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September 2022

Thursday, September 1, 2022

To Do:

- ~~1. Set up KRLVS148, KRLVS149, KRLVS111, and KRLVS112 cultures for RNA purification~~
- ~~2. RNA purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112~~

Results and Data:

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

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

RNA Cultures OD's					
Sample	0 hours	2 hours	4 hours	5 hours	5.6 hours
148-1	0.107	0.168	0.199	0.265	0.286
148-2	0.144	0.195	0.226	0.288	0.307
149-1	0.084	0.142	0.159	0.227	0.262
149-2	0.114	0.150	0.170	0.249	0.302
111-1	0.120	0.163	0.193	0.242	0.252
111-2	0.109	0.142	0.156	0.204	0.218
112-1	0.095	0.138	0.191	0.262	0.299
112-2	0.109	0.129	0.169	0.248	0.304

Clearly my samples took a fair bit longer to grow than normal. I briefly considered that perhaps it was due to the fact I grew up the KRLVS149 and KRLVS112 plates a day earlier than normal, in order to guarantee enough cells. However, this should have, in theory, only affected those cultures then. Otherwise, I placed them in a different location in the shaking incubator than normal, however I highly doubt that this was the reason why they seemed to grow so slowly. Obviously, cells can grow differently from day to day, and I assume it was just that. However, if something is weird about the qRT-PCR results, then perhaps there was something odd going on.

RNA Purification of KRLVS148, KRLVS149, KRLVS111, and KRLVS112

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

12. Wash again with 700 ul Wash buffer
13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

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

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

During the 10 minute spin, three of my tubes seemingly became stuck in the rotor somehow. It took me several moments to extricate them. I forgot to note all three, but sample 5 and 6 were two of the three. The lid broke off on, I believe, sample 5 but I can't quite recall. If there is degradation, I would expect that it came from this issue. Otherwise, nothing notable happened during the purification.

Friday, September 2, 2022

To Do:

1. ~~Process qRT-PCR data of KRLVS148, KRLVS149, KRLVS150, and KRLVS151~~
2. ~~Lab Organization~~

Results and Data:

Wednesday, September 7, 2022

To Do:

- ~~1. cDNA generation of 8/25 RNA samples~~
- ~~2. Set up candidate pKR122 for sequencing~~
- ~~3. Run dishwasher~~

Results and Data:

Generate cDNA (Half) KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/24/22 Pure RNA)

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) 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	X9.5
5X 1st strand buffer	1x	6	57
RNase-free water		2.88	27.36
100 mM DTT	10 mM	3	28.5
10 mM dNTPs	0.5 mM	1.5	14.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	15.49

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

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

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

cDNA Purification with PCR Purification Kit

1. Add 250 uL of Buffer PB to each 50 uL cDNA reaction tube and mix.
2. Place a QIAquick column in a 2mL collection tube.

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

While I was performing my cDNA synthesis I nanodrop'd my candidate pKR122 Miniprep samples, just 1 and 2 based on the diagnostic digest:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR122 1	97.5	1.949	1.064	1.83	2.16
Cand. pKR122 2	131.5	2.631	1.432	1.84	2.15

After I nanodrop'd the samples, I set up sequencing according to the following table:

Sample	Type	Template Name	Primer Name	Template Size (bp)	Concentration (ng/uL)	Plasmid (vol)	H2O (vol)
SS 1	Plasmid	pKR122_1	KROL257	6583	97.5	4.10	5.34
SS 2	Plasmid	pKR122_2	KROL257	6583	131.5	3.04	6.40

Thursday, September 8, 2022

To Do:

- ~~1. DNase treatment of (9/1) RNA samples~~
- ~~2. Second RNA purification of 9/1 samples~~
- ~~3. Nanodrop RNA samples~~
- ~~4. Set up aliquots for cDNA synthesis and RNA gel~~

Results and Data:

Prior to beginning my DNase treatment and the second purification of my RNA samples, I forgot to nanodrop the samples, so I am unsure of the concentration of the total nucleic acids prior to the pure RNA.

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

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

16. Spin max speed 1 min

17. Place flow-through on column again, spin 1 min

Nothing extraordinary occurred while doing the DNase treatment and second RNA purification of my samples. Afterwards, I nanodrop'd my samples:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	385.0	9.625	4.551	2.12	1.41
KRLVS148 2	314.8	7.869	3.746	2.10	2.29
KRLVS149 1	281.3	7.033	3.346	2.10	2.22
KRLVS149 2	251.8	6.294	2.968	2.12	2.01
KRLVS111 1	241.4	6.034	2.955	2.04	1.88
KRLVS111 2	199.4	4.984	2.379	2.09	2.14
KRLVS112 1	266.6	6.665	3.173	2.10	2.21
KRLVS112 2	299.9	7.497	3.531	2.12	2.16

After I nanodrop'd my samples, I set up aliquots for the RNA gel and the cDNA synthesis according to the following tables:

Sample	ng/uL	RNA	Water
KRLVS148 1	385.0	2.60	7.40
KRLVS148 2	314.8	3.18	6.82
KRLVS149 1	281.3	3.55	6.45
KRLVS149 2	251.8	3.97	6.03
KRLVS111 1	241.4	4.14	5.86
KRLVS111 2	199.4	5.02	4.98
KRLVS112 1	266.6	3.75	6.25
KRLVS112 2	299.9	3.33	6.67

Sample	ng/uL	RNA	Water
KRLVS148 1	385.0	7.79	5.71
KRLVS148 2	314.8	9.53	3.97
KRLVS149 1	281.3	10.66	2.84
KRLVS149 2	251.8	11.91	1.59
KRLVS111 1	241.4	12.43	1.07
KRLVS111 2	199.4	15.05	-1.55
KRLVS112 1	266.6	11.25	2.25
KRLVS112 2	299.9	10.00	3.50

Once again, for the sample with too low of a concentration, I simply added 13.5 uL of RNA, since there can't be more volume in the reaction. Given that it worked last time, I am not worried about it. I stored both in strip tubes in my RNA box.

Friday, September 9, 2022

To Do:

1. cDNA synthesis of 9/8 Pure RNA samples

Results and Data:

Generate cDNA (Half) KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/24/22 Pure RNA)

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) 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	X9.5
5X 1st strand buffer	1x	6	57
RNase-free water		2.88	27.36
100 mM DTT	10 mM	3	28.5
10 mM dNTPs	0.5 mM	1.5	14.25
Superscript III (200 U/ul)	10.8 U/ul	1.63	15.49

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' |
| 3 | 42°C | 60' |
| 4 | 70°C | 10' |
| 5 | 4°C | hold |

8. Store at -80°C

Thermocycler had a power failure at Step 3. Hannah let me know, and I asked her to hit 'Ok' on the dialogue box. It specifically said:

Monday, September 12, 2022

To Do:

1. ~~Finish cDNA synthesis~~
2. ~~Purify cDNA samples~~
3. ~~Run gel of 9/8 Pure RNA Samples~~
4. ~~Nanodrop and set up 0.99 ng/uL dilutions of 9/7 cDNA samples~~
5. ~~Nanodrop and set up 0.99 ng/uL dilutions of 9/9 cDNA samples~~

Results and Data:

Finish Generating cDNA of 9/8/22 Pure RNA Samples

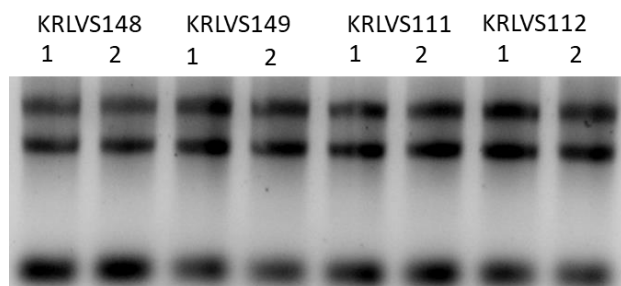
1. Remove RNA from sample by degrading with sodium hydroxide:
 - a. Add 10 ul of 1N NaOH
 - b. Incubate 65°C for 30'
 - c. Neutralize with 10 ul of 1N HCl
 - d. Final volume is 50 ul
2. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!)
3. Elute in 60 ul of 0.1x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

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

1. Make 10 uL aliquots with normalized concentrations, using water to dilute. Then add 2 uL purple loading dye for a final concentration of 1-1.5x.
2. Rinse comb and gel rig with DI water, then ethanol both as well
3. Make fresh agarose gel by adding 0.6g to 60mL fresh 1xTAE and stir with heat until completely dissolved, then place in 50°C water bath until cool enough to touch.
4. Set up gel rig to cast gel, with ladder.
5. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
6. Make fresh 1xTAE, turn gel, add TAE, and remove ladder.
7. Add 12 uL of each sample according to the loading order below.
8. Ran until separated at 113V.
9. Look for distinctive bands (23s, 16s, tRNA), rather than smears.

Gel Loading Order							
Lane1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
RNA 1	RNA 2	RNA 3	RNA 4	RNA 5	RNA 6	RNA 7	RNA 8

While I was removing the RNA from my cDNA synthesis from 9.8, I ran the pure RNA samples on a gel to check for the integrity of the RNA I purified:



The RNA looks good, distinct 23S, 16S, and tRNAs wooo.

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.

After I completed the purification of the cDNA synthesis started on 9.8, I nanodropped all my current cDNA samples, getting the following concentrations:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	15.2	0.461	0.241	1.92	2.05
KRLVS148 2	16.1	0.488	0.253	1.93	2.21
KRLVS149 1	21.1	0.641	0.342	1.88	1.95
KRLVS149 2	21.7	0.656	0.345	1.90	2.12
KRLVS111 1	12.8	0.389	0.198	1.97	2.36
KRLVS111 2	11.1	0.336	0.174	1.93	2.37
KRLVS112 1	19.2	0.582	0.302	1.93	2.12
KRLVS112 2	17.9	0.543	0.283	1.92	2.24

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	30.0	0.908	0.489	1.86	2.05
KRLVS148 2	21.9	0.662	0.361	1.83	1.86
KRLVS149 1	23.9	0.724	0.383	1.89	2.27
KRLVS149 2	25.1	0.759	0.393	1.93	2.16
KRLVS111 1	23.4	0.708	0.376	1.89	2.13
KRLVS111 2	21.6	0.655	0.359	1.82	2.10
KRLVS112 1	22.1	0.669	0.355	1.89	2.38
KRLVS112 2	18.4	0.559	0.293	1.91	2.21

The concentrations and purity look pretty good to me, I think that the purity is better than it has been. When I was doing the purifications, I just left the lids open for a bit longer to encourage ethanol evaporation. After I nanodrop'd the samples, I set up my 0.99 (etc) ng/uL stocks of cDNA according to the following dilutions:

0.99 ng/uL qPCR Stock			
Sample Name	pnc. (ng/u)	DNA uL	EB uL
KRLVS148 1	15.2	3.91	56.09
KRLVS148 2	16.1	3.69	56.31
KRLVS149 1	21.1	2.82	57.18
KRLVS149 2	21.7	2.74	57.26
KRLVS111 1	12.8	4.64	55.36
KRLVS111 2	11.1	5.35	54.65
KRLVS112 1	19.2	3.10	56.90
KRLVS112 2	17.9	3.32	56.68

0.99 ng/uL qPCR Stock			
Sample Name	pnc. (ng/u)	DNA uL	EB uL
KRLVS148 1	30.0	1.98	58.02
KRLVS148 2	21.9	2.71	57.29
KRLVS149 1	23.9	2.49	57.51
KRLVS149 2	25.1	2.37	57.63
KRLVS111 1	23.4	2.54	57.46
KRLVS111 2	21.6	2.75	57.25
KRLVS112 1	22.1	2.69	57.31
KRLVS112 2	18.4	3.23	56.77

After diluting, I prepared water+primer aliquots and added my cDNA to strip tubes for tomorrow.

Tuesday, September 13, 2022

To Do:

1. ~~qRT-PCR of 8/25 RNA samples~~
2. ~~qRT-PCR of 9/8 RNA samples~~
3. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
4. ~~Supplement MHB~~

Results and Data:

qRT-PCR of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/25/22) cDNA

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

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

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

Labelling was as follows:

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

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

Loaded 96-well plate according to the following table:

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

F	A6	B6	C6	D6
G	A7	B7	C7	D7
H	A8	B8	C8	D8

qRT-PCR of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (9/8/22) cDNA

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

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

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

Labelling was as follows:

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

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

Loaded 96-well plate according to the following table:

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

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

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

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

Wednesday, September 14, 2022

To Do:

1. Transform ligation from 8/29 of pKR122 into *E. coli*
2. Patch out KRLVS28 and KRLVS75 on Kan x2
3. Enter GFP strains into worksheet
4. Patch out GFP strains on Kan plates
5. Make LB+ Kan plates

Results and Data:

Transformation of *PrpsU2 tul4* 5'UTR 5X and 3X Ligation (8/29) into *E. coli*

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

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR89	1 uL	100 uL	1
2	(-) control	None	0	Remaining	1
3	Backbone Ligation	BB only ligation	8 uL	100 uL, Rem.	2
4	3x Ligation	3x Ligation	8 uL	100 uL, Rem.	2
5	5x Ligation	5x Ligation	8 uL	100 uL, Rem.	2
Total number of plates					8

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

Reagents

LB Agar + Kanamycin

Melt LB agar and then cool at 56°C

0.5 mL 50 ug/mL Kanamycin

Pour plates (24 mL)

Thursday, September 15, 2022

To Do:

- ~~1. Count transformation plates~~
- ~~2. Make overnights of candidate pKR122~~
- ~~3. Make single use aliquots of GFP strains~~
- ~~4. Patch out KRLVS28 and KRLVS75 on regular CHAH~~

Results and Data:

Transformation Counts			
	3x	5x	BB
100 uL	1	1	0
Remaining	1	5	8

Most of the colonies on the plates were very small, but I picked every colony I (at least thought I) saw. Obviously, it is not a great ratio, but that's all I had.

