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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.

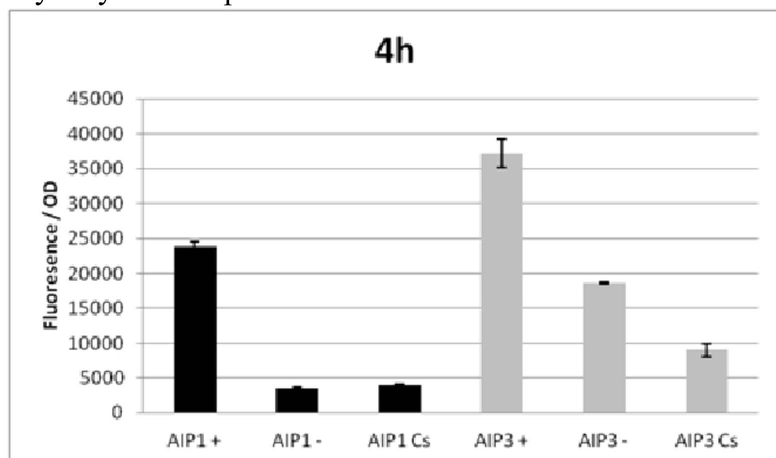


Figure 1

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).

September 2019

Friday, September 13, 2019

To Do:

1. ~~Start on pKR31 (FipB deletion): 100uL PCR~~

Results and Data:

PCR Protocol (updated 7/1/19)

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
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:
   - Molecular grade H2O in 1.5 mL microfuge tube
   - KOD/primestar buffer
   - dNTPs
   - oligo F (10uM)
   - oligo R (10uM)
   - template (eg. LVS gDNA, plasmid, colony, etc.)
   - Note: KOD/primestar 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 enzyme itself or any solution with 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
   - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi H2O to negative control tube
   - Template volume for 1 reaction
9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
   - Add ddi H2O
   - Add dNTPs
   - Add KOD buffer
   - Add KOD enzyme
10. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add 2ate to Master Mix
    - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight

15. 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/kb (adjust based on expected size of product) [left at 40s]
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	pKR31 FR1	LVS gDNA	KROL139, KROL140	538
2	pKR31 FR2	LVS gDNA	KROL141, KROL142	591
3	+ control	LVS gDNA	KROL15, KROL16	441
4	- control	-	KROL15, KROL16	-

Total reaction volume	100
Total number of reactions	4

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	5
ddiH2O			62	310
PrimeSTAR GXL Buffer	5x	1x	20	100
dNTPs	2.5 mM	0.2 mM	8	40
oligo F	10 uM	0.3 uM	3	15
oligo R	10 uM	0.3 uM	3	15
template	100 ng/ul	2 ng/ul	2	10
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	10
		Total volume	100	500

PCR was removed from thermocycler and placed in the Undergraduate Researcher's box in the freezer for storage.

Monday, September 16, 2019

To Do:

1. ~~Autoclave and remove trash.~~
2. ~~Continue pKR31 (FipB deletion): PCR Purification.~~
3. ~~PCR Digest.~~

Results and Data:

Positive and negative controls do not need to be purified.

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

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR31 FR1	NotI, BamHI	15	-
2	pKR31 FR2	NotI, KpnI	15	-
3	pEX backbone	KpnI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 4x (uL)
H_2O	10.8	43.2
10x Buffer*	3.0	12.0
DNA	(15.0)	-
Enzyme 1	(0.6)	2.4
Enzyme 2	(0.6)	2.4
Total	30.0 (13.8 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 (___uL) and 10x buffer (_____uL) to master mix tube (MM).
4. Add indicated amount of DNA (___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 (___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 (____ul).
8. Incubate at 37°C for 1 hour or up to overnight.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation)

Tuesday, September 17, 2019

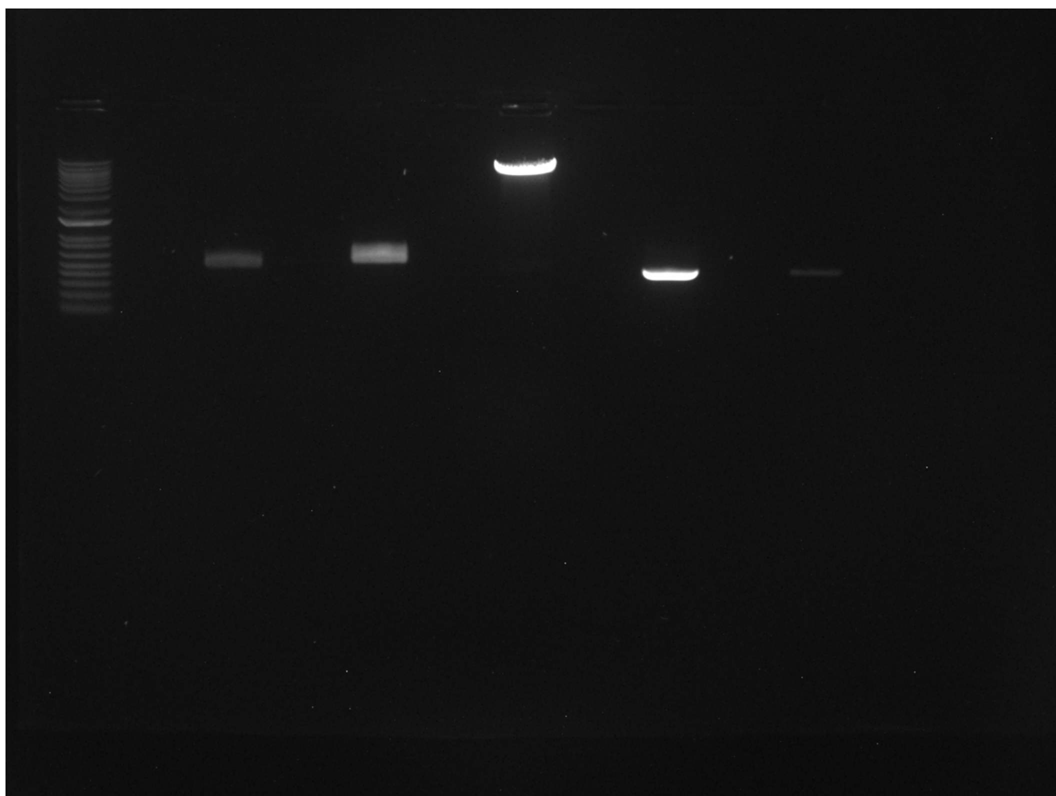
To Do:

