June 10:

- Liz prepared LB liquid media and autoclaved.
    - Protocol: Dissolve 15 g of LB per 1 L of distilled water in a 2 L Erlenmeyer flask. Check that the pH is between 7 and 7.4. Swirl the flasks, then place aluminum foil of the lip of the Erlenmeyer flask. Place autoclave tape on the foil, then place the flasks in the autoclave machine. Select the 20-minute liquid cycle.

June 12:

- Prepared 50 mg/mL Chloramphenicol stock. Stored in -20 degree freezer
- Prepared LB media with 25 mg/L of Chloramphenicol. Stored in 4 degree.

June 14:

- Started on Biobrick fabrication:
    - Grew up 2 colonies from each plate that Dr. Kozminski gave us: XLI-Blue and JM109. Incubated in 37ºC shaker over weekend.

June 17:

- Used DNA mini kit on all cultures to extract bacterial genome

June 18:

- PCR of FtsZ from the extracted genomes.
    - Primers contain annealing sequence and backbone prefix and suffix
- PCR Reaction:
    - For eukaryotic genome, need 200 ng/50 µL reaction.
        - 200 ng • (µL/ X ng) = ____ µL (X = [genome])
    - DNA samples: 1 use 5.46 µL; 2 use 4.18 µL; 3 us 4.71 µL, 4 use 5.63 µL
    - Primers: need 0.4 µM final concentration
        - $M_1V_1 = M_fV_f$
        - (x mol/L) • yL = 0.4 • 10^{-6} mol • 50 • 10^{-6} L
    - We need to put in ~1 µL primer, so how much H20 should I use to solubilize the lyophilized primer? How concentrated should we make the primers?
        - To add 1 µL,
            - X mol/L • 10^{-6} L = 0.4 x 10^{-6} mol • 50 • 10^{-6} L
        - Rougly, (X mol/ L) = 0.02 • 10^{-3} mol/L stock [primer]
    - Based on the calculation that 50 µL H20 would give a concentration of 0.2 mM, we decided to dissolve in 500 µL to get a concentration roughly 10x more diluted.
    - Forward primer: 200.26 µg/500 µL = 400.5 ng/µL; 14.1 • 10^{-9} mol/500 • 10^{-6} L = .0282 • 10^{-3} M
    - Reverse primer: 253.78 µg/500 µL – 507.6 ng/µL; 17.7 • 10^{-9} mol/500 •10^{-6} L = 0.354 • 10^{-3} M
    - Now, volume needed: µL = (0.4 • 10^{-6} mol/L • 50 • 10^{-6} L) / (X mol/L)
        - Forward: 0.71 µL
        - Reverse: 0.56 µL
    - Reaction volumes: 25 µL Master Accuzyme mix, 0.71 µL Forward Primer, 0.56 µL Reverse Primer

	Template Genome	H20

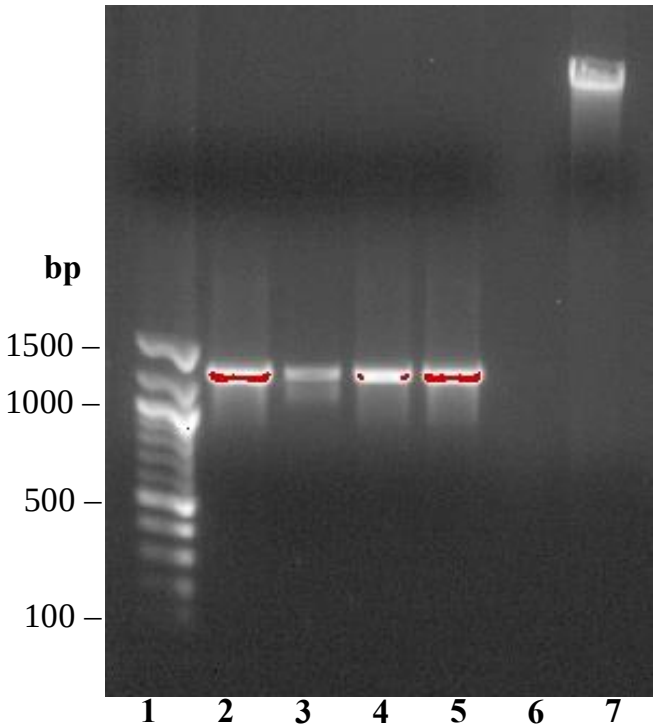
1	5.46 µL	18.27 µL
2	4.18 µL	19.55 µL
3	4.71 µL	19.02 µL
4	5.63 µL	18.1 µL

