Imidacloprid Degradation Notebook

June 25

1. Diluted primers to 100uM H_2O
2. Ran PCR to amplify genes + attach pelB/pET22b adaptor sequences to create megaprimer

PCR Recipe

Tube 1	X1	X4
Template	1uL	4uL
5' primer	1uL	4uL
3' primer	1uL	4uL
dNTP	1uL	4uL
dH_2O	21uL	84uL
Total Volume	25uL	100uL

Tube 2	X1	X14*
5x Buffer	10uL	140uL
dH_2O	14uL	196uL
Phusion enzyme	0.5uL	7uL
Total Volume	25uL	343uL

* 3 genes x 4 temps. = 12 + 1 (-) control + 1 stuff left on sides of tube = 14x

PCR Conditions (30x runs)

98°C	1:00 min
98°C	0:30 min
48°C → 54 °C	0:30 min
72°C	2:00 min
72°C	5:00 min
12°C	HOLD

June 29

1. Ran agarose gel to confirm PCR
    a. 5uL DNA Ladder
    b. 1uL dye + 5uL PCR product
    c. Run 1:00:00 hour @ 100V
2. Gel order: (-) G48 G50 G52 G54 M48 M50 M52 M54 H48 H 50 H52 H54 L

1% Agarose Gel Recipe

0.5g agarose
50mL TAE Buffer
5uL sybersafe

Gene sizes/weights:

CYP6CM1	1566 bp	59,694.9 Da
CYP6G1	1578 bp	59,908.9 Da
HUMCYPdb1	1497 bp	55,801.5 Da
pET22b	5493 bp	

3. PCR Clean-Up
4. Made overnight culture of E. coli with pET22b

<u>June 30</u>

1. Miniprep pET22b overnight culture
2. NanoDrop PCR clean-up + miniprep
    a. M: 52 ug/uL
    b. G: 60 ug/uL
    c. H: 50.3 ug/uL
    d. pET22b: 74.7 ug/uL
3. Ran overnight megaprimer PCR

Whole Plasmid Cloning Recipe

Ingredients	1x	10x*
10x PFX Amp. Buffer	3 uL	30 uL
dNTP (10uM)	0.9 uL	9 uL
$MgSO_4$ (50uM)	0.6 uL	6 uL
DMSO	1.2 uL	12 uL
Platinum PFX	0.6 uL	6 uL
Plasmid (30ng)	X	10x
Megaprimers (225,450,900 nM)	Y	
dH_2O	Z	
Total Volume	30 uL	

* 3 genes x 3 concentrations + 1 (-) = 10 reactions
* 6.3 + X
* X = 0.5 uL (in this case)
* Y = 225/primer concentration 450/[con.], etc.
* Z = 30 – (X + Y)
* Mix = 10x PFX Amp. Buffer + dNTP (10uM) + $MgSO_4$ (50uM) + DMSO + Platinum PFX

Megaprimer & concentration	Mix + X (6.3 + X) X = 0.5 uL	Primer (Y) uL	Z = dH_2O
M 225	6.8	4.33	18.87
M 450	6.8	8.65	14.55
M 900	6.8	17.3	5.9
G 225	6.8	3.74	19.46
G450	6.8	7.48	15.72
G 900	6.8	14.95	8.25
M 225	6.8	4.47	18.73
M450	6.8	8.95	14.25
M 900	6.8	17.9	5.3
Control	6.8	none	23.8

PCR Conditions (25x runs)

68°C	5:00 min
95°C	0:45 min
95°C	0:30 min
55°C	0:30 min
68°C	7:00 min
12°C	HOLD

<u>July 6 (Rohan)</u>

1. Ran PCR product on agarose gel:
    a. H2, H5, H9, M2, M4 , M9, G2, G4, G9, (-), L
2. Ran DPN digest on H4, H9, M4, M9, G4, G9 using 75ul (?) DPN1 for rest of reaction at 37°C for 30:00 min

<u>July 7 (Rohan)</u>

1. Electroporated 6 tubes of DH5α cells using 5uL of each DPN digest reaction
2. Plated onto LB-carbR (since resistance to carb + amp are the same)

<u>July 8 (Rohan)</u>

1. Transferred plates from 37°C to 4°C

<u>July 10 (Rohan)</u>

1. Observed colony growth on plates
    a. 5 colonies for G: 2 450 + 3 900
    b. 6 colonies for H: 2 450 + 4 900
    c. 6 colonies for M: 3 450 + 3 900
2. Ran colony PCR using “out of plasmid primers”
3.

