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March 2023

Friday, March 3, 2023

To Do:

1. ~~Streak out KMLFT104, KRLVS16, and LVS-pF~~
2. ~~Streak out KRLVS28 and KRLVS75~~

Results and Data:

I struck out all of my strains, a couple of the plates were a little wet, but hopefully they're okay.

Monday, March 6, 2023

To Do:

1. ~~Patch out KMLFT104, KRLVS16, and LVS-pF~~
2. ~~Take KRLVS28 and KRLVS75 plates out of the incubator~~
3. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
4. ~~Supplement MHB~~
5. ~~Make yeast overnights~~
6. ~~Set up tubes for tomorrow~~

Results and Data:

Reagents

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, March 7, 2023

To Do:

1. ~~Set up cultures of 1/3/LVS for RNA putification~~
2. ~~Miniprep yeast transformations~~
3. ~~Patch out KRLVS113 and LVS-pF~~
4. ~~Nanodrop yeast minipreps~~
5. ~~PCR of yeast minipreps~~
6. ~~RNA extraction and purification of 1/3/LVS~~
7. ~~Nanodrop RNA samples~~
8. ~~Patch out KRLVS28 and KRLVS75~~

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

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, repeat until all accordant tubes are combined.
7. Discard 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

PCR of Candidate pKR168 Minipreps for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1-6	P*rpsU2_tul4*UTR	MP pKR168	KROL6, KROL257	633
7	- control	-	KROL6, KROL257	-

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

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

Then, I nanodrop'd the total nucleic acid samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KMLFT104 1	968.1	19.361	9.089	2.13	2.31
KMLFT104 2	954	19.081	8.943	2.13	2.29
KMLFT104 3	1063.1	21.262	10.188	2.09	1.85
KRLVS16 1	338	6.759	3.204	2.11	2.16
KRLVS16 2	1066.2	21.324	10.015	2.13	2.34
KRLVS16 3	888.3	17.766	8.174	2.17	2.28
LVS 1	947.5	18.95	8.731	2.17	2.31
LVS 2	601.2	12.023	5.711	2.11	2.27
LVS 3	676.9	13.537	6.522	2.08	2.15

I then struck out KRLVS28 and KRLVS75 for overnights tomorrow.

Wednesday, March 8, 2023

To Do:

1. ~~DNase treatment of 1/3/LVS RNA samples~~
2. ~~Second purification of 1/3/LVS RNA samples~~
3. ~~Nanodrop RNA samples~~
4. ~~Set up aliquots for gel and cDNA synthesis~~
5. ~~Run gel of RNA samples~~
6. ~~Quick and dirty GFP assay of LVS-pF and KRLVS113~~
7. ~~PCR purification of yeast plasmid PCR~~
8. ~~Gel of purified PCR of yeast plasmids~~
9. ~~Set up candidate pKR168 for sequencing~~
10. ~~pH 30 mL MHB to 3.5~~
11. ~~Set up overnights for pH experiment~~

Results and Data:

DNase treatment of KRLVS16, KMLFT104, and LVS

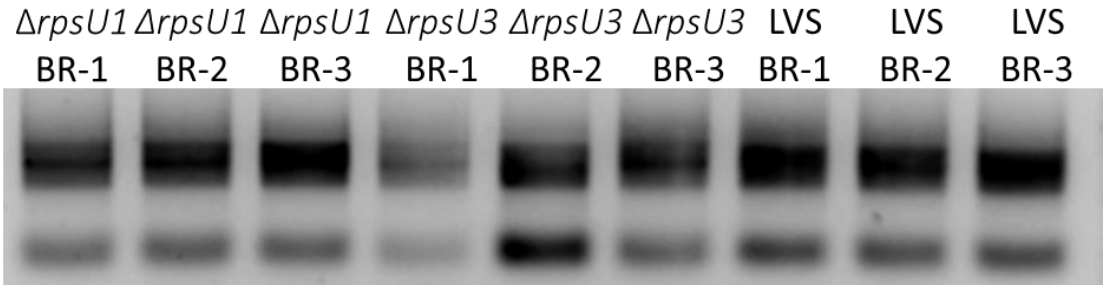
1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
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

Gel of KRLVS16, KMLFT104, and LVS 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 0.6 g to 60 mL 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 6 uL 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 3/8/23 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



I didn't quite let it run long enough, but you can start to see the separation in the 23s and 16s RNA, and none of them are a smudge so it looks all good to me.

PCR Purification of Candidate pKR168 Yeast Miniprep PCR

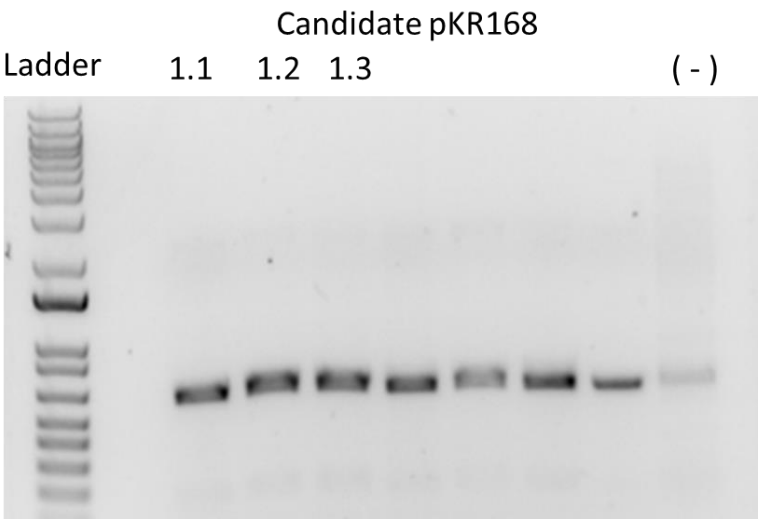
1. Add 100 uL of Buffer PB to each 20 uL PCR reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.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

Loading Order of 2/3/23 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
Ladder		(-)		Cand. 1	Cand. 2	Cand. 3	Cand. 4	Cand. 5	Cand. 6		(+)

Obviously, there is contamination in my negative control. Given that I had contaminations, I did replace everything prior to running the PCR> Clearly, it was the primers which had contamination, so that's not great. However, given the faintness of the negative band, I decided the other six were likely real amplifications, so I decided to sequence half of them.



Then I nanodrop'd the PCR:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR168 1.1	42.9	0.858	0.387	2.22	5.48
Cand. pKR168 1.2	39	0.779	0.37	2.1	5.44
Cand. pKR168 1.3	41.6	0.832	0.375	2.22	5.89
Cand. pKR168 2.1	38.8	0.775	0.337	2.3	6.88
Cand. pKR168 2.2	21.1	0.422	0.148	2.85	-9.39
Cand. pKR168 2.3	38.3	0.766	0.341	2.24	5.68

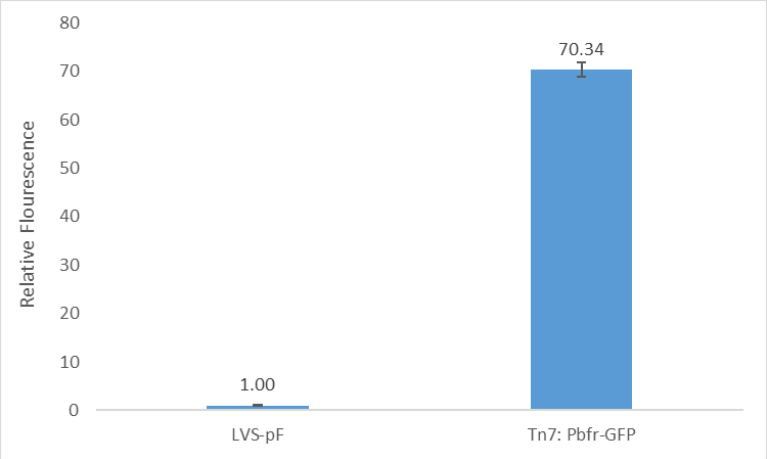
And I set up sequencing of three of the PCR's, based on the highest plasmid concentrations:

Sample	Type	Template Name	Primer	Size (bp)	[Stock] (ng/uL)	PCR (ng)	PCR (uL)	H_2O (uL)
SS1	PCR	Cand. pKR168 1.2	KROL257	633	19.5	15.83	0.81	8.63
SS2	PCR	Cand. pKR168 1.3	KROL257	633	20.2	15.83	0.78	8.66
SS3	PCR	Cand. pKR168 2.1	KROL257	633	19.4	15.83	0.82	8.62
a.Add 2.56 µl of 2.5 µM stock to each reaction								

Modified GFP Assay on Chromosomal Integration of GFP in KRLVS113

1. Scrape up patches of KRLVS113 and LVS-pF into 1xPBS
2. Normalize in 1 mL of 1xPBS to an OD of 1 mL
3. Aliquot 250 ul from each tube in quadruplicate to clear 96-well plate.
4. Go to INBRE lab with 200 uL pipet, pipet tips, black 96-well plate, and flash drive.
5. Read OD600 from clear plate on ID3 plate reader:
    a. Select Absorbance, wavelength=600
    b. Plate type: 96-well standard clearbtm (first option)
6. Transfer 200 ul of each well from clear plate to black plate using the multichannel
7. 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	KRLVS113	KRLVS113	KRLVS113	KRLVS113								
B												
C	LVS-oF	LVS-oF	LVS-oF	LVS-oF								
D												
E												
F												
G												
H												



I took the average of the quadruplicates and normalized both to the raw fluorescence of LVS-pF. It looks like the chromosomal integration with the *bfr* promoter is 70-fold greater than LVS-pF. Obviously, the *tul4* promoter and *rpsU2* 5' UTR will possibly be weaker, however, this looks promising to me for doing an integration of that construct into the Tn7 site. Additionally, I showed Kathryn and she agreed with my estimation. She also had me look at plasmid fluorescence values for comparison, and looking at the *tul4* promoter from Hannah's experiment, she had 1000-fold increase, which is obviously pretty crazy.

Thursday, March 9, 2023

To Do:

1. ~~Media transfer of KRLVS28 and KRLVS75 overnights~~
2. ~~Streak out KRLVS11 and KRLVS148~~
3. ~~B-gal of pH experiment~~

Results and Data:

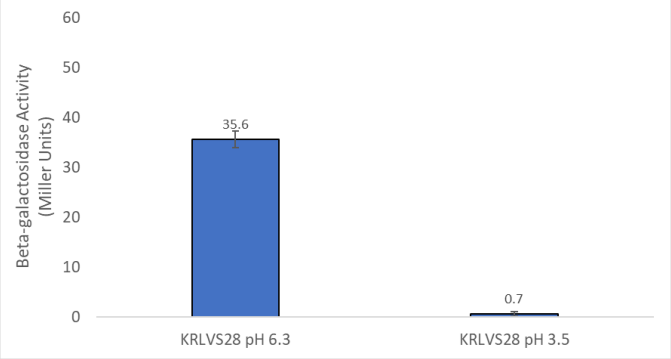
Media Transfer of Overnight Cultures

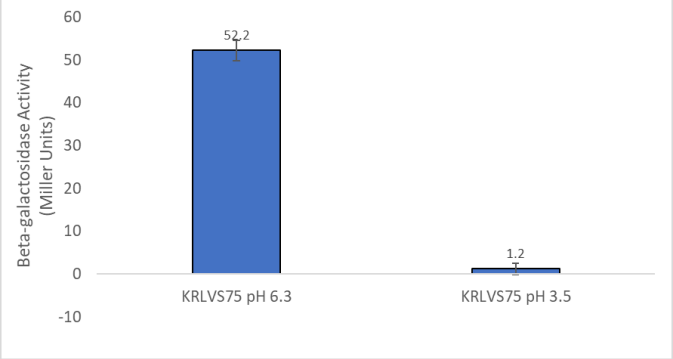
1. Once OD's have reached OD 0.25-0.3 remove from the shaking incubator
2. Transfer contents to 50 mL conical tubes and pellet at 8000 xg for 5 minutes, ensure proper pelleting
3. Pour off supernatant and resuspend each in 300 uL of MHB
4. Addded 175 uL to each replicate tube
5. Shake at 37°C for an additional hour

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

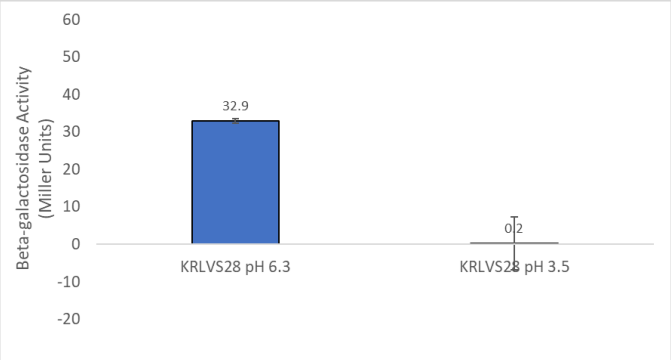
1. Once cultures are ready, spin 1.8 mL of each culture in 2 mL tubes and resuspend in 1 mL 1x PBS then set on ice for 30 minutes

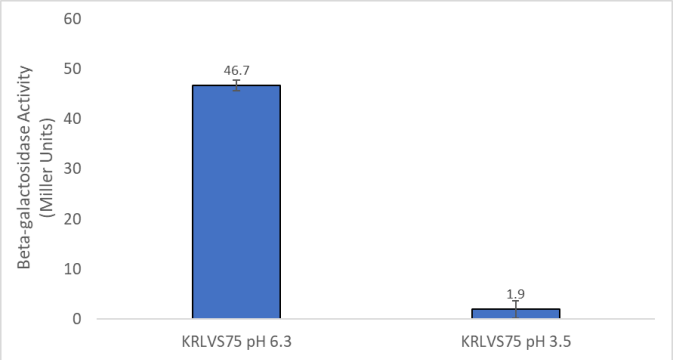
2. Turn on 28°C water bath and put ONPG in water bath
3. Determine Z-buffer needed (0.8ml x 2 x # of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer (2.72 x Xml Z-buffer = µl of BME).
4. Set up reaction tubes with 800µl Z-buffer, put on lids
5. Turn 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.





