

## Table of Contents

November 2022	4
Tuesday, November 1, 2022	4
Wednesday, November 2, 2022	4
Testing the Effect of pH on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and KRLVS193	4
GFP Assay on pH Conditions in KRLVS192 and KRLVS193	5
Thursday, November 3, 2022	6
$\beta$ -galactosidase Assay of KRLVS28 Transposon Insertion Mutants	6
Friday, November 4, 2022	7
Monday, November 7, 2022	8
gDNA Prep of Potential Triple Mutants and KRLVS153	8
Reagents	8
Tuesday, November 8, 2022	8
Receiving and Dissolving Primers	9
PCR of Potential Triple Mutants to Check for <i>rpsU1</i> or <i>rpsU3</i>	9
Reagents	10
Wednesday, November 9, 2022	10
Gel of Potential Triple Mutants	11
Making Single Use Aliquots of KRLVS192 1+2, KRLVS193 1+2, and LVS-pF	11
Reagents	11
Thursday, November 10, 2022	12
qRT-PCR of KRLVS148, KRLVS149, KRLVS150, KRLVS151 (8/5/22) cDNA with <i>LacZ</i>	12
Reagents	13
Friday, November 11, 2022	13
PCR of Potential Triple Mutants to Check for <i>rpsU1</i> or <i>rpsU3</i>	13
Gel of Potential Triple Mutants	14
Monday, November 14, 2022	16
Reagents	16
Tuesday, November 15, 2022	16
MHB v. CDM and CHAH on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and KRLVS193	16
GFP Assay on CDM Conditions in KRLVS192 and KRLVS193	17
Reagents	18
Wednesday, November 16, 2022	18

CDM Iron Conditions on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and KRLVS193	18
GFP Assay on CDM Iron Conditions in KRLVS192 and KRLVS193	19
Reagents	21
Thursday, November 17, 2022	21
CDM Mg Conditions on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and KRLVS193	21
GFP Assay on CDM Mg Conditions in KRLVS192 and KRLVS193	22
Reagents	23
Friday, November 18, 2022	23
Monday, November 21, 2022	24
Tuesday, November 22, 2022	24
Monday, November 28, 2022	25
PCR of Potential Triple Mutants to Check for <i>rpsU1</i> or <i>rpsU3</i>	25
Reagents	26
Tuesday, November 29, 2022	26
Gel of Potential Triple Mutants	26
Reagents	27
Wednesday, November 30, 2022	27
December 2022	28
Thursday, December 1, 2022	28
Friday, December 2, 2022	28
Monday, December 5, 2022	29
Tuesday, December 6, 2022	29
Reagents	29
Wednesday, December 7, 2022	29
Making Electrocompetent Yeast Cells	30
Electroporation of Yeast with pKR168	30
PCR of Potential Triple Mutants to Check for <i>rpsU1</i> or <i>rpsU3</i>	30
Reagents	31
Thursday, December 8, 2022	32
Gel of Potential Triple Mutants	32
Reagents	32
Friday, December 9, 2022	33
CDM Fe and Mg Conditions on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and KRLVS193	33
GFP Assay on CDM Mg and Fe Conditions in KRLVS192 and KRLVS193	33

Sunday, December 11, 2022	34
Reagents	34
Monday, December 12, 2022	35
$\beta$ -galactosidase Assay of KRLVS148 and KRLVS149	35
Reagents	36
Tuesday, December 13, 2022	36
$\beta$ -galactosidase Assay of KRLVS28 and KRLVS75 in MHB and CDM	36
Wednesday, December 14, 2022	37
Growth Curve of LVS and <i>drpsU2</i> in CDM and MHB	37
Reagents	38
Thursday, December 15, 2022	38
Agar overlay for $\beta$ -galactosidase activity in Mutant 4 from MM	38
Ligation of <i>PrpsU2 tul4</i> 5'UTR PCR from gBlock with pKR128 backbone	38
Reagents	39
Friday, December 16, 2022	40
Making electrocompetent yeast	40
Electroporation of Candidate pKR168 + pKR168 Ligation into Yeast	41
Monday, December 19, 2022	42
Effect of 10x and 100x Fe on <i>rpsU1</i> and <i>rpsU3</i> Production in KRLVS192 and 193	42
Agar overlay for $\beta$ -galactosidase activity in Mutant 4 Blue and White Colonies v. LVS	42
GFP Assay on CDM Fe Conditions in KRLVS192 and KRLVS193	42
Wednesday, December 28, 2022	<b>Error! Bookmark not defined.</b>
Thursday, December 29, 2022	<b>Error! Bookmark not defined.</b>
Friday, December 30, 2022	<b>Error! Bookmark not defined.</b>
Bibliography	44

## November 2022

Tuesday, November 1, 2022

### To Do:

1. Patch out KRLVS192, KRLVS193, and LVS-pF
2. Patch out triple mutants

### Results and Data:

I was unable to miniprep my (hopefully) pKR168 from the yeast cultures, as the cultures did not grow. Boo ☹️

Wednesday, November 2, 2022

### To Do:

1. Set up cultures for pH experiment
2. pH experiment on KRLVS192 and KRLVS193
3. GFP assay on pH experiment

### Results and Data:

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

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 (35 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	KRLVS192/3 1			KRLVS192/3 2			KRLVS192/3 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	2 hours	4 hours
KRLVS192 1	0.078	0.111	oops
KRLVS192 2	0.064	0.112	oops
KRLVS192 3	0.068	0.101	oops
KRLVS193 1	0.063	0.128	oops
KRLVS193 2	0.082	0.143	oops
KRLVS193 3	0.075	0.131	oops
LVS-pF	0.067	0.170	oops

I forgot to write down the 4 hour time point, but they were all between 0.205 and 0.270. I let them grow for another 30 minutes prior to media transfer.

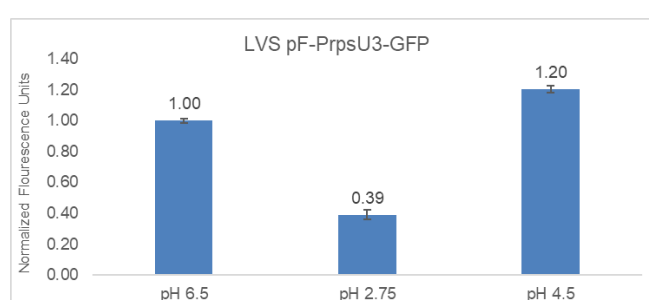
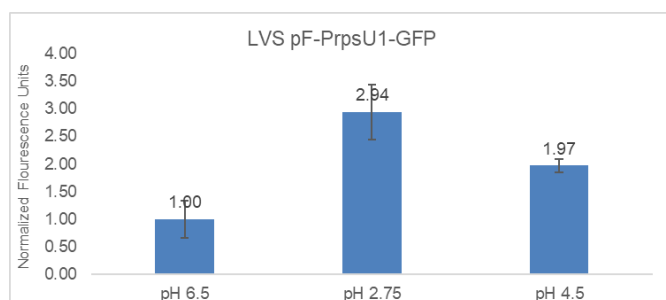
### GFP Assay on pH Conditions in KRLVS192 and KRLVS193

1. Pellet 4 mLs of culture and spin at max speed for 3 minutes
2. Remove all MHB, using 20  $\mu$ L pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250  $\mu$ L 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  $\mu$ L 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
4	KRLVS193 1
5	KRLVS193 2
6	KRLVS193 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1		C1	C1	C1		B4	B4	B4	
B	A2	A2	A2		C2	C2	C2		B5	B5	B5	
C	A3	A3	A3		C3	C3	C3		B6	B6	B6	
D	B1	B1	B1		A4	A4	A4		C4	C4	C4	
E	B2	B2	B2		A5	A5	A5		C5	C5	C5	
F	B3	B3	B3		A6	A6	A6		C6	C6	C6	
G									LVS	LVS	LVS	
H									1xPBS	1xPBS	1xPBS	



KRLVS192 still appears to be pretty variable in its level of fluorescent expression, however, there is still a fold increase at lower pH levels so I'm hoping that that is still promising.

Thursday, November 3, 2022

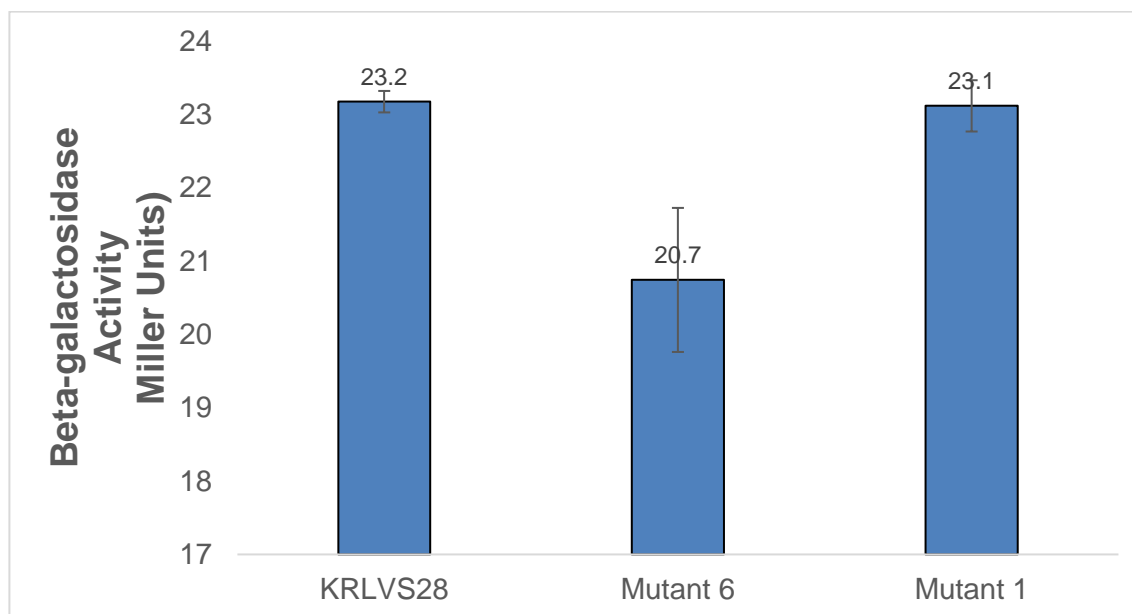
**To Do:**

- ~~1. Prepare cultures for  $\beta$  gal with Brenna~~
- ~~2. Run  $\beta$  gal assay with Brenna~~

