Table of Contents

Introduction 3
Figure 1 3
Thoughts or questions. 3
Protocol 1 3
Reagents 4
File Formatting Protocol 4
January 2019 5
Sequencing Protocol for Plasmids 5
Allelic exchange in F. tularensis LVS with pEX Day 1 (from Protocols folder) 5
Allelic exchange in F. tularensis LVS with pEX Day 2 (from Protocols folder) 5
Allelic exchange in F. tularensis LVS with pEX Day 2 (from Protocols folder) (cont.) 6
Transform Chemically Competent E. coli Cells Protocol 7
QIAprep Spin Miniprep Kit Protocol 8
Allelic exchange in F. tularensis LVS with pEX Day 6 (from Protocols folder) (cont.) 9
Allelic exchange in F. tularensis LVS with pEX Day 6 (from Protocols folder) (cont.) 10
PCR Protocol by John Church (New as of 11/14/18) – modified for PriM Mutants 11
Allelic exchange in F. tularensis LVS with pEX Day 9 (from Protocols folder) (cont.) 12
Testing Plasmids by Plating Dilutions 14
QIAquick Gel Extraction kit Protocol 15
QIAprep Spin Miniprep Kit Protocol 16
LB Broth Protocol (for 500 mL LB) 17
QIAprep Spin Miniprep Kit Protocol 18
PCR Protocol by John Church (New as of 11/14/18) 19
QIAquick PCR Purification Kit Protocol 20
DNA Digest Protocol 21
QIAquick Gel Extraction kit Protocol 21
February 2019 22
PCR Protocol by John Church (New as of 11/14/18) 23
Agarose Gel Protocol – written by Joe Paquette 24
QIAprep Spin Miniprep Kit Protocol 25
100 uL PCR Protocol by John Church (New as of 11/14/18) 27
QIAquick PCR Purification Kit Protocol 28
DNA Digest Protocol 28

Transform chemically competent E. coli cells 29
QIAquick Gel Extraction kit Protocol 31
Ligation of Digested, Purified Plasmid and PCR 31
Transform chemically competent E. coli cells 32
Overnight Cultures Protocol 33
QIAprep Spin Miniprep Kit Protocol 33
Making Electrocompetent Cells (from Allelic Exchange Protocol) 34
Making Electrocompetent Cells (from Allelic Exchange Protocol) 35
Diagnostic Digest on Gel 35
March 2019 37
Copper Assay Protocol 37
Homemade CHAH Plates with Sucrose Protocol 39
OD Growth Curve Protocol for CDM 39
Copper Assay Protocol 40
Allelic Exchange Day 6: 44
Copper Assay Protocol 45
Allelic Exchange Day 6: 46
Allelic Exchange Day 9 (maybe 10): 47
Allelic Exchange Day 10: 47
Colony PCR Protocol 47
PCR Purification 48
DNA Digest 49
FipB Mutant 100 uL PCR 51
QIAquick Gel Extraction kit Protocol 54
Ligations 54
Bibliography 59

Introduction

Thursday, September 20, 2018

To Do:

1. ~~Explain lab notebook formatting~~
2. Media prep

Results and Data:

For each day, copy the previous entry headers and update the date. Save the same ongoing copy of the lab notebook to the lab google drive ONLY. Do not save separate physical copies. At the 1st of each new month, a read-only PDF file of the lab notebook must be saved and given to Dr. Ramsey electronically. **File contents converted to PDF MUST NOT BE EDITED after PDF conversion**. Continue to keep using the same word file until the end of the calendar year. New lab notebook files run in 6 month intervals.

For the To do list, update this each day with new tasks, as tasks are done, use the ~~strikethrough~~ font on the day they are completed and leave them out of the list on the next day.

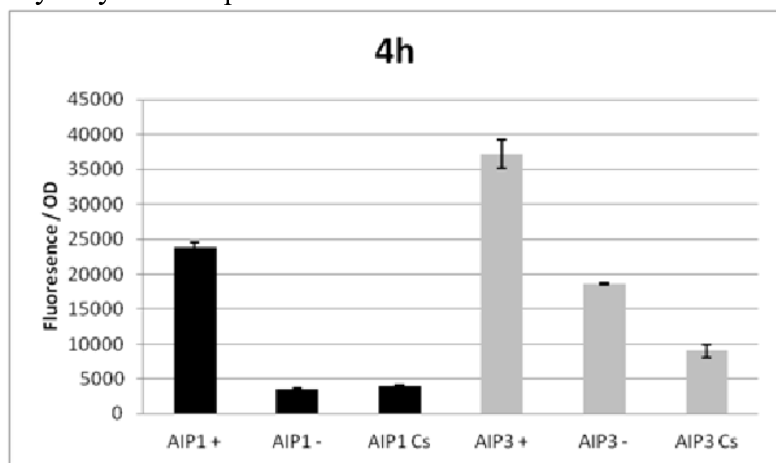


Figure 1

Figures are inserted as inline .png files when possible, .jpg, .gif, .tif otherwise are acceptable. Figure legends are always inserted in Word (right click – 'insert caption') and use "**Heading 2**" text to properly format in the TOC. Table legends are handled the same way. Figure images must be saved in a separate folder where the source data is saved.

Thoughts or questions.

When you have a significant observation, question, confusing point or contradiction that you have identified in your data or protocol, use the "**Heading 3**" text heading on a descriptive brief title or single word heading so you can refer to it in your TOC later.

Protocol 1

1. This is the format for a protocol in your notebook.
2. The protocol title must be formatted in "**Heading 2.**"
3. Reagents which must be made for a specific protocol (buffers, solutions) should be listed in a "Reagents" Section, formatted in "**Heading 3**."
4. The protocol must be in numerical steps.
5. Use standard notation and carefully describe units for your protocol.
6. Use ½" indent for protocol text.

Reagents

Specific buffers

For initial lab notebooks, write as much detail as possible. As time goes by you will be able to refer to written protocols by their heading and/or page number within the document. If you make any modifications to a protocol you must state how and why.

File Formatting Protocol

1. Filenames begin with your initials, an underscore, and the date, formatted as the last 2 digits of the year, the month, then the day, ex: "KMR_180920_Sample_file1v1.xls"
2. This ensures all files will be sorted by their creator and by their date. You must use this file formatting system for all data files (including photos) that will be shared with the lab.

Bibliography data will be saved as (author/date) and using Mendeley at this time with the TOC inserted by order cited at the end of the lab notebook in the Bibliography section.

Example is a recent publication (Ramsey and Dove, 2016).

January 2019

Wednesday, January 2, 2019

To Do:

1. ~~Sequencing of plasmids from December 2018.~~
2. ~~Start Day 1 of Allelic exchange in *F. tularensis* LVS with pEX.~~

Results and Data:

Sequencing Protocol for Plasmids

1. Use SequencingSubmission_template spreadsheet in the Sequencing folder.
2. Fill out according to Plasmid headings.
3. Get required number of micro tubes and label.
4. Add volumes of water first in order to conserve tips.
5. Add plasmid template.
6. Add 4 uL primer to each.
7. Add name and email to the bottom of the submission form.
8. Submit by clipping the form to the clipboard in the sequencing center and placing the micro tubes in the freezer.

Allelic exchange in F. tularensis LVS with pEX Day 1 (from Protocols folder)

Day 1:

-Patch entire plate with recipient LVS strain on CHAH

Thursday, January 3, 2019

To Do:

1. ~~Continue Allelic exchange – Day 2.~~
2. ~~Make Mueller-Hinton broth, CHAH plates, and CHAH with kanamycin plates.~~

Results and Data:

Allelic exchange in F. tularensis LVS with pEX Day 2 (from Protocols folder)

Day 2:

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells, resuspend in sterile 10% sucrose (alternately, cells can be grown to mid-log in MHB)

-Wash cells 3x in 10% sucrose

-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells.

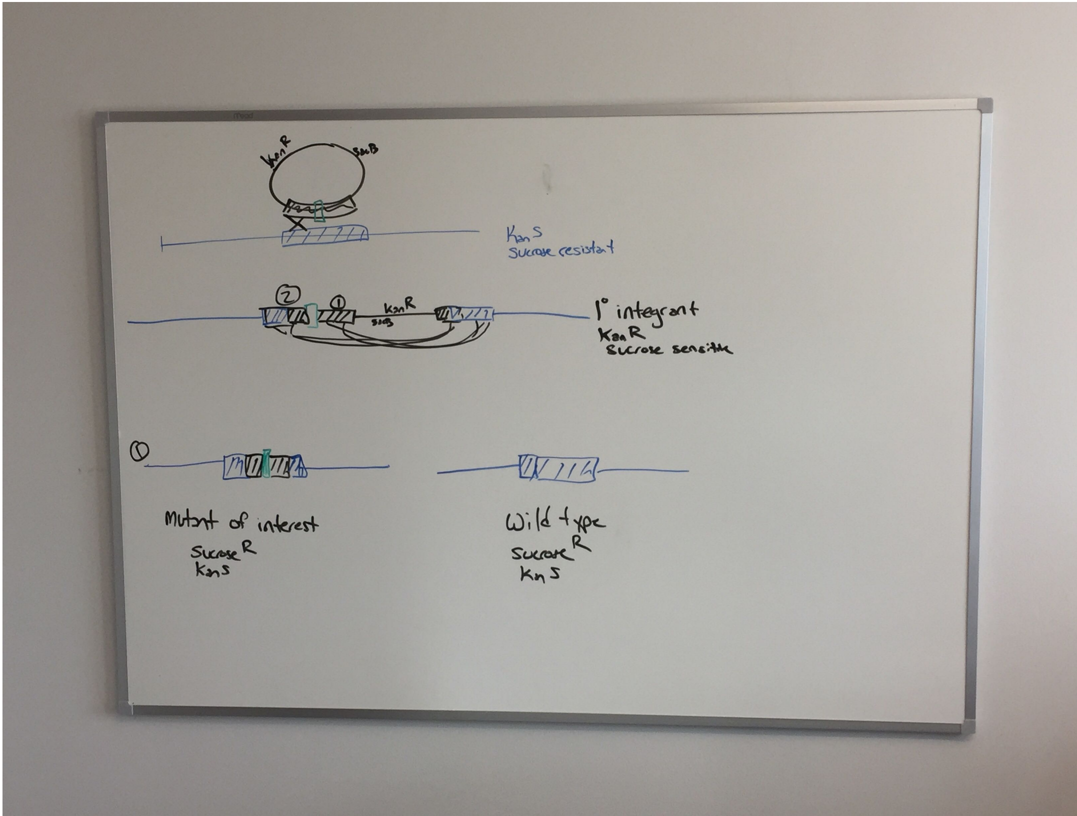
-For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Friday, January 4, 2019

To Do:

1. ~~Analyze results of sequencing.~~
2. ~~Continue Allelic exchange – Day 2 continued.~~
3. ~~Do transformations of *E. coli* with pKL115 plasmid and pKR1.~~

Results and Data:



Above is a depiction of the allelic exchange that is desired.

Sample number	Well	Template Type	Template Name	Primer Name[a]	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	PCR template:	PCR template:	PLASMID template:	Volume H_2O needed
							ng needed =	Volume =	Volume =	
							(A ÷ 100) × 2.5	2x(C ÷ B)µl	2x(~400 ÷ B)µl	(20 less D or E)µl
JTC_1		Plasmid	pKL115_1	KROL6	8168	188.11			4.3	15.7
JTC_2		Plasmid	pKL115_2	KROL6	8168	169.18			4.7	15.3
JTC_3		Plasmid	pKL115_3	KROL6	8168	121.35			6.6	13.4
JTC_4		Plasmid	pKL115_4	KROL6	8168	150.15			5.3	14.7
JTC_5		Plasmid	pKL115_1	KROL7	8168	188.11			4.3	15.7
JTC_6		Plasmid	pKL115_2	KROL7	8168	169.18			4.7	15.3
JTC_7		Plasmid	pKL115_3	KROL7	8168	121.35			6.6	13.4
JTC_8		Plasmid	pKL115_4	KROL7	8168	150.15			5.3	14.7
JTC_9		Plasmid	pKL115_1	P368	8168	188.11			4.3	15.7
JTC_10		Plasmid	pKL115_2	P368	8168	169.18			4.7	15.3
JTC_11		Plasmid	pKL115_3	P368	8168	121.35			6.6	13.4
JTC_12		Plasmid	pKL115_4	P368	8168	150.15			5.3	14.7

The above is a picture of the table used for sequencing. Plasmids 1-4 were used with 3 separate primers, giving 12 different samples. pKL115_1 corresponds to plasmid 1, pKL115_2 corresponds to plasmid 2, and so on. Plasmid 4 showed good results in SnapGene, comparable to the pKL115 plasmid, so plasmid 4 will be used for the electroporations. The tube is now labeled as "pKL115."

Allelic exchange in F. tularensis LVS with pEX Day 2 (from Protocols folder) (cont.)

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C

-For each electroporation, in a 2 mm sterile electroporation cuvette, combine:

5 μL of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/μL)
50 μL electrocompetent cells

-Have recovery media ready
-Electroporate using the following settings: 2.5 kV, 25 μF, and 600 Ω
-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

The electoporations produced arcs, so a control was done using electro competent cells and the plasmid. Arcs still occurred, so it is likely that the plasmid (pKL115) was salty. The experiment was carried out regardless in hopes that the cells are still viable. Next week, more of the plasmid will be made using XL1 Blue cells and using a vacuum instead of centrifuge for certain parts of the mini prep.

-Recover cells for 4-8 hours, shaking at 37°C
-Pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g.
-Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
-Incubate plates at 37°C for 3 days (or until single colonies appear)

Transform Chemically Competent E. coli Cells Protocol

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL, 100 uL, and remaining culture. If transforming a ligation, plate 100 uL and remaining culture.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	(+) control	pKL115	1 uL	20 ul, 100 ul, remaining	3	
2	(-) control	None	0	20 ul, 100 ul, remaining	3	
3	(+) control	pKR1	1 uL	20 ul, 100 ul, remaining	3	
				Total number of plates	9	0

2. Check to be sure you have enough plates with appropriate antibiotic. 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 (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates, spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for

30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

Sunday, January 6, 2019

To Do:

1. ~~Make 4 overnight cultures for miniprep.~~

Results and Data:

Tubes	Purpose	Plate Taken From
1-4	pKL115 Culture	pKL115 (remaining)
5	negative control	negative control (remaining)
6	positive control	pKR1 (remaining)

1. Add 35 mL of LB broth to a 50 mL conical.
2. Add 35 uL of Kanamycin to the conical.
3. Label 6 test tubes (4 for pKR115, 1 for a positive control, and 1 for a negative control).
4. Pipette out the quantity of the conical and aliquot out 5 mL of solution Kanamycin broth into each of the tubes.
5. Inoculate each of the tubes with the corresponding plate.
6. Place in the shaking incubator overnight.
7. Wrap plates in parafilm and put plates in 4 degree.

Monday, January 7, 2019

To Do:

1. ~~Miniprep overnight cultures.~~
2. ~~Day 5 of Allelic exchange.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA
11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.

