June 15th - 18th - Ensuring the cells do not grow on these carbon sources (negative control)

1 500 ml bottle
7 100 ml botttles

Tuesday: 6/15/21 Julie, Abby, David, Nick

- To Make:
  - 5 x 50 mL agar solutions
  - 1x 250 mL M9 solution
  - 1x 10 mL 1M MgSO4 solution
  - 1x 50 mL 1M CaCl2 solution
- Materials:
  - 5X M9 salts
  - Powder CaCl2
  - Powder MgSO4
  - Powder Agar
  - Deionized water
- Protocol:
  - Made 2X M9 solution using the 5X M9 salts
    - Salt instructions: 56.4 grams per 1 L; multiplied 56.4 by ⅖ to yield 2X solution and used this ratio to make 250 mL of the solution
    - 5.64 grams M9/250 mL deionized water
    - This was done in a 500 mL glass bottle [all volumes were roughly double to account for expansion and boiling during the autoclave process]
  - Made 5 identical 2X agar solutions
    - Instructions: 15 grams per 500 mL water
    - Multiplied by 2 to yield 2X solution with 30 grams per 500 mL water
    - Divided each value by 10 to yield 50 mL total in each of the 5 bottles
    - 3.0 grams agar/50 mL deionized water (replicated 5 times)
    - This was done in five 100 mL glass bottles
  - Made 10 mL of 1M MgSO4 solution
    - Measured 6.02 grams of MgSO4 powder using scale
    - Transferred MgSO4 into glass bottle and added 10 mL of deionized water
    - This solution was made in a 100 mL glass bottle
  - Made 50 mL of 1M CaCl2 solution
    - Measured 5.55 grams of CaCl2 powder using scale
    - Transferred CaCl2 into 100 mL glass bottle and added 10 mL of deionized water
    - This solution was made in a 100 mL glass bottle

- The 8 aforementioned glass bottles were sterilized within the autoclave for 30 minutes
- Made 10 mL solutions of each of the carbon sources to be studied at a concentration of 1M in 100 mL beakers (carbon sources: ribitol, D-arabitol, sucrose, and glucose)
- 15ml connocle
    - **Ribitol:** 1.52 grams ribitol/10 mL deionized water
    - **D-arabitol:** 1.53 grams D-arabitol/10 mL deionized water
    - **Glucose:** 1.80 grams glucose/10 mL deionized water
    - **Sucrose:** 3.42 grams sucrose/10 mL deionized water
    - These solutions were then transferred into sterile 15 mL centrifuge tubes through 0.2 µm sterile syringe filters in order to sterilize the solutions
- After autoclaving
    - Per 100 mL of M9-agar - 50 mL M9 to each 50 mL agar solution
        - 100 uL $MgSO_4$ to each agar solution (250 uL) 1
        - 10 uL $CaCl_2$ to each agar solution (25 uL) 2
        - 500 uL carbon source to respective glass bottles (1.25 mL) 3
- Leave the plates in room temperature overnight to dry

Wednesday: 6/16/21 Julie, Abby, David

- Wash cells in PBS to remove LB, resuspend in PBS
    - 500 uL of each culture into centrifuge tubes, spin down 5,000 rpm for 2 minutes
    - Dump out LB
    - Resuspend pellet in PBS in 500 uL
- Perform serial dilutions, spot ~10 uL of each dilution onto agar plates with carbon source to be tested and a positive control (such as glucose)
    - 180 uL of PBS into each well plate
    - 200 uL of liquid culture (washed cells) into first well plate, move over 20 uL each time into next well plate
    - Spot dilute 10 uL
- Allow spots to dry and incubate overnight
    - E. coli 37 degrees C
    - Putida 30 degrees C

Friday: 6/18/21 Julie, Nick, David

- Count colonies

Results

	Glucose	Arabitol	Ribitol	No sugar	Sucrose
E.coli	18 *10^7	30*10^7	30*10^7	30*10^7	20*10^7

	colonies/mL	colonies/mL	colonies /mL	colonies /mL	colonies/mL
Putida	29*10^7 colonies/mL	31 *10^7 colonies/mL	50 *10^7 colonies/mL	27 * 10^7 colonies/mL	48*10^7 colonies/mL

The growth of E.coli and Putida on ribitol, arabitol, and sucrose look like the negative control (no sugar). Very slow growth. For 2 nights, E.coli should have grown a lot, but they didn't, so these cells are very unhappy. Colony counts may be the same, but the growth is very different. These cells did grow so they may have been eating something else. Maybe do it in liquid so you can get an optical density count.

- Cells could be lysing, providing nutrients for the other cells
- Could be left over LB
- Colonies for glucose are much bigger and easier to count → better growth. Colonies of the other cells on arabitol, ribitol and sucrose are very tiny and very hard to count

Monday: 6/21/21 Julie and David

<u>Amplifying ribitol pathway from agrobacterium using platinum superfi 2 enzyme in PCR</u>

Reaction mixture for PCR (50 ul reaction) in PCR tube

- Dilute primers to from 100 uM to 10 uM
- 1 uL forward and reverse primer
- 8 uL of template DNA
- 10 uL enzyme

- Colony PCR
  - Initial denaturation 98 degrees for 2 minutes
  - Denaturing temp 98 degrees for 10 seconds
  - Annealing temp = 60 degrees for 10 seconds
  - Elongation time: 1 min 13 seconds, 72 degrees
  - Final extension 72 c for 5 minutes (extend pieces that may have not been)
  - 35 cycles
- Run PCR products on a gel
  - Run entire reaction on gel with SybrSafe
  - Move band to a tube to store overnight

Tuesday: 6/22/21 Julie, David and Nick

<u>Amplifying plasmid backbone in multiple parts</u>

Reaction mixture for PCR (50 uL reaction) in PCR tube

- 19.5 uL water
- 2.5 uL primer 1
- 2.5 uL primer 2

- .5 uL template
- 25 uL 2x Q5 Enzyme

4 parts amplification:

1. Origin RSF1010 part
    - Primers 2283,2818
    - Template backbone pFLIRS
    - Anneal at 66 degrees temperature, length 6k bp
2. Regulator parts #1 Ltet
    - Primers 2819, 2329
    - Template pFLTR5
3. Regulator parts #2 Cin
    - Primers 2819,2329
    - Template pFCiR5
    - Anneal at 60 degrees temperature; length 1.3kbs
4. Abx part Gent
    - Primers 2822, 1529
    - Template pFCiR5
    - Anneal at 66 degrees temperature; length 1kbs

- Purification for A.Tumefaciens DNA (Ribitol pathway)
    - QIAquick Protocol
    - Step 3: do 5 minutes then vacuum 1 minute and repeat again for that step
      EB 10-12 ul for step 9
    - Concentrate DNA bc too dilute

- DPN1 digestion → Run PCR products on gel
- Gel Creation:
    - After step 1 add each tube to incubate for ____ minutes
    - Add 5 ul of ladder( ) in first well
    - Add 10 ul of loading dye with new pipette to the 50 ul of PCR products and mix it
    - Add PCR product into each consecutive well
    - Run Gel at 100 volts for 30 minutes

- Add 0.5 ul of DPN1 to:
        - Origin RSF1010(still not done yet)
        - Regulator Ltet
        - Regulator Lin
        - Abx(antibiotic)

Monday: 6/23/21 David and Abby

Gel Purification for Ribitol Plasmid Backbone: OriC, Abx part Gent, regulator Ltet regulator Lin

- add 600 uL of Buffer QG (stabilization buffer)
- Melt at 50-55 degrees C for 10 minutes, vortexing at the halfway point
    - Mixture should look yellow
- Add 200 ul of isopropanol to mixture
- Place the sample into a spin column and centrifuge for 1 minute; then remove leftover flow through
    - If the sample volume is too large, repeat the step twice to filter all the leftover flow through
    - If sample mixture greater than 800ul, centrifuge mixture again for 1 minute and discard leftover flowthrough
- Add 750 ul of BUFFER PE to column and centrifuge for 1 minute and then remove flowthrough
- Place the column back into the same tube and centrifuge for 1 minute to remove residual buffer
- Place column into a clean 1,5 ml microcentrifuge tube
- Elute DNA with 11 ul of Buffer EB and centrifuge column for 1 minute
    - In order to get more DNA, remove 11 ul of EB buffer and add it again on column; centrifuge for 1 minute again.

