7.24 the primer was sent to be produced. The prefix is Bam Ⅰ and the suffix is Xba Ⅰ.
At 18:30, recovery the E.col. The mediums were added the Ampicillin and be put under 37℃.
We then use Liquid LB medium to Expand training. But this time we forgot to layout the NC.

7.25 we Picked monoclonal colonies, The strain was inoculated into liquid LB medium. Ampicillin was added to the medium 5 minutes after the inoculation. Ampicillin was not added to the partially cultured medium and was discarded.
Four tube plasmids were extracted, 100 microliters per tube. The concentration is around 150 ng/μl, and the nucleic acid quantitation meter may not be accurate.

7.26 to find the optimum annealing temperature，design the PCR system as below：

MCLAB mix	12.5μL
Primer1	1μL
Primer2	1μL
Template	0.7μL
dd water	9.8μL
Total	25μL

condition：98℃for 2min，【98℃for 10s，56-63℃for 15s，72℃for 20s，】×35，72℃for 5min
Among the above groups, the result of the second group was the best, and the optimum annealing temperature was 59 ° C, and a similar effect was obtained at 60 ° C.
Further amplification of the sequence by using the same condition ：98℃for 2min，【98℃for 10s，59℃for 15s，72℃for 20s，】×35，72℃for 5min
Store the product at 4℃.

7.27 Transformation：50μLDH5α+1.5μLplasmid（150ng/μL）。After the transformation，inoculate the strain to plate mediums which were added Ampicillin. Let the strain grow for a night.
Verified the product from yesterday's PCR, and recovery the gel. The products of recovery is 30μL in total, store them at 4℃.

7.28 The PCR gel recovery product of 7.27 and the PCR product of 7.26 were subjected to enzyme digestion verification. The system is as below：

1		2		3	
10×buffer	10μL	10×buffer	6μL	10×buffer	10μL
BamH Ⅰ	1μL	BamH Ⅰ	4μL	BamH Ⅰ	1μL
EcoR Ⅰ	1μL	EcoR Ⅰ	1μL	EcoR Ⅰ	1μL
dd water	8μL	dd water	1μL	dd water	28μL

PCR gel recovery	30μL	PCR product	18μL	PCR product	10μL
total	50μL	total	30μL	total	50μL

(the BamH Ⅰ and EcoR Ⅰ we used were all fast endonuclease)

1 and 2 were all in 30℃ for 5min by enzyme digestion，then heated to 60 C for 10min to inactivation. Finally, it was stored at 4℃.

We get the valid primers and designed PCR (PCR Ⅱ) to determine the optimal annealing temperature again.

In addition, in order to test whether the ampicillin in the laboratory failed, we used DH5α as a control group, and streaked with PET21α , and diluted to 100-fold and 1000-fold with the transformation product, and then inoculated onto the plate medium.

7.29 We get to know the ampicillin is failed from yesterday's results.

We made a double enzyme confirmation for the product.

7.30 No experiments were conducted today due to the lack of some essential enzymes.

7.31 1、Determination of the gel recovery concentration of the product(PCR Ⅱ)

The concentration is 1975ng/mL and 1368ng/mL.

A260/A280 2.724 *A260/A230 0.872*

2、PCR

Mix	50μL
Primer3	4μL
Primer4	4μL
Template	2.8μL
ddWater	38.2μL
Total	100μL

condition：98℃for 2min，【 98℃for 10s，59℃for 15s，72℃for 20s，】×35，72℃for 5min

Then store the products at 4℃.

The concentrations were determined separately and did a glue recycling.The PCR products were sent to sequencing.

3、Extract plasmids and transform them into PET21α.

4、After the transformation, inoculate the strain to the plate medium.

8.1

1、Measuring the concentration of Pet21α，and the result was about 80ng/μL。

2、Enzyme digestion system：

	1	2
10×buffer	5μL	5μL
PCR(1、2、3、4、gel recovery)	2μL	4μL
PET21α	5μL	2μL

Xho Ⅰ	1μL	1μL
EcoR Ⅰ	1μL	1μL
dd water	36μL	37μL
Total	50μL	50μL

condition：40min，37℃。Heated：15min，80℃。

3、test the product of enzyme digestion。

4、purification：FMO enzyme purification。

5、the system of Connection：

10× bigation buffer	10μL
DNA	40μL
T4 ligase	5μL
dd water	45μL
Total	100μL

	1	2	3	4	5	6
10×buffer	10.5	10.5	10.5	10.5	10.5	10
DNA	40	40	40	40	40	40
T4	10	10	10	10	10	5
dd water	45	45	45	45	45	45
Total	105	105	105	105	105	100

8.3 12:14 Two single colonies were selected from each plate inoculated with the transformed bacteria. There were 14 tubes (12 tubes of bacteria, 2 tubes of blank control).

Bacteria PCR:

	单管	合计
T5super PCR mix	12.5μL	175μL
Primer3	1μL	14μL
Primer4	1μL	14μL
Bacteria	2μL	28μL
dd water	8.5μL	119μL

condition：98℃for 3min，【98℃for 10s，59-64℃for 15s，72℃for 28s，】×29，72℃for 2min

The results are all positive。

The plasmid was extracted. Did a PCR amplification.

