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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 ~~strike through~~ font on the day they are completed and leave them out of the list on the next day.

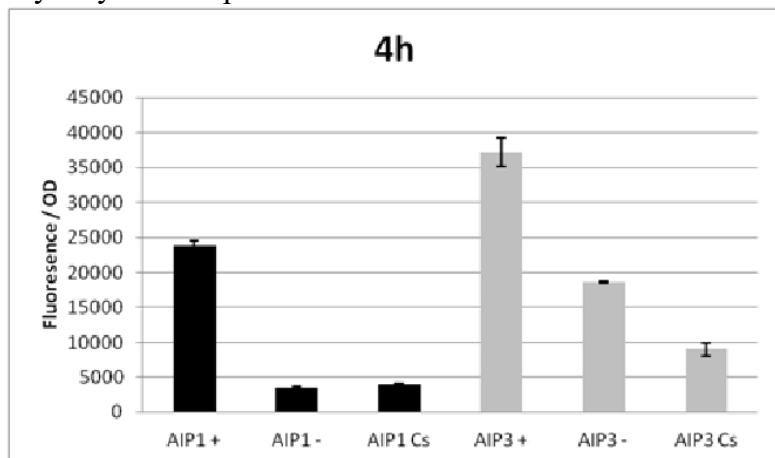


Figure 1

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).

October 2018

Wednesday, October 3, 2018

To Do:

1. Design primers/oligos for overexpression of *rpsU* genes with Joe Paquette.

Results and Data:

Notes on primer design:

Spreadsheet in Team Drive, “DesigningPrimers.xlsx”

Goal: short pieces of DNA homologous to region of amplification with the following specifications

-“Calculated melting temperature” (aka old version) = 72°C

Calculation: $(A+T)*2 + (G+C)*4 = T_m$

Plus or minus 2 is okay (plus being better) but match primer melting temps with each other

-Choose the DNA to be amplified (amplicon) carefully, depending on goal

Today’s primer goal:

Design primers to amplify *rpsUI* (FTL_0456) to clone into overexpression plasmid

-Include a start codon and stop codon

-At the 5’ end, check to be sure there is a promoter and a ribosome binding site (RBS);

these may be on the plasmid already

Monday, October 22, 2018

To Do:

1. Design primers/oligos for overexpression of *rpsU* gene and deletion of FTL_1251

Results and Data:

Design primers to create plasmid for deletion of transporter gene FTL_1251. Deletion will be difficult, due to the close proximity of an *istu2* element (repeated many, many times throughout the genome).

pKR1 pEXΔFTL1251

KROL1 DFTL_1251_FR1F & KROL2 DFTL_1251_FR1R = FR1 = 134 bp

KROL3 DFTL_1251_FR2F & KROL4 DFTL_1251_FR2R = FR2 = 716 bp

Digest FR1 with BamHI / NotI

Digest FR2 with NotI / KpnI

Digest pKL80 with BamHI / KpnI

Confirm deletion with KROL1 and KROL5 = 2411 bp in WT, 766 bp in ΔFTL_1251

Tuesday, October 23, 2018

To Do:

1. Design primers/oligos for overexpression of *rpsU* gene ~~and deletion of FTL_1251~~
2. Review recipe for cystine heart agar (CHA) with hemoglobin with Jamie
3. Review recipe for supplemented Mueller-Hinton broth
4. With undergraduates
 - a. Review LB recipe / make LB
 - b. Sterilize sticks and culture tubes

Cystine heart agar with hemoglobin plates protocol

For 600 mL of CHA total

1. Weigh 30.6 g of cystine heart agar into 1 L flask (non-baffled; 10.2 g/ 100 mL)
2. Add 300 mL of ddiH₂O (type I)

3. Add stirbar to flask
4. Heat on low, stirring, for about 10 minutes (media should be totally dissolved)
5. Autoclave on 30' liquid cycle, filling the water bin up to the height of the media
6. Cool down (ideally to ~55°C)
7. Separately (before), prepare 2% hemoglobin
8. Add 6 g freeze-dried hemoglobin to 300 mL ddiH₂O (type I)
9. Autoclave on 15' liquid cycle, with water in the bin
10. Cool down (ideally to ~55°C)
11. Using sterile technique, pour hemoglobin into CHA
12. Using a 50 mL pipet, aliquot 24 mL of CHAH mixture into each 100 mm plate (should make approximately 25 plates). Try to avoid bubbles!

Reagents

Cystine heart agar
2% hemoglobin
100 mm plates

Mueller-Hinton broth protocol

For 500 mL of MHB total

1. Weigh out 10.5 g Mueller-Hinton broth into 1L square bottle
2. Add 500 mL ddiH₂O (type I)
3. Autoclave on 30' liquid cycle, with water in the bin
4. Cool down (37°C or cooler)
5. Can keep this sterile media indefinitely without supplements
6. Add 5 mL of 10% glucose
7. Add 5 mL of 2.5% iron pyrophosphate
8. Add isovitalax (combine 10 mL from liquid vial with solids, add entire volume to broth)
9. Can keep this supplemented media for 2 weeks, storing at 4°C

Reagents

Mueller-Hinton broth media (dry)
10% glucose (filter-sterilized)
2.5% iron pyrophosphate (1.25 g iron pyrophosphate in 50 mL ddiH₂O [type I], dissolved O/N, fresh solution every 2 weeks)
Isovitalex (commercially available)

LB broth protocol

For 1000 mL of LB total

1. Weigh out 10 g tryptone into a 1 L graduated cylinder with stir bar at bottom
2. Add 10 g of NaCl
3. Add 5 g of yeast extract
4. Add ddiH₂O (type I) to 1 L
5. Mix until dissolved
6. Aliquot out:
 - a. 3 x 200 mL in 1 L flasks

- b. 4 x 75 mL in 100 mL bottles
- 7. Autoclave on 30' liquid cycle, with water in the bin

Sterilize sticks and tubes

***E. coli* competent cells protocol**

Reagents

Solution A

10 mL of 1 M MnCl₂
50 mL of 1 M CaCl₂

200 mL 50 mM 2-morpholino-ethanesulfonic acid (MES) pH 6.3
740 mL ddiH₂O

Solution A plus glycerol

10 mL of 1 M MnCl₂
50 mL of 1 M CaCl₂
200 mL 50 mM 2-morpholino-ethanesulfonic acid (MES) pH 6.3
150 mL Glycerol
590 mL ddiH₂O

Wednesday, October 24, 2018

To Do:

1. Design primers/oligos for overexpression of *rpsU* gene, ~~interruption of FTL_1251, and deletion of *ggt*, FTL_0766~~
2. ~~Make CHA plates with Jamie~~
3. ~~With undergraduates~~
 - a. ~~Wash glassware~~
 - b. ~~Streak out XL1Blue cells~~

Create plasmid to interrupt FTL_1251 (worried about making a clean deletion).

pKR2 pEX_FTL1251-frag

KROL15 FTL1251fragF & KROL16 FTL1251fragR = 456 bp FTL_1251 frag

Digest FTL_1251frag and pKL02 with KpnI / EcoRI

Sequence pKR2 with KROL6

Confirm integration with KROL1 & KROL17 = 765 bp if integrated (no product otherwise)

Design primers to create plasmid for deletion of transporter gene *ggt*, FTL_0766.

pKR3 pEXΔFTL0766

KROL8 DFTL0766_FR1F & KROL9 DFTL0766_FR1R = FR1 = 760 bp

KROL10 DFTL_1251_FR2F & KROL11 DFTL_1251_FR2R = FR2 = 644 bp

Digest FR1 with BamHI / NotI

Digest FR2 with NotI / KpnI

Digest pKL80 with BamHI / KpnI

Sequence plasmid with KROL6, KROL7, KROL14

Confirm deletion with KROL12 and KROL13 = 3260 bp in WT, 1481 bp in Δ FTL_1251

Friday, October 26, 2018

To Do:

1. Design primers/oligos for overexpression of *rpsU* gene
- ~~2. Make CHA plates with Jamie~~
3. With undergraduates
 - ~~a. Wash glassware~~
 - b. Make chemically competent XL1Blue cells

Results and Data:

Aborted *E. coli* competent cells protocol

1. Have 5mL overnight culture from yesterday that Dasith (MRamsey lab) started
2. Add 3 mL of 1 M $MgCl_2$ to 200 mL LB in 1 L baffled flask
3. Inoculate 200 mL LB with $MgCl_2$ with 0.5 mL overnight culture of XL1Blue *E. coli* cells
4. Place flask in shaking incubator, set at 37°C and 225 rpm, start timer
5. At 5:20, took OD600 reading at 1:10 dilution = 0.122, so cells have overgrown.
6. Discard cells

Saturday, October 27, 2018

To Do:

- ~~1. Start TAP purification of LVS RNAP~~
2. Design primers/oligos for overexpression of *rpsU* gene
- ~~3. Wash glassware~~

Results and Data:

Make KHEPES stock solution:

$1 \text{ mol / L} * 276.39 \text{ g/mol} * .05 \text{ L} = 13.82 \text{ g}$, add to about 40 mL ddiH₂O (type I)
pH to 7.9 with 5N HCl (about 5 mL)

Bring volume to 50 mL and filter-sterilize through 0.22 micro filter (30 mL syringe and syringe filter)

Prep Buffer 1 + PI +BB

Final composition	Stock solutions	For 40 mL
20 mM KHEPES pH 7.9	1 M KHEPES	0.8 mL
50 mM KCl	1 M KCl	2 mL
0.5 mM DTT	0.5 M DTT	40 ul
1X Bug Buster	10X Bug Buster	4 mL
10% glycerol	100% glycerol	4 mL
Water		29.16

+ 4 tablets protease inhibitor

Working with samples from 5/25/18 (#4 & #5, 400 mL beta-prime-TAP) and 6/13/18 (C, Δ mgIA beta-prime-TAP), which were stored at -80°C.

2	5/25/18	4	400 mL	0.564
3	5/25/18	5	400 mL	0.556

Resuspend samples in 5 mL Buffer 1 + PI + BB

Add 10 uL DNase I (Epicenter), mix

Incubate at 37°C for 30'

Spin in Nelson lab Beckman floor centrifuge, using rotor SS-34 at 12,500 rpm (18,675xg) for 20 minutes at 4°C

Using rotor SLA-600TC at 11,00 rpm (19,868xg)

Prep IPP150 buffer

Composition	Stock solutions	For 500 ml
10 mM Tris-HCl pH 8	1 M	5 mL
150 mM NaCl	5 M	15 mL
0.1% NP40	10%	5 mL

Add water to 500 mL, filter-sterilize, put on ice to chill.

Remove tubes from Beckman centrifuge. Spin started and stopped a few times bc of circuit breaker issues.

I don't like the poor separation between supernatant and insoluble material.

Transfer material to 2 mL microfuge tubes and spin in Dutta lab minicentrifuge at 4°C for 20'.

Transfer supernatants from microfuge tubes to 50 mL conical (non-sterile).

FORGOT to filter-sterilize!!!! Yikes. Hope there's not too much extra junk.

Estimate volume- about 4.9 mL for each sample.

Adjust buffer concentration by adding to each sample:

10 uL 1M Tris-HCl pH 8.0 / mL sample -> 49 uL

10 uL 10% NP-40 /mL sample -> 49 uL

20 uL 5M NaCl /mL sample -> 98 uL

Remove 50 uL into microfuge tube for control analysis (LYS).