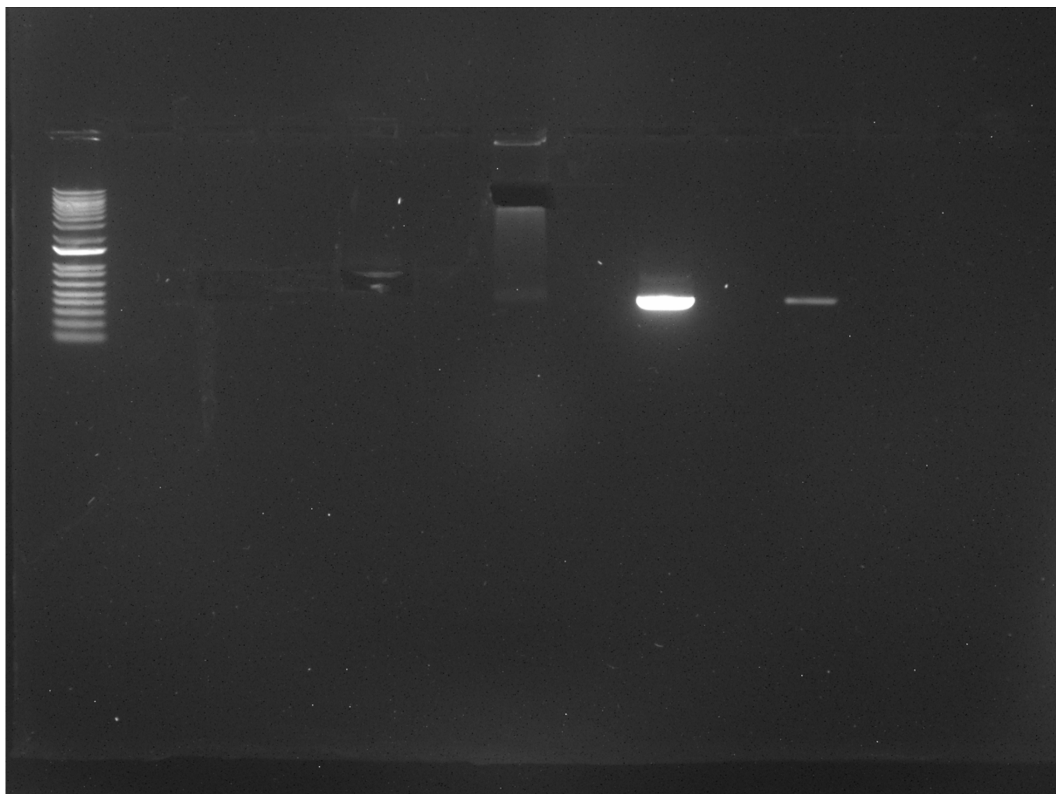
1. ~~CIPP backbone (step 9 from yesterday).~~
2. ~~Run PCR on a gel for digest purification.~~

Results and Data:

30uL samples with 6uL dye.

Tube Number	Lane on Gel	Purpose	Expected Size
1	3	pKR31 FR1	538
2	5	pKR31 FR2	591
3	7	pEX backbone	
4	9	Positive (+) control	Positive Control
5	11	(-) Negative control	Negative Control





Wednesday, September 18, 2019

To Do:

1. ~~Gel extraction and Ligation.~~

Results and Data:

Mass of Empty Microfuge Tube	1.0g	Volume of QB to Add
Total Mass of PCR Tube 1	1.3g	900 uL
Total Mass of PCR Tube 2	1.4g	1200 uL
Total Mass of Plasmid Tube	1.3g	900 uL

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 16ºC.

Thursday, September 19, 2019

To Do:

~~1. Transformation.~~

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 (old cells)	Insert (FR1&FR2) + pEX plasmid	8 uL	100 ul, remaining	2
2	(+) control (old cells)	pKR11	0.5 uL	20 ul, 100 ul, remaining	3
3	(+) control (new cells)	pKR11	0.5 uL	20 ul, 100 ul, remaining	3
4	Plasmid control (should not survive) (new cells)	Digested, purified pEX	1 uL	20 ul, 100 ul, remaining	3
5	(-) control (new cells)	-	0 uL	20 ul, 100 ul, remaining	3
				Total number of plates	14

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.

Note: In the future, the plasmid control with digested, purified plasmid should include 8uL of DNA like the flanking regions and backbone did.

Friday, September 20, 2019

To Do:

1. ~~Check plates.~~

Results and Data:

Plates look as they should. No growth on negative control and good growth on ligations. Overnight cultures will be done next week.

Monday, September 23, 2019

To Do:

1. ~~Overnight cultures.~~

Results and Data:

Overnight Cultures Protocol

By John Church

1. Add ~~45~~ 25 mL of LB broth to a sterile 50 mL conical ~~(makes enough for 8 tubes: 9*5=45)~~ (for 4 tubes)
2. Add ~~45~~ 25 uL of kanamycin (Kan50) to the conical.