Given that this procecure was modified in such a way as to reflect the the protocol of the GFP assay, for reduction of the acidic environment, I am inclined to believe that this test-run is accurate. And, in fact, there just isn't any B-gal activity. Speaking with Kathryn, it is possible that either the protein is down-regualted while the trans ript is up-regulated. Or else that translation is stopping, if that were true I believe that the transcript that is not being transcribed could be being "built-up". Given these results, I believe my last run was also valid, data shown for February 8th:





Friday, March 10, 2023

To Do:

1. ~~Check sequencing results~~
2. ~~Streak out KRLVS112 and KRLVS149~~
3. ~~cDNA synthesis of KMLFT104, KRLVS16, and LVS~~
4. ~~Purify cDNA samples~~
5. ~~Nanodrop cDNA samples~~
6. ~~Dilute cDNA samples~~

Results and Data:

I took a glance at my sequencing results and both candidate 1.2 and 2.1 look good, 1.3 has a gap. Obviously, my sequencing does not fully cover the *lacZ* gene, but I will go forward with it and hopefully it will be fine when I sequence the PCR on the integration.

Generate cDNA (Half) of KMLFT104, KRLVS16, and LVS (3/8/23 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 of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

I then nanodrop'd my cDNA:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KMLFT104 1	13.1	0.398	0.211	1.88	4.54
KMLFT104 2	15.2	0.461	0.261	1.76	2.45
KMLFT104 3	20.3	0.616	0.349	1.76	2.19
KRLVS16 1	9.7	0.293	0.156	1.89	2.92
KRLVS16 2	11.8	0.359	0.175	2.05	3.24
KRLVS16 3	16.7	0.505	0.272	1.85	2.47
LVS 1	16.3	0.495	0.258	1.92	2.95
LVS 2	41.7	1.265	0.627	2.02	2.40
LVS 3	19.2	0.581	0.339	1.71	2.32

Then I diluted my cDNA samples accordingly:

0.99 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KMLFT104 1	13.1	4.54	55.46
KMLFT104 2	15.2	3.91	56.09
KMLFT104 3	20.3	2.93	57.07
LVS 1	16.3	3.65	56.35
LVS 2	41.7	1.43	58.57
LVS 3	19.2	3.10	56.90

2.0 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KRLVS16 1	9.7	12.37	47.63
KRLVS16 2	11.8	10.17	49.83
KRLVS16 3	16.7	7.19	52.81
LVS 1	16.3	7.36	52.64
LVS 2	41.7	2.88	57.12
LVS 3	19.2	6.25	53.75

Monday, March 13, 2023

To Do:

1. ~~Patch out KRLVS111, KRLVS112, KRLVS148, and KRLVS149~~
2. ~~qRT-PCR of KMLFT104, KRLVS16, and LVS samples~~
3. ~~Restriction digest of pKR69, pKR123, and pKR183~~
4. ~~Run gel of restriction digest~~

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	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 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

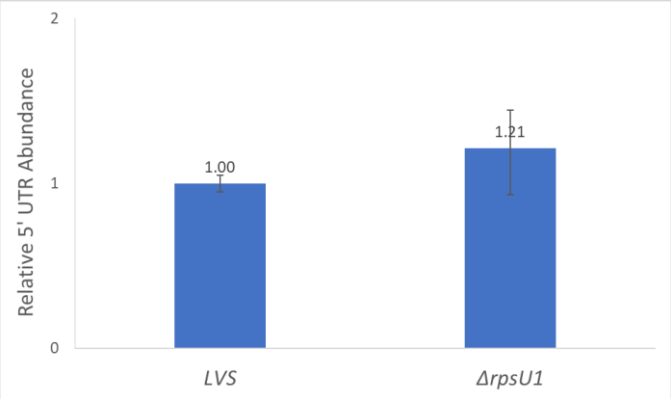
Labelling was as follows:

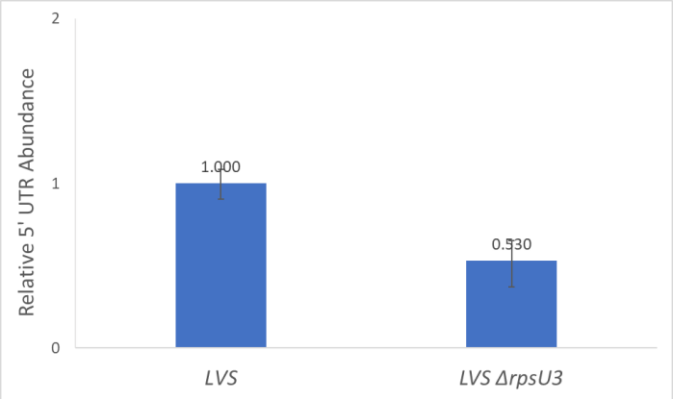
Master Mix Label	Primer Set
A	KROL337/338 *rpsU1* 5'UTR
B	KROL347/348 *rpsU3* 5'UTR
C	KROL63/64 *tul4*
D	KROL211/212 *rpoA*

1	2	3	4	5	6	7	8	9	10	11	12
Δ1-1	Δ1-2	Δ1-3	WT.99-1	WT.99-2	WT.99-3	Δ3-1	Δ3-2	Δ3-3	WT2-1	WT2-2	WT2-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			B7			C5			D7		
B	A2			B8			C6			D8		
C	A3			B9			C7			D9		
D	A4			B10			C8			D10		
E	A5			B11			C9			D11		
F	A6			B12			C10			D12		
G	C1			C3			C11					
H	C2			C4			C12					





With the new concentrations for *d1* the crossing point values are within range, and there is still no significant difference in the values. For the *d3* the crossing point values for *d3* are too high again, but the *rpoA1* were within range, and a better control. However, it looks like there might be a significant difference so Kathryn is having me run the samples again at yet again, a higher concentration.

DNA Digest of P*tul4 rpsU2*UTR, GFP, and pKR69 Backbone w/KpnI, NotI, and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR123	NotI, KpnI	5	10
2	pKR183	NotI, BamHI	5	10
3	pKR69	KpnI, BamHI	5	10

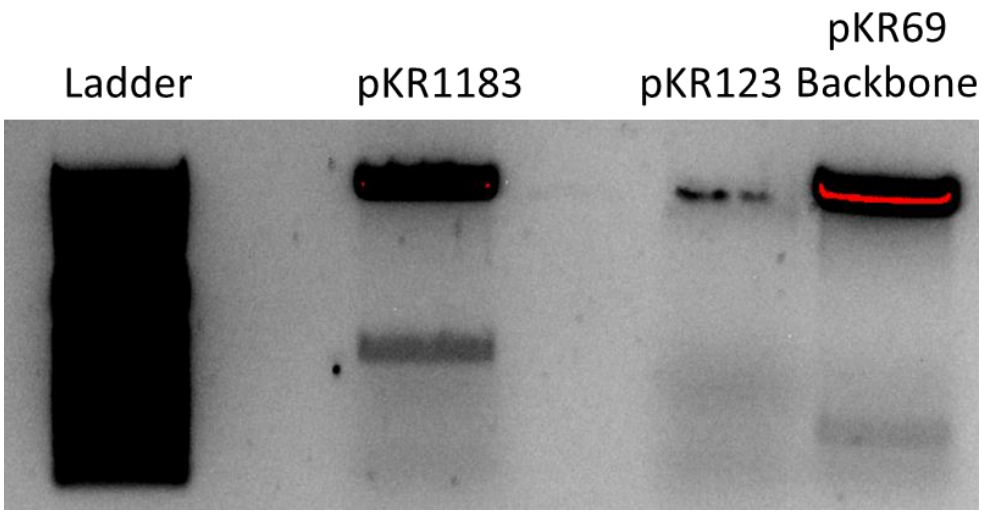
2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**
H_2O	10.8
10x Buffer*	3.0
DNA	(15.0)
Not1	0.6
Kpn1	0.6
Total	30.0 (15.0 actual b/c of DNA)

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

Gel of Digested P*tul4 rpsU2*UTR and GFP + pKR69 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of 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 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.



Visualizing and Cutting Gel

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

Tuesday, March 14, 2023

To Do:

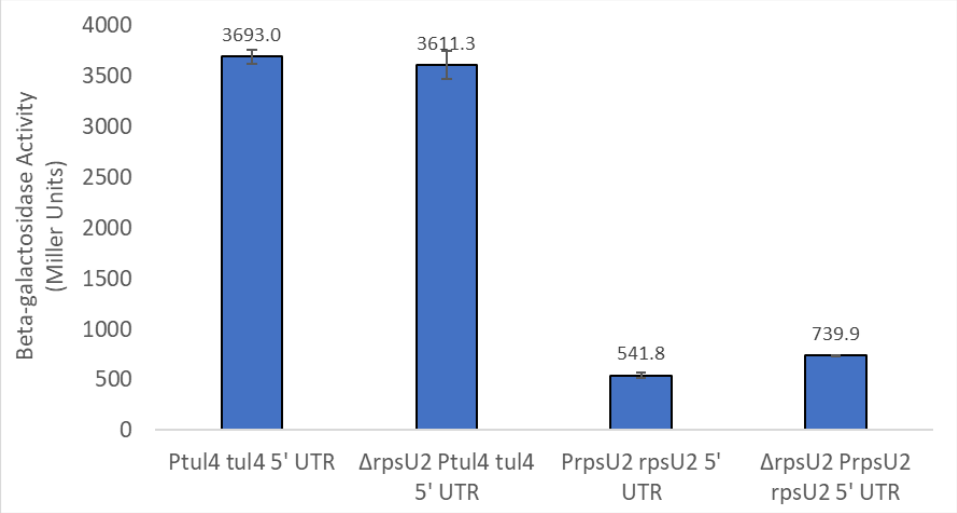
1. ~~Start cultures of KRLVS111, KRLVS112, KRLVS148, and KRLVS149~~
2. ~~Gel purification of restriction digest~~
3. ~~Run B-gal of KRLVS111, KRLVS112, KRLVS148, and KRLVS149~~
4. ~~Patch out KRLVS150 and KRLVS151~~

Results and Data:

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.



Looks good, similar to the other trial, so woo.

Gel Extraction with QIAquick Gel Extraction Kit

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

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
P*tul4 rpsU2* 5'UTR	0.160	480	160
GFP	0.400	1200	400
pKR69 Backbone	0.370	1110	370

Due to the volume of the GFP and Backbone I split it into two tubes, making sure they were equal by eye, prior to add one half of the volume of isopropanol to each.

Additionally, Ben poured the CHAH I made for me. This included the total 500 mL of CHAH+Nat (25 ul of 100 mg/mL Nat) and the total 100 mL CHAH+Hyg (370 ul of 54 mg/mL Hyg).

Wednesday, March 15, 2023

To Do:

1. ~~Patch out KRLVS127~~
2. ~~Patch out KRLVS97 and KRLVS111~~

Results and Data:

When I cheked the concentration of my gel extractions they were NOT good, and with the suboptimal gel situation, I decided to just re-do the digest.

