Notebook Week 5 (June 26-29)

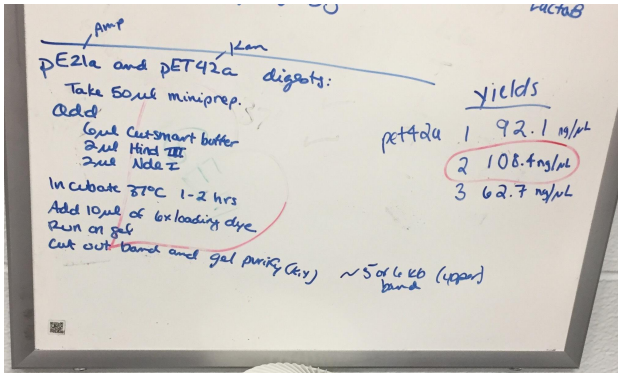
Project: iGEM 2017
Authors: Locke Bonomo, Haylea Northcott, Mike Savioe, Edith Sawyer, Aylin Padir, Catherine Sherman
Dates: 2017-06-26 to 2017-06-29

MONDAY, 6/26

Plan:

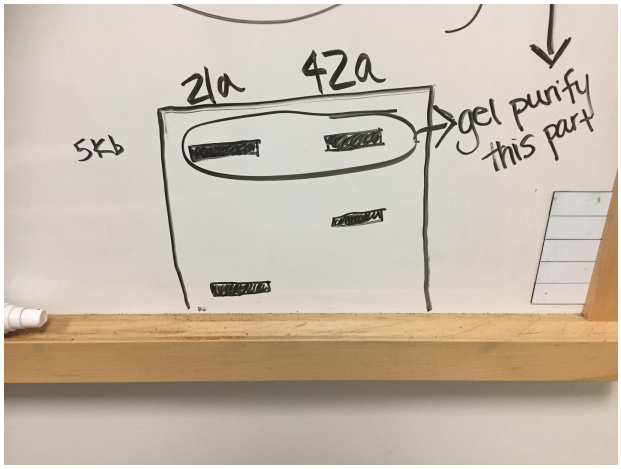
- Complete Safety Forms (Parts 1-3 at least)
- Chromoprotein PCR
- Vector digest
    - Used pet42a mini prep 2 with highest yield and pet21a
    - Digest with HindIII and NdeI
- Running Gel Plan

Image.png.jpeg



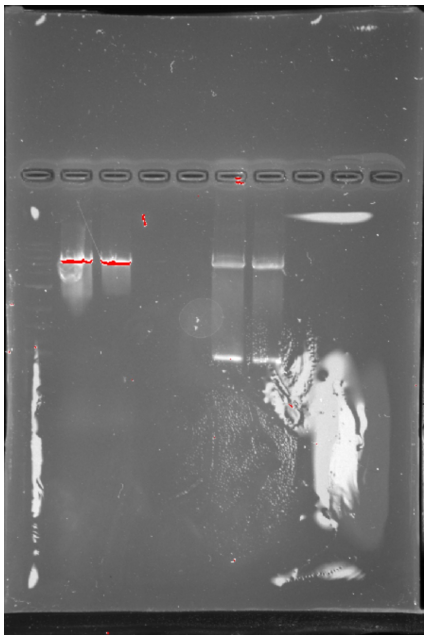
Prediction for the gel based on the restrcition map of Hind III and NdeI on both pet21a and pet42a: gel purifying the first bands on both seperations

Image.png.jpeg



Viewing of gel, second band of pet21a seems to have run off the gel.

gel.PNG



- Gel purification
    - Cut out the first bands of both plasmids (around 5kb)
    - Pet21a- colomns 2 & 3
    - Pet42a- colomns 6&7
- Lead Assay -will be based on data from other results, will likely be for optimizing assay for LB/MRS, after deciding which nanoparticles will be used
- Make concentrated lead
- Make more LB

Lead Assay:

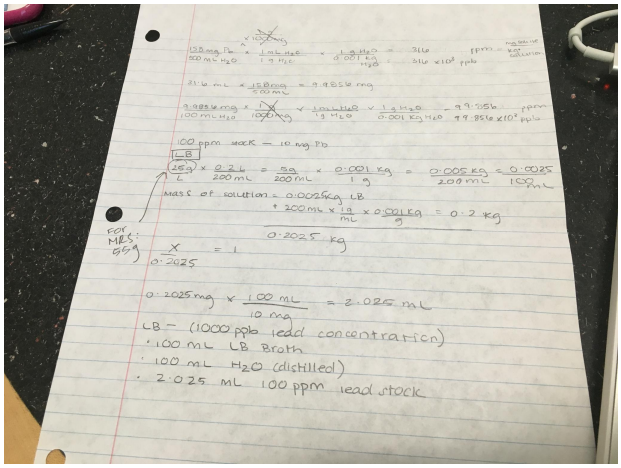
- Set up #1- GSH Optimization w/ 100 AuNPs (repeat from Thursday 6/22/17)
    - Column 1 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 34.6 uL 100 nm AuNPs --> 12 uL GSH
    - Column 1 (E-H): 41.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 12 uL GSH
    - Column 2 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 2 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 10 uL GSH
    - Column 2 (E-H): 43.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 10 uL GSH
    - Column 3 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 4 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 8 uL GSH
    - Column 3 (E-H): 45.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 8 uL GSH
    - Column 4 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 6 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 6 uL GSH
    - Column 4 (E-H): 47.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 6 uL GSH
    - Column 5 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 8 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 4 uL GSH
    - Column 5 (E-H): 49.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 4 uL GSH
    - Column 6 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 10 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 2 uL GSH
    - Column 6 (E-H): 51.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 2 uL GSH
    - Column 7 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 12 uL H20 --> 34.6 uL 100 nm AuNPs
    - Column 7 (E-H): 53.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs

GSH dilution 2 62617.xlsx

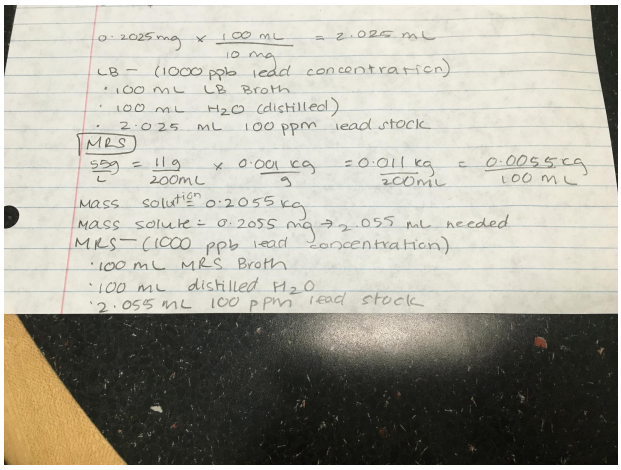
A second trial was done with set up #2 because the data was not uniform, and the second trial showed similar results. We will be continuing our assay with the 20 gold nanoparticles because the results of the second trial of optimized GSH for 100nm particles are so variable. The next set up will be with 20 AuNPs and LB/MRS.
Side note: The lead concentration of the solution we were using appears to be 100 fold off.

Lead Assay Calculations for new dilutions and for LB & MRS tests:

IMG_1916.JPG



IMG_1917.JPG



Notes: 50 mL of LB/MRS was added to an autoclaved 100mL bottle. Next 50 mL of distilled water was added to each bottle. Finally 1.0125/1.0275 mL was added to each bottle (respectively) to make the concentration of lead in each bottle 1ppm or 1000ppb. We decided that instead of diluting the control media ahead of time, dilute it when adding it to the well (i.e. by added half the amount of LB/MRS needed, then adding an equal amount of distilled water).

- Set up #2 of LB/MRS with 20
  - Column 1: 41.5 µL Pb spiked LB --> 10.5 uL LB --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 2: 52 µL Pb spiked LB --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 3: 52 µL diluted LB --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 4: 41.5 µL Pb spiked MRS --> 10.5 uL MRS --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 5: 52 µL Pb spiked MRS --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 6: 52 µL diluted MRS --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 7: 52 µL H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 8: 53.5 µL H20 --> 34.6 uL 20 nm AuNPs

media 62617.xlsx

TUESDAY, 6/27

Plan:

- Growth Curves with LB-Pb and MRS-Pb Media
    - 2 replicates from 1 parent culture (couldn't do 3 replicates because b.subtilis didn't grow enough) Only doing 2 cultures per bacteria, one will be a control, and one with be spiked with 10 ppm of lead. This will give us a starting point to decide how to move forward with amount of lead for thursday.
        - Lactobacillus starter culture OD
            - undiluted- 2.208
            - 1:10 dilued - 1.084
        - B.subtilis starter culture OD
            - undiluted- 0.166
            - checked again 0.189
        - Diluted both cultures back to an OD of 0.1
            - Checked OD
            - Lactobacillus - 0.122
            - B.subtilis- 0.096
    - Checking every 1.5 hours
    - At hour three (exponential phase) we will add either/both DI water, and lead spiked water to get all to the same volume
        - 1 = control
        - 2= 1 ppm Lead spiked water
        - 3= 10 ppm Lead spiked water

Addition of the 100 ppm Stock culture, with calculated dilutions to achieve 10 ppm

Image.png.jpeg

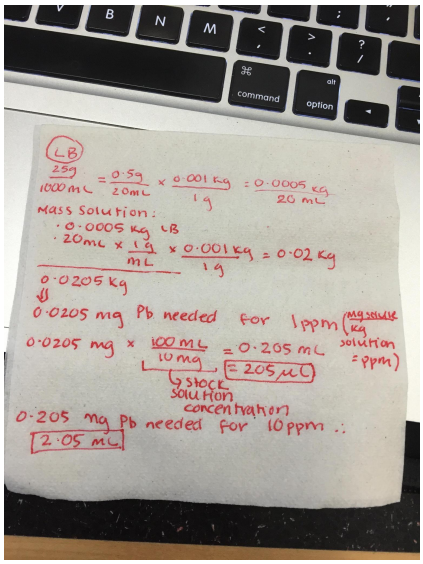
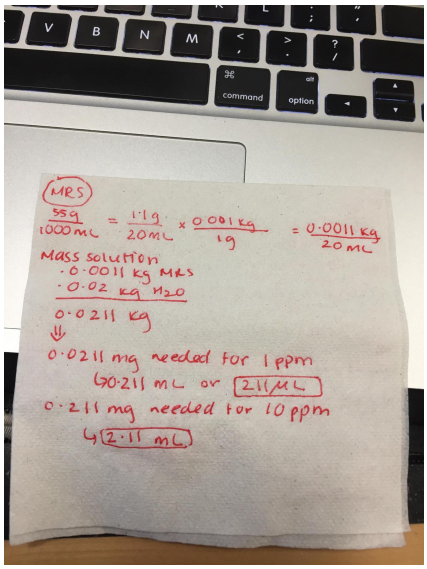


Image-1.png.jpeg



* Only ended up with 10 mL cultures due to lack of growth of B.subtilis, volumes above were just divided by 2 to get correct volume of addition of 100 ppm stock culture*

Growth Curve Analysis

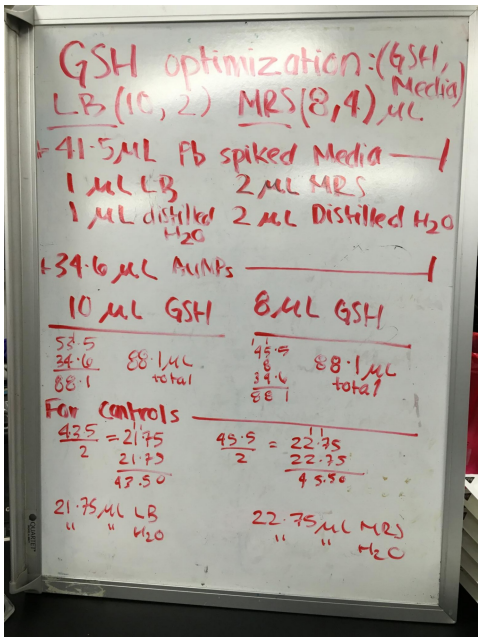
Trial1LeadGrowthCurve.xlsx

Looking at the excel file you can see that both the control growth curve and 10 ppm, were almost identical. This shows that 10 ppm does not affect the growth of the bacteria at all. On thursday we will be upping the amount of lead added at exponential growth to try and find a sensitivity that affects the growth.
Online we found an article (Here) that shows the growth of lactbacillus in varying amount of lead and cadmium.

