Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	245.0 uL
5uM primer set	1 uL	24.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	171.5 uL
Total:	20 uL	465.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5uL of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20 uL of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsu2</i>
C	KROL63/64 <i>tlu4</i>

1	2	3	4	5	6
LVS 1	LVS 2	LVS 3	$\Delta$ rpsu2 1	$\Delta$ rpsu2 2	$\Delta$ rpsu2 3

Loaded 96-well plate according to the following table:

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

A	A1	B1	C1
B	A2	B2	C2
C	A3	B3	C3
D	A4	B4	C4
E	A5	B5	C5
F	A6	B6	C6

Saturday, July 23, 2022

**To Do:**

- ~~1. Make electrocompetent KRLVS153~~
- ~~2. Make electrocompetent KRLVS157~~
- ~~3. Electroporate KRLVS153 with pKR135 and pKR30~~
- ~~4. Electroporate KRLVS157 with pF~~

**Results and Data:**

**Preparing Electrocompetent KRLVS153 and KRLVS157 Cells**

1. Scrape up entire plate of cells into 400  $\mu$ L of sterile 10% sucrose and resuspend
2. Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
3. Spin for 3 minutes at 10,000 rpm
4. Remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
5. Repeat 3x-5x in 10% sucrose
6. After final spin, remove all supernatant.
7. 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  $\mu$ L at a time. It should be about equal amounts of cells as sucrose.
8. For any extra EC cells, aliquot  $\sim 110 \mu$ L / sterile tube (enough for 2 electroporations) and freeze at  $-80^{\circ}\text{C}$

**Electroporating pKR135 and pKR30 into electrocompetent KRLVS153**

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - 3  $\mu$ L of pKR135 and pKR30 plasmid DNA
  - 50  $\mu$ L electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program
5. Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
6. Recover cells for 2 hours, shaking at  $37^{\circ}\text{C}$
7. Plate on CHAH-Nat plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
  - a. Plated 10  $\mu$ L and 100  $\mu$ L of each, straight from recovery tube
8. Incubate plates at  $37^{\circ}\text{C}$  for 3 days (or until single colonies appear)

**Electroporating pF into electrocompetent KRLVS157**

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at  $37^{\circ}\text{C}$
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
  - 3  $\mu$ L of pF plasmid DNA
  - 50  $\mu$ L electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program

5. Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
6. Recover cells for 2 hours, shaking at 37°C
7. Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
  - a. Plated 10 uL and 100 uL of each, straight from recovery tube
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

Sunday, July 24, 2022

**To Do:**

- ~~1. Patch single colonies of pKR143 onto CHAH+Kan plates~~
- ~~2. Patch single colonies of pKR135 onto CHAH+Nat plates~~
- ~~3. Check Hannah's plates in the Burtin Lab incubator to see if there are colonies~~

**Results and Data:**

Pulled out Hannah's plates at 3:20 pm.

Electroporation:

pKR135

Did not see any colonies on the 10 uL plate, but there were quite a few on the 100 uL plate. They were kind of group to one side, but there were some larger, well isolated ones.

pKR143

Pretty much a lawn on the 200uL plate, so used the 20uL plate to make patches. The colonies, again, grouped to one side so I wonder if I had the plates on the edge of the base of the Bunsen burner when they were drying. The colonies were pretty grouped and not super well isolated, but I took what were the most isolated.

Monday, July 25, 2022

**To Do:**

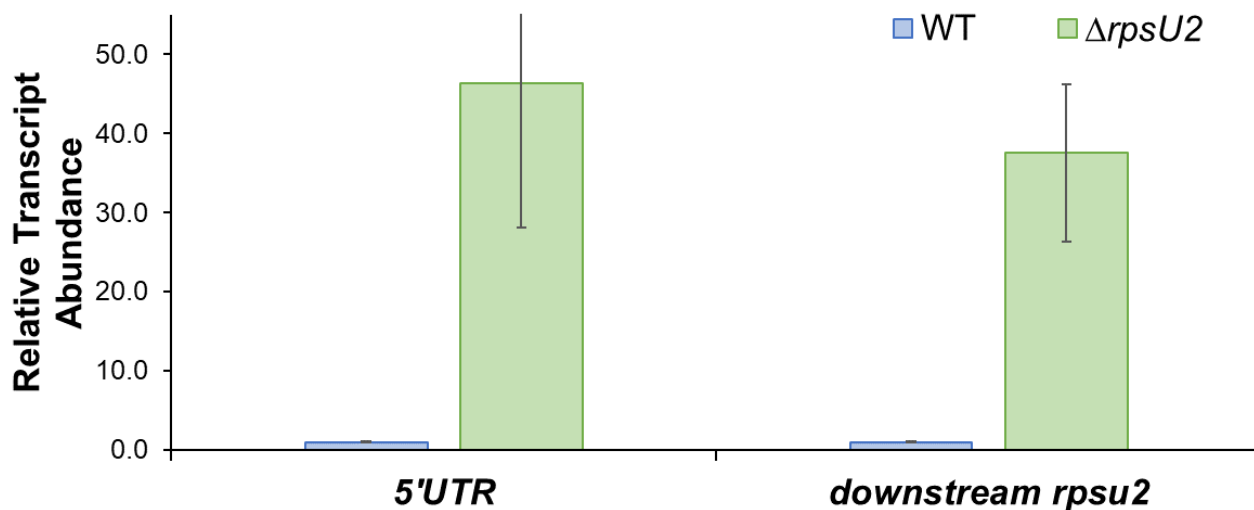
1. ~~Make permanent stocks of LVS + pKR143~~
2. ~~Make permanent stocks of KRLVS24 + pKR135~~

**Results and Data:**

Looked through my qRT-PCR data with Hannah. She checked that all the formulas were correct and explained the different parts of the worksheet to me.  $\Delta C_t$  are the normalized values based on *tul4*. 'Normalizing' WT to 1 using 1.8 (as each cycle should double DNA but doesn't ever quite reach that point, 1.8x each cycle is the value) with negative exponents in order to reduce back from the 40 cycles to the amount of DNA in the first.

The standard deviation for the technical replicants shouldn't be over 0.1, shaded some which were. In red, they couldn't be corrected to 0.1 by removing the outliers. For the two shaded red, removed the outlier as compared to the data for the first replicate which was pretty perfect. I recalled that I did have less master mix than I was expecting at some point during the set-up. I probably should have redone it, but I didn't because I am DUMB. But anyways, that's probably why those two are so off from the rest. I will discuss with Kathryn, but I kind of want to redo it.

Looked at Aisling's and Dan's data, and my qRT-PCR showed a several times larger difference between the relative transcript abundance of  $\Delta rpsu2$  as compared to WT.



**Freezing and Storing Permanent Strains**

1. Per strain, label 2 cryotubes with strain number. Include LVS, strain number, genotype, date.
2. Add 200 uL sterile 75% glycerol to each tube (2 per strain).
3. In a sterile 2 mL tube (1 per strain), add 400 uL of MHB
4. Resuspend patch (all of what you have) in MHB to homogeneity
5. Correct volume to 1700 uL (add 2x 650 uL MHB)
6. Transfer 800 uL to each cryotube (final volume should be 1 mL)
7. Vortex cryotube
8. Quickly spin (mini-fuge) to get liquid to the bottom of the tube
9. Freeze at -80°C in appropriate strain box

Spoke with Kathryn and ultimately, she sees no reason to repeat this experiment again. Instead, next I will be looking at the strains which Aisling and Dan were working on. These strains utilize different combinations of the *rpsU2* and *tul4* promoter/5'UTR. The purpose of this is in order to isolate which component of the gene bS21-2 is interacting with in order to regulate its own expression. These strains are designed to have the downstream primer only at the native locus, *LacZ* is only in the Tn7 site, and 5'UTR is at both the native and Tn7 location.

I confirmed the strain numbers with Aisling and determined that two of the strains were not created, as they were having difficulty creating the plasmid. As such, I will work on creating those strains alongside the following experiments to look at the relative transcript abundance through qRT-PCR. Experiment 1 will be completed in biological triplicate, however experiment 2 will be completed in biological duplicate in order to work up to experiment 3 (and eventually 4), which will be completed in biological triplicate.

Number	KRLVS	Promoter	UTR	rpsU2 status
1	KRLVS149	<i>rpsU2</i>	<i>rpsU2</i>	+
2	KRLVS148	<i>rpsU2</i>	<i>rpsU2</i>	-
3	TBD	<i>rpsU2</i>	<i>tul4</i>	+
4	TBD	<i>rpsU2</i>	<i>tul4</i>	-
5	KRLVS151	<i>tul4</i>	<i>rpsU2</i>	+
6	KRLVS150	<i>tul4</i>	<i>rpsU2</i>	-
7	KRLVS112	<i>tul4</i>	<i>tul4</i>	+
8	KRLVS111	<i>tul4</i>	<i>tul4</i>	-

Experiment 1:		Experiment 3:	
Numbers	Goal	Numbers	Goal
1, 2	Confirm AM and DF results	1, 2, 5, 6	If there is a difference in <i>rpsU2</i> transcript due to the <i>rpsU2</i> UTR
Experiment 2:		Experiment 4:	
Numbers	Goal	Numbers	Goal
1, 2, 7, 8	Confirm lack of impact on <i>tul4</i>	1, 2, 3, 4	If there is a difference in <i>rpsU2</i> transcript due to the <i>rpsU2</i> promoter

**Note for Future Experiment.** After I have these qRT-PCR results, I will use these strains in a  $\beta$ -galactosidase assay in order to examine the protein abundance via activity (due to the *LacZ* fusion). Eventually, I will look at *rpsU1*, *rpsU2*, and *rpsU3* in the context of  $\Delta rpsU1$  or  $\Delta rpsU3$  due to some indication that these homologs also interact in the regulation of bS21-2.

Tuesday, July 26, 2022

**To Do:**

1. ~~Check electroporation of KRLVS153 + pKR135~~
2. ~~Check electroporation of KRLVS153 + pKR30~~
3. ~~Check electroporation of KRLVS157 + pF~~

**Results and Data:**

Electroporations: Did not see single colonies yet, of which I am not surprised given that triple mutant strain tends to grow poorly. Will ask Hannah how long it took single colonies to grow on her plates for either KRLVS153 or KRLVS157, but I am expecting that they took longer, maybe five days? Did see a couple colonies on the negative plate which is obviously very concerning, but I am hoping that there will be very many more colonies on the electroporation plates. That said, given that the other plates were completely clear, I would suspect that the contaminant would show up at the same time on all of the plates, but it was only on the negative so..

## Wednesday, July 27, 2022

## To Do:

1. ~~Streak out KRLVS148 and KRLVS149 to single colony~~
2. ~~Patch out KRLVS148 and KRLVS149 for freezing down~~

**Results and Data:**

Struck for isolation on individual CHAH+Kan plates. Grew patches on same CHAH+Kan plate.

## Thursday, July 28, 2022

## To Do:

1. ~~Check and crosspatch electroporation of KRLVS153 + pKR135~~
2. ~~Check and crosspatch electroporation of KRLVS153 + pKR30~~
3. ~~Check and crosspatch electroporation of KRLVS157 + pF~~
4. ~~Streak out KRLVS148, KRLVS149, KRLVS111, and KRLVS112 to single colony~~
5. ~~Ligation of gel purified pKR122 sample and backbone~~
6. ~~Transformation of ligation of pKR122 sample~~

**Results and Data:**

Received restricted and gel purified *PrpsU2* and *tul4* 5'UTR PCR amplified gBlock fragment and restricted/gel purified pKR68 backbone from Aisling, completed on 2/03/2022. Will attempt to ligate and transform. Her and Dan were having a lot of trouble getting this plasmid, however, Hannah believes that they kept trying from the first gel purification. Additionally, Dan was having difficulty ligating. I will try this out, and if it does not work, then begin from the beginning. Kathryn asked me to double check that we were using the correct restriction sites, which should be KpnI and NotI based on the plasmid map, which is what is stated in Aisling's notebook as what they used.

**Ligation of *PrpsU2 tul4* 5'UTR PCR from gBlock with pKR68 backbone**

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

Tube	Insert	Backbone
1	KpnI, NotI dig., pur. <i>PrpsU2:tul4</i> 5'UTR PCR	KpnI, NotI digested, purified pKR68
2	-	KpnI, NotI digested, purified pKR68

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H <sub>2</sub> O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6.0
Insert	4.0	-	-
Backbone	2.0	2.0	6.0
Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48.0

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. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H<sub>2</sub>O (\_\_\_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.

- Place on bench for 10 minutes.

### Transformation of *PrpsU2 tul4* 5'UTR Ligation into *E. coli*

- Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.
  - Positive control: pF; Backbone ligation: KpnI-NotI digested pKR68

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pF	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pKR68 (digested)	8 uL	10 uL, 200 uL	2
4	Ligation 1	pKR122	8 uL	10 uL, 200 uL	2
Total number of plates					8

- Check to be sure you have enough LB-Kan plates. 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.

Crosspatches: Picked mostly small colonies, but a few large colonies from each transformation and cross-patched 6 colonies. Scraped up entire colony with stick, patched onto antibiotic plate containing the antibiotic from the desire plasmid cassette, then on the opposing antibiotic plate. Hannah suggested I will likely have to streak out patches to single colony again in order to encourage the loss of the other plasmid. Additionally, stated it might take two days for the EV/30 plasmid patches to show on Kan, and the pF to show on Nat.

Friday, July 29, 2022

#### To Do:

- ~~Make overnights of pKR122 in *E. coli*~~
- ~~Check crosspatches~~
- ~~Streak out KRLVS148, KRLVS149, KRLVS150 and KRLVS151 to single colony~~
- ~~Make CHAH + Kan plates~~

#### Results and Data:

Only had two colonies from transformation so, I made two overnights in 5mL LB + 50 ug/mL Kan. Checked crosspatches, given how late I did them in the day yesterday and their slow growth, I am not surprised that there is not much growth yet. From my brief glance, it does look as though they all grew on both antibiotic plates, so tomorrow when I check to make sure I will streak out the plates to single colony in my continued effort to make them lose their plasmids.



## Reagents

### CHAH+Kan

To a 1L flask add:

30.6g of cystine heart agar

300mL of ddiH<sub>2</sub>O (type I)

stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)

Autoclave on 30' liquid cycle, filling the water bin up to the height of the media

Add 2% hemoglobin to water bath, after autoclave add CHA as well

Using sterile technique, pour hemoglobin into CHA

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

## Saturday, July 30, 2022

### To Do:

1. ~~Miniprep pKR122~~
2. ~~Patch out single colonies of KRLVS148 and KRLVS149 in triplicate~~
3. ~~Wrap Hannah's transformation plates in parafilm and put in fridge~~
4. ~~Run dishwasher~~
5. ~~Make CHAH+Kan plates AGAIN because I can't COUNT~~
6. ~~Streak crosspatches to single colony on desired antibiotic plate~~

## Results and Data:

Overnights did not grow up, which I kind of expected because when I was collecting the colonies it seemed like the colonies were maybe just marks on the bottom of the plate, or contamination on the bottom of the plate. As such, I will start over from the PCR step and we'll go from there. Jk. I was looking through Aisling's notebook and she used PIR cells for her ligation so I guess I'll use the correct competent cells and go from there. End me.

## Reagents

### CHAH+Kan

To a 1L flask add:

30.6g of cystine heart agar

300mL of ddiH<sub>2</sub>O (type I)

stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)

Autoclave on 30' liquid cycle, filling the water bin up to the height of the media

Add 2% hemoglobin to water bath, after autoclave add CHA as well

Using sterile technique, pour hemoglobin into CHA

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

Crosspatch: all grew on both antibiotic plates. For single colony plates, I chose to streak the patch from the plate with the desired antibiotic resistance which looked comparably less on the antibiotic plate where we want susceptibility. Aka KRLVS153+pKR135/pKR30 were struck to single colony on Nat (based on the patch which grew much less on its Kan), and KRLVS157+pF was struck to single colony on Kan.

