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May 2023

Monday, May 1, 2023

To Do:

1. ~~PCR to confirm WT vs. d2 in pKR184 integrations (colony and gDNA) and pKR168~~
2. ~~PCR of mutant 1 from pKR184~~
3. ~~Run gel of *rpsU2* check~~
4. ~~Patch out pKR196 colonies to freeze down~~
5. ~~qPCR of Stability Assay sample controls – 3~~

Results and Data:

Colony PCR to Check *rpsU2* Status of Recent Strains

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	-	KROL601, KROL602	-
2	LVS *rpsU2*	LVS gDNA	KROL601, KROL602	193
3	LVS *rpsU2*	LVS Lysate	KROL601, KROL602	193
4	Δ*rpsU2*	Δ*2* Lysate	KROL601, KROL602	-
5	Cand. 1 pKR184 *rpsU2*	Lysate	KROL601, KROL602	193
6	Cand. 1 pKR184 *rpsU2*	gDNA	KROL601, KROL602	193
7	Cand. 5 pKR184 *rpsU2*	Lysate	KROL601, KROL602	-
8	Cand. 5 pKR184 *rpsU2*	gDNA	KROL601, KROL602	-
9	Cand. 3 pKR168 *rpsU2*	gDNA	KROL601, KROL602	-

6. Add DNA or water to PCR tubes
7. Prepare a master mix
8. Add 98 uL of master mix to each tube, pipetting up and down
9. Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	9

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	10
ddiH2O			11.8	118
PrimeSTAR GXL Buffer	5x	1x	4	40
dNTPs	2.5 mM	0.2 mM	1.6	16
oligo F	10 uM	0.3 uM	0.6	6
oligo R	10 uM	0.3 uM	0.6	6
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	4
Cell lysate	-	-	1	
		Total volume	20	190

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. Add DNA or water to PCR tubes
3. Prepare a master mix
4. Add 98 uL of master mix to each tube, pipetting up and down
5. 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	3
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

I diluted pKR184 1:5, meaning I was adding ~100 ng/uL stock to the PCR.

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.

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:

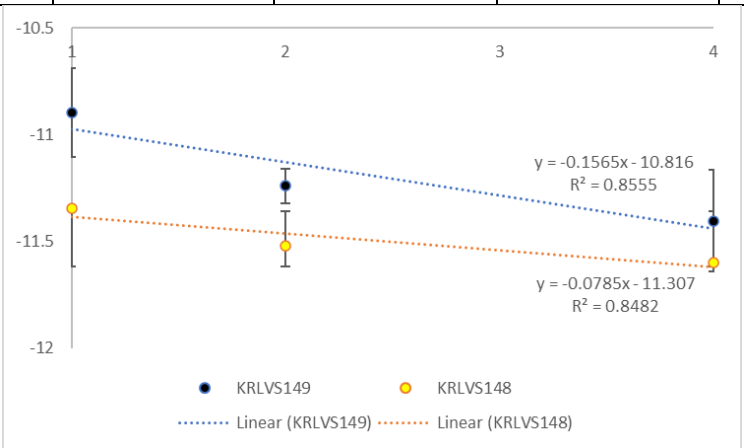
Master Mix Label	Primer Set
A	KROL73/74 23s rRNA

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					



There is technically a slight trend shown, however, the C_p values were massively under the range for the linear values, so it's not a good indicator of anything. Kathryn said there's no reason to rerun.

Tuesday, May 2, 2023

To Do:

1. ~~Restriction digest of pKR184 and pKR196 backbones + PCR of stem loop deletion~~
2. ~~Run gel of restriction digest~~
3. ~~Excise gel fragments~~
4. ~~Put away dishes~~

Results and Data:

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
3	pKR196	PacI, MfeI	5	10

2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**	**Volume in 4x Master Mix (uL)**
H_2O	10.8	43.2
10x Buffer*	3.0	12.0
DNA	(15.0)	-
PacI	0.6	2.4
Mfe1	0.6	2.4
Total	30.0 (15.0 actual b/c of DNA)	60 (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.

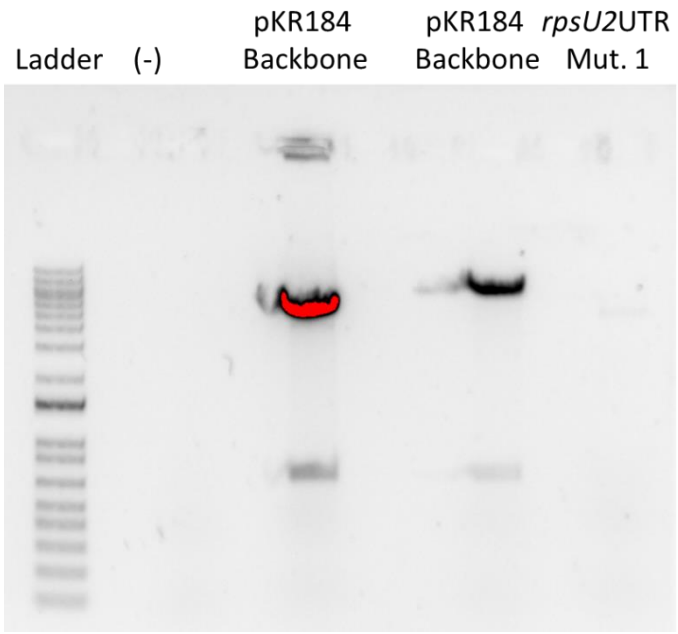
Gel of Digested *rpsU2* UTR Mutant Insert and pKR184 and KRLVS196 Backbone

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.

Component	Size (bp)
rpsU2 5'UTR Mut. 1	648
pKR184 Backbone	4986
pKR196 Backbone	7156

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.