12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Plots | Report

Test type: Nucleic Acid 1/7/2019 11:25 AM Exit

Report Name Report Full Mode Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
tube 1	Default	1/7/2019	11:22 AM	537.03	10.741	5.907	1.82	2.26	50.00	230	4.755	0.013
tube 2	Default	1/7/2019	11:23 AM	695.09	13.902	7.410	1.88	2.32	50.00	230	5.982	-0.008
tube 3	Default	1/7/2019	11:24 AM	644.62	12.892	6.894	1.87	2.33	50.00	230	5.542	-0.030
tube 4	Default	1/7/2019	11:25 AM	548.76	10.975	6.014	1.82	2.27	50.00	230	4.840	0.019

Above is a picture of the nanodropper readings of the plasmid. The plasmid will not be sequenced, since it was cloned from a previous plasmid. The tubes were combined into one tube labeled pKL115 and stored in the Plasmids 1 box in the freezer.

Allelic exchange in F. tularensis LVS with pEX Day 6 (from Protocols folder) (cont.)

-While not great, there was growth on most of the plates. Protocol was continued using colonies that were available on the plates.

Day 6:

-Pick single colonies from electroporation plates and patch onto CHAH-Kan plates (large patches, maximum 6 per plate, ideally 3-4 per plate). Pick at least 4, if there are 4 colonies (preferably from different electroporations to minimize the chance of picking the same integrant).

Tuesday, January 8, 2019

To Do:

1. ~~Continue with allelic exchange – Day 6.~~

Results and Data:

Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

-Plate 100 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate 100 µL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Plate Number:	Plate Type:	Purpose:
1	Sucrose CHAH	LVS Integrant 1 1° (1x10^-2)
2	Sucrose CHAH	LVS Integrant 1 1° (1x10^-3)

3	Sucrose CHAH	LVS Integrant 1 1° (1x10^-4)
4	Sucrose CHAH	LVS Integrant 1 1° (1x10^-5)
5	Sucrose CHAH	LVS Integrant 1 1° (1x10^-6)
6	Sucrose CHAH	LVS Integrant 1 1° (1x10^-7)
7	Normal CHAH	LVS Integrant 1 1° (1x10^-7)
8	Sucrose CHAH	LVS Integrant 2 1° (1x10^-2)
9	Sucrose CHAH	LVS Integrant 2 1° (1x10^-3)
10	Sucrose CHAH	LVS Integrant 2 1° (1x10^-4)
11	Sucrose CHAH	LVS Integrant 2 1° (1x10^-5)
12	Sucrose CHAH	LVS Integrant 2 1° (1x10^-6)
13	Sucrose CHAH	LVS Integrant 2 1° (1x10^-7)
14	Normal CHAH	LVS Integrant 2 1° (1x10^-7)

Wednesday, January 9, 2019

To Do:

1. ~~Continue with allelic exchange – Day 6 continued: make freezer stocks.~~

Results and Data:

Allelic exchange in F. tularensis LVS with pEX Day 6 (from Protocols folder) (cont.)

Day 6:

Make freezer stocks of 1° integrants

-Add 800 uL MHB to cryovial per 1° integrant
-Scrape up 1° integrant cells using loop into cryovial
-Completely resuspend cells in cryovial
-Add 200 uL 75% glycerol, close tube, mix by vortexing, and freeze at -80°C.

Thursday, January 10, 2019

To Do:

1. ~~Combine pKL115 tubes and add to plasmid box.~~

Results and Data:

The pKL115 plasmid is now in the “Plasmids 1” box in the freezer and is labeled “pKL115.”

Friday, January 11, 2019

To Do:

1. ~~PCR for PriM mutants – protocol under PriM folder > PriM mutants plasmids.~~

Results and Data:

Reaction Number	Lane on gel	Primers	Size
1	2	P818 and P707	600
2	3	P708 and P819	782
3	4	KROL19 PriMnoC1FR1F + KROL20 PriMnoC1FR1R	757
4	5	KROL33 PriMnoC1FR1 + KROL34 PriMnoC2FR1R	695
5	6	KROL35 PriMnoC2FR2F + P694	462
6	7	KROL25, KROL26	+ control
7	8	KROL25, KROL26	- Control

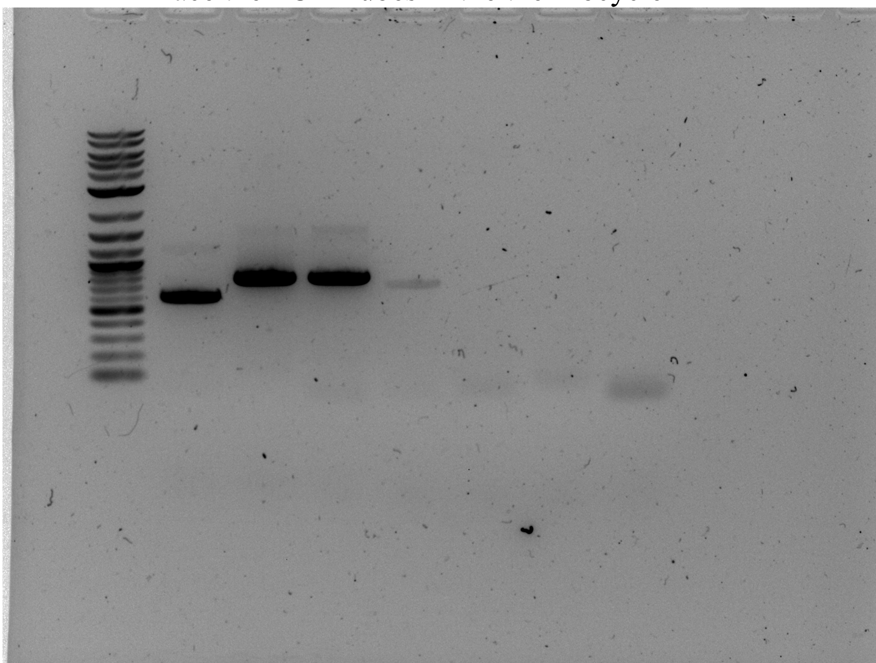
Total reaction volume	20
Total number of reactions	7

Component	Stock concentration	Final concentration	1 rxn volume	Factor 8.8
ddiH2O			4	35.2
KOD buffer	2x	1x	10	88
dNTPs	2 mM	0.4 mM	4	35.2
oligo F	10 uM	0.3 uM	0.6	5.28
oligo R	10 uM	0.3 uM	0.6	5.28
template	100 ng/ul	2 ng/ul	0.4	3.52
KOD	1 U/ul	0.02 U/ul	0.4	3.52
		Total volume	20	176

PCR Protocol by John Church (New as of 11/14/18) – modified for PriM Mutants

1. Acquire and label 7 PCR tubes with initials and designate as Tubes 1-8
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
    - ddi H2O in 1.5 mL microfuge tube
    - uL KOD buffer
    - dNTPs
    - oligo F
    - oligo R
    - template
    - Note: KOD enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
    - DO NOT vortex the KOD enzyme itself or any solution with KOD enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
    - The file is located in the Protocols folder
    - For this protocol, a "Total reaction volume" of 20 uL and 7 "Total number of reactions" were used – the following volumes are based on these specifications
7. Add 0.6 uL of each experiment specific primer (forward and reverse) to PCR Tubes 1-7 (oligos forward and reverse)
8. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add 35.2 uL ddi H2O
    - Add 35.2 uL dNTPs
    - Add 88 uL KOD buffer
    - Add 3.52 uL KOD enzyme

9. Mix the master-mix solution by pipetting up and down
   - Do not vortex to mix
10. Add 18.4 uL of master-mix to PCR Tube 7
11. Add 3.52 uL template to Master Mix
12. Add 18.8 uL master mix to each PCR Tube 1-3 and pipette up and down to mix (conserves tips)
13. Close PCR Tubes 1-7 until the caps are tight (push until the caps do not squeak when you push on them)
14. Place the PCR Tubes in the thermocycler



The PCR gel is shown above. There should be 7 samples shown, but only the first three are visible, so this will be rerun on Sunday to see if there is a different result.

Sunday, January 13, 2019

To Do:

1. ~~Redo and run PCR from Friday 1/11/19 since it did not go as planned.~~
2. ~~Allelic exchange Day 9.~~

Results and Data:

Allelic exchange in F. tularensis LVS with pEX Day 9 (from Protocols folder) (cont.)

Day 9 (maybe 10):

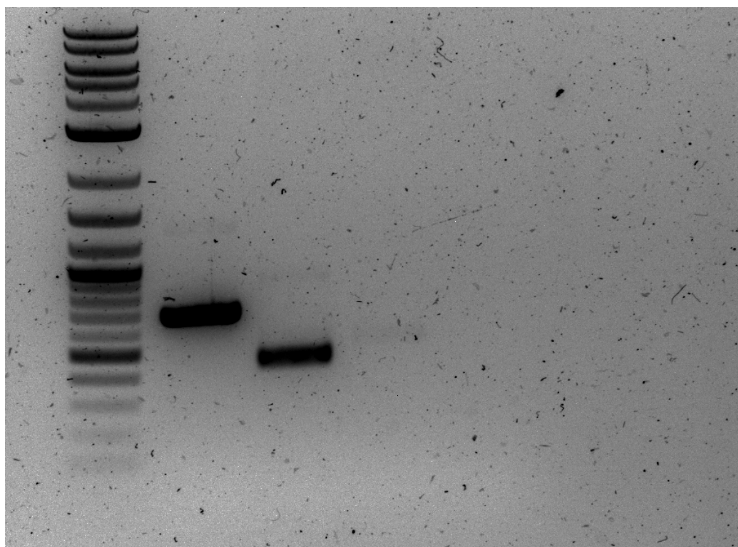
-Pick single colonies (cross-outs) from sucrose plates and patch onto CHAH, 8 – 16 per 1° integrant.

-Optional: cross-patch to CHAH-Kan. Note that it is common to pick up some background cells so some amount of kanamycin-resistant growth is not yet a concern. However, if there is robust kanamycin-resistant growth from all patches from a single 1° integrant on the next day, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

-Note: CHAH-Kan plates were done.

Reaction Number	Lane on gel	Primers	Size
1	2	KROL33 PriMnoC1FR1 + KROL34 PriMnoC2FR1R	695

2	3	KROL35 PriMnoC2FR2F + P694	462
3	4	KROL25, KROL26	+ control
4	5	KROL25, KROL26	- control



Monday, January 14, 2019

To Do:

1. ~~Do 100 uL PCR.~~
2. ~~Overlap extension PCR.~~
3. ~~Make agar+sucrose+kan plates to test plasmids.~~

Results and Data:

Reaction Number	Lane on gel	Primers	Size
1	2	P818 and P707	600
2	3	P708 and P819	782
3	4	KROL19 PriMnoC1FR1F + KROL20 PriMnoC1FR1R	757
4	5	KROL33 PriMnoC1FR1 + KROL34 PriMnoC2FR1R	695
5	6	KROL35 PriMnoC2FR2F + P694	462
7	8	KROL15, KROL16	- Control

Total reaction volume	100
Total number of reactions	6

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	7.7
ddiH2O			20	154
KOD buffer	2x	1x	50	385
dNTPs	2 mM	0.4 mM	20	154

oligo F	10 uM	0.3 uM	3	23.1
oligo R	10 uM	0.3 uM	3	23.1
template	100 ng/ul	2 ng/ul	2	15.4
KOD	1 U/ul	0.02 U/ul	2	15.4
		Total volume	100	770

Testing Plasmids by Plating Dilutions

1. Gather pKR1 and pKL115 plasmids.
2. Get and label 1.5 mL microfuge tubes for the dilutions. 3 each for undiluted, 1:10, 1:100, and 1:1000. Do this for both pKR1 and pKL115 for a total of 24 tubes.
3. Place 400 uL of LB broth into the undiluted tubes and 900 uL into each additional tube.
4. Use a stick to take a small amount of pKR1 and swab it in the undiluted pKR1 tube. Repeat for pKL115.
5. Take 100 uL of the undiluted tube an pipette into the 1:10 tube. Repeat for 1:100 and 1:1000. Repeat for pKL115.
6. Make sucrose and kanamycin agar plates.
7. Gather 3 kanamycin plates and 3 kan+sucrose plates.
8. Divide the plates into halves labeled pKR1 and pKL115. Label the dilutions on the plates as well.
9. Add a 5 uL drop from the dilution tube to the plate under the corresponding label. Repeat twice more so there are 3 drops under each.
10. Do this for each plate.
11. Make sure plates are dry and place in the 37 degree overnight.

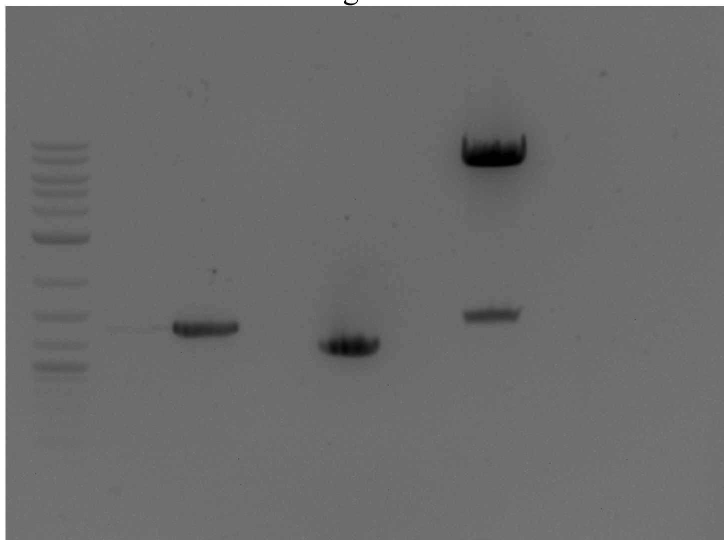
Tuesday, January 15, 2019

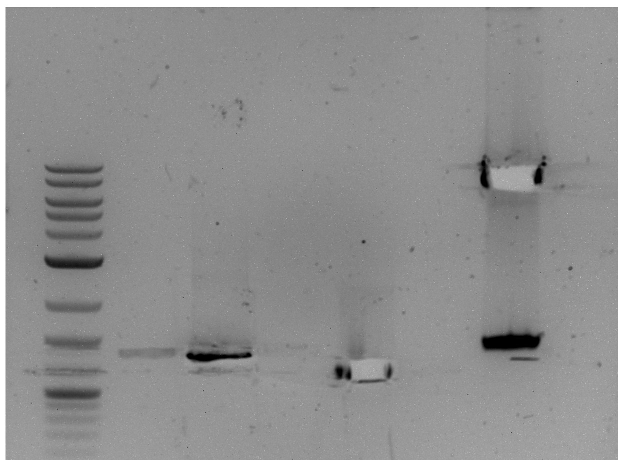
To Do:

1. ~~Look at sucrose+kan plates from 1/14.~~
2. ~~Cut DNA in PCR and digest.~~
3. ~~Purify PCR.~~

Results and Data:

Gel before and after cutting.





