Construction of recombinant plasmids ： pMTL-*Pfba-F/Xpk*、pMTL-*Ptkt-F/Xpk*

1. **PCR amplification on the genome of *Clostridium tyrobutyricum* to obtain the fragment *Pfba*、*Ptkt)*.**
2. **Linearized vector pMTL-*F/Xpk(BD)* was obtained by PCR amplification using plasmid pMTL-*Pthl-F/Xpk(BD)* as template;**
3. **pMTL-*Pfba-F/Xpk(BD)* 、 pMTL-*Ptkt-F/Xpk(BD)* was obtained by Gibson assembly**

Experimenter：Leyi Zhang,Yijiu Lu,Xiaoran Zhao,Yixuan Liu,Qingyue Feng,Ruoxi Liu,Jiale Li

Recorder：Leyi Zhang

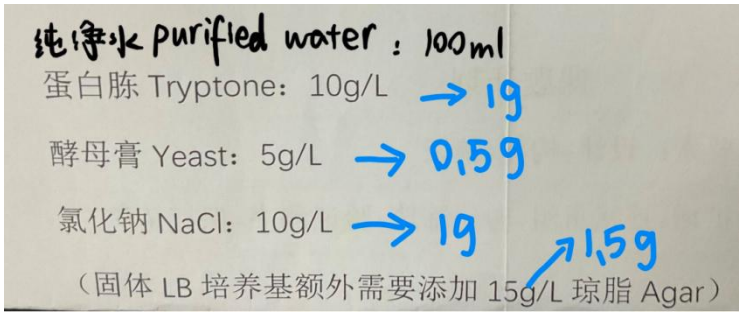
21/07/2023 Friday

Lab goal:

- Preparation of solid & liquid LB nutrient medium
- Replicate target gene & vector Gene (PCR product:*Pfba*)
- Gel Electrophoresis

Steps: *Blending solid LB nutrient medium:*

LB nutrient medium specifically cultivates Escherichia coli. Solid nutrient medium cultivates bacteria in Petri Dish which clearly displays bacterias' status; on the other hand, Liquid nutrient medium cultivates a greater amount of bacteria compared to Solid Nutrient Medium. The vector plasmid contains chloramphenicol resistance gene, and selective solid medium containing chloramphenicol can be prepared to screen the successful transmutation of bacteria.



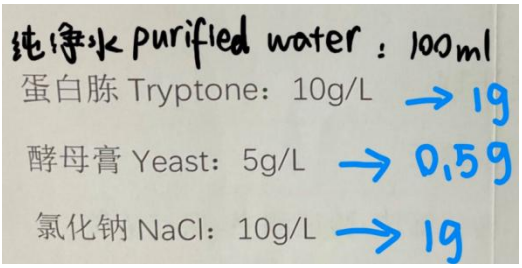
1. Weigh out 1g tryptone, 0.5g yeast, 1g NaCl, 1.5g agar on an electronic scale, adding them in a colonial flask
2. Add 100ml purified water into colonial flask and shake it up
3. Seal the flask with parafilm, newspaper and rubber cord
4. Autoclave the solutions
5. Mix 0.3 grams of chloramphenicol and 10ml of anhydrous ethanol in collection tube

6. Remove the sealed newspaper
7. Dissolve 100ul of antibiotics (Chloramphenicol) into the Nutrient Medium. (Antibiotics:Solution=1:1000)
8. Evenly distribute the nutrient medium into three petri dishes
9. Wait for 20 mins for nutrient Medium to cool, then invert the petri dish to prevent condensate water from dropping
10. Wrap Petri dish with plastic wrap

(step 6 ~ 10 are supposed to be done in biosafety cabinet)

Steps: *Blending liquid LB nutrient medium:*

（Repeat steps 1 to 5, but no agar）



Steps-*PCR*

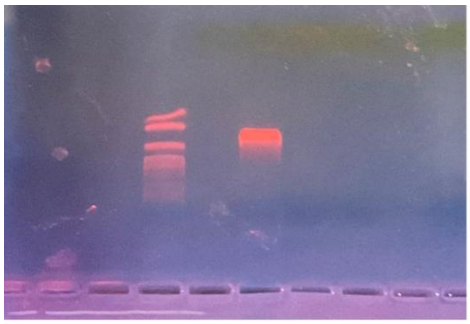
1. Deploy PCR: shown in
2. Run out of PCR: shown in figure as follow

