Notebook Week 9 (July 24-28)

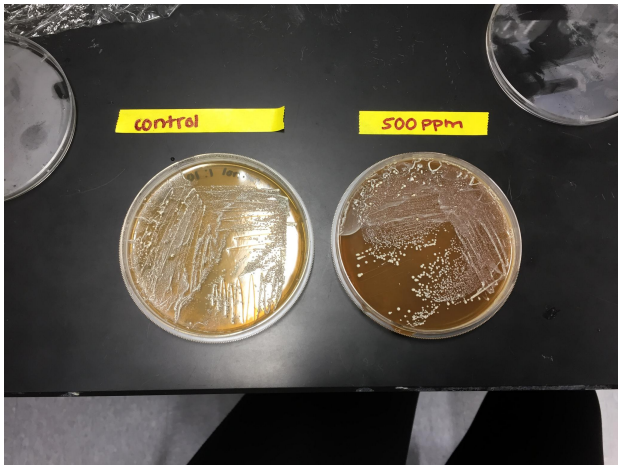
Project: iGEM 2017
Authors: Locke Bonomo
Dates: 2017-07-24 to 2017-07-28

MONDAY, 7/24

To do:

- Email Linda Looft
- Email BU for details about Wednesday
- Check Lactobacillus proof of principal dilution plates from Thursday (Edith took them out of incubator Saturday morning (after 2 days of growth) and placed them in the 4C fridge)
    - 1:10 dilution on both 0 ppm (control) and highest lead concentration 500ppm

IMG_3468.JPG



    - 1:100 dilution on both 0 ppm (control) and highest lead concentration 500 ppm
        - Looks as if the 500ppm had an effect on growth in this streaking but not in the streaking above

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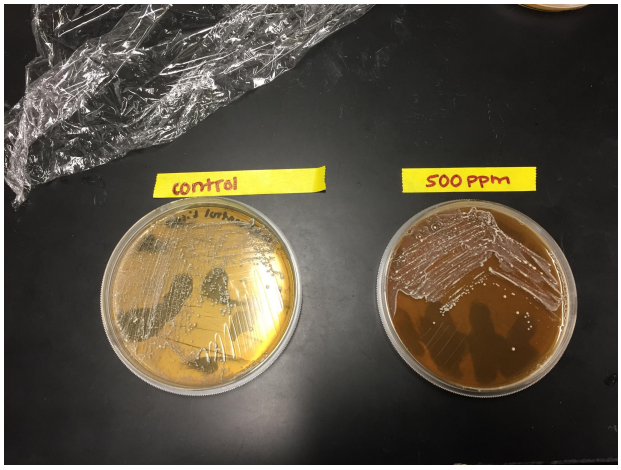


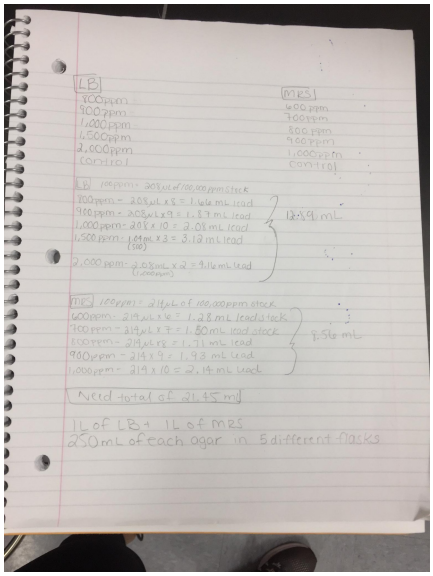
    - Counted colonies on the 4 plates

Lead Plate Counts 7/24

	A	B	C
1	Lead	Dilution	Individual colonies
2	0ppm	1:10	132
3	0ppm	1:100	182
4	500 ppm	1:10	148
5	500 ppm	1:100	33

- Make new lead concentration plates with higher concentrations -> trying to stunt growth to find a limit
    - Calculations below

IMG_3470.JPG



- Respond to Ray, John, & IDEXX (Cat)
- MRS lead assay
    - Plate set up

MRS Lead Assay Set …

	A	B	C
1	Control (0 ppm)	Row A replicate 1	Row A replicate 2
2	50 ppm	" "	" "
3	100 ppm	" "	" "
4	200 ppm	" "	" "
5	300 ppm	" "	" "
6	400 ppm	" "	" "
7	500 ppm	" "	" "

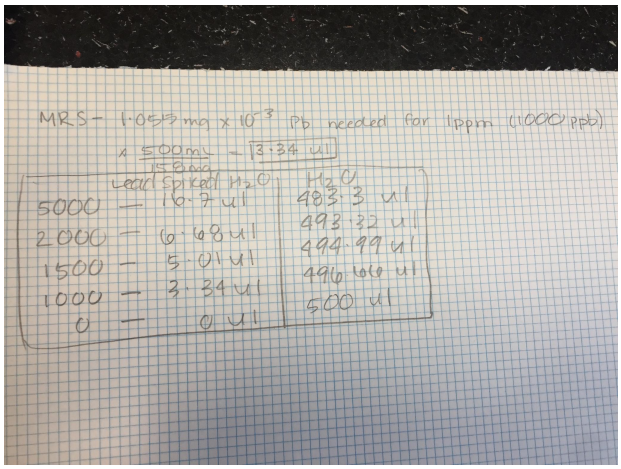
MRS different concentrations in rows.xlsx

MRS assay does not seem to be working at such high concentrations. Plan B, try in the 0-2000ppb range (same range that we currently have a LB assay for).

MRS Lead Assay Set up 2

	A	B	C	D	E
1	Control (0 ppb)	1000 ppb	1500 ppb	2000 ppb	5000 ppb
2	" "	" "	" "	" "	" "
3	" "	" "	" "	" "	" "
4	" "	" "	" "	" "	" "

IMG_5157.JPG



MRS Standard Trial 1.xlsx

- Make overnight culture of lactobacillus with colonies from control plates

Prep and PCR chromoproteins

- Got new primers - Prepared and reconstituted to 100 uM stock, and did PCR of chromoproteins, as explained in Chromoprotein PCR but using different annealing temperatures. Melting and annealing temperatures were calculated using the NEB Tm calculator website (see table)

Melt/Anneal Temperatures for Chromoprotein PCR with new primers

	Temps (C)	amilGFP	fwYellow	efoRed	tsPurple	aeBlue	amilCP
1	5' Tm	58	64	76	63	61	
2	3' Tm	69	73	61	72	70	
3	Avg Tm	63.5	68.5	68.5	67.5	65.5	
4	Anneal Temp	53	59	56	58	56	

Due to the different melting/annealing temperatures, the primers were split into two groups based on the temperatures and two different thermocycling parameter protocols were followed (see below).

- The PCR parameters were identical, aside from the annealing temperatures. The groups and parameters were:
    - Group A: amilGFP, aeBlue
    - Group B: fwYellow, efoRed, tsPurple, amilCP

Chromoprotein PCR Parameters and groups

	Group A	Group B	C
1	95C 1'	95C 1'	x32 cycles
2	54.5C 1'	68C 1' (supposed to be 58C but was misentered)	
3	72C 1'	72C 1'	
4	72C 10'	72C 10'	x1 cycle

Group B was supposed to be 58C but was mis-entered into the thermocycler.

