Construction of recombinant plasmid pMTL-*Pthl-F/Xpk(BD)*

1. **PCR amplification on the genome of *Clostridium acetobutylicum* to obtain the fragment *F/Xpk(BD)*.**
2. **Linearized vector pMTL-*Pthl* was obtained by PCR amplification using plasmid pMTL82151-*Pthl-adhE2* as template;**
3. **pMTL-*Pthl-F/Xpk(BD)* was obtained by Gibson assembly**

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Recorder：Huaize Zhu

10/07/2023 Monday

Lab Goal: Gathering components

- Preparation of LB Nutrient Medium
- Amplification of target and vector genes
- Verify PCR results using gel electrophoresis

Steps-*Preparation of 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.

Solid LB Nutrient Medium

1. Add 1g of Tryptone, 0.5g of Yeast, 1g of NaCl, and 1.5g of Agar into the conical flask
2. Add 100ml of water, then gently shake the solution.
3. Seal the flask with news paper
4. Autoclave the solutions
5. Mix 0.3 grams of chloramphenicol and 10ml of anhydrous ethanol in another conical flask.
6. Separate the solution using filter needles (1ml per centrifuge bottle)
7. Remove the sealed newspaper.
8. Dissolve 100ul of antibiotics (Chloramphenicol) into the Nutrient Medium. (Antibiotics:Solution=1:1000)
9. Evenly distribute the nutrient medium into three petri dishes.
10. Wait for 20 mins for nutrient Medium to cool, then invert the petri dish to prevent condensate water from dropping.

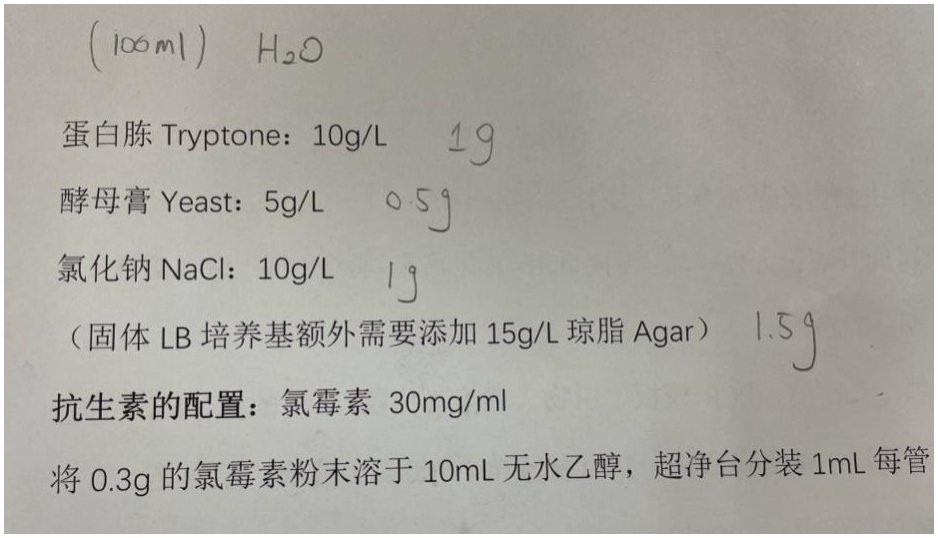
11. Wrap Petri dish with plastic wrap.

⟨Step 7-10 needs to be done in the Biosafety Cabinet⟩

Liquid LB Nutrient Medium

Repeat steps 1 to 8, **BUT DO NOT ADD ANY AGAR**.

