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January 2023

Monday, January 2, 2023

To Do:

1. ~~Patch out KRLVS192, KRLVS193, and LVS-pF~~
2. ~~Prepare tubes for GFP assay~~
3. ~~Start overnight cultures of candidate pKR168 yeast transformation plates in SD-ura~~

Results and Data:

I spoke with Kathryn and she is hoping the transformations will be okay after having to wait a little longer than I anticipated. Additionally, we discussed the RNA stability assay, and she will help me out on January 30th, the last Monday of the month. I am planning to do back-to-back growth days.

I started six yeast overnights in SD-ura at around 1:20. There were two colonies that seemed a bit larger on the remaining plate, so I cultured one of those. Hopefully I get something.

Tuesday, January 3, 2023

To Do:

1. ~~Miniprep candidate pKR168~~
2. ~~Start cultures of magnesium environmental conditions~~
3. ~~Nanodrop minipreps~~
4. ~~Run GFP assay of Mg environmental condition~~

Results and Data:

Miniprep of Candidate pKR168 Plasmid from Yeast

1. Aliquot 3x 1.666 mL of the yeast cells (early log phase, total of 4.5 mL) into 2 ml microfuge tubes and spin down the cells at 600 x g for 3 minutes.
2. Discard the supernatant and add 200 ul Solution 1 to each pellet and add 3 ul Zymolyase. Resuspend pellet by flicking tube or mild vortexing. Note: If running multiple samples, can make a solution 1-enyzme mixture by combining 15 ul of Zymolyase with 1 mL of Solution 1, then adding 200 ul to each pellet. If cells are from stationary phase, add more Zymolyase to ensure efficient lysis.
3. Incubate at 37C for 30 minutes. Then add ~100 ul of glass beads and strap tubes to a vortex set to high speed to help lyse cells, for 30 additional minutes.
4. Add 200 ul Solution 2 to each tube and mix well.
5. Add 400 ul Solution 3 to each tube and mix well. Centrifuge at maximum speed for 3 minutes.
6. Transfer the supernatant from one tube to the Zymo-Spin I Column in a collection tube and centrifuge at >10,000 x g for 30 seconds. Add supernatant from next tube and centrifuge again until entire culture has been added to one column.
7. Discard the flow-through and ensure the flow-through does not come into contact with the column tip.
8. Add 550 ul DNA Wash Buffer to the Spin Column and centrifuge at >10,000 x g for 2 minutes. Discard the flow-through. Spin for 3 more minutes to remove residual ethanol.
9. Place the Spin Column in a clean 1.5 mL microcentrifuge tube and add 15 ul of 0.1xEB. Allow to sit for 5 minutes. Centrifuge at 10,000 x g for 1 minute to elute the plasmid DNA. Put the eluate back on the column, let sit, and centrifuge again

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR168 1	45.6	0.912	0.923	0.99	0.25
Cand. pKR168 2	53.9	1.079	1.168	0.92	0.22
Cand. pKR168 3	42.2	0.845	0.562	1.50	0.76
Cand. pKR168 4	173.8	3.475	1.900	1.83	1.10
Cand. pKR168 5	50.2	1.004	0.539	1.86	1.28

Obviously, there is some pretty bad contamination issues, however, I'll go ahead and PCR all of them and hopefully that'll work. With silent hope that the last two are the plasmid I need given them having marginally less contamination.

Effect of 10x, 100x, and 1000x Mg on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Condition Number	0	1	2	3	4
Experiment Component	Background	Control	Test 1	Test 2	Test 3
Condition	CDM	CDM	1.0 mg/mL Mg	10.0 mg/mL Mg	100.0 mg/mL Mg

1. Resuspend patches of cells in 400uL of CDM checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of respective CDM, either regular or varying concentrations of iron
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, CDM

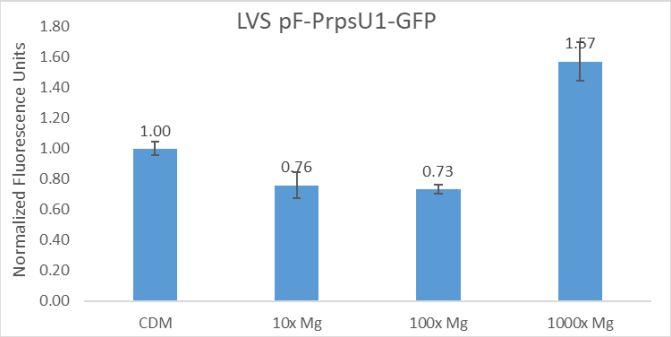
GFP Assay on CDM Fe Conditions in KRLVS192 and KRLVS193

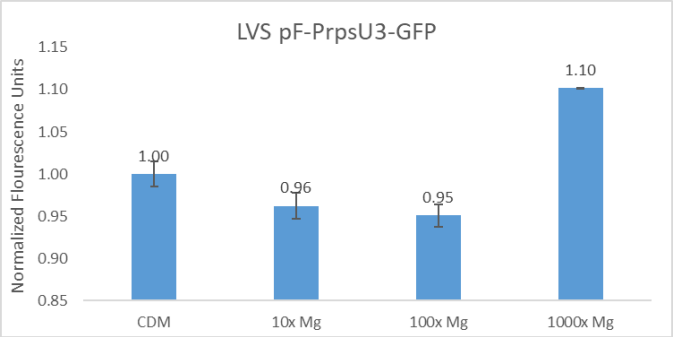
1. Pellet 4 mLs of culture and spin at max speed for 3 minutes
2. Remove all CDM, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS in triplicate as control.
5. Go to INBRE lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
6. Read OD600 from clear plate on ID3 plate reader:
    a. Select Absorbance, wavelength=600
    b. Plate type: 96-well standard clearbtm (first option)
7. Transfer 200 ul of each well from clear plate to black plate using the multichannel
8. Read fluorescence from black plate on ID3 plate reader:
    a. Select fluorescence
    b. Wavelength: 495 to 535
    c. Plate type: CoStar 3789
    d. Gain: Automatic
    e. Integration: 380 ms

Label	Condition
A	CDM
B	1.0 mg/mL Mg
C	10.0 mg/mL Mg
D	100.0 mg/mL Mg

Label	Strain and BR
1	KRLVS192 1
2	KRLVS192 2
3	KRLVS193 1
4	KRLVS193 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1		A3	A3	A3		LVS	LVS	LVS	
B	A2	A2	A2		A4	A4	A4		PBS	PBS	PBS	
C	B1	B1	B1		B3	B3	B3					
D	B2	B2	B2		B4	B4	B4					
E	C1	C1	C1		C3	C3	C3					
F	C2	C2	C2		C4	C4	C4					
G	D1	D1	D1		D3	D3	D3					
H	D2	D2	D2		D4	D4	D4					





Wednesday, January 4, 2023

To Do:

1. ~~PCR of candidate pKR168~~
2. ~~PCR purification of candidate pKR168 PCR~~
3. ~~Run gel of candidate pKR168 PCR~~

Results and Data:

I diluted all of my samples to 40 ng/uL according to the following ratios:

Sample Name	Nucleic Acid (ng/uL)	DNA (uL)	EB (uL)
Cand. pKR168 1	45.6	0.88	0.12
Cand. pKR168 2	53.9	0.74	0.26
Cand. pKR168 3	42.2	0.95	0.05
Cand. pKR168 4	173.8	0.23	0.77
Cand. pKR168 5	50.2	0.80	0.20

PCR of Candidate pKR168 Minipreps for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL6, KROL257	-
2	+ control	MP pKR168	KROL6, KROL257	633
3-7	P*rpsU2_tul4*UTR	MP pKR168	KROL6, KROL257	633

2. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL6, KROL257, (10uM), and candidate pKR168
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 H2O to negative control tube (template volume for 1 reaction)

6. Prepare a master-containing:
    - mgH2O, dNTPs, Primestar buffer, and Primestar enzyme
7. Mix the master-mix solution by pipetting up and down
8. Add 19.0 uL of master-mix to negative control PCR tube
9. Add 19.0 uL 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

Total reaction volume	20
Total number of reactions	7

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	8
ddiH2O			11.4	85.2
PrimeSTAR GXL Buffer	5x	1x	4.0	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
oligo F	10 uM	0.3 uM	0.6	4.8
oligo R	10 uM	0.3 uM	0.6	4.8
template	40.0 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
		Total volume	20	152

PCR Purification of Candidate pKR168 Yeast Miniprep PCR

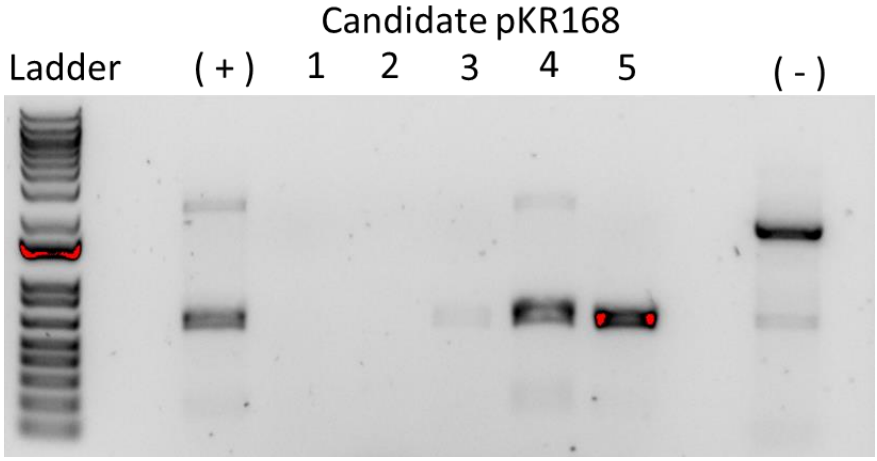
1. Add 100 uL of Buffer PB to each 20 uL PCR reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
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.

Gel of Candidate pKR168 Yeast Miniprep PCR

1. Melt agarose gel until completely dissolved, then place in 56°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. 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.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 10 uL ladder, and 5 uL of each sample.
6. Ran for 45 minutes at 113V.

My gel (pictured below) obviously has contamination in the negative control, but it's weird because that band isn't super present in all of the samples. It is in the positive control, but I expected it to be since it was there before (a discarded candidate pKR168), so I'm not sure what to make of that. There is also a bit of contamination at the band size I'm looking for, but it is very slight. As such, I disregarded candidate 3

as a possibility, however I think that both candidates 4 and 5 look promising. I will sequence the whole plasmid for candidate 4 since it had a high concentration (for yeast), and the PCR for candidate 5. Based on the extra bands, I think candidate 5 is more promising, but we will see.



In preparation for sequencing, I nanodrop'd the PCR for candidate 5, and it was 50.3 ng/uL.