Thursday, March 16, 2023

To Do:

1. ~~Patch out KRLVS126~~
2. ~~Patch out KRLVS96 and KRLVS112~~
3. ~~Streak KRLVS148 and KRLVS150 to single colony~~
4. ~~Make KRLVS97 and KRLVS111 electrocompetent cells~~
5. ~~Electroporate KRLVS97 and KRLVS111~~
6. ~~Make electrocompetent KRLVS127~~
7. ~~Electroporate pKR168 into KRLVS127~~
8. ~~Restriction digest of pKR69, pKR123, and pKR183~~
9. ~~Run gel of restriction digest~~
10. ~~Make personal stocks and single use aliquots of KRLVS150 and KRLVS151~~

Results and Data:

Preparing Electrocompetent KRLVS97, KRLVS111, and KRLVS127 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

Electroporating pF-nat or pKR15 into EC KRLVS97 and KRLVS111 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
   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	EV	KRLVS97	pF-nat	3 uL	10 uL. 100 uL	2
2	o.e. *rpsU2*	KRLVS97	pKR15	3 uL	10 uL. 100 uL	2
3	EV	KRLVS111	pF-nat	3 uL	10 uL. 100 uL	2
4	(-) control	KRLVS97	-	-	200 uL	1
5	(-) control	KRLVS111	-	-	200 uL	1
					Total:	8

Electroporating pKR168 into EC KRLVS127 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:

5 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 4-8 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
    a. Plated 20 uL, 200 uL, and remaining of each
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

Tube	Purpose	Strain	DNA	Vol. of	Vol. Plated	# of Plates
1	d2+pKR168	KRLVS127	Cand. pKR168 1.2	5 uL	20 uL. 200 uL, Rem.	3
2	d2+pKR168	KRLVS127	Cand. pKR168 2.1	5 uL	20 uL. 200 uL, Rem.	3
3	(-) control	KRLVS127	-	-	200 uL	1
					Total:	7

DNA Digest of P*tul4 rpsU2*UTR, GFP, and pKR69 Backbone w/KpnI, NotI, and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR123	NotI, KpnI	15	-
2	pKR183	NotI, BamHI	15	-
3	pKR69	KpnI, BamHI	5	10

2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**
H_2O	10.8
10x Buffer*	3.0
DNA	(15.0)
Not1	0.6
Kpn1	0.6
Total	30.0 (15.0 actual b/c of DNA)

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

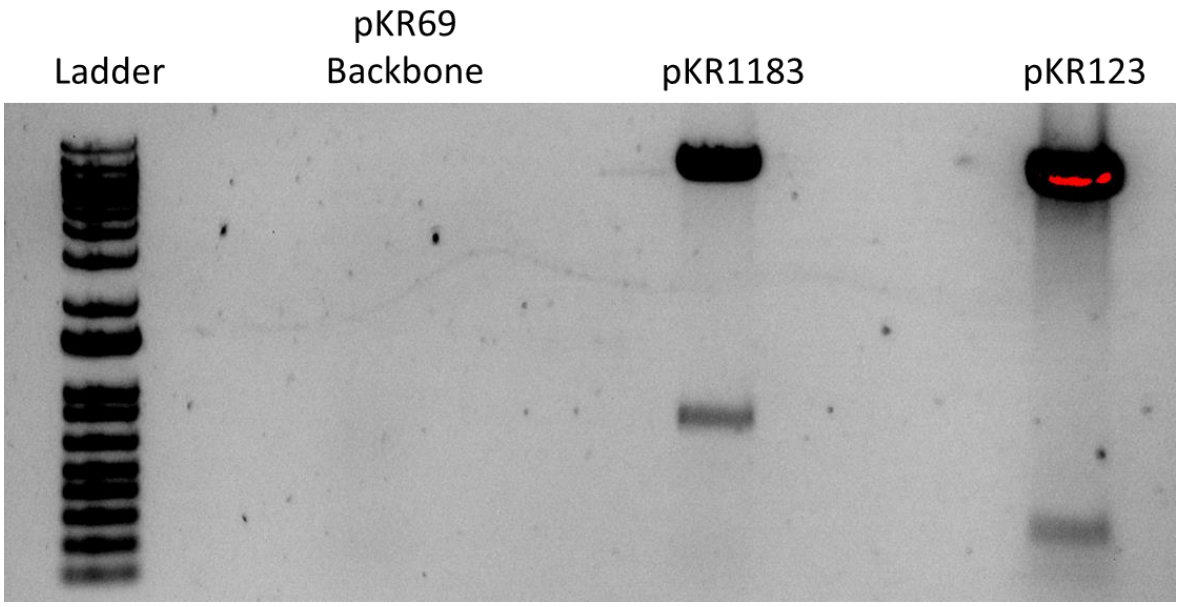
Due to the difficulty I had seeing the GFP and regulatory region with the last gel, I added 15 uL of these plasmid's DNA, also fueled by the low yield from the gel purification. However, I retained the 5 uL of the backbone. Previously I had done low volumes with the expectation that there would be a higher concentration of plasmid than if I was working from a PCR, however given how small these bands are, I thought it better to course correct.

Gel of Digested P*tul4 rpsU2*UTR and GFP + pKR69 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up large gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe dye to gel rig, pour ~120mL of agarose gel, use ladder to mix, then replace ladder and allow to set.

4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.

Loaded: Backbone, GFP, regulatory elements.



Visualizing and Cutting Gel

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

Making Personal Glycerol Stocks and Single Use Aliquots of KRLVS150 and KRLVS151

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 1200uL, mix by pipetting
5. Aliquot 1 mL to cryovial and 50ul of solution to tubes, freeze at -80

Friday, March 17, 2023

To Do:

1. ~~Streak KRLVS149 and KRLVS151 to single colony~~
2. ~~Make electrocompetent KRLVS96 and KRLVS112~~
3. ~~Electroporate KRLVS96 and KRLVS112~~
4. ~~Make electrocompetent KRLVS126~~
5. ~~Electroporate pKR168 into KRLVS126~~
6. ~~Restriction digest of pKR121~~
7. ~~Run gel of restriction digest~~
8. ~~Gel purification of pKR183, pKR123, and pKR121~~
9. ~~Run ligation for pKR184~~
10. ~~Transform ligation into *E. coli*~~
11. ~~Load dishwasher~~

Results and Data:

Preparing Electrocompetent KRLVS96, KRLVS112, and KRLVS126 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

Electroporating pF-nat into EC KRLVS96 and KRLVS112 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	EV	KRLVS96	pF-nat	3 uL	10 uL. 100 uL	2
2	EV	KRLVS112	pF-nat	3 uL	10 uL. 100 uL	2
3	(-) control	KRLVS96	-	-	200 uL	1
4	(-) control	KRLVS112	-	-	200 uL	1
					Total:	6

Electroporating pKR168 into EC KRLVS126 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:
   5 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 4-8 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
   a. Plated 20 uL, 200 uL, and remaining of each
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

Tube	Purpose	Strain	DNA	Vol. of	Vol. Plated	# of Plates
1	WT+pKR168	KRLVS126	Cand. pKR168 1.2	5 uL	20 uL. 200 uL, Rem.	3

2	WT+pKR168	KRLVS126	Cand. pKR168 2.1	5 uL	20 uL. 200 uL, Rem.	3
3	(-) control	KRLVS126	-	-	200 uL	1
					Total:	7

DNA Digest of pKR121 Backbone w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR121	KpnI, BamHI	15	-

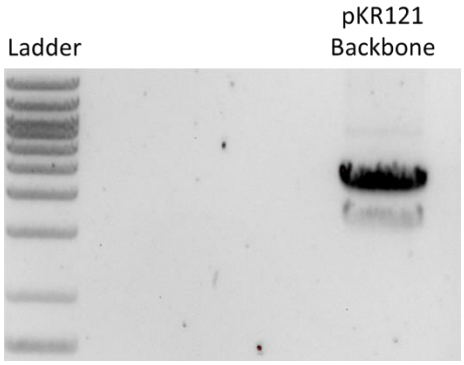
2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**
H_2O	10.8
10x Buffer*	3.0
DNA	(15.0)
Not1	0.6
Kpn1	0.6
Total	30.0 (15.0 actual b/c of DNA)

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

Gel of Digested pKR121 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up large gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe dye to gel rig, pour ~120mL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.



Visualizing and Cutting Gel

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

Gel Extraction with QIAquick Gel Extraction Kit

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

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
P*tul4 rpsU2* 5'UTR	351	1053	351
GFP	190	570	190
pKR121 Backbone	333	999	333

Ligation of Digested P*tul4 rpsU2*UTR and GFP Fragments with Digested pKR69 Backbone

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

Tube	Insert	Backbone
1	P*tul4 rpsU2* UTR (pKR123) + GFP (pKR183)	KpnI, BamHI digested, purified pKR69
2	-	KpnI, BamHI digested, purified pKR69

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)
H_2O	1.13	11.82
pKR121 Backbone	1.68	1.68
pKR123 Ptul4 rpsU2UTR	3.27	-
pKR183 GFP	11.42	-
T4 Ligase Buffer	2.0	2.0
T4 ligase	0.5	0.5
TOTAL	20.0	20.0

3. Obtain ice to assemble and keep the reactions on.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H_2O, 10x buffer, insert, and backbone.
6. Add indicated amount of ligase to the individual tubes.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Left on bench at room temperature for 10 minutes.

Transformation of pKR184 Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. Use 8 uL per ligation and plate 100 uL and remaining.
    a. Positive control: pKR121; Backbone ligation: KpnI-BamHI digested pKR121

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR121	1 uL	200 ul	1
2	(-) control	None	0	200 ul	1
3	Backbone Ligation	BB only ligation	8 uL	10 uL, 200 ul	2
4	pKR184 Ligation	pKR184	8 uL	10 uL, 200 ul	2
				Total number of plates	6

2. Check to be sure you have enough LB-Kan plates. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

Sunday, March 19, 2023

To Do:

1. ~~Check *E. coli* transformation plates~~

Results and Data:

When I came in, there were no visible colonies on the plate, so I placed them into the incubator.

Monday, March 20, 2023

To Do:

1. ~~Patch KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
2. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
3. ~~Supplement MHB~~
4. ~~Streak to single colony pKR168 transformants on CHAH~~
5. ~~Re-dilute KRLVS16 and LVS cDNA samples~~
6. ~~qRT-PCR of KRLVS16 and LVS with just *rpoA1* and the UTR~~
7. ~~Put away dishes~~
8. ~~Set up culture tubes for B-gal tomorrow~~
9. ~~Start overnights of *E. coli* transformants~~

Results and Data:

I re-diluted my cDNA samples according to:

4.0 ng/uL qPCR Stock			
Sample Name	**Conc. (ng/uL)**	**DNA uL**	**EB uL**
KRLVS16 1	9.7	24.74	35.26
KRLVS16 2	11.8	20.34	39.66
KRLVS16 3	16.7	14.37	45.63
LVS 1	16.3	14.72	45.28
LVS 2	41.7	5.76	54.24
LVS 3	19.2	12.50	47.50

qRT-PCR of KRLVS16, and LVS cDNA Samples

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL347/348 as test primers to amplify the *rpsU3* 5'UTR respectively. KROL211/212 as the control, amplifying *rpoA1*.
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 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'

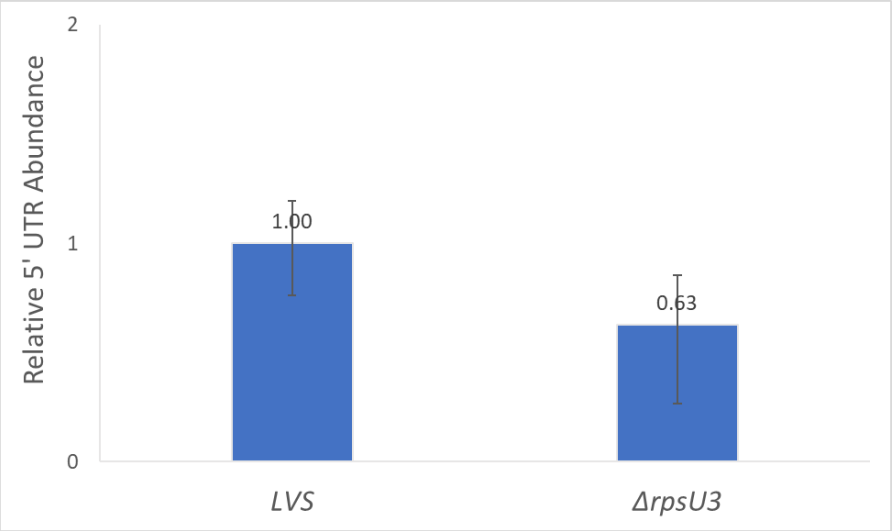
Labelling was as follows:

Master Mix Label	Primer Set
A	KROL347/348 *rpsU3* 5'UTR
B	KROL211/212 *rpoA*

1	2	3	4	5	6
Δ3-1	Δ3-2	Δ3-3	WT4-1	WT4-2	WT4-3

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			B1								
B	A2			B2								
C	A3			B3								
D	A4			B4								
E	A5			B5								
F	A6			B6								
G												
H												



My crossing point values were *still* over the limit for *d3*, however, there is obviously no significant difference on this run. I spoke with Kathryn and given that we have multiple runs that show no significance, we're going with there being no significant levels of autoregulation.

Additionally, I struck out six total colonies for LVS and d2 each of my P*rpsU2 tul4* UTR transformations. Specifically, I struck out three each of either 1.2 or 2.1 in LVS or d2.

Next, I started four overnight cultures of candidate pKR184 transformed *E. coli* in 5 mLs of LB + 5 uL of 50 mg/mL kanamycin.

Tuesday, March 21, 2023

To Do:

1. ~~Patch KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
2. ~~Set up cultures of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 for b-gal~~
3. ~~B-gal of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~

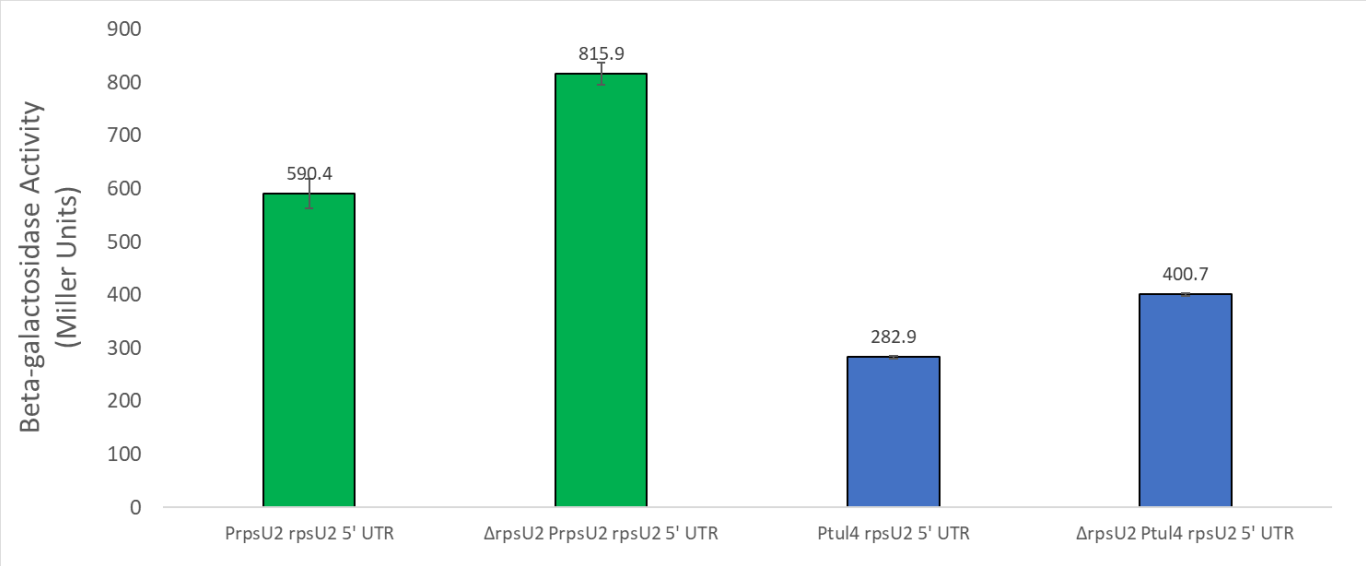
Results and Data:

I spun down my overnight cultures and froze the pellets.