**Results and Data:**

**$\beta$ -galactosidase Assay of KRLVS28 Transposon Insertion Mutants**

1. Grow 6ml cultures until  $OD_{600} = 0.3$
2. Turn on 28°C water bath
3. Determine amount of Z-buffer needed ( $0.8\text{ml} \times 2 \times \#$  of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer ( $2.72 \times X\text{ml Z-buffer} = \_\mu\text{l of BME}$ ).
4. Set up reaction tubes with 800 $\mu\text{l}$  Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. Once cultures reach  $OD_{600} = 0.3$ , place on ice 30 min and put ONPG in water bath
7. After cells have incubated on ice, measure  $OD_{600}$  of bacterial cultures
8. Add 200 $\mu\text{l}$  culture to each reaction tube (add 200 $\mu\text{l}$  culture media to blank tube)
9. Add 30 $\mu\text{l}$  0.1% SDS to each reaction tube
10. Add 60 $\mu\text{l}$   $\text{CHCl}_3$  (chloroform) to each reaction tube
11. Vortex reaction pairs on high for 6 secs (time precisely with timer)
12. Put in water bath for 10 min
13. Prepare repeater pipette with 1M  $\text{Na}_2\text{CO}_3$  (stop)
14. Add 200 $\mu\text{l}$  ONPG in 5 sec intervals (use timer with hours)
15. Shake gently and watch for yellow (goal  $OD_{420}$  is 0.6-0.9)
16. Stop with 500 $\mu\text{l}$  1M  $\text{Na}_2\text{CO}_3$ , record time, vortex at 4 for 10 sec
17. Give all reaction at least 2 hours
18. Remove 1 mL from reaction (avoid chloroform at bottom), measure  $OD_{420}$  and  $OD_{550}$ , using blank reaction as blank in spectrophotometer.



Obviously, neither mutant really resulted in the up-regulation of *rpsU1*.

Friday, November 4, 2022

**To Do:**

1. ~~gDNA prep~~

**Results and Data:**

I realized that I forgot to patch out KRLVS153 yesterday, so I scraped up my patches and pelleted them, and put them in my -80 to get gDNA from on Monday along with KRLVS153.

Monday, November 7, 2022

**To Do:**

- ~~1. gDNA prep of triple deletion strains~~
- ~~2. Make 2.5% iron pyrophosphate~~

**Results and Data:**

**gDNA Prep of Potential Triple Mutants and KRLVS153**

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scrape up patches and resuspend in MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet the debris by centrifugation at 4°C for 10 minutes at max speed. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful not to dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 35uL of 0.1x EB.

I lost some of the sample from mutant 2 and 4, and maybe 5, after the 65°C incubation step. It seemed the lids lifted silently, so when I vortexed them I lost some of the sample. Maybe I should have calculated the concentration of reagents to add after that, but I did not bother. Additionally, after the protein precipitation, sample 8's pellet was large and not all pelleted into the bottom. So after I took the supernatant out of the other samples, I added an additional volume of MPC and spun again, but I had the same issue, so I centrifuged on a shorter spin time a few times and tried to get as much supernatant as I could out without disturbing the pellet. I did get some of the debris in the tube, so I centrifuged again at 4°C and took off what I could into another new tube.

**Reagents**

**2.5% Iron pyrophosphate**

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

Tuesday, November 8, 2022

**To Do:**

- ~~1. Patch out KRLVS192, KRLVS193, and LVS-pF~~
- ~~2. Supplement MHB~~
- ~~3. Nanodrop gDNA~~
- ~~4. Process primers~~
- ~~5. Set up PCR to check for *rpsU1* or *rpsU2* in triple deletion mutants~~



## Results and Data:

I diluted my gDNA samples 1:2 for a total of 3 uL, using 2 uL to nanodrop. Two of my samples, mutants 7 and 8 were still too high so I used the remaining 1 uL and diluted it again, so it was 1:4.

Sample Name	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
Triple Mutant 1	1000.0	10.001	5.385	1.86	1.63
Triple Mutant 2	716.6	7.165	3.873	1.85	1.41
Triple Mutant 3	1695.0	16.949	9.206	1.84	1.33
Triple Mutant 4	1032.2	10.322	5.514	1.87	1.57
Triple Mutant 5	1576.2	15.761	8.521	1.85	1.32
Triple Mutant 7	2832.0	14.160	7.756	1.83	1.40
Triple Mutant 8	2136.2	4.565	2.473	1.85	1.54
KRLVS153	1621.6	16.216	8.708	1.86	1.40

In order to run my PCR, I diluted my gDNA to 100 ng/uL via the following amounts in a total of 200 uL:

Sample Name	Nucleic Acid (ng/uL)	DNA (uL)	EB (uL)
Triple Mutant 1	1000.0	20.0	180.0
Triple Mutant 2	716.6	27.9	172.1
Triple Mutant 3	1695.0	11.8	188.2
Triple Mutant 4	1032.2	19.4	180.6
Triple Mutant 5	1576.2	12.7	187.3
Triple Mutant 7	2832.0	7.1	192.9
Triple Mutant 8	2136.2	9.4	190.6
KRLVS153	1621.6	12.3	187.7

## Receiving and Dissolving Primers

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

## PCR of Potential Triple Mutants to Check for *rpsU1* or *rpsU3*

1. Acquired and labelled PCR tubes: 1, 2, 3, 4, 5, 7, 8, LVS', and negative controls.

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	<i>rpsU2</i>	Mutant 1	KROL601, KROL602	-
2	<i>rpsU2</i>	Mutant 2	KROL601, KROL602	-
3	<i>rpsU2</i>	Mutant 7	KROL601, KROL602	-
4	<i>rpsU2</i>	Mutant 8	KROL601, KROL602	-
5	<i>rpsU2</i>	LVS	KROL601, KROL602	193

6	- control	-	KROL601, KROL602	-
7	<i>rpsU1</i>	Mutant 3	KROL599, KROL600	-
8	<i>rpsU1</i>	Mutant 4	KROL599, KROL600	-
9	<i>rpsU1</i>	Mutant 5	KROL599, KROL600	-
10	<i>rpsU1</i>	LVS	KROL599, KROL600	178
11	- control	-	KROL599, KROL600	-

- Acquire the following components and put them on ice, labeling tubes if necessary:
  - mgH<sub>2</sub>O, 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 H<sub>2</sub>O to negative control tube (template volume for 1 reaction)
- Prepare a master-containing:
  - mgH<sub>2</sub>O, dNTPs, Primestar buffer, respective primers, and Primestar enzyme
- Mix the master-mix solution by pipetting up and down
- Add 19.6 uL of master-mix to negative control PCR tube
- Add 19.6 uL of master mix to each PCR tube and pipet up and down to mix
- Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	6

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH <sub>2</sub> O			12.4	86.8
PrimeSTAR GXL Buffer	5x	1x	4.0	28
dNTPs	2.5 mM	0.2 mM	1.6	11.2
oligo F	10 uM	0.3 uM	0.6	4.2
oligo R	10 uM	0.3 uM	0.6	4.2
template	18.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2.8
Total volume			20	137.2

Used these master mix volumes for both master mixes, with their respective primers.

## 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

Wednesday, November 9, 2022

To Do:

- ~~Run gel of potential triple mutant PCR samples~~
- ~~Put away dishes~~
- ~~Make 75% glycerol~~

4. ~~Make CDM components~~

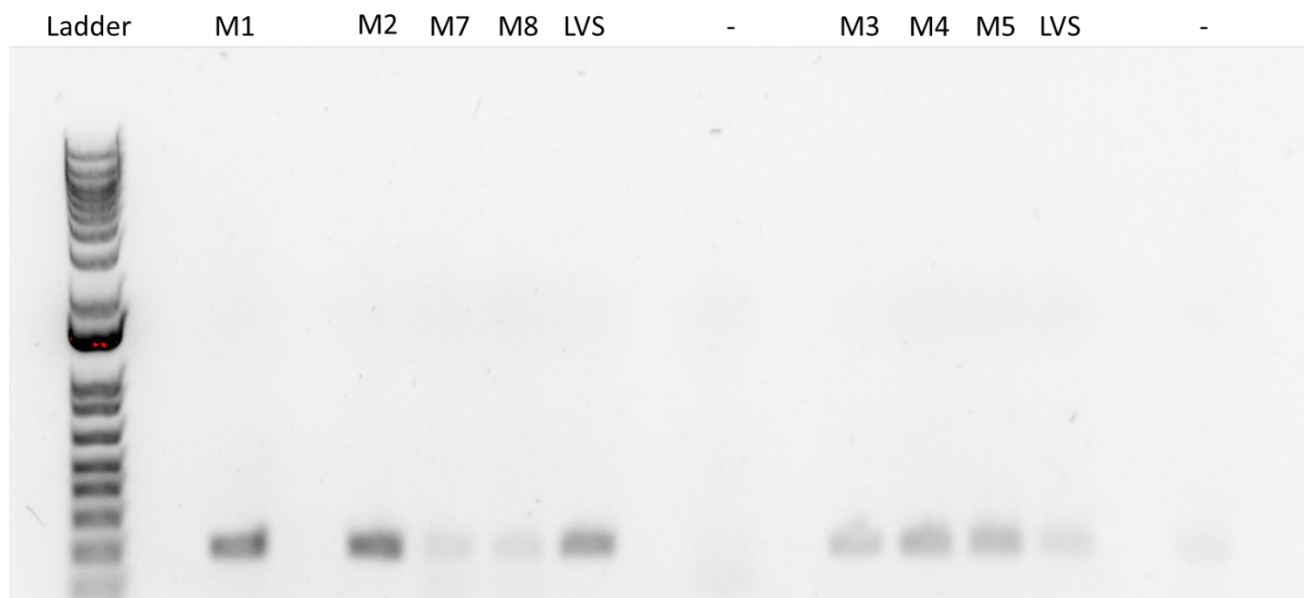
5. ~~Make single use aliquots of KRLVS192 1 and 2, KRLVS193 1 and 2, and LVS-pF~~