Friday: 06/25/21 David, Nick
Plasmid Assembly and Transformation of Sucrose Hydrolase, D-arabitol, and Ribitol

1. Plasmid Assembly:
    a. Use 0.25 ul of Oric, Gent, Ltet and Ribitol dehydrogenase, and add to 1ul of plasmid mixture(Gibson assembly enzymes) for 2ul total volume
        i. Plasmid mixture at 2x []
    b. Use 0.25ul of Oric, Gent, Cin, and Ribitol dehydrogenase and add to 1ul of Plasmid mixture(Gibson assembly enzymes)
    c. Place the 2ul mixture tubes into PCR machine for 1 hour at 50 degrees temperature

2. 2 Transformations of *E.coli* with Ribitol dehydrogenase using Add gene protocol for Ltet and Cin plasmid
    a. Take Strand NEB DH5 alpha of *E.coli* out of -80C and thaw on ice (5 minutes)
    b. Remove agar plates with antibiotic Gent from storage at 4c and incubate at 37C temperature
    c. Mix 20ul of DNA+ assembly mixture into 2 tubes of 100 ul of competent DH5 alpha cells. Mix the bottom of the tube carefully with your fingers a few times.
    d. Incubate competent cell and DNA mixture in ice for 20 minutes

e. Heat shock transformation tube by placing ⅔ of tube Ltet and Cin into a 42C water bath for 45 seconds, varies with the type of competent cells being used
f. Put tubes back in ice for 2 minutes.
g. Add 1000ul of LB media without antibiotics to the DH5 alpha cells and grow in a 37C incubator for one hour.
h. After the incubation period, place the 2 tubes with DH5 alpha cells into a centrifuge for 5000 revs for one minute.

Monday: 06/28/21 Nick
Determine Whether Ribitol Piece has been correctly taken up

1. Ribitol Piece Amplification
    a. In 4 Tubes, combine:
        i. 0.25ul primer 2844
        ii. 0.25ul primer 2845
        iii. 6ul OneTeq Enzyme
        iv. 5.5ul Colony Water (Acts as both template DNA and ddH2O)
            1. Scrape colony on pipette tip
            2. Resuspend in 100ul Nanopure
    b. PCR- 2hrs.
        i. Colony PCR
            Initial denaturation 98 degrees for 2 minutes
            1. Denaturing temp 98 degrees for 10 seconds
            2. Annealing temp = **66 degrees** for 10 seconds
            3. Elongation time: **2 min 25 seconds**, 72 degrees
            4. Final extension 72 c for 5 minutes (extend pieces that may have not been)
            5. 35 cycles
        ii. Run PCR products on a gel
            1. Run entire reaction on gel with SybrSafe
            2. Move band to a tube to store overnight

Tuesday: 06/29/21 David
Redoing transformations

-NHEB alpha cells were contaminated. New plasmid assembly performed

Protocol plasmid assembly:

1. Add 0.25ul of regulator Ltet/Cin and Ribitol dehydrogenase, and Gen antibiotic
2. Add 1ul of 2x enzyme hifi assembly
3. Place on PCR for 59 minutes

Cell Transformation:

1. Add 1.5 ml of NEB5alppha liquid culture to a1.5ml tube, centrifuge for 2 minutes at 5000 revs/min
    i. Drop supernatant
    ii. Add 1.5 ml of NEB5alpha again to each tube, repeat centrifuge step, and drop supernatant
    b. Cell Washing:
        i. Add 1ml of sucrose to 1,5ml tube, mix a few times and centrifuge again for 2minutes at 5000 revs/min
        ii. Drop the supernatant and repeat previous step
2. We proceeded with a voltage directed transformation to make NEB5 alpha cells competent.
    a. NEB5 alpha cells were incubated at 37 C for one hour without antibiotics for resting period
    b. Layla proceeded to place the competent cells in agar plates containing Gent antibiotic
        - Results: No colonies were reported. Either an error in the assembly procedure or the voltage directed protocol for preparing competent cells.
        - E. Coli prefers to be kept in exponential phase

Wednesday: 06/30/21 Nick
Re-doing transformation with a different protocol for preparing competent NEB5 alpha cells

1. 100ul of E.Coli into fresh LB
2. Cool all following components:
3. Spin down culture in cold centrifuge @5kg for 2min.
4. Remove LB supernatent and resuspend in 500ul of cold water
5. Underlay of 500ul cold glycerol-manitol
6. Spin
7. Remove all but pellet
8. Suspend pellet in 50ul glycerol na
9. Add 2ul of plasmid to final mixture
10. Electroshock
11. Peipette approx. 1000ul recovery media into cuvette and put into 1ml of recovery media
12. Plate resulting mixture

1. Layla repeated assembly steps for both plasmids, each containing a CIN and LTET regulator for the Ribitol dehydrogenase gene.

Thursday: 07/01/21 Julie, Nick
Redo transformation

- Transformations of *E.coli* with Ribitol dehydrogenase using Add gene protocol for Ltet and Cin plasmid
    i. Take Strand NEB DH5 alpha of *E.coli* out of -80C and thaw on ice (5 minutes)
    j. Remove agar plates with antibiotic Gent from storage at 4c and incubate at 37C temperature
    k. Mix 2ul of DNA+ assembly mixture into 5 tubes of 100 ul of competent DH5 alpha cells. Mix the bottom of the tube carefully with your fingers a few times.
    l. Incubate competent cell and DNA mixture in ice for 20 minutes
    m. Heat shock transformation tube by placing ⅔ of tube Ltet and Cin into a 42C water bath for 45 seconds, varies with the type of competent cells being used
    n. Put tubes back in ice for 2 minutes.
    o. Add 1000ul of LB media without antibiotics to the DH5 alpha cells and grow in a 37C incubator for one hour.
    p. Plate: 1 = straight from recovery (200 uL). 2 = pellet (100-200 uL)
        i. 1. Ci; Rabitol; Kan
        ii. 2. Lt; Rabitol; Kan
        iii. 3. Ci; Rabitol; Gent
        iv. 4. Lt; Rabitol; Gent
        v. 5. Lt; Ar; Kan
    q. After plating plate 1, place the remainder of 5 tubes with DH5 alpha cells into a centrifuge for 5000 revs for one minute
    r. Dump 600 uL, resuspend, plate 100-200 uL
    s. Incubate overnight

Friday: 07/02/21 Julie, Nick
Colony screening for Ribitol and Arabitol Piece Incorporation

c. Ribitol Piece Amplification

- 100 uL of water in tubes 1-16
- Mastermix: 4 uL of each primer, 96 uL OneTaq Enzyme, 5.5 uL colony water
- 6.5 of mastermix into each PCR tube

d. Arabitol piece amplification

- Mastermix: .5 uL each primer, 12 uL enzyme, 5.5 uL
- 6.5 uL mastermix into each PCR tube
- Add 5.5 ul of colony water into the PCR tube

i. PCR- 2hrs.

1. Colony PCR

Initial denaturation 98 degrees for 2 minutes

a. Denaturing temp 98 degrees for 10 seconds
b. Annealing temp = **66 degrees** for 10 seconds
c. Elongation time: **2 min 25 seconds**, 72 degrees
d. Final extension 72 c for 5 minutes (extend pieces that may have not been)
e. 35 cycles

2. Run PCR products on a gel
    a. Run entire reaction on gel with SybrSafe
    b. Move band to a tube to store overnight

Monday: 07/05/21 Abby
Plasmid Assembly:

- Add 0.25ul of regulator Ltet/Cin and arabitol or sucrose metabolism genes, and Gen antibiotic
    - 1) pF part + Cin + arabintolD + Gent
    - 2) pF part + Ltet + arabintol D + Gent
    - 3) pF part + V (Van) + cscA + Gent
    - 4) pF part + Ltet + cscA + Gent
- Add 1ul of 2x enzyme hifi assembly
- Place on PCR for 59 minutes

Plasmid Extraction:

CRISPR cullture

- Pellet 3 mL bacterial overnight culture

    Place 1.5 mL of culture in the 1.5 mL microcentrifuge tubes and centrifuge at top speed (14,000 rpm) for 1 minute
    Remove the supernatant; place another 1.5 mL in the same tube and centrifuge at top speed again to yield the cell pellet
    Remove the supernatant

-Resuspend the pellets in 250 uL Buffer P1

- Add 250 uL Buffer P2 and invert 4-6 times to mix thoroughly (until the solution becomes clear); wait 4 minutes before proceeding to the next time (do NOT allow the reaction to proceed for more than 5 minutes)
- Add 350 uL Buffer N3 and mix by inverting the tube 4-6 times
- Centrifuge for 10 minutes at 14,000 rpm

    These steps are each conducted on the 4 microcentrifuge tubes
- Apply the supernatant from the previous step (each of the 4 microcentrifuge tubes) to the spin column and centrifuge for a minute at 14,000 rpm
- Discard the flow-through from the spin column and repeat if volume remains in the microcentrifuge tubes
- Add 750 uL Buffer PE(was to the spin columns and centrifuge for 1 minute at 14,000 rpm
- Discard the flow-through
- Centrifuge for 1 minute at 14,000 rpm to remove any residual wash buffer (dry wash)
- Place the prep column in a clean 1.5 mL microcentrifuge tube and elute the DNA with 30 uL Buffer EB by centrifuging for one minute

Transformation into Cloning Strain:

- Take strain NEB DH5 alpha of *E.coli* out of -80C and thaw on ice (5 minutes)
    - 4 tubes required
- Remove agar plates with antibiotic Gent from storage at 4c and incubate at 37C temperature
- Mix 2 ul of DNA+ assembly mixture into 4 tubes of 100 ul of competent DH5 alpha cells. Mix the bottom of the tube carefully with the pipette tip a few times.
- Incubate competent cell and DNA mixture in ice for 20 minutes
- Heat shock transformation tube by placing tubes into a 42C water bath for 45 seconds
- Put tubes back in ice for 2 minutes.
- Add 1000ul of LB media without antibiotics to the DH5 alpha cells and grow in a 37C incubator for one hour.
- After the incubation period, place the 2 tubes with DH5 alpha cells into a centrifuge for 5000 revs for one minute.