## Sunday, July 31, 2022

### To Do:

1. ~~Resuspend patches of KRLVS148 and KRLVS149 and grow up cultures for RNA purification~~
2. ~~Put away dishes~~
3. ~~Make 2.5% iron pyrophosphate~~
4. ~~RNA purification~~

~~5. Nanodrop RNA samples~~

~~6. Patch out single colonies of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 in duplicate~~

## **Results and Data:**

### **Setting Up KRLVS148 and KRLVS149 Cultures for RNA Purification**

1. Scrape each triplicate patch of KRLVS148 and KRLVS149 into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (50uL of sample in 950uL of MHB)
3. Calculate volume to add to tubes of 8 mL MHB for an OD of 0.08 for KRLVS149 and 0.1 for KRLVS148 (calculate for an OD of 0.1 or 0.13 to account for error)
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

RNA Cultures OD's				
	0 hours	2 hours	4 hours	4.6 hours
148-1	0.124	0.159	0.256	0.305
148-2	0.081	0.122	0.196	0.220
148-3	0.109	0.181	0.284	0.325
149-1	0.102	0.156	0.286	0.335
149-2	0.106	0.167	0.305	0.363
149-3	0.102	0.162	0.313	0.383

I calculated for 30% more OD than wanted, as suggested by Hannah, which worked for KRLVS148 (drpsU2) almost perfectly (I knew the second replicate would be a little low but it shouldn't have been THAT low), but obviously was too high for KRLVS149 (rpsU2 +). Tomorrow I will calculate for 0.9 OD for the rpsU2 + cells.

Additionally, I set up my centrifuge tubes and culture tubes for the next two days. I set them in the fridge, obviously, because MHB.

### **RNA Purification of KRLVS148 and KRLVS149**

1. Pellet 1.8mLx2 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through in phenol and methanol waste
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min

18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

My pellets seemed comparably smaller as compared to last time, however, that could be because, I believe, I spun down in 1.5 mL tubes as opposed to 2 mL tubes as I did this time (because I read the protocol better or something lol). However, given the yield I do believe it is possible that they were in fact smaller than last time. Otherwise, nothing notable happened during the purification aside from the cap snapping on sample 6, guess at max speed you really can't just turn the lids inwards. Oh well, noted.

Stored total nucleic acids in -80C freezer in new RNA box. Labelled with the date on the side and, on the lid, the following:

Labelled	Contents
1 RNA	KRLVS148-1
2 RNA	KRLVS148-2
3 RNA	KRLVS148-3
4 RNA	KRLVS149-1
5 RNA	KRLVS149-2
6 RNA	KRLVS149-3

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	539.8	10.795	5.250	2.06	2.31
KRLVS148-2	546.0	10.921	5.156	2.12	2.36
KRLVS148-3	627.8	12.556	6.119	2.05	2.45
KRLVS149-1	844.4	16.888	7.999	2.11	2.46
KRLVS149-2	696.1	13.923	6.765	2.06	2.30
KRLVS149-3	603.6	12.071	5.644	2.14	2.30

## Reagents

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

## August 2022

Monday, August 1, 2022

### To Do:

1. ~~Resuspend patches of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 for RNA purification~~
2. ~~Make patches of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
3. ~~DNase treatment of 7/31/22 RNA samples~~
4. ~~Second RNA purification of 7/31/22 RNA samples~~
5. ~~Nanodrop 7/31/22 RNA samples~~
6. ~~Prepare aliquots of 7/31/22 RNA samples for cDNA synthesis and gel~~
7. ~~Run RNA gel of 7/31/22 RNA samples — will do either tomorrow or Wednesday~~
8. ~~RNA purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
9. ~~Nanodrop KRLVS148, KRLVS149, KRLVS111, and KRLVS112 RNA samples~~
10. Filter sterilize 2.5% iron pyrophosphate
11. Supplement MHB
12. Check Aisling's plates

### Results and Data:

#### DNase treatment of KRLVS148 and KRLVS149

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube
15. Add 100 uL RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

Again, nothing particularly notable happened during this RNA purification, except I dropped sample 6 after the first elution but it appears to have been fine. Just in the centrifuge, not on the floor or anything. Additionally, nanodrop'd the pure RNA:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230	Type
KRLVS148-1	382.1	7.643	3.563	2.14	2.43	RNA
KRLVS148-2	428.6	8.572	4.043	2.12	2.45	RNA
KRLVS148-3	390.5	7.810	3.655	2.14	2.48	RNA
KRLVS149-1	548.5	10.971	5.155	2.13	2.42	RNA
KRLVS149-2	457.1	9.143	4.287	2.13	2.51	RNA
KRLVS149-3	410.8	8.215	4.065	2.02	1.85	RNA

It could be better as for the purity but whatever, I guess. I set up my normalized samples for the RNA gel and cDNA and placed them in my RNA box. Originally was going to run gel today, but decided on later. Did the following dilutions for the gel:

Sample	ng/uL	RNA	Water
KRLVS148-1	382.1	3.93	6.07
KRLVS148-2	428.6	3.50	6.50
KRLVS148-3	390.5	3.84	6.16
KRLVS149-1	548.5	2.73	7.27
KRLVS149-2	457.1	3.28	6.72
KRLVS149-3	410.8	3.65	6.35

And the following dilutions for the cDNA generation:

Sample	ng/uL	RNA	Water
KRLVS148-1	382.1	7.85	5.65
KRLVS148-2	428.6	7.00	6.50
KRLVS148-3	390.5	7.68	5.82
KRLVS149-1	548.5	5.47	8.03
KRLVS149-2	457.1	6.56	6.94
KRLVS149-3	410.8	7.30	6.20

### Setting Up KRLVS148, KRLVS149, KRLVS111, KRLVS112 Cultures for RNA Purification

1. Scrape each duplicate patch of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (50uL of sample in 950uL of MHB)
3. Calculate volume to add to tubes of 8 mL MHB for an OD of 0.08 for KRLVS149 and KRLVS112 and 0.1 for KRLVS148 and KRLVS111 (calculate for an OD of 0.095 or 0.13 to account for error)
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

RNA Cultures OD's				
	0 hours	2 hours	4 hours	5 hours
148-1	0.106	0.153	0.192	0.222
148-2	0.118	0.175	0.258	0.287
149-1	0.081	0.167	0.270	0.354
149-2	0.087	0.182	0.300	0.390
111-1	0.113	0.126	0.185	0.216
111-2	0.121	0.153	0.234	0.274
112-1	0.079	0.140	0.258	0.334
112-2	0.079	0.128	0.237	0.309

Wowee! These cells sure do not want to grow! If this is a persistent issue, with my second repetition I might shoot for a calculated OD of 0.15.

### RNA Purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112

1. Pellet 1.3mLx3 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min

4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

I took a bit longer than normal to transfer the supernatant to the ethanol and I noticed what appeared to be sticky strings, which I assume was cell gunk, even though I couldn't see it after I spun them. I am worried about protein contamination, next time I definitely will not take so long! I hadn't ever seen that before. Stored total nucleic acids in -80C freezer in new RNA box. Labelled with the date on the side and, on the lid, the following:

Labelled	Contents
1 RNA	KRLVS148-1
2 RNA	KRLVS148-2
3 RNA	KRLVS149-1
4 RNA	KRLVS149-2
5 RNA	KRLVS111-1
6 RNA	KRLVS111-2
7 RNA	KRLVS112-1
8 RNA	KRLVS112-2

I nandrop'd the samples of total nucleic acids after the RNA purification. There doesn't seem to be a lot of protein contamination, as I expected, unless I'm mixing up the ratios. There is, however, a lot of phenol/ethanol contamination. Dang! Hopefully I can get that out in the second purification.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	608.5	12.171	5.830	2.09	2.36
KRLVS148-2	805.3	16.107	7.680	2.10	2.41
KRLVS149-1	976.9	19.537	9.291	2.10	2.50
KRLVS149-2	909.7	18.195	8.820	2.06	2.46
KRLVS111-1	542.9	10.858	5.131	2.12	2.23
KRLVS111-2	558.0	11.160	5.312	2.10	2.36
KRLVS112-1	818.0	16.359	7.975	2.05	2.07
KRLVS112-2	690.9	13.817	7.157	1.93	2.59

Aisling's plates had no growth as expected.

## Reagents

### Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

### 2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

Tuesday, August 2, 2022

#### To Do:

- ~~1. Resuspend patches of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 and grow for RNA purification~~
- ~~2. DNase treatment of 8/1/2022 RNA samples~~
- ~~3. Second RNA purification of 8/1/2022 RNA samples~~
- ~~4. Nanodrop 8/1/22 RNA samples~~
- ~~5. Prepare aliquots of 8/1/22 RNA samples for cDNA synthesis and gel~~
- ~~6. RNA purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
- ~~7. Nanodrop KRLVS148, KRLVS149, KRLVS150, and KRLVS151 RNA samples~~
- ~~8. Check electroporation single colony plates and crosspatch~~

## Results and Data:

### DNase treatment of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 RNA Samples

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube
15. Add 100 uL RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

Nothing exciting happened during the second purification of these samples. I nanodrop'd the samples afterwards:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	398.9	7.979	3.729	2.14	2.43
KRLVS148-2	438.9	8.779	4.211	2.08	2.01
KRLVS149-1	379.4	7.587	3.583	2.12	2.27
KRLVS149-2	426.5	8.529	4.001	2.13	2.32
KRLVS111-1	336.7	6.734	3.218	2.09	2.06
KRLVS111-2	301.3	6.026	2.855	2.11	2.24
KRLVS112-1	382.9	7.658	3.585	2.14	2.22
KRLVS112-2	407.9	8.157	3.774	2.16	2.42

Additionally, I set up normalized aliquots of the RNA for my gel and cDNA synthesis, according to the following tables:

Sample	ng/uL	RNA	Water
KRLVS148-1	398.9	3.76	6.24
KRLVS148-2	438.9	3.42	6.58
KRLVS149-1	379.4	3.95	6.05
KRLVS149-2	426.5	3.52	6.48
KRLVS111-1	336.7	4.46	5.54
KRLVS111-2	301.3	4.98	5.02
KRLVS112-1	382.9	3.92	6.08
KRLVS112-2	407.9	3.68	6.32

Sample	ng/uL	RNA	Water
KRLVS148-1	398.9	7.52	5.98
KRLVS148-2	438.9	6.84	6.66
KRLVS149-1	379.4	7.91	5.59
KRLVS149-2	426.5	7.03	6.47
KRLVS111-1	336.7	8.91	4.59
KRLVS111-2	301.3	9.96	3.54
KRLVS112-1	382.9	7.83	5.67
KRLVS112-2	407.9	7.35	6.15

### Setting Up KRLVS148, KRLVS149, KRLVS150, KRLVS151 Cultures for RNA Purification

1. Scrape each triplicate patch of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (50uL of sample in 950uL of MHB)
3. Calculate volume to add to tubes of 8 mL MHB for an OD of 0.08 for KRLVS149 and KRLVS151, and 0.1 for KRLVS148 and KRLVS150 (calculate for an OD of 0.1 or 0.13 to account for error)
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

RNA Cultures OD's				
	0 hours	2 hours	4 hours	5 hours
148-1	0.121	0.179	0.236	0.263
148-2	0.088	0.134	0.205	0.215
148-3	0.107	0.150	0.195	0.224
149-1	0.087	0.171	0.305	0.382



149-2	0.092	0.170	0.311	0.393
149-3	0.079	0.125	0.208	0.283
150-1	0.119	0.162	0.209	0.238
150-2	0.111	0.156	0.207	0.239
150-3	0.114	0.185	0.230	0.272
151-1	0.096	0.179	0.320	0.404
151-2	0.062	0.136	0.249	0.322
151-3	0.092	0.144	0.185	0.235

My patches for KRLVS149 and KRLVS151 were pretty undergrown. Obviously, the worst was the second replicate for KRLVS151. I noticed that there were more air bubbles with these pipet tips, so that may be part of my problem with the extreme inconsistency, as I only noticed partway through measuring. I will be more careful in the future, and hopefully that will fix it.

### RNA Purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151

1. Pellet 1.8mLx2 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through in phenol and methanol waste
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

Stored total nucleic acids in -80C freezer in new RNA box. Labelled with the date on the side and, on the lid, the following:

Labelled	Contents
1 RNA	KRLVS148-1
2 RNA	KRLVS148-2
3 RNA	KRLVS148-3
4 RNA	KRLVS149-1
5 RNA	KRLVS149-2

6 RNA	KRLVS149-3
7 RNA	KRLVS150-1
8 RNA	KRLVS150-2
9 RNA	KRLVS150-3
10 RNA	KRLVS151-1
11 RNA	KRLVS151-2
12 RNA	KRLVS151-3

Nothing notable happened during the purification. However, before I begin the second purification tomorrow I will need to add ethanol to the second wash buffer bottle. Additionally, I nanodrop'd my samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	635.7	12.713	6.212	2.05	2.42
KRLVS148-2	657.2	13.145	6.281	2.09	2.36
KRLVS148-3	688.2	13.763	6.641	2.07	2.36
KRLVS149-1	1693.0	33.861	16.461	2.06	2.45
KRLVS149-2	1929.1	38.581	18.338	2.10	2.48
KRLVS149-3	935.9	18.718	8.834	2.12	2.44
KRLVS150-1	601.7	12.035	5.788	2.08	2.38
KRLVS150-2	638.5	12.770	6.162	2.07	2.37
KRLVS150-3	812.0	16.239	7.801	2.08	2.44
KRLVS151-1	899.9	17.998	8.589	2.10	2.45
KRLVS151-2	518.2	10.363	4.939	2.10	2.05
KRLVS151-3	524.6	10.492	5.000	2.10	2.37

I believe both KRLVS149-1 and -2 are outside of the linear range, which is super annoying. Hopefully I lose some in the second purification, or else I will probably have to dilute and re-nanodrop prior to making the aliquots.

Additionally, I looked at my electroporation plates and they had come to single colony. I cross patched the same as before, by scraping up a colony and patching it on the plate with the desired antibiotic followed by the plate with the undesired antibiotic. This time I only patched out four, as per Hannah's suggestion, in the event that I actually lose the plasmid so I can make a stock. The colonies were very tiny, but I was able to get isolated, though not well isolated, colonies. If I have to bring them to single colony again, I will exchange my stick more. As I completed this in the evening, I do not believe the patches will be ready to view until Thursday.

Wednesday, August 3, 2022

To Do:

1. ~~Run gel of pure RNA samples from 8/1 and 8/2~~
2. ~~Transform pKR122 into PIR cells~~
3. ~~Plate Aisling's cells~~
4. ~~CHAH + Kan and CHAH + Nat~~
5. ~~Check electroporation patch plates~~

## Results and Data:

### Transformation of *PrpsU2 tul4* 5'UTR Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.
  - b. Positive control: pEX; Backbone ligation: KpnI-NotI digested pKR68

**Reaction table**

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pEX	1 uL	100 uL	1
2	(-) control	None	0	100 uL	1
3	Backbone Ligation	pKR68 (digested)	8 uL	10 uL, 200 uL	2
4	Ligation 1	pKR122	8 uL	10 uL, 200 uL	2
Total number of plates					6

- Check to be sure you have enough LB-Kan plates. 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.
- Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

Last time I tried this transformation I used XL1 Blue cells, however, when I looked back at Aisling's notebook I saw that they used PIR cells. I probably should have checked prior to doing the initial transformation, however, I thought it was another pF based plasmid. I saved my ligation from last week, so I will try using that to transform the cells and hopefully I will actually get transformants this time. As with last time, I will only used one positive and one negative plate.