- PCR products saved in freezer
    - Gel and PCR product cleanup will be done tuesday

TUESDAY, 7/25

- Lead Assay MRS Standard Curve for Low Concentrations (Two Trials)

Raw Data:

MRS Standard Trial 2 3 4.xlsx

Edited Data:

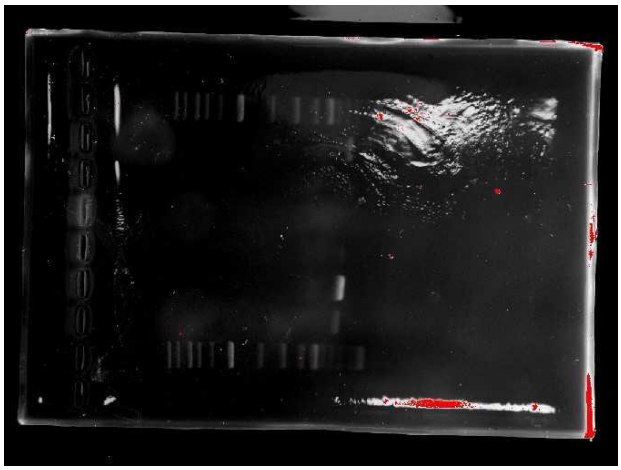
MRS Standard 1 to 3 Media to Water.xlsx

Conclusion: After substantial effort to adjust the assay to perform well with lead-spiked MRS, we have concluded that the MRS media is not suitable for our assay. Attempts to create a standard curve following the same procedure that was used for the LB standard curve (for LB standard curve information see Thursday, 7/13) did not lead to positive results (see Monday, 7/17 MRS Trial 1b). To fix this problem, we referred to the protocol used in the DIY Lead Assay published by Micromachines (Micromachines 2015, 6, 462-472; doi:10.3390/mi6040462). We found that their ratio of NaCl to L-glutathione to phosphate buffer was 10 to 4.1 to 2.6, and so we adjusted our concentrations of each accordingly, without success for the MRS assay (see Wednesday, 7/19 excel document "DIY Lead Assay LB and MRS 719"). Adjustments to the NaCl to L-glutathione to phosphate buffer ratio were made to account for the molarity of the media and the assay was repeated (see Thursday, 7/20 excel document "LB MRS new"). Finally, the pH of the phosphate buffer were made again in accordance with the information provided in the Micromachines DIY Lead Assay. Distinguishing the control MRS (diluted 1:1, Media:DI water) from the 1000 ppb lead-spiked MRS seemed promising when the phosphate buffer was at a pH of 8.6 (see Thursday, 7/20 excel document "LB MRS Basic" and "LB MRS 8.5 9"). Despite this miniature success, the assay was not able to distinguish the amount of lead in the MRS at either high concentrations (50-500 ppm) or low concentrations (1000-2000 ppb), even when diluting the media further to 1:3 Media:DI water (see “DIY Lead Assay MRS Trial 1” and “MRS media dilution” on Friday, 7/21 and “MRS different concentrations in rows” on Monday, 7/24 for high concentrations and “MRS Standard Trial 1” on Monday 7/24 and above file "MRS Standard 1 to 3 Media to Water" excel files for low concentrations).

- Call John
- Email Gary and Alisa
    - Completed
- Streak Lactobacillus and B.subtilis onto varying lead concentrations

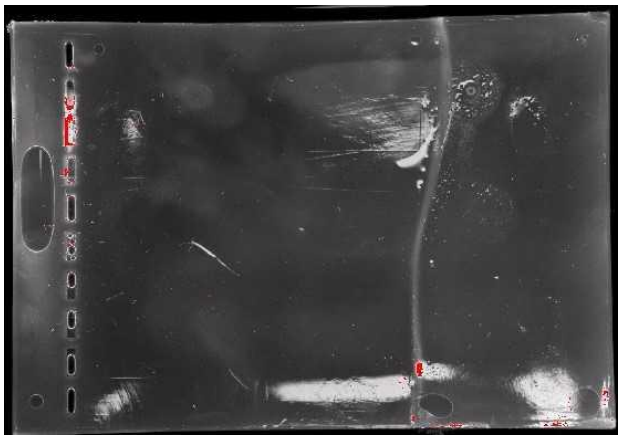
    - Lactobacillus
        - control, 50ppm, 100ppm, 600ppm, 700ppm, 800ppm, 900ppm, 1,000 ppm
    - B.subtilis
        - control, 100 ppm, 300 ppm, 800ppm, 900 ppm, 1,000ppm, 1,500ppm, 2,000 ppm
    - Next steps after studying the growth:
        - Find the limit of lead (where it stunts growth)
        - Start to force-evolve lactobacillus by taking a colony from control, streak to 50ppm, then to 100ppm
            - Similar to the megaplate, but physically moving lactobacillus because it is not motile
            - Pick largest colony that grows on lead plates, as these bacteria may have higher lead resistance/lead-binding capabilities
        - *IDEA:* Take a colony that has already grown on a lead plate and streak in onto a plate of the same concentration to see if it stops growing after it absorbs a maximum capacity of lead
            - Split plate in half
                - i.e Streak a colony from 500ppm and from control only a 500ppm plate
- Pack for BU
    - AuNPs
    - GSH ingredients (1300ul Phosphate buffer, 250ul NaCl, 1000ul L-Glutathione, 2 ml DI H20)
    - Lead Dilutions (20ul 316ppm lead stock (9.7ul for 1500ppb, 6.5ul for 1000ppb), 10ul 1000ppm lead stock(4.1ul for 2000ppb), 2ml DI H2O, 2mL LB)
    - Multichannel Pipetts
    - Tips (1 box of yellow, 1 box of red)
    - Plate
    - Trays
    - Biohazard Cooler
    - Small, New Biohazard Bottle for Lead Waste
- Emailed Professor Scarlett Shell (specializing in microbiology) requesting a meeting
- Talked about modeling ideas on Starlogo Nova
- PCR screening was performed for chromoproteins from day prior. It was discovered that fwyellow, efoRed, tsPurple, and AmilCp were annealed at the incorrect temperature of 68 C, instead of the correct temperature of 58 C

pcr screening 2.jpg



- Gibson Assembly was performed with PCR products of chromoproteins from day prior, LB was used for both positive and negative controls due to running out of S.O.C. media
- PCR was done of chromoproteins again to improve yield
- Results from gel were inconclusive as gel did not appear properly, possibly due to malfunction with voltage machine or other factors

gelpic.jpg



It was later discovered that the gel was made with too high of a concentration of TAE when diluting from a 50X stock. This caused a high amount of resistance in the gel as it ran, which basically "cooked"/degraded the DNA in the gel, making it impossible to see.

WEDNESDAY, 7/26

- Trip to BU to Test Lead Assay (and Protocol) in another facility, and to talk about microfluidics chip.

WPI iGEM LB standard at BU.xls

At BU we had the opportunity to perform our DIY Lead Assay protocol to recreate our standard curve for LB using different equipment. Unfortunately, though the first trial went well and mirrored our LB standard curve (see Thursday, 7/13). When considering possible reasons for error, we considered the possibility that the lead in our samples was "settling" at the bottom of the tray with time, so the samples we used for subsequent trials of the assay may not have been reflective of the starting sample. This will be tested tomorrow, Thursday, 7/27.