QIAquick Gel Extraction kit Protocol

1. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume gel (100 mg gel ~ 100 uL). The maximum amount of gel per spin column is 400 mg.
2. Incubate at 50º C for 10 minutes (or until the gel slice has completely dissolved). Vortex the tube every 2-3 minutes to help dissolve the gel.
3. Add 1 gel volume isopropanol to the sample and mix.
4. Place the QIAquick spin column in a provided 2 mL collection tube. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 uL, load and spin again.
5. Add 500 uL Buffer QG to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 750 uL Buffer PE to QIAquick column and centrifuge for 3 minutes. Discard the flow-through and place the QIAquick column back into the same tube.
7. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, add 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water to the center of the concentration, add 30 uL Buffer EB to the center of the QIAquick membrane and centrifuge for 1 min.

Mass of Empty Microfuge Tube	0.9g	Volume of QB to Add
Total Mass of Tube 1	1.1g	600 uL
Total Mass of Tube 2	1.1g	600 uL
Total Mass of Tube 3	1.1g	600 uL

Wednesday, January 16, 2019

To Do:

1. ~~Repeat Day 6 of Allelic exchange with new plates.~~
2. ~~Supplement Mueller-Hinton broth.~~

Results and Data:

Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 400 uL 1X PBS

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Use multichannel pipette and well plate to make dilutions. Label the first well "Culture" and each additional well 10^-1, 10^-2,…10^-7. This was done for LVS 1, LVS 2, dPmrA 1, dPmrA 2, and KRLVS 3.1.
Perform each dilution by adding 180 uL 1x PBS to each well (except for the culture wells). Set the multichannel pipette to p/m (pipette and mix) and take 20 uL from the culture well and transfer to 1x10^-1. Repeat for each dilution.
~~Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS~~
~~Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS~~

-Plate ~~100 uL~~ 50 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate ~~100 µL~~ 50 uL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Thursday, January 17, 2019

To Do:

1. ~~Help Jamie with minipreps for her and Joe.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube (if in a larger tube).
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA
11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.
12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Friday, January 18, 2019

To Do:

1. ~~Check sucrose CHAH plates.~~
2. ~~Miniprep cultures.~~
3. ~~Make Mueller-Hinton Agar plates to test primary integrants again.~~

Results and Data:

The sucrose CHAH plates unfortunately had growth on them, so it is uncertain what is wrong with the experiment. The Dove, MRamsey, and Nelson labs all use a sucrose solution that is clear, but ours is yellow. While the info sheet says that the solution is supposed to be yellow, there may be some breakdown of the sucrose in the autoclave, causing the yellow color. Since there is little CHA left, the experiment will not be repeated in the near future (but maybe in a week or two).

Monday, January 21, 2019

To Do:

1. ~~Streak out KRLVS 3.1 onto MH sucrose and MH plates.~~

Results and Data:

Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

-Plate ~~100~~ 50 uL of last dilution ($1x10^{-7}$) onto MH plates.

-Plate ~~100~~ 50 µL of each dilution 10^{-2} – 10^{-7} on MH + 10% KMR sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Plate ~~100~~ 50 µL of each dilution 10^{-2} – 10^{-7} on MH + 10% Chris's sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

pKL118

pKR5_noC2: KROL6, P701, and KROL7

Wednesday, January 23, 2019

To Do:

1. ~~Check MH sucrose plates with KRLVS 3.1.~~
2. ~~Make LB.~~

Results and Data:

LB Broth Protocol (for 500 mL LB)

1. Weigh into a 1L graduated cylinder (with stir bar)
   - 5g tryptone
   - 5g NaCl

- 2.5g yeast extract

2. Add type 1 DEI H2O to 1L graduated cylinder
3. Mix until dissolved
4. Aliquot out
   - 2 x 75 mL in 100 flasks
   - 2 x 150 mL in 250 mL bottles
5. Autoclave on LIQ 30 in autoclave (hemoglobin and sucrose are LIQ 20, all others are LIQ 30)

Thursday, January 24, 2019

To Do:

1. ~~Miniprep overnight cultures for Dr. Ramsey.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube (if in a larger tube).
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA
11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.
12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Thursday, January 24, 2019

To Do:

1. ~~Help Dan and Joe make normal plates with heme-histadine, B-NAD, and glucose. Also make kanamycin and sucrose versions.~~

Monday, January 28, 2019

To Do:

1. ~~PCR p822 + p823 and p824 + p825.~~

Results and Data:

Total reaction volume	20
Total number of reactions	4

Factor

Component	Stock concentration	Final concentration	1 rxn volume	5.5
ddiH2O			4	22
KOD buffer	2x	1x	10	55
dNTPs	2 mM	0.4 mM	4	22
oligo F	10 uM	0.3 uM	0.6	(3.3)
oligo R	10 uM	0.3 uM	0.6	(3.3)
template	100 ng/ul	2 ng/ul	0.4	2.2
KOD	1 U/ul	0.02 U/ul	0.4	2.2
		Total volume	20	110

Tube Number	Purpose	Primers
1	pKL125 FR1	p822 + p823
2	pKL125 FR2	p824 + p825
3	positive control	KROL 19 + KROL 20
4	negative control	KROL 19 + KROL 20

PCR Protocol by John Church (New as of 11/14/18)

1. Acquire and label 4 PCR tubes with initials and designate as Tubes 1-4
    - The tubes comes in strips of 8 and they can be split into 4 tube pieces so that the first 3 tubes are used and the fourth is unused
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
    - ddi H2O in 1.5 mL microfuge tube
    - uL KOD buffer (door of freezer)
    - dNTPs (door of freezer)
    - oligo F (KROL 10 uM box in freezer)
    - oligo R (KROL 10 uM box in freezer)
    - template (UGR researchers)
    - Note: KOD enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
    - DO NOT vortex the KOD enzyme itself or any solution with KOD enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
    - The file is located in the Protocols folder
    - For this protocol, a "Total reaction volume" of 20 uL and 4 "Total number of reactions" were used – the following volumes are based on these specifications
7. Add 0.6 uL of each experiment specific primer (forward and reverse) to PCR Tubes 1 and 2 (oligos forward and reverse)

- The amount added should be calculated by taking the total volume for 1 reaction (in worksheet) and subtracting the volumes for 1 reaction that have not yet been added to the master-mix

8. Add 0.6 uL of each control primer (oligos forward and reverse) to PCR Tubes 3 and 4
9. ~~Add 0.4 uL ddi H2O to PCR Tube 4 so that all 4 PCR Tubes have an even amount of solution~~ Negligible amount of water.
10. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add 22 uL ddi H2O
    - Add 22 uL dNTPs
    - Add 55 uL KOD buffer
    - Add 2.2 uL KOD enzyme
11. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
12. Add 18.4 uL of master-mix to PCR Tube 4
13. Add 1.8 uL template to Master Mix
14. Add 18.8 uL master mix to each PCR Tube 1-3 and pipette up and down to mix (conserves tips)
15. Close PCR Tubes 1-4 until the caps are tight (push until the caps do not squeak when you push on them)
16. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes,
    - 94 degrees C for 20 seconds
    - 50 degrees C for 30 seconds
    - 68 degrees C for 1 minute [modified because product is ~800 bp which is over 1 kbp] (KOD polymerase functions properly at 68 degrees C; TAC polymerase is different temp)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Tuesday, January 29, 2019

To Do:

1. ~~Spread dilutions onto heme infused agar plates.~~
2. ~~PCR purification.~~
3. ~~PCR digest.~~

Results and Data:

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
2. Place a QIAquick column in the provided 2 mL tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.

5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add 50 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Master Mix Solution	1x (uL)	3x (uL)
H2O	10.8	75.6
10x Cutsmart Buffer	3.0	21.0
DNA	(15.0)	-
BamH2 – HF	0.6	-
Kpn2 - HF	0.6	-
Total	30.0 (15.0 actual b/c of DNA)	

Tube	DNA	DNA Volume (uL)	H2O Volume (uL)
1	pKL125 FR1	15	-
2	pKL125 FR2	15	-
3	pEX backbone	5	10
4	pKR4 FR1	15	
5	pKR4 FR2	15	
6	pKL115	5	10

DNA Digest Protocol

1. To master mix tube (MM), add 32.4 uL H2O and 9.0 uL Cutsmart Buffer.
2. To individual DNA tubes, add 15 uL of each DNA type (Purified PCR and pKL80 plasmid).
3. Add 1.8 uL of each enzyme to the MM.
4. Mix it by pipetting up and down.
5. Add 15 uL of MM to individual tubes.
6. Incubate at 37° C overnight.

Wednesday, January 30, 2019

To Do:

1. ~~PCR gel purification.~~

Results and Data:

QIAquick Gel Extraction kit Protocol

1. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume gel (100 mg gel ~ 100 uL). The maximum amount of gel per spin column is 400 mg.
    a. Since the gel in tube 2 is above 400 mg, it will be put into a conical and dissolved, then added to a spin tube multiple times
2. Incubate at 50° C for 10 minutes (or until the gel slice has completely dissolved). Vortex the tube every 2-3 minutes to help dissolve the gel.
3. Add 1 gel volume isopropanol to the sample and mix.
4. Place the QIAquick spin column in a provided 2 mL collection tube. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 uL, load and spin again.

5. Add 500 uL Buffer QG to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 750 uL Buffer PE to QIAquick column and centrifuge for 3 minutes. Discard the flow-through and place the QIAquick column back into the same tube.
7. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, add 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water to the center of the concentration, add 30 uL Buffer EB to the center of the QIAquick membrane and centrifuge for 1 min.

Mass of Empty Microfuge Tube	1.0g	Volume of QB to Add
Total Mass of Tube 1	1.4g	1200 uL
Total Mass of Tube 2	1.5g	1500 uL
Total Mass of Tube 3	1.4g	1200 uL
Total Mass of Tube 4	1.4g	1200uL
Total Mass of Tube 5	1.3g	900uL
Total Mass of Tube 6	1.4g	1200uL

Thursday, January 31, 2019

To Do:

1. ~~Transform ligations.~~

Results and Data:

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	Ligation rxn 1	Insert + pEX plasmid	8 uL	100 ul, remaining	2
2	Ligation rxn 2	pEX	8 uL	100 ul, remaining	2
3	Ligation rxn 3	Insert + pKL115	8 uL	100 ul, remaining	2
4	Ligation rxn 4	pKL115	8 uL	100 ul, remaining	2
5	(+) control	pKL115	1 uL	20ul, 100 ul	2
6	(-) control	None	0	20ul, 100 ul	2
				Total number of plates	12

February 2019

Friday, February 1, 2019

To Do:

1. ~~PCR of KROL94 + KROL95 and KROL96 + KROL97.~~
2. ~~Run PCR on a gel.~~

Results and Data:

Total reaction volume	20
Total number of reactions	4

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5.5
ddiH2O			4	22
KOD buffer	2x	1x	10	55

dNTPs	2 mM	0.4 mM	4	22
oligo F	10 uM	0.3 uM	0.6	3.3
oligo R	10 uM	0.3 uM	0.6	3.3
Template (LVS gDNA)	100 ng/ul	2 ng/ul	0.4	2.2
KOD	1 U/ul	0.02 U/ul	0.4	2.2
		Total volume	20	110

Tube Number	Purpose	Primers
1	test	KROL94 + KROL95
2	test	KROL96 + KROL97
3	positive control	KROL 19 + KROL 20
4	negative control	KROL 19 + KROL 20

PCR Protocol by John Church (New as of 11/14/18)

1. Acquire and label 4 PCR tubes with initials and designate as Tubes 1-4
    - The tubes come in strips of 8 and they can be split into 4 tube pieces so that the first 3 tubes are used and the fourth is unused
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
    - ddi H2O in 1.5 mL microfuge tube
    - uL KOD buffer (door of freezer)
    - dNTPs (door of freezer)
    - oligo F (KROL 10 uM box in freezer)
    - oligo R (KROL 10 uM box in freezer)
    - template (UGR researchers; LVS gDNA)
    - Note: KOD enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
    - DO NOT vortex the KOD enzyme itself or any solution with KOD enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
    - The file is located in the Protocols folder
    - For this protocol, a "Total reaction volume" of 20 uL and 4 "Total number of reactions" were used – the following volumes are based on these specifications
    - Note: 20 ul reaction volumes can be used for test PCRs and 100 uL reactions can be used for others.
7. Add 0.6 uL of each experiment specific primer (forward and reverse) to PCR Tubes 1 and 2 (oligos forward and reverse)
    - The amount added should be calculated by taking the total volume for 1 reaction (in worksheet) and subtracting the volumes for 1 reaction that have not yet been added to the master-mix

8. Add 0.6 uL of each control primer (oligos forward and reverse) to PCR Tubes 3 and 4
9. ~~Add 0.4 uL ddi H2O to PCR Tube 4 so that all 4 PCR Tubes have an even amount of solution~~
   Negligible amount of water.
10. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add 22 uL ddi H2O
    - Add 22 uL dNTPs
    - Add 55 uL KOD buffer
    - Add 2.2 uL KOD enzyme
11. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
12. Add 18.4 uL of master-mix to PCR Tube 4
13. Add 1.8 uL template to Master Mix
14. Add 18.8 uL master mix to each PCR Tube 1-3 and pipette up and down to mix (conserves tips)
15. Close PCR Tubes 1-4 until the caps are tight (push until the caps do not squeak when you push on them)
16. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes,
    - 94 degrees C for 20 seconds
    - 50 degrees C for 30 seconds
    - 68 degrees C for 2 minutes [modified because product is ~1800 bp which is over 1 kbp] (KOD polymerase functions properly at 68 degrees C; TAC polymerase is different temp)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Agarose Gel Protocol – written by Joe Paquette

(Note: all ddiH2O is type I)

1. Mix 25x TAE buffer with ddiH_2O to obtain 1x TAE solution.

   - To make 1 L of 1x TAE, add 40 mL 25x TAE into 1 L graduated cylinder and fill to 1 L with ddiH2O.

2. Add 1 g agarose powder to 100 mL 1x TAE buffer in 250 mL.
3. Add stirbar to container.
4. Heat to dissolve the agarose while stirring (don't let over boil, should look like clear liquid, no solids).
5. Once the sugar has dissolved, make a 1x concentration of SYBR safe in the 1% agarose solution.
   - To make, add 10µL of 10,000x SYBR safe dye to the 1% agarose solution
6. Let the 1% agarose solution cool to approximately 50-55°C.
7. Apply autoclave tape to the edges of the gel cast (ensure the tape is tightly bound).
8. Pour 1% agarose - 1x SYBR safe solution into the cast and insert a comb to mold wells in the gel.
9. Let sit until the 1% agarose – 1x SYBR safe solution has cooled and solidified into a gel.

