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DNA Ladder


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January 2022

pKR95 Cloning Plan

1. Run a 100 ul PCR:

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	Flanking Region 1	SA gDNA	KROL419, 420	1015
2	Flanking Region 2	SA gDNA	KROL421, 422	969
3	- control	-	KROL421, 422	-

2. PCR purify samples 1 and 2, elute in 35 ul 0.1xEB; hold onto sample 3 in -20C
3. Digest for ~ 1hr at 37C, total volume of 30 ul:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	Purified PCR 1 (FR1)	KpnI, NotI	15	-
2	Purified PCR 2 (FR2)	NotI, BamHI	15	-
3	Backbone (pIMAY-Z)	BamHI, KpnI	5	10

4. Run entire digest on 1% agarose gel SKIPPING LANES BETWEEN SAMPLES. Also run negative control from PCR
5. If band are appropriate sizes (pIMAY-Z should be ~8.7kb), cut out with razor blade and gel purify with QIAgen kit, eluting in 35 ul of 0.1xEB

6. Ligate overnight, 20 ul, at 16C. Adjust water so that each insert is 4 ul:

Tube	Insert 1	Insert 2	Backbone
1	Gel purified FR1	Gel purified FR2	Gel purified pIMAY-Z
2	-	-	Gel purified pIMAY-Z

7. Transform into E. coli IM08B. Use pIMAY-Z from the plasmid box as a positive control. Plate on LB-chloramphenicol
8. Check plates. If ratios of ligation 1 and ligation 2 look good (>2-fold), pick single colonies into LB-chlor and grow at 37C overnight. The next day, miniprep.
9. Send minipreps out for sequencing.

Jan 31, 2022

- First day back in lab; worked on my honors project following the cloning plan Hannah made
  - Made 15 LB-Chloramphenicol plates for the *E. coli* transformation (made them early so they would be ready)
    - Ended up making the LB-agar with too high of a concentration of chloramphenicol (100 ul/mg)
    - Hannah made me new plates with correct concentration
  - Performed a 100 ul PCR on the SA gDNA using PCR protocol
    - SA gDNA that Hannah made was only 73 ng/ul so we had to accommodate for that

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	Flanking Region 1	SA gDNA	KROL419, 420	1015
2	Flanking Region 2	SA gDNA	KROL421, 422	969
3	- control	-	KROL421, 422	-

- Using the primer table above, I ran three reactions following the Primestar mix calculations
  - I messed up the first round of the PCR (used wrong primer volumes for the tubes; Accidentally pipetted the master mix volumes into the tubes instead of the 1 reaction volumes) so we do not have a lot of the gDNA from *S. aureus* left.

Total reaction volume	100
Total number of reactions	3

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	4
ddiH2O			61.3	245.
PrimeSTAR GXL Buffer	5x	1x	20.0	80
dNTPs	2.5 mM	0.2 mM	8.0	32
oligo F	10 uM	0.3 uM	3.0	12
oligo R	10 uM	0.3 uM	3.0	12
template	73 ng/ul	2 ng/ul	2.7	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	8
		Total volume	100	389.0

February 2022

Feb 2, 2022

- Ran a digest for the PCR from Jan 31st, following digest protocol exactly
    - Ran PCR purification beforehand for tubes 1 and 2 (FR1 and FR2)

DNA Digests Protocol

- Add indicated amounts of H_2O and 10x buffer to the master mix tube (MM).
- Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
- Add the indicated amount of each enzyme (__uL) to the master mix tube (MM).
- Mix the master mix by pipetting up and down.
- Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (___ul).
- Incubate at 37°C for 1 hour or up to overnight.
- If using a digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to the backbone tube, mix by pipetting and put back in the 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-

ligation). If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.

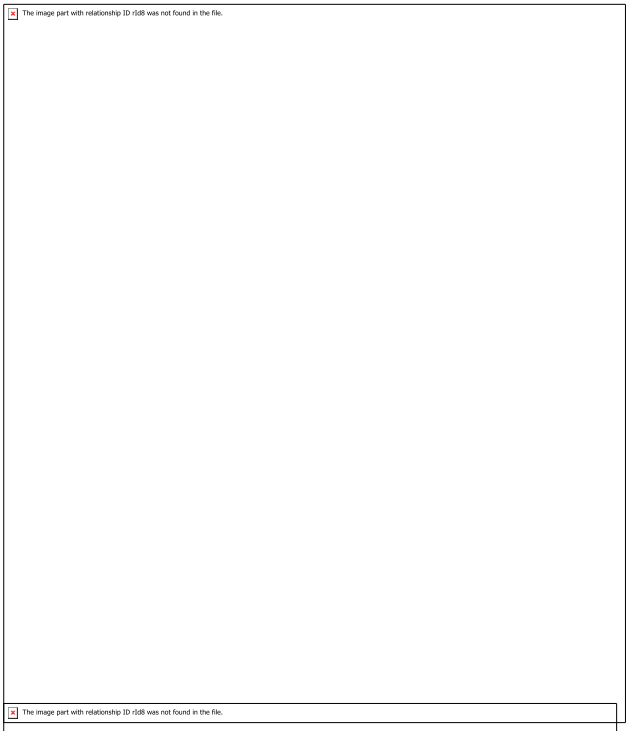
- Tube one: SA gDNA Flanking region 1
- Tube two: SA gDNA Flanking region 2
- Tube three: pIMAY-Z backbone

For cloning:

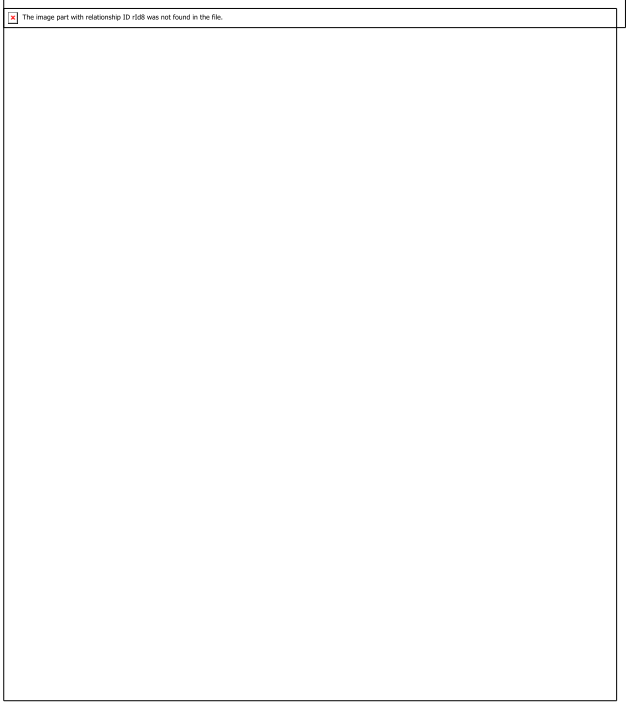
Number of samples	**3**
Master mix factor	**4**

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix (uL)
H_2O	10.8	43.2
10x Buffer*	3	12
DNA	(15)	
Enzyme 1	0.6	
Enzyme 2	0.6	
Total	30.0 (15.0 actual b/c of DNA)	55.2

- The table above was the mixture for each tube
  - Master mix contained only the water and buffer
  - Tube 1 (FR1) used KpnI and NotI + 15 ul of the PCR product
  - Tube 2 (FR2) used NotI and BamHI + 15 ul of the PCR product
  - Tube 3 (pIMAY-Z) used KpnI and BamHI + 5 ul pIMAY-Z + 10 ul H2O
- Ran DNA digests on a gel with the negative control from January 31st
  - Lane 1: Ladder
  - Lane 2: DNA Digest 1 (SA gDNA FR1) ~1015 bp
  - Lane 3: DNA Digest 2 (SA gDNA FR2) ~968 bp
  - Lane 4: DNA Digest 3 (pIMAY-Z backbone) ~8.7 kb
  - Lane 5: (-) control from PCR
  - Accidently forgot to do the CIP protocol so the ladder was inserted into the dye an hour before the other lanes were filled (flushed most of the ladder out though)



- I accidently put in 10 ul of the 6x loading dye instead of the ladder so it was not visible in this picture although Hannah and I agreed the bands seemed to be in the correct position so we went and cut them out for the gel extraction
  - Lane 2 was supposed to be around 1015 bp
  - Lane 3 was supposed to be around 969 bp
  - Lane 4 was supposed to be around 8,700 bp (we only used 5 ul of the backbone and it was very light so we may use 15 ul next time)
  - Lane 5 was supposed to have no bands



- This gel is after we removed the bands with the razor blade; the ladder is much more visible

Feb 3, 2022

- Performed a gel extraction for the bands obtained from the digest gel ran on Feb 2

QIAquick Gel Extraction

  - Weigh out the gels taken from the digest gel electrophoresis

        - FR1 was 0.53g; too big, so Hannah split the fragment into two different tubes, weighing 0.31g and 0.16g
        - FR2 was 0.33g
        - Backbone was 0.32g
            - All volumes were too big for 1.5ml tubes, had to transfer to 2ml tubes
    - Add three volumes buffer QG to 1 volume gel in each tube
        - FR1a - 930 ul FR1b - 480 ul
        - FR2 - 990 ul
        - BB - 960 ul
    - Incubate at 42 degrees for 10 minutes, vortexing every 2-3 minutes
        - Turned on heat block to too high a temperature during melting step for about 6 minutes, might have ruined the digest, we will see after transformation
    - Once gel has dissolved, add one volume 100% isopropanol to each tube
        - FR1a - 310 ul FR1b - 160 ul
        - FR2 - 330 ul
        - BB - 320 ul
    - Begin to transfer the volumes from each tube to the spin columns, 750ul at a time; spin at 13,000 rpm for 1 minute, discarding the flow through after
        - Had to spin each reaction multiple times since there was too much for the spin columns to hold
    - Add 500ul buffer QG to each spin column and spin for another minute, discarding the flow through
    - to wash, add 750 ul buffer PE to the column and spin for 1 minute, discarding the flow through; to removes residual buffer, spin for 3 minutes after wash
    - Place spin column in clean 1.5 ml tube, adding 35 ul 0.1x EB to elute DNA, let stand for 1 minute, then spin for 1 minute

Feb 7, 2022

- Performed ligation reactions on the gel extraction samples from the 3rd

Tube	Insert 1	Insert 2	Backbone
1	Gel purified FR1	Gel purified FR2	Gel purified pIMAY-Z
2	-	-	Gel purified pIMAY-Z

	Number of samples	**2**
	Master mix factor	**3**
Components	**Volumes in 1 reaction (uL)**	**Volumes in Master Mix (uL)**
H2O	7.5	22.5

10x ligase buffer	2.0	6
Insert	8.0	
Backbone	2.0	6
Ligase	0.5	1.5
Total	20	36

Ligation Protocol

1. Obtain ice to assemble and keep the reactions on. This is important, as the reaction happens at 16˚C and the ligase buffer (which contains ATP) needs to be kept cold in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
4. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Place in the thermocycler overnight at 16°C.
    a. We left the ligation reactions out for 10 minutes at room temperature instead of overnight so we could perform the transformation right after

Chemical Transformation in E. coli *Protocol*

Tube #	Purpose	DNA	Volume of DNA	Final Volume to Plate	# of chlor plates
1	(+) control		1 ul	20 ul, 100 ul	2
2	(-) control		0 ul	20 ul, 100 ul	2
3	Ligation 1		8 ul	100 ul, remaining	2
4	Backbone Ligation		8 ul	100 ul, remaining	2

1. Obtain DNA and thaw on ice if necessary.
    - I did not vortex the pIMAY-Z backbone (straight from big -20) which Hannah said is very important
        i. All the plasmid backbone was on bottom of tube
2. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)

3. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
4. 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.
    - I added DNA first and gently added the cells, but pipetted to mix (slowly and gently) which I should not have done
5. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
6. Place tubes with cells and DNA onto a 42°C heat block for 30 seconds (heat shock step).
    - Made sure I did only 30 seconds, used timer to check
    - I added first tube at 0 seconds, added second at 10 seconds, third at 20 seconds, and took the first tube off at 30 seconds, second off at 40 seconds, third off at 50 seconds, and finally added fourth tube at 60 seconds and removed at 90 seconds (should just add two at a time next time, one pair at 0 seconds and second pair within 5 seconds)
7. After heat shock, place tubes back on ice until the next step (don't keep them here too long).
    - On ice for less than 3 minutes
8. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
9. Using autoclave tape, tape microfuge tubes down in a shaking incubator set to 37°C. (save the tape!)
10. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
11. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in a 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.
    - Should add beads before pipetting culture onto the plate next time.