**Gel of 8/1 and 8/2 Pure RNA Samples**

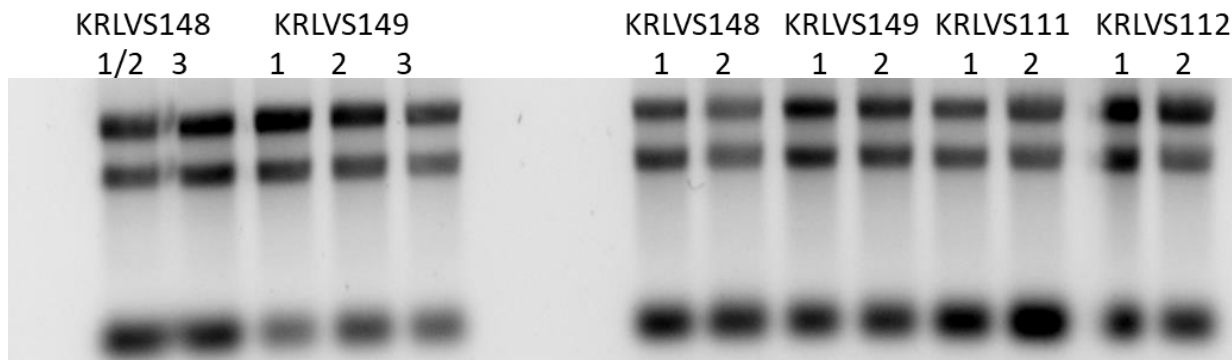
- Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x and run on gel.
- Rinse comb and gel rig with DI water, then ethanol both as well
- Make fresh agarose gel by adding 1.2g to 120mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
- Set up gel rig to cast gel, with ladder.
- Add 12uL of Sbyr Safe dye to rig, pour gel, use ladder to mix, then replace ladder and allow to set.
- Add new 1xTAE, turn gel, add TAE, and remove ladder.
- Add 12 uL of each sample according to the loading order below.
- Ran until separated at 113V.
- Look for distinctive bands (23s, 16s, tRNA), rather than smears.

Loading Order of 8/1/22 RNA					
Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6

Loading Order of 8/2/22 RNA							
Lane 11	Lane 12	Lane 13	Lane 14	Lane 15	Lane 16	Lane 17	Lane 18
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8

I ran these samples on one large gel, since there was not a ladder which had 14 wells on the small gel rig. These are the pure RNA samples of the KRLVS148 and KRLVS149 control strains and KRLVS148, KRLVS149, KRLVS111, and KRLVS122, also control.

I accidentally loaded KRLVS148-1 and -2 from 8/1/22 on top of each other again. I might need to ask Kathryn for some sort of clip lamp so I can see the wells better. I might also try turning the gel rig around so it's closer to the edge of the counter next time. Obviously this is not ideal, however, hopefully neither of them are degraded so I can confirm that they're both in tact...



Aaand, it looks good! There are distinct 23S, 16S, and tRNAs, and no smearing/degradation.

### Plating Day 42 of 4°C Samples of Aislings Project

1. Place ~20 beads on each plate aseptically
2. Pipette 300 ul of each sample onto each plate
  - a. Three samples from 4C room
  - b. 2 plates per sample = 6 plates total.
3. Shake in each direction
4. Let each plate sit for at least 2-3 minutes so all of the liquid is absorbed
5. Remove the beads within the flame into the 50 mL tube and add ethanol if it is not covering all the beads.
6. Place in the incubator to pull out on Friday

Finally, I took a glance at my electroporation plates, and they definitely need another day of growth, so I will look at them tomorrow and streak for isolation.

### Reagents

#### CHAH+Kan

To a 1L flask add:

30.6g of cystine heart agar

300mL of ddiH2O (type I)

stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)

Autoclave on 30' liquid cycle, filling the water bin up to the height of the media

Add 2% hemoglobin to water bath, after autoclave add CHA as well

Using sterile technique, pour hemoglobin into CHA

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

**CHAH+Nat**

To a 1L flask add:

30.6g of cystine heart agar

300mL of ddiH<sub>2</sub>O (type I)

stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)

Autoclave on 30' liquid cycle, filling the water bin up to the height of the media

Add 2% hemoglobin to water bath, after autoclave add CHA as well

Using sterile technique, pour hemoglobin into CHA

For 5 ug/ml, add 30 ul of 100 mg/mL nourseothricin to 600 mL of media MADE HALF FLASK

**Thursday, August 4, 2022****To Do:**

- ~~1. DNase treatment of 8/2/2022 RNA samples~~
- ~~2. Second RNA purification of 8/2/2022 RNA samples~~
- ~~3. Nanodrop pure RNA samples~~
- ~~4. Prepare RNA gel and cDNA aliquots of pure RNA samples~~
- ~~5. Run RNA gel of 8/4/22 pure RNA samples~~
- ~~6. Check transformation plates and make overnights~~
- ~~7. Check electroporation plates and make isolation streaks~~

**Results and Data:****DNase treatment of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 RNA Samples**

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube
15. Add 100 uL RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

Nothing particularly notable happened during the purification, aside from me dropping one of the tubes back onto the rack, but I didn't observe any droplets accumulating on the side of the tube, so I think it was okay. 12 samples, predictably, still took a long time so in order to speed up the process a little bit I opened up all the tubes at each washing step to quickly add the wash buffers, obviously still switching tips between each sample. Based on the results, this didn't seem to be a problem.

Accordingly, I nanodrop'd my samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	393.5	7.871	3.754	2.10	2.25
KRLVS148-2	374.4	7.488	3.593	2.08	2.22
KRLVS148-3	400.4	8.008	3.827	2.09	2.21
KRLVS149-1	471.2	9.424	4.473	2.11	2.29
KRLVS149-2	740.2	14.804	7.109	2.08	2.25
KRLVS149-3	526.6	10.531	4.985	2.11	2.41
KRLVS150-1	426.7	8.535	4.039	2.11	2.42
KRLVS150-2	366.1	7.322	3.474	2.11	2.32
KRLVS150-3	438.6	8.773	4.142	2.12	2.30
KRLVS151-1	530.0	10.601	5.029	2.11	2.37
KRLVS151-2	367.1	7.343	3.481	2.11	2.40
KRLVS151-3	397.0	7.939	3.870	2.05	1.87

Additionally, I prepared diluted aliquots for both the RNA gel and cDNA synthesis. I realized with my last two sample aliquots I was making the cDNA aliquots in 1.5 mL centrifuge tubes when I ought to have just done them in strip tubes, so I did this time. I labelled them 4-1, 4-2, etc. to indicate the date the pure RNA was synthesized, and which sample it was. Diluted as follows for gel and cDNA, respectively:

Sample	ng/uL	RNA	Water
KRLVS148-1	393.5	3.81	6.19
KRLVS148-2	374.4	4.01	5.99
KRLVS148-3	400.4	3.75	6.25
KRLVS149-1	471.2	3.18	6.82
KRLVS149-2	740.2	2.03	7.97
KRLVS149-3	526.6	2.85	7.15
KRLVS150-1	426.7	3.52	6.48
KRLVS150-2	366.1	4.10	5.90
KRLVS150-3	438.6	3.42	6.58
KRLVS151-1	530.0	2.83	7.17
KRLVS151-2	367.1	4.09	5.91
KRLVS151-3	397.0	3.78	6.22

Sample	ng/uL	RNA	Water
KRLVS148-1	393.5	7.62	5.88
KRLVS148-2	374.4	8.01	5.49
KRLVS148-3	400.4	7.49	6.01
KRLVS149-1	471.2	6.37	7.13
KRLVS149-2	740.2	4.05	9.45
KRLVS149-3	526.6	5.70	7.80
KRLVS150-1	426.7	7.03	6.47
KRLVS150-2	366.1	8.19	5.31
KRLVS150-3	438.6	6.84	6.66
KRLVS151-1	530.0	5.66	7.84
KRLVS151-2	367.1	8.17	5.33
KRLVS151-3	397.0	7.56	5.94

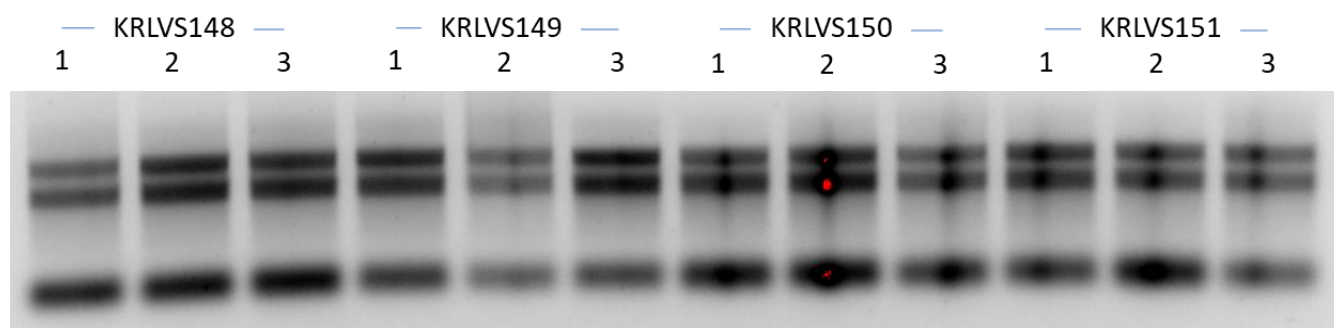
When I initially made the gel dilutions, the formulas broke so I placed the wrong amount of water in 1-8, however, I calculated the difference and adjusted each tube. However, some of these were very small amounts (0.05 uL, 0.2 uL, etc.) so I expected the RNA gel may appear with different brightnesses.

### Gel of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 RNA Samples

1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x.
2. Rinse comb and gel rig with DI water, then ethanol both as well
3. Make fresh agarose gel by adding 2.0g to 200mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
6. Make fresh 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 12 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA), rather than smears.

Loading Order of RNA Gel											
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8	RNA 9	RNA 10	RNA 11	RNA 12

When loading the gel, I flipped it around so that the wells were closer to me and I could sit while loading, and that seemed to help with my ability to see the wells. However, this was also the wider ladder which I always can see better, so I may still need to request a lamp from Kathryn.



I ran the gel for ~20 minutes. I did accidentally run the electrodes the wrong way for a maximum of 10 seconds. As seen above, the RNA looks good with distinct 23S, 16S, and tRNA bands. There is some smearing in the centers of quite a few of them, I am unsure whether this is an artifact of the gel or some degradation, however I am inclined to believe that it was an artifact of the gel given that the bands are otherwise quite distinct, and do not look like the one degraded lane from AM/DF's RNA gel. I think it was from the brief moment of running the gel the wrong way, or else I may have stabbed into the bottom of the wells a little bit, I noticed that I had inserted my pipet quite deeply on a few of them, based on loading speed (was not unloading properly due to the tip touching a solid surface).

I also checked my transformation plates and, once again, there were no colonies. I think maybe the restriction digest isn't set up right because it seems like pKR89 uses different ones, and that is what Aisling used, rather than pKR69. I will consult with Kathryn or Hannah and try again next week.

Next, I checked my second round of patch plates from the electroporation. They all had growth on both plates, however, KRLVS153 + pKR135 and KRLVS157 + pF were substantially less grown on the Kan and Nat plates, respectively. KRLVS153 + pKR30 had similar growth on both plates as per the last patches. I struck to single colony again, doing my best to estimate which patch had the comparatively least growth on the undesired antibiotic plate.



## Friday, August 5, 2022

1. ~~Generate cDNA of 8/1, 8/2, and 8/4 Pure RNA samples~~
2. ~~Pull out Aisling's Day 42 plates~~
3. ~~Pull out single colony electroporation plates~~

### Results and Data:

I pulled out Day 42 of Aisling's plates and left them on the bench over the weekend. I did not see any colonies grown on the plates as of yet. I set them on the undergrad bench, and will check them again on Monday and take photos of any plates with colonies.

I pulled my electroporation second single colony streak plates out of the incubator to grow over the weekend on the bench, because I will not be able to come pull them out of the incubator on Sunday. I glanced at them and in the expected areas of dense cells, there is some growth already but obviously no single colonies. With how cold the building has been, I worry they may not be ready by Monday, but hopefully putting them back in the incubator will be okay, given how humid it is and how fresh the plates where I do not expect the plates to dry out.

### Generate cDNA (Half) of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/2/22 Pure RNA)

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	1.5 ul	25 ng/ul
RNase-free water	up to 13.5 ul	

2. To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes. Total volume of cDNA and water is 13.5 uL.
3. Add the 1.5 uL (NS)<sub>5</sub> oligo to the tubes.
4. Incubate using program JSScDNA1 in the thermocycler:

Step	Temp	Time
1	70°C	10'
2	25°C	10'
3	4°C	hold

5. Prepare master mix at 1.5 + #reactions.

Component	Final Concentration	Volume	X27.5
5X 1st strand buffer	1x	6	165
RNase-free water		2.88	79.2
100 mM DTT	10 mM	3	82.5
10 mM dNTPs	0.5 mM	1.5	41.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	44.83

6. Aliquot 15 ul of master mix into each PCR tube from the first reaction (total volume now 30ul)
7. Incubate using program JSScDNA2

Step	Temp	Time
1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold

8. Remove RNA from sample by degrading with sodium hydroxide:
  - a. Add 10 ul of 1N NaOH
  - b. Incubate 65°C for 30'
  - c. Neutralize with 10 ul of 1N HCl



- d. Final volume is 50  $\mu$ L
9. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
10. Elute in 60  $\mu$ L of 0.1x EB
11. Check concentration by Nanodrop
12. Store cDNA at  $-80^{\circ}\text{C}$

I lost volume on my 8/2-8 sample (KRLVS112 BR-2) prior to adding the NS<sup>5</sup> primers, however since I didn't know how long these primers could sit on the RNA, I proceeded with the protocol. Given that there was a previous error in the volumes of this step, broadly, (due to the protocol having the wrong math) I believe that it will go forward okay, but may have less cDNA which is fine given how little I need for the qRT-PCR. I hope.

### cDNA Purification with PCR Purification Kit

1. Add 250  $\mu$ L of Buffer PB to each 50  $\mu$ L cDNA reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750 $\mu$ L of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 60 $\mu$ L of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

I realized looking at this protocol, that last time I did a cDNA synthesis purification, I technically added too much Buffer PB (final volume was 50 not 60 $\mu$ L so I added 50 $\mu$ L extra), but obviously it was fine. So that's a little reassuring.

Due to the loss of volume of sample 8 from 8.2.22, I reapplied the eluted cDNA to the column and eluted again to compensate. Lastly, I nanodrop'd all of my samples:

Sample Name	Nucleic Acid (ng/ $\mu$ L)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	28.4	0.568	0.325	1.75	1.65
KRLVS148-2	34.1	0.682	0.420	1.63	1.74
KRLVS148-3	30.6	0.612	0.334	1.83	2.18
KRLVS149-1	25.6	0.513	0.280	1.83	1.95
KRLVS149-2	35.3	0.706	0.387	1.82	1.85
KRLVS149-3	26.3	0.526	0.301	1.75	2.21

Sample Name	Nucleic Acid (ng/ $\mu$ L)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	14.7	0.293	0.173	1.69	1.41
KRLVS148-2	21.0	0.419	0.233	1.80	2.09
KRLVS149-1	30.9	0.617	0.358	1.73	1.49
KRLVS149-2	28.2	0.564	0.320	1.76	1.91
KRLVS111-1	30.4	0.608	0.342	1.78	1.95
KRLVS111-2	21.7	0.435	0.246	1.77	2.22
KRLVS112-1	30.4	0.608	0.353	1.72	1.80
KRLVS112-2	27.5	0.549	0.307	1.79	2.05

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148-1	22.5	0.450	0.255	1.77	2.20
KRLVS148-2	28.1	0.563	0.319	1.76	2.19
KRLVS148-3	24.7	0.493	0.291	1.69	2.19
KRLVS149-1	29.8	0.597	0.336	1.78	2.25
KRLVS149-2	21.8	0.437	0.250	1.75	1.57
KRLVS149-3	25.5	0.510	0.285	1.79	1.98
KRLVS150-1	20.9	0.418	0.239	1.75	2.12
KRLVS150-2	27.6	0.551	0.325	1.69	1.51
KRLVS150-3	19.1	0.383	0.213	1.80	2.18
KRLVS151-1	24.1	0.483	0.291	1.66	1.79
KRLVS151-2	28.1	0.562	0.320	1.76	2.24
KRLVS151-3	26.3	0.526	0.294	1.79	2.01

I found the purity to be of a poorer quality than I am used to. Perhaps it has to do with how long it took me to move through all the samples, or else, something to do with how long the PB was on the column? I'll have to run these by Kathryn, I'm not quite sure what happened there. That said the purity is also not the WORST, so I think that I can move forward with the qRT-PCR without problem. Lastly, it seems sample 8 from 8.2.22 had a perfectly fine concentration, not even the lowest, so that is good.