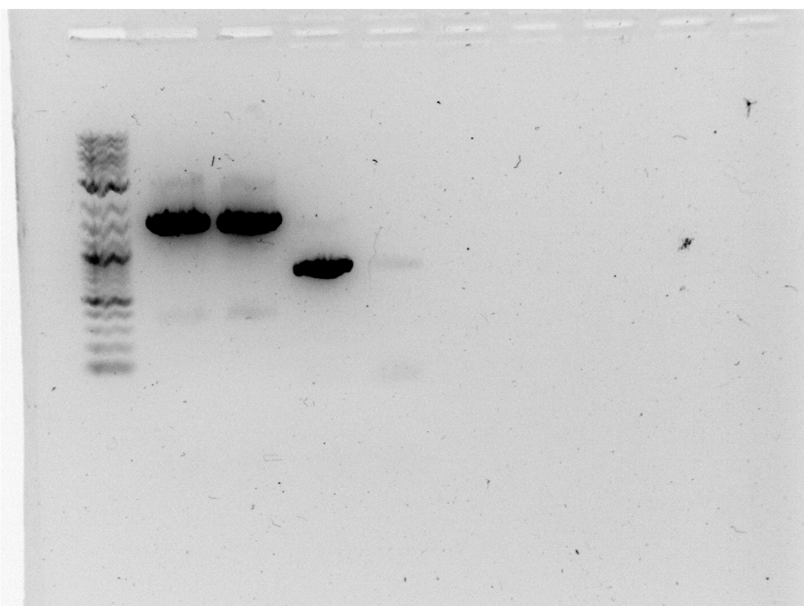
10. Carefully remove the comb.
11. Pour the 100 ml of 1x TAE buffer solution into the gel tank (add just enough to slightly submerge the gel itself).
12. Obtain 5 µL of each PCR sample.
13. Make 1x loading dye in 6 µL of solution.

    - To make 6 µL of 1x loading dye, combine 5 µL PCR sample with 1 µL of 6x loading dye

14. Load 10 µL of 2x log ladder into the first well.
15. Load 5 µL of the PCR-dye mixture into the wells in sequential order.
16. Insert the electrodes and run the gel at about 113 volts.

Materials needed:

- 1.0g of agarose powder
- SYBR safe dye
- 1 packet of 25x TAE buffer mix



Above is the gel that was run to test primers KROL94 + KROL95 and KROL96 + KROL97. The ladder is shown in lane 1, KROL94 + KROL95 are shown in lane 2, KROL96 + KROL97 are shown in lane 3, the positive control is shown in lane 4 and the negative control is absent from lane 5. The products were expected to be about 1800 bp, so the PCR and primers appear successful.

Monday, February 4, 2019

To Do:

1. ~~Take OD600 readings for Jamie.~~
2. ~~Miniprep pelleted cultures.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).

2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube (if in a larger tube).
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA
11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.
12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.		Cursor abs.
	340 raw											
pKR4-1	Default	2/4/2019	12:10 PM	795.08	15.902	8.602	1.85	2.38	50.00	230	6.682	0.014
pKR4-2	Default	2/4/2019	12:11 PM	900.34	18.007	9.741	1.85	2.39	50.00	230	7.531	0.369
pKR4-3	Default	2/4/2019	12:12 PM	789.05	15.781	8.516	1.85	2.38	50.00	230	6.624	0.025
pKR4-4	Default	2/4/2019	12:13 PM	491.43	9.829	5.487	1.79	2.33	50.00	230	4.214	0.010
pKL125-1	Default	2/4/2019	12:15 PM	322.70	6.454	3.479	1.86	2.35	50.00	230	2.749	0.005
pKL125-2	Default	2/4/2019	12:17 PM	212.22	4.244	2.284	1.86	2.31	50.00	230	1.837	0.023
pKL125-3	Default	2/4/2019	12:18 PM	259.81	5.196	2.791	1.86	2.34	50.00	230	2.225	-0.006
pKL125-4	Default	2/4/2019	12:19 PM	232.00	4.640	2.507	1.85	2.28	50.00	230	2.031	0.038

The plasmids were placed in the Plasmids 1 box and labeled with the date and plasmid.

Wednesday, February 6, 2019

To Do:

1. ~~100 uL PCR of KROL94 + KROL95 and KROL96 + KROL97.~~
2. ~~Sequencing for pKL125.~~

Results and Data:

Tube Number	Purpose	Primers	Expected Size
1	pF_FTL_1251	KROL94 + KROL95	1800bp
2	pF_FTL_0766	KROL96 + KROL97	1800bp
3	positive control	KROL 19 + KROL 20	757 bp
4	negative control	KROL 19 + KROL 20	- control

Total reaction volume	100
Total number of reactions	4

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5.5

ddiH_2O			20	110
KOD buffer	2x	1x	50	275
dNTPs	2 mM	0.4 mM	20	110
oligo F	10 uM	0.3 uM	3	16.5
oligo R	10 uM	0.3 uM	3	16.5
template	100 ng/ul	2 ng/ul	2	11
KOD	1 U/ul	0.02 U/ul	2	11
		Total volume	100	550

100 uL PCR Protocol by John Church (New as of 11/14/18)

1. Acquire and label 4 PCR tubes with initials and designate as Tubes 1-4
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
   - ddi H_2O in 1.5 mL microfuge tube
   - uL KOD buffer (door of freezer)
   - dNTPs (door of freezer)
   - oligo F (KROL 10 uM box in freezer)
   - oligo R (KROL 10 uM box in freezer)
   - template (UGR researchers; LVS gDNA)
   - Note: KOD enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
   - DO NOT vortex the KOD enzyme itself or any solution with KOD enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
   - The file is located in the Protocols folder
   - For this protocol, a “Total reaction volume” of 100 uL and 4 “Total number of reactions” were used – the following volumes are based on these specifications
   - Note: 20 ul reaction volumes can be used for test PCRs and 100 uL reactions can be used for others.
7. Add 3 uL of each experiment specific primer (forward and reverse) to PCR Tubes 1 and 2 (oligos forward and reverse)
8. Add 3 uL of each control primer (oligos forward and reverse) to PCR Tubes 3 and 4
9. Add 2 uL ddi H_2O to PCR Tube 4 so that all 4 PCR Tubes have an even amount of solution
   - This is to account for the absence of template in the negative control
10. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add 110 uL ddi H_2O
    - Add 110 uL dNTPs
    - Add 275 uL KOD buffer
    - Add 11 uL KOD enzyme
11. Mix the master-mix solution by pipetting up and down

- Do not vortex to mix

12. Add 92 uL of master-mix to PCR Tube 4
13. Add 8.8 uL template (LVS gDNA) to Master Mix
14. Add 94 uL master mix to each PCR Tube 1-3 and pipette up and down to mix (conserves tips)
15. Close PCR Tubes 1-4 until the caps are tight (push until the caps do not squeak when you push on them)
16. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes,
    - 94 degrees C for 20 seconds
    - 50 degrees C for 30 seconds
    - 68 degrees C for 2 minutes [modified because product is ~1800 bp which is over 1 kbp] (KOD polymerase functions properly at 68 degrees C; TAC polymerase is different temp)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Friday, February 8, 2019

To Do:

1. ~~PCR purification.~~
2. ~~DNA digest.~~
3. ~~Digest purification.~~
4. ~~Transformations for Jamie.~~

Results and Data:

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
2. Place a QIAquick column in the provided 2 mL tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add 50 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

DNA Digest Protocol

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pF_FTL_1251	NotI, BamHI	15	-

2	pF backbone	NotI, BamHI	5	10
3	pF_FTL_0766	NotI, BamHI	15	-

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 5x (uL)
H_2O	10.8	54.0
10x Buffer*	3.0	15.0
DNA	(15.0)	-
NotI	0.6	3.0
BamHI	0.6	3.0
Total	30.0 (15.0 actual b/c of DNA)	

*Cutsmart Buffer is used for all the "HF" enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

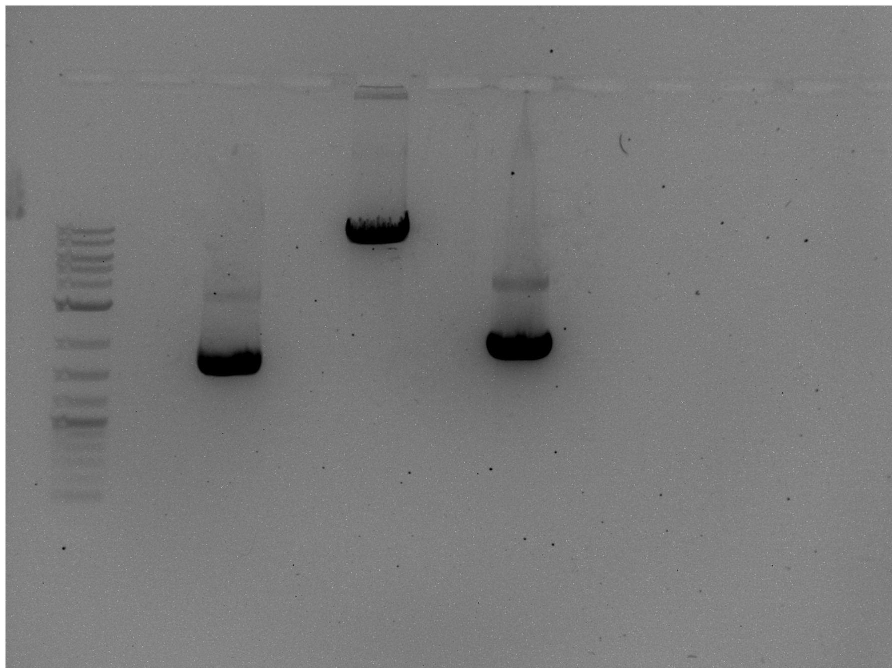
3. Add indicated amounts of H_2O (54.0 uL) and 10x buffer Cutsmart (15.0 uL) to master mix tube (MM).
4. Add indicated amount of DNA (15.0 uL) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler.
5. Add indicated amount of each enzyme (3.0 uL) to the master mix tube (MM).
6. Mix the master mix by pipetting up and down.
7. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (15.0 ul).
8. Incubate at 37°C ~~overnight~~. For 1 hour

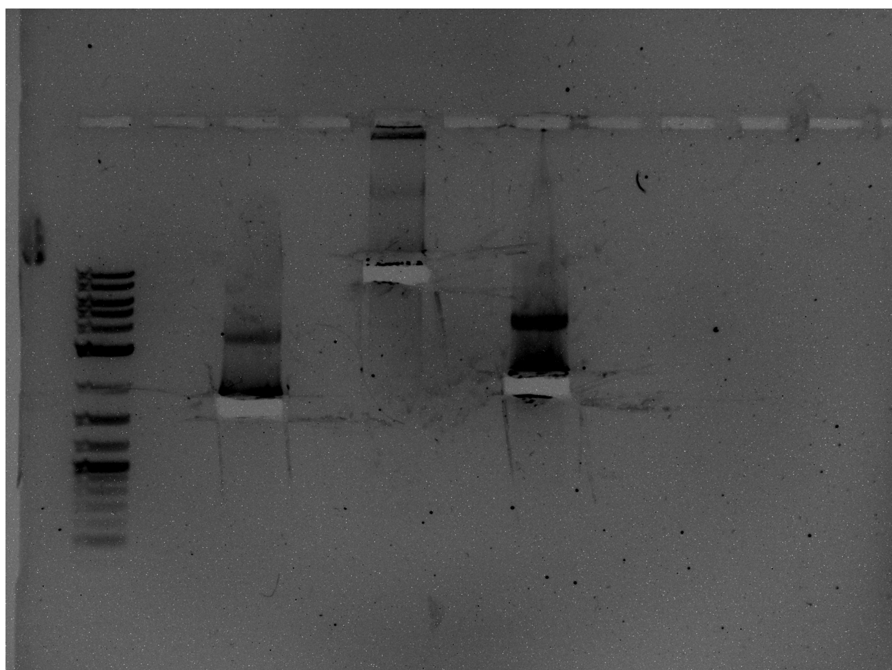
Transform chemically competent E. coli cells

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL, 100 uL, and remaining culture. If transforming a ligation, plate 100 uL and remaining culture.
2. Check to be sure you have enough plates with appropriate antibiotic. 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 (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates, spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for

30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

- After running incubating and running on a gel, the gel was cut, put in tubes, and placed in the fridge.





Monday, February 11, 2019

To Do:

1. ~~Digest Purification for pF_FTL_125, pF_FTL_0766, and pF backbone.~~

Results and Data:

QIAquick Gel Extraction kit Protocol

1. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume gel (100 mg gel ~ 100 uL). The maximum amount of gel per spin column is 400 mg.
2. Incubate at 50º C for 10 minutes (or until the gel slice has completely dissolved). Vortex the tube every 2-3 minutes to help dissolve the gel.
3. Add 1 gel volume isopropanol to the sample and mix.
4. Place the QIAquick spin column in a provided 2 mL collection tube. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 uL, load and spin again.
5. Add 500 uL Buffer QG to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 750 uL Buffer PE to QIAquick column and centrifuge for 3 minutes. Discard the flow-through and place the QIAquick column back into the same tube.
7. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, add 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water to the center of the concentration, add 30 uL Buffer EB to the center of the QIAquick membrane and centrifuge for 1 min.

Mass of Empty Microfuge Tube	1.0g	Volume of QB to Add
Total Mass of Tube 1	1.2g	600 uL
Total Mass of Tube 2	1.3g	900 uL
Total Mass of Tube 3	1.2g	600 uL

Ligation of Digested, Purified Plasmid and PCR

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

Tube	Insert	Backbone
1	pF_FTL_1251	digested, purified pF
2	pF_FTL_0766	digested, purified pF
3	-	digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)
H_2O	11.5	11.5	15.5
10x ligase buffer	2.0	2.0	2.0
Insert	4.0	4.0	-
Backbone	2.0	2.0	2.0
Ligase	0.5	0.5	0.5
TOTAL	20.0	20.0	20.0

1. 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.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.

3. To the individual tubes, add indicated amounts of H_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
4. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Place in the thermocycler overnight at 16°C.

Tuesday, February 12, 2019

To Do:

1. ~~Transform plasmids.~~

Results and Data:

Transform chemically competent E. coli cells

1. Set up reaction table. **Always include a positive and negative control for each antibiotic (and for each backbone)**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL, 100 uL, and remaining culture. If transforming a ligation, plate 100 uL and remaining culture.
   - Note: The positive control should be a regular plasmid, not the digested/purified one. In this experiment, the digested purified plasmid was used and there was no growth.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates	Number of carbenicillin-containing plates
1	(+) control	pF	1 uL	20 ul, 100 ul, remaining	3	
2	(-) control	None	0	20 ul, 100 ul, remaining	3	
3	Reaction 1	pF_FTL_1251	8 uL	100 ul, remaining	2	
4	Reaction 2	pF_FTL_0766	8 uL	100 ul, remaining	2	
5	(+) control (3)	pEX	1 uL	20 uL, 100 uL, remaining	3	
6	(-) control (3)	None	0	20 ul, 100 uL, remaining	3	
7	Reaction 3	pKR9	1 uL	20 uL, 100 uL, remaining	3	
				Total number of plates	19	0

2. Check to be sure you have enough plates with appropriate antibiotic. 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 (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates, spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.
14. Incubate overnight.