Steps-*Gel Electrophorus:*

1. Mix 0.5g of Agarose and 50 ml of TAE solution. (Agarose: TAE=1:100)
2. Heat the Solution using microwave oven (medium high/ 1~2min)
3. Add nucleic acid dye to help see more clearly
4. Connect the Gel-tank with comb (8 keys, each key creates 50 ul trough) , then pour the solution to the tank
5. Wait around 15min until the Gel is dry (Feels no heat when touching the bottom of the Gel-tank) and then gently

6. remove the comb
7. Place the Gel in the electrophoresis tank: trough close to cathode (Black) and ensure that the gel is soaked in running
8. buffer
9. Load Marker (12000bp), and sample
10. Program and run Gel Electrophorus (110V/30mins): shown in *Figure*
11. Leave the machine running after visualizing little bubbles coming out of Cathode

Results: Success: 2/5



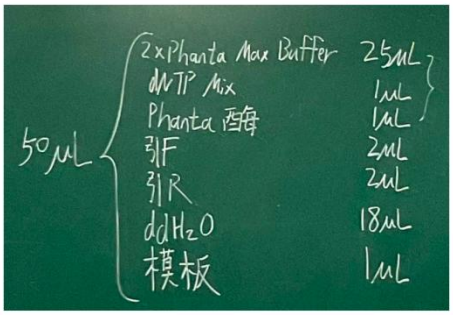
24/07/2023 Monday

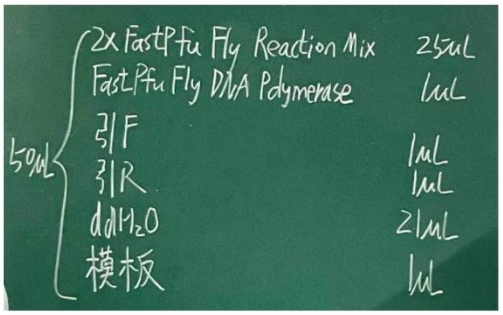
Lab goal:

- Replicate target gene & vector Gene (PCR,product:fragment *Ptkt*，linear plasmid pMTL-*F/Xpk(BD*))
- Gel Electrophoresis
- Gel Recovery

Steps-*PCR:*

Deploy PCR: shown in figure as follow:





1. Deploy PCR
2. Run out of PCR

Steps-*Gel Electrophorus:*

DNA is negatively charged and is attracted to the positive electrode when an electric field is applied. In porous gels, the longer the DNA, the slower the migration rate, enabling the separation of DNA of different sizes.

The experimental steps are as follows:

1. Weigh 0.25 g of agarose, 25mL 1xTAE buffer into a conical flask, shake to mix well, and put in Microwave over medium heat until boiling.
2. Add 2.5μL of nucleic acid dye and shake evenly.
3. Pour 25mL of solution into a small rubber plate and insert it into a comb.

4. Use the tip of pipette to pick out the air bubbles and stand at room temperature for 15min and wait for it to solidify.
5. Pull out the comb and remove the gel and place it in the electrophoresis tank.
6. Add 10 μL of 6x nucleic acid dye to 50 μL of PCR product and pipette evenly. Take 30 μL in two divided into two wells.
7. 115V electrophoresis for 30min.
8. After electrophoresis, remove the gel.
9. Cut the part of gel, containing the intent fragment, under ultraviolet light and remove as much excess gel as possible. Chop and place it in a 1.5mL centrifuge tube.
10. Add equal volume of gel of (submerged ever the gel) Buffer GDP, 55 °C water bath for 6min, and mix inverted once during the water bath.
11. Brief centrifugation: Place the FastPure DNA Mini Columns-G adsorption column in a collection tubes. Transfer <=700 μL of sol solution to the adsorption column, centrifuge at 12,000r for 60 s.
12. Discard the filtrate, place the adsorption column in the collection tube, add 300μL of Buffer GDP to the adsorption column, and let stand for 1min, centrifuge at 12,000 rpm for 60 s.
13. Discard the filtrate, place the adsorption column in a collection tube, add 700μL of Buffer GW (with absolute ethanol) to the adsorption column, and centrifuge at 12000rpm for 60s.
14. Repeat step 13.
15. Discard the filtrate, place the adsorption column in the collection tube, and centrifuge at 12000rpm for 2min.
16. Place the adsorption column in a 1.5mL centrifuge tube, add 21 μL ddH2O to the inner membrane of the adsorption column, place 2min 12000rpm, and centrifuge for 1min.
17. Discard the adsorption column and store the DNA at -20 °C