I labelled each tube with the number from the KRLVS number, the biological replicant number, and cDNA on the lid. And then the date of the second RNA purification and the date of cDNA synthesis.

Monday, August 8, 2022

**To Do:**

1. Dilute cDNA samples
2. qRT-PCR of KRLVS148 and KRLVS149 (8/4/22) cDNA samples
3. qRT-PCR of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 (8/4/22) cDNA samples

**Results and Data:**

Diluted cDNA samples to 1.5 ng/uL. According to Hannah, we do not want the crossing point to be over 25, so if that becomes a problem I should do a more diluted concentration, however, since I haven't run these strains before I will be doing the same concentration as before. I diluted according to the following:

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KRLVS148-1	28.4	3.17	56.83
KRLVS148-2	34.1	2.64	57.36
KRLVS148-3	30.6	2.94	57.06
KRLVS149-1	25.6	3.52	56.48
KRLVS149-2	35.3	2.55	57.45
KRLVS149-3	26.3	3.42	56.58

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KRLVS148-1	14.7	6.12	53.88
KRLVS148-2	21.0	4.29	55.71
KRLVS149-1	30.9	2.91	57.09
KRLVS149-2	28.2	3.19	56.81
KRLVS111-1	30.4	2.96	57.04
KRLVS111-2	21.7	4.15	55.85
KRLVS112-1	30.4	2.96	57.04
KRLVS112-2	27.5	3.27	56.73

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KRLVS148-1	22.5	4.00	56.00
KRLVS148-2	28.1	3.20	56.80
KRLVS148-3	24.7	3.64	56.36
KRLVS149-1	29.8	3.02	56.98
KRLVS149-2	21.8	4.13	55.87
KRLVS149-3	25.5	3.53	56.47
KRLVS150-1	20.9	4.31	55.69
KRLVS150-2	27.6	3.26	56.74
KRLVS150-3	19.1	4.71	55.29
KRLVS151-1	24.1	3.73	56.27
KRLVS151-2	28.1	3.20	56.80
KRLVS151-3	26.3	3.42	56.58

**qRT-PCR of KRLVS148 and KRLVS149 (8/4/22) cDNA**

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL343/344 and KROL504/505 as test primers, specifically to amplify the 5'UTR and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying *tul4*.

- Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*3=18 reactions)
- Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	245.0 uL
5uM primer set	1 uL	24.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	171.5 uL
Total:	20 uL	465.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5µL of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20 µL of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

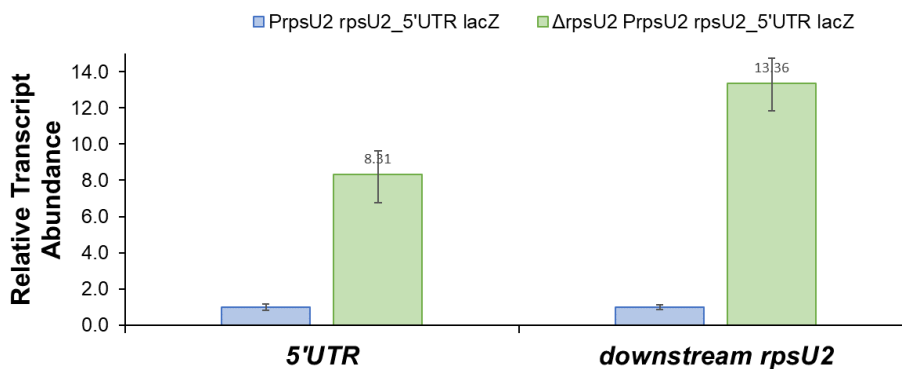
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsu2</i>
C	KROL63/64 <i>tul4</i>

1	2	3	4	5	6
148-1	148-2	148-3	149-1	149-2	149-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9
A	A1			B1			C1		
B	A2			B2			C2		
C	A3			B3			C3		
D	A4			B4			C4		
E	A5			B5			C5		
F	A6			B6			C6		



**qRT-PCR of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/4/22) cDNA**

- Each experiment will need at least one test primer and one control primer for each sample
  - KROL343/344 and KROL504/505 as test primers, specifically to amplify the 5'UTR and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying *tul4*.
- Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*3=18 reactions)
- Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	315.0 uL
5uM primer set	1 uL	31.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	252.0 uL
Total:	20 uL	598.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5µL of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20 µL of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

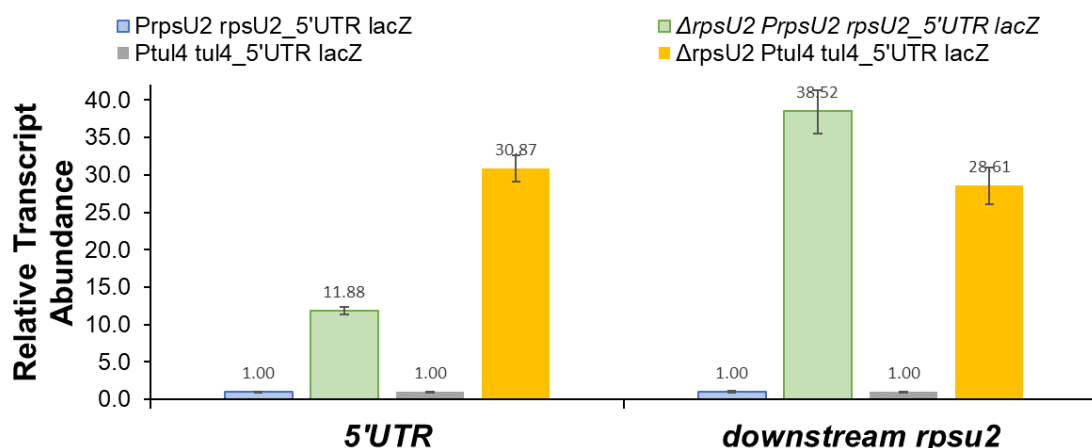
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsu2</i>
C	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8
148-1	148-2	149-1	149-2	111-1	111-2	112-1	112-2

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9
A	A1			B1			C1		
B	A2			B2			C2		
C	A3			B3			C3		
D	A4			B4			C4		
E	A5			B5			C5		
F	A6			B6			C6		
G	A7			B7			C7		
H	A8			B8			C8		



For complete results go to [August 12<sup>th</sup>](#).

Tuesday, August 9, 2022

#### To Do:

- ~~1. qRT-PCR of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 (8/4/22) cDNA samples 1~~
- ~~2. qRT-PCR of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 (8/4/22) cDNA samples 2~~
- ~~3. Remake pKR122 plasmid map on SnapGene~~
- ~~4. Set up PCR of gBlock for pKR122~~
- ~~5. Run dishwasher~~
- ~~6. Dump Kumasi stain on Kathryn's gel after 4:40 and replace with graduated cylinder contents~~
- ~~7. LB+Kan~~
- ~~8. Put in waste requests~~
- ~~9. Patch out electroporation experiment plates~~

#### Results and Data:

I cross-patched the electroporation plates from the second isolation streak. As before, I patched first onto the plate with the antibiotic of the plasmid we are trying to maintain, followed by the other antibiotic plate. As per Kathryn, I replaced the isolation streaks in the incubator.

#### qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/5/22) cDNA - 1

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL343/344 and KROL504/505 as test primers, specifically to amplify the 5'UTR and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying tul4.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*3=18 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	245.0 uL
5uM primer set	1 uL	24.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	171.5 uL
Total:	20 uL	465.5 uL

4. Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
5. Add 3.5μL of cDNA into appropriately labelled tubes.

- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20  $\mu$ L of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

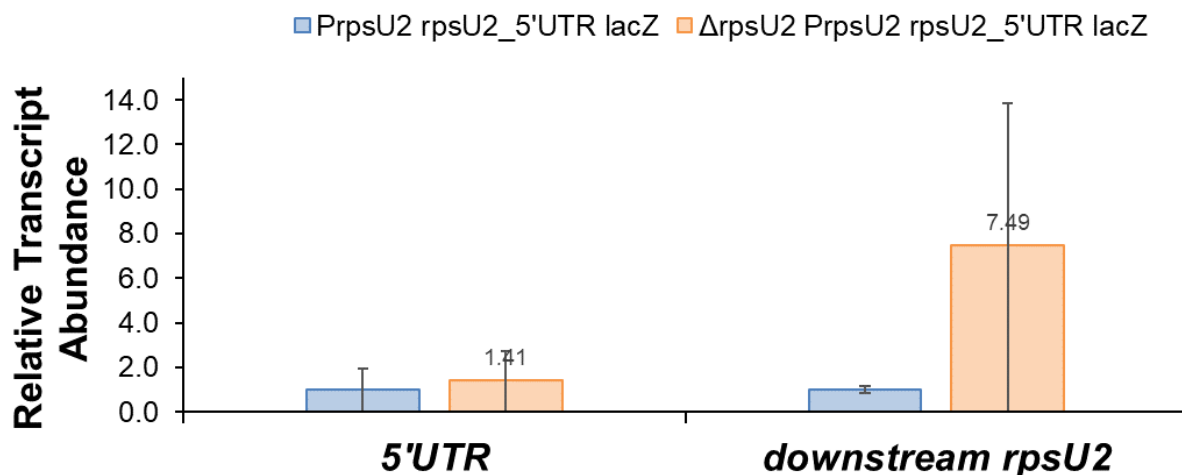
Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsu2</i>
C	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9
A	A1			B1			C1		
B	A2			B2			C2		
C	A3			B3			C3		
D	A4			B4			C4		
E	A5			B5			C5		
F	A6			B6			C6		

There was potentially a problem with the electronic multichannel pipet (well, user error), as it seemed it had sucked up a bubble so I ejected and re-pipetted a few times, as it seemed some volume was still in the tip. This was during the A series, so hopefully it will be okay but if there is large error that is why. Otherwise, B and C series seemed to go fine. I may redo this tomorrow if there are issues, but again, we will see.



As predicted, this data looks pretty terrible, as per the error bars. Hopefully this was just from pipetting error, but since I have to redo the qRT-PCR anyways to include the extra primer, hopefully it looks better. If it doesn't, then I assume it was an issue with the inconsistency in the culture growth.

For complete results go to [August 16<sup>th</sup>](#).

**qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/5/22) cDNA - 2**

- Each experiment will need at least one test primer and one control primer for each sample
  - KROL343/344 and KROL504/505 as test primers, specifically to amplify the 5'UTR and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying *tul4*.
- Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*3=18 reactions)
- Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	245.0 uL
5uM primer set	1 uL	24.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	171.5 uL
Total:	20 uL	465.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5µL of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20 µL of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

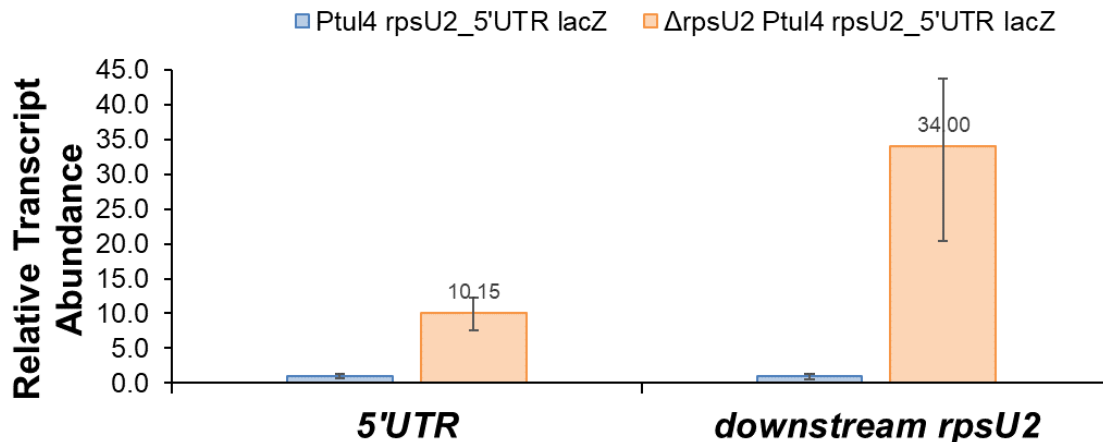
Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsu2</i>
C	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9
A	A7			B7			C7		
B	A8			B8			C8		
C	A9			B9			C9		
D	A10			B10			C10		
E	A11			B11			C11		
F	A12			B12			C12		





For complete results go to [August 16<sup>th</sup>](#).

### PCR of *PrpsU2\_tul4*UTR gBlock for pKR122

1. Acquired and labelled PCR tubes: 122 and negative control. \*omitted positive b/c primers verified
2. Acquire the following components and put them on ice, labeling tubes if necessary:
  - mgH<sub>2</sub>O, Primestar buffer, dNTPs, KROL472, KROL350, (10uM), and *PrpsU2\_tul4*UTR gBlock
3. Vortex each component (aside from enzyme)
4. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
5. Add ddi H<sub>2</sub>O to negative control tube (template volume for 1 reaction)
6. Prepare a master-containing:
  - mgH<sub>2</sub>O, dNTPs, Primestar buffer, and Primestar enzyme
7. Mix the master-mix solution by pipetting up and down
8. Add appropriate volume of master-mix to negative control PCR tube
9. Add appropriate volume of master mix to each PCR tube and pipet up and down to mix
10. 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 30 seconds > Go back to step 2 > Repeat 32x > 68 degrees C for 5 minutes > 12 degrees C for infinity

Total reaction volume	100
Total number of reactions	2

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH <sub>2</sub> O			62.0	184.0
PrimeSTAR GXL Buffer	5x	1x	20.0	60.0
dNTPs	2.5 mM	0.2 mM	8.0	24.0
oligo F	10 uM	0.3 uM	3.0	9.0
oligo R	10 uM	0.3 uM	3.0	9.0
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	6.0
Total volume			100	294

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL472, KROL350	-
2	<i>PrpsU2_tul4UTR</i>	gBlock	KROL472, KROL350	252

## Reagents

LB Agar + Kanamycin

Melt LB agar and then cool at 56°C

0.5 mL 50 ug/mL Kanamycin

Pour plates (24 mL)

Wednesday, August 10, 2022

### To Do:

1. ~~Put away dishes~~
2. ~~PCR purification of *PrpsU2\_tul4UTR* gBlock fragment~~
3. ~~Digest gBlock amplification and pKR89 backbone with NotI/KpnI~~
4. ~~Run gel of restriction digest and negative control from PCR~~
5. ~~Cut gel and purify samples~~
6. ~~Ligate backbone and PCR fragment~~
7. ~~Transform into *E. coli*~~

## Results and Data:

### PCR Purification of Amplified *PrpsU2\_tul4UTR* gBlock

1. Add 500 uL of Buffer PB to each 100 uL PCR reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 35 uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

I eluted my PCR product in 35 uL 0.1x EB in the event that I need to re-do the digest (accounting for pipetting error), as per previous guidance from Hannah. Additionally, I purified the negative control since I needed a balance anyways, however I eluted the negative in 30 uL EB so that I can add 6 uL of loading dye and load the same amount as I will from my digest. I kept the negative control w/dye on ice.

