Week of 5/28 to 6/1

Week of 6/4 to 6/8

Week of 6/11 to 6/15

Week of 6/18 to 6/22

Week of 6/25 to 6/29

Week of 7/2 to 7/6

Week of 7/9 to 7/13

Week of 7/16 to 7/20

*Day 1: Monday, July 16th*

Prep of BHET LC/MS Assay and Protein Gel Samples:

Grew up in 4 mL of LB Broth:

1. BL21(SS-)
    a. **[Flask 1 & 1B]**
2. BL21(SS-):PETase(+) with 4 µl of Kanamycin, 4 µl of Carbomycin
    a. **pMB 393 [Flask 2 & 2B]**
3. BL21(SS-):PETase(+):yebF(+) with 4 µl of Kanamycin, 4 µl of Carbomycin
    a. **pMB 386 [Flask 3 & 3B]**
4. BL21(SS-):MHETase(+) with 4 µl of Kanamycin, 4 µl of Carbomycin
    a. **pMB 412 [Flask 4 & 4B]**
5. BL21(SS-):MHETase(+):yebF(+) with 4 µl of Kanamycin, 4 µl of Carbomycin
    a. **pMB 402 [Flask 5 & 5B]**
6. Felix's GFP Cells with 4 µl of Kanamycin
    a. **[Flask 6 & 6B]**
7. BL21(SS-) with 4 µl of Kanamycin, 4 µl of Carbomycin ("Blank")

Put in the incubator to grow up overnight. Took 1 mL out and transferred into 250 mL flasks. Two sets of conditions:

1. 50 mL of **Minimal Media**, 0.4% Glucose, 0.5 mM IPTG, 200 mg/L BHET, and Antibiotics
    a. 49 mL Minimal Media
    b. 1 mL 20% Glucose

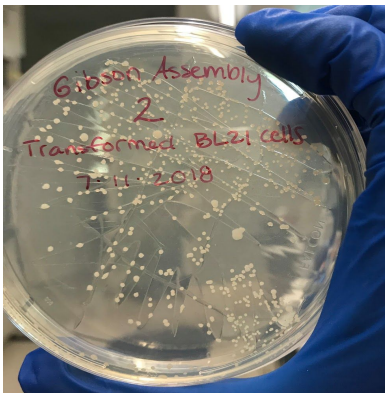
c. 25 µL 1.0M IPTG (Except in Flasks 1 and 6)
d. 10 mg BHET (Except in Flask 6)
e. Antibiotics
    i. For Flask 1, none
    ii. For Flasks 2-5, 50 µL of Kanamycin, 50 µl of Carbomycin
    iii. For Flask 6, 50 µL of Kanamycin

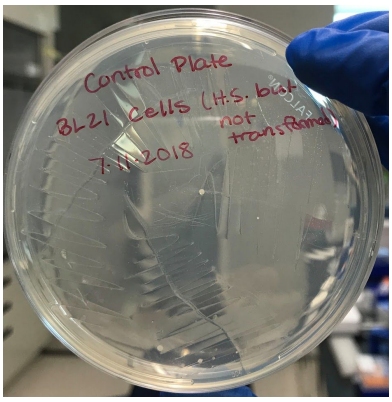
2. 50 mL of **Terrific Broth**, 0.4% Glucose, 0.5 mM IPTG, 200 mg/L BHET, and Antibiotics
    - 49 mL Terrific Broth
    - 1 mL 20% Glucose
    - 25 µL 1.0M IPTG (Except in Flasks 1 and 6)
    - 10 mg BHET (Except in Flask 6)
    - Antibiotics
        - For Flask 1, none
        - For Flasks 2-5, 50 µL of Kanamycin, 50 µl of Carbomycin
        - For Flask 6, 50 µL of Kanamycin

Put in the shaker at 37ºC for overnight growth. To be checked in the morning.

SIDE NOTES:

- Cultures from last week were taken out. They had been growing for three days in minimal media with antibiotics. No IPTG or Glucose so cells aren't reliable and were not taken for further formal testing. They were the same cell types as above.
    - Instead taken from beakers, put into 50 mL Falcon tubes.
- Checked on Gibson Assembly Transformations done into BL21 Cells on Kanamycin Plates. Were in the incubator for the weekend.
    - Colonies are looking good. Had very, very minor growth on the control plate. Could be that heat shock introduced a possible mutation. See pictures below:


*Day 2: Tuesday, July 17th*

The 4 mL culture tubes were checked in the morning for growth. The pMB cultures were good but the cultures of BL21 and Felix's GFP were not at an appropriate optical density.

- So, I took the pMB cultures and put them into their appropriate 250 mL flasks with all the right ingredients and set them up in the 37º C shaker at 10:45 am. And made two new culture tubes of 4 mL LB to grow up BL21 cells from a Test Plate and Felix's GFP. I added 4 µL of Gentamicin to Felix's GFP culture tube and put them in the 37º C incubator at the same time.
- By the end of the work day the BL21 cells were confluent so 1 mL went into their prepared flasks.
- Felix's cells were redone using a glycerol stock of them in 6 mL with 6 µL of kanamycin and left to grow for a couple of hours.

*Day 3: Wednesday, July 18th*

10 of the 250 mL flasks were taken out (Flasks 1 - 5 and 1B - 5B) of the shaker to begin lyophilization which would be done overnight.

- Contents were transferred into 50 mL conical Falcon tubes and spun down in the centrifuge for 10 minutes at 4000 rpm to pellet the cells.
- Supernatant was then separated and transferred into another 50 mL conical Falcon tube and put into the -80º C fridge for 30 minutes to freeze contents.
    - Note: To save our samples we split the roughly 50 mL of supernatant into two tubes of around 25 mL each.
- Frozen tubes were then put into the Crawford Lab's lyophilization machine for overnight processing. To be analyzed by LC/MS in the coming days.

SIDE NOTES:

Notes on improvements we can make for the future...

- We need to be careful of media we use.
- When growing our cells we should streak cells from glycerol stocks on plates first, then pick colonies to use, grow them up in culture and then move them to the flasks for testing.
- Induce at 0.4 OD rather than prior to them growing, in other words don't fully prepare flask with contents. Let the cells be introduced, grow to 0.4 OD, and then induce.