- = 50 µL total in 4 separate reactions

- Place in thermocycler; program to:

Step	Temp.	Time	Cycles
Initial Denaturation	95-98ºC	3 min	1
Denaturation	95-98ºC	15s	
Annealing	51ºC	15s	25-35
Extension	72ºC	1.5-2min/kb (FtsZ length = 1.71 kb, 1.75-2.34 mins)	

- Run Gel Electrophoresis to confirm PCR amplification
  - Do a PCR clean-up and nanodrop before running on gel.
  - Prepare 1% Agarose gel:
    - Mix 0.5 g Agarose and 50 mL 1x TAE Buffer in flask. Microwave for 90 seconds. Once agarose is cool enough to touch, pour into EtBr flask and add 1 µL EtBr
      - DO NOT add EtBr into any other glassware except the glassware designated EtBr.
    - Swirl to mix EtBr into Agarose and pour onto taped plated. Use pipette tip to get rid of all bubbles. Place comb in gel. Allow to harden for 30 min – 1 hr. Put gel into electrophoresis box, fill to 1 cm above gel line with 1x TAE buffer. Prep samples to load by adding loading dye enough to dilute the 6x dye down to 1x. Load 12 µL samples into gel, proceeded by a ladder and including controls.
    - Ladder: 100 bp DNA Ladder N3231S; 8 µL H20, 2 µL 6x dye, 2 µL DNA ladder



Lane 1 contains 100 bp DNA ladder. Lanes 2-5 contain PCR amplification products of FtsZ gene. Lane 6 is empty and lane 7 contains genomic DNA for comparison.

June 19:

- Restriction Digest of Amplicons/Vector and Transformation of other backbones.
- Restriction Digest of FtsZ gene

- Stock concentration of linearized backbone: 25 ng/µL. For ligation, we need $0.025 \cdot 10^{-12}$ mol vector • 5 (4 ligation reactions +1 for good measure) = $0.125 \cdot 10^{-12}$ mol needed in total
- $0.125 \cdot 10^{-12}$ mol • (499.45 g/1 mol nucleotides) • 2070 nucleotides = $0.126 \cdot 10^{-6}$ g.
- We need 126 ng vector DNA, so we'll use 200 ng. So, 200 ng • (25 ng/µL) = 8 µL vector DNA for digest
- Because a normal restriction digest uses 1 µg DNA, we can quarter the total volume of the digest from 50 µL to 12.5 µL
- Digest for vector volumes: 8 µL vector, 0.25 µL EcoRI-HF, 0.25 µL PstI-HF, 1.25 µL NEB Cutsmart Buffer, 0.2 µL BSA, 2.55 µL H20 = 12.5 µL total
- Digest for insert volumes: 1 µL FtsZ fragment, 0.25 µL EcoRI-HF, 0.25 µL PstI-HF, 1.25 µL NEB Cutsmart Buffer, 0.2 µL BSA, 9.55 µL H20 = 12.5 µL total
- The intended backbone after ligation is FtsZ on a chloramphenicol resistant backbone (psB1C3)
- Final concentrations of DNA in digests
  - Vector: 16 ng/mL
  - 1: 7.12 ng/mL; 2: 2.48 ng/mL; 3: 5.90 ng/mL; 4: 5.98 ng/mL

- In calculation volumes of vector and insert to add to ligation reaction, we used the following equations:

Volumes of vector and insert to add to ligation reaction:

Vector: $$0.025 * 10^{-12} mol * \left(\frac{1*10^{-6} L}{X*10^{-9} g}\right) * \left(\frac{499.45\ g}{1\ mol}\right) * length\ of\ vector = \_\_\_\_\_ L\ vector$$

Insert: $$0.075 * 10^{-12} mol * \left(\frac{1*10^{-6} L}{X*10^{-9} g}\right) * \left(\frac{499.45\ g}{1\ mol}\right) * length\ of\ insert = \_\_\_\_\_ L\ insert$$

  - Length of Vector = 2070 bp; Insert = 1200 bp

June 20:

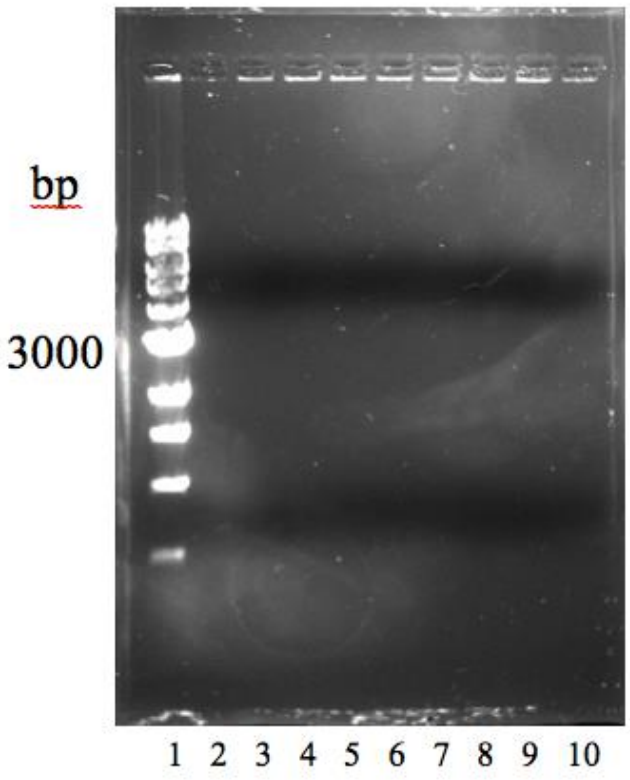
- Transformations of lyophilized plasmid backbones into competent DH5α cells failed.
- 10 µL DI H2O was pipetted into well, pipette up and down and let sit for 5 minutes while prepping cells. All 10 µL were transferred into competent cells, while only 2 µL should have been transferred to the 50 µL of competent cells. Place on ice for 20 minutes, 42ºC waterbath for 30 seconds, ice for 2 minutes, place in 1 mL LB broth, 37ºC shaker for 1 hour. Plate on chloramphenicol plates, incubate.
- Ligation of digests
  - Reaction mixture: 2 µL DNA Ligase Buffer, 1 µL T4 Ligase, 1.6 µL vector, appropriate amount of insert based on equation, H2O to total 20 µL volume
  - Incubated reaction for 30 minutes at room temperature. Transformed 5 µL of each ligation reaction: 4 ligation reactions, GFP-Amp resistance, 5 transformation efficiency-Amp, 1 control

June 21:

- 5 transformation efficiency plates – no data; cannot compare cell count due to lawn of growth.
- 4 biobrick plates – GFP, IPTG-inducible promoter, RBS, Terminator
    - Grown on ampicillin plates
    - Colonies present; there were 3 colonies to inoculate from each plate
- 4 ligation plates – FtsZ and backbone
    - There was no growth on any of the plates, but the plates were not labeled so we did not know if they were grown on the correct plates.
    - We also were not sure which backbone was used, so we must redo the ligation.
- Restriction enzymes were mistakenly left in the 4ºC fridge for a day.
- Quadrupled the volumes from restriction digest on 6/19
    - Digest for vector volumes: 16 µL vector, 1 µL EcoRI, 1 µL PstI, 5 µL NEB Cutsmart Buffer, 0.5 µL BSA, 10.5 µL H20
    - Digest for insert volumes: 10 µL fragment DNA, 1 µL EcoRI, 1 µL PstI, 0.5 µL BSA, 5 µL NEB Cutsmart Buffer
    - Thermocycler digest program: incubate for 30 min at 37ºC, inactivated enzymes 20 min at 80ºC

June 24:

- On Friday the 21st, we redid the ligations because we could not be sure as to which backbone was used the first time. We must redo the ligations because an incorrect volume of DNA was used. The same volume as the initial ligations was used, even though we had a different initial concentration.
- Ligation reactions were carried out with the psB1C3 backbone and FtsZ
    - Briefly microfuged the reaction mixture and let sit for 30 minutes. Transferred from Eppendorf to 0.2 mL MicroAmp tubes and transferred them to thermocycler (80º for 20 minutes)
    - Obtained new concentrations of FtsZ DNA and calculated how much was needed for 50 ng of circular DNA for transformations. Transferred 14.37 µL of ligated DNA into Ependorf tubes with 50 µL of competent cells labeled 1, 2, 3, 4 and control. Incubated on ice for 20 minutes, heat shocked at 42ºC for 30 seconds, placed on ice for 2 minutes, added 1 mL LB broth to each tube, incubated for 2 hours at 37ºC and streaked LB plated with cultures.
- Miniprepped cultures with plasmids for GFP, Promoter, RBS, Terminator. Nanodropped plasmid samples: H2O to clear, Buffer EB as blank
- Did a restriction digest of each plasmid and ran gel electrophoresis



