NOTEBOOK

FJNU-China

XUEYI'S

NOTEBOOK

WEEK1 05 July-11 July Pre-preparation of circuit and remove KANA

First human practice

05.07

1. Prepared LB solid plates and liquid medium.

2. Coating cultured BW25113 for 12h

3. Prepared Ampicillin and Kanamycin for working concentration

4. Prepared Kanamycin plate, underline cultured pET28a for 12h

06.07

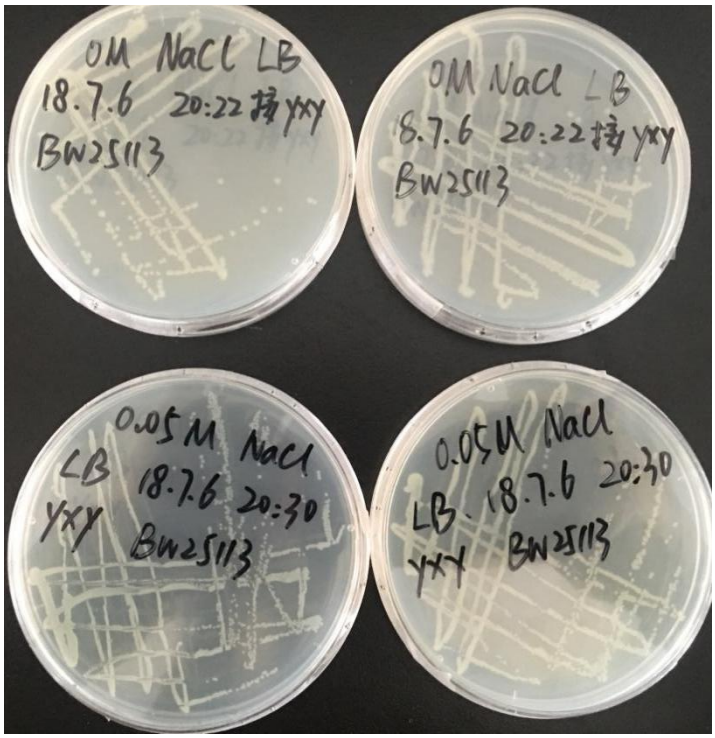
1. Shook single BW25113 colony for 12h

2. Shook single pET28a in Kanamycin liquid medium for 12 hours

3. Prepared 4 LB plates, two of them are salt-free, the rest of them with 0.05M NaCl, and then underline cultured BW25113 for 12h.

07.07

1. BW25113 grew normally in salt-free plates and 0.05M plates.



2. Underline cultured the bacteria with hns-synthesis plasmid we designed before on ampicillin plate.

3. Held a summer camp with Quanzhou 7th High school and Fuzhou 8th High school for 2 days, we had a really good time.

08.07

1. The second day of summer camp with Fuzhou 8th high school and Quanzhou 7th high school, taught them how to extract plasmid from bacteria.

2. Made 8 tubes of competent cells with Junru.

3. Retained the species of hns-top10 with 15% glycerol.

09.07

1. Discussed how to design the primer of eGFP and lacI gene with our P.I.

2. Visited the previous member of iGEM team FAFU-CHINA, gained

experience about wiki-making and experiments.

3. Extracted 2 tubes of hns-top10 plasmid and used nanodrop to detect the concentration of hns-top10 and pET28a plasmid. (The concentration is too low. Maybe it is because the shaking time is not enough for hns-top10 strain.)

4. Transform 4ul pcp20 plasmid into the competent cells.

Protocol:

1.Defrost cells on ice. Take competent hns-/- and tnaA cells
2. Add 4ul of plasmid DNA.
3. Put on ice for 30min.
4. Heat shock at 42℃ in a water bath for 90s
5. Return to ice for 3-5min
6. Add 1ml of LB medium
7. Incubate at 37℃ in shaking table for 1 hour.

5. Coating cultured the incubated cells on ampicillin plates overnight.

10.07

1. Pick 16 single colonies from the ampicillin plates, underline cultured on kanamycin plates to test if successfully remove the resistance of kanamycin.

2. Extracted 4 tubes of hns-top10 plasmid and 2 tubes of pET28a plasmid. I detected the concentration of plasmid using nanodrop.

July 12, second team meeting with our PI

Week2 13 July-20 July Pre-preparation of circuit and remove KANA

13.07

1. I picked the colony which didn't grow on kanamycin but grew on ampicillin into medium without resistence, shaking cultured at 43℃ for

12h and underline cultured on plate.

2. I extracted plasmid with Hanrong.

Hns-top10①：114.5ng/ul	pET28a①：34.8ng/ul
260/230：1.88	260/230：1.92
260/280：1.84	260/280：1.53
Hns-top10②：115.9ng/ul	pET28a②：31.5ng/ul
260/230：1.89	260/230：1.99
260/280：1.91	260/280：1.47

3. I picked the single colony into amp and kana liquid medium to check the kanamycin if successfully been removed.

14.07

1. PCR lacI with 15ul system.

Mix 7.5ul	95℃ 3min
F primer 1ul	94℃ 35s
R primer 1ul	61℃ 30s
Plasmid 0.7ul	72℃ 3min X30
ddH2O 5.8ul	72℃ 10min

2. I got the plasmid of eGFP and transformed into competent BW25113

3. I prepared 4 kanamycin plates.

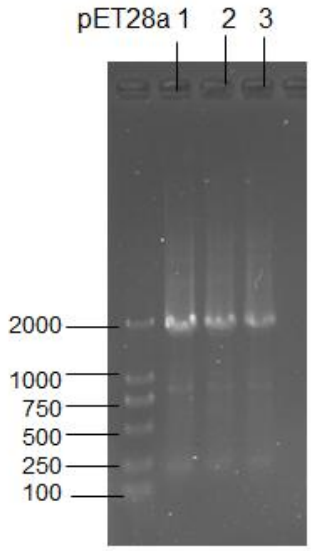
4. $HNS^{-/-}$ strain grew in kanamycin, change the new ampicillin and try again.

15.07

1. I made competent cells of HNS-/-, and transformed pcp20 into it.

2. I picked the single colony of eGFP into liquid medium with kanamycin, shaking culture for 12h.

3. I changed the Tm of lacI-PCR into 67, 69℃, and choose the 69℃ to expand system.



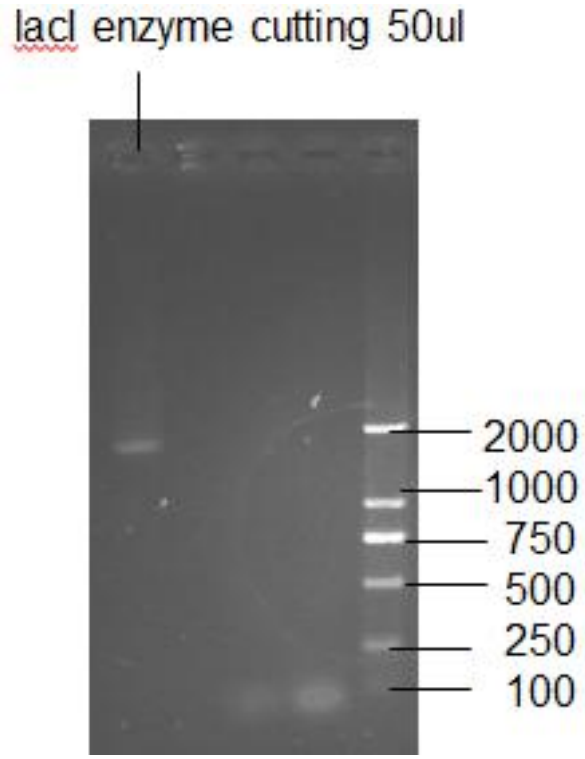
16.07-18.07

1. I expand the system of lacI-PCR into 50ul. After gel electrophoresis I found the single band of lacI, then made cycle pure and detected the concentration.

lacI fragment: 35.865 ng/ul

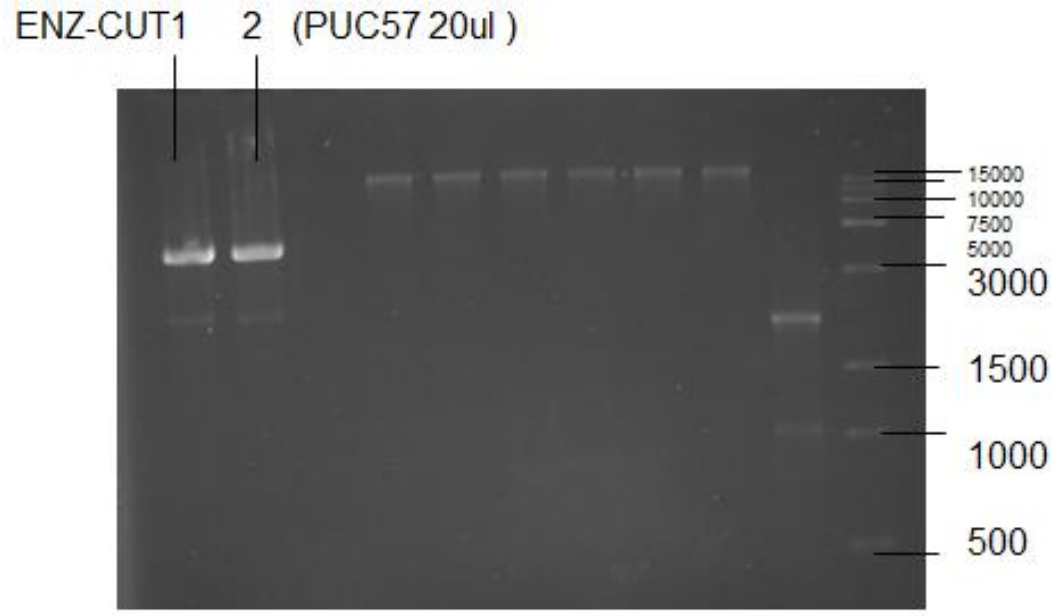
260/230：1.51

260/280：2.02

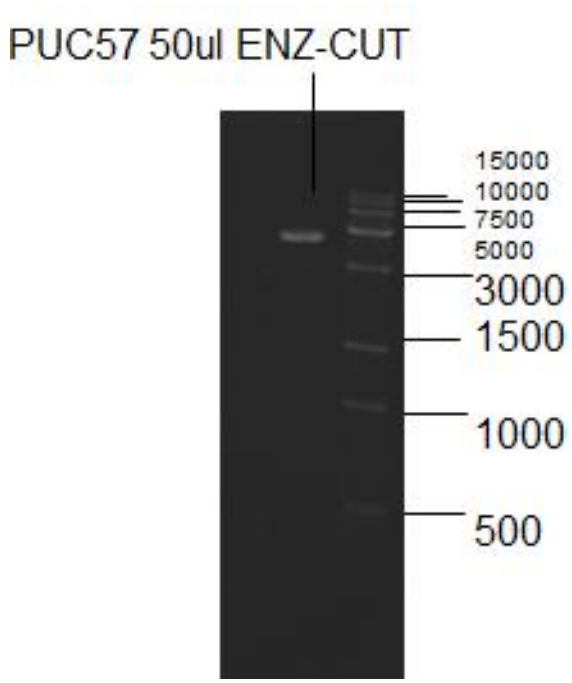


2. I used the enzyme Nsi1 and BsiW1 to cut the circuit we synthesis in 20ul and check the efficiency of enz-cutting with gel electrophoresis

Plasmid 14ul
Nsi1 1ul
BsiW1 1ul
Cut-smart 2ul
ddH2O 2ul
37℃ in aquatic bath for 5h



There were two bands on the gel and I suspected the time is too short to cut the plasmid completely. When I expanded it into 50ul, I left it for almost 5.5h.



Then I added all enz-cut-syn together and did the gel extraction. I detected the final concentration after enz-cutting.

PUC57 enz-cut: 104.441ng/ul
260/230: 0.35
260/280: 1.95

3. I picked 3 single eGFP colony into liquid medium, shaking culture for 12h.

19. July, third team meeting with our PI

19.07

1. I extracted plasmid contained eGFP with Hanrong.

eGFP①：32.377ng/ul
260/230：1.40
260/280：2.30
eGFP②：61.717ng/ul
260/230：1.64
260/280：2.03

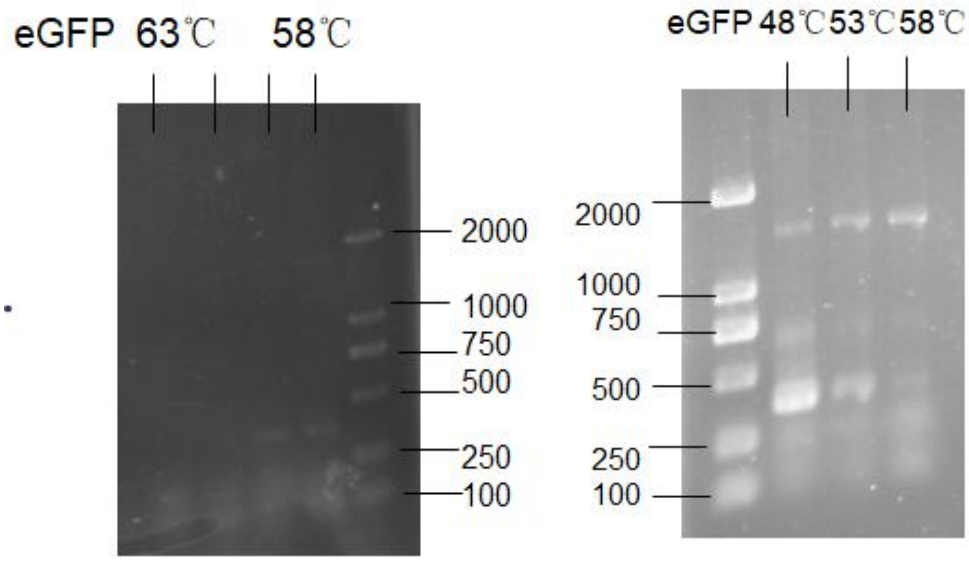
Week 3 20.July-28.July Gibson assembly and construct circuit

20.07-21.07

1. PCR eGFP (15ul)

Mix7.5ul	95℃ 3min
F primer 0.5ul	94℃ 35s
R primer 0.5ul	58/61/63/65/68℃ 30s
Plasmid 0.7ul	72℃ 3min X30
ddH2O 5.8ul	72℃ 10min

Setting the different Tm, but I didn't find the single band of eGFP.



2. I made 10 tubes of competent BW25113.

3. Our team prepared the paperwork for the visa interview and discussed the poster used in the iGEM Eurasian Meetup 2018.

22.07-26.07

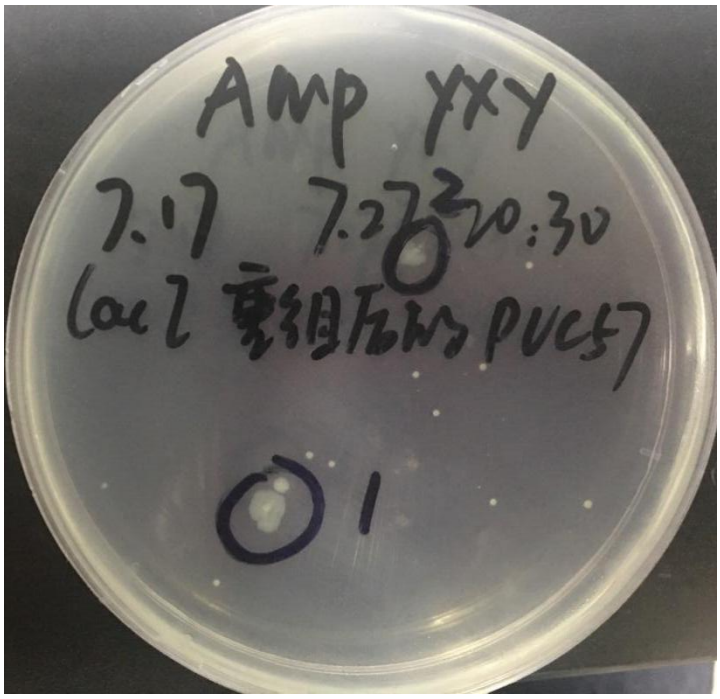
We went to Guangzhou for visa's interview and Shenzhen to attended the iGEM Eurasian Meetup 2018. We found something useful to our project and focus on how to improve our project.

27.07-28.07

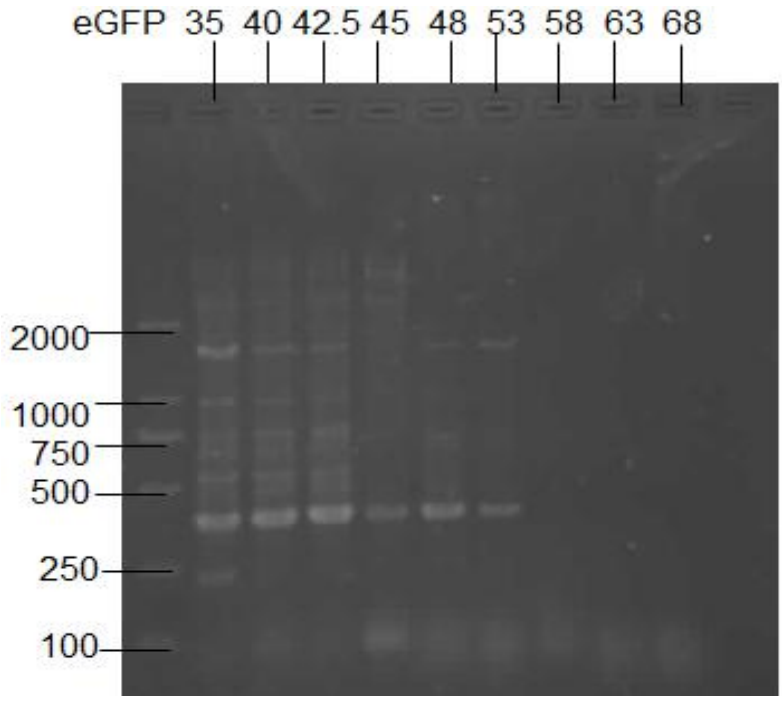
1. I did the T4 ligation for lacI and the circuit we synthesized and transformed 4ul recombined plasmid into competent BW25113 immediately, coating cultured on amp plate overnight.

Protocol：

1. Set a 10ul system as followed.
   lacI:9.191ng/ul*7.55ul
   circuit:52.22ng/ul*0.5ul
   T4 ligase: 1ul
   T4 buffer(10x): 1ul
2. 22℃ in aquatic bath for 3h
3. Add 4ul plasmid into competent BW25113
4. Put on ice for 30min.
5. Heat shock at 42℃ in a water bath for 90s
6. Return to ice for 5min
7. Add 1ml of LB liquid medium
8. Incubate at 37℃ in shaking table for 1 hour.
9. Centrifugalize with 10000rpm, 1min
10. Remove 900ul of supernatant, and suspend the rest liquid
11. Drawn 100ul coating culture on amp plate.



2. I compared all the PCR products in different Tm, choose 42.5 to expand the system.



29 July, forth meeting with our PI

Week5 29.July-04.Aug Verify the Gibson assembly and construct circuit

30.07

1. I extracted plasmid of recombine and eGFP with Xinyue and Hanrong.

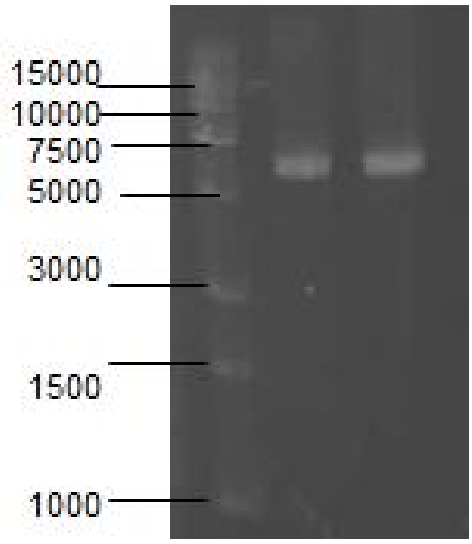
eGFP①：49.667ng/ul
260/230：1.64
260/280：1.94
eGFP②：23.119ng/ul
260/230：1.28
260/280：1.82
eGFP③：69.949ng/ul
260/230：1.52
260/280：1.81
eGFP④：28.781ng/ul
260/230：1.33
260/280：1.94

lacI-hns-syn①：104.353ng/ul
260/230：1.95
260/280：2.04
lacI-hns-syn②：117.127ng/ul
260/230：1.91
260/280：2.08
lacI-hns-syn③：109.531ng/ul
260/230：1.91
260/280：2.04
lacI-hns-syn④：131.563ng/ul
260/230：2.00
260/280：2.05

2. Cut the recombined plasmid with Kpn1 and Dra3 (50ul)

Plasmid 20ul
Kpn1 1ul
Dra3 1ul
Cut-smart 5ul
ddH2O 23ul

37℃ in aquatic bath for 5h



31.07

1. I did the cycle pure of cutting recombine plasmid and detected the final concentration.

Plasmid cycle pure: 18.048ng/ul
260/230: 1.40
260/280: 1.78

2. PCR eGFP (50ul)

Mix 25ul	95℃ 3min
F primer 1ul	94℃ 35s
R primer 1ul	42.5℃ 30s
Plasmid 1.1ul(49.667ng/ul)	72℃ 3min X30
ddH2O 21.9ul	72℃ 10min

3. Gel extraction of eGFP

4. I made 10 tubes of competent cells of BW25113

01.08

1. Cut eGFP with Kpn1 and Dra3 (30ul)

Plasmid 13ul (22.238ng/ul)
Kpn1 1ul
Dra3 1ul
Cut-smart 3ul
37℃ in aquatic bath for 5h

2. I did the cycle pure of eGFP after cutting and detected the final concentration.

eGFP cycle pure: 19.086ng/ul

3. T4 ligation overnight. (10ul)

Fragment: 5.9ul
Backbone: 2.1ul
T4ase: 1ul
T4 buffer: 1ul
16℃ in aquatic bath overnight

02.08

1. I transformed combined plasmid with eGFP into competent BW25113

Protocol:

1. Add 4ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃, 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 10000rpm, 1min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on amp plate.

2. I prepared change the backbone of the plasmid from PUC57 into pRB1S, same as the backbone of PLA, which from the strain of BM4R.

03.08

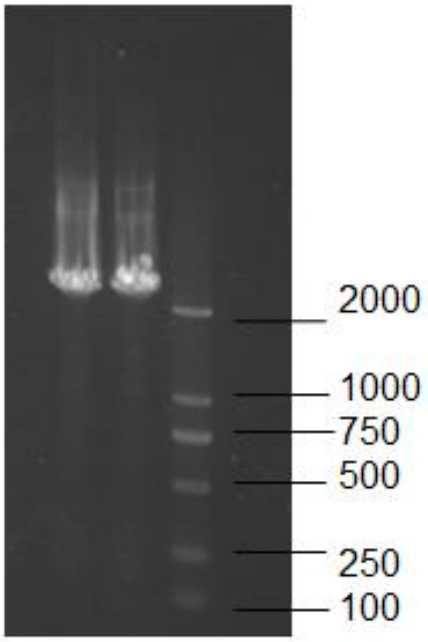
1. I picked two single colony of hns-syn-eGFP. After shaking cultured for 16h, I found the medium are still clear.

2. PCR BM4R

I used the plasmid extracted by Xinyue a few days ago. (First 15ul and expanded into 50ul after confirming the PCR's parameters)

Mix7.5ul
F primer 0.5ul
R primer 0.5ul
Plasmid 0.7ul

ddH2O 5.8ul
95℃ 3min
94℃ 35s
63℃ 30s
72℃ 3min X30
72℃ 10min



3. Gel extraction of fragment of BM4R and concentration detected

Backbone BM4R①: 422.576ng/ul
260/230: 0.98
260/280: 1.98
Backbone BM4R②: 432.178ng/ul
260/230: 2.03
260/280: 2.00

04.08 Fifth team meeting with our PI

04.08

1. Colony PCR of hns-syn-eGFP. There is no band on the gel. This determines I failed to insert the eGFP into our circuit, thus, our team decided to use GFP to character our circuit.

Week 5 5.Aug-11.Aug Verify the Gibson assembly and construct circuit

05.08

1. PCR hns-syn (50ul)

Mix 25ul	95℃ 3min
F primer 1ul	94℃ 35s
R primer 1ul	42.5℃ 30s
Plasmid 0.8ul	72℃ 3min X30
ddH2O 22.2ul	72℃ 10min

2. Gel extraction of hns-syn and concentration detected

Fragment hns-syn: 134.057ng/ul

260/230: 0.67

260/280: 1.90

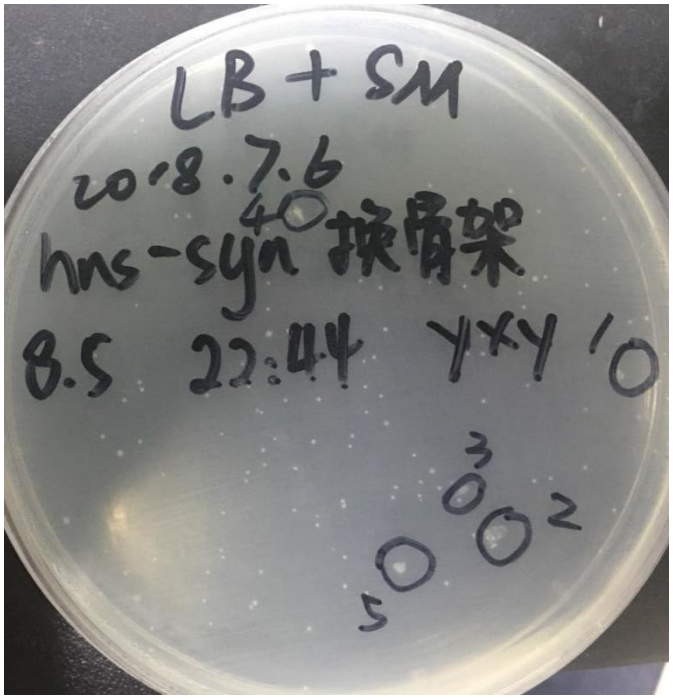
3. I firstly learned the Gibson assembly with Junru.