3. Label 4 glass sterile test tubes for each new plasmid and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB + Kan 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. ~16-22 hours

Tubes	Purpose
1-2	pKR31 100 uL
3-4	pKR31 remaining

Tuesday, September 24, 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.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
pKR31 1	Default	9/24/2019	9:45 AM	126.56	2.531	1.308	1.93	2.34	50.00	230	1.082	-0.001
pKR31 2	Default	9/24/2019	9:46 AM	177.19	3.544	1.908	1.86	1.82	50.00	230	1.943	0.372
pKR31 3	Default	9/24/2019	9:48 AM	184.78	3.696	1.926	1.92	2.34	50.00	230	1.580	0.001
pKR31 4	Default	9/24/2019	9:49 AM	153.90	3.078	1.580	1.95	2.36	50.00	230	1.305	0.003

Wednesday, September 25, 2019

To Do:

1. ~~Set up sequencing submission worksheet.~~

Results and Data:

Thursday, September 26, 2019

To Do:

1. ~~Sequencing for pKR31.~~

Results and Data:

Sequencing was done before 10am and the plasmid tubes were placed back into the Undergraduate Researchers box (labeled 1, 2, 3, and 4 with pKR31).

Friday, September 27, 2019

To Do:

1. ~~Check sequencing results of pKR31.~~

Results and Data:

Monday, September 30, 2019

To Do:

1. ~~Streak out 2 plates of dPmrA LVS for allelic exchange.~~

Results and Data:

2 plates were struck out: one is older cells, one is new cells.
Placed in 37 degree incubator overnight.

October 2019

Tuesday, October 1, 2019

To Do:

1. ~~Day 2 of Allelic Exhange: scrape up plates and make electrocompetent cells.~~

Results and Data:

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-5x in 10% sucrose *by pelleting at 10,000rpm for 3 minutes and resuspending*

-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

The tubes were placed in the -80 in the MRamsey lab. The tubes labeled with a 1 are older and the tubes labeled with a 2 are from a new tube of LVS.

Friday, October 4, 2019

To Do:

1. ~~Day 2 of Allelic Exhange: electroporations.~~

Results and Data:

3 electroporations, 1 control

Tube	Purpose
1	Plasmid
2	Plasmid
3	Plasmid
4	Control

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C *(get this done first and quick)*

-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 (*Arc on first EP, none on others)*

GenePulser in Nelson Lab: Switch on back right, 4-Preset Protocols, 1-Bacteria, 2-E coli 2mm, 2.5kV, insert cuvetter with knob in groove, pulse

-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

Use 1000uL pipette to take 1mL of MHB and put into cuvette, mix, and place back into tube

-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)

Monday, October 7, 2019

To Do:

1. ~~Day 5 of Allelic Exhange: check plates.~~
2. ~~Scrape out LVS and dPmrA plates to make more EC cells tomorrow.~~

Results and Data:

One of the plates from EP1 had lots of spreading contamination so it was thrown away. The other plates do not have clear single colonies yet so they were placed back in the incubator for another night. The controls had no growth as well.

Tuesday, October 8, 2019

To Do:

1. ~~Day 6 of Allelic Exhange: picking single colonies.~~
2. ~~Make EC cells from LVS and dPmrA plates.~~
3. ~~Electroporations.~~

Results and Data:

There was still no growth on the EP plates so they were placed back in the incubator for one more day and more EPs were set up.

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-5x in 10% sucrose *by pelleting at 10,000rpm for 3 minutes and resuspending*

-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

Tube	Purpose
1	Plasmid
2	Plasmid
3	Plasmid
4	Control

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot ~~4 mL~~ 7 mL MHB into glass test tubes for recovery, warm in shaker at 37°C *(get this done first and quick)*

-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 (*Arc on first EP, none on others)*

GenePulser in Nelson Lab: Switch on back right, 4-Preset Protocols, 1-Bacteria, 2-E coli 2mm, 2.5kV, insert cuvetter with knob in groove, pulse

-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

Use 1000uL pipette to take 1mL of MHB and put into cuvette, mix, and place back into tube

-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)

Wednesday, October 9, 2019

To Do:

1. ~~Plasmid design with Jamie for pKR38.~~

Results and Data:

Mutation changes phenylalanine to a leucine. Affects ABC transporter. KROL 266, 267, 268, and 269.

Thursday, October 10, 2019

To Do:

1. ~~Check electroporation plates.~~

Results and Data:

Electroporations all have considerable growth that looks like *Francisella* but grows faster. A CHA-Kan plate was struck out with 6 single colonies to check growth.

Friday, October 11, 2019

To Do:

1. ~~Check EP plates.~~
2. ~~Fill ddi H2O carboy.~~
3. ~~Autoclave plate waste.~~

Results and Data:

EP plates confirmed to have something other than Francisella.

Tuesday, October 15, 2019

To Do:

1. ~~Reconstitute new primers.~~
2. ~~20 uL PCR.~~

Results and Data:

To reconstitute new primers, centrifuge, add 10*nmol of 0.1x EB to tube, and dilute 1:10 (30uL primer, 270 uL H2O) before transferring to 1.5 mL tube.

