Introduction:
Our goal for this year's iGEM project was to come up with cheap and effective ways to do synthetic biology in order for it to be more accessible to everyone.
One of the issues we had to tackle was coming up with a protein purification method that did not rely on expensive equipment. We decided to look at RTX (repeats-in-toxin) as a potential extraction method. RTX is a calcium-precipitable tag that consists of repeats of the 9 amino acid consensus sequence GGAGNDTLY. This sequence can be repeated any number of times but we observed an 8-mer and a 17-mer sequence in our experiments.
We obtained the sequence from Oren Shur at Columbia University, from which we made a glycerol stock.
This sequence contains an RTX 17-mer tag (BRT_{17}) connected to a maltose binding protein (MBP) and a GFP protein. The GFP protein allows us to see a green-colored pellet when RTX is precipitated in calcium.
The idea of RTX being a simple protein purification solution comes from its ability to precipitate at certain calcium concentrations with the protein of choice, and then be resolubilized with EDTA or other chemicals that will dissociate it from calcium. This way, the protein can be precipitated, the supernatant can be removed, and only the protein of interest is left in the pellet.

Methods:

9/3/13 Tuesday

- RTX:
    - Used BRT-17 glycerol stock to make overnight in 10mL LB buffer with 10μL of 1000x Ampicillin

9/4/13 Wednesday

- Supplemented 250mL of LB media with 100 μg/mL of Ampicillin (250μL) and 0.2% glucose (0.52 grams)
- Inoculate LB media with 10mL of overnight culture grown in LB
    - Grow culture at 37°C and 225 rpm to an OD600 of approximately 0.5
        - OD600:

T=0 min	A= 0.1156
T=20 min	A= 0.1245
T=60 min	A= 0.2306
T= 105 min	A= 0.6031

        - Induce with 0.3mM IPTG
            - $M_1V_1=M_2V_2$: (1M IPTG) x (0.075mL) = (0.3mM IPTG) x (250mL LB)
            - Add 75μL 1M IPTG to 250mL solution for 0.3mM IPTG final concentration

        - Since this culture contains pMAL_BRT_{17}_GFP, it will need to be transferred to a 25°C shaker and the cells will be allowed to express for 16 hours before being harvested.
            - Placed in 25°C shaker at 12:30PM on Wednesday 9/4/13

9/5/13 Thursday

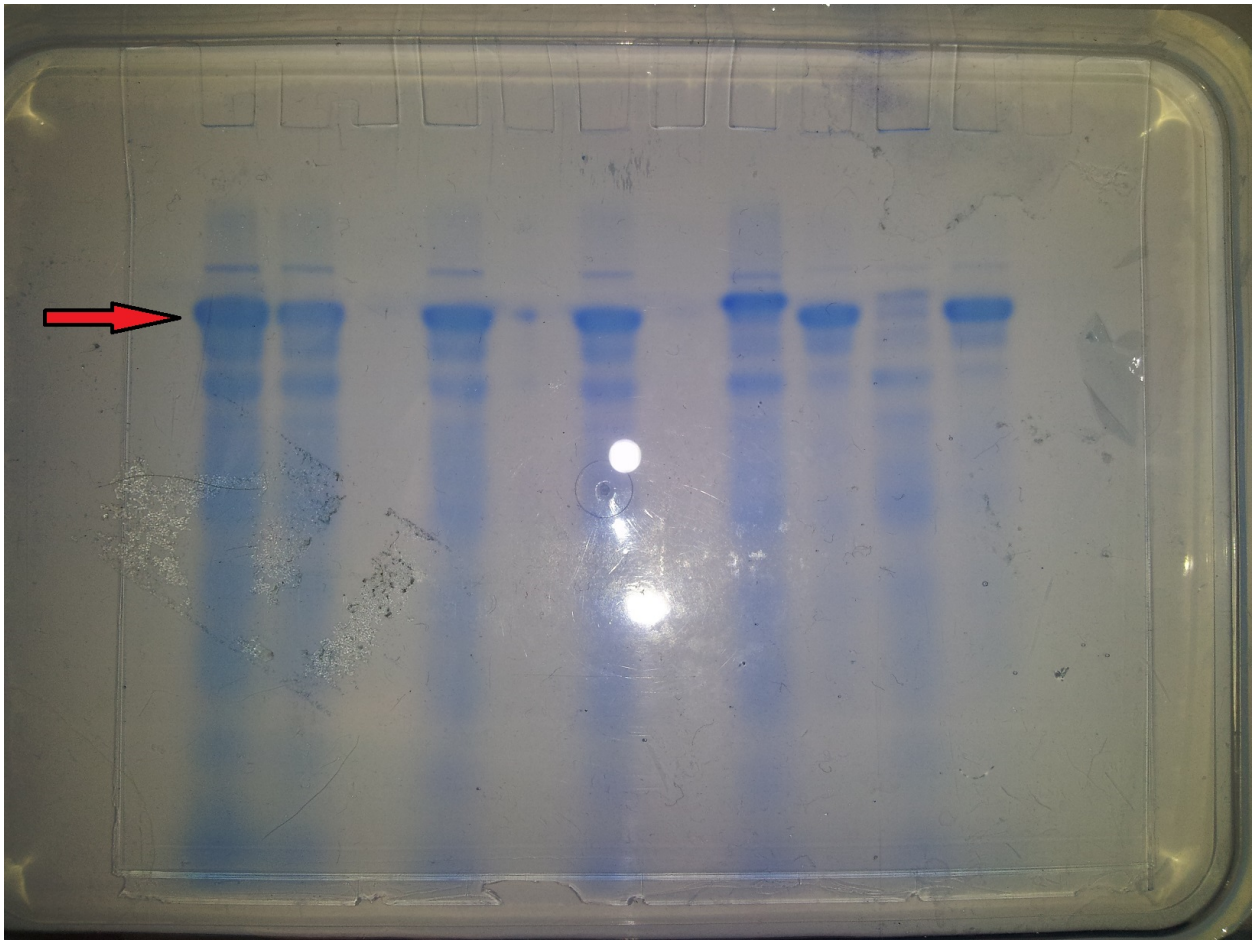
- Took out at 9:30AM Thursday 9/5/13 (Incubation time: 21 hours)
    - Spun down cells at 13k RPM for 1.5 hours
        - Decanted supernatant
        - Froze cells at -70°C overnight