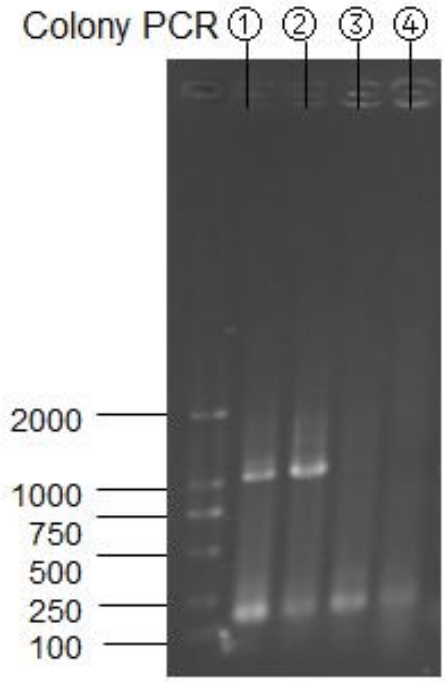
Protocol:

1. According the equation of fragment/backbone=3 and $V_{fragment}+V_{backbone}=4ul$, calculate the volume of backbone and fragment.
2. Mix the correspond volume and 6ul Gibson enzyme together, and 50℃ in metal bath for 60 min.
3. When assembly left 5 minutes, get the competent cells from -80℃ refrigerator.
4. Take all of assembly products (10ul) into competent cells, and put on ice for 30 min.
5. Heat shock at 42℃ in water bath for 90s.
6. Back to ice for 2 min.
7. Add 800ul SOC medium into cells (this step must complete in bechtop).
8. Incubate at 37℃, 120rpm in shaking table for 1 hour.
9. Centrifugalize with 3000 rpm, 5min.
10. Remove 900ul of supernatant, and suspend the rest liquid.
11. Drawn 100ul coating culture on plate with streptomycin, 37℃ 12h.

06.08

1. I picked 5 single colonies into liquid medium with streptomycin, shaking cultured 37℃ for 16h.





2. Colony PCR to check the assembly if success.

Mix 7.5ul
Primer F 0.5ul
Primer R 0.5ul
Colony 3ul
ddH_2O 3.5ul

Two of them have clearly bands, retained the species of pRB1S-hns-syn

with 15% glycerol.

3. I extracted plasmid of pRB1s-hns-syn.

pRB1s①：184.268ng/ul
260/230：2.10
260/280：2.00
pRB1s②：148.083ng/ul
260/230：2.06
260/280：2.02
pRB1s③：153.168ng/ul
260/230：2.06
260/280：1.99
pRB1s④：199.548ng/ul
260/230：2.01
260/280：1.98

07.08

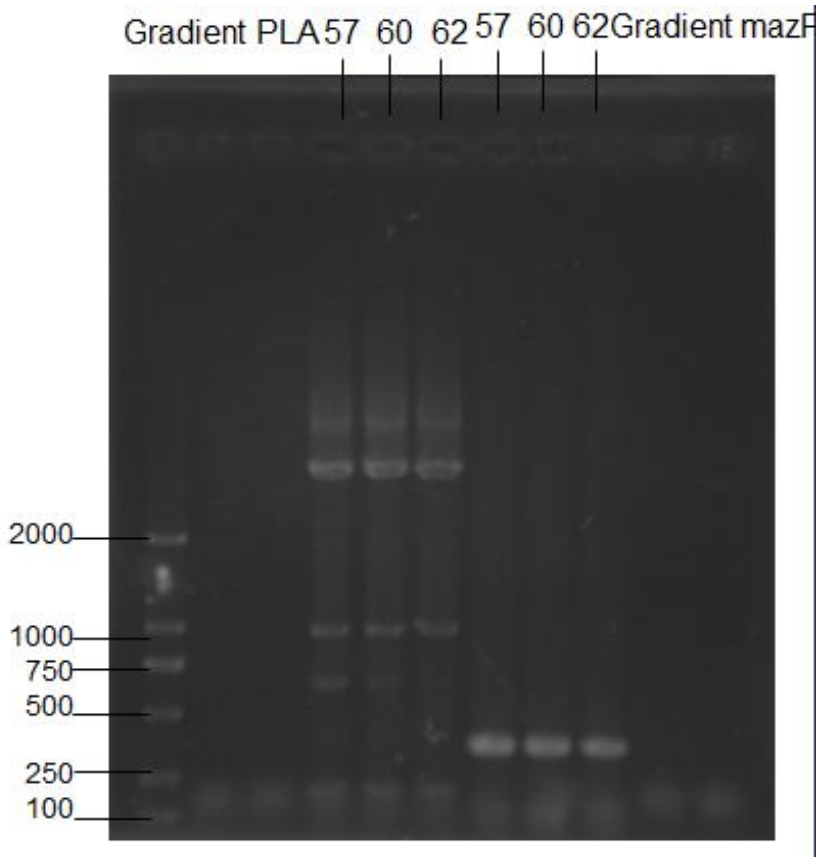
1. Gradient PCR PLA and mazF (15ul)

Mix7.5ul
F primer 0.5ul
R primer 0.5ul
Plasmid 0.7ul
ddH2O 5.8ul
95℃ 3min

94℃ 35s	72℃ 3min X30
57/60/62℃ 30s	72℃ 10min

2. PCR PLA and mazF (50ul, Tm=60℃)

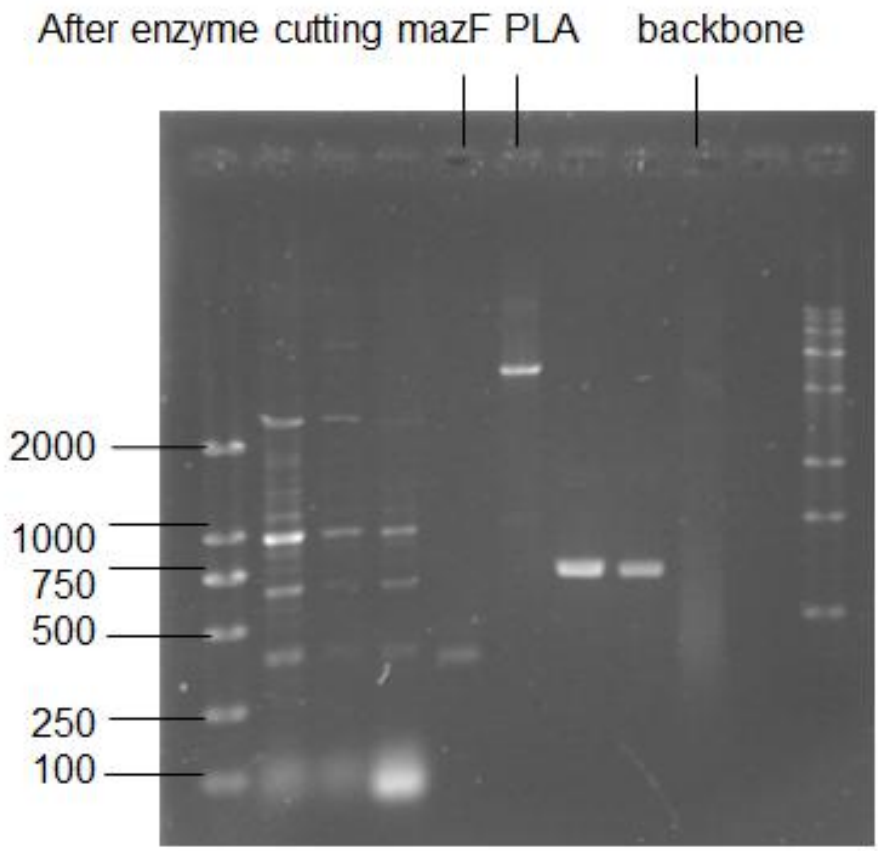
Mix 25ul	95℃ 3min
F primer 1ul	94℃ 35s
R primer 1ul	60℃ 30s
Plasmid 0.8ul	72℃ 3min X30
ddH2O 22.2ul	72℃ 10min



3. I did gel extraction to purified PLA and cycle pureed mazF.

PLA: 38.757ng/ul
260/230：0.13
260/280：1.96
mazF: 11.879ng/ul
260/230：0.84
260/280：1.70

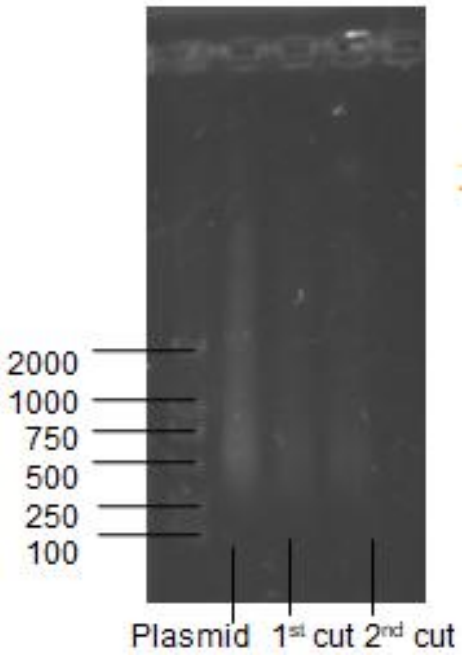
4. Enzyme cutting pRB1s, PLA and mazF with Kpn1 and Dra3.



09.08

1. I did a gel electrophoresis to verify the enzyme cutting efficiency of PLA, mazF and pRB1s, then cycle pureed.

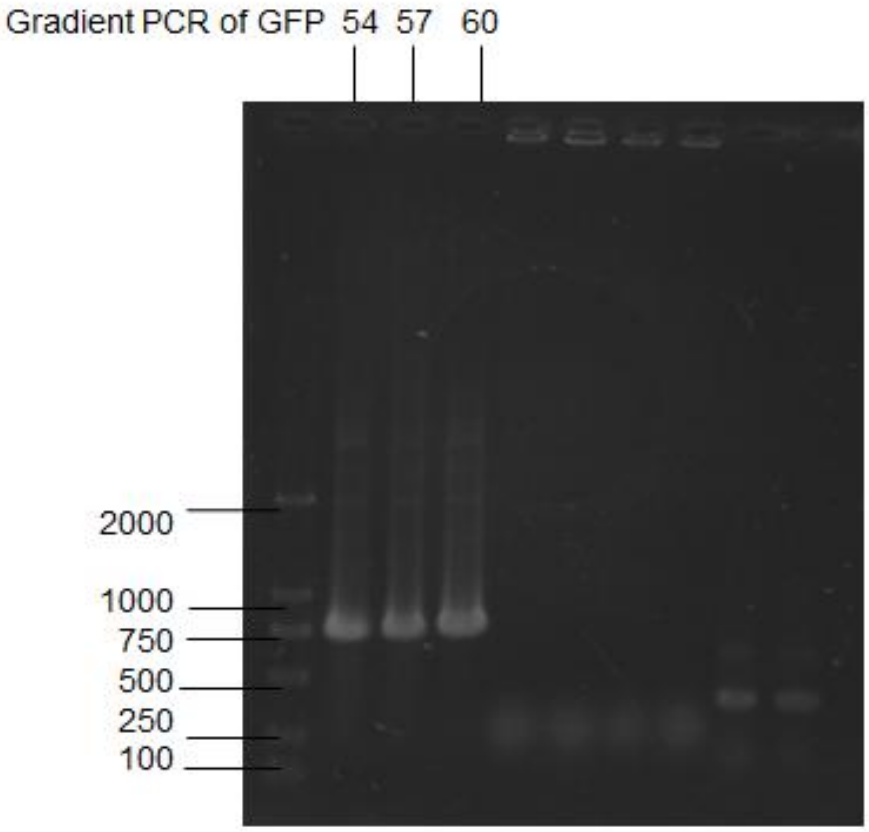
But the plasmid did not have any band, so I ran a gel electrophoresis with plasmid. Then I found that there was something wrong with the plasmid.



2. I picked two tubes of pRB1s-hns-syn, shaking cultured at 37℃ 220rpm, 12h.

3. GFP primer arrived. I did a gradient PCR of GFP, and expanded the volume from 15ul to 50ul.

4. Prepared LB solid plates and liquid medium with Xinyue.



08.10

1.Gel extraction of GFP and concentration detected.

GFP①：299.986ng/ul
260/230：1.44
260/280：1.94

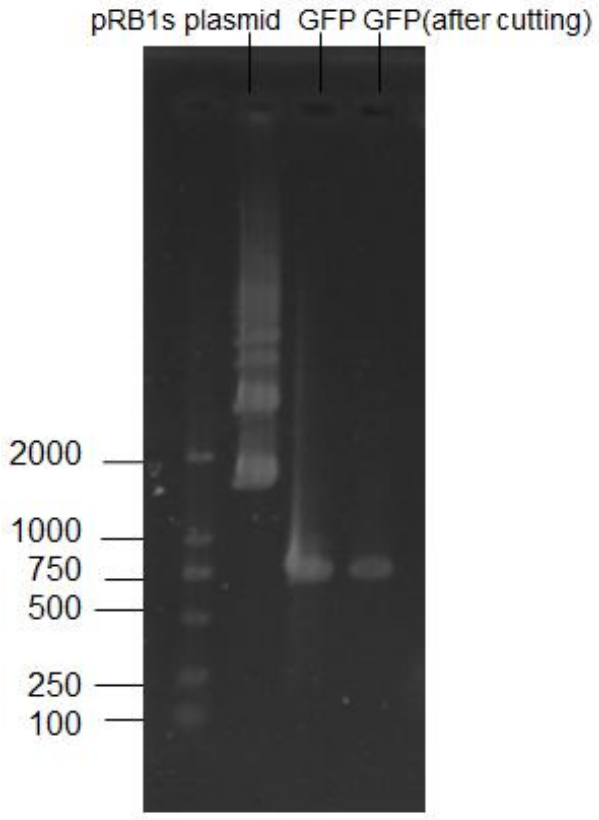
2. I designed GFP enzyme cutting system (50ul)

Plasmid 7ul
Kpn1 1ul
Dra3 1ul
Cut-smart 5ul
ddH2O 36ul
37℃ in aquatic bath for 5h

3. I extracted plasmid of pRB1s-hns-syn, during waiting for enzyme

cutting.

pRB1s-hns-syn①：716.495ng/ul
260/230：2.22
260/280：2.00



4. I did cycle pure after enzyme cutting and designed T4 ligation system.

Fragment: 4.42ul
Backbone(PUC57 after-cutting): 3.58ul
T4ase: 1ul
T4 buffer: 1ul
22℃ in aquatic bath 3h

5. I transformed combined plasmid into competent cells immediately.

Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃， 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid

9. Drawn 100ul coating culture on amp plate.

Week 6 11.Aug-16.Aug Poster prepare and construct circuit

11.08-12.08

1. I picked 5 single colonies into liquid medium with ampmycin, shaking cultured 37℃ for 16h.

2.Colony PCR to check the insert if success.

Mix 7.5ul
Primer F 0.5ul
Primer R 0.5ul
Colony 3ul
ddH_2O 3.5ul

There was no band on the gel, maybe had picked false postive colony. Then I picked another 3 single colonies, it didn't have any bands after colony PCR.

3. I cut pRB1s-hns-syn using Spe1 and Xba1 to check the assembly if success.(50ul)

pRB1s-hns 3ul
Spe1 1ul
Xba1 1ul
Cut-smart 5ul
ddH_2O 40ul

The band on the gel was a smear, so I can't determine it clearly.

12.08

1. I failed to compelete T4 ligation maybe because the propotion of frangment and backbone is not enough. Then I changed the propotion and did the ligation again.

Fragment: 7.39ul
Backbone(PUC57 after-cutting): 0.61ul
T4ase: 1ul

T4 buffer: 1ul
16℃ in aquatic bath overnight

2. I transformed the combined plasmid into competent cells immediately.

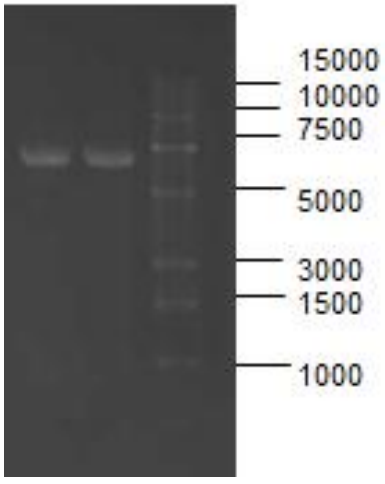
Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃, 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on amp plate.

13.08

1. I picked 8 single colonies into liquid medium with ampmycin, shaking cultured 37℃ for 16h.

2. Another reason for the ligation failure is the backbone have mistaken belt. So I cut and pured the backbone PUC57 with kpn1 and Dra3 again.

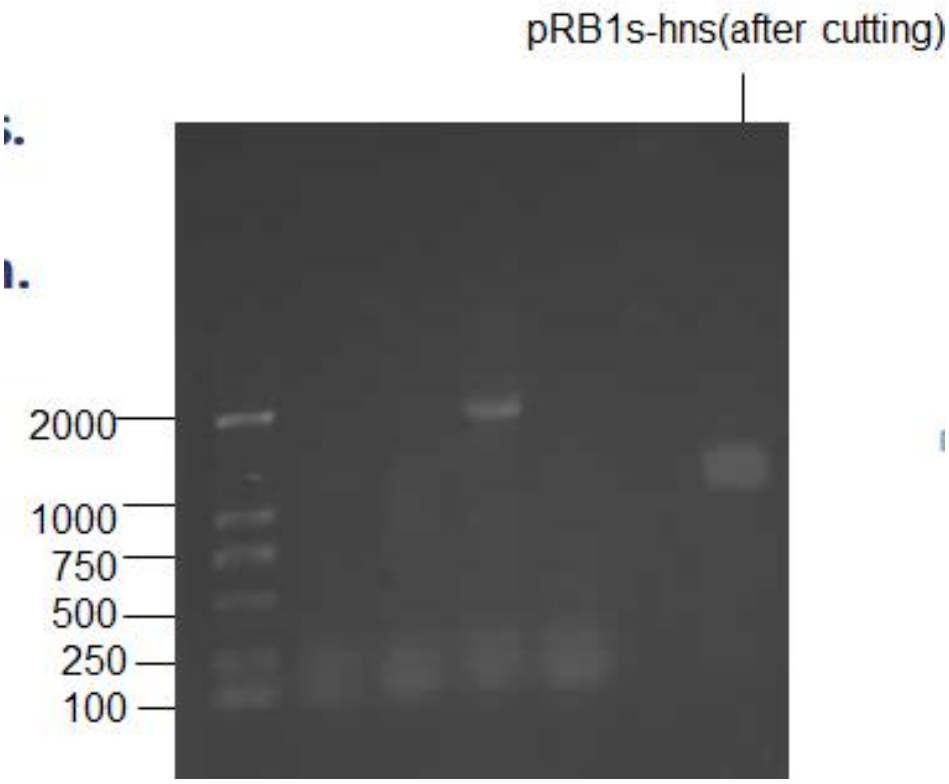


14.08

1. I made 10 tubes of competent cells.

2. I ran the pRB1s-enz cutting on the gel again and it's still a smear. I

thought if the enzyme cutting time should be extend. (And we ran out of the Spe1, maybe I didn't add enough enzyme to the system.)



15.08

1. I found the gibson plate I used to change backbone and picked another 5 colonies, shaking culture at 37℃ 12h.

2. Colony PCR to check the new colony if assembly succeed.

Mix 7.5ul
Primer F 0.5ul
Primer R 0.5ul
Colony 3ul
ddH_2O 3.5ul

There was no band on the gel.

3.I finish the design of our project title and navigation of wiki.

16.08

1. I designed the poster which will used in CCIC and finished the

expriment record.

Week 7 17.Aug-24.Aug Communicate with XMU and construct circuit

17.08-18.08

We went to Xiamen University and discussed our projects to each other.

19.08

We visited Environmental Protection Agency and got advices from specialist to better our project.

20.08

1. Cut the plasmid, GFP and PLA with Kpn1 and Dra3 (50ul)

Plasmid (173.416ng/ul) 8ul GFP (359.13ng/ul) 4ul PLA (38.861ng/ul) 30ul
Kpn1 1ul
Dra3 1ul
Cut-smart 5ul
ddH2O up to 50ul
37℃ in aquatic bath for 5h

2. I prepared poster for 5th ccic in Shanghai.

21.08

1. I cycle pured the plasmid, GFP and PLA and detected the congcentration.

2. T4 ligation for GFP and PLA

Fragment: 2.36ul
Backbone(PUC57 after-cutting): 5.64ul
T4ase: 1ul
T4 buffer: 1ul
22℃ 3h in aquatic bath

3. I cut pRB1s-hns-syn using Spe1 and Xba1 to check the assembly if success.(50ul)

pRB1s-hns (716.495ng/ul) 2ul

Spe1 1ul
Xba1 1ul
Cut-smart 5ul
ddH_2O 41ul

3. I did gibson aseembly again.

Protocol:

1. According the equation of fragment/backbone=3 and $V_{fragment}+V_{backbone}=4ul$, calculate the volume of backbone and fragment.(B=0.66ul,F=3.34ul)
2. Mix the correspond volume and 6ul Gibson enzyme together, and 50℃ in metal bath for 60 min.
3. When assembly left 5 minutes, get the competent cells from -80℃ refrigerator.
4. Take all of assembly products (10ul) into competent cells, and put on ice for 30 min.
5. Heat shock at 42℃ in water bath for 90s.
6. Back to ice for 2 min.
7. Add 800ul SOC medium into cells (this step must complete in bechtop).
8. Incubate at 37℃, 120rpm in shaking table for 1 hour.
9. Centrifugalize with 3000 rpm, 5min.
10. Remove 900ul of supernatant, and suspend the rest liquid.
11. Drawn 100ul coating culture on plate with streptomycin, 37℃ 12h.

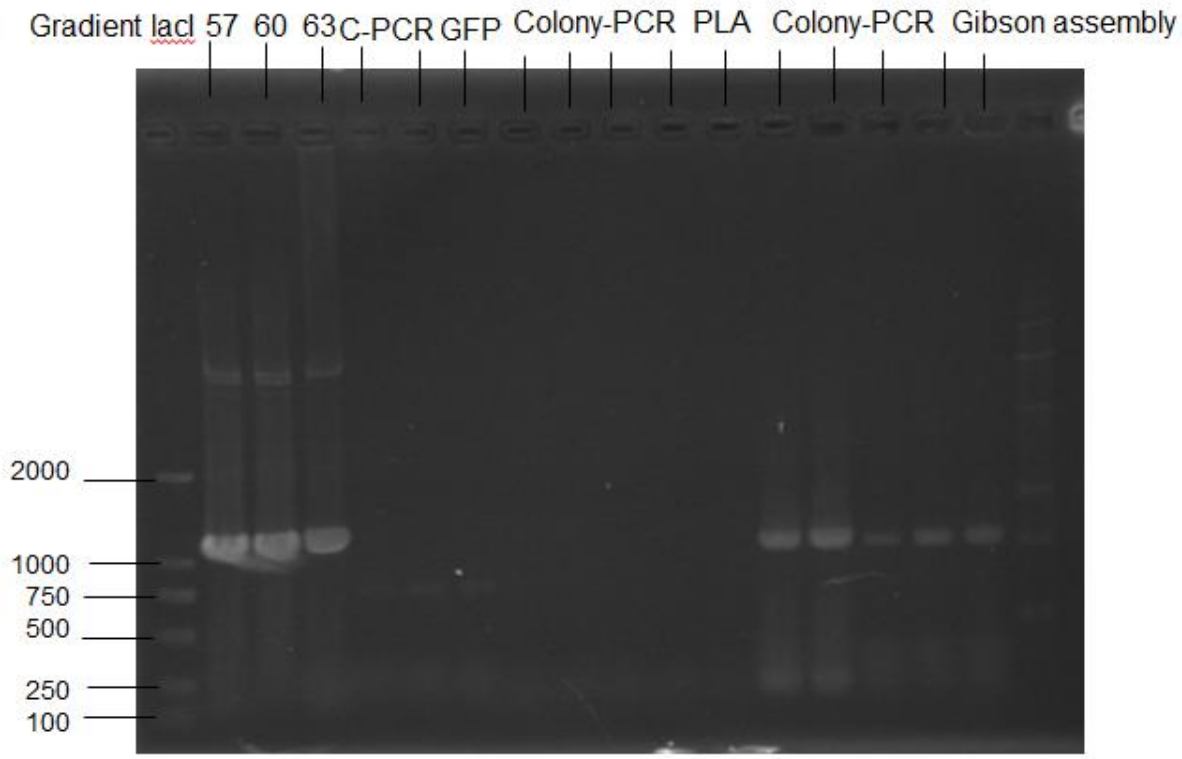
4. I transformed combined plasmid into competent cells immediately.

Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃, 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on amp plate.

22.08

1. I picked 5 single colonies of GFP, 5 single colonies of PLA and 5 single colonies of gibson assembly into liquid medium with ampmycin, shaking cultured 37℃ for 16h.



23.08 team meeting with our PI and training for the presentation on the 5th ccic.

Week 8 25.Aug- 2. Sep Attend 5th ccic in shanghai.

24.08-31.08

We went Shanghai attending the 5th ccic.

Week 9 01.Sep-8.Sep Finish circuit construction and data processing

04.09

1. I did gel extraction of GFP and detected the conentration of GFP

06.09

1. T4 ligation for GFP

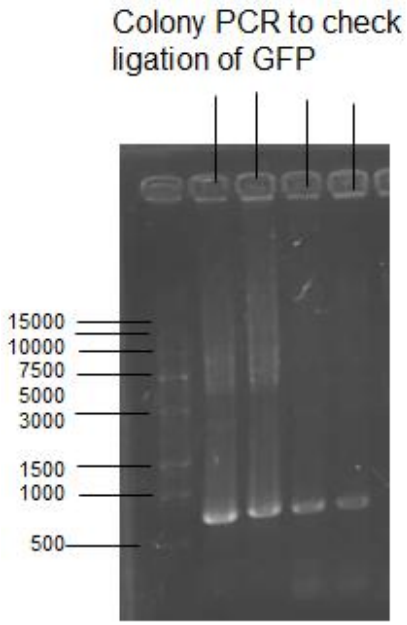
Fragment: 5.74ul
Backbone(PUC57 after-cutting): 1.26ul
T4ase: 1ul
(5x)T4 buffer: 2ul
22℃ 3h in aquatic bath

2. I transformed combined plasmid into competent cells immediately.

Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃， 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on amp plate.

3. I picked single colonies of GFP



07.09

1. Retained the species of hns-synthesis with 15% glycerol.

2. I prepared 3 gradient of salt concentration of LB plates to detected the flourensence level. And also prepared 3 gradient of salt comcentration of liquid medium.

Team meeting.