- Lead Assays
    - GSH optimization for LB+Pb and MRS+Pb at 405,450,570nm

30 15.xlsx

IMG_1931.JPG

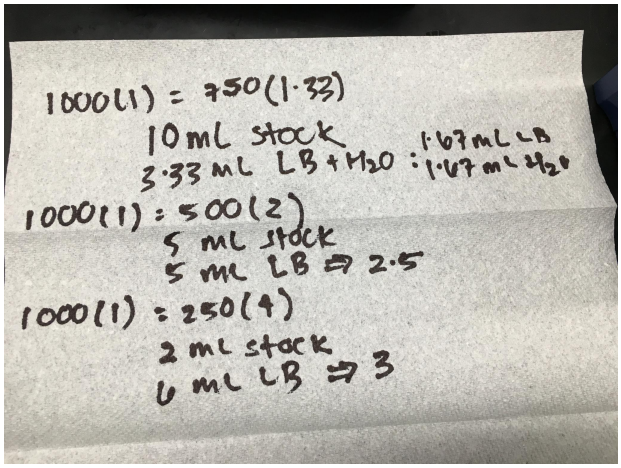


- Repeat assay #2 from 6/26/17
- Try at lower concentrations of Pb in both LB and MRS
  - Note: control also needs to be diluted to make sure pigment of media with and without lead is the same

The lead assay for MRS and LB GSH optimization was successfully completed. The optimized GSH concentration for LB is 8µL and the concentration for MRS is 10µL. Another small lead assay was also completed with the LB and MRS controls, and the LB and MRS with their respective optimized GSH concentrations. Three trials need to be done to make sure the test is repeatable and to develop the standard curve. The next step is to see how the assay works with LB and MRS at smaller concentrations of lead. If this works right away it will be all set to start using to test how much lead the bacteria can take up. If it does not work right away we can adjust the concentrations of GSH to see if we can make it more effective at lower concentrations of lead, but in the mean time we can still use the assay when measuring large concentrations of lead.

media GSH dilution.xlsx

IMG_1932.JPG



all media.xlsx

- Gibson Assembly

Gibson Assembly Notes:

Gibson assembly (1/2 reactions) of chromoproteins (no PCR), transformed into competent E. coli:

  - 6 chromoproteins, + 2 neg controls with just vector => 8 plates
  - 2 fragment assembly page 12-13
  - 1:2 ratio vector to insert (3-5 uL of vector, ~5 uL insert, 10 uL MM, top off w/ water)
  - SOC Media in fridge on drawer for cell recovery
- Chromoproteins -> centrifuge max 5' (dry)
- add 50 uL elution buffer from miniprep kit
- vortex on high 2' to dissolve
- spin tube 30s
- Should be ~10 ng/uL (nanodrop)

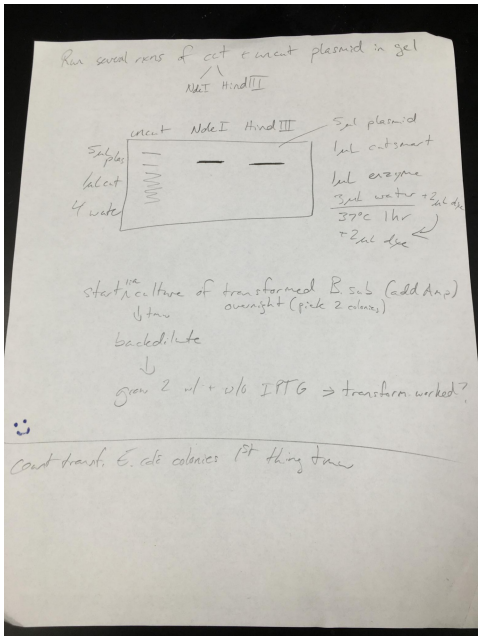
Table3

	A	B	C	D	E	F	G
1	Sample:	aeBlue	amilCP	amilGFP	efoRed	fwYellow	tsPurple
2	Concentration (ng/uL)	10.4	9.4	9.4	8.4	11.2	
3	Volumes (uL)	3	3	3	3	3	

- Then: followed Gibson Plasmid Assembly and Transformation into E. coli
  - 1/2 reactions (10uL total), so halve all volumes in protocol
  - no DI water used

The freezer box was left out overnight so the Gibson assembly will be tried, but may need to be redone. 8 transformations were done, with all 6 of the chromoproteins and the negative controls. They were incubated for an hour and then plated. The blue colors are in pet21a and the yellows and reds are in pet42a. There are 8 plates total, and 6 of the plates should have color. There are no replicates. Because of the freezer box mishap, an enzyme digest was run to check if the enzymes will need to be re-ordered.

IMG_1930.JPG



- Error Prone PCR Research Review
- Discuss Collaborations (Skype calls sometime? What are we doing? Options?)

Collaborations include a scheduled meeting with a Germany team for Thursday 6/29/17 at 9am (our time). The BU Hardware was also emailed for possible collaborations involving the lead assay and the mechanics of turning it into an automated chip.

Research was also done into other 2016 iGEM teams that worked with heavy metals. We found that the team from University of Pittsburg, as well as 2 China teams worked on this topic. Pitt worked with detection through toehold switches. One of the China teams worked with Mercury detection in fish through the gut, and the other team worked with lead paint detection and herbs.
http://2016.igem.org/Team:Pittsburgh
http://2016.igem.org/Team:HSiTAIWAN
http://2016.igem.org/Team:Lanzhou
http://2016.igem.org/Team:FAU_Erlangen/Safety

Grew overnight culture of transformed B. Subtillus to test tomorrow.