Wednesday, May 3, 2023

To Do:

1. ~~Set up cultures for GFP assay~~
2. ~~Run GFP assay of pKR184 integrants~~

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				4				LVS			
B	2				5				PBS			
C	3				6							
D												
E												
F												
G												
H												

Thursday, May 4, 2023

To Do:

1. ~~Set up cultures for GFP assay on replicative GFP~~
2. ~~Restriction digest of mut. 1 stem loop deletion~~
3. ~~Run gel of restriction digest~~
4. ~~Freeze down permanent strains of WT and d2 + pKR196~~

Results and Data:

DNA Digest of *rpsU2* Mut. 1 PCR 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. Set up master mix table:

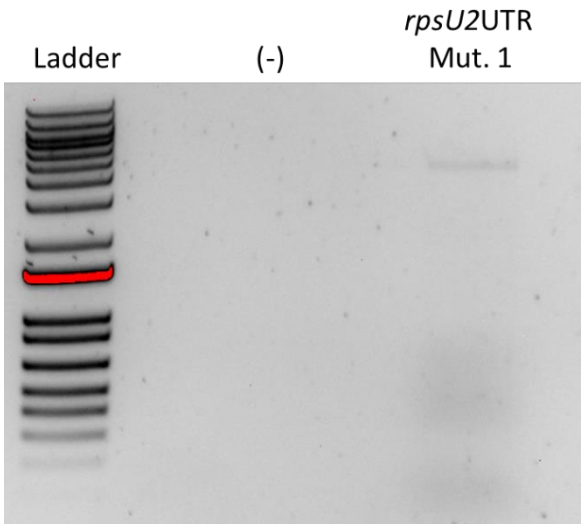
Components	Volumes in 1 reaction (uL)
H_2O	10.8
10x Buffer*	3.0
DNA	(15.0)
PacI	0.6
Mfe1	0.6
Total	30.0 (15.0 actual b/c of DNA)

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 *rpsU2* UTR Mutant 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 6uL 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 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.

Component	Size (bp)
rpsU2 5'UTR Mut. 1	648



Friday, May 5, 2023

To Do:

1. ~~Electroporate pKR168 into WT~~

Results and Data:

Electroporating pKR168 into EC 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 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	Number of Plates
1	pKR168+WT	KRLVS126	pKR168	5 uL	20 uL. 200 uL, rem	3
2	(-) control	KRLVS126	-	-	200 uL	1
					Total:	4

Sunday, May 7, 2023

To Do:

1. ~~PCR of mutant 1 fragment from pKR184~~
2. ~~PCR purification of mutant 1 fragment~~
3. ~~Restriction digest of mutant 1 fragment~~
4. ~~Run gel of restriction digest~~
5. ~~Gel purification of restriction digest +backbone gel excisions~~
6. ~~Ligation of pKR184 and pKR196 backbones with stem loop deletion insert~~
7. ~~Transformation of ligations into *E. coli*~~
8. ~~Make LB+Kan plates~~

Results and Data:

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. Add DNA or water to PCR tubes
3. Prepare a master mix
4. Add 98 uL of master mix to each tube, pipetting up and down
5. 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	3
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

I used my 1:5 diluted pKR184, meaning I was adding ~100 ng/uL stock to the PCR.

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 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. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**
H_2O	10.8
10x Buffer*	3.0
DNA	(15.0)

PacI	0.6
Mfe1	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.

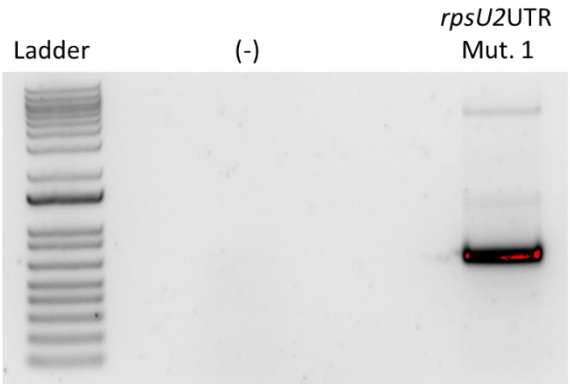
Gel of Digested *rpsU2* UTR Mutant Insert

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 6uL 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 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.

Component	Size (bp)
rpsU2 5'UTR Mut. 1	648

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	556	1668	556
pKR184 Backbone	388	1164	388
pKR196 Backbone	349	1047	349

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

1. Reaction table:

Tube	Insert	Backbone
1	*rpsU2* UTR Mut. 1 PCR (pKR191)	PacI, MfeI digested, purified pKR184
2	-	PacI, MfeI digested, purified pKR184
3	*rpsU2* UTR Mut. 1 PCR (pKR197)	PacI, MfeI digested, purified pKR196
4	-	PacI, MfeI digested, purified pKR196

2. 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. Assemble reactions on ice, making master mix.
4. Add backbone and insert to each tube, and add 14 uL of master mix to each.
5. Incubated at room temperature for 10 minutes.

Transformation of pKR191 and pKR197 Ligation into *E. coli*

1. 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	pKR191 Ligation	pKR191 Ligation	8 uL	100 uL, remaining	2
4	Backbone Ligation	BB only ligation	8 uL	100 uL, remaining	2
5	pKR197 Ligation	pKR197 Ligation	8 uL	100 uL, remaining	2
				Total number of plates	9

2. Warm plates at 37°C, and obtain DNA and allow to thaw, along with appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
3. Add indicated volume of indicated DNA on ice and gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
4. Incubate cells on ice for 20 minutes.
5. Heat shock cells at 42°C for 30 seconds.
6. After heat shock, place tubes back on ice until next step, for not too long.
7. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
8. Allow cells to recover for 1 hour at 37°C, shaking.
9. Plate indicated amount of cells on appropriate antibiotic plates (LB-Kan)

Reagents

LB Agar + Kanamycin

Melt LB agar and then cool at 56°C

0.5 mL 50 ug/mL Kanamycin

Pour plates

Monday, May 8, 2023

To Do:

1. ~~Make 200 mL LB in a baffled flask~~
2. ~~Start XL1B overnight for electrocompetent cells~~

Results and Data:

I checked my pKR168+WT plates, and, as I suspected, I did not see any colonies. I left them in the incubator to check the next day. Additionally, I didn't see transformants on my *E. coli* plates, so I decided to leave them in the incubator and check in the morning.

Reagents

LB (200 mL)

Add to 500 mL baffled flask

2 g NaCl
2 g Tryptone
1 g Yeast extract

Add 200 mL ddi H2O
Autoclave Liquid 30'

Tuesday, May 9, 2023

To Do:

1. ~~Start 200 mL culture of *E. coli*~~
2. ~~Patch out potential pKR168 + WT integrations~~
3. ~~Make chemically competent XL1 Blue *E. coli*~~

Results and Data:

Chemically Competent *E. coli*

1. Add 3 mL of sterile 1M MgCl2 to 200 mL LB in 1 L flask. Add antibiotic if appropriate.
2. Inoculate 200 mL LB with 0.5 mL of culture grown overnight.
3. Incubate 200 mL culture at 37°C, shaking until culture reaches an OD600 of 0.5.
4. Monitor culture growth by assessing OD600 using the spectrophotometer at 3, 4, and 5 hours
5. When cultures approach correct OD, cool down centrifuge to 4°C (INBRE high speed centrifuge)
6. When culture reaches an OD600 of approximately 0.5, transfer culture volume to sterile tubes (4x 50 mL conical) to pellet bacteria
7. Place tubes in cool centrifuge and pellet bacteria by spinning 10 minutes at 8000 rpm
8. Pour off supernatant and add 15 mL cold solution A to each 50 mL conicol
9. VERY gently resuspend cell pellet by pipetting up and down
10. Incubate resuspended cells on ice for at least 20 minutes (can stay on ice for up to 3 hours).