07/06/21 David M, Nick

Screening for Sucrose and Arabitol Piece Incorporation into NEB5 alpha competent cells

- Place 100 uL of purified water in tubes 1-16
- Extract colonies from plates #1Sucrose pathway and place each in tubes1-8
    - Make sure to indicate and label the colonies used with a marker in the petri dish
- Repeat extraction step from plate #4 Arabitol pathway and place colonies in tubes 9-16

- Mastermix: 4 uL of each forward and reverse primer, 96 uL OneTaq Enzyme, 5.5 uL colony water
- 6.5 of mastermix into each PCR tube
- Mastermix: .5 uL each primer, 12 uL enzyme, 5.5 uL
- 6.5 uL mastermix into each PCR tube
- Add 5.5 ul of colony water intothe PCR tube

Run gel at 100V for 30min
1 Load: 2-9 Arabitol: 10-17Sucrose
Take out gel, leave buffer in, DARKROOM- UV302 (FOR ETHIDIUM BROMIDE)- AUTOEXPOSURE- SAVE AS- LAYLA- IGEM2021

Look for 1.3kb and 1.4kb bands
Thursday: 07/08/21 Nick

Miniprep and Plasmid Extraction (Tuesday's successful colonies were cultures; Ar #5,6; Su #2,5)

Miniprep for Plasmid Extraction (4 Each of 4 samples)

1. 1.5mL bacteria overnight culture to microcentrifugetube
2. Spin @14000rpm for 1 minute to obtain pellet
3. Rid of supernactant
4. Pippette in another 1.5mL to tube;
5. Spin @14000rpm for 1 minute to obtain pellet
6. Rid of supernactant
7. Resuspend pellet in 250uL P1 Buffer
8. Add 250uL P2 Buffer
9. Invert Tube 4-6 times
10. Let Sit for 4 minutes (no more than 5 minutes)
11. Add 350uL N3 Buffer
12. Immediately mix thoroughly by inverting tube 4-6 times
13. Spin @13,000rpm for 10 minutes
14. Pipette 800uL supernatent into spin column
15. Spin @14000rpm for 1 minute
16. Discard Filterate
17. Pippette 750uL Buffer PE (Wash Butter)
18. Spin @14000rpm for 1 minute
19. Discard Filtrate
20. (Dry) Spin @14000rpm for 1 minute to remove any remaining wash buffer
21. Transfer spin column to clean 1.5mL microcentrifuge tube
22. Add 30uL Buffer EB
23. Let stand for 1 minute
24. Spin @14000rpm for 1 minute
25. Pippette and reuse 30uL filtrate back to center of spin column
26. Spin @14000rpm for 1 minute

07/12/21

David Murcia

Cell Transformation Pseudomonas. Putida and preparation of E.coli culture for Transformation

Goal: In order to transfor*m* E.coli, we will need to prepare a subculture and dilute it in 5ml of agar so it is ready to be transformed at the preferred exponential growth phase. P.putida

Aim:

100 ul of overnight culture and placed into LB

Take 5000 ul of lb media and open flame it use and flame and close
To 5 mls add 100 ul of e.coli
2 hours

#PfCiAb5:
ADD 3UL of plasmid PfLtAb5

3. Add 1.5 ml of P.putida liquid culture to a1.5ml tube, centrifuge for 2 minutes at 5000 revs/min
    i. Drop supernatant
    Cell Washing: Be fast cells hate sucrose
    ii. Add 1ml of sucrose to 1,5ml tube, mix a few times gently with pipette tip and centrifuge again for 2 minutes at 5000 revs/min
    iii. Drop the supernatant and repeat previous step
    iv. After washing twice, drop supernatant and then add 1/10th of 150ul of sucrose to 1.5ml tube and make sure to mix solution, resuspending
4. We proceeded with a voltage directed transformation to make NEB5 alpha cells competent.
    a. Add 50 ul of 1.5 ml tube solution to the electric compartment right at the center and add 1 ul of PfCiAb5 to the center as well. Then place in transformation machine. **Results less than 2 are not good.**
    b. Place 1 mil of lb into the electric compartment and mix, take it out and place it in a new 1.5 ml tubes so cells are prepared to rest
    c. Cap goes off place it i electroshock machine and click pulse and take it out

    d. Pseudomonas. Putida cells were incubated at 37 C for 2 hours without antibiotics for resting period

    4. B Repeat step 4 now using 1 ul of PfLtAb5 plasmid instead for step d.

Ribitol, Arabitol and Sucrose
-Ribitol
-Arabitol: we have CIN and need to check Ltet
-Sucrose: Ltet, missing Cin

5. Add 1 ml of E.coli liquid culture to 2 1.5ml tubes, centrifuge for 2 minutes at 5000 revs/min
    i. Drop supernatant
    Cell Washing: Be fast cells hate sucrose
    ii. Add 1ml of sucrose to 1,5ml tube, mix a few times gently with pipette tip and centrifuge again for 2 minutes at 5000 revs/min
    iii. Drop the supernatant and repeat previous step

iv. After washing twice, add 1/10th of 200 ul of sucrose and make sure to mix solution, resuspending

6. We proceeded with a voltage directed transformation to make E.coli cells competent.
    a. Add 50 ul of 1.5 ml tube to the electric compartment right at the center and add 1 ul to the center as well.
    b. Place 1 mil of lb into the electric compartment and mix, take it out and place it in a new 1.5 ml tubes so cells are prepared to rest
    c. Cap goes off place it i electroshock machine and click pulse and take it out
    d. Add 1ml of agar and mix solution with electro machine and then place in new 1.5 ml tube.

    e. NEB5 alpha cells were incubated at 37 C for one hour without antibiotics for resting period

Plating of P.putida and E.coli colonies into agar
Note: Place beads first on agar and then add liquid culture []s
1 . Obtain 200ul of liquid E.coli cultures and place it in an agar plate having Gent antibiotic and perform beads plate streaking.
2. Place rest of liquid culture into centrifuge for 1 minute at 5k revs/min, leave up to 1/4th of liquid solution and make sure to resuspend with pipette and then place liquid culture on an agar plate and repeat bead streaking step.

Place on centrifuge on 5k rpm 1 minute and leave 1/4th of solution and resuspend.

PUTIDA NEED HIGHER [ ] OF GENT

2 tubes 2.5, 2.5 and 1.tenth 250ul

Putida likes to grow at 37 c and

07/13/21 Screening Sucrose: Ltet plasmid(pFLtAra5) incorporation David, Sarah 07/13/21

- Place 100 uL of purified water in each tubes 1-8; mix w/ pipet
- Extract colonies from plates #1Sucrose pathway and place each in tubes1-8, changing the pipette tip each time.
    - Make sure to indicate and label the colonies used with a marker in the petri dish
    - Annotate w/ sharpie over colonies scooped
- Mastermix: 2 uL of each forward and reverse primer, 48 uL OneTaq Enzyme into a large tube and then transfer 6.5 ul of master mix from the large tube to each 1-8 new PCR tubes

- Use the multiple pipette and add 2.75ul of the colony water into the new 1-8 PCR tubes
- Add 5.5 ul of colony water into the PCR tube

Run gel at 100V for 30min
1 Load: 2-9 Arabitol: 10-17Sucrose
Take out gel, leave buffer in, DARKROOM- UV302 (FOR ETHIDIUM BROMIDE)- AUTOEXPOSURE- SAVE AS- LAYLA- IGEM2021

Wednesday: 6/15/21 David, Sarah, Abby

- To Make:
    - 5 x 50 mL agar solutions
    - 1x 250 mL M9 solution
    - 1x 10 mL 1M MgSO4 solution
    - 1x 50 mL 1M CaCl2 solution
- Materials:
    - 5X M9 salts
    - Powder CaCl2
    - Powder MgSO4
    - Powder Agar
    - Deionized water
- Protocol:
    - Made 2X M9 solution using the 5X M9 salts
        - Salt instructions: 56.4 grams per 1 L; multiplied 56.4 by ⅖ to yield 2X solution and used this ratio to make 250 mL of the solution
        - 5.64 grams M9/250 mL deionized water
        - This was done in a 500 mL glass bottle [all volumes were roughly double to account for expansion and boiling during the autoclave process]
    - Made 5 identical 2X agar solutions
        - Instructions: 15 grams per 500 mL water
        - Multiplied by 2 to yield 2X solution with 30 grams per 500 mL water
        - Divided each value by 10 to yield 50 mL total in each of the 5 bottles
        - 3.0 grams agar/50 mL deionized water (replicated 5 times)
        - This was done in five 100 mL glass bottles
    - Made 10 mL of 1M MgSO4 solution
        - Measured 6.02 grams of MgSO4 powder using scale
        - Transferred MgSO4 into glass bottle and added 10 mL of deionized water
        - This solution was made in a 100 mL glass bottle
    - Made 50 mL of 1M CaCl2 solution
        - Measured 5.55 grams of CaCl2 powder using scale
        - Transferred CaCl2 into 100 mL glass bottle and added 10 mL of deionized water

