**iGEM UIUC 2021**

**Notebook -** General overview of what we did over the course of this project.

**Day 1 Wednesday 6/9**

Objectives:

- Make LB Media: There's pre-mixed media. Directions on bottle
    a. For 900 mL: 9 g tryptone, 4.5 g yeast extract, 4.5 g NaCl.
    b. For 200 mL: 2 g tryptone, 2 g yeast extract, 2 g NaCl.
    c. Store at 4C - Lasts for 1 year
- Making LB Agar for Plates LB powder + agar (1 or 2% agar)
    a. For 200 mL: 2 g tryptone, 1 g yeast extract, 1 g NaCl, 3 g agar.
    b. Autoclave
    c. Store at 4C - Lasts for 2 months
- Make 100 mL of 1M CaCl2, 100 mL of 50% Glycerol
    a. 1M CaCl2
        ■ Add 14.7 g CaCl2 to ~80 mL dH2O, mix, fill to 100 mL, autoclave
    b. 50% Glycerol (https://2015.igem.org/wiki/images/a/ab/Exeter_Glycerol_recipe.pdf)
        ■ See if there is any available in the lab, or combine 50 mL of dH20 with 50 mL of pure glycerol
    c. Autoclave
- Autoclave micropipette tips (gravity cycle, 20-25 mins)
- Autoclave dH2O (large quantity)
- Autoclave Flasks
-

LB Media Procedure:

1. Take a flask (or bottle) , then put 9g tryptone, 4.5g yeast extract, 4.5g NaCl mix and add 900ml DI water. Shake the flask to make sure there is no big chunk inside.
2. Autoclave the flask for 25 min . Loosen cap cover with foil. Put tape somewhere.

LB Agar Plate Procedure:

1. Take a flask (or bottle) , then put 2g tryptone, 1g yeast extract, 1g NaCl mix, 3 g agar and add 200ml DI water. Shake the flask to make sure there is no big chunk inside.
2. Autoclave the flask for 25 min . Loosen cap cover with foil. Put tape somewhere.
3. Gently pour the LB agar medium into 2-3 petri dishes. The liquid should be ⅓ to ½ of the petri dish. Leave the lids slightly off for 30 minutes inside the flow hood. Finally wrap it in parafilm and then store upside down in 4 degree celsius.

Starter culture: (From Anshu's Plate)

a. select a single colony of *E. coli* from a fresh LB plate and inoculate in 15 mL LB grow overnight in 37C shaker

        - 125 mL flask

- Competent Cell Prep Procedure:
    1. Autoclave:
        - LB media
        - 900 mL 100mM CaCl2
            - Add 13.23 g of CaCl2 *2H2O to ~700 mL of DI water, mix, fill to 900 mL, autoclave
        - 900 mL 100mM MgCl2
            - Add 18.27 g of MgCl2*6H20 to ~700 mL of DI water, mix, fill to 900 mL, autoclave
        - 100 mL 85mM CaCl2 with 15% glycerol v/v
            - Mix 8.5 mL of 1M CaCl2, 30 mL of 50% Glycerol and 61.5 mL of dH20
        - Autoclave dH2O (large quantity)
        - microfuge tubes
        - centrifuge tubes
        - Autoclave flasks
    2. Chill overnight at 4C:
        - the autoclaved 100mM CaCl2,
        - 100mM MgCl2
        - 85mM CaCl2 with 15% glycerol
        - Chill the centrifuge bottles x4
        - Eppendorf Tubes for dilutions and storing

**Day 2 Thursday 6/10:**

Starter culture: (From Anshu's Plate)

b. select a single colony of *E. coli* from a fresh LB plate and inoculate in 15 mL LB grow overnight in 37C shaker
    - 125 mL flask

- Competent Cell Prep Procedure:
    3. Autoclave:
        - LB media
        - 900 mL 100mM CaCl2
            - Add 13.23 g of CaCl2 *2H2O to ~700 mL of DI water, mix, fill to 900 mL, autoclave
        - 900 mL 100mM MgCl2

        - Add 18.27 g of MgCl2*6H20 to ~700 mL of DI water, mix, fill to 900 mL, autoclave
    - 100 mL 85mM CaCl2 with 15% glycerol v/v
        - Mix 8.5 mL of 1M CaCl2, 30 mL of 50% Glycerol and 61.5 mL of dH20
    - microfuge tubes
    - centrifuge tubes
4. Chill overnight at 4C:
    - the autoclaved 100mM CaCl2,
    - 100mM MgCl2
    - 85mM CaCl2 with 15% glycerol
    - Chill the centrifuge bottles x4
    - Eppendorf Tubes for dilutions and storing