Primer Labels:

OutOfPET-2F
OutOfPET_CYP6CM1-2R
OutOfPET_CYP6G1-2R
OutOfPET_HUMCYP-2R

PCR Recipe

Ingredients	1x	20x*
10x Taq Buffer	2.5 uL	50 uL
dNTP 's	0.5 uL	10 uL
Forward Primer	0.5 uL	10 uL
Reverse Primer	0.5 uL	10 uL
Template	Colony	
Taq	0.2	4 uL
dH_2O	20.8 uL	416 uL
Total Volume	25 uL	500 uL

* 17 colonies + 3 (-) = 20 reactions

PCR Conditions (30x runs)

98°C	1:00 min
98°C	0:30 min
62°C	0:30 min
68°C	2:00 min
68°C	5:00 min
12°C	HOLD

<u>July 12 (Rohan)</u>

1. Ran 2 gels to check for fragment amplification
    a. Gel 1: L, M41, M42, M43, M91, M92, M93, H41, H42, H91, H92, H93, H94
        i. No bands whatsoever. Lots of primer dimer. Try with T7 primers next time

<u>July 13 (Rohan)</u>

1. Retrying colony PCR withT7 primers instead
    a. Same recipe as last time

PCR Conditions (30x runs)

95°C	2:00 min
95°C	0:30 min
50°C	0:30 min
68°C	2:00 min
68°C	5:00 min
10°C	HOLD

2. Ran gel: L, G41, G91, G92, H41, H91, H92, M41, M42, M91, M92

July 14

1. Made overnight culture of pET22b *E. coli*

July 15

1. Plasmid prep to elute pET22b
2. Nanodrop prep: 123.4 ng/uL
3. Ran whole plasmid (WP) PCR
    a. Same recipe as last WP PCR

Megaprimer & concentration	Mix + X (6.3 + X) X = 0.3 uL	Primer (Y) uL	Z = dH_2O
M 225	6.6	4.33	23.37
M 450	6.6	8.65	21.05
M 900	6.6	4.3 (none left)	12.4
G 225	6.6	3.74	25.96
G450	6.6	7.48	22.22
G 900	6.6	14.95	14.75
M 225	6.6	4.47	25.23
M450	6.6	8.95	20.75
M 900	6.6	None left	11.80
Control	6.6	None	23.40

* Plasmid = 123.4 ng/uL x 0.3 uL (X) = 30ng

PCR Conditions (revised) (30x runs) (30uL volume)

94°C	1:00 min
95°C	0:30 min
55°C	0:30 min
68°C	7:00 min
68°C	10:00 min
10°C	HOLD

July 16

1. Digested pET22b with Nco1 for 1:15 hours @ 37°C

Digest Recipe

Cutsmart Buffer	5 uL
Nco1	1 uL
Plasmid (1 ug)*	8.1 uL
dH_2O	35.9 uL

* 0.1234 ug/ul x 8.1 uL = 1ug plasmid

2. PCR clean digest (eluted with 30uL elution buffer)
3. Nanodrop PCR clean: 14.5 ng/uL
4. Made more megaprimers
    a. Not enough template so I used 1uL template for each
    b. Optimal PCR temps: G @ 54°C, H @ 48°C, M @ 52°C, control @ 51.7°C

Tube 1	1x
Template	1 uL
5' primer	1 uL
3' primer	1 uL
dNTP	1 uL
dH_2O	21 uL
Total	25 uL

Tube 2	1x
5x Buffer	10 uL
dH_2O	14 uL
Phusion	0.5 uL
Total	24.5 uL

PCR Conditions (30x runs) (50uL volume)

98°C	1:00 min
98°C	0:30 min
48, 52, 54°C	0:30 min
72°C	2:00 min
72°C	5:00 min
12°C	HOLD

5. PCR clean (eluted all in 50uL elution buffer)
6. Nanodrop PCR clean:
    a. M: 30.1 ng/uL
    b. G: 26.3 ng/uL
    c. H: 19.0 ng/uL

July 17

1. Ran gel of PCR clean: L, M, H, G, pET22b, M(-), H (-), G (-)
    a. 5 uL PCR mix + 1uL dye
    b. 5 uL DNA ladder (GeneDirex 1kb
    c. 1:00 hour @ 100V
2. Ran WP PCR

Megaprimer & concentration	Mix + X (6.3 + X) X = 2.06 uL	Primer (Y) uL	Z = dH_2O
M 450	8.36	14.95	6.69
M 450	8.36	21.64*	0*
M 450	8.36	17.1	4.54
Control	8.36	none	21.64