        - This solution was made in a 100 mL glass bottle
    - The 8 aforementioned glass bottles were sterilized within the autoclave for 30 minutes
    - Made 10 mL solutions of each of the carbon sources to be studied at a concentration of 1M in 100 mL beakers (carbon sources: ribitol, D-arabitol, sucrose, and glucose)
    - 15ml conical
        - **Ribitol:** 1.52 grams ribitol/10 mL deionized water
        - **D-arabitol:** 1.53 grams D-arabitol/10 mL deionized water
        - **Glucose:** 1.80 grams glucose/10 mL deionized water
        - **Sucrose:** 3.42 grams sucrose/10 mL deionized water
        - These solutions were then transferred into sterile 15 mL centrifuge tubes through 0.2 µm sterile syringe filters in order to sterilize the solutions
    - After autoclaving
        - 250 uL $MgSO_4$ to each agar solution
        - 125 mL M9 to each agar solution
        - 25 uL $CaCl_2$ to each agar solution
        - 1.25 mL carbon source to respective glass bottles
    - Plate each of these solutions given the 4 carbon sources
        - 10 plates with ribitol
        - 10 plates with D-arabitol
        - 10 plates of sucros
        - 9 plates of glucose (1 less plate due to supplies)
    - Leave the plates in room temperature overnight to dry

07/15/21 Screening Sucrose: Ltet plasmid(pFLtAra5) incorporation Sarah 07/15/21

- Place 100 uL of purified water in each tubes 1-8; mix w/ pipet
- Extract colonies from plates #1Sucrose pathway and place each in tubes1-8, changing the pipette tip each time.
    - Make sure to indicate and label the colonies used with a marker in the petri dish
    - Annotate w/ sharpie over colonies scooped
- Mastermix: 2 uL of each forward and reverse primer, 48 uL OneTaq Enzyme into a large tube and then transfer 6.5 ul of master mix from the large tube to each 1-8 new PCR tubes
- Use the multiple pipette and add 2.75ul of the colony water into the new 1-8 PCR tubes (wrong)
- Add 5.5 ul of colony water into the PCR tube

Run gel at 100V for 30min
1 Load: 2-9 Arabitol: 10-17Sucrose
Take out gel, leave buffer in, DARKROOM- UV302 (FOR ETHIDIUM BROMIDE)- AUTOEXPOSURE- SAVE AS- LAYLA- IGEM2021

Streaked new plates (3):

I. Positive control: arabitol, sucrose, wild type
II. Arabatol and WT
III. Sucrose and WT

07/16/21 Plating Transformed Cells on Plate with Arabitol AND Inducer; MiniPrep of pFLtAb5 - Abby Putida growing on sucrose (wild type and transformed)

- Mix together 25 uL of the inducer with 75 uL of water in a 1.5 mL conical tube
    - Total volume = 100 uL
- Using glass beads, spread the solution evenly across the plate containing arabitol and wait ~10-20 minutes for the solution to sink into the agar plate and dry
    - Remove glass beads once the solution has completely dried into the plate
- Divide the plate into two equal sections
    - Streak one side with wild-type P. putida
    - Streak the other side of the plate with the transformed P. putida
    - Labeled which cell type was streaked on either side of the plate
- Replicated this process on another plate of sucrose as crossover was observed between the two sides of the plated P. putida on sucrose
- Place plates in 30 C incubator
- Inducers
    - OHC14 → Cin
    - ATC → Ltet
- Streaked the transformed E. coli cells on the corresponding carbon source with and without inducer present following the same protocol for mixing the 100 uL inducer solution as well as streaking the cells onto the appropriate plates

MiniPrep

- Pellet 3 mL of the bacterial culture
    - Place 1.5 mL of the liquid culture into a 1.5 mL conical tube and centrifuge for 1 minute at 14,000 rpm
    - Remove the supernatant and repeat this process
- Resuspend the pellet in 250 uL of Buffer P1
- Add 250 uL of Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear
    - Do not allow this lysis reaction to proceed for more than 5 minutes
- Add 350 uL Buffer N3 and mix immediately by inverting the tube 4-6 times

- Centrifuge for 10 minutes at 14,000 rpm
- Applied the supernatant from the previous steps into a spin column and centrifuged for 1 minute at 14,000 rpm
- Washed the spin column with 750 uL of Buffer PE followed by centrifugation for 1 minute at 14,000 rpm
- Dump the solution and centrifuge once more to make sure any residual solution has been removed (dry run)
- Transfer the spin column into a fresh 1.5 mL conical tube
- Add 30 uL of Buffer EB, let it stand for 1 minute, and centrifuge for 1 minute

Results

- Both varieties P. putida that had been plated on sucrose (wild-type and transformed) grew overnight
    - This may be the result of crossover that was observed in the streaking between either side of the plate
    - The wild-type was predicted to not grow and the inducer was not present to promote the expression of the sucrose metabolism genes
    - Growth may be the result of leaky expression of this promoter and crossover may explain why the cells were able to grow on both sides of the plate

07/19/21 David Murcia

Follow up results from plating transformed cells of putida and E.coli :
Cell Transformation Pseudomonas. Putida with Plasmid PfLtAb5

E.coli Subculture transformation: need to do it during exponential face which takes a

-100 ul of overnight culture and placed into LB
-Take 5000 ul of lb media and open flame it use and flame and close
to 5 mls add 100 ul of e.coli

2 hours

#PfCiAb5:

ADD 3UL of plasmid PfLtAb5

1. Add 1.5 ml of P.putida liquid culture to a1.5ml tube, centrifuge for 2 minutes at 5000 revs/min
    i. Drop supernatant
    Cell Washing: Be fast cells hate sucrose
    ii. Add 1ml of sucrose to 1,5ml tube, mix a few times gently with pipette tip and centrifuge again for 2 minutes at 5000 revs/min
    iii. Drop the supernatant and repeat previous step
    iv. After washing twice, drop supernatant and then add 1/10th of1.5 ml = 150ul of sucrose make sure to mix solution, resuspending

2. We proceeded with a voltage directed transformation to make NEB5 alpha cells competent.

f. Add 50 ul of 1.5 ml tube to the electric compartment right at the center and add 3ul ul of PfLtAb5 to the center as well. And
g. Place 1 mil of lb into the electric compartment and mix, take it out and place it in a new 1.5 ml tubes so cells are prepared to rest
h. Cap goes off place it i electroshock machine and click pulse and take it out
i. Add 1ml of agar and mix solution with an electro machine and then place in new 1.5 ml tube.
j. NEB5 alpha cells were incubated at 37 C for one hour without antibiotics for resting period

3. B Repeat step 4 now using 1 ul of PfLtAb5 plasmid instead for step d.

07/21/21 Transforming P. putida with the 5 plasmids - Abby

- Add 1.5 mL of MG1655 E. coli liquid culture to a 1.5 mL tube, centrifuge for 2 minutes at 5000 revs/min
- Dump supernatant
- Add another 1.5 mL of the liquid culture to the same tube and centrifuge again for 2 minutes at 5000 rpm
- Dump supernatant
- *Do the 4 previous steps parallel with one another in 2 separate 1.5 mL tubes in order to make use of all 6 mL of the liquid culture*

  Cell Washing: Be fast cells hate sucrose
  - Add 1ml of sucrose to 1.5 mL tube, mix a few times gently with pipette tip and centrifuge again for 2 minutes at 5000 revs/min
  - Dump the supernatant and repeat previous step
  - After washing twice, drop supernatant and then add 250 ul of sucrose make sure to mix solution, resuspending
- We proceeded with a voltage directed transformation to make NEB5 alpha cells competent.
  k. Add 50 ul from the 1.5 mL tube containing the final resuspension to the electric cuvette right at the center and add 1uL of each plasmid and 3 uL of pFLtAb5 to the center as well.
  l. Take the cap off, place in the electroshock machine, and click pulse
  m. Place 1 mL of LB into the electric compartment and mix, take it out and place it in a new 1.5 ml tubes so cells are prepared to recover
  n. MG1655 E. coli (wild-type) cells were incubated at 37 C for one hour without antibiotics for resting period

Adding inducer to respective liquid cultures of P. putida

- Cin → OHC14

- Ltet → aTc
- Add 5 uL of inducer to the 5 mL liquid cultures of P. putida
    - Concentration is 1 uL of inducer per 1 mL of liquid culture
- Place cultures in 30 degree incubator and allow to grow overnight

Plating transformed E. coli

- Using the glass beads, spread 200 uL of each solution of transformed cells onto an LB plate with antibiotic
- Allow to grow overnight in the 37 degree incubator