**Day 3 Friday 6/11:**

- Need cryovial tubes for stock (use Eppendorf 1.5 ml for competent cell storage instead→ keep volume low enough to ensure that competent cells retain competence)
- Competent Cell Prep
- Store Glycerol Stock of *E. coli* at -80C
- Store Glycerol Stock of competent cells at -80C

Glycerol Stock of *E. coli* (0.5-0.7 mL of starter)

b. Plates should be only a week to 10 days old
c. Can do in Eppendorf tubes
d. 1:1 ratio

Competent Cells Procedure:
http://mcb.berkeley.edu/labs/krantz/protocols/calcium_comp_cells.pdf

1. Inoculate 1 L of LB media with 10 mL starter culture (made on Thursday) and grow in 37°C shaker.
    a. Measure the OD600 every hour, then every 15-20 minutes when the OD gets above 0.2. Check OD600 0.35-0.4 (approximately 3 hours)
    b. Measure using spectrophotometer
    c. Set centrifuge to chill at 4C
    d. Make sure cap is loose!!

(approximately 2 hours below)

2. Split the LB into 20 parts (50 mL in each of the conical tubes we chilled Thursday) and place cells on ice to chill for 20-30 min (Stop excess growth) swirling occasionally to ensure even cooling

3. (Spin #1) Harvest the cells by centrifugation at 3000g (~4000 rpm) for 15 minutes at 4°C.
4. Decant the supernatant and gently resuspend each pellet in about 5 mL of ice cold MgCl2. Combine all suspensions into one centrifuge bottle. Make sure to prepare a blank bottle as a balance.
5. (Spin #2) Harvest the cells by centrifugation at 2000g (~3000 rpm) for 15 minutes at 4°C.
6. Decant the supernatant and resuspend the pellet in about 10 mL of ice cold CaCl2. Keep this suspension on ice for at least 20 minutes. Start putting 1.5 mL microfuge tubes on ice if not already chilled.
7. (Spin #3) Harvest the cells by centrifugation at 2000g (~3000 rpm) for 15 minutes at 4°C. At this step, rinse a 50 mL conical tube with ddH2O and chill on ice.
8. Decant the supernatant and resuspend the pellet in ~2.5 mL of ice cold 85 mM CaCl2, 15% glycerol. Transfer the suspension to the 50 mL conical tube. Combine into one conical tube.
9. (Spin #4) Harvest the cells by centrifugation at 1000g (~2100 rpm in the Beckman GH-3.8 rotor) for 15 minutes at 4°C
10. Decant the supernatant and resuspend the pellet in 1.8 mL of ice cold 85 mM CaCl2, 15% glycerol. The final OD600 of the suspended cells should be ~ 200-250.
11. Aliquot 50 µL into sterile 1.5 mL microfuge tubes and snap freeze with dry ice. Store frozen cells in the -80°C freezer.
    a. Store competent cells in a separate box from frozen stocks

**Later:**

1.) Grow DH5 Alpha for plasmid propagation, storing plasmid, miniprep work (Need 1 150 ul competent cell tube)
    a.) Consider ordering DH5 alpha competent cells
    b.) Need this only once we get the plasmid
2.) Transformation
https://2019.igem.org/wiki/images/5/5a/T--Toronto--transformation.pdf
3.) Plasmid Purification
4.) Run one protein purification and if it works, scale up. If the process does not work, look into the cell free system.
    a.) Cell-free system TX-TL
    https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3960857/#__sec2title
        i.) Prepare reagents for Cell-free system

Plasmid containing a his-tag.
Bl21.
Boil cells in 10% sds, at 100 degrees for 5 minutes using coomassie blue.
Take 5-10 uL and load gel, run gel.

Goal: Get a thick band.

DH5 alpha make lots of plasmid (Anshu has)
Need to make as well

Bl21 optimized protein expression
(We have this may be sufficient we will know after doing miniprep).