Week10 08.Sep-15.Sep part-making and data analysis

08.09

1. I shaking cultured our engineered bacteria in 0M, 0.05M, and 0.3M at 25℃ and 37℃ with 3 parallel testing and got the first data process.

control	260973	239803	
0M 25	342859	336081	346245
0M 37	742567	774674	803238
0.05M 25	390072	374539	352269
0.05M 37	865996	793921	932560
0.3M 25	281656	285673	288798
0.3M 37	1154822	1379244	1153108

10.09

1. I added another temperature data at 32.5 and got the second data.

(Control the OD_{600}=7 even the bacteria under deifferent temperature.

Facility: Molecular Devices SpectraMax i3x)

0M 32.5	440986	481276	446379
0.05M 32.5	418776	406582	422616
0.3M 32.5	346489	349108	355089

11.09

1. I extracted plasmid of hns-syn and detected the concentration of plasmid.

2. Enzyme-cutting the plasmid with EcoR1 and Spe1, gel extraction and nano-detection.

3. T4 ligation for salt-temperature circuit and pSB1C3.

Fragment: 5.65ul
Backbone(PUC57 after-cutting): 1.35ul

T4ase: 1ul
(5x)T4 buffer: 2ul
16℃ overnight in aquatic bath

12.09

1. I prepared the LB plate with Chl.

2. I transformed combined plasmid into competent cells immediately.

Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃, 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on Chl plate.

3. I picked three single colonies and did plasmid extraction.

4. Enzyme-cutting to check if T4 ligation succeed.

13.09

1. Line cultured the hns-syn on the different salt concentration of plates.

15.09

Team meeting

Week 11 16.Sep-23.Sep learn to use Fluorescence microscope

17.09

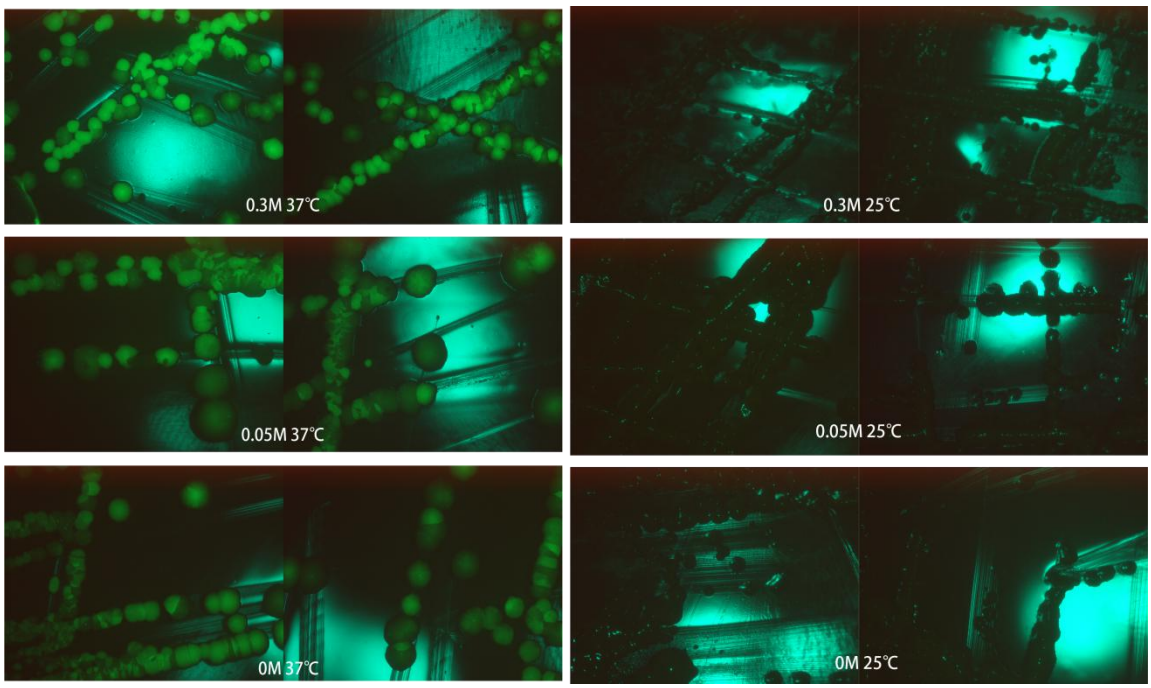
1. I contacted with the teacher who in charge with facility management,

learning how to use fluorescence microscope.

2. I observed the GFP level under fluorescence microscope and collected

the pictures of

GFP.



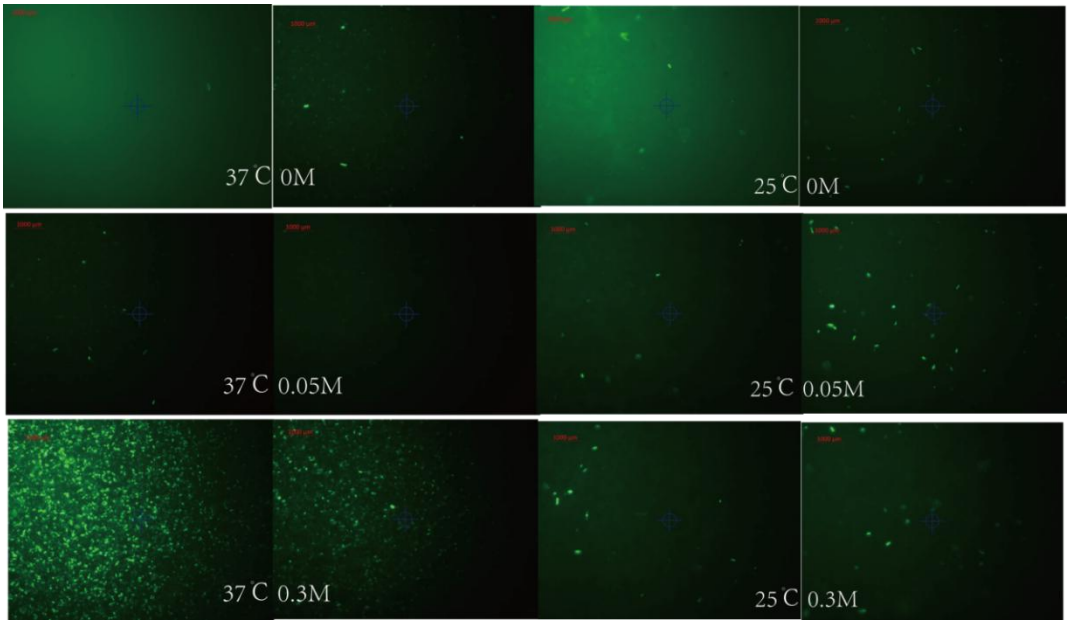
18.09

1. The pictures are not ideal enough and prepared the medium again.

19.09

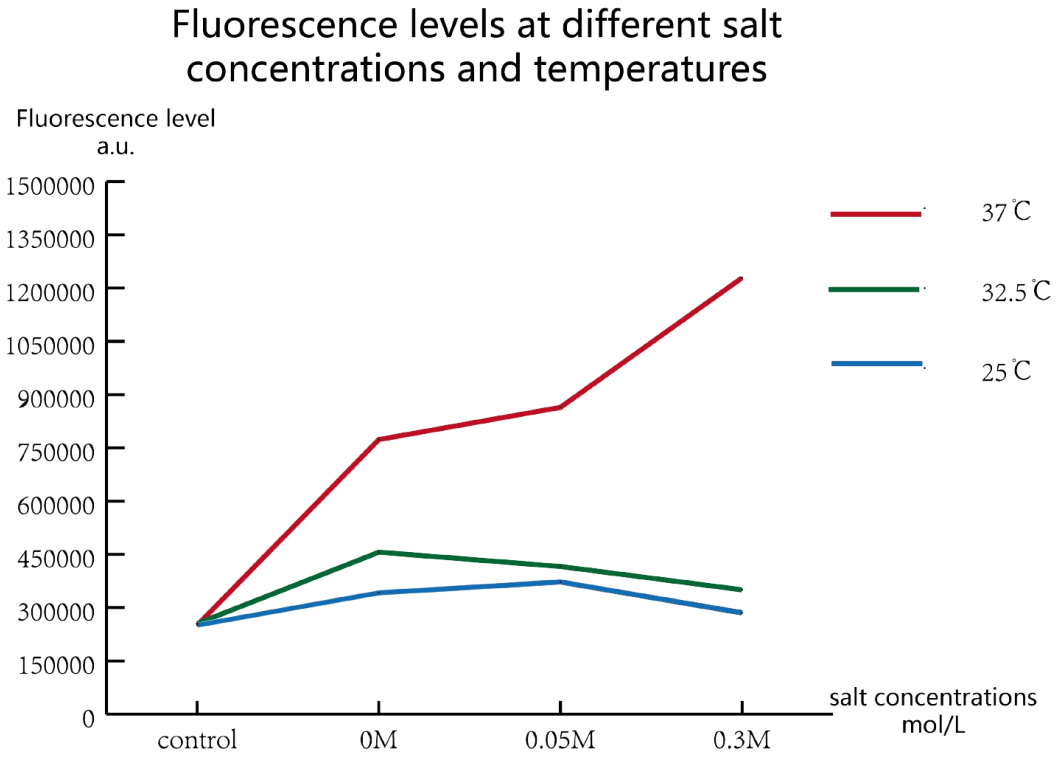
1. Shaking cultured the strain at different salt concentration and temperature.

2. Took the photograph of GFP again.



20.09

1. I dealed with the data and draw the line of fluorescence level and salt temperature.



21.09

1. I did gel extraction of PLA, and detected the concentration.

2. T4 ligation PLA and hns-syn.

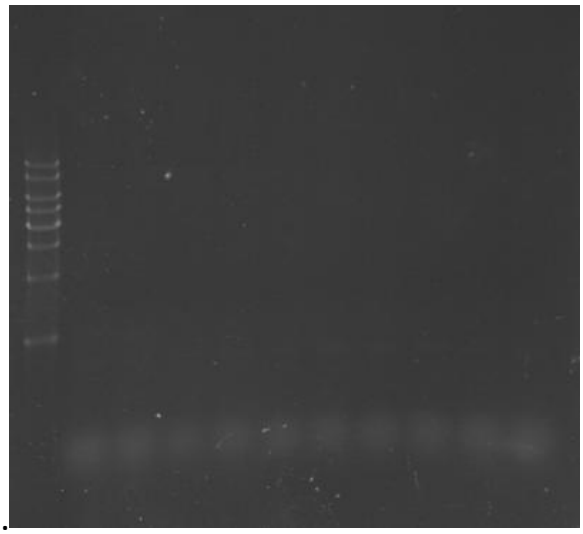
Fragment: 5.60ul
Backbone: 1.40ul
T4ase: 1ul
(5x)T4 buffer: 2ul
22℃ 3h in aquatic bath

3. I transformed combined plasmid into competent cells immediately.

Protocol:

1. Add 10ul plasmid into competent BW25113
2. Put on ice for 30min.
3. Heat shock at 42℃ in a water bath for 90s
4. Back to ice for 5min
5. Add 1ml of LB liquid medium
6. Incubate at 37℃, 220 rpm in shaking table for 1 hour.
7. Centrifugalize with 3000rpm, 5min
8. Remove 900ul of supernatant, and suspend the rest liquid
9. Drawn 100ul coating culture on amp plate.

4. I picked six single colonies to check the T4 ligation if succeed.



There is no bands on the gel, maybe because the legth of plasmid is too long to ligate. And my expriments stopped here, because short of time.

25.09 Team meeting.

26.09-10.17 wiki making, experiment notes finishing, part submitting.

10.18-10.22 presentation preparing and poster designing

10.23 prepare for Gaint Jamboree.

YUXIAN & GUANXUAN

NOTEBOOK

using protocal: The effect of different concentrations of arabinose on the number of deaths in mazf

1、Transfer mazf to LB medium plate，let the bacteria grow 12h.

2、Select Single colonies grown on the plate selected to 5 mL LB liquid medium and culture them in the conditions of 37℃ and 220rpm

3、Transfer 500 microliter mazf solution into 50 ml medium and cultured it under the condition of 37℃ and 22rpm until the solution OD reach 0.6

5、Take out 5ml of the cultured bacteria solution into the test tube . Add 50 microliter Arabic sugar solution in different concentrations to the mazf. Prepare three copies of the above samples as parallel experiments.

6、Put the above samples to culture at 37℃ and 22rpm

, extract part of the bacteria solution at intervals, conduct gradient dilution of the obtained bacteria solution, and then put 5 microliter of each diluted sample point on the culture medium.

7、After the bacteria solution on the medium was fully absorbed, cultured the medium at 37 ℃.

Using protocal: Lethal experiments on staphylococcus epidermidis by different concentrations of PLA

1、Transfer Staphylococcus epidermidis to medium plate，let the bacteria grow 20h.

2 、Take single colony to 5 mL of nutrient gravy liquid medium and culture them.

3、Transfer 500 microliter Staphylococcus epidermidis solution into 50 ml medium and cultured it under the condition of 37℃ and 22rpm until solution reaches a certain concentration

4、Mix staphylococcus epidermis with PLA of different concentrations to make the total system 5 ml. Culture the sample under the condition of 22rpm、37℃.

5、Extract part of the bacteria solution at intervals.

6、Wash the sample of staphylococcus epidermis with buffer solution
7、Conduct gradient dilution of the obtained bacteria solution, and then put 5 microliter of each diluted sample point on the culture medium.
8、After the bacteria solution on the medium was fully absorbed, cultured the medium at 37 degrees Celsius.

Week1: 1/7-7/7 experiment preparation

05.07
①Prepared liquid and solid culture media of staphylococcus epidermidis.
②Prepared ampicillin and kana antibiotics.
③Cultured staphylococcus epidermidis.

06.07
①Prepared ZY medium.
②Inoculated BM4R and pea32b into solid medium

07.07
Transferred single colonies of staphylococcus epidermidis to the liquid medium.

Week2: 8/7-14/7
1、Made 16s PCR of staphylococcus epidermidis(15μl)
2、The lethal experiment of PLA against Staphylococcus epidermidis:
The initial OD value of E.coli =0.5 kbs and Staphylococcus epidermidis =0.5 kbs; Concentration of PLA=0 20 50 100 mmol/L

08.07
① Transferred staphylococcus epidermidis to a conical flask containing 50ML of culture medium.

②Transferred single colonies of BM4R to the liquid medium.
③Extracted genome of staphylococcus epidermidis.

The concentration of genome as follows:
Ep 1
44.342ng/μl，260/230 1.93，260/280 2.11
ep 2
55.360ng/μl，260/230 1.34，260/280 1.70
④Transferred BM4R to autoinduction medium
2018.7.9
①Made 16s PCR of staphylococcus epidermidis mix

Experimental procedures of PCR
15ul system：

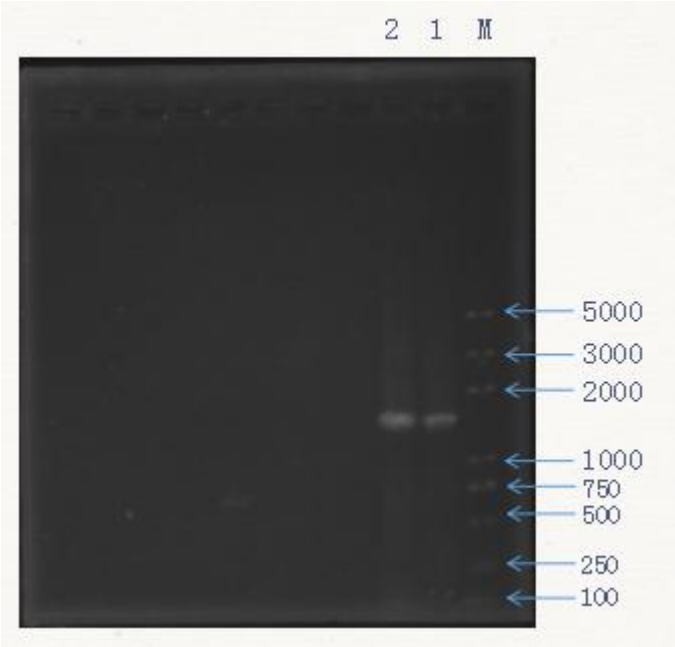
2xPCR Mix	7.5ul
Bacterial fluid	2.0ul
10 μM For. Primer	0.5ul
10 μM Ror. Primer	0.5ul
dd water	4.5ul

PCR Protocol ：

Initiation	95°，3min
Denaturation	94°，35s
Annealing	55°，35s
Extension	72°，1min30s
	35 cycles

Final Extension	72°，10min

Result:



110V 180mA

2018.7.10

①Prepared 500 mmol/L PLA

②Prepared liquid beef peptone medium

2018.7.12

①Prepared liquid beef peptone medium

②prepared PLA solution (200 mmol/L)

2018.7.13

①Transferred e. coli and staphylococcus epidermidis to 50ml liquid medium.

②Selected staphylococcus epidermidis colonies to 5ml liquid medium.

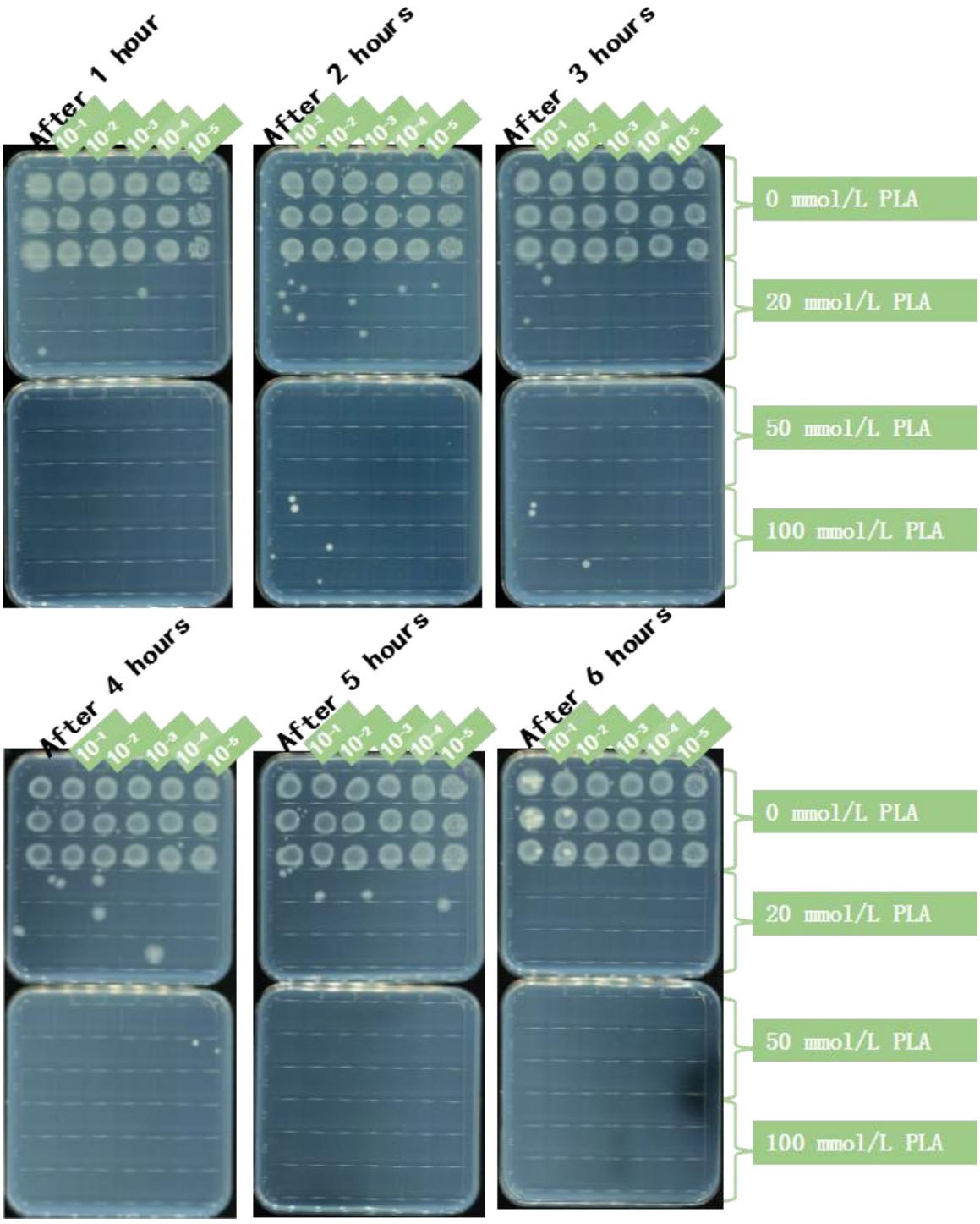
③The lethal experiment of PLA against *E.coli*

(1)Used different concentration of PLA to react with *E.coli*

(2)5 μL *E.coli* with different concentration of PLA were added to the grid every 1hours for a total of 6 hours after being washed and diluted with buffer.

The initial OD value of *E.coli* =0.5 kbs

Concentration of PLA=0 20 50 100 mmol/L


After 1 hour
After 2 hours
After 3 hours
After 4 hours
After 5 hours
After 6 hours
10-1
10-2
10-3
10-4
10-5
0 mmol/L PLA
20 mmol/L PLA
50 mmol/L PLA
100 mmol/L PLA

2018.7.14

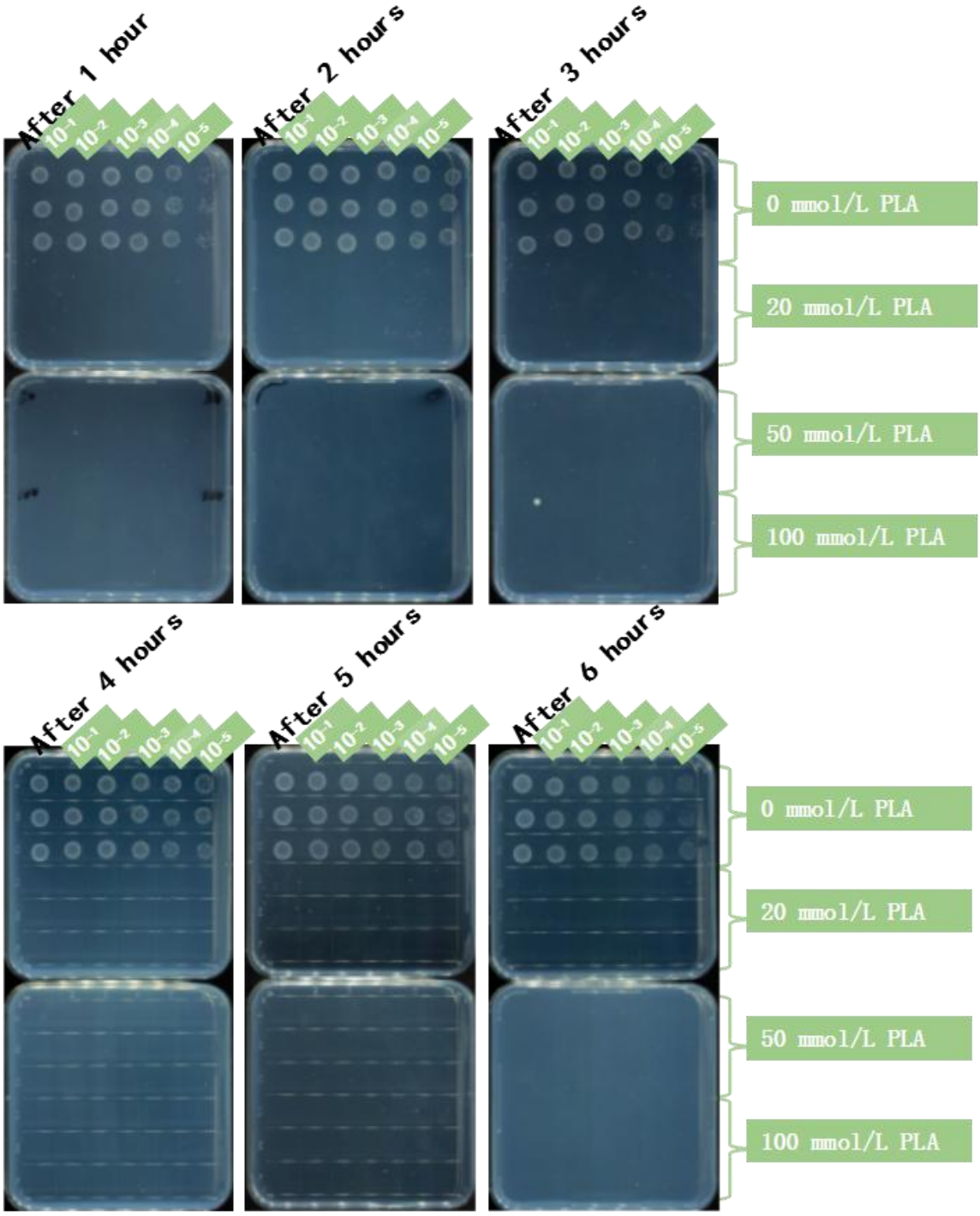
①The lethal experiment of PLA against *Staphylococcus epidermidis*

(1) Added different concentrations of PLA solution to staphylococcus epidermidis solution and culture at 37℃ and 22rpm.

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 6 hours after being washed and diluted with buffer.

The initial OD value of *Staphylococcus epidermidis* =0.5 kbs

Concentration of PLA=0 20 50 100 mmol/L


After 1 hour
After 2 hours
After 3 hours
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}
0 mmol/L PLA
20 mmol/L PLA
50 mmol/L PLA
100 mmol/L PLA
After 4 hours
After 5 hours
After 6 hours
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}
0 mmol/L PLA
20 mmol/L PLA
50 mmol/L PLA
100 mmol/L PLA

Week3: 15/7-21/7

1、Made 16s PCR of staphylococcus epidermidis(50μl)

2、The lethal experiment of PLA against Staphylococcus epidermidis: (The initial OD value of Staphylococcus epidermidis =1 kbs; Concentration of PLA=0 20 50 100 mmol/L)

2018.7.16

①Made 16s PCR of staphylococcus epidermidis mix

②Prepared of LB solid medium.

③Sterilized the PCR tube and EP tube at high temperature

④Prepared Nutrient broth medium and LB medium.

⑤Inoculated Staphylococcus epidermidis into solid medium

⑥ Isolated single colonies of staphylococcus epidermidis into liquid medium and cultured it in the condition of 220rpm, 37℃.