Prepare for first immunoprecipitation:

Need:

IgG Sepharose 6 Fast Flow (GE Healthcare, 17-0969-01) beads, kept at 4°C

Poly-Prep Chromatography Columns (Bio-rad 7311550), 1 per sample, snap bottom stopper off

Non-sterile 50 mL conicals for flow-through collection

Qiagen tip holders (blue or purple plastic pieces to hold column over conical tube)

In cold room

Cut tip of 1mL pipette tip slightly

Resuspend beads completely

Pipette 200 uL IgG Sepharose beads into each column, placing column on top of Qiagen tip holder placed on 50 mL conical for flow-through

Wash beads with 10 mL of IPP150 buffer

When all the IPP150 buffer has passed through the column, cap the bottom of the column (use parafilm if necessary)

Add 1 sample per polyprep column, add top cap to close column (make sure it is not dripping liquid)

Incubate samples with beads, rocking at 4°C, for 2 hours

Prep TEV Cleavage Buffer

Composition	Stock solutions	For 40 mL
-------------	-----------------	-----------

10 mM Tris-HCl pH 8	1 M	400 uL
150 mM NaCl	5 M	1.2 mL
0.1% NP40	10.00%	400 uL
0.5 mM EDTA	0.5 M	40 uL
1 mM DTT	0.5 M	80 uL
Water		37.88 mL

After 2 hour incubation, open columns and let lysate pass through column, discarding flow through
 Wash columns 3x with 10 mL 1PP150 buffer, discarding flow through
 Wash columns with 10 mL TEV Cleavage buffer, discarding flow through
 Cap column bottoms, add 1 mL TEV Cleavage buffer and 10 uL AcTEV Protease (ThermoFisher 12575015) to each. Had an issue with leaking of sample 3, added 500 ul more buffer and 5 ul more protease, now volume is higher than other two samples
 Incubate rocking at 4°C overnight

Test new CHA and standard CHA plates:

Streak out:

LVS *rpoC*-TAP (JCLVS61.8)

LVS Δ *mglA* (JCLVS62.1)

LVS Δ *pigR* (JCLVS106)

And incubate O/N at 37°C

Sunday, October 28, 2018

To Do:

1. Finish TAP purification of LVS RNAP
2. Run protein gel with samples
3. Fix protein gels for staining
4. Design primers/oligos for overexpression of *rpsU* gene
5. Autoclave media for O/N cultures of beta prime TAP
6. Start O/N cultures of beta prime TAP

Results and Data:

Prep FRESH IPP150 Calmodulin Binding Buffer (no detergent)

Need ~45 mL per sample

Composition	Stock solutions	For 150 mL
10 mM β -mercapto ethanol	14.3 M	104.9 uL
10 mM Tris-HCl pH 8	1 M	1.5 mL
150 mM NaCl	5 M	4.5 mL
1 mM Mg-acetate	1 M	150 uL
1 mM Imidazole	5 M	30 uL
2 mM CaCl ₂	1 M	300 uL
Water		to 150 mL

Prep FRESH IPP150 Calmodulin Elution Buffer (no detergent)

Need ~1 mL per sample

Composition	Stock solutions	For 5 mL
10 mM β -mercapto ethanol	14.3 M	3.5 μ L
10 mM Tris-HCl pH 8	1 M	50 μ L
150 mM NaCl	5 M	150 μ L
1 mM Mg-acetate	1 M	5 μ L
1 mM Imidazole	5 M	1 μ L
2 mM EGTA	0.5 M	20 μ L
Water		4,770.5 μ L

In cold room, open columns incubated O/N and place on clean 50 mL conical tubes

Wash columns with 200 μ L TEV cleavage buffer, eluting into same tube.

KEEP eluted material

Discard column with IgG beads

Set up new empty columns on washing 50 mL conicals

Completely resuspend Calmodulin beads

Cut tip of 1 mL tip and use to add 200 μ L calmodulin beads per column

Wash calmodulin beads with 10 mL calmodulin binding buffer

Bring samples from IgG column (on ice) back to bench and estimate volume.

Add 3.3 μ L of 1 M CaCl_2 to each 1.1 mL sample

Sample 1 & 2 -> ~900 μ L, so add 1 μ L, sample 3 -> 1 mL, so add 1.1 μ L

In cold room, plug columns with calmodulin beads.

Add samples to columns

Add 3 mL IPP150 Calmodulin Binding Buffer to each column

Incubate for 1 hour, rocking at 4°C

Unplug columns, let liquid flow through (discard)

Wash 3 times with 10 mL IPP150 Calmodulin Binding Buffer, discarding flow-through

Wash columns once with 200 μ L IPP150 Calmodulin Elution Buffer, discarding flow-through

Place columns on clean new 50 mL conical (Yikes! I think I didn't place sample #2 on clean conical! May be dirty protein).

Elute protein by running 200 μ L IPP150 Calmodulin Elution Buffer over column five times (final protein volume ~ 1mL)

Run gel to check protein purity:

Combine 10 μ L of each protein sample with 10 μ L 2x sample loading buffer

Use 10% NuPAGE Bis-Tris gel, run in 500 mL MES buffer (25 mL 20x stock with 475 mL type I ddiH₂O with 1.25 mL antioxidant):

Lane	Volume	Contents
1	20	1x SLB
2	10	1:10 diluted Benchmark Ladder
3	20	#1, LVS Δ mg1A Beta-prime-TAP purification
4	20	#2, LVS Beta-prime-TAP purification

5	20	#3, LVS Beta-prime-TAP purification
6	20	1x SLB
7	20	1x SLB
8	10	Benchmark Ladder
9	20	#1, LVS Δ mg1A Beta-prime-TAP purification
10	20	#2, LVS Beta-prime-TAP purification
11	20	#3, LVS Beta-prime-TAP purification
12	20	1x SLB

Run at ~135 V using Bloom power source for ~20 minutes. Unlikely to be properly calibrated and things seem a little fast at 20', so drop down to ~115 V. Ran for about 1 hr 16' (about right).

Prep fixative solution for both silver stain and Coomassie stain:

Silver stain fixative:

40 mL ethanol
10 mL acetic acid
50 mL ddiH₂O (type I)

Coomassie stain fixative:

50 mL methanol
10 mL acetic acid
40 mL ddiH₂O (type I)

Cut gel in half, keeping side with 1:10 ladder dilution for silver stain.

Rinse gels in type I ddiH₂O, then incubate in fixing solution at room temperature rocking overnight in tip box.

Prepare Mueller-Hinton media:

Mix all of the media from Dove lab (50 mL conical ~30g) into 1.4 L of ddiH₂O (type I). Distribute into 1x 500 mL in 1 L bottle and 3x 300 mL in 1L baffled flasks
Autoclave 30'

Make 30 mL 2.5% iron pyrophosphate:

Dissolve 0.75 g iron pyrophosphate in ddiH₂O (type I).
Filter-sterilize with syringe filter

Make 250 mL of 10% glucose stock:

25 g glucose, make up to 250 mL, filter-sterilize

Mix together 3 vials of Isovitelex:

Combine 10 mL from resuspension vial with solids, aliquot to sterile 50 mL conical

Supplement all the Mueller-Hinton broth (sMHB):

5 mL 2.5% iron pyrophosphate
5 mL 10% glucose
10 mL Isovitelex
Per 500 mL in 1 L bottle
3 mL 2.5% iron pyrophosphate

3 mL 10% glucose
 6 mL Isovitelex
 Per 300 mL in 1 L flask

Scrape up cells from plates struck last night:

Aliquot 3 mL sMHB into 2 sterile 50 mL conicals, ~20 mL into another

Scrape up cells from beta-prime-TAP plate (old CHA) using loop and resuspend in sMHB to high density

Scrape up cells from Δ pigR plate (old CHA) using loop and resuspend in sMHB to decent density

Check density of resuspended cultures and dilute to final OD600 of 0.005 in 300 mL sMHB in baffled flask for growth overnight.

Strain	Dilution	Measured OD600	Calc OD600	Volume for 0.005 in 300 mL
Beta prime TAP	1:20	0.410	8.2	183 uL
Δ pigR	1:10	0.287	2.87	523 uL

Put in 37°C shaking incubator at 6:10 PM

Re-measured OD600 values using MRamsey lab spec. Different values!

Strain	Dilution	Measured OD600	Calc OD600	Cal OD600 in 300 mL
Beta prime TAP	1:20	0.368	7.36	0.0045
Δ pigR	1:10	0.237	2.37	0.0041

Don't bother adjusting. Will just take a little longer to get to same OD600.

Monday, October 29, 2018

To Do:

1. Stain protein gels
2. Ship protein to Maria
- 3.
4. Design primers/oligos for overexpression of *rpsU* gene

Results and Data:

Check OD600 of cultures grown O/N:

Strain	8:35 AM	10:30		
1: beta-prime-TAP	0.171	0.196		
2: beta-prime-TAP	0.188	0.217		
3: Δ pigR	0.095	0.104		

Daniel and John:

PCR:

Test:

Daniel P822 and P823

John P824 and P825

Carly P820 and P821

Control: P780 and P781

Tuesday, October 30, 2018**To Do:**

1. Check lab for cleanup items
2. ~~Finish Coomassie stain gel~~
3. ~~Make TAE running buffer~~
4. ~~Pour 1% agarose gel~~
5. ~~Run agarose gel with PCR products~~
6. ~~Visualize PCR products in agarose gel~~

Ship protein

Plan for growth experiment:

3 mL cultures

Tube number	Condition
1	MHB
2	MHB
3	MHB + 30 uL 2.5% iron pyrophosphate
4	MHB + 30 uL 2.5% iron pyrophosphate
5	MHB + 30 uL 10% glucose
6	MHB + 30 uL 10% glucose
7	MHB + 60 uL isovitelex
8	MHB + 60 uL isovitelex
9	MHB + all supplements
10	MHB + all supplements

MHB = Mueller Hinton Broth supplemented by Kathryn on 10/28

Target a starting OD600 of 0.08. We'd like to inoculate with about 50 uL.