Thursday, January 5, 2023

To Do:

1. ~~Set up sequencing for candidate pKR168~~
2. ~~Load dishwasher~~
3. ~~Streak out KRLVS148 and KRLVS111~~
4. ~~Patch out KRLVS75~~
5. ~~Make 2% hemoglobin~~
6. ~~Put away dishes~~

Results and Data:

I diluted Candidate 5's PCR by half in order to pipet a larger volume (labelled as candidate 2, whoops). I set up my sequencing acroding to the following:

#	Type	Sample	Primer	Size (bp)	[Stock] (ng/µl)	PCR (ng)	PCR (uL)	Plasmid (uL)	H_2O (uL)
SS1	Plasmid	Cand. pKR168 1	KROL257	9861	173.8			2.30	7.14
SS2	PCR	Cand. pKR168 2	KROL257	633	25.15	15.83	0.63		8.81
a.Add 2.56 µl of 2.5 µM stock to each reaction									

Reagents

2% Hemoglobin (5x 150 mL)

Add 3g hemoglobin (kept in fridge) to 500 mL flask (add stir bar)
Add 150 mL Type 1 ddiH_2O
Stir for at least 10 minutes, until clumps are all dissolved
Autoclave Liquid20 in water bath

Friday, January 6, 2023

To Do:

1. ~~Think about RNA stability assay~~
2. ~~Make glycerol stocks of KRLVS75~~
3. ~~Streak KRLVS28 and KRLVS75 to single colony~~
4. ~~Streak out KRLVS149 and KRLVS112~~
5. ~~Patch out KRLVS9 and KRLVS16 at room temperature; ask for KMLFT104 (d*rpsU1*)~~
6. ~~Pour out hemoglobin and suffer~~

Results and Data:

I got my sequencing results back from Janet. Evidently, the first sample (Candidate pKR168 4) did not provide any signal. Sample 2, Candidate pKR168 5, had a single point mutation in the *lacZ*. Due to the fact that the PCR was contaminated, Kathryn wants me to redo the PCR, get a clean one, and submit 4 and 5 (and potentially 3) for sequencing again, all in PCR form.

Making Personal Glycerol Stocks and Single Use Aliquots of KRLVS75

1. Prepare 1600uL of MHB in a 2mL tube
2. Take at least half of a thickly spread plate and add cells to the MHB tube
3. Resuspend until there are no clumps in the MHB
4. Add 400ul of 75% glycerol to the 800uL mix by pipetting
5. Aliquot 1 mL to cryovial and 50ul of solution to tubes, freeze at -80

Monday, January 9, 2023

To Do:

1. ~~Make 2% hemoglobin~~
2. ~~Make 2.5% iron pyrophosphate~~
3. ~~Patch out KRLVS28 and KRLVS75 at room temperature~~
4. ~~Patch out KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
5. ~~PCR of candidate pKR168 minipreps~~
6. ~~PCR purification~~
7. ~~Run gel of candidate pKR168 miniprep PCR~~
8. ~~Make glycerol stocks of KRLVS111 and KRLVS112~~
9. ~~Make glycerol stocks of KRLVS9, KRLVS16, and KMLFT104~~
10. ~~Supplement MHB~~
11. ~~Set up cultures for tomorrow~~

Results and Data:

PCR of Candidate pKR168 Minipreps for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL6, KROL257	-
2	+ control	MP pKR168	KROL6, KROL257	633
3-7	P*rpsU2_tul4*UTR	MP pKR168	KROL6, KROL257	633

2. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL6, KROL257, (10uM), and candidate pKR168
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 H2O to negative control tube (template volume for 1 reaction)
6. Prepare a master-containing:
    - mgH2O, dNTPs, Primestar buffer, and Primestar enzyme
7. Mix the master-mix solution by pipetting up and down
8. Add 19.0 uL of master-mix to negative control PCR tube
9. Add 19.0 uL 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

Total reaction volume	20
Total number of reactions	7

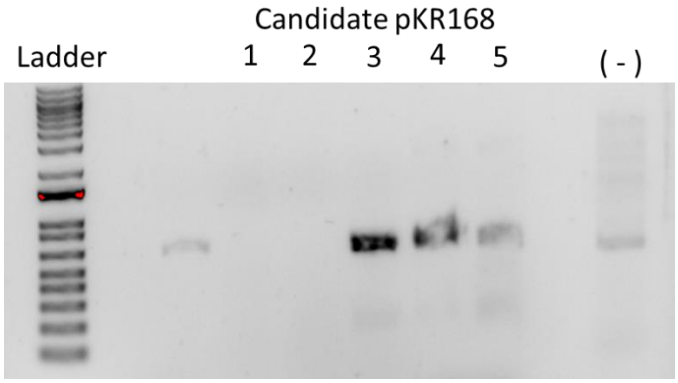
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	8
ddiH2O			11.4	85.2
PrimeSTAR GXL Buffer	5x	1x	4.0	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
oligo F	10 uM	0.3 uM	0.6	4.8
oligo R	10 uM	0.3 uM	0.6	4.8
template	40.0 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
		Total volume	20	152

PCR Purification of Candidate pKR168 Yeast Miniprep PCR

1. Add 100 uL of Buffer PB to each 20 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.

Gel of Candidate pKR168 Yeast Miniprep PCR

1. Melt agarose gel until completely dissolved, then place in 56°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. 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.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 10 uL ladder, and 5 uL of each sample.
6. Ran for 45 minutes at 113V.



I replaced each component from the PCR, and made sure to not open any of the DNA until I had added water to each tube and the master mix. Clearly contamination is getting in there somehow but WHEREEEEEEEEEEE? End me lol. Anyways, Kathryn told me to just go ahead and sequence candidates 3, 4, and 5.

Reagents

2% Hemoglobin (3x 300 mL)

Add 6g hemoglobin (kept in fridge) to 1L flask (add stir bar)
Add 300 mL Type 1 ddiH_2O
Stir for at least 10 minutes, until clumps are all dissolved
Autoclave Liquid20 in water bath

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH2O (type 1), dissolved overnight, fresh solution every 2 weeks

Mueller Hinton Broth (500 mL)
To supplement add:
5mL of 10% glucose
5mL of 2.5% iron pyrophosphate
isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)
Can keep this supplemented media for 2 weeks, storing at 4 ℃

Tuesday, January 10, 2023

To Do:

1. ~~Set up cultures of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
2. ~~Set up sequencing~~
3. ~~Patch out KRLVS28 and KRLVS75~~
4. ~~Run B-gal of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
5. ~~Run dishwasher~~
6. ~~Set up cultures for tomorrow~~

Results and Data:

I nanodrop'd my promising PCR reactions:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR168 3	6.7	0.134	0.073	1.82	1.22
Cand. pKR168 4	50.2	1.004	0.548	1.83	2.20
Cand. pKR168 5	46.1	0.923	0.497	1.86	2.33

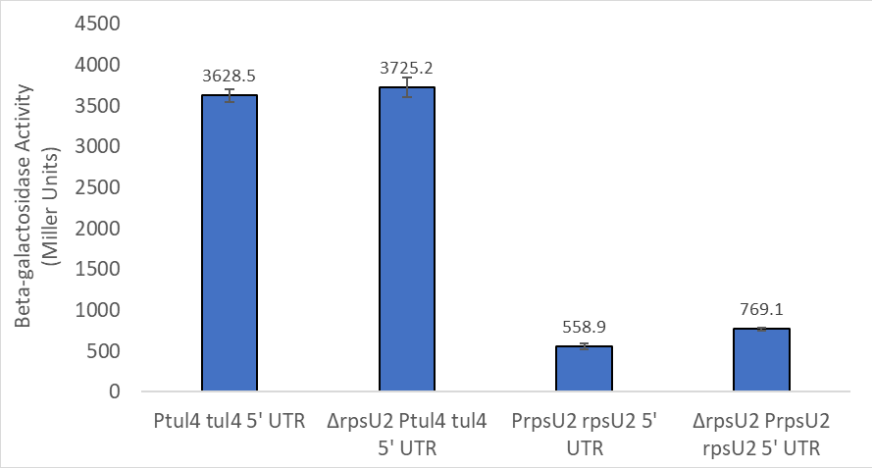
However, given that the concentration of candidate 3 was so low, I did not see a reason to ultimately sequence it, as it did not seem to amplify, and I don't super trust the gel unfortunately. Regardless, I sequenced candidates 4 and 5:

Sample	Type	Template	Primer	Size (bp)	[Stock] ng/uL	PCR (ng)	PCR (uL)	H2O (uL)
SS1	PCR	Cand. pKR168 4	KROL257	633.00	25.10	15.83	0.63	8.81
SS2	PCR	Cand. pKR168 5	KROL257	633.00	23.05	15.83	0.69	8.75
a.Add 2.56 µl of 2.5 µM stock to each reaction								

β-galactosaidase Assay of KRLVS111, KRLVS112, KRLVS148, and KRLVS149

1. Grow 7ml cultures until OD600 = 0.3
2. Turn on 28°C water bath
3. Determine amount of Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = _µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. Once cultures reach OD_{600} = 0.3, place on ice 30 min and put ONPG in water bath
7. After cells have incubated on ice, measure OD_{600} of bacterial cultures
8. Add 200µl culture to each reaction tube (add 200µl culture media to blank tube)
9. Add 30µl 0.1% SDS to each reaction tube
10. Add 60µl CHCl3 (chloroform) to each reaction tube
11. Vortex reaction pairs on high for 6 secs (time precisely with timer)
12. Put in water bath for 10 min
13. Prepare repeater pipette with 1M Na_2CO_3 (stop)
14. Add 200µl ONPG in 5 sec intervals (use timer with hours)
15. Shake gently and watch for yellow (goal OD420 is 0.6-0.9)

16. Stop with 500µl 1M Na_2CO_3, record time, vortex at 4 for 10 sec
17. Give all reaction at least 2 hours
18. Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and OD_{550}, using blank reaction as blank in spectrophotometer.



I overshot the OD420 on all but WT *rpsU2*:*rpsU2*; however the numbers are pretty similar between the *rpsU2*:*rpsU2* as I got in my last experiment so I think it's okay.

Wednesday, January 11, 2023

To Do:

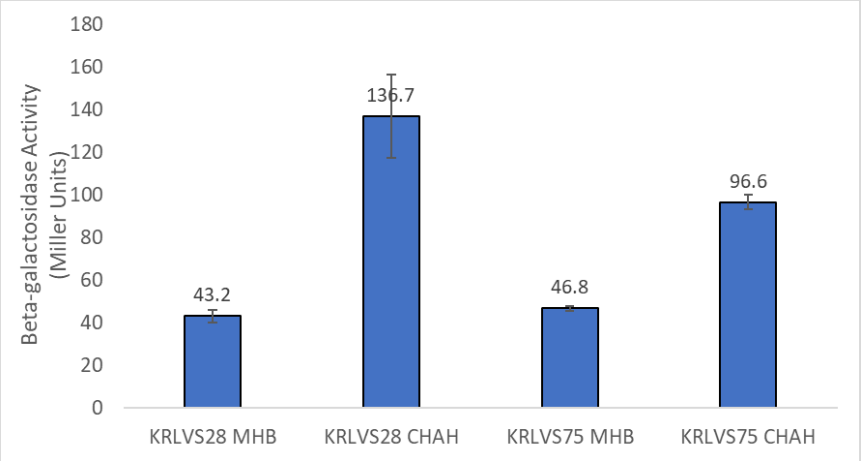
1. ~~Set up cultures of KRLVS28 and KRLVS75~~
2. ~~Put away dishes~~
3. ~~pH 50 mL MHB to pH 3.5~~
4. ~~Run B-gal of MHB v CHAH~~
5. ~~Supplement more MHB~~
6. ~~Set up overnight cultures of KRLVS28 and KRLVS75~~

Results and Data:

β-galactosaidase Assay of KRLVS28 and KRLVS75 in MHB and CHAH

1. Grow 7ml cultures until OD600 = 0.3
2. Turn on 28°C water bath
3. Determine amount of Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = _µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. Once cultures reach OD_{600} = 0.3, place on ice 30 min
7. Otherwise, resuspend patches in MHB and normalize to OD ~0.3 in 0.5 mL and place on ice for 30 minutes
8. Place ONPG in water bath
9. After cells have incubated on ice, measure OD_{600} of bacterial cultures
10. Add 200µl culture to each reaction tube (add 200µl culture media to blank tube)
11. Add 30µl 0.1% SDS to each reaction tube
12. Add 60µl CHCl3 (chloroform) to each reaction tube
13. Vortex reaction pairs on high for 6 secs (time precisely with timer)
14. Put in water bath for 10 min

15. Prepare repeater pipette with 1M Na_2CO_3 (stop)
16. Add 200µl ONPG in 5 sec intervals (use timer with hours)
17. Shake gently and watch for yellow (goal OD420 is 0.6-0.9)
18. Stop with 500µl 1M Na_2CO_3, record time, vortex at 4 for 10 sec
19. Give all reaction at least 2 hours
20. Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and OD_{550}, using blank reaction as blank in spectrophotometer.