## **Results and Data:**

### **Gel of Potential Triple Mutants**

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cooled
2. Set up large gel rig to cast gel, with ladder.
3. Add 12uL of Sbyr Safe and pour ~120uL gel, use ladder to mix, then add 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																
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	LD		1		2	7	8	LVS		-		3	4	5	LVS	-



### **Making Single Use Aliquots of KRLVS192 1+2, KRLVS193 1+2, and LVS-pF**

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

### **Reagents**

#### **75% Glycerol**

To 250 mL bottle add:

75 mL 100% glycerol

25 mL water

Pipet up and down until mixed

Autoclave Liquid 30'

400 mg/mL Glucose

To 50 mL beaker add:

18 g glucose

~20 mL of water

Stir on heat until complete dissolved

Add solution to graduated cylinder and add water to final volume of 45 mL

Filter sterilize

0.5 mg/mL  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

To 50 mL beaker add:

22.5 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

45 mL water

Stir until complete dissolved

33.75 mg/mL  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

To 50 mL beaker add:

1.52 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

~40 mL water

Stir until complete dissolved

Add solution to graduated cylinder and add water to final volume of 45 mL

Thursday, November 10, 2022

To Do:

1. ~~qRT-PCR of *lacZ* region of *rpsU2* 5'UTR samples~~
2. ~~Ask Camberg lab for ura media~~
3. ~~Make SC ura media~~
4. ~~Make yeast overnights~~

## Results and Data:

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

1. Each experiment will need at least one test primer and one control primer for each sample
  - a. KROL399/400 as test primers to amplify *LacZ*. KROL63/64 the control, amplifying *tul4*.
2. Each cDNA sample will be used in a reaction with each primer set meaning #Samples\*#Primer Sets (12\*2=24 reactions)
3. Set up a master mixes, labelling the tubes as A, B, C, etc.:

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

4. Obtain a strip of strip tubes per each primer set with the appropriate number of tubes in the strip, according to the number of cDNA samples. Label strip tubes with letter of primer master mix, and the sample number.
5. Add 3.5uL of cDNA into appropriately labelled tubes.
6. As these will be in technical triplicate, each tube will be an individual master mix, totaling 70 uL. Thusly, add corresponding primer master mix to tubes containing DNA at a volume of 66.5 uL.

7. Pipette 20  $\mu$ L of each primer set strip tubes, in triplicate, using multichannel pipet.
8. Spin plate down and run in LightCycler using the 'KRamsey\_Lab\_old\_stepone' program according to 'qRT-PCR\_LightCycler\_Machine.docx'

Labelling was as follows:

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

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

Loaded 96-well plate according to the following table:

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

## Reagents

SC-ura Media (100 mL)

To 250 mL bottle add:

0.19 g of SC-ura media

100 mL of water

Mix and autoclave Liquid 15'

Friday, November 11, 2022

To Do:

1. ~~Miniprep yeast plasmid~~
2. ~~PCR of potential triple mutants again~~
3. ~~Gel of PCR of potential triple mutants~~

## Results and Data:

My yeast overnights didn't grow even a little bit.

## PCR of Potential Triple Mutants to Check for *rpsU1* or *rpsU3*

1. Acquired and labelled PCR tubes: 1, 2, 3, 4, 5, 7, 8, LVS', and negative controls.

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	<i>rpsU2</i>	Mutant 1	KROL601, KROL602	-
2	<i>rpsU2</i>	Mutant 2	KROL601, KROL602	-
3	<i>rpsU2</i>	Mutant 7	KROL601, KROL602	-
4	<i>rpsU2</i>	Mutant 8	KROL601, KROL602	-
5	<i>rpsU2</i>	LVS	KROL601, KROL602	193
6	<i>rpsU2</i>	KRLVS47	KROL601, KROL602	-
7	- control	-	KROL601, KROL602	-
8	<i>rpsU1</i>	Mutant 3	KROL599, KROL600	-
9	<i>rpsU1</i>	Mutant 4	KROL599, KROL600	-

10	<i>rpsU1</i>	Mutant 5	KROL599, KROL600	-
11	<i>rpsU1</i>	LVS	KROL599, KROL600	178
12	<i>rpsU1</i>	KRLVS47	KROL599, KROL600	-
13	- control	-	KROL599, KROL600	-

- Acquire the following components and put them on ice, labeling tubes if necessary:
  - mgH<sub>2</sub>O, Primestar buffer, dNTPs, KROL599, KROL600, KROL601, KROL602, (10uM), and candidate triple mutants
- 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 H<sub>2</sub>O to negative control tube (template volume for 1 reaction)
- Prepare a master-containing:
  - mgH<sub>2</sub>O, dNTPs, Primestar buffer, respective primers, and Primestar enzyme
- Mix the master-mix solution by pipetting up and down
- Add 19.6 uL of master-mix to negative control PCR tube
- Add 19.6 uL of master mix to each PCR tube and pipet up and down to mix
- Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	7

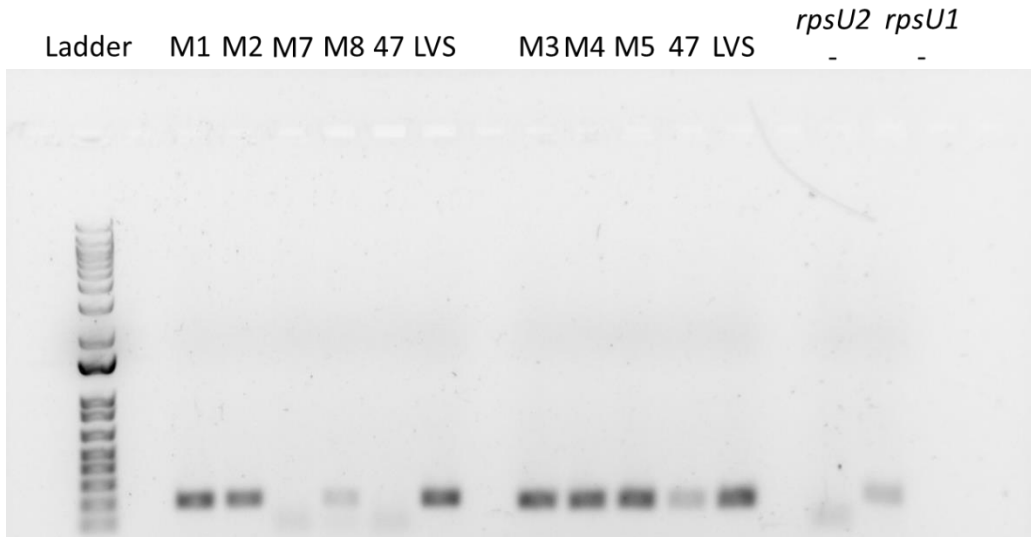
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	8
ddiH <sub>2</sub> O			12.4	99.2
PrimeSTAR GXL Buffer	5x	1x	4.0	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
oligo F	10 uM	0.3 uM	0.6	4.8
oligo R	10 uM	0.3 uM	0.6	4.8
template	18.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
Total volume			20	156.8

### Gel of Potential Triple Mutants

- Melt agarose gel until completely dissolved, then place in 50°C water bath until cooled
- Set up large gel rig to cast gel, with ladder.
- Add 12uL of Sbyr Safe and pour ~120uL gel, use ladder to mix, then add ladder and allow to set.
- Turn gel, add used 1xTAE, remove ladder.
- Loaded 10 uL ladder, and 15 uL of each sample according to the loading order below.
- Ran for 45 minutes at 113V.

Loading Order of Diagnostic Digest of Candidate pKR122																
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
LD		1	2	7	8	47	LVS		3	4	5	47	LVS		-	-

Given my issues with my last gel, I made sure to pour this one much thicker. Additionally, I added less of each PCR reaction to each individual well.



It does look like my *rpsU2* primers are forming primer dimers, given the lower band in the negative, also seen in the (positive) negative control, and mutant 8. It looks like I still have LVS contamination in the *rpsU1* primer set mix, though I am unsure of how it got there given that I got new aliquots of each component, apart from the enzyme. Additionally, I added each component to both master mixes at the same time (ie. water into both, followed by buffer into both, etc.) It's not just spillover either, given that the negative was so separate. My best guess is it might be the diluted primers, so I might re-dilute and run the PCR on the *rpsU1* set again.