- We used LB - chloramphenicol plates instead of LB-Kan plates
    - IMPORTANT - was talking to Hannah when plating the tubes, might have plated tube #3 on the 100 ul negative control plate by accident; compared the volumes in all tubes, did not seem like I messed up since tubes 3 and 4 had similar volumes and tubes 1 and 2 were also similar
    - In the future, vortex the plasmid before using it for the positive control
- I also calculated the amounts of certain things we need for the beta-gal on Wednesday; we have enough of everything, just need to make more before next beta-gal
    - Z-buffer - 23 ml
    - BME - 62.56 ul
    - 1% SDS - 0.78 ml
    - Na2CO3 (stop) - 11.5 ml
    - ONPG - 5.2 ml

Plasmidsaurus Link

https://www.plasmidsaurus.com/instructions/

- For when I do the PCR with the primers (KROL 493 and KROL 494): Wildtype should be 2159 and the delta_rpsu 2000
    - Primers:
        - KROL 493 drpsU_SA_ck_F
            - taaaagattataacttggcccaattatta

        - KROL 494 drpsU_SA_ck_R
            - acttaaactaaacatggtatcgctcc

Feb 8, 2022

- Helped prepare for tomorrow's beta-gal assay
- Aisling added 6 ml to 12 different test tubes
    - I prepared 12 sterile 1.5 microcentrifuge tubes with 500 ul MHB
    - 12 cuvettes with 950 ul MHB
        - Both went into the fridge

- ***Beta-Gal 2/9 Assay Prep:***
    - WARM EVERYTHING UP AT 37 DEGREES
    - Loop and scrape up all cells from designated patches on the plates
        - Label which patches were used on the back of the plates
    - Add the cells to the 1.5ml sterile tubes filled with 500ul MHB from earlier
        - Use blue pipette set at 400ul to resuspend cells, vortex if clumps still remain
    - Take 50ul of the sample tubes and add to empty cuvettes that have 950ul MHB already added
        - Cover with parafilm and invert 3x to mix
    - Check the OD600 (using the prepared blank)
    - Use spreadsheet to calculate V1
        - C1V1=C2V2
        - delta_rpsu strains should be 0.13
        - LVS strains should be 0.10
            - If OD600 is not high enough, repeat previous steps until high enough (using all cells from patches so should be high enough)
    - Add calculated V1 to the 6ml MHB tubes Aisling prepared
    - Take 500ul from the 6ml tubes, gently swirling to mix, and add to the empty cuvettes
    - Put the tubes into the shaker for around 3.5 hours, starting timer counting up
    - Check OD600
- **COVID Exposure**
    - To all that read this, I sincerely apologize for not being able to be there, it was out of my control and I really don't want anyone to get themselves or others sick because I did not speak up about an exposure

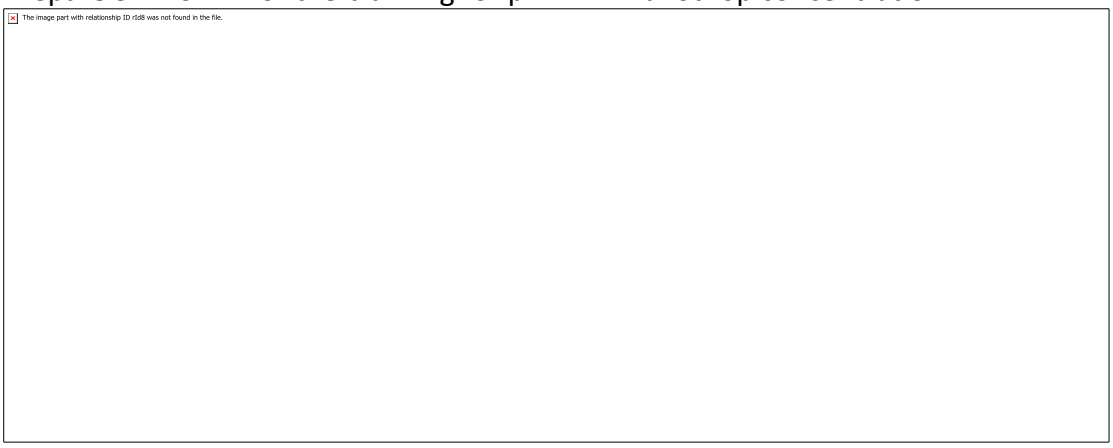
Feb 10, 2022

- I tested negative! Aisling successfully completed the beta-gal with help from Hannah
- The two control plates from the transformation grew colonies so they were parafilmed by Hannah so I will calculate the transformation efficiency
- The ligation plates did not grow (Hannah left them out in room temp after no growth was seen) so I will redo them starting at the digest step
    - I will use 15 ul of the backbone for the digest this time, making sure the concentration is at least 70 ng/ul
- Primers came so I will get them ready at some point, maybe today when I go in

Receiving Primers Protocol

By: Hannah Trautmann

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so dessicated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for the intended purpose of the primer.
    a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in the appropriate freezer box.
    b. If a sequencing primer, make a 2.5 uM stock by diluting 5 uL of the 100 uM stock into 195 uL of 0.1x EB. Label with a blue sticker and put in the appropriate freezer box.
7. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

- I made the 100 mM and 10 mM aliquots for primers KROL493 and KROL494
- Packed up the plate bin for the autoclave
    - Going to wait for another bag before taking down to the autoclave
- For tomorrow:
    - Prepare 5 ml of TE for the blanking for pIMAY-Z nanodrop concentration
    - 
    - 50 ml ratio calculations are:
        - 1M Tris-HCL 500 ul
        - 0.5M EDTA 100 ul
        - ddH2O 49.4 ml
    - Redo recent experiments starting with the DNA Digest from the PCR purification

Feb 11, 2022

- Redoing the digest from Feb 2nd
- Made the 50 ml 0.1x TE buffer to measure concentration of the pIMAY-Z plasmid for the digest (needs to be ~70 ng/ul) and for the transformation efficiency calculation
    - 49.8 ng/ul

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	Purified PCR 1 (FR1)	KpnI, NotI	15	-
2	Purified PCR 2 (FR2)	NotI, BamHI	15	-
3	Backbone (pIMAY-Z)	BamHI, KpnI	25.8	-

- Using 25.8 ul of the backbone this time instead of 5 ul
- The following table is just for the back bone, Hannah calculated how much backbone we'd need to use for the digest (no master mix, just one reaction tube)

Components	**Volumes in 1 reaction (uL)**	**Volumes in Master Mix (uL)**
H2O	0	
10x Buffer*	3	
BackBone	25.8	
Enzyme 1	0.6	
Enzyme 2	0.6	
Total	30	

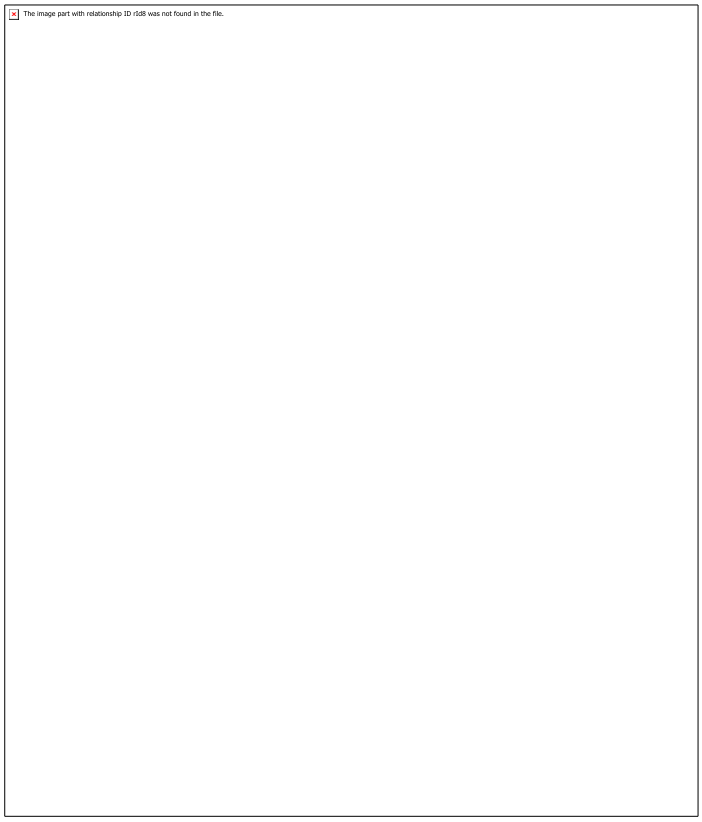
- The two inserts can be made with a master mix following the restriction enzyme worksheet calculator

For cloning:		
	Number of samples	**2**
	Master mix factor	**3**
Components	**Volumes in 1 reaction (uL)**	**Volumes in Master Mix (uL)**
H2O	10.8	32.4
10x Buffer*	3	9
DNA	15	
Enzyme 1	0.6	
Enzyme 2	0.6	
Total	30.0 (15.0 actual b/c of DNA)	41.4

- Following the digest protocol, I let all tubes incubate at 37 degrees for 1 hour

    - The backbone will be treated with QuickCIP to remove phosphates from the ends to prevent joining of the ends
- From the tables:
    - Master mix contained only the water and buffer
    - Tube 1 (FR1) used KpnI and NotI + 15 ul of the PCR product
    - Tube 2 (FR2) used NotI and BamHI + 15 ul of the PCR product
    - Tube 3 (pIMAY-Z) used KpnI and BamHI + 25.8 ul of the pIMAY-Z plasmid
- Ran DNA digests on a gel
    - Lane 1: Ladder
    - Lane 2: DNA Digest 1 (SA gDNA FR1) ~1015 bp
    - Lane 3: DNA Digest 2 (SA gDNA FR2) ~968 bp
    - Lane 4: DNA Digest 3 (pIMAY-Z backbone) ~8.7 kb

Lane	Contents	Expected bp
1	Ladder	—----------
2	DNA Digest 1 (SA gDNA FR1)	1,015
3	DNA Digest 2 (SA gDNA FR2)	968
4	DNA Digest 3 (pIMAY-Z)	8,700



- The gel I ran from the digest looked good; last time the backbone band was really dull, which is why we increased volume of the backbone more than five fold
    - This new band is significantly darker than the previous gel, so it worked
- ***Agarose gel***
    - 5 g agarose
    - 500 ml ddih2o
    - Spun and heated at 300 degrees until clear
    - Updated the tape on agarose flask
- Performed the QIAquick gel extraction to obtain the inserts and backbone from the gel I ran, following the QIAquick gel extraction protocol I have in this notebook
    - Made sure to follow exactly and not touching the heat block settings; the temperature was 42 degrees
- Did not start the ligation since I could not come in tomorrow to put overnight ligation reactions in the freezer, going to do ligation and transformation next week
- ***Transformation efficiency***
    - 100 ul positive control plate from Feb 7th (pIMAY-Z plasmid straight from -20 freezer)
    - TE = colony count/ug/dilution
        - Colonies = 107 (88 small, 14 medium, 5 large)
        - ug = 49.8 ng/ul converted to 0.0498 ug
        - 1.101 ml total, 100 ul of cells = 100/1,101 -> 0.0908
    - TE = 107/0.0498/0.0908 = 2.4 x 10^4 cells per ug
    - Used same equation to calculate the expected amount of transformed colonies where the ug is 20 ng (Hannah wrote “Input 20 ng of DNA + dilution + plating 1 ml/1.1 ml”)
    - 2.4x10^4 = x/20 ng/(1 ml/1.1 ml) -> 2.4x10^4 = x/0.02 ug/0.909
        - x = 2.4 x 10^4 x 0.022 = 528 expected colonies
        - 528 is expecting every last insert is ligated into the backbones; Hannah said 10% is a reasonable expectation so 53 colonies can be expected during a successful transformation

Feb 14, 2022

- Performed the second ligation with a higher backbone concentration

	Sample Name	Desired Ratio	Sample Concentration (ng/ul)
Backbone	pIMAY-Z	1	7.6
Insert 1	FR1	5	10.12
Insert 2	FR2	5	5.48

desired amount of backbone (ng)=	50
ul needed of backbone	6.578947368

Reaction Mix	**ul needed**	BB only control

Backbone	6.58	6.58
Insert 1	2.84	-
Insert 2	5.01	-
T4 Ligase buffer	2.00	2.00
T4 ligase	0.50	0.50
water	3.06	10.92
total	20 ul	20.00

Tube	Insert 1	Insert 2	Backbone
1	Gel purified FR1	Gel purified FR2	Gel purified pIMAY-Z
2	-	-	Gel purified pIMAY-Z

- The concentrations of the digests are as follows:
  - Backbone - 7.6 ng/ul (we expected a concentration around here)
  - FR1 - 101.2 ng/ul (really high; Hannah had not seen this before)
  - FR2 - 54.8 ng/ul (also high)
  - The flanking region concentrations were too high, so I diluted 2 ul of each insert in 18 ul of ddiH2O so the amounts were pipetteable.
- ***Sterilizing the cuvettes***
  - Once ethanol has dried, put in the cancer microwave for 10 minutes
    - Should be preset for 10 minutes on high
  - Put caps back on before removing from microwave and put in the "Ready for EP" cuvette box

Feb 15, 2022

- Performed the transformation of chemically competent *E. coli* cells (IMO8B cells); referred back to the protocol in my notebook (under Feb 7, 2022)

Tube #	Purpose	DNA	Volume of DNA	Final Volume to Plate	# of chlor plates
1	(+) control		1 ul	20 ul, 100 ul	2

2	(-) control	0 ul	20 ul, 100 ul	2
3	Ligation 1	8 ul	100 ul, remaining	2
4	Backbone Ligation	8 ul	100 ul, remaining	2

- Same set up as the transformation done on the 7th, but with the digest/ligations that were done yesterday (8 plates labeled referring to their contents from the table)
    - Higher concentration of backbone and I made sure to learn from the mistakes I made last time
        - I put the gel extraction tubes on the heat block at 42 degrees instead of ~92 degrees
        - Vortexed pIMAY-Z plasmid before adding to the positive control tube
        - All tubes were heat shocked for exactly 30 seconds
- I have class at 2 so Hannah had to plate the transformation tubes on the 8 LB-chloramphenicol plates
    - I labeled the plates and put beads on them, so Hannah just has to pipette the cultures onto the respective plates
- Only the positive control plates grew last time, hoping for the other plates to grow this time
    - Since positive control plates grew, it can be inferred that the chloramphenicol concentration may be ok, but maybe too high since other plates had no growth, will talk to Hannah about it tomorrow.

Feb 16, 2022

- To do list:
    - ~~Check plates~~
    - ~~Make more 50% sucrose (lab task)~~
    - ~~Make more 1X TAE (New)~~
- Looked at all plates that Hannah plated for me yesterday; she added two new controls (10 ul of stock IMO8B and 5 ul IMO8B on no antibiotic)
    - No growth on any of the plates besides the No Antibiotic Control 5 ul
        - There was a lawn (very high cell density), indicating that the chloramphenicol concentration may be too high (25 ug/ml right now) or that the promoter for the Chlor-Resistance gene may not be strong enough
        - Going to wait longer for cells to grow since the positive control plates took two days to grow last time
        - When plating *S. aureus,* we will reduce chlor concentration from 25 ug/ml to 10 ug/ml in the LB plates and in XGAL plates (look up concentration in paper Hannah sent me)
        - Will test those 10 ug/ml chlor plates with the XGAL plates by streaking out with our stock of *S. aureus*
            - XGAL stock is 50 ug/ml, need to dilute before putting into plates
            - There should be no growth on these plates
        - XGAL needs to be protected by light; when making these plates, wrap in tin foil

Making 50% Sucrose

- Combine 360 g of sucrose and enough type I ddiH_2O to make 720 mL.
- Autoclave for 20'.
- Suggested method: in 1 L beaker, add ~100 g increments of sucrose into ~250 mL type I ddiH_2O, heating slightly (below boiling point!) and waiting for each addition to be completely dissolved before adding next mass of sucrose. Measure final volume in graduated cylinder and add type I ddiH_2O until volume is 720 mL.