- Count B.subtilis colonies on lead plates

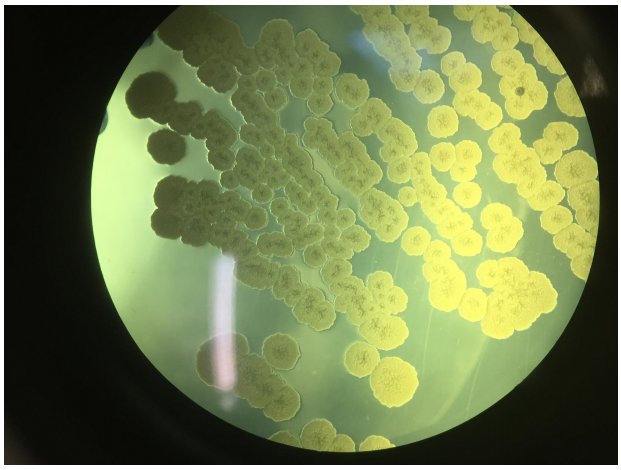
Table5

	A	B	C
1	Amount of lead	Plate 1	Plate 2
2	Control (0 ppm)	131	172
3	100ppm	242	110
4	200ppm	142	183
5	800ppm	89	59
6	900ppm	54	70
7	1,000 ppm	69	60
8	1,500 ppm	159	140
9	2,000 ppm	94	115

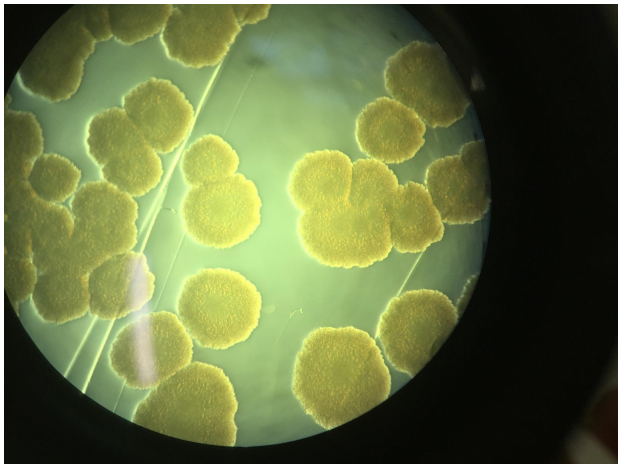
    - See excel file below for graphs and average analysis