* Concentration was too low so I lowered the amount of MP and didn't add H_2O

PCR Conditions (30x runs) (30uL volume)

94°C	1:00 min
94°C	0:30 min
55°C	0:30 min
68°C	7:00 min
68°C	10:00 min
12°C	HOLD

3. Ran gel of PCR: L, M, H, G, no control (messed up well)

July 20 (Rohan)

1. Made 50 uL liquid cell culture of CYP6G1 and CYP6CM1 from plates Mike brought (CYPs and reductase in PSB1C3 → chloramphenicol resistant) + grew overnight

July 21 (Rohan)

1. Inoculated cell cultures in 1L LB (10 mL in 1L LB) + added 1mL chloramphenicol
2. Induced cultures at OD: 0.6 with 1mM IPTG
3. Separated each culture into 50mL aliquots and shook overnight in 16°C, 20°C, 26°C, 30°C, and 37°C.

July 22 (Rohan)

1. Spun down 1.5mL of each culture and stored in -80°C
2. Spun down 1.5mL of each culture and stored in -20°C

July 23

1. Prepared samples for SDS-PAGE
    a. Resuspend pellet with 400uL 20mM phosphate and 500mM NaCl
    b. Sonicate each sample 4 times for 10 seconds on lowest setting
    c. Centrifuge @ 4°C for 30:00 min.
    d. Transfer 50uL supernatant into 1.5mL tube (soluble fraction) + discard rest of s.n.
    e. Resuspend pellet with Buffer 3 (containing urea)
    f. Centrifuge @ 4°C for 30:00 min.
    g. Transfer 50uL supernatant into 1.5 mL tube
    h. Add 10uL SDS sample buffer to all 1.5mL tubes

July 24

1. Set-up SDS apparatus
    a. Place small glass plate in front of larger glass plate
    b. Place both plats into bracket
2. Prepared 40% polyacrylamide gel + ran gel + imaged gel
    a. Order:
        i. dH_2O
        ii. SDS
        iii. Tris (separating/stacking)
        iv. Polyacrylamide
        v. APS
        vi. TEMED
    b. Storing conditions:
        i. TEMED: 4°C
        ii. APS + Ladder: -20°C
        iii. SDS: room temp.

40% Polyacrylamide SDS-PAGE gel recipe:

Ingredients	Stacking	Separating
Gel percentage (%)	5	10
40% Polyacrylamide (mL)	0.5	2.5
1M Tris(mL) Stacking: pH 6.8 Separating: pH 8.8	0.5	2.5
10% Ammonium persulfate (mL)	0.04	0.1
10% SDS (mL)	0.04	0.1
TEMED (mL)	0.004*	0.004*
dH_2O (mL)	2.92	4.8

Total volume (mL)	4	10

SDS Gel Making Protocol:

1. Make Separating and Stacking gel mixtures
2. Take 1mL of separating mixture into 1.5mL tube
3. Add 4uL TEMED to 1.5mL tube
4. Add 500uL separating mixture + TEMED in between glass plates
5. Add isopropanol to even out gel line
6. Wait ~ 30 min for gel to solidify
7. Add 8-10uL TEMED into remaining separating solution
8. Add separating + TEMED soln. between glass plates up to 1cm away from top
9. Add isopropanol to even out gel line
10. Wait ~ 30 min for gel to solidify
11. Add 8uL TEMED to stacking mixture and add in between glass plates (overflow it)
12. Add comb and wait ~30 min for gel to solidify
13. Add SDS Running buffer, load wells (10uL) and run gel

SDS-PAGE Gel Running Conditions

1. 80V until proteins are halfway down gel
2. 160V until 2cm away from bottom
3. 200V until blue dye runs off

SDS-PAGE Gel Washing + Staining Protocol

1. Wash gel with dH_2O 3 times and microwave for 90 seconds (45 seconds per gel)
2. Remove dH_2O and add blue dye + stir for 10 min
3. Wash off dye and wash with dH_2O 3 times
4. Add 5 kimwipes (clump into ball) and place in corner of Tupperware
5. Microwave for 3:00 min
6. Stir for 10 min
7. Image