Making 1X TAE New

- For 2 L:
  - Mix 40 ml 50X TAE into 1,960 ml H2O
    - I added 40 ml 50x TAE to a 1 L graduated cylinder, filled with water until 1 L, poured that into the 1X TAE NEW container, then added another liter of water

Feb 17, 2022

- To do list:
  - ~~Count plates from ligation transformation~~

Counted plates from second ligation transformation

- No growth except on the (+) control plates again
  - 102 on the 100 ul, 2 on the 20 ul
- I may have not been pipetting accurately and this could have been the reason for the failed ligation; the tube for the insert ligation had a slightly higher volume than the backbone ligation tube
  - Need to talk to Kathryn about troubleshooting
  - I should try constructing a set-up which allows me to pipette more easily
    - Tube rack is elevated around eye level so I can see the volumes being pipetted in and out of tip
    - Always double check that the volumes are correct
- Kathryn said to re-do the ligation because they are finicky and need to have exact amounts
  - This IMO8B strain has a lower transformarion efficiency than the *E. coli* strains previously used in lab
  - We are going to do a 3X ligation
- XGAL concentration and volume for plates (both papers sent by Hannah say 100 ug/ml when cultivating *S. aureus,* whereas one paper says 100 ug/ml in *E.coli* and another says 50 ug/ml*)*
- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Melford) was used at 50 µg/ml in *E. coli* and 100 µg/ml in *S. aureus*.
  - For *E. coli*: 250 ml XGAL into 250ml LB broth
  - For *S. aureus*: 500 ml XGAL into 250ml LB broth

Feb 18, 2022

- To do:
  - ~~Make the LB-chlor/XGAL plates~~

- ~~Check my plates~~

- The insert ligation plate had a large colony on it, but the backbone ligation plate had a small colony so there's a high chance the backbone religated
  - I kept the insert ligation plate just in case it does have a successful ligation; I am still going to re do the ligation though

LB-chlor/XGAL plates

- 500 ml LB (which I stupidly let boil over)
- Chlor stock concentration is 50 mg/ml

XGAL stock concentration is 50 mg/ml

- After calculations, I will add 100 ul of chloramphenicol and 1000 ul XGAL to the 500 ml of LB (used the C1V1=C2V2 equation to obtain proper volumes)
- XGAL is sensitive to light so the plates need to be wrapped in foil once they solidified
  - While plates were setting, I kept them under a box
  - Also had the LB flask wrapped in foil while it was mixing and when I poured the plates
  - Wrapped the plates in stacks of six (24 plates total)

Feb 22, 2022

- To do list:
  - ~~3X and 5X ligation on LB-chlor/XGAL plates~~
  - ~~Put plates into the fridge~~

Third Ligation with 3X Ligation

The desired amount of backbone was reduced from 50 ng to 40 ng so I would have enough gel purified backbone for all three reaction tubes

ul needed of backbone 5.26 ul

Tube	Insert 1	Insert 2	Backbone
1 (BB ligation)	-	-	Gel Purified pIMAY-Z
2 (3X ligation)	Gel Purified FR1	Gel Purified FR2	Gel Purified pIMAY-Z
3 (5X ligation)	Gel Purified FR1	Gel Purified FR2	Gel Purified pIMAY-Z

- 3X ligation

	Sample Name	Desired Ratio	Sample Concentration (ng/ul)	size (kb)
Backbone	pIMAY-Z	1	7.6	8815
Insert 1	FR1	3	10.12	1015
Insert 2	FR2	3	5.48	969

Reaction Mix	**ul needed**
Backbone	5.26
Insert 1	1.37
Insert 2	2.41

T4 Ligase buffer	2.00
T4 ligase	0.50
water	8.46
total	20 ul

- 5X ligation

	Sample Name	Desired Ratio	Sample Concentration (ng/ul)	size (kb)
Backbone	pIMAY-Z	1	7.6	8815
Insert 1	FR1	5	10.12	1015
Insert 2	FR2	5	5.48	969

Reaction Mix	**ul needed**	BB only control
Backbone	5.26	5.26
Insert 1	2.28	-
Insert 2	4.01	-
T4 Ligase buffer	2.00	2.00
T4 ligase	0.50	0.50
water	5.95	12.24
total	20 ul	20.00

- I followed the ligation protocol noted higher up in this lab notebook
    - Since previous ligations have not gone right, I made sure to pipette the exact amounts of each reaction part
    - This included elevating the PCR tube rack to near eye level so I could see the volumes I pipetted more easily
    - Whenever I pipetted an amount, I would put the liquid back and pipette again to make sure I got the right amount
    - All tubes had the same volume before I added the ligase, and I added the 0.5 ul directly into the reaction volumes and pipetted with the 18 ul after
        - This made some bubbles at the top of the reactions after the mixing, but almost all the reaction volume was liquid at the bottom

Feb 23, 2022

- To do list:
    - ~~Sterilize some cuvettes~~
    - ~~Perform transformation for the new ligation from yesterday (BB, 3X, and 5X)~~
    - ~~Autoclave plate waste~~
    - ~~Fill carboy~~

Third Transformation

Tube #	Purpose	Volume of DNA	Final Volume to Plate	# of chlor/XGAL plates
1	(+) control	1 ul	20 ul, 100 ul	2
2	(-) control	0 ul	20 ul, 100 ul	2
3	3X Ligation	8 ul	100 ul, remaining	2
4	5X Ligation BB	8 ul	100 ul, remaining	2
5	Ligation	8 ul	100 ul, remaining	2

- 5 reaction tubes this time since we added the 3X ligation
- Added volumes of DNA and the cells to the reaction tubes on ice and close to the flame
    - Made sure to vortex the pIMAY-Z plasmid beforehand and I did not mix the cells and DNA once they were together in the tube
- PRO TIP: Hannah saw me put my arm over an open container that is supposed to stay sterile; this could result in contamination since my skin and jacket are covered in microbes
    - Rearrange my area before hand and rehearse moving around to make sure I am not going over the flam or an open container
- Just a reminder: "For "remaining" volume, spin tubes at max speed in a 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."

For Autoclaving Plate waste

- Make sure both bags of plates are double bagged and taped close
- Liquid 20 (20') setting on autoclave (no water)

2/23/22 KRLab Meeting

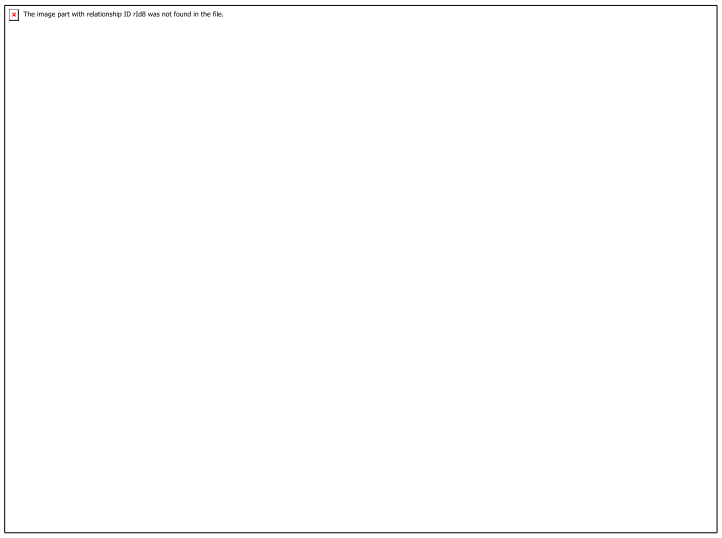
- pKR122 can be put on back burner
- We are going to do a qt-PCR for the strains used in the last beta-gal
    - This will be a fun time; going to start on Monday, March 7th

Feb 24, 2022

- To do:
    - ~~Clear bin from the autoclave accident yesterday~~
    - ~~Check the transformation plates~~
- Transformation plates
    - There was no growth on any plates which was to be expected since they have taken at least two days to grow in the past transformations
    - Roads will be bad tomorrow so I probably won't come in; cannot leave the plates in the incubator over the weekend, Hannah said they can be left out in room temp for three days, will check again on Monday
        - Hannah said don't panic if there's no growth on Monday, room temperature incubation can take up to five days

XGAL Function

- XGAL is a reporter chemical for b-galactosidase/lac z activity
- Only cells with lac z activity will turn blue; they must have the plasmid within them since pIMAY-Z has the chloramphenicol resistance gene



- Positive control should be blue
- Negative should have no growth
- 3X should be blue
- 5X should be blue
- BB should have no growth

Feb 28, 2022

- To do:
  - Check Plates
  - Streak out stock IMO8B on a Chlor/XGAL plate
  - Prepare cultures for MiniPrep tomorrow

Plates from third ligation

- \+ control plates:
  - 20 ul: 12 blue colonies – means that the transformation worked, and the plasmid was expressed in the cells
  - 100 ul: 72 blue colonies and 7 white colonies – some cells were transformed, and some were not, but the cells without the plasmid should not have grown
- – control plates:
  - 20 ul: 0 colonies
  - 100 ul: 24 white colonies – once again, these white colonies should not have grown
- 3X Ligation:
  - 100 ul: 13 white colonies, no plasmid in the cells
  - Remaining ul: 12 white colonies, no plasmid in the cells
- 5X Ligation:
  - 100 ul: 12 white colonies, no plasmid in the cells
  - Remaining ul: 5 white colonies, no plasmid in the cells
- BB Ligation:
  - 100 ul: 17 white colonies, 1 blue colony, and 2 yellow colonies – most of the cells did not have the plasmid, only 1 colony had religated plasmids, and the 2 yellow colonies means that I contaminated the plate with another species of bacteria
- In the end, the ligation failed, and I was not sufficiently aseptic

Plasmid Construction Troubleshooting

- NEED TO MIX EVERYTHING BY EITHER VORTEXING OR PIPETTING BEFORE ADDING TO THE REACTION TUBES!!!
- Since there were white colonies on the chlor plates, Kathryn and Hannah think 10 ug/ml may not be a high enough concentration
    - Testing this out by streaking stock IMO8B cells on a10 ug/ml chlor/100 ug/ml XGAL plate
- We are also going to try the ligation and transformation again, still doing a 3X and 5X
    - I do not have enough plasmid backbone left so I need to collect some from the + control plates
        - MiniPrep
    - Along with this, we are going to do an identical transformation with 5-alpha *E. coli* cells, which are highly competent and sensitive to chloramphenicol
        - I will do the IMO8B cells first, then do the 5-alpha cells separately afterwards
- IMO8B stock streaking
    - I struck out 50 ul of the stock IMO8B cells onto a 10 ug/ml chlor/100 ug/ml XGAL plate to see if the chloramphenicol concentration is high enough; if colonies show up, we will most likely increase the concentration of chloramphenicol in the next plates
- I ran out of backbone for the next ligation and did not have enough of the pIMAY-Z plasmid, so I must MiniPrep some of the backbone from the + control colonies from my last transformation
    - Started by adding 5 ml of LB to two vials, then adding 1 ul of chloramphenicol to the tubes (the culture tubes must have the same concentration of chloramphenicol as the plates; got 1 ul volume from a C1V1=C2V2 calculation)
    - Used a sterile stick to pick up blue colonies from the + control plates, violently shook the stick around in each of the tubes, then stuck them in the shaking incubator overnight

Large Batch CHA Powder

- Add the following to the Cysteine Heart Agar container:
    - 75 g Proteose Peptone
    - 75 g Beef Heart Infusion
    - 75 g Glucose
    - 112.5 g Agar
    - 7.5 g NaCl

March 2022

March 1, 2022

- To do list:
    - MiniPrep the overnight cultures from yesterday

MiniPrep Spin Kit Protocol

1. Pellet all 5 ml bacterial overnight culture by spinning at >8000 rpm for 3 min at room temp
    a. Put in 2 ml tubes; spin and discard the supernatant; must do this 3 times for 5 ml
2. Resuspend pelleted cells in 250 ul Buffer p1 from fridge

3. Add 250 ul buffer p2 and mix by inverting tube 4-6 times; do not let this step proceed for more than 5 minutes
4. Add 350 ul buffer n3 and mix immediately by inverting 4-6 times
5. Centrifuge for 10 minutes at 13,000 rpm
6. Apply 750 ul from step 5 into spin column and spin for 1 minute, then discard flowthrough
    a. Do this until all the solution from the previous step is gone
7. Wash with 500 ul buffer PB; spin for 1 min and discard flow through
8. Wash again with 750 ul buffer PE; spin for 1 minute, discard flowthrough, and do these two more times
9. Centrifuge for 3 min to remove residual buffer
10. Place column in a 1.5 ml tube and elute DNA with 50 ul 0.1X EB; let stand for 1 min before spinning for 1 min

RNA Plan

- Planning for Monday March 7 (KRLVS 149 and KRLVS 149)
    - On Saturday, 148 must be streaked out to single colonies for the RNA on Monday
    - On Sunday, cells must be streaked to single colonies for RNA; need single colonies (149)
    - On Wednesday, I will streak out 148 since delta_rpsu takes a long time to grow
    - On Thursday, we will streak out 149

March 2, 2022

- To do list:
    - ~~Perform digest on the plasmids recovered from the positive control plates from the third transformation~~
    - Fourth ligation
    - ~~Check the stock IMO8B test plate from Monday; determine whether the chloramphenicol concentration is high enough~~
    - ~~Streak out KRLVS 148~~
- ~~pIMAY-Z from + control digest~~
    - Check concentration

DNA Digests Protocol

1. Add indicated amounts of H_2O and 10x buffer to the master mix tube (MM).
2. Add indicated amount of DNA or DNA + water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
3. Add the indicated amount of each enzyme (__uL) to the master mix tube (MM).
4. Mix the master mix by pipetting up and down.
5. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (___ul).
6. Incubate at 37°C for 1 hour or up to overnight.
7. If using a digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to the backbone tube, mix by pipetting and put back in the 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-

ligation). If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.