BsubtilisHighLead2000ppmColonyCount.xlsx

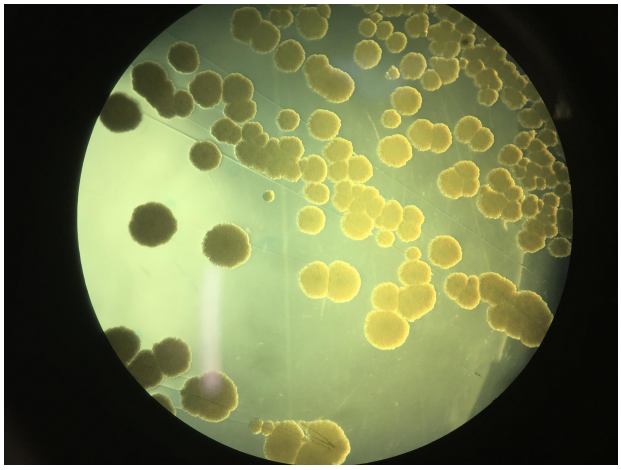
B.sub control 0ppm



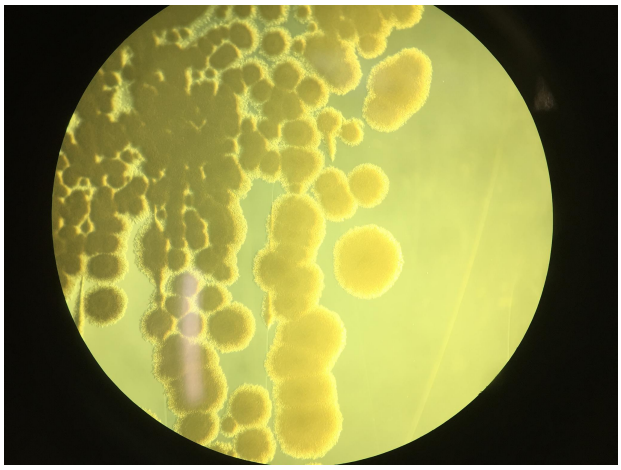
100ppm



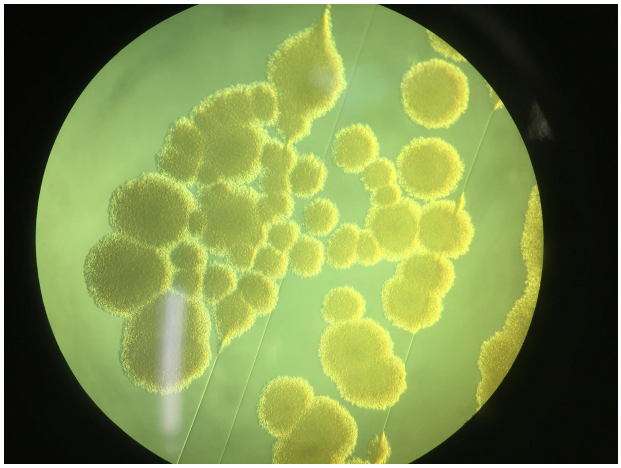
200 ppm



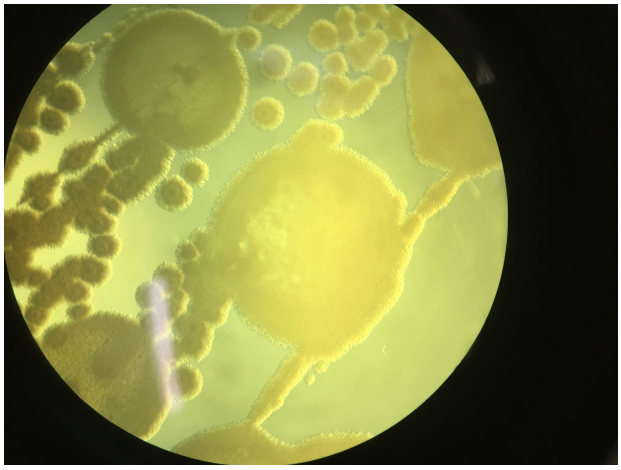
800 ppm



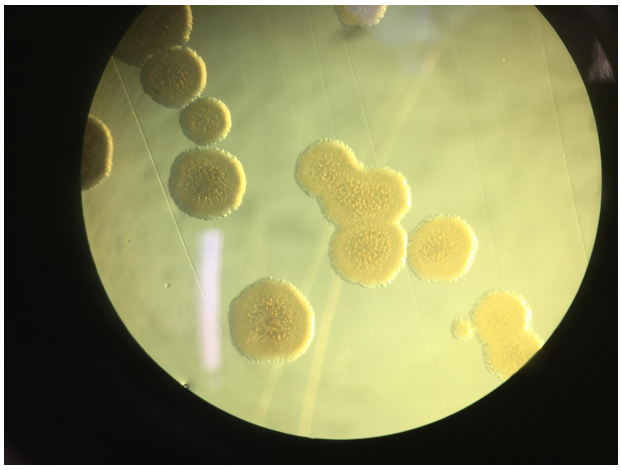
900 ppm



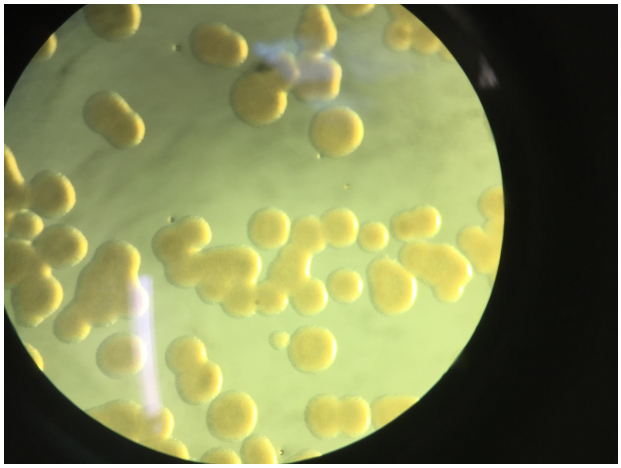
1000 ppm



1500 ppm

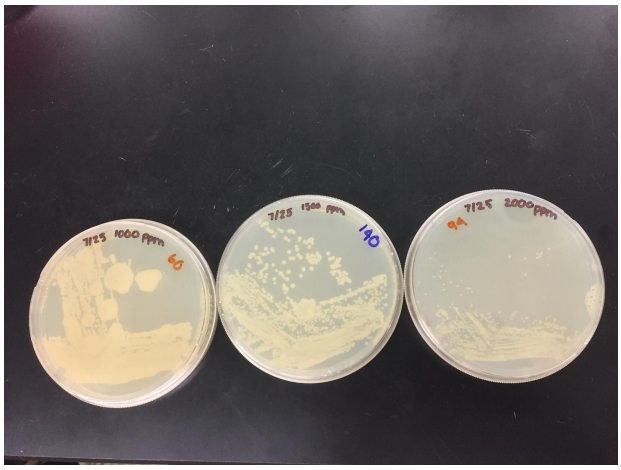


2000 ppm



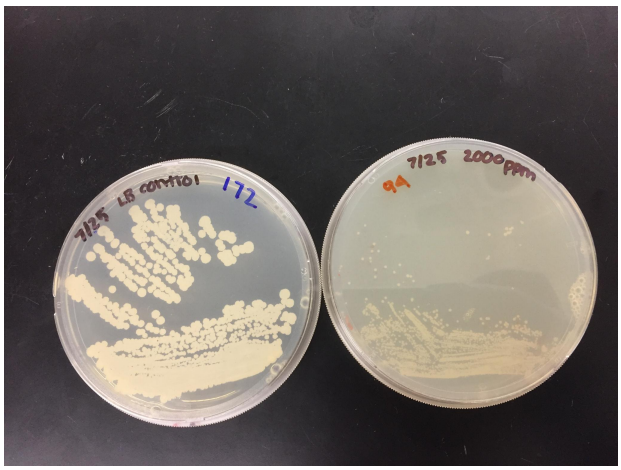
Seen in the series of photos above, as lead concentrations increase from 0 ppm (control) through 1500 ppm, B.subtilis colonies begin to form a ring around each colony. The colonies also begin to appear more fuzzy as lead concentrations increase. We hypothesize that this ring and fuzzy appearance is the result of lead that has bound to the outside of each B.subtilis cell. However, the colonies in the 2,000 ppm photo look different from every other plate. We hypothesize that this could be because they are not B.subtilis or because they evolved to live in the high lead concentration.

B. sub 1000 vs 1500 vs 2000 ppm



As the lead concentration increases from 1000 ppm to 1500 ppm to 2000 ppm the colonies decrease in size.

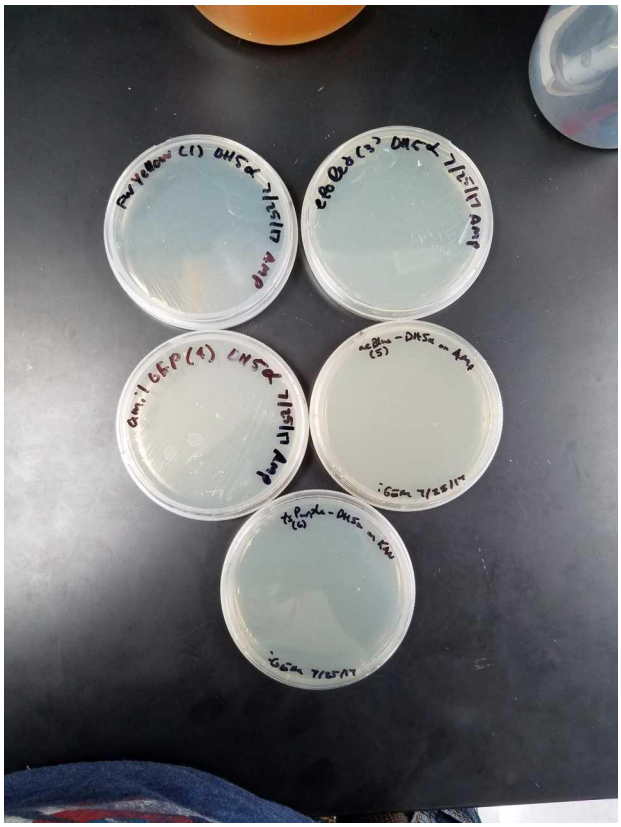
B.sub 0 vs 2000 ppm



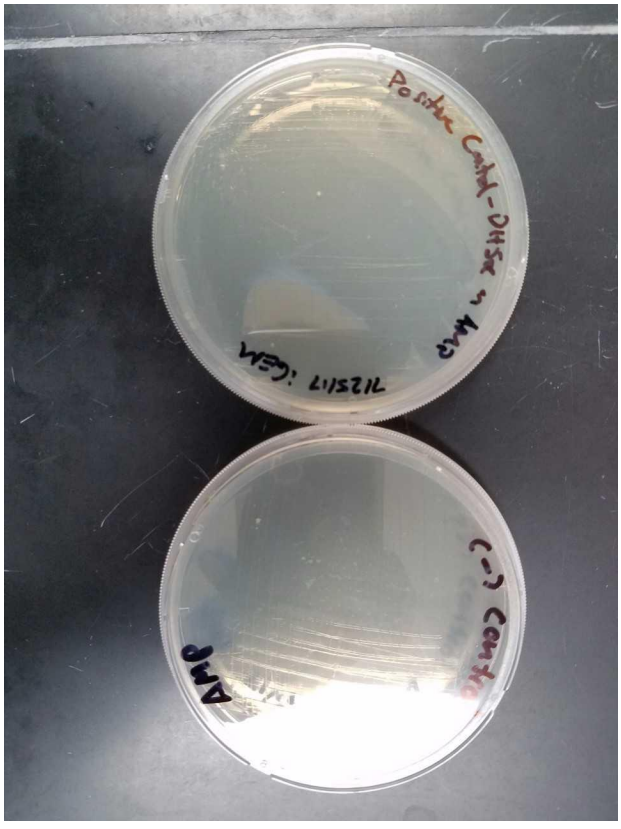
As lead concentration increases form 0ppm to 2000ppm the size of the colonies decreases and the color of the colonies seem to darken.