**Day 4 Monday 6/14:**

- Make Agar Plates https://www.addgene.org/protocols/pouring-lb-agar-plates/
  - Make LB media with agar (remember to autoclave).
  - Amp (or whatever antibiotic for plasmid)

Materials

- Petri Dishes
- Bacto Agar (BD 214010)
- LB Broth (Sigma L3522)
- Antibiotic, usually at 1000X, add 1 uL/mL of media

Protocol

- 400 mL makes about one sleeve of petri dishes
- Add 25g LB Broth per Liter to an appropriately sized beaker (~10 g LB broth for 400 mL)
- Add Water and Stir until clumps are gone
- Add 15 g Agar per Liter , this will not dissolve (~6 g Agar
- Transfer to a erlenmeyer flask at least 2x the volume that you are making
- Autoclave for at least a 20 minute liquid cycle
- Once the liquid is cool to the touch (doesn't burn your hand to hold) add antibiotic and mix by swirling
- Pour into plates, covering the surface and avoiding bubbles
- Let plates cool with the lids ajar
- Invert plates, place in sleeve and mark sleeve as LB/Amp or LB/Km depending on the antibiotic

- Locate test plasmid for transformation

**Day 5 Tuesday 6/15:**

- Transformation to check if comp cells work
  https://2019.igem.org/wiki/images/5/5a/T--Toronto--transformation.pdf
  - Spread Plate

**Day 6 Wednesday 6/16:**

- Check plates for growth
- Practice miniprep

https://2019.igem.org/Team:Toronto/Experiments

https://2019.igem.org/Team:IIT_Chicago/Experiments

PET Degradation
https://www.sciencedirect.com/science/article/pii/S0141391019300126?via%3Dihub

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3380294/

https://www.nature.com/articles/s41598-020-79031-5
The "bulk" absorbance method was first validated on UV spectrophotometers. Absorbance measurements between 240 and 260 nm have been typically used for detection of TPA, MHET, and BHET4,7,9,11,12,13,22. The enzyme reaction buffer in this study, however, contains DMSO, which absorbs strongly between 220–250 nm but negligibly at 260 nm (Supplementary Fig. 2). Therefore 260 nm was selected as the detection wavelength for the bulk absorbance method. Linear absorbance profiles at 260 nm were established for TPA and MHET, which are the major products of the reaction (Fig. 2A, Supplementary Fig. 3). Absorbance spectra between 220–350 nm for TPA and MHET are shown in Supplementary Fig. 4. Based on the absorbance profiles, MHET and TPA have distinct extinction coefficients at 260 nm of 5500 M−1 cm−1 and 4200 M−1 cm−1, respectively. According to the Beer-Lambert Law, the extinction coefficient of any combination of these products must fall between these two values.

PET films for purchase:
https://www.nature.com/articles/s41598-020-79031-5
https://www.professionalplastics.com/PET_Sheet_ClearPolyester

For measuring catalytic activity:
https://microbialcellfactories.biomedcentral.com/articles/10.1186/s12934-020-01355-8

**Day 7 Thursday 6/17:**

- Bacteria did not grow on petri dishes or in the lb broth.
- Competent cells grew on "stolen" petri dish that had amp.
- Made new lb + agar solution. Made 200 ml of lb + agar solution with 3 grams of agar.

- 100 ug/ ml is the concentration of ampicillin we're targeting.
- 500 mg of ampicillin to 5 ml of water + vortex to make 100 ug/ml ampicillin stock.
- Add 1 ml of ampicillin stock solution per ml of the (lb + agar).

**Day 8 Friday 6/18:**

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PET film
https://www.goodfellowusa.com/catalog/GFCat4J.php?ewd_token=Fhw4naDzTlYbJC9jvxEeiVDgLg5rQq&n=GZBQDfZ2p9eGXOfQTkrRwYIzvCDFbA

**Day 9 Tuesday 6/22:**

- **Check the two plates grown on Friday.**
- **Figure out plate issues make more??**
- **Meet with Anshu tomorrow**

**Next Steps with plasmid**

**Day 10 Thursday 6/24:**

1. Streak William's Plates with addGene Plasmid (X)
2. Make reagents for protein purification (can be moved to Friday)
3. Autoclave many flasks for starter colonies
4. Make LB agar plates
5. Ask Lucienne if there is 20 degree incubator (X)

**Day 11 Friday 6/25:**

6. Make 4 starters (4 colonies from plate)
7. Test plates (X)

**Day 12 Saturday 6/26:**

a. Make Glycerol Stocks of each of three, Do plasmid Purification, PCR check, Run gel.
b. Store purified plasmid in -20C
c. PCR check:
    i. Forward Primer:
        1. T7 Promoter
            a. TAATACGACTCACTATAGGG

ii. Reverse Primer:
    1. T7 Terminator
        a. GCTAGTTATTGCTCAGCGG
iii. PCR Product Length: 1053 bp
    1. PCR Extension Time 1 min
iv. PCR Gel 1%

d. Transform excess plasmid into BL21 (Day 3)