Calculation:

$$(3,000 \text{ uL}) * (0.08) / (50 \text{ uL}) = 4.8 \text{ OD600}$$

Resuspended, diluted 1:50 and measured OD600

Initially = 0.068 -> 3.4

Added more cells, diluted 1:50 and measured OD600

OD = 0.122 -> 6.1

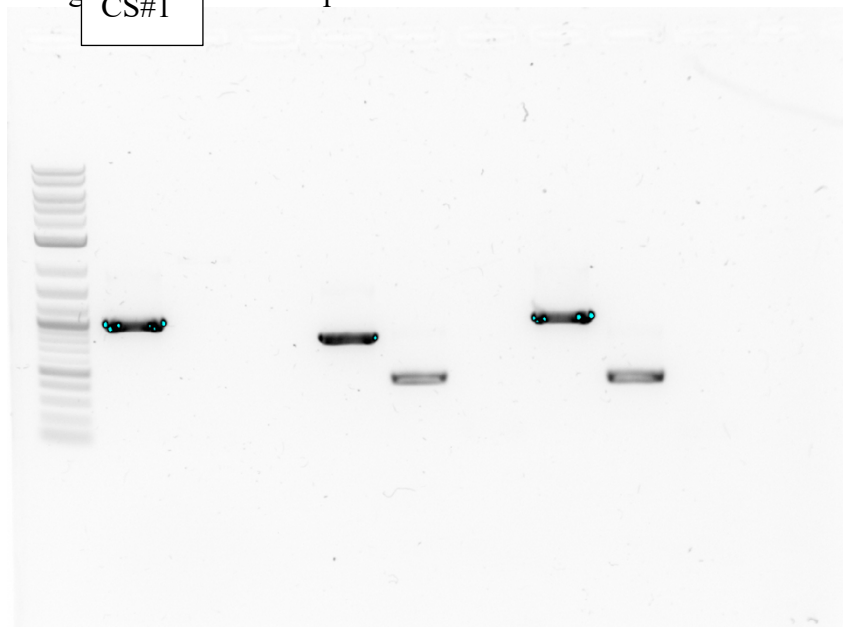
If we add 50 uL at OD600 6.1 to 3mL culture, final OD should be: 0.102

Loading agarose gel (left to right)

Lane	Contents	Expected size
1	2-log NEB ladder	varies
2	CS #1 P820 P821	994 bp
3	CS #2 P780 P781	443 bp
4	CS #3 P780 P781	0 bp
5	JC #1 P825 P825	772 bp
6	JC #2 P780 P781	443 bp
7	JC #3 P780 P781	0 bp
8	KR #1 P820 P821	994 bp
9	KR #2 P780 P781	443 bp

10 KR #3 P780 P781 0 bp
11 – 15 Empty

Run g CS#1 V for unspecified time



If the band in lane 6 is approximately 2x the intensity of the 1 kb band (122 ng), then we loaded 244 ng. We loaded 5 uL of PCR product, so our concentration of PCR product is: 48.8 ng/uL. That means that in a 50 uL reaction, we have 2.44 ug of our PCR product.

Wednesday, October 31, 2018

To Do:

1. Start O/N cultures for competent cells
2. Streak out plates for LVS and beta-prime TAP growth tomorrow

November 2018

Thursday, November 1, 2018

To Do:

1. Perform competent E. coli cells protocol with UGR (day 3)
2. With Jamie: start large LVS cultures to pellet and use for protein isolation next week
- 3.

Results and Data:

Day 3

1. Add 3 mL of sterile $MgCl_2$ to 200 mL LB in 1 L flask. Add antibiotic if appropriate.
We used 1 L flask that is not baffled
2. Inoculate 200 mL LB with 0.5 mL of culture grown overnight.
3. Incubate 200 mL culture at 37°C, shaking until culture reaches an OD600 of 0.5.
4. Monitor culture growth by assessing OD600 using the spectrophotometer:

At 3, 4, and 5 hours or at appropriate times between, measure OD600

Use a cuvette with 1 mL LB as a blank

Add 1 mL culture from flask to a cuvette

Use MRamsey lab spectrophotometer set to a wavelength of 600nm

After ~ 6 hours, OD600 = 0.55 and we split culture into 4 50 mL conicals to spin down

5. When cultures approach correct OD, cool down centrifuge to 4°C (Dutta lab benchtop centrifuge)
6. When culture reaches an OD600 of approximately 0.5, transfer culture volume to sterile tubes (4x 50 mL conical) to pellet bacteria
7. Place tubes in cool centrifuge and pellet bacteria by spinning (in Dutta lab benchtop centrifuge, 15 minutes at 4000 rpm).
8. Remove tubes from centrifuge, decant the supernatant into a waste bottle, and keep cell pellets on ice
9. Add a total of 60 mL cold solution A to cell pellets from the original 200 mL culture. If you are using four 50 mL conical tubes, each tube should contain 15 mL cold solution A.
10. VERY gently resuspend cell pellet by pipetting up and down. Don't completely dispense liquid with each cycle, to prevent creating bubbles/froth. The cells should be completely homogeneous when done (no clumps or chunks)
11. Incubate resuspended cells on ice for at least 20 minutes (can stay on ice for up to 3 hours).
12. Place tubes in cool centrifuge and pellet bacteria by spinning (in Dutta lab benchtop centrifuge, 15 minutes at 4000 rpm).
13. While cells are spinning, prepare tubes for final competent cell aliquots: label and pre-chill on ice.
14. Remove tubes from centrifuge, decant the supernatant into a waste bottle, and keep cell pellets on ice
15. Add a total of 12 mL cold solution A + 15% glycerol to cell pellets from the original 200 mL culture. If you are using four 50 mL conical tubes, each tube should contain 3 mL cold solution A + 15% glycerol.
16. VERY gently resuspend cell pellet by pipetting up and down as previously.
17. Aliquot competent cells in 550 uL volumes into sterile pre-cooled 1.5 mL microcentrifuge tubes.
18. If available, freeze cells immediately upon aliquoting using using dry ice.
19. Store competent cells at -80°C.

At first use, test competency of cells by transforming with a known amount of supercoiled plasmid and record the transformation efficiency.

Added 3 mL 1M MgCl₂

Inoculate with 0.5 mL of culture of XL1 Blue cells grown overnight (was grown in 5 mL volume without antibiotic

Time	8:10	11:30	12:40	1:55
OD500	start	0.136	0.324	0.55

Monday, November 5, 2018

To Do:

1. Make kanamycin stock solution
2. Pour kanamycin-containing plates

Results and Data:

John Church:

Weigh out 1 g of kamamycin into non-sterile 50 mL conical

Add 15 mL of type I ddiH2O, dissolve kanamycin
 Pour into a graduated cylinder, add type I ddiH2O until volume reaches 20 mL.
 Filter-sterilize into sterile 50 mL conical. Use a syringe filter.
 Aliquot 500 uL amounts of sterile kanamycin solution into 1.5 mL microfuge tubes (40 tubes).

Made more LB-agar (500 mL)
 Poured LB-agar plates + kanamycin
 Poured LB-agar plates + carbenicillin

Tuesday, November 6, 2018

To Do:

1. With Jamie: Purify RNAP
2. With undergrads: transform competent cells (Carly and Daniel)

Do control version of competent cell transformation:

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 transformation 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	pKL02	1 uL	20 ul, 100 ul, remaining	3	
2	(+) control	pKL80	1 uL	20 ul, 100 ul, remaining	3	
3	(+) control	pF	1 uL	20 ul, 100 ul, remaining	3	
4	(-) control	None	0	20 ul, 100 ul, remaining	3	
5	(+) control	pUC19	1 uL	20 ul 1:10, 20 ul, 100 ul, remaining		4
6	(-) control	None	0	20 ul, 100 ul, remaining		3
Total number of plates					12	7

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.
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.

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, November 7, 2018

To Do:

1. With Jamie: Finish purifying RNAP

2. Run gel to look at purification
3. With undergrads: check transformation efficiency, start O/N cultures of plasmids (all)

Transformation efficiency calculation numbers:

DNA amount, pg	50
DNA amount, ug	0.05
DNA amount, mg	0.00005
Volume plated	100
Total volume	1100
Dilution	0.091

Results from UGR transformation:

colonies	mass (mg)	dilution	transformation efficiency
8	0.00005	0.090909091	1.76E+06

Results from my transformation:

colonies	mass (mg)	dilution	transformation efficiency
36	0.00005	0.090909091	7.92E+06

Monday, November 12, 2018

To Do:

1. Start making plasmids pKR1, pKL2, pKL3
 - a. PCR flanking regions
 - b. Digest ends
 - c. PCR-purify digests
 - d. Ligate ON?
2. Streak out strains for this week's experiments
 - a. LVS
 - b. B-TAP
 - c. rpoH-VSV-G (KMLFT3)

Set up PCR:

Reactions

Number	Primers	Target	Expected size	For plasmid
1	KROL1 DFTL_1251_FR1F KROL2 DFTL_1251_FR1R	FTL_1251 FR1	134 bp	pKR1
2	KROL3 DFTL_1251_FR2F KROL4 DFTL_1251_FR2R	FTL_1251 FR2	716 bp	pKR1
3	KROL15 FTL1251fragF KROL16 FTL1251fragR	FTL_1251 internal	456 bp	pKR2
4	KROL8 DFTL0766_FR1F KROL9 DFTL0766_FR1R	FTL_0766 FR1	760 bp	pKR3
5	KROL10 DFTL0766_FR2F KROL11 DFTL0766_FR2R	FTL_0766 FR2	644 bp	pKR3
6	P820 and P821	Positive control	994 bp	Control

7	P820 and P821	Negative control	-	Control
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Reaction setup:

Total reaction volume	100			
Total number of reactions	6			
				Factor
Component	Stock concentration	Final concentration	1 rxn volume	7.15
ddiH ₂ O			20	143
KOD buffer	2x	1x	50	357.5
dNTPs	2 mM	0.4 mM	20	143
oligo F	10 uM	0.3 uM	3	individual
oligo R	10 uM	0.3 uM	3	individual
template	100 ng/ul	2 ng/ul	2	(12.3)
KOD	1 U/ul	0.02 U/ul	2	14.3
		Total volume	100	715

(Use 50 uL volume for both controls!)

Use STN1 program:

1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C 1'
5. Goto step 2, 32X
6. 68°C 5'
7. 12°C hold

PCR-purify rxns 1 - 5, eluting in 35 uL 0.1X EB

Set up digests:

Rxn #	DNA	Enzyme 1	Enzyme 2	To make
1	PCR 1: FTL 1251 FR1	BamHI-HF	NotI-HF	pKR1
2	PCR 2: FTL 1251 FR2	KpnI-HF	NotI-HF	pKR1
3	PCR 3: FTL 1251 internal	KpnI-HF	EcoRI-HF	pKR2
4	PCR 4: FTL 0766 FR1	BamHI-HF	NotI-HF	pKR3
5	PCR 5: FTL 0766 FR2	KpnI-HF	NotI-HF	pKR3
6	pKL80	BamHI-HF	KpnI-HF	pKR1,3
7	pKL02	KpnI-HF	EcoRI-HF	pKR2

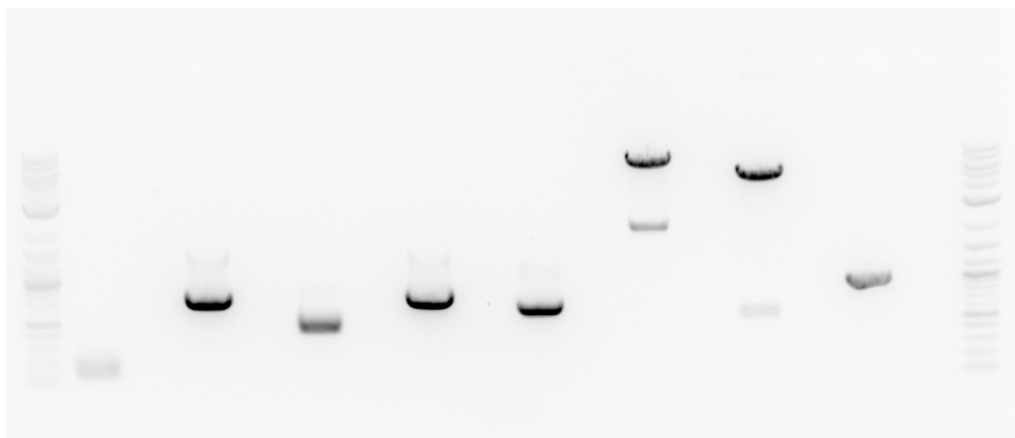
Master Mix:

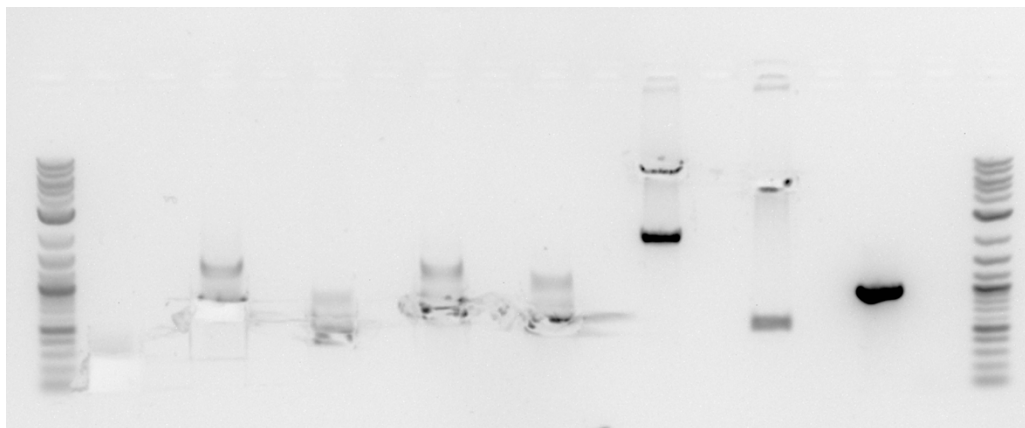
Component	x1	x8
H ₂ O	5.8	46.4
10X CutSmart buffer	3	24
DNA	20 uL (5 uL plasmid)	indiv
Enzyme 1	0.6	indiv
Enzyme 2	0.6	indiv
Total volume	30	

Incubate at 37°C for 35'

Add 6 ul of 6x Orange-G loading dye and run entire digest on gel:

Lane	Vol	Contents
1	10	NEB 2-log ladder
2	35	Digest 1
3	-	-
4	35	Digest 2
5	-	-
6	35	Digest 3
7	-	-
8	35	Digest 4
9	-	-
10	35	Digest 5
11	-	-
12	35	Digest 6
13	-	-
14	35	Digest 7
15	-	-
16	5	PCR 6
17	5	PCR 7
18	10	NEB 2 log ladder
19		
20		





Put gel slices at 4°C O/N

Tuesday, November 13, 2018

To Do:

1. Start making plasmids pKR1, pKL2, pKL3
 - a. Gel-purify digests
 - b. Ligate
 - c. Transform

Set up ligations:

Ligation #	Insert 1	Insert 2	Backbone
1	FTL_1251 FR1	FTL_1251 FR2	pKL80, cut
2	FTL_1251 internal	-	pKL02, cut
3	FTL_0766 FR1	FTL_0766 FR2	pKL80, cut
4	-	-	pKL80, cut
5	-	-	pKL02, cut

Master Mix

Component	x1	x6
H ₂ O	7.5 ul	45
10x ligase buffer	2 ul	12
Insert 1	4 ul	indiv
Insert 2	4 ul	indiv
Backbone	2 ul	indiv
Ligase	0.5 ul	3
Total	20 ul	

Add 10 uL master mix to individual tubes with inserts + backbone (0.1xEB if no DNA)

Incubate O/N at 16°C

Wednesday, November 14, 2018

To Do:

1. Pellet LVS and betaprime TAP cells for future RNAP purification
2. Continue making plasmids pKR1, pKL2, pKL3
 - a. Transform

Pelleted 2x300 mL LVS and B'-TAP cultures in 6x 50mL conicals each, washed with 12 mL Buffer 1 (no PI, no BugBuster):

Composition	Stock solutions	For 100 ml
20 mM KHEPES pH 7.9	1 M KHEPES	2 mL
50 mM Kcl	1 M KCl	5 mL
0.5 mM DTT	1 M DTT	0.1 mL
10 % Glycerol	100% Glycerol	10 ml
	+ Water	To 100 mL

Pelleted again, freeze @ -80°C

Transform ligations:

#	Volume	Reaction	Plate
1	8 uL	Ligation 1: pKR1	100 uL, remaining
2	8 uL	Ligation 2: pKR2	100 uL, remaining
3	8 uL	Ligation 3: pKR3	100 uL, remaining
4	8 uL	Ligation 4: pKL80 cut only	100 uL, remaining
5	8 uL	Ligation 5: pKL02 cut only	100 uL, remaining
6	0.5 uL	pKL80 plasmid	20 uL, 100 uL

Add 100 uL cells to indicated DNA in sterile 1.5 mL Eppendorf tube (<50 uL for control)

Incubate on ice for ~10', HS 42°C 30", recover in 1 mL LB shaking at 37°C for ~1hr

Plated 100 uL volume. Thought I spun down remainder of culture in tubes, decanted liquid, realized cells hadn't pelleted! Spin down and plate remaining culture, but variable per transformation and some only a bit more than 100 uL.

Put at 37°C O/N.

Thursday, November 15, 2018

To Do:

1. Continue making plasmids pKR1, pKL2, pKL3
 - a. Pick colonies, grow O/N

Transformation results

Reaction	# colonies on 100 uL plate	Reaction
1	1	Ligation 1: pKR1 (pKL80 backbone)
2	46	Ligation 2: pKR2 (pKL02 backbone)
3	4	Ligation 3: pKR3 (pKL80 backbone)
4	0	Ligation 4: pKL80 cut only
5	4	Ligation 5: pKL02 cut only

Plenty of colonies on pKL80 control plates

Ratios look promising. Pick 4 colonies per reaction and start 5 mL cultures O/N in LB Kan50 (Have more colonies on "1mL" plate from ligation 1, so pick some from that plate!)

Monday, November 26, 2018

To Do:

- a. Miniprep plasmids
- b. Check plasmid concentration and prepare to send to sequence

Minipreped, eluted in 50 uL of 0.1x EB

1-1 -> 1-4 (potential pKR1)

2-1 -> 2-4 (potential pKR2)

3-1 -> 3-4 (potential pKR3)

pKL80 x 2

Check concentration by Nanodrop:

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.
1-1	571.15	11.423	6.35	1.8	2.3	50	230	4.965
1-2	455.53	9.111	5.042	1.81	2.32	50	230	3.933
1-3	496.26	9.925	5.472	1.81	2.32	50	230	4.271
1-4	709.02	14.18	7.616	1.86	2.35	50	230	6.024
2-1	793.2	15.864	8.545	1.86	2.32	50	230	6.826
2-2	805.54	16.111	8.585	1.88	2.33	50	230	6.92
2-3	862.47	17.249	9.219	1.87	2.33	50	230	7.416
2-4	815.73	16.315	8.733	1.87	2.32	50	230	7.039
3-1	623.47	12.469	6.646	1.88	2.38	50	230	5.248
3-2	637.4	12.748	6.785	1.88	2.36	50	230	5.402
3-3	557.81	11.156	6.225	1.79	2.31	50	230	4.837
3-4	551.97	11.039	6.113	1.81	2.32	50	230	4.76
pKL80	549.03	10.981	6.042	1.82	2.36	50	230	4.656
pKL80	636.48	12.73	6.737	1.89	2.39	50	230	5.333

Set up tubes for sequencing. Need 24 uL total (2x12ul) with 5 pmol primer (4 ul of 2.5 uM) and calculated amount of plasmid:

Sample ID ^b	Well		A.	B.	E.	F.
(Indicate <u>your</u> plate well or s-tube number if necessary)	(GSC use <u>only</u>)		Template Size	Template Stock Conc.	PLASMID template:	Volume PCR-H ₂ O
			(bases)	(ng/μl)	Volume =	needed
		Template Type ^c			(~400 ÷ B)μl	(10 less D or E)μl
KR1		Plasmid	7695	571	0.7	9.3
KR2		Plasmid	7695	571	0.7	9.3
KR3		Plasmid	7695	455	0.9	9.1
KR4		Plasmid	7695	455	0.9	9.1
KR5		Plasmid	7695	496	0.8	9.2
KR6		Plasmid	7695	496	0.8	9.2
KR7		Plasmid	5535	793	0.5	9.5
KR8		Plasmid	5535	793	0.5	9.5
KR9		Plasmid	5535	805	0.5	9.5
KR10		Plasmid	5535	805	0.5	9.5
KR11		Plasmid	5535	862	0.5	9.5
KR12		Plasmid	5535	862	0.5	9.5

KR13		Plasmid	8249	623	0.6	9.4
KR14		Plasmid	8249	623	0.6	9.4
KR15		Plasmid	8249	637	0.6	9.4
KR16		Plasmid	8249	637	0.6	9.4
KR17		Plasmid	8249	558	0.7	9.3
KR18		Plasmid	8249	558	0.7	9.3

Need to make better sample submission sheet: this is the code for what is in each sample:

Sample ID	Template	Primer
KR1	pKR1_1-1	KROL6
KR2	pKR1_1-1	KROL7
KR3	pKR1_1-2	KROL6
KR4	pKR1_1-2	KROL7
KR5	pKR1_1-3	KROL6
KR6	pKR1_1-3	KROL7
KR7	pKR2_2-1	KROL6
KR8	pKR2_2-1	KROL7
KR9	pKR2_2-2	KROL6
KR10	pKR2_2-2	KROL7
KR11	pKR2_2-3	KROL6
KR12	pKR2_2-3	KROL7
KR13	pKR3_3-1	KROL6
KR14	pKR3_3-1	KROL7
KR15	pKR3_3-2	KROL6
KR16	pKR3_3-2	KROL7
KR17	pKR3_3-3	KROL6
KR18	pKR3_3-3	KROL7

Tuesday, November 27, 2018

To Do:

- a. Send plasmids out to sequence
- b. Purify RNAP with Jamie (start)

1. Check for stock solutions; make Buffer 1 + PI +BB if not enough in freezer and make fresh TEV cleavage buffer.

Make more Buffer 1 + PI +BB:

Buffer 1 + PI +BB

Final composition	Stock solutions	For 40 mL
20 mM KHEPES pH 7.9	1 M KHEPES	0.8 mL
50 mM KCl	1 M KCl	2 mL

0.5 mM DTT	0.5 M DTT	40 ul
1X Bug Buster	10X Bug Buster	4 mL
10% glycerol	100% glycerol	4 mL
Water		29.16

+ 4 tablets protease inhibitor

- Write out samples in table:
(JW will prep 1&2, I will prep 3&4)

Sample#	Date prepared	Labeled	Volume of cells	OD600 at harvest
1	11/14/18	LVS 2	300 mL	0.490 or 0.463
2	11/14/18	B'-TAP 2	300 mL	0.427 or 0.441
3	11/14/18	LVS 2	300 mL	0.490 or 0.463
4	11/14/18	B'-TAP 2	300 mL	0.427 or 0.441

- Resuspend samples in 5 mL Buffer 1 + PI + BB by pipetting with 5 mL pipette. Be sure no clumps remain
- Add 10 uL DNase I (Lucigen/Epiceter), mix by pipetting
- Incubate at 37°C for 30'
- During incubation, cool down Dutta lab microcentrifuge