Making Single Use Aliquots of KRLVS192 and KRLVS193

1. Prepare 800uL of MHB in a 1.5mL 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 to the 800uL mix by pipetting
5. Aliquot 50ul of solution to tubes, freeze at -80

Friday, September 16, 2022

To Do:

- ~~1. Miniprep candidate pKR122~~
- ~~2. Run diagnostic digest of candidate pKR122~~
- ~~3. Run gel of diagnostic digest~~
- ~~4. Make single use aliquots of KRLVS28 and KRLVS75~~
- ~~5. Make EC cells from KRLVS28 and KRLVS75~~
- ~~6. Streak KRLVS192 and KRLVS193 to single colony on CHAH + Kan~~
- ~~7. CHAH Kan~~

Results and Data:

Miniprep of Candidate pKR122 from *E. coli*

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

9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Diagnostic Digest of Candidate pKR122 Miniprep w/KpnI and NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR122	KpnI, NotI	2.0	-
2	Cand. pKR122 1	KpnI, NotI	2.0	-
3	Cand. pKR122 2	KpnI, NotI	2.0	-
4	Cand. pKR122 3	KpnI, NotI	2.0	-
5	Cand. pKR122 4	KpnI, NotI	2.0	-
6	Cand. pKR122 5	KpnI, NotI	2.0	-
7	Water	KpnI, NotI	-	2.0

2. Set up master mix table:

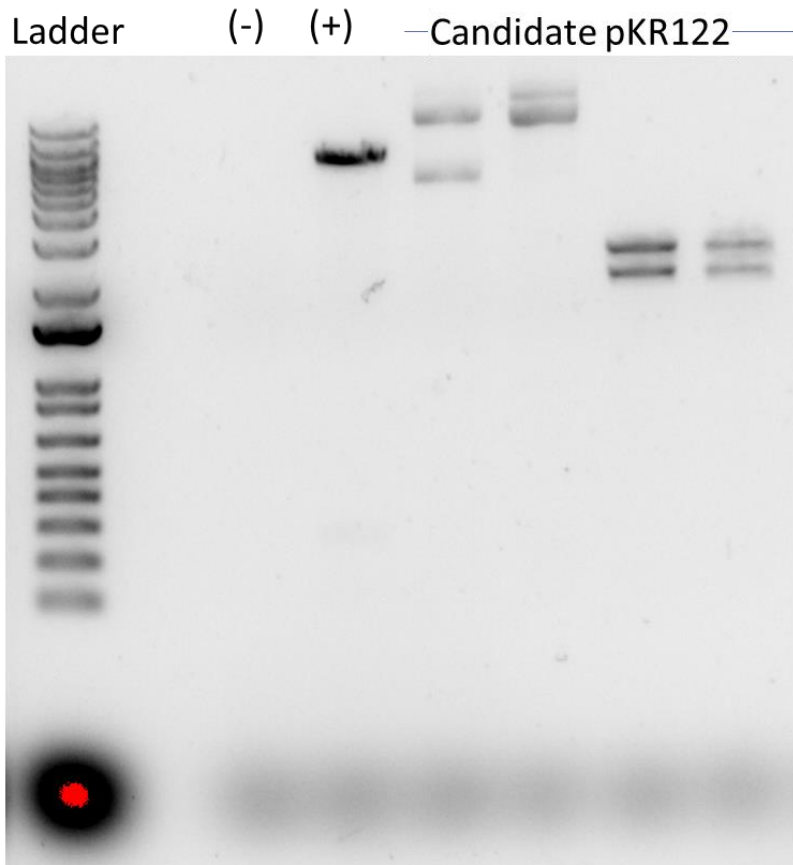
Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H ₂ O	15	120
10x Buffer (Cutsmart)	2	16
DNA	(2)	
KpnI	0.5	4
NotI	0.5	4
Total	20.0 (18.0 actual b/c of DNA)	144

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

Gel of Diagnostic Digest of Candidate pKR122 Miniprep

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

Loading Order of Diagnostic Digest of Candidate pKR122									
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Ladder		(-)	(+)	1	2	3	4	5	



Preparing Electrocompetent KRLVS28 and KRLVS75 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 $\sim 1 \times 10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.

I resuspended both strains in 120 uL of 10% sucrose. This will allow me to do one positive control and one transposon reaction for each, as well as leave a little bit to streak on a plate for the negative control.

Making Personal Glycerol Stocks of KRLVS28 and KRLVS75

1. Make 1 cryovial for each strain, label!
2. Prepare 1.6mL of MHB in a 2mL centrifuge tube
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 400ul of 75% glycerol to the 1.6mL mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. For single use stocks pipet 50ul of solution to tubes

Monday, September 19, 2022

To Do:

1. ~~Electroporate pKR141 into EC cells~~
2. ~~Make patches of KRLVS192 and KRLVS193~~

Results and Data:

Electroporating pKR141 into electrocompetent KRLVS28 and KRLVS75

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 pKR141 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 3 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
 - a. Plated 10 µL, 100 µL, 200 µL, and 1 mL of cells spun down and resuspended in ~100 µL of liquid
8. Incubate plates at 37°C for 3 days

I went through the protocol that Aisling used when she used this plasmid, and for the most part it was the same as the standard electroporation protocol, however, the recovery time was very specifically three hours so that is what I will do. Additionally, since we do not know the transposon efficiency, I plated a variety of volumes for both strains.

Both of my electroporations arced which isn't fantastic, but has previously been okay so here's hoping. Additionally, KRLVS28 + pKR141 has a whole bunch of cells, visibly. But KRLVS75 + pKR141 had very few cells, so I may have to redo that electroporation, but I have about 70 µL of competent cells left so hopefully that'll work out.

Tuesday, September 20, 2022

To Do:

1. ~~Digest pKR128 and *PrpsU2_tul4* 5'UTR PCR fragment with NotI/KpnI~~
2. ~~Run digest on gel and excise fragments~~
3. ~~Make YEPD plates~~

Results and Data:

DNA Digest of *PrpsU2_tul4*UTR gBlock PCR and pKR128 Backbone w/KpnI and NotI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	<i>PrpsU2_tul4</i> UTR	NotI, KpnI	15	-
2	pKR128	NotI, KpnI	5	10

2. Set up master mix table:

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

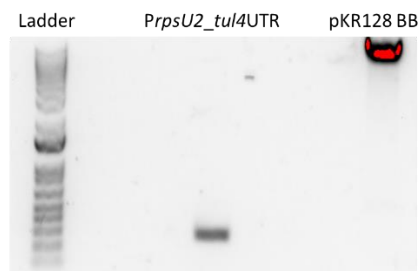
3. Add 15 uL of Master Mix to individual tube for digest.
4. Mix by pipetting up and down.
5. Incubate at 37°C for 1 hour.
6. After digest: add 1 uL of QuickCIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes, then put at 80°C for 2 minutes to inactivate the enzyme.

Gel of Digested *PrpsU2_tul4*UTR PCR Fragment + pKR89 Backbone

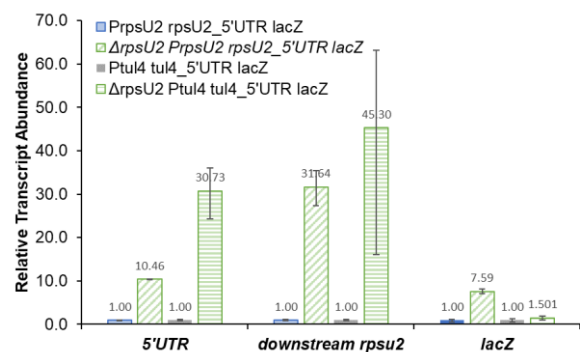
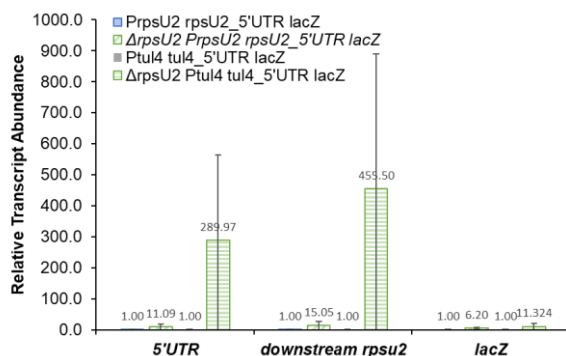
1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
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. Placed fragments in the fridge to purify tomorrow.



Additionally, I finally processed my qRT-PCR data. My 9.12 cDNA samples came out predictably, but my 9.7 cDNA samples came out super weird, Kathryn agreed in the meeting that I ought to re-dilute my cDNA samples and re-run the qRT-PCR.



Reagents

YPD Plates

1. For 250 mL of YPD, weigh out the components and add to a 500 mL flask:
 - a. 2.5 g Yeast extract
 - b. 5.0 g Protease-Peptide

- c. 5.0 g Glucose
- d. 5.0 g Agar
2. Add 250 mL type I ddiH2O
3. Stirred on plate until combined
4. Autoclave 15 minutes on the liquid cycle
5. Pour plates
6. Let dry for 2-3 days prior to bagging, can be stored for 3 months at room temperature or longer in the fridge

Wednesday, September 21, 2022

To Do:

- ~~1. Set up cultures for temperature environment experiment in duplicate~~
- ~~2. Conduct temperature environment experiment in duplicate~~
- ~~3. Run GFP assay on temperature conditions~~
- ~~4. Patch out yeast~~
- ~~5. Purify gel extracts~~

Results and Data:

Testing the Effect of Temperature on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Experimental Layout			
Condition Number	1	2	3
Experiment Component	Control	Test 1	Test 2
Condition	37°C	4°C	42°C

1. Begin by streaking out relevant GFP tagged strains to single colony on selective antibiotic plates, 3 days prior to experiment if no growth defect is expected
2. Patch out each strain in duplicate/triplicate per condition on CHAH+selective antibiotic the day prior to the experiment
3. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution in the spectrophotometer
4. Normalize samples to an OD of 0.08-0.10 in 8 mL of MHB.
5. Shake cells at 37°C to an OD of 0.25-0.30, typically around 4 hours.
6. Once desired OD is reached, split tubes between conditions: 4°, 37°, and 42°C; shaking for an additional hour
 - a. To accommodate the resources in the cold room, add tube volume to baffled flask and place on nutator for aeration
7. Then follow the GFP reporter assay protocol, starting at Step 4, normalizing each sample to its own OD, then to the fluorescence of standard condition, cells continued to shake at 37°C

OD Readings			
Sample	0 hours	2 hr 45'	4 hours
KRLVS192 1	0.094	0.200	0.311
KRLVS192 2	0.083	0.172	0.282
KRLVS192 3	0.079	0.178	0.275
KRLVS192 4	0.092	0.197	0.307
KRLVS192 5	0.084	0.202	0.292
KRLVS192 6	0.082	0.185	0.293
KRLVS193 1	0.082	0.177	0.291
KRLVS193 2	0.074	0.188	0.269
KRLVS193 3	0.097	0.168	0.288

KRLVS193 4	0.092	0.203	0.319
KRLVS193 5	0.074	0.150	0.248
KRLVS193 6	0.096	0.197	0.300

Sample Label Condition											
4°C		37°C		42°C		4°C		37°C		42°C	
192 1	192 2	192 3	192 4	192 5	192 6	193 1	193 2	193 3	193 4	193 5	193 6

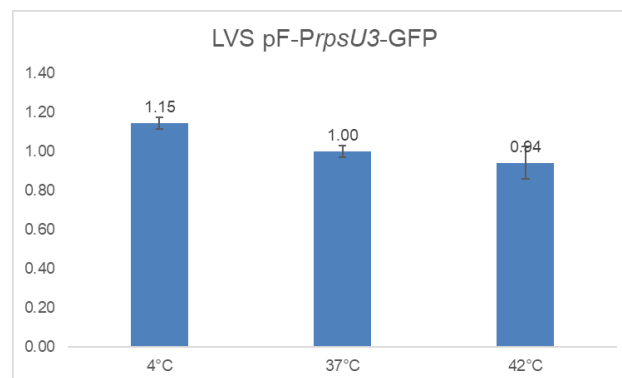
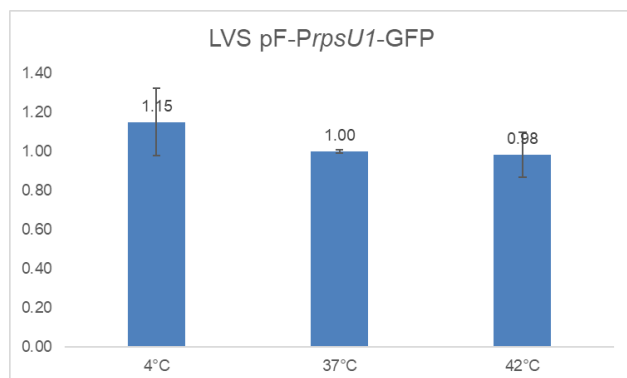
GFP Assay on Temperature Conditions in KRLVS192 and KRLVS193

1. Take 1 mL from each culture tube into microcentrifuge tubes. 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 microfuge tube in triplicate into a clear 96-well plate (not tissue culture treated). Pipette PBS in triplicate as a 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)
 - c. Copy and paste results into an excel file on the plate reader's computer
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
 - f. Copy and paste results into excel file, then save onto flash drive
9. Analyze by dividing fluorescence for each well by OD600 for each well. Normalize each well to the wild-type strain such that wild-type is set to 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	192 1		192 1		192 1		193 1		193 1		193 1	
B	192 2		192 2		192 2		193 2		193 2		193 2	
C	192 3		192 3		192 3		193 3		193 3		193 3	
D	192 4		192 4		192 4		193 4		193 4		193 4	
E	192 5		192 5		192 5		193 5		193 5		193 5	
F	192 6		192 6		192 6		193 6		193 6		193 6	
G	1xPBS		1xPBS		1xPBS							
H												

Spoke to Hannah and she suggested always doing a single replicate of LVS, which I will do with my next experiment, in order to know if the fluorescent cells need to be condensed. This will mean that I subtract the LVS fluorescence from each sample, and then divide the test replicates by the control replicates in order to get my fluorescent values. This subtraction helps to eliminate the basal fluorescent input by LVS, in order to more clearly see whether fold changes are significant or not.