<u>July 27</u>

1. Prepared SDS gels

<u>July 28</u>

1. Ran CYP6G1 soluble + insoluble fractions on SDS-PAGE
2. Imaged gels:

July 29

1. Prepared 50mL overnight cell cultures for CYP6CM1, CYP6G1 and HUMCYPdb1

July 30

1. Digested pET22b with Sac1 and Nco1 @37°C for 1:30 hours

Cutsmart	2uL
Nco1	1uL
Sac1	1L
Plasmid	8.1uL
8.9 dH_2O	8.9uL

2. Performed Gibson Assembly on digest to insert CYP6G1 and HUMCYP into pET vector

Gibson Master Mix	5uL
Digested Plasmid (50ng)	1uL
Primers (44ng)	2.31uL HUMCYP 1.67uL 6G1
dH_2O	Add to make 5uL total plasmid + primer
Total	10uL

3. Incubated Gibson mix in 37°C for 1:00 hour
4. Transformed 100uL competent DH5α *E. coli* with 5uL Gibson mix

Transformation Protocol

1. In 1.5mL tube:
2. Add 5uL insert to 100uL cells
3. Ice for 30:00 min
4. Heat @ 42°C for 0:45 min
5. Ice for 5:00 min
6. Add 1 mL LB (w/o abx.)
7. Incubate + shake @ 37°C for 1:00 hour
8. Spread 100uL transformant on plates (ampR)

5. Transferred 10mL of overnight cultures to 1L lB (6G1 + HUMCYP)
6. Measured OD values until 0.645 6G1 and 0.633 HUMCYP
7. Induced with 1mM IPTG, separated into 5 flasks (per gene) and shook overnight in 16°C, 20°C, 26°C, 30°C, and 37°C

July 31

1. Observations:
    a. No colony growth on G plate
    b. 1 colony growth on H plate
2. Prepared previous overnight cultures (6G1 + HUMCYP) for SDS with revised protocol (to get more insoluble protein on gel) + ran gel (only 6G1, not HUMCYP)
3. Ran colony PCR on the 1 HUMCYP colony

Revised SDS-PAGE sample preparation

1. Resuspend pellet with 400uL 300mM NaCl and 50mM HEPES 5% glycerol pH 7.4
2. Sonicate each sample 4 times for 10 seconds on lowest setting
3. Centrifuge @ 4°C for 30:00 min.
4. Transfer 50uL supernatant into 1.5mL tube (soluble fraction) + discard rest of s.n.
5. Resuspend pellet with 100uL dH_2O
6. Transfer 50uL supernatant into 1.5 mL tube
7. Add 10uL SDS sample buffer to all 1.5mL tubes
8. Boil @ 98°C for 10:00 min + spin down

August 1

1. Transformed 100uL competent DH5α *E. coli* with remaining 5uL Gibson mix + plated overnight
    a. (+) controls: undigested pET22b/cells/ampR LB plate + cells/LB plate

August 2

2. Observations: no growth on G or H plates but (+) controls grew fine

August 4

1. Ran SDS on H and G + stained
    a. Spilled G (-) controls
    b. Didn't normalize cell numbers so H (-) bands have less protein
2. Left shaking overnight

August 5

1. Imaged previous SDS gels

August 5

1. Imaged HUMCYP colony PCR product

August 7

1. Prepared overnight culture of AgCPR and CYP6CM1

August 8

1. Inoculated 10mL overnight culture in 500mL LB with 50uL chloramphenicol
2. Grew until OD:
    a. AgCPR: 0.703
    b. CYP6CM1: 0.618

August 10

1. Normalized cell count to OD 2.5
    a. V_2 = 1.5mL final volume; C_2 =OD 2.5
    b. $V_1 = [(2.5)(1.5)] / [(3)(C_1)]$ → amount undiluted to add
2. Prepared samples for SDS-PAGE

August 11

1. Ran SDS-PAGE + imaged

August 12

1. Diluted megaprimer method 2 primers to 100uM + made 10x working stock
2. Ran PCR to create megaprimers using previous protocol + recipe
3. Imaged gel:

August 17

1. Ran "mutagenesis" PCR to insert pelB upstream of the CYP's + reductase

Recipe:

5x Phusion Buffer	5
Template (100ng)	depends
Primer (6uM)	1.5
Ampligase (5U/uL)	1.5
NAD+ (50mM)	0.25
Phusion	0.25
dNTP (10mM)	0.5
DMSO	0.75
dH_2O	11.75
Total	25 uL

PCR Conditions (29x runs) (25uL)

98°C	0:30 min
98°C	0:15 min
55°C	1:00 min
72°C	3:00 min
72°C	10:00 min
12°C	HOLD

2. Ran overnight DPN1 digest on PCR product

August 18

1. Transformed 2ul into 50ul DH5alpha cells and added 1mL LB
2. Plated 50uL on chlor plates

August 19

1. Re-ran mutagenesis PCR with same conditions/reagents (except used new NAD+)
2. Added 0.5 uL DPN1 per 25uL PCR reaction + incubated O/N