This is promising! I did not think ahead very well, so the way that I prepared the resuspended patches and then checked the OD600 meant that it was under the linear range so these are not super valid. I will re-do with a larger volume so I can get an accurate OD600.

Additionally, I set up 4 biological replicate cultures for each condition of both strains at OD 0.005 (hopefully), and put them in at 4:15.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

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

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

Thursday, January 12, 2023

To Do:

1. ~~Patch out KRLVS9, KRLVS16, and KMLFT104~~
2. ~~Check sequencing results~~

Results and Data:

My overnights were all way too overgrown. Next time, I will make a serial dilution of my stock resuspension so that I'm adding a larger, and hopefully, more accurate volume. As such, obviously I won't be doing the B-gal today.

Alternatively, since we still don't have SnapGene I used BLAST Global Alignment. It did not allow me to use our plasmid file, so I pasted in the sequence that would have been amplified, up to the number of base pairs sequenced from the PCR. I had to use the .seq files, as the .ab1 files did not work. Based on the

alignment, there seems to be insertions/deletions throughout the amplified region. I'll double check once we have SnapGene again since I'm less familiar with BLAST, but it looks like I'll have to redo the ligation again.

Friday, January 13, 2023

To Do:

1. ~~Streak out KRLVS28 and KRLVS75~~
2. ~~Patch out KRLVS9 at room temperature~~
3. ~~Make 10% glucose~~

Results and Data:

My KRLVS16 and KMLFT104 aliquots were contaminated, so I assume I've found some contaminated glycerol.

Was finally able to check my sequencing data again with SnapGene, and Kathryn looked with me. The only errors were outside the Tn7 site and were also near the beginning or ending of the sequence where the data is trash sooo it seems that I have the plasmid finally!!!! I'll try to transform both of them into LVS to cover my bases and if I have any leftover, I'll try to transform them back into yeast so I can get more, hopefully.

Reagents

10% Glucose (250 mL)

To a beaker add:

25 g glucose

200 mL ddi water

Stir until dissolved and correct volume to 250 mL

Filter sterilize

Monday, January 16, 2023

To Do:

1. ~~Patch out KRLVS28 and KRLVS75 x2~~
2. ~~Set up tubes for tomorrow~~

Results and Data:

Both of my isolation streaks had a single colony of contamination which is really annoying. However, there were single colonies so I could see that most of it was okay... buuuut if something goes wrong I'm blaming that. I also checked my d2 patch plates and put it in the incubator- it didn't seem contaminated? So I will check again tomorrow, and then check my glycerol stocks/single use aliquots later to see if the glycerol that I used was actually contaminated like I assumed (lol using old glycerol from rotation students...). Hopefully they're fine.

I additionally set up my culture tubes for tomorrow morning with 7 mL of MHB.

Tuesday, January 17, 2023

To Do:

1. ~~Set up cultures for B-gal of MHB v. CHAH~~
2. ~~Run B-gal of MHB v. CHAH~~
3. ~~Set up overnight cultures for B-gal of Standard vs Low MHB pH~~

Results and Data:

β-galactosaidase Assay of KRLVS28 and KRLVS75 in MHB and CHAH

1. Grow 7ml cultures until OD600 = 0.3
2. Turn on 28°C water bath
3. Determine amount of Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = _µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. Once cultures reach OD_{600} = 0.3, place on ice 30 min
7. Otherwise, resuspend patches in MHB and normalize to OD ~0.3 in 3 mL MHB, and place on ice for 30 minutes
8. Place ONPG in water bath
9. After cells have incubated on ice, measure OD_{600} of bacterial cultures
10. Add 200µl culture to each reaction tube (add 200µl culture media to blank tube)
11. Add 30µl 0.1% SDS to each reaction tube
12. Add 60µl CHCl3 (chloroform) to each reaction tube
13. Vortex reaction pairs on high for 6 secs (time precisely with timer)
14. Put in water bath for 10 min
15. Prepare repeater pipette with 1M Na_2CO_3 (stop)
16. Add 200µl ONPG in 5 sec intervals (use timer with hours)
17. Shake gently and watch for yellow (goal OD420 is 0.6-0.9)
18. Stop with 500µl 1M Na_2CO_3, record time, vortex at 4 for 10 sec
19. Give all reaction at least 2 hours
20. Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and $OD_{550,}$ using blank reaction as blank in spectrophotometer.

Preparing Overnight Cultures of KRLVS28 and KRLVS75

1. Resuspend a very small loopful of strains in quadruplicate in 800 uL of MHB and check OD 1:10

2. Calculate for a final OD of 0.003 in 8 mL of MHB
3. If addition is less than ~50 uL then dilute the sample prior to addition to the culture
4. Shake at 37°C and allow to grow for 16-17 hours

My overnights were set in the shaking incubator at 5:45 pm.

Wednesday, January 18, 2023

To Do:

1. ~~Media transfer to low pH~~
2. ~~Run B-gal of Standard v. Low MHB pH~~
3. ~~Put plate waste in bag~~
4. ~~Load and unload dishwasher~~

Results and Data:

Media Transfer of Overnight Cultures

1. Once OD's have reached OD 0.25-0.3 remove from the shaking incubator
2. Transfer contents to 50 mL conical tubes and pellet at 8000 xg for 5 minutes, ensure proper pelleting
3. Pour off supernatant and resuspend each in 1 mL of appropriate media
4. Add to tubes of appropriate media such that the final volume equals the total volume spun down
5. Shake at 37°C for an additional hour

β-galactosaidase Assay of KRLVS28 and KRLVS75 in Standard and Low pH MHB

1. Once cultures are ready, place on ice 30 min
2. Turn on 28°C water bath and put ONPG in water bath
3. Determine amount of Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = _µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. After cells have incubated on ice, measure OD_{600} of bacterial cultures
7. Add 200µl culture to each reaction tube (add 200µl culture media to blank tube)
8. Add 30µl 0.1% SDS to each reaction tube
9. Add 60µl CHCl3 (chloroform) to each reaction tube
10. Vortex reaction pairs on high for 6 secs (time precisely with timer)
11. Put in water bath for 10 min
12. Prepare repeater pipette with 1M Na_2CO_3 (stop)
13. Add 200µl ONPG in 5 sec intervals (use timer with hours)
14. Shake gently and watch for yellow (goal OD420 is 0.6-0.9)
15. Stop with 500µl 1M Na_2CO_3, record time, vortex at 4 for 10 sec
16. Give all reaction at least 2 hours
17. Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and $OD_{550,}$ using blank reaction as blank in spectrophotometer.

Monday, January 23, 2023

To Do:

1. ~~Streak out KRLVS148~~
2. ~~Patch out personal glycerol stocks to check for contamination~~
3. ~~Patch out KRLVS9, KRLVS16, and KMLFT104~~

Results and Data:

Spoke with Kathryn regarding the B-gal results fort he pH experiment, and she agreed that the B-gal was probably deactivated. She had hoped that with the larger volume of buffer that the pH would not affect it, however clearly it did. We discussed two possibilities for handling this issue. I first suggested washing the cells, spinning them down and resuspending them. However, she commented that this may be a bit more laborious than just neutralizing the pH. I will test how much NaOH is required to neutralize the pH of a certain volume of MHB in order to see if that would be feasible. Obviously, we cannot add too much volume.

Otherwise, we also discussed the RNA stability assay and she raised concerns about being able to safely extract RNA from the flash frozen cultures. The protocol we are using is from Mycobacterium, so obviously their cell walls are quite robust. We will flash freeze a little bit of LVS and see if we can spin them down. Additionally, I will do one run through of the protocol prior to doing both time course experiments to ensure that the RNA will be okay. If it is okay, I might ask Kathryn with the next shipment of liquid nitrogen if I can freeze the cultures from my bS21-1, -2, and -3 experiments since those will have a bunch of samples.

Tuesday, January 24, 2023

To Do:

1. ~~Streak out KRLVS149~~
2. ~~Patch out KRLVS192, KRLVS193, and LVS-pF~~
3. ~~Check glycerol stock check patch plates~~
4. ~~Patch out KRLVS9~~
5. ~~Patch out KMLFT104~~
6. ~~Make CDM (50 mL)~~
7. ~~Make CDM with 200 uM Spermine (50 mL)~~

Results and Data:

I checked my patch plates from my overnight and it seemed like the glycerol was fine, as well as all of my personal stocks aside from KRLVS16. As such, I patched out from my d2 stocks, and my d1 stocks in order to make electrocompetent cells and to extra confirm my single use aliquots are okay. If they are not, then I saved my patch plates in order to make new personal glycerol stocks.

Reagents

CDM (50mL)

To 100 mL beaker add:

4 mL Combined Amino Acid Stock

Then add:

200 uL Thiamine HCl
200 uL Spermine tetrahydrochloride
200 uL DL-Calcium pantothenate

Then add:

1.71 mL 5N NaCl
0.5 mL KH2PO4
200 uL K2HPO4

Then:
0.5 mL 400 mg/mL Glucose
Then:
38 mL Water
Then:
200 uL FeSO4•7H2O
200 uL MgSO4•7H2O
pH media to a pH between 6.3 and 6.5
Add water to 50 mL total
Filter sterilize

Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	4
Thiamine HCl	4°C	2	1	0.004	0.2
Spermine tetrahydrochloride	4°C	2	8.74	0.0700	0.4
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.2
NaCl	RT	3	292.2	10.0	1.71
KH2PO4	RT	3	100	1	0.5
K2HPO4	RT	3	250	1	0.2
Glucose	RT	Separate	400	4	0.5
FeSO4•7H2O	RT	Separate	0.5	0.002	0.2
MgSO4•7H2O	RT	LAST	33.75	0.1	0.2
Water	-	-	-	-	41.89

Wednesday, January 25, 2023

To Do:

1. ~~Set up cultures for environmental condition assay with spermine~~
2. ~~Make CHAH+Kan plates~~
3. ~~Run GFP assay for effects of spermine concentration on *rpsU1* and *rpsU3* production~~
4. ~~Make new personal stocks KRLVS16~~
5. ~~Patch out KRLVS16~~

Results and Data:

Effect of 200µM Spermine on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193

Condition Number	0	1	2
Purpose	Background	Control	Test 1
Condition	CDM	CDM	200µM Spermine

1. Resuspend patches of cells in 400uL of CDM checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of respective CDM, either regular or varying concentrations of iron
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, CDM