Additionally, when I was performing the actual GFP assay and transferring volume to the black plate from the white plate, I had some issues with pipetting. I drew up some liquid, saw a large air bubble and dispensed, and then continued to have issues getting the right volume for a couple of the technical replicates. Hannah recommended that I keep the pipet tips at the very bottom of the wells. While my standard deviations were a bit high for some of the technical replicates, I think they were probably okay.



I performed a t-test on the samples, and there was no significant difference between either 4° or 42° with 37°. So dang, not temperature! It's definitely going to be a CDM media change lol.

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)
<i>PrpsU2 tul45</i> UTR	160	480	160
pKR128 Backbone	371	1130	371

I stored the gel purified samples in my cloning box, labelled as per typical with the description of the fragment, dig., gp, and the date.

Thursday, September 22, 2022

To Do:

1. Apply overlay to electroporation plates
2. Make MHB with 10% NaCl
3. Sterilize water
4. Make yeast overnight for EC cells
5. Run overnight ligation of pKR128 backbone with *PrpsU2 tul4 5'*UTR gel purified fragment

Results and Data:

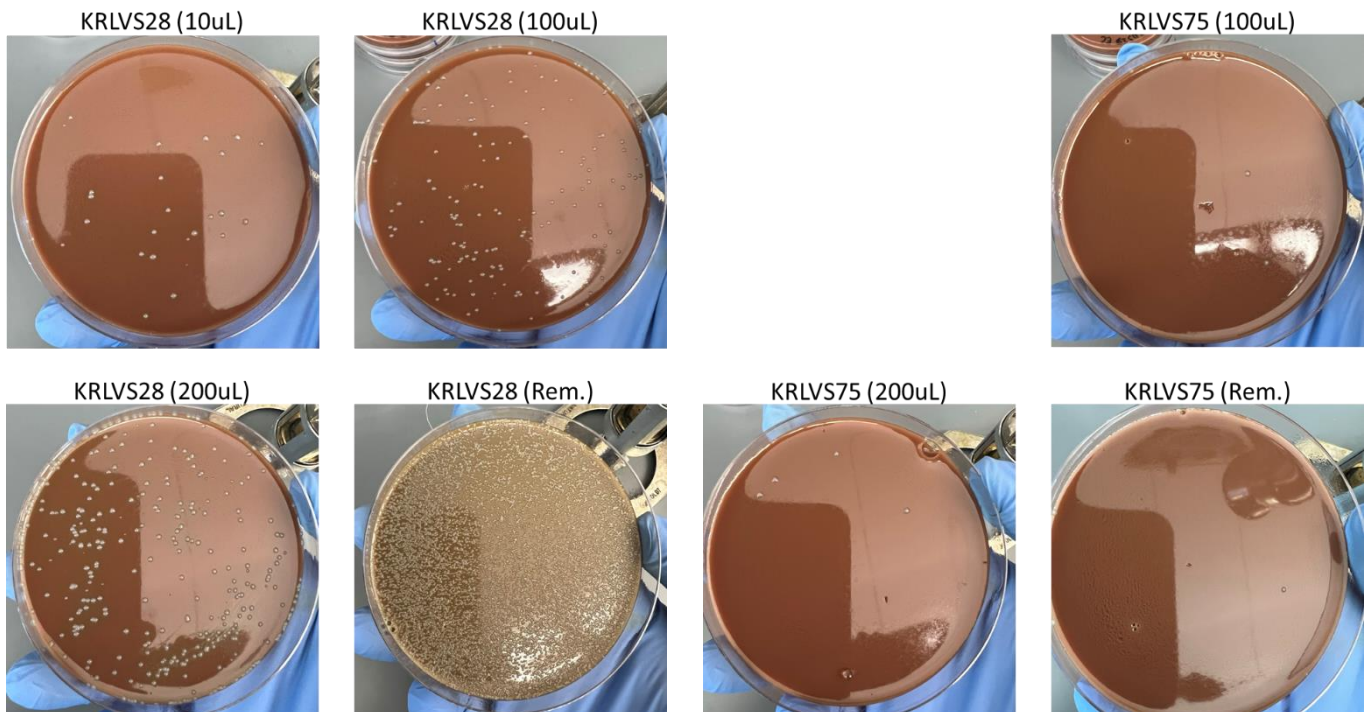
Agar overlay for β -galactosidase activity in KRLVS28 and KRLVS75 + pKR141

1. Prior to overlay protocol, incubate intended cells on CHAH until single colonies form.
2. On the day of the experiment:
 - a. Prepare soft agar overlay solution by adding the following components to a flask:
 - 60 mL of diH₂O
 - 0.42 g of agar
 - b. Add stir bar
 - c. Stir for 5 min
 - d. Autoclave Liquid 30
3. Place agar in water bath at 56°C until cool enough to touch. Add 120 uL x-gal to agar.
4. Overlay 8mL of the agar solution to each plate. Protect from light by wrapping in foil. Incubate lid side up.
5. The next day look for any blue colonies
 - a. Dark blue indicates high β -galactosidase activity, light blue indicates some, white indicates none
6. Isolate colonies of interest

I made the overlay in the morning and autoclaved it in the Instant Pot. Due to class I asked Hannah to remove it and my salty MHB, and to play the overlay in the water bath. It was stored at 56°C for a few hours due to my class and then the rain/thunderstorms keeping me away from Avedisian. There didn't seem to be any problem with the overlay from eye, and although not all the agar dissolved it dissolved while being autoclaved.

Just realized I accidentally used agarose instead of agar, yikes. There shouldn't be an issue since agarose is the purified version of agar, but I am dumb and that was expensive. At least I didn't have to use too much?

I did actually get colonies on the KRLVS75 plates, though I had substantially fewer cells, so I will do the overlay on each plate that has colonies (only the 10 uL didn't, which... makes sense). Regardless, KRLVS28 had quite a few more colonies.



For the future, as long as I have the proper number of cells, I believe that a few plates at 200 uL will suffice, if I need to do it again, which, I probably will because of KRLVS75 lmao.

Prior to adding the overlay, I allowed the soft agar to cool for a period of time, after removing it from the waterbath and adding the x-gal, after I stirred it with the x-gal. I wrapped it in foil during this period of waiting. Once it didn't burn my hand to touch, I added the 8 mL of overlay to the plates. I tried to add it gently as to not disturb the colonies, however I did not move the serological pipet at first and did swirl the first few plates in order to spread the overlay. After the first few, I moved the serological pipet in order to distribute the overlay without swirling the plates. After I applied the overlay, I covered my stack of plates with foil, and left them on my bench to set for several minutes prior to putting them into the incubator.

Ligation of *PrpsU2 tul4* 5'UTR PCR from gBlock with pKR128 backbone

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

Tube	Insert	Backbone
1	KpnI, NotI dig., pur. <i>PrpsU2:tul4</i> 5'UTR PCR	KpnI, NotI digested, purified pKR128
2	-	KpnI, NotI digested, purified pKR128

2. Set up master mix table:

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

3. Obtain ice to assemble and keep the reactions on. This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold in order to avoid degradation.
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 H₂O (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
6. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Incubate at 16°C overnight.

Making electrocompetent cells – Day 1

1. Have a thick patch of *S. cerevisiae* parental strain grown up on a YPD plate in advance. Grow 250 mL overnight cultures of yeast strain in YEPD media at 30C, shaking at 250 rpm. Start from plates and resuspend to OD of approximately 0.06 if from an older plate or 0.01 if from a new plate.

After speaking with Hannah yesterday about the differences in her experiments between OD 0.06 and 0.01, decided to go with OD 0.01 rather than 0.06, since if they undergrow they can continue growing. I calculated for 0.0115, to cover the 30%, could not check to make sure it was right because OD <0.05

Reagents

Mueller Hinton Broth with 10% NaCl (100 mL)

To make, add to a 250 mL bottle:

- 2.1g of Mueller Hinton Broth mix
- 10 g NaCl
- 100 mL of diH₂O

Autoclave on Liquid 30'

Can keep un-supplemented media at room temperature indefinitely

To supplement add:

1mL of 10% glucose

1mL of 2.5% iron pyrophosphate

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

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

Friday, September 23, 2022

To Do:

- ~~1. Patch out promising colonies from electroporation overlays~~
- ~~2. Prepare electrocompetent yeast cells~~
- ~~3. Electroporate yeast with ligation~~

Results and Data:

When I got in, around 13 hours after I started the culture, the OD was 0.94, so I let it grow for another hour and fifteen minutes, at which point it was OD 1.31 so I went forward with the protocol.

Unfortunately, I did not realize there was an o-ring in the 250 mL centrifuge bottles and a quarter of my sample was sucked out of the bottle. I cleaned the centrifuge and then continued with that volume.

Making electrocompetent cells

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

Electroporation of cells

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

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	Number of Plates
1	pKR122	Lig. 1	8 uL	200 uL, Rem.	2
2	Backbone	Lig. BB	8 uL	200 uL, Rem.	2
3	(+) control	pKR128	3 uL	200 uL, Rem.	2
4	(-) control	-		200 uL	1
				Total:	7

Decided to plate 200 uL and remaining rather than 100 uL and 500 uL after speaking with Hannah and reviewing her colony counts and success rate. I misinterpreted the amount of plasmid to add for my positive control, so rather than adding 1 ug, I just added 3 uL of the stock plasmid. For the negative control, I just plated 200 uL of the straight competent cells, which was a mistake, the cells were too thick and made the plate sticky. I don't think it will be an accurate representation.

On a different note, I realized as I was putting away the remaining -ura plates that they technically expired in June, so this might not actually work at all, but we'll see. The plates certainly weren't dry or anything, but I couldn't quite find any information on other people using them for longer, and in my quick glances at potential recipes I wasn't quite sure what was expiring. Who knows.

Next, I looked at my overlay plates, and there definitely seemed to be some dark blue colonies, as well as some white and lighter colonies. I wasn't completely confident on identifying the dark blue colonies on my own this first time (I probably should have struck out the strain that Caterina and I used to test the overlay method, as a positive control for comparison, whoops). Kathryn came by to look at the plates with me and she picked out some colonies that she thought were interesting and marked the soft agar for me. The plan is for me to patch them out and freeze them down. After I freeze them down, I can do some validation experiments. This may include another overlay, but will definitely include cross-patching on CHAH and CHAH+x-gal so we can see which do not grow, because if the colonies do not grow then we know that they are producing a bunch of *lacZ*.

I patched out the colonies that Kathryn indicated on my plate. I am leaving them out at room temperature over the weekend. The patches were labelled as follows:

Patch Number	Electroporation	Description
1	KRLVS28 + pKR141	Very small dark blue colony
2	KRLVS28 + pKR141	Very small dark blue colony
3	KRLVS28 + pKR141	Average size colony with dark blue margin and white center
4	KRLVS28 + pKR141	Very small colony with dark blue margin and white center
5	KRLVS28 + pKR141	Average size dark blue colony
6	KRLVS28 + pKR141	Large colony with dark margin and light center
7	KRLVS28 + pKR141	Small dark blue colony
8	KRLVS28 + pKR141	Moderately small dark blue colony
9	KRLVS28 + pKR141	Very small colony with dark blue margin and light center
10	KRLVS28 + pKR141	White colony, for control

Kathryn determined that the plates with few colonies were not good to use because their colony color could not really be compared to any other colonies on the plate. This means that I will definitely have to redo the KRLVS75 electroporation.

Monday, September 26, 2022

To Do:

1. ~~Re-dilute cDNA from 8/25~~
2. ~~qRT-PCR of 8/25 RNA cDNA samples — Redo~~
3. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
4. ~~Supplement MHB~~
5. ~~Make overnights from yeast transformation~~

Results and Data:

Due to the weird results I got on the previous qRT-PCR of the 9/7 cDNA samples, I decided I would need to re-run the qRT-PCR, but I thought it would be best to re-dilute the cDNA samples. Just in case, it was an issue here rather than a mechanical issue. I got the following nanodrop concentrations:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS148 1	16.4	0.498	0.273	1.82	1.70
KRLVS148 2	18.7	0.567	0.325	1.74	1.82
KRLVS149 1	21.9	0.665	0.375	1.77	1.88
KRLVS149 2	24.0	0.727	0.404	1.80	1.82
KRLVS111 1	14.2	0.431	0.235	1.83	1.87
KRLVS111 2	12.4	0.377	0.204	1.85	1.69
KRLVS112 1	21.6	0.655	0.382	1.72	1.72
KRLVS112 2	18.1	0.549	0.316	1.73	1.78

After getting these new concentrations, I re-diluted the cDNA according to the following calculated table:

0.99 ng/uL qPCR Stock			
Sample Name	Conc. (ng/uL)	DNA uL	EB uL
KRLVS148 1	16.4	3.62	56.38
KRLVS148 2	18.7	3.18	56.82
KRLVS149 1	21.9	2.71	57.29
KRLVS149 2	24	2.48	57.52
KRLVS111 1	14.2	4.19	55.81
KRLVS111 2	12.4	4.79	55.21
KRLVS112 1	21.6	2.75	57.25
KRLVS112 2	18.1	3.28	56.72

qRT-PCR of KRLVS148, KRLVS149, KRLVS111, KRLVS112 (8/25/22) cDNA

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

Component	Volume per Reaction	Master Mix (#Samplesx3.5+3.5)
PowerUp SYBR Green MM	10 uL	315.0 uL
5uM primer set	1 uL	31.5 uL
1.5 ng/uL Stock cDNA	1 uL	
ddiH ₂ O	8 uL	252.0 uL
Total:	20 uL	598.5 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	KROL343/344 5'UTR
B	KROL504/505 ds <i>rpsU2</i>
C	KROL399/400 <i>lacZ</i>
D	KROL63/64 <i>tul4</i>

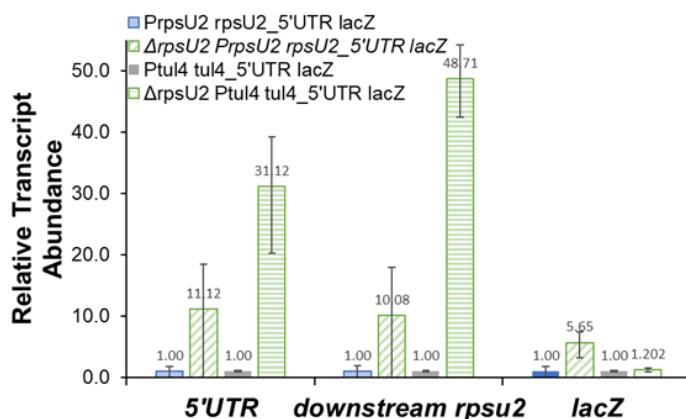
1	2	3	4	5	6	7	8
148-1	148-2	149-1	149-2	111-1	111-2	112-1	112-2

Loaded 96-well plate according to the following table:

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

As I was setting up the qRT-PCR, I ran out of "C" Master Mix for the last sample, C8, so I quickly made a little bit extra (20 uL Sybr Green MasterMix, 2 uL KROL399/400, 16 uL of water), but this does make me worry for the rest of these samples. Which pretty much sucks a lot because *lacZ* is really the only one we super care about in this case. Hopefully it'll be fine, I may perish if I have to repeat the qPCR or RNA purification. :")

Weirdly enough, it was in downstream of *rpsU2* (master mix for "B" which had issues, specifically KRLVS148 both had larger standard deviations than should have been between technical replicates). Regardless, this data looks substantially better.