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	pKR58 FR1	LVS gDNA	KROL266, KROL267	863

2	pKR58 FR2	LVS gDNA	KROL268, KROL269	1176
3	Check primers	LVS gDNA	KROL270, KROL271	1300
4	+ control	LVS gDNA	KROL15, KROL16	441
5	- control	-	KROL15, KROL16	-

Total reaction volume	20
Total number of reactions	4

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5
ddiH2O			12.4	62
PrimeSTAR GXL Buffer	5x	1x	4	20
dNTPs	2.5 mM	0.2 mM	1.6	8
oligo F	10 uM	0.3 uM	0.6	3
oligo R	10 uM	0.3 uM	0.6	3
template	100 ng/ul	2 ng/ul	0.4	2
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
		Total volume	20	100

PCR Protocol (updated 7/1/19)

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
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:
   - Molecular grade H2O in 1.5 mL microfuge tube
   - KOD/primestar buffer
   - dNTPs
   - oligo F (10uM)
   - oligo R (10uM)
   - template (eg. LVS gDNA, plasmid, colony, etc.)
   - Note: KOD/primestar 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 enzyme itself or any solution with 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
   - Also setup table below to specify which primers and source DNA will be used

7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi H2O to negative control tube
    - Template volume for 1 reaction
9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add ddi H2O
    - Add dNTPs
    - Add KOD buffer
    - Add KOD enzyme
10. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
    - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. 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/kb (adjust based on expected size of product) [1min 30s]
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Wednesday, October 16, 2019

To Do:

1. ~~Run gel from 20 uL PCR.~~
2. ~~100 uL PCR.~~

Results and Data:

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.

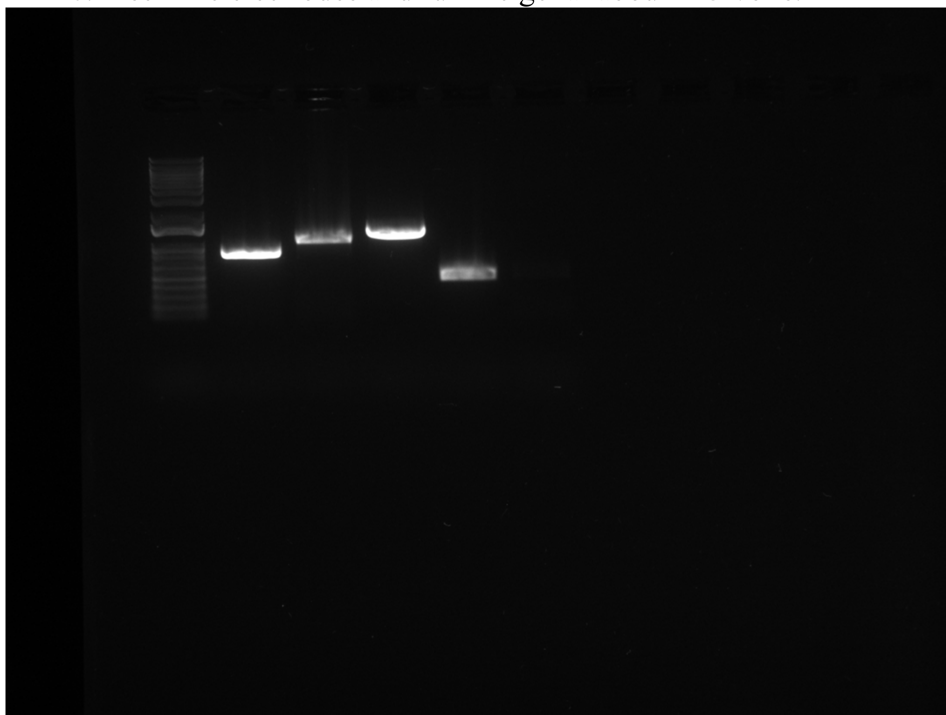
    - To make 1 L of 1x TAE, add 40 mL 25x TAE into 1 L graduated cylinder and fill to 1 L with ddiH2O.

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.



Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	pKR58 FR1	LVS gDNA	KROL266, KROL267	863
2	pKR58 FR2	LVS gDNA	KROL268, KROL269	1176
3	+ control	LVS gDNA	KROL15, KROL16	441
4	- control	-	KROL15, KROL16	-

Total reaction volume	100
Total number of reactions	4

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	5
ddiH2O			62	310
PrimeSTAR GXL Buffer	5x	1x	20	100
dNTPs	2.5 mM	0.2 mM	8	40
oligo F	10 uM	0.3 uM	3	15
oligo R	10 uM	0.3 uM	3	15
template	100 ng/ul	2 ng/ul	2	10
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	10
		Total volume	100	500

PCR Protocol (updated 7/1/19)

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
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:
   - Molecular grade H2O in 1.5 mL microfuge tube
   - KOD/primestar buffer
   - dNTPs
   - oligo F (10uM)
   - oligo R (10uM)
   - template (eg. LVS gDNA, plasmid, colony, etc.)
   - Note: KOD/primestar 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 enzyme itself or any solution with 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
   - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi H2O to negative control tube
   - Template volume for 1 reaction
9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
   - Add ddi H2O
   - Add dNTPs
   - Add KOD buffer
   - Add KOD enzyme

10. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
    - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. 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/kb (adjust based on expected size of product) [1min 30s]
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Friday, October 18, 2019

To Do:

1. ~~PCR purification.~~
2. ~~PCR round B.~~

Results and Data:

Purify PCR, combine samples, dilute 1:10 and use this as template for another PCR (round B). Use start and end primers (A and D).

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.