Wednesday, May 10, 2023

To Do:

1. ~~Patch out KRLVS271 and KRLVS272 (LVS and d2 pF-Ptul4-rpsU2UTR-GFP)~~
2. ~~Make 2.5% iron pyrophosphate~~
3. ~~Start dishwasher~~

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

Thursday, May 11, 2023

To Do:

1. ~~Supplement MHB~~
2. ~~Set up cultures for GFP assay of replicating plasmids~~
3. ~~Streak KRLVS148 to single colony~~
4. ~~Colony PCR of potential pKR168 integrants~~
5. ~~Ligation of pKR191 and pKR197~~
6. ~~GFP assay of replicating plasmids~~

Results and Data:

Colony PCR of Candidate pKR168 Integrations into LVS

1. Resuspend part of patch in 50 uL of sterile water using toothpick, then lyse cells at 95°C for 10'
2. Dilute lysates 1:10
3. Reaction table:

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

4. Assemble master mix according to below.
5. Add 19.0 uL of master mix to 1 uL lysed cells
6. Use STN1 program, modifying 68°C step to 1' per kb

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

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

1. Reaction table:

Tube	Insert	Backbone
1	*rpsU2* UTR Mut. 1 PCR (pKR191)	PacI, MfeI digested, purified pKR184
2	-	PacI, MfeI digested, purified pKR184
3	*rpsU2* UTR Mut. 1 PCR (pKR197)	PacI, MfeI digested, purified pKR196
4	-	PacI, MfeI digested, purified pKR196

2. 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. Assemble reactions on ice, making master mix.
4. Add backbone and insert to each tube, and add 14 uL of master mix to each.
5. Incubated at room temperature for 10 minutes.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

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

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

Friday, May 12, 2023

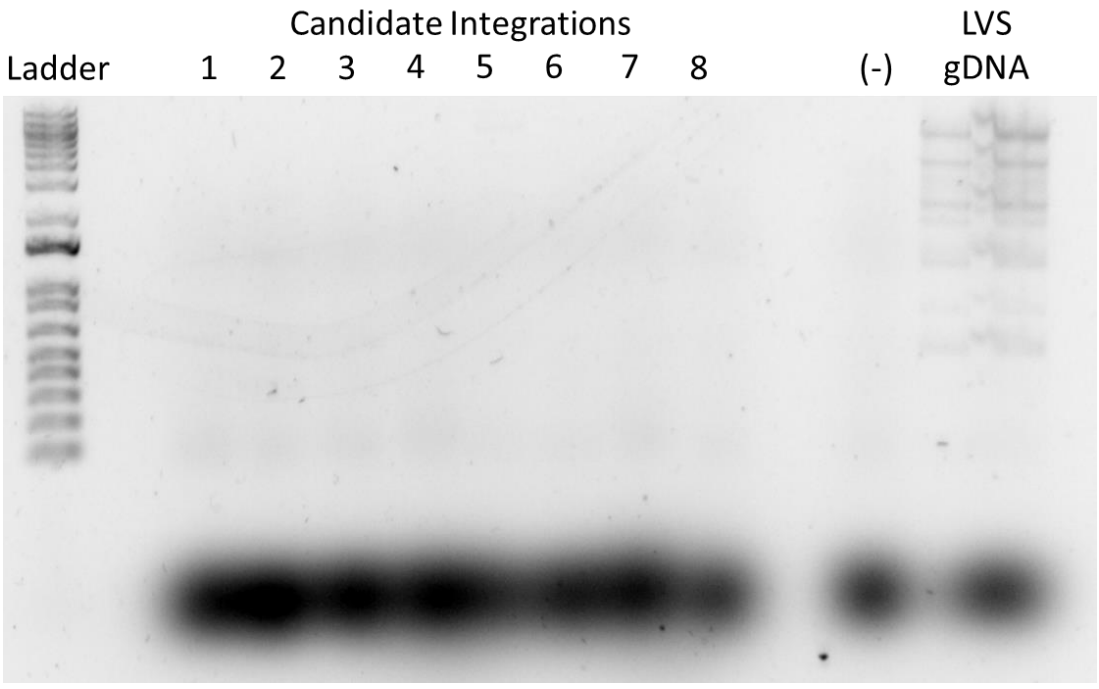
To Do:

1. ~~Streak KRLVS149 to single colony~~
2. ~~Run gel on colony PCR~~

Results and Data:

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.



Monday, May 15, 2023

To Do:

1. ~~Patch out KRLVS148 and KRLVS149 for RNA stability assay~~
2. ~~Transform pKR191 and pKR197 ligations into *E. coli*~~
3. ~~Set up for stability assay~~

Results and Data:

Transformation of pKR191 and pKR197 Ligation into *E. coli*

1. Reaction table:

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(-) control	None	0	100 uL	1
2	(+) control	pF	1 uL	100 uL	1
3	Backbone Ligation	BB only ligation	8 uL	100 uL, remaining	2
4	pKR191 Ligation	pKR191 Ligation	8 uL	100 uL, remaining	2
5	Backbone Ligation	BB only ligation	8 uL	100 uL, remaining	2
6	pKR197 Ligation	pKR197 Ligation	8 uL	100 uL, remaining	2
				Total number of plates	10

2. Warm plates at 37°C, and obtain DNA and allow to thaw, along with appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
3. Add indicated volume of indicated DNA on ice and gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
4. Incubate cells on ice for 20 minutes.
5. Heat shock cells at 42°C for 30 seconds.
6. After heat shock, place tubes back on ice until next step, for not too long.
7. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
8. Allow cells to recover for 1 hour at 37°C, shaking.
9. Plate indicated amount of cells on appropriate antibiotic plates (LB-Kan)

Tuesday, May 16, 2023

To Do:

1. ~~Set up cultures for RNA stability assay~~
2. ~~RNA stability assay~~

Results and Data:

RNA Stability Assay

1. Normalize OD's to 0.08 or 0.1 for wild-type and d2
2. Prepare 0.5 mg/mL rifampin
3. Once OD ~0.3, transfer 7mL of each replicate into 15 mL tubes, one for each time point and allow to shake for an additional 30 minutes
4. Flash freeze 0' time point in liquid nitrogen, then add 7 uL 0.5 mg/mL rif, flash freezing each 15 mL tube after either 1, 2, 4, or 8 minutes.
5. Repeat for each biological replicate, can start second time course during 4 minute wait, cultures can be stored in the -80 for up to two months

Sample Experimental Configuration																													
Strain	KRLVS148															KRLVS149													
Replicate	1					2					3					1					2					3				
rif (min)	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8

To prepare the rifampin, I prepared 50 mg/mL by dissolving 5 mg in 100 uL of DMSO, then serially diluted to 0.5 mg/mL in a final volume of 250 uL. ond

Wednesday, May 17, 2023

To Do:

1. ~~Patch out WT+hp and leave at room temperature~~

Results and Data:

Patched out the plate and left it at room temperature, asked Kira to streak out KRLVS148 on Thursday, and KRLVS149 on Friday.