07/22/21 Streaking transformed E. coli and plating P. putida with inducer - Abby

- Take 1 mL of the cell culture and place it in a 1.5 mL tube
    - Centrifuge this for 1 minute at 5000 rpm
    - Dump the supernatant and add 1 mL PBS; centrifuge for 1 minute at 5000 rpm
    - Dump the supernatant and repeat the previous step
- Perform a 10-fold dilution in a 96-well plate with wild type P. putida and the 2 P. putida samples that had inducer added (pFLtAb5 and pFCiAb5) and were rinsed with PBS
    - Place 200 uL of the sample in the first well and 180 uL of PBS in each of the remaining wells
    - Transfer 20 uL of the initial solution into the second well
    - Continue with the serial dilution until the 12th column
    - Row F → wild-type
    - Row G → pFCiAb5
    - Row H → pFLtAb5
- On arabitol plates, add the appropriate inducer using the glass beads
    - Add 25 uL of the appropriate inducer to 150 uL of water within a 1.5 mL tube
        - Cin → OHC14
        - Ltet → aTc
    - Using a micropipette, transfer this volume onto the arabitol plates and spread with glass beads
- On these plates, using the 8-tip transfer pipette, spot plate the appropriate row of serial dilutions, ensuring that the appropriate inducer is being aligned with the transformed bacteria
    - Spot volume: 10 uL
    - Spot 3-4 of the wild-type P. putida on each plate and all 12 rows of the respective P. putida with the inducer

- On LB plates, streak one colony of each of the MG1655 E. coli samples plated the previous day in order to better examine their growth

07/23/21 - Plating transformed E. coli

- Add inducer to each of the sugar plates with respect to which plasmid will be placed on it
    - Mix 50 uL of the inducer with 300 uL of Nanopure water
    - Ie. Cin → OHC14
    - Ltet → aTc
    - Add 115 uL of aTc to each plate using the Ltet promoter and 115 uL of OHC14 to each plate with the Cin promoter plasmid
- Let the inducer dry
- Mark the plate with which side will contain wild-type E. coli and which will contain the transformed cells
- Take a colony from the growth exhibited on the LB plates generated on 07/22/21 and streak on the corresponding sugar/inducer combination alongside wild-type for a means of comparison
- Take a colony from each of these various streaks and plate them on a plate with glucose alongside the wild type
- Place each of the plates in the 37 C incubator for growth

Monday David, Nick 07/26/21

Insight from previous results: We need to allow more time for putida and E.coli to grow and make the enzymes needed to survive in their environment based on previous results where both the wild type and the transformed cells were not growing, leading us to believe cells needed more time to make enzymes necessary to survive their environment.

Goal of the day: Putida subculturing and preparing E.coli for next day subculturing

E.coli preparation: We need to transfer our transformed bacteria into new liquid cultures and need to add the same Gent antibiotic in order to make sure our bacteria are keeping our plasmids.

Method: We are doing the following steps 5 times for the respective assembled pfplasmids: 2pfLtRi5, pfLtAb5, pfciAb5, and pfLtAb5

1. Take 5 mls of liquid agar with a mechanical pipette. And add 5ul of antibiotic 20 Gent per 5ml of agar agar to a clean tube for a ratio of 1ul of antibiotic per 1ml of solution.
2. Use wooden sticks for streaking bacteria.

Pseudomonas preparation

1. Add 5ml of liquid agar into the new tube. Repeat step for new tube
2. Add 5ul of Gent 100 antibiotic to the tube for pfltAb5 culture.

3. Add 100ul of liquid culture of WT into the new liquid culture. Repeat step with PfLtAb5 culture to new tube.
4. Add 5ul aTc inducer
5. Place cultures in 30 degree incubator and allow to grow overnight

Tuesday Nick, David 07/27/21

Subculture E.Coli

3. Take 5 mls of liquid agar with a mechanical pipette. And add 5ul of antibiotic 20 Gent per 5ml of agar agar to a clean tube for a ratio of 1ul of antibiotic per 1ml of solution.
4. Use wooden sticks for streaking bacteria.

Repeat Experiment from 07/22

- Take 1 mL of the cell culture and place it in a 1.5 mL tube
    - Centrifuge this for 1 minute at 5000 rpm
    - Dump the supernatant and add 1 mL PBS; centrifuge for 1 minute at 5000 rpm
    - Dump the supernatant and repeat the previous step
- Perform a 10-fold dilution in a 96-well plate with wild type P. putida and the 2 P. putida samples that had inducer added (Wild Type and pFLtAb5) and were rinsed with PBS (make sure to dispose of tips between subsequent dilutions- *pseudomonas* sticks to end of pipette tip)
    - Place 200 uL of the sample in the first well and 180 uL of PBS in each of the remaining wells
    - Transfer 20 uL of the initial solution into the second well
    - Continue with the serial dilution until the 12th column
    - Row A → wild-type
    - Row C → pFLtAb5
- On arabitol plates, add the appropriate inducer using the glass beads
    - Add 25 uL of the appropriate inducer to 75 uL of water within a 1.5 mL tube
        - Ltet → aTc
    - Using a micropipette, transfer this volume onto the arabitol plates and spread with glass beads
- On these plates, using the 8-tip transfer pipette, spot plate the appropriate row of serial dilutions, ensuring that the appropriate inducer is being aligned with the transformed bacteria
    - Spot volume: 10 uL
    - Spot 3-4 of the wild-type P. putida on each plate and all 12 rows of the respective P. putida with the inducer

Wednesday Nick 07/28/21

- Prepared 1mM mixture aTc
    - Mix 231.45 micrograms with 500ul Ethanol

Pseudomonas preparation

- Add 5ml of liquid agar into the new tube. Repeat step for new tube
- Add 5ul of Gent 100 antibiotic to the tube for pfltAb5 culture.
- Add 100 ul of liquid culture of WT into the new liquid culture. Repeat step with PfLtAb5 culture to new tube.

Thursday 07/29/21 Abby

Preparing agar plates

- After autoclaving
    - 125 mL M9 to each 125 mL agar solution
    - 250 uL $MgSO_4$ to each agar solution
    - 25 uL $CaCl_2$ to each agar solution
    - 1.25 mL carbon source to respective glass bottles
- Plate each of these solutions given the 4 carbon sources
    - 10 plates with ribitol
    - 10 plates with D-arabitol
    - 10 plates of sucrose
    - 10 plates of glucose
- Leave the plates in room temperature overnight to dry

NEB10β Liquid Culture

- Label 5 glass tubes with wild type and each of the 4 plasmids contained by the cells
- Add 5 mL of LB to 5 separate tubes
- Add 5 uL of the antibiotic Gent to each of these tubes
    - The appropriate volume of Gent to add is 1 uL per every 1 mL
- Perform a liquid culture from the plates into the prepared volume of LB and antibiotic

Induce pFLtAb5 P. putida

- Add 5 uL of the inducer aTc to the 5 mL liquid culture
    - Ratio: 1 uL:1 mL

Induce E. coli with respective inducers

- Add 5 uL of the inducer (OHC14 or aTc) to the respective 5 mL liquid culture of E. coli

Friday 07/30/21 Abby and David- Transforming SDU plasmid, serial dilution, plating previously transformed cells with inducer in liquid culture

Add inducer to plates (Abby)

- Make a solution with 50 uL OHC14 and 300 uL of nanopure water
    - Spread 175 uL of this solution on each of the two plates with Cin inducers using glass beads
- Make three solutions with 3 uL of the 1 mM aTc inducer and 100 uL of nanopure water
    - Spread each of these solutions on each of the three plates containing a plasmid with the Ltet inducer using glass beads
- Allow these plates to dry for at least an hour (we want to mitigate the presence of ethanol as this may be serving as a carbon source for the plated cells, thus allowing them to grow where they shouldn't)

Perform a Serial Dilution of 7 liquid cell cultures (Abby)

A → P. putida pFLtAb5
B → P. putida wild type
D → E. coli NEB10B wild type
E → E. coli pFCiRi5
F → E. coli pFLtRi5
G → E. coli pFLtSc5
H → E. coli pFCiAb5

- Take 1 mL of each liquid culture and centrifuge at 5000 rpm for 2 minutes
- Dump the supernatant and add 1 mL of PBS, centrifuging again
- Dump the supernatant and add another 1 mL of PBS, centrifuge this solution and then resuspend
- Take 200 uL of this solution and add it to well 1 in each of the respective rows
- Add 180 uL of PBS in each of the following rows (go to column 12 for all except the wild-type of each cell type, for which we will be doing to column 3)
- Serially dilute 20 uL of the solution across the row until the spot dilution is complete

Add antibiotic to plates (David)

- Add 25 uL of Kanamycin to 75 uL of nanopure
- Add this solution to one of the ampicillin plates
- Plate the (hopefully) transformed cells with the plasmids from Denmark
- Place 2 liquid NEB5 alpha cultures from 2ml tubes and centrifuge them at 5k revs/min for 2 minutes.
- Dump ½ of the supernatant of each tube and mix each tube with the tip of the pipette to have an even solution.
- Place the even solution #2 in the Kan/Amp plasmid and the #1 in the Amp plasmid, then use marbles to evenly spread the liquid culture of NEB5alpha cells in the plate

Tuesday 08/03/21 David

Results from last week: Transformation for both conjugation and Crispr plasmid did not work, so we are re-doing those steps

Plasmid Extraction

- Cut center of each plasmid and add each one to a respective 2ml tube, label them #1 conjugation, #2 Crispr, and add about 45 ul of Buffer EB. Try to make sure that paper is somewhat humid but not completely soaked. If we soak it entirely, the plasmid will be too diluted.
- Let the tubes rest for about 10 minutes,