### DNA Digest of *PrpsU2\_tul4UTR* gBlock PCR and pKR89 Backbone w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	<i>PrpsU2_tul4UTR</i>	NotI, KpnI	15	-
2	pKR89	NotI, KpnI	5	10

2. Set up master mix table:

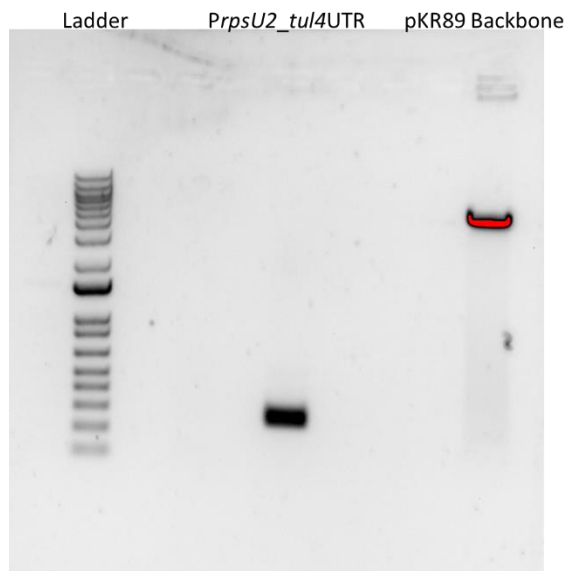
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H <sub>2</sub> O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
NotI	0.6	1.8
KpnI	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

3. Add 15 uL of Master Mix to individual tube for digest.
4. Mix by pipetting up and down.
5. Incubate at 37°C for 1 hour.
6. After digest: add 1 uL of QuickCIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the enzyme.

Completed incubation in PCR tubes in Thermocycler because incubator was at a low temperature. For the backbone QuickCIP step, I also completed all incubations in the thermocycler.

### Gel of Digested *PrpsU2\_tul4*UTR PCR Fragment + pKR89 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sybr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.



### Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen
2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube.

When I visualized the gel after cutting out the backbone (which I cut out second), I noticed a fair bit of the band was still present on the gel, so I removed the gel piece from the 2 mL tube, carefully, and took an image of it. From this, I cut a small sliver from the bottom of that piece, and then cut out the majority of the band which had remained in the gel.

### Gel Extraction with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel.
3. Incubate at 42°C for 10 minutes or until gel is dissolved. Vortex every 2-3min to help dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Load sample into QIAquick column and centrifuge for 1 min at 13,000rpm. Discard flow through.
6. Add 500uL Buffer QG. Centrifuge for 1 min at 13,000rpm. Discard flow through.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge 1 min at 13,000rpm. Discard flow through. Place column back in tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer EB .01x and let stand for 1-4 minutes. Centrifuge for 1 min at 13,000rpm.

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
<i>PrpsU2 tul45'</i> UTR	301	903	301
pKR89 Backbone	570	1710	570

Due to the weight of the backbone gel piece I added half the amount of Buffer QG for the melting step, and added the second half to a new tube. Once the gel piece was melted, I mixed the backbone one, took 835uL and added it to the second tube of QG, mixed it, and then mixed the same amount in the first tube again, and then made sure to split the amount evenly amongst the two tubes. I then added half the total isopropanol volume to each and mixed as per normal. I ran the protocol forward with tube columns for the backbone, and eluted on one column, then re-eluted the same over the second column. Due to the need for a counterbalance, I went ahead and re-eluted the fragment over its own column again.

### Ligation of Digested *PrpsU2\_tul4*UTR PCR Fragment with Digested pKR89 Backbone

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

Tube	Insert	Backbone
1	NotI, KpnI dig., puri. <i>PrpsU2_tul4</i> UTR PCR	NotI, KpnI digested, purified pKR89
2	-	NotI, KpnI digested, purified pKR89

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H <sub>2</sub> O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6.0
Insert	4.0	-	-
Backbone	2.0	2.0	6.0
Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48.0

3. Obtain ice to assemble and keep the reactions on.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H<sub>2</sub>O, 10x buffer, insert, and backbone.
6. Add indicated amount of ligase to the individual tubes.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Place on bench for 10 minutes.

### Transformation of *PrpsU2\_tul4*UTR + pKR89 Backbone Ligation into *E. coli*

1. Set up reaction table. Use 8 uL per ligation and plate 20 uL and 200 uL.
  - a. Positive control: pKR89; Backbone ligation: KpnI-NotI digested pKR89

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR89	1 uL	100 uL	1
2	(-) control	None	0	100 uL	1
3	Backbone Ligation	pKR89 (digested)	8 uL	20 uL, 200 uL	2
4	Ligation 1	pKR122	8 uL	20 uL, 200 uL	2
Total number of plates					6

2. Check to be sure you have enough LB-Kan plates. Warm plates at 37°C, if kept at 4°C.
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, gently pipette 100 uL of cells into each tube onto DNA
7. Incubate cells on ice for 20 minutes.
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
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Tape tubes in shaking incubator set to 37°C and allow to recover for 1 hr, shaking.
12. Plate indicated amount of cells on LB-Kan, spreading until plates look dry.

Used PIR cells due to the origin of this plasmid only being compatible with PIR competent *E. coli* cells. Due to the difficulty of making this plasmid, and potentially low copy number, I played 20 uL and 200 uL rather than 10 uL and 200 uL as I may normally. Additionally, I plated 100 uL each of the positive and negative control.

Thursday, August 11, 2022

#### To Do:

1. ~~Put away tube caps~~
2. ~~Count transformation plates~~
3. ~~Check electroporation plates~~
4. ~~Make glycerol stocks of potential triple mutant strains with no *rpsU* gene~~
5. ~~Make overnights~~

#### Results and Data:

I finally went through all of my qRT-PCR data outputs and ran them through the calculation sheet on excel. I determined my error with the first data sheet output, which I had shown Kathryn and Hannah at our meeting. I accidentally normalized to KRLVS148 which is the  $\Delta rpsU2$  strain in the Tn7 *PrpsU2 rpsU2* 5'UTR *LacZ* series of strains. Due to this mix up I changed all the calculation sheets to genotype rather than KRLVS strain number, which I should have done before. I showed Kathryn the outputs and she said that the two Tn7 *PrpsU2 rpsU2* 5'UTR *LacZ* looked as expected and the Tn7 *Ptul4 tul4* 5'UTR *LacZ* series also looked as expected, despite my initial worries. However, Kathryn realized I was missing a primer set that I should have included, looking at *LacZ* specifically, so I will redo the 8.2 and 8.4 qRT-PCR and get complete results. I won't both with the 8.1, since I am repeating the Tn7 *PrpsU2 rpsU2* 5'UTR *LacZ* series so many times regardless.

Hannah looked up the primers for the *LacZ* region and either KROL395/396 or KROL399/400 will work. However, Aisling used KROL399/400. In order to be able to compare more directly with her results, I will also use KROL399/400.

There was one colony on my transformation plate, as well as one on the backbone. I spoke with Kathryn and went over each step of my transformation. Due to the low copy number predicted with this plasmid, I ought to have plated 200 uL and remaining for the ligation and backbone only. Additionally, she suggested I plate 20 uL and 100 uL for the positive control, and plate the remaining for the negative

control. I will miniprep my one colony tomorrow and run a diagnostic digest on it, and hopefully it is correct. Otherwise, I will try again next week with these new parameters.

After checking my plates, I placed them back into the incubator until later in order to make the overnight as late in the day as possible, as per usual. For my single overnight, I added the colony to 5mL of LB + 50 ug/mL Kan, which was 5 uL in 5 mL.

I checked my electroporation experiment patch plates, and there were patches which appear to have lost the plasmid on each of the experiments. Kathryn looked at them as well, and I will freeze down the potential strains which are now, potentially, free of any *rpsU* gene, and next week I will crosspatch again from the glycerol stock to ensure that this is correct. Additionally, she would like me to crosspatch from fresh colonies that have grown on my last isolation streak plate.

### Making Glycerol Stocks Protocol

1. Make 1 cryovials for each strain (permanent stocks), label!
2. Prepare 0.8 mL of MHB in a 1.5 mL centrifuge tube
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 200ul of 75% glycerol to the cryovial followed by 800 uL of resuspended cells
6. Freeze at -80

I made one cryovial for each patch, some of them made very light stocks. Regardless I stored then in my Glycerol Stocks #2 box. I labelled each cryovial with:

triple mut./del. + (plasmid)  
(patch#)  
BG: KRLVS\_\_\_\_  
8/11/22

In order to indicate that it was a triple deletion mutant; whether it had pKR135, pF-Nat, or pF; which patch number it was in order to easily dispose of one should a patch grow on the original plasmid correspondent antibiotic plate, the background from the electroporation, and to know when it was made.

Friday, August 12, 2022

#### To Do:

- ~~1. qRT-PCR of 8.2 RNA samples with all three primer sets~~
- ~~2. Streak out KRLVS111 and KRLVS148~~
- ~~3. Miniprep candidate pKR122~~
- ~~4. Diagnostic digest of candidate pKR122~~
- ~~5. Run gel of diagnostic digest~~

### Results and Data:

#### Miniprep of Candidate pKR122 from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.

8. Wash the QIAprep column with 750uL Buffer PE. Centrifuge 30-60s x3 and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

I split my culture between two columns and eluted in 30 uL, letting the elution buffer sit on the column for 4 minutes. There was some volume loss, so there is slightly less than 60 uL total in the tube.

### Diagnostic Digest of Candidate pKR122 Miniprep w/KpnI and NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	pKR122	KpnI, NotI	2.0	-
7	Water	KpnI, NotI	-	2.0

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H <sub>2</sub> O	15	45
10x Buffer (Cutsmart)	2	6
DNA	(2)	
KpnI	0.5	1.5
NotI	0.5	1.5
Total	20.0 (18.0 actual b/c of DNA)	54

3. Add indicated amounts of H<sub>2</sub>O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

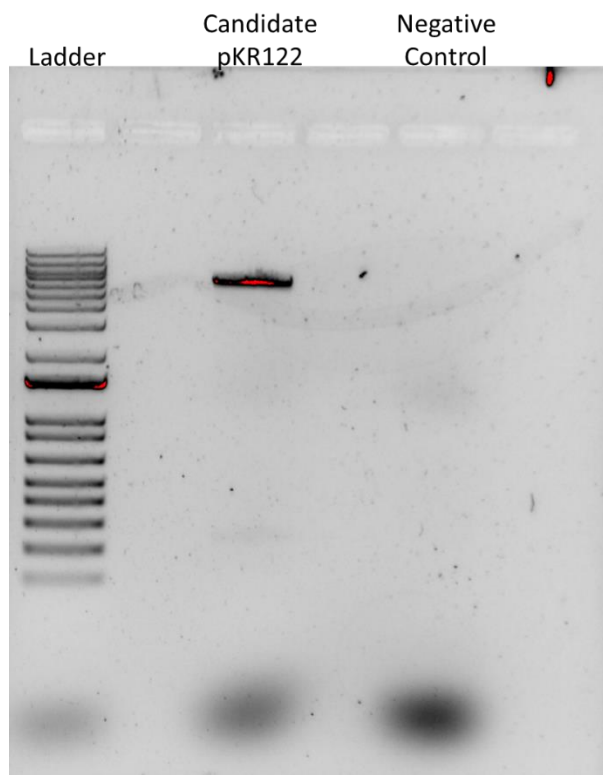
### Gel of Diagnostic Digest of Candidate pKR122 Miniprep

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cooled
2. Set up small gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe and pour ~60uL gel, use ladder to mix, then place ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 10 uL ladder, and 24 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Diagnostic Digest of Candidate pKR122					
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Ladder		pKR122(?)		(-) Control	

I probably should have nanodrop'd and diluted my miniprep, however it does look like I have a very light band, so I overexposed it. I think I might have it, I'll confer with Kathryn. But I do think that this is promising, I might just nanodrop and redo the diagnostic digest on Monday.





### qRT-PCR of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/4/22) cDNA

- Each experiment will need at least one test primer and one control primer for each sample
  - KROL343/344, KROL399/400, and KROL504/505 as test primers, specifically to amplify the 5'UTR, *lacZ*, and downstream of the *rpsu2* gene respectively. KROL63/64 as the control, amplifying *tul4*.
- Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (8\*4=32 reactions)
- Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	315.0 uL
5uM primer set	1 uL	31.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	252.0 uL
Total:	20 uL	598.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5μL of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
- Pipette 20 μL of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'



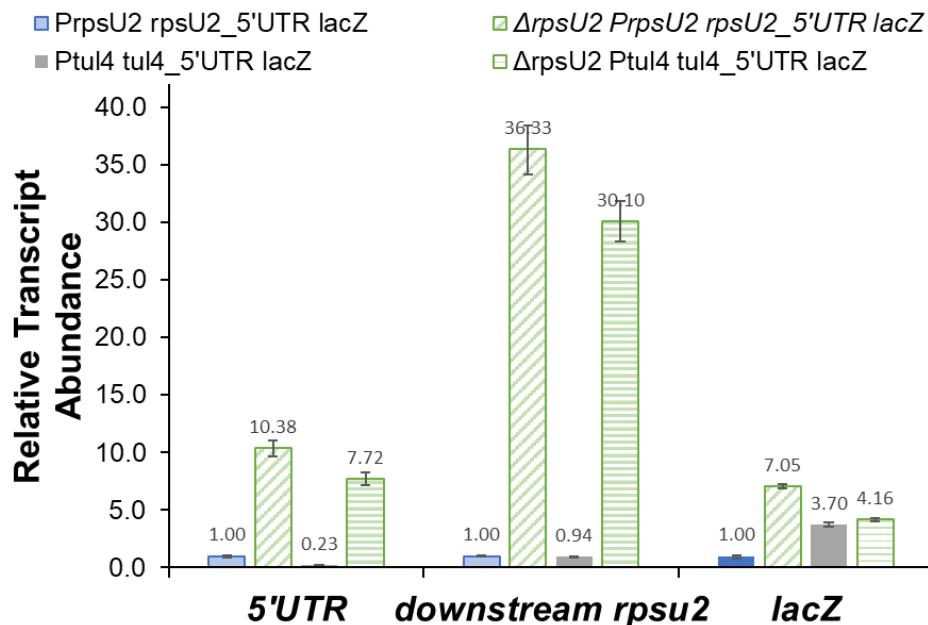
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds- <i>rpsU2</i>
C	KROL399/400 <i>LacZ</i>
D	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8
148-1	148-2	149-1	149-2	111-1	111-2	112-1	112-2

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			B1			C1			D1		
B	A2			B2			C2			D2		
C	A3			B3			C3			D3		
D	A4			B4			C4			D4		
E	A5			B5			C5			D5		
F	A6			B6			C6			D6		
G	A7			B7			C7			D7		
H	A8			B8			C8			D8		



5'UTR: Present at both loci in the *rpsU2:rpsU2* strains, and only at the native locus in *tul4:tul4* strains.

Downstream *rpsU2*: Present at only the native locus in all strains

*lacZ*: Present in only the Tn7 site in all strains

The relationship of increased transcript abundance appears to be maintained in the *rpsU2:rpsU2* strain at both the native and Tn7 site from the WT to  $\Delta$ *rpsU2* context. This relationship observed from the *tul4:tul4* strains is present only at the native locus, as seen with the nearly identical transcript abundance at the *lacZ* primer site, which is only present at the Tn7 site.

Saturday, August 13, 2022

**To Do:**

~~1. Streak out KRLVS149 and KRLVS112~~

**Results and Data:**

Struck out KRLVS148, KRLVS149, KRLVS112, KRLVS150, and KRLVS151 on CHAH+Kan.