Wednesday, February 13, 2019

To Do:

1. ~~Make overnight cultures from plates.~~

Results and Data:

Overnight Cultures Protocol

By John Church

1. Add 30 mL of LB broth to 2 50 mL conicals.
2. Add 30 uL of kanamycin to each conical.
3. Label 4 test tubes for each reaction of new plasmids and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB media into each of the tubes.
5. Pick 4 colonies from each reaction and inoculate the tube.
    - Do this by using a stick and picking an isolated colony from plate
6. Place in the 37° shaking incubator overnight.

Tubes	Purpose
1-4	pF_FTL_1251
5-8	pF_FTL_0766
9-10	pKR9

Thursday, February 14, 2019

To Do:

1. ~~Miniprep overnight cultures.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA

11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.
12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Nanodrop results table:

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
pF_FTL_1251(1)	Default	2/14/2019	12:17 PM	276.45	5.529	3.010	1.84	2.15	50.00	230	2.568	0.272
pF_FTL_1251(2)	Default	2/14/2019	12:18 PM	289.47	5.789	3.104	1.87	2.40	50.00	230	2.408	-0.001
pF_FTL_1251(3)	Default	2/14/2019	12:19 PM	243.07	4.861	2.602	1.87	2.41	50.00	230	2.020	0.006
pF_FTL_1251(4)	Default	2/14/2019	12:20 PM	267.55	5.351	2.935	1.82	1.95	50.00	230	2.746	0.416
pF_FTL_0766(5)	Default	2/14/2019	12:21 PM	201.21	4.024	2.153	1.87	2.42	50.00	230	1.663	0.012
pF_FTL_0766(6)	Default	2/14/2019	12:23 PM	207.00	4.140	2.238	1.85	2.26	50.00	230	1.828	0.123
pF_FTL_0766(7)	Default	2/14/2019	12:24 PM	252.05	5.041	2.723	1.85	2.35	50.00	230	2.146	0.086
pF_FTL_0766(8)	Default	2/14/2019	12:25 PM	244.39	4.888	2.660	1.84	2.16	50.00	230	2.260	0.203
pKR9(9)	Default	2/14/2019	12:26 PM	201.73	4.035	2.174	1.86	2.22	50.00	230	1.816	0.089
pKR9(10)	Default	2/14/2019	12:27 PM	154.83	3.097	1.706	1.82	1.75	50.00	230	1.770	0.369

Friday, February 15, 2019

To Do:

~~1. Make electrocompetent cells.~~

Results and Data:

Making Electrocompetent Cells (from Allelic Exchange Protocol)

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells, resuspend in 300 uL sterile 10% sucrose (alternately, cells can be grown to mid-log in MHB)

-Centrifuge for 3 minutes at 10,000 rpm

-Wash cells 5x in 10% sucrose

-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells.

-For any extra EC cells, aliquot ~110 μL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Monday, February 18, 2019

To Do:

~~1. Make hemoglobin.~~

Results and Data:

19g hemoglobin to 950 mL H2O to 2 L flask

Tuesday, February 19, 2019

To Do:

~~1. Strike out primary integrant dilutions onto CHA 10%, 15%, and 20% sucrose plates with controls.~~

Results and Data:

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting ~~25~~ 50 uL cells into ~~250~~ 450 uL 1X PBS

-Plate ~~100~~ 50 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate ~~100~~ 50 μL of each dilution 10^{-2} – 10^{-7} on CHAH + 10%, 15%, 20% sucrose plates, . If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to

be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Wednesday, February 20, 2019

To Do:

1. ~~Work on dPmrA and LVS electrocompetent cells for Jamie.~~

Results and Data:

Making Electrocompetent Cells (from Allelic Exchange Protocol)

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells, resuspend in 300 uL sterile 10% sucrose (alternately, cells can be grown to mid-log in MHB)

-Centrifuge for 3 minutes at 10,000 rpm

-Wash cells 5x in 10% sucrose

-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells.

-For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Thursday, February 21, 2019

To Do:

1. ~~Make 2 LB, 1 CHA medias.~~
2. ~~Digest pKL133~~

Results and Data:

Friday, February 22, 2019

To Do:

1. ~~Scrape up plate, dilute, and plate out.~~
2. ~~Run digest on gel.~~

Results and Data:

Diagnostic Digest on Gel

1. Make gel with dye
2. Add 6x orange dye to digest tubes
3. Load ladder into gel
4. Load each sample into gel
5. Image gel

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

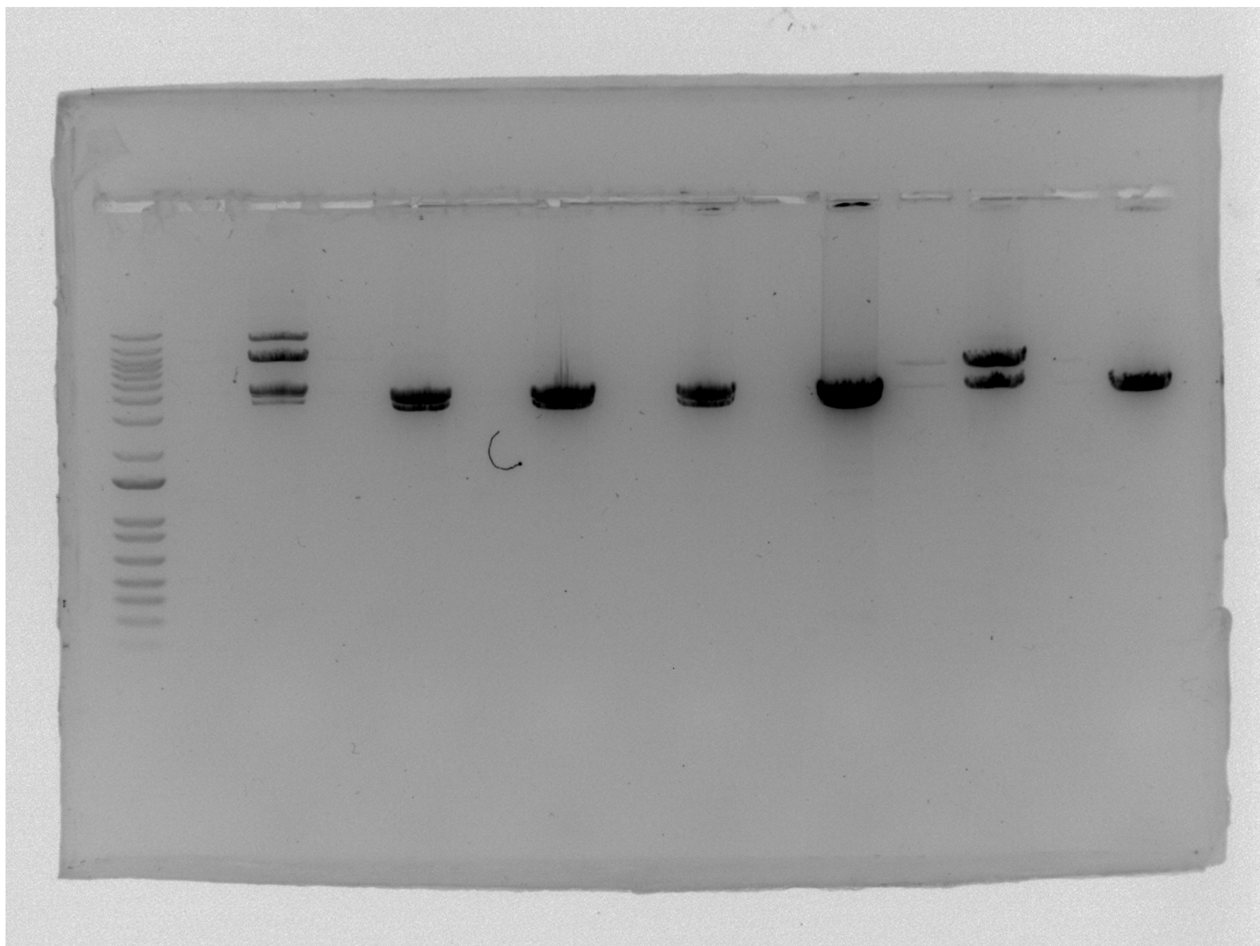
Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 250 uL 1X PBS

-Plate ~~100~~ 50 uL of last dilution ($1x10^{-7}$) onto CHAH plate and kan + CHAH plate.

-Plate ~~100~~ 50 µL of each dilution 10^{-2} – 10^{-7} on CHAH + 10%, CHA + 15%, and CHA + 20% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.



The gel appears to need to run longer, so Jamie put it back on.

Monday, February 25, 2019

To Do:

1. ~~Lab meeting.~~
2. ~~Set up sequencing for Tuesday.~~

Results and Data:

Since KROL137 has not come in as of 2/26/19, sequencing will have to wait until Thursday. The sequencing strip tubes and plasmids being sequenced were put in the undergrad cloning box.

Tuesday, February 26, 2019

To Do:

1. ~~Start LVS CDM with Joe.~~

Results and Data:

Wednesday, February 27, 2019

To Do:

1. ~~Finish and submit sequencing.~~
2. ~~Continue with LVS CDM.~~

Results and Data:

Could not get methionine crystals to dissolve.

Thursday, February 28, 2019

To Do:

1. ~~Continue with LVS CDM.~~

Results and Data:

March 2019

Friday, March 1, 2019

To Do:

1. ~~Continue with LVS CDM.~~

Results and Data:

Monday, March 4, 2019

To Do:

1. ~~Lab meeting.~~
2. ~~PriM team meeting for copper assay.~~

Results and Data:

Tuesday, March 5, 2019

To Do:

1. ~~Start copper assay.~~

Results and Data:

Copper Assay Protocol

1. Label 36 sterile culture tubes for copper dilution.
   - 9 tubes for each dilution (0, 40, 60, and 100uM)
   - 4 strains with 3 tubes for each strain (triplicates)
2. Make 25 mL of each dilution in sterile tubes by diluting 0.1M stock solution in supplemented MHB broth.
3. Use aseptic technique to add 2 mL of each MH/copper broth concentration to each tube.
4. Inoculate 2 mL of supplemented MH broth with OD 0.1 strains.
   - Strains: WT, LVS dPmrA, LVS dPmrA dPriM
5. Incubate at 37°C overnight and take ODs after 4 hours and 24 hours
6. After 3.5-4 hours, remove the tubes from the incubator
7. Using aseptic technique for the culture tubes, add 1 mL to labeled cuvettes and take OD600 readings for each sample
8. Place back in the shaking incubator and repeat OD600 readings after 24 hours

Note: The OD600 readings at 24 hours were diluted so these values have been multiplied by 4

Tube Number	Dilution	Strain	OD600 Reading at 3.5-4 hours	OD600 Reading at 24 hours
1	0 uM	1 - WT	0.255	1.676
2	0 uM	1 - WT	0.245	1.740
3	0 uM	1 - WT	0.230	1.844
4	0 uM	2 – LVS dPmrA	0.207	1.668
5	0 uM	2 – LVS dPmrA	0.214	1.604
6	0 uM	2 – LVS dPmrA	0.228	1.672
7	0 uM	3 – LVS dPmrA dPriM	0.227	1.544
8	0 uM	3 – LVS dPmrA dPriM	0.263	1.388
9	0 uM	3 – LVS dPmrA	0.249	1.424

		dPriM		
10	40 uM	1 - WT	0.242	1.496
11	40 uM	1 - WT	0.255	1.600
12	40 uM	1 - WT	0.249	1.528
13	40 uM	2 – LVS dPmrA	0.178	0.604
14	40 uM	2 – LVS dPmrA	0.180	0.588
15	40 uM	2 – LVS dPmrA	0.208	0.592
16	40 uM	3 – LVS dPmrA dPriM	0.214	0.652
17	40 uM	3 – LVS dPmrA dPriM	0.266	0.656
18	40 uM	3 – LVS dPmrA dPriM	0.229	0.588
19	60 uM	1 - WT	0.226	1.204
20	60 uM	1 - WT	0.239	1.076
21	60 uM	1 - WT	0.241	1.288
22	60 uM	2 – LVS dPmrA	0.159	0.576
23	60 uM	2 – LVS dPmrA	0.148	0.564
24	60 uM	2 – LVS dPmrA	0.158	0.444
25	60 uM	3 – LVS dPmrA dPriM	0.196	0.476
26	60 uM	3 – LVS dPmrA dPriM	0.206	0.524
27	60 uM	3 – LVS dPmrA dPriM	0.215	0.560
28	100 uM	1 - WT	0.200	0.660
29	100 uM	1 - WT	0.233	0.832
30	100 uM	1 - WT	0.230	0.856
31	100 uM	2 – LVS dPmrA	0.134	0.360
32	100 uM	2 – LVS dPmrA	0.143	0.240
33	100 uM	2 – LVS dPmrA	0.148	0.348
34	100 uM	3 – LVS dPmrA dPriM	0.186	0.380
35	100 uM	3 – LVS dPmrA dPriM	0.189	0.336
36	100 uM	3 – LVS dPmrA dPriM	0.192	0.360

Wednesday, March 6, 2019

To Do:

1. ~~Continue LVS CDM.~~
2. ~~Take 24 hour OD600s (above).~~

Results and Data:

Thursday, March 7, 2019

To Do:

1. ~~Continue LVS CDM.~~

Results and Data:

Friday, March 8, 2019

To Do:

1. ~~Continue LVS CDM.~~

Results and Data:

Monday, March 18, 2019

To Do:

1. ~~Help Hannah with homemade CHA (making CHA with ddi H2O and molecular H2O).~~

Results and Data:

Homemade CHAH Plates with Sucrose Protocol

1. 5g beef heart infusion
2. 5g proteose peptone
3. 5g glucose
4. 0.5g L-cystine
5. 7.5g agar
6. Add to 250ml of ddiH2O
7. Repeat and add to 250ml of molecular grade H2O
8. Autoclave both flasks on a 30’ cycle.
9. Prepare 2 flasks of 250ml hemoglobin w/ sucrose
10. Combine hemoglobin (20’ cycle) with the 250ml of CHA in 500ml and pour plates

Tuesday, March 19, 2019

To Do:

1. ~~Work with Joe to do growth curve of CDM.~~

Results and Data:

OD Growth Curve Protocol for CDM

1. Resuspend LVS aseptically in 4 50 mL volumes of MHB and CDM (2 replicates in 125mL baffled flasks of MHB, 2 replicates in CDM).
2. Set to an OD of 0.1.
3. Incubate in 37° shaking incubator, taking ODs at 2 hour time intervals.