## Monday, November 14, 2022

### To Do:

1. ~~Patch out KRLVS192, KRLVS193, LVS-pF~~
2. ~~Make KH<sub>2</sub>PO<sub>4</sub>~~
3. ~~Make CDM~~

### Results and Data:

#### Reagents

KH<sub>2</sub>PO<sub>4</sub> (45 mL)

To 100 mL beaker add:

4.5 g KH<sub>2</sub>PO<sub>4</sub>

~35 mL water

Stir until mixed

Add water to a total volume of 45 mL

CDM (150mL)

To 250 mL beaker add:

12 mL Combined Amino Acid Stock

Then add:

600 uL Thiamine HCl

600 uL Spermine tetrahydrochloride

600 uL DL-Calcium pantothenate

Then add:

5.13 mL 5N NaCl

1.5 mL KH<sub>2</sub>PO<sub>4</sub>

600 uL K<sub>2</sub>HPO<sub>4</sub>

Then:

1.5 mL 400 mg/mL Glucose

Then:

115 mL Water

Then:

600 uL FeSO<sub>4</sub>•7H<sub>2</sub>O

600 uL MgSO<sub>4</sub>•7H<sub>2</sub>O

pH media to a pH between 6.3 and 6.5

Add water to 150 mL total

Filter sterilize

## Tuesday, November 15, 2022

### To Do:

1. ~~Patch out KRLVS192, KRLVS193, and LVS-pF~~
2. ~~Set up cultures for MHB v. CDM, CHAH, and overgrowth experiment~~
3. ~~GFP assay of MHB v. CDM and CHAH~~
4. ~~Make differing iron CDM solutions~~

### Results and Data:

#### MHB v. CDM and CHAH on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193

Condition Number	0	0	1	2
Experiment Component	Background	Background	Test 1	Test 2
Condition	CDM	MHB	CDM	MHB



1. Resuspend patches of 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 in culture tubes.
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, non-buffered MHB

OD Readings			
Sample	0 hours	2 hours	4 hours
KRLVS192 1 C	0.094	0.193	0.345
KRLVS192 2 C	0.083	0.192	0.345
KRLVS193 1 C	0.084	0.186	0.330
KRLVS193 2 C	0.099	0.201	0.333
LVS-pF C	0.104	0.235	0.427
KRLVS192 1 M	0.086	0.162	0.278
KRLVS192 2 M	0.089	0.150	0.242
KRLVS193 1 M	0.089	0.164	0.294
KRLVS193 2 M	0.077	0.151	0.278
LVS-pF M	0.084	0.150	0.264

I additionally started cultures of KRLVS192 1 and 2, and KRLVS193 1 and 2 in 5mL MHB OD<sub>600</sub>=0.05 at 3:30 pm in order to test stationary phase with the assay the next day.

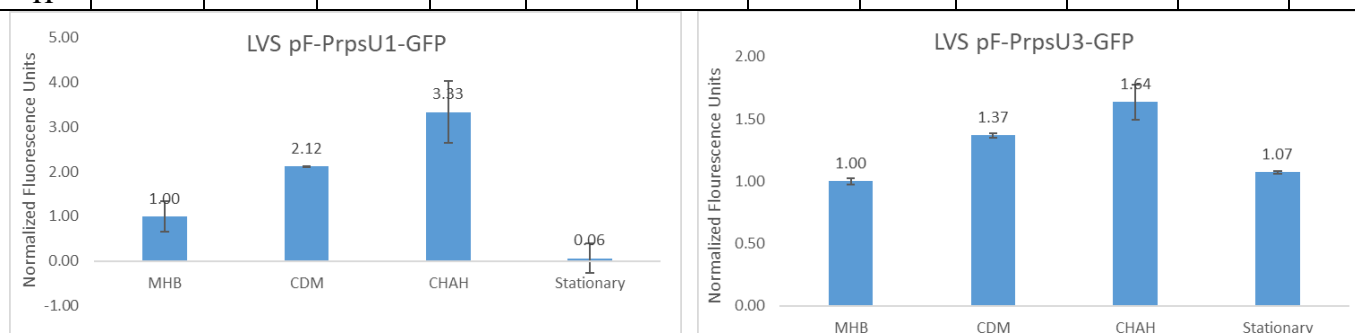
### GFP Assay on CDM Conditions in KRLVS192 and KRLVS193

1. Pellet 2 mLs of culture (MHB and CDM, obviously not CHAH) and spin at max speed for 3 minutes
  - a. Scrape up patches of CHAH and resuspend in PBS
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 OD<sub>600</sub> 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	MHB
B	CDM
C	CHAH

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		B3	B3	B3		C1	C1	C1	
B	A2	A2	A2		B4	B4	B4		C2	C2	C2	
C	A3	A3	A3		B1	B1	B1		C3	C3	C3	
D	A4	A4	A4		B2	B2	B2		C4	C4	C4	
E	A.LVS	A.LVS	A.LVS		B.LVS	B.LVS	B.LVS		C.LVS	C.LVS	C.LVS	
F												
G												
H												



## Reagents

Component	Location	Group	[Stock] (mg/mL)	[Final]	No iron (mL)	Quarter Iron (mL)	Double Iron (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8	2.8	2.8
Thiamine HCl	4°C	2	1	0.004	0.14	0.14	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14	0.14	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14	0.14	0.14
NaCl	RT	3	292.2	10.0	1.197	1.197	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35	0.35	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14	0.14	0.14
Glucose	RT	Separate	400	4	0.35	0.35	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0	0	0.035	0.28
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14	0.14	0.14
Water	-	-	-	-	29.603	29.568	29.323

Wednesday, November 16, 2022

### To Do:

1. Prepare cultures for iron concentration
2. Patch out KRLVS192, KRLVS193, and LVS\_pF
3. Ship triple mutant 7 for whole genome sequencing
4. Put away dishes
5. Make CDM with varying Magnesium concentrations
6. Make 200 mL MHB
7. Run GFP assay on iron and stationary phase

## Results and Data:

### CDM Iron Conditions on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193

Condition Number	0	1	2	3	4
Experiment Component	Background	Control	Test 1	Test 2	Test 3
Condition	CDM	CDM	0 mg/mL Fe	0.0005 mg/mL Fe	0.004 mg/mL Fe

1. Resuspend patches of cells in 400uL of CDM, with 0 mg/mL Fe, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of respective CDM, either regular or varying concentrations of iron
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, CDM

OD Readings			
Sample	0 hours	2 hours	4 hours
KRLVS192 1 C	0.103	0.233	0.444
KRLVS192 2 C	0.087	0.213	0.383
KRLVS193 1 C	0.083	0.207	0.403
KRLVS193 2 C	0.026	0.067	0.131
KRLVS192 1 0	0.095	0.223	0.424
KRLVS192 2 0	0.084	0.217	0.435
KRLVS193 1 0	0.083	0.211	0.418
KRLVS193 2 0	0.085	0.207	0.398
KRLVS192 1 ¼	0.092	0.228	0.441
KRLVS192 2 ¼	0.089	0.224	0.427
KRLVS193 1 ¼	0.083	0.223	0.419
KRLVS192 2 ¼	0.093	0.227	0.432
KRLVS192 1 2x	0.095	0.241	0.458
KRLVS192 2 2x	0.086	0.210	0.383
KRLVS193 1 2x	0.083	0.223	0.434
KRLVS193 2 2x	0.078	0.210	0.363
LVS-pF CDM	0.080	0.203	0.376

These cultures obviously grew up very fast for LVS which makes me kind of nervous. I had been a little worried about my plates since there's still an unknown source of contamination from when Brenna was in lab, so I had theorized maybe it was the glycerol (after already making new single use aliquots). However, Morgan checked the glycerol on a plate (plated 50uL) and didn't see growth so it's definitely fine, probably. Regardless, because I was nervous I had Hannah check out the plates when she came in and she said that she didn't see any contamination, and all of the cultures grew quickly so if it was a different contaminant then it would have had to enter all the cultures. The doubling time was about ~2 hours here, with like no lag time. I did change up my plating and scraping protocol to try to have more active cells, but I don't think they changed it that much. (Also though... I used the same single use aliquots for the experiment on Wednesday and they grew as expected in MHB, so it's probably not the single use aliquots). It's maybe fine, I looked for a previous growth experiment in CDM and couldn't find any, so I'll ask Kathryn if she wants one.

### GFP Assay on CDM Iron Conditions in KRLVS192 and KRLVS193

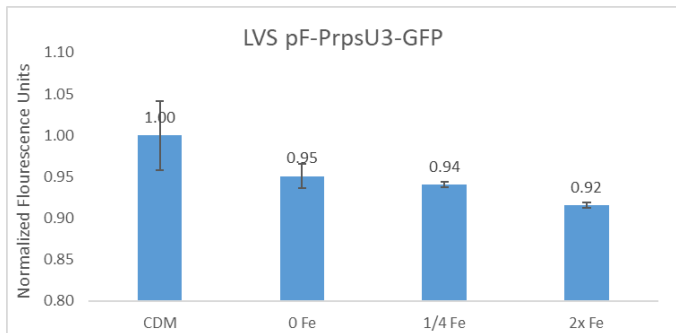
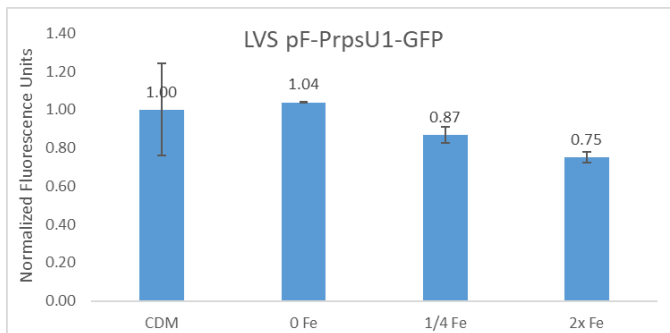
1. Pellet 2 mLs of culture and spin at max speed for 3 minutes
2. Remove all CDM, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.
4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS in triplicate as control.
5. Go to INBRE lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
6. Read OD600 from clear plate on ID3 plate reader:

- a. Select Absorbance, wavelength=600
  - b. Plate type: 96-well standard clearbtm (first option)
7. Transfer 200 ul of each well from clear plate to black plate using the multichannel
8. Read fluorescence from black plate on ID3 plate reader:
  - a. Select fluorescence
  - b. Wavelength: 495 to 535
  - c. Plate type: CoStar 3789
  - d. Gain: Automatic
  - e. Integration: 380 ms

Label	Condition
A	CDM
B	0 mg/mL Fe
C	0.0005 mg/mL Fe
D	0.004 mg/mL Fe
E	Stationary Phase

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

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



## Reagents

Component	Location	Group	[Stock] (mg/mL)	[Final]	No Mg (mL)	Quarter Mg (mL)	Double Mg (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8	2.8	2.8
Thiamine HCl	4°C	2	1	0.004	0.14	0.14	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14	0.14	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14	0.14	0.14
NaCl	RT	3	292.2	10.0	1.197	1.197	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35	0.35	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14	0.14	0.14
Glucose	RT	Separate	400	4	0.35	0.35	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0	0.14	0.14	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0	0.035	0.28
Water	-	-	-	-	29.603	29.568	29.323

### Mueller Hinton Broth (200 mL)

To make, add to a 500 mL bottle:

4.2g of Mueller Hinton Broth mix

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

Autoclave on Liquid 30'

Can keep un-supplemented media at room temperature indefinitely

Thursday, November 17, 2022

#### To Do:

1. Supplement MHB
2. Set up cultures for magnesium environmental condition test
3. Autoclave plates and take out glass
4. Run GFP assay on magnesium

## Results and Data:

### CDM Mg Conditions on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193

Condition Number	0	1	2	3	4
Experiment Component	Background	Control	Test 1	Test 2	Test 3
Condition	CDM	CDM	0 mg/mL Mg	0.025 mg/mL Fe	0.2 mg/mL Fe

1. Resuspend patches of cells in 400uL of CDM, with 0 mg/mL Mg, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of respective CDM, either regular or varying concentrations of iron
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, CDM

OD Readings		
Sample	0 hours	4 hours
KRLVS192 1 C	0.087	0.356
KRLVS192 2 C	0.080	
KRLVS193 1 C	0.076	
KRLVS193 2 C	0.093	

KRLVS192 1 0	0.090	0.244
KRLVS192 2 0	0.081	
KRLVS193 1 0	0.072	
KRLVS193 2 0	0.086	
KRLVS192 1 ¼	0.095	0.380
KRLVS192 2 ¼	0.090	
KRLVS193 1 ¼	0.076	
KRLVS192 2 ¼	0.104	
KRLVS192 1 2x	0.084	0.318
KRLVS192 2 2x	0.073	
KRLVS193 1 2x	0.082	
KRLVS193 2 2x	0.098	
LVS-pF CDM	0.081	0.0349

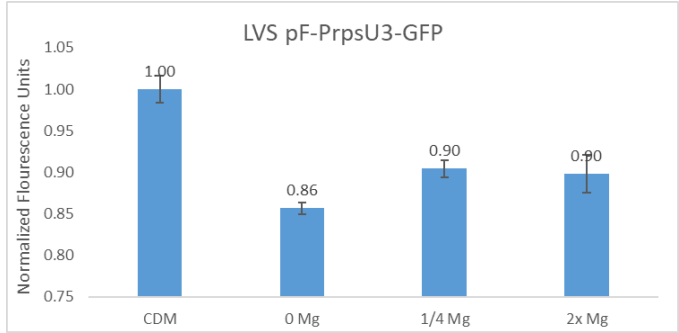
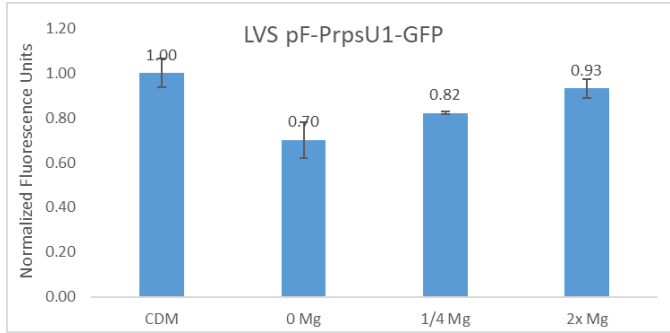
### GFP Assay on CDM Mg Conditions in KRLVS192 and KRLVS193

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

Label	Condition
A	CDM
B	0 mg/mL Mg
C	0.025 mg/mL Mg
D	0.2 mg/mL Mg

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

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



## Reagents

Mueller Hinton Broth (200 mL)

To supplement add:

1 mL of 10% glucose

1 mL of 2.5% iron pyrophosphate

2x isovitalex (combine 2mL from liquid vial with solids, add entire volume to broth)

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

Friday, November 18, 2022

To Do:

~~1. Streak out KRLVS9 (*drpsU2*) and LVS~~

## Results and Data:

Monday, November 21, 2022

To Do:

- ~~1. Patch out *drpsU2* and LVS from single colony streak~~

**Results and Data:**

Tuesday, November 22, 2022

To Do:

- ~~1. Streak out *drpsU2* and LVS to single colony and leave out on bench over holiday~~

**Results and Data:**



Monday, November 28, 2022

**To Do:**

- ~~1. Patch out *rpsU2* from single colony streak~~
- ~~2. Make 2.5% iron pyrophosphate~~
- ~~3. Supplement MHB~~
- ~~4. Run dishwasher~~
- ~~5. Run PCR of *rpsU1* mutants~~

**Results and Data:**

**PCR of Potential Triple Mutants to Check for *rpsU1* or *rpsU3***

1. Acquired and labelled PCR tubes: 1, 2, 3, 4, 5, 7, 8, LVS', and negative controls.

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	<i>rpsU1</i>	Mutant 3	KROL599, KROL600	-
2	<i>rpsU1</i>	Mutant 4	KROL599, KROL600	-
3	<i>rpsU1</i>	Mutant 5	KROL599, KROL600	-
4	<i>rpsU1</i>	LVS	KROL599, KROL600	178
5	<i>rpsU1</i>	KRLVS47	KROL599, KROL600	-
6	- control	-	KROL599, KROL600	-

2. Acquire the following components and put them on ice, labeling tubes if necessary:
  - mgH<sub>2</sub>O, Primestar buffer, dNTPs, KROL599, KROL600, KROL601, KROL602, (10uM), and candidate triple mutants
3. Vortex each component (aside from enzyme)
4. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
5. Add ddi H<sub>2</sub>O to negative control tube (template volume for 1 reaction)
6. Prepare a master-containing:
  - mgH<sub>2</sub>O, dNTPs, Primestar buffer, respective primers, and Primestar enzyme
7. Mix the master-mix solution by pipetting up and down
8. Add 19.6 uL of master-mix to negative control PCR tube
9. Add 19.6 uL of master mix to each PCR tube and pipet up and down to mix
10. Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	6

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH <sub>2</sub> O			12.4	86.8
PrimeSTAR GXL Buffer	5x	1x	4.0	28.0
dNTPs	2.5 mM	0.2 mM	1.6	11.2
oligo F	10 uM	0.3 uM	0.6	4.2
oligo R	10 uM	0.3 uM	0.6	4.2
template	18.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2.8
		Total volume	20	137.2

## Reagents

### 2.5% Iron pyrophosphate

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

### 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, November 29, 2022

### To Do:

1. ~~Run gel on triple mutant PCR~~
2. ~~Make CDM~~
3. ~~Put away dishes~~

## Results and Data:

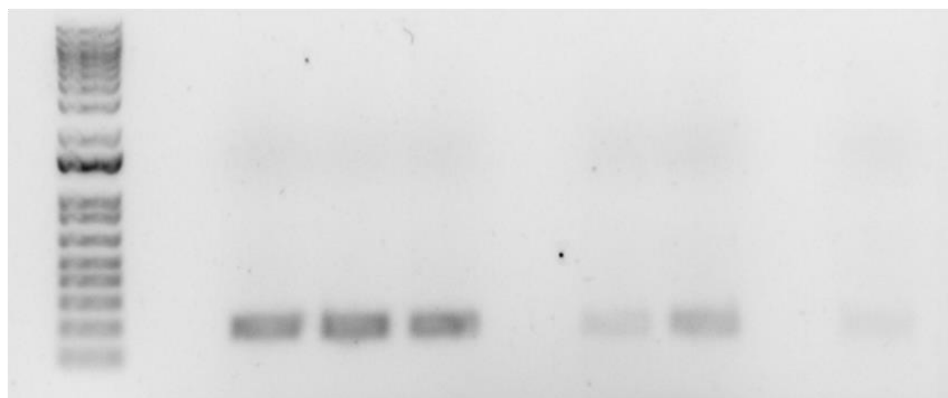
My cultures of yeast from my electroporation of pKR168 were once again not grown when I got in. I let them grow for an additional 7 hours but did not see any change in turbidity. Hannah suggested I try making one 5 mL overnight culture and then add to a 20 mL culture in the morning, so I will try that and also prepare to do a new yeast electroporation.

## Gel of Potential Triple Mutants

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 ~60mL gel, use ladder to mix, then add ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 10 uL ladder, and 15 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Triple Deletion Mutant PCR									
1	2	3	4	5	6	7	8	9	10
Ladder		Mut. 3	Mut. 4	Mut. 5		KRLVS47	LVS		(-)

Ladder      M3 M4 M5      47 LVS      (-)



Obviously, there is still contamination in the negative of LVS gDNA. I hadn't changed my water since in my last gel the *drpsU2* were fine, however Hannah pointed out it could have gotten contaminated between making the master mixes (though I technically added the water to both at the same time). I also didn't change out my enzyme last time, so I'll use fresh water and a fresh enzyme when I do it again.