After I set up the qRT-PCR, I counted my yeast transformation plates from Friday. I was having difficulty distinguishing the colonies on the remaining plate, so Hannah double checked them for me.

Volume Plated	BB Only	Ligation
200 uL	0	7
Remaining	0	8

Hannah suggested I screen two colonies to start since there were no backbone colonies. Thus, I inoculated two tubes containing 5 mL SD-ura media with one colony each and stored the two ligation plates in the fridge.

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

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

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

Tuesday, September 27, 2022

To Do:

- ~~1. Patch out LVS, KRLVS192, and KRLVS193~~
- ~~2. Miniprep candidate pKR122 plasmids from yeast~~
- ~~3. Freeze down patches from electroporation overlay assay~~

Results and Data:

Miniprep of Candidate pKR122 Plasmid from Yeast

1. Aliquot 3x 1.5 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-enzyme mixture by combining 15 ul of Zymolyase with 1 mL of Solution 1, then adding 200 ul to each pellet. If cells are from stationary phase, add more Zymolyase to ensure efficient lysis.
3. Incubate at 37C for 30 minutes. Then add ~100 ul of glass beads and strap tubes to a vortex set to high speed to help lyse cells, for 30 additional minutes.
4. Add 200 ul Solution 2 to each tube and mix well.
5. Add 400 ul Solution 3 to each tube and mix well. Centrifuge at maximum speed for 3 minutes.
6. Transfer the supernatant from one tube to the Zymo-Spin I Column in a collection tube and centrifuge at >10,000 x g for 30 seconds. Add supernatant from next tube and centrifuge again until entire culture has been added to one column.
7. Discard the flow-through and ensure the flow-through does not come into contact with the column tip.

8. Add 550 ul DNA Wash Buffer to the Spin Column and centrifuge at $>10,000 \times g$ 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 $10,000 \times g$ for 1 minute to elute the plasmid DNA. Put the eluate back on the column, let sit, and centrifuge again

Freezing Down KRLVS28 Transposon Integrants

1. Make 1 cryovials for each mutant patch, label!
2. Prepare 0.8 mL of MHB in a 1.5mL tube
3. Scrape up plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 200ul of 75% glycerol to the 0.8mL mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80

Not all of my patches grew up, so the glycerol stocks are labelled as such:

Tube Number	Electroporation	Description
1	KRLVS28 + pKR141	Very small dark blue colony
2	KRLVS28 + pKR141	Average size colony with dark blue margin and white center
3	KRLVS28 + pKR141	Average size dark blue colony
4	KRLVS28 + pKR141	Large colony with dark margin and light center
5	KRLVS28 + pKR141	Small dark blue colony
6	KRLVS28 + pKR141	Moderately small dark blue colony
7	KRLVS28 + pKR141	Very small colony with dark blue margin and light center
8	KRLVS28 + pKR141	White colony, for control

Wednesday, September 28, 2022

To Do:

- ~~1. Set up cultures for NaCl environmental condition test~~
- ~~2. Conduct NaCl environmental condition protocol~~
- ~~3. GFP assay for NaCl environmental conditions~~

Results and Data:

Testing the Effect of Salinity on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Experimental Layout				
Condition Number	0	1	2	3
Experiment Component	Background	Control	Test 1	Test 2
Condition	0% NaCl	0% NaCl	0.85% NaCl	3% NaCl

1. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution in the spectrophotometer
2. Normalize samples to an OD of 0.08-0.10 in 40 mL of MHB+Kan in baffled flasks.
3. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
4. Prepare %NaCl MHB by:
 - a. 3% NaCl
 - i. 6 mL 10% NaCl MHB
 - ii. 14 mL MHB
 - b. 0.85%

- i. 1.7 mL 10% NaCl MHB
 - ii. 18.3 mL MHB
5. Centrifuge 10 mL of each strain/replicate and resuspend in 10 mL of MHB, 0.85% NaCl MHB, or 3% NaCl MHB.
6. Shake cells at 37°C for an additional 1-1.5 hours to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, normalizing to the OD, then to the fluorescence of each strain to standard condition, MHB with no NaCl supplementation

Experimental Set-Up												
Strain	KRLVS192 BR-1			KRLVS192 BR-2			KRLVS193 BR-1			KRLVS193 BR-2		
Culture Vol.	40 mL			40 mL			40 mL			40 mL		
%NaCl	0.0%	0.85%	3.0%	0.0%	0.85%	3.0%	0.0%	0.85%	3.0%	0.0%	0.85%	3.0%
Separated Vol.	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL

I included a 12 mL culture of LVS alongside KRLVS192 and KRLVS193, in order to exclude background from LVS+PBS. I started the LVS at the same OD as KRLVS192 and KRLVS193, spun down 10 mL of the culture, resuspended in 10 mL of fresh MHB and shook at 37°C for the additional growing time.

OD Readings			
Sample	0 hours	2 hours	4 hours
KRLVS192 1	0.103	0.175	0.301
KRLVS192 2	0.082	0.151	0.287
KRLVS193 1	0.095	0.176	0.338
KRLVS193 2	0.096	0.277	0.324
LVS	0.103	0.161	0.292

It looks like KRLVS193 2 is contaminated based on the growth rate, that certainly is not LVS. The MHB had kan in it, and clearly that OD is not LVS, so it must be kan resistant *E. coli* (maybe not resistant, since the concentration of kan for LVS is much lower)? I cleared off plates from my bench this morning, though I thought those were only yeast. I don't think it's yeast contamination, unless yeast can grow in MHB? I will ask Kathryn how to move forward, whether I should only test KRLVS192 or what.

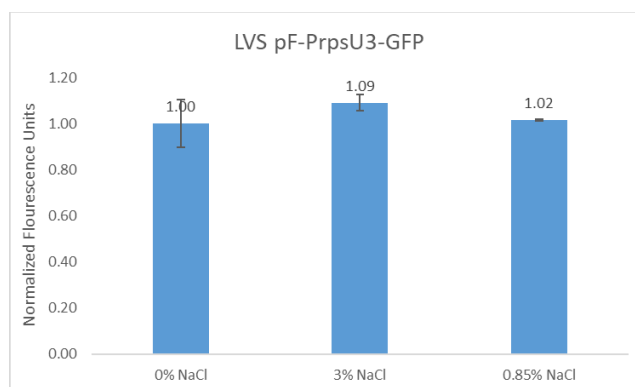
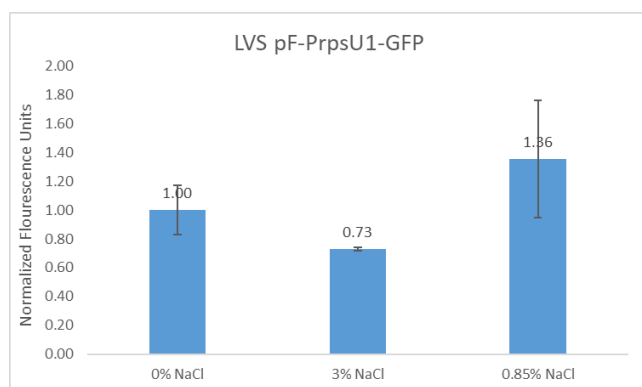
I spoke with Kathryn and she recommended that I make technical replicates for KRLVS193, clearly I do not have enough to do 10 mL cultures, so instead I will do 6 mL cultures for each replicate. This means that I will spin down 6 mL of culture and resuspend in 6 mL of culture.

GFP Assay on Salinity Conditions in KRLVS192 and KRLVS193

1. Take 1 mL from each culture tube into microcentrifuge tubes. 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 microfuge tube in triplicate into a clear 96-well plate (not tissue culture treated). Pipette LVS in PBS in triplicate as a 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)
 - c. Copy and paste results into an excel file on the plate reader's computer
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
 - f. Copy and paste results into excel file, then save onto flash drive

	1	2	3	4	5	6	7	8	9	10	11	12
A	192 1		192 1		192 1		193 1		193 1		193 1	
B	192 2		192 2		192 2		193 2		193 2		193 2	
C	192 3		192 3		192 3		193 3		193 3		193 3	
D	192 4		192 4		192 4		193 4		193 4		193 4	
E	192 5		192 5		192 5		193 5		193 5		193 5	
F	192 6		192 6		192 6		193 6		193 6		193 6	
G	LVS		LVS		LVS							
H	1xPBS		1xPBS		1xPBS							



According to the t-test there was no significant difference in the fluorescence of either KRLVS192 or KRLVS193 when exposed to increasing concentrations of salinity.

Thursday, September 29, 2022

To Do:

1. ~~Set up candidate plasmids for sequencing~~
2. ~~Supplement MHB~~
3. ~~Put away dishes~~
4. ~~Patch out LVS, KRLVS192, and KRLVS193~~

Results and Data:

I was finally able to nanodrop my miniprep samples, however, I realized after thawing my tubes that my first pKR122 candidate did not have any volume. I 'm not entirely sure what happened, however the best approximation that I can think of is that in my rush to get to class I ejected my tip in the re-elution step prior to adding the volume back into the column. I re-blanked and sampled (the unlabeled sample). Clearly, the contamination was super bad, maybe I didn't bead beat well enough? Or sucked up some debris, I wasn't able to sequence it due to the purity and will have to redo.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Candidate pKR122	18.0	0.359	0.497	0.72	0.43
	23.4	0.469	0.375	1.25	0.53

OD Readings		
Sample	0 hours	4 hours
KRLVS192 1	0.074	0.260
KRLVS192 2	0.073	0.243
LVS	0.050	0.274

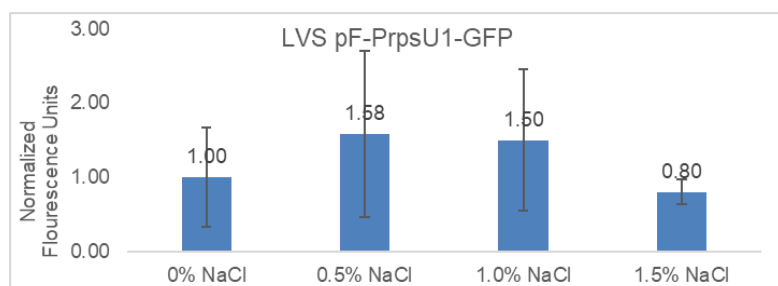
GFP Assay on Salinity Conditions in KRLVS192 and KRLVS193

1. Take 1 mL from each culture tube into microcentrifuge tubes. 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 microfuge tube in triplicate into a clear 96-well plate. Pipette LVS in PBS in triplicate as a control.
5. Go to INBRE lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
6. Read OD600 from clear plate on ID3 plate reader:
 - a. Select Absorbance, wavelength=600
 - b. Plate type: 96-well standard clearbtm (first option)
7. Transfer 200 ul of each well from clear plate to black plate using the multichannel
8. Read fluorescence from black plate on ID3 plate reader:
 - a. Select fluorescence
 - b. Wavelength: 495 to 535
 - c. Plate type: CoStar 3789
 - d. Gain: Automatic
 - e. Integration: 380 ms

Label	Condition
A	0% NaCl
B	0.5% NaCl
C	1.0% NaCl
D	1.5% NaCl

Label	Strain and BR
1	KRLVS192 1
2	KRLVS192 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1		A1		A1		LVS		LVS		LVS	
B	A2		A2		A2		1xPBS		1xPBS		1xPBS	
C	B1		B1		B1							
D	B2		B2		B2							
E	C1		C1		C1							
F	C2		C2		C2							
G	D1		D1		D1							
H	D2		D2		D2							



October 2022

Monday, October 3, 2022

To Do:

1. ~~Make MHB~~
2. ~~Make CHAH+xgal plates~~
3. ~~Make CHAH+nat plates~~
4. ~~Make 5N NaOH~~
5. ~~Start overnights of candidate plasmid containing yeast colonies~~

Results and Data:

5 pm: started two 5 mL cultures in SD -ura media of my yeast transformants, shaking at 30°C overnight

Reagents

Mueller Hinton Broth (500 mL)

To make, add to a 1 L bottle:

- 10.5g of Mueller Hinton Broth mix
- 500 mL of diH₂O

Autoclave on Liquid 30'

Can keep un-supplemented media at room temperature indefinitely

CHAH+Nat or Xgal

To x2 500mL flask add:

- 15.3g of cystine heart agar
 - 150mL of ddiH₂O (type I)
- stirbar

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

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

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

Using sterile technique, pour hemoglobin into CHA

For 5 ug/ml nat, add 15 ul of 100 mg/mL nourseothricin to 300 mL of media

For 100 ug/mL X-gal, add 600 uL of 50 mg/mL X-gal to 300 mL of media

5N NaOH

To 250mL beaker add:

- 20.0g of NaOH pellets
- stirbar

Place beaker on stir plate and begin to stir prior to adding 80 mL of diH₂O

Once dissolved, removed from stir plate and allow to cool

Once cool, correct volume to 100 mL

Tuesday, October 4, 2022

To Do:

1. ~~Patch out LVS, KRLVS192, and KRLVS193~~
2. ~~Miniprep candidate pKR122 from yeast~~
3. ~~Autoclave plate waste~~
4. ~~Supplement MHB~~
5. ~~Set up for environmental experiment~~
6. ~~PCR of candidate pKR122 plasmids from yeast~~

Results and Data:

My cultures were not as grown up as I think they should have been. If I have to repeat the miniprep again, I will probably start the cultures much earlier. I started them at 5 pm last night and took them out at 7:15 or so this morning, but I believe I read somewhere the overnight yeast cultures could go for 16-24 hours.