Time point	Media Type	OD600
2 hours	CDM-1	0.146
	CDM-2	0.149
	MHB-1	0.130
	MHB-2	0.168
4 hours	CDM-1	0.302
	CDM-2	0.288
	MHB-1	0.277
	MHB-2	0.333
6 hours	CDM-1	0.430
	CDM-2	0.434
	MHB-1	0.419
	MHB-2	0.496
24 hours	CDM-1	3.88
	CDM-2	4.84
	MHB-1	2.46

	MHB-2	2.42

Wednesday, March 20, 2019

To Do:

1. ~~Start new Copper Assay.~~
2. ~~Take 24 hr timepoint for CDM growth curve.~~
3. ~~Make drip plates using remainder of CDM and MHB.~~

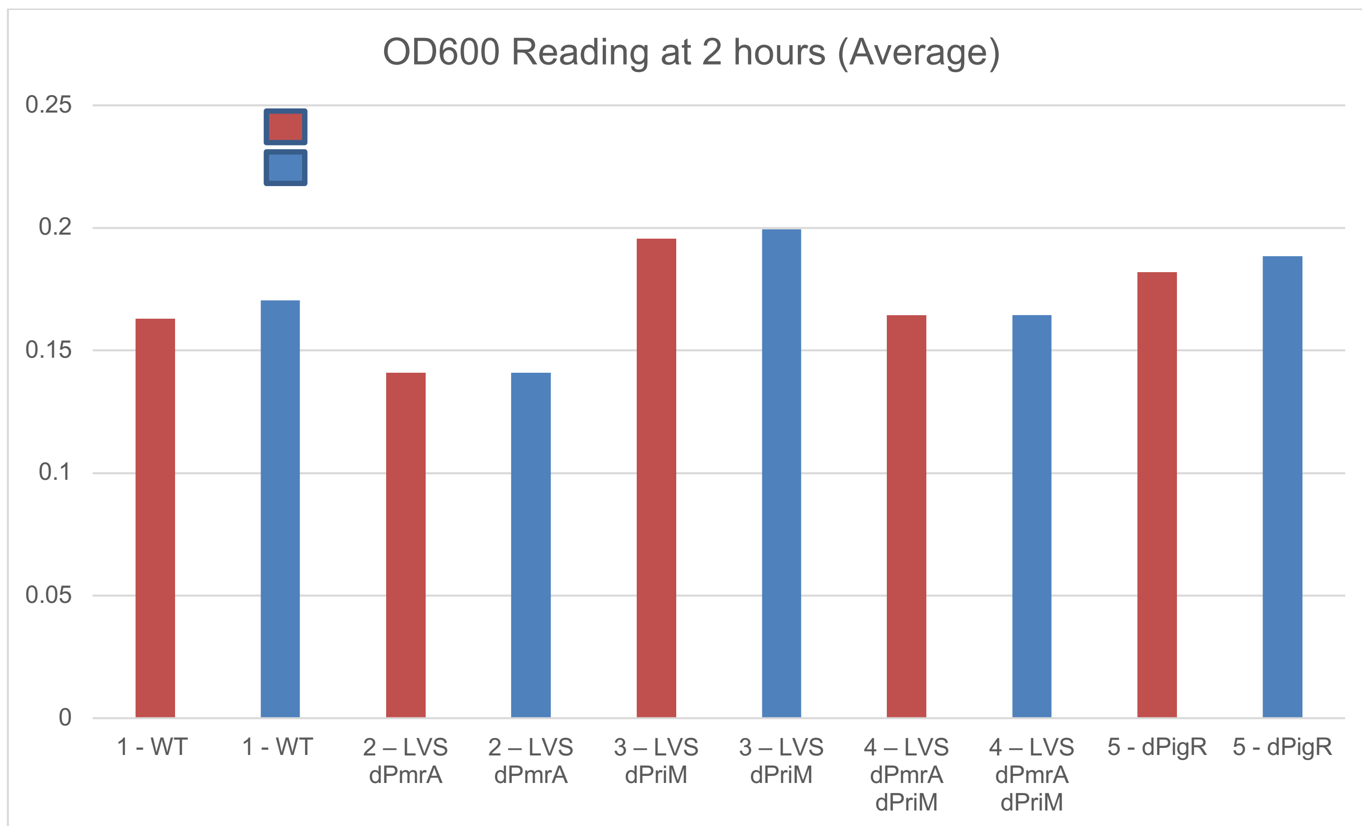
Results and Data:

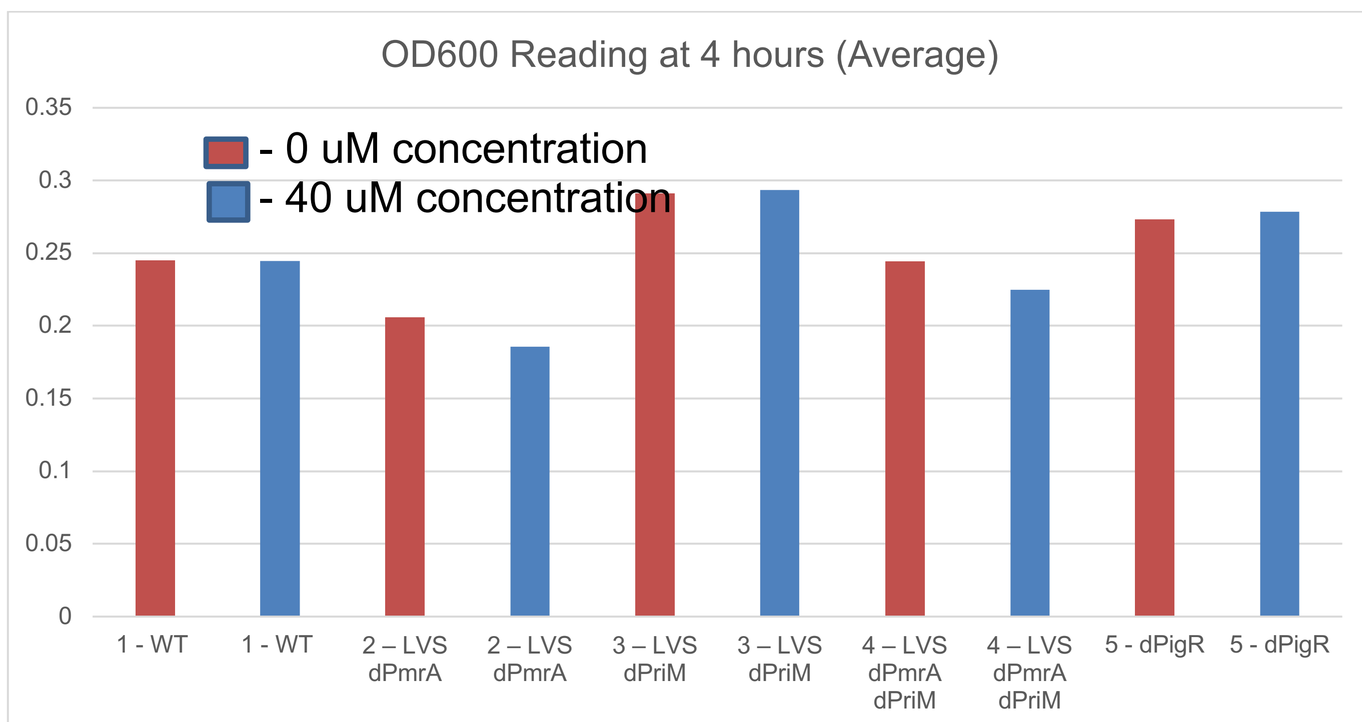
Copper Assay Protocol

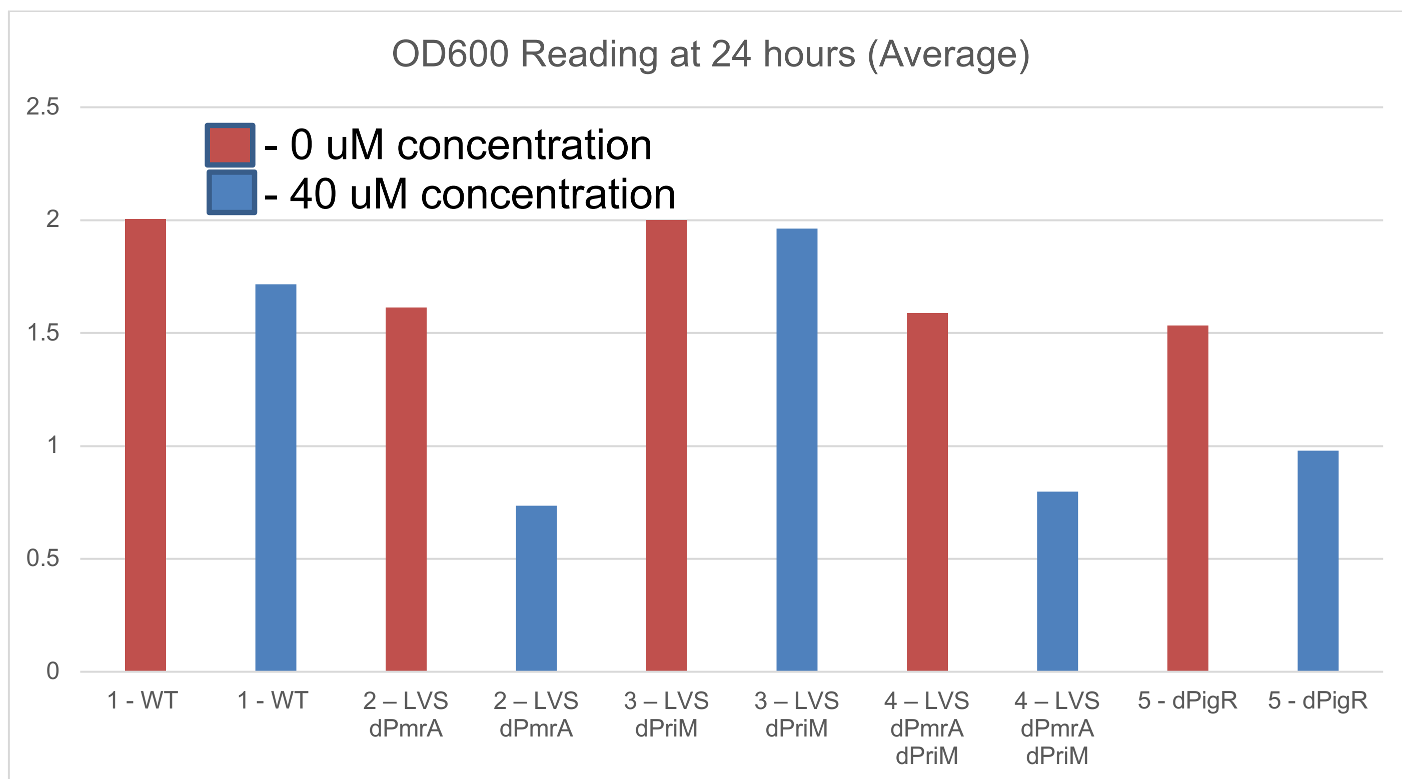
1. Label 30 sterile culture tubes for copper dilution.
   - 0uM and 40uM dilutions of copper
   - 5 strains with triplicates for each
2. Resuspend plate cultures of each strain in s-MHB
3. Make 60 mL of 80uM dilution in sterile tubes by diluting 0.1M stock solution in supplemented MHB broth.
   - (80uM)(60mL)=(V)(0.1M) > V=48uL
   - 80 because it will be split when added to culture tubes.
4. Use aseptic technique to add 3 mL of each MH/copper broth concentration to each tube.
5. Inoculate 2 mL of supplemented s-MHB broth with OD 0.1 strains.
   - Strains: WT, LVS dPmrA, LVS dPmrA dPriM, dPigR
6. Incubate at 37°C overnight and take ODs after 4 hours and 24 hours
7. After 3.5-4 hours, remove the tubes from the incubator
8. Using aseptic technique for the culture tubes, add 1 mL to labeled cuvettes and take OD600 readings for each sample
9. Place back in the shaking incubator and repeat OD600 readings after 24 hours

Tube Number	Dilution	Strain	OD600 Reading at 2 hours	OD600 Reading at 4 hours	OD600 Reading at 24 hours
1	0 uM	1 - WT	0.163	0.243	2.06
2	0 uM	1 - WT	0.165	0.246	1.964
3	0 uM	1 - WT	0.161	0.246	1.992
4	40 uM	1 - WT	0.173	0.247	1.748
5	40 uM	1 - WT	0.170	0.245	1.72
6	40 uM	1 - WT	0.168	0.242	1.684
7	0 uM	2 – LVS dPmrA	0.141	0.209	1.252
8	0 uM	2 – LVS dPmrA	0.138	0.202	1.82
9	0 uM	2 – LVS dPmrA	0.144	0.206	1.768
10	40 uM	2 – LVS dPmrA	0.140	0.185	0.696
11	40 uM	2 – LVS dPmrA	0.141	0.185	0.752
12	40 uM	2 – LVS dPmrA	0.142	0.187	0.756

13	0 uM	3 – LVS dPriM	0.200	0.298	2.052
14	0 uM	3 – LVS dPriM	0.194	0.288	1.992
15	0 uM	3 – LVS dPriM	0.193	0.287	1.96
16	40 uM	3 – LVS dPriM	0.203	0.293	2.024
17	40 uM	3 – LVS dPriM	0.206	0.306	2.172
18	40 uM	3 – LVS dPriM	0.189	0.282	1.692
19	0 uM	4 – LVS dPmrA dPriM	0.166	0.246	1.544
20	0 uM	4 – LVS dPmrA dPriM	0.161	0.241	1.596
21	0 uM	4 – LVS dPmrA dPriM	0.166	0.246	1.628
22	40 uM	4 – LVS dPmrA dPriM	0.165	0.227	0.848
23	40 uM	4 – LVS dPmrA dPriM	0.164	0.226	0.776
24	40 uM	4 – LVS dPmrA dPriM	0.164	0.222	0.768
25	0 uM	5 - dPigR	0.188	0.280	1.572
26	0 uM	5 - dPigR	0.180	0.271	1.496
27	0 uM	5 - dPigR	0.178	0.268	1.532
28	40 uM	5 - dPigR	0.190	0.280	1.024
29	40 uM	5 - dPigR	0.186	0.276	0.964
30	40 uM	5 - dPigR	0.189	0.279	0.948


OD600 Reading at 2 hours (Average)
0.25
0.2
0.15
0.1
0.05
0
1 - WT
1 - WT
2 – LVS dPmrA
2 – LVS dPmrA
3 – LVS dPriM
3 – LVS dPriM
4 – LVS dPmrA dPriM
4 – LVS dPmrA dPriM
5 - dPigR
5 - dPigR


OD600 Reading at 4 hours (Average)
- 0 uM concentration
- 40 uM concentration
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1 - WT
1 - WT
2 – LVS dPmrA
2 – LVS dPmrA
3 – LVS dPriM
3 – LVS dPriM
4 – LVS dPmrA dPriM
4 – LVS dPmrA dPriM
5 - dPigR
5 - dPigR



Thursday, March 21, 2019

To Do:

1. ~~Pour CHA plates with Jamie.~~
2. ~~24 hours timepoint for copper assay.~~

Results and Data:

Friday, March 22, 2019

To Do:

1. ~~Meet with Dr. Ramsey about CDM growth curve results, copper assay results, and moving forward with allelic exchange (notes in planner).~~
2. ~~Seminar.~~

Results and Data:

Monday, March 25, 2019

To Do:

1. ~~Plan week ahead for allelic exchange with Joe.~~
2. ~~Lab meeting.~~

Results and Data:

Tuesday, March 26, 2019

To Do:

1. ~~Work on CDM growth curve results, copper assay results, and prepare media for allelic exchange.~~
2. ~~Make CHAH-Kan plates from batch and CHAH and CHAH+sucrose plates from homemade media.~~

Results and Data:

Wednesday, March 27, 2019

To Do:

1. ~~Work on CDM growth curve results, copper assay results, and prepare media for allelic exchange.~~

Results and Data:

Thursday, March 28, 2019

To Do:

1. ~~Start pKL115 (Tube 1) allelic exchange with 1° integrant - Plate primary integrant on CHA plates.~~
2. ~~Pour agarose gel for Jamie.~~

Results and Data:

Since we were able to get sucrose selection to work using homemade CHA plates, we will proceed with allelic exchange using 1° integrants from the -80 to make mutants.
Note: We used tube 1 from -80 stocks.