β-galactosaidase Assay of KRLVS148, KRLVS149, KRLVS150, and KRLVS151

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.



Obviously, the strain with the promoter for *tul4* has level overall protein output than the construct containing both the promoter and UTR for *rpsU2*. This could mean a number of things, but potentially the *tul4* promoter isn't as strong as the *rpsU2* promoter. I don't believe our data up to this point supports that, however. I wonder if perhaps there is some regulatory process which promoters protein regulation based on the *rpsU2* promoter, however, we will certainly try to test that. Though, if its some combination of both the promoter and UTR then I image we will still see lower protein output. Super wacky thought, which might be why the *rpsU2* promoter seems so biologically unfavorable: regulation at the promoter increases the abundance, while regulation of the UTR might down-regulate? Probably not though, that would be insane and certainly would not be something I could necessarily tease out in the next couple of months.

Wednesday, March 22, 2023

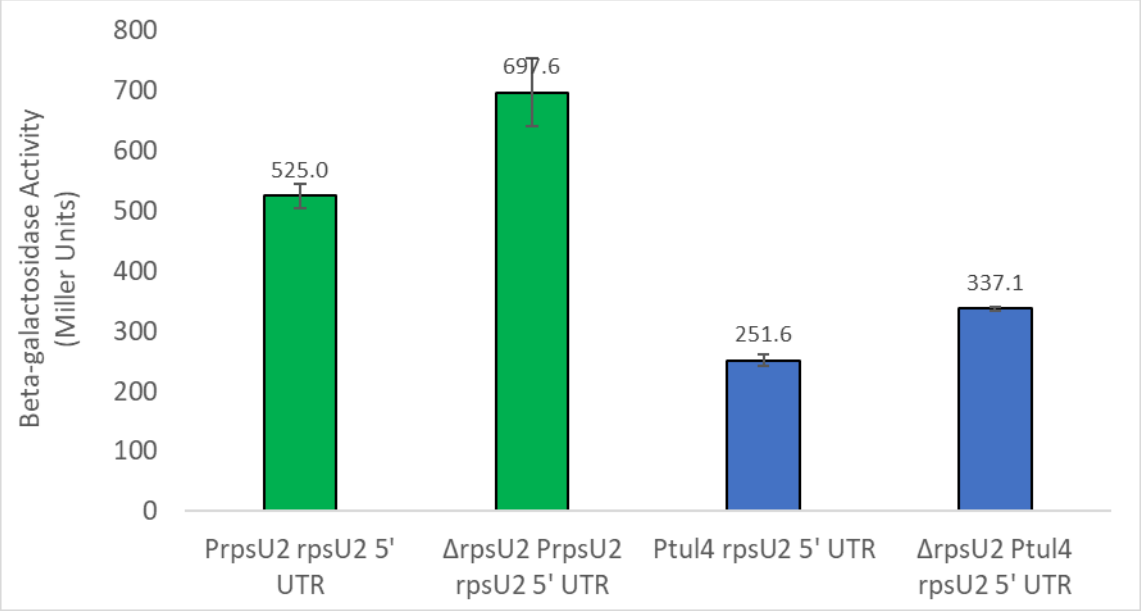
To Do:

1. ~~Set up cultures of KRLVS148, KRLVS149, KRLVS150, and KRLVS151 for b-gal~~
2. ~~B-gal of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~

Results and Data:

β-galactosaidase Assay of KRLVS148, KRLVS149, KRLVS150, and KRLVS151

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 was a little worried about the fold change being a little different in this run, since my B-gal data has been pretty consistent, however, Hannah said it was probably okay.

Thursday, March 23, 2023

To Do:

1. ~~Cross patch single colonies from pKR168 integrant plates on Hyg and Kan~~
2. ~~Prepare lysates of potential pKR168 integrants~~
3. ~~Freeze down promising first quadrant patches based on colony PCR~~

Results and Data:

I prepared lysates of each potential integrant and stored them in my working strains box in the -80.

Making Glycerol Stocks of Potential pKR168 Integrant Strains

1. Prepare 800uL 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 200ul of 75% glycerol cryovial, then add 800 uL of resuspended cells
5. Vortex until mixed and minifuge, freeze at -80

Sample #	Description
1	Δ*rpsU2* + pKR168 1.2
2	Δ*rpsU2* + pKR168 1.2
3	Δ*rpsU2* + pKR168 1.2
4	Δ*rpsU2* + pKR168 2.1
5	Δ*rpsU2* + pKR168 2.1
6	LVS + pKR168 2.1
7	LVS + pKR168 2.1
8	LVS + pKR168 1.2
9	LVS + pKR168 2.1
10	LVS + pKR168 1.2
11	LVS + pKR168 1.2

Friday, March 24, 2023

To Do:

1. ~~Miniprep *E. coli* transformants~~
2. ~~Colony PCR from patch quadrant of potential pKR168 integrants~~
3. ~~Run gel of colony PCR~~

Results and Data:

Colony PCR of Candidate pKR168 Integration into LVS and d*rpsU2*

1. Take small amounts of each patch and resuspend in 50 µL sterile water using a sterile toothpick.
2. Heat samples at 95°C for 10’ to lyse and kill cells
3. Dilute lysates 1:10
4. Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation.
5. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL472, KROL319	-
2	LVS gDNA	LVS gDNA	KROL472, KROL319	-
3-8	P*rpsU2_tul4*UTR *LacZ*	Lysate	KROL472, KROL319	3328
9-14	d2 P*rpsU2_tul4*UTR *LacZ*	Lysate	KROL472, KROL319	3328

6. Acquire the following components and put them on ice, labeling tubes if necessary:

- mgH2O, Primestar buffer, dNTPs, KROL472, KROL319 (10uM), LVS gDNA, LVS lysate, and candidate pKR168 integrant lysates

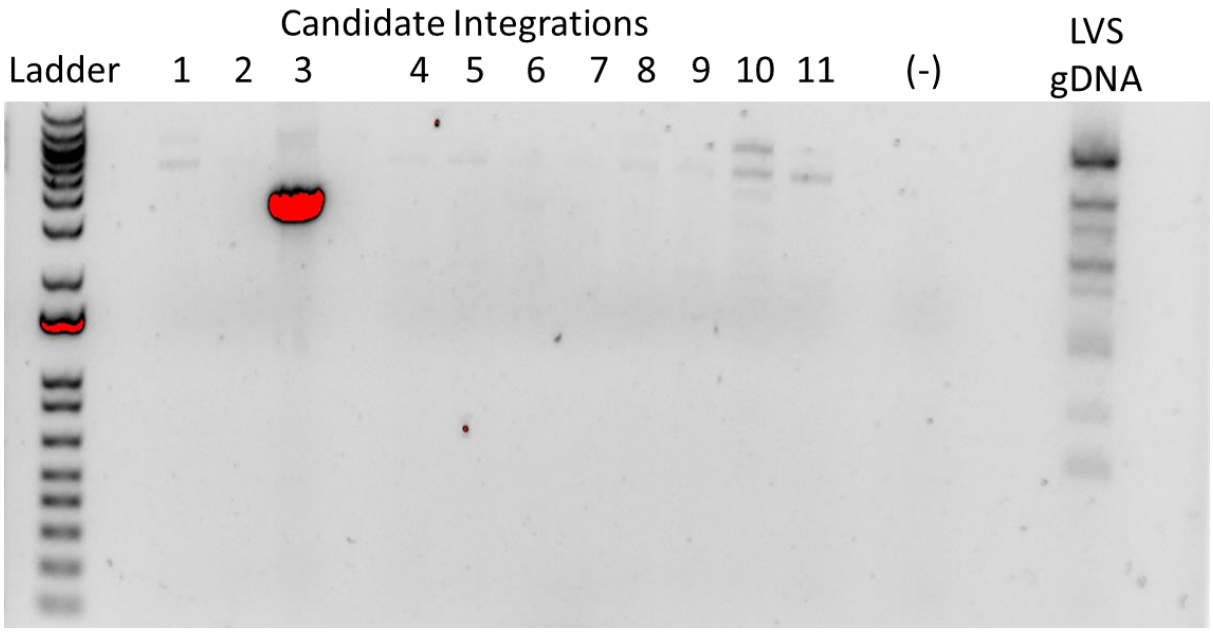
7. Vortex each component (aside from enzyme)
8. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
9. Add ddi H2O to negative control tube (template volume for 1 reaction)
10. Prepare a master-containing:
    - mgH2O, dNTPs, Primestar buffer, and Primestar enzyme
11. Mix the master-mix solution by pipetting up and down
12. Add 19.0 uL of master-mix to negative control PCR tube
13. Add 19.0 uL of master mix to each PCR tube and pipet up and down to mix
14. Place the PCR Tubes in the thermocycler according to the following program:
    a. 94°C 2'
    b. 94°C 20"
    c. 50°C 30"
    d. 68°C (1' per kb)
    e. Go to step 2, rep 32x
    f. 68°C 5'
    g. Hold 12°C

Total reaction volume	20
Total number of reactions	14

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	15
ddiH2O			11.8	177
PrimeSTAR GXL Buffer	5x	1x	4	60
dNTPs	2.5 mM	0.2 mM	1.6	24
oligo F	10 uM	0.3 uM	0.6	9
oligo R	10 uM	0.3 uM	0.6	9
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	6
Cell lysate	-	-	1	
		Total volume	20	285

Gel of Colony PCR of P*rpsU2 tul4* 5'UTR *lacZ* Integrant into LVS and d*2*

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 12 uL of Sbyr Safe dye to gel rig, pour ~120mL 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 36 uL of each sample.
6. Ran for 45 minutes at 113V.



Miniprep of Candidate pKR184 from *E. coli*

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

Monday, March 27, 2023

To Do:

1. ~~Patch out candidate d2 strain for gDNA prep~~
2. ~~Patch out KRLVS126 and KRLVS127~~
3. ~~Check miniprep concentrations~~
4. ~~Diagnostic digest of minipreps~~
5. ~~Run gel of diagnostic digest of minipreps~~

Results and Data:

Based on my gel, I patched out candidate integrant #3 for gDNA prep.

DNA Digest of Candidate pKR184 w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	Water	KpnI, BamHI	-	15
2	pKR121	KpnI, BamHI	5	10
3-6	Cand. pKR184	KpnI, BamHI	5	10

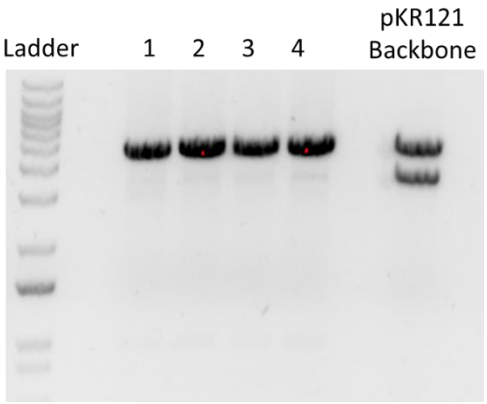
2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in 7x Master Mix (uL)
H_2O	20.8	145.6
10x Buffer*	3.0	21.0
DNA	(5.0)	-
KpnI	0.6	4.2
BamH1	0.6	4.2
Total	30.0 (25.0 actual b/c of DNA)	175.0

3. Add 25 uL of Master Mix to individual tube for digest.
4. Mix by pipetting up and down.
5. Incubate at 37°C for 1 hour.

Gel of Diagnostic Digest of Candidate pKR184 Minipreps

7. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
8. Set up gel rig to cast gel, with ladder.
9. Add 6 uL of Sbyr Safe dye to gel rig, pour ~60mL of agarose gel, use ladder to mix, then replace ladder and allow to set.
10. Turn gel, add used TAE, remove ladder.
11. Loaded 10 uL ladder, and 36 uL of each sample.
12. Ran for 45 minutes at 113V.