Total reaction volume	100
Total number of reactions	3

Factor

Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH2O			62	248
PrimeSTAR GXL Buffer	5x	1x	20	80
dNTPs	2.5 mM	0.2 mM	8	32
oligo F	10 uM	0.3 uM	3	12
oligo R	10 uM	0.3 uM	3	12
template	100 ng/ul	2 ng/ul	2	8
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	8
		Total volume	100	400

PCR Protocol (updated 7/1/19)

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
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:
    - Molecular grade H2O in 1.5 mL microfuge tube
    - KOD/primestar buffer
    - dNTPs
    - oligo F (10uM)
    - oligo R (10uM)
    - template (eg. LVS gDNA, plasmid, colony, etc.)
    - Note: KOD/primestar 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 enzyme itself or any solution with 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
    - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi H2O to negative control tube
    - Template volume for 1 reaction
9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add ddi H2O
    - Add dNTPs
    - Add KOD buffer

- Add KOD enzyme

10. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
    - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. 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/kb (adjust based on expected size of product)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	PCR product	LVS gDNA	KROL266, KROL269	2018
2	+ control	LVS gDNA	KROL15, KROL16	441
3	- control	-	KROL15, KROL16	-

Monday, October 21, 2019

To Do:

1. ~~Purify PCR.~~
2. ~~Digest PCR.~~
3. ~~Run gel for PCR.~~

Results and Data:

Positive and negative controls do not need to be purified.

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

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	P-pKR58	KpnI, BamHI	15	-
2	pEX backbone	KpnI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
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.

3. Add indicated amounts of H_2O (___uL) and 10x buffer (_____uL) to master mix tube (MM).
4. Add indicated amount of DNA (___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 (___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 (____ul).
8. Incubate at 37°C for 1 hour or up to overnight.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation)

Tuesday, October 22, 2019

To Do:

1. ~~CIP backbone.~~
2. ~~Run on gel and cut.~~

Results and Data:

CIP backbone (step 9 from yesterday).

30uL samples with 6uL dye.

Number	Lane on Gel	Purpose	Expected Size
1	3	pKR58	591
2	5	pEX backbone	6726

3	7	Round A Positive (+) control	441
4	8	Round A (-) Negative control	Negative Control
5	9	Round B Positive Control	441
6	10	Round B Negative Control	Negative Control

Agarose Gel Protocol – written by Joe Paquette

(Note: all ddiH2O is type I)

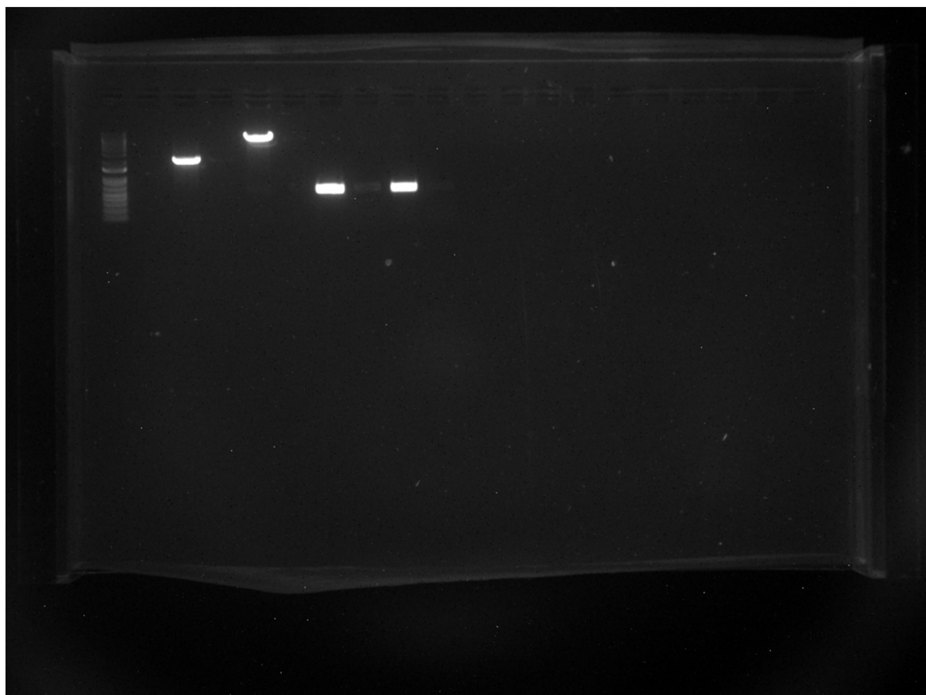
17. Mix 25x TAE buffer with ddiH_2O to obtain 1x TAE solution.

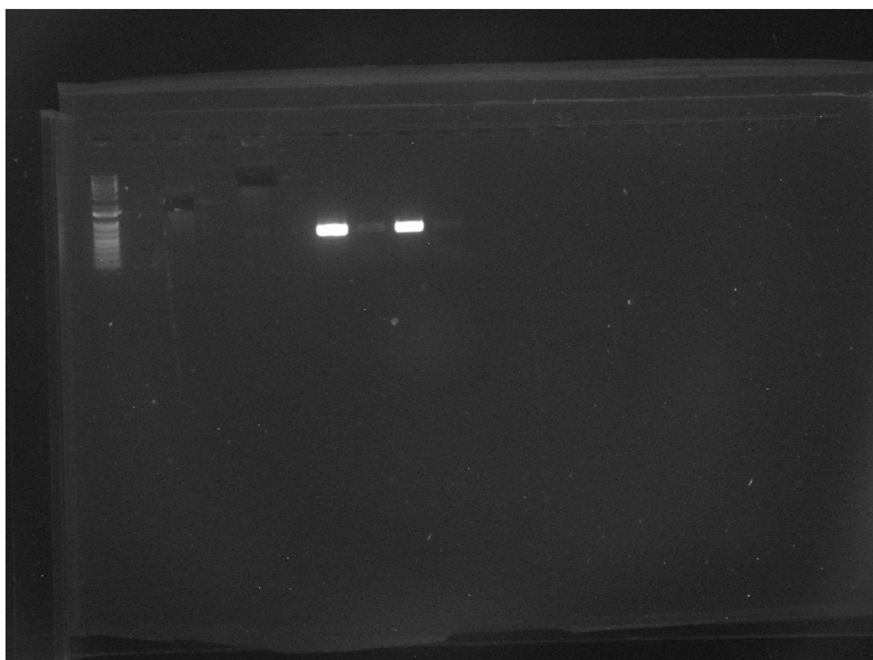
    - To make 1 L of 1x TAE, add 40 mL 25x TAE into 1 L graduated cylinder and fill to 1 L with ddiH2O.