*Day 3: Wednesday, June 18th*

Sequence Verification of Gibson Assembly Plasmids:

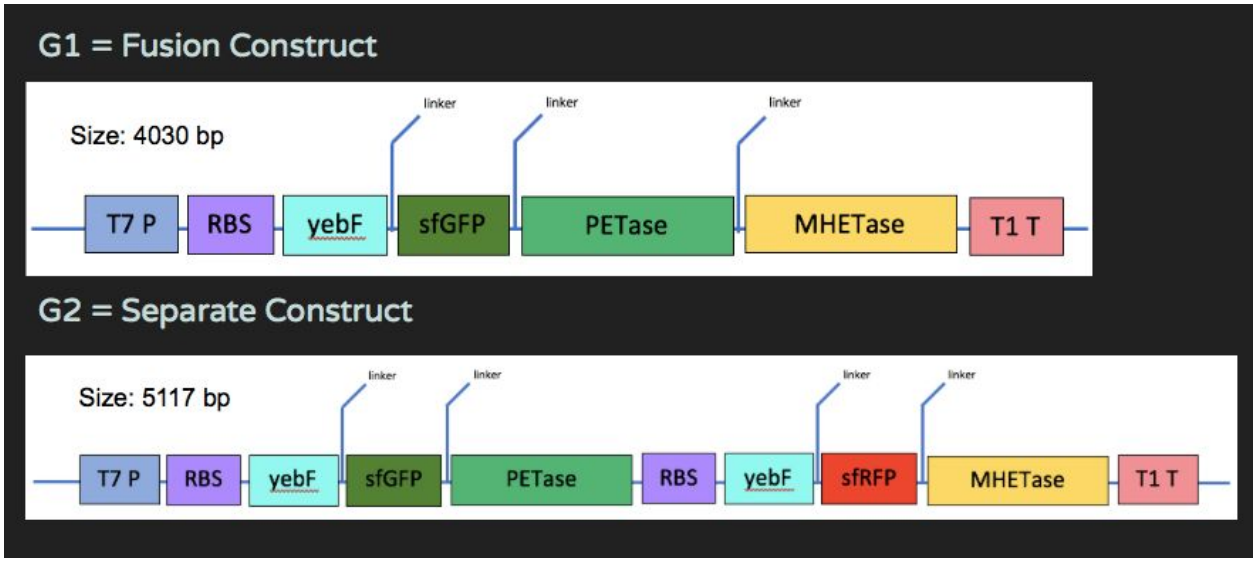
Last week two successful Gibson Assemblies were done into 10 beta cells:

| Gibson Assembly 1 | Gibson Assembly 2 |
|---|---|

Fusion


Separate


For reference, this is what the Gibson Assembly constructs should look like. MHETase was inserted. The total insert of each should be contained in a 2 kb plasmid, giving a total size of around 6 kb.




Six colonies from each plate where chosen to be Mini-Prepped (See: Mini-Prep of Gibson Assembly Trial #2) and the plasmids garnered from each of the colonies went further tested to analyze the construction of the plasmids that were present.

- DNA concentration of each Mini-Prepped colony plasmid:
  - G11 = 104.2 ng/mL
  - G12 = 111.1 ng/mL
  - G13 = 100.7 ng/mL

    - G14 = 107.8 ng/mL
    - G15 = 107.5 ng/mL
    - G16 = 91.7 ng/mL
    - G21 = 76.3 ng/mL
    - G22 = 85.3 ng/mL
    - G23 = 88.4 ng/mL
    - G24 = 99.5 ng/mL
    - G25 = 89.7 ng/mL
    - G26 = 80.0 ng/mL
- All primers were diluted to working stocks of 10 µM

Samples were prepared for Sanger Sequencing following GeneWiz general protocol with some modifications. Each PCR tube had a total volume of 15 µL and consisted of:

- 10 µL of plasmid DNA (from a specific Mini-Prep above)
- 2.5 µL of distilled H2O
- 2.5 µL of a certain primer
    - Primer used:
        - yebF-4 (634)
            - Used just this primer to check for MHET in all of the plasmids as preliminary testing so we know what direction to take our sequencing in.

PCR Strip Key

| Tube Number | Plasmid Code | Primer Number |
|---|---|---|
| 1 | G11 | yebF-4 (634) |
| 2 | G12 | yebF-4 (634) |
| 3 | G13 | yebF-4 (634) |
| 4 | G14 | yebF-4 (634) |
| 5 | G15 | yebF-4 (634) |
| 6 | G16 | yebF-4 (634) |
| 7 | G21 | yebF-4 (634) |
| 8 | G22 | yebF-4 (634) |
| 9 | G23 | yebF-4 (634) |
| 10 | G24 | yebF-4 (634) |

| | | |
|---|---|---|
| 11 | G25 | yebF-4 (634) |
| 12 | G26 | yebF-4 (634) |

Put prepared sequencing samples in the -20º C fridge for submission later.

*Day 4: Thursday, July 19th*

PCR Screen of Gibson Assembly G1 and G2:

Samples were prepared for PCR Screen to determine if MHET has been successfully assembled in. We are looking for 1.1 kb for G1 and 0.7 kb for G2. Each PCR tube had a total volume of 25 µL and consisted of:

- 1 µL of template DNA (from a specific Mini-Prep above, which was diluted to 10 µL and taken from that stock)
- 12.5 µL of FAST (green stuff)
- 9.5 µL of distilled H2O
- 1 µL of a certain forward primer (from working stocks of 10 mM)
- 1 µL of a certain reverse primer (from working stocks of 10 mM)
    - Primer used:
        - For G1
            - G13F (647) and yebF-4 (634)
        - For G2
            - G26F (654) and yebF-4 (634)

PCR Strip Key

| Tube Label | Plasmid Code | Primer Number |
|---|---|---|
| A | G11 | G13F (647) and yebF-4 (634) |
| B | G12 | G13F (647) and yebF-4 (634) |
| C | G13 | G13F (647) and yebF-4 (634) |
| D | G14 | G13F (647) and yebF-4 (634) |
| E | G15 | G13F (647) and yebF-4 (634) |
| F | G16 | G13F (647) and yebF-4 (634) |
| G | G21 | G26F (647) and yebF-4 (634) |
| H | G22 | G26F (647) and yebF-4 (634) |

| | | |
|---|---|---|
| I | G23 | G26F (647) and yebF-4 (634) |
| J | G24 | G26F (647) and yebF-4 (634) |
| K | G25 | G26F (647) and yebF-4 (634) |
| L | G26 | G26F (647) and yebF-4 (634) |

PCR Reaction Conditions:

- Thermocycle
    - 1. 95ºC for 30 seconds
    - 2. 95ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 15 seconds
    - 3. Return to Step #2 34 times
    - 4. 72ºC for 2 minutes

Made 2 small agarose gels and loaded them with 5 µL of PCR Reaction in addition to a ladder. Each strip (A-F) and (G-L) got their own gel and ladder.