- Do the IDEA experiment where the plate is split in half and take one colony from a freshly grown on lead LB and then a control colony and plate on a concentration of lead
    - 100ppm, 800ppm, and 2,000ppm
- Move forward with Starlogo Nova Modeling
    - Biosensor: Have pbrR repressor, with lead molecules colliding (binding) and have it produce circles of certain color based on how many lead molecules collided showing the chromoproteins while also changing the terrain color to the mix of the colors of the circles.
- Colonies from Gibson assembly were counted. Positive control had 149 colonies. Only one plate had growth, AmilCp, with 10 colonies counted.

platesA.jpg



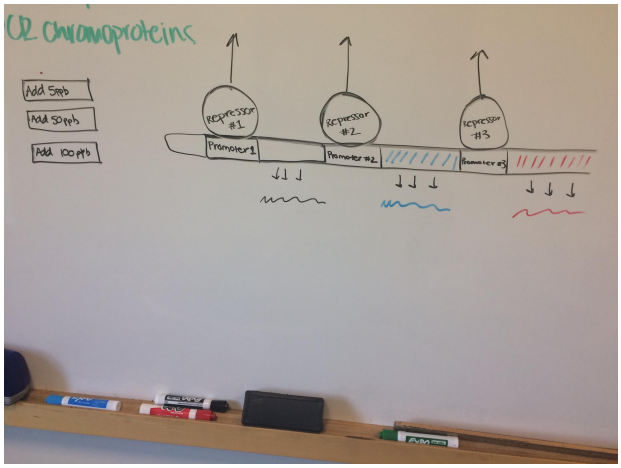
platesB.jpg



platesC.jpg

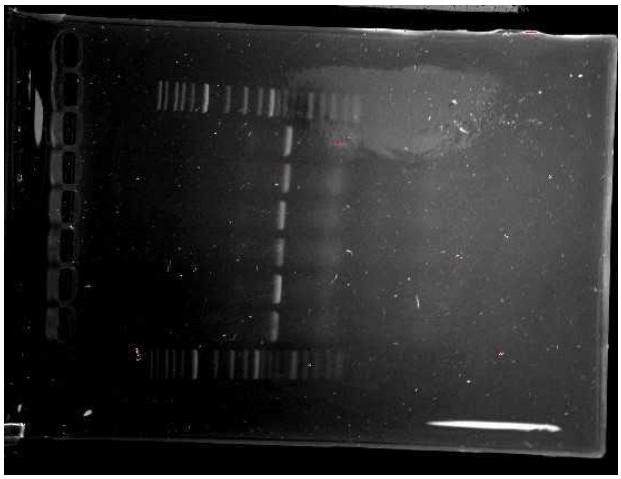
- StarLogo model brainstorm

model brainstorm



- Made overnight cultures of RFP for mini-prep tomorrow
- Ran gels to check size of PCR products

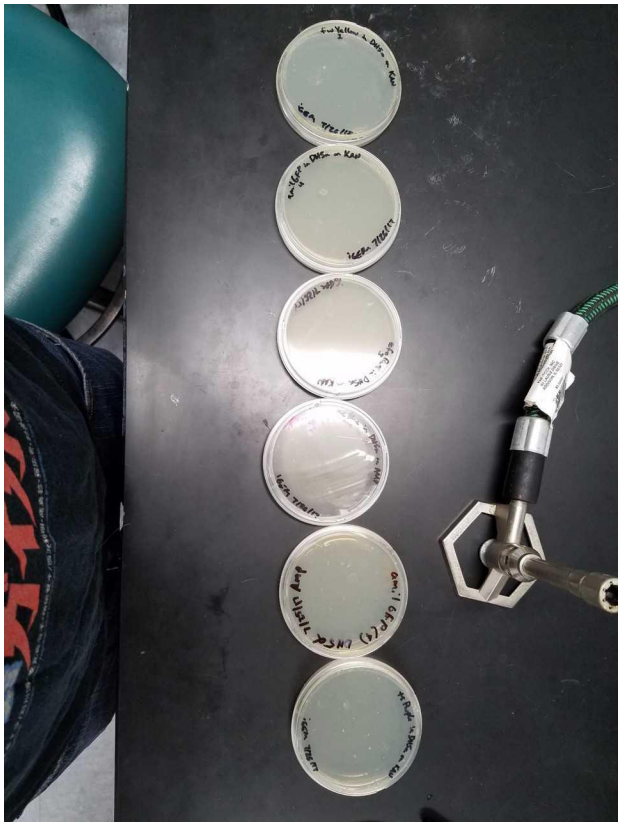
good PCR.jpg



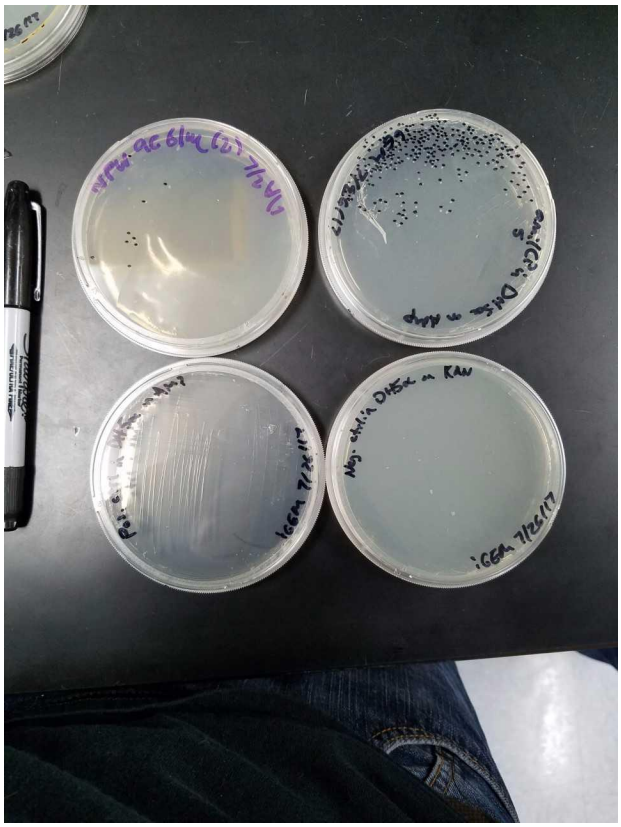
Sizes were appropriate at ~700 bp for all products. All bands were bright.

- Performed Gibson assembly on PCR'd chromoprotein genes
    - Problems arose when plating the plate for AmilCP as the wrong transformed material was plated

themplates.jpg



thoseplates.jpg



AeBlue plate has 10 colonies, AmilCp had 243 Colonies, the positive control had 23 colonies, and the negative control had 0.

THURSDAY, 7/27

- LB Lead Assay
    - Test to see whether vortexing the samples prior to each trial reduces error (with the assumption that otherwise the lead in the sample would "settle" to the bottom, and therefore the sample used for the assay may not be reflective of the original sample)

LB Standard Curve Check with Vortex.xlsx

    - Run a new standard curve experiment using the modified GSH solution and GSH, AuNPs, and sample volumes (see Wednesday, 7/19 and Thursday, 7/20)

LB Standard Curve New.xlsx

        - Could the length of time after the sample was made be affecting the assay?