25/07/2023 Tuesday

Lab goal:

- Gel Recovery
- Create and transform competent cells

PCR product is: linear pMTL-*F/Xpk(BD*)

Receptor cell type is E. coli JM109

Steps-*Gel Recovery:*

1. Cut the part of gel, containing the intent fragment, under ultraviolet light and remove as much excess gel as possible. Chop and place it in a 1.5mL centrifuge tube.
2. Add equal volume of gel of (submerged ever the gel) Buffer GDP, 55 °C water bath for 6min, and mix inverted once during the water bath.
3. Brief centrifugation: Place the FastPure DNA Mini Columns-G adsorption column in a collection tubes. Transfer <=700 μL of sol solution to the adsorption column, centrifuge at 12,000r for 60 s.
4. Discard the filtrate, place the adsorption column in the collection tube, add 300μL of Buffer GDP to the adsorption column, and let stand for 1min, centrifuge at 12,000 rpm for 60 s.

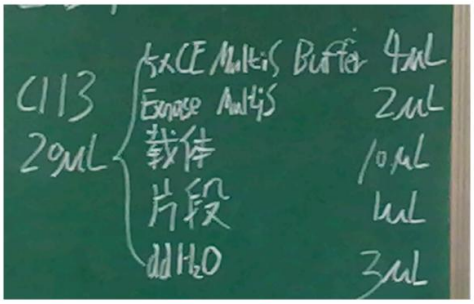
5. Discard the filtrate, place the adsorption column in a collection tube, add 700μL of Buffer GW (with absolute ethanol) to the adsorption column, and centrifuge at 12000rpm for 60s.
6. Repeat step 5.

7. Discard the filtrate, place the adsorption column in the collection tube, and centrifuge at 12000rpm for 2min.
8. Place the adsorption column in a 1.5mL centrifuge tube, add 21 μL ddH2O to the inner membrane of the adsorption column, place 2min 12000rpm, and centrifuge for 1min.
9. Discard the adsorption column and store the DNA at -20 °C.

Steps-*Create and transform competent cells*

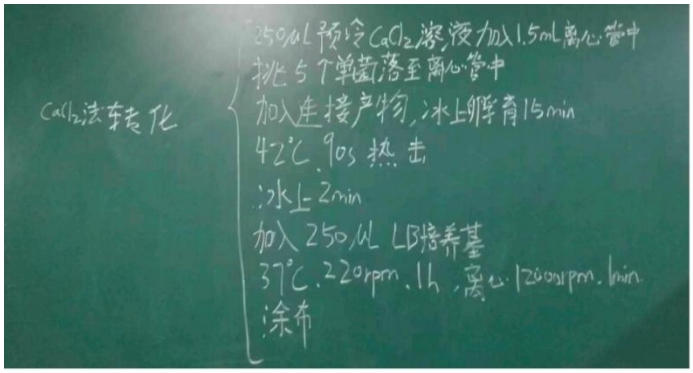
After the recipient cell is treated by a special method (use CaCl2 in our experiment), the permeability of the cell membrane is temporarily changed, and it becomes a competent cell that can allow the entry of foreign DNA molecules.

The experimental system is as follows:



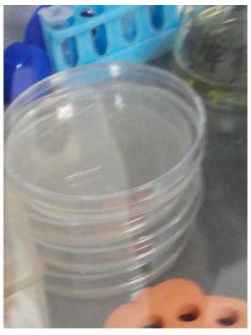
1. Deploy 20 μL： shown in figure 4 （4 μL 5x CE Maltis Buffer、2 μL Exnase multis、10 μL carrier、1 μL part、3 μL ddH2O）

2. Run out of PCR: 37°C 30min
3. The PCR tubes are cooled at 4 °C for later use



(Step 4,5,6,9,11 are supposed to work in sterile environment.)