Revised Transformation Protocol

1. Add 5uL DNA to 50uL cells
2. Ice for 30 min
3. Heat for 45 sec at 42°C
4. Add 250uL LB
5. Incubate and shake at 37°C for 1 hour
6. Spread 300uL on plates

August 20

1. Heat inactivated DPN1 at 80°C for 20 min
2. Transformed 50uL DH5alpha with 5 uL DNA
3. (+) control: 6CM1 PSB1C3 template
    a. Added 1.5uL b/c its very concentrated (312.2ng/ul)
4. Plated all 300uL on chlor LB plates and incubated at 37°C O/N

August 21

1. Saw colony growth on plates → ran colony PCR on all colonies and controls

Colony PCR Conditions

98°C	1:00 min
98°C	0:30 min
53°C	0:30 min
72°C	2:30 min
72°C	5:00 min
12°C	HOLD

** The above conditions do not allow for proper lysis of cells (1 min) which is why it may not have worked.

2. Ran Gel

August 24

1. Ran mutagenesis PCR #2 (same everything)
    a. 0.4766ul G template
    b. 0.32 uL M template
    c. 0.27 uL A template
    d. M- M G A
2. DPN1 digested O/N

August 25

1. Transformed using previous revised protocol
2. Plated and grew O/N
3. Re-rean colony PCR from Mut. PCR #1
    a. Same reagents (except primers)
    b. Revised conditions
    c. Primers: OutofPET 6CM1/6G1 and VR as reverse primers

98°C	2:00 min
98°C	0:30 min
50°C	0:30 min
72°C	3:00 min
72°C	5:00 min
12°C	HOLD

August 26

1. Ran gel on colony PCR (same colonies, revised primers/conditions)
    a. L M- M G- G1 G2 G3 G4 A- A1 A2 A3
    b. 6uL DNA + 1 uL dye
2. Made 3 O/Ns of PSB1C3 DH5alpha
3. Transformed DH5alpha cells with combined Mut #1 and Mut #2 PCR DNA via electroporation
    a. Desalt DNA first
4. Plated on chlor plates an grew overnight

August 27

1. Ran HPLC: 5 samples
    a. IMI
    b. IMI + NADPH
    c. Cell lysate
    d. Cell lysate + IMI
    e. Cell lysate + IMI + NADPH
2. Made 2mM stock IMI solution (0.0024g IMI + 5mL H2O)
3. Made 2mM stock NADPH solution (0.0074g + 5mL H2O)
    a. Heat IMI+ H2O to fully dissolve
4. Incubated at 37°C for 1:00 hour
5. Followed HPLC protocol from: http://ac.els-cdn.com/S0965174809001258/1-s2.0-S0965174809001258-main.pdf?_tid=44fbdc3e-4dde-11e5-ac81-00000aab0f6c&acdnat=1440805454_515dd4ad0cd8617e2833d01a13b3a1dc
    a. Grow cells O/N

b. Pellet + Resuspend in 1mL resuspension buffer
    i. pH 7.6 (physiological pH)
    ii. Tris 50mM
    iii. DTT 0.1mM
    iv. NaCl 150mM
c. Sonicate
d. Make 1mL working stock solutions:
    i. 1mL H20 to control
    ii. 0.8mL H20 to IMI
    iii. 0.6mL H20 to IMI+NADPH
    iv. 0.2mL IMI to IMI and IMI+NADPH
    v. 0.2mL NADPH to IMI+NADPH
e. Filter dH2O through 0.45um paper
f. Run HPLC

HPLC Conditions

a. Injected 90uL + flow rate of 0.7mL/min @ room temp
b. Time trial linear gradient (in H2O):
    a. 0%-90% ACN 6 min
    b. 90% ACN 4 min
    c. 90%-0% ACN 1 min
    d. 0% ACN 4 min
c. C18 column, 150mm
d. 270nm wavelength
    a. Printed graphs

6. Ran colony PCR from electroporated cell colonies (10 colonies/gene)

<u>August 28</u>

1. Ran gels from colony PCR

3. Ran mutagenesis #3 (same protocol/conditions)
    a. New Ampligase, Phus, 5x Buffer
    b. Used diluted template (100ng)
    ➔ Didn't proceed with experiment (waste of time/money)