**Reagents to make:**

- **300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0 (400 mL)**
    - **120 mL 1 M NaCl**
    - **120 mL 1 M imidazole**
    - **80 mL Tris HCl**
    - **Adjust pH to 8.0 using NaOH**
- **100 mM NaCl, 20 mM Tris HCl, pH 7.5 (400 mL)**
    - **40 mL 1 M NaCl**
    - **80 mL Tris HCl**
    - **Adjust pH to 7.5 using NaOH**
- **Make Stock solutions and medium:**
    - **2xYT medium (1 L)**
        - Measure ~900ml of distilled $H_2O$.
        - Add 16g Bacto Tryptone.
        - Add 10g Bacto Yeast Extract.
        - Add 5g NaCl.
        - Adjust pH to 7.0 with 5N NaOH.
        - 5N NaOH(200 g in 1 L), 8g in 40 mL
        - Adjust to 1L with distilled $H_2O$.
        - Sterilize by autoclaving.
        - Alternatively, find 2xYT broth. https://us.vwr.com/assetsvc/asset/en_US/id/7455389/contents
    - **1 M NaCl (1 L)**
        - Dissolve 58.44 g NaCl in 900 mL of $dH_2O$, adjust to 1 L $dH_2O$
        - 400 mL, 23.38 g NaCl
    - **1 M imidazole (1 L)**
        - Dissolve 68.077g imidazole in 900 mL of $dH_2O$, adjust to 1 L $dH_2O$
        - 400 mL, 27.2 g
    - **100 mM Tris HCl, pH ~7.5-8.0**
        - **http://protocol-place.com/basic-lab-techniques/stock-solutions/tris-hcl-1-m-ph-7-4/ (for 1 M Tris - HCl 7.4)**
        - **https://theolb.readthedocs.io/en/latest/buffers/100mm-tris-buffer.html**
        - **Make at least 400 mL**

- **Warning:** Hydrochloric acid is corrosive to the eyes, skin, and mucous membranes. Acute (short-term) inhalation exposure may cause eye, nose, and respiratory tract irritation and inflammation and pulmonary edema in humans.

**Day 13 Monday 6/28:**

Make buffers for protein purification

a. **300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0 (400 mL)**
   i. **120 mL 1 M NaCl**
   ii. **120 mL 1 M imidazole**
   iii. **80 mL Tris HCl**
   iv. **Adjust pH to 8.0 using NaOH**
b. **100 mM NaCl, 20 mM Tris HCl, pH 7.5 (400 mL)**
   i. **40 mL 1 M NaCl**
   ii. **80 mL Tris HCl**
   iii. **Adjust pH to 7.5 using NaOH**

2. Find restriction enzymes
   a. Nde1 and Xho1
3. Do digest
4. Run a DNA Gel
5. Make 3x isPETase starters for purification and 2x Anshu's

**Addgene Restriction Digest Protocol:**

[Molecular Biology Protocol - Restriction Digest of Plasmid DNA]

**Day 14 Tuesday 6/29:**

**Protein Purification 1**

- Transform Anshu's plasmid?
- Starter culture
  1. Single colonies from transformation were then inoculated into a starter culture of Luria Broth (LB) media containing 100 µg/mL ampicillin and grown at 37°C overnight. The starter culture was inoculated at a 100- fold dilution into a 2xYT medium containing 100 µg/mL ampicillin and grown at 37°C until the optical density measured at 600 nM (OD600) reached 0.6-0.8
  2. 8. Protein expression was then induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.
     a. Kicks in protein production
     b. Leave smaller volume uninduced to use a control
  3. Cells were maintained at 20°C for 18 to 24 hours following IPTG induction,