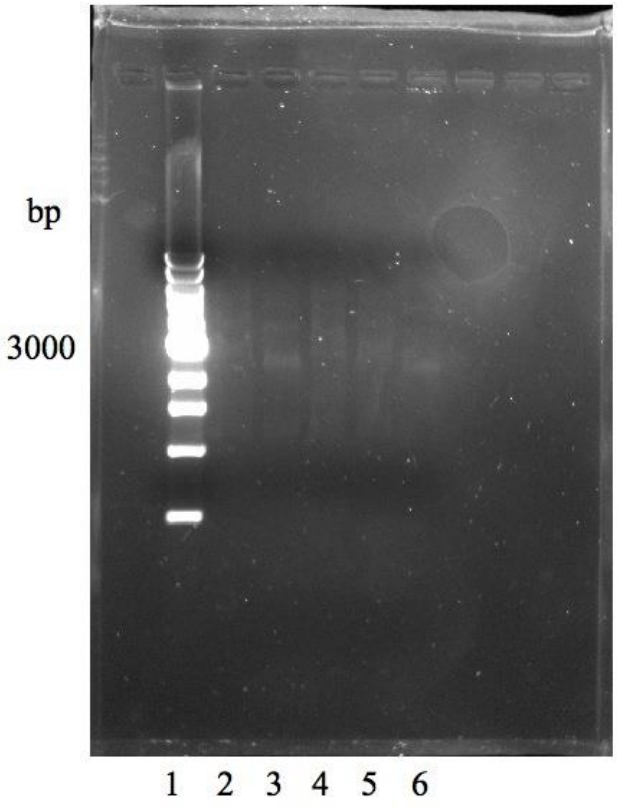
Lane 1 contains a 1 kb ladder, lanes 2-4 contain digests of the RBS, lanes 5-7 contain promoter digests and lanes 8-10 contain GFP digests. Another gel was run for the terminator and no images were obtained for this digest either.

June 25:

- No images from gels yesterday. Shaun, Elyse and Matt re-plated the transformation products on ampicillin and chloramphenicol plates because they were previously plated with loop instead of using all 50 µL.
- Redid restriction digest for the single culture with the highest concentrations from the nanodrop. The gel image showed no bands in any of the lanes. We plan on re-digesting and redoing the gel against a non-digested plasmid to see if we see anything.
- The re-plated transformants had no colones. There may not have been enough cells plated.
- We determined that if the gel does not show the bands we are expecting, we should replate the transformants of GFP, promoter, RBS and terminator. On 6/26, we will then grow up another culture and the next day we will miniprep. We should also check the fidelity of the enzymes in the MR5 lab. If the gel does not return the expected results, the restriction enzymes still work. We will confirm he fragment lengths with the length given by the biobrick's existing page on the registry. If they work, we will add LB media to the existing cultures to grow them up more. We'll then make a glycerol stock of each culture and miniprep the rest while waiting for the digest/ligations.
- Chris Langguth and Liz ran the gel as discussed above, but no bands were visualized besides the ladder. As previously mentioned, we plan to re-plate the transformants of GFP, RBS, promoter and terminator on ampicillin plates. On 6/26, we will check the plates for growth. If we have colonies, we will collect 3 per plate and grow them up in culture. We will then mini-prep again.

June 26:

- The LB plates with ampicillin showed growth for the GFP, RBS, promoter and terminator. Our transformants worked! However, we did not have a control plate. This was concerning due to the observed growth on the control plate for the FtsZ and psB1C3 backbone on LB and amp lates.
- LB plates with ampicillin and FtsZ and psB1C3 backbone showed growth on all plates, including the control. The backbone should have conferred resistance to chloramphenicol, so there should not be any growth on these plates. This may be because FtsZ was incubated with the wrong backbone during its ligation, that the backbone was mislabeled by iGEM (unlikely), or there was an issue in labeling the plates. However, the control also showed growth.
- Taking this all into account, the plan was as follows: with GFP, RBS, promoter and terminator – pick colonies from each plate to grow up in 5 mL of LB + Amp and culture overnight. Mini-prep these samples on 6/27.
- With FtsZ – we have to throw out all of the plates and start over. We will perform a digest of FtsZ and psB1C3 backbone, then ligate to finally transform competent cells to plate for growth overnight.
- Shaun performed digest of FtsZ with the psB1C3 backbone in MR5 with Kelly lab's restriction enzymes. There was a concern that our enzymes may not be working properly since we have not had a successful confirmation product with them yet and they were also mistakenly left in the 4ºC fridge rather than the -20ºC fridge previously. Shaun will confirm that our enzymes work by digesting his own backbone with Kelly lab's enzymes as well as our own. We will run products in parallel on gel and if we obtain the same results, then our enzymes work (which it turns out they did!)
- Richard, Bethany, Surbhi and Chris L performed a ligation of FtsZ and the psB1C3 backbone. The power went out in the middle of our ligation, however and knocked out the 4ºC fridge and incubators.