⑦Prepared 75% alcohol

The experimental steps

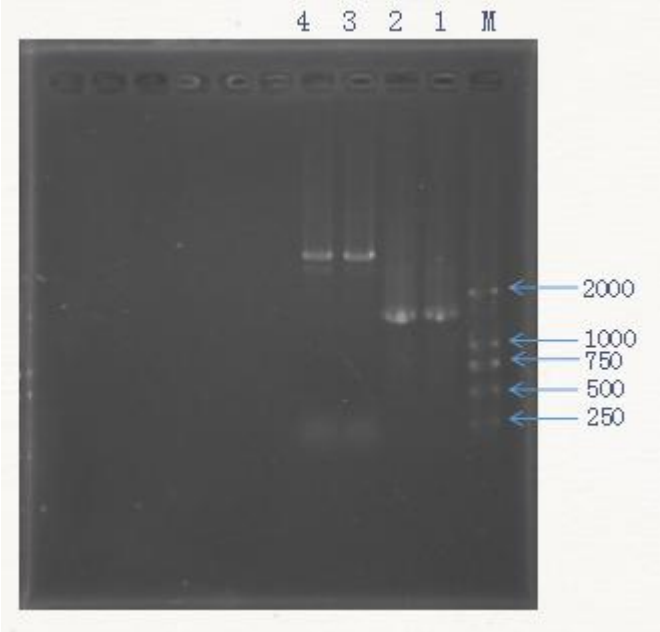
50 ul system（backbone）:

2xSuper Mix	25ul
PEA32	0.45ul
10 μM For. Primer	1.0ul
10 μM Ror. Primer	1.0ul
dd water	22.55ul

PCR Protocol :

Initiation	95°，3min
Denaturation	94°，35s
Annealing	55°，35s

Extension	72°，1min30s
	35 cycles
Final Extension	72°，10min



110V 180mA

2018.7.17

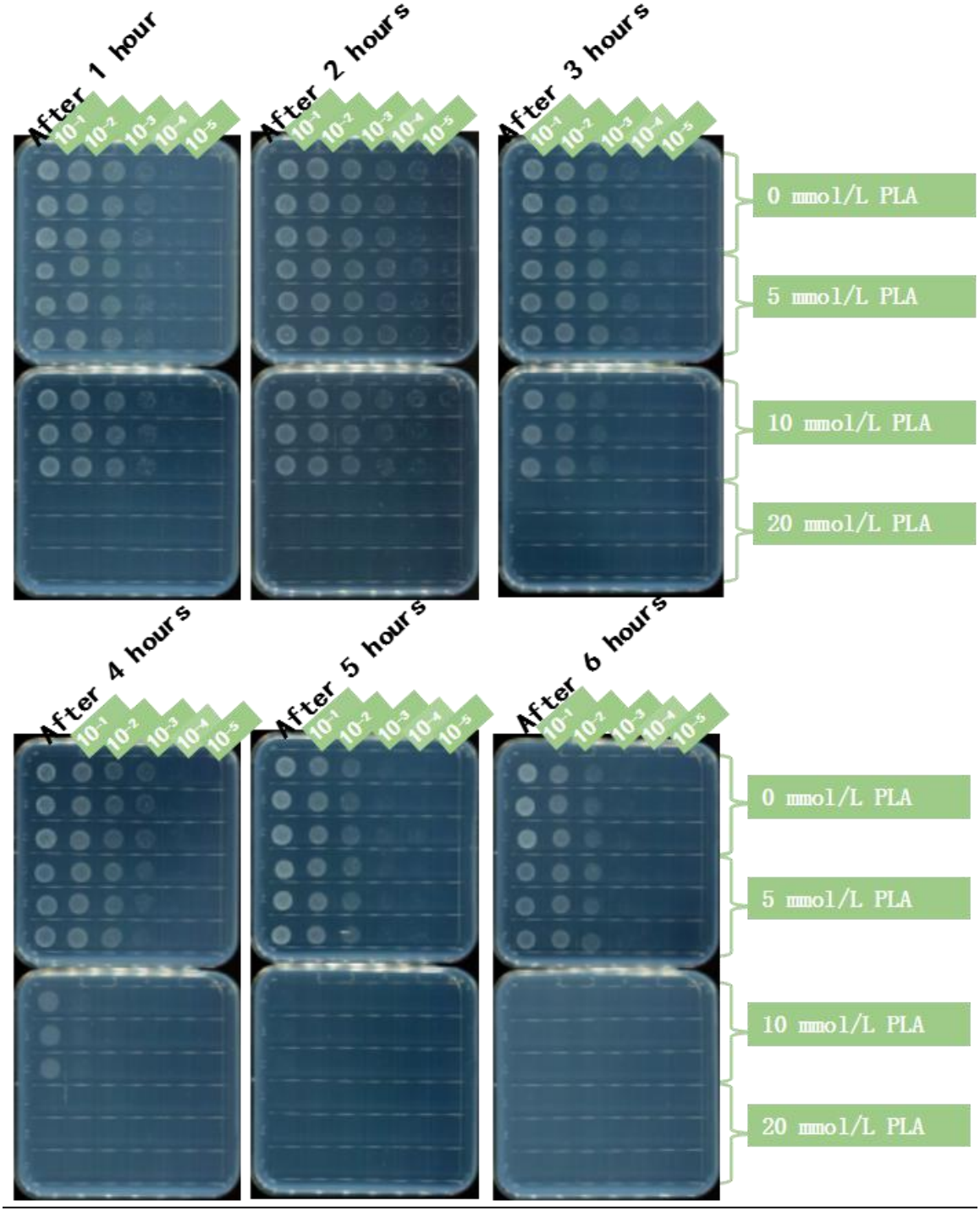
The lethal experiment of PLA against *staphylococcus epidermidis*

(1) Added different concentrations of PLA solution to staphylococcus epidermidis solution and culture at 37℃ ,22rpm.

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer

The initial OD value of staphylococcus epidermidis =1 kbs

Concentration of PLA=0 5 10 20 mmol/L


After 1 hour
After 2 hours
After 3 hours
After 4 hours
After 5 hours
After 6 hours
10-1
10-2
10-3
10-4
10-5
0 mmol/L PLA
5 mmol/L PLA
10 mmol/L PLA
20 mmol/L PLA

2018.7.18

①Statistics of staphylococcus epidermidis sample data.

② Inoculated staphylococcus epidermidis into 50ml liquid medium and cultured at 220rpm, 37℃.

Week4: 22/7-28/7

1、Measured OD value of the samples every 1 hour for total 8 hours.

（OD of initial staphylococcus epidermidis =1 kbs

Concentration of PLA=0 12.5 15 17.5 mmol/L)

2018.7.26

Isolated Staphylococcus epidermidis into liquid medium and cultured at 220rpm, 37℃.

2018.7.27

Prepared the growth curve of Staphylococcus epidermidis by the method of OD.

2018.7.28

Used spectrophotometer to do the lethal experiment of PLA against *staphylococcus epidermidis*

(1) Used different concentration of PLA to react with *staphylococcus epidermidis*

(2)Measured OD value of the samples every 1 hour for total 8 hours.

OD of initial staphylococcus epidermidis =1 kbs

Concentration of PLA=0 12.5 15 17.5 mmol/L

	2018.7.28			
	0	12.5	15	17.5
1h	0.9210	0.3892	0.3136	0.5162
2h	0.9392	0.5544	0.3282	0.5846
3h	0.9362	0.5606	0.2842	0.5966
4h	0.9306	0.6148	0.2934	0.5436
5h	0.8784	0.6140	0.4290	0.4536
6h	0.8988	0.5510	0.3170	0.5116
7h	0.9470	0.5880	0.2992	0.4584
8h	0.8400	0.6236	0.2146	0.4302

Week5: 29/7-4/8

1、Measured OD value of the samples every 1 hour for total 8 hours.
（OD of initial staphylococcus epidermidis =1 kbs
Concentration of PLA=0 12.5 15 17.5 mmol/L)

2、The lethal experiment of PLA against Staphylococcus epidermidis: (The initial OD value of Staphylococcus epidermidis =1 kbs; Concentration of PLA=0 11.25 12.5 15 mmol/L)

3、The lethal experiment of PLA against Staphylococcus epidermidis: (The initial OD of staphylococcus epidermidis =0.5 kbs; Concentration of PLA=0 5 10 12.5mmol/L)

2018.7.29

①Isolated staphylococcus epidermidis colonies into liquid medium

②Transferred 5ml staphylococcus epidermidis to 60ml liquid medium and cultured it at 37℃ and 22rpm.

2018.7.30

Used spectrophotometer to do the lethal experiment of PLA against *staphylococcus epidermidis*

(1) Used different concentration of PLA to react with *staphylococcus epidermidis*

(2)Measured OD value of the samples every 1 hour .

OD of initial staphylococcus epidermidis =1 kbs

Concentration of PLA=0 12.5 15 17.5 mmol/L

2018.7.30 PLA浓度 / 时间	0	12.5	15	17.5
1h	0.95	0.64	0.32	0.27
2h	0.87	0.42	0.43	0.33
3h	0.94	0.47	0.43	0.28
4h	0.92	0.55	0.44	0.31
5h	0.93	0.57	0.41	0.32
6h	0.92	0.49	0.42	0.38
20h	0.69	0.39	0.39	0.33

2018.7.31

Used spectrophotometer to do the lethal experiment of PLA against *staphylococcus epidermidis*

(1) Used different concentration of PLA to react with *staphylococcus epidermidis*

(2)Measured OD value of the samples every 1 hour for total 6 hours.

OD of initial staphylococcus epidermidis =1 kbs

Concentration of PLA=0 12.5 15 17.5 100 mmol/L

2018.7.31 PLA浓度 / 时间	0	12.5	15	17.5	100
1h	0.99	0.59	0.50	0.42	0.40
2h	0.93	0.45	0.50	0.42	0.42
3h	0.86	0.54	0.35	0.45	0.50
4h	0.85	0.58	0.49	0.47	0.51
5h	0.88	0.59	0.56	0.45	0.43
6h	0.76	0.52	0.52	0.49	0.47

2018.8.1

①Prepared PLA solution and culture medium

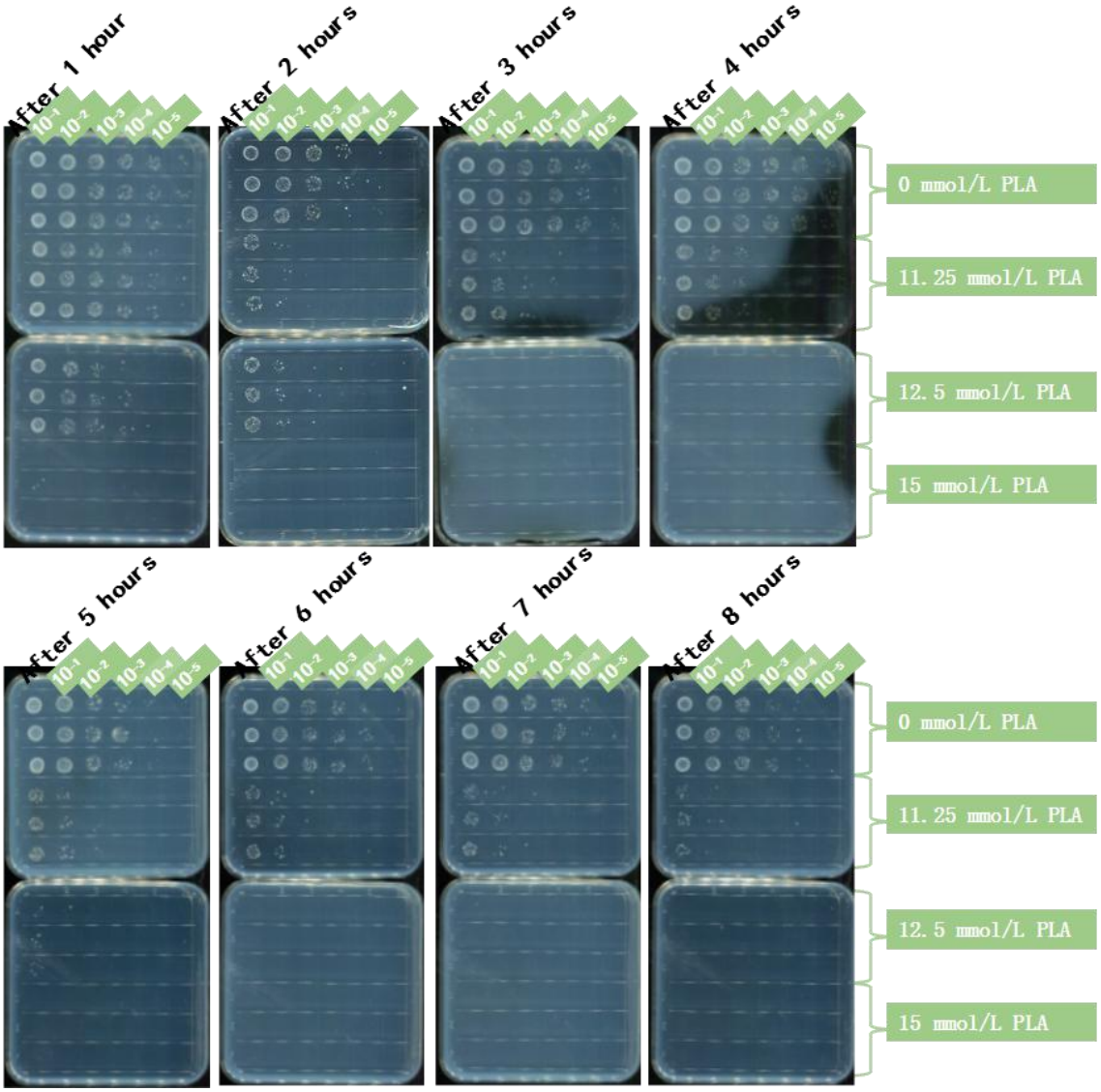
②The lethal experiment of PLA against *staphylococcus epidermidis*

(1) Added different concentrations of PLA solution to staphylococcus epidermidis solution and cultured at 37℃, 22rpm.

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer

OD of *staphylococcus epidermidis* =1 kbs

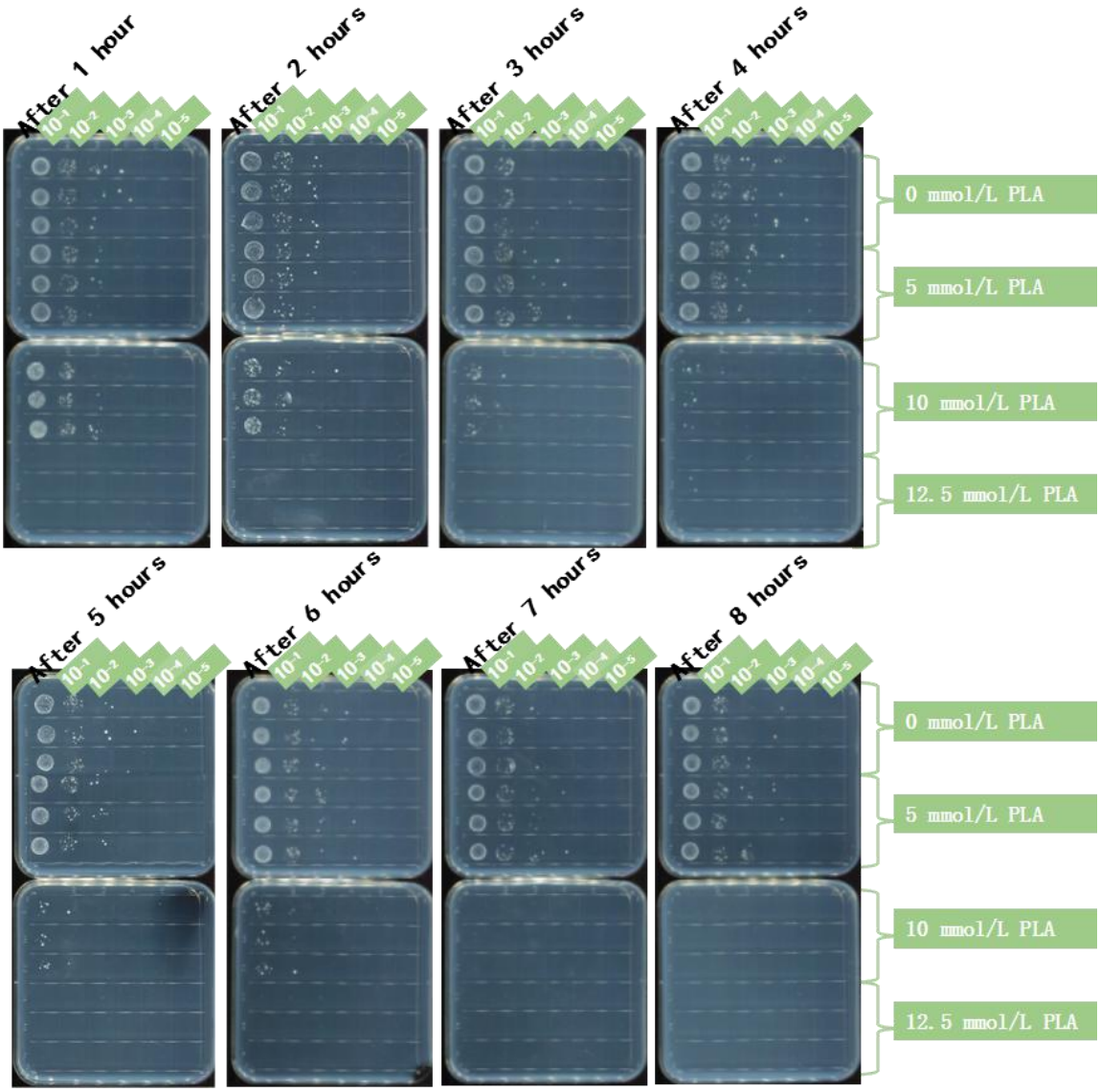
Concentration of PLA=0 11.25 12.5 15mmol/L



2018.8.2
Sorted data

2018.8.3
Transferred epidermal staphylococcus bacteria solution to 50ML liquid medium and cultured it at 37 degrees Celsius and 220rpm

2018.8.4
①Prepared PLA solution and culture medium
②The lethal experiment of PLA against *staphylococcus epidermidis*
(1)Added different concentrations of PLA solution to staphylococcus epidermidis solution and cultured at 37℃, 220rpm.
(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer
OD of *staphylococcus epidermidis* =0.5 kbs
Concentration of PLA=0 5 10 12.5mmol/L


After 1 hour
After 2 hours
After 3 hours
After 4 hours
After 5 hours
After 6 hours
After 7 hours
After 8 hours
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
0 mmol/L PLA
5 mmol/L PLA
10 mmol/L PLA
12.5 mmol/L PLA

Week6: 5/8-11/8

1、The lethal experiment of PLA against Staphylococcus epidermidis:

（The initial OD value of Staphylococcus epidermidis =1 kbs; Concentration of PLA=0 5 10 mmol/L）

2、The lethal experiment of PLA against Staphylococcus epidermidis:

（The initial OD value of Staphylococcus epidermidis =2 kbs; Concentration of PLA=0 5 12.5 20 mmol/L）

2018.8.5

①Prepared PLA solution and culture medium

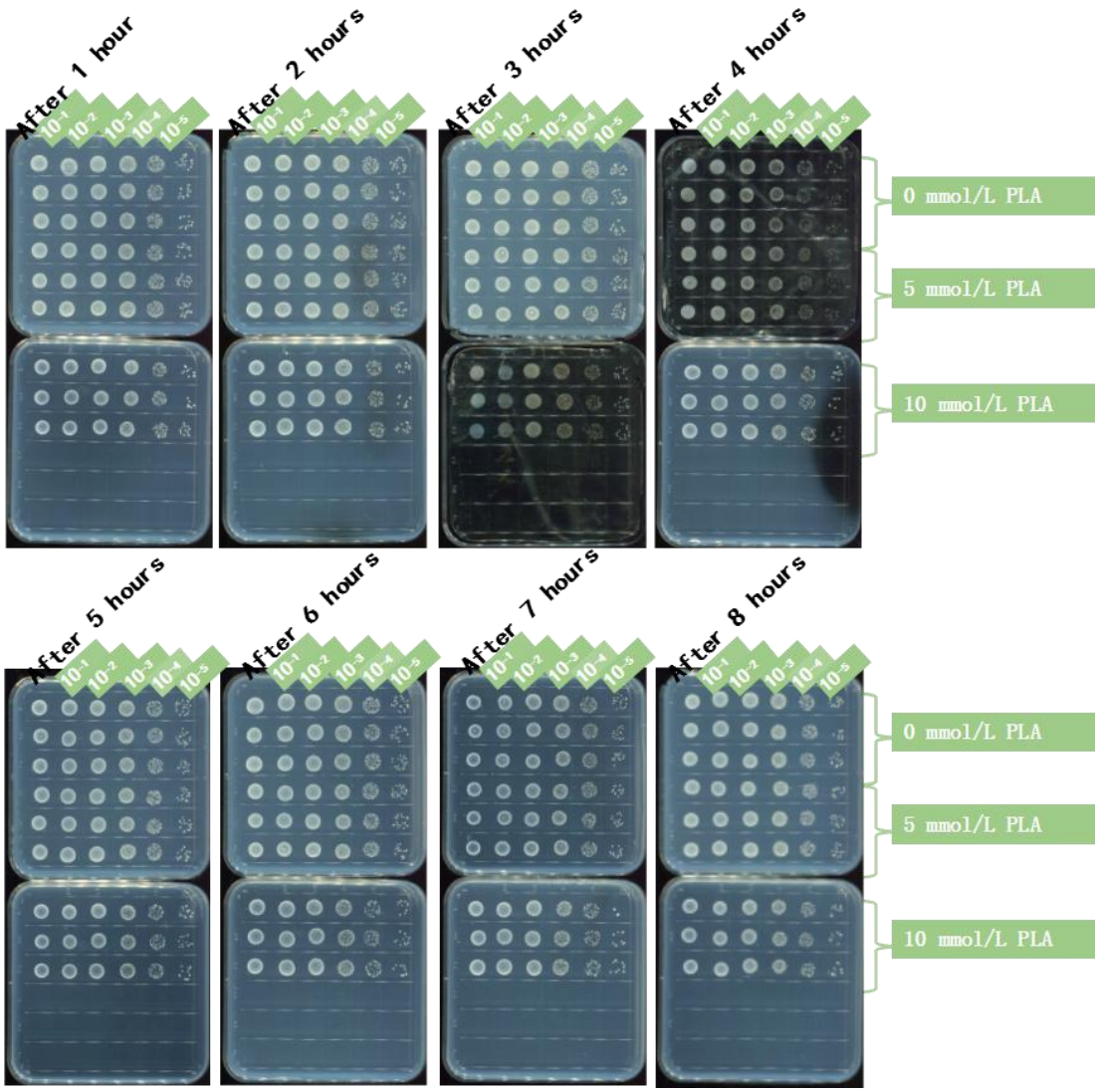
②The lethal experiment of PLA against *staphylococcus epidermidis*

(1) Added different concentrations of PLA solution to staphylococcus epidermidis solution and culture at 37℃, 220rpm.

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer.

OD of *staphylococcus epidermidis* =1 kbs

Concentration of PLA =0 5 10 mmol/L


After 1 hour
After 2 hours
After 3 hours
After 4 hours
10-1
10-2
10-3
10-4
10-5
0 mmol/L PLA
5 mmol/L PLA
10 mmol/L PLA
After 5 hours
After 6 hours
After 7 hours
After 8 hours
10-1
10-2
10-3
10-4
10-5
0 mmol/L PLA
5 mmol/L PLA
10 mmol/L PLA

2018.8.6

①Prepared LB solid medium

②Inoculated BM4R and pea32b into solid medium

2018.8.7

①Transferred 5ml staphylococcus epidermidis solution to 40ml liquid medium.

②Selected staphylococcus epidermidis colonies and cultured them in liquid medium

③Prepared of liquid beef peptone medium

④Inoculated staphylococcus epidermidis in solid medium

2018.8.8

①Transferred the bm4r into the liquid medium

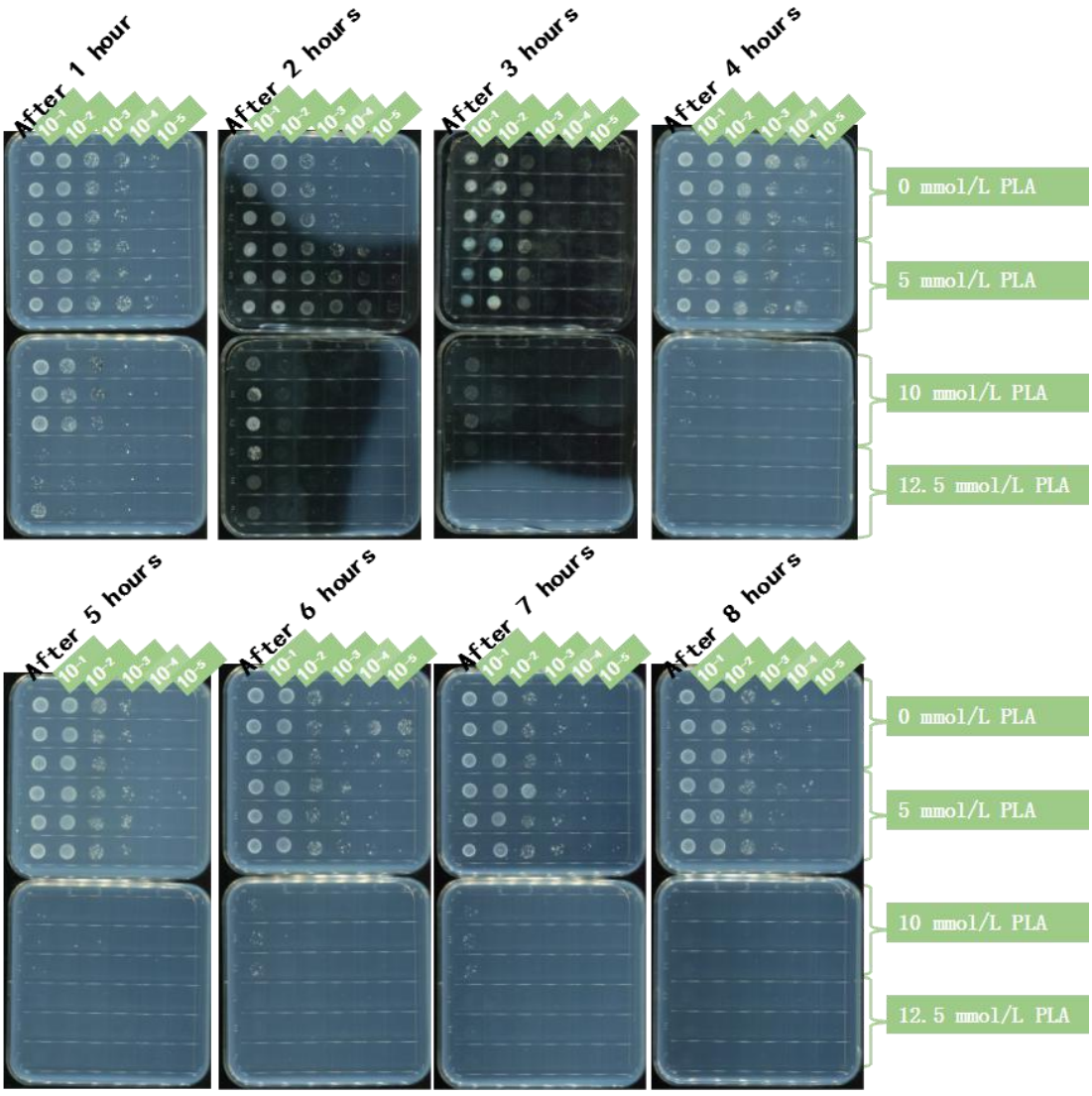
②The lethal experiment of PLA against *staphylococcus epidermidis*

(1) Used different concentration of PLA to react with *staphylococcus epidermidis*

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer.