- The concentrations from the pIMAY-Z MiniPrep cultures are as follows:
    - Tube 1: 60.5 ug/ml
    - Tube 2: 74.0 ug/ml

Components	Volumes in 1 reaction (uL)
H_2O	0
10x Buffer*	3
DNA	25.8
Enzyme 1	0.6
Enzyme 2	0.6
Total	30.0 (15.0 actual b/c of DNA)

- The table above is for the digest run with both enzymes which I will be cutting out

Components	Volumes in 1 reaction (uL)
H_2O	19.4
10x Buffer*	3
DNA	7
Enzyme 1	0.6
Enzyme 2	0
Total	30.0 (15.0 actual b/c of DNA)

- This is the table for the linear plasmid digests, using one of the two enzymes
    - One digest with BamHI and one digest with KpnI

Components	Volumes in 1 reaction (uL)
H_2O	20
10x Buffer*	3
DNA	7
Enzyme 1	0
Enzyme 2	0
Total	30.0 (15.0 actual b/c of DNA)

- This is the table I used for the digest with no enzymes
- Only did the QuickCIP protocol on the full digest tube since that's the plasmid we want to cut for the ligation
- For the stock IMO8B cell 10 ug/ml plate, there were two white colonies that grew
    - Going back up to 25 ug/ml of chlor in the next set of plates

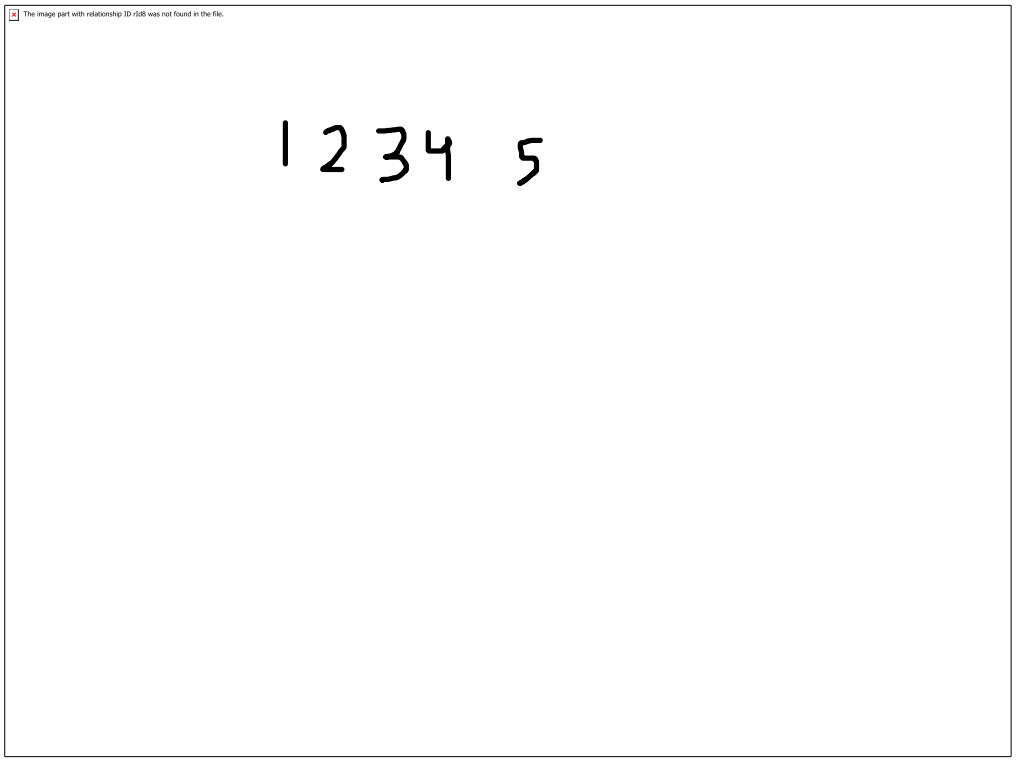
Streaking out KRLVS 148

- Pipetted 100 ul (whole tube) onto corner of the plate; used sterile stick to streak it out in a small area
- Used a clean stick to go twice into first zone and then struck out again in second quadrant
- Repeated the same process for the third and fourth quadrants, then put into standing incubator

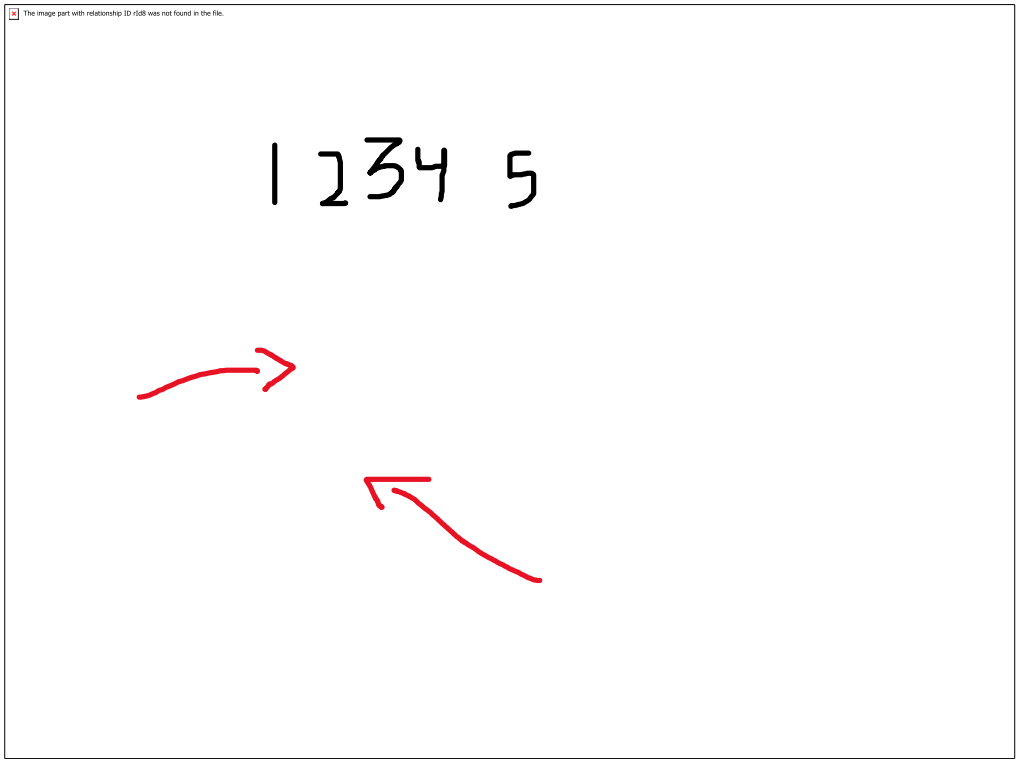
New Chlor/XGAL Plates

- Since stock IMO8B grew on the 10 ug/ml chlor plates, we are increasing the concentration from 10 ug/ml t0 25 ug/ml
    - 500 ml LB
        - 250 ul of the 50 mg/ml stock Chloramphenicol
        - 1000 ul of the 50 mg/ml stock XGAL
    - Made 25 plates total; two are very full since I tried to go light on most of them
- **ACCIDENTLY PUT TWO RED STRIPES ON THESE PLATES INSTEAD OF ONE RED STRIPE**
    - **This makes the plates look like 10 ug/ml chlor when they are really 25 ug/ml**
- Gel table

Lane #	Contents
1	10 ul Ladder
2	30 ul No Enzyme Plasmid Digest
3	30 ul BamHI Plasmid Digest
4	30 ul KpnI Plasmid Digest
5	30 ul Both Enzyme Plasmid Digest



- First picture of the gel; I cut the full digest plasmid out for gel extraction (0.19 g) and left the rest of the lanes to migrate longer (15 minutes at 50 V, 20 minutes at 110 V)



- The single enzyme bands are the same distance, which is expected, while the no enzyme digest lane has two bands

- The two bands are due to supercoiling; the higher up band is a plasmid with a single strand nick (this causes more friction with the agarose) while the band further down is a supercoiled plasmid which has less friction with the agarose

March 3, 2022

- To do list:
  - ~~Perform fourth ligation~~

Ligation Protocol

1. Obtain ice to assemble and keep the reactions on. This is important, as the reaction happens at 16˚C and the ligase buffer (which contains ATP) needs to be kept cold in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
4. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Place in the thermocycler overnight at 16°C.

Fourth Ligation

- Made sure I was pipetting the correct amounts of everything, comparing the volumes between all tubes to make sure they all had the same by the end
  - Had Aisling pipette the 0.5 ul of the T4 Ligase just to be cautious with my shaky hand
  - I vortexed everything I could and I mixed the ligase into the reaction tubes using 18 ul pipette

Tube	Insert 1	Insert 2	Backbone
1 (BB ligation)	-	-	Gel Purified pIMAY-Z
2 (3X ligation)	Gel Purified FR1	Gel Purified FR2	Gel Purified pIMAY-Z
3 (5X ligation)	Gel Purified FR1	Gel Purified FR2	Gel Purified pIMAY-Z

Desired amount of backbone: 50 ng; Backbone concentration from digest: 12.5 ng/ul

	Sample Name	Desired Ratio	Sample Concentration (ng/ul)	size (kb)
Backbone	pIMAY-Z	1	12.5	8815
Insert 1	FR1	5	10.12	1015
Insert 2	FR2	5	5.48	969
	Sample Name	Desired Ratio	Sample Concentration (ng/ul)	size (kb)
Backbone	pIMAY-Z	1	12.5	8815
Insert 1	FR1	3	10.12	1015
Insert 2	FR2	3	5.48	969

- The table directly below is for the 5X and Backbone ligation and the table below that is for the 3X Ligation

Reaction Mix	ul needed	BB only control
Backbone	4.00	5.26
Insert 1	2.84	-
Insert 2	5.01	-
T4 Ligase buffer	2.00	2.00
T4 ligase	0.50	0.50
water	5.64	12.24
total	20 ul	20.00

Reaction Mix	ul needed
Backbone	4.00
Insert 1	1.71
Insert 2	3.01
T4 Ligase buffer	2.00
T4 ligase	0.50
water	8.78
total	20 ul

March 4, 2022

- To do list:
  - Perform the transformations on on the IMO8B and 5-alpha *E. coli*

5-alpha E. coli *Protocol*

- Thaw the tubes of competent cells on ice for 10 minutes (5E box in -80 freezer)
  - Need to thaw 5 tubes since each tube contains the right amount for one transformation
- Add appropriate amounts of plasmid DNA to each tube, pipetting gently
  - 1 ul pIMAY-Z plasmid for the positive control
  - 0 ul for the negative control
  - 8 ul ligation to the 3X, 5X, and backbone tubes
- Place tubes on ice for 30 minutes, do not mix
- Heat shock at 42 degrees for exactly 30 seconds, place on ice for 5 minutes right after
- Add 950 ul SOC (right next to the parafilm)
- Incubate in shaking incubator for 1 hour and make sure the plates are warmed also
- Mix tubes by flicking, then apply correct volume of cells to each plate
- Put in incubator or keep at room temp over weekend

- IMO8B transformation

Tube #	Purpose	Volume of DNA	Final Volume to Plate	# of chlor/XGAL plates
1	(+) control	1 ul	20 ul, 100 ul	2
2	(-) control	0 ul	20 ul, 100 ul	2
3	3X Ligation	8 ul	100 ul, remaining	2
4	5X Ligation BB	8 ul	100 ul, remaining	2
5	Ligation	8 ul	100 ul, remaining	2

- 5-alpha transformation
  - This will be completed after the IMO8B transformation to reduce the chances of cross contamination

Tube #	Purpose	Volume of DNA	Final Volume to Plate	# of chlor/XGAL plates
1	(+) control	1 ul	20 ul, 100 ul	2
2	(-) control	0 ul	20 ul, 100 ul	2
3	3X Ligation	8 ul	100 ul, remaining	2
4	5X Ligation BB	8 ul	100 ul, remaining	2
5	Ligation	8 ul	100 ul, remaining	2

- Performed the IMO8B transformation as I have in the past; following the tables I created and using cautious aseptic technique
  - While the IMO8B cultures were incubating for the 1 hour, I prepared the 5-alpha cells, making sure the wipe everything down and I even changed gloves
- For the 5-alpha cells:
  - Thawed on ice and added indicated volumes of DNA to each tube
  - Let them sit on ice for 30 minutes before the 30 second heat shock
    - Made sure each tube was heat shocked for 30 seconds
  - Put back on ice for 5 minutes after heat shock; I then added 950 ul of the SOC to each tube before putting into the shaking incubator for 1 hour

March 5, 2022

5-alpha Transformation plates

- It worked! All colonies were small and uniform in morphology
- – control
  - 0 colonies on either plate
- + control
  - 60 blue colonies on the 20 ul plate, ~150 blue colonies on the 100 ul plate
- 3X Ligation
  - 28 blue colonies on the 100 ul plate, ~110 blue colonies on the remaining plate

    - 5X Ligation
        - 30 blue colonies on the 100 ul plate, 119 blue colonies on the remaining plate
    - BB Ligation
        - 0 colonies on either plate
- I wrapped the 3X and 5X plates in parafilm, put back in the foil and placed them in the fridge