<u>September 1</u>

1. Made glycerol stocks of:
    a. CYP/ptac/LacI constructs
    b. CYP/tac constructs
    c. CYP/pelB/ptac constructs

2. Made O/N of:
    a. 6 6G1 cultures
    b. 6 AgCPR cultures
    c. 6 pSB1C3 no insert cultures

<u>September 2</u>

1. Made resuspension buffer, IMI stock and NADPH stock for HPLC + ran HPLC
2. Autocalved 20 250mL dry E. flasks
3. Made O/N of ptac/pelB M/H/A for HPLC in 5mL cultures
4. Made O/N of ptac/pelB A/M for SDS in 50mL cultures

Resuspension Buffer (250mL dH_2O, pH 7.6) Recipe

50mM Tris	1.514g
150mM NaCl	2.1915g
0.5mM DTT	0.01928g

IMI Stock

- 1.97mM IMI: 0.0024g
- 5mL dH_2O

NADPH Stock

- 1.41mM NADPH: 0.0055g
    - Ran out (normally use 0.0074g to get 1.99mM NADPH)

HPLC Protocol

1. Make O/N's of required samples
2. Spin down 5mL of each O/N + resuspend with resuspension buffer
3. Add protease inhibitors (10uL)
4. Lyse cells (sonicate)
5. Make appropriate sample mixtures
    a. 0.4mL of cell types
    b. 0.2mL of IMI and NADPH
    c. Add resuspension buffer to final volume of 1.2mL
6. Incubate at 37°C for 1:00 hour
7. Spin down samples at 21,000g for 25 min
8. Filter 0.6mL supernatant through o.1um paper
9. Run HPLC:
    a. [IMI]: 0.2, 0.4, 0.8 mM
    b. [NADPH]: 0.4mM
    c. Flow rate: 0.7mL/min
    d. Injection: 90uL
    e. UV wavelength: 270nm

HPLC observations:

1. DTT shows early release (very polar). Also degrades and loses reducing ability rather quickly (day or so)
2. Filter dH_2O so no microorganisms get stuck on the column and grow in it
3. No difference in peak height (only in peak width: increases as [IMI] increases)
    a. Wider peak because takes more ACN to release all IMI
4. High pressure damages the column, doest affect the flow rate
5. IMI leaves at around 5.2 min
6. No effect seen from 6g1+IMI+NADPH sample:
    a. Trouleshoot:
        i. Increase incubation time (allow for more degradation)
        ii. Add Mg^{2+} to res. Buffer to help CYPs
        iii. Add more/different protease inhibitors
        iv. Spin down more cells
7. The hydroxylation of C4 and/or C5 makes 4-, 5-hydroxyIMI more polar. therefore, less ACN is required for the release of metabolites from column and therefore the retention time is lower (expect metabolite peaks to the left of IMI peak)

<u>September 3</u>

1. Autoclaved flasks and LB
2. Ran HPLC on the following mixtures:
    a. Cell: A, M, H, AM, AH
    b. Cell+IMI: AM, AH
    c. Cell+IMI+NADPH: M, H, AM, AH
    d. Use same HPLC protocol as last time
3. Transferred 1mL O/N SDS cultures to 100mL flasks (added Chlor)
4. Grew cultures A + M until OD 0.8 then transferred 50mL to 30°C and grew O/N
5. Made O/N of ptac/pelB/CYP G/H/G-/H- at 30°C in 50mL + 50mL chlor
6. Made O/N of LacI/ptac/pelB/CYP G/H/A/M and w/o pelB AMGH at 30°C in 50mL + 50mL chlor

<u>September 4</u>

1. Normalized ptac(+) M/A and prepapred for SDS prep
2. OD'd ptac(+) G/H and LacI(+)/(-) and grew O/N at 30°C

<u>September 5</u>

1. Normalized cell counts to OD 2.5 and prepared ALL for SDS
    a. Made 12uL samples using previous SDS prep protocol (should have made larger samples)

September 7

1. Ran SDS-PAGE on all LacI constructs using new gel recipe (Meghans)
    a. 0.75mm gel bracket
    b. No positive results

Separating (1 gel) Recipe

- 2mL 30% acrylamide
- 2.95mL PAGE-mix
- 50uL 10% APS
- 2uL TEMED

Stacking (1 gel) Recipe

- 0.325mL 30% acrylamide
- 2.175mL STACK-mix
- 25uL 10% APS
- 2.5uL TEMED

September 8

1. Ran ptac (+)/(-) constructs on SDS-PAGE
    a. No positive results