Heat Transformation of NEB5alpha cells

- Get a bucket of Ice and fill it in, add 2 50ul NEB5 alpha cells tubes into the bucket of ice(label them according to the plasmid they will have) that have been prepared for heat transformation.
- Make sure throughout the process that the tubes of cells stay in the bucket except for the heat step
- Wait about 6-7 minutes and add 2 ul of each plasmid into the respective labeled tubes.
- Gently mix the solution with the pipette for each tube. Let the tubes sit for about 15 minutes.
- After 15 minutes, bring the tubes with the bucket and transfer them to a water bath for 45 seconds.
- Then place the tubes in the bucket and add 100ul of recovery LB media to each tube.
- The 2 tubes of cells will then be incubated at 37 C for 1.5 hours
- Then they will be added

Thursday 08/05/2021 Abby - re-trying transformation of SDU plasmids, checking plasmid sequences, and plating NEB10β on plates with Leucine

Plasmid Extraction

- Add 50 uL of buffer to the microcentrifuge tubes containing the cut out pieces of paper containing plasmid
- Allow this to sit in about 50 degrees for 10-15 minutes before transforming the competent cells with 5-10 uL of this solution

Plating cells with pFLtSc5 plasmid

- Add 28.5 uL of leucine to 75 uL nanopure
- Add 3 uL of concentrated aTc to 100 uL nanopure
- Add both solutions to the sucrose plate using glass beads and allow to dry in between

- Add leucine solution to a glucose plate
- Perform a serial dilution up to the 6th column in the 96-well plate
    - Take 1 mL of the cell culture and place it in a 1.5 mL tube
        - Centrifuge this for 2 minutes at 5000 rpm
        - Dump the supernatant and add 1 mL PBS; centrifuge for 1 minute at 5000 rpm
        - Dump the supernatant and repeat the previous step
    - Perform a 10-fold dilution in a 96-well plate with the pFLtSc5 E. coli cells that were rinsed with PBS
    - Place 200 uL of the sample in the first well and 180 uL of PBS in each of the remaining wells
    - Transfer 20 uL of the initial solution into the second well
    - Continue with the serial dilution
- 706 → A
- 712 → B
- Plate these dilutions using the electronic pipette on the respective place and place in the incubator (37)

Transforming cells with the extracted plasmid

- Remove the cells from the freezer and allow to sit on ice
- Add 5 uL of the respective plasmid to each of the (labeled) microcentrifuge tubes, gently stirring with the tip of the pipette
- Allow to sit on ice for 15 minutes before adding to the water bath for 45 seconds
- After the heat shock in the water bath, allow to sit on ice for around 1 minute before adding 1 mL of LB to each tube
- Allow the cells to recuperate

Adding antibiotics to plates + plating

- Retrieve 2 ampicillin plates
- Add 25 uL of kanamycin to 75 uL of nanopure and spread this on one of the plates using the glass beads
- Take the transformed cells and centrifuge for 2 minutes at 5000 rpm
- Dump half of the resulting supernatant
- Resuspend the cells in the remaining supernatant and plate the respective plasmid on the antibiotic plate using glass beads
    - CRISPR → ampicillin resistance
    - Conjugative → ampicillin AND kanamycin resistance
- Place these plates in the 37 degree incubator

08/06/21 David Re-doing Transformations

1. Add 50ul of elution buffer EBr and let it sit for about 20 minutes. Get the 2 dna plasmid tubes and this time we are vortexing them to obtain a better extraction.

2. Tubes are also placed under rest at 55 celsius for about 5 minutes, when the 5 alpha cells are placed in their ice buckets for about 5-6 minutes.

   Why are we using an elution buffer?
   Elution buffers are used as a method to obtain soluble DNA for further manipulation. DNA is soluble under low-ionic strength

Heat Transformation of NEB5alpha cells

- Get a bucket of Ice and fill it in, add 2 50ul NEB5 alpha cells tubes into the bucket of ice(label them according to the plasmid they will have) that have been prepared for heat transformation.
- Make sure throughout the process that the tubes of cells stay in the bucket except for the heat step.
- Wait about 6-7 minutes and add 5 ul of each plasmid into the respective labeled tubes.
- Gently mix the solution with the pipette for each tube. Let the tubes sit for about 15 minutes.
- After 15 minutes, bring the tubes with the bucket and transfer them to a water bath for 45 seconds.
- Then place the tubes in the bucket and add 100ul of recovery LB media to each tube.
- The 2 tubes of cells will then be incubated at 37 C for 1.5 hours
- Then they will be added.

Neb5beta need leucine to be added into minimal media due to being an auxotroph

08/19/21 David

Preparing cultures of A. Neb5alpha CRISPR DSU Plasmid, B. pfltetR5(E.coli) #1 PR706 plasmid and C. Putida cultures wild type, D. Negative control E.coli

1. Grab 4 tubes and label them with respective names: Negative E.coli, WT putida, CRISPR DSU NEB5alpha, pfltR5 E.coli
2. Add 5 mls of Lb into labeled tubes
3. Add g20 to E.coli pfltR5 plasmid and negative control E.coli cultures, then add 5 ul of ampicillin to DSU Crispr Neb5alpha plasmids.
   a. G20 [] is 1ul per 1 ml, so if we have 5mls you add 5 uls for a total ratio of 0.001 per ml of solution.

4. We are doing two strains at a time. First negative control E.coli and pfLtR5 strains. Grab those freezer vials.
   a. Be mindful they have to be on ice, can't melt and cultures will be unstable

    b. Grab a stick, make sure to turn on the fire and sterilize tubes, use the stick to inoculate the tubes. Make sure tubes remain in ice for most of the time
5. Now do igem DSU Neb5alpha plasmid
6. Repeat the same step with WT P.putida. This time we will also inoculate the strain into an agar plate

Tomorrow's checklist:

-Test different [ ]s of sugars : 10-50 times volumes of agar plates.
-We need negative control: having bacteria without our plasmid, hopefully showing it can't grow
-Things need to be done in parallel with the testing strain.

Things we will be doing:

Transformation of putida wild type to do transformation of pfletR5(tomorrow), preparing cultures today.

Comments: After sending plasmid pfltR5 to be sequenced, data seems to show that our plasmid was assembled correctly, allowing us to continue our experiments knowing we are working with a functional fragment.

08/20/21 David Murcia

1. Take 100 ul into 5ml of media of pftlR5 and Negative control E.coli liquid
2. Add 5ul of g20
3. For every 1 ml we add 50 ul of the carbon source into the minimal media
4. Add 200 ul of sugar per mil

Plasmid Extraction DSU IGEM CRISPR PLASMID NEB5alpha cells

- Pellet 3 mL bacterial overnight culture
    Place 1.5 mL of culture in the 1.5 mL microcentrifuge tubes and centrifuge at top speed (14,000 rpm) for 1 minute
    Remove the supernatant; place another 1.5 mL in the same tube and centrifuge at top speed again to yield the cell pellet
    Remove the supernatant
- Resuspend the pellets in 250 uL Buffer P1
- Add 250 uL Buffer P2 and invert 4-6 times to mix thoroughly (until the solution becomes clear); wait 4 minutes before proceeding to the next time (do NOT allow the reaction to proceed for more than 5 minutes)
- Add 350 uL Buffer N3 and mix by inverting the tube 4-6 times
- Centrifuge for 10 minutes at 14,000 rpm
- Apply the supernatant from the previous step (each of the 4 microcentrifuge tubes) to the spin column and centrifuge for a minute at 14,000 rpm
- Discard the flow-through from the spin column and repeat if volume remains in the microcentrifuge tubes

- Add 750 uL Buffer PE to the spin columns and centrifuge for 1 minute at 14,000 rpm
- Discard the flow-through
- Centrifuge for 1 minute at 14,000 rpm to remove any residual wash buffer (dry wash)
- Place the prep column in a clean 1.5 mL microcentrifuge tube and elute the DNA with 30 uL Buffer EB, then wait 1-2 minutes and centrifuge the solution for about 1 minute at 14k revs/min.

How does Plasmid purification work?

Plasmid purification in bacteria works by lysing the cell's components. This is done using a detergent solution and a strong base like NAOH. The detergent basically breaks the membranes and allows for the NAOH to denature the proteins part of those domains.

-In this part of the process, basically the chromosomal and plasmid DNA becomes denatured(open strands)

-We then remove the supernatant lysate through centrifugation, filtration or magnetic clearing.

-Then we can also use potassium acetate to allow for only the covalently closed plasmid dna to Reannel and stay solubilized

Preparing agar plates

- After autoclaving
    - 50mL M9 to each 200 mL pure water solution
    - 250 uL $MgSO_4$ to solution
    - 25 uL $CaCl_2$ to solution
    - Label glass bottle with each [] and name of each solute added, date, and Name

Checklist for tomorrow

1.Make minimal media and the plasmid extraction.
2.Negative control e.coli has PfltS5
3. We will have 3-4 different concentrations. Can do by 10 fold
4. Always start at overnight, not wait 2 days for a culture.

08/23/21 David, Nick

Goals of today: We should be able to complete transformations of Putida as well as take subcultures of our negative control and pFTLRi5/pFLTS5 E.coli cultures, and inducing them at the exponential phase for about 2 hours to then place them into different types of [ ]s of sugars and spotting them.