a. Ask where 20C or rm temp incubator

**Day 15 Wednesday 6/30:**

1. Protein Purification Day 2
    a. Soluble and Insoluble portions
    b. Run SDS PAGE Gels (3 lanes + ladder)

**Protein Purification 2**

1. Harvested by centrifugation, and stored at -80°C until purification
2. Harvested cells were resuspended in a lysis buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris HCl, pH 8.0,) and lysed using a bead beater (BioSpec Products, Inc.)
    a. Or sonicator
3. Lysate was clarified by centrifugation at 40,000 x g for 45 minutes. ]
    a. Soluble and Insoluble portions
    b. Run SDS PAGE Gels (3 lanes + ladder)
4. Clarified lysate was then applied to a 5 mL HisTrap HP (GE Healthcare) Ni-NTA column using an ÄKTA Pure chromatography system (GE Healthcare) and eluted using 300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0
5. Resulting fractions containing proteins of interest were applied to a Sephacryl S-100 26/60 HR (GE Healthcare) size exclusion column equilibrated with 100 mM NaCl, 20 mM Tris HCl, pH 7.5 for biochemical assays, or the fractions were applied to a Superdex 75 pg 16/60 (GE Healthcare) size exclusion column equilibrated with 100 mM NaCl, 20 mM Tris HCl, pH 7.5 for crystallography.
6. Protein in eluted fractions from Ni-NTA and size exclusion columns were assessed using SDS-PAGE with Coomassie staining and Western blot using primary antibody against the hexa-histidine epitope t
7. ag (Invitrogen).
8. Total protein was assessed by BCA assay (2). For proteins that did not express, or expressed in inclusion bodies, using the above described expression protocol, additional E. coli expression cell lines were tested, including Rosetta 2 (DE3) (Novagen), BL21 (DE3), and Lemo21 (DE3) (New England Biolabs), as was expression by autoinduction at 30°C in ZYP-5052 media (1). All studies reported were performed with freshly purified protein. The use of lyophilized protein was attempted, however, specifically for MHETase, inconsistent enzyme inhibition behaviors were observed.

**Day 16 Thursday 7/1:**

**Anshu's Practice Protein Purification**

1. Do smaller culture first 100 mL 2 flasks 50 mL
2. Determine if in soluble or insoluble

3. 1-2L of actual protein purification

**Revised Schedule:**

**Day 15 Wednesday 6/30:**

1. Make a starter culture from each of the plates

**Day 16 Thursday 7/1:**

1. Using the starter culture:
    a. Make glycerol stocks
    b. Purify plasmid using Miniprep
    c. Once plasmid is purified, use it for protein purification? Or do restriction digestion and run gel?

**Day 22 Monday 7/12:**

**Add 1 uL of antibiotic to 1 mL of final amount. For example, 10 mL of starter, add 10 uL of amp.**

**Protein Purification 1**

- Starter culture

1.) Single colonies from transformation were then inoculated into a starter culture of Luria Broth (LB) media containing 100 µg/mL ampicillin and grown at 37°C overnight. The starter culture was inoculated at a 100- fold dilution into a 2xYT medium containing 100 µg/mL ampicillin and grown at 37°C until the optical density measured at 600 nM (OD600) reached 0.6-0.8

2.) Protein expression was then induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.
    b. Kicks in protein production
    c. Leave smaller volume uninduced to use a control

3.) Cells were maintained at 20°C for 18 to 24 hours following IPTG induction,
    d. Ask where 20C or rm temp incubator

**Day 23 Tuesday 7/13:**

**Protein Purification 2**

9. Harvested by centrifugation, and stored at -80°C until purification
10. Harvested cells were resuspended in a lysis buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris HCl, pH 8.0,) and lysed using a bead beater (BioSpec Products, Inc.)
    a. Or sonicator
11. Lysate was clarified by centrifugation at 40,000 x g for 45 minutes. ]
    a. Soluble and Insoluble portions

b. Run SDS PAGE Gels (3 lanes + ladder)

12. Clarified lysate was then applied to a 5 mL HisTrap HP (GE Healthcare) Ni-NTA column using an ÄKTA Pure chromatography system (GE Healthcare) and eluted using 300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0
13. Resulting fractions containing proteins of interest were applied to a Sephacryl S-100 26/60 HR (GE Healthcare) size exclusion column equilibrated with 100 mM NaCl, 20 mM Tris HCl, pH 7.5 for biochemical assays, or the fractions were applied to a Superdex 75 pg 16/60 (GE Healthcare) size exclusion column equilibrated with 100 mM NaCl, 20 mM Tris HCl, pH 7.5 for crystallography.
14. Protein in eluted fractions from Ni-NTA and size exclusion columns were assessed using SDS-PAGE with Coomassie staining and Western blot using primary antibody against the hexa-histidine epitope t
15. ag (Invitrogen).
16. Total protein was assessed by BCA assay (2). For proteins that did not express, or expressed in inclusion bodies, using the above described expression protocol, additional E. coli expression cell lines were tested, including Rosetta 2 (DE3) (Novagen), BL21 (DE3), and Lemo21 (DE3) (New England Biolabs), as was expression by autoinduction at 30°C in ZYP-5052 media (1). All studies reported were performed with freshly purified protein. The use of lyophilized protein was attempted, however, specifically for MHETase, inconsistent enzyme inhibition behaviors were observed.