Re-streak B. Subtillus on to new plates to make sure they're more active. This should be redone once a week to ensure higher growth rates.

The case study was roughly outlined. For the case study, we can use the iGEM team as the story line. The link is: Case Study

Research was also completed about how the FDA classifies probiotics and dietary supplements:

https://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm228269.htm
https://www.fda.gov/Food/DietarySupplements/ProductsIngredients/default.htm
https://www.loc.gov/law/help/restrictions-on-gmos/usa.php
https://www.fda.gov/newsevents/testimony/ucm426541.htm
https://www.fda.gov/NewsEvents/Testimony/ucm468833.htm
https://www.fda.gov/downloads/newsevents/meetingsconferencesworkshops/ucm472502.pdf

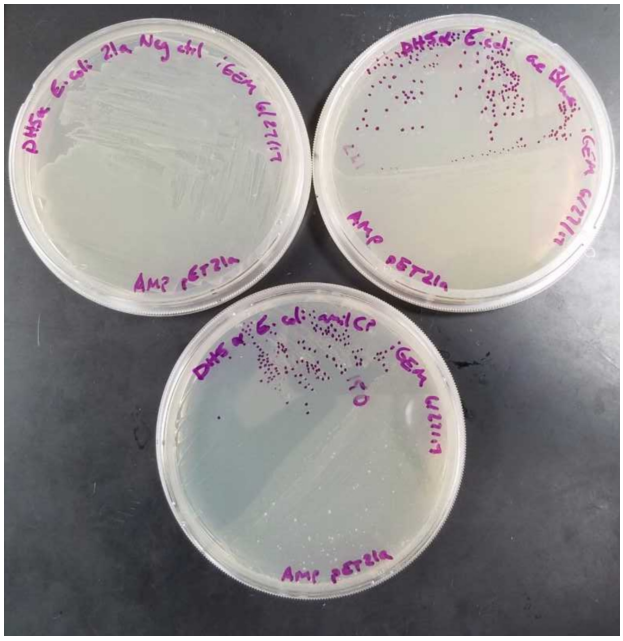
WEDNESDAY, 6/28

Plan:

- Check on transformed B. Subtillus & E. Coli

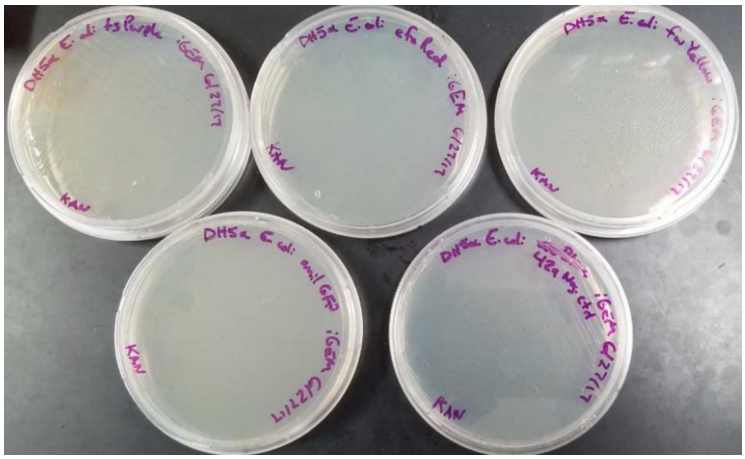
Only 21a plates grew, incl neg control. No 42a plates grew. Counted colonies on plates that grew.
Plates:

image.png



Amp plates with all transformations pET21a
Counted colonies - 150 on amilCP plate; 197 on aeBlue. Didn't count lawns (negative control was all lawn)

image.png



Kan plates with all pET42a transformations.
No colonies grew on any plates.

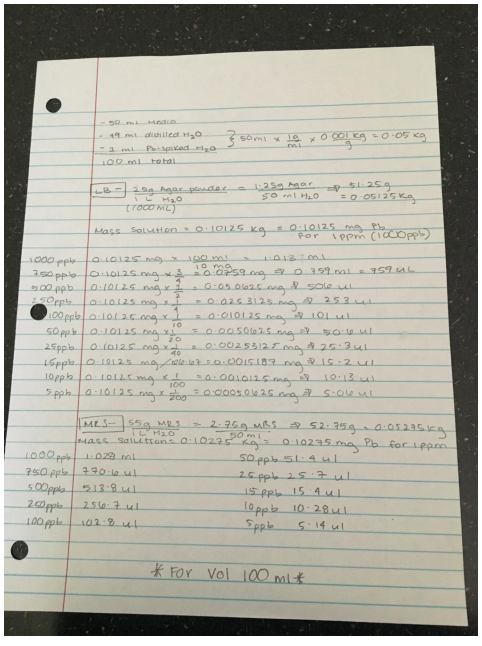
Just to see if they grow - Labeled new Amp/Kan plates and plated another 100 uL of the cells that were left over from yesterday (still in SOC media). Put in 37C incubator.

- Set up liquid cultures of B. Subtillus for growth curves Thursday

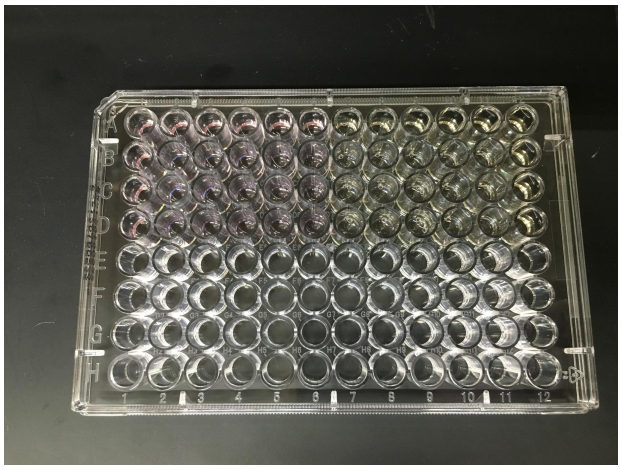
- Lead assay standard curve (3 replicates, specific time points)

The media dilution of 1000, 750, 500, and 250ppb was redone this morning because yesterday's dilution data came out a little bit odd. The data this morning for the same levels came out better than yesterday, but the results were not uniform. Because the bacteria seem to be thriving in high amounts of lead (results of yesterday's growth curve), the next step is to figure out how the assay would work at higher concentrations of lead. From this a standard curve for higher concentrations could be developed. We then ran another lead assay with high concentrations of lead, 1,500, 2,000, and 5,000ppb. This went well, and helped us figure out exactly which time points are important to the different kinds of media. MRS should be read immediately through ~5min at 570nm, and LB should be read from ~8-13min at 405nm. This assay will be done again in 3 trials tomorrow to create a curve for higher lead concentrations.