March 6, 2022

IMO8B Transformation plates

- These plates did work; colony size and morphology varied, just like these plates in the past
    - – control
        - 0 colonies on either plate
    - + control
        - 30 blue colonies on the 20 ul plate, 51 blue colonies on the 100 ul plate
    - 3X Ligation
        - There was a very dense blue lawn on the 100 ul plate, but nothing grew on the remaining plate (weird)
    - 5X Ligation
        - ~217 blue colonies on the 100 ul plate, and a very dense blue lawn on the remaining plate
    - BB Ligation
        - Unfortunately, there was growth; 1 large blue colony on the 100 ul plate, 21 large blue colonies on the remaining plate
- I put both the 5X ligation plates and the 100 ul 3X ligation plate in the fridge just in case

RNA Sequencing Protocol Prep

- Aisling and I prepared the plates for RNA isolation over this past week and patched out the single colonies on plates for tomorrow; each plate has six patches
- We also prepared eight sterile 1.5 tubes with 300 ul MHB, eight culture tubes with 7 ml MHB

Culture Growth for RNA Isolation

- Scrape up patches from the plates and mix into the 1.5 ml tubes containing 300 ul MHB; add another 700 ul MHB and make sure cells are resuspended
- Check OD600
    - Dilute cells by adding 100 ul of resuspended cells into 900 ul MHB (1:10 Dilution) and check OD600, using a MHB cuvette to blank
    - The actual OD600 can be calculated by multiplying the measured OD600 by the dilution factor
- **Calculate the volume of cells needed to get an OD600 of 0.08 in 10 ml**
    - Say the OD600 of the resuspended cells is 4.0 and we want it to be 0.08,
        - (10 ml) (0.08)/(4.0) = 0.2 ml resuspended cells needed
- Swirl the tubes to distribute cells and take 1 ml out to measure the OD600 again, making sure its 0.08

- Place tubes in the shaking incubator for around 2 hours to see that cells are close to doubling, then taking out to measure the OD600 after around 4-6 hours when the OD600 is between 0.3-0.4
- **Once the tubes are between 0.3-0.4, transfer 1.8 ml into 2 ml tube, spinning at max speed at 4 degrees for 3 minutes to pellet cells**
- Resuspend cell pellet in 1 ml TRI-Reagent

March 7, 2022

- Aisling and I completed the first steps of the RNA culturing protocol
- Kathryn had to make the MHB-Kan culture tubes; I scraped up all the patches from the plates and suspended them in 300 ul MHB in the sterile 1.5 ml tubes
  - Tubes 1-4 are the ΔrpsU2 strain (148.1)
  - Tubes 5-8 are the LVS strain (149.1)

RNA Culturing

Tube	OD600 measured	Actual OD600	Vol. of cells	OD600 0 hrs
1	0.118	1.18	793	0.100
2	0.268	2.68	349	0.098
3	0.318	3.18	294	0.109
4	0.344	3.44	272	0.087
5	0.345	3.45	271	0.098
6	0.284	2.84	330	0.095
7	0.332	3.32	282	0.082
8	0.363	3.63	258	0.112

- We prepared the culture tubes by calculating the volume of cells needed and removing that same volume of MHB-Kan media from the appropriate culture tube and adding that volume of cells so that each tube would have the same volume even with different volumes of cells needed to get the desired OD600 at T=0
  - For ΔrpsU2, we wanted the OD600 to be around 0.117 at T=0, so the equation we used to get the volume of cells was V = (0.117) (8,000 ul) / (measured OD600 x 10)
  - For LVS the same equation was used, except we wanted to the T=0 OD600 to be around 0.9, so V = (0.9) (8,000 ul) / (measured OD600 x 10)
- The table above shows the measured OD600s of each tube and the calculated volume of cells needed to get the desired OD600
- When measuring the T=0 OD600 for each tube, Aisling and I forgot to swirl the culture tubes, so the OD600s we got for T=0 was not accurate
  - Hannah said to juts check again at 2 hours so we wouldn't use anymore of the cultures
- After two hours (2:04:06 I went over to take them out), I checked the culture OD600s again, where they should've doubled in value

- Aisling forgot to hit start on the shaking incubator
- That may be the reason the cultures did not double in OD600 value

Tube	OD600 measured	actual OD600	Vol. of cells	OD600 0 hrs	OD600 2 hrs
1	0.118	1.18	793	0.100	0.134
2	0.268	2.68	349	0.098	0.132
3	0.318	3.18	294	0.109	0.126
4	0.344	3.44	272	0.087	0.122
5	0.345	3.45	271	0.098	0.127
6	0.284	2.84	330	0.095	0.126
7	0.332	3.32	282	0.082	0.152
8	0.363	3.63	258	0.112	0.138

Since the culture OD600 values did not double, I am going to check again in an hour (

Preparation for the RNA Isolation

- To do:
  - Get heat block to 60 degrees
  - After 2-hour OD600 readings, set large centrifuge to 4 degrees
  - Prepare the working area
    - Moving the centrifuge into hood, wiping everything down with water, ethanol, and RNAse-away

RNA Isolation Protocol

1. Purify Nucleic Acids
   a. Resuspend cell pellets in 1 ml TRI-Reagent (Under hood)
   b. Incubate at 60 degrees on heat block for 10 minutes (to lyse cells)
   c. Spin at 4 degrees for 10 minutes at max speed (to get rid of cell debris)
      i. The rest of the steps in this part of the protocol are all under a hood
   d. Transfer the supernatant to new 2 ml tube (this will contain the nucleic acids)
   e. Add equal volume 100% ethanol to the tubes (this will be around 950 ul of ethanol, 1900 ul total)
   f. Pass sample over Directzol column, 600 ul at a time, spin 30 seconds at max speed, and discard flowthrough; do this until all the sample has been passed through the column
   g. Place the columns in new collection tubes before washing twice with 400 ul RNA PreWash buffer, discarding the flow through in phenol/methanol waste
   h. Add 700 ul of wash buffer, letting it sit for 3 minutes, then spinning for 2 minutes at max speed
   i. Place column in new collection tube
   j. Spin again at max speed for 3 minutes before placing column in clean 1.5 ml tube

k. Add 90 ul RNase-free water, letting it sit for 2 minutes before spinning at max speed for 1 minute
l. Place the flow through on the column again and spinning for 1 minute again
m. Store nucleic acids at -80 degrees until tomorrow when Aisling and Kathryn begin the DNase step

2. DNase Treatment

NOTE

ON MARCH 31, MY LAB NOTEBOOK WAS CORRUPTED AND I ONLY HAD A VERSION UPDATED TO MARCH 7TH. I FILLED IN WHAT DATA I HAD AND WHAT I COULD REMEMBER BEFORE APRIL 1ST.

March 8, 2022

- Prepared 4 50 ml overnight culture flasks for the midi-prep tomorrow
  - Each flask contained
    - One colony from the 5X ligation plate
    - 25 ul of the 50 mg/ml chloramphenicol stock
    - 50 ml LB media

March 9, 2022

- First Midi-Prep

MidiPrep Protocol

1. Grow 5 mL culture of LB+abx overnight from a single colony.
2. In the morning, dilute 100 ul of the overnight culture into 25 mL LB media + abx for high-copy plasmids (50-100 mL for low copy). Grow until reaches about 0.5-0.6 OD600.
3. Harvest by centrifuging at 6000 x g for 15 minutes at 4C.
4. Completely resuspend pellet in 4 mL Buffer P1.
5. Add 4 mL Buffer P2, mix by inverting 4-6 times and incubate at room temperature for up to 5 minutes.
6. During the incubation, screw the cap onto the outlet nozzle of the QIAfilter cartridge, and plate it in a convenient tube or rack.
7. Add 4 mL prechilled Buffer P3 to the lysate, and mix immediately by inverting 4-6 times.
8. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temp for up to 10 minutes without the plunger.
9. Equilibrate the QIAGEN-tip by applying 4 mL Buffer QBT and allowing to empty by gravity flow.
10. Remove the cap from the QIAfilter Cartridge Outlet nozzle. Gently insert the plunger into the QIAfilter Cartridge and filter the cell lysate into the equilibrated QIAGEN-tip. Allow the lysate to enter the resin by gravity flow.
11. Wash the QIAGEN-tip with 2x 10 mL Buffer QC.
12. Elute the DNA with 5 mL Buffer QF.
13. Precipitate the DNA by adding 3.5 mL room-temperature isopropanol, mix and centrifuge at 15,000 x g for 30 min at 4C. Carefully decant the supernatant.
14. Wash the DNA pellet with 2 mL room temperature 70% ethanol and centrifuge at 5,000 x g for 10 min (4C is okay). Carefully decant the supernatant.

15. Air-dry the pellet for 5-10 min and redissolve the DNA in 0.1X EB. Start with 50 uL and add more until it is not super viscous.

    - This midi-prep did not work, the DNA concentrations were too low for sequencing

March 10, 2022

- Prepared 4 50 ml overnight culture flasks for the midi-prep tomorrow
    - Each flask contained
        - One colony from the 5X ligation plate
        - 25 ul of the 50 mg/ml chloramphenicol stock
        - 50 ml LB media

March 11, 2022

- Second Midi-Prep

MidiPrep Protocol

16. Grow 5 mL culture of LB+abx overnight from a single colony.
17. In the morning, dilute 100 ul of the overnight culture into 25 mL LB media + abx for high-copy plasmids (50-100 mL for low copy). Grow until reaches about 0.5-0.6 OD600.
18. Harvest by centrifuging at 6000 x g for 15 minutes at 4C.
19. Completely resuspend pellet in 4 mL Buffer P1.
20. Add 4 mL Buffer P2, mix by inverting 4-6 times and incubate at room temperature for up to 5 minutes.
21. During the incubation, screw the cap onto the outlet nozzle of the QIAfilter cartridge, and plate it in a convenient tube or rack.
22. Add 4 mL prechilled Buffer P3 to the lysate, and mix immediately by inverting 4-6 times.
23. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temp for up to 10 minutes without the plunger.
24. Equilibrate the QIAGEN-tip by applying 4 mL Buffer QBT and allowing to empty by gravity flow.
25. Remove the cap from the QIAfilter Cartridge Outlet nozzle. Gently insert the plunger into the QIAfilter Cartridge and filter the cell lysate into the equilibrated QIAGEN-tip. Allow the lysate to enter the resin by gravity flow.
26. Wash the QIAGEN-tip with 2x 10 mL Buffer QC.
27. Elute the DNA with 5 mL Buffer QF.
28. Precipitate the DNA by adding 3.5 mL room-temperature isopropanol, mix and centrifuge at 15,000 x g for 30 min at 4C. Carefully decant the supernatant.
29. Wash the DNA pellet with 2 mL room temperature 70% ethanol and centrifuge at 5,000 x g for 10 min (4C is okay). Carefully decant the supernatant.
30. Air-dry the pellet for 5-10 min and redissolve the DNA in 0.1X EB. Start with 50 uL and add more until it is not super viscous.

    - This midi-prep did not work, the DNA concentrations were too low for sequencing
    - Saw the DNA pellet at first, but lost it at some point

March 12-20

- Spring break

March 21, 2022

- Was not feeling well so I did not come in

March 22, 2022

- Prepped 4 5ml overnight culture tubes for miniprep
    - Each tube contained:
        - A colony from the 5X ligation plate
        - 2.5 ul of the 50 mg/ml chloramphenicol stock
        - 5 ml LB media

March 23, 2022

- Ran a mini prep on the overnight cultures

MiniPrep Spin Kit Protocol

1. Pellet all 5 ml bacterial overnight culture by spinning at >8000 rpm for 3 min at room temp
    a. Put in 2 ml tubes; spin and discard the supernatant; must do this 3 times for 5 ml
2. Resuspend pelleted cells in 250 ul Buffer p1 from fridge
3. Add 250 ul buffer p2 and mix by inverting tube 4-6 times; do not let this step proceed for more than 5 minutes
4. Add 350 ul buffer n3 and mix immediately by inverting 4-6 times
5. Centrifuge for 10 minutes at 13,000 rpm
6. Apply 750 ul from step 5 into spin column and spin for 1 minute, then discard flowthrough
    a. Do this until all the solution from the previous step is gone
7. Wash with 500 ul buffer PB; spin for 1 min and discard flow through
8. Wash again with 750 ul buffer PE; spin for 1 minute, discard flowthrough, and do these two more times
9. Centrifuge for 3 min to remove residual buffer
10. Place column in a 1.5 ml tube and elute DNA with 50 ul 0.1X EB; let stand for 1 min before spinning for 1 min

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit
1		Science	3/23/2022 11:52:01 AM	173.5	ng/µl
2		Science	3/23/2022 11:53:15 AM	190.1	ng/µl
3		Science	3/23/2022 11:53:52 AM	191.1	ng/µl
4		Science	3/23/2022 11:54:33 AM	159.5	ng/µl

Prepped all the sequencing samples and sent them out to plasmidsaurus

March 24, 2022

- I checked the cDNA Nanodrop concentrations from the isolation protocol Aisling did

ssDNA calculations

	Sample ID	Nucleic Acid	Unit	A260 (Abs)	Factor
1	cDNA sample 1	17.5	ng/µl	0.528	33.3
2	cDNA sample 2	20.3	ng/µl	0.61	33.3
3	cDNA sample 3	21.5	ng/µl	0.647	33.3
4	cDNA sample 4	18.3	ng/µl	0.55	33.3
5	cDNA sample 5 (the redo)	16.1	ng/µl	0.486	33.3
6	cDNA sample 6	15.3	ng/µl	0.462	33.3

March 25, 2022

- Figured out what I would need for the electroporation
- Materials:
  - LB Media, no abx
  - LB plates, no abx
  - LB media, 10% glycerol and 500 mM sucrose
    - For 100 ml:
    - 1 g of NaCl
    - 1 g of Tryptone
    - 0.5 g of Yeast Extract
    - 17.5 g of sucrose (for 500 mM sucrose in 100 ml)
    - 13.3 ml of 75% glycerol (for 10% glycerol)
    - 86.7 ml of ddiH2O
  - Sterile ddiH2O
    - Ice cold
  - 10% glycerol in LB
    - Ice cold
  - LB plates, 25 ug/ml chloramphenicol, 100 ug/ml XGAL (3 plates per electroporation)
  - LB media, 500 mM sucrose
- I calculated the volumes and concentrations of all materials I'd need
  - 100 ml LB 10% glycerol 500 mM sucrose
    - 1 g Nacl
    - 1 g tryptone
    - 0.5 g yeast extract
    - 17.11 g sucrose
    - 13.3 ml 75% glycerol
    - 86.7 ml ddiH2O
  - 45 ml 10% glycerol
    - 39 ml ddiH2O
    - 6 ml 75% glycerol
  - 50 ml LB 500 mM sucrose
    - 8.57 g sucrose
    - 50 ml LB media