Monday, May 22, 2023

To Do:

1. ~~Patch out KRLVS148 and KRLVS149~~
2. ~~Transformation of pKR191 and pKR197 into *E. coli*~~

Results and Data:

For RNA stability assay, qPCR primer sets: *rpsO*, *tul4*, *lacZ*, 5' UTR.

Email CoP to ask about lead time

Transformation of pKR191 and pKR197 Ligation into *E. coli*

1. Reaction table:

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(-) control	None	0	200 uL	1
2	(+) control	pF	1 uL	200 uL	1
3	Backbone Ligation	BB only ligation	8 uL	20 uL, 200 uL	2
4	pKR191 Ligation	pKR191 Ligation	8 uL	20 uL, 200 uL	2
5	Backbone Ligation	BB only ligation	8 uL	20 uL, 200 uL	2
6	pKR197 Ligation	pKR197 Ligation	8 uL	20 uL, 200 uL	2
				Total number of plates	10

2. Warm plates at 37°C, and obtain DNA and allow to thaw, along with appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
3. Add indicated volume of indicated DNA on ice and gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
4. Incubate cells on ice for 20 minutes.
5. Heat shock cells at 42°C for 30 seconds.
6. After heat shock, place tubes back on ice until next step, for not too long.
7. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
8. Allow cells to recover for 1 hour at 37°C, shaking.
9. Plate indicated amount of cells on appropriate antibiotic plates (LB-Kan)

Tuesday, May 23, 2023

To Do:

1. ~~Make electrocompetent WT+hp~~
2. ~~Transformation of pKR168 into WT~~
3. ~~Receive primers~~
4. ~~PCR of NotI-GFP-NotI~~
5. ~~PCR purification~~
6. ~~Overnight restriction digest~~
7. ~~Set up for RNA stability assay~~
8. ~~Start overnights of pKR191 and pKR197~~

Results and Data:

Preparing Electrocompetent 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 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 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	WT+pKR168	KRLVS126	Cand. pKR168 2.2	5 uL	20 uL. 200 uL, Rem.	3
2	WT+pKR168	KRLVS126	Cand. pKR168 2.3	5 uL	20 uL. 200 uL, Rem.	3
3	(-) control	KRLVS126	-	-	200 uL	1
					Total:	7

Receiving GFP-NotI-R 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 NotI-GFP Fragment from pKR184

1. Acquired and labelled PCR tubes

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	GFP flanked by NotI sites	pKR184	KROL524, KROL667	739
2	- control	-	KROL524, KROL667	-

2. Add DNA or water to PCR tubes
3. Prepare a master mix
4. Add 98 uL of master mix to each tube, pipetting up and down
5. 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	3
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

I used a 1:4 dilution of pKR184 for the PCR, which should be about ~100 ng/uL.

PCR Purification of NotI-GFP-NotI 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 NotI-GFP-NotI and pKR11 w/NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	NotI-GFP-NotI PCR	NotI	15	-
2	pKR11	NotI	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)
NotI	1.2
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 overnight
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.

Additionally, I set up four overnights each for candidate pKR191 and pKR197 in 5 mL LB + 5 uL kanamycin.

Wednesday, May 24, 2023

To Do:

1. ~~Start cultures of KRLVS148 and KRLVS149~~
2. ~~Miniprep of transformation overnights~~
3. ~~Run gel of restriction digest~~
4. ~~Make rifampicin~~
5. ~~RNA stability assay of KRLVS148 and KRLVS149~~

Results and Data:

RNA Stability Assay

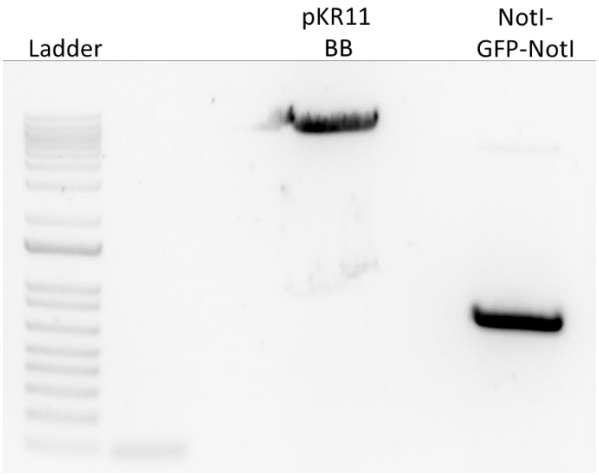
1. Normalize OD's to 0.08 or 0.1 for wild-type and d2
2. Prepare 0.5 mg/mL rifampin
3. Once OD ~0.3, transfer 7mL of each replicate into 15 mL tubes, one for each time point and allow to shake for an additional 30 minutes
4. Flash freeze 0' time point in liquid nitrogen, then add 7 uL 0.5 mg/mL rif, flash freezing each 15 mL tube after either 1, 2, 4, or 8 minutes.
5. Repeat for each biological replicate, can start second time course during 4 minute wait, cultures can be stored in the -80 for up to two months

Sample Experimental Configuration																												
Strain	KRLVS148															KRLVS149												
Replicate	1					2					3					1					2					3				
rif (min)	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8

To prepare the rifampin, I prepared 50 mg/mL by dissolving 5 mg in 100 uL of DMSO, then serially diluted to 0.5 mg/mL in a final volume of 250 uL.

Gel of Digested NotI-GFP-NotI PCR and pKR11 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.

Miniprep of Candidate pKR191 and pKR197 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.