7. Add 1.5 ml of P.putida liquid culture to a1.5ml tube, centrifuge for 2 minutes at 5000 revs/min

i. Drop supernatant
   Cell Washing: Be fast cells hate sucrose:
ii. Add 1ml of sucrose to 1,5ml tube, mix a few times gently with pipette tip and centrifuge again for 2 minutes at 5000 revs/min
iii. Drop the supernatant and repeat previous step
iv. After washing twice, drop supernatant and then add 1/10th of original Putida volume (1.5ml) so 150ul of sucrose, make sure to mix solution, resuspending

8. We proceeded with a voltage directed transformation to make putida cells competent.
   a. Add 50 ul of 1.5 ml tube to the electric compartment right at the center and add 1 ul of PfltR5 to the center as well.
   b. Place 1 ml of lb into the electric compartment and mix, take it out and place it in a new 1.5 ml tubes so cells are prepared to rest
   c. Transformed P.putida cells were incubated at 30 C for 2 hours without antibiotics for resting period

4. B Repeat step 4 now using 1 ul of PfLtAb5 plasmid instead for step d

Liquid culture varying []s: helps answer whether the [] of sugar is being provided affecting results
Solid culture: is spotting is exponential phase + inducing them at the same time
Subculturing:

Label and set up tubes:

-Use both overnight cultures of E.coli, PfLtSc5, and PfLtRi5 .
- Add 5ul of g10 and 5ul of inducer to the 5ml culture. We always do 1 ul per 1ml.
-Make sure to label each tube with their name, reagents used and date
-Place tubes in incubation for 2 hours and set a timer.

For the liquid cultures: We will be doing 4[ ]s of sugars in each respective liquid media:

-25 ul per 5ml
-250ul per 5ml
-500ul per 5ml
- 1ml per 5ml
We will be having a total of 16 tubes. We are testing two sugars with two respective bacterial cultures. One is the target bacteria and the other is the negative control, where we basically do

not expect them to grow on the carbon source since they have not been transformed with the correct plasmid.

For the first set of 8 tubes we will be using the PfLtR5 E.coli cultures as our test target and the PfltS5 as our negative control. Ribose is the carbon source.

For the second set of eight tubes, pFLtSc5 E. Coli cultures will serve as the positive control whilst pFLtRi5 will serve as the negative control. No growth is expected per the negative control with growth for the positive control. Sucrose concentration is the independent variable.

Each of the sixteen tubes will contain the following:
-5mL M9 Media
-50ul Respective Liquid Culture in Exponential Phase
-5.7ul Leucine (1.14ul/mL times 5mL)
-0.6ul aTc Inducer
-Respective Sugar Concentration

Testing for Sugar Growth
Perform a Serial Dilution of 2 Liquid Cultures

- Take 1 mL of each liquid culture and centrifuge at 5000 rpm for 2 minutes
- Dump the supernatant and add 1 mL of PBS, centrifuging again
- Dump the supernatant and add another 1 mL of PBS, centrifuge this solution and then resuspend
- Take 200 uL of this solution and add it to well 1 in each of the respective rows
- Add 180 uL of PBS in each of the following rows (go to column 12 for all except the wild-type of each cell type, for which we will be doing to column 3)
- Serially dilute 20 uL of the solution across the row until the spot dilution is complete

Each of the two plates (sucrose and ribitol) were first plated with the following mixture:
-100ul Nanopure
-28.5ul Leucine
-3ul aTc Inducer
The plates were then left open to dry for one hour
Plate 4 most concentrated dilutions of negative control and all 12 of the positive control

Prepare Plates

Two plates were made,

200ul P. Putida pFLtRi5 and 200ul concentrated (Spin remainder of initial 1mL sample for 2min @5000rpm, resuspend in supernatant, dispose of 400ul, plate 200ul of remaining solution)

For the liquid culture: do 50 in 5mls of the bacteria solute
08/24/21 David, Nick

[Nick]Liquid cultures with the varying sugar concentrations appeared similar across all concentrations for both sucrose and ribitol colonies and between positive and negative (the opposite sugar colony (e.g. ribitol was negative control for sucrose)) controls. Because of this, the results were inconclusive.

[David]
Our previous experiment involved the process of growing the respective strain cul

Basically we have grown our bacteria in regular agar with no inducer for carbon source and have let them go on an exponential phase for 2 hours and then let them grow overnight. At that point they are at a stationary phase. We then make a subculture in a less diluted agar vial and we let them grow at exponential phase, add the inducer and then let them grow overnight. Our results show that the cultures did not grow on their respective carbon sources. Indicating a few parameters to have in mind. One of those could the whether we have provided the cultures enough carbon [] to grow or wether the timing between the exponential and before the exponential had any effect on their growth

Inducing: after 2 hours

The experiment involving plating both sugar colonies in exponential rather than stationary phase appeared to work (e.g. negative did not grow at all- see photos below); however, due to the least concentrated colonies exhibiting growth and the most not growing, we will streak both colonies onto a Gent plate to verify E. Coli growth containing our respective plasmids.

Struck out both of yesterday's "Testing for Sugar Growth" colonies on a Gent plate to verify correct E. Coli Growth

Preparing P.putida PLtR5/PfLtS5 liquid cultures [Not adding carbon source inducer]

1. Take 5ml of LB media and place it in a glass tube. Then add 5ul of G100 antibiotic
2. Usee wooden stick to extract P.putida colonies and gently place it in the respective tube mixing lightly.

3. Place labeled tube in a 30 C incubator. Cultures will be allowed to grow overnight to reach the stationary phase.

Comment: We are waiting on results from today, in order to determine how to move forward

08/25/21 David Growing 5 cultures of PfLtAB5, PfLtSc5,PfCiAb5, PfCiR5, and PfLtR5

1. Get 5 glass tubes, add 5ml of agar and 5ul of Gent 20

Comments from 08/26/21 results: Both of our pftls5b and pftlR5 cultures do seem to be e.coli after plating them again in agar with g20 no specific carbon source. Results do show that the strain is the same due to the pattern, shape and growth compared to known standard e.coli neb 5 alpha strains.

The spotting step needs to be worked on as we should be observing a dilution pattern in each successive step of the process in order to be able to quantify CFU units.

Since our spotting experiment was successful in testing the E.coli Neb 5 Alpha strains working on their respective carbon sources: Sucrose and Ribitol using plasmids pfltSC5 and pfLtR5, we will be doing the same experiment on a massive scale testing all of our different plasmids as well. Since we don't have WT strain, we will use different strands from our plasmids that can survive on different carbon sources. We are expanding it due to results being promising.

Inoculation of strains:

1. Repeat each step for each plasmid one at a time. Make sure to inoculate gently and dispose of wooden sticks. Strains are sensitive to changes in temperature, make sure they are in ice most of the time.
2. Let strands grow overnight into stationary phase

Tomorrow's checklist/notes:

1. We will be taking a subculture of the grown cultures and repeating same experiment by inducing them before exponential phase

08/260/21 David

ATC LTEL Inducer: very concentrated due to having issues with bacteria not being able to grow on ethanol solvent.

A.PfLtAB5

B. PfLtSc5
C.PfCiAb5
D. PfCiR5
E.PfLtR5

Performing Subcultures of NEB5 alpha E.coli Strains

1. Take 100 ul of overnight culture
2. Add 5ul of G20
3. Add 0.6 ul of ATC LT regulator to respective plasmid
4. Add 5ul of CIN to cultures having CIN regulator

PLATES:
Testing for Sugar Growth
Perform a Serial Dilution of 2 Liquid Cultures

- Take 1 mL of each liquid culture and centrifuge at 5000 rpm for 2 minutes
- Dump the supernatant and add 1 mL of nanopure, re-suspend and centrifuge for 5k/2mns
- Dump the supernatant and add another 1 mL of nanopure, resuspend, centrifuge this solution. Dump supernatant.
- Add another 1ml of nanopure and resuspend. Now should be well washed
- Take 200 uL of this solution and add it to well 1 in each of the respective rows
- Add 180 uL nanopure in each of the following rows (go to column 12 for all except the wild-type of each cell type, for which we will be doing to column 3)
    - 2-12 tubes add 180 ul of nanopure except column 1

- Serially dilute 20 uL of the solution across the row until the spot dilution is complete

3 rows of 4 circles = 12 total
4 most negative place in same plt
Pull 10 ul from total 200 when doing spotting

Mix very well well resuspending them 8 times

Preparation of solid M9 media plates

Each of the 5 plates were first plated with the following mixture and following the structure of the below table:

-100ul Nanopure: used for having large volume for expansion and spreading things better.
-28.5ul Leucine
-3ul aTc Inducer
-There is no antibiotic at this step
-25 ul of OHC14/Cin inducer to plates that will use the Cin regulator(2)
-Add each of those concentrations to the respective plate and use marbles to evenly spread the solution on the plate.