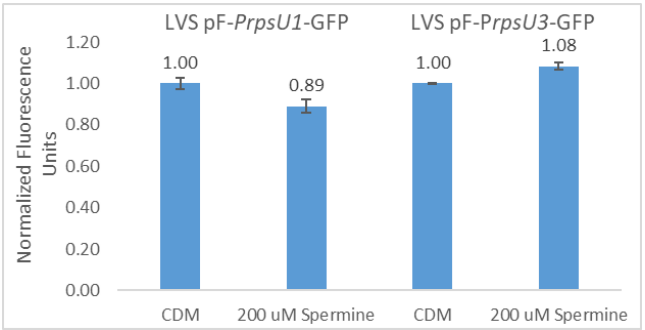
GFP Assay on CDM Spermine Conditions in KRLVS192 and KRLVS193

1. Pellet 4 mLs of culture and spin at max speed for 3 minutes
2. Remove all CDM, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS in triplicate as control.
5. Go to INBRE lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
6. Read OD600 from clear plate on ID3 plate reader:
    a. Select Absorbance, wavelength=600
    b. Plate type: 96-well standard clearbtm (first option)
7. Transfer 200 ul of each well from clear plate to black plate using the multichannel
8. Read fluorescence from black plate on ID3 plate reader:
    a. Select fluorescence
    b. Wavelength: 495 to 535
    c. Plate type: CoStar 3789
    d. Gain: Automatic
    e. Integration: 380 ms

Label	Condition
A	CDM
B	200µM Spermine

Label	Strain and BR
1	KRLVS192 1
2	KRLVS192 2
3	KRLVS193 1
4	KRLVS193 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1		LVS	LVS	LVS					
B	A2	A2	A2		PBS	PBS	PBS					
C	B1	B1	B1									
D	B2	B2	B2									
E	A3	A3	A3									
F	A4	A4	A4									
G	B3	B3	B3									
H	B4	B4	B4									



Making Personal Glycerol Stocks and Single Use Aliquots of KRLVS16

1. Prepare 1200uL of MHB in a 2mL tube
2. Take at least half of a thickly spread plate and add cells to the MHB tube
3. Resuspend until there are no clumps in the MHB

4. Add 300ul of 75% glycerol to the 800uL mix by pipetting
5. Aliquot 1 mL to cryovial and 50ul of solution to tubes, freeze at -80

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

Thursday, January 26, 2023

To Do:

1. ~~Make electrocompetent KRLVS16 and KMLFT104~~
2. ~~Do d2 electroporations~~

Results and Data:

Electroporate pKR168 (Tn7 Transposon) into KRLVS127 (Helper Plasmid)

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
   5 µL of Tn7 transposon plasmid
   50 uL electrocompetent cells containing helper plasmid
3. Have recovery media ready
4. Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω (setting Ec2)
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 4-8 hours, shaking at 37°C
7. Plate 20 µL, 200 µL, and remaining on CHAH-Kan plates
8. Incubate plates at 37°C for 4 days

Tube	Purpose	Strain	DNA	Vol. of DNA	Vol. Plated	# of Plates
1	Δ2+pKR168	KRLVS127	Cand. pKR168 4	5 uL	20 uL. 200 uL, Rem.	3
2	Δ2+pKR168	KRLVS127	Cand. pKR168 5	5 uL	20 uL. 200 uL, Rem.	3
3	(-) control	KRLVS127	-	-	200 uL	1
					Total:	7

Preparing Electrocompetent KRLVS9, KRLVS16, and KMLFT104 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 ~1x10^{11} cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
8. For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Friday, January 27, 2023

To Do:

1. ~~Check KRLVS148 and KRLVS149 patch plates~~
2. ~~Streak out KRLVS16, KMLFT104, and LVS~~
3. ~~Electroporate candidate pKR168 into LVS with helper plasmid~~
4. ~~Electroporate pKR6, pKR7, and pKR8 into KRLVS16, and KMLFT104~~

Results and Data:

Electroporate pKR168 (Tn7 Transposon) into KRLVS126 (Helper Plasmid)

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
   5 µL of Tn7 transposon plasmid
   50 uL electrocompetent cells containing helper plasmid
3. Have recovery media ready
4. Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω (setting Ec2)
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 4-8 hours, shaking at 37°C
7. Plate 20 µL, 200 µL, and remaining on CHAH-Kan plates
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

Tube	Purpose	Strain	DNA	Vol. of DNA	Vol. Plated	# of Plates
1	WT+pKR168	KRLVS126	Cand. pKR168 4	5 uL	20 uL. 200 uL, Rem.	3
2	WT+pKR168	KRLVS126	Cand. pKR168 5	5 uL	20 uL. 200 uL, Rem.	3
3	(-) control	KRLVS126	-	-	200 uL	1
					Total:	7

Electroporating pKR6, pKR7, and pKR8 into EC KRLVS16 and KMLFT104 Cells

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
   3 µL of plasmid DNA
   50 µL 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)

Tube #	Purpose	Strain	DNA	Vol. of DNA	Vol. Plated	Number of Plates
1	Δ1+pF-1	KMLFT104	pKR6	3 uL	10 uL. 100 uL	2
2	Δ1+pF-2	KMLFT104	pKR7	3 uL	10 uL. 100 uL	2
3	Δ1+pF-3	KMLFT104	pKR8	3 uL	10 uL. 100 uL	2

4	Δ3+pF-1	KRLVS16	pKR6	3 uL	10 uL. 100 uL	2
5	Δ3+pF-2	KRLVS16	pKR7	3 uL	10 uL. 100 uL	2
6	Δ3+pF-3	KRLVS16	pKR8	3 uL	10 uL. 100 uL	2
7	(-) control	KMLFT104	-	-	200 uL	1
8	(-) control	KRLVS16	-	-	200 uL	1
					Total:	14

Monday, January 30, 2023

To Do:

1. ~~Set up cultures for RNA stability assay~~
2. ~~Make rifampicin~~
3. ~~Supplement MHB~~
4. ~~Take isolation streaks out of the incubator~~
5. ~~Label tubes for RNA stability assay~~
6. ~~Patch out electroporations~~
7. ~~Run RNA stability assay~~

Results and Data:

RNA Stability Assay on KRLVS148 and KRLVS149

1. Start cultures at an appropriate OD (0.08 for KRLVS149, 0.10 for KRLVS148) in 10 mL of culture and grow to mid-log (OD 0.3-0.4), typically ~4-4.6 hours
    a. Keep track of culture volume after each OD check, need a minimum of 7.5 mL at the end
2. While cultures are growing, label and prepare cryovials
3. Once desired OD is reached, add rifampin to tubes at a final concentration of 0.5 ug/mL, adding to replicates in 10 second intervals
4. Flash freeze 1.8 mL of culture in cryovials with liquid nitrogen at 0, 1, 2, and 4 minutes for each replicate, cultures can be stored in the -80 for a couple months

Experimental Configuration																								
Strain	KRLVS148												KRLVS149											
Replicate	1				2				3				1				2				3			
rif (min)	0	1	2	4	0	1	2	4	0	1	2	4	0	1	2	4	0	1	2	4	0	1	2	4

KRLVS148 3, two-minute time point is +10 seconds, on accident. There were some issues in taking the second aliquots after the first and space of time between. Kathryn and I brain-stormed some ideas for next time to make it a bit smoother.

Additionally, I patched out my electroporations. It seemed there were only two colonies on my Tn7 LVS pKR168 plates, though another might have been growing, so I'll check on that again. I'm screening four colonies for the d2 Tn7 pKR168. I patched out one colony from each of my pF transformations, because there were limited plates. But, I think I'll patch out again tomorrow from the plates left on the bench in order to get my .2's.

Reagents

0.5 mg/mL Rifampicin

To 15 mL Conical tube add:

2.5 mg of rifampicin

5 mL DMSO

Vortex until totally dissolved

Aliquot into labelled 1.5 mL centrifuge tubes

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

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

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

Tuesday, January 31, 2023

To Do:

1. ~~Patch out KRLVS16, KMLFT104, and LVS~~
2. ~~Patch out additional colonies from pF transformations~~
3. Check volume needed of NaOH to neutralize 5 mL of MHB pH 3.5

Results and Data:

My patches for the Tn7 pKR168 exchange turned out to not be Francisella, which I had unfortunately thought would happen as the colonies did not look quite right. As such, I will have to re-ligate the plasmid in yeast, miniprep again, and electroporate back into Francisella. However, I will be making sure to make fresh electrocompetent cells on the day of to ensure that they're good. I have a lot planned the next three days, but I might just pull an early day so that I can do the ligation and transformation on Friday so that I have colonies next week.

Alternatively, my pF plasmid transformation patches looked good. Since I patched them out so late they weren't quite grown enough, so I put them at room temperature to make glycerol stocks tomorrow. I also patched out again from the transformation plates now that I have more Kan, to make .2's in the strains. I also patched those out kind of late, so we'll see how well they've grown by the time I'm doing stuff, but I may make the permanent stocks during the DNase step on Thursday, putting them at room temperature.

February 2023

Wednesday, February 1, 2023

To Do:

1. ~~Make 2.5% iron pyrophosphate~~
2. ~~Wash hemoglobin flasks~~
3. ~~Load dishwasher~~
4. ~~RNA extraction of RNA stability assay samples~~
5. ~~Make strains and glycerol stocks from electroporations~~

Results and Data:

RNA Purification of RNA Stability Assay Samples

1. Pellet 2 mL 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

Labelled	Contents
1 RNA	KRLVS148 1 0’
2 RNA	KRLVS148 2 0’
3 RNA	KRLVS148 3 0’
4 RNA	KRLVS148 1 1’
5 RNA	KRLVS148 2 1’
6 RNA	KRLVS148 3 1’
7 RNA	KRLVS148 1 2’
8 RNA	KRLVS148 2 2’
9 RNA	KRLVS148 3 2’
10 RNA	KRLVS148 1 4’
11 RNA	KRLVS148 2 4’
12 RNA	KRLVS148 3 4’

Additionally, I nanodrop’d my total nucleic acid samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1 0'	377.7	9.444	4.535	2.08	2.20
KRLVS148 2 0'	336.6	8.415	3.975	2.12	1.78
KRLVS148 3 0'	280.8	7.021	3.335	2.11	2.32
KRLVS148 1 1'	281.3	7.034	3.384	2.08	2.25
KRLVS148 2 1'	294.0	7.351	3.490	2.11	2.28
KRLVS148 3 1'	259.6	6.491	3.093	2.10	2.29
KRLVS148 1 2'	387.8	9.695	4.644	2.09	2.23
KRLVS148 2 2'	293.1	7.328	3.462	2.12	1.95
KRLVS148 3 2'	307.6	7.690	3.675	2.09	2.25
KRLVS148 1 4'	403.3	10.084	4.847	2.08	2.20
KRLVS148 2 4'	352.8	8.819	4.246	2.08	2.13
KRLVS148 3 4'	155.3	3.883	1.863	2.08	1.48

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

Thursday, February 2, 2023

To Do:

1. ~~Start cultures for RNA isolation from KRLVS16, KMLFT104, and LVS~~
2. ~~DNase treatment of RNA stability assay samples~~
3. ~~Second RNA purification of stability assay RNA samples~~
4. ~~Put away dishes~~
5. ~~RNA extraction of KRLVS16, KMLFT104, and LVS~~

Results and Data:

Setting Up KRLVS16, KMLFT104, and LVS Cultures for RNA Purification

1. Scrape each triplicate patch of KRLVS16, KMLFT104, and LVS into 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
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

DNase treatment of RNA Stability Assay 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