Prep 30 mL TEV cleavage buffer (x2; Jamie prepped 30 mL, I prepped 30 mL)

- Aliquot samples into 2 mL microfuge tubes
- Spin tubes at 4°C for 20 minutes at max speed
- Pool lysate from same samples in microfuge tubes into new (nonsterile) 50 mL conical tubes, leaving behind insoluble material at bottom (okay to leave some behind; better than taking too much)
- Filter-sterilize lysates using 30mL syringe and syringe filter into new (nonsterile) 50mL conical tube. Specifically, pull plunger out from 30mL syringe, thread tip of syringe onto syringe use filter (use MillexGP 0.22 micrometer filter, has 33mm diameter), pour/pipette lysate into syringe, insert plunger and recover filtered lysate into new (nonsterile) 50mL conical tube
- Obtain a good estimate of sample volume using pipette. **~5 mL**
- Adjust buffer concentration by adding to each sample:
- 10 uL 1M Tris-HCl pH 8.0 / mL sample -> **50 uL**
- 10 uL 10% NP-40 /mL sample -> **50 uL**
- 20 uL 5M NaCl /mL sample -> **100 uL**
- Remove 50 uL into microfuge tube for control analysis (LYS).
- Cut tip of 1mL pipette tip slightly
- Resuspend IgG Sepharose beads completely
- Pipette 200 uL IgG Sepharose beads into each column, placing column on top of Qiagen tip holder placed on 50 mL conical for flow-through
- Wash beads with 10 mL of IPP150 buffer
- When all the IPP150 buffer has passed through the column, cap the bottom of the column
- Add 1 sample per polyprep column, add top cap to close column (make sure column is closed completely and is not dripping liquid)
- Incubate samples with beads, rocking at 4°C, for 2 hours

24. After 2 hour incubation, open columns and let lysate pass through column. ~2hr 50"
25. Save 50 uL aliquot of flow through in microfuge tube (FT1), discard remaining.
26. Wash columns 3x with 10 mL 1PP150 buffer, discarding flow through
27. Wash columns with 10 mL TEV Cleavage buffer, discarding flow through
28. Cap column bottoms, add 1 mL TEV Cleavage buffer and 10 uL AcTEV Protease to each.
29. Incubate rocking at 4°C overnight- make sure columns are closed completely and are not dripping liquid.

Wednesday, November 28, 2018

To Do:

- a. Purify RNAP with Jamie (finish)
 1. Open columns incubated O/N and place on new clean 50 mL conical tubes, letting liquid elute out
 2. Wash columns with 200 uL TEV cleavage buffer, eluting into same tube.
Managed to first wash column with Calmodulin binding buffer instead of TEV cleavage buffer! Re-wash with TEV cleavage buffer.
 3. KEEP eluted material, transfer 20 uL aliquot of elution in microfuge tube (E1).
 4. Discard column with IgG beads
Pipette 200 uL 1x SLB into the columns with beads and resuspend as best as possible, saving spent beads in new tube (IB)
 5. Set up new empty columns on "washing" 50 mL conicals
 6. Completely resuspend Calmodulin beads by vortexing
 7. Cut tip of 1 mL tip and use to add 200 uL calmodulin beads per column
 8. Wash calmodulin beads with 10 mL calmodulin binding buffer
 9. After wash liquid is through columns, plug bottom of columns closed.
 10. Bring samples from IgG column (on ice) back to bench and estimate volume.
 11. Add 3.3 uL of 1 M CaCl₂ to each 1.1 mL sample
Sample X -> 1 mL uL, so add 3.2 uL. (should have only added 3!)
 12. Bring samples back to cold room and add samples to columns
 13. Add 3 mL IPP150 Calmodulin Binding Buffer to each column
 14. Add top cap to close columns (make sure columns are closed completely and are not dripping liquid)
 15. Incubate for 1 hour, rocking at 4°C
 16. Unplug columns, let liquid flow through
 17. Save 50 uL aliquot of flow through in microfuge tube (FT2), discard remaining.
 18. Wash 3 times with 10 mL IPP150 Calmodulin Binding Buffer, discarding flow-through
 19. Wash columns once with 200 uL IPP150 Calmodulin Elution Buffer, discarding flow-through.
 20. Place columns on clean new 50 mL conical.
 21. Elute protein by running 200 uL IPP150 Calmodulin Elution Buffer over column five times (final protein volume ~ 1mL)

Run protein samples on 10% NuPAGE Bis-Tris gels with MES buffer

Dilute ladder (1ul) into 9ul 1x sample loading buffer

Lane	Volume	Contents
1	10ul	Benchmark unstained ladder (1:10)
2	20ul	3: LVS- IgG Beads
3	20ul	3: LVS- E1
4	20ul	3: LVS- FT2
5	20ul	3: LVS- Calmodulin Beads
6	20ul	3: LVS- elution final
7	20ul	4: Beta-prime elution final

8	20ul	4: Beta-prime Calmodulin Beads
9	20ul	4: Beta-prime FT2
10	20ul	4: Beta-prime E1
11	20ul	4: Beta-prime IgG Beads
12	10ul	1x Sample Loading Buffer (SLB)

(Jamie took over so I could go home!)

Check sequencing results:

1-3 is correct = pKR1

2-1 and 2-3 are correct = pKR2

3-2 may be correct, but the sequencing with KROL7 had duplicate peaks for all three sequenced plasmids.

Try to re-sequence with M13 reverse and also sequence 3-4 with KROL6 and M13 reverse.

Thursday, November 29, 2018

To Do:

- a. Aliquot anti-VSV-G antibody, 2° antibodies
- b. ~~Make hemoglobin~~
- c. ~~Make CHA plates~~
- d. ~~Streak out LVS for EC cells~~
- e. ~~Streak out ΔFTL_1548 for EC cells~~

Send out samples to sequence:

Sample number	Template Type	Template Name	Primer Name ^a	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/μl)	<u>PLASMID template:</u>	<u>Volume H₂O needed</u>
						Volume =	
						$2 \times (\sim 400 \div B) \mu\text{l}$	$(20 \text{ less D or E}) \mu\text{l}$
KR19	Plasmid	pKR3_3-2	M13R	8249	637	1.3	18.7
KR20	Plasmid	pKR3_3-4	KROL6	8249	552	1.4	18.6
KR21	Plasmid	pKR3_3-4	M13R	8249	552	1.4	18.6

Make 1.2 L of 2% hemoglobin to aliquot into 100 mL bottles – 24 g freeze-dried hemoglobin

Jamie silver-stained our protein purification gels:

Friday, November 30, 2018

To Do:

- a. ~~Make new iron pyrophosphate~~
- b. ~~Make 10% sucrose~~
- c. ~~Make EC cells: LVS and ΔFTL_1548~~
- d. ~~Electroporate pKR1, pKR2, pKR3~~

Received sequencing results- both 3-2 and 3-4 are correct! -> pKR3

Made 10% sucrose, new iron pyrophosphate

Washed LVS 3x with 10% sucrose, enough for 6 electroporations. Not enough ΔFTL_1548 to work with (is there a growth defect?).

Electroporations:

Number	Strain	Plasmid
--------	--------	---------

1	LVS	pKR1
2	LVS	pKR1
3	LVS	pKR2
4	LVS	pKR2
5	LVS	pKR3
6	LVS	pKR3

Recover in 5 mL MH+ supplemented today for ~5 hours at 37°C shaking (MRamsey lab incubator). Pelleted in sterile 1.5mL tubes, plated each electroporation on two CHA-Kan5 plates (poured today).

December 2018

Monday, December 3, 2018

To Do:

- Check electroporation plates
- Make stocks of KMLFT114.1 LVS Δ FTL_1548
- Streak out Δ FTL_1548 and LVS (original stock) for more EC cells and single-use LVS stocks

Looked at electroporation plates. On one of the plates from electroporation 6, there are 4 colonies. Patch out to new CHA-Kan plate (6-1 -> 6-4). No colonies on any of the other plates. Scrape up KMLFT114.1 LVS Δ FTL_1548 cells and make single-use glycerol stocks (~500 ul, split into 9 tubes). Use one tube to patch strain out tonight. Patch out LVS for single-use stocks and EC cells.

Tuesday, December 4, 2018

To Do:

- Check electroporation plates
- Make EC KMLFT114.1 LVS Δ FTL_1548
- Make EC LVS and single-use LVS stocks

Looked at electroporation plates- no new single colonies.

Made EC KMLFT114.1 LVS Δ FTL_1548 and EC LVS (didn't make single-use LVS stocks, wanted to maximize EC cells)

Struck out more original LVS strain to make single-use stocks tomorrow

Wednesday, December 5, 2018

To Do:

- Check electroporation plates
- Make single-use LVS stocks
- New electroporations

Looked at electroporation plates- single colonies coming up on all plates now. Not sure if these are background or transformants- they look distinct less like background. Patch 6 from each electroporation to a new CHA-Kan plate to check

Electroporate pKR1 – 3 into cells lacking FTL_1548

Electroporations:

Number	Strain	Plasmid
--------	--------	---------

7	LVS ΔFTL 1548	pKR1
8	LVS ΔFTL 1548	pKR2
9	LVS ΔFTL 1548	pKR3

Recover at 37°C shaking (MRamsey lab incubator) in 4 mL MH+ supplemented today from ~10:45 - ~4pm (~5 hrs).

Pellet in sterile 2mL tubes, plated each electroporation on two CHA-Kan5 plates (poured 11/30). Incubate at 37°C.

Scrape up cells from LVS plate, resuspend in 3 mL Mueller Hinton. Add 750 ul of 75% glycerol and aliquot 100 ul into sterile 1.5 mL tubes -> -80°C for single-use stocks.

Thursday, December 6, 2018

To Do:

- Check patch plates from potential integrants
- PCR to check for integration if growth

8:15am: the patch plates went into the incubator before noon yesterday. Seems like all the patches show some evidence of growth, suggesting the cells are really KanR. The amount of growth is less than I would expect for that incubation time (although the colonies were small). Maybe these integration events lead to growth defect? Would be somewhat surprising, as it's only the first integration event and it's in two different loci. There were colonies much earlier on the pKR3 plasmid plate (#6), for integration into FTL_0766 (*ggt*). It is possible that those cells have integration of the plasmid into the *groEL* locus, which wouldn't result in a growth defect.