## Reagents

### CDM (150mL)

To 250 mL beaker add:

12 mL Combined Amino Acid Stock

Then add:

600 uL Thiamine HCl

600 uL Spermine tetrahydrochloride

600 uL DL-Calcium pantothenate

Then add:

5.13 mL 5N NaCl

1.5 mL KH<sub>2</sub>PO<sub>4</sub>

600 uL K<sub>2</sub>HPO<sub>4</sub>

Then:

1.5 mL 400 mg/mL Glucose

Then:

115 mL Water

Then:

600 uL FeSO<sub>4</sub>•7H<sub>2</sub>O

600 uL MgSO<sub>4</sub>•7H<sub>2</sub>O

pH media to a pH between 6.3 and 6.5

Add water to 150 mL total

Filter sterilize

## Wednesday, November 30, 2022

### To Do:

- ~~1. Patch out KRLVS28 and KRLVS75 for Morgan~~
- ~~2. Patch out yeast~~
- ~~3. Start 5 mL overnight culture of yeast with pKR168 hopefully~~

## Results and Data:

I was going to set up my cultures for the growth curve today, however I realized that my *drpsU2* plate was contaminated and when I looked back at the single colony streak I realized that the colonies were not in fact LVS. It appears to be the same contaminant that Brenna had issues with, and Morgan had an issue with so I'm really nervous about it.

I started a 5 mL culture of pKR168 in the new SD -ura, allowing the media to warm to 30°C. I will add it to a 20 mL culture in the morning.

## December 2022

Thursday, December 1, 2022

### To Do:

1. ~~Help Morgan start cultures for B-gal~~
2. ~~Miniprep pKR168 ☹️ nvm~~
3. ~~Help Morgan take OD600 for her cultures~~
4. ~~Help Morgan set up for B-gal ☹️ nvm mostly~~
5. ~~Help Morgan with B-gal ☹️ nvm~~

### Results and Data:

In the morning I help Morgan set up her cultures for the B-gal. She had done most of the techniques that go into it, so I mostly helped with questions/math/etc.

Alternatively, my yeast cells did not grow up enough to do a miniprep, ie they did no grow at all. I gave them a few extra hours in the incubator prior to tossing them.

I started helping Morgan with setting up for the B-gal, however I got ill and Hannah took over for me.

Friday, December 2, 2022

### To Do:

1. ~~Help Morgan with transformation efficiency math~~
2. ~~Help Morgan with transformation~~
3. ~~Help Morgan with gel~~
4. ~~Image gel for Morgan~~

### Results and Data:

## Monday, December 5, 2022

## To Do:

1. ~~Clean hemoglobin flasks~~

**Results and Data:**

## Tuesday, December 6, 2022

## To Do:

1. ~~Make 35 mL of 10x Iron CDM~~
2. ~~Make 250 mL of YPD~~
3. ~~Autoclave x @ 200 mL of water~~
4. ~~Start overnight culture of yeast~~
5. ~~Put away dishes~~

**Results and Data:**

Started my yeast overnight at an OD of 0.013. Obviously, I could not check that this was accurate. However, I think that it will grow slower due to the fact that the plate is old. However, I have pretty much all day tomorrow so it can grow for longer if need be.

## 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

Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.04	2.8
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14
Water	-	-	-	-	26.803

pH'd to 6.5

## Wednesday, December 7, 2022

## To Do:

1. ~~Patch out *drpsU2*~~
2. ~~Streak KRLVS149 to single colony~~
3. ~~Streak out LVS to single colony @room temperature~~
4. ~~Make electrocompetent yeast~~

5. ~~Electroporate yeast with pKR168~~
6. ~~Make 20x Iron~~
7. ~~PCR of triple deletion strains~~

## **Results and Data:**

I checked my cells at 9:50 am, which means they were in the incubator for ~17.5 hours and it was at an OD of 0.89, so clearly a week old plate does grow slower, so I probably could have started it at a higher OD600.

## **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	100, 200 uL, Rem.	3
2	(+) control	pKR128	3 uL	200 uL	1
3	(-) control	-		200 uL	1
Total:					5

## **PCR of Potential Triple Mutants to Check for *rpsU1* or *rpsU3***

1. Acquired and labelled PCR tubes: 1, 2, 3, 4, 5, 7, 8, LVS', and negative controls.

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	<i>rpsU1</i>	Mutant 3	KROL599, KROL600	-
2	<i>rpsU1</i>	Mutant 4	KROL599, KROL600	-
3	<i>rpsU1</i>	Mutant 5	KROL599, KROL600	-
4	<i>rpsU1</i>	LVS	KROL599, KROL600	178
5	<i>rpsU1</i>	KRLVS47	KROL599, KROL600	-
6	- control	-	KROL599, KROL600	-

2. Acquire the following components and put them on ice, labeling tubes if necessary:
  - mgH<sub>2</sub>O, Primestar buffer, dNTPs, KROL599, KROL600, KROL601, KROL602, (10uM), and candidate triple mutants
3. Vortex each component (aside from enzyme)
4. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes

5. Add ddi H<sub>2</sub>O to negative control tube (template volume for 1 reaction)
6. Prepare a master-containing:
  - mgH<sub>2</sub>O, dNTPs, Primestar buffer, respective primers, and Primestar enzyme
7. Mix the master-mix solution by pipetting up and down
8. Add 19.6 uL of master-mix to negative control PCR tube
9. Add 19.6 uL of master mix to each PCR tube and pipet up and down to mix
10. Place the PCR Tubes in the thermocycler on STN 1

Total reaction volume	20
Total number of reactions	6

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	7
ddiH <sub>2</sub> O			12.4	86.8
PrimeSTAR GXL Buffer	5x	1x	4.0	28.0
dNTPs	2.5 mM	0.2 mM	1.6	11.2
oligo F	10 uM	0.3 uM	0.6	4.2
oligo R	10 uM	0.3 uM	0.6	4.2
template	18.0 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2.8
Total volume			20	137.2

## 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

Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.08	2.8
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14
Water	-	-	-	-	26.803

pH'd to 6.5

Thursday, December 8, 2022

**To Do:**

1. ~~Streak out KRLVS148 to single colony~~
2. ~~Streak out LVS to single colony @room temperature~~
3. ~~Patch out KRLVS192, KRLVS193, and LVS pF~~
4. ~~Run gel of *rpsU1* triple deletion PCR~~
5. ~~Make 20x Mg CDM~~

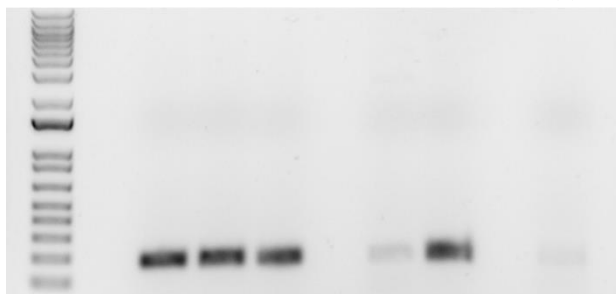
**Results and Data:**

**Gel of Potential Triple Mutants**

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 ~60mL gel, use ladder to mix, then add ladder and allow to set.
4. Turn gel, add used 1xTAE, remove ladder.
5. Loaded 10 uL ladder, and 15 uL of each sample according to the loading order below.
6. Ran for 45 minutes at 113V.

Loading Order of Triple Deletion Mutant PCR									
1	2	3	4	5	6	7	8	9	10
Ladder		Mut. 3	Mut. 4	Mut. 5		KRLVS47	LVS		(-)

Ladder M3 M4 M5 47 LVS (-)



Obviously, there is still some contamination in the negative controls. However, based on how intense the bands are between the positive and the three mutants, I think that it is safe to assume that each of the three mutants did snatch *rpsU1* back from the plasmid.

**Reagents**

Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.004	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	2.0	2.8
Water	-	-	-	-	26.803



pH'd to 6.5. After I added the 2.8 mL of magnesium sulfate the media precipitated. I stirred it for awhile and then took it to pH. Once it reached a pH of 6.8-6.5 it went back into solution. I also realized that I was making 20x of the ions rather than 10x, as I meant to. Regardless, in the future, if there is an issue with precipitation it seems bringing it down to the proper pH helps.

Friday, December 9, 2022

**To Do:**

1. ~~Start cultures of KRLVS192 and KRLVS193 in iron and magnesium~~
2. ~~Streak KRLVS28 and KRLVS75 to single colony~~
3. ~~Check yeast plates for colonies~~
4. ~~Take plates out of the incubator for Hannah~~
5. ~~GFP assay of iron and magnesium of KRLVS192 and KRLVS193~~

**Results and Data:**

**CDM Fe and Mg Conditions on *rpsU1* and *rpsU3* Production in KRLVS192 and KRLVS193**

Condition Number	0	1	2	3
Experiment Component	Background	Control	Test 1	Test 2
Condition	CDM	CDM	0.08 mg/mL Fe	2.0 mg/mL Mg

1. Resuspend patches of cells in 400uL of CDM, with 0 mg/mL Mg, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 7 mL of respective CDM, either regular or varying concentrations of iron
3. Shake cells at 37°C to an OD of 0.3-0.4 typically 4 and a half hours.
4. Then follow the GFP reporter assay protocol, starting at Step 4, subtracting LVS in PBS, and normalizing to the fluorescence of each strain to standard condition, CDM

OD Readings	
Sample	0 hours
KRLVS192 1 C	0.073
KRLVS192 2 C	0.076
KRLVS193 1 C	0.059
KRLVS193 2 C	0.083
KRLVS192 1 Fe	0.082
KRLVS192 2 Fe	0.066
KRLVS193 1 Fe	0.080
KRLVS193 2 Fe	0.074
KRLVS192 1 Mg	0.073
KRLVS192 2 Mg	0.073
KRLVS193 1 Mg	0.088
KRLVS192 2 Mg	0.071
LVS-pF CDM	0.070

I checked my yeast plates and saw small colonies on the positive control, but did not see any on my electroporation plates. I took them out of the incubator and set them on the bench for over the weekend, and I'll check again on Monday.