IMG_1934.JPG



IMG_1935.JPG



super high media.xlsx

- Reach out to FDA about classification of our probiotic

FDA Contact Emails:
for food additives: premarkt@fda.hhs.gov
for dietary supplements: ODSP@fda.hhs.gov
for drug regulation: druginfo@fda.hhs.gov
https://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ContactCDER/ucm071014.htm

- Outline Case study (rough outline completed (6/27))

- Interlab Graphs and Finalized Data

Worcester_WPI_InterLab_2017_Measurements.xlsx

- Redo Gibson Assembly

Performed another gibson assembly, following the same protocol but doing transformation all in the same day (rather than assembly, then transformation the next day).

- Redo Enzyme Digest

Problem: Gel made with water not TAE buffer

- Worked on final presentation, have a new theme

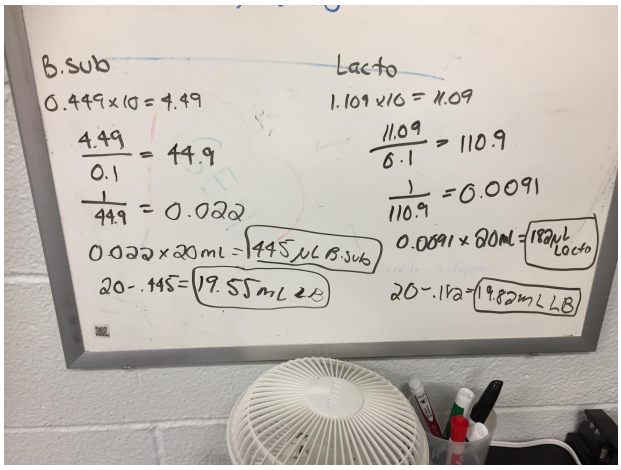
THURSDAY, 6/29

Plan:

- Growth Curves with LB, MRS- Pb Media w/ varying amounts of lead
    - 1 parent culture for both B.subtilis and Lactobacillus
        - each parent culture will make 3 sub cultures from -> control, 50 ppm, 100ppm
        - All shaking at 37 degrees, checked every 1.5 hours
        - This will be a test to see how much lead the bacteria can bind in, we believe that the 50 ppm should alter the growth but due to non difference with the 10 ppm where other literature saw difference, we will also be testing 100 ppm.
    - Starter OD (1:10 dilution)
        - Lactobacillus- 1.109
            - actual OD = 11.09
        - B.subtilis- 0.449

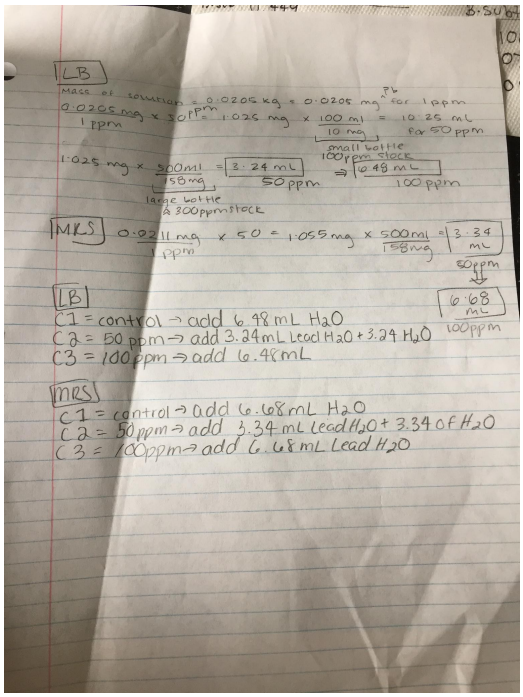
- actual OD = 4.49
- Dilution to get each 3 subcultures to 0.1

IMG_3181.JPG



- Will add lead at hour 3 when both cultures hit expotential phase. This is the amounts of lead and water added to each culture to achieve a consitent volume across all subcultures. Culture 2 will be 50 ppm and Culture 3 will be 100ppm.

IMG_3180.JPG



Growth Curves continued to have funky data at hour 7, so stopped.
Growth was not alter in the 50 ppm or 100ppm lead.. more research needs to be conducted to figure out why this is
A paper grew them in the lead overnight, maybe that is the new route we take instead of introducing the lead at exponential growth.
The excel file with all OD's and growth curve charts can be found below

Trial2LeadGrowthCurve.xlsx

- Skype with German Team @ 9am

Skype with Germany went well. We are going to research high salt concentrations in water in the US and see if it is as much of a problem here as it is in Germany. Their project involves engineering the proton pumps in yeast to absorb the salt and remove it from the water. They will be researching lead water pollution problems in Germany, and we will contact each other with our findings within the next week or two. In addition, each team will be considering how we can use pieces of each other's projects together, and where we can further collaborate.