I nanodrop'd the other three PCR reactions I had from my candidate pKR168 minipreps from yeast in preparation for sequencing:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR168 1.1	57.1	1.143	0.639	1.79	1.86
pKR168 2.2	20.9	0.418	0.215	1.94	1.64
pKR168 2.3	46.1	0.923	0.507	1.82	1.96

Additionally, I nanodrop'd my candidate pKR184 minipreps from *E. coli* in preparation for sequencing:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR184 1	482.1	9.643	5.112	1.89	2.33
Cand. pKR184 2	521.7	10.434	5.520	1.89	2.36
Cand. pKR184 3	465.3	9.305	4.887	1.90	2.32
Cand. pKR184 4	484.8	9.696	5.151	1.88	2.25

Tuesday, March 28, 2023

To Do:

1. ~~Set up candidate pKR168 PCR for sequencing~~
2. ~~Set up pKR184 for sequencing~~
3. ~~gDNA prep of candidate d2 strain~~
4. ~~Dilute gDNA to 100 ng/uL~~
5. ~~Make personal glycerol stocks of KRLVS126 and KRLVS127~~

Results and Data:

I set up my candidate pKR168 and pKR184 for sequencing. I followed the same PC sequencing set-up I have been using for pKR168. For pKR184, I used two primers to cover the regulatory elements and the GFP, in the plasmid:

Sample	Type	Template	Primer	Template (bp)	[Template] (ng/uL)	PCR ng/uL Needed	PCR (uL)	Plasmid (uL)	Water (uL)
SS1	Plasmid	Cand. pKR184 1	KROL257	5672	482.1			0.83	8.61
SS2	Plasmid	Cand. pKR184 2	KROL257	5672	521.7			0.77	8.67
SS3	Plasmid	Cand. pKR184 3	KROL257	5672	465.3			0.86	8.58
SS4	Plasmid	Cand. pKR184 4	KROL257	5672	484.8			0.83	8.61
SS5	Plasmid	Cand. pKR184 1	KROL362	5672	482.1			0.83	8.61
SS6	Plasmid	Cand. pKR184 2	KROL362	5672	521.7			0.77	8.67
SS7	Plasmid	Cand. pKR184 3	KROL362	5672	465.3			0.86	8.58
SS8	Plasmid	Cand. pKR184 4	KROL362	5672	484.8			0.83	8.61
SS9	PCR	Cand. pKR168 1.1	KROL257	633	57.1	15.83	0.28		9.16
SS10	PCR	Cand. pKR168 2.2	KROL257	633	20.9	15.83	0.76		8.68
SS11	PCR	Cand. pKR168 2.3	KROL257	633	46.1	15.83	0.34		9.10
a.Add 2.56 µl of 2.5 µM stock to each reaction									

gDNA Prep of Cand. pKR168 Integrant

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of candidate pKR168 integrant into d2 and resuspended in MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.

10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 35uL of 0.1x EB.

Making Personal Glycerol Stocks and Single Use Aliquots of KRLVS150 and KRLVS151

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 1200uL, mix by pipetting
5. Aliquot 1 mL to cryovial and 50ul of solution to tubes, freeze at -80

Wednesday, March 29, 2023

To Do:

1. ~~Patch out KRLVS126 and KRLVS127~~
2. ~~PCR of candidate d2 strain gDNA~~
3. ~~PCR purification of candidate pKR168 integration~~
4. ~~Run gel of PCR~~
5. ~~Check sequencing results~~

Results and Data:

PCR of Candidate pKR168 Integrations for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	Tn7 P*rpsU2 tul4*UTR	Cand.3 gDNA	KROL 252, KROL253	5000
2	Tn7 LVS	LVS	KROL 252, KROL253	350
3	- control	-	KROL 252, KROL253	-

2. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL252, KROL253 (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 19.6 uL of master-mix to negative control PCR tube
9. Add 19.6 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	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	2.4
oligo R	10 uM	0.3 uM	0.6	2.4
template	40.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
		Total volume	20	78.4

I checked my sequencing results and the additional three pKR168 plasmids look good. There might be a gap in 2.2, though, that's near the end of the sequence, so I'm not sure if I entirely trust it. So, I will move forward with the other two first.

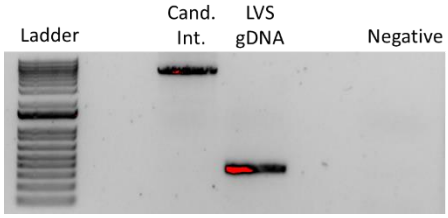
Additionally, the sequences for pKR184 look good. There is a bit of a discrepancy between the two sequencing reactions, potential insertions appearing on the sequencing that begins earlier, which makes me trust the second reaction more for those later sequences, but I double checked with Kathryn to be sure. She confirmed my thoughts so I they're all good(:

PCR Purification of Candidate pKR168 Integration into d2 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 Integration into d2 PCR

1. Melt agarose gel until completely dissolved, then place in 56°C water bath until cool enough.
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.



Thursday, March 30, 2023

To Do:

1. ~~Set up sequencing of candidate pKR168 integration PCR~~
2. ~~Make electrocompetent KRLVS126 and KRLVS127~~
3. ~~Electroporate pKR184 into KRLVS127~~
4. ~~Make 10% sucrose~~

Results and Data:

I nanodrop'd the PCR of my potential pKR168 Tn7 integration mutant in d2:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Δ2 pKR168 Tn7	51.2	1.025	0.544	1.89	4.01

Preparing Electrocompetent KRLVS126 and KRLVS127 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

Electroporating pKR184 into EC KRLVS127 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 4-8 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
   a. Plated 20 uL, 200 uL, and remaining of each
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	d2+pKR184	KRLVS127	pKR184	3 uL	20 uL. 200 uL, Rem.	3
2	(-) control	KRLVS127	-	-	200 uL	1
					Total:	4

Reagents

10% Sucrose

Dissolve 25g sucrose in a total of 250mL of ddiH2O (type 1)
Filter sterilize

Friday, March 31, 2023

To Do:

1. ~~Electroporate pKR184 into KRLVS126~~

Results and Data:

Electroporating pKR184 into EC KRLVS126 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 4-8 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
   a. Plated 20 uL, 200 uL, and remaining of each
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	d2+pKR184	KRLVS126	pKR184	3 uL	20 uL. 200 uL, Rem.	3
2	(-) control	KRLVS126	-	-	200 uL	1
					Total:	4

FTL_1362 rpsU3

FTL_0456 rpsU1

FTL_1047 rpsU2

April 2023

Wednesday, April 5, 2023

To Do:

1. ~~Restriction digest of pKR184 and pF~~
2. ~~Run gel of restriction digest~~
3. ~~Order primers and qPCR primers~~

Results and Data:

DNA Digest of pF Backbone and pKR184 Insert w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR184	KpnI, BamHI	-	15
2	pF	KpnI, BamHI	5	10

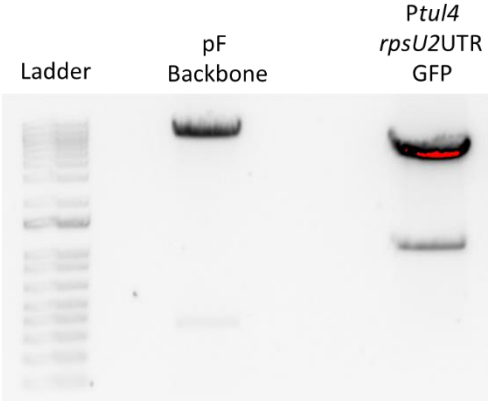
2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in 3x Master Mix (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(5.0)	-
KpnI	0.6	1.8
BamH1	0.6	1.8
Total	30.0 (25.0 actual b/c of DNA)	75.0

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

Gel of Digested pF Backbone + pKR184 Insert

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6 uL of Sbyr Safe dye to gel rig, pour ~60mL 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 36 uL of each sample.
6. Ran for 45 minutes at 113V.



Visualizing and Cutting Gel

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

Thursday, April 6, 2023

To Do:

1. ~~Make 2.5% iron pyrophosphate~~
2. ~~Gel purification of restriction digest~~
3. ~~Colony PCR from patch quadrant of potential pKR184 integrants~~
4. ~~Run gel of colony PCR~~
5. ~~Freeze down promising first quadrant patches based on colony PCR~~

Results and Data:

Colony PCR of Candidate pKR184 Integration into LVS and d*rpsU2*

1. Take a small amount of each patch and resuspend in separate aliquots of 50 µL sterile water using a sterile toothpick.
2. Heat samples at 95°C for 10' to lyse and kill cells
3. Dilute lysates 1:10
4. Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation.
5. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL326, KROL402	-
2	LVS gDNA	LVS gDNA	KROL326, KROL402	-
3-6	P*tul_ rpsU2*UTR *gfp*	Lysate	KROL326, KROL402	995
7-10	d2 P*tul_ rpsU2*UTR *gfp*	Lysate	KROL326, KROL402	995

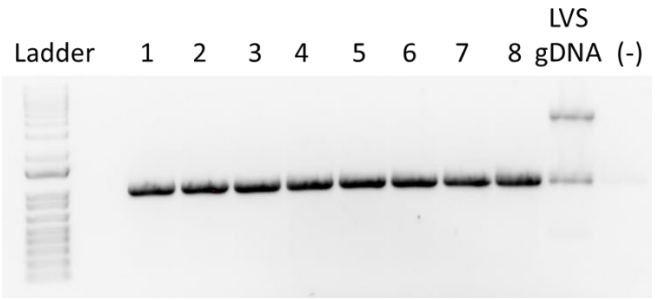
6. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL472, KROL319 (10uM), LVS gDNA, LVS lysate, and candidate pKR168 integrant lysates
7. Vortex each component (aside from enzyme)
8. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
9. Add ddi H2O to negative control tube (template volume for 1 reaction)
10. Prepare a master-containing:
    - mgH2O, dNTPs, Primestar buffer, and Primestar enzyme
11. Mix the master-mix solution by pipetting up and down
12. Add 19.0 uL of master-mix to negative control PCR tube
13. Add 19.0 uL of master mix to each PCR tube and pipet up and down to mix
14. Place the PCR Tubes in the thermocycler according to the following program:
    a. 94°C 2'
    b. 94°C 20"
    c. 50°C 30"
    d. 68°C (1' per kb)
    e. Go to step 2, rep 32x
    f. 68°C 5'
    g. Hold 12°C

Total reaction volume	20
Total number of reactions	10

Component	Stock concentration	Final concentration	1 rxn volume	Factor 11
ddiH2O			11.8	129.8
PrimeSTAR GXL Buffer	5x	1x	4	44
dNTPs	2.5 mM	0.2 mM	1.6	17.6
oligo F	10 uM	0.3 uM	0.6	6.6
oligo R	10 uM	0.3 uM	0.6	6.6
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	4.4
Cell lysate	-	-	1	
		Total volume	20	209

Gel of Colony PCR of Candidate pKR184 Integrations into LVS and d*2*

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enouh..
2. Set up large gel rig to cast gel, with ladder.
3. Add 12 uL of Sbyr Safe dye to gel rig, pour ~120mL 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 15 uL of each sample.
6. Ran for 45 minutes at 113V.



The ladder is difficult to see, however, I was looking for a 3000-ish bp band and it looks like the bands are in the 3000/4000 range, so I think it's good.

Gel Extraction with QIAquick Gel Extraction Kit

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

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)

P*tul4 rpsU2* 5'UTR GFP	298	894	298
pF Backbone	286	858	286

Making Glycerol Stocks of Potential pKR184 Integrant Strains

1. Prepare 800uL 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 200ul of 75% glycerol cryovial, then add 800 uL of resuspended cells
5. Vortex until mixed and minifuge, freeze at -80

Sample #	Description
1	LVS + pKR184
2	LVS + pKR184
3	LVS + pKR184
4	LVS + pKR184
5	Δ*rpsU2* + pKR184
6	Δ*rpsU2* + pKR184
7	Δ*rpsU2* + pKR184
8	Δ*rpsU2* + pKR184

Monday, April 17, 2023

To Do:

1. ~~Start dishwasher~~
2. ~~Electroporate pKR168 into KRLVS126 (LVS)~~
3. ~~Ligate gel purification~~
4. ~~Transform ligation of pF+Ptul4 rpsU2UTR GFP into *E. coli*~~
5. ~~Receive and dilute primers~~
6. ~~Run PCR for rpsU2UTR mutant 1~~

Results and Data:

Electroporating pKR168 into EC KRLVS126 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:
   5 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 4-8 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
   a. Plated 20 uL, 200 uL, and remaining
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	pKR168 1.1	5 uL	20 uL. 200 uL, Rem.	3
2	(-) control	KRLVS126	-	-	200 uL	1
					Total:	4

Ligation of Digested pKR184 Insert with Digested pF Backbone

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

Tube	Insert	Backbone
1	P*tul4 rpsU2* UTR GFP (pKR184)	KpnI, BamHI digested, purified pF
2	-	KpnI, BamHI digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)
H2O	11.5	11.5
10x ligase buffer	2.0	2.0
Insert	4.0	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

3. Obtain ice to assemble and keep the reactions on.
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, 10x buffer, insert, and backbone.
6. Add indicated amount of ligase to the individual tubes.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Left on bench at room temperature for 10 minutes.

Transformation of pKR196 Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. Use 8 uL per ligation and plate 10 uL and 100 uL.
    b. Positive control: pF; Backbone ligation: KpnI-BamHI digested pKR184

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(-) control	None	0	200 ul	1
2	Backbone Ligation	BB only ligation	8 uL	10 uL, 100 ul	2
3	pKR196 Ligation	pKR196 Ligation	8 uL	10 uL, 100 ul	2
				Total number of plates	5

2. Check to be sure you have enough LB-Kan plates. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

Receiving pKR184/pKR196 Mut. 1 Primer

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
    a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in "Oligos Spec Sheet" binder and shipping sheet in Invoices box.