**GFP Assay on CDM Mg and Fe Conditions in KRLVS192 and KRLVS193**

1. Pellet 2 mLs of culture and spin at max speed for 3 minutes
2. Remove all CDM, using 20 ul pipette to remove small amount at bottom of tube.
3. Add 1 mL of 1XPBS and resuspend the cells.

4. Aliquot 250 ul from each tube in triplicate to clear 96-well plate. Add PBS in triplicate as control.
5. Go to INBRE lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
6. Read OD600 from clear plate on ID3 plate reader:
  - a. Select Absorbance, wavelength=600
  - b. Plate type: 96-well standard clearbtm (first option)
7. Transfer 200 ul of each well from clear plate to black plate using the multichannel
8. Read fluorescence from black plate on ID3 plate reader:
  - a. Select fluorescence
  - b. Wavelength: 495 to 535
  - c. Plate type: CoStar 3789
  - d. Gain: Automatic
  - e. Integration: 380 ms

Label	Condition
A	CDM
B	0.08 mg/mL Fe
C	2.0 mg/mL Mg

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

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

Sunday, December 11, 2022

**To Do:**

1. ~~Patch out KRLVS148 and KRLVS149~~
2. ~~Make 2.5% iron pyrophosphate~~
3. ~~Check LVS and drpsU2 plates and put in incubator if necessary~~

**Results and Data:**

I checked my yeast plates and it almost looked like there may actually be colonies, so I put them back into the incubator.

**Reagents**

2.5% Iron pyrophosphate

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

Monday, December 12, 2022

**To Do:**

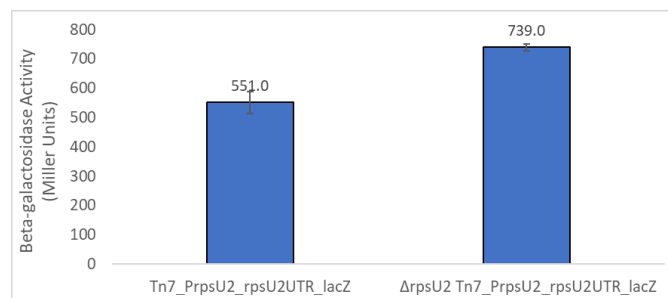
1. ~~Set up cultures for B-gal of KRLVS148 and KRLVS149~~
2. ~~Patch out KRLVS28 and KRLVS75~~
3. ~~Patch out transformants for Hannah~~
4. ~~Streak out mutant 4 to single colony from freezer stock~~
5. ~~B-gal of KRLVS148 and KRLVS149~~
6. ~~Filter sterilize 2.5% iron pyrophosphate~~
7. ~~Supplement MHB~~
8. ~~Prepare tubes for tomorrow~~
9. ~~Make 4 mg/mL ONPG~~
10. ~~Start yeast overnights hopefully nvm ☹️~~

**Results and Data:**

I checked my yeast plates again, and what I thought were potentially colonies were in fact just condensation. Given that I can't seem to get colonies from these plates to grow, I think I need to just re-do the ligation electroporation and start from scratch sort of. I can't remember if I still have gel purified stuff, but I'll check my box.

**β-galactosidase Assay of KRLVS148 and KRLVS149**

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



This was about a 34% increase from WT to *drpsU2*, and corresponds with the previous data shown by Aisling. So, that's looking good. I had to throw out one of the technical replicates from the first biological replicate due to the fact I accidentally added a bunch of stop buffer. Oops.

## 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

4 mg/mL ONPG (40 mL)

To a 50 mL beaker add:

160 mg of ONPG (stored at -20°C

40mL of Z-buffer

Cover in foil and stir until dissolved

Transfer to a 50 mL conical tube wrapped in foil and store in the -20°C

Tuesday, December 13, 2022

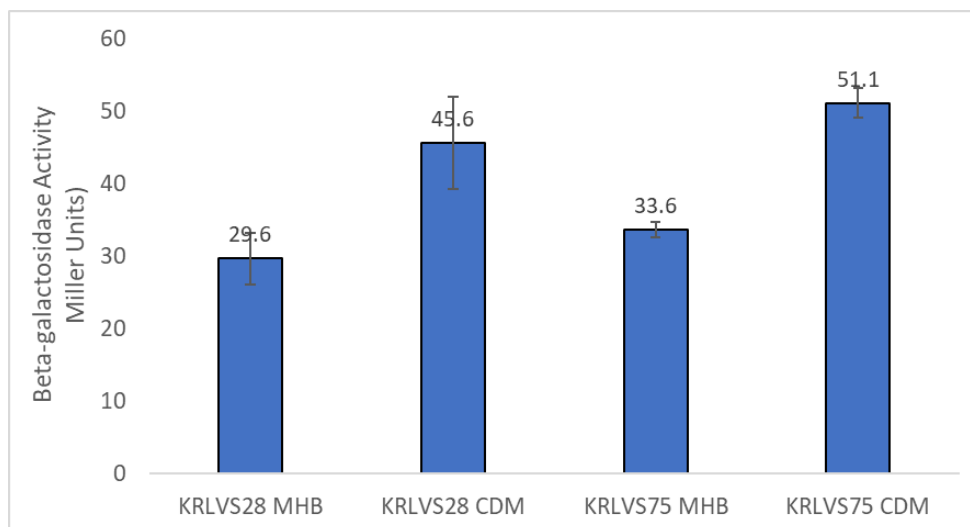
To Do:

- ~~1. Set up cultures of KRLVS28 and KRLVS75~~
- ~~2. Patch out LVS and *drpsU2*~~
- ~~3. B-gal of KRLVS28 and KRLVS75 in MHB and CDM~~

## Results and Data:

### $\beta$ -galactosidase Assay of KRLVS28 and KRLVS75 in MHB and CDM

1. Grow 6ml cultures until  $OD_{600} = 0.3$
2. Turn on 28°C water bath
3. Determine amount of Z-buffer needed ( $0.8\text{ml} \times 2 \times \#$  of cultures plus 1, the 2 is for running duplicates, the 1 is for a blank replicate). Add BME to Z-buffer ( $2.72 \times X\text{ml Z-buffer} = \text{\_}\mu\text{l of BME}$ ).
4. Set up reaction tubes with 800 $\mu\text{l}$  Z-buffer, put on lids
5. Turn on spec and gather cuvettes
6. Once cultures reach  $OD_{600} = 0.3$ , place on ice 30 min and put ONPG in water bath
7. After cells have incubated on ice, measure  $OD_{600}$  of bacterial cultures
8. Add 200 $\mu\text{l}$  culture to each reaction tube (add 200 $\mu\text{l}$  culture media to blank tube)
9. Add 30 $\mu\text{l}$  0.1% SDS to each reaction tube
10. Add 60 $\mu\text{l}$   $\text{CHCl}_3$  (chloroform) to each reaction tube
11. Vortex reaction pairs on high for 6 secs (time precisely with timer)
12. Put in water bath for 10 min
13. Prepare repeater pipette with 1M  $\text{Na}_2\text{CO}_3$  (stop)
14. Add 200 $\mu\text{l}$  ONPG in 5 sec intervals (use timer with hours)
15. Shake gently and watch for yellow (goal  $OD_{420}$  is 0.6-0.9)
16. Stop with 500 $\mu\text{l}$  1M  $\text{Na}_2\text{CO}_3$ , record time, vortex at 4 for 10 sec
17. Give all reaction at least 2 hours
18. Remove 1 mL from reaction (avoid chloroform at bottom), measure  $OD_{420}$  and  $OD_{550}$ , using blank reaction as blank in spectrophotometer.



When compared to the GFP fluorescence assay, *rpsU1* showed a 2.12 fold increase, while *rpsU3* showed a 1.37 fold increase. This B-gal is showing, for both *rpsU* a 1.5 ish fold increase. This suggests that in regards to *rpsU1* the protein abundance is slightly less, while for *rpsU3* the protein abundance is slightly more when normalized to MHB. However, the error bars are also none too small.