Check growth of plates later today. Plan on checking the potential pKR2 plasmid integrants by PCR tomorrow (these would be the final strain; integration of pKR2 should inactivate the FTL_1251 gene. Confirm integration with KROL1 & KROL17 = 765 bp if integrated (no product otherwise)

Friday, December 7, 2018

To Do:

- Check patch plates from potential integrants
- PCR to check for integration if growth

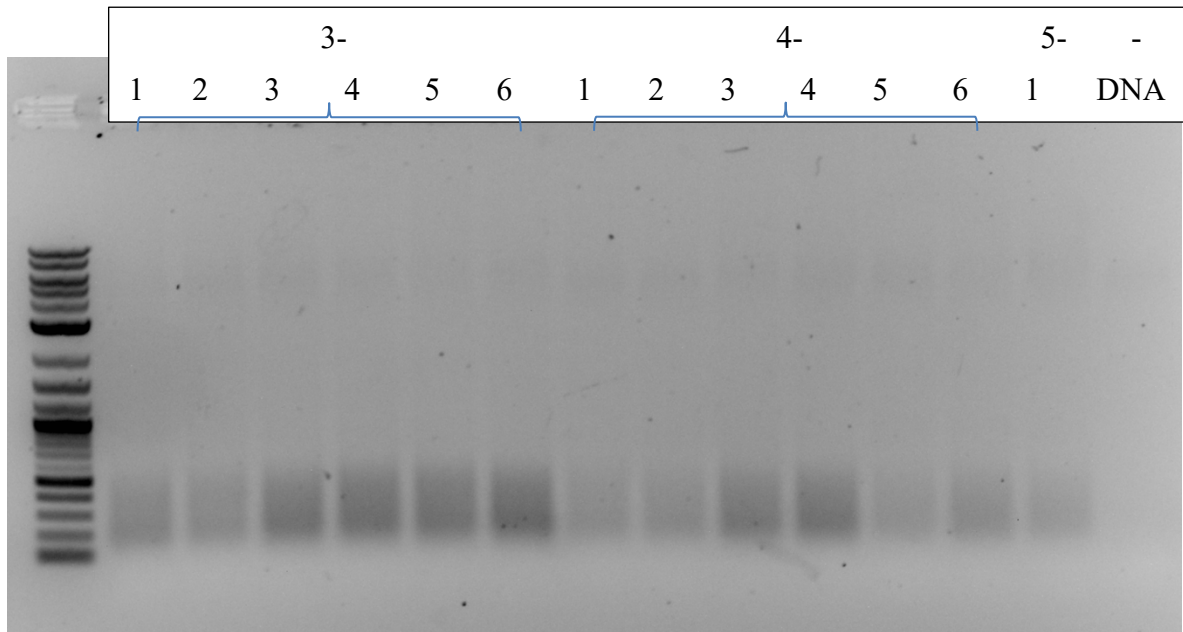
Looks like there is growth on the majority of patches. Check for integration of pKR2 in cells from electroporations 3 & 4 (for which all 6 patches grew). If this integration is positive, then I have more confidence that the cells from other electroporations are actually 1° integrants as well.

Reaction	DNA
1 – 6	Colony PCR from cells from EP3 -> 3-1 through 3-6
7 – 12	Colony PCR from cells from EP4 -> 4-1 through 4-6
13	Negative control- colony PCR from cells from EP5 (5-1)
14	Negative control, no template

Factor

Component	Stock concentration	Final concentration	1 rxn volume	
ddiH2O			4	16.5
KOD buffer	2x	1x	10	66
dNTPs	2 mM	0.4 mM	4	165
KROL1	10 uM	0.3 uM	0.6	66
KROL17	10 uM	0.3 uM	0.6	9.9
template	100 ng/ul	2 ng/ul	0.4	-
KOD	1 U/ul	0.02 U/ul	0.4	6.6
Total volume			20	330

STN1 with 1' extension
Run entire PCR on gel:



If pKR2 had integrated properly, we would expect to see amplification of a band at 765 bp. No hint of such a band, and the smearing pattern is consistent in all lanes with cells (including cells lacking the pKR2 plasmid). This indicates that these are not the correct integrants. [Side note- what the heck are these KanR cells, then?!?!]

Need to re-do

Monday, December 10, 2018

To Do:

- a. Pour plates (CHA-Kan, CHA-sucrose)
- b. Streak strains for:
 - a. More EC ΔFTL_1548
 - b. 1° pKR3 integration, plating on sucrose
 - c. ΔrelAΔspoT for glycerol stock for Jerry Nau

Tuesday, December 11, 2018

To Do:

- a. Electroporations

- a. ~~pKR1&2 into LVS~~
- b. ~~pKR1, 2, 3 into LVS Δ FTL_1548~~
- b. ~~Plate LVS 1° pKR3 integrations on sucrose~~

Electroporations:

Number	Strain	Plasmid	Notes
10	LVS	pKR1	
11	LVS	pKR1	
12	LVS	pKR2	
13	LVS	pKR2	
14	LVS Δ FTL 1548	pKR1	
15	LVS Δ FTL 1548	pKR1	Arced
16	LVS Δ FTL 1548	pKR2	
17	LVS Δ FTL 1548	pKR2	
18	LVS Δ FTL 1548	pKR3	
19	LVS Δ FTL 1548	pKR3	Arced

Perform EP10-13 (already have EC LVS). Recover in 4 mL MH from 11:50 – ~4:30 (~5.5 hours), plate each electroporation on 2 CHA-Kan plates

Make EC LVS Δ FTL_1548 from plate struck yesterday. Enough for 5 vials of 110 uL.

Resuspend some amount of cells from the LVS 1° pKR3 integrants (6-1, 6-2, 6-3, 6-4) in 1x PBS. Dilute ~1:10 serially in 5 tubes (1.5 mL microfuge, 30 ul in to 270 ul). Plate 100 uL of each dilution on CHA-10% sucrose plates poured yesterday, dilutions 10^{-1} – 10^{-5} .

Perform EP14-19. Recover in 4 mL MH from 1:30 - ~5:30 (~4 hours), plate each electroporation on 2 CHA-Kan plates.

Wednesday, December 12, 2018

To Do:

- a. ~~Make Δ relA Δ spoT for glycerol stock for Jerry Nau~~

Thursday, December 13, 2018

To Do:

- a. ~~Check for colonies on electroporation plates~~
- b. ~~Check for colonies on sucrose counter-selection plates~~
- e. ~~Make more sucrose plates~~
- d. Re-plate 1° integrants on sucrose, include last dilution on CHA only

No visible colonies on electroporation plates yet (AM).

Sucrose counter-selection plates- lawn to almost a lawn up to the 10^{-5} dilution plates. Doesn't look good. Try to re-make the plates and re-plate dilutions, include plating last dilution on CHA only plates. Re-made plates, but too late to let them dry and re-plate. Re-plate tomorrow.

Friday, December 14, 2018

To Do:

- Check for colonies on electroporation plates
- Check for colonies on sucrose counter-selection plates
- Re-plate 1° integrants on sucrose, include last dilution on CHA only

Electroporation results:

Number	Strain	Plasmid	Notes	Colonies
10	LVS	pKR1		0
11	LVS	pKR1		3
12	LVS	pKR2		5
13	LVS	pKR2		1
14	LVS Δ FTL 1548	pKR1		0
15	LVS Δ FTL 1548	pKR1	Arced	0
16	LVS Δ FTL 1548	pKR2		0
17	LVS Δ FTL 1548	pKR2		1
18	LVS Δ FTL 1548	pKR3		1
19	LVS Δ FTL 1548	pKR3	Arced	24 (!?)

Re-plate LVS pKL3 1° integrants on sucrose plates. Resuspended some cells in 270 μ L 1x PBS. Diluted 1:100 in next tube, 1:100 again (10 / 990 μ L 1x PBS), then 1:10 for 10^{-5} – 10^{-7} . Plated 100 μ L of 10^{-4} – 10^{-7} . Also managed to find a single colony on the old sucrose plates- cross-patched to CHA and CHA-Kan; see if the sucrose (particularly with that cell density) was at all selective.

Saturday, December 15, 2018

To Do:

- PCR for 1° integration of pKR2

Looks like there is growth for lots of patches! Check for integration of pKR2 in cells from electroporations 12, 13, & 17. If this integration is positive, then I have more confidence that the cells from other electroporations are actually 1° integrants as well.

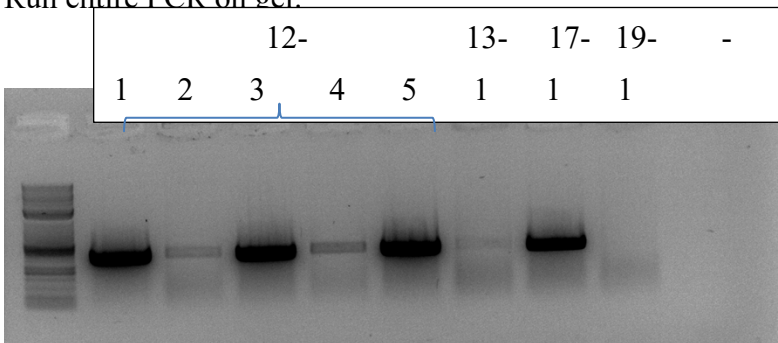
Reaction	DNA
1 – 5	Colony PCR from cells from EP12 -> 12-1 through 12-5
6	Colony PCR from cells from EP13 -> 13-1
7	Colony PCR from cells from EP17 -> 17-1
8	Negative control- colony PCR from cells from EP11 (11-1)

9	Negative control, no template
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Component	Stock concentration	Final concentration	1 rxn volume	Factor
				11
ddiH2O			4	44
KOD buffer	2x	1x	10	110
dNTPs	2 mM	0.4 mM	4	44
KROL1	10 uM	0.3 uM	0.6	6.6
KROL17	10 uM	0.3 uM	0.6	6.6
template	100 ng/ul	2 ng/ul	0.4	indiv
KOD	1 U/ul	0.02 U/ul	0.4	4.4
Total volume			20	220

STN1 with 1' extension

Run entire PCR on gel:



Confirm integration with KROL1 & KROL17 = 765 bp if integrated (no product otherwise)
 Looks great! It's quite clear that at least 12-1, 12-13, 12-5, and 17-1 have the expected integration of pKR2!

pKR2 is a single-integration inactivation plasmid; these cells now should have inactive versions of FTL_1251, which encodes a transporter we think is involved in utilization of glutathione as a cysteine source.

Other electroporations:

Still no colonies on EP14 & 15 plates, LVS ΔFTL_1548 pKR1

Patches:

LVS pKR1 11-1, 11-2, 11-3 : all grew, 11-1 and 11-3 more robust growth.