18. Add 1 g agarose powder to 100 mL 1x TAE buffer in 250 mL.
19. Add stirbar to container.
20. Heat to dissolve the agarose while stirring (don't let over boil, should look like clear liquid, no solids).
21. 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
22. Let the 1% agarose solution cool to approximately 50-55°C.
23. Apply autoclave tape to the edges of the gel cast (ensure the tape is tightly bound).
24. Pour 1% agarose - 1x SYBR safe solution into the cast and insert a comb to mold wells in the gel.
25. Let sit until the 1% agarose – 1x SYBR safe solution has cooled and solidified into a gel.
26. Carefully remove the comb.
27. Pour the 100 ml of 1x TAE buffer solution into the gel tank (add just enough to slightly submerge the gel itself).
28. Obtain 5 µL of each PCR sample.
29. 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

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





Wednesday, October 23, 2019

To Do:

1. ~~Gel extraction.~~
2. ~~Ligation.~~

Results and Data:

Mass of Empty Microfuge Tube	1.0g	Volume of QB to Add
Total Mass of PCR Tube 1	1.3g	900 uL
Total Mass of Plasmid Tube	1.3g	900 uL

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	8.0	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

3. Obtain ice to assemble and keep the reactions on. 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 16ºC.

Thursday, October 24, 2019

To Do:

1. ~~Transformations.~~

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 + pEX plasmid	8 uL	100 ul, remaining	2
2	(+) control	pKR11	0.5 uL	20 ul, 100 ul, remaining	3
3	(-) control	None	-	20 ul, 100 ul, remaining	3
4	Plasmid control (should not survive)	Digested, purified pEX	8 uL	20 ul, 100 ul, remaining	3
				Total number of plates	11

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.

Friday, October 25, 2019

To Do:

1. ~~Check transformations and wrap to be placed in cold room over the weekend.~~

Results and Data:

CHA-Kan plates were used erroneously, so there was significant growth on the plates and single colonies cannot be picked so another transformation will be done on Monday.

Monday, October 28, 2019

To Do:

1. ~~Transform chemically competent E. coli cells.~~
2. ~~Poor LB-Kan plates.~~

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 + pEX plasmid	8 uL	100 ul, remaining	2
2	(+) control	pKR41	0.5 uL	20 ul, 100 ul	2
3	(-) control	None	-	20 ul, 100 ul	2
4	Plasmid control (should not survive)	Digested, purified pEX	8 uL	100 ul, remaining	2
				Total number of plates	8

2. Check to be sure you have enough LB-Kan 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.

Tuesday, October 29, 2019

To Do:

1. ~~Redo ligation.~~

Results and Data:

Plates had growth on digested, purified pEX, but not on the ligation reaction plate so ligations will be redone.

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	8.0	-
Backbone	2.0	2.0
Ligase	0.5	0.5
TOTAL	20.0	20.0

3. Obtain ice to assemble and keep the reactions on. 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 16ºC.

Wednesday, October 30, 2019

To Do:

1. ~~Transformations.~~

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 + pEX plasmid	8 uL	100 ul, remaining	2
2	(+) control	pKR41	0.5 uL	20 ul, 100 ul	2
3	(-) control	None	-	20 ul, 100 ul	2
4	Plasmid control (should not survive)	Digested, purified pEX	8 uL	100 ul, remaining	2
				Total number of plates	8

2. Check to be sure you have enough LB-Kan 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.

Thursday, October 31, 2019

To Do:

1. ~~Overnight cultures.~~

Results and Data:

The ligation reaction worked and the rest of the plates look as they should.

Overnight Cultures Protocol

By John Church

1. Add ~~45~~ 25 mL of LB broth to a sterile 50 mL conical (makes enough for 8 tubes: 9*5=45)
2. Add ~~45~~ 25 uL of kanamycin (Kan50) to the conical.
3. Label 4 glass sterile test tubes for each new plasmid and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB + Kan 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. ~16-22 hours

November

Friday, November 1, 2019

To Do:

1. ~~Miniprep pKR58.~~
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.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
pKR58_1	Default	11/1/2019	10:52 AM	693.99	13.880	7.376	1.88	2.35	50.00	230	5.902	0.026
pKR58_2	Default	11/1/2019	10:53 AM	594.77	11.895	6.581	1.81	2.30	50.00	230	5.171	0.019
pKR58_3	Default	11/1/2019	10:54 AM	578.53	11.571	6.404	1.81	2.30	50.00	230	5.030	0.014
pKR58_4	Default	11/1/2019	10:55 AM	575.45	11.509	6.374	1.81	2.30	50.00	230	5.004	-0.005

Monday, November 4, 2019

To Do:

1. ~~Sequence pKR58.~~

Results and Data:

See 191104_JTC.xlsx

This sequencing was withdrawn because more sequencing primers are needed.

Wednesday, November 6, 2019

To Do:

1. ~~Sequencing for pKR58.~~

Results and Data:

See 191106_SequencingSubmission.xlsx

Thursday, November 7, 2019

To Do:

1. ~~Make EC cells.~~

Results and Data:

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)

-Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL

-Spin for 3 minutes at 10,000 rpm

-remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose

-Repeat 3x-5x in 10% sucrose

-After final spin, remove all supernatant.