Wednesday, December 14, 2022

**To Do:**

1. ~~Set up cultures for growth curve of LVS and *drpsU2* in CDM~~
2. ~~Patch out yeast~~
3. ~~Run dishwasher~~
4. ~~Make iron and magnesium sulfate with higher concentration~~
5. ~~Put away dishes~~

**Results and Data:**

**Growth Curve of LVS and *drpsU2* in CDM and MHB**

1. Resuspend patches of cells in 400uL of CDM or MHB, checking OD in a 1:20 dilution
2. Normalize samples to an OD of 0.08-0.10 in 8 mL of CDM or MHB
3. Shake cells at 37°C for 24 hours

Sample	OD Readings					
	0 hours	2 hours	3.5 hours	5 hours	6 hours	25 hours
LVS 1 MHB	0.061	0.085	0.158	0.207	0.259	1.612
LVS 2 MHB	0.071	0.146	0.211	0.298	0.378	1.708
LVS 3 MHB	0.097	0.137	0.238	0.308	0.377	1.720
<i>drpsU2</i> 1 MHB	0.077	0.115	0.162	0.212	0.254	0.780
<i>drpsU2</i> 2 MHB	0.066	0.109	0.151	0.203	0.234	0.720
<i>drpsU2</i> 3 MHB	0.078	0.122	0.167	0.242	0.270	0.772
LVS 1 CDM	0.073	0.179	0.311	0.436	0.526	3.136
LVS 2 CDM	0.072	0.172	0.262	0.369	0.425	3.540
LVS 3 CDM	0.075	0.159	0.260	0.358	0.448	3.620
<i>drpsU2</i> 1 CDM	0.077	0.192	0.285	0.405	0.502	2.256
<i>drpsU2</i> 2 CDM	0.074	0.165	0.266	0.369	0.453	2.016
<i>drpsU2</i> 3 CDM	0.075	0.168	0.273	0.384	0.473	2.124

## Reagents

50 mg/mL  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

To 50 mL beaker add:

250 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

~5 mL water

Stir until complete dissolved, filter sterilize

500 mg/mL  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

To 50 mL beaker add:

5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

~7 mL water

Stir until complete dissolved and add water to final volume of 5 mL

Thursday, December 15, 2022

### To Do:

1. ~~Check 24 hour time point of growth curve~~
2. ~~Soft agar overlay of mutant 4~~
3. ~~Make CDM with iron and magnesium sulfate x10, x100, and x1000~~
4. ~~Make CDM (150 mL)~~
5. ~~Make YPD in 250 mL flask~~
6. ~~Autoclave 2x200 mL water~~
7. ~~Ligation of *PrpsU2 tul4* 5' UTR PCR with pKR128 backbone (from 9/21/22)~~
8. ~~Set up yeast overnight~~

## Results and Data:

### Agar overlay for $\beta$ -galactosidase activity in Mutant 4 from MM

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:
    - 20 mL of  $\text{dH}_2\text{O}$
    - 0.14 g of agar
  - b. Stir and autoclave Liquid 30
3. Place in water bath at  $56^\circ\text{C}$  until cool enough to touch. Add 20  $\mu\text{L}$  x-gal to agar.
4. Overlay 10mL of the agar solution to each plate. Protect from light by wrapping in foil. Incubate lid side up.

### 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 ( $\mu\text{L}$ )	Reaction 2 ( $\mu\text{L}$ )	Master Mix (3x)
$\text{H}_2\text{O}$	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 H<sub>2</sub>O (\_\_\_uL), 10x buffer (\_\_\_\_uL), insert (\_\_\_uL), and backbone (\_\_\_uL).
6. Add indicated amount of ligase (\_\_\_uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Incubate at 16°C overnight.

## 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

### CDM (150mL)

To 250 mL beaker add:

12 mL Combined Amino Acid Stock

Then add:

600 uL Thiamine HCl

600 uL Spermine tetrahydrochloride

600 uL DL-Calcium pantothenate

Then add:

5.13 mL 5N NaCl

1.5 mL KH<sub>2</sub>PO<sub>4</sub>

600 uL K<sub>2</sub>HPO<sub>4</sub>

Then:

1.5 mL 400 mg/mL Glucose

Then:

115 mL Water

Then:

600 uL FeSO<sub>4</sub>•7H<sub>2</sub>O

600 uL MgSO<sub>4</sub>•7H<sub>2</sub>O

pH media to a pH between 6.3 and 6.5

Add water to 150 mL total

Filter sterilize

10x Iron Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final] (mg/mL)	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.02	1.4
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14
Water	-	-	-	-	28.203

100x Iron Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final] (mg/mL)	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	50	0.2	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14
Water	-	-	-	-	29.463

1000x Iron Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final] (mg/mL)	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	50	2	1.4
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	0.1	0.14
Water	-	-	-	-	28.203

10x Magnesium Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final] (mg/mL)	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.002	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	33.75	1.0	1.4
Water	-	-	-	-	28.203

100x Magnesium Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.002	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	500	10.0	0.7
Water	-	-	-	-	28.903

1000x Magnesium Concentration					
Component	Location	Group	[Stock] (mg/mL)	[Final]	Volume to Add (mL)
Combined Amino Acid Stock	4°C	1	12.5X	1X	2.8
Thiamine HCl	4°C	2	1	0.004	0.14
Spermine tetrahydrochloride	4°C	2	8.74	0.0350	0.14
DL-Calcium pantothenate	4°C	2	0.5	0.002	0.14
NaCl	RT	3	292.2	10.0	1.197
KH <sub>2</sub> PO <sub>4</sub>	RT	3	100	1	0.35
K <sub>2</sub> HPO <sub>4</sub>	RT	3	250	1	0.14
Glucose	RT	Separate	400	4	0.35
FeSO <sub>4</sub> •7H <sub>2</sub> O	RT	Separate	0.5	0.002	0.14
MgSO <sub>4</sub> •7H <sub>2</sub> O	RT	LAST	500	100.0	7
Water	-	-	-	-	22.603

Friday, December 16, 2022

#### To Do:

- ~~1. Make electrocompetent yeast~~
- ~~2. Electroporation yeast with leftover candidate pKR168 and new ligation~~
- ~~3. Patch out KRLVS192, KRLVS193, and LVS-pF~~
4. Set up cultures for GFP assay on Monday
- ~~5. Check soft agar overlay~~

### Results and Data:

#### Making electrocompetent yeast

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



6. Wash in 20 ml of ice cold 1M sorbitol and centrifuge again, as above.
7. Resuspend in smallest volume of ice cold 1M sorbitol, starting with 200 ul and up to 500 ul as needed.

### Electroporation of Candidate pKR168 + pKR168 Ligation into Yeast

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

Tube #	Purpose	DNA	Vol. of DNA	Vol. Plated	# of Plates
1	pKR168	MP plasmid	3 uL	200 uL, Rem.	2
2	pKR168	Lig. 1	8 uL	200 uL, Rem.	2
3	Backbone	Lig. BB	8 uL	200 uL, Rem.	2
4	(+) control	pKR128	3 uL	200 uL	1
5	(-) control	-		200 uL	1
				Total:	8

After the meeting, I looked at the overlay plate with Kathryn, and there are a mix of blue and “white” colonies. The assumption is that there is a mixed population. I will be streaking to single colony a blue colony, a white colony, and LVS and doing an overlay on Monday to see if they are separate populations, and to determine if the “white” colonies are white when compared to LVS.

Additionally, it seemed my 1000x iron CDM did eventually dissolve, but I can’t image that it’s good to use after sitting out all night- may have been contaminated. Additionally, the iron is not completely dissolved as evidenced by the uhhh terrible color lmao. Turbidity is growth or iron, so yeah, just not gonna happen.

## Monday, December 19, 2022

### To Do:

- ~~1. Set up cultures for 10x, 100x, and 1000x Fe environmental condition~~
- ~~2. Run plates in autoclave~~
- ~~3. Run dishwasher~~
4. Put away dishes
5. Overlay of LVS + blue and white colonies from mutant 4
6. Check yeast electroporation plate
7. GFP assay of iron conditions on KRLVS192 and KRLVS193

### Results and Data:

#### Effect of 10x and 100x Fe on *rpsU1* and *rpsU3* Production in KRLVS192 and 193

Condition Number	0	1	2	3
Experiment Component	Background	Control	Test 1	Test 2
Condition	CDM	CDM	0.02 mg/mL Fe	0.2 mg/mL Fe

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

#### Agar overlay for $\beta$ -galactosidase activity in Mutant 4 Blue and White Colonies v. LVS

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:
    - 30 mL of diH<sub>2</sub>O
    - 0.21 g of agar
  - b. Stir and autoclave Liquid 15
3. Place in water bath at 56°C until cool enough to touch. Add 30 uL x-gal to agar.
4. Overlay 10mL of the agar solution to each plate. Protect from light by wrapping in foil. Incubate lid side up.

#### GFP Assay on CDM Fe Conditions in KRLVS192 and KRLVS193

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

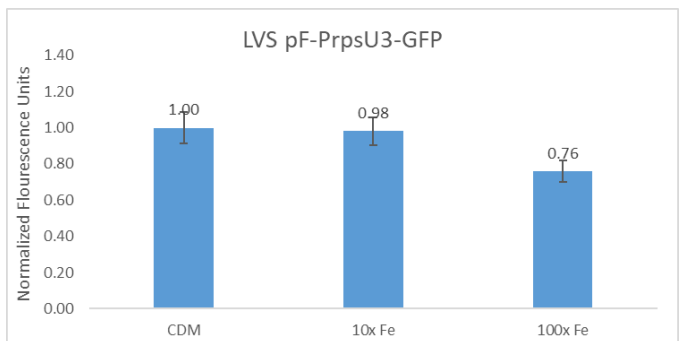
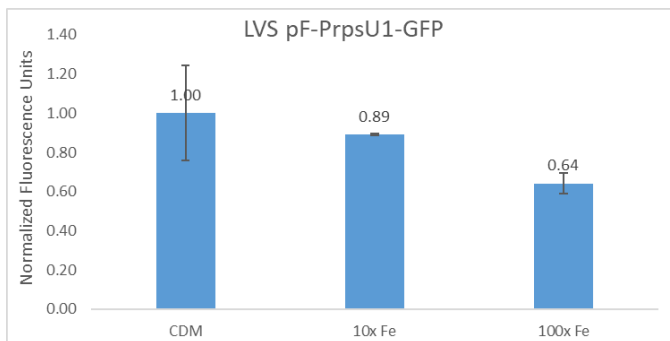
- d. Gain: Automatic  
e. Integration: 380 ms

Label	Condition
A	CDM
B	0.02 mg/mL Fe
C	0.20 mg/mL Fe

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

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

I checked my yeast transformation plates. There were no backbone colonies, there were some ligation colonies, and it didn't seem that there were any plasmid colonies. The positive control was well grown though.



## Bibliography

Ramsey, K. M. and Dove, S. L. (2016) ‘ A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor ’, *Molecular Microbiology*. doi: 10.1111/mmi.13418.