Friday, March 29, 2019

To Do:

1. ~~Make CHA+Kan plates (and regular CHA if needed)~~
2. ~~Allelic exchange day 6: plate 1° integrant on homemade 10% sucrose plates.~~

Results and Data:

Allelic Exchange Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 200 uL 1X PBS ("John and Joe" stock)

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

-Plate 100 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate 100 μL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Modnay, April 1, 2019

To Do:

1. ~~Lab meeting – presentation with Joe.~~
2. ~~Inspect CHA sucrose plates for growth.~~

Results and Data:

Sucrose plates do not have growth, so we will give them another day.

Tuesday, April 2, 2019

To Do:

1. ~~Check plates.~~
2. ~~Plan moving forward.~~

Results and Data:

The CHA sucrose plates still had no growth, while the CHA control had good growth. Upon inspecting the plates in the other lab bay, it was clear that they had dried out. This may be the cause for why we had no growth on the sucrose plate. To move forward, we will begin a copper assay Wednesday and finish it Thursday, and then streak out the primary integrant again on Thursday.

Wednesday, April 3, 2019

To Do:

1. ~~Prep for copper assay: make concentrations of copper, store in fridge, set up tubes, and streak plates.~~
2. ~~Help Jamie make glycerol stocks.~~

Results and Data:

Thursday, April 4, 2019

To Do:

1. ~~Begin copper assay.~~

Results and Data:

Copper Assay Protocol

1. Label 45 sterile culture tubes for copper dilution.
   - 0uM, 20uM, and 40uM dilutions of copper
   - 5 strains with triplicates for each (WT, dPmrA, dPmrA dPriM, dPriM, dPigR)
2. Resuspend plated cultures of each strain in s-MHB
3. Make 40 mL of 80uM and 40uM concentrations in sterile tubes by diluting 0.1M stock solution in supplemented MHB broth.
   - (80uM)(50mL)=(V)(0.1M) -> V=40 uL volume 80 uM and 20 uL for 40 uM
   - 80 and 40 because the concentration will be split in half when 3 mL of cell culture is added.
4. Use aseptic technique to add 3 mL of each MH/copper broth concentration to each tube.
5. For each strain, make a liquid culture by resuspending plated cultures in 2 mL sMHB in 2mL tubes.
6. Dilute 1:20 and take OD of suspended culture.
7. Use this to make a dilution so that the cultures will be 0.1 OD in 6 mL cultures.
8. Incubate at 37°C overnight and take ODs after 4 hours and 24 hours, using dilutions to take ODs when necessary

Tube Number	Concentration	Strain	OD600 Reading at 0 hours	OD600 Reading at 4 hours	OD600 Reading at 7 hours	OD600 Reading at 24 hours
1	0 uM	1-WT	0.103	0.360	0.620	
2	0 uM	1-WT	0.100	0.350	0.610	
3	0 uM	1-WT	0.094	0.338	0.608	
4	0 uM	2 – LVS dPmrA	0.087	0.245	0.464	
5	0 uM	2 – LVS dPmrA	0.082	0.261	0.490	
6	0 uM	2 – LVS dPmrA	0.090	0.271	0.512	
7	0 uM	3 – LVS dPriM	0.123	0.437	0.698	
8	0 uM	3 – LVS dPriM	0.122	0.421	0.722	
9	0 uM	3 – LVS dPriM	0.116	0.406	0.704	
10	0 uM	4 – LVS dPmrA dPriM	0.088	0.300	0.522	
11	0 uM	4 – LVS dPmrA dPriM	0.119	0.273	0.544	
12	0 uM	4 – LVS dPmrA dPriM	0.071	0.303	0.484	
13	0 uM	5 – LVS dPigR	0.088	0.315	0.602	
14	0 uM	5 – LVS dPigR	0.096	0.352	0.642	
15	0 uM	5 – LVS dPigR	0.095	0.335	0.626	
16	20 uM	1-WT	0.108	0.345	0.610	
17	20 uM	1-WT	0.070	0.380	0.672	

18	20 uM	1-WT	0.107	0.343	0.598	
19	20 uM	2 – LVS dPmrA	0.095	0.249	0.428	
20	20 uM	2 – LVS dPmrA	0.096	0.253	0.440	
21	20 uM	2 – LVS dPmrA	0.098	0.250	0.444	
22	20 uM	3 – LVS dPriM	0.127	0.398	0.694	
23	20 uM	3 – LVS dPriM	0.130	0.404	0.704	
24	20 uM	3 – LVS dPriM	0.128	0.399	0.708	
25	20 uM	4 – LVS dPmrA dPriM	0.105	0.301	0.508	
26	20 uM	4 – LVS dPmrA dPriM	0.100	0.290	0.494	
27	20 uM	4 – LVS dPmrA dPriM	0.103	0.299	0.506	
28	20 uM	5 – LVS dPigR	0.103	0.323	0.626	
29	20 uM	5 – LVS dPigR	0.115	0.354	0.672	
30	20 uM	5 – LVS dPigR	0.115	0.351	0.668	
31	40 uM	1-WT	0.115	0.353	0.612	
32	40 uM	1-WT	0.112	0.348	0.592	
33	40 uM	1-WT	0.112	0.350	0.598	
34	40 uM	2 – LVS dPmrA	0.097	0.223	0.352	
35	40 uM	2 – LVS dPmrA	0.091	0.214	0.322	
36	40 uM	2 – LVS dPmrA	0.103	0.237	0.364	
37	40 uM	3 – LVS dPriM	0.131	0.412	0.684	
38	40 uM	3 – LVS dPriM	0.127	0.399	0.662	
39	40 uM	3 – LVS dPriM	0.129	0.411	0.692	
40	40 uM	4 – LVS dPmrA dPriM	0.096	0.276	0.438	
41	40 uM	4 – LVS dPmrA dPriM	0.105	0.290	0.468	
42	40 uM	4 – LVS dPmrA dPriM	0.104	0.289	0.468	
43	40 uM	5 – LVS dPigR	0.110	0.352	0.638	
44	40 uM	5 – LVS dPigR	0.114	0.354	0.620	
45	40 uM	5 – LVS dPigR	0.082	0.376	0.670	

Friday, April 5, 2019

To Do:

1. ~~Take 24 hour copper assay results.~~
2. ~~Allelic exchange: plate 1° integrant onto homemade sucrose plates – pKR4 for Jamie.~~

Results and Data:

Allelic Exchange Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 200 uL 1X PBS (“John and Joe” stock)

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

-Plate 100 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate 100 μL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Jamie will plate pKL115 on Saturday since we plated pKR4 for her.

Monday, April 8, 2019

To Do:

1. ~~Lab meeting.~~
2. ~~Reviewed copper assay results with Joe.~~

Results and Data:

Tuesday, April 9, 2019

To Do:

1. ~~Allelic exchange – patch single colonies.~~
2. ~~DNA digest.~~

Results and Data:

Allelic Exchange Day 9 (maybe 10):

-Pick single colonies (cross-outs) from sucrose plates and patch onto CHAH using sticks, 8 – 16 (12) per 1° integrant.

~~-Optional:~~ cross-patch to CHAH-Kan. Note that it is common to pick up some background cells so some amount of kanamycin-resistant growth is not yet a concern. However, if there is robust kanamycin-resistant growth from all patches from a single 1° integrant on the next day, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Wednesday, April 10, 2019

To Do:

1. ~~Colony PCR.~~
2. ~~Joe will do PCR purification and digest.~~

Results and Data:

Allelic Exchange Day 10:

-Take small amounts of each cross-out patch using toothpicks and resuspend in sterile water.

-Heat samples at 95°C for 10' to lyse and kill cells

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. For controls, use LVS cells or LVS gDNA, the pEX-based allelic exchange vector, and water only. Example colony PCR (use a master mix):

Colony PCR Protocol

1. Take small amounts of each cross-out patch and resuspend in 50 uL of sterile water
2. Heat samples at 95ºC for 5' to lyse and kill cells.
3. Dilute samples 1:10 in sterile water.
4. Use 1-2 uL of heat-killed cells as template in colony PCR to check for desired mutation
   a. Controls = LVS cells, LVS gDNA, pEX-based allelic exchange factor, and water only.
      i. For LVS cells, follow steps 1-3 to prepare for PCR (same as cross-out colonies).

Total reaction volume (uL)	20		

Total number of reactions		5		
Component	**Stock concentration**	**Final concentration**	**1 rxn volume (uL)**	**Master Mix (uL)**
ddiH2O			4	72
KOD buffer	2x	1x	10	180
dNTPs	2 mM	0.4 mM	4	72
oligo F	10 uM	0.3 uM	0.6	10.8
oligo R	10 uM	0.3 uM	0.6	10.8
template	-	-	1.00	-
KOD	1 U/ul	0.02 U/ul	0.4	7.2
		Total volume	20.6	352.8

Tube Number	Lane on Gel	DNA	Primers	Purpose	Size
1	2	Lysate 1	P697 + P698	diagnostic	499 bp
2	3	Lysate 2	P697 + P698	diagnostic	
3	4	Lysate 3	P697 + P698	diagnostic	
4	5	Lysate 4	P697 + P698	diagnostic	
5	6	Lysate 5	P697 + P698	diagnostic	
6	7	Lysate 6	P697 + P698	diagnostic	
7	8	Lysate 7	P697 + P698	diagnostic	
8	9	Lysate 8	P697 + P698	diagnostic	
9	10	Lysate 10	P697 + P698	diagnostic	
10	11	Lysate 11	P697 + P698	diagnostic	
11	12	Lysate 12	P697 + P698	diagnostic	
12	13	LVS cells	P697 + P698	(+) control	
13	14	LVS gDNA	P697 + P698	(+) control	
14	15	pKL115 backbone	P697 + P698	(+) control	
15	16	water	P697 + P698	(-) control	

PCR Purification

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix.
   a. 1 volume of PCR reaction = 20 uL
2. Place a QIAquick column in a provided 2mL collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 seconds. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2mL collection tube for 3 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5mL microfuge tube.

7. To elute DNA, add 50uL 0.1x Buffer EB to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

DNA Digest

Tube	PCR Product	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	1	PvuII	15	-
2	2	PvuII	15	-
3	3	PvuII	15	-
4	4	PvuII	15	-
5	5	PvuII	15	-
6	6	PvuII	15	-
7	7	PvuII	15	-
8	8	PvuII	15	-
9	9	PvuII	15	-
10	10	PvuII	15	-
11	11	PvuII	15	-
12	12	PvuII	15	-
13	13	PvuII	3	12
14	14	PvuII	3	12

1. Set up master mix table:

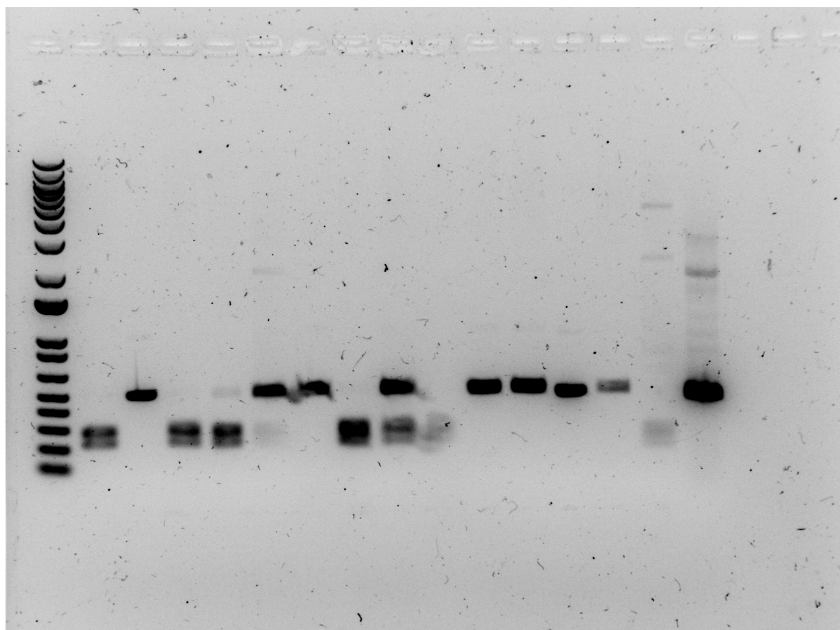
Components	**Volumes in 1 reaction (uL)**	**Volumes in Master Mix 3x (uL)**
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

*Cutsmart Buffer is used for all the "HF" enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

<u>From Joe's Lab Notebook:</u>

Gel image:



Notes:

- *It appears that lanes 2, 4, 5, and 8 (samples 1, 3, 4, and 7, respectively) contain DNA with the desired mutation*
- *Patches (mutants) 1, 3, and 7 were streaked for single colonies on CHAH plates*

Thursday, April 11, 2019

To Do:

1. ~~Start over with FipB mutant: begin with 100 uL PCR.~~

Results and Data:

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5.5
ddiH2O			20	110
KOD buffer	2x	1x	50	275
dNTPs	2 mM	0.4 mM	20	110
oligo F	10 uM	0.3 uM	3	16.5
oligo R	10 uM	0.3 uM	3	16.5
template	100 ng/ul	2 ng/ul	2	11
KOD	1 U/ul	0.02 U/ul	2	11
		Total volume	100	550

Reaction Number	Primers	Size
1	KROL139- KROL140	538
2	KROL141- KROL142	591
3	KROL15, KROL16	+ control
4	KROL15, KROL16	- Control