lanes 2-5 contain the FtsZ ligation products and lane 6 contains the control

- Liz made 20 mg/L chloramphenicol LB media. Kozminski recommended using a lower concentration when plating and then a higher concentration after selection occurred. We borrowed plates from Kozminski because Liz thought she may have messed up the chloramphenicol stock.

June 27:

- Josh replated the IPTG and the ligation control. Neither plate grew, suggesting that our stock concentration of ampicillin was incorrect.
- Richard, Surbhi and Chris L performed a transformation of the FtsZ and psB1C3 backbone ligation product. For insert 1, 13.9 µL of ligated solution was added to 50 µL of competent cells. For the rest of the inserts, 14.2 µL of ligation solution were added to 50 µL of competent cells. Eppendorf tubes were left on ice for 20 minutes, incubated in 42ºC water bath for 45 seconds, and incubated on ice again for 2 minutes. 1 mL of LB media was added to the cell and they were left in the shaker/incubator for 2 hours at 37ºC.
- The ampicillin concentration has been too low, so cells were not successfully selected for. This explains the growth of control cells and FtsZ and psB1C3 transformed cells. After talking to Wormington and Kozminski, we think we were adding amp while the media was too hot, thereby degrading the antibiotic. We threw away all our ampicillin stock and were given 5 amp plates from Kozminski, which were used to replate the GFP, RBS, promoter and terminator.

- Plates were streaked by Shaun and stored in the 37ºC incubator. After 14 hours, Bethany, Elyse, Chris Cai and Jesscame to pick colonies and culture in LB brother with ampicillin (5 mL LB media, 5 µL ampicillin.
- Kozminski gave us a new media protocol:
    - LB medium (3L): 30 g Tryptone, 15 g Yeast Extract, 30 g NaCl. Add to 2.9 L MilliQ water, adjust pH to 7.0 with 5 N NaOH. Sterilize by autoclaving. For LB agar, same recipe as for LB medium, but also add 60g Agar. If antibiotics are required, add to final concentration indicated as the agar is cooling (e.g., ampicillin concentration should be 100 µg/mL = 100 mg/L)