PCR of Mutant 1 Fragment from pKR184

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	P*tul4 rpsU2*UTR Mut. 1	pKR184	KROL 635, KROL560	658
2	- control	-	KROL 635, KROL560	-

2. Acquire the following components and put them on ice, labeling tubes if necessary:

- mgH2O, Primestar buffer, dNTPs, KROL252, KROL253 (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 19.6 uL of master-mix to negative control PCR tube
9. Add 19.6 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	100
Total number of reactions	2

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			62.0	186
PrimeSTAR GXL Buffer	5x	1x	20.0	60
dNTPs	2.5 mM	0.2 mM	8.0	24
oligo F	10 uM	0.3 uM	3.0	9
oligo R	10 uM	0.3 uM	3.0	9
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	6
		Total volume	100	294

Tuesday, April 18, 2023

To Do:

1. ~~Patch out promising frozen stocks of pKR184 integrants~~
2. ~~PCR purify PtuI4 rpsU2UTR stem loop deletion PCR~~
3. ~~Restriction digest of backbone and *rpsU2* UTR mutant~~
4. ~~Run gel of restriction digest and excise bands~~
5. ~~Gel purification of restriction digest~~
6. ~~Ligation of gel purification of restriction digest~~

Results and Data:

PCR Purification of *rpsU2* UTR Stem Loop Deletion PCR

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

DNA Digest of *rpsU2* Mut. 1 PCR and pKR184 Backbone w/PacI and MfeI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	*rpsU2* UTR Mut. 1 PCR	PacI, MfeI	15	-
2	pKR184	PacI, MfeI	5	10

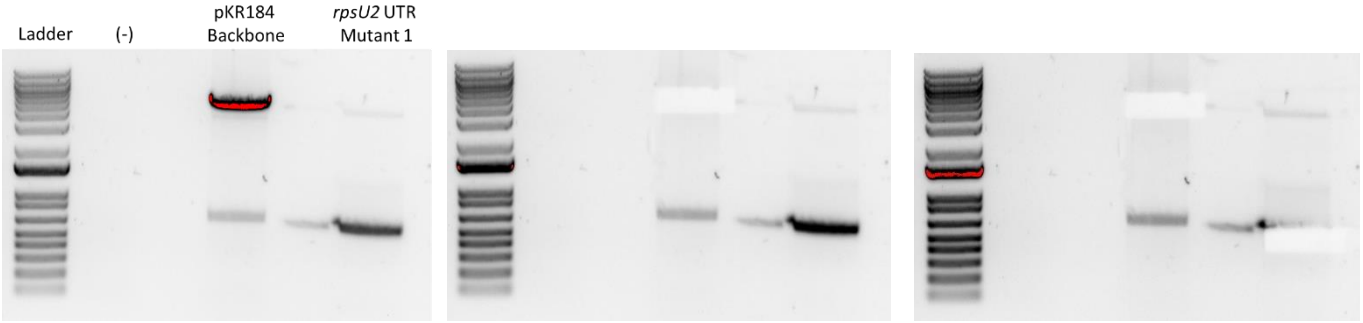
2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**	**Volume in 3x Master Mix (uL)**
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Not1	0.6	1.8
Kpn1	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	45 (15 uL for each reaction)

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

Gel of Digested *rpsU2* UTR Mutant Insert and pKR184 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe dye to gel rig, pour ~120mL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.



Expecting band sizes of ~5600 for the backbone and ~650 for the insert. Which looks like what I got to me. I cut it accordingly. I missed some of the insert, however, given that there was some spillover which could have either been insert or the unwanted insert from the backbone, I did not go back to cut out more. That said, my insert looks like it's a bit smaller than the region we are removing from the backbone, which is good, since it should be about ~50 base pairs smaller. The stain blob was not visible in the uncropped gel picture, but it was there in real life, so I know the negative ran and is just clean.

Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen

2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube. Stored gel pieces in fridge overnight.

Gel Extraction with QIAquick Gel Extraction Kit

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

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
rpsU2 5'UTR Mut. 1	265	795	265
pKR184 Backbone	329	987	329

Additionally, I checked my transformation plates from yesterday and did not see any growth. The cells I started with seemed a little light, so hopefully that was the issue. I will do an overnight ligation along with my pKR191 ligation and re-transform tomorrow. Additionally, I placed my plates back into the incubator in case there is growth tomorrow.

Ligation of Digested Mutant 1 + pKR184 Backbone and pKR184 Insert + pF Backbone

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

Tube	Insert	Backbone
1	*rpsU2* UTR Mut. 1 PCR	PacI, MfeI digested, purified pKR184
2	-	PacI, MfeI digested, purified pKR184
3	P*tul4 rpsU2* UTR GFP (pKR184)	KpnI, BamHI digested, purified pF
4	-	KpnI, BamHI digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	4.5 x Master Mix (uL)
H2O	11.5	51.75
10x ligase buffer	2.0	9.0
Insert	4.0	-
Backbone	2.0	-
Ligase	0.5	2.25
TOTAL	20.0	63

3. Obtain ice to assemble and keep the reactions on the ice.
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, 10x buffer, insert, and backbone.
6. Add indicated amount of ligase to the individual tubes.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Incubated at 16°C overnight.

Wednesday, April 19, 2023

To Do:

1. ~~Transform pKR191 ligation into *E. coli*~~
2. ~~Transform pKR196 ligation into *E. coli*~~
3. ~~gDNA prep of potential pKR184 integrants~~
4. ~~PCR of gDNA of potential pKR184 integrants~~

Results and Data:

Transformation of pKR191 and pKR196 Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. Use 8 uL per ligation and plate 100 uL and remaining.

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(-) control	None	0	100 ul	1
2	Backbone Ligation	BB only ligation	8 uL	100 uL, remaining	2
3	pKR196 Ligation	pKR196 Ligation	8 uL	100 uL, remaining	2
4	Backbone Ligation	BB only ligation	8 uL	100 uL, remaining	2
5	pKR191 Ligation	pKR196 Ligation	8 uL	100 uL, remaining	2
				Total number of plates	9

2. Check to be sure you have enough LB-Kan plates. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

gDNA Prep of Cand. pKR184 Integrants in LVS and Δ*rpsU2*

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of candidate pKR184 integrant into WT/d2 and resuspended in MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.

11. Pellet debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 70uL of 0.1x EB.

Then I nanodrop'd my gDNA samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR184+WT Cand. 1	1289.2	25.783	13.282	1.94	1.36
pKR184+Δ2 Cand. 5	1279.3	25.585	13.310	1.92	1.39

I diluted both 1:10, with 10 uL of gDNA in 90 uL of 0.1xEB.

PCR of Candidate pKR184 Integrations for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	LVS P*tul4 rpsU2*UTR GFP	Cand 1 gDNA	KROL 252, KROL253	3000
2	P*tul4 rpsU2*UTR GFP	Cand 5 gDNA	KROL 252, KROL253	3000
3	Tn7 LVS	LVS	KROL 252, KROL253	350
4	- control	-	KROL 252, KROL253	-

2. Acquire the following components and put them on ice, labeling tubes if necessary:
   - mgH2O, Primestar buffer, dNTPs, KROL252, KROL253 (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 19.6 uL of master-mix to negative control PCR tube
9. Add 19.6 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	4

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	5
ddiH2O			12.4	62
PrimeSTAR GXL Buffer	5x	1x	4.0	20
dNTPs	2.5 mM	0.2 mM	1.6	8

oligo F	10 uM	0.3 uM	0.6	3
oligo R	10 uM	0.3 uM	0.6	3
template	40.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
		Total volume	20	98

Thursday, April 20, 2023

To Do:

1. ~~PCR purification of pKR184 integrate gDNA PCR~~
2. ~~Run gel of PCR purification~~
3. ~~Submit potential pKR184 integrant candidates for sequencing~~
4. ~~Primer efficiencies of GFP qPCR primers~~
5. ~~Make *E. coli* overnights~~

Results and Data:

PCR Purification of pKR184 Integrants gDNA PCR for Sequencing

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

Next, I nanodrop'd my PCR samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR184+WT Cand. 1	63.1	1.263	0.632	2.00	1.74
pKR184+d2 Cand. 5	61.2	1.225	0.633	1.94	1.72

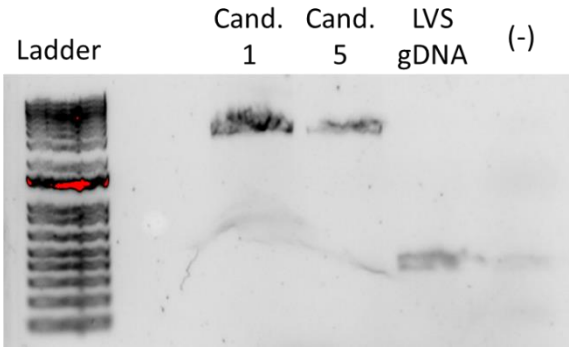
And set them up for sequencing:

Sample	Template	Template Name	Primer	Template (bp)	Template (ng/uL)	PCR (ng)	PCR (uL)	H2O (uL)
SS1	PCR	pKR184+WT Cand. 1	KROL252	3000	63.1	75.00	1.19	8.25
SS2	PCR	pKR184+WT Cand. 1	KROL253	3000	63.1	75.00	1.19	8.25
SS3	PCR	pKR184+WT Cand. 1	KROL257	3000	63.1	75.00	1.19	8.25
SS4	PCR	pKR184+WT Cand. 1	KROL362	3000	63.1	75.00	1.19	8.25
SS5	PCR	pKR184+d2 Cand. 5	KROL252	3000	61.2	75.00	1.23	8.21
SS6	PCR	pKR184+d2 Cand. 5	KROL253	3000	61.2	75.00	1.23	8.21
SS7	PCR	pKR184+d2 Cand. 5	KROL257	3000	61.2	75.00	1.23	8.21
SS8	PCR	pKR184+d2 Cand. 5	KROL362	3000	61.2	75.00	1.23	8.21
a.Add 2.56 µl of 2.5 µM stock to each reaction								

Gel of Potential pKR184 Integrants into WT and d2 gDNA PCR for Sequencing

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe dye to gel rig, pour ~120mL of agarose gel, use ladder to mix, then replace ladder and allow to set.

4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.



There's obviously a bit of contamination in the negative, however, I was loading pretty quickly and this band isn't in any of the other lanes, so I'm pretty sure it was just carryover from LVS. The band size I was expecting is around 3000 bp. It looks like its around that range (10 bands from the bottom of the ladder), though there's not great resolution since I didn't have time to run it as long as I should have. Regardless, I already saw the proper bands in the colony PCR, this gel was just for checksies.

Additionally, I used the 2nd replicate of LVS cDNA from my second rep of my *rpsU1*/*rpsU3* autoregulation experiment to make the serially diluted cDNA for my primer efficiencies. I made enough for 4 reactions today, GFP plus *tul4* control. And then a separate tube for 7 reactions for tomorrow. Ergo, *secG*, *rpsO*, and the *tul4* control. As follows:

1.5 ng/uL qPCR Stock for Primer Efficiency			
Sample Name	**Conc. (ng/uL)**	**DNA uL**	**EB uL**
LVS 2	41.7	2.88	77.12
LVS 2	41.7	5.04	134.96

Real-Time Primer Efficiency Test of GFP qRT-PCR Primers for cDNA

1. Dilute cDNA to 1.5 ng/uL and make 10-fold serial dilutions (1.5ng/ul, 0.15ng/ul, 0.015ng/ul, 0.0015ng/ul)
2. Add 17.5ul of each DNA sample into strip tubes, for each primer set
3. Set up master mixes for each primer set:

10 ul of SYBR green	x 17.5	= 175
1 ul of 5uM primer set	x 17.5	= 17.5
4 ul ddH2O	x 17.5	= 70
	TOTAL =	262.5

4. Add 52.5 uL of accordant primer master mix to each strip tube
5. Using multi-channel pipette, transfer 20 uL of each primer set + diluted cDNA to 96-well plate in triplicate
6. Place adhesive film onto plate and centrifuge 3 minutes at 500xg to draw all liquid to the bottom
7. Place in light cycler and run the following program:

Old StepOne Plus (2-step amplification)

95°C 10'
95°C 15"
60°C 60"
Go to step 2, 39x (total 40 cycles)

95°C 10”
65°C 60”
97°C 60”
Melt curve (95°C 10”, 65°C 60”, 97°C 60” with continuous ramp)

8. Plot a standard curve, as well as a melting curve. Ideally you want the calculated primer efficiency to be between 1.8 and 2.0. Ensure melting curve has only one peak.
9. Labelling was as follows:

Master Mix Label	Primer Set
A	KROL431/432 GFP
B	KROL433/434 GFP
C	KROL435/436 GFP
D	KROL63/64 *tul4*

1	2	3	4
1.5 ng/uL	0.15 ng/uL	0.015 ng/uL	0.0015 ng/uL

10. Loaded 96-well plate according to the following table:

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

I started to do the standard curves and melting curves, however, I didn’t really have standard curves fro any of my GFP samples. My *tul4* looked good, which is when it dawned on me that LVS does not have GFP.

Then, because Aisling and I both had LVS on our negatives, and Kathryn saw a patch of a negative control grow on Kan, we thought the kanamycin plates might not be good. As such, I’m goanna hold off on streaking out potential integrant colonies since there may have been no pressure. Additionally, I am patching out LVS on a made kan plate, a CHAH plate with added kanamycin, and a regular CHAH plate to diagnose if there is an issue.

Friday, April 21, 2023

To Do:

1. ~~Pellet overnights~~
2. ~~Put away dishes~~
3. ~~Check sequencing results~~
4. ~~Patch out potential integrants of pKR168 into LVS~~

Results and Data:

I checked my sequencing results, and although KROL257 did not have signal for either of my candidates, I had enough coverage otherwise. Both candidate 1 and 5 integrated successful, so pKR184 is in both WT and d2!