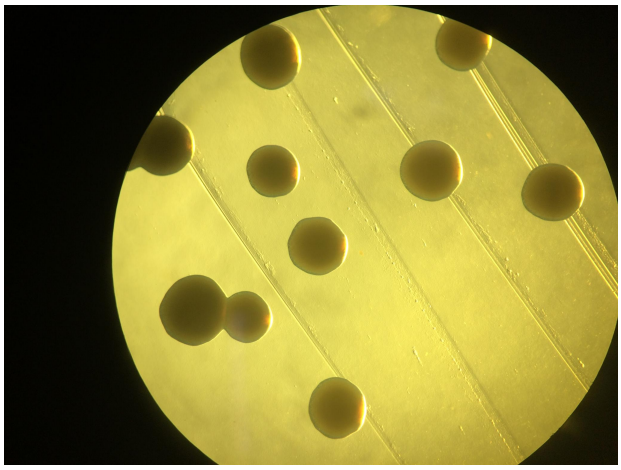
LB fresh sample.xlsx

- Count lactobacillus colonies on lead plates
    - See excel file for numbers and graphs

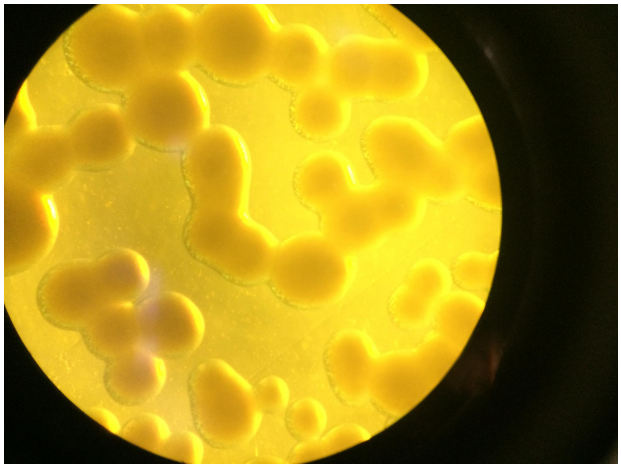
LactobacillusHighLeadPlatesColonyCount.xlsx

    - Photos of plates (below)

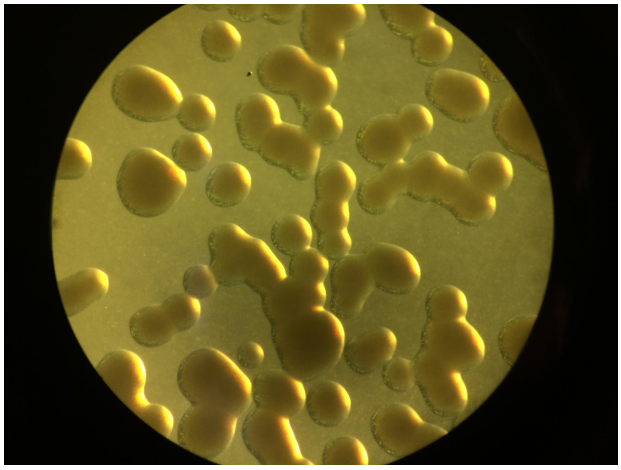
50ppm lactobacillus



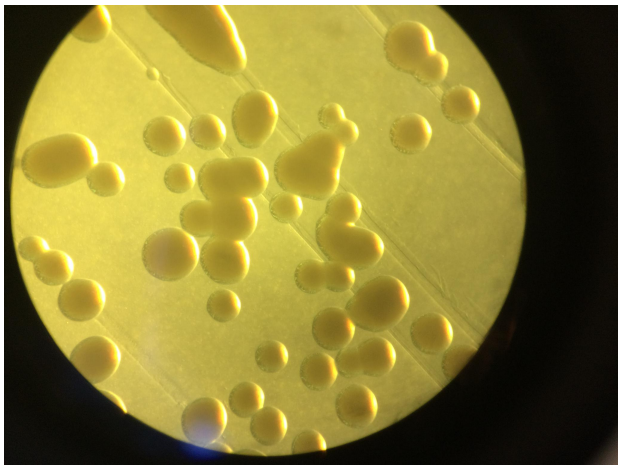
100 ppm



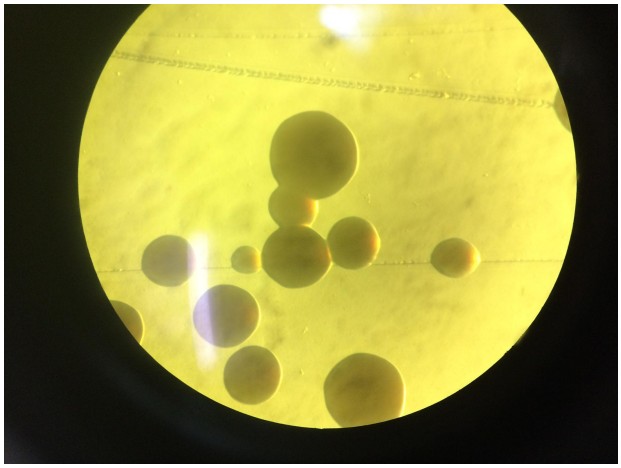
600 ppm



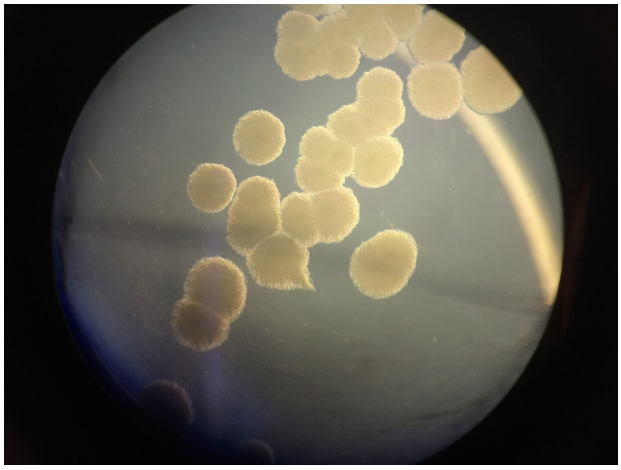
700 ppm



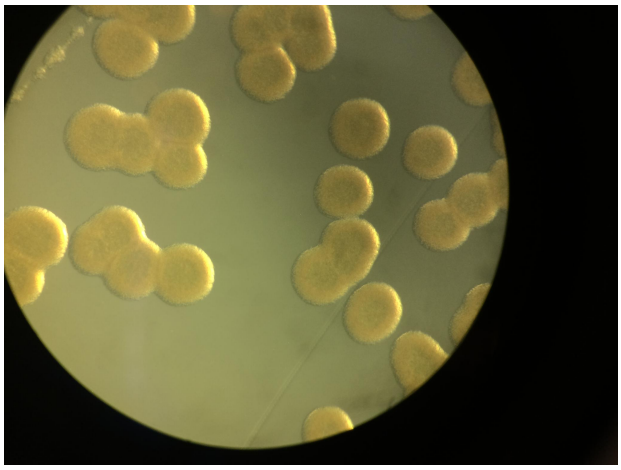
800 ppm



900 ppm



1000 ppm



The series of photos above are of lactobacillus on different concentrations of lead media ranging from 50ppm to 1000ppm. The zoom and focus were primarily chosen to try and emphasize the ring around each colony. As seen in the photos above, the ring around each colony evolves differently than as seen in the photos of B.subtilis. The photos of lactobacillus show that the ring does not seem to get larger as the lead concentration of each plate increases.