9/6/13 Friday

- Resuspended frozen cells in 12.5mL 50mM tris-HCl
- Lysed cells using a french press
- Clarified cell lysate by spinning down at 4500 RPM for 45 minutes
- Made 5 $CaCl_2$ concentrations: 100mM, 10mM, 1mM, 0.1mM, 0mM (control)
    - Added 100µL $CaCl_2$ to 900µL of clarified cell lysate to obtain correct molarities
    - Incubated for 2 minutes
    - Spun down for 2 min at 13.2k RPM, , took 2µL sample from supernatant for SDS-PAGE
    - Washed once with 1mL 50mM tris-HCl for each concentration, spun down, decanted, washed in 1mL 50mMtris-HCl with EDTA of same molarity as $CaCl_2$ concentration for each of 5 samples. Took 2µL samples for SDS-PAGE
    - Results after adding 100µL $CaCl_2$ to 900µL of clarified cell lysate and spinning down for 2 min

As can be seen here, the 0mM did not form any precipitate, the 0.1mM, and 1mM $CaCl_2$ concentrations precipitated slightly, and the 10mM and 100mM precipitated significantly

- 2μL of each supernatants were taken after $CaCl_2$ addition, and after tris-HCl with EDTA resuspension
- SDS-PAGE gel was run with 2μL of samples in each lane added to 3μL of ddH_2O and 5μL of running buffer. Each mixture was then boiled for 10 minutes, vortexed and loaded onto 4% PAGE
    - 7μL was loaded into each lane
    - Gel was run at 100V for 1.5 hours
    - Results:



Lane 1: Clarified cell lysate
Lane 2: 0mM supernatant after addition of 0mM $CaCl_2$ and 2min spin at 13.2k RPM
Lane 3: 0mM supernatant after wash and resuspension in 50mM tris-HCl with 0mM EDTA
Lane 4: 0.1mM supernatant after addition of 0.1mM $CaCl_2$ and 2min spin at 13.2k RPM
Lane 5: 0.1mM supernatant after wash and resuspension in 50mM tris-HCl with 0.1mM EDTA
Lane 6: 1mM supernatant after addition of 1mM $CaCl_2$ and 2min spin at 13.2k RPM
Lane 7: 1mM supernatant after wash and resuspension in 50mM tris-HCl with 1mM EDTA
Lane 8: 10mM supernatant after addition of 10mM $CaCl_2$ and 2min spin at 13.2k RPM
Lane 9: 10mM supernatant after wash and resuspension in 50mM tris-HCl with 10mM EDTA
Lane 10: 100mM supernatant after addition of 100mM $CaCl_2$ and 2min spin at 13.2k RPM
Lane 11: 100mM supernatant after wash and resuspension in 50mM tris-HCl with 100mM EDTA

- Interpretation:
    - The large protein band at the top (marked by a red arrow) is the RTX construct
    - As expected lanes 1 and 2 look identical, which demonstrates that the negative control worked fine.
    - Lane 3 has no protein product because no pellet precipitated, which caused the tris-HCl wash to remove everything from the tube.
    - Lane 4 shows a large RTX band, which means no significant amount of RTX precipitates in the presence of 0.1mM $CaCl_2$

    - Lane 5 further corroborates the results of lane 4. Only a small amount of RTX is resolubilized in EDTA since only a small pellet was initially precipitated. The wash removed all other proteins
    - Lane 6 shows that most of the RTX does not precipitate in 1mM $CaCl_2$. This is also shown in the lack of a significant pellet in the microfuge tube
    - Lane 7 has almost no protein product for the same reason as lane 5
    - Lane 8 has an aberration in the presence of a band above where RTX should be. There is however no band where RTX should be, indicating that it precipitated in the presence of 10mM $CaCl_2$
    - Lane 9 has mostly RTX in it, which indicates that 10mM EDTA is sufficient to resolubilize the RTX pellet
    - Lane 10 is also missing a significant band at RTX indicating it has precipitated
    - Lane 11 contains mostly RTX for the same reason as lane 9
- Conclusion:
    - Based on the results of the SDS-PAGE and pellet formation in microfuge tubes, the data indicates that RTX paired to maltose binding protein and GFP is an effective protein purification solution as long as the calcium concentration is 10mM or above.
    - Further tests could be run with calcium concentrations below 10mM to assess the lowest effective concentration, but our data demonstrates that this amount is no lower than 1mM.