OD of *staphylococcus epidermidis* =2 kbs

Concentration of PLA=0 5 12.5 20 mmol/L



③ Transferred the BM4R and pea28b bacteria solution to the ZY medium and cultured it in the conditions of 37℃, 220rpm

2018.8.9

①Transformed the bm4r and take out the sample every 1h

② Samples stored in - 20 ℃ condition, prepared for HPLC test concentration

Week7: 12/8-18/8

1、The lethal experiment of PLA against Staphylococcus epidermidis:

（The initial OD of bacteria containing mazf =0.6

Concentration of arabinose =0% 5% 10% 15% 20% 50%）

2018.8.14

Selected single colonies of bacteria with mazf into the liquid medium and cultured it in the conditions of 37℃, 220rpm

2018.8.15

① Transferred the mazf bacteria solution to the conical flask which contains 50ml liquid medium and cultured it in the condition of 37℃, 220rpm

② Selected staphylococcus epidermidis single colonies into liquid medium

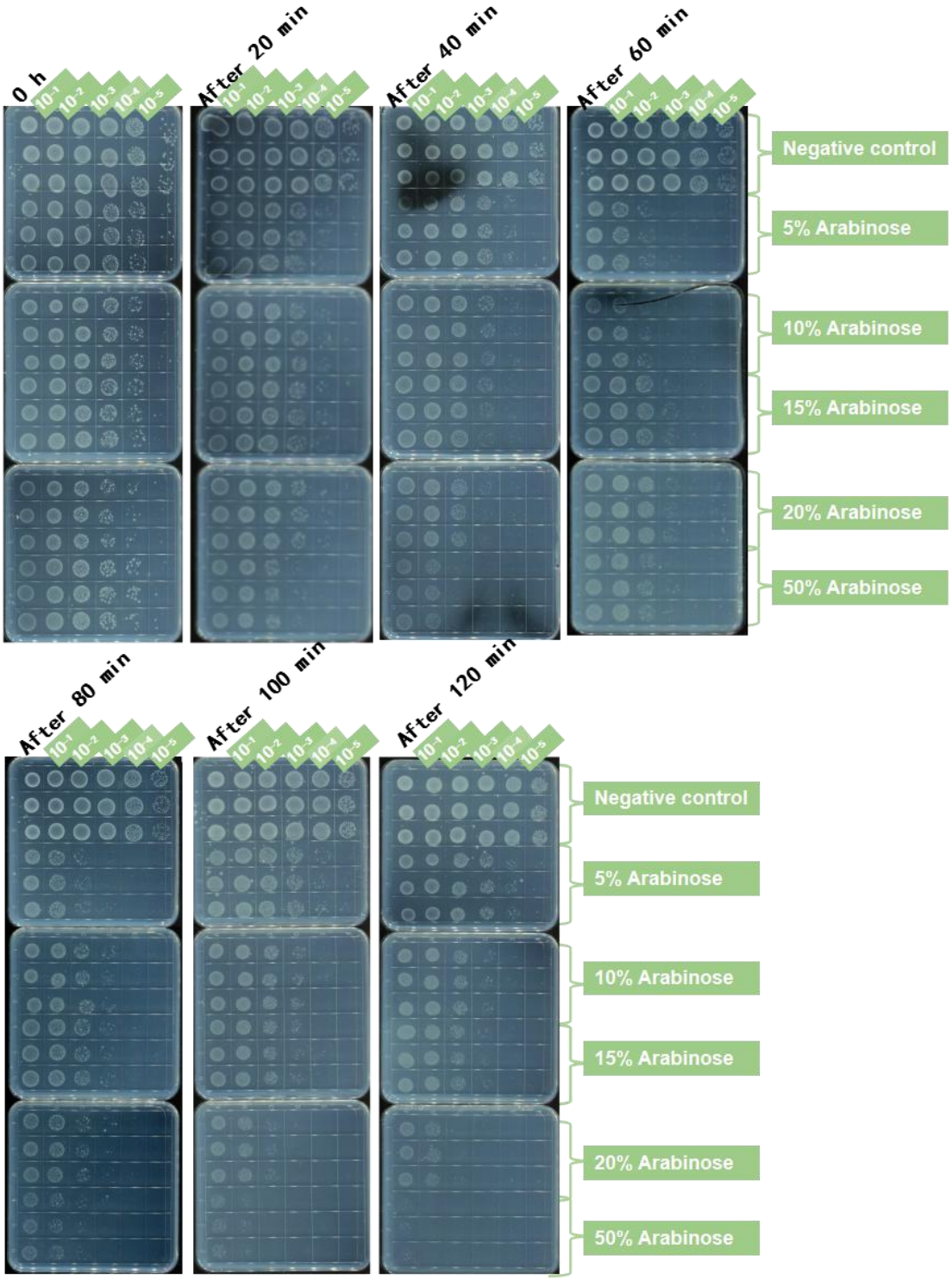
③The lethal experiment of different concentration of arabinose against engineered bacteria containing mazf gene.

(1)Added engineered bacteria containing the mazf gene to arabinose in different concentrations to react

(2)5 μL samples were added to the grid every 20 minutes for a total of 2 hours.

OD of bacteria containing mazf =0.6

Concentration of arabinose =0% 5% 10% 15% 20% 50%



2018.8.16

①Transferred the Staphylococcus epidermidis solution to 50ml liquid medium and cultured it in the condition of 37℃, 220rpm.

②Sorted data

Week8: 19/8-25/8

1、The lethal experiment of PLA against Staphylococcus epidermidis:

(The initial OD of bacteria containing mazf =0.6

Concentration of arabinose =0% 5% 10% 15% 20% 50%)

2018.8.20

①Selected single colonies of bacteria with mazf into the liquid medium and cultured it in the conditions of 37℃, 220rpm

②Prepared LB medium

2018.8.21

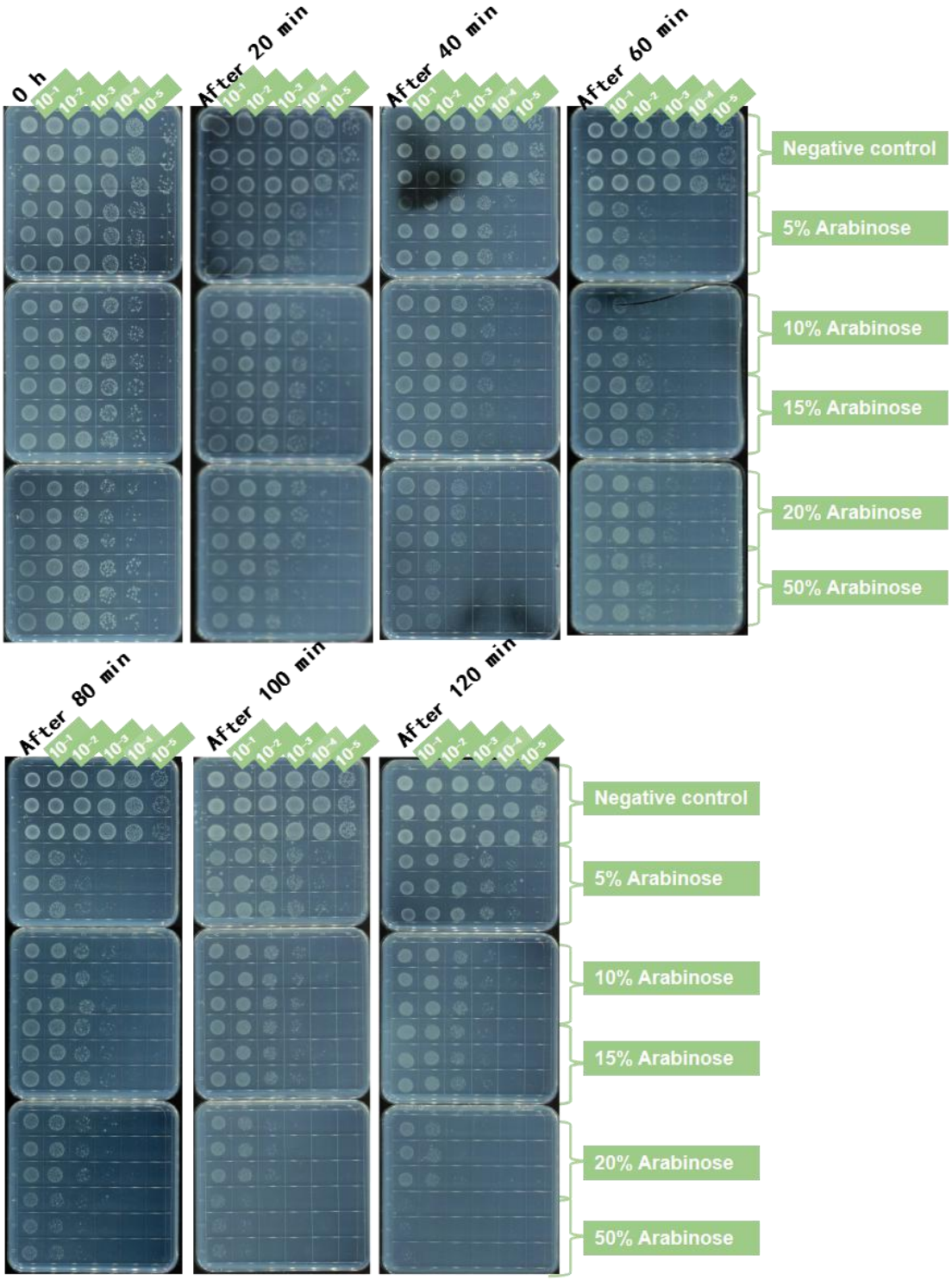
The lethal preliminary experiment of different concentration of arabinose against engineered bacteria containing mazf gene.

(1)Added engineered bacteria containing the mazf gene to arabinose in different concentrations to react

(2)5 μL samples were added to the grid every 1h for a total of 2 hours.

OD of bacteria containing mazf=0.6

Concentration of the Arab sugar =0% 5% 10% 15% 20% 50%



2018.8.22

The lethal experiment of different concentration of arabinose against engineered bacteria containing mazf gene.

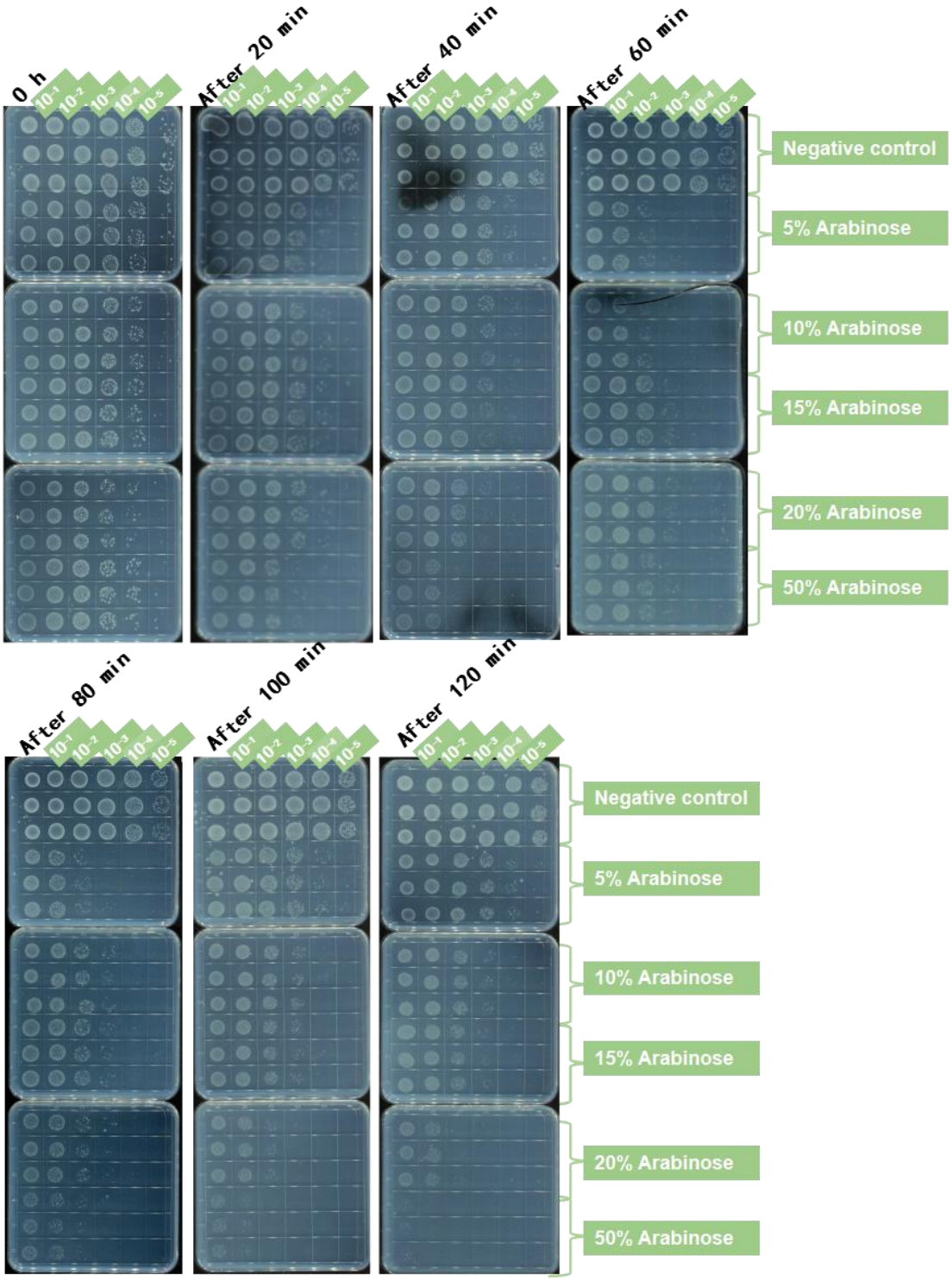
(1)Added engineered bacteria containing the mazf gene to arabinose

in different concentrations to react

(2)5 μL samples were added to the grid every 20 minutes for a total of 2 hours.

OD of bacteria containing mazf =0.6

Concentration of arabinose =0% 5% 10% 15% 20% 50%



Week9: 26/7-1/9

No experimental arrangement

Week10: 2/9-8/9

1、The lethal experiment of PLA against Staphylococcus epidermidis:

（The initial OD value of Staphylococcus epidermidis =1 kbs; Concentration of PLA=7　8.5　10.5 mmol/L）

2018.9.2

Prepared a solid medium for nutrient gravy

2018.9.3

① Inoculated Staphylococcus epidermidis in solid medium and cultured at 37℃.

②Prepared Liquid culture medium and PLA solution

2018.9.4

①Inoculated single colonies of staphylococcus epidermidis on solid medium into liquid medium

②Transferred the staphylococcus epidermidis solution to 50ml liquid medium and cultured it in the condition of 37℃，220 rpm.

2018.9.5

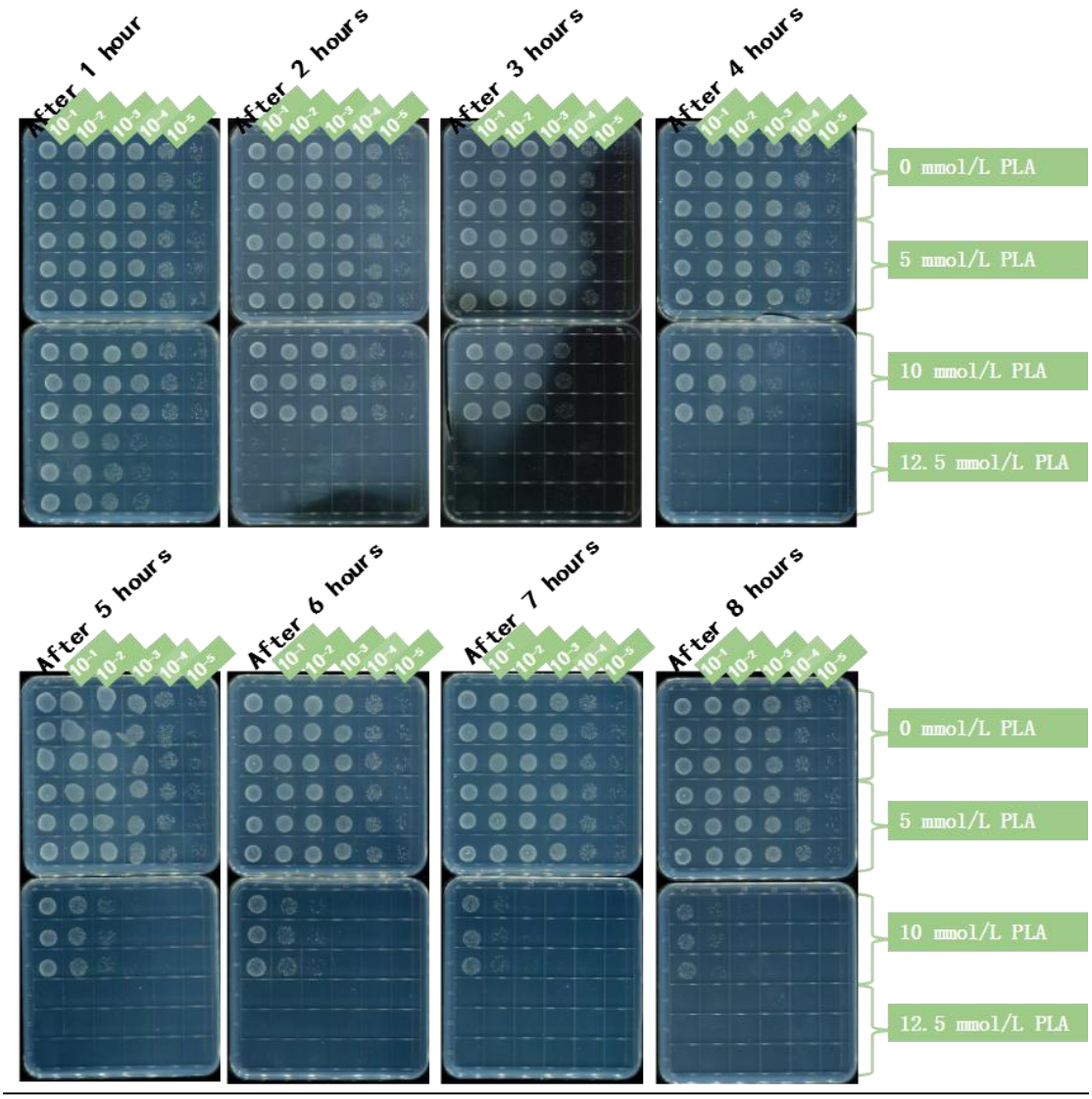
The lethal experiment of PLA against *staphylococcus epidermidis*

(1)Added different concentrations of PLA solution to staphylococcus epidermidis solution and cultured at 37℃, 220rpm.

(2)5 μL *staphylococcus epidermidis* above with different concentration of PLA were added to the grid every 1hours for a total of 8 hours after being washed and diluted with buffer

OD of *staphylococcus epidermidis* =1 kbs

Concentration of PLA =7　8.5　10.5mmol/L



2018.9.7

Processed experimental data

TIME（week1）

Sent D-lactate dehydrogenase gene (d-ldh), transaminase gene (Tyrb) and glutamate dehydrogenase (rocG) to the company for synthesis

TIME(week1)

PCR was performed using d-lactate dehydrogenase gene (d-ldh), transaminase gene (Tyrb) and glutamate dehydrogenase (rocG)

PCR 反应体系

Table1-3 Reaction system of PCR

PCR 反应体系	
5×reaction buffer	10 μL
5×high GC buffer	10 μL
10 mM dNTP	1 μL
forward prime	2 μL
reverse prime	2 μL
Q_5	0.5 μL
template	1 μL
ddH_2O	23.5 μL

表 1-4　PCR 扩增条件

Table1-4 Reaction conditions of PCR

PCR 扩增条件		
98 ℃	2 min	1 个循环
98 ℃	30 S	
63℃/65℃	30 S	29 个循环
72 ℃	45 S	
72 ℃	10 min	

- We purified and recovered the products of homologous recombinant sequences amplified by PCR，and the purification method was performed according to the instructions of for the SanPrep column DNA gel recovery kit. Then, the purification was confirmed by agarose gel electrophoresis and be stored in the condition of -20 degree centigrade.

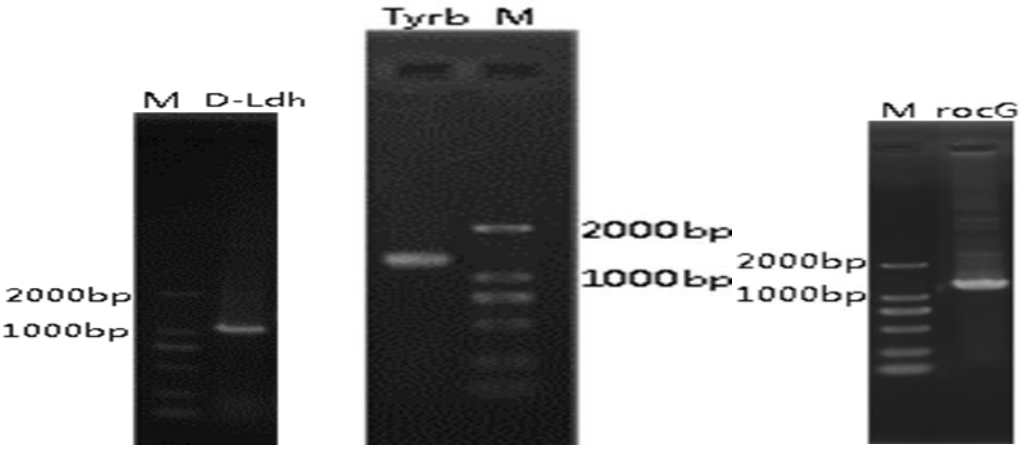


图 1-2　Tyrb、*rocG* 扩增片段

Fig.1-2 PCR amplification for *Tyrb/rocG*

Note: M: Marker: DL2000; *Ldh*: PCR products D*ldh*

TIME(week2)

①Prepared of *E. coli* receptive cells

②Extracted plasmid vector (pRB1s)

TIME(week2-week3)

Construction of recombinant plasmid vectors

⑴Cut the plasmid vector pRB1s by double enzyme digestion, and the restriction enzymes we used were NcoI、 BglⅡ

⑵The above products were purified and recovered using SanPrep column DNA gel recovery kit.

⑶The plasmids obtained by double enzyme digestion were connected with d-lactate dehydrogenase (d-ldh) gene fragments obtained by PCR amplification using Gibson system

⑷After the connection was completed, the products were transferred into the *E.coli* BW25113 receptor respectively, and the clones were screened using the medium containing streptomycin resistance.

表 1-5 system of double digests

Table1-5 The reaction system of double enzyme digestion

酶切反应体系	
10×3.1 buffer	5 μL
XhoI	1 μL
BglⅡ	1 μL
pRB1s	2~2.5 μg
ddH_2O	补加至 50 μL
反应条件	37 ℃维持 3-5 h

表 1-7　Gibson 反应体系

Table1-7 The reaction system of Gibson

Gibson 反应体系	
双酶切胶回收纯化后载体 pRB1s/pRB1s-D*ldh* PCR 扩增纯化产物 D*ldh/Tyrb*	质粒跟基因片段按照摩尔比 1：3，两者体积和为 4 μL
Gibson 体系	6 μL
反应条件	50 ℃反应 1 h

⑸After cultivating all night， we transferred monoclonal recombinants that could be grown on a medium containing streptomycin to a liquid LB medium containing streptomycin resistance, then cultured it in the condition of 37℃，220 rpm for 8 hours.

⑹We took 1 uL bacteria solution at clean bench platform for PCR (1-8) verification, and then sent the correct bacteria solution with gel electrophoresis to the bioengineer in Shanghai for sequencing.

表 1-8　PCR 反应体系

Table1-8 Reaction system of PCR

PCR 反应体系	
2× PCRmix	7.5 μL
Forward prime	0.5 μL
Reverse prime	0.5 μL
Template	1 μL
ddH_2O	5.5 μL

⑹After comparing the sequencing results, the engineering strain whose sequencing result are correct was named as BW/ prb1s-dld. We saved the strains in 12.5% of glycerol, and stored in the condition of frozen - 80 ℃.

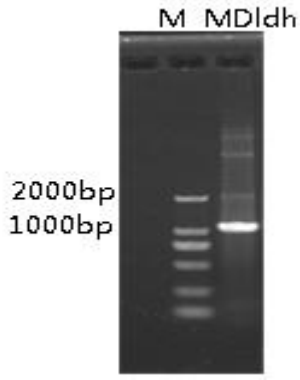


图 1-3　菌落 PCR 验证

Fig.1-3 PCR verification of Bacterial colony

Note: M: Marker: DL2000; D*ldh*: PCR products

Time (week 4)

Construction of recombinant plasmid vectors

⑴Digested the recombinant vector prb1s-dldh with single enzyme， and the enzyme of single enzyme digestion is Bgl Ⅱ.

⑵Then, we used SanPrep column DNA gel recovery kit to purify and recover the products above.

⑶ After that, we used Gibson system to connect the plasmids after enzyme cutting and the fragments of transaminase (Tyrb) obtained by PCR amplification.

⑷After the connection was completed, the products were transferred into the *E.coli* BW25113 receptor respectively, and the clones were screened using the medium containing streptomycin resistance.