Thursday, May 25, 2023

To Do:

1. ~~Nanodrop minipreps~~
2. ~~Set up pKR191 and pKR197 for sequencing~~
3. ~~Streak out KRLVS272 to single colony~~
4. ~~RNA purification and extraction of RNA stability assay samples~~
5. ~~Nanodrop total nucleic acids of stability assay samples~~

Results and Data:

I nanodrop'd my minipreps from yesterday. Candidate pKR191:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR191 Cand. 1	419.3	8.387	4.468	1.88	2.34
pKR191 Cand. 2	506.6	10.132	5.414	1.87	2.27
pKR191 Cand. 3	431.8	8.636	4.604	1.88	2.32
pKR191 Cand. 4	473.4	9.468	5.104	1.86	2.27

Candidate pKR197:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR197 Cand. 1	200.2	4.003	2.207	1.81	1.73
pKR197 Cand. 2	240.3	4.805	2.574	1.87	2.39
pKR197 Cand. 3	244.6	4.892	2.621	1.87	2.41
pKR197 Cand. 4	205.6	4.111	2.202	1.87	2.41

Additionally, I set up sequencing:

Sample	Type	Template	Primer	Template (bp)	Stock (ng/uL)	Template (uL)	Water (uL)
SS1	Plasmid	Cand. pKR191 1	KROL257	5634	419.3	0.95	8.49
SS2	Plasmid	Cand. pKR191 2	KROL257	5634	506.6	0.79	8.65
SS3	Plasmid	Cand. pKR191 3	KROL257	5634	431.8	0.93	8.51
SS4	Plasmid	Cand. pKR191 4	KROL257	5634	473.4	0.84	8.60
SS5	Plasmid	Cand. pKR191 1	KROL362	5634	419.3	0.95	8.49
SS6	Plasmid	Cand. pKR191 2	KROL362	5634	506.6	0.79	8.65
SS7	Plasmid	Cand. pKR191 3	KROL362	5634	431.8	0.93	8.51
SS8	Plasmid	Cand. pKR191 4	KROL362	5634	473.4	0.84	8.60
SS9	Plasmid	Cand. pKR197 1	KROL257	7842	200.2	2.00	7.44
SS10	Plasmid	Cand. pKR197 2	KROL257	7842	240.3	1.66	7.78
SS11	Plasmid	Cand. pKR197 3	KROL257	7842	244.6	1.64	7.80
SS12	Plasmid	Cand. pKR197 4	KROL257	7842	205.6	1.95	7.49
SS13	Plasmid	Cand. pKR197 1	KROL362	7842	200.2	2.00	7.44
SS14	Plasmid	Cand. pKR197 2	KROL362	7842	240.3	1.66	7.78
SS15	Plasmid	Cand. pKR197 3	KROL362	7842	244.6	1.64	7.80
SS16	Plasmid	Cand. pKR197 4	KROL362	7842	205.6	1.95	7.49
a.Add 2.56 µl of primer							

RNA Purification of RNA Stability Assay 2 Samples

1. Thawed flash frozen cultures on ice and pelleted 7mL of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent and transferred to 2 mL tube
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Completed ZymoPrep RNA kit according to our standard procedure
6. Add 90 uL RNase-free water, let sit on column 2 min
7. Spin max speed 1 min
8. Place flow-through on column again, spin 1 min
9. Store nucleic acids at -80°C if not moving directly to the next step

Labelled	Contents	Labelled	Contents	Labelled	Contents
1 RNA	149 1 0’	9 RNA	149 2 4’	17 RNA	148 1 2’
2 RNA	149 1 1’	10 RNA	149 2 8’	18 RNA	148 1 4’
3 RNA	149 1 2’	11 RNA	149 3 0’	19 RNA	148 1 8’
4 RNA	149 1 4’	12 RNA	149 3 1’	20 RNA	148 3 0’
5 RNA	149 1 8’	13 RNA	149 3 2’	21 RNA	148 3 1’
6 RNA	149 2 0’	14 RNA	149 3 4’	22 RNA	148 3 2’
7 RNA	149 2 1’	15 RNA	149 3 8’	23 RNA	148 3 4’
8 RNA	149 2 2’	16 RNA	148 1 0’	24 RNA	148 3 8’

Sample	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
RNA 1	961.2	24.030	11.391	2.11	2.31
RNA 2	1126.9	28.172	13.197	2.13	2.23
RNA 3	1059.1	26.476	12.367	2.14	2.25
RNA 4	905.1	22.628	10.747	2.11	2.31
RNA 5	925.0	23.126	10.964	2.11	2.30
RNA 6	832.5	20.812	9.872	2.11	2.30
RNA 7	1026.4	25.660	12.127	2.12	2.03
RNA 8	1127.2	28.180	13.374	2.11	2.31
RNA 9	1115.7	27.893	13.290	2.10	2.32
RNA 10	1012.6	25.316	11.932	2.12	2.27
RNA 11	811.3	20.282	9.647	2.10	2.29
RNA 12	913.2	22.831	10.872	2.10	2.28
RNA 13	958.4	23.960	11.355	2.11	2.27
RNA 14	650.4	16.260	7.915	2.05	2.19
RNA 15	583.2	14.579	7.106	2.05	2.18
RNA 16	1205.4	30.136	14.365	2.10	2.21
RNA 17	812.0	20.299	9.703	2.09	2.17
RNA 18	1154.7	28.868	13.807	2.09	2.13
RNA 19	867.0	21.675	10.427	2.08	2.20
RNA 20	871.1	21.778	10.494	2.08	2.13
RNA 21	971.0	24.276	11.611	2.09	2.23
RNA 22	1026.3	25.658	12.206	2.10	2.20
RNA 23	853.1	21.328	10.156	2.10	1.96
RNA 24	757.1	18.928	8.969	2.11	2.16

Friday, May 26, 2023

To Do:

1. ~~Streak out KRLVS271 to single colony~~
2. ~~Check integrant plates~~

Results and Data:

Colonies on electroporation plates were not big enough to patch out.

Tuesday, May 30, 2023

To Do:

1. ~~Patch out KRLVS271 and KRLVS272~~
2. ~~Patch out potential integrants~~
3. ~~Check sequencing results~~

Results and Data:

I looked at my sequencing and it seems like pKR191 Candidate 4 and pKR197 Candidate 1 are good and dandy.

Wednesday, May 31, 2023

To Do:

1. ~~Run dishwasher~~
2. ~~Take plates out of incubator~~

Results and Data:

I took plates out of the incubator and looked at them.