Figure1:

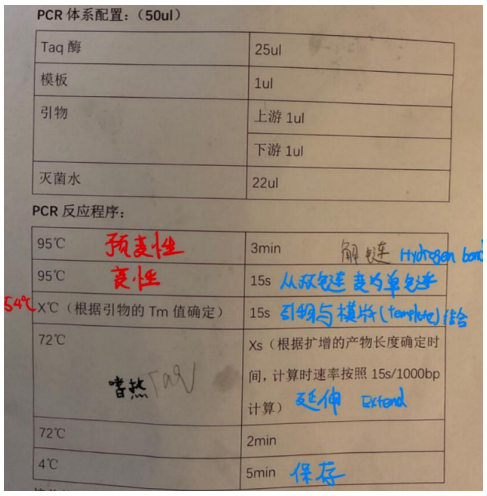


Escherichia coli grows better in aerobic situations, so the bacterial liquid are usually placed in the shaker.

Steps-*Polymerase Chain Reaction (PCR)*:

1. DNA vector and DNA fragments follows the same system*
2. Deploy the system according to the *procedure* (50ul) 【*Figure 2*】
3. 22 ul of Taq enzyme, 1ul of templated DNA, 1 ul of forward primer, 1 ul of reverse primer, and 22 ul of double distilled water.
4. Program and run PCR (30 cycles)

Figure2:



PCR 体系配置：（50ul）

Taq 酶	25ul
模板	1ul
引物	上游 1ul
	下游 1ul
灭菌水	22ul

PCR 反应程序：

95℃ 预变性	3min 解链 Hydrogen bond
95℃ 变性	15s 从双链变为单链
54℃ X℃（根据引物的 Tm 值确定）	15s 引物与模板(template)结合
72℃ 嗜热 Taq	Xs（根据扩增的产物长度确定时间，计算时速率按照 15s/1000bp 计算） 延伸 Extend
72℃	2min
4℃	5min 保存

Steps-*Gel Electrophorus*:

5. Mix 0.6g of Agarose and 60ml of TAE solution. (Agarose:TAE=1:100)
6. Heat the Solution using microwave oven (Medium High/1min)
7. Connect the Gel-tank with comb (8 keys, each key creates 50ul trough) Then pour the solution to the tank.
8. Wait until the Gel is dry (Feels no heat when touching the bottom of the Gel-tank) Then gently remove the comb.
9. Place the Gel in the electrophoresis tank. Trough close to cathode (Black). Ensure that the gel is soaked in running buffer (use your hand)
10. Load Marker (12000bp), and sample.
11. Program and run Gel Electrophorus (110V/30mins) 【Figure 3】
12. Leave the machine running after visualizing little bubbles coming out of Cathode.

Figure 3:

Results-*LB Nutrient Medium*:

Figure four shows Liquid LB nutrient medium; Figure five shows Solid LB nutrient medium.

Figure 4:

Figure 5:

Results-*PCR*:

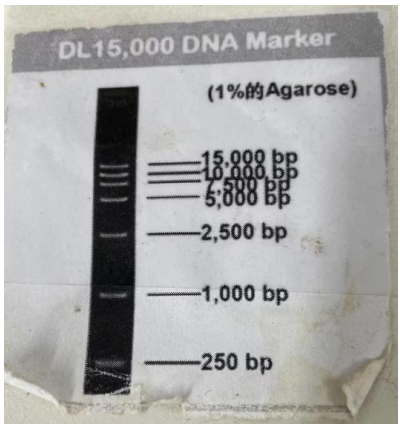
The PCR product has not been verified yet, and whether the product was successfully replicated and the concentration is unknown.

11/07/2023 Tuesday

Lab goal:Gathering components

- Gather PCR results
- Gel recovery and concentration test

Figure 6:



The picture above shows the DNAmarker used.