Freeze down single vial of all 3 1° integrants and patch out 11-1 and 11-2 onto CHA

LVS ΔFTL_1548 pKR3 18-1, 19-1 -> 19-5; all grew, 18-1 and 19-4 less robust growth

Freeze down single vial of: 18-1, 19-1, 19-2, 19-4 and patch out 18-1 and 19-1 onto CHA

Tuesday, December 18, 2018

To Do:

- a. PCR for crossout of pKR3 in cells from EP6

Reaction	DNA
1 – 8	Colony PCR from cells from 6-1-1 -> 6-1-8
9 - 16	Colony PCR from cells from 6-2-1 -> 6-2-8
17 – 24	Colony PCR from cells from 6-3-1 -> 6-3-8
25 – 32	Colony PCR from cells from 6-4-1 -> 6-4-8
33	LVS gDNA
34	pKR3 (1:10)
35	- DNA

Total reaction volume	20
Total number of reactions	35

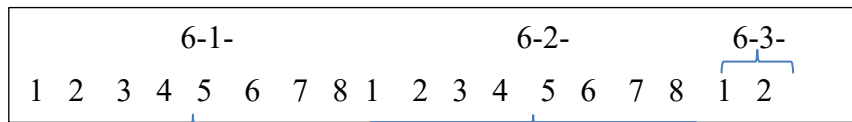
Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH2O			4	39.6
KOD buffer	2x	1x	10	158.4
dNTPs	2 mM	0.4 mM	4	396
KROL12	10 uM	0.3 uM	0.6	158.4
KROL13	10 uM	0.3 uM	0.6	23.76
template	100 ng/ul	2 ng/ul	0.4	23.76
KOD	1 U/ul	0.02 U/ul	0.4	indiv
Total volume			20	15.84
				792

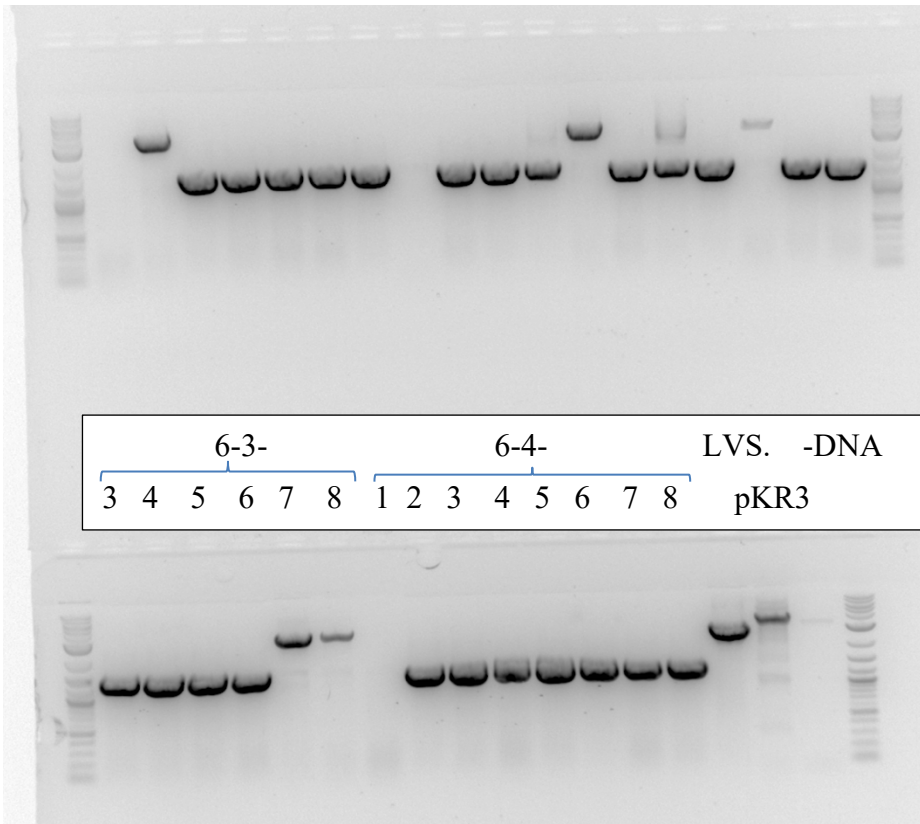
Confirm deletion with KROL12 and KROL13 = 3260 bp in WT, 1481 bp in ΔFTL_0766

Wednesday, December 19, 2018

To Do:

- a. Run PCR to check for Δggt strains (pKR3)





Looks like there are plenty of FTL_0766 deletion mutants! Pick 6-1-3, 6-2-1, 6-3-1, 6-4-2, streak to single colonies

Thursday, December 20, 2018

To Do:

- a. —Electroperations

Friday, December 21, 2018

To Do:

- a. —Pick single colonies of potential Δ ggt mutants, patch to CHA and CHA-Kan
- b. —PCR to check for crossout of pKR1 and pKR3

Have patches from:

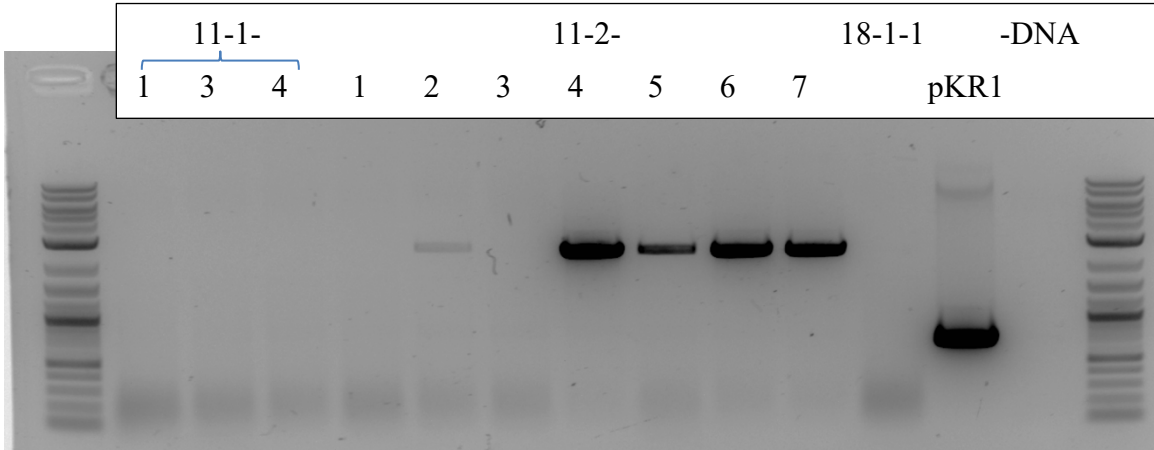
LVS pKR1 – to make Δ FTL_1251 EP 11-1-1, 11-1-3, 11-1-4 (remaining 5 didn't grow)
 EP 11-2-1 -> 11-2-7 (11-2-8 didn't grow)

LVS Δ FTL_1548 pKR3 – to make Δ FTL_1548 Δ ggt EP 18-1-1, 18-1-3 -> 18-1-8 (18-1-2 didn't grow)
 EP 19-1-1, 19-1-3 -> 19-1-8 (19-1-2 didn't grow)

Check by PCR:

To confirm Δ FTL_1251, use KROL1 and KROL5 = 2411 bp in WT, 766 bp in Δ FTL_1251, have 10 samples plus negative control (18-1-1), pKR1 (1:10), - DNA = 13 samples

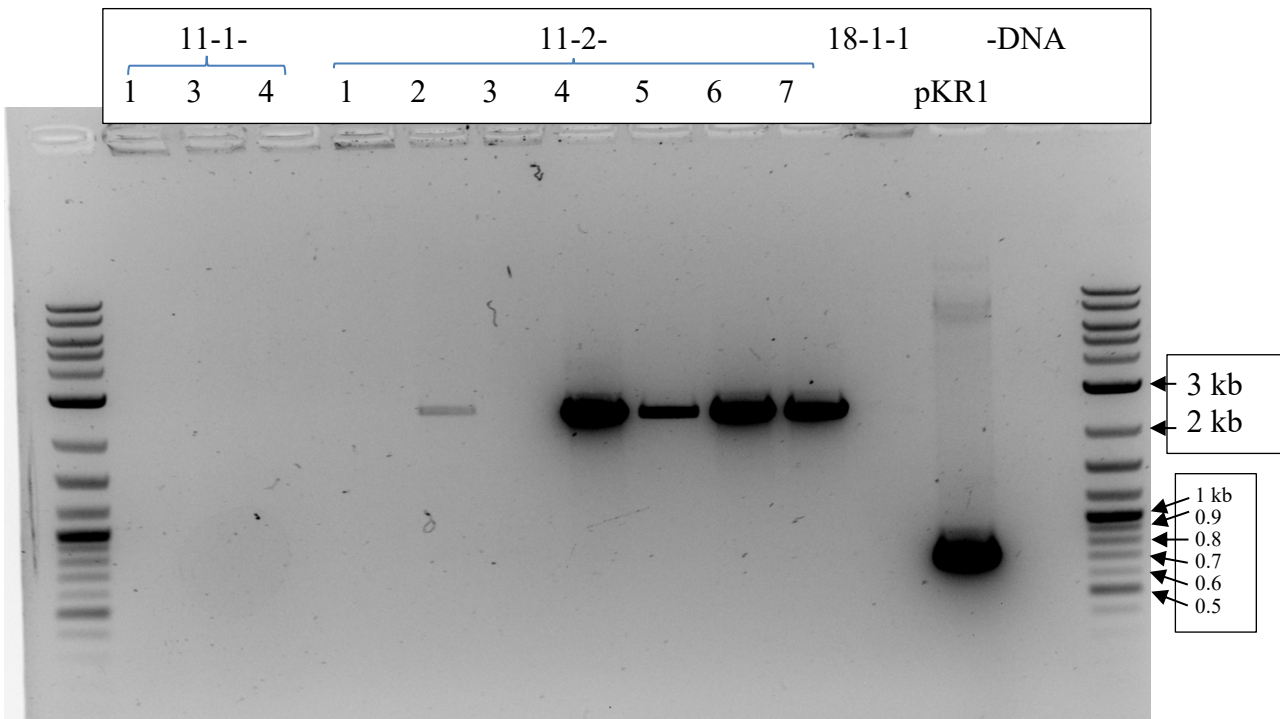
To confirm Δ ggt, use KROL12 and KROL13 = 3260 bp in WT, 1481 bp in Δ FTL_1251, have 14 samples plus negative control (11-1-1), pKR3 (1:10), - DNA = 17 samples



Clearly this PCR is not diagnostic. Expected 2411 bp in WT; Could be seeing WT in:
 11-2-2, 4, 5, 6, 7

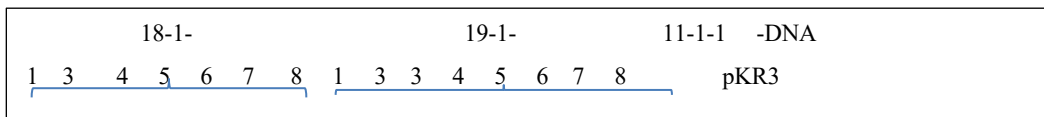
But there is no band in the other lanes- should be either WT or deletion (at 766 bp). And there shouldn't be amplification from the plasmid DNA!

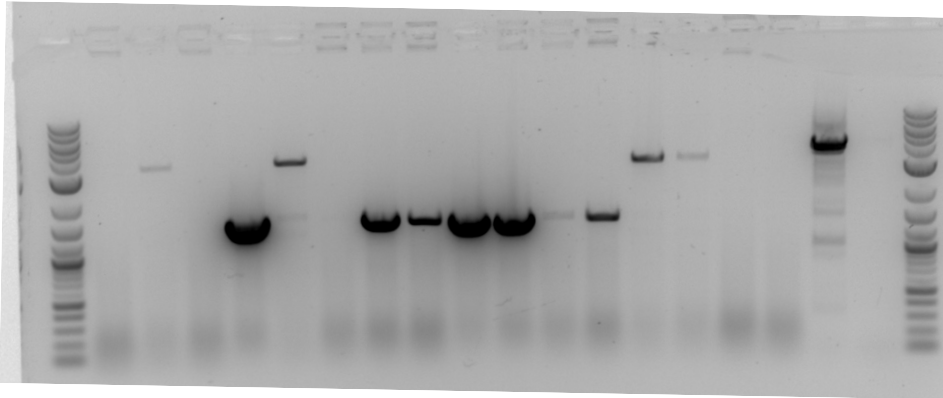
After being run further:



The bands in 11-2-2, 4, 5, 6, 7 are all running in a location consistent with 2411 bp
 The band in pKR1 could be running near the deletion size (766 bp) although the primer KR0L5 shouldn't be able to anneal to pKR1! ☹

Try a different PCR tomorrow- maybe 18 and 5, also 4 and 18?