FipB Mutant 100 uL PCR

1. Acquire and label 4 PCR tubes with initials and designate as Tubes 1-4
    - The tubes comes in strips of 8 and they can be split into 4 tube pieces so that the first 3 tubes are used and the fourth is unused
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
    - ddi H2O in 1.5 mL microfuge tube
    - uL KOD buffer
    - dNTPs
    - oligo F
    - oligo R
    - template
    - Note: KOD enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
    - DO NOT vortex the KOD enzyme itself or any solution with KOD enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
    - The file is located in the Protocols folder
    - For this protocol, a "Total reaction volume" of 100 uL and 4 "Total number of reactions" were used – the following volumes are based on these specifications
7. Add 0.75 uL of each experiment specific primer (forward and reverse) to PCR Tubes 1 and 2 (oligos forward and reverse)
    - The amount added should be calculated by taking the total volume for 1 reaction (in worksheet) and subtracting the volumes for 1 reaction that have not yet been added to the master-mix
8. Add 0.75 uL of each control primer (oligos forward and reverse) to PCR Tubes 3 and 4
9. Add 0.5 uL ddi H2O to PCR Tube 4 so that all 4 PCR Tubes have an even amount of solution
    - Template volume for 1 reaction
10. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add 27.5 uL ddi H2O
    - Add 27.5 uL dNTPs
    - Add 68.75 uL KOD buffer
    - Add 2.75 uL KOD enzyme
11. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix

12. Add 23 uL of master-mix to PCR Tube 4
13. Add 2.25 uL template to Master Mix
    - Factor template volume minus 1 template reaction volume.
14. Add 23.5 uL master mix to each PCR Tube 1-3 and pipette up and down to mix (conserves tips)
15. Close PCR Tubes 1-4 until the caps are tight (push until the caps do not squeak when you push on them)
16. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes,
    - 94 degrees C for 20 seconds
    - 50 degrees C for 30 seconds
    - 68 degrees C for 45 seconds [modified because product is ~500 bp which is over 1 kbp] (KOD polymerase functions properly at 68 degrees C; TAC polymerase is different temp)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Friday, April 12, 2019

To Do:

1. ~~PCR purification.~~
2. ~~PCR digest.~~
3. ~~Run PCR on gel.~~

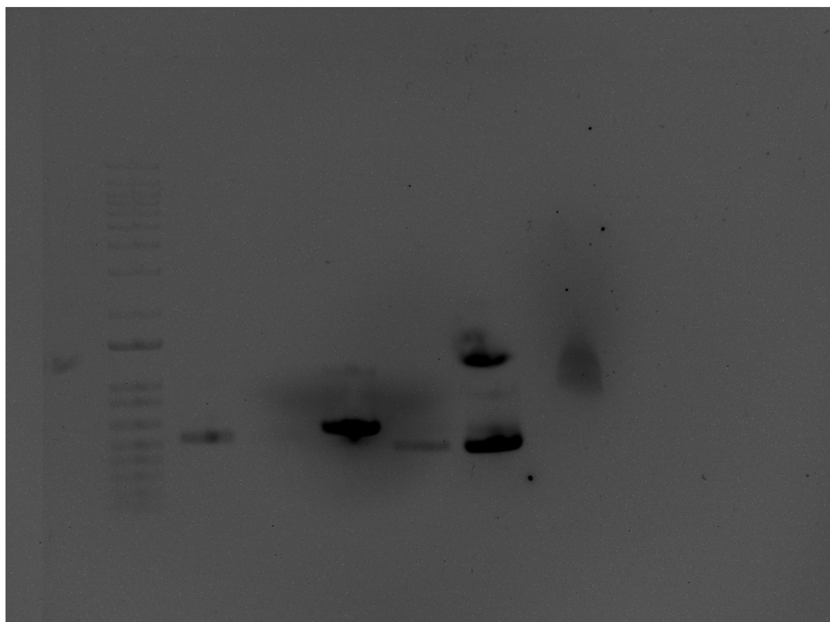
Results and Data:

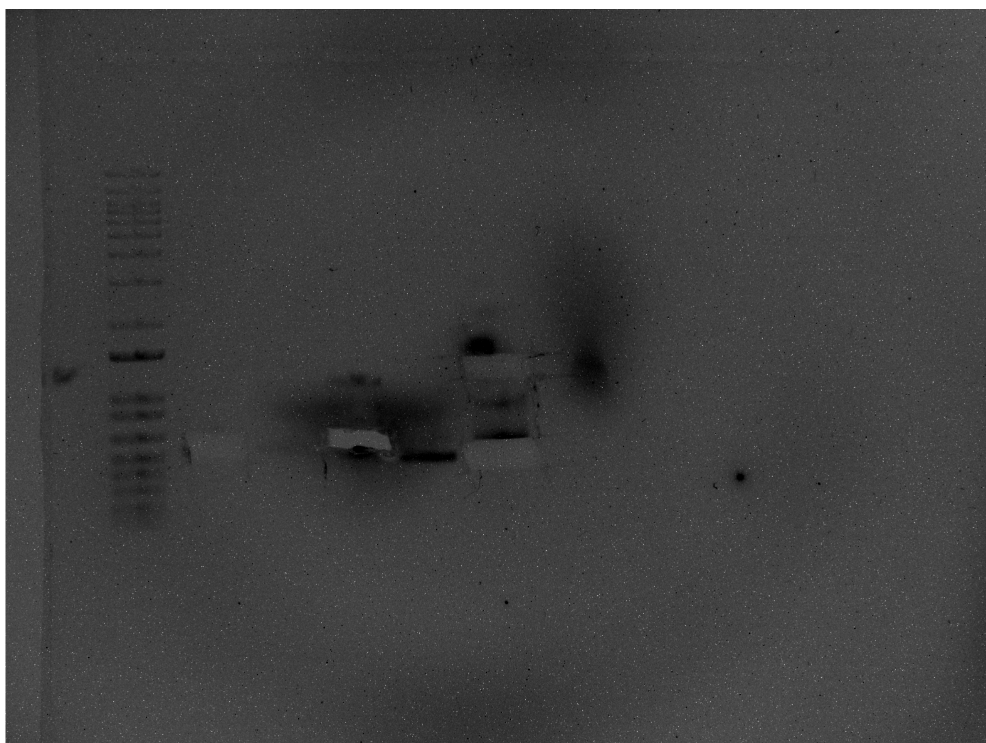
For purification, only the experimental tubes need to be purified. The positive and negative control do not.

After digesting tubes 1 and 2, run on a gel. Use the digested, purified plasmids and the positive and negative controls that were PCR'd.

Load 30 uL samples with 6 uL of dye (want to get as much of plasmid as possible)

Tube Number	Lane on Gel	Purpose	Expected Size
1	2	FR1	538
2	4	FR2	591
3	6	Positive Control	Positive Control
4	8	Negative Control	-
-	-	Digested pEX backbone	Already have from a previous gel





Images of gel before and after. Bands were small. The positive control was also cut out, but this is not necessary.

Monday, April 15, 2019

To Do:

1. ~~Lab meeting.~~
2. ~~Gel extraction.~~

Results and Data:

QIAquick Gel Extraction kit Protocol

1. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume gel (100 mg gel ~ 100 uL). The maximum amount of gel per spin column is 400 mg.
2. Incubate at 50º C for 10 minutes (or until the gel slice has completely dissolved). Vortex the tube every 2-3 minutes to help dissolve the gel.
3. Add 1 gel volume isopropanol to the sample and mix.
4. Place the QIAquick spin column in a provided 2 mL collection tube. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 uL, load and spin again.
5. Add 500 uL Buffer QG to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 750 uL Buffer PE to QIAquick column and centrifuge for 3 minutes. Discard the flow-through and place the QIAquick column back into the same tube.
7. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, add 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water to the center of the concentration, add 30 uL Buffer EB to the center of the QIAquick membrane and centrifuge for 1 min.

Ligations

Protocol by John Church

The purpose of this is to ligate the cut plasmid backbone to the strand of PCR DNA.

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

Tube	Insert	Backbone
1	digested, purified FR1, digested, purified FR1	digested, purified pEX backbone
2	-	digested, purified pEX backbone

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)
H_2O	11.5-4.0= 7.5	15.5
10x ligase buffer	2.0	2.0
Insert	4.0FR1+4.0FR2	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

3. Obtain ice to assemble and keep the reactions on. 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 PCR tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
6. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.

7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Place in the ~~thermocycler~~ cold room incubator overnight at 16°C.

Tuesday, April 16, 2019

To Do:

1. ~~Transform plasmid.~~

Results and Data:

Transform chemically competent E. coli cells

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL, 100 uL, and remaining culture. If transforming a ligation, plate 100 uL and remaining culture.
    a. Note: The plasmid used for the positive control plates should be the regular circular plasmid, not the digest/purified one.

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	Ligation rxn 1	Insert (FR1&FR2) + pEX plasmid	8 uL	100 ul, remaining	2
2	(+) control	pEX	1 uL	20 ul, 100 ul, remaining	3
3	(-) control	Insert + pKL115	0 uL	20 ul, 100 ul, remaining	3
				Total number of plates	8

2. Check to be sure you have enough plates with appropriate antibiotic. 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 (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates, spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

Wednesday, April 17, 2019

To Do:

1. ~~Overnight cultures.~~

Results and Data:

Overnight Cultures Protocol

By John Church

1. Add 30 mL of LB broth to 2 50 mL conicals.
2. Add 30 uL of kanamycin to each conical.
3. Label 4 test tubes for each reaction of new plasmids and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB media into each of the tubes.
5. Pick 4 colonies from each reaction and inoculate the tube.
    - Do this by using a stick and picking an isolated colony from plate
6. Place in the 37° shaking incubator overnight.

Tubes	Purpose
1-4	pKR31

Thursday, April 18, 2019

To Do:

1. ~~Miniprep pKR31.~~
2. ~~Nanodrop.~~

Results and Data:

QIAprep Spin Miniprep Kit Protocol

1. Pellet 5 mL bacterial overnight culture by centrifuging at 10000 x g for 1 minute at room temperature (15-25 deg C). Pipette 1.5 mL culture into the tube, spin, discard the supernatant, and repeat until all 5 mL are gone (3x).
2. Resuspend pelleted bacterial cells in 250 uL Buffer P1 (stored at 4 deg C fridge) and transfer to a microcentrifuge tube
3. Add 250 uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue, the solution will turn colorless.
5. Centrifuge for 10 minutes at 13,000 rpm (or max speed) in a tabletop microcentrifuge
6. Apply 800 uL supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30-60 seconds and discard the flow-through.
7. Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 seconds and discard the flow through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Wash with 0.75 mL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Repeat 2x more (updated protocol in hopes of removing sodium to prevent arcing).
10. Centrifuge for 3 minutes to remove residual wash buffer. Ethanol in DNA
11. Place the QIAprep 2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 0.1x 50 uL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 minute, and centrifuge for 1 minute.
12. Analyzing on a gel would show us how pure the chromosome is because supercoiled plasmid DNA moves faster.

Nanodrop:

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Tube 1	Default	4/18/2019	11:42 AM	832.45	16.649	8.833	1.88	2.35	50.00	230	7.099	-0.089
Tube 2	Default	4/18/2019	11:43 AM	946.47	18.929	10.059	1.88	2.36	50.00	230	8.029	-0.031
Tube 3	Default	4/18/2019	11:44 AM	907.53	18.151	9.570	1.90	2.35	50.00	230	7.720	-0.050
Tube 4	Default	4/18/2019	11:46 AM	1393.36	27.867	14.769	1.89	2.36	50.00	230	11.808	0.008

Wednesday, May 8, 2019

To Do:

1. ~~Streak out strains for characterization assay.~~
2. ~~PCR with primers P697 and P698.~~

Results and Data:

Total reaction volume	20
Total number of reactions	3

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	5
ddiH2O			4	~~20~~
KOD buffer	2x	1x	10	~~50~~
dNTPs	2 mM	0.4 mM	4	~~20~~
oligo F	10 uM	0.3 uM	0.6	~~3.0~~
oligo R	10 uM	0.3 uM	0.6	~~3.0~~
Template	100 ng/ul	2 ng/ul	~~0.4~~	2.0
KOD	1 U/ul	0.02 U/ul	0.4	~~2.0~~
		Total volume	20	100

Tube Number	Template	Purpose	Primers
1	LVS cells	Positive control	P697 + P698
2	LVS gDNA	Positive control	P697 + P698
3	water	Negative control	P697 + P698

Thursday, May 9, 2019

To Do:

1. ~~Run PCR from yesterday on gel.~~
2. ~~Growth curve assay to characterize strains.~~

Results and Data:

Note: This was done with Joe, so any missing data may be in his notebook.

Growth Curve Assay

1. Scrape up overnight cultures and resuspend in 500uL supplemented MHB.
2. Add 50uL of cells to 950uL of MHB in a cuvette to find the OD of the cultures.
3. Prepare 8mL of s-MHB in each sterile test tube.
4. Aliquot cells into sterile tubes so that an 8mL culture has an OD of 0.1.
5. Add 1mL of the culture to a cuvette to record the true initial OD. (7mL should remain)
6. Place tubes in shaker at 37ºC and record OD readings at 2, 4.5, 7, and 24-hour time points.

Tube Number	Cuvette Number	Strain	OD600 at 0 hours	OD600 at 2 hours	OD600 at 4.5 hours	OD600 at 7 hours	OD600 at 22.5 hours
1-1	1	KRLVS 11.1	0.095	0.175	0.360	0.545x	1.96
1-2	2	KRLVS 11.1	0.087	0.162	0.345	0.515	1.86
1-3	3	KRLVS 11.1	0.092	0.170	0.360	0.500	1.86

2-1	4	KRLVS 11.2	0.099	0.181	0.350	0.535	1.90
2-2	5	KRLVS 11.2	0.099	0.177	0.350	0.550	1.92
2-3	6	KRLVS 11.2	0.097	0.174	0.340	0.510	1.84
3-1	7	KRLVS 13.1	0.083	0.154	0.315	0.460	2.16
3-2	8	KRLVS 13.1	0.125	0.155	0.315	0.470	2.24
3-3	9	KRLVS 13.1	0.086	0.158	0.315	0.460	2.32
4-1	10	KRLVS18.1	0.100	0.201	0.405	0.620	2.02
4-2	11	KRLVS18.1	0.122	0.236	0.510	0.720	1.88
4-3	12	KRLVS18.1	0.107	0.207	0.410	0.620	1.94
5-1	13	KRLVS 18.2	0.093	0.182	0.380	0.605	2.26
5-2	14	KRLVS 18.2	0.090	0.184	0.380	0.600	2.00
5-3	15	KRLVS 18.2	0.090	0.178	0.365	0.590	1.92
6-1	16	LVS	0.084	0.140	0.270	0.325	2.00
6-2	17	LVS	0.085	0.138	0.265	0.395	2.20
6-3	18	LVS	0.081	0.135	0.260	0.410	2.32
7-1	19	ΔpmrA	0.094	0.154	0.300	0.440	2.30
7-2	20	ΔpmrA	0.093	0.166	0.310	0.485	1.88
7-3	21	ΔpmrA	0.099	0.166	0.315	0.485	1.94

Thursday, May 9, 2019

To Do:

1. ~~Take 24 hour timepoint for growth curve.~~
2. ~~DNA extraction of KRLVS 11.1, 11.2, 13.1, 18.1, and 18.2 using MasterPure Complete DNA and RNA Purification Kit.~~

Results and Data:

I started the MasterPure Purification Kit, Joe finished it.

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.