June 2023

Thursday, June 1, 2023

To Do:

1. ~~Work on poster~~
2. ~~Work on joint lab meeting presentation~~

Friday, June 2, 2023

To Do:

1. ~~Work on poster~~

Saturday, June 3, 2023

To Do:

1. ~~Colony PCR of candidate pKR168 integrants~~
2. ~~DNase treatment of RNA Stability Samples – 1~~
3. ~~Second RNA extraction of RNA Stability Samples – 1~~
4. ~~Nanodrop RNA Stability Samples – 1~~
5. ~~Set up aliquots of RNA Stability Samples for gel and cDNA synthesis~~
6. ~~Run gel of colony PCR~~

Results and Data:

Colony PCR of Candidate pKR168 Integrations into LVS

1. Resuspend cells in 50 uL of sterile water using toothpick, then lyse cells at 95°C for 10', dilute 1:10
2. Reaction table:

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL472, KROL319	-
2	LVS gDNA	LVS gDNA	KROL472, KROL319	-
3-18	P*rpsU2* *tul4*UTR *lacZ*	Lysate	KROL472, KROL319	3328

3. Assemble master mix according to below.
4. Add 19.0 uL of master mix to 1 uL lysed cells
5. Use STN1 program, modifying 68°C step to 1' per kb

Total reaction volume	20
Total number of reactions	18

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	19
ddiH2O			11.8	224.2
PrimeSTAR GXL Buffer	5x	1x	4	76
dNTPs	2.5 mM	0.2 mM	1.6	30.4
oligo F	10 uM	0.3 uM	0.6	11.4
oligo R	10 uM	0.3 uM	0.6	11.4
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	7.6
Cell lysate	-	-	1	
		Total volume	20	361

DNase treatment of RNA Stability Assay samples

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol

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

Then I nanodrop'd my pure RNA samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS149 1 0'	601.2	15.030	7.252	2.07	2.26
KRLVS149 1 1'	699.2	17.479	8.548	2.04	2.23
KRLVS149 1 2'	569.3	14.233	7.027	2.03	2.23
KRLVS149 1 4'	620.2	15.506	7.614	2.04	2.32
KRLVS149 1 8'	492.1	12.301	6.052	2.03	2.21
KRLVS149 2 0'	501.7	12.543	6.062	2.07	2.21
KRLVS149 2 1'	711.3	17.782	8.689	2.05	2.20
KRLVS149 2 2'	1106.1	27.653	14.270	1.94	1.66
KRLVS149 2 4'	550.8	13.770	6.807	2.02	2.21
KRLVS149 2 8'	702.4	17.559	8.548	2.05	2.20
KRLVS149 3 0'	422.1	10.552	5.060	2.09	2.20
KRLVS149 3 1'	584.9	14.623	7.169	2.04	2.19
KRLVS149 3 2'	659.1	16.477	8.054	2.05	2.26
KRLVS149 3 4'	375.6	9.390	4.522	2.08	2.22
KRLVS149 3 8'	434.0	10.850	5.296	2.05	2.04
KRLVS148 1 0'	597.5	14.936	7.418	2.01	2.27
KRLVS148 1 2'	542.6	13.565	6.635	2.04	2.20
KRLVS148 1 4'	543.6	13.591	6.753	2.01	2.11
KRLVS148 1 8'	572.4	14.309	7.132	2.01	2.20
KRLVS148 3 0'	641.6	16.039	7.842	2.05	2.16
KRLVS148 3 1'	446.2	11.156	5.392	2.07	2.19
KRLVS148 3 2'	592.1	14.803	7.244	2.04	2.11
KRLVS148 3 4'	529.2	13.231	6.522	2.03	2.18

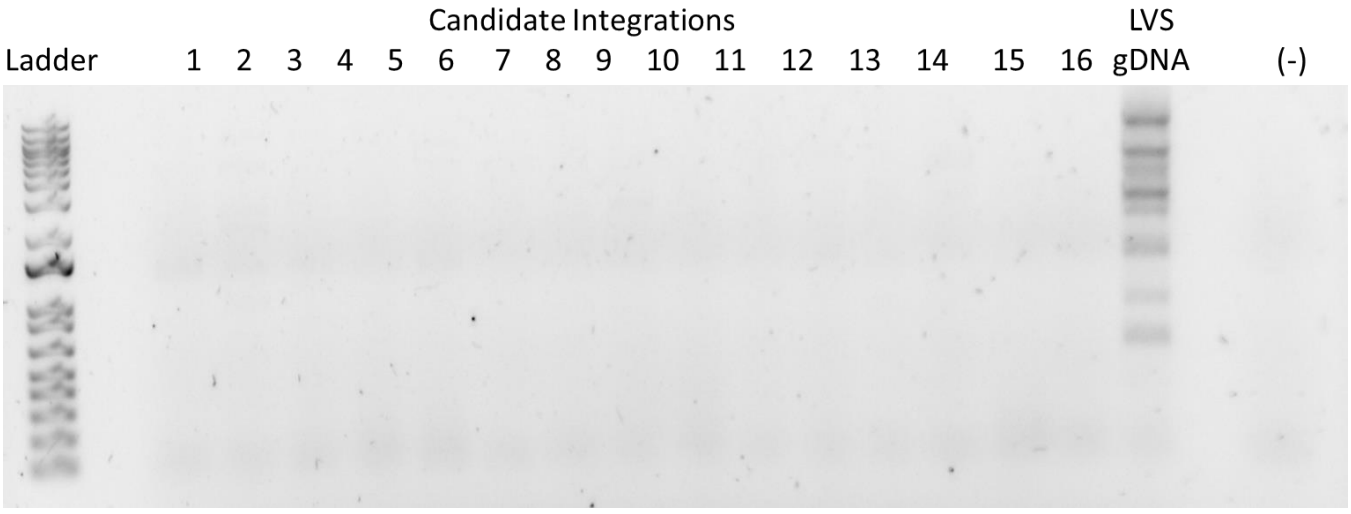
Then, I set up my aliquots for the RNA gel and cDNA synthesis:

Gel			
Sample	ng/uL	RNA	Water
KRLVS149 1 0'	601.2	1.33	8.67
KRLVS149 1 1'	699.2	1.14	8.86
KRLVS149 1 2'	569.3	1.41	8.59
KRLVS149 1 4'	620.2	1.29	8.71
KRLVS149 1 8'	492.1	1.63	8.37
KRLVS149 2 0'	501.7	1.59	8.41
KRLVS149 2 1'	711.3	1.12	8.88
KRLVS149 2 2'	1106.1	0.72	9.28
KRLVS149 2 4'	550.8	1.45	8.55
KRLVS149 2 8'	702.4	1.14	8.86
KRLVS149 3 0'	422.1	1.90	8.10
KRLVS149 3 1'	584.9	1.37	8.63
KRLVS149 3 2'	659.1	1.21	8.79
KRLVS149 3 4'	375.6	2.13	7.87
KRLVS149 3 8'	434.0	1.84	8.16
KRLVS148 1 0'	597.5	1.34	8.66
KRLVS148 1 2'	542.6	1.47	8.53
KRLVS148 1 4'	543.6	1.47	8.53
KRLVS148 1 8'	572.4	1.40	8.60
KRLVS148 3 0'	641.6	1.25	8.75
KRLVS148 3 1'	446.2	1.79	8.21
KRLVS148 3 2'	592.1	1.35	8.65
KRLVS148 3 4'	529.2	1.51	8.49