Results-*PCR results:*

First trial: Failed (Vector: 1/3) (Fragment: 1/3)

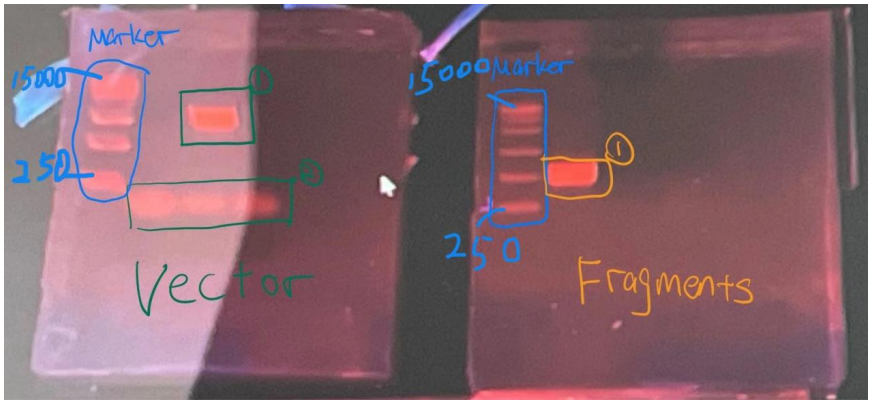
Vector 1: Successful.

Vector 2: Failed.

Reasoning: Results are shorter (have fewer base pairs) than marker (250bp). Vector is designed to have 8000 base pairs. It is estimated that these results may be an unused primer that didn't bind to the template strand due to its low specificity. Primers (30bp) may possibly pass the marker's range and ultimately presents a shallow mark on the Gel.

Fragment 1: Successful.

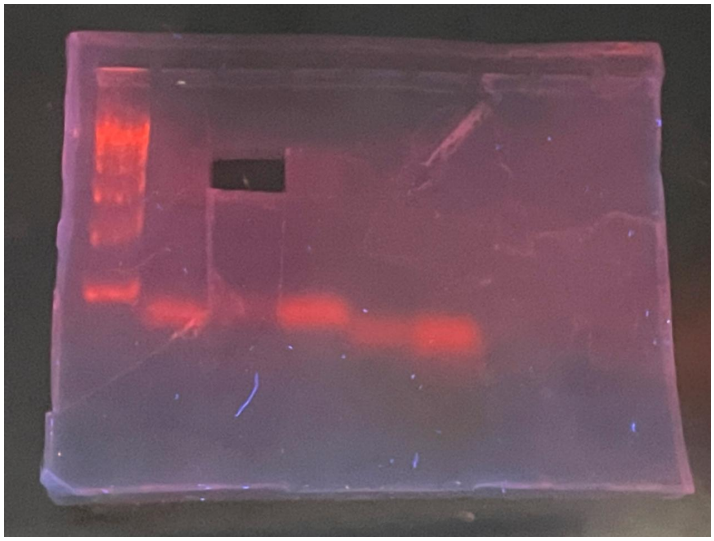
Figure 7:



Second Trial: Failed (Vector: 0/3) (Fragment: 0/3)

Reasoning: Sample DNA completely dispersed in Gel. The marker does not demonstrate clear linear marks. Due to the lack of consideration for the possibility of DNA dispersion caused by prolonged gel soaking, the experimenters went to lunch. This failure is because the Gel wasn't taken out immediately after the program is finished.

Figure 8:



Third Trial: Successful (Vector: 3/3) (Fragment: 0/3) [Later will be referred as P-Vector-1]

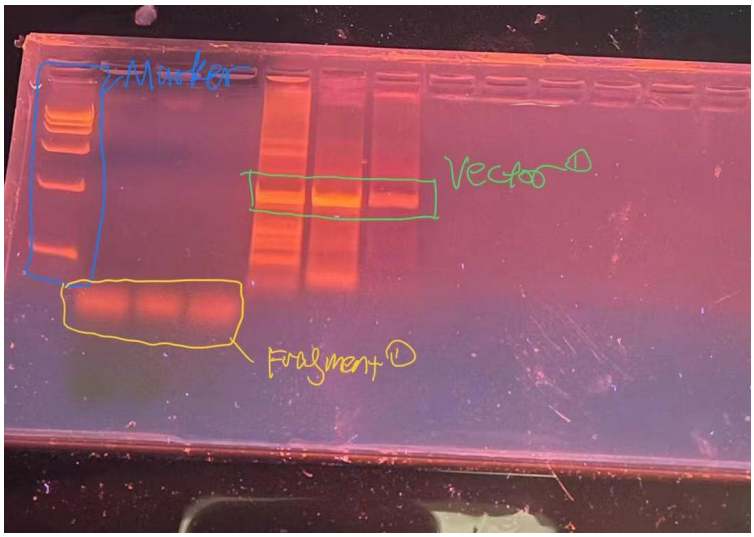
Fragment 1: Failed.