RNA Purification of KRLVS16, KMLFT104, and LVS

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

Labelled	Contents
1 RNA	KMLFT104 1
2 RNA	KMLFT104 2
3 RNA	KMLFT104 3
4 RNA	KRLVS16 1
5 RNA	KRLVS16 2
6 RNA	KRLVS16 3
7 RNA	LVS 1
8 RNA	LVS 2
9 RNA	LVS 3

Friday, February 3, 2023

To Do:

1. ~~Streak out KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
2. ~~Streak out KRLVS28 and KRLVS75~~
3. ~~DNase treatment of KRLVS16, KMLFT104, and LVS RNA samples~~
4. ~~Second RNA purification of KRLVS16, KMLFT104, and LVS RNA samples~~

Results and Data:

DNase treatment of KRLVS16, KMLFT104, and LVS

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

Monday, February 6, 2023

To Do:

1. ~~Patch out KRLVS28 and KRLVS75~~
2. ~~Supplement MHB~~
3. ~~Nanodrop stability assay RNA samples~~
4. ~~Prepare aliquots of stability assay RNA samples for cDNA synthesis and gel~~
5. ~~Nanodrop KRLVS16, KMLFT104, and LVS RNA samples~~
6. ~~Prepare aliquots of KRLVS16, KMLFT104, and LVS RNA samples for cDNA synthesis and gel~~
7. ~~RNA extraction and purification of RNA Stability Assay samples 2~~

Results and Data:

I nanodrop'd my KMLFT104, KRLVS16, and LVS RNA samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRMLFT104 1	638.3	15.957	7.575	2.11	2.04
KRMLFT104 2	350.0	8.751	4.196	2.09	2.21
KRMLFT104 3	300.4	7.509	3.552	2.11	1.62
KRLVS16 1	296.5	7.413	3.512	2.11	2.28
KRLVS16 2	445.9	11.148	5.161	2.16	2.17
KRLVS16 3	350.4	8.761	4.144	2.11	2.09
LVS 1	252.8	6.319	3.026	2.09	2.16
LVS 2	338.2	8.455	4.048	2.09	2.17
LVS 3	362.4	9.059	4.295	2.11	1.87

Additionally, I diluted the samples for both the gel and cDNA according to:

Gel			
Sample	ng/uL	RNA	Water
KRMLFT104 1	638.3	1.57	8.43
KRMLFT104 2	350.0	2.86	7.14
KRMLFT104 3	300.4	3.33	6.67
KRLVS16 1	296.5	3.37	6.63
KRLVS16 2	445.9	2.24	7.76
KRLVS16 3	350.4	2.85	7.15
LVS 1	252.8	3.96	6.04
LVS 2	338.2	2.96	7.04
LVS 3	362.4	2.76	7.24

cDNA			
Sample	ng/uL	RNA	Water
KRMLFT104 1	638.3	4.70	8.80
KRMLFT104 2	350.0	8.57	4.93
KRMLFT104 3	300.4	9.99	3.51
KRLVS16 1	296.5	10.12	3.38
KRLVS16 2	445.9	6.73	6.77
KRLVS16 3	350.4	8.56	4.94
LVS 1	252.8	11.87	1.63
LVS 2	338.2	8.87	4.63
LVS 3	362.4	8.28	5.22

Additionally, I nanodrop'd the first half of my stability assay samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1 0'	187.6	4.690	2.173	2.16	2.29
KRLVS148 2 0'	242.4	6.061	2.849	2.13	2.31
KRLVS148 3 0'	171.7	4.293	2.019	2.13	2.32
KRLVS148 1 1'	242.6	6.064	2.869	2.11	2.13
KRLVS148 2 1'	284.5	7.113	3.391	2.10	2.22
KRLVS148 3 1'	230.5	5.762	2.718	2.12	2.21
KRLVS148 1 2'	280.7	7.018	3.321	2.11	1.69
KRLVS148 2 2'	186.1	4.653	2.185	2.13	2.22
KRLVS148 3 2'	194.8	4.871	2.323	2.10	1.98
KRLVS148 1 4'	320.8	8.020	3.741	2.14	2.18
KRLVS148 2 4'	234.6	5.865	2.795	2.10	2.23
KRLVS148 3 4'	87.1	2.178	1.063	2.05	2.21

Additionally, I diluted the samples for both the gel and cDNA according to:

Gel			
Sample	**ng/uL**	**RNA**	**Water**
KRLVS148 1 0'	187.6	4.26	5.74
KRLVS148 2 0'	242.4	3.30	6.70
KRLVS148 3 0'	171.7	4.66	5.34
KRLVS148 1 1'	242.6	3.30	6.70
KRLVS148 2 1'	284.5	2.81	7.19
KRLVS148 3 1'	230.5	3.47	6.53
KRLVS148 1 2'	280.7	2.85	7.15
KRLVS148 2 2'	186.1	4.30	5.70
KRLVS148 3 2'	194.8	4.11	5.89
KRLVS148 1 4'	320.8	2.49	7.51
KRLVS148 2 4'	234.6	3.41	6.59
KRLVS148 3 4'	87.1	9.18	0.82

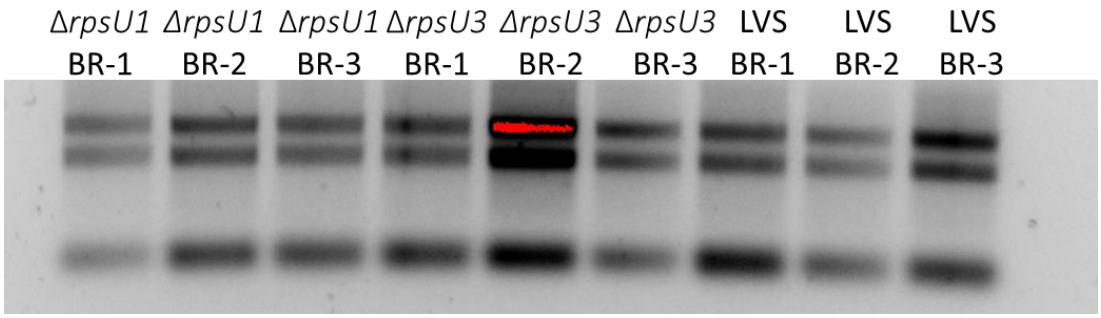
cDNA			
Sample	**ng/uL**	**RNA**	**Water**
KRLVS148 1 0'	187.6	14.23	-0.73
KRLVS148 2 0'	242.4	11.01	2.49
KRLVS148 3 0'	171.7	15.55	-2.05
KRLVS148 1 1'	242.6	11.01	2.49
KRLVS148 2 1'	284.5	9.38	4.12
KRLVS148 3 1'	230.5	11.58	1.92
KRLVS148 1 2'	280.7	9.51	3.99
KRLVS148 2 2'	186.1	14.35	-0.85
KRLVS148 3 2'	194.8	13.71	-0.21
KRLVS148 1 4'	320.8	8.32	5.18
KRLVS148 2 4'	234.6	11.38	2.12
KRLVS148 3 4'	87.1	30.65	-17.15

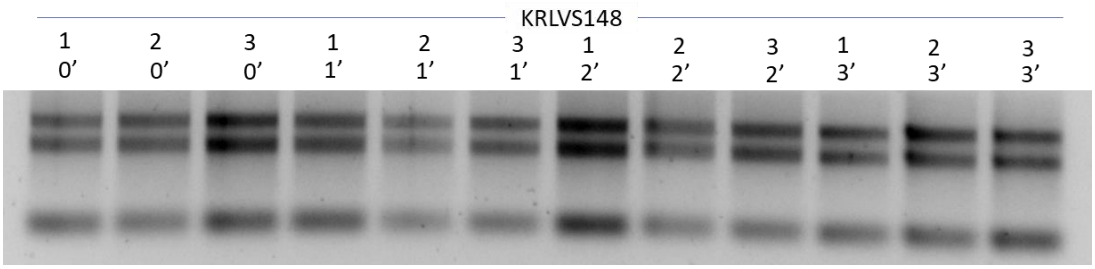
Gel of KRLVS16, KMLFT104, and LVS and Stability Assay Pure RNA Samples

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 1.2g 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 12uL of Sbyr Safe dye to rig, pour gel, use ladder to mix, then replace ladder and allow to set.
6. Add new 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+5s), rather than smears.

Loading Order of 2/3/23 RNA								
Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8	RNA 9

Loading Order of 2/2/23 RNA											
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





RNA Purification of RNA Stability Assay Samples - 2

1. Pellet 2 mL 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

Labelled	Contents
1 RNA	KRLVS149 1 0’
2 RNA	KRLVS149 2 0’
3 RNA	KRLVS149 3 0’
4 RNA	KRLVS149 1 1’
5 RNA	KRLVS149 2 1’
6 RNA	KRLVS149 3 1’
7 RNA	KRLVS149 1 2’
8 RNA	KRLVS149 2 2’
9 RNA	KRLVS149 3 2’
10 RNA	KRLVS149 1 4’
11 RNA	KRLVS149 2 4’
12 RNA	KRLVS149 3 4’

Tuesday, February 7, 2023

To Do:

1. ~~Generate cDNA of KRLVS16, KMLFT104, and LVS RNA~~
2. ~~Purify KRLVS16, KMLFT104, and LVS cDNA samples~~
3. ~~DNase Treatment of 2nd RNA Stability Assay samples – 2~~
4. ~~Second RNA purification of stability assay RNA samples~~
5. ~~Nanodrop stability assay RNA samples~~
6. ~~Check volume NaOH needed to neutralize acidic MHB~~
7. ~~Make MHB pH 3.5~~
8. ~~Make ONPG~~
9. ~~Prepare tubes for pH stuff tomorrow~~
10. ~~Start overnight cultures of KRLVS28 and KRLVS75~~

Results and Data:

Generate cDNA (Half) of KMLFT104, KRLVS16, and LVS (2/3/23 Pure RNA)

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
$(NS)_5$ 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 $(NS)_5$ 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	X10.5
5X 1st strand buffer	1x	6	63
RNase-free water		2.88	30.24
100 mM DTT	10 mM	3	31.5
10 mM dNTPs	0.5 mM	1.5	15.75
Superscript III (200 U/ul)	10.8 U/ul	1.63	17.12

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 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. 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 o f Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

DNase treatment of RNA Stability Assay Samples - 2

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

Additionally, I nanodrop'd the second half of my pure RNA samples from the stability assay:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS149 1 0'	195.7	4.893	2.327	2.10	2.25
KRLVS149 2 0'	247.8	6.194	3.009	2.06	2.08
KRLVS149 3 0'	180.7	4.518	2.206	2.05	2.17
KRLVS149 1 1'	216.7	5.417	2.625	2.06	2.11
KRLVS149 2 1'	221.1	5.527	2.828	1.95	1.69
KRLVS149 3 1'	187.5	4.689	2.320	2.02	1.92
KRLVS149 1 2'	254.1	6.353	3.247	1.96	1.62
KRLVS149 2 2'	357.2	8.931	4.637	1.93	1.33
KRLVS149 3 2'	268.9	6.724	3.651	1.84	1.34
KRLVS149 1 4'	214.7	5.368	2.805	1.91	1.53
KRLVS149 2 4'	261.5	6.538	3.306	1.98	1.72
KRLVS149 3 4'	106.9	2.673	1.320	2.03	1.92

I checked the volume needed to neutralize 5 mL of pH 3.5 MHB. 30 uL neutralized it to 7.12, so I might do 27 uL in each of my tubes so it's closer to 6.3 or so. Additionally, I started my overnights at 5:10 pm at 0.003, so they should be ready in 16 hours or so, around 9 am.