cDNA			
Sample	ng/uL	RNA	Water
KRLVS149 1 0'	601.2	6.15	7.35
KRLVS149 1 1'	699.2	5.29	8.21
KRLVS149 1 2'	569.3	6.50	7.00
KRLVS149 1 4'	620.2	5.97	7.53
KRLVS149 1 8'	492.1	7.52	5.98
KRLVS149 2 0'	501.7	7.37	6.13
KRLVS149 2 1'	711.3	5.20	8.30
KRLVS149 2 2'	1106.1	3.35	10.15
KRLVS149 2 4'	550.8	6.72	6.78
KRLVS149 2 8'	702.4	5.27	8.23
KRLVS149 3 0'	422.1	8.77	4.73
KRLVS149 3 1'	584.9	6.33	7.17
KRLVS149 3 2'	659.1	5.61	7.89
KRLVS149 3 4'	375.6	9.85	3.65
KRLVS149 3 8'	434.0	8.53	4.97
KRLVS148 1 0'	597.5	6.19	7.31
KRLVS148 1 2'	542.6	6.82	6.68
KRLVS148 1 4'	543.6	6.81	6.69
KRLVS148 1 8'	572.4	6.46	7.04
KRLVS148 3 0'	641.6	5.77	7.73
KRLVS148 3 1'	446.2	8.29	5.21
KRLVS148 3 2'	592.1	6.25	7.25
KRLVS148 3 4'	529.2	6.99	6.51

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.



Monday, June 5, 2023

To Do:

1. ~~Put away dishes~~
2. ~~cDNA synthesis of RNA Stability samples~~
3. ~~Run gel of RNA stability assay samples~~
4. ~~Load dishwasher~~

Results and Data:

Generate cDNA (Half) of RNA Stability Assay 2 samples - 1

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	X24.5
5X 1st strand buffer	1x	6	147
RNase-free water		2.88	70.56
100 mM DTT	10 mM	3	73.5
10 mM dNTPs	0.5 mM	1.5	36.75
Superscript III (200 U/ul)	10.8 U/ul	1.63	39.94

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

Gel of RNA Stability Assay 2 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 spray down with ethanol
3. Make fresh agarose gel by adding 1.2 g to 120 mL fresh 1xTAE and stir with heat until completely dissolved, then place in 56°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 12 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.

Friday, June 16, 2023

To Do:

1. ~~Streak out KRLVS271 and KRLVS272~~

Results and Data:

Monday, June 19, 2023

To Do:

1. ~~Patch out KRLVS271 and KRLVS272~~
2. ~~Purify cDNA samples~~

Results and Data:

Generate cDNA (Half) of KMLFT104, KRLVS16, and LVS (3/8/23 Pure RNA)

1. 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
2. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
3. Elute in 60 ul of 0.1x EB
4. 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.

Tuesday, June 20, 2023

To Do:

1. ~~Take KRLVS271 out of the autoclave~~
2. ~~Generate cDNA of RNA stability assay~~

Results and Data:

I diluted my RNA samples to 333 ng/uL by:

cDNA			
Sample	**ng/uL**	**RNA**	**Water**
KRLVS149 1 0'	601.2	7.48	6.02
KRLVS149 1 1'	699.2	6.43	7.07
KRLVS149 1 2'	569.3	7.90	5.60
KRLVS149 1 4'	620.2	7.25	6.25
KRLVS149 1 8'	492.1	9.13	4.37
KRLVS149 2 0'	501.7	8.96	4.54
KRLVS149 2 1'	711.3	6.32	7.18
KRLVS149 2 2'	1106.1	4.06	9.44
KRLVS149 2 4'	550.8	8.16	5.34
KRLVS149 2 8'	702.4	6.40	7.10
KRLVS149 3 0'	422.1	10.65	2.85
KRLVS149 3 1'	584.9	7.69	5.81
KRLVS149 3 2'	659.1	6.82	6.68
KRLVS149 3 4'	375.6	11.97	1.53
KRLVS149 3 8'	434.0	10.36	3.14
KRLVS148 1 0'	597.5	7.52	5.98
KRLVS148 1 2'	542.6	8.28	5.22
KRLVS148 1 4'	543.6	8.27	5.23
KRLVS148 1 8'	572.4	7.85	5.65
KRLVS148 3 0'	641.6	7.01	6.49
KRLVS148 3 1'	446.2	10.07	3.43
KRLVS148 3 2'	592.1	7.59	5.91
KRLVS148 3 4'	529.2	8.49	5.01

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	X24.5
5X 1st strand buffer	1x	6	147
RNase-free water		2.88	70.56
100 mM DTT	10 mM	3	73.5
10 mM dNTPs	0.5 mM	1.5	36.75
Superscript III (200 U/ul)	10.8 U/ul	1.63	39.94

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

Thursday, June 22, 2023

To Do:

1. ~~Electroporate pKR191 into WT and d2 cells~~
2. ~~Generate cDNA of RNA stability assay samples~~
3. ~~Purify cDNA of RNA stability assay samples~~
4. ~~qPCR of RNA stability assay samples – *rpsU2* UTR~~

Results and Data:

Electroporating pKR191 into EC 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 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 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	Number of Plates
1	pKR191+WT	KRLVS126	pKR191	3 uL	200 uL, rem	2
2	pKR191+d2	KRLVS127	pKR191	3 uL	200 uL, rem	2
3	(-) control	KRLVS126	-	-	200 uL	1
4	(-) control	KRLVS127	-	-	200 uL	1
					Total:	6

Generate cDNA (Half) of KMLFT104, KRLVS16, and LVS (3/8/23 Pure RNA)

1. 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

2. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
3. Elute in 60 ul of 0.1x EB
4. 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.