Monday, August 15, 2022

**To Do:**

1. Put away dishes
2. Make 2.5% iron pyrophosphate
3. Patch KRLVS148 and KRLVS111
4. Replenish 5uM stocks of primer sets 63/64, 343/344, and 504/505
5. qRT-PCR of 8.4 RNA samples with *lacZ* and *tul4*
6. Set up sequencing of pKR122 candidate

**Results and Data:**

I could not patch out KRLVS148 or KRLVS111 as there were no single colonies. I will do that RNA purification early next week.

**Diluting qRT-PCR 5uM Primer Sets**

1. Thaw 100uM stock of each primer in primer set
2. Vortex vigorously to ensure complete thawing
3. Label tube with primer set and 5 uM on a red sticker if creating a new primer set
4. Add 270uL of 0.1xEB to tube
5. Add 15 uL of 100uM stock of each primer to tube and vortex vigorously, then minifuge.
6. Store in appropriate qPCR primer box in -20

**qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/5/22) cDNA with *LacZ***

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL499/500 as test primers to amplify *LacZ*. KROL63/64 the control, amplifying *tul4*.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (12\*2=24 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	455.0 uL
5uM primer set	1 uL	45.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	364.0 uL
Total:	20 uL	864.5 uL

4. Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
5. Add 3.5uL of cDNA into appropriately labelled tubes.
6. As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
7. Pipette 20 uL of each primer set strip tubes, in triplicate, using multichannel pipet.
8. Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

Master Mix Label	Primer Set
A	KROL399/400 <i>LacZ</i>
B	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			A7			B1			B7		
B	A2			A8			B2			B8		
C	A3			A9			B3			B9		
D	A4			A10			B4			B10		
E	A5			A11			B5			B11		
F	A6			A12			B6			B12		

I forgot that I would need to also do KRLVS148 and KRLVS149 for all primer sets again due to the error in pipetting resulting in highly variable results, after Kathryn informed me that I wouldn't need to do all the primer sets again for these, just *lacZ* and *tul4* to normalize to. For results see [August 16<sup>th</sup>](#).

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Candidate pKR122	254.5	5.089	2.715	1.87	2.27

Sample number	Template Type	Template Name	Primer Name <sup>a</sup>	Template Size	Concentration	Vol. Plasmid	Volume H <sub>2</sub> O	Primer
SS 1	Plasmid	pKR122_1	KROL257	6583	254.5	1.57	7.87	2.56

I checked using SnapGene and it looked as though KROL257 would more than cover the *rpsU2* promoter and *tul4* 5'UTR. Additionally, there are 321 bp between the end of the primer and the start of the promoter sequence. I figured I wouldn't really need the sequence from the *lacZ* region nor the Tn7 site since there were not included in the insert.

## Reagents

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

Tuesday, August 16, 2022

To Do:

1. Filter sterilize 2.5% iron pyrophosphate
2. Supplement MHB
3. Patch KRLVS148 and KRLVS150
4. qRT-PCR of KRLVS148 and KRLVS149 from 8.4 samples

## Results and Data:

### qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/5/22) cDNA with *LacZ*

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL343/344 and KROL504/505 as test primers to amplify the 5'UTR and downstream of *rpsU2*. KROL63/64 the control, amplifying *tul4*.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (12\*2=24 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	245.0 uL
5uM primer set	1 uL	24.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	196.0 uL
Total:	20 uL	465.5 uL

- Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
- Add 3.5  $\mu$ L of cDNA into appropriately labelled tubes.
- As these will be in technical triplicate, each tube will be an individual master mix, totaling 70  $\mu$ L. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5  $\mu$ L.
- Pipette 20  $\mu$ L of each primer set strip tubes, in triplicate, using multichannel pipet.
- Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

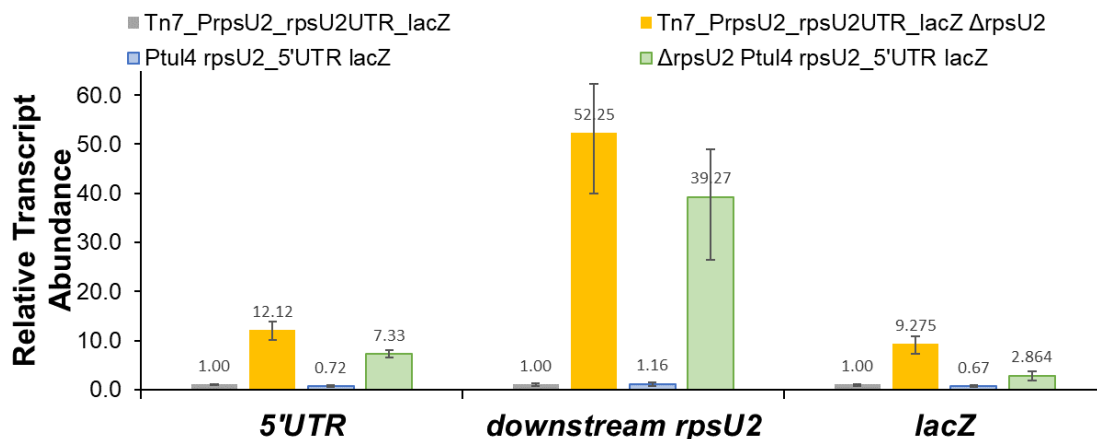
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 <i>rpsU2</i>
C	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9
A	A1			B1			C1		
B	A2			B2			C2		
C	A3			B3			C3		
D	A4			B4			C4		
E	A5			B5			C5		
F	A6			B6			C6		



5'UTR: Present at both sites in all strains

Downstream *rpsU2*: Present only at the native locus in all strains

*lacZ*: Present only at the Tn7 sites for all strains.

Given that the relationship from the WT to the  $\Delta$ *rpsU2* context is maintained in the *tul4:rpsU2* context, it would seem that, perhaps, the *rpsU2* promoter is not where bs21 interacts in order to influence its own transcription, as switching it out for the known, steady transcription promoter of *tul4* had no effect on the relationship.

## Reagents

### Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

### 2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

Wednesday, August 17, 2022

### To Do:

1. Patch KRLVS149 and KRLVS15
2. Transform pKR122 ligation (8/10) into *E. coli*
3. Run dishwasher

## Results and Data:

I received my sequencing results from the one colony I was able to miniprep from my last transformation. As shown in the image below, there was an insertion in the lipoprotein after the *rpsU2* promoter and prior to the *lacZ* gene. Due to the location, this created an obvious frameshift mutation which would nullify the ability for *lacZ* to operate functionally.



Due to the frameshift mutation, Kathryn believes that the plasmid is likely toxic, since it is known that *lacZ* is toxic to the cell. As such, I will have to repeat my transformation. Due to the toxicity Kathryn said that I should grow the cells on plates at 30°C, however I can still recover at 37°C. Additionally, she said that I could use the ligation that I made last week, I'm assuming because obviously the ligation did work.

## Transformation of *PrpsU2\_tul4UTR* + pKR89 Backbone Ligation into *E. coli*

1. Set up reaction table. Use 8 uL per ligation and plate 200 uL and remaining.
  - a. Positive control: pKR89; Backbone ligation: KpnI-NotI digested pKR89

### Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR89	1 uL	100 ul	1
2	(-) control	None	0	Reaming	1
3	Backbone Ligation	pKR89 (digested)	8 uL	200uL, remaining	2
4	Ligation 1	pKR122	8 uL	200uL, remaining	2
Total number of plates					6

2. Check to be sure you have enough LB-Kan plates. Warm plates at 37°C, if kept at 4°C.
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, gently pipette 100 uL of cells into each tube onto DNA
7. Incubate cells on ice for 20 minutes.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes into tube rack.
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Tape tubes in shaking incubator set to 37°C and allow to recover for 1 hr, shaking.
12. Plate indicated amount of cells on LB-Kan, spreading until plates look dry. Incubate at 30°C.

As per Kathryn last week, I will plate 200uL and remaining of both the ligation and backbone, since this is a low copy number plasmid. As before, I will be using the PIR1 competent cells due to the low copy number origin.

Thursday, August 18, 2022

**To Do:**

1. ~~Set up cultures of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
2. ~~Streak KRLVS148 and KRLVS151 for isolation~~
3. ~~Put away dishes~~
4. ~~RNA purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
5. ~~Nanodrop RNA purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
6. ~~Count transformation plates~~
7. ~~Make overnights of pKR122 transformants (hopefully)~~

**Results and Data:**

**Setting Up KRLVS148, KRLVS149, KRLVS150, KRLVS151 Cultures for RNA Purification**

1. Scrape each triplicate patch of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (50uL of sample in 950uL of MHB)
3. Calculate volume to add to tubes of 8 mL MHB for an OD of 0.08 for KRLVS149 and KRLVS151, and 0.1 for KRLVS148 and KRLVS150 (calculate for an OD of 0.1 or 0.13 to account for error)
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

RNA Cultures OD's				
Sample	0 hours	2 hours	4 hours	5 hours
148-1	0.120	0.173	0.220	0.250
148-2	0.114	0.151	0.204	0.248
148-3	0.103	0.151	0.214	0.225
149-1	0.103	0.148	0.249	0.320
149-2	0.084	0.143	0.237	0.287
149-3	0.077	0.152	0.267	0.317
150-1	0.116	0.178	0.263	0.274
150-2	0.100	0.141	0.195	0.217
150-3	0.109	0.159	0.225	0.264
151-1	0.094	0.192	0.326	0.376
151-2	0.091	0.180	0.312	0.375
151-3	0.083	0.155	0.260	0.311

Due to the fume hoods going down in Avedisian Hall, I had to take my cultures and do the RNA purification in CBLS. Due to this, after the four hour check, I placed the cultures back into our shaking

incubator for 10 minutes, took 9 minutes to then transport the cultures to CBLS, and returned them into a new shaking incubator for 41 minutes. As such, the cells were taken out of a stable temperature and the shaking environment was, seemingly, different as the shaking appeared to be much slower in the second incubator. Additionally, my final OD readings were with an atypical spectrophotometer

### RNA Purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151

1. Pellet 1.8mLx2 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL/spin, 30/spin at max speed, discarding flow-through
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through in waste
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

Stored total nucleic acids in -80C freezer in new RNA box. Labelled with the date on the side and, on the lid, the following:

Labelled	Contents	Labelled	Contents
1 RNA	KRLVS148-1	7 RNA	KRLVS150-1
2 RNA	KRLVS148-2	8 RNA	KRLVS150-2
3 RNA	KRLVS148-3	9 RNA	KRLVS150-3
4 RNA	KRLVS149-1	10 RNA	KRLVS151-1
5 RNA	KRLVS149-2	11 RNA	KRLVS151-2
6 RNA	KRLVS149-3	12 RNA	KRLVS151-3

Due to the change in location, different centrifuges were used. For the 4°C step, the centrifuge was set to 4°C, 21,900 xg, 10 minutes. For this step, it is typically run at 4°C, 25,830 xg, 10 minutes. For all other centrifugation steps, the max speed on the table top centrifuge was 14,000 rpm, whereas typically these steps are completed at 15,060 rpm. Nanodrop'd samples, shown below:



Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	458.2	9.164	4.296	2.13	1.54
KRLVS148 2	552.8	11.056	5.230	2.11	2.45
KRLVS148 3	515.9	10.317	4.873	2.12	2.44
KRLVS149 1	709.8	14.197	6.867	2.07	2.50
KRLVS149 2	789.7	15.795	7.555	2.09	2.41
KRLVS149 3	950.4	19.007	8.952	2.12	2.52
KRLVS150 1	601.1	12.023	5.747	2.09	2.40
KRLVS150 2	524.7	10.494	4.955	2.12	2.45
KRLVS150 3	526.4	10.528	5.027	2.09	2.46
KRLVS151 1	1496.9	29.938	13.911	2.15	2.54
KRLVS151 2	1064.4	21.287	10.217	2.08	2.60
KRLVS151 3	723.2	14.464	6.855	2.11	2.53

Transformation Plates		
Plasmid	200 uL	Remaining
pKR122	0	5?
Backbone	1	3

I checked my transformation plates, and it was very hard for me to tell if there were colonies, so the above is tentative. By the time I checked them they had been at 30°C for 24 hours. Given the lower temperature, maybe they weren't visible yet, so I did put the plates back into the incubator overnight. Additionally, I made cultures of LB + 50ng/uL Kan of what I thought were colonies on the ligation plate. I'll check again tomorrow, and we'll see what's going on.

Friday, August 19, 2022

**To Do:**

- ~~1. Streak KRLVS149 and KRLVS112 for isolation~~
- ~~2. DNase treatment of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
- ~~3. Miniprep pKR122 candidates~~
- ~~4. Check transformation plates again~~
- ~~5. Second purification of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
- ~~6. Nanodrop Pure RNA samples~~

**Results and Data:**

**Miniprep of Candidate pKR122 from *E. coli***

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column with 750uL Buffer PE. Centrifuge 30-60s x3 and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

I split my culture between two columns and eluted in 30 uL, letting the elution buffer sit on the column for 2 minutes. There was some volume loss, so there is 60 uL total. I also nanodrop'd it:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Candidate pKR122	50.8	1.017	0.541	1.88	1.71

### DNase treatment of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 RNA Samples

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube
15. Add 100 uL RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

I nanodrop'd the samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	361.9	7.237	3.693	1.96	1.48
KRLVS148 2	262.3	5.246	2.554	2.05	1.98
KRLVS148 3	292.7	5.855	2.794	2.10	2.27
KRLVS149 1	396.6	7.932	3.698	2.14	2.42
KRLVS149 2	357.8	7.155	3.373	2.12	2.37
KRLVS149 3	367.2	7.344	3.450	2.13	2.42
KRLVS150 1	388.2	7.765	3.684	2.11	2.42
KRLVS150 2	328.4	6.568	3.114	2.11	2.48
KRLVS150 3	361.7	7.233	3.478	2.08	2.15
KRLVS151 1	701.9	14.039	6.764	2.08	2.45
KRLVS151 2	422.5	8.451	3.975	2.13	2.46
KRLVS151 3	365.2	7.305	3.425	2.13	2.48

When I was using the nanodrop, I was having some issues, where after running a few samples, I would get a very odd concentration, run it again and get a concentration as though there was nothing on the pedestal. When this happened, I shut down the nanodrop program and restarted it, re-blanked it, and seemed to have no issue. I also experienced this issue when I was nanodropping my miniprep sample in the morning.

Monday, August 22, 2022

**To Do:**

1. ~~Patch KRLVS148 and KRLVS111~~
2. ~~Set up aliquots for gel and cDNA~~
3. ~~Run RNA gel~~
4. ~~Generate cDNA of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
5. ~~Diagnostic digest of candidate pKR122~~
6. ~~Run gel of diagnostic digest~~

**Results and Data:**

I set up the aliquots for both the RNA gel and cDNA synthesis according to the following volumes, calculated using excel, respectively:

Sample	ng/uL	RNA	Water
KRLVS148-1	361.9	4.14	5.86
KRLVS148-2	262.3	5.72	4.28
KRLVS148-3	292.7	5.12	4.88
KRLVS149-1	396.6	3.78	6.22
KRLVS149-2	357.8	4.19	5.81
KRLVS149-3	367.2	4.08	5.92
KRLVS150-1	388.2	3.86	6.14
KRLVS150-2	328.4	4.57	5.43
KRLVS150-3	361.7	4.15	5.85
KRLVS151-1	701.9	2.14	7.86
KRLVS151-2	422.5	3.55	6.45
KRLVS151-3	365.2	4.11	5.89

Sample	ng/uL	RNA	Water
KRLVS148-1	361.9	8.29	5.21
KRLVS148-2	262.3	11.44	2.06
KRLVS148-3	292.7	10.25	3.25
KRLVS149-1	396.6	7.56	5.94
KRLVS149-2	357.8	8.38	5.12
KRLVS149-3	367.2	8.17	5.33
KRLVS150-1	388.2	7.73	5.77
KRLVS150-2	328.4	9.14	4.36
KRLVS150-3	361.7	8.29	5.21
KRLVS151-1	701.9	4.27	9.23
KRLVS151-2	422.5	7.10	6.40
KRLVS151-3	365.2	8.21	5.29

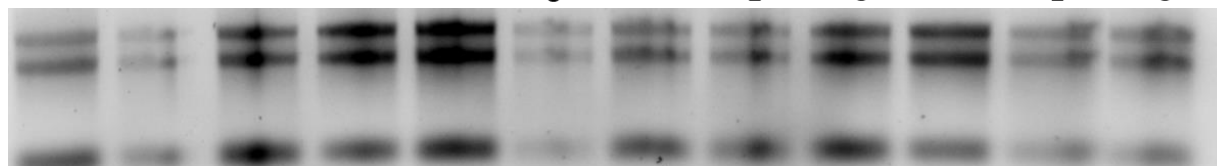
**Gel of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 RNA Samples**

1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x.
2. Rinse comb and gel rig with DI water, then ethanol both as well
3. Make fresh agarose gel by adding 1.2g to 120mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
6. Make fresh 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 12 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA), rather than smears.