Miniprep of Candidate pKR196 from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.

2. Placed pellets removed of supernatant in the -20.

Satrurday, April 22, 2023

To Do:

1. ~~Colony PCR of first quadrant from streaks of potential integrants~~
2. ~~Receive and dilute primers~~
3. ~~Primer efficiencies of *secG* and *rpsO* qPCR primers~~
4. ~~Miniprep *E. coli* overnights~~
5. ~~Run gel of colony PCR~~

Results and Data:

Colony PCR of Candidate pKR168 Integrations into LVS

1. Take a small amount of each patch and resuspend in separate aliquots of 50 µL sterile water using a sterile toothpick.
2. Heat samples at 95°C for 10’ to lyse and kill cells
3. Dilute lysates 1:10
4. Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation.
5. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL472, KROL319	-
2	LVS gDNA	LVS gDNA	KROL472, KROL319	-
3-10	P*rpsU2 tul4*UTR *lacZ*	Lysate	KROL472, KROL319	3328

6. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL472, KROL319 (10uM), LVS gDNA, LVS lysate, and candidate pKR168 integrant lysates
7. Vortex each component (aside from enzyme)
8. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
9. Add ddi H2O to negative control tube (template volume for 1 reaction)
10. Prepare a master-containing:
    - mgH2O, dNTPs, Primestar buffer, and Primestar enzyme
11. Mix the master-mix solution by pipetting up and down
12. Add 19.0 uL of master-mix to negative control PCR tube
13. Add 19.0 uL of master mix to each PCR tube and pipet up and down to mix
14. Place the PCR Tubes in the thermocycler according to the following program:
    a. 94°C 2'
    b. 94°C 20"
    c. 50°C 30"
    d. 68°C (1' per kb)
    e. Go to step 2, rep 32x
    f. 68°C 5'
    g. Hold 12°C

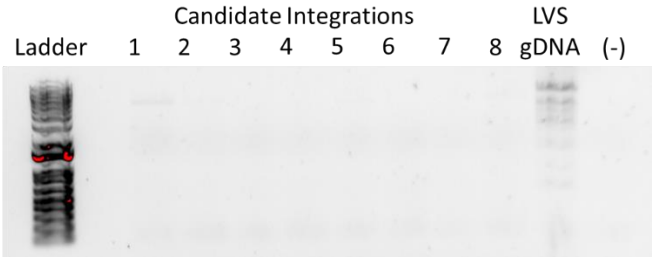
Total reaction volume	20
Total number of reactions	10

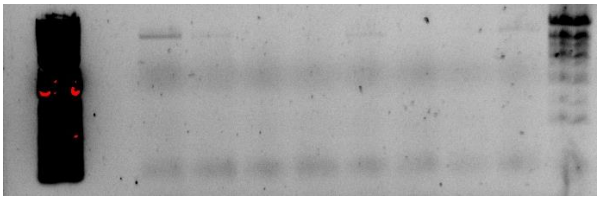
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	11

ddiH2O			11.8	129.8
PrimeSTAR GXL Buffer	5x	1x	4	44
dNTPs	2.5 mM	0.2 mM	1.6	17.6
oligo F	10 uM	0.3 uM	0.6	6.6
oligo R	10 uM	0.3 uM	0.6	6.6
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	4.4
Cell lysate	-	-	1	
		Total volume	20	209

Gel of Colony PCR of Candidate pKR168 Integrations into LVS

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enouh..
2. Set up large gel rig to cast gel, with ladder.
3. Add 12 uL of Sbyr Safe dye to gel rig, pour ~60mL 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 15 uL of each sample.
6. Ran for 45 minutes at 113V.





*4 and 5 are switched. I was expecting a band about ~3000 bp. My lysates seemed a little less dense than usual, so I decreased the brightness and you can see some bands in this range potentially. There's not really the greatest resolution since I didn't run it quite long enough. Regardless, I'll gDNA prep 1 and 8, and save down 1, 2, 4, and 8 to check if I don't see what I want from the gDNA PCR.

Receiving RNA Stability Control qPCR Primers

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
    a. If qPCR primer pairs, make a combined 5 uM stock by diluting 15 uL of the 100 uM stock each of the forward and reverse qPCR primer in 270 uL of 0.1x EB. Label with red sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in "Oligos Spec Sheet" binder and shipping sheet in Invoices box.

Real-Time Primer Efficiency Test of *secG* and *rpsO* qRT-PCR Primers for cDNA

1. Dilute cDNA to 1.5 ng/uL and make 10-fold serial dilutions (1.5ng/ul, 0.15ng/ul, 0.015ng/ul, 0.0015ng/ul)
2. Add 17.5ul of each DNA sample into strip tubes, for each primer set
3. Set up master mixes for each primer set:

10 ul of SYBR green	x 17.5	= 175
1 ul of 5uM primer set	x 17.5	= 17.5
4 ul ddH2O	x 17.5	= 70
	TOTAL =	262.5

4. Add 52.5 uL of accordant primer master mix to each strip tube
5. Using multi-channel pipette, transfer 20 uL of each sample to 96-well plate in triplicate
6. Place adhesive film onto plate and centrifuge 3 minutes at 500xg to draw all liquid to the bottom
7. Place in light cycler and run the following program:
   Old StepOne Plus (2-step amplification)
   95°C 10’
   95°C 15”
   60°C 60”
   Go to step 2, 39x (total 40 cycles)
   95°C 10”
   65°C 60”
   97°C 60”
   Melt curve (95°C 10”, 65°C 60”, 97°C 60” with continuous ramp)
8. Plot a standard curve, as well as a melting curve. Ideally you want the calculated primer efficiency to be between 1.8 and 2.0. Ensure melting curve has only one peak.
9. Labelling was as follows:

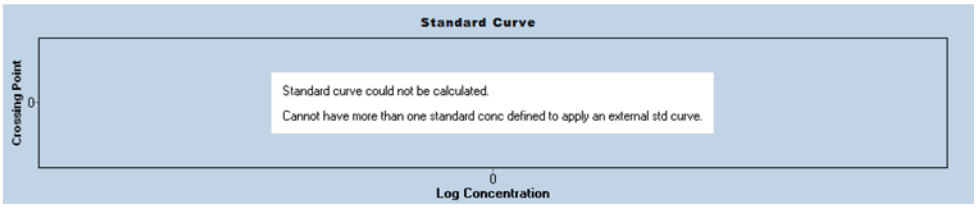
Master Mix Label	Primer Set
A	KROL640/641 *secG*
B	KROL642/643 *secG*
C	KROL644/645 *secG*
D	KROL646/647 *rpsO*
E	KROL648/649 *rpsO*
F	KROL650/651 *rpsO*
G	KROL63/64 *tul4*

1	2	3	4
1.5 ng/uL	0.15 ng/uL	0.015 ng/uL	0.0015 ng/uL

10. Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			C1			E1			G1		
B	A2			C2			E2			G2		
C	A3			C3			E3			G3		
D	A4			C4			E4			G4		
E	B1			D1			F1					
F	B2			D2			F2					
G	B3			D3			F3					
H	B4			D4			F4					

A.



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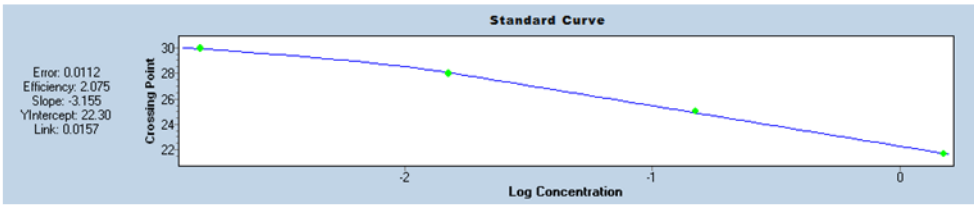

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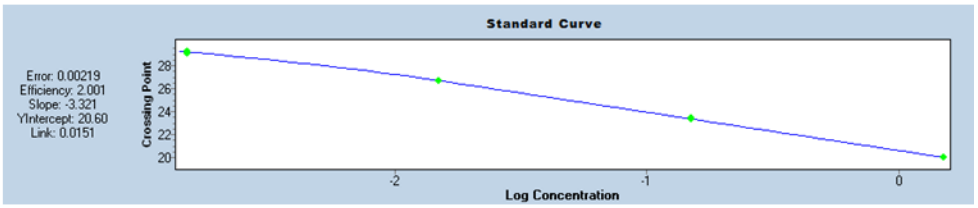

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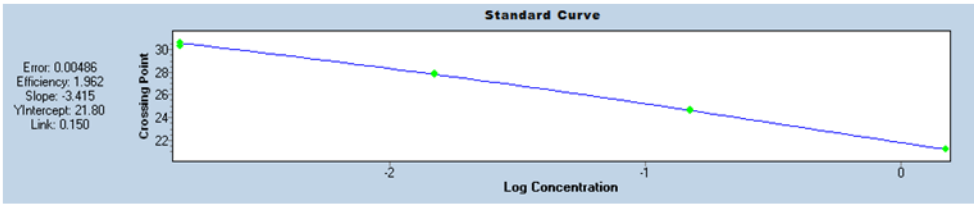
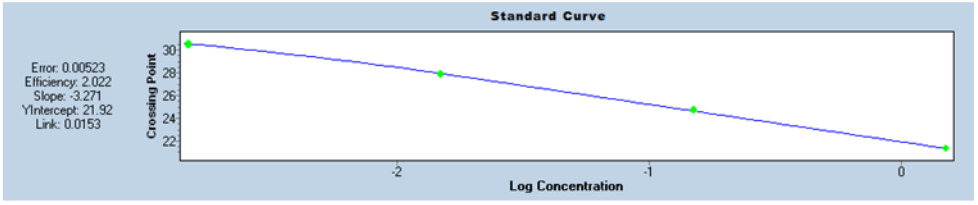
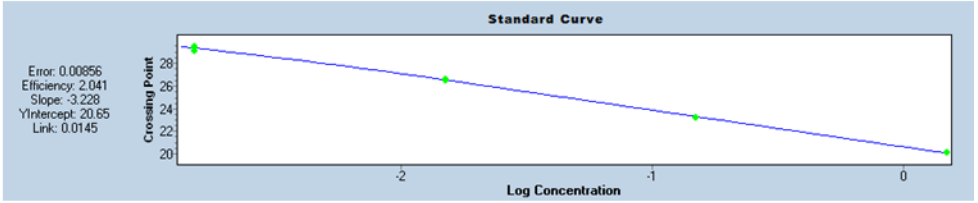


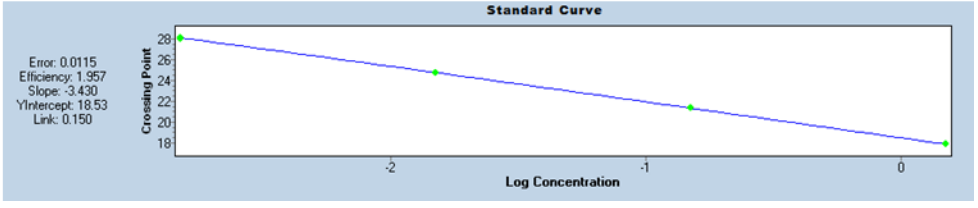
E.



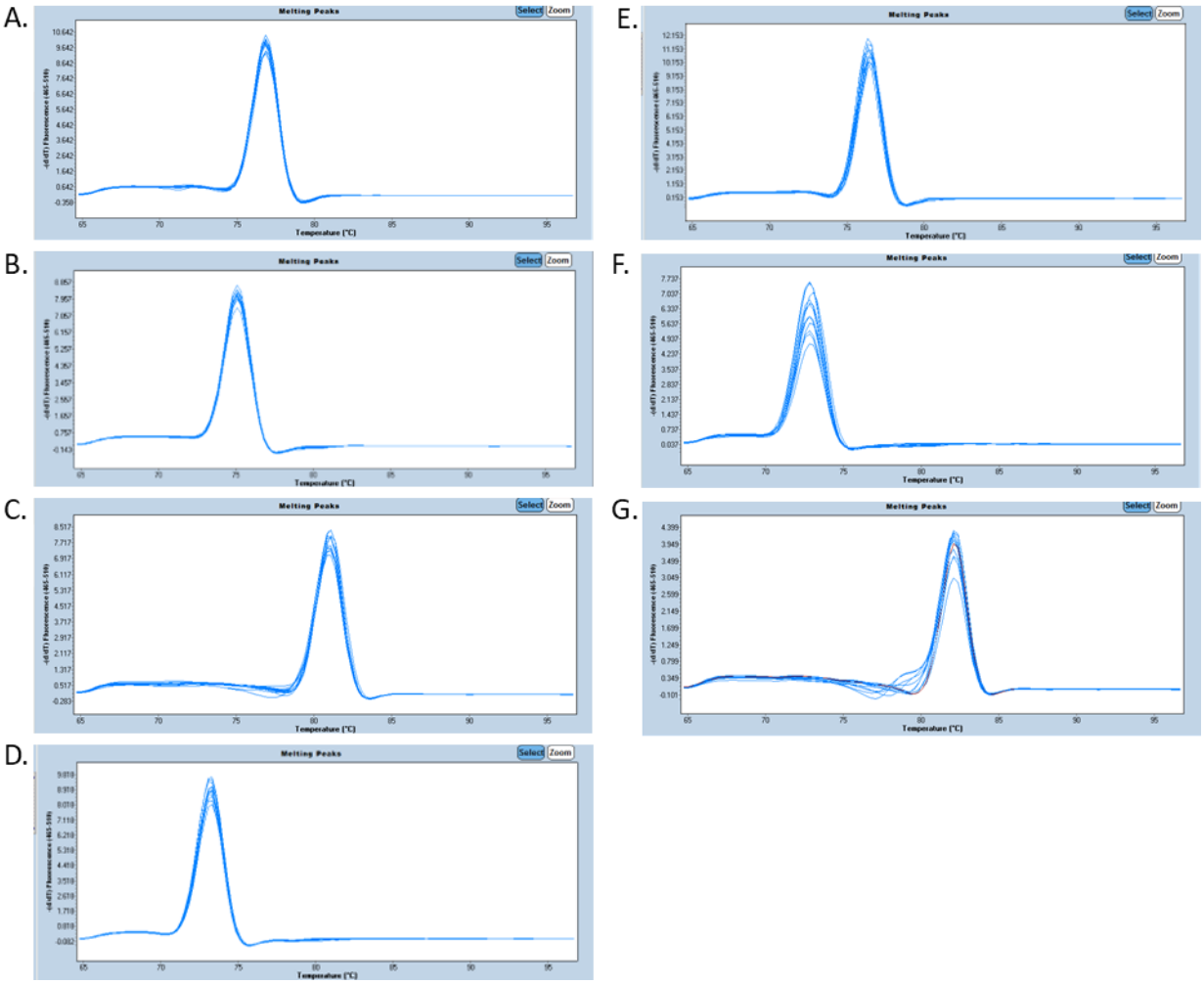
F.