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. KROL504/505 as test primers to amplify *rpsU2* 5' UTR
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	840.0 uL
5uM primer set	1 uL	84.0 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH_2O	8 uL	672.0 uL
Total:	20 uL	1596.0 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	KROL504/505 *rpsU2* 5' UTR

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

13	14	15	16	17	18	19	20	21	22	23
149 1 8'	149 2 8'	149 3 8'	148 1 0'	148 3 0'	148 3 1'	148 1 2'	148 3 2'	148 1 4'	148 3 4'	148 1 8'

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					

Monday, June 26, 2023

To Do:

1. ~~Patch out transformants of pKR191 + WT or d2~~

Results and Data:

pKR122: pKR89 with NotI/KpnI and KROL472 and 350 amplified gBlock cut with NotI/KpnI

Tuesday, June 27, 2023

To Do:

1. ~~Gel extraction of pKR11 backbone and NotI-GFP-NotI~~
2. ~~Nanodrop gel extraction~~
3. ~~PCR of PrpsU2_tul4UTR gBlock for pKR122~~

Results and Data:

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)
NotI-GFP-NotI	201	603	201
pKR11 Backbone	147	441	147

I ran through this protocol with Johanyx, showing her basics of pipetting etc. Additionally, we nanodrop'd my gel extraction samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR11 Backbone	20.8	0.417	0.216	1.93	0.10
NotI-GFP-NotI	108.9	2.177	1.179	1.85	0.62

PCR of P*rpsU2_tul4*UTR gBlock for pKR122

1. Acquired and labelled PCR tubes: 122 and negative control. *omitted positive b/c primers verified
2. Acquire the following components and put them on ice, labeling tubes if necessary:
    - mgH2O, Primestar buffer, dNTPs, KROL472, KROL350, (10uM), and P*rpsU2_tul4*UTR gBlock
3. Vortex each component (aside from enzyme)
4. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
5. Add ddi 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 appropriate volume of master-mix to negative control PCR tube
9. Add appropriate volume of master mix to each PCR tube and pipet up and down to mix

10. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes, > 94 degrees C for 20 seconds > 50 degrees C for 30 seconds > 68 degrees C for 30 seconds > Go back to step 2 > Repeat 32x > 68 degrees C for 5 minutes > 12 degrees C for infinity

Total reaction volume	100
Total number of reactions	2

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	3
ddiH2O			62.0	184.0
PrimeSTAR GXL Buffer	5x	1x	20.0	60.0
dNTPs	2.5 mM	0.2 mM	8.0	24.0
oligo F	10 uM	0.3 uM	3.0	9.0
oligo R	10 uM	0.3 uM	3.0	9.0
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	6.0
		Total volume	100	294

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL472, KROL350	-
2	P*rpsU2_tul4*UTR	gBlock	KROL472, KROL350	252

Wednesday, June 28, 2023

To Do:

1. ~~PCR purification of gBlock amplification~~
2. ~~DNA digest of PCR and pKR89 backbone~~
3. ~~Run gel of DNA digest~~
4. ~~Excise gel fragments from DNA digest~~

Results and Data:

PCR Purification of Amplified P*rpsU2_tul4*UTR gBlock

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

DNA Digest of P*rpsU2_tul4*UTR gBlock PCR and pKR89 Backbone w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	P*rpsU2_tul4*UTR	NotI, KpnI	15	-

2	pKR89	NotI, KpnI	5	10

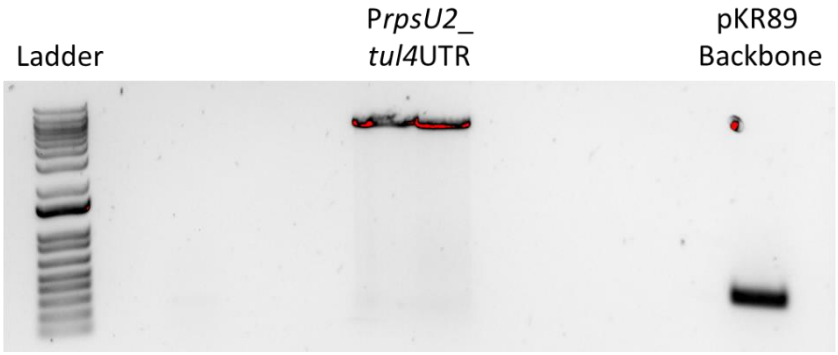
2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_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)	

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, then put at 80C for 2 minutes to inactivate the enzyme.

Gel of Digested P*rpsU2_tul4*UTR PCR Fragment + pKR89 Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of 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.

Thursday, June 29, 2023

To Do:

1. ~~Gel extraction of pKR89 backbone and P*rpsU2 tul4* 5'UTR gBlock PCR~~

Results and Data:

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.

I observed Johanyx completing this protocol, and stored the gel purifications in my cloning box. She has the table with the weights listed for fragments + volume of QG and isopropanol.

Friday, June 30, 2023

To Do:

1. ~~Ligation of pKR122 components~~
2. ~~Transformation of pKR122 ligation~~
3. ~~Prepare lysates of potential WT and d2 + pKR191 mutants for colony PCR~~
4. ~~Freeze down strains of WT and d2 + pKR191~~

Results and Data:

Ligation of Digested P*rpsU2 tul4* UTR gBlock + pKR89 Backbone

1. Reaction table:

Tube	Insert	Backbone
1	P*rpsU2 tul4* UTR gBlock PCR	NotI, KpnI digested, purified pKR89
2	-	NotI, KpnI digested, purified pKR89

2. Master mix table:

Component	Reaction 1 (uL)	2.5 x Master Mix (uL)
H2O	11.5	28.75
10x ligase buffer	2.0	5
Insert	4.0	-
Backbone	2.0	5
Ligase	0.5	1.25
TOTAL	20.0	35

3. Assemble reactions on ice, making master mix.
4. Add backbone and insert to each tube, and add 14 uL of master mix to each.
5. Incubated at room temperature for 10 minutes.

Transformation of pKR191 and pKR197 Ligation into *E. coli*

1. 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	pKR191 Ligation	pKR191 Ligation	8 uL	100 uL, remaining	2
				Total number of plates	5

2. Warm plates at 37°C, and obtain DNA and allow to thaw, along with appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
3. Add indicated volume of indicated DNA on ice and gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
4. Incubate cells on ice for 20 minutes.

5. Heat shock cells at 42°C for 30 seconds.
6. After heat shock, place tubes back on ice until next step, for not too long.
7. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
8. Allow cells to recover for 1 hour at 37°C, shaking.
9. Plate indicated amount of cells on appropriate antibiotic plates (LB-Kan)

Preparing Lysates for Colony PCR of Candidate pKR191 Integration into LVS and d*rpsU2*

1. Resuspend cells in 50 uL of sterile water using toothpick, then lyse cells at 95°C for 10', dilute 1:10
2. Reaction table:

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 Mut1 *gfp*	Lysate	KROL326, KROL402	995
7-10	d2 P*tul_ rpsU2*UTR Mut1 *gfp*	Lysate	KROL326, KROL402	995

3. Assemble master mix according to below.
4. Add 19.0 uL of master mix to 1 uL lysed cells
5. Use STN1 program, modifying 68°C step to 1' per kb

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 pKR191 Integrations into LVS and d2

7. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enouh..
8. Set up large gel rig to cast gel, with ladder.
9. 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.
10. Turn gel, add used TAE, remove ladder.
11. Loaded 10 uL ladder, and 15 uL of each sample.
12. Ran for 45 minutes at 113V.

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.