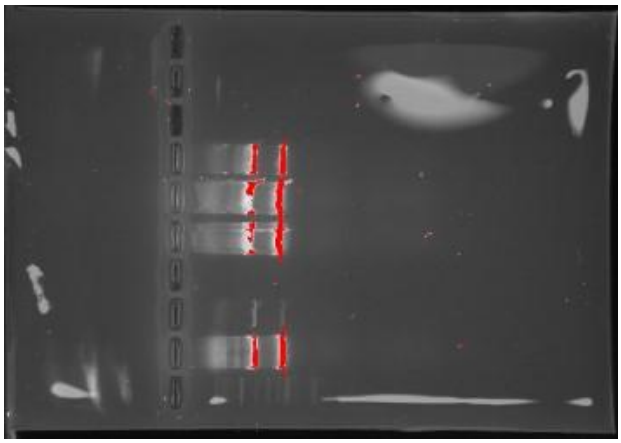
- Count b.subtilis from IDEA experiment

B.subtilis IDEA Colony Counts

	A	B	C
1	Amount of Lead	Plate 1	Plate 1
2	100ppm		
3	Control	42	32
4	Lead	22	29
5	800ppm		
6	Control	54	85
7	Lead	49	52
8	2,000ppm		
9	Control	29	38
10	Lead	63	41

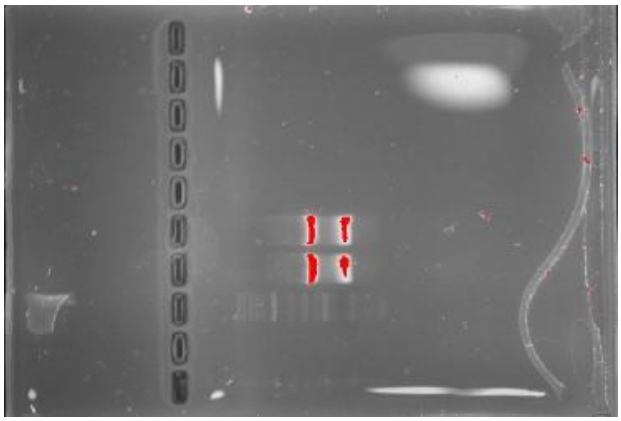
- Mini-prep RFP BBa 04450 - for restriction digest with ppBr promoter
    - 2 mini-preps
        - Yields
            - 86.00 ng/ul
            - 28.00 ng/ul
- Restriction digest
    - 50uL of miniprep
    - 6uL of cutsmart buffer
    - 2 ul XbaI
    - 2ul of SpeI

thumbnail_Capture.jpg



- Gel purify the restriction digest plasmid pieces (larger band, on top) RFP is the smaller lower band
- PCR
    - PREP
        - pBBpBRr-GFP-3 (template)
            - spin max speed for 5 mins
            - 50 uL of AE buffer
            - Vortex a lot, then sit 5 mins
            - Quick spin
        - Primers
            - spin max speed 5 mins
            - Make 100uM stock
                - 5' add 269 uL
                - 3' add 221 uL
            - Dilute to 10uM stock
                - 1 uL of 100uM stock and 9 uL of water
- PCR reaction
    - 1uL of template
    - 1.25 uL of each 10uM primer dilutions (5' and 3')
    - 22.5 uL of water
    - 25 uL of Taq master mix (green)
- PCR Protocol Used
    - 95 for 2 min x1
    - (95 for 30second, 50 for 45s, and 72 for 1.5 mins) x32
    - 72 for 10 mins x1
    - Hold at 10 for infinity
- Run 5uL of the PCR on a gel to check size

thumbnail_PCR.jpg



    - Larger top band is our desired ppbR gene, bottom band seems to be the primers annealing to a random middle portion of the gene
    - Will run the rest of the PCR reaction on another gel tomorrow and gel purify the top band for the gibson assembly
- Performed PCR cleanup for chromoproteins
    - Didn't cleanup amilCP and aeBlue PCRs because we have colonies on those plates - will run experiments to see whether they contain the vector + insert

PCR Cleanup

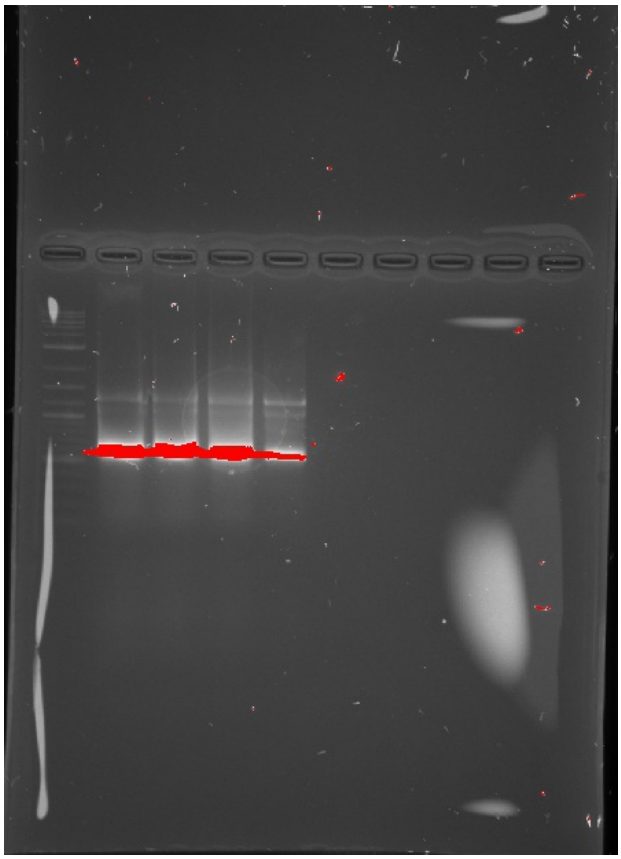
	Tube #	Gene Name	Concentration (ng/uL)
1	1	fwYellow	65.4
2	3	efoRed	33.2
3	4	amilGFP	34.0
4	6	tsPurple	82.9

- Start overnight liquid cultures of (3) colonies from each plate of amilCP and aeBlue (6 cultures total) (note; before miniprep on Friday save 100 uL of culture for IPTG experiment)
    - Tomorrow: Save 100 ul of miniprep and add IPTG to see if cells express chromoprotein -> cultures should turn blue
        - Setup: 100 uL minprep culture, plus 4 mL LB, 40 uL IPTG, grow all day.
    - Tomorrow: miniprep and digest each of the 6 cultures; run on gel to see if both vector and insert (2 bands) are present in any of the cells.
- Start liquid culture for pet42a for miniprep.
    - Tomorrow: Restriction digest 10 uL reaction: 5 uL miniprep, 1 uL cutsmart, 1 uL xba1, 1 uL Hindi3, and 2 uL DI water. Perform for pet21a as control. Control should be 1 band the size of the vector. Chromoprotein should have 2 bands, 1 the size of the vector the other the size of the insert.