Reagents

4 mg/mL ONPG (40 mL)
- To 100 mL beaker add:
  - 120 mg ONPG
  - 40 mL Z-Buffer
- Cover with foil and stir overnight
- Store at -20°C in 50 mL conical tubes covered in foil

Wednesday, February 8, 2023

To Do:

1. ~~Media transfer of overnight cultures into fresh MHB or MHB pH 3.5~~
2. ~~Run B-gal of KRLVS28 and KRLVS75 pH~~
3. ~~Prepare aliquots of stability assay RNA samples for cDNA synthesis and gel~~
4. ~~Make YPD and autoclave 2x 200mL water~~
5. ~~Patch out yeast~~

Results and Data:

Media Transfer of Overnight Cultures

1. Once OD's have reached OD 0.25-0.3 remove from the shaking incubator
2. Transfer contents to 50 mL conical tubes and pellet at 8000 xg for 5 minutes, ensure proper pelleting
3. Pour off supernatant and resuspend each in 1 mL of appropriate media
4. Add to tubes of appropriate media such that the final volume equals the total volume spun down
5. Shake at 37°C for an additional hour

For the media transfer, I ended up pelleting the two cultures of each replicate together, at a total of 12 mL. After pelleting, I resuspended in 600 uL and added 250 uL to each second 5 mL culture (either fresh MHB or MHB pH 3.5). Due to the fact that there's always a little extra volume leftover from the supernatant, it was definitely more than 600 uL but I thought starting lower given the OD's being around 0.35 would be good, to stop them from overgrowing in the additional hour of growth.

Additionally, I diluted the second half of my stability assay samples for the gel and cDNA synthesis:

Gel			
Sample	ng/uL	RNA	Water
KRLVS149 1 0'	195.7	4.09	5.91
KRLVS149 2 0'	247.8	3.23	6.77
KRLVS149 3 0'	180.7	4.43	5.57
KRLVS149 1 1'	216.7	3.69	6.31
KRLVS149 2 1'	221.1	3.62	6.38
KRLVS149 3 1'	187.5	4.27	5.73
KRLVS149 1 2'	254.1	3.15	6.85
KRLVS149 2 2'	357.2	2.24	7.76
KRLVS149 3 2'	268.9	2.98	7.02
KRLVS149 1 4'	214.7	3.73	6.27
KRLVS149 2 4'	261.5	3.06	6.94
KRLVS149 3 4'	106.9	7.48	2.52

cDNA			
Sample	ng/uL	RNA	Water
KRLVS149 1 0'	195.7	13.64	-0.14
KRLVS149 2 0'	247.8	10.77	2.73
KRLVS149 3 0'	180.7	14.78	-1.28
KRLVS149 1 1'	216.7	12.32	1.18
KRLVS149 2 1'	221.1	12.08	1.42
KRLVS149 3 1'	187.5	14.24	-0.74
KRLVS149 1 2'	254.1	10.51	2.99
KRLVS149 2 2'	357.2	7.47	6.03
KRLVS149 3 2'	268.9	9.93	3.57
KRLVS149 1 4'	214.7	12.44	1.06
KRLVS149 2 4'	261.5	10.21	3.29
KRLVS149 3 4'	106.9	24.98	-11.48

β-galactosaidase Assay of KRLVS28 and KRLVS75 in Standard and Low pH MHB

1. Once cultures are ready, place on ice 30 min, neutralize 5 mL low pH MHB with 27 uL NaOH
2. Turn on 28°C water bath and put ONPG in water bath
3. Determine Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. After cells have incubated on ice, measure OD_{600} of bacterial cultures
7. Add 200µl culture to each reaction tube (add 200µl culture media to blank tube)
8. Add 30µl 0.1% SDS to each reaction tube
9. Add 60µl CHCl3 (chloroform) to each reaction tube
10. Vortex reaction pairs on high for 6 secs (time precisely with timer)
11. Put in water bath for 10 min
12. Prepare repeater pipette with 1M Na_2CO_3 (stop)
13. Add 200µl ONPG in 5 sec intervals (use timer with hours)
14. Shake gently and watch for yellow (goal OD420 is 0.6-0.9)
15. Stop with 500µl 1M Na_2CO_3, record time, vortex at 4 for 10 sec
16. Give all reaction at least 2 hours
17. Remove 1 mL from reaction (avoid chloroform at bottom), measure OD_{420} and $OD_{550,}$ using blank reaction as blank in spectrophotometer.

Once again, the B-gal activity was compromised despite neutralizing the media. Ergo, the OD420 values were reading as equivalent-ish to the blank in the pH 3.5 samples, but not those grown in standard MHB. I notified Kathryn, and we will brainstorm what to do.

Reagents

YPD (250 mL)

To 500 mL baffled flask add:

2.5 g Yeast extract
5.0 g Protease-Peptone
5.0 g Glucose

Mix thoroughly and autoclave Liquid 15'

Store at room temperature

Thursday, February 9, 2023

To Do:

1. ~~Streak out KRLVS148 and KRLVS111~~
2. ~~Generate cDNA of stability assay RNA samples~~
3. ~~Purify stability assay cDNA samples~~
4. ~~Run gel of stability assay RNA samples – 2~~
5. ~~Ligation of pKR168 plasmid~~
6. ~~Start yeast overnights~~

Results and Data:

Generate cDNA (Half) of Stability Assay Samples

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
$(NS)_5$ 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 $(NS)_5$ 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	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

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 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 o f Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Gel of Stability Assay Pure RNA Samples - 2

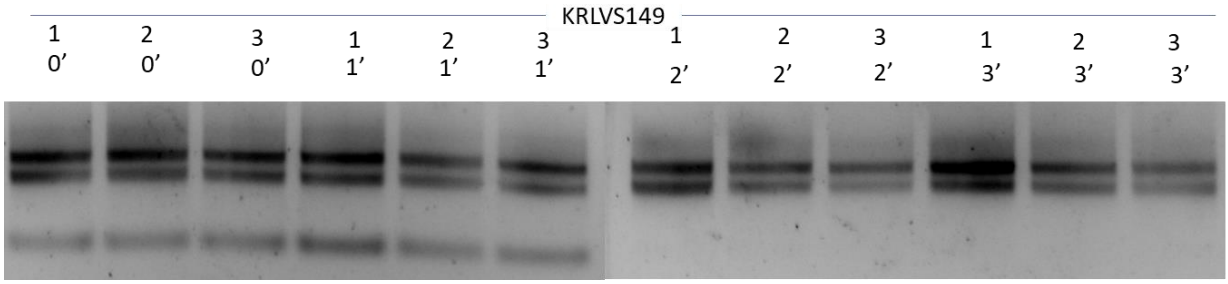
1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x and run on gel.
2. Rinse comb and gel rig with DI water, then ethanol both as well

3. Make fresh agarose gel by adding 0.6 g to 60mL 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 rig, pour gel, use ladder to mix, then replace ladder and allow to set.
6. Add new 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 10 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA+5s), rather than smears.

Loading Order of 2/3/23 RNA					
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6

Loading Order of 2/3/23 RNA					
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
RNA 7	RNA 8	RNA 9	RNA 10	RNA 11	RNA 12

I ran the gel a little too long, so the samples from the second comb ran out of dye at the band for the 5s RNA and tRNA's, I assume, based on the fact the 23s and 16s bands are intact and fine. I ran the gel for 30 minutes, so if I were to use this method again I might check after 15 or 20 minutes instead. Regardless:



Ligation of P*rpsU2 tul4* 5'UTR PCR from gBlock with pKR128 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. P*rpsU2:tul4* 5'UTR PCR 9.22	KpnI, NotI digested, purified pKR128
2	KpnI, NotI dig., pur. P*rpsU2:tul4* 5'UTR PCR 8.22	KpnI, NotI digested, purified pKR128
3	-	KpnI, NotI digested, purified pKR128

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)	Master Mix (3x)
H_2O	11.5	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	2.0	6.0
Insert	4.0	4.0	-	-
Backbone	2.0	2.0	2.0	6.0
Ligase	0.5	0.5	0.5	1.5
TOTAL	20.0	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.
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_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
6. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Incubate at 16°C overnight.

Friday, February 10, 2023

To Do:

1. ~~Streak out KRLVS149 and KRLVS112~~
2. ~~Start dishwasher~~
3. ~~Put away dishes~~
4. ~~Nanodrop and prepare aliquots of 1.5 ng/uL KMLFT104, KRLVS16, and LVS cDNA~~
5. ~~Autoclave plate waste~~

Results and Data:

When I got in and checked the OD of my yeast, it was below the linear range of the spec. I put it back in to grow, and I will check it again after seminar, but I am not confident that the yeast will have grown more, but we will see. Additionally, I placed my ligation into my cloning box.

When I grabbed the culture to check the OD again after seminar, I realized that the incubator was much warmer than 30°C, so clearly that's why the yeast did not grow.

Additionally, I nanodrop'd my autoregulation of *rpsU1* and *rpsU3* cDNA:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KMLFT104 1	8.0	0.242	0.138	1.76	2.15
KMLFT104 2	7.1	0.215	0.125	1.72	2.26
KMLFT104 3	18.1	0.547	0.308	1.78	1.99
KRLVS16 1	19.5	0.591	0.342	1.73	2.14
KRLVS16 2	20.7	0.628	0.358	1.75	1.90
KRLVS16 3	6.5	0.198	0.114	1.74	1.50
LVS 1	8.4	0.256	0.135	1.89	2.35
LVS 2	12.6	0.383	0.214	1.79	1.94
LVS 3	4.9	0.148	0.089	1.66	1.76

They looked good enough to me. I also diluted them for qPCR. Typically, with the *rpsU2* regulation stuff I was diluting to something like 0.9962 ng/uL. However, given that I expected *rpsU1* and *rpsU3* to have lower transcript overall I diluted them to 1.5 ng/uL, according to the following:

1.5 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KMLFT104 1	8.0	11.25	48.75
KMLFT104 2	7.1	12.68	47.32
KMLFT104 3	18.1	4.97	55.03
KRLVS16 1	19.5	4.62	55.38
KRLVS16 2	20.7	4.35	55.65
KRLVS16 3	6.5	13.85	46.15
LVS 1	8.4	10.71	49.29
LVS 2	12.6	7.14	52.86
LVS 3	4.9	18.37	41.63

Monday, February 13, 2023

To Do:

1. ~~Patch out KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~
2. ~~qRT-PCR of KMLFT104, KRLVS16, and LVS cDNA samples~~
3. ~~Nanodrop RNA Stability Assay cDNA samples~~

Results and Data:

qRT-PCR of KMLFT104, KRLVS16, and LVS cDNA Samples

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL337/338 and KROL347/348 as test primers to amplify the *rpsU1* 5'UTR and *rpsU3* 5'UTR 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 (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	350.0 uL
5uM primer set	1 uL	35.0 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH_2O	8 uL	280.0 uL
Total:	20 uL	665.0 uL

4. Obtain strip tubes per primer set with appropriate number of tubes, 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'

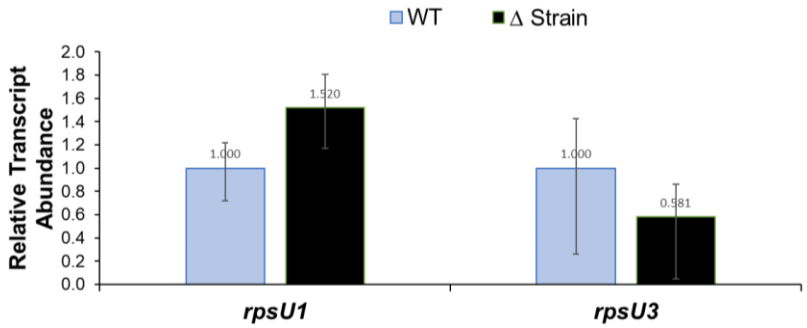
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL337/338 *rpsU1* 5'UTR
B	KROL347/348 *rpsU3* 5'UTR
C	KROL63/64 *tul4*

1	2	3	4	5	6	7	8	9
Δ*rpsU1*-1	Δ*rpsU1*-2	Δ*rpsU1*-3	Δ*rpsU3*-1	Δ*rpsU3*-2	Δ*rpsU3*-3	LVS-1	LVS-2	LVS-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			B4			C1			C7	C8	C9
B	A2			B5			C2					
C	A3			B6			C3					
D	A7			B7			C4					
E	A8			B8			C5					
F	A9			B9			C6					
G												
H												



Given the large error bars, there is no significant difference, so although it almost looks like there might be regulation, negative and positive respectively, there probably isn't. Not significantly or detectably.