Loading Order of RNA Gel											
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8	RNA 9	RNA 10	RNA 11	RNA 12

— KRLVS148 —      — KRLVS149 —      — KRLVS150 —      — KRLVS151 —

1      2      3      1      2      3      1      2      3      1      2      3



**Generate cDNA (Half) KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/19/22 Pure RNA)**

- Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	1.5 ul	25 ng/ul
RNase-free water	up to 13.5 ul	

- To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes. Total volume of cDNA and water is 13.5 uL.
- Add the 1.5 uL (NS)<sub>5</sub> oligo to the tubes.
- Incubate using program JSScDNA1 in the thermocycler:

Step	Temp	Time
1	70°C	10'
2	25°C	10'
3	4°C	hold

- Prepare master mix at 1.5 + #reactions.

Component	Final Concentration	Volume	X13.5
5X 1st strand buffer	1x	6	81
RNase-free water		2.88	38.88
100 mM DTT	10 mM	3	40.5
10 mM dNTPs	0.5 mM	1.5	20.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	22.01

- Aliquot 15 ul of master mix into each PCR tube from the first reaction (total volume now 30ul)
- Incubate using program JSScDNA2

Step	Temp	Time
1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold

- Remove RNA from sample by degrading with sodium hydroxide:
  - Add 10 ul of 1N NaOH
  - Incubate 65°C for 30'
  - Neutralize with 10 ul of 1N HCl
  - Final volume is 50 ul
- Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
- Elute in 60 ul of 0.1x EB
- Check concentration by Nanodrop
- Store cDNA at -80°C

**cDNA Purification with PCR Purification Kit**

- Add 250 uL of Buffer PB to each 50 uL cDNA reaction tube and mix.
- Place a QIAquick column in a 2mL collection tube.
- Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
- Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm.
- Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
- Place the QIAquick column in a fresh 1.5mL centrifuge tube.
- Elute: add 60uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	25.9	0.518	0.287	1.81	2.22
KRLVS148 2	43	0.860	0.468	1.84	2.02
KRLVS148 3	4.7	0.095	0.051	1.86	1.41
KRLVS149 1	38.7	0.773	0.437	1.77	2.09
KRLVS149 2	3.8	0.076	0.045	1.66	2.34
KRLVS149 3	4.6	0.092	0.047	1.95	0.67
KRLVS150 1	8.1	0.162	0.090	1.79	1.07
KRLVS150 2	9.8	0.195	0.119	1.64	1.38
KRLVS150 3	8.6	0.173	0.115	1.50	1.31
KRLVS151 1	4.6	0.092	0.072	1.28	1.16
KRLVS151 2	6.2	0.125	0.086	1.46	1.12
KRLVS151 3	13.6	0.273	0.187	1.46	0.66

I continued to have problems with the nanodrop where I would start getting weird outputs, would close out of the program, re-blank, and resume taking sample concentrations.

### Diagnostic Digest of Candidate pKR122 Miniprep w/KpnI and NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	pKR122	KpnI, NotI	2.0	-
7	Water	KpnI, NotI	-	2.0

2. Set up master mix table:

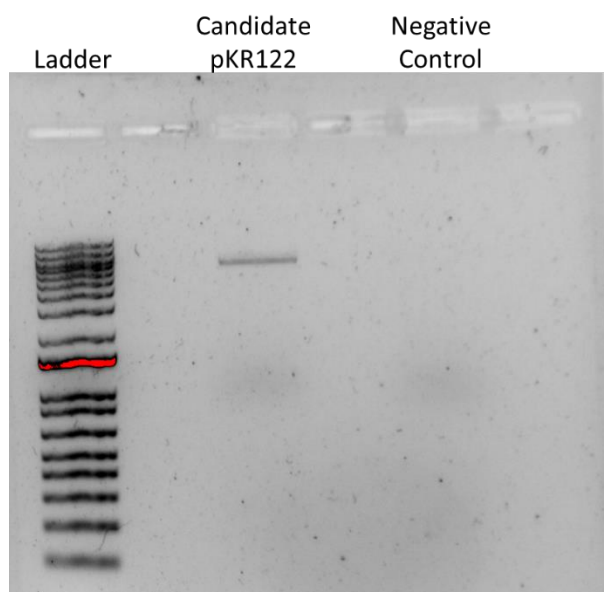
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H <sub>2</sub> O	15	45
10x Buffer (Cutsmart)	2	6
DNA	(2)	
KpnI	0.5	1.5
NotI	0.5	1.5
Total	20.0 (18.0 actual b/c of DNA)	54

3. Add indicated amounts of H<sub>2</sub>O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

### Gel of Diagnostic Digest of Candidate pKR122 Miniprep

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cooled
2. Set up small gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe and pour ~60uL gel, use ladder to mix, then place ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 10 uL ladder, and 24 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Diagnostic Digest of Candidate pKR122					
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Ladder		pKR122(?)		(-) Control	



Clearly, this was a re-ligated backbone, as there is no insertion fragment excised from the restriction digest. I did overexpose the gel, in case the band was very light again, but I did not see it. Additionally, given the much lower concentration of this miniprep, I didn't suspect that would be an issue regardless.

Tuesday, August 23, 2022

**To Do:**

1. ~~Patch KRLVS149 and KRLVS112~~
2. ~~Nanodrop RNA samples again~~
3. ~~Set up aliquots for new cDNA synthesis reaction~~
4. ~~Generate cDNA of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~

**Results and Data:**

I re-nanodrop'd the pure RNA samples using the INBRE Core lab nanodrop:

Sample Name	Nucleic Acid (ng/uL)	A260	A280	260/280	260/230
KRLVS148 1	248.9	6.222	2.939	2.12	2.36
KRLVS148 2	210.1	5.252	2.609	2.01	2.31
KRLVS148 3	283.2	7.079	3.456	2.05	2.29
KRLVS149 1	320.3	8.008	3.903	2.05	2.38
KRLVS149 2	291.5	7.287	3.474	2.10	2.44
KRLVS149 3	288.0	7.201	3.457	2.08	2.61
KRLVS150 1	321.6	8.041	3.977	2.02	2.44
KRLVS150 2	280.2	7.005	3.456	2.03	2.40
KRLVS150 3	270.0	6.749	3.342	2.02	2.38
KRLVS151 1	628.2	15.705	7.310	2.15	2.60
KRLVS151 2	335.2	8.381	4.047	2.07	2.60
KRLVS151 3	278.8	6.971	3.337	2.09	2.55

Accordingly, I re-calculated the dilutes for cDNA synthesis and diluted according to the following:

Sample	ng/uL	RNA	Water
KRLVS148-1	248.9	12.05	1.45
KRLVS148-2	210.1	14.28	-0.78
KRLVS148-3	283.2	10.59	2.91
KRLVS149-1	320.3	9.37	4.13
KRLVS149-2	291.5	10.29	3.21
KRLVS149-3	288.0	10.42	3.08
KRLVS150-1	321.6	9.33	4.17
KRLVS150-2	280.2	10.71	2.79
KRLVS150-3	270.0	11.11	2.39
KRLVS151-1	628.2	4.78	8.72
KRLVS151-2	335.2	8.95	4.55
KRLVS151-3	278.8	10.76	2.74

For KRLVS148-2, I only used 13.5 uL due to the fact that the reaction has to be 13.5 uL of RNA with 1.5 uL of NS<sub>5</sub> Primers. Given my previous calculations which would have put my RNA at a much lower concentration, I anticipate that this sample will have a lower concentration of cDNA than the others, but given how little I need for the qPCR, I do not anticipate this being an issue.

### Generate cDNA (Half) KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/19/22 Pure RNA)

- Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	1.5 ul	25 ng/ul
RNase-free water	up to 13.5 ul	

- To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes. Total volume of cDNA and water is 13.5 uL.
- Add the 1.5 uL (NS)<sub>5</sub> oligo to the tubes.
- Incubate using program JSScDNA1 in the thermocycler:

Step	Temp	Time
1	70°C	10'
2	25°C	10'
3	4°C	hold

- Prepare master mix at 1.5 + #reactions.

Component	Final Concentration	Volume	X13.5
5X 1st strand buffer	1x	6	81
RNase-free water		2.88	38.88
100 mM DTT	10 mM	3	40.5
10 mM dNTPs	0.5 mM	1.5	20.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	22.01

- Aliquot 15 ul of master mix into each PCR tube from the first reaction (total volume now 30ul)
- Incubate using program JSScDNA2

Step	Temp	Time
1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold



8. Remove RNA from sample by degrading with sodium hydroxide:
  - a. Add 10 ul of 1N NaOH
  - b. Incubate 65°C for 30'
  - c. Neutralize with 10 ul of 1N HCl
  - d. Final volume is 50 ul
9. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
10. Elute in 60 ul of 0.1x EB
11. Check concentration by Nanodrop
12. Store cDNA at -80°C

### cDNA Purification with PCR Purification Kit

1. Add 250 uL of Buffer PB to each 50 uL cDNA reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 60uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Wednesday, August 24, 2022

#### To Do:

- ~~1. Set up cultures of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
- ~~2. Make MHB~~
- ~~3. Streak out cells from pKR122 electroporation plate~~
- ~~4. RNA purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
- ~~5. Run dishwasher~~

### Results and Data:

#### Setting Up KRLVS148, KRLVS149, KRLVS111, KRLVS112 Cultures for RNA Purification

1. Scrape each duplicate patch of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (30uL of sample in 570uL of MHB)
3. Calculate volume to add to tubes of 7 mL MHB for an OD of 0.08 for KRLVS149 and KRLVS112, and 0.1 for KRLVS148 and KRLVS111 (calculated OD of 0.095 or 0.13 for error)
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at 2 hours initially

RNA Cultures OD's				
Sample	0 hours	2 hours	4 hours	5 hours
148-1	0.147	0.164	0.227	0.261
148-2	0.127	0.175	0.245	0.278
149-1	0.093	0.170	0.293	0.353
149-2	0.085	0.160	0.289	0.372
111-1	0.132	0.166	0.217	0.248
111-2	0.119	0.154	0.186	0.203
112-1	0.065	0.122	0.228	0.275
112-2	0.092	0.187	0.338	0.417

I forgot to set up my MHB the night before as I normally do, but in addition to that, there was simply not enough MHB for me to operate the experiment in triplicate at the typical 8 mL of MHB per culture tube. I thought there was unsupplemented MHB, but there was not, so I figured out the best option in order to move the experiment forward was to use 7 mL of MHB and conduct the experiment in duplicate as I had done before. Obviously, this means I might have to repeat it again in duplicate, but I would have had to repeat it anyways... Furthermore, once I started to scrape up the patches I realized I would not have had enough cells to work in triplicate anyways as all my patches for KRLVS149 and KRLVS112 were very light, and one of each was basically unusable due to the lack of growth. If I do have to repeat this experiment, I think that I will patch them out at the end of the same day I patch out the *ΔrpsU2* cells, because I think maybe the *lacZ* is impairing their growth marginally despite containing bs21.

After setting up my cultures, I made two bottles of MHB. Because Hannah was also making CHAH plates, I added the bottles to her autoclave load.

I then took my electroporation plate from my last attempt at electroporating the ligation of my *PrpsU2 tul4* 5'UTR amplified gBlock fragment with the digested pKR89 backbone and struck out from a clump of colonies which seemed to have been dragged together with the beads when I was spreading them. My hope is the plasmid was maintained in the cells and that I will be able to separate it out from a potentially mixed population of backbone only and plasmid cells. I grew these at 30°C as I did with the original plate. My plan is to then make overnight cultures tomorrow night and miniprep on Friday. If I have time, then I will also run a diagnostic digest and set up sequencing on Monday.

### RNA Purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112

1. Pellet 1.8mLx2 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through in phenol and methanol waste
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

Stored total nucleic acids in -80C freezer in new RNA box. Labelled with the date on the side and, on the lid, the following:

Labelled	Contents
1 RNA	KRLVS148-1
2 RNA	KRLVS148-2
3 RNA	KRLVS149-1
4 RNA	KRLVS149-2
5 RNA	KRLVS111-1
6 RNA	KRLVS111-2
7 RNA	KRLVS112-1
8 RNA	KRLVS112-2

## Reagents

Mueller Hinton Broth (500 mL)

To make, add to a 1 L bottle:

10.5g of Mueller Hinton Broth mix

500 mL of diH<sub>2</sub>O

Autoclave on Liquid 30'

Can keep un-supplemented media at room temperature indefinitely

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

## Thursday, August 25, 2022

To Do:

1. ~~Put away dishes~~
2. ~~Nanodrop RNA purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
3. ~~DNase treatment of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
4. ~~Second purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
5. ~~Nanodrop KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
6. ~~Set up aliquots for gel and cDNA~~
7. ~~Run RNA gel~~
8. ~~Nanodrop KRLVS148, KRLVS149, KRLVS150, and KRLVS151 cDNA samples~~

## Results and Data:

I nanodrop'd my samples from yesterday. I did not experience any issues with the nanodrop as I had been, but I was also being particularly careful. Additionally, I set it to the RNA setting, which it should have been at anyways. It looks good to me.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	388.5	9.713	4.653	2.09	2.19
KRLVS148 2	442.2	11.054	5.560	1.99	2.42
KRLVS149 1	567.2	14.179	6.940	2.04	2.51
KRLVS149 2	538.8	13.469	6.435	2.09	2.42
KRLVS111 1	308.4	7.710	3.648	2.11	2.37
KRLVS111 2	242.3	6.058	2.930	2.07	2.24
KRLVS112 1	382.2	9.555	4.591	2.08	2.40
KRLVS112 2	566.9	14.173	7.023	2.02	2.38

**DNase treatment of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 RNA Samples**

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
6. Place spin column in new collection tube
7. Wash twice with 400 uL RNA PreWash buffer, 30s at max speed, discarding flow-through in phenol and methanol waste
8. Add 700 ul of Wash buffer, let sit on column for 3 min
9. Spin max speed for 2 min
10. Wash again with 700 ul Wash buffer
11. Spin max speed for 2 min
12. Place column in new collection tube
13. Spin max speed for 3 min
14. Place column in clean 1.5 mL tube
15. Add 100 uL RNase-free water, let sit on column 2 min
16. Spin max speed 1 min
17. Place flow-through on column again, spin 1 min

On the first and second sample, I messed up and prior to the dry 3 minute spin, I accidentally added 100 uL of nuclease free water to each of the columns. I immediately sucked up what I could and after the spin I took the flow through and reapplied it to the column prior to adding the column to the final collection tube. Obviously, this may have caused issues in the contamination of these samples. Additionally, I dropped sample 8 out of the RNase free area I had set up, but the column remained in the 2 mL collection tube. I RNase'd Away the outer of the tube and column and continued on. I nanodrop'd the samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	191.4	4.786	2.265	2.11	2.34
KRLVS148 2	191.4	4.786	2.265	2.11	2.34
KRLVS149 1	314.4	7.861	3.681	2.14	2.43
KRLVS149 2	364.3	9.108	4.318	2.11	2.50
KRLVS111 1	108.1	2.703	1.294	2.09	2.41
KRLVS111 2	151.3	3.782	1.805	2.10	2.37
KRLVS112 1	226.9	5.673	2.671	2.12	2.46
KRLVS112 2	342.6	8.564	4.041	2.12	2.47

Additionally, I set up aliquots for the RNA gel and cDNA according to the following tables, respectively:

Sample	ng/uL	RNA	Water
KRLVS148 1	191.4	5.22	4.78
KRLVS148 2	191.4	5.22	4.78
KRLVS149 1	314.4	3.18	6.82
KRLVS149 2	364.3	2.74	7.26
KRLVS111 1	108.1	9.25	0.75
KRLVS111 2	151.3	6.61	3.39
KRLVS112 1	226.9	4.41	5.59
KRLVS112 2	342.6	2.92	7.08

Sample	ng/uL	RNA	Water
KRLVS148 1	191.4	15.67	-2.17
KRLVS148 2	191.4	15.67	-2.17
KRLVS149 1	314.4	9.54	3.96
KRLVS149 2	364.3	8.23	5.27
KRLVS111 1	108.1	27.75	-14.25
KRLVS111 2	151.3	19.83	-6.33
KRLVS112 1	226.9	13.22	0.28
KRLVS112 2	342.6	8.76	4.74

For the low concentration samples of RNA, I added just 13.5 uL, hopefully it will be enough.