FRIDAY, 7/28

- Do inventory on lead plates and make more of the lower concentrations that are needed (50,100,etc.) and make higher concentrations in both LB and MRS
- Run gel with rest of PCR reaction and gel purify for gibson assembly
    - gel purify the top band for gibson assembly

PCR take 2.PNG



- Do Gibson Assembly of RFP plasmid and pbrR
    - 10 uL reactions
    - Fragment Assembly
        - MasterMix- 5 uL
        - RFP vector- 3 uL
        - pBR PCR- 2uL

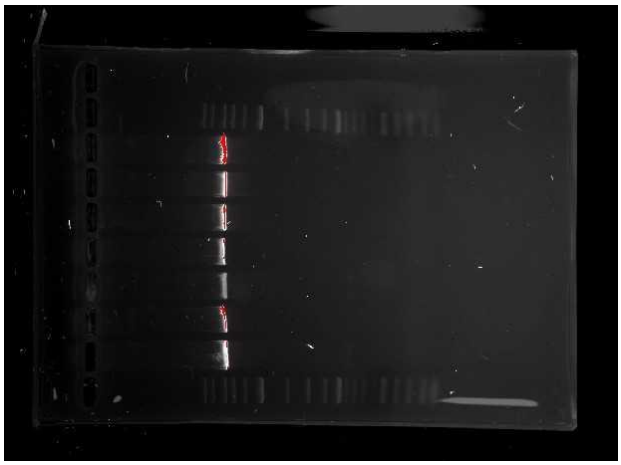
    - Positive Control
        - MasterMix- 5uL
        - Postive Control Tube- 5uL
    - Negative Control
        - MasterMix- 5uL
        - RFP vector- 3uL
        - Water- 2uL
- Haylea & Edith- Meeting with Professor Shell in Gateway to talk about colony morphology and how to analyze colony counting data and steps moving forward with force evolving the probiotic
    - Helped get ideas about how to move forward and what issues we could be dealing with
        - Re-do Growth Curves with lead, go back to smaller concentrations from literature
            - Starting at a lower OD when diluting back so that there is less bacterial growth in lag phase
            - Change starting OD of liquid growth curves to 0.01
        - Re-do Lead Plates, smaller concentrations
            - Make 10 fold serial dilutions(take ODs) and plate them by spreading across the whole plate and counting and moving forward with a specific dilution and then plating the same dilution (ensuring the same OD) to lead plates and count colonies to compare to the set amount of colonies that should be there
- Miniprep all aeBlue, amilCP, 21a, and 42a cultures setup yesterday (8 total)

Nanodrop results of miniprep cultures for clones

	Clone	DNA Concentration (ng/uL)
1	aeBlue 1	50.6
2	aeBlue 2	43.3
3	aeBlue 3	56.0
4	amilCP 1	66.7
5	amilCP 2	54.7
6	amilCP 3	57.0
7	pET21a	64.6
8	pET42a	58.1

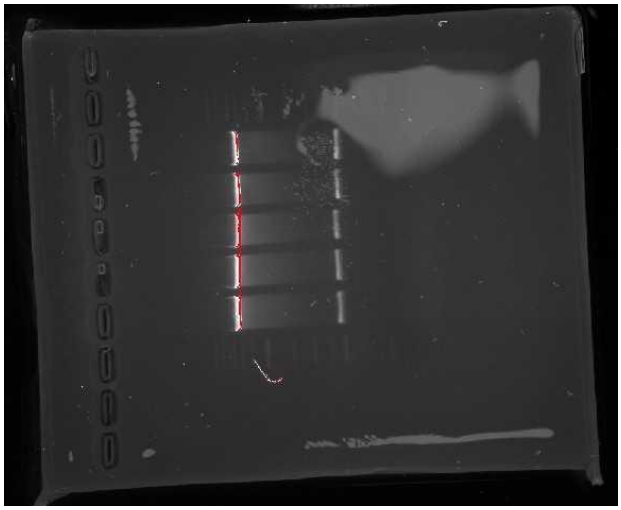
- Restriction digest 10 uL reaction: 5 uL miniprep, 1 uL cutsmart, 1 uL xba1, 1 uL Hindi3, and 2 uL DI water. Perform for pet21a as control. Control should be 1 band the size of the vector. Chromoprotein should have 2 bands, 1 the size of the vector the other the size of the insert.
    - First lane is ladder, followed by 3 lanes of aeBlue samples (1-3), followed by 3 lanes of amilCP samples (1-3), with a ladder following that lane. They appear all of similar size. Since all bands are equal in size and appearance to the negative control, and the fact that there is only one band rather than two for the chromoprotein clones, we conclude that the colonies tested contain an empty vector with no chromoprotein insert. The gibson assembly will need to be reevaluated and performed again.

gelcomparison.jpg



- IPTG experiment: 100 uL minprep culture, plus 4 mL LB, 40 uL IPTG, grow all day.
    - The tubes exhibited no color change (Blue production). This coincides with our results from the restriction digest (no chromoprotein gene).
- Gel purified Pet42a

pet42adigest2.jpg



- Nanodrop concentration was 14.3 ng/uL
- Lead Assay
    - Due to the large variability in the data for the LB assay between trials, all of the assays involving LB were reviewed prior to proceeding with another assay

Notes:

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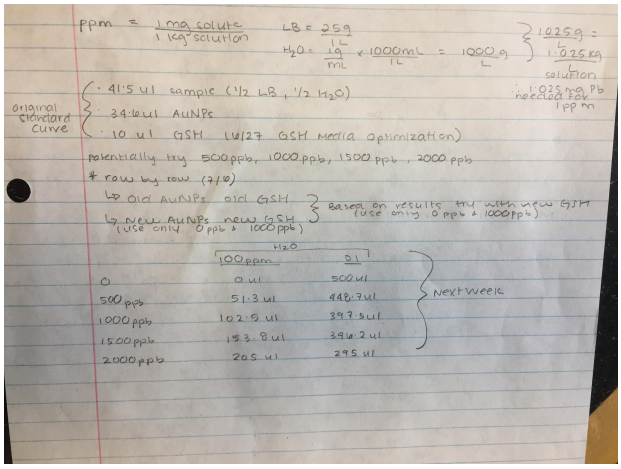

- After reviewing the data from the LB Standard Curve (Thursday, 7/13), when the outliers are excluded from the sample averages, the error bars overlap immensely, therefore a new standard should be made

LB Standard Curve Analysis (2).xlsx

- Before starting the process of performing another standard curve for LB, an assay was run to test compare the AuNPs we've been using (have had large period of time without being refrigerated) to fresh AuNPs (have always been refrigerated), and to compare the original GSH solution to the most recent GSH solution

DIY Lead Assay 72817.xlsx

- See "Questions for Pediatricians" document for Human Practices updates
- Check Gibson Assembly plate for growth and count colonies
  - No colonies on negative control and gibson assembly plate but there was growth on the positive control (60 colonies)
  - Left in incubator for another day to see if anything will grow
  - No growth

IMG_3539.JPG