Reasoning: Results are shorter (have fewer base pairs) than marker (250bp). Fragments are designed to have 1500 base pairs. We mistakenly added ethanol to the PCR system which we are spouse to add double distilled water.

Vector: Successful !

Reasoning: Results show clear and deep marks on the Gel.

Figure 8:



Steps-Gel *Recovery*:

[P-Vector-1}

Under E-Gel Imager cut off the part of the gel that contained the target DNA. Remove as many gel as possible to seek for higher concentration.

1. Transfer the gel that contained the target DNA into centrifuge tube.
2. Measure the weight of the gel. Then add x ul of Buffer GDP. (xng=xul)
3. Heat the centrifuge tube using water bath to melt the gel. (55 °C; 7mins)
4. Place DNA Mini Absorbing Columns-G inside the Collection Tube. 【Figure 9】

Figure 9:

5. Transfer the melt Gel and GDP solution into the Absorbing Column (700 ul max)
6. Centrifuge the solution (12000 rpm; 1min)
7. Discard the filtrate in the collection tube, then add 300 ul of Buffer.
8. Let the centrifuge tube stand for one minute, then centrifuge the solution (12000 rpm; 1min)
9. Discard the filtrate in the collection tube, then add 700 ul of Buffer GW with Anhydrous Ethanol into the Absorbing Column. Centrifuge the solution (12000 rpm; 1min).
10. Repeat Step 10
11. Discard the filtrate in the collection tube, then centrifuge. (12000 rpm; 2mins)
12. Discard the filtrate and the collection tube. Place the absorbing column into a centrifuge tube.
13. Add 30 ul of double distilled water. Make sure the double distilled water covers the membrane of the absorbing column

Let the centrifuge tube stand for two minutes, *then close the centrifuge tube's cover*, and centrifuge (12000 rpm; 1mins)

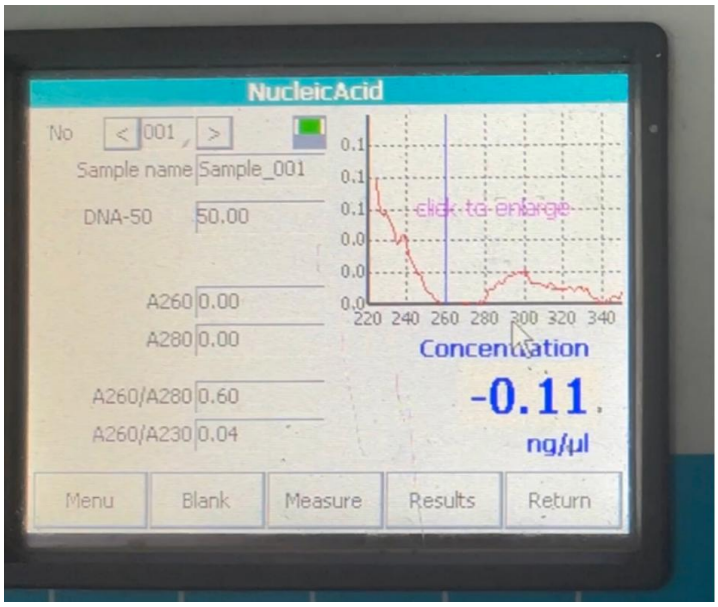
Results-Gel Recovery:

To test the concentration, we first use double distilled water to set up a "blank". Testing results would be based on the "blank" we set up. Normally, it is expected that the concentration of target DNA reaches at least 20ng/ul. A successful PCR

recovery would have a concentration of over 100ng/ul. P-Vector-1 Gel recovery is an absolute failure because the results show that our Gel recovery product is cleaner than water.