March 28, 2022

- Made the 45 ml 10% glycerol, 100 ml LB 10% glycerol 500 mM sucrose, and 200 ml autoclaved water
- Streaked out a 50 ul aliquot of *S. aureus* to single colonies on an LB agar plate for the overnight cultures I'll prepare tomorrow

March 29, 2022

- Prepared 6 overnight cultures from the single colonies of *S. aureus*
    - 5 tubes for the electroporation
    - 1 tube to make sure the cells grow on LB, but not on LB+CX

March 30, 2022

- Ran the preparation of electrocompetent cells protocol

Preparation of Electrocompetent Cells

- *S. aureus* must be struck out to single colonies two days prior to the electroporation in order to have overnight cultures ready the day of electroporation

1. Prepare overnight cultures of electrocompetent *S. aureus* cells in 10 ml LB media at 37 °C in the shaking incubator
2. These overnight cultures will be diluted down to an OD600 of 0.5 in warmed media, then put back into shaking incubator for another 40 minutes
    a. The dilution will be done using the C1V1=C2V2 equation with the concentrations being the OD600s; V2 will be 10 ml and C2 will be 0.5; subtract V1 amount from the 10 ml of LB so total volume equals 10 ml
    b. At this step, starting cooling down the big centrifuge
    c. If OD600 of any of them is greater than 0.6, dilute it so that it will be less than 0.6

IF (OD600 Values)	THEN (Dilution)
0.6-0.9	1:2
0.9-1.5	1:4
1.6-	1:10

3. To harvest cells, spin down at 7000 x g for 5 minutes at 4°C in 50 ml conicles and discard supernatant
4. An equal volume of ice-cold sterile water will be added, and the cells spun down again at the same settings (this is done twice)
5. The cells will then be centrifuged and resuspended in in 1/5 volume (2 ml) of sterile ice-cold 10% glycerol LB media (this is done twice)
6. Finally, the cells will be resuspended in 1/200 (50 ul) sterile ice-cold 10% glycerol LB media and can be stored at -70 °C

- I followed this protocol exactly
- I added 80 ul of LB 500 mM sucrose media into one of the 50 ul aliquots
    - Pipetted 20 ul onto a regular LB plate and 110 ul onto a LB+CX plate

March 31, 2022

- I checked the control plates from yesterday
    - No growth on the LB+CX plate and a nice lawn on the LB plate
    - The cell aliquots can grow and there was no contamination

April 2022

April 1, 2022

- To do list:
  - ~~Present during lab meeting~~
  - ~~Make 26+ LB+CX plates for next week~~
- The LB+CX plates were made by:
  - 500 ml LB agar
  - 250 ul of the 50 mg/ml chloramphenicol stock
  - 1000 ul of the 50 mg/ml XGAL stock

April 4, 2022

- To do list:
  - ~~Electroporation~~

Electroporation of S. aureus

1. Thaw cells and spin for 3 minutes at 5000 x g, resuspending in 80 ul 10% glycerol-500 mM sucrose LB media
2. Plasmid DNA is then added to the cells (1 ug), and then the volume is transferred to a 1 mm electroporation cuvette
   a. 5.76 ul of the DF95-1 plasmid
   b. The DNA and cells incubated at room temp for about 15 minutes before electroporation
3. After the pulse (at 21 kV/cm, 100 ohms, and 25 µF), 1 ml of LB with 500 mM sucrose is added to the cuvettes and pipetted up and down to mix before being transferred to a tube and incubated at an angle (45 degrees) for 1 hour at 30 °C
   a. I changed the kV to 2.1/mm to match the plan, could not change ohms
4. Two 100 ul aliquots are to be pipetted on LB chlor/xgal plates (25 ug/ml chlor, 100 ug/ml XGAL) and incubated overnight at 37 °C
   a. I forgot to incubate one plate at 30 C for two days
5. The remaining cells are to be spun down at 7000 x g for 5 minutes, supernatant removed, and resuspended in 100 ul of the LB 500 mM sucrose before the entire volume is pipetted onto another plate for overnight incubation

- I followed this protocol exactly except for a step or two
  - One of the tubes had a pellet that kept moving around and resuspended when I tried to remove the supernatant, so I spun it down for a minute

Tube	Contents	Plasmid	MS
1	E.C. S.A. aliquot 1	pIMAY-Z-ΔrpsU	Arc (0.0)
2	E.C. S.A. aliquot 2	pIMAY-Z-ΔrpsU	Arc (0.0)
3	E.C. S.A. aliquot 3	pIMAY-Z-ΔrpsU	Arc (0.0)
4	E.C. S.A. aliquot 4	pIMAY-Z-ΔrpsU	Arc (0.0)
5	E.C. S.A. aliquot 5	----------	Arc (0.0)

- I do not know why all of them Arc'd, but I resuspended the cells in 1 ml 500 mM LB media in the cuvette directly after the pulse and then transferred the entire volume to a recovery tube to incubate for 1 hour at 30 degrees

- Once the cells were finished recovering, I plated the two 100 ul aliquots on LB+CX plates, then concentrated the remaining cells and plating another 100 ul on another LB+CX
    - All plates were incubated at 37 degrees overnight to be looked at tomorrow

April 5, 2021

- To do:
    - ~~Check plates~~
    - ~~Talk to Hannah about electroporation~~
    - ~~Prepare overnight cultures for prep of electrocompetent cells~~
- Sadly, no plates had any growth so I will have to do the electroporation again
    - All 15 plates were grown at 37 C overnight
    - None of them had any signs of growth, so I put them back into the incubator
- I will need more electrocompetent aliquots, so I prepped 8 overnight cultures of *S. aureus* from the single colony plate I made last week

Electroporation Troubleshooting

- Hannah and I discussed the following for my next electroporation:
    - I will do an extra wash of both water and 10% glycerol next time I prep the electrocompetent cells
        - So 3 washes with ice-cold water, 3 washed with ice-cold 10% glycerol
    - There is a *S. aureus* setting on the electroporator (StA), so I will use that in the future
        - This works with the 0.2 cm cuvettes
    - Allow cells to incubate with DNA at room temperature for 30 min
    - I will do 4 electroporations with different parameters to see which one works, then I will update the protocol
- Kathryn edited the allelic exchange protocol so I will follow that tomorrow

April 6, 2022

- To do list:
    - ~~Prepare electrocompetent cells~~
    - Transform IMO8B cells
    - Remake some of the media

Preparation of Electrocompetent Cells

- Streak out *S. aureus* to single colony two days prior to making electrocompetent cells.
- Have all materials ready before starting preparation of electrocompetent cells.

7. For every 6 – 8 aliquots of electrocompetent cells to be made, inoculate one glass culture tubes with 10 mL LB media with a single *S. aureus* colony.
8. Grow overnight at 37°C in the shaking incubator.
    a. I had 8 overnight cultures
9. Check the density of the overnight culture.
    a. Combine in a cuvette 900 uL LB and 100 uL from the overnight culture. Mix by pipetting.
    b. Check OD600, using a cuvette with LB as a blank.
    c. Multiply the observed OD600 by 10 to determine the density of the overnight culture.
10. For each aliquot of electrocompetent cells to be made, made a 10 mL culture of LB and cells from the overnight culture, diluted to OD600 of 0.5.

a. Calculate how much of the overnight culture is needed to obtain an OD600 = 0.5 in 10 mL. Use the C1V1=C2V2 equation with the concentrations being the OD600s; V2 will be 10 ml and C2 will be 0.5.
b. Add the appropriate amount of LB to glass culture tubes (10 mL – volume of overnight culture to add).
    i. I used two of the overnight cultures to make 10 diluted cultures
    ii. The two cultures I used had an OD600 of 4.74 (after multiplying by 10), so I took out 1.05 ml of LB from each tube and added the same volume of cells (tubes 1-5 got 1.05 ml from overnight tube 1, tubes 6-10 got 1.05 ml from overnight culture 2)
c. Add calculated volume of overnight culture to glass tube. Put tubes into shaking 37°C incubator for another 40 minutes.

Start cooling down large centrifuge for next steps.

11. To harvest cells, spin down entire culture (10 mL) at 7000 x g for 5 minutes at 4°C in 50 ml conical tubes and discard supernatant
12. Add equal volumes of ice-cold sterile water (10 mL), and spin down again at the same settings. Discard the supernatant and repeat (total of two washes with water).
13. After two washes with water, resuspend cells in 2 mL (1/5 volume) of sterile ice-cold 10% glycerol. Discard the supernatant and repeat (total of two washes with 10% glycerol).
    a. At this step, the pellets started to move when they sat on ice for an extended period of time (tubes 7-10 since they were the last ones I got to)
14. After two washes with 10% glycerol, resuspend cells in 50 uL (1/200) sterile ice-cold 10% glycerol and transfer cells to sterile microfuge tubes.
15. Store aliquots of cells at -70 °C
    a. In DF+AM box (5F)
16. If using a new strain: Plate out (spreading) one aliquot on two plates.
    a. 45 ul on LB 25 ug/ml chloramphenicol plate
    b. 5 ul on LB

- Made 10 aliquots total, plated one aliquot on an LB plate and a LB+CX plate

Chemical Transformation in E. coli *Protocol*

Tube #	Purpose	DNA	Volume of DNA	Final Volume to Plate	# of chlor plates
1	(-) control		0 ul	20 ul, 100 ul	2
2	pKR95		1 ul	20 ul, 100 ul	2

1. Obtain DNA and thaw on ice if necessary.
2. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
3. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
4. 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.

5. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
6. Place tubes with cells and DNA onto a 42°C heat block for 30 seconds (heat shock step).
7. After heat shock, place tubes back on ice until the next step (don't keep them here too long).
    - On ice for less than 3 minutes
8. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
9. Using autoclave tape, tape microfuge tubes down in a shaking incubator set to 37°C. (save the tape!)
10. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
11. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in a 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.

April 7, 2022

- To do list:
    - ~~Prep overnight cultures of the IMO8B cells from transformation for mini-prep tomorrow~~

April 8, 2022

- To do list:
    - ~~Mini-prep~~
    - ~~Make more LB+CX plates~~
    - ~~Make 10% glycerol 500 mM sucrose~~
    - ~~Make more autoclaved ddiH2O for Hannah~~

MiniPrep Spin Kit Protocol

1. Pellet all 5 ml bacterial overnight culture by spinning at >8000 rpm for 3 min at room temp
    a. Put in 2 ml tubes; spin and discard the supernatant; must do this 3 times for 5 ml
2. Resuspend pelleted cells in 250 ul Buffer p1 from fridge
3. Add 250 ul buffer p2 and mix by inverting tube 4-6 times; do not let this step proceed for more than 5 minutes
4. Add 350 ul buffer n3 and mix immediately by inverting 4-6 times
5. Centrifuge for 10 minutes at 13,000 rpm
6. Apply 750 ul from step 5 into spin column and spin for 1 minute, then discard flowthrough
    a. Do this until all the solution from the previous step is gone
7. Wash with 500 ul buffer PB; spin for 1 min and discard flow through
8. Wash again with 750 ul buffer PE; spin for 1 minute, discard flowthrough, and do these two more times
9. Centrifuge for 3 min to remove residual buffer
10. Place column in a 1.5 ml tube and elute DNA with 50 ul 0.1X EB; let stand for 1 min before spinning for 1 min

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit
1	pKR95 miniprep 1	Science	4/8/2022 12:53:27 PM	189.8	ng/µl

	pKR95 miniprep		4/8/2022			
2	2	Science	12:55:07 PM	185.3	ng/µl	

- I must pay closer attention to detail; I have been using the wrong columns for MiniPreps (the purple PCR/Gel columns versus the blue MiniPrep ones)
- I also made the media for the electroporation on Monday

Media for Second Electroporation

10% glycerol and 500 mM sucrose

- For 50 ml:
- 9.3 g of sucrose (for 500 mM sucrose in 50 ml)
- 6.67 ml of 75% glycerol (for 10% glycerol)
- Add water until 50 ml of ddiH2O
- Filter-sterilize, keep at 4°C

LB media, 500 mM sucrose (50 ml)

- 50 ml LB media
- 9.3 g sucrose

LB+CX Plates

- For 500 ml (~25 plates)
  - 500 ml LB agar
  - 250 ul of the 50 mg/ml chloramphenicol stock
  - 1 ml of the 100 mg/ml XGAL (warmed to same temperature as the LB)
  - Keep away from sunlight by wrapping the flask in tin foil when mixing and pouring, along with wrapping the plates in foil too once they have set

April 11, 2022

- To do list:
  - ~~Electroporation~~
- pKR95 new concentration:

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit
	pKR95 New		4/11/2022		
1	Conc	Science	9:49:03 AM	154	ng/µl

- To have 1 ug of DNA in the electroporation tubes, I added 6.49 ul to each tube.

Electroporation

1. Start cooling down large centrifuge and be sure shaking incubator is set to 30°C, put the plates in the standing incubator to warm.
2. Thaw cells on ice (~ 5mins).
3. Spin cells for 3 minutes at 5000 x g at 4°C.
4. Discard supernatant and resuspend in 80 ul 10% glycerol-500 mM sucrose.
5. Add 1 ug plasmid DNA to the cells. Should be in a volume of 5 – 10 uL, so DNA concentrations should be 100-200 ng/uL). Very carefully mix by pipetting, making sure not to include air bubbles
6. Transfer cells and DNA to a 1 mm electroporation cuvette.
7. Set electroporator to 2.1 kV and pulse. Take note of the time constant.