- Lead Assay Curve for high concentrations
    - LB at 405 nm: 8:00, 9:30, 11:00, 12:30

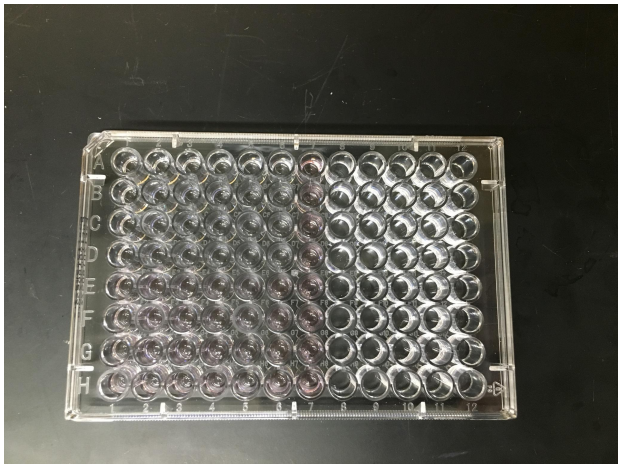
LB Trial 1:

LB high trial 1.xlsx

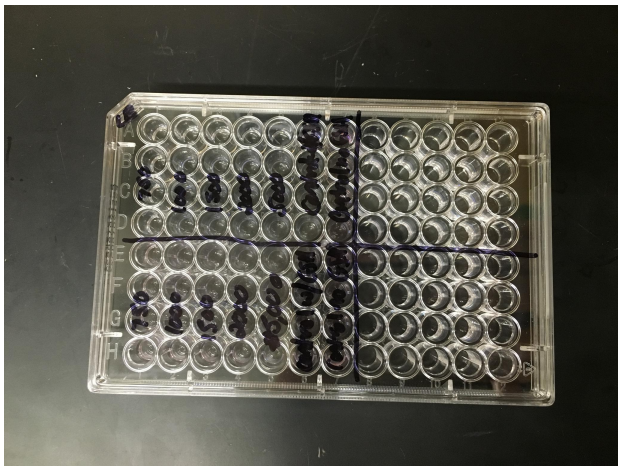
LB Trial 2:

LB high trial 2.xlsx

IMG_1941.JPG



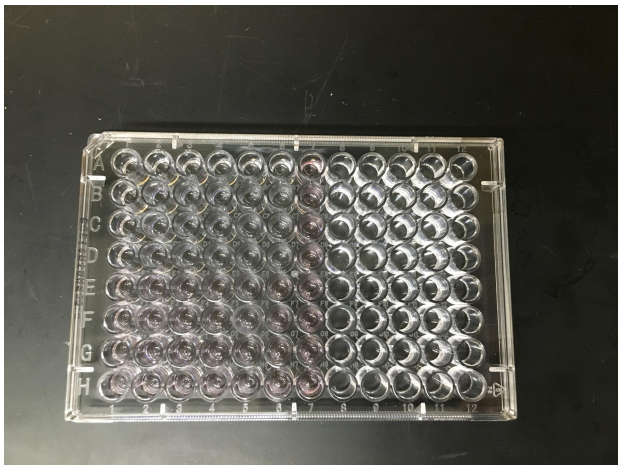
IMG_1938.JPG



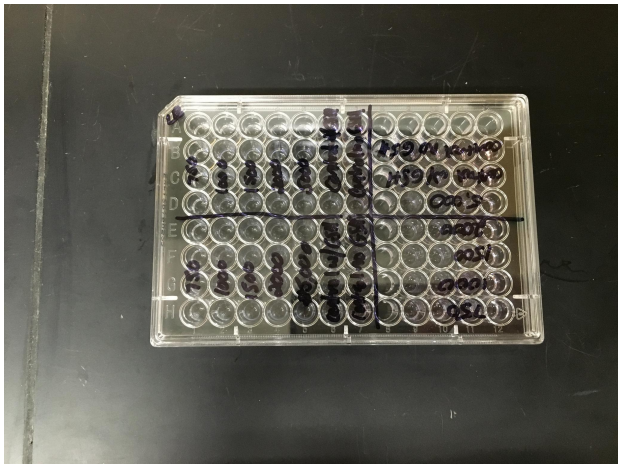
LB Trial 3:

LB high trial 3.xlsx

IMG_1941.JPG



IMG_1944.JPG



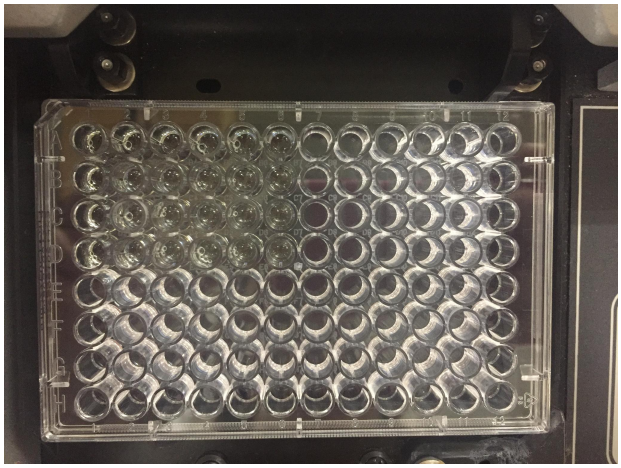
In general, the numbers seemed a bit off from what was seen yesterday. The lower concentrations were very clustered, and had to differentiate between in some cases, and in others the 2,000ppb had a higher absorbance than the 5,000ppb. Additional readings were taken so there was one at 3:00, 6:00, 8:00, 9:30, 11:00, 12:30, and 14:00. A standard curve was made for time points 8:00, 9:30, 11:00, and 12:30, with and without the 750ppb measurement. The standard curves including the 750nm measurement are off, but the ones that do not include it are very linear. The standard curve for 12:30min without the 750ppb measurement was the best and had an R value of .98.