-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.

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

Note: There are now 6 tubes of LVS and 5 of dPmrA in the EC cells box.

Friday, November 8, 2019

To Do:

1. ~~Check sequencing.~~

Results and Data:

The sequencing was checked and Tube 1 was thrown out while 2, 3, and 4 were kept. They were combined into one tube labeled pKR58 and placed in the plasmids working stock box. 5uL was also put into a tube and placed in the permanent stock in the -80.

Tuesday, November 12, 2019

To Do:

1. ~~Electroporations into LVS.~~

Results and Data:

*Electroporate plasmid into EC cells** (about 1 hour)

-For each electroporation, aliquot 4-7 mL supplemented 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

-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)

*Always including a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

Tube	Purpose
1	LVS Plasmid
2	LVS Plasmid
3	LVS Plasmid
4	LVS Control

Went into the incubator at 9:45am. Tube 2 arced and had a low time, the rest were fine (>3).

Wednesday, November 13, 2019

To Do:

1. ~~Check EP plates.~~
2. ~~Work on presentation.~~

Results and Data:

Thursday, November 14, 2019

To Do:

3. ~~Electroporations in dPmrA.~~

Results and Data:

*Electroporate plasmid into EC cells** (about 1 hour)

-For each electroporation, aliquot 4-7 mL supplemented 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 (Bacteria: EC2)

-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

-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)

*Always including a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

Tube	Purpose
1	dPmrA Plasmid
2	dPmrA Plasmid
3	dPmrA Plasmid
4	dPmrA Control

Tube 2 arced and had a low time, the rest did not.

Friday, November 15, 2019

To Do:

1. ~~Pick colonies from LVS EP.~~
2. ~~Check dPmrA EP.~~

Results and Data:

Day 5 (maybe 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).
This plate was left out for the weekend.

The dPmrA cells appear to have some sort of contamination on them, but there is no growth on the negative controls.

Monday, November 18, 2019

To Do:

1. ~~Freeze down 1° integrants.~~
2. ~~Streak out 1° integrants for sucrose selection since plates are not ready today.~~
3. ~~Streak out EC cells to check if there is contamination.~~

Results and Data:

EC cells from 11.7.19.

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.

Tuesday, November 19, 2019

To Do:

1. ~~Electroporations into dPmrA.~~

Results and Data:

Tube	Purpose
1	dPmrA Control
2	dPmrA Plasmid
3	dPmrA Plasmid

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot ~~4~~ 7 mL MHB into glass test tubes for recovery, warm in shaker at 37°C *(get this done first and quick)*

-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 (*Arc on first EP, none on others)*

GenePulser in Nelson Lab: Switch on back right, 4-Preset Protocols, 1-Bacteria, 2-E coli 2mm, 2.5kV, insert cuvetter with knob in groove, pulse

-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

Use 1000uL pipette to take 1mL of MHB and put into cuvette, mix, and place back into tube

-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)

Bacteria: EC2

Wednesday, November 20, 2019

To Do:

1. ~~Check EP plates.~~

Results and Data:

Thursday, November 21, 2019

To Do:

1. ~~Electroporations into dPmrA.~~

Results and Data:

Tube	Purpose
1	dPmrA Plasmid
2	dPmrA Plasmid
3	dPmrA Plasmid
4	dPmrA Control

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot ~~4~~ 7 mL MHB into glass test tubes for recovery, warm in shaker at 37°C *(get this done first and quick)*
-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 (*Arc on first EP, none on others)*
GenePulser in Nelson Lab: Switch on back right, 4-Preset Protocols, 1-Bacteria, 2-E coli 2mm, 2.5kV, insert cuvetter with knob in groove, pulse
-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
Use 1000uL pipette to take 1mL of MHB and put into cuvette, mix, and place back into tube
-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)

Bacteria: EC2

Friday, November 22, 2019

To Do:

1. ~~Check EP plates.~~

Results and Data:

No growth yet.

Monday, November 25, 2019

To Do:

1. ~~Check EP plates.~~

Results and Data:

The first set of plates (11.19.19) had no growth and were thrown out. The second set (11.21.19) had one single colony which was patched onto CHA-Kan and placed into the incubator with the rest of the plates.

Tuesday, November 26, 2019

To Do:

1. ~~Freeze down single colony of dPmrA.~~
2. ~~Count colonies for Jamie.~~

Results and Data:

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.

Monday, December 2, 2019

To Do:

1. ~~Streak out LVS 1° integrants for sucrose selection.~~

Results and Data:

Streak onto REGULAR CHA plates.

Tuesday, December 3, 2019

To Do:

1. ~~Sucrose selection on LVS pKR58.~~

Results and Data:

Used 300ul of PBS to resuspend cells.

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.

Wednesday, December 4, 2019
To Do:

1. ~~Streak out 2 plates of dPmrA for making EC cells.~~

Results and Data:

Thursday, December 5, 2019
To Do:

1. ~~Make EC cells.~~
2. ~~Check plates for growth.~~

Results and Data:
Prepare electrocompetent (EC) cells
-Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
-Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
-Spin for 3 minutes at 10,000 rpm
-remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
-Repeat 3x-5x in 10% sucrose
-After final spin, remove all supernatant.
-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
-For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Friday, December 6, 2019
To Do:

1. ~~Electroporations.~~
2. ~~Cross patch sucrose selection.~~

Results and Data:
Strain 1 of pKR58 in LVS did not work. Strain 2 did because there were colonies on the 10^-7 control plate but none on the sucrose 10^-7 plate.