Miniprep of Candidate pKR122 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-enzyme mixture by combining 15 ul of Zymolyase with 1 mL of Solution 1, then adding 200 ul to each pellet. If cells are from stationary phase, add more Zymolyase to ensure efficient lysis.
3. Incubate at 37C for 30 minutes. Then add ~100 ul of glass beads and strap tubes to a vortex set to high speed to help lyse cells, for 30 additional minutes.
4. Add 200 ul Solution 2 to each tube and mix well.
5. Add 400 ul Solution 3 to each tube and mix well. Centrifuge at maximum speed for 3 minutes.
6. Transfer the supernatant from one tube to the Zymo-Spin I Column in a collection tube and centrifuge at >10,000 x g for 30 seconds. Add supernatant from next tube and centrifuge again until entire culture has been added to one column.
7. Discard the flow-through and ensure the flow-through does not come into contact with the column tip.
8. Add 550 ul DNA Wash Buffer to the Spin Column and centrifuge at >10,000 x g for 2 minutes. Discard the flow-through. Spin for 3 more minutes to remove residual ethanol.
9. Place the Spin Column in a clean 1.5 mL microcentrifuge tube and add 15 ul of 0.1xEB. Allow to sit for 5 minutes. Centrifuge at 10,000 x g for 1 minute to elute the plasmid DNA. Put the eluate back on the column, let sit, and centrifuge again

I worry I may have lost pellets in my sample 1 and one of my sample 2 tubes. I was trying to be quick in pouring off the supernatant and pipetting out the last bit of the supernatant, but with how small the pellets were I worry they may have resuspended. But also, I have a hard time seeing pellets in 2 mL tubes anyways, and given how small they were, maybe that was it. Also, I added too many beads to some of the tubes, so that's a nightmare. Additionally, given my large level of protein contamination, as I was re-reading the protocol, I realized that perhaps in the transfer to a column stage, protein contamination may have gotten in during the spins while the tubes were just sitting there with all components in place, so I immediately transferred the supernatant of all tubes into a fresh tube which I used to apply the supernatant to the column with.

After I re-eluted my miniprep, I nanodrop'd my samples. Unfortunately, I had to use 2 uL to test the concentrations, because I messed up adding the first 1 uL so there is only 13 uL left which will hopefully be okay. Regardless I got:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR122 1	54.3	1.087	0.949	1.15	0.75
pKR122 2	19.2	0.384	0.272	1.41	0.52

The protein contamination is marginally better, though the ethanol contamination most certainly is not any better. Obviously, pKR122 1 has a very good concentration which is super exciting. I will do a PCR of it tonight and prepare my samples for sequencing tomorrow. I'll need to check with Hannah to see if we have competent cells with the helper plasmid, but if we do then I might go ahead and do that electroporation on Friday.

For my PCR, I diluted all of my samples to the lowest concentration, 18.0 ng/uL as such:

Sample Name	Nucleic Acid (ng/uL)	DNA	EB
pKR122 1.1	18.0	2.2	0
pKR122 1.2	54.3	0.73	1.47
pKR122 2	19.2	2.1	0.1

PCR of Candidate pKR122 Minipreps for Sequencing

1. Acquired and labelled PCR tubes: 122 and negative control. *omitted positive b/c primers verified

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL6, KROL257	-
2-4	<i>PrpsU2_tul4</i> UTR	MP pKR122	KROL6, KROL257	633

2. Acquire the following components and put them on ice, labeling tubes if necessary:
 - mgH₂O, Primestar buffer, dNTPs, KROL6, KROL257, (10uM), and candidate pKR122
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 17.8 uL of master-mix to negative control PCR tube
9. Add 17.8 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

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			10.2	51
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	18.0 ng/ul	2 ng/ul	2.2	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
Total volume			20	98

Hannah showed me how she modified the PCR master mix components for doing her PCR for sequencing, so I worked off of that, diluting samples to my lowest concentration and calculating 2.2 uL

total for a final concentration of 2 ng/uL in a 20 uL reaction. Additionally, I looked at the plasmid she was working on, and the primers she used to see how much of her region she sequenced, and then I saw that if I used the same primers on my plasmid it would sequence the same area which uhhh makes sense since they were probably working off of the same backbone but whatever.

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 °C

Wednesday, October 5, 2022

To Do:

1. Set up cultures for pH environmental condition test
2. Set up cultures to test 0.5% and 1.0% NaCl in KRLVS192 again
3. Freeze down SUA of KRLVS192.2 and KRLVS193.2 from left over
4. Buffer 22 mL of MHB each at pH 3.0, 5.0 and 10.0
5. Make hemoglobin
6. Conduct pH and NaCl environmental condition protocol
7. GFP assay for pH and NaCl environmental conditions
8. PCR purify PCR of yeast minipreps
9. Run gel of 5 μ L of PCR of yeast minipreps
10. Set up appropriate PCR samples for sequencing

Results and Data:

Testing the Effect of pH on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Experimental Layout					
Condition Number	0	1	2	3	4
Experiment Component	Background	Control	Test 1	Test 2	Test 3
Condition	Unaltered pH	Unaltered pH	pH 3	pH 5	pH 10

1. Prepare (3) MHB (22 mL) by buffering in INBRE, and filter sterilizing
2. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution
3. Normalize samples to an OD of 0.08-0.10 in 25 mL of MHB in baffled flasks.
4. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
5. Centrifuge each culture at 8,000 xg for 5 minutes and resuspend in 1 mL of MHB, calculate how much to add to 5 mL MHB, MHB pH 3, MHB pH 5, or MHB pH 10 via:
$$\text{OD} \times \text{Total vol} = x \text{ OD units}; \text{OD} \times 5\text{mL} = y \text{ OD units}; y \text{ OD units} / x \text{ OD units} = \text{vol to add}$$
6. Shake cells at 37°C for an additional 1-1.5 hours to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, non-buffered MHB

[illegible]

OD Readings			
Sample	0 hours	2 hours	4.5 hrs
KRLVS192 1	0.076	0.124	0.305
KRLVS192 2	0.078	0.149	0.319
KRLVS193 1	0.078	0.137	0.325
KRLVS193 2	0.076	0.114	0.283
LVS	0.096	0.092	

I thought at the two hour mark that perhaps I switched around the OD of LVS to start, and that it was just growing very slowly. However, at the four and half hour time point, I realized that last night I put kanamycin into the MHB. When I buffered the MHB, I ended up buffering to pH 3.13, pH 5.33, pH 10.12 because I was too worried about time to get it more accurate. Also, when I got to the media switching stage, I calculated the average of the OD's and used the calculation, ultimately adding 200 uL of the pellet resuspended in 1 mL MHB. When I pulled them out, pH 10.12 was noticeably less turbid.

Testing the Effect of Salinity on *rpsU1* Production in KRLVS192

Experimental Layout				
Condition Number	0	1	2	3
Experiment Component	Background	Control	Test 1	Test 2
Condition	0% NaCl	0% NaCl	0.5% NaCl	1.0% NaCl

1. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution in the spectrophotometer
2. Normalize samples to an OD of 0.08-0.10 in 12 mL of MHB+Kan in tubes.
3. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
4. **Using pre-prepared 0.5% and 1.0% NaCl MHB
5. Centrifuge each culture at 8,000 xg for 5 minutes and resuspend in 200 mL of MHB, calculate how much to add to 5 mL 0.5% NaCl MHB or 1.0% NaCl MHB via:

$$\text{OD} \times \text{Total volume} = x \text{ OD units}$$

$$\text{OD} \times 5\text{mL} = y \text{ OD units}$$

$$y \text{ OD units} / x \text{ OD units} = \text{volume (mL) to add}$$
6. Shake cells at 37°C for an additional 1.5 hours to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, normalizing to the OD, then to the fluorescence of each strain to standard condition, MHB with no NaCl supplementation

Experimental Set-Up (KRLVS192)				
Strain	BR 1		BR 2	
Culture Vol.	12 mL		12 mL	
%NaCl	0.5%	1.0%	0.5%	1.0%
Separated Vol.	5 mL	5 mL	5 mL	5 mL

OD Readings			
Sample	0 hours	2 hours	4.5 hrs
KRLVS192 1	0.063	0.117	0.300
KRLVS192 2	0.062	0.134	0.338

For the additional NaCl conditions, I used the control condition from the pH experiment, but obviously was unable to use LVS for this one as well. When it came to the media switching stage, I resuspended the pellet in 500 uL of MHB and added 200 uL of that resuspension to each tube.

GFP Assay on pH Conditions in KRLVS192 and KRLVS193

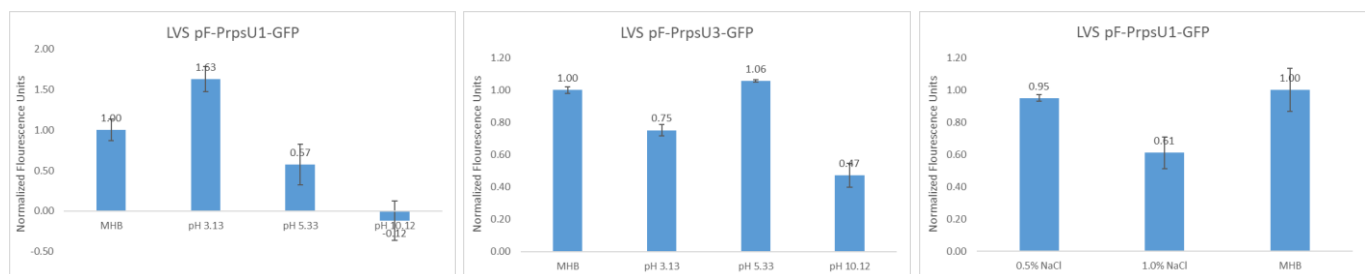
1. Take 1 mL from each culture tube into microcentrifuge tubes. Spin at max speed for 3 minutes
2. Remove all MHB, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS 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	Condition
A	Unaltered pH
B	pH 3
C	pH 5
D	pH 10
E	0.5% NaCl
F	1.0% NaCl

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

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

Due to the fact I didn't have a control LVS, for processing the data, I looked at my two previous experiments that had LVS, and once they were normalized to the OD, the values were very close so I averaged the fluorescence and used that to subtract from each fluorescence. *rpsU1*: MHB, pH 3; $p=0.01$

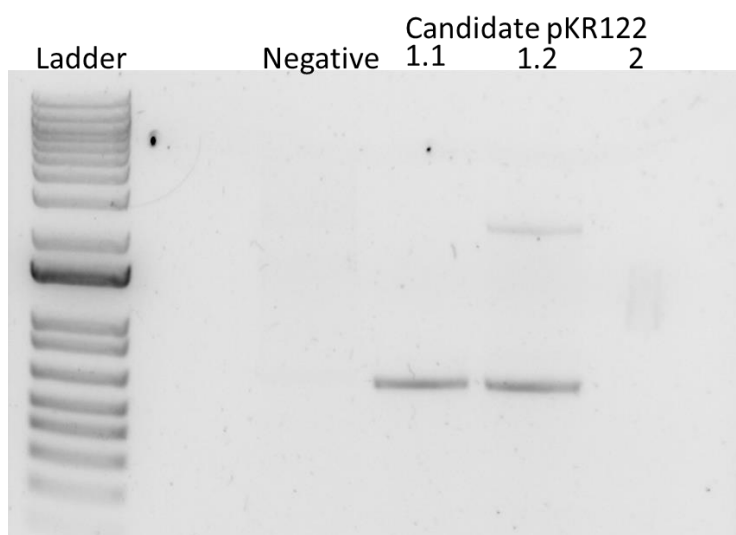


PCR Purification of Candidate pKR122 Yeast Miniprep PCR

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 pKR122 Yeast Miniprep PCR

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 10 uL ladder, and 5 uL of each sample.
6. Ran for 45 minutes at 113V.