<u>Gel Map</u>

G1 Gel

- Lane 1 = Ladder (7 µL)
- Lane 2 = G11 (Tube A)
- Lane 3 = G12 (Tube B)
- Lane 4 = G13 (Tube C)
- Lane 5 = G14 (Tube D)
- Lane 6 = G15 (Tube E)
- Lane 7 = G16 (Tube F)

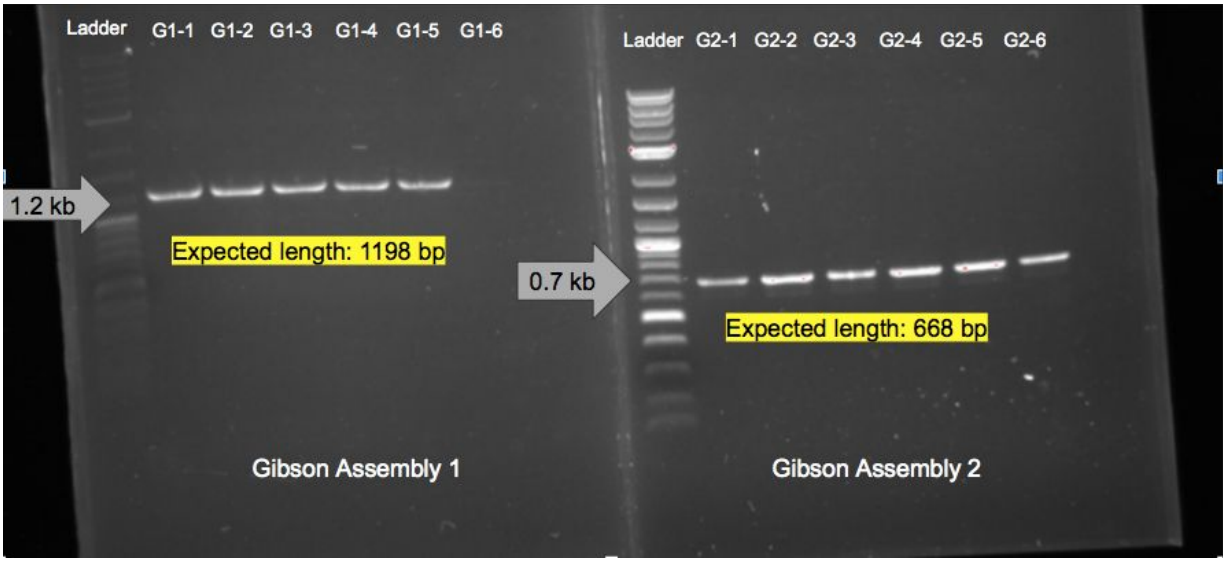
G2 Gel

- Lane 1 = Ladder (7 µL)
- Lane 2 = G21 (Tube G)
- Lane 3 = G22 (Tube H)
- Lane 4 = G23 (Tube I)
- Lane 5 = G24 (Tube J)
- Lane 6 = G25 (Tube K)
- Lane 7 = G26 (Tube L)

Gels were run for 45 minutes and then examined for bands.

- Visible bands around 1.1 kb on Gel 1 were excised and stored in -20ºC for purification tomorrow.
- Visible bands around 0.7 kb on Gel 2 were excised and stored in -20ºC for purification tomorrow as well.

Image: Bands of G1 PCR (left) and G2 PCR (right). Note that the only sample with no visible band was G16, Lane F.




SIDE NOTES:

- Supernatant that was lyophilized was taken out and stored in the -80ºC freezer. The dry components are to be resuspended in a solvent.
    - BHET solubility in water is 17.61 g/L
    - TPA solubility in water is 0.0015 g/100 mL at 20 °C
    - EG has miscible solubility in water

*Day 5: Friday, July 20th*

Gel Purification of PCR Gibson Assembly 1 and 2:

All bands extracted from the Gibson Assembly were purified for further sequencing according to the QIAquick Gel Extraction Protocol. With two modifications:

- Before final elution step, the column was placed in incubator for 5 minutes to boil off residual ethanol in the column.
- Before final elution step, heat nuclease free water was used to elute DNA up to 60ºC.

After gel extraction and purification, DNA concentrations were taken and PCR strips were assembled.

- DNA concentration of each gel extraction:

- A G11 = 39.5 µg/mL
- B G12 = 12.2 µg/mL
- C G13 = 145.4 µg/mL
- D G14 = 95.6 µg/mL
- E G15 = 168.3 µg/mL
- G G21 = 7.9 µg/mL
- H G22 = 9.5 µg/mL
- I G23 = 33.7 µg/mL
- J G24 = 117.5 µg/mL
- K G25 = 166.8 µg/mL
- L G26 = 16.4 µg/mL

Samples were prepared for Sanger Sequencing following GeneWiz general protocol with some modifications. Each PCR tube had a total volume of 15 µL and consisted of:

- 10 µL of plasmid DNA (from a specific Mini-Prep above)
- 2.5 µL of distilled H2O
- 2.5 µL of a certain primer
  - Primer used:
    - yebF-4 (634)

PCR Strip Key

| Tube Number | Plasmid Code | Primer Number |
|---|---|---|
| 1 | G11 | yebF-4 (634) |
| 2 | G12 | yebF-4 (634) |
| 3 | G13 | yebF-4 (634) |
| 4 | G14 | yebF-4 (634) |
| 5 | G15 | yebF-4 (634) |
| 6 | G21 | yebF-4 (634) |
| 7 | G22 | yebF-4 (634) |
| 8 | G23 | yebF-4 (634) |
| 9 | G24 | yebF-4 (634) |
| 10 | G25 | yebF-4 (634) |
| 11 | G26 | yebF-4 (634) |

SIDE NOTES:

Preparing BHET Assay Samples:

- Resuspend the lyophilized sample as best as possible in a 50-50 water and methanol solution.

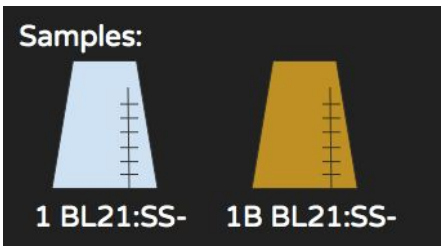
Protein Gel Layout

- Tube & Lane 1: Ladder
- Tube & Lane 2: MM BL21 (Supernatant 1)
- Tube & Lane 3: MM PET+/SS- (Supernatant 2 - pMB 393)
- Tube & Lane 4: MM PET+/yebF+ (Supernatant 3 - pMB 386)
- Tube & Lane 5: MM MHET+/SS- (Supernatant 4 - pMB 412)
- Tube & Lane 6: MM MHET+/yebF+ (Supernatant 5 - pMB 402)
- Tube & Lane 7: TB BL21 (Supernatant 1B)
- Tube & Lane 8: TB PET+/SS- (Supernatant 2B - pMB 393)
- Tube & Lane 9: TB PET+/yebF+ (Supernatant 3B - pMB 386)
- Tube & Lane 10: TB MHET+/SS- (Supernatant 4B - pMB 412)
- Tube & Lane 11: TB MHET+/yebF+ (Supernatant 5B - pMB 402)
- Tube & Lane 12: MM Felix's GFP (Supernatant 6 - FR193)
- Tube & Lane 13: TB Felix's GFP (Supernatant 6B - FR193)

Week of 7/23 to 7/27

*Day 1: Monday, July 23rd*

Last week, on Day 5, as we were heading into the BHET Assay it was discovered that our supernatant samples hadn't been lyophilized completely. Samples were put into the lyophilization machine once again to be lyophilized over the weekend. By Monday morning, only half of the samples had been lyophilized as shown below.