表 1-6 单酶切反应体系

Table1-6 The reaction system of single enzyme digestion

酶切反应体系	
10×3.1buffer	5 μL
BglⅡ	1 μL
pRB1s	2~2.5 μg
ddH_2O	补加至 50 μL
反应条件	37 ℃维持 3-5 h

表 1-7 Gibson 反应体系

Table1-7 The reaction system of Gibson

Gibson 反应体系	
双酶切胶回收纯化后载体 pRB1s/pRB1s-D*ldh*	质粒跟基因片段按照摩尔比 1：3，两者体积和为 4 μL
PCR 扩增纯化产物 D*ldh/Tyrb*	
Gibson 体系	6 μL
反应条件	50 ℃反应 1 h

⑸ After cultivating all night， we transferred monoclonal recombinants that could grow on streptomycin-resistant medium to LB liquid medium which contain streptomycin resistance, then cultured it in the condition of 37℃， 220 rpm for 8 hours.

⑹We took 1 uL bacteria solution at clean bench platform for PCR verification, and then sent the correct bacteria solution with gel electrophoresis to the bioengineer in Shanghai for sequencing. The PCR validation reaction system of colony was shown in table 1-8.

表 1-8 PCR 反应体系

Table1-8 Reaction system of PCR

PCR 反应体系	
2× PCRmix	7.5 μL
Forward prime	0.5 μL
Reverse prime	0.5 μL

Template	1 μL
ddH_2O	5.5 μL

(7) After comparing the sequencing results, the engineering strains whose sequencing result are correct was named as BWpRB1s-D*ldh-Tyrb*. We saved the strains in 12.5% of glycerol, and stored in the condition of frozen - 80 ℃.

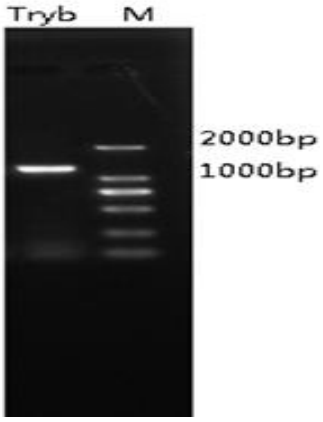


图 1-3 菌落 PCR 验证

Fig.1-3 PCR verification of Bacterial colony

Note: M: Marker: DL2000; *Tyrb*: PCR products

Time(week5)

Induction culture of engineering bacteria

(1) selected the engineered bacteria and original strains (BW/ prb-dldh, bwprb1s-dldh-tyrb, BW25113) from the seed tube and inoculated on LB solid medium containing streptomycin resistance and activated overnight.

(2) Pick a single colony to 5 mL LB liquid medium containing streptomycin and cultured it in the condition of 37 ℃、 220 RPM for around 8 hours.

(3) Inoculated 500UL bacterial solution into 3 bottles of 50mL self-induction medium and cultured it in the condition of 37℃，220 rpm for 16 h。

SDS-PAGE verifies protein expression

(1) After the induction culture was completed, 3 bottles of bacteria solution of each group were mixed separately and 10 mL of bacteria solution was taken and centrifuged at 8000 RPM for 3 min.

(2) Measured the OD value of the remaining bacterial liquid by uv spectrophotometer (the

dilution of the bacterial liquid is 10 times)and added the volume of high pure water, which is consistent with the measured OD600 value, into the centrifuge tube, so that the amounts of bacteria in the tube can reach 10OD.

⑶Mixed the bacteria solution, take 1.2ml bacteria solution into 1.5ml EP tube and put it on ice. Finally, the bacterial cells were broken by using the ultrasonic crushing instrument, and the crushing conditions were shown in table 1-9.

表 1-9　超声破碎的条件

Table1-9 Conditions of Sonic disruption

项目	数值
工作时间	5s
停顿时间	5s
破碎总时间	5min
功率	35%
控制温度	30℃

⑷After ultrasonic crushing, centrifugation was performed for 2 min under 12,000 x g condition.
⑸Transferred supernatant to a new 1.5ml EP tube, and re-added the precipitation with 1.2ml high pure water and mixed.
⑹Took 10 uL of supernatant and 10 uL of precipitate respectively in two new 1.5EP tubes with 10 uL 2 x protein loading buffer.
⑺After boiling water for 10 min, we centrifuged the samples again for 2 min with 12,000 x g, and then took 10 uL samples (the final amount of sample was 50 mOD) for Polyacrylamide Gel Electrophoresis (sds-page) to observe the expression of d-lactic acid dehydrogenase and transaminase.

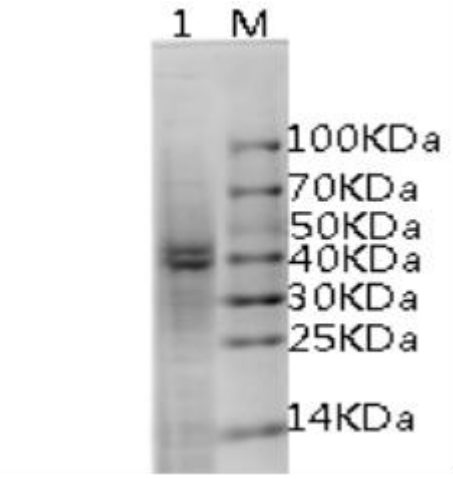


图 1-10　A：BW/pRB1s-MD*ldh-Tryb* 的 SDS-PAGE 蛋白表达

Fig.1-10　The SDS-PAGE results of the recombinant strain A:BW/pRB1s-MD*ldh-Tryb*、

Note:1:protein of D*ldh*、*Tyrb*

D-乳酸脱氢酶蛋白大小为 40 KDa 左右，转氨酶蛋白大小为 43KDa 左右

Test of whole cell catalytic efficiency of engineered bacteria

⑴Took 100ul recombinant bacteria after night induction, after centrifugation, we discarded supernatant and added 1 mLddH2 to the precipitation.

⑵Used ultraviolet spectrophotometer to measure the OD value of bacteria solution (the dilution of bacteria solution was 10 times measured), and obtained the bacteria solution containing 50 OD according to the measured OD value.

⑶The above samples were centrifuged with a high-speed freezing centrifuge (12,000rpm for 5 min), the supernatant was discarded, and the bacteria were collected and added with the substrate phenylalanine （100 mM pH7.5 Tris-HCl ）of 50mm. Then carried out whole cell catalytic experiments in the condition of 37℃， 220 rpm. Set 3 replications for each strain of engineered bacteria.

⑷Took samples every one hour and used HPLC to measure the content of d-pla in the conversion solution and the production rate of unit bacteria D-PLA.

TIME week 6)

Construction of recombinant plasmid vectors

⑴ Recovered and purified the PCR carrier fragment (prb1s-dldh-tyrb). The purification method was performed according to the instructions of SanPrep column DNA gel recovery kit.

⑵Connected the rocG fragment and the carrier fragment prb1s-mdldh-tyrb by Gibson method。

表 1-17　Gibson 反应体系

Table1-17 The reaction system of Gibson

Gibson 反应体系	
双酶切纯化后载体 pRB1s-*Tyrb*/pRB1s-MD*ldh-Tyrb* PCR 扩增纯化产物 MD*ldh/rocG*	质粒跟基因片段按照摩尔比 1：3， 两者体积和为 4 μL
Gibson 体系	6 μL
反应条件	50 ℃反应 1 h

⑶Put the ligand products into *E.coli* BW25113 receptor, and the monoclonal recombinants were

obtained after overnight culture.

⑷The monoclonal was selected for preliminary PCR validation, which was then sent to Shanghai for gene sequencing validation. The recombinants after successful verification were saved in 12.5% of glycerol, and stored in the condition of frozen - 80 ℃.

菌落 PCR 验证

Fig.1-8 PCR verification of Bacterial colony

M: DL5000,1: *rocG*,2: Prb1s-D*ldh*-*Tyrb*: PCR products

Time (week7)

Engineering bacteria induction culture

⑴selected the engineered bacteria and original strains (BW/ prb-dldh, bwprb1s-dldh-tyrb, BW25113) from the seed tube and inoculated on LB solid medium containing streptomycin resistance and activated overnight.

⑵ Pick a single colony to 5 mL LB liquid medium containing streptomycin and cultured it in the condition of 37 ℃、 220 RPM for around 8 hours.

⑶ Inoculated 500UL bacterial solution into 3 bottles of 50mL self-induction medium and cultured it in the condition of 37℃，220 rpm for 16 h

SDS-PAGE verifies protein expression

⑴After the induction culture was completed, 3 bottles of bacteria solution of each group were mixed separately and 10 mL of bacteria solution was taken and centrifuged at 8000 RPM for 3 min.

⑵Measured the OD value of the remaining bacterial liquid by uv spectrophotometer (the dilution of the bacterial liquid is 10 times)and added the volume of high pure water, which is consistent with the measured OD600 value, into the centrifuge tube, so that the amounts of

bacteria in the tube can reach 10OD.

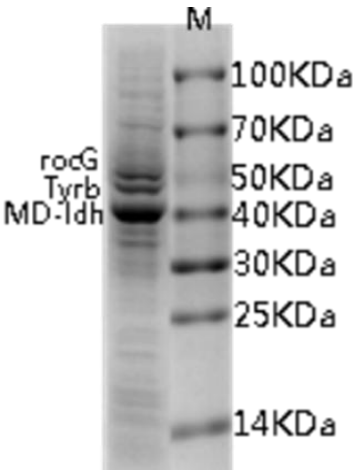
⑶Mixed the bacteria solution, take 1.2ml bacteria solution into 1.5ml EP tube and put it on ice. Finally, the bacterial cells were broken by using the ultrasonic crushing instrument, and the crushing conditions were shown in table 1-9.

表 1-9　超声破碎的条件

Table1-9 Conditions of Sonic disruption

项目	数值
工作时间	5s
停顿时间	5s
破碎总时间	5min
功率	35%
控制温度	30℃

⑷After ultrasonic crushing, centrifugation was performed for 2 min under 12,000 x g condition.
⑸Transferred supernatant to a new 1.5ml EP tube, and re-added the precipitation with 1.2ml high pure water and mixed.
⑹Took 10 uL of supernatant and 10 uL of precipitate respectively in two new 1.5EP tubes with 10 uL 2 x protein loading buffer.
⑺After boiling water for 10 min, we centrifuged the samples again for 2 min with 12,000 x g, and then took 10 uL samples (the final amount of sample was 50 mOD) for Polyacrylamide Gel Electrophoresis (sds-page) to observe the expression of d-lactic acid dehydrogenase and transaminase.



BW/pRB1s-MD*ldh-Tryb-rocG* 的 SDS-PAGE 蛋白表达

Fig.1-10 The SDS-PAGE results of the recombinant strain: BW/pRB1s-MD*ldh-Tryb-rocG*

Test of whole cell catalytic efficiency of engineered bacteria

⑴Took 100ul recombinant bacteria after night induction, after centrifugation, we discarded supernatant and added 1 mLddH2 to the precipitation.

⑵Used ultraviolet spectrophotometer to measure the OD value of bacteria solution (the dilution of bacteria solution was 10 times measured), and obtained the bacteria solution containing 50 OD according to the measured OD value.

⑶The above samples were centrifuged with a high-speed freezing centrifuge (12,000rpm for 5 min), the supernatant was discarded, and the bacteria were collected and added with the substrate phenylalanine （100 mM pH7.5 Tris-HCl ）of 50mm. Then carried out whole cell catalytic experiments in the condition of 37℃， 220 rpm. Set 3 replications for each strain of engineered bacteria.

⑷Took samples every one hour and used HPLC to measure the content of d-pla in the conversion solution and the production rate of unit bacteria D-PLA.

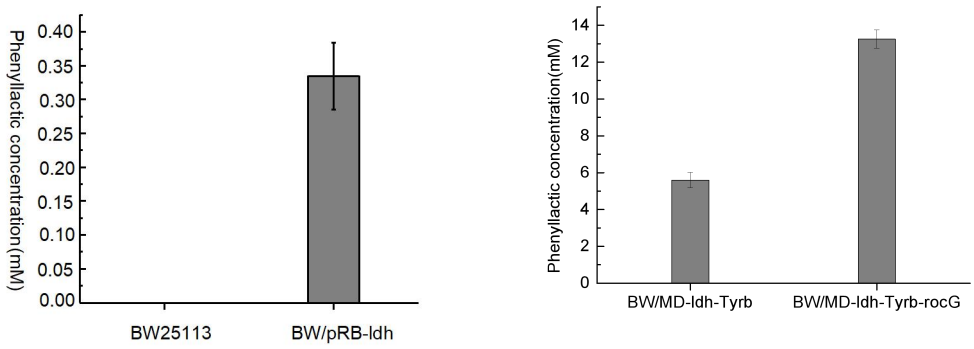


图 1-11　全细胞催化生成 D-PLA 的浓度

Fig.1-11 The whole-cell bioconversion of the recombinant strain for D-PLA concentration

BW/pRB1s-MD*ldh-Tyrb*= BW/MD-ldh-Tyrb

BW/pRB1s-MD*ldh-Tyrb-rocG*= BW/MD-ldh-Tyrb-rocG

TIME(week8)

Obtained different amounts of d-lactate dehydrogenase gene by PCR

⑴Inoculated the previously constructed recombinant bacteria (BW/ prb1s-mdldh-tyrb-rocg) into solid LB tablets containing 50mg/mL streptomycin for activation and cultured it in the condition of 37 c overnight.

⑵ Single colonies were selected and transferred to 5 mL LB liquid medium containing streptomycin for 8 h culture and 2 mL bacteria solution was taken for plasmid extraction。

⑶ Performed PCR amplification of d-lactate dehydrogenase gene at the start codon downstream of its primer carrying different amounts of arginine rare codon (AGG or AGA).

表 2-2　PCR 扩增条件

Table2-2 Reaction conditions of PCR

PCR 扩增条件		
98 ℃	2 min	1 个循环
98 ℃	30 S	
66℃	30 S	29 个循环
72 ℃	45 S	
72 ℃	10 min	

⑷Recovered the amplified PCR products with glue and purified it. The purification method was performed according to the instructions of SanPrep column DNA gel recovery kit.

⑸Used agarose gel electrophoresis to determine whether the product was purified.　Then, the desired products was stored in the condition of -20c.

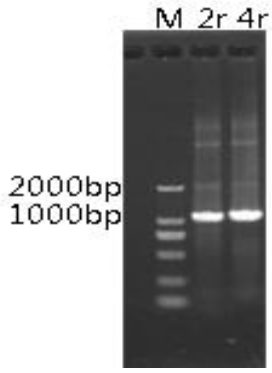


图 2-1　D*ldh-2r*、D*ldh-4r* 扩增片段；

Fig.2-1　PCR amplification for D*ldh-2r*、D*ldh-4r* ;

Note: M: Marker: DL2000;2r= D*ldh-2r*:2Arg;4r= D*ldh-4r*:4Arg PCR products;

TIME(week9)

Construction of recombinant plasmid vectors

⑴ Cut the plasmid vector by double enzyme digestion and obtained carrier segment （pRB1s-*Tyrb-rocG*），the restriction enzymes we used are XhoI Ⅰ and Nco Ⅰ.

⑵Recovered the carrier fragment (prb1s-tyrb-rocg) obtained by Double Digests and purified it. The purification method was performed according to the instructions of SanPrep column DNA gel recovery kit.

⑶ Connected the retrieved and purified Dldh fragments with rare codon bases to the carrier fragment prb1s-tyrb-rocg from Double Digests by using the Gibson method

⑷ After the connection was completed, the ligands were transferred into the *E.coli* BW25113 receptor respectively. The clonons were screened using solid medium containing streptomycin resistance.

⑸ Obtained monoclonal recombinants by overnight culture. After that, selected the monoclonal for preliminary PCR verification. If the preliminary verification was successful, sent it to Shanghai for gene sequencing verification.

⑹The recombinant after successful verification was stored in 12.5% glycerol in -80 degree refrigerator.

⑺The reconstructed recombinant bacteria were named BW/pRB1s-2rMD*ldh-Tyrb-rocG*、BW/pRB1s-4rMD*ldh-Tyrb-rocG* respectively.

表 2-3 双酶切反应体系

Table2-3 The reaction system of enzyme digestion

酶切反应体系	
10×3.1 buffer	5 μL
XhoI	1uL
NcoI	1 μL
pRB1s-MD*ldh-Tyrb-rocG*	2~2.5 μg
ddH_2O	补加至 50 μL
反应条件	37 ℃维持 3-5 h

表 2-4 Gibson 反应体系

Table2-4 The reaction system of Gibson

Gibson 反应体系	
PCR 纯化后载体 pRB1s-*Tyrb-rocG*	质粒跟基因片段按照摩尔比 1：3，
PCR 扩增纯化产物 rMD*ldh*	两者体积和为 4 μL
Gibson 体系	6 μL

反应条件	50 ℃反应 1 h

图 2-1　菌落 PCR 验证

Fig.2-1 B: PCR verification of the transformants

Note: M: Marker: DL2000; M: DL2000,1:2rD*ldh*,2:4rD*ldh*

TIME（week 9）

Induction culture of engineering bacteria

⑴selected the engineered bacteria and original strains (BW/ prb-dldh, bwprb1s-dldh-tyrb, BW25113) from the seed tube and inoculated on LB solid medium containing streptomycin resistance and activated overnight.

⑵ Pick a single colony to 5 mL LB liquid medium containing streptomycin and cultured it in the condition of 37 ℃、 220 RPM for around 8 hours.

⑶ Inoculated 500UL bacterial solution into 3 bottles of 50mL self-induction medium and cultured it in the condition of 37℃，220 rpm for 16 h.

Verify the expression of 2rMDldh and 4rMDldh by SDS-PAGE

⑴After the induction culture was completed, 3 bottles of bacteria solution of each group were mixed separately and 10 mL of bacteria solution was taken and centrifuged at 8000 RPM for 3 min.

⑵Measured the OD value of the remaining bacterial liquid by uv spectrophotometer (the dilution of the bacterial liquid is 10 times)and added the volume of high pure water, which is consistent with the measured OD600 value, into the centrifuge tube, so that the amounts of bacteria in the tube can reach 10OD.

⑶Mixed the bacteria solution, take 1.2ml bacteria solution into 1.5ml EP tube and put it on

ice. Finally, the bacterial cells were broken by using the ultrasonic crushing instrument, and the crushing conditions were shown in table 1-9.

表 1-9　超声破碎的条件

Table1-9 Conditions of Sonic disruption

项目	数值
工作时间	5s
停顿时间	5s
破碎总时间	5min
功率	35%
控制温度	30℃

⑷After ultrasonic crushing, centrifugation was performed for 2 min under 12,000 x g condition.
⑸Transferred supernatant to a new 1.5ml EP tube, and re-added the precipitation with 1.2ml high pure water and mixed.
⑹Took 10 uL of supernatant and 10 uL of precipitate respectively in two new 1.5EP tubes with 10 uL 2 x protein loading buffer.
⑺After boiling water for 10 min, we centrifuged the samples again for 2 min with 12,000 x g, and then took 10 uL samples (the final amount of sample was 50 mOD) for Polyacrylamide Gel Electrophoresis (sds-page) to observe the expression of d-lactic acid dehydrogenase and transaminase.

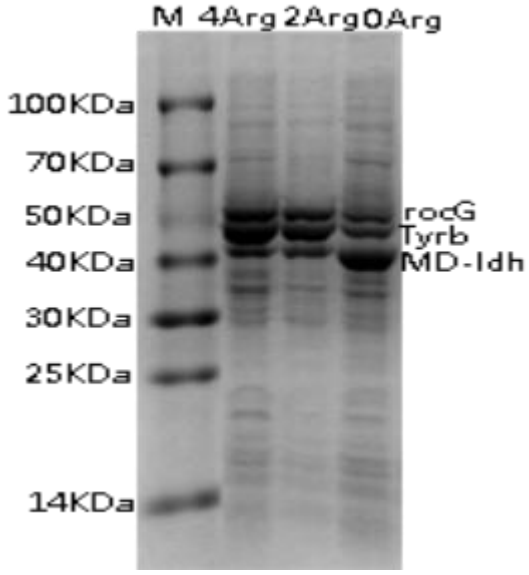


图 2-2　重组菌的 SDS-PAGE 蛋白表达

Fig.2-2 The SDS-PAGE results of the recombinant strain

Note：M=Maker;0Arg: BW/pRB1s-MD*ldh*-Tryb-*rocG*

2Arg: BW/pRB1s-2rMD*ldh*-Tryb-*rocG*

4Arg: BW/pRB1s-4rMD*ldh*-Tryb-*rocG*

Test of whole cell catalytic efficiency of engineered bacteria

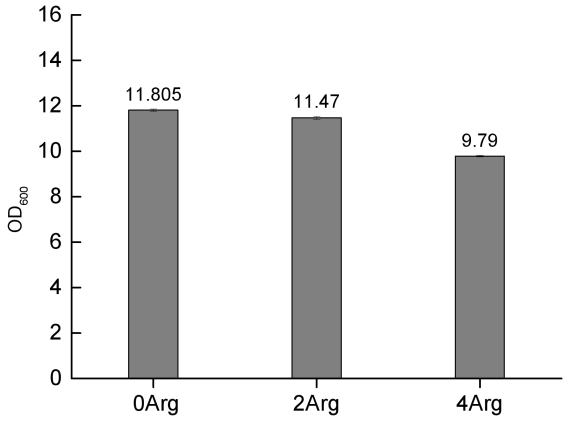


图 2-3　Arg 数量对重组菌生物量的影响

Fig. 2-3 Effect of Arg number on the recombinant stain biomass

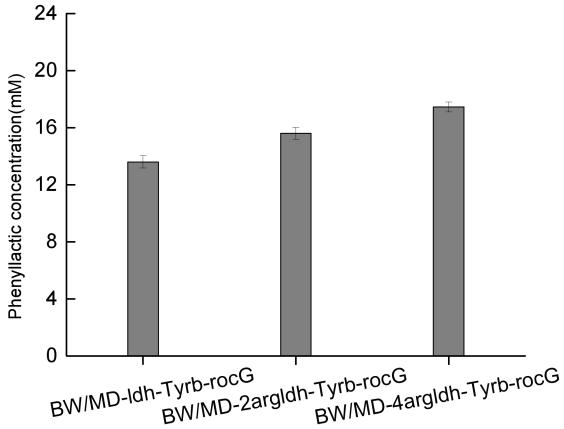


图 2-4　全细胞催化生成 D-PLA 的浓度

Fig. 2-4 The whole-cell bioconversion of the recombinant strain for D-PLA concentration

XINYUE'S

NOTEBOOK

Week 1 27.05-03.06

Cultivate *E. coli BW25113* and extract genome.

27.05

Streak *E. coli BW25113* on LB plates and inoculate for 12-16 hours at 37℃ for subsequent genome extraction.

28.05

E. coli BW25113 successfully grows.

Isolate a single colony from the LB plate, and inoculate a culture of 5ml LB medium. Incubate for 12-16 hours at 37℃ with vigorous shaking.

29.05-31.05

Extract genome of *E. coli* Bw25113 with TRAN EasyPure Bacteria Genomic DNA Kit using the following protocol.

Protocol:

1. Preparing materials
   a) Transfer 1~5 ml cell culture (≤2×109) to a 1.5 ml tube and centrifuge the tube at 12,000×g for 1 minute. Discard the supernatant completely.
   b) Add 100 µl LB2 and 20 µl Proteinase K into the tube. Resuspend the cell pellet by vortexing or pipetting. c) Incubate at 55°C for 15 minutes.
2. Centrifuge the tube briefly and transfer all the lysate to a spin column. Centrifuge at 12,000×g for 30 seconds. Discard the flow through.
3. Add 500 µl CB2, Centrifuge at 12,000×g for 30 seconds. Discard the flow through.
4. Repeat step 4. 6
5. Add 500 µl WB2 (check to ensure you have added ethanol) and centrifuge at 12,000×g for 30 seconds. Discard the flow through.
6. Repeat step 6.
7. Centrifuge the empty column at maximum speed (≥12,000×g) for 2 minutes to remove residual WB2.
8. Place the spin column in a sterile 1.5 ml microcentrifuge tube. Add 50-200 µl of Elution Buffer (preheated to 65°C) or sterile, distilled water (pH >7.0, preheated to 65°C) to the column matrix. Incubate at room temperature for 1 minute. Centrifuge at 12,000×g for 1 minute to elute the isolated genomic DNA. Optional: To get more DNA by repeating step 9.
9. Store the isolated DNA at -20°C.

Results:

Tube 1

70.098 ng/µl	A260	1.4020	260/230	0.80	260/280	1.69

Tube 2

53.885 ng/µl	A260	1.0777	260/230	0.13	260/280	1.75

Tube 3

40.985 ng/µl	A260	0.8197	260/230	0.11	260/280	1.36

Tube 4

89.370 ng/µl	A260	1.7874	260/230	0.66	260/280	1.56

Week 2 04.06-10.06

Cultivate *E. coli BM4R* and extract plasmid.

Cultivate *Clostridium acetobutylicum*

04.06

Streak *E. coli* BM4R on LB plates containing streptomycin antibiotic and inoculate for 12-16 hours at 37℃ for subsequent plasmid extraction.

05.06

E. coli BM4R successfully grew.

Isolate a single colony from the LB plate, and inoculate a culture of 5ml LB medium containing streptomycin antibiotic. Incubate for 12-16 hours at 37℃ with vigorous shaking.

06.06

Extract plasmid of *E. coli* BM4R with E.Z.N.A.® Plasmid Mini Kit I using the following protocol.

Protocol:

1. Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture. Add 1ml of bacteria solution to the culture tube.
2. Centrifuge at 10,000 x g for 1 minute at room temperature.
3. Decant or aspirate and discard the culture media.
4. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.
   Note: RNase A must be added to Solution I before use.
5. Transfer suspension into a new 1.5 mL microcentrifuge tube.
6. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary.
   Note: Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO2 in the air.
7. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.
   Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.
8. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.

9. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
10. Transfer the cleared supernatant from Step 8 by CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
11. Centrifuge at maximum speed for 1 minute.
12. Discard the filtrate and reuse the collection tube.
13. Add 500 µL HBC Buffer.
    Note: HBC Buffer must be diluted with 100% isopropanol before use.
14. Centrifuge at maximum speed for 1 minute.
15. Discard the filtrate and reuse collection tube.
16. Add 700 µL DNA Wash Buffer.
    Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use.
17. Centrifuge at maximum speed for 1 minute.
18. Discard the filtrate and reuse the collection tube.
19. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
    Note: It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.
20. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
21. Add 30-100 µL Elution Buffer or sterile deionized water directly to the center of the column membrane.
    Note: The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If using sterile deionized water, make sure that the pH is around 8.5.
22. Let sit at room temperature for 1 minute.
23. Centrifuge at maximum speed for 1 minute.
    Note: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.
24. Store DNA at -20°C.