Reasoning: This is because we made a dramatic mistake during step 10. We are supposed to add the Buffer GW with anhydrous ethanol to purify our product by washing away ions, salt, and proteins. However, we added the Buffer GW without anhydrous ethanol. This means the solution is no longer organic, and can take DNA across the membrane of the absorbing column. Buffer GW not only washed away ions, salt, and proteins it also washed away DNA molecules which is our product.

Figure 10:



12/07/2023 Wednesday

Lab goal: Gathering Components

- PCR and results
- Gel Recovery and Concentration Test
- PCR recoveries

Steps-*PCR&Gel Recovery*

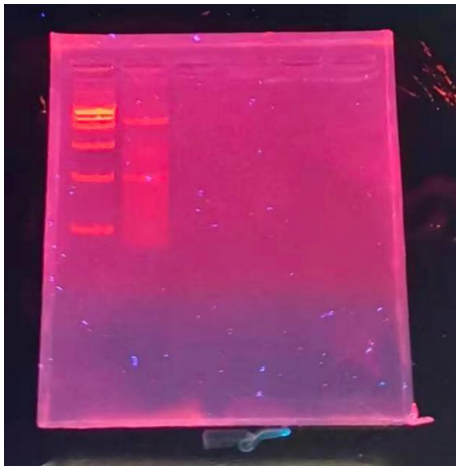
Experiment steps carried out in these categories are exactly same as Monday and Tuesday.

Results-*PCR*

Fourth Trial: Successful (Fragment: 1/1) *[Later be referred to P-Fragment-1]*

Reasoning: Results show clear and deep marks on the Gel. We can move on to the next step: Gel recovery and concentration test.

Figure 11:



Results-*Gel Recovery and Concentration Test*

We conducted concentration on the Gel recovery results. Concentrations above 20ng/ul are acceptable, and above 50ng/ul are good. Gel recovery for P-Fragment-1 was another failure.

Reasoning: It is estimated that Gel electrophorus has possibly decreased PCR final product's concentration. Thus we decided to carry out direct PCR recoveries instead of Gel recoveries.

Figure 12:

Steps-*PCR Recoveries:*

The purpose of Gel Electrophorus was to verify whether the PCR is successful. In fact, Gel recovery will decrease the concentration of the PCR final product. If Gel Electrophorus shows that PCR has been successful once, PCR carried out using the same material and machine can default to successful. Thus, it is acceptable to recover the PCR product directly without Gel Electrophorus to seek for higher concentration. However, we decided to use 3ul of the PCR results to run Gel Electrophorus and use the rest to carry out PCR Recoveries .

Here are the experimental steps：

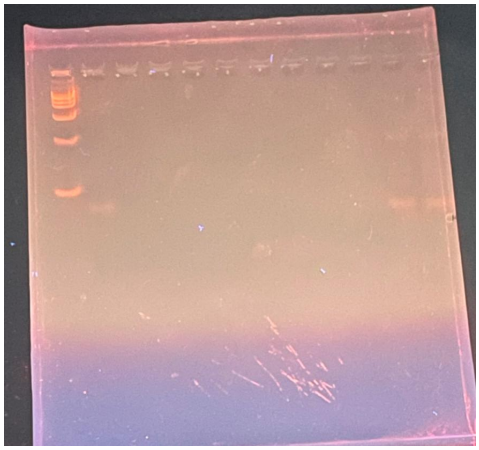
1. Add 5 times the volume of Buffer GDP to the PCR product, then invert and shake well
2. Place DNA Mini Absorbing Columns-G inside the Collection Tube.