After I ran the gel, I nanodrop'd the PCR samples that had a band at the appropriate size, though I was a little concerned about the pKR122 1.2 sample due to the second band on the gel.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR122.1	38.7	0.774	0.424	1.83	1.99
Cand. pKR122.2	70.8	1.417	0.768	1.84	2.03

I then set up sequencing according to the following:

Sample#	Type	Name	Primer	Size (bp)	Conc. (ng/μl)	Vol. PCR	Volume H ₂ O
SS1	PCR	Cand. pKR122 1	KROL257	633	10	1.58	7.86
SS2	PCR	Cand. pKR122 2	KROL257	633	10	1.58	7.86

Reagents

2% Hemoglobin

Add 6 g hemoglobin (kept in fridge) to 500 mL flask (add stir bar)

Add 300 mL Type 1 ddiH₂O

Stir for at least 10 minutes, until clumps are all dissolved

Autoclave Liquid20 in water bath

Thursday, October 6, 2022

To Do:

1. ~~Streak out LVS-pF, KRLVS192, and KRLVS193~~
2. ~~Get ready for H₂O₂ condition experiment~~

Results and Data:

Friday, October 7, 2022

To Do:

1. ~~Set up cultures for H₂O₂ stress environmental condition~~
2. ~~Set up culture for pH 8 environmental condition~~
3. ~~Perform H₂O₂ stress environmental condition experiment~~
4. ~~Perform pH 8 environmental condition experiment~~
5. ~~GFP assay for H₂O₂ stress and pH 8 environmental condition experiment~~
6. ~~Crosspatch potential triple deletion strains to confirm~~
7. ~~Validate transposon insertion colonies for high production of β -Galactosidase~~

Results and Data:

Testing the Effect of H₂O₂ Stress on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Experimental Layout				
Condition Number	1	2	3	4
Experiment Component	Control	Test 1	Test 2	Test 3
Condition [H ₂ O ₂]	MHB	0.02 mM	0.1 mM	0.5 mM

1. Patch out each strain in duplicate per condition on CHAH+selective antibiotic the day prior to the experiment
2. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution in the spectrophotometer
3. Normalize samples to an OD of 0.08-0.10 in 8 mL of MHB.
4. Shake cells at 37°C to an OD of ~0.3 typically 4-4.5 hours.
5. Add 0.02, 0.1, or 0.5 mM H₂O₂ to each tube (calculated to ~6.2 mL, after OD checks) and shake cells at 37°C for 30 additional minutes.
6. Follow the GFP reporter assay protocol, starting at Step 4 for each timepoint, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, the time zero point

Testing the Effect of pH 8 on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193

Experimental Layout			
Condition Number	0	1	2
Experiment Component	Background	Control	Test 1
Condition	MHB	MHB	pH 8

1. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 12 mL of MHB+Kan in tubes.
3. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
4. Buffer 22 mL's to pH 8
5. Centrifuge each culture at 8,000 xg for 5 minutes and resuspend in 1 mL and add to a total of 5mL
6. Shake cells at 37°C for an additional 1.5 hours to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, subtract LVS, normalizing to the OD, then to the fluorescence of each strain to standard condition, MHB without altered pH

Experimental Set-Up				
Strain	KRLVS192 1	KRLVS192 2	KRLVS193 1	KRLVS193 1
Culture Vol.	5 mL	5 mL	5 mL	5 mL
pH	8	8	8	8
Separated Vol.	5 mL	5 mL	5 mL	5 mL

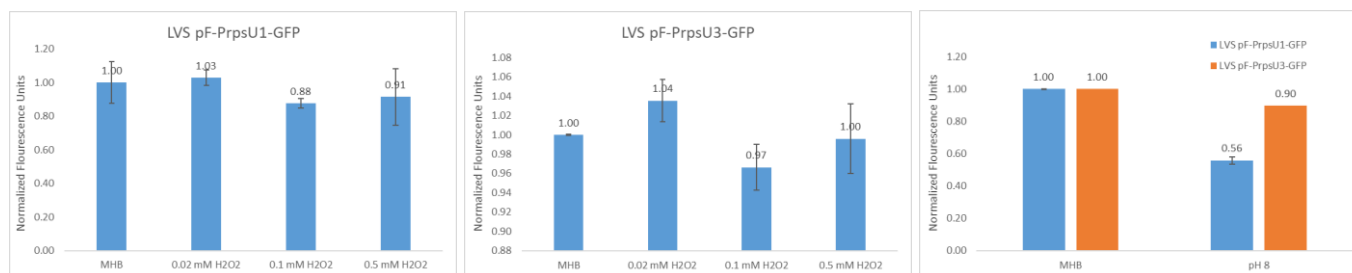
GFP Assay on H2O2 Stress and pH 8 Environ. Conditions in KRLVS192 and KRLVS193

1. Take 1 mL from each culture tube into microcentrifuge tubes. 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 microfuge tube in triplicate into a clear 96-well plate. Pipette LVS in PBS in triplicate as a 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. FL > Wavelength: 495 to 535 > Plate type: CoStar 3789 > Gain: Automatic > Integration: 380 ms

Label	Condition
A	MHB
B	0.02 mM H2O2
C	0.1 mM H2O2
D	0.5 mM H2O2
E	pH 8

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

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



It doesn't seem that oxidative stress will result in the upregulation of either bs21-1 or bs21-3. Same with pH 8. Also clearly, H2O2 seems to mostly decrease the production.

I also looked at my sequencing results and it looks like Candidate pKR122 1.2 is good, doesn't include gaps or insertions like pKR122 1.1. However, pKR122 1.2 is included that extra band in the gel, so I am a little sus of it. But from what I was seeing it was fine...? Will discuss with Kathryn.

I then cross-patched the transposon *lacZ* electroporation stocks that I froze down. I patched onto CHAH and CHAH+xgal. I numbered according to the previous labelling scheme:

Tube Number	Electroporation	Description
1	KRLVS28 + pKR141	Very small dark blue colony
2	KRLVS28 + pKR141	Average size colony with dark blue margin and white center
3	KRLVS28 + pKR141	Average size dark blue colony
4	KRLVS28 + pKR141	Large colony with dark margin and light center
5	KRLVS28 + pKR141	Small dark blue colony
6	KRLVS28 + pKR141	Moderately small dark blue colony
7	KRLVS28 + pKR141	Very small colony with dark blue margin and light center
8	KRLVS28 + pKR141	White colony, for control

I then cross-patched the triple deletion plasmid loss electroporation stocks that I froze down back in August, whoops! I labelled them accordingly:

Label	Electroporation
1	KRLVS153 + pF-nat
2	KRLVS153 + pF-nat
3	KRLVS157 + pF
4	KRLVS157 + pF
5	KRLVS157 + pF
6	KRLVS153 + pKR135
7	KRLVS153 + pKR135
8	KRLVS153 + pKR135

Tuesday, October 11, 2022

To Do:

- ~~1. Make and sterilely filter 2.5% iron pyrophosphate~~
- ~~2. Supplement MHB~~
- ~~3. Make yeast overnights~~

Results and Data:

After speaking to Kathryn, we determined it might be best to just do a new yeast miniprep on some of my remaining colonies, given the larger band in the gel might be a duplication. This would potentially have no effect, but it also might. I wasn't sure whether it was more worth it to try out that plasmid or to just prepare another.

Additionally, she mentioned that just because the original pKR122 didn't work, I shouldn't save over it, so I created a new plasmid entry for the yeast version of the plasmid and it is now named pKR168.

Due to the difficulty I seem to be having with getting this yeast plasmid, I made three overnights in 5mL SD -ura from my yeast transformation plate in the fridge.

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

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

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

Wednesday, October 12, 2022

To Do:

- ~~1. Miniprep pKR168~~
- ~~2. Patch out KRLVS192, KRLVS193, and LVS~~
- ~~3. Nanodrop pKR168 minipreps~~
- ~~4. PCR of pKR168 candidates~~
- ~~5. PCR purification of pKR168 sequencing PCR~~
- ~~6. Run gel on 5 uL of PCR~~
- ~~7. Nanodrop PCR of pKR168 candidates~~

Results and Data:

Miniprep of Candidate pKR168 Plasmid from Yeast

1. Aliquot 3x 1.666 mL of the yeast cells (early log phase, total of 4.5 mL) into 2 ml microfuge tubes and spin down the cells at 600 x g for 3 minutes.
2. Discard the supernatant and add 200 ul Solution 1 to each pellet and add 3 ul Zymolyase. Resuspend pellet by flicking tube or mild vortexing. Note: If running multiple samples, can make a solution 1-enzyme 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.

- 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.
- Add 200 ul Solution 2 to each tube and mix well.
- Add 400 ul Solution 3 to each tube and mix well. Centrifuge at maximum speed for 3 minutes.
- Transfer the supernatant from one tube to the Zymo-Spin I Column in a collection tube and centrifuge at $>10,000 \times g$ for 30 seconds. Add supernatant from next tube and centrifuge again until entire culture has been added to one column.
- Discard the flow-through and ensure the flow-through does not come into contact with the column tip.
- Add 550 ul DNA Wash Buffer to the Spin Column and centrifuge at $>10,000 \times g$ for 2 minutes. Discard the flow-through. Spin for 3 more minutes to remove residual ethanol.
- 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 $10,000 \times g$ for 1 minute to elute the plasmid DNA. Put the eluate back on the column, let sit, and centrifuge again

I repeated what I did last time, transferring all the supernatant from one sample series into a single tube while I did the spins.

I nanodrop'd the samples, after leaving them sit on ice for awhile while I met with Kathryn. The concentrations are abysmal, as is the contamination, but I will see if I can get amplification.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR168 1	7.8	0.156	0.103	1.52	0.37
pKR168 2	8.8	0.177	0.133	1.32	0.64
pKR168 3	5.0	0.100	0.062	1.61	0.53

For the PCR, I diluted each sample to the lowest concentration, 5.0 ng/uL, according to the following table:

Sample Name	Nucleic Acid (ng/uL)	DNA (uL)	EB (uL)
pKR168 1	7.8	5.13	2.87
pKR168 2	8.8	4.55	3.45
pKR168 3	5.0	8.00	0.00

PCR of Candidate pKR122 Minipreps for Sequencing

- Acquired and labelled PCR tubes: 122 and negative control. *omitted positive b/c primers verified

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	- control	-	KROL6, KROL257	-
2-4	<i>PrpsU2_tul4</i> UTR	MP pKR168	KROL6, KROL257	633

- Acquire the following components and put them on ice, labeling tubes if necessary:
 - mgH₂O, Primestar buffer, dNTPs, KROL6, KROL257, (10uM), and candidate pKR122
- Vortex each component (aside from enzyme)
- Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
- Add ddi H₂O to negative control tube (template volume for 1 reaction)
- Prepare a master-containing:
 - mgH₂O, dNTPs, Primestar buffer, and Primestar enzyme
- Mix the master-mix solution by pipetting up and down

8. Add 12 uL of master-mix to negative control PCR tube
9. Add 12 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 (45 second extension time)

Total reaction volume	20
Total number of reactions	4

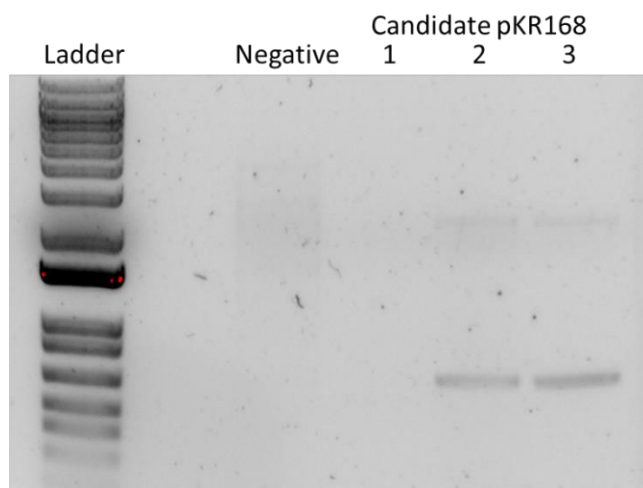
Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			4.4	22
PrimeSTAR GXL Buffer	5x	1x	4.0	20
dNTPs	2.5 mM	0.2 mM	1.6	8
oligo F	10 uM	0.3 uM	0.6	3
oligo R	10 uM	0.3 uM	0.6	3
template	5.0 ng/ul	2 ng/ul	8.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
Total volume			20.0	58

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 30 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 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 10 uL ladder, and 5 uL of each sample.
6. Ran for 45 minutes at 113V.



Obviously, I got that 3,000bp band in the samples which actually amplified. This is what I did, and sent to Kathryn because I'm not re-writing it:

I just ran the gel for my newest yeast minipreps for pKR168. I'm seeing that heavy, unexpected band in both of the samples that actually amplified, and I also saw a smear that looked like it might be in that area in the negative control so I got worried that there was maybe contamination. I looked at what I had previously PCR'd to see if there could be some contamination from that DNA. The last several things have been gBlocks and back in June or July the inducible strains. Theoretically, there is a binding site for KROL257 but not KROL6 so potentially if there was contamination it could be from that, though given I sequenced off of KROL257 I would have expected to see something about that in the sequencing results. Additionally, just to be thorough, I checked all the other yeast plasmids to see if there was a 3,000bp fragment from those primers (I was pretty sure there wasn't, and didn't see how it might survive in the miniprep kit anyways, but thought I'd check). That said, I don't know that it is contamination given that in my previous gel only one lane had that band, and the non-amplified fourth lane sample from this gel did not have this band (unless it did, I see a very light smudge, maybe). I did make these PCR reactions from a master mix, and also I only used a 45 second extension time so I'm not entirely sure how we got a 3,000bp band anyways.

Regardless, I did nanodrop my PCR while the gel was running and got this:

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Cand. pKR168 1 PCR	14.7	0.294	0.145	2.02	0.82
Cand. pKR168 2 PCR	68.4	1.367	0.717	1.91	2.00
Cand. pKR168 3 PCR	63.3	1.266	0.664	1.91	2.30

Thursday, October 13, 2022

To Do:

1. ~~Set up cultures for UV stress environmental condition~~
2. ~~Set up sequencing for candidate pKR168~~
3. ~~Perform UV stress environmental condition experiment~~
4. ~~GFP assay for UV stress environmental condition experiment~~

Results and Data:

Testing the Effect of UV Stress on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Experimental Layout

Condition Number	1	2	3	4
Experiment Component	Control	Test 1	Test 2	Test 3
Condition (UV)	MHB	5 $\mu\text{J}/\text{cm}^2 \times 100$	10 $\mu\text{J}/\text{cm}^2 \times 100$	15 $\mu\text{J}/\text{cm}^2 \times 100$

1. Patch out each strain in duplicate per condition on CHAH+selective antibiotic the day prior to the experiment
2. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution in the spectrophotometer
3. Normalize samples to an OD of 0.08-0.10 in 10 mL of MHB.
4. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
5. Adjust the UVP Crosslinker to a time of 1 minute and an energy of either 5 $\mu\text{J}/\text{cm}^2 \times 100$, 10 $\mu\text{J}/\text{cm}^2 \times 100$, or 15 $\mu\text{J}/\text{cm}^2 \times 100$ and irradiate appropriate tubes.
6. Replace tubes in incubator and allow to shake for 30 additional minutes.
7. Follow the GFP reporter assay protocol, starting at Step 4 for each aliquot, subtract LVS, and normalize to the fluorescence of each strain to standard condition, zero irradiation

Sample	0 hours	4.5 hours
KRLVS192 1	0.096	0.308
KRLVS192 2	0.072	0.240
KRLVS193 1	0.083	0.289
KRLVS193 2	0.085	0.306
LVS-pF	0.087	0.311

For the 4.5 hour time mark I only checked the OD on the MHB labelled tubes, since I made a master mix, more or less, of each culture they should be growing around the same so I didn't see a reason to check every single replicate.