The other half had condensed back down into a smaller volume of liquid. They were put back into the machine.

Due to overcrowding of the incubator, Felix's GFP cells, designated for Flask 6 and 6B of the Protein Gel Experiment, had to be slotted in later. A 4.75 mL liquid LB culture of Felix's cells were grown in 4.75 µL of kanamycin. To be back diluted into proper flasks tomorrow.

*Day 1: Monday, July 23rd*

<u>Redo of BHET LC/MS Assay and Protein Gel Samples:</u>

After discussion with Pol and Jaymin, previous preparation of BHET LC/MS and Protein Gel samples from Flasks 1-5 and 1B-5B were discarded due to their inconsistency and our desire for more concise and current testing samples. New protocol was devised, similar to the previous week's but with less variables. Overall, a more directed test with less redundancy.

Grew up in 3 mL of LB Broth:

1. BL21
    a. **BL21 SS- [Flask 1 & 6]**
2. BL21(SS-):PETase(+) with 3 µl of Kanamycin, 3 µl of Carbomycin
    a. **pMB 393 [Flask 2 & 7]**
3. BL21(SS-):PETase(+):yebF(+) with 3 µl of Kanamycin, 3 µl of Carbomycin
    a. **pMB 386 [Flask 3 & 8]**
4. PET+:MHET+:Fusion (Gibson Assembly 1, Colony 1) with 3 µl of Kanamycin
    a. **G1-1 [Flask 4 & 9]**
5. PET+:MHET+:Separate (Gibson Assembly 2, Colony 1) with 3 µl of Kanamycin
    a. **G2-1 [Flask 5 & 10]**

Put in the incubator to grow up overnight.

*Day 2: Tuesday, July 24th*

Back diluted culture tubes 1-5 from previous day to acquire cells at a smaller concentration and OD. Took 1 mL of cells and put them into 250 mL flasks with corresponding numbers.

Flasks 1-5:

- Cells in **50 mL of Terrific Broth, 0.4% Glucose, and Antibiotics**
    - 49 mL Terrific Broth
    - 1 mL 20% Glucose
    - Antibiotics

        - For Flask 1, none
        - For Flasks 2-3, 50 µL of Kanamycin, 50 µl of Carbomycin
        - For Flask 4-5, 50 µL of Kanamycin
- Cells left to grow and establish themselves for a while in 37ºC. When 0.6 OD was reached samples were induced with 0.1 mM IPTG.
    - 12.5 µL 1.0 mM IPTG in Flasks 1-5

For Flasks 6-10:

- Cells in **50 mL of Terrific Broth, 0.4% Glucose, and Antibiotics**
    - 49 mL Terrific Broth
    - 1 mL 20% Glucose
    - Antibiotics
        - For Flask 1, none
        - For Flasks 7-8, 50 µL of Kanamycin, 50 µl of Carbomycin
        - For Flask 9-10, 50 µL of Kanamycin
- Cells left to grow and establish themselves for a while in 37ºC. When 0.6 OD was reached samples were induced with 0.1 mM IPTG and BHET.
    - 5 µL 1.0 mM IPTG in Flasks 6-10
    - 5 mg of BHET in Flasks 6-10
- Samples were then transferred to 16ºC to grow overnight for better protein expression.

For Flask 11:

- Felix's GFP Cells in **50 mL of Terrific Broth and Antibiotics**
    - 49 mL Terrific Broth
    - Antibiotics
        - 50 µL of Kanamycin
- Cells left to grow and establish themselves for a while in 37ºC.

*Day 3: Wednesday, July 25th*

In the afternoon, samples were spun down into a pellet for 11 minutes at 4000 rpm and 4ºC, supernatant was extracted and poured into conical 50 mL Falcon tubes, with a KimTech tissue wipe taped top instead of a cap.

- Supernatants were divided into 20 total 50 mL tubes, with tubes 1-10 containing about 12 mL of supernatant.
- 12 mL samples were then lyophilized at this volume. A lower volume of 12 mL was chosen since it generated a better surface area to volume ratio, allowing for quicker lyophilization as we needed to be done by Thursday.
- Tubes were frozen down in the -80
- Tubes were put in the lyophilization machine at 0.030 mBar for freeze drying and concentration overnight.

    - *Goal: Have lyophilized supernatant samples of colonies that grew WITH BHET and supernatant samples of colonies that DID NOT grow with BHET so that BHET can be added to the produced enzyme in the supernatant.*
- Tubes 1A-10A contained the remaining supernatant and was stored at -80ºC for further analysis.
- Pellets were kept in separate tubes labeled 1PS-10PS and put in the cold room
- Felix's GFP cells, Flask 11, were also transferred to 50 mL Falcon tubes and spun down into a pellet and then separated from supernatant. Sample was labeled and put in the cold room.

Before pellets were stored, basic GFP assessment was done via the lightbox in the gel room. Plans to quantify GFP fluorescence by Flow Cytometry were made.

*Day 3: Wednesday, July 25th*

<u>Full Sequencing of Gibson Assembly in BL21 Transformed Cells G11 and G22:</u>

- DNA concentration of Mini-Prepped colony plasmid:
    - G11 = 104.2 ng/mL
    - G22 = 85.3 ng/mL
        - Due to the fact that we needed a lot of plasmid and that GeneWiz recommends a certain concentration, both plasmids were diluted. G11 was diluted to 80 µL, initially it was 35 µL. G22 was diluted to 110 µL, initially it was 35 µL.

- All primers were diluted to working stocks of 10 µM

Samples were prepared for Sanger Sequencing following GeneWiz general protocol with some modifications. Each PCR tube had a total volume of 15 µL and consisted of:
- 10 µL of plasmid DNA (from a specific Mini-Prep above)
- 2.5 µL of distilled H2O
- 2.5 µL of a certain primer

PCR Strip Key

| Tube Number | Plasmid Code | Primer Number |
|---|---|---|
| 1 | G11 | yebF-1 (631) |
| 2 | G11 | yebF-2 (632) |
| 3 | G11 | yebF-3 (633) |

| | | |
|---|---|---|
| 4 | G11 | G11F (645) |
| 5 | G11 | G12F (646) |
| 6 | G11 | G13F (647) |
| 7 | G11 | yebF-4 (634) |
| 8 | G22 | yebF-1 (631) |
| 9 | G22 | yebF-2 (632) |
| 10 | G22 | yebF-3 (633) |
| 11 | G22 | G11F (645) |
| 12 | G22 | G22F (648) |
| 13 | G22 | G23F (649) |
| 14 | G22 | G12F (646) |
| 15 | G22 | G25F (650) |
| 16 | G22 | G26F (651) |
| 17 | G22 | yebF-4 (634) |

Submitted prepared sequencing samples.