LB Standard Curves.xlsx

- MRS at 570 nm: ASAP through 4:30/5:00
  - Trial 1

IMG_4580.JPG

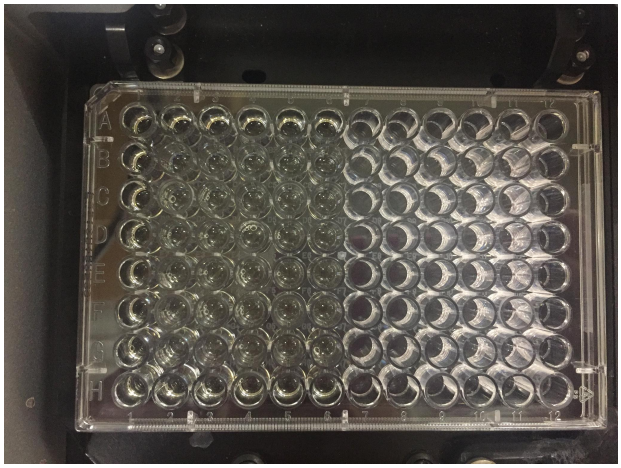
IMG_4581.JPG



MRS high standard 1.xlsx

- Trial 2

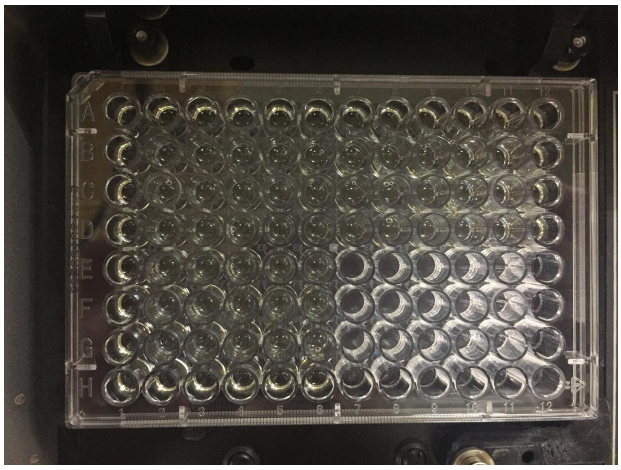
IMG_4582.JPG



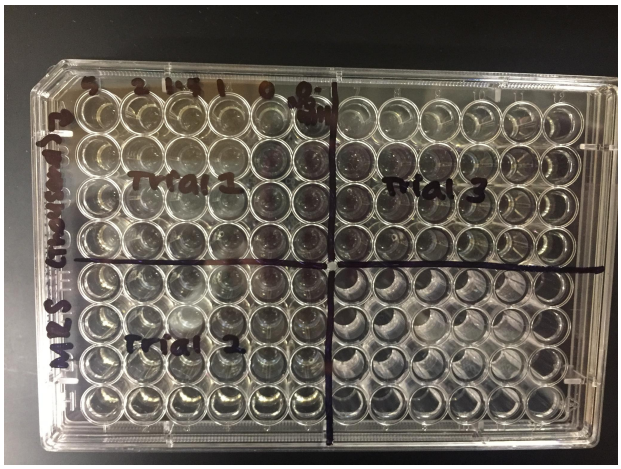
MRS high standard 2.xlsx

- Trial 3

IMG_4583.JPG



IMG_4584.JPG



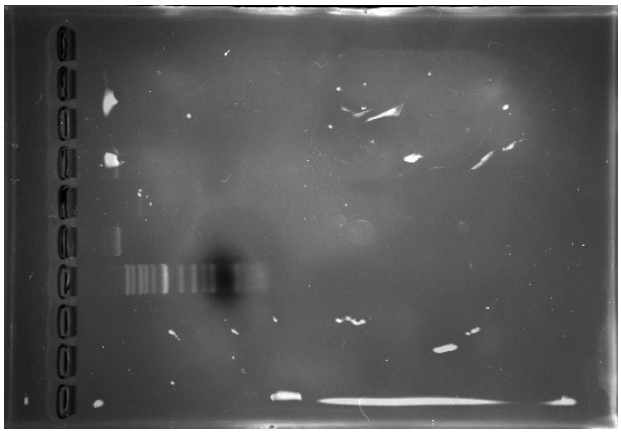
MRS high standard 3.xlsx

- Continue working on presentation so that it is ready for our presentation to Farny Monday 6/10
- Restriction digest (again):

Redid restriction digest correctly this time. Followed the same protocol as yesterday for the digest reaction. The gel was made with 100 mL 1X TAE and 1 g of agarose, for a 1% agarose gel. The results will tell us whether or not the enzymes HindIII-HF and NdeI have respectively "survived" being left out overnight out of the freezer.

Results:

Enzyme test sensitive imaging 6_29.PNG



In order from bottom to top: 2-log ladder, no-enzyme control, NdeI, HindIII-HF.
The results indicate that HindIII-HF may not be functional. NdeI may be functional because the band is lower than the control, but somewhat less volume was loaded into this well (NdeI) due to bubbles in the tube - the lower band may be a result of lower DNA concentration in the volume loaded.

- Add IPTG to cultures of our transformed Bacillus subtilis to see if it's transformed

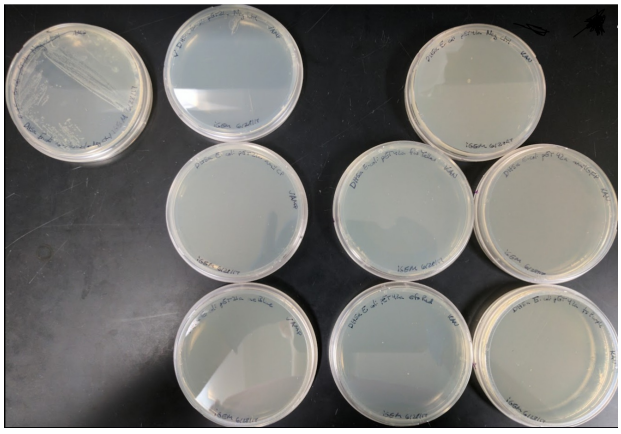
At 8AM, 3X 100 uL of the B. subtilis culture started yesterday was added to 3X 5 mL of LB (no amp) to "refresh" it. To one culture, 5uL IPTG was added to induce GFP expression on the putatively transformed plasmid in the subtilis. Another culture received 50 uL IPTG. The third culture received no IPTG. We should see expression after 4 hours incubating shaking at 37C.

Checked after 4.5 hours - made one slide using 8 uL of each culture, and no fluorescence was seen in any of them.