GFP Assay on UV Stress Environmental Conditions in KRLVS192 and KRLVS193

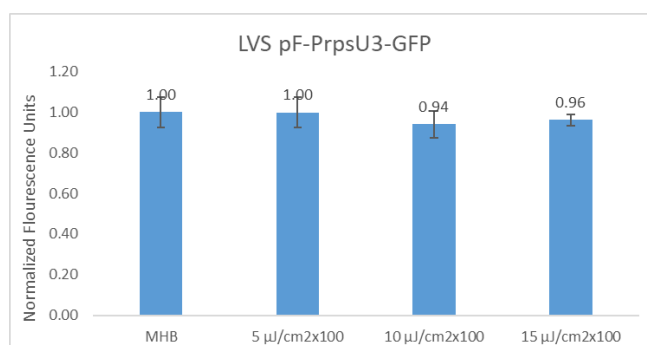
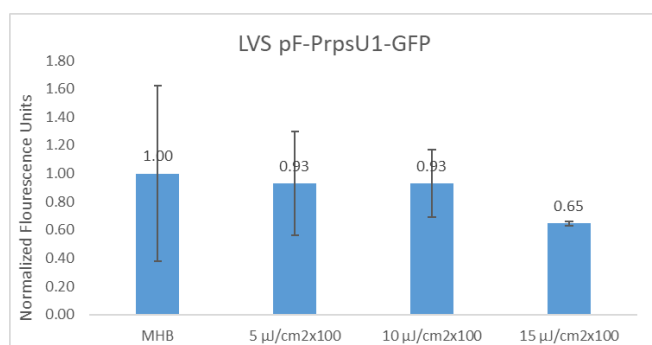
1. Take 1 mL from each culture tube into microcentrifuge tubes. Spin at max speed for 3 minutes
2. Remove all MHB, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each microfuge tube in triplicate into a clear 96-well plate. Pipette LVS in PBS in triplicate as a 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. FL > Wavelength: 495 to 535 > Plate type: CoStar 3789 > Gain: Automatic > Integration: 380 ms

Label	Condition
A	MHB
B	5 $\mu\text{J}/\text{cm}^2 \times 100$ for 1 min
C	10 $\mu\text{J}/\text{cm}^2 \times 100$ for 1 min
D	15 $\mu\text{J}/\text{cm}^2 \times 100$ for 1 min

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

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

A	A1	A1	A1		A3	A3	A3		LVS	LVS	LVS	
B	A2	A2	A2		A4	A4	A4		1xPBS	1xPBS	1xPBS	
C	B1	B1	B1		B3	B3	B3					
D	B2	B2	B2		B4	B4	B4					
E	C1	C1	C1		C3	C3	C3					
F	C2	C2	C2		C4	C4	C4					
G	D1	D1	D1		D3	D3	D3					
H	D2	D2	D2		D4	D4	D4					



I'm a bit concerned about the error bar on the MHB for *rpsU1*, I think I'll check my old graphs to see if *rpsU1* always has more variability between replicates, like I noticed with the salinity and was it pH?

While my cells were growing, I set up sequencing reactions for my pKR168 candidate plasmids according to the following:

Sample#	Type	Template	Primer	Temple (bp)	Temp. (ng/uL)	ng needed	Vol. PCR	Vol. H2O
SS1	PCR	Cand. pKR168 2	KROL257	633	10	15.83	1.58	7.86
SS2	PCR	Cand. pKR168 3	KROL257	633	10	15.83	1.58	7.86

Friday, October 14, 2022

To Do:

1. ~~Analyze sequencing results~~
2. ~~Look at my triple deletion plates~~
3. ~~Help Breena with electroporations~~
4. ~~Put away dishes~~

Results and Data:

I went forward with sequencing the two candidate plasmids I miniprep'd despite the heavier 3,000 bp band, at the behest of Kathryn. Both of the sequences have gaps/insertions, and candidate pKR168 2 additionally has a mismatch. Yikes!!!!!! Hopefully if Kathryn is about just accepting these candidates with the heavier band, then I'll be able to go forward with the previous plasmid that had that band but was correct.

Additionally, I showed Brenna how to electroporate using the leftover competent cells I had in my box. I'm a little nervous about how old they were, but obviously there was no other option given that I'm a dummy and she wasn't able to make her own. But also, I believe all of hers arced, but I thought that when I last didn't they didn't both arc for me, so that was a little weird? That said, she had colloquium so I ended up plating the recovered cells for her. Previously, KRLVS75 didn't have enough cells, however the turbidity last time was noticeably lighter than it was this time, so I went with the volumes that were optimal for KRLVS28 last time, and plated 100 uL and 200 uL for both. For the negative plates, I just plated remaining.

Additionally, I finally looked at the triple deletion plates. Some of them looked like they may have had light growth, but I'm going to count them as no growth given how long I let the cells grow. I patched last Friday and left them out on my bench, Aisling put them in the 30°C incubator on Monday, I pulled them out on Wednesday, and left them on my bench again until today. Whoops. Regardless:

Label	Electroporation	Expectation (nat/kan)	Observation (nat/kan)
1	KRLVS153 + pF-nat	Growth/No Growth	Growth/No Growth
2	KRLVS153 + pF-nat	Growth/No Growth	Growth/No Growth
3	KRLVS157 + pF	No Growth/Growth	No Growth/Growth
4	KRLVS157 + pF	No Growth/Growth	No Growth/Growth
5	KRLVS157 + pF	No Growth/Growth	No Growth/Growth
6	KRLVS153 + pKR135	Growth/No Growth	Growth/Growth
7	KRLVS153 + pKR135	Growth/No Growth	Growth/No Growth
8	KRLVS153 + pKR135	Growth/No Growth	Growth/No Growth

It would seem that all but Sample 6 are triple deletion mutants with absolutely no *rpsU* genes. Sample 6 probably has both plasmids.

Monday, October 17, 2022

To Do:

- ~~1. Check Brenna's electroporation plates and move to incubator if necessary~~
- ~~2. Make CHAH+Hyg plates~~

Results and Data:

I didn't see any colonies grown on Brenna's plates so I popped them into the incubator. I'm not sure that we will be able to see any colonies tomorrow either, but we will check. If there is no growth, then we'll leave them to grow for an additional day. If there is additionally no growth on Wednesday, then I think that I will have her patch out KRLVS28 and KRLVS75 ON REGULAR CHAH and make new competent cells on Thursday and do the electroporations again on Friday. That said, we might still do that if there are not very many colonies. However, I think a similar thing happened to me the last time I did this, where I left them out at room temperature and didn't see any growth, then saw colonies after an additional day or two in the incubator.

Reagents

54 mg/mL Hygromycin

To 100mL beaker add:

0.27g of Hygromycin B powder (-20°C)

5 mL diH₂O (type I)

stirbar

Stir until dissolved

Pre-wet the filter by passing 5-10mL diH₂O through it

Filter sterilize the 54 mg/mL Hyg

Store at 4°C

CHAH+Hyg

To 500mL flask add:

4.08g of cystine heart agar

40mL of ddiH₂O (type I)

stirbar

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

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

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

Using sterile technique, pour hemoglobin into CHA

For 200 ug/ml nat, add 296 ul of 54 mg/mL hygromycin to 80 mL of media

Tuesday, October 18, 2022

To Do:

- ~~1. Patch out KRLVS192 and LVS-pF on Kan plates~~
- ~~2. Patch out KRLVS127 on Hyg plates (drpsU2 with helper plasmid)~~
- ~~3. Make embedded rpsU1 and rpsU2 primers~~

Results and Data:

Came into lab and it turns out that there was a contamination issue going on with the plates that Aisling made yesterday, we ultimately deduced it was probably the hemoglobin. I patched out plates, and checked them after a couple hours of growth and they had contaminants growing. My Hyg plates did not. This means I am pushing my experiments back a day, but the yeast transformation should go forward just fine.

Otherwise, I struck out KRLVS127 onto a Hyg plate, since it seemed that they were not contaminated. Additionally, I showed Brenna how to streak out LVS and she struck out KRLVS28, KRLVS75, and two of the transposon mutants on xgal plates.

Lastly, I ordered some oligos for checking the potential triple deletion strains.

KROL599	rpsU1_tripmut_check_F	KROL599 rpsU1_tripmut_check_F	aataaatgctcttcttgagaaattctt
KROL600	rpsU1_tripmut_check_R	KROL600 rpsU1_tripmut_check_R	Agatgagcacaaccatttgatataag
KROL601	rpsU2_tripmut_check_F	KROL601 rpsU2_tripmut_check_F	ccaagcgtagaattaaagaaagagaa
KROL602	rpsU2_tripmut_check_R	KROL602 rpsU2_tripmut_check_R	acctttaactattatattgctcttatga

Wednesday, October 19, 2022

To Do:

1. Patch out KRLVS126 on Hyg plates (LVS with helper plasmid)
2. Patch out KRLVS192, KRLVS193, and LVS-pF
3. Patch out yeast
4. Make 250 mL of YPD in a baffled flask
5. Autoclave 2x 200 mL of sterile water
6. Make 5N HCl
7. pH MHB
8. Get ready for pH pt.2

Results and Data:

I struck out KRLVS193 this morning along with the other things I was patching, however I decided it would probably be better to do the overgrowth alongside my mini MHB vs. CDM next week since I will have so many tubes tomorrow, so I won't actually do that tomorrow. Additionally, I think I'll do the plate vs. MHB at the same time. So it will be a different media experiment, essentially. MHB vs. CDM vs. CHAH agar.

Reagents

YPD (250 mL)

To 500 mL baffled flask add:

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

Mix thoroughly and autoclave Liquid 15'

Store at room temperature

Mueller Hinton Broth (500 mL)

To supplement add:

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

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

5N HCl

In fume hood add to a beaker with stir bar:

- ~40 mL of ddiH₂O
- 41.5 mL of 37% HCl
- stir

Add to graduated cylinder and correct to 100 mL

Stir again until combined
Add to 200 mL glass bottle

Thursday, October 20, 2022

To Do:

1. ~~Set up cultures for pH experiment pt. 2~~
2. ~~Conduct pH experiment pt. 2~~
3. ~~GFP assay on pH experiment pt. 2~~
4. ~~Make CHAH+Kan plates~~
5. ~~Make electrocompetent KRLVS126 and KRLVS127~~
6. ~~Start yeast overnight~~

Results and Data:

pH Dose Response Curve of *rpsU1* Production

Experimental Layout								
Condition Number	1	2	3	4	5	6	7	8
Experiment Component	Control	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7
Condition	Unaltered pH	pH 2.75	pH 3.5	pH 4.25	pH 5.75	pH 6.5	pH 7.25	pH 8.0

1. Prepare (7) MHB (17 mL) by buffering in INBRE, and filter sterilizing
2. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution
3. Normalize samples to an OD of 0.08-0.10 in 42 mL of MHB in baffled flasks.
4. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
5. Centrifuge each culture at 8,000 xg for 5 minutes and resuspend in 800 uL of MHB, adding 100 uL of cells into each control/test tube
6. Shake cells at 37°C for an additional 1-1.5 hours to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, non-buffered MHB

Experimental Set-Up								
Strain	KRLVS192 BR-1, -2, and -3 each							
Culture Vol.	42 mL							
pH	U.A.	2.75	3.5	4.25	5.75	6.5	7.25	8
Separated Vol.	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

OD Readings		
Sample	0 hours	4 hours
KRLVS192 1	0.073	0.223
KRLVS192 2	0.085	0.168
KRLVS192 3	0.074	0.089
LVS-pF	0.081	0.234

GFP Assay on pH Dose Response Curve of *rpsU1* Production

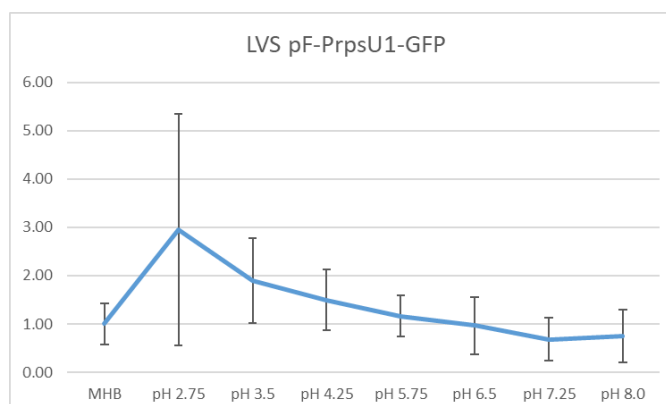
1. Take 1 mL from each culture tube into microcentrifuge tubes. Spin at max speed for 3 minutes
2. Remove all MHB, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS 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	Condition
A	Unaltered pH
B	pH 2.75
C	pH 3.5
D	pH 4.25
E	pH 5.75
F	pH 6.5
H	pH 7.25
I	pH 8.0

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1		E1	E1	E1		LVS	LVS	LVS	
B	A2	A2	A2		E2	E2	E2		PBS	PBS	PBS	
C	B1	B1	B1		F1	F1	F1					
D	B2	B2	B2		F2	F2	F2					
E	C1	C1	C1		H1	H1	H1					
F	C2	C2	C2		H2	H2	H2					
G	D1	D1	D1		I1	I1	I1					
H	D2	D2	D2		I2	I2	I2					



Spoke with Kathryn and obviously my error bars are pretty big, and when she looked at the fluorescence they weren't much larger than LVS. Due to the low growth, going to repeat with just 2.75, 4.5, and 6.5 (don't need regular MHB with it as well) in triplicate, and condense the samples for the assay.

Preparing Electrocompetent KRLVS28 and KRLVS75 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 $\sim 1 \times 10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.