*Day 4: Thursday, July 26th*

Lyophilized samples were not sufficiently condensed. Each tube was frozen again using dry ice for 30 minutes and then put in the lyophilized machine again at 0.030 mBar for freeze drying and concentration.

In the end, the lyophilization machine was only able to condense the samples into a more concentrated liquid form. Took the samples as they were and froze the tubes that contained BHET, 1- 5, in the -80ºC fridge. Samples not exposed to BHET earlier, tubes 6-10, were kept at room temperature and 5 mg/L of BHET was added before putting samples in the incubator to shake at 37ºC overnight.

Results from the full sequencing of the Gibson Assemblies G11 and G22, done yesterday, came in and the sequence for G22 was unsatisfactory, potentially due to an error in DNA preparation.

*Day 4: Thursday, July 26th*

Second Full Sequencing of Gibson Assembly in BL21 Transformed Cells G22 and G23:

DNA concentration of Mini-Prepped colony plasmid:

- G22 = 85.3 ng/mL
- G23 = 33.7 µg/mL
    - Due to the fact that we needed a lot of plasmid and that GeneWiz recommends a certain concentration, both plasmids were diluted. 70 µL of ddH2O was added to G22 and G23.
- All primers were diluted to working stocks of 10 µM

Samples were prepared for Sanger Sequencing following GeneWiz general protocol with some modifications. Each PCR tube had a total volume of 15 µL and consisted of:

- 10 µL of plasmid DNA (from a specific Mini-Prep above)
- 2.5 µL of distilled H2O
- 2.5 µL of a certain primer

PCR Strip Key

| Tube Number | Plasmid Code | Primer Number |
|---|---|---|
| 1 | G22 | yebF-1 (631) (#1) |
| 2 | G22 | yebF-2 (632) (#2) |
| 3 | G22 | yebF-3 (633) (#3) |
| 4 | G22 | G11F (645) (#4) |
| 5 | G22 | G22F (648) (#5) |
| 6 | G22 | G23F (649) (#6) |
| 7 | G22 | G12F (646) (#7) |
| 8 | G22 | G25F (650) (#8) |
| 9 | G22 | G26F (651) (#9) |
| 10 | G22 | yebF-4 (634) (#10) |
| 11 | G23 | yebF-1 (631) (#1) |
| 12 | G23 | yebF-2 (632) (#2) |

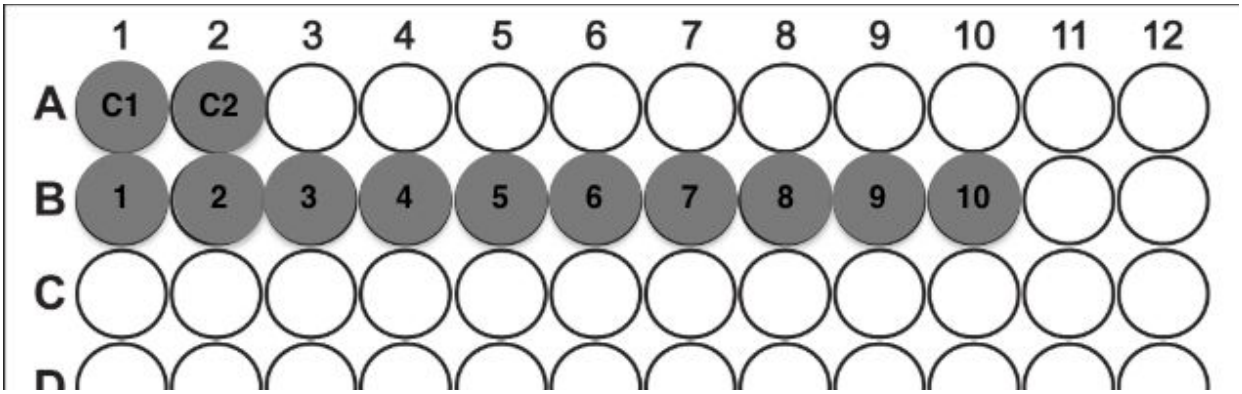
| | | |
|---|---|---|
| 13 | G23 | yebF-3 (633) (#3) |
| 14 | G23 | G11F (645) (#4) |
| 15 | G23 | G22F (648) (#5) |
| 16 | G23 | G23F (649) (#6) |
| 17 | G23 | G12F (646) (#7) |
| 18 | G23 | G25F (650) (#8) |
| 19 | G23 | G26F (651) (#9) |
| 20 | G23 | yebF-4 (634) (#10) |

Stored prepared sequencing sample for submission.

*Day 5: Friday, July 26th*

LC/MS Assay Plate Construction:

In the afternoon, plate for LC/MS was created as followed.




A1 (Control 1) contained 100 µL of BHET Solution (5 mg of BHET in 5 mL of ddH2O)
A2 (Control 2) contained 100 µL of TPA dissolved in Tris Buffer (1 mg/mL)
B1-10 contained the 100 µL of the lyophilized concentrated supernatant from Flasks 1-10.

- B1-5 had concentrated supernatant with post separation BHET introduction.
- B6-10 had concentrated supernatant with pre-separation BHET introduction.

Plate was then sealed with a foil wrap and put in the LC/MS machine. Results to be uploaded and interpreted.

SIDE NOTES:

- Need additional controls for the LC/MS functional assay to establish how effective and distinct our curves are from Controls 1 and 2.
    - Need supernatant without any BHET.
- BioBrick Submission Report
    - Types of Submissions:
        - Basic Parts
        - Composite Parts
        - Construction Intermediates
    - Need to submit two things:
        - Original BioBrick (Gibson Assembly Fusion? G1?)
        - Improvement of a Part (Moreno's Constructs or Jaymin's SS)
    - All submissions are sent in plasmid pSB1CS and flanked by BioBrick standard prefix (2 options) and suffix (1 option)
        - Cannot have following restriction enzyme sites in DNA sequence:
            - EcoR1 (gaattc)
            - Xba1 (tctaga)
            - Spe1 (actagt)
            - Pst1 (ctgcag)
            - Not1 (gcggccgc)
- Check of possible submissions:
    - T7 MHETase RFP Pa. Prot.
        - EcoR1 at 3038
        - Xba1 at 871 and 616
        - Pst1 at 1633 and 3044
    - T7 MHETase RFP Sm. Heme.
        - EcoR1 at 2912
        - Xba1 at 871 and 616
        - Pst1 at 1633 and 2699
    - T7 MHETase RFP Sm. Prot. 1
        - EcoR1 at 3062
        - Xba1 at 871 and 616
        - Pst1 at 1633 and 3068
    - T7 MHETase RFP Sm. Prot. 2
        - EcoR1 at 3062
        - Xba1 at 871 and 616
        - Pst1 at 1633 and 3068
    - T7 MHETase RFP
        - EcoR1 at 2615
        - Xba1 at 871 and 616
        - Pst1 at 1633 and 2621
    - T7 MHETase RFP yebF
        - EcoR1 at 2966
        - Xba1 at 1708 and 1963
        - Pst1 at 2725 and 2972
    - T7 PETase GFP Pa. Prot.
        - EcoR1 at 1598 and 2111
        - Pst1 at 2117
    - T7 PETase GFP Sm. Heme.
        - EcoR1 at 1598 and 1985
        - Pst1 at 1991 and 1772
    - T7 PETase GFP Sm. Prot. 1
        - EcoR1 at 1598 and 2135
        - Pst1 at 1674 and 2141
    - T7 PETase GFP Sm. Prot. 2
        - EcoR1 at 1598 and 2135
        - Pst1 at 2141
    - T7 PETase GFP
        - EcoR1 at 1598 and 1688
        - Pst1 at 1694
    - T7 PETase GFP yebF
        - EcoR1 at 1082 and 2039
        - Pst1 at 2015
    - T7 PETase MHETase yebF Fusion (IDEAL)