Results:

Tube 1							
30.353 ng/µl	A260	0.6071	260/230	1.87	260/280	1.81	
Tube 2							
21.080 ng/µl	A260	0.4216	260/230	1.86	260/280	1.78	
Tube 3							
27.458 ng/µl	A260	0.5492	260/230	1.62	260/280	1.83	
Tube 4							
38.101 ng/µl	A260	0.7602	260/230	1.47	260/280	1.73	

07.06

Cultivate *Clostridium acetobutylicum*

PYG medium

polypeptone	5.0	g
tryptone	5.0	g
yeast extract	10.0	g
glucose	10.0	g
salt solution(the formula is as follows)	40.0	ml
distilled water	960.0	ml
agar	15.0	g
PH 7.2		
Salt solution		
CaCl2	0.2	g
MgSO4 ▪ 7H20	0.4	g
K2HPO4	1.0	g
KH2PO4	1.0	g
NaHCO3	10.0	g
NaCl	2.0	g
distiled water	1.0	L

Protocol:

1. Prepare 20ml liquid medium according to the above formula.
2. Place the medium in an anaerobic culture flask, and place a blue lid, press the iron ring to fix.
3. Open the valve of the bottle filled with the nitrogen.
4. Unscrew the switch of the pumping device and open the vacuum pump.
5. Slowly insert the needle into the flask while inflating.
6. Inflate for 40 seconds, when the liquid level is almost stable, open the suction switch, pump for 60 seconds, and then inflate.
7. Repeat the above steps 4-5 times.
8. Pull out the needle while inflating.
9. First off the gas pump, close the inflation valve.
10. Sterilize the medium at 115℃ for 30 minutes.
11. Pipette 1ml of *Clostridium acetobutylicum* solution into the medium with a 2ml syringe.
12. Cultivate *Clostridium acetobutylicum at 37 ℃.*

Week 3 11.06-17.06

Week 4 18.06-24.06

Week 5 25.06-01.07

Cultivate *Clostridium acetobutylicum* at 37℃

Result:

Clostridium acetobutylicum did not multiply.

Maybe there is no polypeptone in the medium, I replaced it with bacterial peptone.

Week 6 02.07-08.07

Get fragments of atoD, atoA and adhE2 by PCR

Try to extract genome of *Clostridium acetobutylicum*

06.07

Do a PCR pre-experiment to amplify atoD, atoA and backbone using the following protocol:

Genome *BW25113*: 40.985 ng/μl Plasmid *BM4R:* 38.101 ng/μl

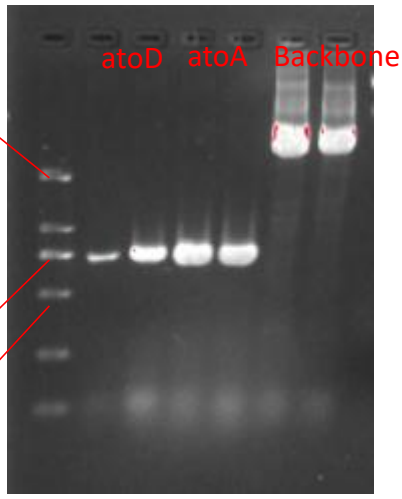
PCR Protocol I

Initiation	95 °C, 5 minutes	
Denaturation	94 °C, 35 seconds	
Annealing	64 °C, 35 seconds	×30 cycles
Extension	72 °C, 4 minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 μl
template	0.5 μl
10 μM Forward Primer	0.5 μl
10 μM Reverse Primer	0.5 μl
ddH2O	6 μl
Total volume	15 μl

Three PCR reactions were prepared: atoD, atoA, backbone.



The PCR products were run on a 1% Agarose gel for at 150V and 180mA. Each well was loaded with 4 μl of the PCR product and the first well was loaded with 4 μl of 2000 marker. The atoD and atoA were as expected, while the backbone was miscellaneous band.

07.07

atoD and atoA were amplified using the following protocol:

Genome *BW25113*: 40.985 ng/μl

PCR Protocol Ⅰ

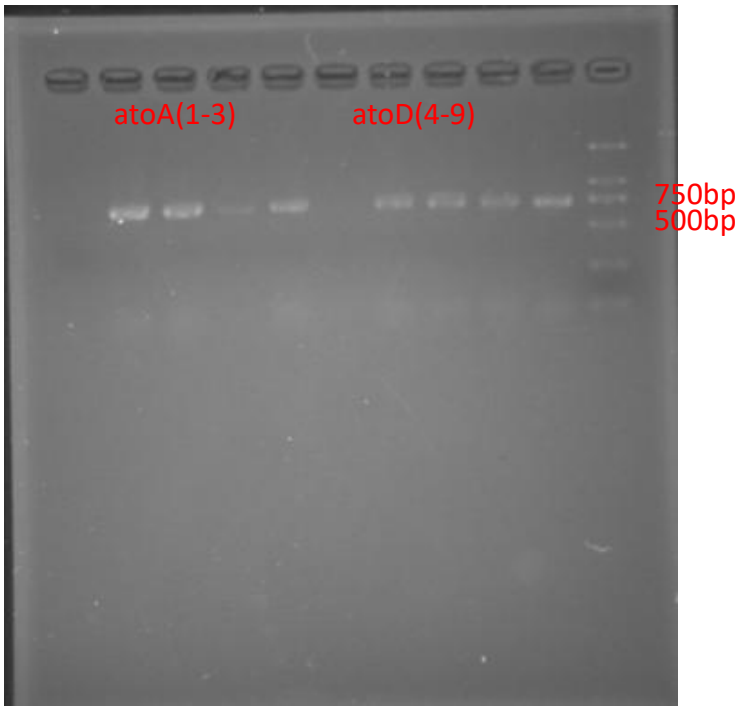
Initiation	95 °C, 5 minutes	
Denaturation	94 °C, 35 seconds	
Annealing	64 °C, 35 seconds	×30 cycles
Extension	72 °C,2 minutes	
Final Extension	72 °C, 10 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 µl
template	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
ddH2O	6 µl
Total volume	15 µl

Two PCR reactions were prepared:

The PCR products were run on a 1% Agarose gel. Five bands of atoD were as expected and one band was blank. Two banks of atoA were expected and two banks were blank.



Extract genome of *Clostridium acetobutylicum* with TRAN EasyPure Bacteria Genomic DNA Kit. Protocol is as above.

Result:

Failed.

6.415 ng/μl A260 0.1283 260/230 0.09 260/280 1.14

Clostridium acetobutylicum is a Gram-positive bacterium, but the genome is extracted according to the protocol of Gram-positive bacteria.

08.07

Increase annealing temperature to amplify backbone.

Plasmid *BM4R:* 38.101 ng/μl

PCR Protocol Ⅱ

Initiation	95 °C, 5 minutes	
Denaturation	94 °C, 35 seconds	
Annealing	67 °C, 35 seconds	×30 cycles
Extension	72 °C, 4 minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 μl
template	0.5 μl
10 μM Forward Primer	0.5 μl
10 μM Reverse Primer	0.5 μl
ddH2O	6 μl
Total volume	15 μl

Extract genome of *Staphylococcus epidermidis* with TRAN EasyPure Bacteria Genomic DNA Kit with Yuxian Chen.

Results:

Tube 1

44.342 ng/μl A260 0.8868 260/230 1.93 260/280 2.11

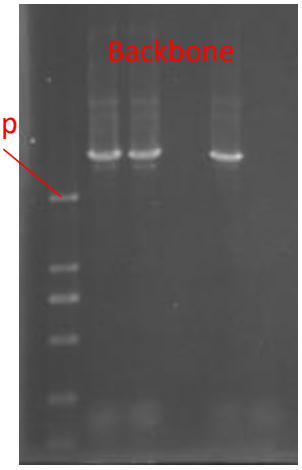
Tube 2

55.360 ng/μl A260 1.1072 260/230 1.34 260/280 1.70

Week 7 09.07-15.07

Cycle pure PCR product of atoD and atoA, gel extraction of adhE2

09.07



The PCR products of backbone in 67 °C were run on a 1% agarose gel at 120V. The backbone was miscellaneous band. The first well was loaded with 4 µl of 2000 marker and the second, third, and the forth was loaded with 4 µl of backbone.

Extract genome of *Clostridium acetobutylicum* with TRAN EasyPure Bacteria Genomic DNA Kit using the following protocol.

Protocol:

1. Preparing materials
   a) Transfer 1~5 ml cell culture (≤2×109) to a 1.5 ml tube and centrifuge the tube at 12,000×g for 1 minute. Discard the supernatant completely.
   b) Add 200 µl RB2 (including 4mg lysozyme) into the tube. Incubate at 37℃with no less than 60 minutes.
   c) Add 100 µl LB2 and 20 µl Proteinase K into the tube. Resuspend the cell pellet by vortexing or pipetting.
   d) Incubate at 55°C for 15 minutes.
2. Centrifuge the tube briefly and transfer all the lysate to a spin column. Centrifuge at 12,000×g for 30 seconds. Discard the flow through.
3. Add 500 µl CB2, Centrifuge at 12,000×g for 30 seconds. Discard the flow through.
4. Repeat step 4. 6
5. Add 500 µl WB2 (check to ensure you have added ethanol) and centrifuge at 12,000×g for 30 seconds. Discard the flow through.
6. Repeat step 6.
7. Centrifuge the empty column at maximum speed (≥12,000×g) for 2 minutes to remove residual WB2.
8. Place the spin column in a sterile 1.5 ml microcentrifuge tube. Add 50-200 µl of Elution Buffer (preheated to 65°C) or sterile, distilled water (pH >7.0, preheated to 65°C) to the column matrix. Incubate at room temperature for 1 minute. Centrifuge at 12,000×g for 1 minute to elute the isolated genomic DNA. Optional: To get more DNA by repeating step 9.
9. Store the isolated DNA at -20°C.

Result:

6.707 ng/µl	A260	0.1341	260/230	0.47	260/280	1.07

Failed.
It's unknown for reason.

10.07
Increase annealing temperature to amplify backbone.
Plasmid *BM4R:* 38.101 ng/µl

PCR ProtocolⅢ

Initiation	95 °C, 5 minutes	
Denaturation	94 °C, 35 seconds	
Annealing	69 °C, 35 seconds	×30 cycles
Extension	72 °C, 4 minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 µl
template	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
ddH2O	6 µl
Total volume	15 µl

The PCR products of backbone in 67 °C were run on a 1% agaros gel at 120V. There were no banks.

11.07 Typhoon

12.07
Cycle pure PCR product of atoD and atoA with E.Z.N.A.® Cycle Pure Kit using the following protocol.

Protocol:

1. Perform agarose gel/ethidium bromide electrophoresis to analyze PCR product.
2. Determine the volume of your PCR reaction.
3. Transfer the sample into a clean 1.5 mL microcentrifuge tube (not provided).
4. Add 4-5 volumes CP Buffer. For PCR products smaller than 200 bp, add 5 volumes CP Buffer and 0.4 volumes 100% isopropanol.

Note: Volume refers to the size of your PCR reaction. For example, if your PCR reaction is 100 µL and is smaller than 200 bp, you would use 500 µL CP Buffer and 40 µL isopropanol.

5. Vortex to mix thoroughly. Briefly centrifuge to collect any drops from the inside of the lid.
6. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube (provided).
7. Add the sample from Step 5 to the HiBind® DNA Mini Column.
8. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature.
9. Discard the filtrate and reuse collection tube.
10. Add 700 µL DNA Wash Buffer.
    Note: DNA Wash Buffer must be diluted with 100% ethanol before use.
11. Centrifuge at maximum speed for 1 minute.
12. Discard the filtrate and reuse collection tube.
13. Repeat Steps 10-12 for a second DNA Wash Buffer wash step.
14. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column.
    Note: This step is critical for removal of trace ethanol that may interfere with downstream applications.
15. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube.
16. Add 30-50 µL Elution Buffer, TE Buffer, or sterile deionized water directly to the center of column matrix.
17. Let sit at room temperature for 2 minutes.
18. Centrifuge at maximum speed for 1 minute.
    Note: This represents approximately 80-90% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.
19. Store DNA at -20°C.

Results:

atoD

64.758 ng/µl	A260	1.2952	260/230	1.64	260/280	1.89

Tube 2

99.083 ng/µl	A260	1.9817	260/230	2.04	260/280	2.05

13.07

Try to amplify adhE2 using the following protocol:

Adhe2 ①: 6.415 ng/µl Adhe2 ②: 6.707 ng/µl

PCR ProtocolⅣ

Initiation	95 °C, 5 minutes	
Denaturation	94 °C, 35 seconds	
Annealing	61 °C, 35 seconds	×30 cycles
Extension	72 °C, 2 minutes	
Final Extension	72 °C, 10 minutes	
Hold at 4 °C		

The PCR mix prepared for 15 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 µl
template	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
ddH2O	6 µl
Total volume	15 µl

The PCR products of adhE2 were run on a 1% agaros gel at 120V. There were no banks.

Gel extraction of backbone using the following protocol.

1. Perform agarose gel electrophoresis to fractionate DNA fragments.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 mL microcentrifuge tube.
4. Add 3 volume Binding Buffer.
5. Incubate at 60℃ for 7 minutes or until the gel has completely melted. Vortex or shake the tube every 2-3 minutes.
6. Insert a Column in a 2 mL Collection Tube.
7. Centrifuge at 12,000 x g for 1 minute at room temperature. Repeat 3 times. Discard the filtrate and reuse collection tube.
8. Add 650 µL SPW Wash Buffer.
9. Centrifuge at 12,000 x g for 1 minute at room temperature. Discard the filtrate and reuse collection tube.
10. Centrifuge the empty Column for 2 minutes at maximum speed to dry the column matrix.
11. Transfer the HiBind DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
12. Add 30µL deionized water directly to the center of the column membrane.
13. Let sit at room temperature for 2 minutes. Centrifuge at maximum speed for 1 minute.
14. Store DNA at -20℃.

Result:

13.9 ng/µl A260 0.278 260/230 1.91 260/280 0.45

14.07

Cultivate *Clostridium acetobutylicum*

PYG medium

polypeptone	5.0	g
tryptone	5.0	g
yeast extract	10.0	g

glucose	10.0	g
salt solution(the formula is as follows)	40.0	ml
distilled water	960.0	ml
agar	15.0	g
PH 7.2		
Salt solution		
CaCl2	0.2	g
MgSO4 ▪ 7H20	0.4	g
K2HPO4	1.0	g
KH2PO4	1.0	g
NaHCO3	10.0	g
NaCl	2.0	g
distilled water	1.0	L

Protocol:

1. Prepare two 10ml liquid medium according to the above formula.
2. Place the medium in an anaerobic culture flask, and place a blue lid, press the iron ring to fix.
3. Open the valve of the bottle filled with the nitrogen.
4. Unscrew the switch of the pumping device and open the vacuum pump.
5. Slowly insert the needle into the flask while inflating.
6. Inflate for 40 seconds, when the liquid level is almost stable, open the suction switch, pump for 60 seconds, and then inflate.
7. Repeat the above steps 4-5 times.
8. Pull out the needle while inflating.
9. First off the gas pump, close the inflation valve.
10. Sterilize the medium at 115℃ for 20 minutes.
11. Pipette all *Clostridium acetobutylicum* solution into the two flasks with a 2ml syringe.
12. Cultivate *Clostridium acetobutylicum at 37 ℃.*

15.07

Try to get adhE2 by PCR.

Result:

There were no stripes.

Week 8 16.07-22.07

Week 9 23.07-29.07

Week 10 30.07-05.08

Try to extract genome of *Clostridium acetobutylicum*

Get the fragments of atoD and atoA

31.07

Extract plasmid of eGFP and pUV5 with E.Z.N.A.® Plasmid Mini Kit I using the protocol as above with Xueyi Chen.

01.08

Try to extract genome of *Clostridium acetobutylicum* cultured a few days before with TRAN EasyPure Bacteria Genomic DNA Kit using the protocol as above.

Results:

Failed

0.220 ng/μl A260 0.0044 260/230 0.04 260/280 0.32

02.08-03.08

Making a standard curve for 2-PE by HPLC with Hanrong Zhu.

03.08

Do a PCR pre-experiment to amplify atoD, atoA of constructing part to add restriction sites for them using the following protocol.

Genome *BW25113*: 40.985 ng/μl

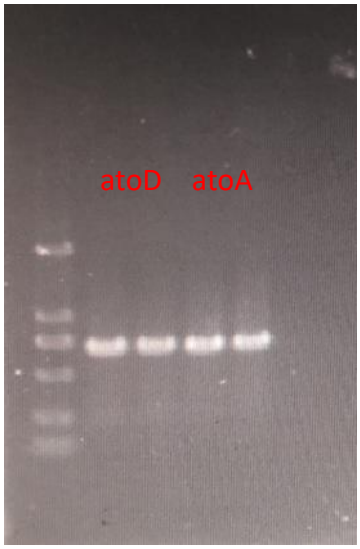
PCR Protocol Ⅴ

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	59 °C, 30 seconds	×30 cycles
Extension	72 °C, 1 minutes	
Final Extension	72 °C, 7 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 μl
template	0.5 μl
10 μM Forward Primer	0.5 μl
10 μM Reverse Primer	0.5 μl
ddH2O	6 μl
Total volume	15 μl

Result：



Expand the system to amplify atoD, atoA of constructing part using the following protocol.

Genome *BW25113*: 40.985 ng/μl

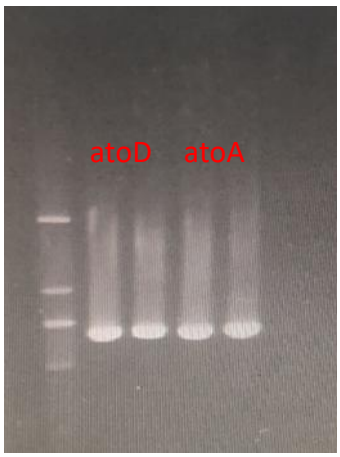
PCR Protocol Ⅵ

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	59 °C, 30 seconds	×30 cycles
Extension	72 °C, 1 minutes	
Final Extension	72 °C, 7 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 μl
template	0.5 μl
10 μM Forward Primer	1μl
10 μM Reverse Primer	1 μl
ddH2O	22.5 μl
Total volume	50 μl

Result:



04.08

Cycle pure PCR product of atoD and atoA of constructing part with E.Z.N.A.® Cycle Pure Kit using the protocol as above.

Results:

atoD:

182.565 ng/μl	A260	3.6513	260/230	2.17	260/280	2.01

atoA:

178.327 ng/μl	A260	3.5665	260/230	2.23	260/280	2.01

Do a PCR pre-experiment to amplify atoA and backbone of constructing pRB1a-atoD-atoA using the following protocol.

Genome *BW25113*: 40.985 ng/μl Plasmid *BM4R*: 50 ng/μl

PCR ProtocolⅧ

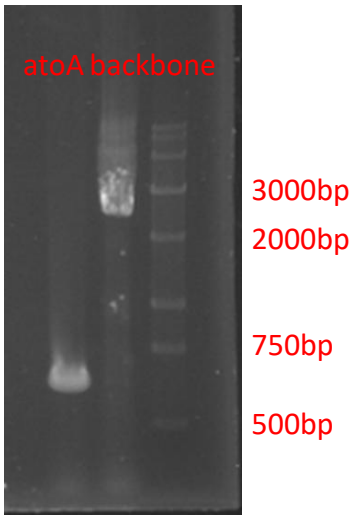
Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	63 °C, 30 seconds	×30 cycles
Extension	72 °C, 4 minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 15 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 μl
template	0.5 μl
10 μM Forward Primer	0.5 μl

10 μM Reverse Primer	0.5 μl
ddH2O	6 μl
Total volume	15 μl

The PCR products of atoA and backbone of constructing pRB1a-atoD-atoA were run on a 1% agaros gel at 120V. The bank of atoA was as expected, while the bank of backbone is miscellaneous.



Expand the system to amplify atoA of constructing pRB1a-atoD-atoA using the following protocol:
Genome *BW25113*: 40.985 ng/μl

PCR ProtocolⅧ

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	63°C, 30 seconds	×30 cycles
Extension	72 °C, 1:30 minutes	
Final Extension	72 °C, 10 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 μl
template	0.5 μl
10 μM Forward Primer	1μl
10 μM Reverse Primer	1 μl
ddH2O	22.5 μl
Total volume	50 μl

The backbone of constructing pRB1a-atoD-atoA was amplified by gradient PCR using the protocol as above.

05.08

Cycle pure PCR product of atoA of constructing pRB1a-atoD-atoA with E.Z.N.A.® Cycle Pure Kit using the protocol as above.

Result:

141.371 ng/μl A260 2.8274 260/230 2.29 260/280 2.06

The backbone of pRB1a-atoD-atoA was amplified for gel extraction using the following protocol:

Plasmid *BM4R*: 50 ng/μl

PCR ProtocolⅨ

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	63°C, 30 seconds	×30 cycles
Extension	72 °C, 4 minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 μl
template	0.5 μl
10 μM Forward Primer	1μl
10 μM Reverse Primer	1 μl
ddH2O	22.5 μl
Total volume	50 μl

Week 11 06.08-12.08

Make tnaA-/- competent cells and construct pRB1s-atoD-atoA by Gibson assembly, get atoD and atoA of connected part.

06.08

Gel extraction of the backbone.

Result:

246.721 ng/μl A260 4.9344 260/230 0.86 260/280 1.99

07.08

Make tnaA-/- competent cells according to the following protocol.

Protocol:

1. Pick up the activated single colonies into 5 mL of LB medium and incubate at 37 ℃, 220 rpm to logarithmic growth phase（OD600=0.4 - 0.6）.（OD value is detected every two hours）
2. In an aseptic ultra-clean bench, transfer 1.4 mL of cells in logarithmic growth phase to a sterile 1.5 mL centrifuge tube. Return to ice for 15min.
3. Centrifuge for 1 min at 4 ° C, 5000 rpm.
4. In an aseptic ultra-clean bench, discard the supernatant, and pour the centrifuge tube into the filter paper to blot out the remaining residue, and spray the suspension with 1 mL of ice-cold 0.1 M CaCl2 solution, ice bath 30 Min.
5. Centrifuge for 1 min at 4 ° C, 5000 rpm.
6. Repeat step 4, pour the supernatant, add 100 uL of pre-cooled 0.1 M CaCl2 solution, gently squirt and resuspend the cells, and ice bath for 30 min.
7. Centrifuge for 1 min at 4 ° C, 5000 rpm.
8. In an aseptic ultra-clean bench, pour the supernatant, add 100 uL of ice-cold 0.1M CaCl2-15% glycerol buffer solution, gently resuspend the cells, and store in a refrigerator at -80 °C.

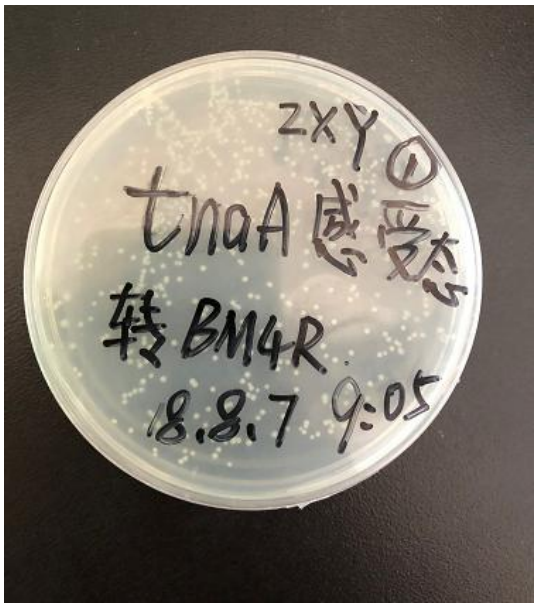
Verify the efficiency of the tnaA-/- competent cells

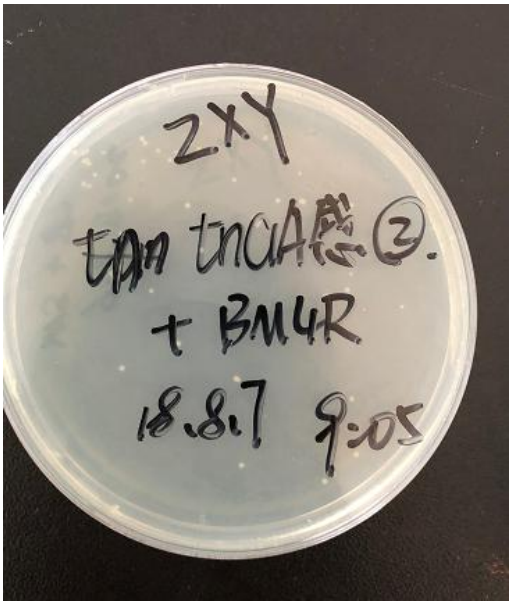
Protocol:

1. Pick 100 uL of tnaA-/- competent cells on the ice, and then add 2 uL plasmid of *BM4R* to it. Shake them gently and stand still on ice for 30 min.
2. Heat shock tubes at 42°C for 45 seconds and incubate on ice for 5min.
3. Pipette 800µl SOC media to each transformation.
4. Incubate at 37°C for 1 hours, shaking at 120rpm.
5. Pipette 150 uL of tnaA-/- competent cells on the plate.
6. Incubate for 12 hours at 37°C.

Result:

The first tnaA-/- competent cell is more efficient and the second is weaker.





08.08

Construct pRB1s-*atoD*-*atoA* by Gibson assembly.

Protocol:

The reaction system of Gibson

Component	**Volume**
atoD fragment (64.75 ng/μl 663bp)	1.42μl
atoA fragment (141.371 ng/μl 651bp)	0.64 μl
Backbone (246.721 ng/μl 3462bp)	1.94μl
Gibson system	6 μL
Reaction conditions	Reaction at 50 °C for 1 h

1. When the Gibson junction was left for 5 min, take out the prepared 100 uL E. coli BW25113 competent from the -80 degree refrigerator and left to stand in the ice .
2. The Gibson-linked product was added to the competent cell and gently mixed, ice-bathed for 30 min, and allowed to stand.
3. Heat shock at 42*C in a waterbath for 90secs. Return to ice for 2min.
4. Add 800 uL of SOC medium. 37 ° C, 120 rpm shock recovery for 1 h.
5. The resuscitated cells were centrifuged at 3000 rpm for 5 min, the supernatant was aspirated, and 1-200 uL was left, and plated on a LB solid medium plate containing streptomycin and cultured overnight at 37 °C.

The plasmid of BW25113 was digested with spe1 and xba1 to obtain the fragment for connecting part.