8. Immediately after pulsing, add 1 ml of LB with 500 mM sucrose to the cuvette. Mix by pipetting. Transfer to transfer to a sterile glass culture tube and incubated at an angle (~45°) for 1 hour at 30 °C

Tube	Contents	DNA	MS
1	E-1 pKR95	6.49 ul pKR95	4.6
2	E-2 pKR95	6.49 ul pKR95	4.0
3	E-3 (-) control	--- (6.49 ul sterile H2O)	4.9

9. Plate out 100 ul of recovered cells on two LB chlor/X-gal plates. Incubate one plate overnight at 37 °C and the other plate at 30°C for 48 h.
10. Spin down remaining cells in a sterile microfuge tube at 7000 x g for 5 minutes. Remove supernatant and resuspend in 100 ul LB 500 mM sucrose. Plate entire volume onto another chlor/X-gal LB plate for overnight incubation at 37°C

- I followed the protocol with Dr. Ramsey's new edits along with using the new media I made on Friday. I also made sure to incubate the plates at the correct temperature (100 ul 37 C. 100 ul 30 C, 100 ul rem 37 C) for each electroporation this time.
- Also worked a lot on my poster, incorporating all the comments and edits Dr. Ramsey and Hannah gave me
    - Edited the KMRedit rather than the draft I had been working on.

April 12, 2022

- To do list:
    - ~~Check the electroporation plates~~
    - Make the LB+X plates
    - Prepare overnight cultures and plates if there are direct integrants from electroporation
- Electroporation plates:
    - 37°C Plates
        - E-1 100 ul = 0 colonies, E-1 100 ul rem = 0 colonies
        - E-2 100 ul = 0 colonies, E-2 100 ul rem = 0 colonies
        - E-3 100 ul = 0 colonies, E-3 100 ul rem = 0 colonies
    - 30°C
        - E-1 100 ul = 0 colonies
        - E-2 100 ul = 0 colonies
        - E-3 100 ul = 0 colonies
    - The 37°C Plates did not grow (there were no direct integrants), we were confident since no electroporation pulse resulted in an arc.
        - In the monk paper, the discussion stated that some strains of *S. aureus* could not have direct integration due to low electroporation efficiency (perhaps due to a thicker cell wall although we were thinking the cells would not have thicker cell walls until after the second homologous recombination event has occurred)
    - The 30°C plates will have slow integration and will therefore need 48 hours so it is not surprising there were no colonies; will check again tomorrow
        - Need to talk to Hannah about the plan for the rest of this week tomorrow

LB+X Plates

- For 500 ml (~25 plates)
  - 500 ml LB agar
  - 1 ml of the 50 mg/ml XGAL stock (warmed to the same temperature as the LB agar)
  - Keep protected from light

April 13, 2022

- To do list:
  - ~~Check the 30°C plates~~
  - ~~Electroporation~~
- The 30°C plates had no growth, so I am going to do another electroporation with different parameters

Electroporation

1. Start cooling down large centrifuge and be sure shaking incubator is set to 30°C, put the plates in the standing incubator to warm.
2. Thaw cells on ice (~ 5mins).
3. Spin cells for 3 minutes at 5000 x g at 4°C.
4. Discard supernatant and resuspend in 80 ul 10% glycerol-500 mM sucrose.
5. Add 1 ug plasmid DNA to the cells. Should be in a volume of 5 – 10 uL, so DNA concentrations should be 100-200 ng/uL). Very carefully mix by pipetting, making sure not to include air bubbles
6. Transfer cells and DNA to a 1 mm electroporation cuvette.
7. Set electroporator to 2.1 kV and pulse. Take note of the time constant.
8. Immediately after pulsing, add 1 ml of LB with 500 mM sucrose to the cuvette. Mix by pipetting. Transfer to transfer to a sterile glass culture tube and incubated at an angle (~45°) for 1 hour at 30 °C
9. Plate out 100 ul of recovered cells on two LB chlor/X-gal plates. Incubate one plate overnight at 37 °C and the other plate at 30°C for 48 h.
10. Spin down remaining cells in a sterile microfuge tube at 7000 x g for 5 minutes. Remove supernatant and resuspend in 100 ul LB 500 mM sucrose. Plate entire volume onto another chlor/X-gal LB plate for overnight incubation at 37°C

Tube	DNA (ug)	DNA Incubation	EP	MS
1	1 (6.49 ul pKR95)	30 min	2.1 kV / 0.1 cm cuvette	4.8
2	1 (6.49 ul pKR95)	30 min	1.8 kV / 0.2 cm cuvette (StA)	2.5
3	1.54 (10 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	4.6
4	----- (6.49 ul ddiH2O)	30 min	2.1 kV / 0.1 cm cuvette	0.9

- This table has all the various parameters I included for each electroporation
  - Tube 2 was in a 0.2 cm cuvette, and we used the StA setting on the electroporator
- I followed everything in the protocol the same except adding the new parameters
- Since I will not be here tomorrow or Friday, Hannah or Aisling will need to check my plates and either:
  - Fast Integration Plates (37°C):
    - Streak out colonies from the fast integration plates on new LB+CX plates; incubate at 37°C overnight
    - Wrap in parafilm and put in fridge

- Slow Integration Plates (30°C):
  - Wrap plates with colonies in parafilm and put in fridge

April 18, 2022

- To do list:
  - ~~Check plates~~
  - ~~Test S.A. cells on 10 ug/ml LB+CX plates~~
  - ~~Streak out S.A. on LB plate to use patches for glycerol stocks~~

- Electroporation plates
  - There was no growth on any of the electroporation plates, 30°C or 37°C
  - Need to talk to Hannah about what to do next

Electroporation Troubleshooting

- Hannah suggested that we use LB+CX plates with a lower concentration of chloramphenicol
  - I still have some 10 ug/ml LB+CX plates that I can test out
- I suggested that I plate some of the electroporated cells on regular LB plates to see whether the cells would grow at all after the electroporation

- I aliquoted 25 ul of one of Hannah's S.A. glycerol stocks onto a LB+CX (10 ug/ml chlor, 100 ug/ml XGAL) plate to see whether that concentration is high enough

- I also aliquoted the other 25 ul of the glycerol stock onto a LB no abx plate; one big patch to make some more S.A. stocks for later use in my project

April 19, 2022

- To do list:
  - ~~Check the LB+CX plate (10 ug/ml) for growth~~
  - ~~Make the new S.A. glycerol stocks~~

Making Glycerol Stocks Protocol – adapted for S. aureus

1. Make 2 cryovials for each strain (permanent stocks), label! Additionally, make 15 x 50 uL single use stocks

2. Prepare 2.6 mL of LB in a 50 mL conical (adjust if you are also making single use stocks)

3. Take at least half of a thickly spread plate and add cells to the LB tube

4. Resuspend until there are no clumps in the LB

5. Add 400ul of 75% glycerol to the 2.6 mL mix by pipetting

6. Aliquot 1mL per cryovial, freeze at -80 (2 cryovials)

7. For single use stocks follow the same protocol but pipet 50ul of solution to tubes (make 15 single use stocks)

- Before starting this protocol, prepare and label all tubes needed
    - The two cryovial stubes
    - The 15 single use aliquot tubes

- The 10 ug/ml LB+CX plate had no growth on it, indicating that the chloramphenicol concentration is high enough
- I made the two cryovial S.A. tubes along with 15 single use aliquots
    - I put them in the -80 in the new DF Glycerol Stocks box (4F)
    - Made sure to update the -80 inventory sheet

April 20, 2022

- To do list:
    - ~~Test electroporation on the 10 ug/ml LB+CX plates with the final two aliquots~~
    - ~~Prepare 2 x 250 ml LB media for Maxi-Prep on Friday~~

Electroporation

1. Start cooling down large centrifuge and be sure shaking incubator is set to 30°C, put the plates in the standing incubator to warm.
2. Thaw cells on ice (~ 5mins).
3. Spin cells for 3 minutes at 5000 x g at 4°C.
4. Discard supernatant and resuspend in 80 ul 10% glycerol-500 mM sucrose.
5. Add 1 ug plasmid DNA to the cells. Should be in a volume of 5 – 10 uL, so DNA concentrations should be 100-200 ng/uL). Very carefully mix by pipetting, making sure not to include air bubbles
6. Transfer cells and DNA to a 1 mm electroporation cuvette.
7. Set electroporator to 2.1 kV and pulse. Take note of the time constant.
8. Immediately after pulsing, add 1 ml of LB with 500 mM sucrose to the cuvette. Mix by pipetting. Transfer to transfer to a sterile glass culture tube and incubated at an angle (~45°) for 1 hour at 30 °C
9. Plate out 100 ul of recovered cells on two LB chlor/X-gal plates. Incubate one plate overnight at 37 °C and the other plate at 30°C for 48 h.
10. Spin down remaining cells in a sterile microfuge tube at 7000 x g for 5 minutes. Remove supernatant and resuspend in 100 ul LB 500 mM sucrose. Plate entire volume onto another chlor/X-gal LB plate for overnight incubation at 37°C

Tube	DNA (ug)	DNA Incubation	EP	MS
1	1 (6.49 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	4.9
2	------ (6.49 water)	0 min	2.1 kV / 0.1 cm cuvette	4.8

- For this electroporation, I only had two aliquots to use so I prepared them under the conditions listed in the protocol

- I will be plating these electroporated cells on 10 ug/ml chlor LB+CX plates since there was no growth on the 10 ug/ml LB+CX plate from yesterday
- I will also be plating a LB + no abx plate with the pKR95 electroporation to see whether the cells can grow at all after electroporation
    - 3 ul of E-1 in 100 ul of LB onto a LB + no abx plate; grown overnight at 37°C

LB Media

Protocol written by KMR

Preparing LB media

1. For 250 mL of LB, weigh out the following components and add to a 500 mL bottle:
    a. 2.5 g NaCl
    b. 2.5 g Tryptone
    c. 1.25 g Yeast extract
2. Add 250 mL type I ddiH20
3. Close tightly and shake to mix
4. Loosen cap and add a small piece of autoclave tape with the date
5. Autoclave on 30-minute liquid cycle
6. Sterile media can be stored indefinitely

- Our autoclaves are broken so had to use one in CBLS
    - Also autoclaved some tubes for Dr. Bertin after my cycle

April 21, 2022

- To do list:
    - ~~Check the electroporation plates for growth~~
    - ~~Prepare 2 x 5 ml overnight cultures from the most recent transformation plate for the maxi-prep on Friday~~

    - There was no growth on the 37°C plates, although the LB test plate had a lawn, so we know that the cells are not dying after the electroporation

    - I also prepared the 2 x 5 ml overnight cultures of the transformed IMO8B cells for the Maxi-Prep tomorrow

        - I will add 625 ul of the overnight cultures to the two 250 ml LB, growing until they reach an OD600 of 0.4-0.5 (roughly 5.5 hours)

April 22, 2022

- To do list:
    - Maxi-Prep the overnight cultures (aim to start daytime cultures around 7 am)
    - Check electroporation plates

- Electroporation plates had no growth once again; running this Maxi-Prep to see if a much higher DNA concentration will change anything next time I do the electroporation

Maxi-Prep Protocol

1. Harvest cells by spinning down at 6000 x g for 15 minutes at 4°C
    a. When doing this, transfer the 250 ml daytime cultures (which should be grown to an OD600 of 0.4-0.5) to 250 ml centrifuge canisters
2. Remove supernatant and resuspend cells in 5 ml of Buffer P1; after this step, transfer cell solution to 50 ml tubes
3. Add 5 ml of Buffer P2 to each tube and mix by rigorously inverting 4-6 times and incubate at room temp for 3 minutes
4. Add 5 ml Buffer S3 to each tube, immediately mixing by inverting 4-6 times
5. Centrifuge the tubes for 45 minutes at 14365 x g at 4°C and then transfer the supernatant containing the plasmid to a new 50 ml tube
6. Centrifuge again for 25 minutes at 14365 x g at 4°C, transferring the supernatant to another new tube
    a. During this step, set up vacuum manifold, making sure all holes are plugged besides the ones to be used
7. Add 5 ml Buffer BB to each tube, mixing by inverting 4-6 times and then transfer to the tube extenders, which are to be attached to the CompactPrep columns on the vacuum manifold
8. Use the vacuum manifold to draw the lysate through the column, making sure all the lysate is through
    a. Once all lysate has been pulled through the column, immediately shut off the vacuum and transfer CompactPrep column to a 2 ml collection tube
9. Wash DNA via microcentrifuge
    a. Add 700 ul of Buffer PE to the column, spinning for 1 minute at 10000 x g, then removing the flowthrough and spinning again for 3 minutes to remove residual buffer
10. Elute DNA
    a. Add 50 ul 0.1x EB to the columns, letting stand for 1 minute before spinning for 1 minute
    b. Then, pipetting the flowthrough and putting on the column again, letting stand 1 minute before spinning for 1 minute

pKR95 Maxi-Prep Concentrations

#	Sample ID	Username	Date and Time	Nucleic Acid	Unit
1	MaxiPrep 1	Science	4/22/2022 3:01:43 PM	1040.2	ng/µl
2	MaxiPrep 2	Science	4/22/2022 3:02:34 PM	992.7	ng/µl

A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
20.805	10.762	1.93	2.38	DNA
19.854	10.329	1.92	2.44	DNA

- Whole lotta plasmid; Hannah will check the absorbance ratios on Monday

April 25, 2022

- To do list:
  - ~~Streak out *S. aureus* cells to single colonies for preparation of electrocompetent cells~~
  - ~~Check the electroporation plates that were left out over the weekend~~

- Electroporation plates still had no growth (surprise surprise)
- Used one of the glycerol stocks I made on Tuesday for the single colony plate

April 26, 2022

- To do list:
  - ~~Prepare two overnight cultures for electrocompetent cell prep~~

April 27, 2022

- To do list:
  - ~~Make electrocompetent cells~~
  - ~~Electroporation with increased DNA concentration~~

Preparation of Electrocompetent Cells

- Streak out *S. aureus* to single colony two days prior to making electrocompetent cells.
- Have all materials ready before starting preparation of electrocompetent cells.