Additionally, I nanodrop'd the cDNA samples I synthesized yesterday. The ethanol contamination is super not ideal:

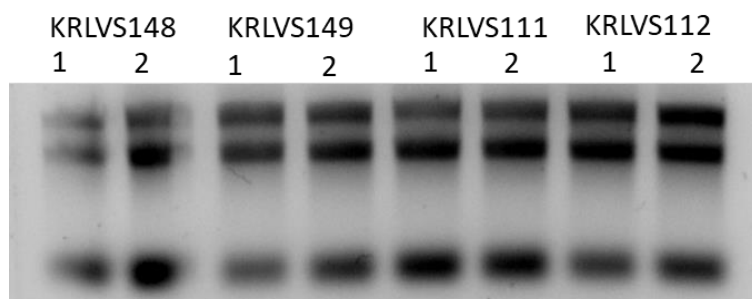
Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	2.8	0.086	0.044	1.96	1.56
KRLVS148 2	3.1	0.094	0.063	1.48	1.33
KRLVS148 3	17.5	0.531	0.304	1.75	1.95
KRLVS149 1	19.3	0.584	0.338	1.73	2.01
KRLVS149 2	14.1	0.427	0.244	1.75	2.07
KRLVS149 3	17.9	0.541	0.310	1.75	0.78
KRLVS150 1	15.3	0.463	0.265	1.75	1.88
KRLVS150 2	11.1	0.335	0.187	1.79	1.93
KRLVS150 3	16.4	0.497	0.283	1.76	1.99
KRLVS151 1	3.5	0.106	0.063	1.69	1.67
KRLVS151 2	18.2	0.550	0.317	1.74	1.72
KRLVS151 3	11.3	0.341	0.194	1.76	1.98

### Gel of KRLVS148, KRLVS149, KRLVS111, and KRLVS112 RNA Samples

1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x.
2. Rinse comb and gel rig with DI water, then ethanol both as well
3. Make fresh agarose gel by adding 2.0g to 200mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
6. Make fresh 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 12 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA), rather than smears.

Gel Loading Order							
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8

When I was turning the gel within the gel rig, my thumb slipped and I bapped the gel a bit on the side so I avoided using the last two lanes.



Contains the distinct bands I would expect: 23s, 16s, and tRNAs. The first lane obviously ran a little weird, but I noticed that the well itself seemed to be a little oddly formed, so I attribute it to that.

Friday, August 26, 2022

**To Do:**

1. ~~Put away caps~~
2. ~~Set up 1.5 ng/uL aliquots of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 cDNA samples~~
3. ~~qRT-PCR of 8/19 RNA samples - 1~~
4. ~~qRT-PCR of 8/19 RNA samples - 2~~
5. Streak out KRLVS148, KRLVS149, KRLVS111, and KRLVS112

**Results and Data:**

Due to the fact I had been taking my nanodrop readings incorrectly before on the DNA setting for both RNA and cDNA, my calculates for my 1.5 ng/uL stocks would not have actually been 1.5 ng/uL. As such, I re-processed one of my cDNA samples under the DNA sample type in order to back-calculate the concentration that I have been using for qRT-PCR since it seems to be at a sweet spot with values right above the minimum crossing point and below the maximum. The calculation was as follows:

ssDNA Conc. (ng/uL)	DNA Conc. (ng/uL)	(60*1.5)/DNA Conc.	(ssDNA Conc.*Vol. According to DNA Conc.)/60
17.5	26.5	3.40	0.99

Going forward I will making my stocks according to this concentration, as done with these cDNA samples as follows:

0.99 ng/uL qPCR Stock			
Sample Name	conc. (ng/u)	DNA uL	EB uL
KRLVS148 1	2.8	21.23	38.77
KRLVS148 2	3.1	19.17	40.83
KRLVS148 3	17.5	3.40	56.60
KRLVS149 1	19.3	3.08	56.92
KRLVS149 2	14.1	4.22	55.78
KRLVS149 3	17.9	3.32	56.68
KRLVS150 1	15.3	3.88	56.12
KRLVS150 2	11.1	5.35	54.65
KRLVS150 3	16.4	3.62	56.38
KRLVS151 1	3.5	16.98	43.02
KRLVS151 2	18.2	3.27	56.73
KRLVS151 3	11.3	5.26	54.74

**qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/19/22) cDNA - 1**

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL343/344, KROL504/505, and KROL499/500 as test primers to amplify the 5'UTR, downstream of *rpsU2*, and *lacZ* respectively. KROL63/64 the control, amplifying *tul4*.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*4=24 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	455.0 uL
5uM primer set	1 uL	45.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	364.0 uL
Total:	20 uL	864.5 uL

4. Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
5. Add 3.5µL of cDNA into appropriately labelled tubes.
6. As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
7. Pipette 20 µL of each primer set strip tubes, in triplicate, using multichannel pipet.
8. Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds <i>rpsU2</i>
C	KROL399/400 <i>lacZ</i>
D	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			B1			C1			D1		
B	A2			B2			C2			D2		
C	A3			B3			C3			D3		
D	A4			B4			C4			D4		
E	A5			B5			C5			D5		
F	A6			B6			C6			D6		

### qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/19/22) cDNA - 2

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL343/344, KROL504/505, and KROL499/500 as test primers to amplify the 5'UTR, downstream of *rpsU2*, and *lacZ* respectively. KROL63/64 the control, amplifying *tul4*.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (6\*4=24 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	455.0 uL
5uM primer set	1 uL	45.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH <sub>2</sub> O	8 uL	364.0 uL
Total:	20 uL	864.5 uL

4. Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
5. Add 3.5µL of cDNA into appropriately labelled tubes.

6. As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.
7. Pipette 20 µL of each primer set strip tubes, in triplicate, using multichannel pipet.
8. Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

Master Mix Label	Primer Set
A	KROL343/344 5'UTR
B	KROL504/505 ds <i>rpsU2</i>
C	KROL399/400 <i>lacZ</i>
D	KROL63/64 <i>tul4</i>

1	2	3	4	5	6	7	8	9	10	11	12
148-1	148-2	148-3	149-1	149-2	149-3	150-1	150-2	150-3	151-1	151-2	151-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A7			B7			C7			D7		
B	A8			B8			C8			D8		
C	A9			B9			C9			D9		
D	A10			B10			C10			D10		
E	A11			B11			C11			D11		
F	A12			B12			C12			D12		



Monday, August 29, 2022

**To Do:**

1. ~~Ligate *PrpsU2 tul4* 5'UTR gBlock fragment with digested pKR89~~
2. ~~Transform ligation into *E. coli*~~

**Results and Data:**

I first nanodrop'd my gel purifications and received the following concentrations:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR89 KpnI NotI Dig. GP	15.2	0.304	0.152	2.01	0.04
<i>PrpsU2 tul4</i> 5'UTR Dig. GP	29.8	0.596	0.309	1.93	0.11

I used the molar ratio calculator to determine the volumes for my ligation. However, due to the high concentration of my insert fragment, I would have had to pipet 0.33 uL. As such, I added 15 uL 0.1x EB and re-nanodrop'd this sample. I then got 15.1 ng/uL which was still too high, added another 15 uL of 0.1x EB for a final concentration of 10.2 ng/uL which I used accordingly.

**Ligation of *PrpsU2 tul4* 5'UTR PCR from gBlock with pKR68 backbone**

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

Tube	Insert	Backbone
1	KpnI, NotI dig., pur. <i>PrpsU2:tul4</i> 5'UTR PCR	KpnI, NotI digested, purified pKR68
2	-	KpnI, NotI digested, purified pKR68

2. Set up master mix table:

Component	3X (uL)	5X (uL)	BB only (uL)
H <sub>2</sub> O	13.66	13.30	14.21
Ligation Buffer	2	2	2
Backbone	3.29	3.29	3.29
Insert	0.55	0.91	-
Ligase	0.5	0.5	0.5
TOTAL	20.00	20.00	20.00

9. 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.
10. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
11. To the individual tubes, add indicated amounts of H<sub>2</sub>O (\_\_\_uL), 10x buffer (\_\_\_uL), insert (\_\_\_uL), and backbone (\_\_\_uL).
12. Add indicated amount of ligase (\_\_\_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
13. After all of the components have been added, mix each tube with a pipette set to 18 uL.
14. Place on bench for 10 minutes.

**Transformation of *PrpsU2 tul4* 5'UTR Ligation into *E. coli***

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.
  - a. Positive control: pF; Backbone ligation: KpnI-NotI digested pKR68

**Reaction table**

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR89	1 uL	100 ul	1

2	(-) control	None	0	Remaining	1
3	Backbone Ligation	BB only ligation	8 uL	100 uL, Rem.	2
4	3x Ligation	3x Ligation	8 uL	100 uL, Rem.	2
5	5x Ligation	5x Ligation	8 uL	100 uL, Rem.	2
Total number of plates					8

2. Check to be sure you have enough LB-Kan plates. 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
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

I ended up leaving the cells in recovery for an extra ten minutes, not for any reason, it just took me a little time to set everything up. The plates were once again fairly wet despite the nearly two hours they were in the incubator, I assume the fridge and humidity are both to blame. Given how the plates came out last time, I made sure to dry them for a longer amount of time with the beads. Additionally, previously when running the transformation, in order to plate the remaining I spun for 3 min @13,000 rpm. However, this time, given the difficulties I've been having, I spun for 3 min @10,000 rpm, since obviously these cells are a bit more fragile. I didn't previously think it was an issue given that the transformation should have already commenced, but hey, no reason to not treat the cells gently.

Tuesday, August 30, 2022

**To Do:**

- ~~1. Patch out KRLVS148 and KRLVS111 in the morning~~
- ~~2. Make and filter sterilize 2.5% iron pyrophosphate~~
- ~~3. Supplement MHB~~
- ~~4. Count transformation plates~~
- ~~5. Make overnights of transformation plates and the one in the fridge~~
- ~~6. Patch out KRLVS149 and KRLVS112 in the afternoon~~

**Results and Data:**

I patched out KRLVS148 and KRLVS111 at 10:45 am. I forgot I'm only doing it in duplicate, so I patched out three patches of KRLVS111, and two patches of KRLVS148 on CHAH+Kan plates. Additionally, I made two patches of LVS for Kathryn on regular CHAH.

Volume Plated	3x Ligation	5x Ligation	Backbone
200 uL	0	0	0
Remaining	0	0	1

Due to the fact I didn't see any colonies on my transformation plates, I made overnights only off of the plate I struck out from my last transformation. I made four overnights. I will check my plates again tomorrow. I might ask if I could try out the original configuration of this plasmid, not low copy number.

## Reagents

### Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

### 2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

Wednesday, August 31, 2022

#### To Do:

- ~~1. Miniprep the overnights of potential pKR122 candidates~~
- ~~2. Run a diagnostic digest of candidate pKR122 samples~~
- ~~3. Run gel of diagnostic digest~~

## Results and Data:

### Miniprep of Candidate pKR122 from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column with 750uL Buffer PE. Centrifuge 30-60s x3 and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

### Diagnostic Digest of Candidate pKR122 Miniprep w/KpnI and NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H <sub>2</sub> O Volume (uL)
1	Cand. pKR122-1	KpnI, NotI	2.0	-
2	Cand. pKR122-2	KpnI, NotI	2.0	-
3	Cand. pKR122-3	KpnI, NotI	2.0	-
4	Cand. pKR122-4	KpnI, NotI	2.0	-
5	P-Mut pKR122	KpnI, NotI	2.0	-
6	Water	KpnI, NotI	-	2.0

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H <sub>2</sub> O	15	105
10x Buffer (Cutsmart)	2	14
DNA	(2)	

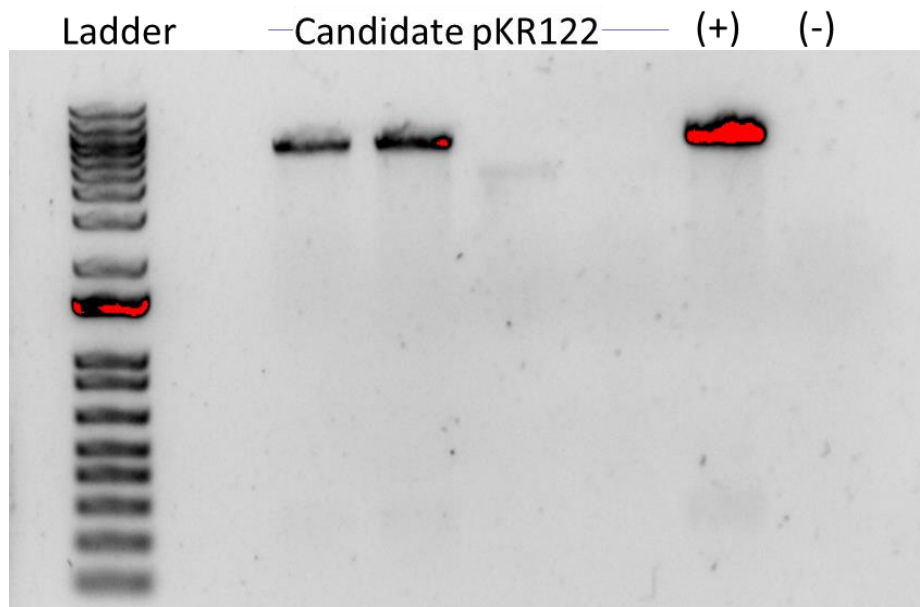
KpnI	0.5	3.5
NotI	0.5	3.5
Total	20.0 (18.0 actual b/c of DNA)	126

3. Add indicated amounts of H<sub>2</sub>O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

### Gel of Diagnostic Digest of Candidate pKR122 Miniprep

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cooled
2. Set up small gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe and pour ~60uL gel, use ladder to mix, then place ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 10 uL ladder, and 15 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Diagnostic Digest of Candidate pKR122									
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Ladder		Cand. 1	Cand. 2	Cand. 3	Cand. 4	P-Mut. +	(-) Cont.		HT



It is very faint, but on the overexposed image of the gel, there is a band consistent with the fragment size according to the ladder and the positive control (the plasmid with the point mutation).

## 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.