3. Centrifuge (12000rpm; 1min), then discard the solution in the collection tube
4. Discard the filtrate in the collection tube, then add 300 ul of Buffer.
5. Let the centrifuge tube stand for one minute, then centrifuge the solution (12000 rpm; 1min)
6. Discard the filtrate in the collection tube, then add 700 ul of Buffer GW with Anhydrous Ethanol into the Absorbing Column. Centrifuge the solution (12000 rpm; 1min).
7. Repeat Step 10
8. Discard the filtrate in the collection tube, then centrifuge. (12000 rpm; 2mins)
9. Discard the filtrate and the collection tube. Place the absorbing column into a centrifuge tube.
10. Add 30 ul of double distilled water. Make sure the double distilled water covers the membrane of the absorbing column
11. Let the centrifuge tube stand for two minutes, then close the centrifuge tube's cover, and centrifuge (12000 rpm; 1mins)
12. Results-Gel Electrophoruses:

Unfortunately, Gel Electrophoruses results weren't very present. We couldn't see anything except for the marker.

Reasoning: It is estimated that maybe 3 ul wasn't enough. Or, it is possible that we accidentally puncture the Gel while loading the sample.

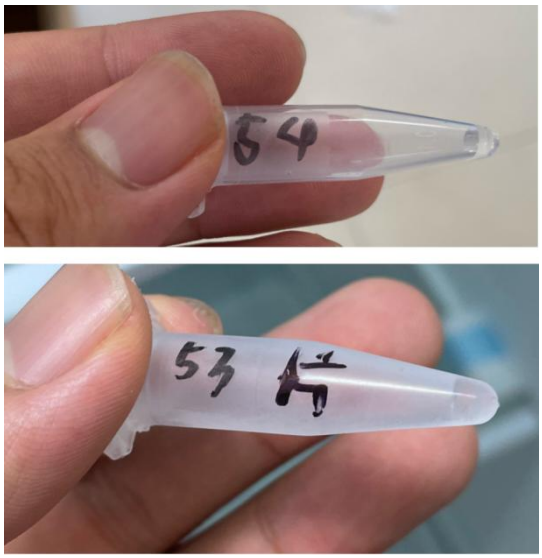
Figure 13:



Results-*PCR recoveries*

We directly recovered the PCR product, and tested the concentration. It turns out to be very successful. Vector Concentration: 54ng/ul; Fragment concentration: 53ng/ul

Figure 14:



13/07/2023 Thursday

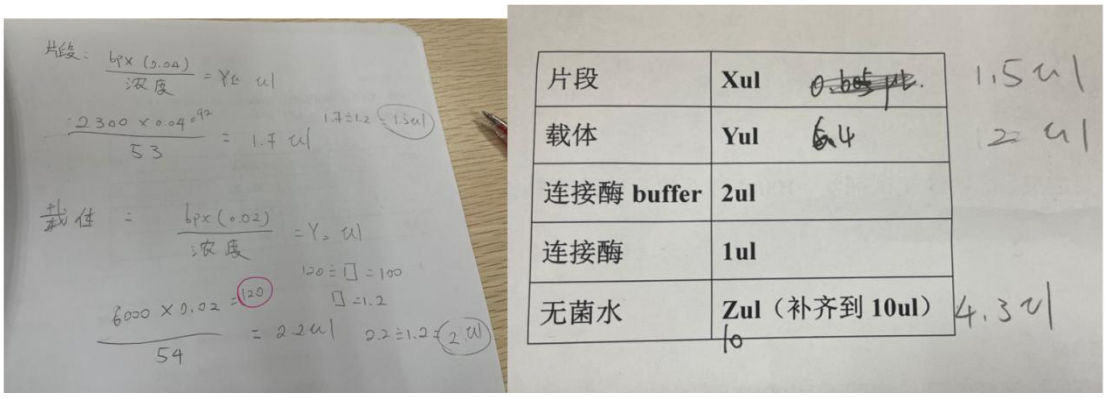
Lab goal: Building Plasmid and Plasmid transcription.