The plates were then left open to dry for one hour
Plate 4 most concentrated dilutions of negative control and all 12 of the positive control
Plan of how we will be testing our sugars and setting up our negative controls:

Target Neb5-alpha strain	Corresponding Negative Control	Type of sugar used on both control and target
A. PfLtAb5 #714	**PfLtSc5 #712**	**Arabitol**
B. PfLtSc5 #712	**PfLtAb5 #714**	**Sucrose**
C. PfCiAb5 #710	**PfCiR5 #708**	**Arabitol**
D. PfCiR5 #708	**PfCiAb5 #710**	**Ribitol**
E. PfLtR5 #706	**PfltAb5 #714**	**Ribitol**

8/30/21 Nick

Comments Continued from 8/26 previous experimental comments:
Upon further growth and subsequent examination, colony color and morphology do not appear to be representative of E. Coli. As such, it is hypothesized that contamination led to the resulting dilution series observation which must be amended. It did not follow logic that the spotting procedure must be “refined” as the dilution pattern simply did not occur. That was the reasoning for restriking the seemingly Neb 5 Alpha growth which now is confirmed to not be the supposed bacteria.

We will be beginning to start a new experiment.

Bacteria: P. Putida (this will make sure the experiment works on scale prior to transferring to E. Coli which did not respond to the liquid culture in case something in Putida vs Coli caused Coli not to work in liquid but Putida to now potentially work on solid plating). The purpose of this

experiment is to gauge whether or not the sugar concentration makes any difference in the ability of the induced cellular machinery to allow for colony growth. This experiment is the next step to that taken with the liquid cultures; however, it will now be performed with plates instead.

If the liquid cultures showed equivalent growth in the the least concentrated cultures among both positive and negative controls, it may have been postulated that the induced cellular machinery was already operating at peak efficiency and therefore could not further promote bacterial growth. The most concentrated liquid cultures exhibited visible cell debris which ascertains the concentration of sugar to have been too much. The mechanism of this excess sugar inhibiting growth may have been due to a wide variety of reasons.

Because the liquid cultures did not display nearly any growth between the least sugar-concentrated positive and negative controls and that the cell debris in the most sugar-concentrated cultures is of undeterminable nature, this experiment will be performed on plates instead.

As such, six (6) plates will be made. The experiment will consist of only P. Putida. Sugar concentrations of each of the three Putida plasmids will have two plates. For example, Ltet ribitol will have both a positive and negative control. The negative control will have the previously-used concentration of sugar at 102.5ul whereas the positive control will have 10x the sugar concentration. A 20x and 50x plate were also proposed. The 50x plate will not be made due to the PI's recommendation that such a high concentration would be too much and be of little value. The 20x plate remained an option; however, this experiment seeks to use sugars as a selection marker. As such, the degree of colony growth is irrelevant; the only outcome to be measured is whether or not any growth occurs. It was deemed highly unlikely that the ideal sugar level of growth would have been within such a small magnitude; as such, this experiment will use 10x concentration as an initial, pilot look and confirmation that sugar concentration makes a difference.
NOTE: The liquid culture experiment went up to 40x sugar concentration.
NOTE: Because positive control will have so much solution, a handheld glass spreader will be placed in the center of the plate and slowly spun. Moreover, the plate must be left for a day in order for the solution to fully sink into the plate.

Comments on Future:
Selection Marker and Transformation Efficiency are two different things. We have not yet been able to prove consistency in our plasmids allowing for selectable growth. Prior to the final step of testing transformation efficiency of sugar markers in comparison to use with antibiotics, it must first be confirmed that replication and verification that sugar selection is plausible.

6 Plates will be made:

3 Negative Controls (Putida with previously used sugar level) and 3 Experimental (Putida with 10x sugar concentration).

Each 150ul Plate will be spread with beads and each 1250ul Plate will be spread by handheld sterile glass spreader (NOTE: To sterilize: in between uses, dip flat end into ethanol and hold over open flame until additional flame ebbs away, hold to let cool before use) of the following mixture:

Negative Controls:
3ul aTc Inducer (Ltet) or 25ul OHC14 Inducer (Cin)
22ul Nanopure H2O (only for Ltet to get to 150ul of spreadable solution)
125ul Respective Sugar

Positive Controls:
3ul aTc Inducer (Ltet) or 25ul OHC14 Inducer (Cin)
1.25ml Respective Sugar

08/31/21 David

Goal of the day: To prepare cultures for pFltRi5(#720), pfLtAb5(#718), pfCiAb5(#717), and wild type P.putida liquid cultures for tomorrow's experiment where we will be repeating our induction of the sugar pathways and follow up with the spotting method to obtain CFU units

1. Grab 4 tubes for liquid cultures. Label each tube into respective putida culture with date and antibiotic used.
2. Add 5 ml of LB media to each tube.
3. Add 5ul of G100
4. Use wooden sticks to transfer bacterial cultures to their respective tubes
5. Place cultures for overnight growth at 30 C in incubator

09/02/21 David(subculture preparation), Abby

Goal of the day: Preparing P.putida subcultures with inducer added and proceeding with spotting after 2 hours once they are into exponential phase.

1. **Performing Subcultures of *P.putida* pFltRi5(#720), pfLtAb5(#718), and wild type**

    5. Take 100 ul of overnight culture
    6. Add 5ul of G20
    7. Add 0.6 ul of ATC LT regulator to respective plasmid
    8. Add 5ul of CIN to cultures having CIN regulator
    9. The negative control has nothing added to it.

Performing a serial dilution of the pFLtRi5, pFLtAb5, and wild type

- Take 1 mL of the cell culture and place it in a 1.5 mL tube
- Note: took two rounds of 1 mL of cell culture in order to establish a cell pellet that was visible and we will still be resuspending in 1 mL of PBS
    - Centrifuge this for 1 minute at 5000 rpm
    - Dump the supernatant and add 1 mL PBS; centrifuge for 1 minute at 5000 rpm
    - Dump the supernatant and repeat the previous step
    - Resuspend in 1 mL of PBS as the final step
- Perform a 10-fold dilution in a 96-well plate with wild type P. putida and the 2 P. putida (pFLtAb5 and pFLtRi5) and were rinsed with PBS
    - Place 200 uL of the sample in the first well and 180 uL of PBS in each of the remaining wells
    - Transfer 20 uL of the initial solution into the second well
    - Continue with the serial dilution until the 12th column
        - Row A → Wild-type
        - Row B → pFLtRi5
        - Row C → pFLtAb5
- Prepare liquid cultures in LB agar
- Prepare 90 uL of OHC14 inducer

09/08/21 Nick

This experiment will test for a proportional increase in induced enzymatic activity within our transformed cells.

1. **Grow up 5mL PfLtR5 and WT (NEB5alpha without any plasmid as a control- will show no ribitol enzyme bc doesn't have our plasmid- or any plasmid) overnight**
2. **Pellet 5mL and resuspend in potassium phosphate**
    a. Obtain two 2mL Eppendorf tube. Spin 1.5mL cultured overnight strains PfLtR5 #706 and Wild Type @16873rcf for 1minute, dump supernatant, in respective tube for each strain- repeat until all 5mL overnight culture have been pelleted at bottom of 2mL tube (we can spin at top speed b/c it does not matter whether the cells remain alive because they will be later lysed regardless). The combined pellet from the 3 spin cycles should remain.
    b. Resuspend the pellet in 500ul 50mM, pH 7.0 potassium phosphate.

3. Add 500uL of cell extract to tubes with beads and lyse
    a. Add 500ul respective resuspensions to two of the prepared tubes with silica spheres
    b. Vortex on Fisherbrand Vortex Genie 2 @4000 for 10min (in fumehood, caps should be facing outwards)
    c. Spin both tubes @18673rcf for 1min

4. Prepare Assay Mixture (Total Volume 1.3mL)

a. 5mM NAD (100ul in 2mL Final Volume of 0.1M NAD created=5mM)
b. 0.1M Ribitol (200ul in 2mL Final Volume=0.1M)
c. 100mM Potassium Phosphate pH 7

5. Read 2mL Final Volume Samples in cuvette in Nanodrop: Take continuous readings over 5min
    i. Settings: 20 intervals of 15sec each for a total of 300sec; measure from 190-340nm wavelengths and monitor at 340nm
    b. 3 Total samples (Add NAD cell extract immediately before readings)
        i. PfLtR5 #706 : 1.3mL Final Volume +50ul Cell Extract (1mL 100mM Potassium Phosphate, 200ul Ribose, 100ul 0.1M NAD, 650ul Nanopure, 50ul Extract)
        ii. PfLtR5 #706 : 1.3mL Final Volume +100ul Cell Extract (1mL 100mM Potassium Phosphate, 200ul Ribose, 100ul 0.1M NAD, 600ul Nanopure, 100ul Extract)
        iii. WT : 2mL Final Volume + 50ul Cell Extract (1mL 100mM Potassium Phosphate, 200ul Ribose, 100ul 0.1M NAD, 650ul Nanopure, 50ul Extract)

Filled 15 MP Biomedicals Lysing Matrix tubes with 0.74g Lysing Matrix B (0.1mm silica spheres)

Weigh out 0.0663g Sigma Beta-Nicotinamide Adenine Dinucleotide (NAD) and add to 1000ul Nanopure H2O to a final concentration of 0.1M (the maximum solubility of NAD)