        - EcoR1 at 1082 and 3869
        - Xba1 at 2611 and 2866
        - Pst1 at 3628 and 3875
    - EcoR1 at 1082 and 4956
    - Xba1 at 3707 and 3962
    - Pst1 at 4724 and 4962
    - T7 PETase MHETase yebF Separate (DIFFERENT PROTEINS)
- Kevin's Suggestion
    - Mutate to change codon to same amino acid but not so it registers as an "illegal" sequence.
    - Make a Gene Block and let IDT synthesize it over a month.

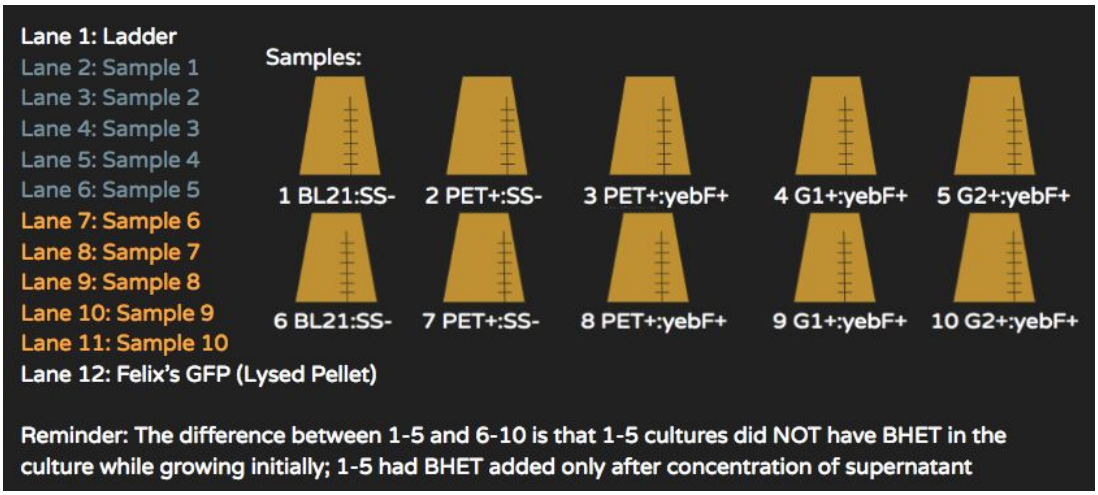
Week of 7/30 to 8/3

*Day 1: Monday, July 30th*

Streaked Felix's GFP cells on Kanamycin LB Plates since we needed fresh cells for the week.

<u>Protein Gel of BHET Assay Sample Preparation</u>

Plan:




All samples used have been lyophilized. Since samples 1-5 were cloudy with BHET, they were spun down before supernatant liquid was extracted for gel.

Samples were prepped as followed in a PCR strip, with each tube containing 50 µL of the sample supernatant and 15 µL of 4x Laemmli Sample Buffer.

The strip was then incubated in the PCR machine for 5 minutes at 95ºC. Strip was then stored at -80ºC because Felix's GFP cells were not ready.

Second Lyophilization of BHET LC/MS and Protein Gel Samples

Since certain assays and tests were to be repeated, we needed to take new aliquots from our stocks of frozen supernatant and lyophilize them. The 50 mL conical tubes with ~ 37 mL of supernatant were taken out of the -80ºC freezer.

After two hours of thawing at room temperature the samples were still mostly frozen so tubes were put in a 42ºC water bath to speed up thawing. After they had been thawed, 10 mL of each tube was taken out and frozen at -80ºC in a separate conical tube to be lyophilized next day.

*Day 2: Tuesday, July 31st*

Frozen tubes of 10 mL of supernatant were taken out and capped with a single side of a KimTech wipe. Kept on dry ice for a couple hours before being put in the lyophilization machine at low pressure for overnight processing.

Protein Gel of BHET LC/MS Samples

A colony of Felix's cells were taken from a streaked kanamycin agar plate and grown up in 4 mL of LB with 4 µL of kanamycin. After about 5 hours, cells were spun down, supernatant was dumped and pellet was resuspended in 4x Laemmli Sample Buffer which contains SDS.

Two 4-20% Stainfree Tax Gel gels were then run with 20 µL of samples in each well.

Gel 1 Layout

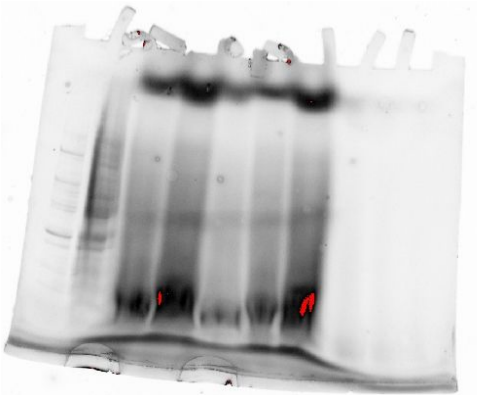
Lane 1: StainFree Ladder (10 µL)
Lane 2: Felix's GFP Cells
Lane 3: Sample 1 (BL21:SS-)
Lane 4: Sample 2 (PET+:SS-)
Lane 5: Sample 3 (PET+:yebF+)
Lane 6: Sample 4 (G1-1)
Lane 7: Sample 5 (G2-1)
Gel 2 Layout

Lane 1: StainFree Ladder (10 µL)
Lane 2: Sample 6 (BL21:SS-)
Lane 3: Sample 7 (PET+:SS-)
Lane 4: Sample 8 (PET+:yebF+)
Lane 5: Sample 9 (G1-1)
Lane 6: Sample 10 (G2-1)