**Day 26 Saturday 7/16/2021:**

Conditions:

1.) 130 volts
2.) Make and store lysis buffer at -20 (can be stored for months).
3.) Load 2 uL of colorful protein marker.
4.) Dilute samples more with lysis buffers.
5.) Stop SDS page when gel has run and is almost 1 cm from the bottom.

1. **Run diluted samples on new SDS Gel**
2. **Make Buffers (Bio Rad Protocol)**
    a. **Lysis Buffer (Anshu's)**
        i. **https://drive.google.com/drive/folders/1tlBVl_ILJ3r7HBdLgMq5LdMeaNQFS-zL**
        ii.
            1. 50 mM NaH2PO4 6.90 g NaH2PO4·H2O (MW 137.99 g/mol)
            2. 300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol)
            3. 10 mM imidazole 0.68 g imidazole (MW 68.08 g/mol)
            4. Adjust pH to 8.0 using NaOH.

b. **1x Running Buffer (dilute stock 10x)**
c. **2x SDS-PAGE Laemmli Buffer (30mL) (BIORAD) MAKE WHILE SONICATING**
    i. **https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf**

3. **Sample Preparation**
    1. Resuspend cell pellet in 5 ml of lysis buffer for native purification.
    2. Sonicate 6 x 10 s with 10 s pauses at 200–300 W. Keep lysate on ice at all times. Use a sonicator with a microtip probe.
    3. Centrifuge lysate at 10,000 x g at 4°C for 20–30 min. Decant the supernatant (crude extract A, soluble protein) and save on ice.
    4. Resuspend the pellet in 5 ml lysis buffer. This is a suspension of the insoluble matter (crude extract B, insoluble protein).
4. **SDS-PAGE analysis**
    a. **SDS-PAGE BIORAD page 60**
        i. **https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf**

**Day 27 Monday 7/18/2021:**

- Look at gel, look for protein of interest
    - Consider re-running gel, redoing SDS page
- Make starter of transformed BL21 cells for SDS page
- Make additional 2xYT or Terrific Broth?

**Day 28 Tuesday 7/19/2021:**

- Made additional starters of competent cells and transformed cells

**Day 29 Wednesday 7/20/2021:**

- Next steps:
    - Talk to Anshu
    - Run our sample FPLC - Nickel Column
        - Run an SDS PAGE gel after
        - Need to get help from someone to run it
    - SDS-PAGE (+) control
        - Ask Anshu for it (weekend)
    - Boil samples for longer

**Objectives for Week of 9/20/2021**

- Prepare buffers for FPLC (page 114 of Qiagen Protein Manual)(**Friday 9/23**)https://drive.google.com/file/d/1azT6yEq5meTxyn9lynsAUaU4GAgJYjlQ/view?usp=sharing
- Make starter culture (1 to 99 mL 2xYT, 10 mL to 990 mL 2xYT), at least 10 mL LB Broth (**Thursday 9/22**)
    - Remake 2xYT buffer (at least 2-3 L) (**Thursday 9/22**)
- Induction - Make subculture, should be 1-2 L (**Friday 9/23)**
    - If we make larger than 1 L, may be better because we need to use some cells for SDS
    - Set aside for SDS?
- SDS Page (**Saturday 9/24**)
- Review FPLC protocol, Protocol 13 in Qiagen manual (pages 83-84) https://drive.google.com/file/d/1azT6yEq5meTxyn9lynsAUaU4GAgJYjlQ/view?usp=sharing