Mix	25μL
Primer3	2μL
Primer4	2μL
Template	3μL
dd water	18μL

Solid medium with tryptophan and Ampicillin。

Tryptone	2g
Yeast extract	1g

NaCl	2g
Agar	3-4g
Tryptophan	0.4g

Enzyme digestion system:

Buffer	2.5μL
PET21α-FMO	10μL
Nde I	0.5μL
PST I	0.5μL
dd water	11.5μL
Total	25μL

MCLAB Mix	12.5μL
Primer3	2μL
Primer4	2μL
Template	1.4μL
dd water	7.1μL

Enzyme digestion: (μL)

	1(Empty plasmid Single digestion)	2 (PET21 α Double digestion)	*3 (PET21α)*	4 (FMO PCR)	5 (NC)
10 × green buffer	2.5	2.5	2.5	2.5	2.5
EcoR I	1	1		1	1
PST I		1		1	1
Sample	4	4	4	4	
ddwater	11.5	11.5	11.5	11.5	11.5

Enzyme digestion system:

	Recovery	recovery1	FMO1	FMO2	FMO3
10 × green buffer	5μL	5μL	5μL	5μL	5μL
PCR	4μL	4μL	4μL	4μL	4μL
PET21α	2μL	2μL	2μL	2μL	2μL
Xho I	1μL	1μL	1μL	1μL	1μL
EcoR I	1μL	1μL	1μL	1μL	1μL
dd water	37μL	37μL	37μL	37μL	37μL
Total	50μL	50μL	50μL	50μL	50μL

Condition: 37℃ 40min, 80℃ 15min

The product was recovered and verified by PSTI digestion., 37℃, 20min。

10×green buffer	2.5μL
Pst I	0.5μL
Product	4μL
dd water	18μL

8.6 PCR system

	1	2	3	4
Mix	12.5μL	12.5μL	12.5μL	12.5μL
Primer3	2μL		2μL	
Primer4	2μL		2μL	
Template	1.4μL	1.4μL	1.4μL	1.4μL
dd water	7.1μL	11.1μL	7.1μL	11.1μL
Plasmid	+	+	-	-

Enzyme digestion system：

	FMO Glue recovery product	PET-21α	NC
10×G buffer	5μL	5μL	1.5μL
对应样品	30μL	15μL	3μL
Xho Ⅰ	1μL	1μL	0.5μL
EcoR Ⅰ	1μL	1μL	0.5μL
dd water	13μL	28μL	9.5μL
Total	50μL	50μL	15μL

The first two sets of experimental products were purified by column.

Connection：

	3×	5×	7×
10×T4 buffer	4μL	4μL	4μL
T4	3μL	3μL	3μL
FMO Fragment	24μL	25μL	24.5μL
PET-21α	8μL	5μL	3.5μL
dd water	1μL	3μL	5μL

8.11

Indole Medium: add 75 mg indole per liter into the medium. And add 1.5% IPTG in proportion.

After incubation at 37 ° C for 3 h, IPTG was added and incubation was continued for 3 h.

The transformation was carried out with BL21, and some colony was selected and compiled into No. 1-7, and the results of No. 1 and No. 2 were better.

The colony was transferred into liquid medium. After about 10 hours, the miedium turned blue.

500 μL of the bacterial solution was taken and added to 4.5 mL of ampicillin-containing liquid LB for a while. After 3 hours, IPTG was added, and after 6 hours, hydrazine was added. At the same time, in order to verify whether the expression is leaked, the above process was repeated with wild LB21.

In addition, it is also planned to: 1 further improve the expression conditions (try to add IPTG and indole after different time after inoculation); 2 *expression contrast, and verify with a spectrophotometer.*

8.13

PET21α-FMO indigo expression concentration and IPTG induced concentration

After incubation at 37 ° C for 3 h, IPTG and 75mg/mL indole was added and incubation was continued for 6 h.

Indigo was detected by an ultraviolet spectrophotometer.

IPTG(mM)	0.25	0.325	0.5	0.625	0.75
Indigo(A)	0.3	0.374	0.383	0.365	0.252

8.15

PET21α-FMO indigo expression concentration and indole concentration

After incubation at 37 ° C for 3 h, 0.5mM IPTG and indole was added and incubation was continued for 6 h.

Indigo was detected by an ultraviolet spectrophotometer.

Indole(mg/mL)	0	37.5	75	150	300	600	NC
Indigo(A)	0.610	1.006	1.417	1.916	1.709	0.062	0.093

8.22

PET21α-FMO indigo expression concentration and indole concentration

After incubation at 37 ° C for 3 h, 0.5mM IPTG and 75mg/mL indole was added and incubation was continued for 0 0.5 1 2 4 6 8 24 h.

Indigo was detected by an ultraviolet spectrophotometer.

	0	0.5	1	2	4	6	8	24
FMO1（iptg+,indo+)	0.1817	0.3213	0.4537	0.7173	0.5063	0.5727	0.624	0.69
FMO2（iptg-,indo-)	0.2463	0.2043	0.3487	0.3027	0.2807	0.3387	0.3177	0.2973
FMO（iptg+,indo+)	0.347	0.4373	0.501	1.304	1.0757	0.977	0.9213	1.0733