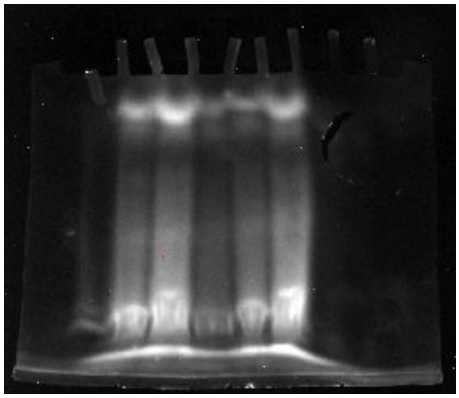
Results:

*Gel 1*

StainFree Read

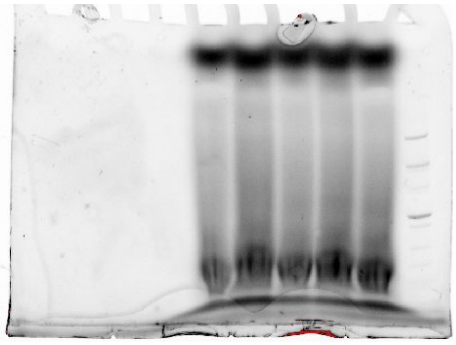


SYBR Green Read

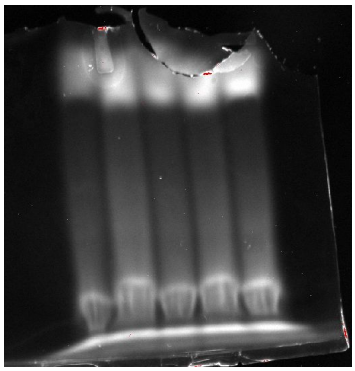


*Gel 2*

StainFree Read



SYBR Green Read



*Day 3: Wednesday, August 1st*

Samples were decently lyophilized overnight. Some were more liquidy than others but in general the amount of supernatant in each tube went down. However, since we have time until LC/MS next week the tubes were put back in the lyophilization machine to further dry them out.

Question about how to dissolve samples once dried as TPA is not soluble in water. A chemistry paper (Terephthalic Acid Solubility, Harper et. al, 1970) proposes basic solutions of Toluene, Anisole, 3-Pentanone, 1,4-Dioxane, and N,N-Dimethylformamide as possible solvents. However, basic solutions are not ideal for LC/MS machine according to Pol, so investigation into mixtures of methanol and water are underway as samples lyophilize again.

Another paper (Determination of Terephthalic Acid Isopropylamide in Urine With a Liquid Chromatography/Mass Spectrometry Method, Baumann et. al, 2008) said wrote:

*" A total of 1.78mg terephthalic acid isobutylamide was dissolved in 25% methanolic solution. This solution was stored at -20ºC. A total of 100 mL of stock solution was diluted with 900mL bidistilled water and 50 mL of this mixture was used as an internal standard for sample preparation. A 1-mg/mL solution of terephthalic acid isopropylamide was prepared in a 50% methanol/water mixture. The stock solution was diluted with bidistilled water to obtain 100- and 10-mg/mL solutions."*

*Day 4: Thursday, August 2nd*

Streaked new plates with BL21 (No SS), pMB 393, pMB 386, G1-1 and G2-1 all from glycerol stocks we had.

With all the free time we had, we decided to clean out the fridge and get rid of any samples that had expired. In doing this we discovered that our G1-1 and G2-1 samples we'd been taking from our glycerol stocks were in 10-beta cells rather than the desired BL21 *E. coli*. This resulted in a reevaluation of our project and the current lyophilized samples 4, 5, 9, and 10.

Reestablishing Fusion and Separate Constructs in BL21

Inoculated 4 mL of LB Media with 4 µL of Kanamycin and some cells from glycerol stocks G1-1, G2-1, G2-2, and G2-3. Grew up cells for 16-20 hours (put them in at 3:00pm today) mini prepped them.

*Day 5: Friday, August 3rd*

Mini Prep of G1-1, G2-1, G2-2, and G2-3

Followed the QIAprep Mini Prep Protocol and eluted DNA with water so we did not have to worry about desalination.

Measured the DNA concentration of each final miniprep elution using the Nanodrop. Results below:

- G1-1 = 45.0 µg/mL
- G2-1 = 149.5 µg/mL
- G2-2 = 21.5 µg/mL
- G2-3 = 90.9 µg/mL

Electroporation of BL21 Cells with Gibson Assembly Plasmids

Took the plasmid that we got from the mini prep and used that in electroporation of BL21 cells.

For each transformation, a 5 mL cell culture of BL21 cells was grow up until 0.4 - 0.6 OD. Cells were then spun down in 15 mL falcon tubes at 4000 rpm for 5 mins. Supernatant was removed and cells were resuspended in 1 mL ice-cold sterile water. For all subsequent steps, cells were kept on ice.

Cells were spun down and resuspended again 1 mL ice-cold sterile water. Cells were spun down a third time and finally resuspended in 50 µL of sterile water.

DNA (10-100 ng) was added to the cell solution and then transferred entirely to a chilled 1mm electrocuvette. Sample was electroporated at 1800V.

Cells were then recovered in 1 mL of LB in Eppendorf tube for 30 minutes. After 30 minutes, cells were plated on solid agar plates with the appropriate antibiotic.

Plated all 4 cultures of newly transformed BL21 cells on agar plates, both kan and carb/kan plates. 16 plates were done with 8 plated with 20 µL and the other duplicate set of 8 plated with 100 µL.

- BL21 transformed with G1-1 should be the “fusion” construct
- BL21 transformed with G2-1, G2-2, and G2-3 should be the “separate” construct

<u>Protein Gel of Lyophilized Samples</u>

For lyophilized samples, we resuspended 10 mg of each sample dry powdery substance in 500 µL of 1x PBS. Stored in -80ºC fridge.

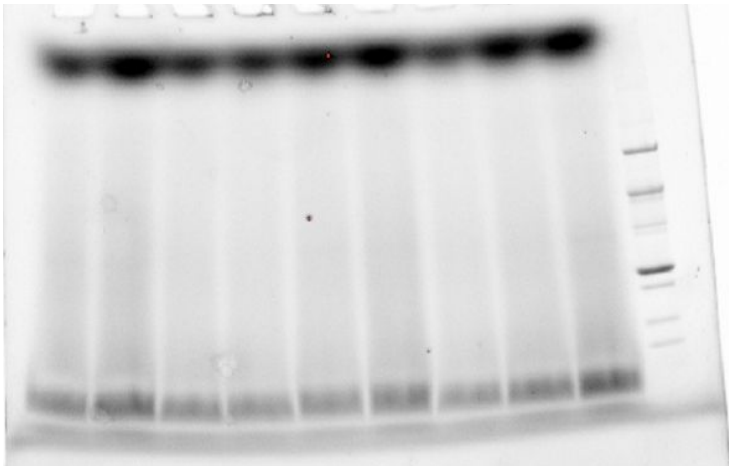
From each of these resuspended samples, 50 µL was taken to run a protein gel following normal protocol. Image turned up negative. Results below. GFP screen of the gel was strange, results concluded to be ambiguous.