**Objectives for Week of 9/27/2021**

- Prepare buffers for FPLC (from the paper)
    - Make additional 5 M NaOH
        - **http://biochemicalc.nuim.ie/wp-content/pdf/5M-NaOH-Preparation.pdf**
        - 5N NaOH(200 g NaOH in 1 L), 8g NaOH in 40 mL
    - **LYSIS:** 300 mM NaCl, 10 mM imidazole, 20 mM Tris HCl, pH 8.0
        - **300 mM NaCl, 10mM imidazole, 20 mM Tris HCl, pH 8.0 (400 mL)**
            - **120 mL 1 M NaCl**
            - **4 mL 1 M imidazole**
            - **80 mL Tris HCl**
            - **Adjust pH to 8.0 using NaOH**
            - **Fill with DI Water to 400 mL**
            - **Sterilize using bottle top filter**
    - **ELUTION**: 300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0
        - **300 mM NaCl, 300 mM imidazole, 20 mM Tris HCl, pH 8.0 (400 mL)**
            - **120 mL 1 M NaCl**
            - **120 mL 1 M imidazole**
            - **80 mL Tris HCl**
            - **Adjust pH to 8.0 using NaOH**
            - **Fill with DI Water to 400 mL**
            - **Sterilize using bottle top filter**
    - 100 mM NaCl, 20 mM Tris HCl, pH 7.5 - For size exclusion step

        - **100 mM NaCl, 20 mM Tris HCl, pH 7.5 (400 mL)**
            - **40 mL 1 M NaCl**
            - **80 mL Tris HCl**
            - **Adjust pH to 7.5 using NaOH**
            - **Fill with DI Water to 400 mL**
            - **Sterilize using bottle top filter**
    - **STORE ALL BUFFERS AT 4 degrees Celsius**
- Lysis of cells (**Friday 10/1**)
    - Harvested cells were resuspended in a lysis buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris HCl, pH 8.0,) and lysed using a bead beater (BioSpec Products, Inc.).
    - Lysate was clarified by centrifugation at 40,000 x g for 45 minutes.
- Consider looking at the protocols below for more info on measuring protein concentration using Nanodrop
  http://2016.igem.org/wiki/images/7/7c/TUE_Protein_Purification.pdf

**Objectives for Week of 10/04/2021**

- Design and order primer through IDT (**Tuesday**)
- Write protocol for PCR and digest/ligation (**Tuesday**)
- FPLC Training (**Thursday**)
    - Prepare lysate
        - Resuspend pellet (each pellet weighs ~0.5 g, add 1.5 ml of lysis buffer to each conical tube, 35 tubes = 52.5 ml of lysis buffer needed)
        - Sonicate
        - Centrifuge for 45 minutes at 40,000 g
- Run PCR
- Purify PCR Product
- Do a restriction digest with the PCR product and the plasmid
    - Ligate the PCR product and plasmid together
- *Next steps*:
    - Transform the product into competent cells
    - Conduct FPLC for mutant proteins

**Objectives for Week of 10/11/2021**

- Prepare and run Nanodrop Activity Assay (**Saturday**)
    - Make reaction buffer (50 mM glycine-NaOH pH 9, 50 mM NaCl, 10% DMSO v/v)
        - 0.5 M glycine-NaOH pH 9 (100 mL)
            - Add 80 mL of dH2O to bottle

        - Add 3.75 g of Glycine to the solution
        - Add 0.35 g of NaOH to the solution
        - Adjust final concentration until concentration is at desired pH (9) using 5 N NaOH or HCl
        - Fill to 100 mL
    - 0.5 M NaCl (100 mL)
        - Add 80 mL of dH2O to bottle
        - Add 2.922 g of NaCl
        - Fill to 100 mL
    - **To make 100 mL of reaction buffer**:

| |
|---|
| 1. Add 10 mL of 0.5 M glycine-NaOH pH 9 |
| 2. Add 10 mL of 0.5 M NaCl |
| 3. Add 10 mL of DMSO |
| 4. Fill to 100 mL with autoclaved dH2O. |

    - Buy hole punch
- FPLC (**Sunday**)
    - Prepare lysate
        - Resuspend pellet (each pellet weighs ~0.5 g, add 1.5 ml of lysis buffer to each conical tube, 35 tubes = 52.5 ml of lysis buffer needed)
        - Sonicate
        - Centrifuge for 45 minutes at 40,000 g
- BCA Assay (**Monday**)

**Objectives for Week of 10/18/2021**

- SDS PAGE (**Monday**)
- Run PCR
- Purify PCR Product
- Do a restriction digest with the PCR product and the plasmid
    - Ligate the PCR product and plasmid together
- *Next steps*:
    - Transform the product into competent cells
    - IPTG Induction

**End of Notebook!**