Additionally, I nanodrop'd the first half of my RNA Stability Assay cDNA samples, and there were some pretty bad ethanol contamination:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1 0'	6.9	0.208	0.118	1.76	1.75
KRLVS148 2 0'	22.3	0.677	0.372	1.82	2.28
KRLVS148 3 0'	18.1	0.549	0.302	1.82	2.51
KRLVS148 1 1'	18.8	0.569	0.331	1.72	1.83
KRLVS148 2 1'	18.1	0.549	0.315	1.74	1.88
KRLVS148 3 1'	13.8	0.419	0.221	1.90	3.17
KRLVS148 1 2'	10.3	0.313	0.185	1.69	2.33
KRLVS148 2 2'	15.9	0.481	0.271	1.78	3.13
KRLVS148 3 2'	13.3	0.402	0.232	1.73	3.36
KRLVS148 1 4'	16.5	0.501	0.29	1.73	2.91
KRLVS148 2 4'	21.6	0.654	0.352	1.86	2.75
KRLVS148 3 4'	12.5	0.378	0.202	1.87	3.96

I've never had contamination like that before, it's kind of crazy. I'll ask if I can re-do the purification.

Tuesday, February 14, 2023

To Do:

1. ~~Nanodrop second half of cDNA samples~~
2. ~~Make 2.5% iron pyrophosphate~~

Results and Data:

I nanodrop'd the second half of my stability assay samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS149 1 0'	0.0	-0.001	-0.014	0.07	0.01
KRLVS149 2 0'	0.5	0.015	0.010	1.44	-0.37
KRLVS149 3 0'	19.9	0.605	0.331	1.82	2.80
KRLVS149 1 1'	8.9	0.270	0.156	1.73	3.09
KRLVS149 2 1'	12.7	0.384	0.224	1.71	2.64
KRLVS149 3 1'	7.9	0.238	0.130	1.83	4.18
KRLVS149 1 2'	1.4	0.042	0.029	1.46	-5.44
KRLVS149 2 2'	8.6	0.261	0.143	1.82	4.64
KRLVS149 3 2'	14.8	0.449	0.251	1.79	2.53
KRLVS149 1 4'	6.3	0.192	0.102	1.88	5.66
KRLVS149 2 4'	8.7	0.264	0.137	1.93	4.84
KRLVS149 3 4'	8.0	0.242	0.121	2.00	2.42

Obviously, some of the samples are not feasible. The very low/non-existent cDNA samples do not correlate to the low RNA samples, so my best guess at what happened is that the enzyme I used was a little wonky, but also that doe'n't super make sense to me because it was in a master mix... But also the purity of the cDNA is real sus...

Met with Kathryn to discuss the qPCR results. With the range of the standard deviations, the values are not significantly different. I confirmed the primer efficiencies, however, looking at the primers, Kathryn realized that the *rpsU1* primers are situated in a proposed terminator structure/sequence within the *cspC*, which obviously could be impacting or could not be impacting our detection of transcript abundance dependent on whether it is active or not. But there is not really a way to know whether or not it is. However, looking at the RNASeq data, the level of transcript is pretty steady between the *cspC* and *rpsU1* so it may not really be an issue based on that. So we feel good about thi primer set in this scenario.

Reagents

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH2O (type 1), dissolved overnight, fresh solution every 2 weeks

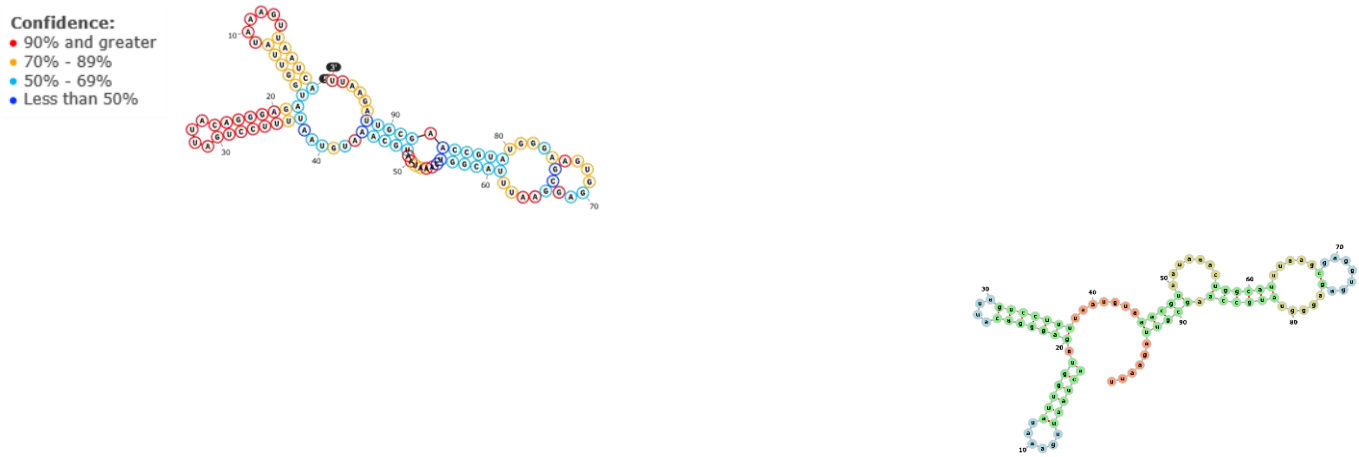
Wednesday, February 15, 2023

To Do:

1. ~~Filter sterilize 2.5% iron pyrophosphate~~
2. ~~Aliquot KRLVS149 stability assay RNA samples for cDNA synthesis~~
3. ~~Re-cDNA synthesis of KRLVS149 RNA stability assay samples~~
4. ~~Purify cDNA samples~~
5. ~~Nanodrop cDNA samples~~

Results and Data:

Hannah showed me the RNA secondary structure website as well as how to show the secondary structure on SnapGene. MXfold2 is very easy, you just put in the DNA nucleotides and it will automatically convert to RNA. It displays colors to indicate regions on the structure, as well as numbers to show distance from the 5' end. SnapGene also shows color for how probable a certain structure is, but she prefers to use MXfold2. That said, Hannah made me a SnapGene file with the 5' UTR of *rpsU2*. And then I put the sequence into MXfold2 (after Hannah showed me how), shown respectively:



(SnapGene on the left, MXfold2 on the right). The structures are very similar, with a slight difference in the rotation in 3D space, according to Hannah. I would agree given that the 10 and 30 bp stem loops are flipped. But otherwise, they look very similar. I found MXfold2 to be easier to use.

I prepared KRLVS149 RNA samples in strip tubes according to the following, for cDNA synthesis:

cDNA			
Sample	**ng/uL**	**RNA**	**Water**
KRLVS149 1 0'	195.7	13.64	-0.14
KRLVS149 2 0'	247.8	10.77	2.73
KRLVS149 3 0'	180.7	14.78	-1.28
KRLVS149 1 1'	216.7	12.32	1.18
KRLVS149 2 1'	221.1	12.08	1.42
KRLVS149 3 1'	187.5	14.24	-0.74
KRLVS149 1 2'	254.1	10.51	2.99
KRLVS149 2 2'	357.2	7.47	6.03
KRLVS149 3 2'	268.9	9.93	3.57
KRLVS149 1 4'	214.7	12.44	1.06
KRLVS149 2 4'	261.5	10.21	3.29
KRLVS149 3 4'	106.9	24.98	-11.48

Generate cDNA (Half) of Stability Assay Samples

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
$(NS)_5$ 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 $(NS)_5$ 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	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

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 ul

9. Purify, elute, nanodrop, 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 column. Centrifuge 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 o f Buffer 0.1x EB. Let column stand for 1'. Centrifuge for 1' at 13,000rpm.

Additionally, I nanodrop'd my cDNA samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS149 1 0'	2.8	0.086	0.038	2.23	-8.40
KRLVS149 2 0'	14.9	0.451	0.270	1.67	2.70
KRLVS149 3 0'	16.5	0.500	0.307	1.63	2.47
KRLVS149 1 1'	16.9	0.513	0.278	1.84	2.47
KRLVS149 2 1'	14.1	0.427	0.240	1.78	3.05
KRLVS149 3 1'	14.6	0.443	0.275	1.61	2.19
KRLVS149 1 2'	15.2	0.460	0.258	1.79	2.77
KRLVS149 2 2'	10.8	0.326	0.168	1.94	5.37
KRLVS149 3 2'	10.7	0.325	0.176	1.84	3.25
KRLVS149 1 4'	6.9	0.210	0.118	1.78	3.67
KRLVS149 2 4'	9.2	0.279	0.171	1.63	3.13
KRLVS149 3 4'	11.1	0.335	0.187	1.80	2.91

Obviously, I actually got a workable concentration in all of them this time, however the first sample is looking weird and does not instill great confidence, so I might have to go into duplicates.

Thursday, February 16, 2023

To Do:

1. ~~Streak out KMLFT104, KRLVS16, and LVS @room temperature~~
2. Supplement MHB

Results and Data:

I spoke to Hannah about the ethanol contamination in my cDNA samples, and she showed me how the low concentrations can set up a worse ratio just because the peaks are lower, which makes sense. I'll still let the samples evaporate some, and re-nanodrop, but then I think I will go ahead and move forward.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose
5mL of 2.5% iron pyrophosphate
isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)

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

Tuesday, February 21, 2023

To Do:

1. ~~Leave KRLVS148 with caps open to evaporate ethanol hopefully and re-nanodrop~~

Results and Data:

Hannah gave me a tip for the *rpsU2* 5' UTR plasmid stuff based on what she's been working on, if the insert is 100 bp or less then do the 5x and 3x ligations and don't bother with diluting the DNA sample, just do all DNA.