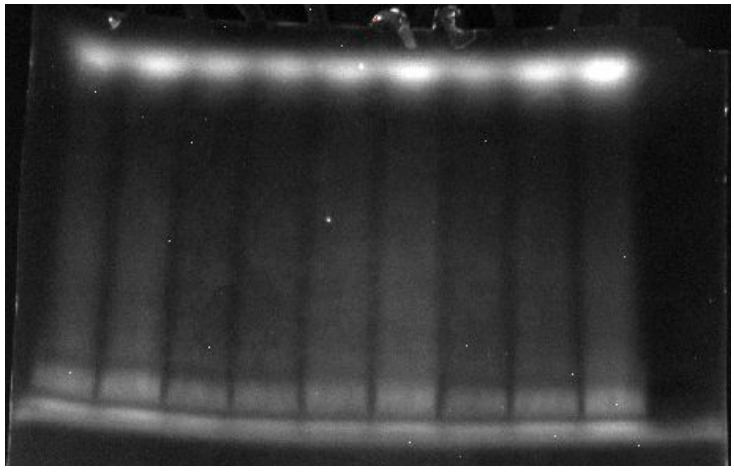
From left to right:

- Lane 1 = Flask 10
- Lane 2 = Flask 9
- Lane 3 = Flask 8
- Lane 4 = Flask 7
- Lane 5 = Flask 5
- Lane 6 = Flask 4
- Lane 7 = Flask 3
- Lane 8 = Flask 2
- Lane 9 = Flask 1
- Lane 10 = Ladder

Note: Flask 6 was emitted because it is essentially the same as Flask 1 in Lane 9.

Results





Week of 8/6 to 8/8

*Day 1: Monday, August 6th*

Preparing BL21 Transformed Samples for Future Functional Assays

Took out some glycerol stocks (BL21 SS-, pMB 393, and pMB 386) and swiped some cells from the successful BL21 Transformed with G1-1, G2-1, G2-2, and G2-3 plates (all kanamycin). Grew up those cells in 5 mL of LB with 5 µL of the appropriate antibiotics.

Take cultures out of incubator and used the glass bead method to spread on selective plates.

I. Cultures used:
   A. BL21 (No SS)
   B. pMB 393 (BL21:PET+:SS-)
   C. pMB 386 (BL21:PET+:yebF+)
   D. BL21 G1-1 (PET/MHET Fusion)
   E. BL21 G2-1 (PET/MHET Separate)
   F. BL21 G2-2 (PET/MHET Separate)
   G. BL21 G2-3 (PET/MHET Separate)

II. Plates used. Notes to correspond to cultures above, use letter key.
   A. No antibiotics, just LB
   B. Kanamycin and carbomycin
   C. Kanamycin and carbomycin
   D. Kanamycin
   E. Kanamycin
   F. Kanamycin
   G. Kanamycin

Put plates into 37ºC incubator for 24 hours.

*Day 2: Tuesday, August 7th*

Generating Glycerol Stocks of BL21 Gibson Assembly Transformation Cells

Took single colonies from each plate. Grew up cells up in 4 mL of LB with 4 µL of appropriate antibiotics. Once cells got to 0.4 OD, 500 µL were taken out and combined with 500 µL of 50% glycerol. Labeled:

- BL21 Trans. G1-1 Plasmid (Fus.) 8/7/18
- BL21 Trans. G2-1 Plasmid (Sep.) 8/7/18
- BL21 Trans. G2-2 Plasmid (Sep.) 8/7/18
- BL21 Trans. G2-3 Plasmid (Sep.) 8/7/18

Side note, cultures of the following cells were grown up from glycerol stocks. See further experiments.

- pMB 393
- pMB 386
- BL21 SS-

Stored in the -80ºC fridge.

Future Assay Prep and Schedule

- LC/MS
    - Run 9 samples for testing:
        - Lyophilized samples 1-3 (3 samples)
        - Lyophilized samples 6-8 (3 samples)
        - Supernatants of lyophilized samples 1-3 with BHET growing for extended period of time (3 samples)
    - Prep of samples on 8/7/18:
        - Took 5 mL of 1x PBS and resuspended 100 mg of lyophilized supernatant 1, 2, and 3 and dispensed into separate tubes. These after getting a sample for LC/MS will be left out as a long-term assay.
        - Weighed 40 mg of BHET. Crushed up the disks to generate a larger surface area to volume ratio and added them to each tube.
        - Took 5 mL of 1x PBS and added 40 mg of BHET as a standard.
        - Stored at room temperature, slanted.
    - Sample Key
        - 1.) 1x PBS and BHET [Long Term]
        - 2.) Sample 1, BL21 with BHET [Long Term]

- 3.) Sample 2, PET+:SS- with BHET [Long Term]
- 4.) Sample 3, PET+:yebF with BHET [Long Term]
- 5.) Sample 1, BL21
- 6.) Sample 2, PET+:SS-
- 7.) Sample 3, PET+:yebF
- 8.) Sample 6, BL21 with BHET [Colony Exposure]
- 9.) Sample 7, PET+:SS- with BHET [Colony Exposure]
- 10.) Sample 8, PET+:yebF with BHET [Colony Exposure]

Fluorescence of Plates

See photos in file.

PET Pellet Long-Term Functional Assay

After we made glycerol stocks we had 4.5 mL of LB with each of the corresponding colonies, except in the pMB 386, pMB 393 and BL21 tubes because they did not have anything taken out for glycerol stocks; they had original 5 mL of LB with cells.

2 µL of IPTG was added to tubes to get to ~0.5 mM IPTG concentration.

Pre-weighted PET Pellets were added to each culture tube and then lids were shut tight and let sit at room temperature for long-term assay.

Culture Tube PET Key:

- For:
    - BL21 Trans. G1-1 Plasmid (Fus.) 8/7/18 = 0.1647 g
    - BL21 Trans. G2-1 Plasmid (Sep.) 8/7/18 = 0.1658 g
    - BL21 Trans. G2-2 Plasmid (Sep.) 8/7/18 = 0.1628 g
    - BL21 Trans. G2-3 Plasmid (Sep.) 8/7/18 = 0.1642 g
    - pMB 393 = 0.1616 g
    - pMB 386 = 0.1638 g
    - BL21 SS- = 0.1711 g

Tubes with everything in them were weighed individually as well:

- BL21 Trans. G1-1 Plasmid (Fus.) 8/7/18 = 8.4005 g
- BL21 Trans. G2-1 Plasmid (Sep.) 8/7/18 = 8.6260 g
- BL21 Trans. G2-2 Plasmid (Sep.) 8/7/18 = 8.4714 g
- BL21 Trans. G2-3 Plasmid (Sep.) 8/7/18 = 8.2626 g
- pMB 393 = 9.0378 g
- pMB 386 = 8.8833 g
- BL21 SS- = 9.0247 g