G.



They all look good except for KROL640+641 *secG*, since I couldn't get it to generate a standard curve. The melting curves all look good too, with only one peak.



Miniprep of Candidate pKR196 from *E. coli*

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

Monday, April 24, 2023

To Do:

1. ~~gDNA prep of potential WT + pKR168 integrants~~
2. ~~Run PCR of potential WT + pKR168 integrants~~
3. ~~Run gel of potential WT + pKR168 integrants~~
4. ~~Freeze down promising strains based on colony PCR~~
5. ~~Set up pKR191 and pKR196 for sequencing~~
6. ~~Set up potential pKR168 integration into WT for sequencing~~

Results and Data:

gDNA Prep of Cand. pKR168 Integrants in LVS

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of candidate pKR184 integrant into WT/d2 and resuspended in MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 70uL of 0.1x EB.

Then I nanodrop'd my gDNA samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR168 + WT Cand. 1	1043.1	20.862	11.244	1.86	1.45
pKR168 + WT Cand. 8	1547.5	30.951	16.754	1.85	1.30

I diluted them to ~100 ng/uL by diluting 10 uL of Candidate 1 in 90 uL 0.1xEB. Then, 6 uL of Candidate 8 in 94 uL of 0.1xEB.

PCR of Candidate pKR168 Integrations for Sequencing

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	Tn7 P*rpsU2 tul4*UTR	Cand.1 gDNA	KROL 252, KROL253	5000
2	Tn7 P*rpsU2 tul4*UTR	Cand.8 gDNA	KROL 252, KROL253	5000
3	Tn7 LVS	LVS	KROL 252, KROL253	350
4	- control	-	KROL 252, KROL253	-

2. Add ddi H2O to negative control tube (template volume for 1 reaction)
3. Prepare master-mix
4. Add 19.6 uL of master mix to each PCR tube and pipet up and down to mix
5. Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	4

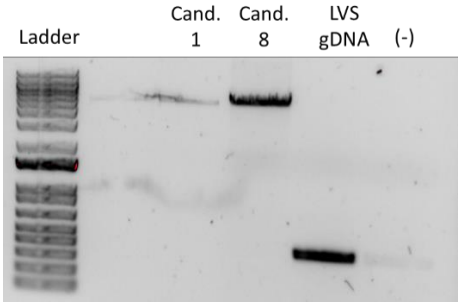
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	5
ddiH2O			12.4	62
PrimeSTAR GXL Buffer	5x	1x	4.0	20
dNTPs	2.5 mM	0.2 mM	1.6	8
oligo F	10 uM	0.3 uM	0.6	3
oligo R	10 uM	0.3 uM	0.6	3
template	40.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
		Total volume	20	98

PCR Purification of Candidate pKR168 Integration into d2 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 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Gel of Candidate pKR168 Integration into d2 PCR

1. Melt agarose gel until completely dissolved, then place in 56°C water bath until cool enough.
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 was expecting a ~5000 bp band if the integration had occurred, and a ~300 bp if not, so it looks good.

Making Glycerol Stocks of Potential pKR168 Integrants into LVS

1. Prepare 800uL 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 200ul of 75% glycerol cryovial, then add 800 uL of resuspended cells
5. Vortex until mixed and minifuge, freeze at -80

Then I nanodrop'd my minipreps from Saturday, pKR191 candidate minipreps:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR191 Cand. 1	466.3	9.327	4.956	1.88	2.35
pKR191 Cand. 2	497.5	9.950	5.320	1.87	2.33
pKR191 Cand. 3	477.0	9.541	5.102	1.87	2.33
pKR191 Cand. 4	405.0	8.101	4.318	1.88	2.33

Candidate pKR196 minipreps:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR196 Cand. 1	279.0	5.581	2.965	1.88	2.40
pKR196 Cand. 2	226.1	4.522	2.414	1.87	2.40
pKR196 Cand. 3	226.5	4.531	2.441	1.86	2.30
pKR196 Cand. 4	228.9	4.579	2.448	1.87	2.39

And, finally, my candidate pKR168 integration into WT gDNA PCR:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR168 + WT Cand. 1	71.0	1.420	0.771	1.84	2.20
pKR168 + WT Cand. 8	65.0	1.300	0.678	1.92	2.20

Then I set-up sequencing:

Sample	Type	Template	Primer	Size (bp)	[Stock] ng/uL	PCR (ng)	PCR (uL)	Plasmid (uL)	H_2O (uL)
SS1	PCR	pKR168 Tn7 WT	KROL177	5000	65.0	125.00	1.92		7.52
SS2	PCR	pKR168 Tn7 WT	KROL178	5000	65.0	125.00	1.92		7.52
SS3	PCR	pKR168 Tn7 WT	KROL179	5000	65.0	125.00	1.92		7.52
SS4	PCR	pKR168 Tn7 WT	KROL180	5000	65.0	125.00	1.92		7.52
SS5	PCR	pKR168 Tn7 WT	KROL181	5000	65.0	125.00	1.92		7.52
SS6	PCR	pKR168 Tn7 WT	KROL182	5000	65.0	125.00	1.92		7.52
SS7	PCR	pKR168 Tn7 WT	KROL252	5000	65.0	125.00	1.92		7.52
SS8	PCR	pKR168 Tn7 WT	KROL253	5000	65.0	125.00	1.92		7.52
SS9	Plasmid	Cand. pKR191 1	KROL257	5634	466.3			0.86	8.58
SS10	Plasmid	Cand. pKR191 2	KROL257	5634	497.5			0.80	8.64
SS11	Plasmid	Cand. pKR191 3	KROL257	5634	477.0			0.84	8.60
SS12	Plasmid	Cand. pKR191 4	KROL257	5634	405.0			0.99	8.45
SS13	Plasmid	Cand. pKR191 1	KROL362	5634	466.3			0.86	8.58
SS14	Plasmid	Cand. pKR191 2	KROL362	5634	497.5			0.80	8.64
SS15	Plasmid	Cand. pKR191 3	KROL362	5634	477.0			0.84	8.60
SS16	Plasmid	Cand. pKR191 4	KROL362	5634	405.0			0.99	8.45
SS17	Plasmid	Cand. pKR196 1	KROL257	7842	279.0			1.43	8.01
SS18	Plasmid	Cand. pKR196 2	KROL257	7842	226.1			1.77	7.67
SS19	Plasmid	Cand. pKR196 3	KROL257	7842	226.5			1.77	7.67
SS20	Plasmid	Cand. pKR196 4	KROL257	7842	228.9			1.75	7.69
SS21	Plasmid	Cand. pKR196 1	KROL362	7842	279.0			1.43	8.01
SS22	Plasmid	Cand. pKR196 2	KROL362	7842	226.1			1.77	7.67
SS23	Plasmid	Cand. pKR196 3	KROL362	7842	226.5			1.77	7.67
SS24	Plasmid	Cand. pKR196 4	KROL362	7842	228.9			1.75	7.69
a.Add 2.56 µl of 2.5 µM stock to each reaction									

Tuesday, April 25, 2023

To Do:

1. ~~Patch out pKR184 integrants and LVS~~
2. ~~Patch out d2 and LVS~~
3. ~~Make 2.5% iron pyrophosphate~~
4. ~~qPCR of Stability Assay sample controls – 1~~
5. ~~Supplement MHB~~

Results and Data:

For both my *secG* and *rpsO* primer sets I used the 0.99 ng/uL stocks that I have of cDNA already made from my last run. I prepared 3 PCR strips for each primer set I will need to run to reduce freeze/thaw of my stocks. For the 23s rRNA, I used a 0.2 ng/uL dilution. I did this by diluting the 0.99 ng/uL stock in each PCR tube, as such:

0.2 ng/uL qPCR Stock			
Sample Name	**Conc.**	**DNA uL**	**EB uL**
KRLVS148 1 0'	0.99	0.71	2.79
KRLVS148 2 0'	0.99	0.71	2.79
KRLVS148 3 0'	0.99	0.71	2.79
KRLVS148 1 1'	0.99	0.71	2.79
KRLVS148 2 1'	0.99	0.71	2.79
KRLVS148 3 1'	0.99	0.71	2.79
KRLVS148 1 2'	0.99	0.71	2.79
KRLVS148 2 2'	0.99	0.71	2.79
KRLVS148 3 2'	0.99	0.71	2.79
KRLVS148 1 4'	0.99	0.71	2.79
KRLVS148 2 4'	0.99	0.71	2.79
KRLVS148 3 4'	0.99	0.71	2.79

qRT-PCR of RNA Stability Assay cDNA Samples – 1

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL642/643 and KRLVS650/651 as test primers to amplify *secG* and *rpsO*.
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	875.0 uL
5uM primer set	1 uL	87.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH_2O	8 uL	700.0 uL
Total:	20 uL	1662.5 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	KROL644/645 *secG*

1	2	3	4	5	6	7	8	9	10	11	12
148 1 0'	148 2 0'	148 3 0'	148 1 1'	148 2 1'	148 3 1'	148 1 2'	148 2 2'	148 3 2'	148 1 4'	148 2 4'	148 3 4'

13	14	15	16	17	18	19	20	21	22	23	24
149 1 0'	149 2 0'	149 3 0'	149 1 1'	149 2 1'	149 3 1'	149 1 2'	149 2 2'	149 3 2'	149 1 4'	149 2 4'	149 3 4'

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			A9			A17					
B	A2			A10			A18					
C	A3			A11			A19					
D	A4			A12			A20					
E	A5			A13			A21					
F	A6			A14			A22					
G	A7			A15			A23					
H	A8			A16			A24					

Reagents

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 ℃

Wednesday, April 26, 2023

To Do:

1. ~~Start cultures of pKR184 integrants and LVS in MHB~~
2. ~~GFP assay of pKR184 integrants and LVS in MHB~~

Results and Data:

GFP Assay on pKR184 Integrants into WT and d2

1. Pellet 1 mLs of culture and spin at max speed for 3 minutes
2. Remove all MHB, 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	Strain and BR	Label	Strain and BR
1	KRLVS184+WT 1	4	KRLVS184+d2 1
2	KRLVS184+WT 2	5	KRLVS184+d2 2
3	KRLVS184+WT 3	6	KRLVS184+d2 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	1											
B	2											
C	3											
D	4											
E	5											
F	6											
G	LVS											
H	PBS											

Thursday, April 27, 2023

To Do:

1. ~~Check sequencing results~~

Results and Data:

The WT integrant of pKR168 does not look right- somehow it got the promoter for *tul4*. pKR191 did not have the stem loop deletion- realized I PCR'd way too much DNA so I had both products in that PCR. pKR196 looked good.

Friday, April 28, 2023

To Do:

1. ~~Make electrocompetent LVS and d2~~
2. ~~Electroporate pKR196 into WT~~
3. ~~Electroporate pKR196 into d2~~
4. ~~qPCR of Stability Assay sample controls - 2~~

Results and Data:

qRT-PCR of RNA Stability Assay cDNA Samples – 2

1. Each experiment will need at least one test primer and one control primer for each sample
    a. KROL642/643 and KRLVS650/651 as test primers to amplify *secG* and *rpsO*.
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	875.0 uL
5uM primer set	1 uL	87.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH_2O	8 uL	700.0 uL
Total:	20 uL	1662.5 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	KROL650/651 *rpsO*

1	2	3	4	5	6	7	8	9	10	11	12
148 1 0'	148 2 0'	148 3 0'	148 1 1'	148 2 1'	148 3 1'	148 1 2'	148 2 2'	148 3 2'	148 1 4'	148 2 4'	148 3 4'

13	14	15	16	17	18	19	20	21	22	23	24
149 1 0'	149 2 0'	149 3 0'	149 1 1'	149 2 1'	149 3 1'	149 1 2'	149 2 2'	149 3 2'	149 1 4'	149 2 4'	149 3 4'

Loaded 96-well plate according to the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			A9			A17					
B	A2			A10			A18					
C	A3			A11			A19					
D	A4			A12			A20					
E	A5			A13			A21					
F	A6			A14			A22					
G	A7			A15			A23					
H	A8			A16			A24					

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.