I barely had enough KRLVS126 and did not have enough KRLVS127.

Friday, October 21, 2022

To Do:

1. ~~Make electrocompetent yeast~~
2. ~~Electroporate candidate pKR168 into yeast~~
3. ~~Make CHAH+Xgal~~

Results and Data:

Making Electrocompetent Yeast Cells

1. Take OD600 (dilute 1:10) until it reaches about 1.3-1.5 OD
2. Place entire volume of culture in sterile 250 mL centrifuge bottle
3. Centrifuge at 3000 xg for 5 min at 4C. Discard supernatant.
4. Wash with ~ 200 mL of ice-cold water twice with identical centrifugation.
5. Wash in 20 ml of ice cold 1M sorbitol and centrifuge again, as above.
6. Resuspend in smallest volume of ice cold 1M sorbitol, starting with 200 ul and up to 500 ul

Electroporation of Yeast with pKR168

1. Add 3 ul of plasmid to 100 ul of electrocompetent cells. Mix via pipette. Incubate on ice for 5 min.
2. Transfer to prechilled 0.2 cm cuvette
3. Electroporate on presetting SC2 (1500 V, 5 msec)
4. Immediately add the cells to 1 mL of cold 1M sorbitol
5. Plate onto -ura selective plates. For plasmid transformation, plate 10 ul and 100 ul. Put in incubator set to 30C. Colonies will be visible within 2-3 days for successful transformations.

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	Number of Plates
1	pKR168	pKR168	3 uL	10 uL, 100 uL.	2
2	(+) control	pKR128	3 uL	200 uL	1
3	(-) control	-		200 uL	1
Total:					4

Reagents

CHAH+Xgal

To 500mL flask add:

- 15.3g of cystine heart agar
- 150mL of ddiH2O (type I)
- stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)
Autoclave on 30' liquid cycle, filling the water bin up to the height of the media
Add 2% hemoglobin to water bath, after autoclave add CHA as well
Using sterile technique, pour hemoglobin into CHA
For 100 ug/mL X-gal, add 600 uL of 50 mg/mL X-gal to 300 mL of media

Due to the fact Brenna has multiple plates with contamination, I struck out from the permanent glycerol stocks to see if they got contaminated. Initially I told Kathryn and Hannah that she did patch from the original glycerol stocks, but I'm actually not sure now, I can't really remember. Regardless, I labelled the plates and put them into the incubator so hopefully it will turn out okay. There are two tubes for each, so hopefully at least one of each is okay, ultimately.

Monday, October 24, 2022

To Do:

1. ~~Patch out KRLVS192, KRLVS193, and LVS-pF~~
2. ~~Check glycerol stock plates for contamination~~
3. ~~Check yeast plates~~
4. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
5. ~~Supplement MHB~~
6. ~~Patch out transposon inserted into KRLVS28 mutants~~
7. ~~Make 100 mL CDM~~

Results and Data:

I checked the plates I struck out from KRLVS28 and KRLVS75 on Friday to check for contamination, and had Kathryn double check and they look okay. Additionally, I looked at my yeast plates and had Hannah double check them for me. My positive control did work, but my electroporation with candidate pKR168 did not work. I spoke with Hannah and we determined I probably should have plated remaining as well, so I will do that when I do my next electroporation.

Additionally, I spoke to Kathryn about Brenna's schedule and she had me patch out the mutants for her to do colony streaking tomorrow.

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

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

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

CDM (100mL)

To 100 mL graduate cylinder add:

8 mL Combined Amino Acid Stock

400 uL Thiamine HCl

400 uL Spermine tetrahydrochloride

400 uL DL-Calcium pantothenate

3.42 mL 5N NaCl

1 mL KH₂PO₄

400 uL K₂HPO₄

1 mL 400 mg/mL Glucose

400 uL FeSO₄•7H₂O

400 uL MgSO₄•7H₂O

Add water to 100 mL

Filter sterilize into autoclaved bottle

I had to make the K₂HPO₄ fresh, and I may have switched the volumes between the two potassiums... Additionally, I used 2 mL of 20% glucose instead of 1 mL of 40% glucose. It was super hard to filter.

Tuesday, October 25, 2022

To Do:

- ~~1. Patch out KRLVS192 in triplicate and LVS-pF~~
- ~~2. Set up cultures for CDM vs MHB~~

Results and Data:

Testing CDM, CHAH, and Overgrowth on the Up-Regulation of *rpsU1* and *rpsU3*

Experimental Layout				
Condition Number	1	2	3	4
Experiment Component	Control	Test 1	Test 2	Test 3
Condition	MHB	CDM	CHAH	Overgrowth

1. Resuspend cells in 400uL of MHB or CDM, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of MHB or CDM.
3. Shake cells at 37°C to an OD of 0.30-0.40 typically 4.5 hours.
4. Resuspend patches in 1xPBS and normalize to an OD of 0.30
5. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, non-buffered MHB

OD Readings		
Sample	0 hours	4 hours
KRLVS192 1 MHB	0.110	0.408
KRLVS192 2 MHB	0.098	0.304
KRLVS193 1 MHB	0.088	0.230
KRLVS193 2 MHB	0.079	0.274
LVS-pF MHB	0.090	0.294
KRLVS192 1 CDM	0.043	
KRLVS192 2 CDM	0.072	
KRLVS193 1 CDM	0.072	
KRLVS193 2 CDM	0.100	
LVS-pF CDM	0.103	

I didn't write down the OD's for the CDM samples, however they were all below 0.05, so clearly they died. I spoke to Kathryn, and I should have made fresh iron and magnesium sulfate- whoops. Obviously, I cut the experiment here and did not continue.

Wednesday, October 26, 2022

To Do:

- ~~1. Set up cultures to test pH in KRLVS192~~
- ~~2. Patch out KRLVS148 and KRLVS149~~
- ~~3. Patch out KRLVS28 and KRLVS75~~
- ~~4. Patch out yeast~~
- ~~5. Buffer MHB to pH 2.75, 4.5, and 6.5~~
6. Make YPD, 250 mL
7. Autoclave 200 mL H₂O x2
8. Run protocol for pH environmental condition test
9. GFP assay on pH environmental condition

Results and Data:

Testing the Effect of pH on *rpsU1* Production in KRLVS192

Condition Number	0	1	2	3
Experiment Component	Background	Test 1	Test 2	Control
Condition	Unaltered pH	pH 2.75	pH 4.5	pH 6.5

1. Prepare (3) MHB (17 mL) by buffering in INBRE, and filter sterilizing
2. Scrape up patches of cells and resuspend in 400uL of MHB, checking OD in a 1:20 dilution
3. Normalize samples to an OD of 0.08-0.10 in 18 mL of MHB in baffled flasks.
4. Shake cells at 37°C to an OD of 0.25-0.30 typically 4 hours.
5. Pellet cells at 8,000 xg for 5 minutes and resuspend in 300 uL of MHB and split evenly
6. Shake cells at 37°C for an additional 0.5-1 hour to mid-log (OD 0.3-0.4)
7. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, non-buffered MHB

Experimental Set-Up									
Strain	KRLVS192 1			KRLVS192 2			KRLVS192 3		
Culture Vol.	18 mL			18 mL			18 mL		
pH	2.75	4.5	6.5	2.75	4.5	6.5	2.75	4.5	6.5
Separated Vol.	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

OD Readings			
Sample	0 hours	3 hours	4.5 hrs
KRLVS192 1	0.079	0.212	0.284
KRLVS192 2	0.085	0.176	0.304
KRLVS192 3	0.089	0.192	0.319
LVS-pF	0.091	0.224	0.337

I started my cultures in culture tubes, but they had only been shaking for a few minutes prior to Hannah and Kathryn letting me know that they probably wouldn't grow well due to low aeration, so I transferred them to baffled flasks.

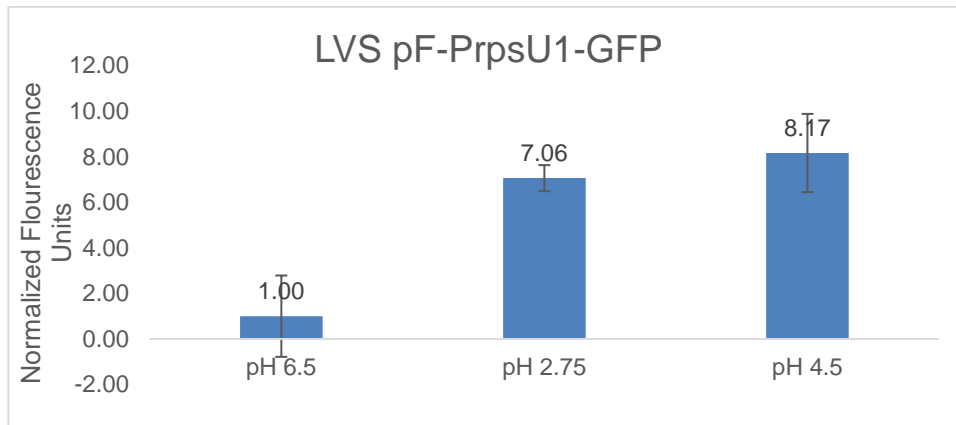
GFP Assay on pH Conditions in KRLVS192

1. Pellet 4 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	Condition
A	pH 6.5
B	pH 2.75
C	pH 4.5

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1		C1	C1	C1					
B	A2	A2	A2		C2	C2	C2					
C	A3	A3	A3		C3	C3	C3					
D	B1	B1	B1		LVS	LVS	LVS					
E	B2	B2	B2		1xPBS	1xPBS	1xPBS					
F	B3	B3	B3									
G												
H												



Pretty neat.

Reagents

YPD (250 mL)

To 500 mL baffled flask add:

2.5 g Yeast extract

5.0 g Protease-Peptone

5.0 g Glucose

Mix thoroughly and autoclave Liquid 15'

Store at room temperature

Thursday, October 27, 2022

To Do:

1. Supplement MHB
2. Patch out KRLVS148
3. Set up yeast overnight

Results and Data:

My patches of KRLVS148 and KRLVS149 were not nearly grown enough to make cultures. I put them back in the incubator in order to make single use aliquots for tomorrow. I'm thinking of patching out and leaving them at room temperature over the weekend so that I can do the assay on Monday instead.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

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

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

Friday, October 28, 2022

To Do:

1. ~~Make electrocompetent yeast~~
2. ~~Transform pKR168 into yeast~~
3. ~~Electroporate pKR141 into KRLVS28 and KRLVS75 for Brenna~~
4. ~~Patch out KRLVS149~~
5. Make glycerol stocks and SUA of KRLVS148 and KRLVS149

Results and Data:

Making Electrocompetent Yeast Cells

1. Take OD600 (dilute 1:10) until it reaches about 1.3-1.5 OD
2. Place entire volume of culture in sterile 250 mL centrifuge bottle
3. Centrifuge at 3000 xg for 5 min at 4°C. Discard supernatant.
4. Wash with ~200 mL of ice-cold water twice with identical centrifugation.
5. Wash in 20 ml of ice cold 1M sorbitol and centrifuge again, as above.
6. Resuspend in smallest volume of ice cold 1M sorbitol, starting with 200 ul and up to 500 ul

Electroporation of Yeast with pKR168

1. Add 3 ul of plasmid to 100 ul of electrocompetent cells. Mix via pipette. Incubate on ice for 5 min.
2. Transfer to prechilled 0.2 cm cuvette
3. Electroporate on presetting SC2 (1500 V, 5 msec)
4. Immediately add the cells to 1 mL of cold 1M sorbitol
5. Plate onto -ura selective plates. For plasmid transformation, plate 10 ul and 100 ul. Put in incubator set to 30°C. Colonies will be visible within 2-3 days for successful transformations.

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	Number of Plates
1	pKR168	pKR168	3 uL	100, 200 uL, Rem.	3
2	(+) control	pKR128	3 uL	200 uL	1
3	(-) control	-		200 uL	1
				Total:	5

Electroporating pKR141 into electrocompetent KRLVS28

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 pKR141 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 3 hours, shaking at 37°C
7. Plate on CHAH-Kan plates
 - a. Plated 10 uL, 100 uL, 200 uL, and remaining volume of cells spun down and resuspended in ~100 uL of liquid
8. Incubate plates at 37°C for 3 days

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	Number of Plates
1	pKR168	pKR168	3 uL	10 uL, 100 uL, 200 uL, Rem.	4
2	(-) control	-	-	200 uL	1
				Total:	5

Monday, October 31, 2022

To Do:

- ~~1. Make CHAH+Nat~~
- ~~2. Make glycerol stocks of KRLVS148 and KRLVS149~~
- ~~3. Resuspend Zymolyase~~
- ~~4. Make soft agar overlay for Brenna~~
- ~~5. Make overnight of yeast electroporation~~
- ~~6. Patch out yeast colony~~

Results and Data:

Making Personal Glycerol Stocks of KRLVS148 and KRLVS149

1. Make 1 cryovial for each strain, label!
2. Prepare 1.6mL of MHB in a 2mL centrifuge tube
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 400ul of 75% glycerol to the 1.6mL mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. For single use stocks pipet 50ul of solution to tubes

For making my yeast overnights for the miniprep tomorrow, I started one culture in 6 mL of SD -ura and let it grow for an hour, then added 1 mL to each of 6 tubes already containing 4 mL of SD -ura, for a total of 5 mL. Additionally, for the patch, I meant to only grab half the colony to make my overnight but I ended up accidentally grabbing most of it, so after I added it to the tube I immediately patched the stick on a SD -ura plate, then grabbed up the little bit that was left of the colony and patched that out in order to make a stock strain tomorrow.

Reagents

Zymolyase (1000 U)

Add 200 uL Zymolyase storage buffer to lyophilized zymolyase

Pipet up and down, do not vortex

CHAH+Nat

To 500mL flask add:

15.3g of cystine heart agar

150mL of ddiH2O (type I)

stirbar

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

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

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

Using sterile technique, pour hemoglobin into CHA

For 5 ug/ml nat, add 15 ul of 100 mg/mL nourseothricin to 300 mL of media