4. Add 250 μL of pre-cooled $CaCl_2$ to a 1.5 mL centrifuge tube
5. Pick 5 pre-cultured single colonies into centrifuge tubes with pipette tips
6. Add the ligation product and incubate the centrifuge tube on ice for 15 min
7. Place the centrifuge tube in a 42 °C water bath with 90s heat hit
8. Remove the centrifuge tube and place on ice for 2min （shown in figure as follow）
9. Add 250 μL of LB medium to the centrifuge tube and stir well
10. Place the centrifuge tube in a shaker, 37°C at 220 rpm for 1 h, and centrifuge at 12000 rpm for 1 min
11. Inoculate the bacteria on solid medium (coating) （shown in figureas follow）

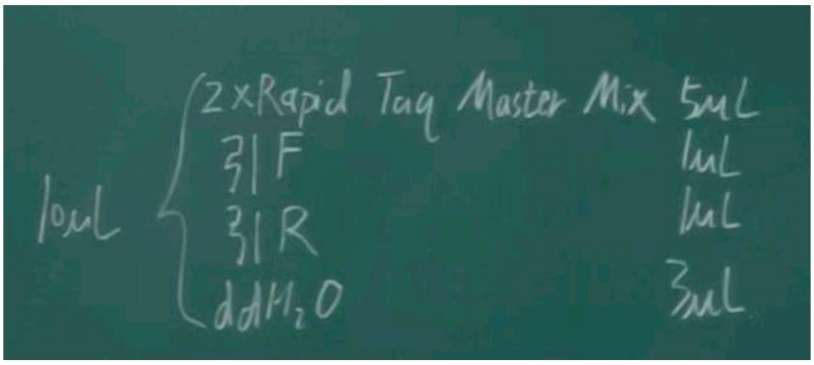


26/07/2023 Wednesday

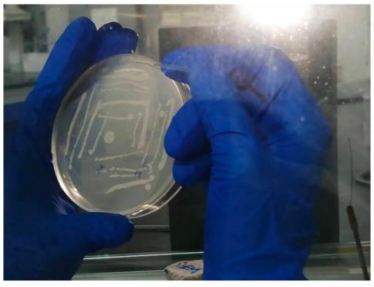
Lab goal:

- Colony PCR:target plasmid pMTL-*Pfba-F/Xpk*
- Make nucleic acid glue & gel electrophoresis

Steps-colony PCR:

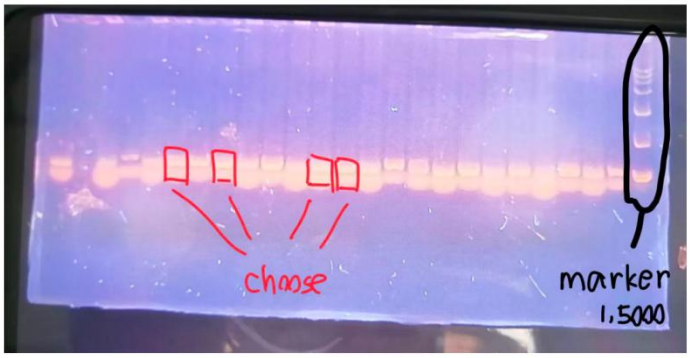


1. Deploy 50 μL PCR solution (shown in the figure above)
2. Add 25 μL of 2x Rapid Taq Master Mix, 5 μL of F-primer, 5 μL of R-primer, and 15 μL of ddH_2O to the 1.5 mL centrifuge tube, respectively.
3. Centrifuge quickly in order to collect the residual liquid on the tube wall and lid
4. Add 10 μL of PCR system sequentially to five PCR tubes （shown in figure 2）
5. In the biosafety cabinet, pick up 5 colonies and add each to the five PCR tubes, then use pipette to help mix well (shown in figure as follow)
6. Run out of PCR


7. Prepare a gel (meddle size): 10 μL of PCR in each, 120V 30min

8. **Result:**success！



9. In biosafety cabinet, add 5 mL of LB liquid medium to the shaker tube. Use the tip to pick up the corresponding colony and drive into the shaker tube.）

27/07/2023 Thursday

Lab goal:

- Plasmid extraction
- restriction enzymes：template plasmids：pMTL-*Pfba-F/Xpk*；pMTL-*Ptkt-F/Xpk*

Steps-*Plasmid extraction*:

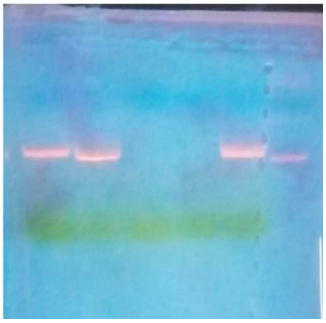
1. Add 2ml of logarithmic E. coli solution to a centrifuge tube, centrifuge at 10,000 rpm (11,500x g) for 1min, discard the medium, and absorb the residual liquid with a paper towel.
2. Add 250 ul Buffer P1 (RNase A added) to the centrifuge tube with the bacterial pellet and mix well with a pipette. 3.
3. Add 250 ul Buffer P2 to step 2 and mix 8-10 times upside down gently until the solution becomes viscous and translucent, that is, the bacteria are fully lysed.
4. Add 350 p1 Buffer P3 to step 3, immediately gently upside down 8-10 times to allow the solution to completely neutralize Buffer P2, centrifuge at 12,000 rpm (13,400x g) for 10 min after a white flocculent pellet appears.
5. Place the sorption column in a 2 ml collection tube.
6. Carefully transfer the Step 4 supernatant to the adsorption column with a pipette, centrifuge at 12,000 rpm (13,400 xg) for 60sec, drain the waste from the collection tube, and place the adsorbent column back into the collection tube.
7. Add 600 ul Buffer PW2 (diluted with absolute ethanol) to the adsorption column and centrifuge 30-60sec at 12,000 rpm (13,400x g). Discard the waste liquid and put the adsorption column back into the collection tube.
8. Repeat step 7.
9. Put the adsorption column back into the collection tube, centrifuge the adsorption column at 12,000 rpm (13,400x 9) for 1 min to dry the adsorption column and completely remove the residual rinse solution in the adsorption column.

10. Place the adsorption column in a new sterilized 1.5 ml centrifuge tube. Add 21ul ddH_2O to the center of the membrane attached to the column. Centrifuge at room temperature for 2 min and rinse DNA by centrifugation at 12,000 rpm (13,400 x g) for 1 min.
11. Discard the adsorption column and store the DNA product at -20°C to prevent DNA degradation.

Steps-*Restriction enzymes*:

1. Prepare 50μL digestion system.
2. Add 5 μL of 10x Quick Cut Green Buffer, 1 μL of XhoI, 1 μL of plasmid, ddH_2O to 50 μL to the PCR tube.
3. Put the PCR tube into the thermal cycler and set the program to: 37 °C for 1h + 80 °C for 20min.
4. Glue making.
5. Add 25 μL of digestion product to each sample well. 120V electrophoresis for 30min.

Results:



28/07/2023 Friday

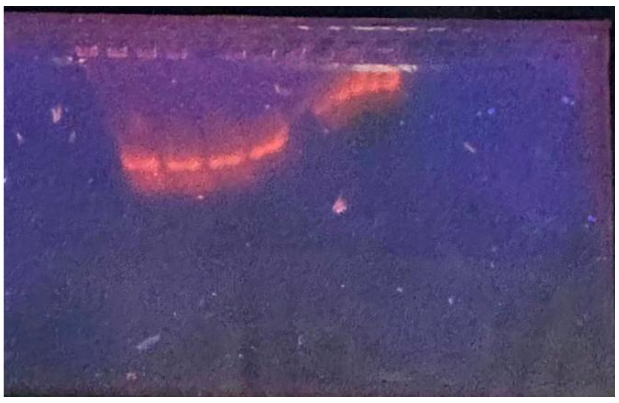
Lab goal:

- Make nucleic acid glue & gel electrophoresis
- Colony PCR （validation target plasmids were: pMTL-Ptkt-F/Xpk）

Steps-*colony PCR:*

1. Deploy 50 μL PCR solution Add 25 μL of 2x Rapid Taq Master Mix, 5 μL of F-primer, 5 μL of R-primer, and 15 μL of ddH_2O to the 1.5 mL centrifuge tube, respectively.
1. Centrifuge quickly in order to collect the residual liquid on the tube wall and lid
2. Add 10 μL of PCR system sequentially to five PCR tubes
3. In the biosafety cabinet, pick up 5 colonies and add each to the five PCR tubes, then use pipette to help mix well
4. Run out of PCR
5. Prepare a gel (meddle size): 10 μL of PCR in each, 120V 30min

Results：

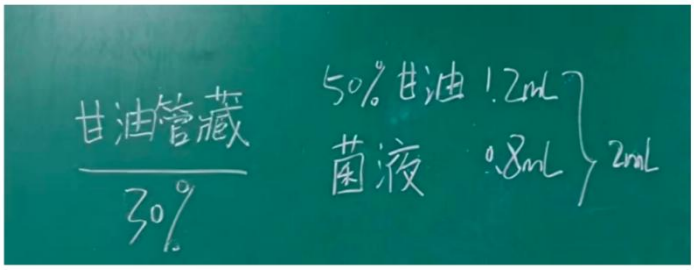


31/07/2023 Monday

Lab goal:

- Glycerin tube storage
- Plasmid extraction
- Restriction enzyme & run the gel
- Deploy solution

Steps-*Gglycerin tube storage*:



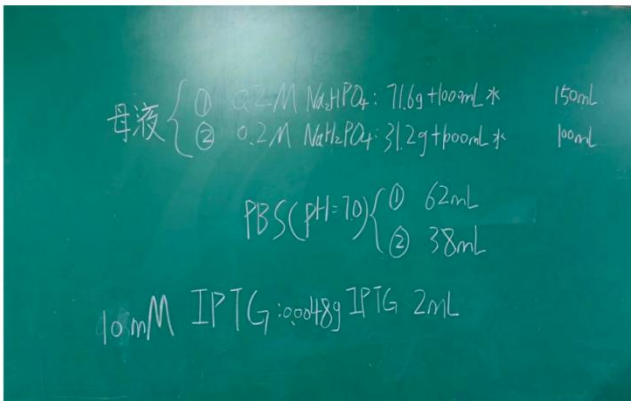
1. Deploy the solution: 1.2ml glycerol and 0.8ml
2. Storage the solution in -20°C

Steps-*restriction enzymes & run the gel*

1. Prepare 50μL digestion system.
2. Add 5 μL of 10x Quick Cut Green Buffer, 1 μL of XhoI, 1 μL of plasmid, ddH2O to 50 μL to the PCR tube.
3. Put the PCR tube into the thermal cycler and set the program to: 37 °C for 1h + 80 °C for 20min.
4. Glue making.
5. Add 25 μL of digestion product to each sample well. 120V electrophoresis for 30min.

results:

Steps-*Deploy solution*:



01/08/2023 Tuesday

Lab goal:

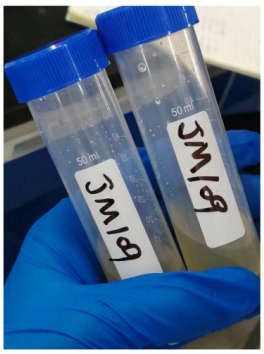
- Make competent cells
- IPTG induction, SDS-PAGE

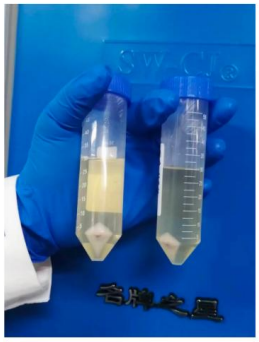
Steps-*Preparation o competent cells*:

After $CaCl_2$ treatment, bacterial cell membrane permeability changes and transforms into competent cells, which are easy to accept foreign DNA.

The experimental steps are as follows:

1. Pick bacteria activation, scribing inoculation, 37 °C culture for 12h.
2. Insert the activated bacteria into LB liquid medium at 1% inoculum to OD_{600}=0.5. Pre-cool the solution with the centrifuge tube at the same time.
3. Pour the bacteria into a 50mL centrifuge tube, ice bath for 10min, 4000rpm 4°C centrifuge for 10min.
4. Discard the supernatant, add 10 mL of PBS, and aspirate and resuspend. Centrifuge at low speed for 5 min.
5. Repeat step 4.
6. Discard the supernatant, add 10mL of $CaCl_2$ solution, puff and resuspend, and take an ice bath for 30min. Centrifuge at 5,000 rpm for 5 min and discard the supernatant.
7. Repeat step 6.
8. Add 2mL of $CaCl_2$ solution and take an ice bath for 10-30min. Add 2 mL of glycerol-labeled aliquots





Steps-*IPTG induction*:

IPTG binds to lac repressor, inhibits its binding to lac operon, thereby inducing sequence expression within lac operon.

IPTG solution was prepared as follows:

1. Add 1 μL of 50mM IPTG solution to 50mL LB liquid medium in ultra-clean. (Make the concentration of IPTG in culture 1 mM)
2. Place in a shaker to culture overnight.
3. Configure 2 L of 1x protein buffer, weigh 3.02g Tris, 18.8g Gly, 1g SDS, and add to the container. Weigh 2 L up H_2O with a graduated cylinder and add to the container. Mix well and set aside

Results-*Final product*

Even though Electrophoresis results show that there are four strains accepted the target plasmid, we choose to transplant the two with the brightest marks on the Gel.