I left the caps open on a quarter of my stability assay cDNA samples while thawing and nanodrop'd them:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1 0'	10.2	0.309	0.132	2.34	9.68
KRLVS148 2 0'	19.2	0.582	0.286	2.03	3.75
KRLVS148 3 0'	15.3	0.463	0.228	2.03	5.27
KRLVS148 1 1'	15.5	0.471	0.258	1.83	2.07
KRLVS148 2 1'	13.7	0.415	0.198	2.09	4.16
KRLVS148 3 1'	11.5	0.348	0.161	2.16	24.09

Obviously, my 260/230 ratios got higher. Hannah let me know that ethanol actually peaks at 230, meaning that ethanol contamination would drive the ratio lower. Obviously, allowing the ethanol to evaporate drove the ratios even higher. I looked up what this higher ratio could mean, but I couldn't find very good information on a high ratio, just a low ratio. From what I could gather, it seems there's some proteinases which could have carried over from the RNA isolation? Otherwise, I'm not sure. Rather than messing with them anymore, I reached out to Kathryn to meet with her about it.

Hannah and Janet said that it might just be more cDNA than 230, or outside of the linear range.

Read: Olson E. R. (1993). Influence of pH on bacterial gene expression. *Molecular microbiology*, *8*(1), 5–14. https://doi.org/10.1111/j.1365-2958.1993.tb01198.x

Wednesday, February 22, 2023

To Do:

1. ~~Patch out KMLFT104, KRLVS16, and LVS~~
2. ~~Patch out yeast~~

Results and Data:

Gonna replace lacZ with GFP into the Tn7 site, create a NotI between the first six amino acids of bs21-2 and the ala ala ala linker to GFP. Tul4 promoter, bs21-2 UTR, fix six aa of bs21-2, and then GFP. psiI or pacI in the tul4 promoter is GFP strong enough from a chromosomal integration

pKR91 (*bfr* promoter, and made up strong 5' UTR), KRLVS113, checked in macrophage, could see by eye in patch but not colony, could see plenty of signal in fluorescence microscopy. Can try it out from a patch and diluting to 0.35 in both KRLVS113 and LVS-pF

Thursday, February 23, 2023

To Do:

1. ~~Set up cultures of KMLFT104, KRLVS16, and LVS~~
2. ~~Make YPD~~
3. ~~Start yeast overnight~~

Results and Data:

I started my cultures for RNA purification but I accidentally overgrew them... so :")

Reagents

YPD (250 mL)

To 500 mL baffled flask add:

2.5 g Yeast extract
5.0 g Protease-Peptone
5.0 g Glucose

Mix thoroughly and autoclave Liquid 15'

Store at room temperature

Friday, February 24, 2023

To Do:

1. ~~Streak out KRLVS111, KRLVS112, KRLVS148 and KRLVS149~~
2. ~~Streak out KRLVS28 and KRLVS75~~
3. ~~Make electrocompetent yeast cells~~
4. ~~Electroporate ligation into pKR168~~

Results and Data:

Making electrocompetent yeast

1. ~~Have a thick patch of S. cerevisiae parental strain grown up on a YPD plate in advance. Grow 250 mL overnight cultures of yeast strain in YEPD media at 30C, shaking at 250 rpm. Start from plates and resuspend to OD of approximately 0.06 if from an older plate or 0.01 if from a new plate.~~
2. Take OD600 (dilute 1:10) until it reaches about 1.3-1.5 OD
3. Place entire volume of culture in sterile 250 mL centrifuge bottle
4. Centrifuge at 3000 xg for 5 min at 4C. Discard supernatant.
5. Wash with ~200 mL of ice-cold water twice with identical centrifugation.
6. Wash in 20 ml of ice cold 1M sorbitol and centrifuge again, as above.
7. Resuspend in smallest volume of ice cold 1M sorbitol, starting with 200 ul and up to 500 ul as needed.

Electroporation of Candidate pKR168 + pKR168 Ligation into Yeast

1. Add 3 ul of plasmid to 100 ul of electrocompetent cells. For ligations, add 8 ul. Mix via pipette. Incubate on ice for 5 min.
2. Transfer to prechilled 0.2 cm cuvette
3. Electroporate on presetting SC2 (1500 V, 5 msec)
4. Immediately add the cells to 1 mL of cold 1M sorbitol
5. Plate onto -ura selective plates. For ligations, plate 200 ul and remaining. Put in incubator set to 30C. Colonies will be visible within 2-3 days for successful transformations.

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	# of Plates
1	pKR168	Lig. 1	8 uL	200 uL, Rem.	2
2	pKR168	Lig. 2	8 uL	200 uL, Rem.	2
3	Backbone	Lig. BB	8 uL	200 uL, Rem.	2
4	(-) control	-		200 uL	1
				Total:	7

I had more cells in my remainder than I have previously. My time constants were 1.45, 1.5, and 1.5.

Additionally, I tried out making a potential chromosomal integration GFP plasmid.

Sunday, February 26, 2023

To Do:

1. ~~Dilute KRLVS148~~
2. ~~qRT-PCR of stability assay samples – 1.1~~
3. ~~qRT-PCR of stability assay samples – 1.2~~
4. ~~Put away dishes~~

Results and Data:

I diluted my KRLVS148 samples according to my most recent Nanodrop concentration readings, as follows:

0.99 ng/uL qPCR Stock			
Sample Name	**Conc.**	**DNA uL**	**EB uL**
KRLVS148 1 0'	10.2	5.83	54.17
KRLVS148 2 0'	19.2	3.10	56.90
KRLVS148 3 0'	15.3	3.88	56.12
KRLVS148 1 1'	15.5	3.83	56.17
KRLVS148 2 1'	13.7	4.34	55.66
KRLVS148 3 1'	11.5	5.17	54.83
KRLVS148 1 2'	10.3	5.77	54.23
KRLVS148 2 2'	15.9	3.74	56.26
KRLVS148 3 2'	13.3	4.47	55.53
KRLVS148 1 4'	16.5	3.60	56.40
KRLVS148 2 4'	21.6	2.75	57.25
KRLVS148 3 4'	12.5	4.75	55.25

qRT-PCR of RNA Stability Assay cDNA Samples – 1.1

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL399/400 and KRLVS504/505 as test primers to amplify *LacZ* and downstream of *rpsU2* 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 (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_2O	8 uL	196.0 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.
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	KROL399/400 *lacZ*
B	KROL504/505 ds *rpsU2*
C	KROL63/64 *tul4*

1	2	3	4	5	6
148 1 0’	148 2 0’	148 3 0’	148 1 1’	148 2 1’	148 3 1’

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					
B	A2			B2			C2					
C	A3			B3			C3					
D	A4			B4			C4					
E	A5			B5			C5					
F	A6			B6			C6					
G												
H												

qRT-PCR of RNA Stability Assay cDNA Samples – 1.2

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL399/400 and KRLVS504/505 as test primers to amplify *LacZ* and downstream of *rpsU2* 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 (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_2O	8 uL	196.0 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.
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	KROL399/400 *lacZ*
B	KROL504/505 ds *rpsU2*
C	KROL63/64 *tul4*

7	8	9	10	11	12
148 1 2’	148 2 2’	148 3 2’	148 1 4’	148 2 4’	148 3 4’

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A7			B7			C1					
B	A8			B8			C2					
C	A9			B9			C3					
D	A10			B10			C4					
E	A11			B11			C5					
F	A12			B12			C6					
G												
H												

Monday, February 27, 2023

To Do:

1. ~~Patch out KRLVS111, KRLVS112, KRLVS148, and KRLVS149~~
2. ~~Patch out KRLVS28 and KRLVS75~~
3. ~~Patch out LVS-pF and KRLVS113~~
4. ~~qRT-PCR of stability assay samples – 2.1~~
5. ~~qRT-PCR of stability assay samples – 2.2~~
6. ~~Check yeast electroporations~~

Results and Data:

I diluted the KRLVS149 stability assay samples according to:

0.99 ng/uL qPCR Stock			
Sample Name	**Conc.**	**DNA uL**	**EB uL**
KRLVS149 1 0'	2.8	21.23	38.77
KRLVS149 2 0'	14.9	3.99	56.01
KRLVS149 3 0'	16.5	3.60	56.40
KRLVS149 1 1'	16.9	3.52	56.48
KRLVS149 2 1'	14.1	4.22	55.78
KRLVS149 3 1'	14.6	4.07	55.93
KRLVS149 1 2'	15.2	3.91	56.09
KRLVS149 2 2'	10.8	5.50	54.50
KRLVS149 3 2'	10.7	5.55	54.45
KRLVS149 1 4'	6.9	8.61	51.39
KRLVS149 2 4'	9.2	6.46	53.54
KRLVS149 3 4'	11.1	5.35	54.65

qRT-PCR of RNA Stability Assay cDNA Samples – 2.1

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL399/400 and KRLVS504/505 as test primers to amplify *LacZ* and downstream of *rpsU2* 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 (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)
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ddiH_2O	8 uL	196.0 uL
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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.
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Labelling was as follows:

Master Mix Label	Primer Set
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Loaded 96-well plate according to the following table:

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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												
H												

qRT-PCR of RNA Stability Assay cDNA Samples – 2.2

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL399/400 and KRLVS504/505 as test primers to amplify *LacZ* and downstream of *rpsU2* 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 (12*2=24 reactions)
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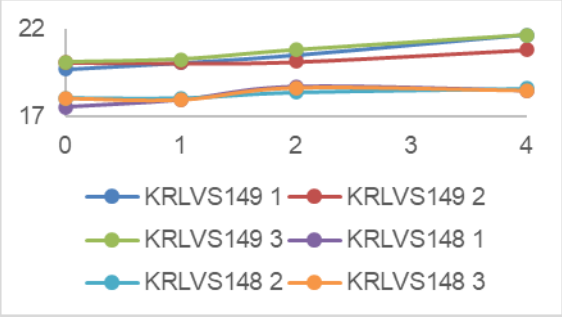
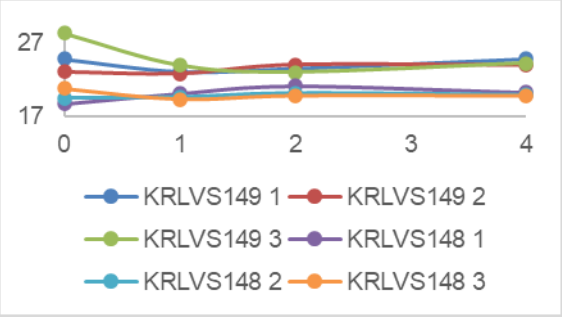
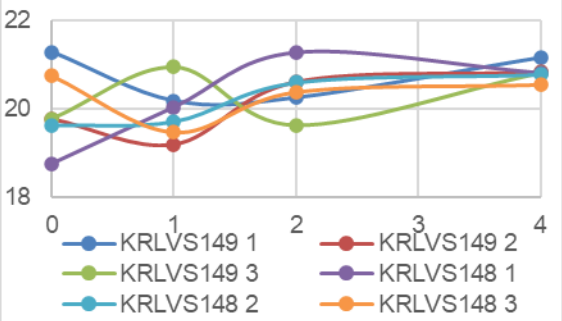
C	A9			B9			C3					
D	A10			B10			C4					
E	A11			B11			C5					
F	A12			B12			C6					
G												
H												

We discussed during the meeting some different ways to look at this data, since we aren’t actually positive that *tul4* can be used as a control. So instead, Kathryn had me plot each replicate C_P value over time for each amplified region, in order *lacZ*, 5’ UTR, *tul4*:







I would say *lacZ* and the 5’ UTR region of *rpsU2* look pretty stable to me, but *tul4* looks kind of strange.

Otherwise, I looked at my transformation plates and I did have quite a few transformants- yay! However, there’ going to be some inclement weather tomorrow morning, so I put them in the fridge.

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.