Tube	Purpose
1	dPmrA Plasmid
2	dPmrA Plasmid
3	dPmrA Control

*Electroporate plasmid into EC cells**
-For each electroporation, aliquot ~~4~~ 7 mL MHB into glass test tubes for recovery, warm in shaker at 37°C *(get this done first and quick)*
-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 (*Arc on first EP, none on others)*

GenePulser in Nelson Lab: Switch on back right, 4-Preset Protocols, 1-Bacteria, 2-E coli 2mm, 2.5kV, insert cuvetter with knob in groove, pulse

-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

Use 1000uL pipette to take 1mL of MHB and put into cuvette, mix, and place back into tube

-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)

Bacteria: EC2

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.

Monday, December 9, 2019

To Do:

1. ~~Colony PCR.~~
2. ~~Purify Colony PCR.~~
3. ~~Check EP plates.~~

Results and Data:

No growth on EP plates.

Day 10:

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

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

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use ~~LVS cells~~, 17LVS gDNA, the 18pEX-based allelic exchange vector, and 19water only.

Example colony PCR using KOD (use a master mix):

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants. Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

-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).

Samples 1-16 correspond to plate, 17 is LVS gDNA, 18 is pEX plasmid, and 19 is water.

KROL266 and KROL269

4 controls: ~~LVS~~, gDNA, plasmid PCR, negative

16 samples + 4 controls = 20 total samples

Use 50uL sample of reaction volume.

Total reaction volume	50
Total number of reactions	20

Component	Stock concentration	Final concentration	1 rxn volume	Factor 21
ddiH2O			31	651
PrimeSTAR GXL Buffer	5x	1x	10	210
dNTPs	2.5 mM	0.2 mM	4	84
oligo F	10 uM	0.3 uM	1.5	31.5
oligo R	10 uM	0.3 uM	1.5	31.5
template	100 ng/ul	2 ng/ul	1	21
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	~~1~~	~~21~~
		Total volume	50	1050

Note: The template does not go into the master mix, but everything else does. Use 2uL of template. Label 16 tubes (nonsterile) and fill with 50uL of molecular grade H2O. Another 16 will be needed for the dilution.

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~~ 30 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.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Sample 1	Default	12/9/2019	3:16 PM	128.36	2.567	1.393	1.84	2.32	50.00	230	1.108	0.028
Sample 2	Default	12/9/2019	3:17 PM	78.86	1.577	0.870	1.81	2.39	50.00	230	0.659	0.001
Sample 3	Default	12/9/2019	3:17 PM	135.29	2.706	1.448	1.87	2.42	50.00	230	1.118	0.005
Sample 4	Default	12/9/2019	3:18 PM	156.00	3.120	1.705	1.83	2.42	50.00	230	1.289	0.023
Sample 5	Default	12/9/2019	3:19 PM	118.54	2.371	1.292	1.84	2.39	50.00	230	0.992	0.036
Sample 6	Default	12/9/2019	3:20 PM	116.99	2.340	1.260	1.86	2.46	50.00	230	0.952	0.008
Sample 7	Default	12/9/2019	3:21 PM	128.83	2.577	1.392	1.85	2.40	50.00	230	1.075	0.014
Sample 8	Default	12/9/2019	3:22 PM	140.56	2.811	1.537	1.83	2.43	50.00	230	1.155	0.019
Sample 9	Default	12/9/2019	3:23 PM	127.31	2.546	1.391	1.83	2.42	50.00	230	1.051	0.002
Sample 10	Default	12/9/2019	3:24 PM	144.06	2.881	1.571	1.83	2.38	50.00	230	1.212	0.035
Sample 11	Default	12/9/2019	3:26 PM	170.64	3.413	1.859	1.84	2.26	50.00	230	1.513	0.078
Sample 12	Default	12/9/2019	3:27 PM	121.73	2.435	1.320	1.84	2.46	50.00	230	0.991	0.107
Sample 13	Default	12/9/2019	3:28 PM	59.71	1.194	0.645	1.85	2.06	50.00	230	0.578	0.050
Sample 14	Default	12/9/2019	3:29 PM	123.50	2.470	1.330	1.86	2.45	50.00	230	1.010	0.024
Sample 15	Default	12/9/2019	3:30 PM	128.88	2.578	1.389	1.86	2.39	50.00	230	1.077	0.023
Sample 16	Default	12/9/2019	3:31 PM	10.39	0.208	0.117	1.78	1.40	50.00	230	0.148	0.025

Sample 16 Redrop	Default	12/9/2019	3:32 PM	10.02	0.200	0.124	1.62	1.48	50.00	230	0.136	0.020
Sample 17	Default	12/9/2019	3:33 PM	136.99	2.740	1.486	1.84	2.36	50.00	230	1.160	0.009
Sample 18	Default	12/9/2019	3:34 PM	148.44	2.969	1.598	1.86	2.44	50.00	230	1.218	0.004
Sample 19	Default	12/9/2019	3:35 PM	-1.73	-0.035	-0.017	2.08	0.35	50.00	230	-0.098	-0.015

Tuesday, December 10, 2019

To Do:

1. ~~Sequencing.~~
2. ~~Check EP plates.~~
3. ~~Streak single colony to CHA-Kan.~~

Results and Data:

KROL270 for sequencing
Size: 1928bp
EP plates had a single colony that was struck onto CHA-Kan.

Wednesday, December 11, 2019

To Do:

1. ~~Check sequencing.~~
2. ~~Check EP plates.~~
3. ~~Make freezer stock of primary integrant from dPmrA(2).~~
4. ~~Restreak LVS(2) primary integrant onto regular CHA.~~
5. ~~Repick single colonies from sucrose selection plates from 12/3/19.~~

Results and Data:

Sequencing was all wild type.
No more growth on EP plates, so they were thrown out.
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.

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.