Some missing amplification here, too. ☹

But, can clearly see WT clones are 18-1-3, 18-1-6, 19-1-6, 19-1-7

Deletion strains are 18-1-5, 18-1-8, 19-1-1, 19-1-3 (amplified 2x accidentally), 19-1-4 (?), 19-1-5

Streak out to single colony:

18-1-5, 18-1-8, 19-1-1, 19-1-3, 19-1-5

Saturday, December 22, 2018

To Do:

- a. PCR to check for crossout of pKR1 and pKR3

Reactions

Reaction Set	Primers	Target	WT size	Deletion size	Number of expt. samples	Number of control samples
A	KROL12, KROL13	ggt locus	3260 bp	1481 bp	10	3
B	KROL18 KROL5	FTL 1251 locus	2462 bp	674 bp	10	3
C	KROL18 KROL4	FTL 1251 locus	2425 bp	637 bp	10	3

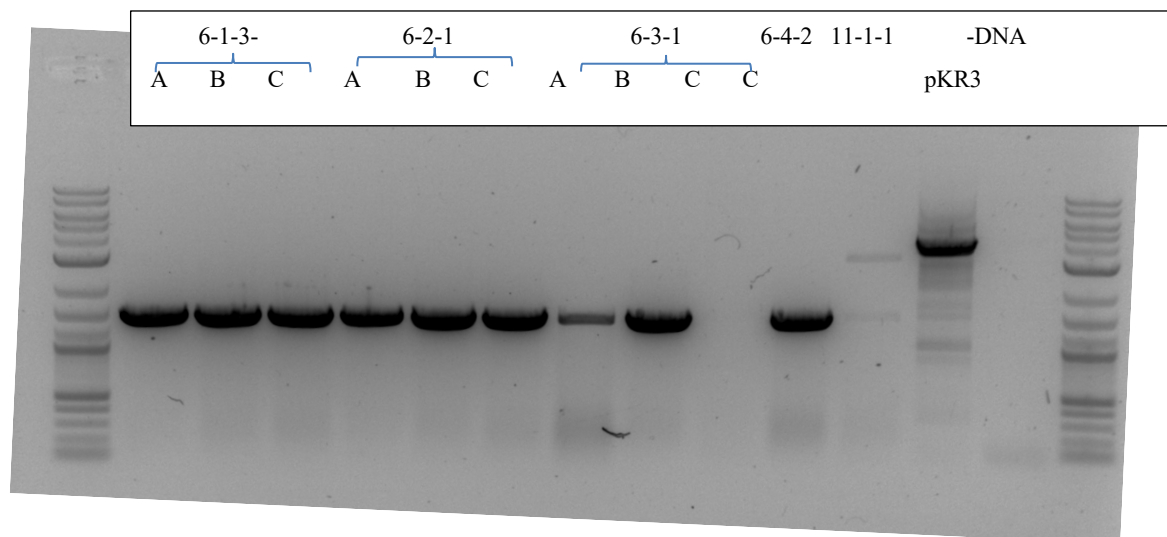
Total reaction volume	20
Total number of reactions (A, B, C)	13
Number of master mixes	3

Component	Stock concentration	Final concentration	1 rxn volume	Factor	A, B, or C
ddiH2O			4	61.5	19.5
KOD buffer	2x	1x	10	246	358.8
dNTPs	2 mM	0.4 mM	4	615	
KOD	1 U/ul	0.02 U/ul	0.4	24.6	
oligo F	10 uM	0.3 uM	0.6		
oligo R	10 uM	0.3 uM	0.6		11.7
template	100 ng/ul	2 ng/ul	0.4		indiv
Total volume			20	1131.6	382.2

Reaction	Sample number	Sample
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A	1-3	EP 6-1-3 A->C
A	4-6	EP 6-2-1 A->C
A	7-9	EP 6-3-1 A->C
A	10	EP 6-4-2 C
A	11	EP 11-1-1
A	12	pKR3
A	13	-DNA
B	1-3	EP 11-1-1, 3, 4
B	4-10	EP 11-2-1 -> 7
B	11	EP 6-1-3 A
B	12	pKR1
B	13	-DNA
C	43103	EP 11-1-1, 3, 4
C	43200	EP 11-2-1 -> 7
C	11	EP 6-1-3 A
C	12	pKR1
C	13	-DNA

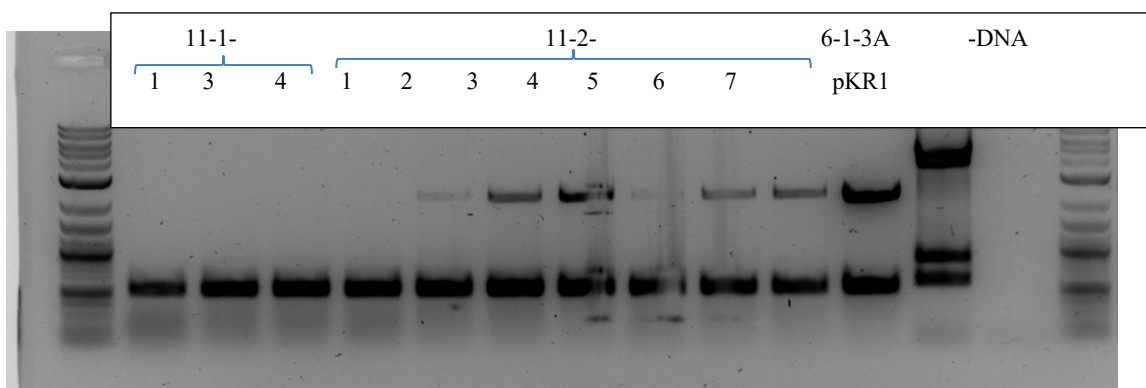
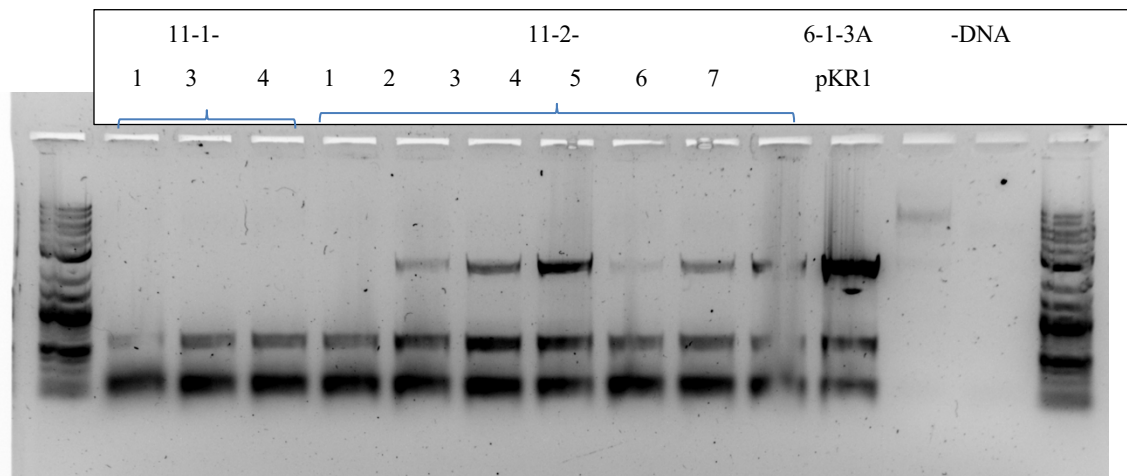
EP6 = 10 patches



All look correct! Not sure why the 11-1-1 band is so faint this time; there is a faint band at the deletion size as well, but that could be from using the same tip to load- the previous band is very intense!

Freeze down:

KRLVS



Tomorrow: PCR to check EP 18, 19 for crossout

Wednesday, December 26, 2018

To Do:

- a. PCR to check for crossout of pKR3
- b. ~~Pour new CHA plates~~

Cells didn't grow well at RT; re-streak onto new CHA

Thursday, December 27, 2018

To Do:

- c. PCR to check for crossout of pKR3

Reactions

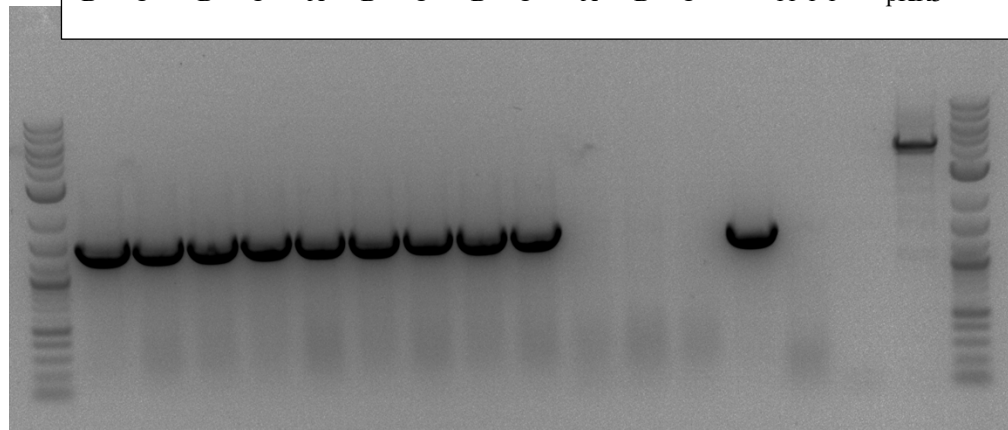
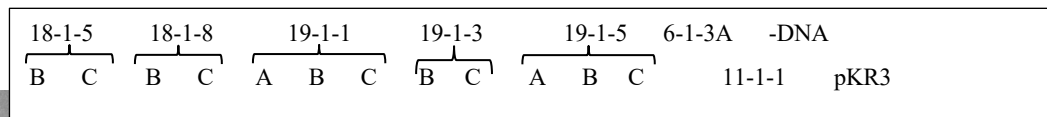
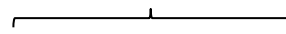
Reaction Set	Primers	Target	WT size	Deletion size	Number of expt. samples	Number of control samples
A	KROL12, KROL13	ggt locus	3260 bp	1481 bp	12	3

Total reaction volume	20
Total number of reactions (A)	

Component	Stock concentration	Final concentration	1 rxn volume	Factor
				18
ddiH2O			4	72
KOD buffer	2x	1x	10	180
dNTPs	2 mM	0.4 mM	4	72
KOD	1 U/ul	0.02 U/ul	0.4	7.2
oligo F	10 uM	0.3 uM	0.6	10.8
oligo R	10 uM	0.3 uM	0.6	10.8
template	100 ng/ul	2 ng/ul	0.4	
	Total volume		20	360

STN1 with 3:20' extension

Reaction	Sample number	Sample
A	1,2	EP 18-1-5 B->C
A	3,4	EP 18-1-8 B->C
A	5-7	EP 19-1-1 A->C
A	8,9	EP 19-1-3 B->C
A	10-12	EP 19-1-5 A->C
	13	EP 6-1-3A (+)
A	14	EP 11-1-1 (-)
A	15	pKR3
A	16	-DNA



To purchase:

Waterbath

Power strips

Tissues

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.