- Connecting Vector and Fragment
- Conversion to Competent state, and transcription.

Steps-*Connecting Vector and Fragment*:

1. Calculate the system, then add all the components into a PCR tube.
2. Place the tubes into the water bath (37°C /30mins) Product would be referred as "Target plasmid"

Figure 15:



片段	Xul
载体	Yul
连接酶 buffer	2ul
连接酶	1ul
无菌水	Zul（补齐到 10ul）

Steps-*Competent State*

1. Add 250 ul of cold Calcium Florine into the centrifuge tube.
2. Pick 5 bacteria colonies from the petri dish and add them to a centrifuge tube, stirring
3. Add 10 ul of Target plasmid into the centrifuge tube.
4. Place it on the ice for 15mins or more. At this stage the bacteria has already entered Competent state. Shaking, vibrating, or any severe action will kill the cell.

5. Using water bath, 42°C, 90second. Allow plasmid enter the cell.
6. Put it back onto ice for 2 mins or more. The bacteria are now back to normal stage.
7. Add 250 ul of liquid LB nutrient medium. Then place it into the table concentrator (37°C; 1hour)
8. Finally, we coated the bacteria onto the solid LB nutrient medium. Then, place it the incubator. (37°C; 21 hours)

Results:

We have “imported” the target plasmid into the bacteria; however, we are still unsure weather the process was successful or not. Thus, it is necessary to verify our results.

14/07/2023 Friday

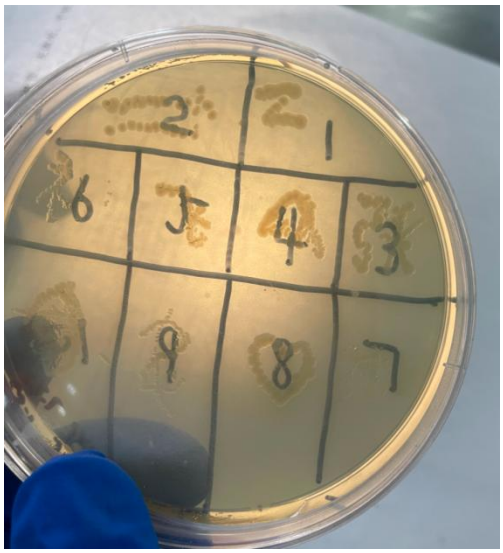
Lab goal: Verifying the final product

- PCR
- Gel Electrophoruses

Steps-*Bacteria PCR & Gel Electrophoruses*:

1. Coordinate the system according to the instruction in a PCR tube.
2. Using pipette gun pick 1 bacteria colony from the petri dish and transplant them onto another (Marked) 【Figure 1】
3. Move pipette gun, solid LB nutrient medium, PCR tube into Biosafety Cabinet. Then, prob the pipette gun into the PCR tube, mix well.
4. Program and run PCR.
5. After PCR program, carry out gel electrophoruses to verify the success of Plasmid transcription.

Figure 16:



Results-*Gel Electrophoruses*:

Successful (6/16) Meaning that a decent amount of bacterias accepted our target plasmid.

Stage Conclusion-Week 1

The purpose of the experiment is to import F/xpk enzyme's gene sequence into *Clostridium Tyrobutyricum* to establish NOG pathways. We selected two gene fragments expressing the same enzymes coming from *Clostridium acetobutylicum,* and *Bifidobacterium Adolescentis*. Particularly C. *acetobutylicum* has high homology with the template strain (*C. Tyrobutyricum*); on the other hand, *B. Adolescentis* has more efficient expression.

This week we successfully established the target gene from *C. acetobutylicum* for our template strain (*C. Tyrobutyricum*). Note that, we have NOT imported the the established target gene into template strain. We still have to verify the results using SDS-PAGE, HPLC, and MFA.

The experiment next week will be very similar to this week, except for the gene fragment will be coming from *B. Adolescentis* instead of C. *acetobutylicum.*