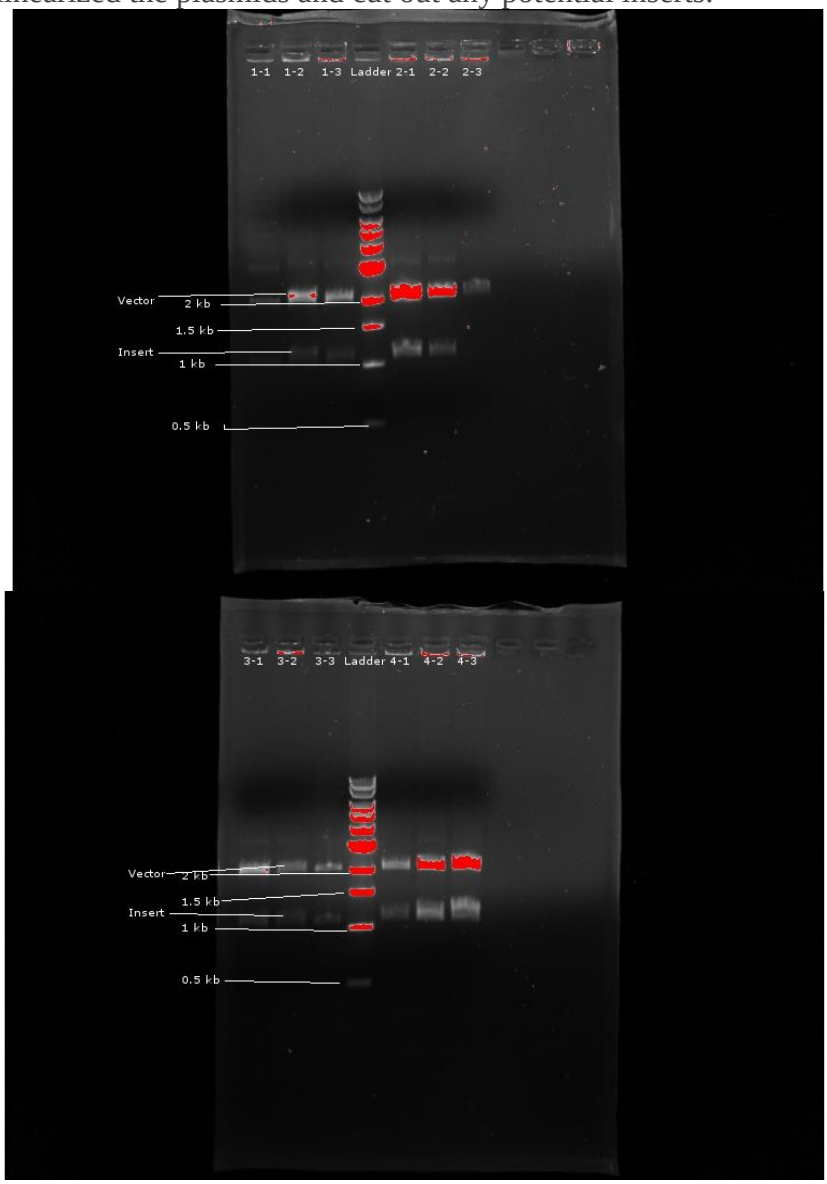
June 28:

- There was no growth on any of the plates, including the GFP, RBS, promoter, terminator and control. We analyzed potential problems:
    - For GFP, RBS, promoter and terminator: plated on Chloramphenicol at 25 µg/mL and there was no growth, but when plated on ampicillin at 100 µg/mL (made by Liz) there was growth. When grown on 100 µg/mL ampicillin plates from Kozminski, there was no growth.
    - For the FtsZ and psB1C3 backbon, nothing grew on the chloramphenicol 25 µg/mL plates, but there was growth on the 100 µg/mL ampicillin plates.
    - From this, we assumed that there was either a problem with the competent cells or with our transformation protocol.

June 29:

- FtsZ plates showed colonies and there were no colonies on the controls. There were more colonies on the original 25 µg/mL chloramphenicol plates, but we used a different streaking method with glass rods.
- Elyse and Jess inoculated colonies from the chloramphenicol plates. 3 colonies from each of four plates were selected and placed in tubes with 5 mL LB media and 2.5 µL chloramphenicol stock. The tubes were then placed in a 37ºC shaker.
- Chris L, Richard, Josh, Matt and Surbhi re-suspended DNA from previous iGEM kits:
    - BBa_R0011 (IPTG-inducible promoter from 2010 kit); BBa_B0034 (RBS from 2010 kit); BBa_B0015 (terminator from 2010 kit); BBa_E0040 (GFP from 2011 kit)
    - Added 2 µL of each plasmid to 50 µL of competent cells and did three samples each. We also included a control tube of competent cells. All plates were streaked with 200 µL of appropriate transformed cells using glass pipettes.
- Bethany, Elyse, Chris and Jess miniprepped transformants of FtsZ on the psB1C3 backbone.
    - Miniprep tips: In the first step, since there is 5 mL in each culture, pellet 4 mL total. When you remove the supernatant the second time, use a smaller pipette to get whatever is left in the tube after you have already removed 2 mL with a P1000. In step 10 of our protocol, add 25 µL EB Buffer to the center of the spin column, let stand for 5 minutes and centrifuge for one minute. For the buffers, use the P1 buffer that is in the fridge and not the one in the kit. Also, put the EB Buffer in the 37ºC incubator prior to use – this will help to increase the final yield.
    - Highest concentration obtained for FtsZ after miniprep was 64.3 ng/µL

- Josh, Richard and Matt performed a restriction digest and carried out gel electrophoresis to verify that the transformed cells contain FtsZ on the psB1C3 backbone. Digesting them linearized the plasmids and cut out any potential inserts.



- Elyse and Shaun submitted FtsZ for sequencing.
- Plated three of each of the four biobricks using a 9” glass pipet to spread the culture on the plates.