17. For every 6 – 8 aliquots of electrocompetent cells to be made, inoculate one glass culture tubes with 10 mL LB media with a single *S. aureus* colony.
18. Grow overnight at 37°C in the shaking incubator.
19. Check the density of the overnight culture.
    a. Combine in a cuvette 900 uL LB and 100 uL from the overnight culture. Mix by pipetting.
    b. Check OD600, using a cuvette with LB as a blank.
    c. Multiply the observed OD600 by 10 to determine the density of the overnight culture.
20. For each aliquot of electrocompetent cells to be made, made a 10 mL culture of LB and cells from the overnight culture, diluted to OD600 of 0.5.
    a. Calculate how much of the overnight culture is needed to obtain an OD600 = 0.5 in 10 mL. Use the C1V1=C2V2 equation with the concentrations being the OD600s; V2 will be 10 ml and C2 will be 0.5.
    b. Add the appropriate amount of LB to glass culture tubes (10 mL – volume of overnight culture to add).
    c. Add calculated volume of overnight culture to glass tube. Put tubes into shaking 37°C incubator for another 40 minutes.

Start cooling down large centrifuge for next steps.

21. To harvest cells, spin down entire culture (10 mL) at 7000 x g for 5 minutes at 4°C in 50 ml conical tubes and discard supernatant
22. Add equal volumes of ice-cold sterile water (10 mL), and spin down again at the same settings. Discard the supernatant and repeat (total of two washes with water).
23. After two washes with water, resuspend cells in 2 mL (1/5 volume) of sterile ice-cold 10% glycerol. Discard the supernatant and repeat (total of two washes with 10% glycerol).
    a. At this point, I increased the speed to 8000 x g and the time to 7 minutes because the pellets began to move and became difficult to separate from the supernatant
24. After two washes with 10% glycerol, resuspend cells in 50 uL (1/200) sterile ice-cold 10% glycerol and transfer cells to sterile microfuge tubes.
25. Store aliquots of cells at -70 °C

26. If using a new strain: Plate out (spreading) one aliquot on two plates.
    a. 45 ul on LB 25 ug/ml chloramphenicol plate
    b. 5 ul on LB

Electroporation

1. Start cooling down large centrifuge and be sure shaking incubator is set to 30°C, put the plates in the standing incubator to warm.
2. Thaw cells on ice (~ 5mins).
3. Spin cells for 3 minutes at 5000 x g at 4°C.
4. Discard supernatant and resuspend in 80 ul 10% glycerol-500 mM sucrose.
5. Add 1 ug plasmid DNA to the cells. Should be in a volume of 5 – 10 uL, so DNA concentrations should be 100-200 ng/uL). Very carefully mix by pipetting, making sure not to include air bubbles
6. Transfer cells and DNA to a 1 mm electroporation cuvette.
7. Set electroporator to 2.1 kV and pulse. Take note of the time constant.
8. Immediately after pulsing, add 1 ml of LB with 500 mM sucrose to the cuvette. Mix by pipetting. Transfer to transfer to a sterile glass culture tube and incubated at an angle (~45°) for 1 hour at 30 °C
9. Plate out 100 ul of recovered cells on two LB chlor/X-gal plates. Incubate one plate overnight at 37 °C and the other plate at 30°C for 48 h.
10. Spin down remaining cells in a sterile microfuge tube at 7000 x g for 5 minutes. Remove supernatant and resuspend in 100 ul LB 500 mM sucrose. Plate entire volume onto another chlor/X-gal LB plate for overnight incubation at 37°C

Tube	DNA (ug)	DNA Incubation	EP	MS
1	10.4 (10 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	3.3
2	5.2 (5 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	3.6

- I messed up and only had enough plates for 2 electroporations (6 10 ug/ml chlor LB+CX plates)
    - Hannah said it would be more important to test out two high concentration electroporations rather than include a negative control

April 28, 2022

- To do list:
    - ~~Check the electroporation plates~~
    - ~~Make more LB+CX plates~~

- No growth on the 37°C plates ☹

LB+CX Plates

- For 500 ml
    - 500 ml LB agar
    - 1 ml of the 50 mg/ml XGAL stock
    - 100 ul of the 50 mg/ml chloramphenicol stock

April 29, 2022

- To do list:

- ~~Check electroporation plates~~

- No growth on the 30°C plates ☹
  - Need to talk to Hannah and Kathryn about next steps; maybe doing a 4-5 hour recovery incubation period instead of the 1, along with trying the other variable electroporations done a couple weeks before with the high DNA concentration

XGAL Recipe

X-gal	50 mg/ml	-20°C	-20°C	0.5 g powder in 10 mL DMF (in the hood), vortex, foil

Electroporation Attempts

Tube	Contents	DNA (ug)	MS
1	E-1 pKR95	1 (6.49 ul) pKR95	4.6
2	E-2 pKR95	1 (6.49 ul) pKR95	4.0
3	E-3 (-) control	--- (6.49 ul sterile H2O)	4.9

25 ug/ml chlor LB+CX plates (2.1 kV / 0.1 cm cuvette)
Unsuccessful (no growth on 37°C or 30°C plates)

Tube	DNA (ug)	DNA Incubation	EP	MS
1	1 (6.49 ul pKR95)	30 min	2.1 kV / 0.1 cm cuvette	4.8
2	1 (6.49 ul pKR95)	30 min	1.8 kV / 0.2 cm cuvette (StA)	2.5
3	1.54 (10 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	4.6
4	----- (6.49 ul ddiH2O)	30 min	2.1 kV / 0.1 cm cuvette	0.9

25 ug/ml chlor LB+CX plates + different variables
Unsuccessful (no growth on 37°C or 30°C plates)

Tube	DNA (ug)	DNA Incubation	EP	MS
1	1 (6.49 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	4.9
2	------ (6.49 water)	0 min	2.1 kV / 0.1 cm cuvette	4.8

10 ug/ml chlor LB+CX plates
Unsuccessful (no growth on 37°C or 30°C plates)

Tube	DNA (ug)	DNA Incubation	EP	MS
1	10.4 (10 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	3.3
2	5.2 (5 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	3.6

10 ug/ml chlor LB+CX plates + much more plasmid
Unsuccessful (no growth on 37°C or 30°C plates)

May 2, 2022

- To do list:
  - ~~Electroporations~~
  - ~~Make new medias~~

Electroporation Plan

	Monk	Ramsey	Grosser	Mix 1	Mix 2	Mix 3	(-)
		Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Thaw	5 min	5 min	Make day of	5 min	5 min	5 min	5 min
Spin Down	5,000 x g; 3 min	5,000 x g; 1 min	-------	5000 x g; 3 min	5,000 x g; 1 min	5,000 x g; 1 min	5,000 x g; 3 min
Resuspend	80 ul 10% glycerol, 500 mM sucrose	50 ul 10% glycerol, 500 mM sucrose	70 ul 10% glycerol	50 ul 10% glycerol, 500 mM sucrose	80 ul 10% glycerol, 500 mM sucrose	50 ul 10% glycerol, 500 mM sucrose	50 ul 10% glycerol, 500 mM sucrose
Cuvette	0.1 cm	0.1 cm	0.2 cm	0.1 cm	0.1 cm	0.2 cm	0.1 cm
DNA (ug)	0.5-1	<5	1	10	<5	<5	---
Voltage	2.1 kV	2.1 kV	1.8 kV	2.1 kV	2.1 kV	1.8 kV	1.8 kV
Recovery	1 hr; 30°C	1-1.5 hrs; 37°C	3 hr; 37°C	3 hrs; 30°C	2.5-3 hrs; 30°C	2.5-3 hrs; 30°C	3 hrs; 30°C
Media	LB+500 mM sucrose 1 mL	TSB+500 mM sucrose 1 mL	B2 500 ul	TSB+500 mM sucrose 1 mL	TSB+500 mM sucrose 500 ul	TSB+500 mM sucrose 1 mL	LB+500 mM sucrose 1 mL

LB media, 500 mM sucrose (50 ml)

- 50 ml LB media
- 8.56 g sucrose
- Mix and sterilize in instant pot

10% Glycerol + 500 mM sucrose (50 ml)

- 8.56 g sucrose
- 6.67 ml 75% glycerol
- Add water until 50 ml
- Filter sterilize, keep at 4°C

TSB, 500 mM sucrose

- 8.56 g sucrose
- 1.5 g TSB mix
- Initially add 35 ml water, heat and mix until everything dissolves, then add water until 50 ml
- Mix and sterilize in instant pot

Electroporation

1. Start cooling down large centrifuge and be sure shaking incubator is set to 30°C, put the plates in the standing incubator to warm.
2. Thaw cells on ice (~ 5mins).
3. Spin cells for 3 minutes at 5000 x g at 4°C.
4. Discard supernatant and resuspend in 80 ul 10% glycerol-500 mM sucrose.
5. Add 1 ug plasmid DNA to the cells. Should be in a volume of 5 – 10 uL, so DNA concentrations should be 100-200 ng/uL). Very carefully mix by pipetting, making sure not to include air bubbles
6. Transfer cells and DNA to a 1 mm electroporation cuvette.
7. Set electroporator to 2.1 kV and pulse. Take note of the time constant.
8. Immediately after pulsing, add 1 ml of LB with 500 mM sucrose to the cuvette. Mix by pipetting. Transfer to transfer to a sterile glass culture tube and incubated at an angle (~45°) for 1 hour at 30 °C
9. Plate out 100 ul of recovered cells on two LB chlor/X-gal plates. Incubate one plate overnight at 37 °C and the other plate at 30°C for 48 h.
10. Spin down remaining cells in a sterile microfuge tube at 7000 x g for 5 minutes. Remove supernatant and resuspend in 100 ul LB 500 mM sucrose. Plate entire volume onto another chlor/X-gal LB plate for overnight incubation at 37°C

Electroporation Tubes

Tube 1 (R)	**Tube 2 (G)**	**Tube 3 (M1)**	**Tube 4 (M2)**	**Tube 5 (M3)**	**Tube 6 (-con)**
5 min	Make day of	5 min	5 min	5 min	5 min
5,000 x g; 1 min	-------	5000 x g; 3 min	5,000 x g; 1 min	5,000 x g; 1 min	5,000 x g; 3 min
50 ul 10% glycerol, 500 mM sucrose	70 ul 10% glycerol	50 ul 10% glycerol, 500 mM sucrose	80 ul 10% glycerol, 500 mM sucrose	50 ul 10% glycerol, 500 mM sucrose	50 ul 10% glycerol, 500 mM sucrose
0.1 cm	0.2 cm	0.1 cm	0.1 cm	0.2 cm	0.1 cm
<5	1	10	<5	<5	---
2.1 kV	1.8 kV	2.1 kV	2.1 kV	1.8 kV	1.8 kV
2.5-3 hrs; 30°C	3 hr; 30°C	3 hrs; 30°C	2.5-3 hrs; 30°C	2.5-3 hrs; 30°C	3 hrs; 30°C
TSB+500 mM sucrose 1 mL	TSB 500 ul	TSB+500 mM sucrose 1 mL	TSB+500 mM sucrose 500 ul	TSB+500 mM sucrose 1 mL	LB+500 mM sucrose 1 mL

- I plated them all after a 3-hour recovery incubation:
  - 100 ul at 37°C
  - 100 ul at 30°C
  - 100 ul rem at 37°C
  - 18 plates total (3 plates per electroporation)

May 3, 2022

- To do list:
  - ~~Check plates~~

- ~~Meet with Kathryn about presentation~~

- 37°C Plates:
  - R: no growth on either plate (100 ul or 100 ul rem)
  - G: no growth on either plate (100 ul or 100 ul rem)
  - M1: no growth on either plate (100 ul or 100 ul rem)
  - M2: no growth on either plate (100 ul or 100 ul rem)
  - M3: no growth on the 100 ul; bit of a lawn on the remaining 100 ul (promising for the 30°C)
    - There looked to be a couple large white spots on the plate, but Hannah explained that this was probably just uneven spreading of cells after I pipetted them onto the plate
  - (-) control: no growth on either plate (100 ul or 100 ul rem)
- Meeting with Kathryn
  - She said my presentation was lovely
  - We decided to briefly introduce the idea of allelic exchange, but not explain it unless someone asks so I can cut down on time
  - I think I have good flow through the concepts within my presentation, and without the allelic exchange section, I am probably around 3 ½ minutes

May 4, 2022

- To do list
  - ~~Check 30°C plates~~
  - ~~Some honors conference thing~~

- 30°C plates
  - Unfortunately, there was no growth on any of the plates
  - Although, the Mix 3 plate from the 37°C plate stack looks very interesting as there is a visible lawn
  - I left all plate stacks out at room temp

May 9, 2022

- To do list:
  - ~~Finish *S. aureus* allelic exchange protocol~~
  - ~~Some cleaning things~~

- Hannah saw a blue colony on the E-2 plate from April 27th electroporation (this was the normal electroporation protocol, just with much higher DNA concentration)

E-2	5.2 (5 ul pKR95)	0 min	2.1 kV / 0.1 cm cuvette	3.6

- The plate was very contaminated, but Hannah went forward with the Slow Integration steps from the protocol.

Last Day

- Cleaned out my freezer boxes
    - Small -20°C
        - Threw out the unsuccessful Midi-Prep samples along with the unsuccessful ligation samples
        - Left successful ligation samples, most recent digest samples (just so plasmid components are still available), along with the Maxi-Prep samples which I had used in the most recent electroporation attempts
    - DF + AM -80°C
        - Did not throw out anything
    - DF + AM RNA -80°C
        - Did not throw out anything
    - DF Glycerol Stocks -80°C
        - Did not throw out anything
        - This box contains *S. aureus* permanent stocks and single use aliquots
- Unfortunately, I was cleaning out the dirty plates and threw out the most recent electroporation plates
    - They did not have any growth, but the E-2 from April 27th took almost a week and a half to grow a blue colony at room temperature so I should not have thrown those out since it has only been a week
    - Sorry Hannah!