- Check transformation plates of our E. coli from yesterday

Checked plates from the Gibson assembly and transformation yesterday - results: no transformants grew. Both negative controls for no insert were clear of colonies as well. The negative control for no plasmid or insert (to check the ampicilin plates) grew several colonies, so the bad amp plates will need to be thrown out and new plates should be poured.

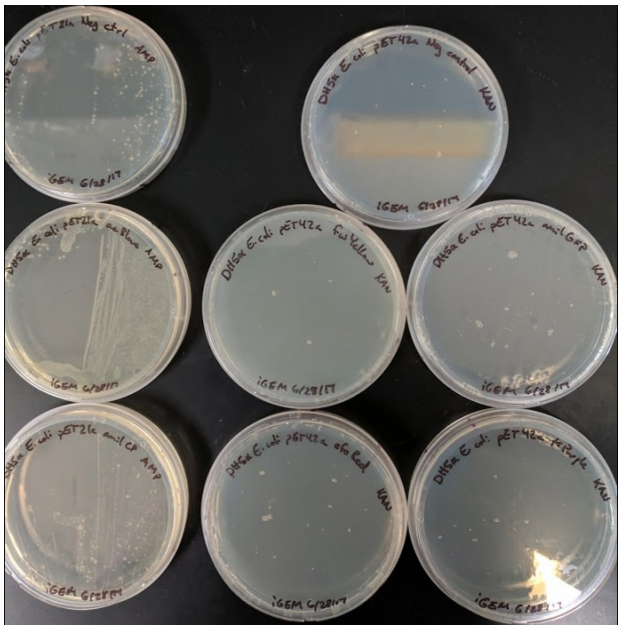
image.png



Gibson assembly & transformation from yesterday. No colonies grew, except for a negative control on an amp plate (not supposed to grow).

Yesterday we also streaked 100 ul each of the leftover cells from Tuesday onto plates (this was done before we realized the amp plates might be bad), and nothing grew except for colonies on the (bad) amp plates.

image.png



Leftover transformants from Tuesday. Nothing grew except for colonies/lawns on the bad amp plates. These colonies probably don't actually contain a pET21a plasmid.

Overall: The gibson assembly and transformation will need to be redone after break.

- Things to consider over break: How lead is tested when it is sent to a lab, How to continue with lead assay, Presentation, Research on salt in water pollution for German team, http://igem.rwth-aachen.de/, FDA contact, Wiki Stuff

DIY Lead Assay

Introduction

Materials

- Gold Nanoparticles
- Lead Nitrate
- GSH
- NaCl (1 M)
- Phosphate Buffer (50 mM, pH=7)
- D.I Water
- 15 mL conical tubes
- 1.5 centrifuge tube

Procedure

Make Glutathione Liquid

1. Make 100mM solution of L-Glutathione

   30.72 g / 100mM
   Add 0.5 g of the Glutathione powder to 16.3 mL of D.I water
   Make 1 mL alaquotes and store in freezer

Make Phosphate Buffer

2. Make a 1 M stock of Phosphate Buffer
3. Add to a graduated cylinder 65.82 g/L of Sodium Monobasic and 93.1 g/L of sodium dibasic in 100 mL of water
4. Test pH with pH meter and adjust using 10 normal NaOH
5. Top graduated cylinder to 1 L

Make NaCl solution

6. Make 1 M stock of NaCl
7. Add 11.86 g into 200 mL of water into a bottle

Make GSH solution

8. Make dilute of the phosphate buffer to 50 mmol, add 1 mL of stock to 19 mL of D.I water
9. In a 15 mL conical tube add 110 µL of NaCl, 620 µL of Phosphate buffer, 480 µL of L-Glu liquid and 790 µL of D.I water.
10. Vortex thoroughly for 5 seconds

Prepare Lead Spiked Water Stock

11. Measure out 100 mg of Lead nitrate to get 1000 ppb
12. Dilute as necessary to achieve concentrations of 5 ppb, 10 ppb, 15 ppb, 30 ppb, 50ppb, and 100 ppb.

    5 ppb - 1 µL Pb in 20 mL of D.I
    10 ppb - 1 µL Pb in 10 mL of D.I
    15 ppb - 1 µL Pb in 6.67 mL of D.I
    30 pbb - 1 µLL Pb in 3.3 mL of D.I
    50 ppb- 1 µL Pb in 2 mL of D.I
    100 ppb - 1 µL Pb in 1 mL of D.I

Prepare Cadmium Spiked Water Stock

13. Measure out 100 mg of Cadmium nitrate tetrahydrate to get 1000 ppb
14. Dilute as necessary to achieve concentrations of 5 ppb, 10 ppb, 15 ppb, 30 ppb, 50ppb, and 100 ppb.

    50 ppb- 1 µL Cd in 2 mL of D.I
    100 ppb - 1 µL Cd in 1 mL of D.I

Well Preparation and Plate Reading

15. In wells A1-A7, place samples 5 ppb-control
16. In each well add 12 µL of GSH and 34.6 µL of AuNP along with 41.5 µL of the lead spiked water
17. Mix contents with pipette by pipetting up and down
18. Place replicates in B1-B7, C1-C7, and D1-7
19. In wells A, B, C, D8-9 add 12 µL of GSH and 34.6 µL of AuNP along with 41.5 µL of the cadmium spiked water (50-100 ppb)
20. Place in plate reader at Absorbance $A_6$10
21. Incubate for 10 min and read over period of time
22. Perform second well test and record color change every 1 mins f

Lead/Cadmium AuNP Assay 96-well plate

	A	B	C	D	E	F	
1	5 ppb Pb (1 uL Pb-H2O/20 mL DI H2O)	10 ppb Pb (1uL/10mL)	15 Pb (1uL/6.67mL)	30 Pb (1uL/3.33mL)	50 Pb (1uL/2mL)	100 Pb (1uL/1mL)	Cor
2	"	"	"	"	"	"	Cor
3	"	"	"	"	"	"	Cor
4	"	"	"	"	"	"	Cor
5							
6							
7							
8							

23.