Protocol:

10×Cut-smart Buffer 5 ul

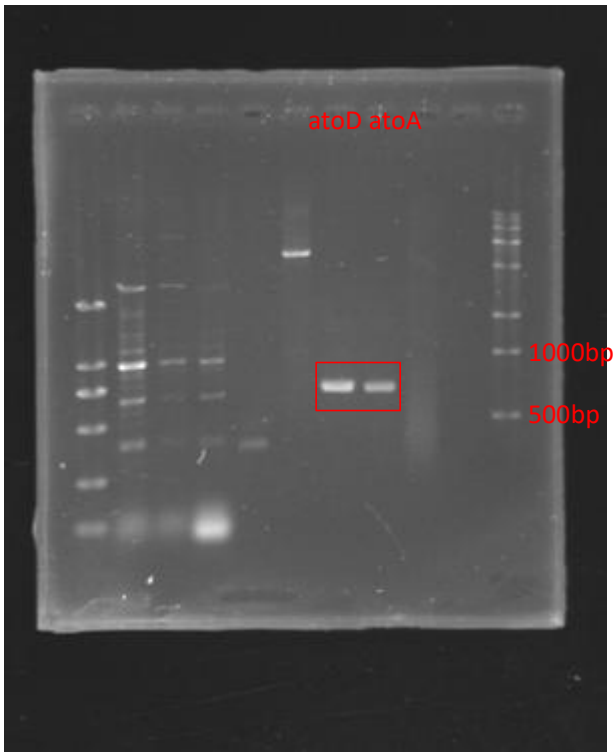
Xba Ⅰ 1 ul

Spe Ⅰ 1 ul

Plasmid	40 ul
ddH2O	3 ul

37℃ in aquatic bath for 5h and 80℃ for 30min.

Result:



Cycle pure digested product of atoD and atoA with E.Z.N.A.® Cycle Pure Kit using the protocol as above.

Results：

atoD

31.990 ng/μl	A260	0.6398	260/230	1.68	260/280	2.04

atoA:

37.808 ng/μl	A260	0.7562	260/230	1.64	260/280	1.93

Construct pSB1C3-atoD and pSB1C3-atoA by T4 ligation

Protocol:

T4 ligase	6 ul
atoD fragment	6.59 ul
backbone	1.41 ul
T4ase	1 ul
10×T4 Buffer	1 ul

T4 ligase	6 ul
atoA fragment	6.39 ul
backbone	1.61 ul

T4ase 1 ul

10×T4 Buffer 1 ul

16 °C in aquatic bath overnight.

09.08

Transformed connection product into DH5 α competent cells.

Protocol:

1. Thaw competent cells on ice
2. Pipette 10µl of connection product into DH5α competent cells
3. Close 1.5ml tubes, incubate on ice for 30min
4. Heat shock tubes at 42°C for 90 seconds
5. Incubate on ice for 3-5min
6. Pipette 800µl SOC media to transformation
7. Incubate at 37°C for 1 hours, shaking at 120rpm
8. Spin down cells at 5000r/min for 1mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates
9. Incubate transformations overnight (14-18hr) at 37°C

Picked 5 single colonies from the plate of gibson assembly into LB liquid medium with streptomycin resistance and incubated for 12 hours under a 37 degree shaker.

10.08

Use bacterial solution PCR to verify whether the Gibson connection is successful.

Protocol:

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	63°C, 30 seconds	×30 cycles
Extension	72 °C, 1:30 minutes	
Final Extension	72 °C, 10 minutes	
	Hold at 4 °C	

Component	**Volume**
2×*EasyTaq* PCR SuperMix	7.5 µl
Bacteria solution	2 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
ddH2O	4.5 µl
Total volume	15 µl

Result:
There were no stripes.

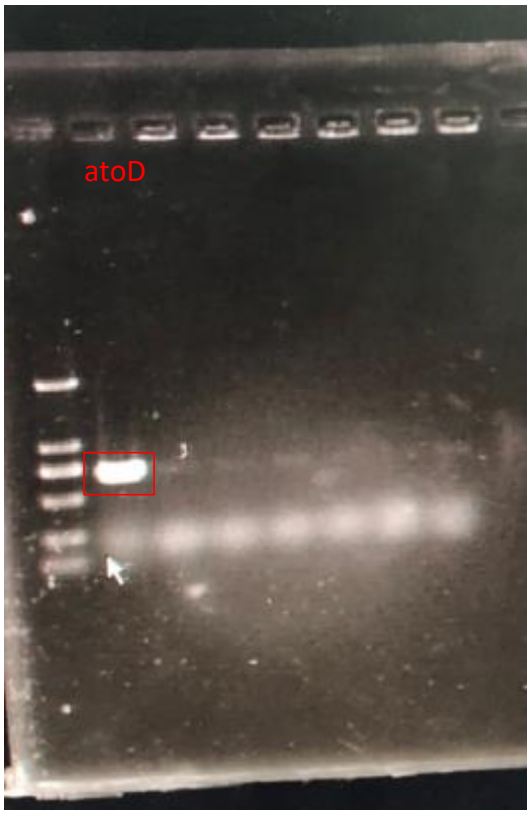
Picked one single colony from the plate 1 of T4 connection of pSB1C3-atoD into LB liquid medium with chloramphenicol resistance, and incubated for 12 hours under a 37 degree shaker.
There were no bacteria on the plate of pSB1C3-atoA.
Picked 8 single colonies from the plate of Gibson assembly into LB liquid medium with streptomycin resistance and incubated for 12 hours under a 37 degree shaker.

11.08
Use bacterial solution PCR to verify whether the Gibson connection is successful using the protocol as above.

Result:
There were no strips.

Use bacterial solution PCR to verify whether pSB1C-atoD of T4 connection is successful using the protocol as above.

Result:
There was one very light stripe. That verified the success of constructing pSB1C3-atoD.



Repeated the process of constructing pSB1C3-atoA by T4 ligation using the protocol as above.

12.08

Picked 5 single colonies from the plate of pSB1C3-atoA into LB liquid medium with chloramphenicol resistance and incubated for 12 hours under a 37 degree shaker.

Week 12 13.08-19.08

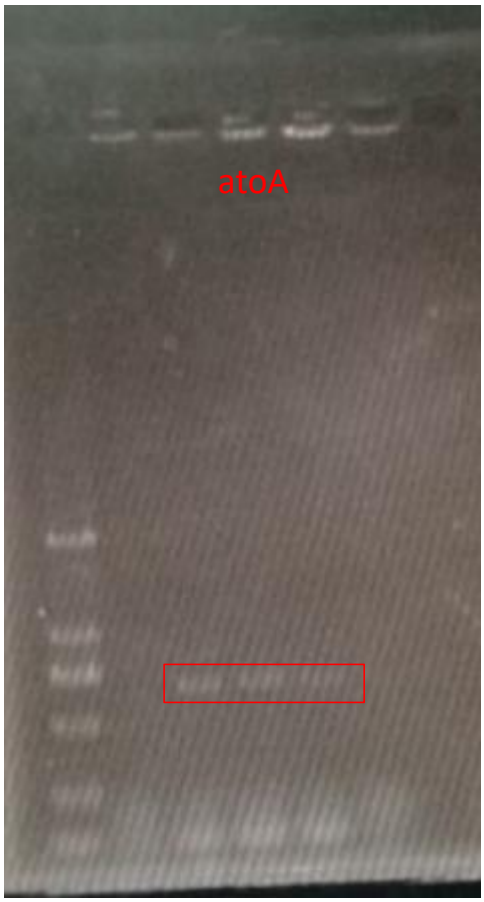
Construct pSB1C3-atoD and pSB1C3-atoA by T4 connection

14.08

Use bacterial solution PCR to verify whether pSB1C-atoA of T4 connection is successful using the protocol as above.

Result:

Bacteria of pSB1C3-atoA from number 2-4 were successfully connected.



15.08

Preserve the constructed bacteria of pSB1C3-atoA and pSB1C3-atoD.

Pipette 700 ul of bacteria solution into seed tube, and then added 300ul of 15% glycerin.

Extract plasmid of pSB1C3-atoA and pSB1C3-atoD with E.Z.N.A.® Plasmid Mini Kit I using the protocol as above.

Results:

pSB1C3-atoD

112.889 ng/μl	A260	2.2578	260/230	1.80	260/280	1.93
112.821 ng/μl	A260	2.2564	260/230	1.62	260/280	1.92

pSB1C3-atoA 2

8.475 ng/μl	A260	0.1749	260/230	0.80	260/280	1.69
12.175 ng/μl	A260	0.2435	260/230	0.73	260/280	1.27

pSB1C3-atoA

31.015 ng/μl	A260	0.6203	260/230	1.78	260/280	1.70
42.682 ng/μl	A260	0.8536	260/230	1.85	260/280	1.75

pSB1C3-atoA 4

24.790 ng/μl	A260	0.4958	260/230	2.17	260/280	1.94
37.808 ng/μl	A260	0.4722	260/230	0.22	260/280	1.93

17.08-19.08

We went to Xiamen to communicate with Xiamen University.

Week 13 20.08-26.08

Construct pRB1s-atoD-atoA by Gibson assembly

20.08

Make tnaA-/- competent cells according to the protocol as above.

21.08

Repeat the process of constructing pRB1s-*atoD-atoA* by Gibson assembly.

Protocol:

The reaction system of Gibson

Component	**Volume**
atoD fragment (64.75 ng/μl 663bp)	1.42μl
atoA fragment (141.371 ng/μl 651bp)	0.64 μl
Backbone (246.721 ng/μl 3462bp)	1.94μl
Gibson system	6 μL
Reaction conditions	Reaction at 50 °C for 1 h

1. When the Gibson junction was left for 5 min, take out the prepared 100 uL E. coli BW25113 competent from the -80 degree refrigerator and left to stand in the ice .
2. The Gibson-linked product was added to the competent cell and gently mixed, ice-bathed for 30 min, and allowed to stand.
3. Heat shock at 42*C in a waterbath for 90secs. Return to ice for 2min.
4. Add 800 uL of SOC medium. 37 ° C, 120 rpm shock recovery for 1 h.

5. The resuscitated cells were centrifuged at 3000 rpm for 5 min, the supernatant was aspirated, and 1-200 uL was left, and plated on a LB solid medium plate containing streptomycin and cultured overnight at 37 °C.

22.08

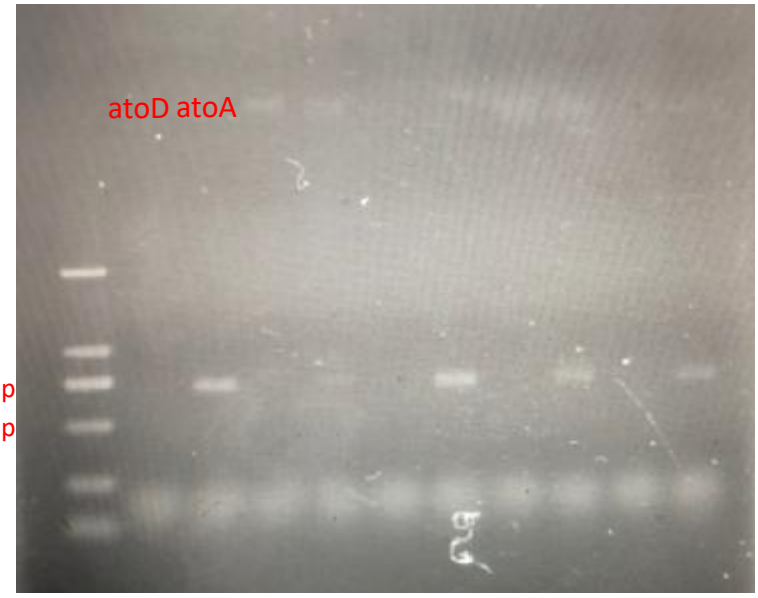
Picked 5 single colonies from the plate of pSB1C3-atoA into LB liquid medium with streptomycin resistance and incubated for 12 hours under a 37 degree shaker.

23.08

Use bacterial solution PCR to verify whether pRB1s-*atoD-atoA* is successfully constructed.

Result:

The construction of pRB1s-*atoD-atoA* is unsuccessful.



Week 14 27.08-02.09

Week 15 03.09-09.09

Get the fragment of adhE2

03.09

Remove 50ul of bacteria from the preservation tube, incubated for 12 hours under a 37 degree shaker.

05.09

Extract plasmid of pSB1C3-atoA and pSB1C3-atoD with E.Z.N.A.® Plasmid Mini Kit I using the protocol as above.

Results:

pSB1C3-atoD						
88.830 ng/μl	A260	1.7766	260/230	1.56	260/280	1.98
pSB1C3-atoD 2-6						
24.803 ng/μl	A260	0.4961	260/230	1.10	260/280	1.98
pSB1C3-atoA 3						
13.548 ng/μl	A260	0.2710	260/230	1.16	260/280	1.67
pSB1C3-atoA 4						
22.053 ng/μl	A260	0.4411	260/230	1.25	260/280	2.07

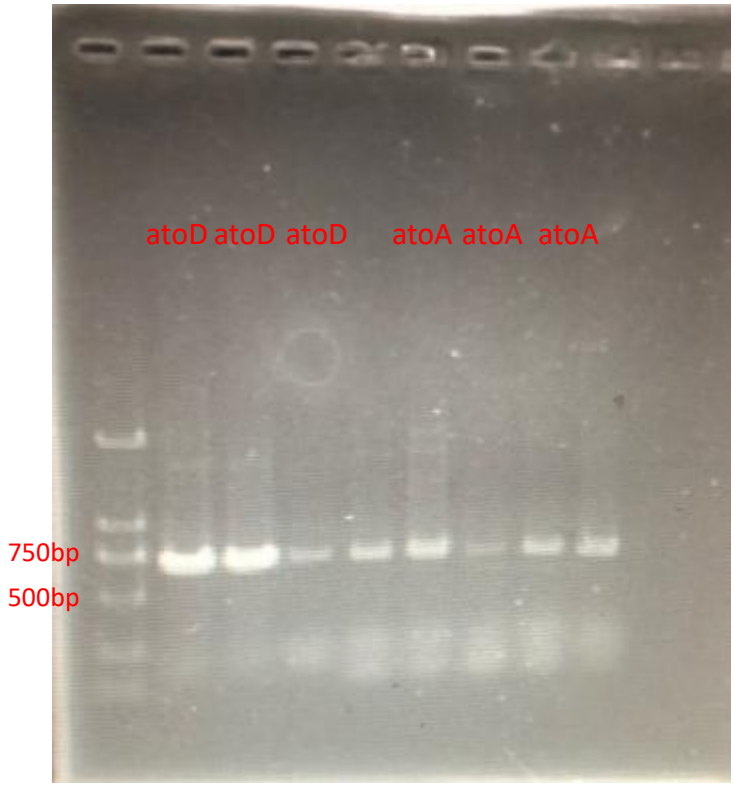
Streak *PUC57-adhE2* on LB plates and inoculate for 12-16 hours at 37℃.

07.09

Verify the success of connection of pSB1C3-atoD and pSB1C3-atoA by PCR using the protocol as above.

Result:

Successfully



Isolate two single colonies from the LB plate, and inoculate a culture of 5ml LB medium. Incubate for 12-16 hours at 37℃ with vigorous shaking.

08.09

Extract plasmid of pUC57 with E.Z.N.A.® Plasmid Mini Kit I using the protocol as above.

Results:

pUC57 1

296.08 ng/μl
pUC57 2
299.014 ng/μl
pUC57 3

268.127 ng/μl	A260	5.3625	260/230	2.19	260/280	1.96

pUC57 4

256.801 ng/μl	A260	5.1360	260/230	2.10	260/280	1.95

09.09

Get atoA by PCR with new primers using the following protocol.

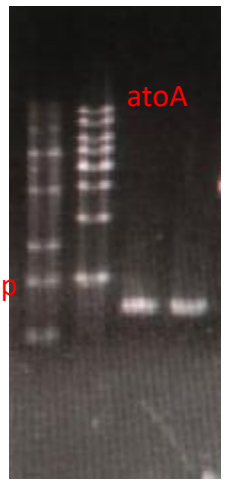
PCR Protocol

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	64°C, 30 seconds	×30 cycles
Extension	72 °C, 1 minutes	
Final Extension	72 °C, 7 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 μl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 μl
template	0.5 μl
10 μM Forward Primer	1μl
10 μM Reverse Primer	1 μl
ddH2O	22.5 μl
Total volume	50 μl

Result:



Get adhE2 by PCR with new primers using the following protocol.

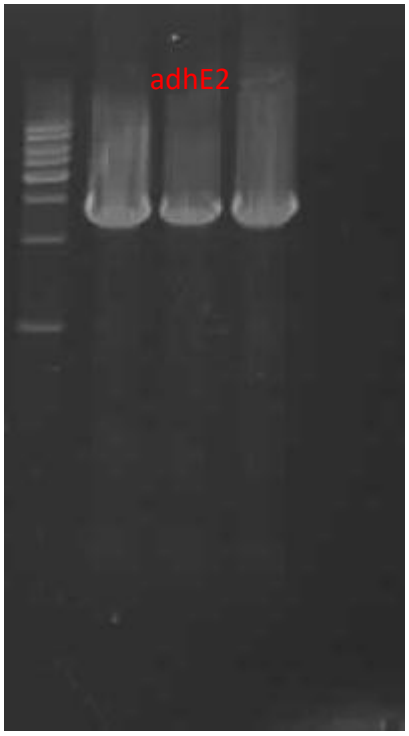
PCR Protocol

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	59°C, 30 seconds	×30 cycles
Extension	72 °C, 2 minutes 40 seconds	
Final Extension	72 °C, 13 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 µl
template	0.5 µl
10 µM Forward Primer	1µl
10 µM Reverse Primer	1 µl
ddH2O	22.5 µl
Total volume	50 µl

Result:



Get backbone by PCR with new primers using the following protocol.

PCR Protocol

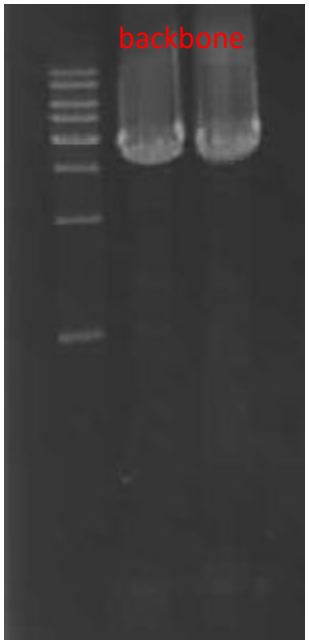
Initiation	94 °C, 5 minutes
Denaturation	94 °C, 30 seconds

Annealing	64°C, 30 seconds	×30 cycles
Extension	72 °C, 4minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 µl
template	0.5 µl
10 µM Forward Primer	1µl
10 µM Reverse Primer	1 µl
ddH2O	22.5 µl
Total volume	50 µl

Result:



Cycle pure atoA with E.Z.N.A.® Cycle Pure Kit using the protocol as above.

Result:

24.736 ng/µl A260 0.4947 260/230 0.24 260/280 1.89

Week 15 10.09-16.09

Construct pRB1s-atoD-atoA-adhE2 and composite part

10.09

Gel extraction of adhE2 and backbone using the protocol as above.

Results:

adhE2:

253.668 ng/µl	A260	5.0734	260/230	1.37	260/280	1.97

Backbone:

106.398 ng/µl	A260	2.1280	260/230	1.34	260/280	1.98

11.09

Construct pRB1s-*atoD-atoA-adhE2* by Gibson assembly.

Protocol:

The reaction system of Gibson

Component	**Volume**
atoD fragment (23.638 ng/µl 663bp)	1. 2µl
atoA fragment (24.736 ng/µl 651bp)	1.1 µl
adhE2 fragment (253.668 ng/µl 2577bp)	0.5 µl
Backbone (106.398 ng/µl 3462bp)	1.4 µl
Gibson system	6 µL
Reaction conditions	Reaction at 50 °C for 1 h

1. When the Gibson junction was left for 5 min, take out the prepared 100 uL E. coli BW25113 competent from the -80 degree refrigerator and left to stand in the ice .
2. The Gibson-linked product was added to the competent cell and gently mixed, ice-bathed for 30 min, and allowed to stand.
3. Heat shock at 42*C in a waterbath for 90secs. Return to ice for 2min.
4. Add 800 uL of SOC medium. 37 ° C, 120 rpm shock recovery for 1 h.
5. The resuscitated cells were centrifuged at 3000 rpm for 5 min, the supernatant was aspirated, and 1-200 uL was left, and plated on a LB solid medium plate containing streptomycin and cultured overnight at 37 °C.

12.09

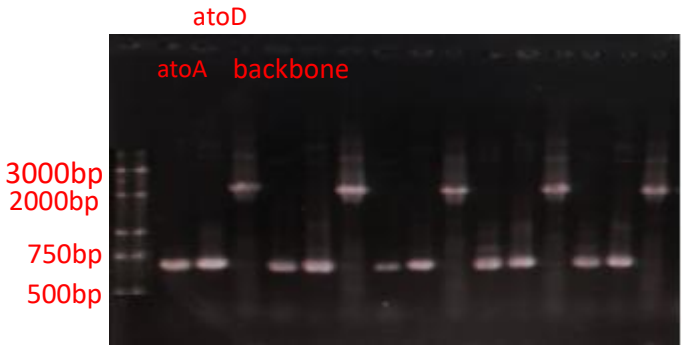
Picked 5 single colonies from the plate of gibson assembly into LB liquid medium with streptomycin resistance and incubated for 12 hours under a 37 degree shaker.

13.09

Use bacterial solution PCR to verify whether the Gibson connection is successful using the protocol as above.

Result:

Successfully



14.09

Get the fragment of connected part by PCR using the following protocol.

PCR Protocol

Initiation	94 °C, 5 minutes	
Denaturation	94 °C, 30 seconds	
Annealing	58°C, 30 seconds	×30 cycles
Extension	72 °C, 4minutes	
Final Extension	72 °C, 15 minutes	
	Hold at 4 °C	

The PCR mix prepared for 50 µl is reported as follows:

Component	**Volume**
2×*EasyTaq* PCR SuperMix	25 µl
template	0.5 µl
10 µM Forward Primer	1µl
10 µM Reverse Primer	1 µl
ddH2O	22.5 µl
Total volume	50 µl

Result:

Gel extraction of the fragment of connected part.

Result:

190.508 ng/μl	A260	3.8102	260/230	0.84	260/280	1.98

The fragment was digested with spe1 and xba1 to obtain the fragment for connecting part.

Protocol:

10×Cut-smart Buffer	5 ul
Xba Ⅰ	1 ul
Spe Ⅰ	1 ul
Plasmid	10 ul
ddH2O	3 ul

37℃ in aquatic bath for 5h and 80℃ for 30min.

Cycle pure digested product.

Result:

44.912 ng/μl	A260	0.8982	260/230	2.18	260/280	1.94

T4 ligase	6 ul
atoA fragment	6.39 ul
backbone	1.61 ul
T4ase	1 ul
10×T4 Buffer	1 ul

16 °C in aquatic bath overnight.

09.08

Transformed connection product into DH5 α competent cells.

Protocol:

1. Thaw competent cells on ice
2. Pipette 10μl of connection product into DH5α competent cells
3. Close 1.5ml tubes, incubate on ice for 30min
4. Heat shock tubes at 42°C for 90 seconds
5. Incubate on ice for 3-5min
6. Pipette 800μl SOC media to transformation
7. Incubate at 37°C for 1 hours, shaking at 120rpm
8. Spin down cells at 5000r/min for 1mins and discard 800μL of the supernatant. Resuspend the cells in the remaining 100μL, and pipette each transformation onto petri plates
9. Incubate transformations overnight (14-18hr) at 37°C

Picked 5 single colonies from the plate of gibson assembly into LB liquid medium with streptomycin resistance and incubated for 12 hours under a 37 degree shaker.

15.09

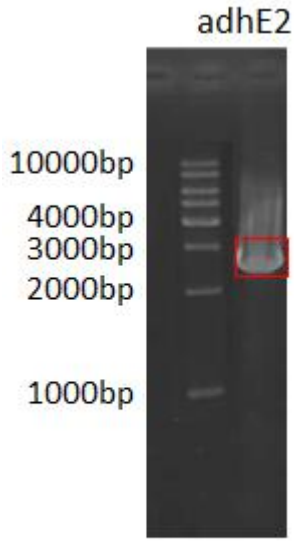
Extracted plasmid of pSB1C3-adhE2 using the protocol as above.

Result:

31.912 ng/µl	A260	0.7982	260/230	1.63	260/280	1.74

Verify the successful connection of pSB1C3-adhE2 by PCR using the protocol as above.

Result:



Successfully

Week 15 17.09-23.09

Week 15 24.09-30.09

LUYING'S

NOTEBOOK

Reagent and equipment preparation

1. Prepared in PBS:

Weigh the following components, potassium dihydrogen phosphate (KH2PO4) 0.24 g, disodium hydrogen phosphate (Na2HPO4) 1.44 g, sodium chloride (NaCl) 8.0 g, potassium chloride (KCl) 0.2 g, dissolved in 900 mL double steaming In the water. After adjusting the pH to 7.4 with 1 M hydrochloric acid, the volume was adjusted to 1000 mL and stored under high pressure steam sterilization.

2. Nucleic acid electrophoresis buffer (50xTAE Buffer):

Tris Base 242.0 g, Na2EDTA·2H2O 37.2, g, 57.1 mL of acetic acid, and stirred well by adding 800 mL of double distilled water. Finally, the solution was made up to 1 L with double distilled water and stored at room temperature. Use 50 times diluted with double distilled water before use.

3. Preparation of antibiotics:

Ampicillin: Weigh 0.5 g of ampicillin, dissolve in 9 mL of ddH20, and mix by shaking. After completely dissolving, the volume was adjusted to 10 mL, and the mixture was filtered through a 0.22 μm water filter.

Streptomycin: Weigh 0.5 g streptomycin, dissolve in 9 mL ddH20, and mix by shaking. After complete dissolution. The volume was adjusted to 10 mL, and the mixture was filtered through a 0.22 μm water filter.

Kanamycin: Weigh 0.5 g of kanamycin, dissolve in 9 mL of ddH20, and mix by shaking. Completely dissolved. After the solution, the volume was adjusted to 10 mL, and the mixture was filtered through a 0.22 μm water filter.

After all antibiotics were prepared, they were dispensed into 1.5 mL sterile centrifuge tubes and stored in a -20 °C freezer. The working concentration of each antibiotic is 50μg/mL.The resistance carried by the host bacteria and the plasmid is added to the medium.

4. LB (Luria-Bcrtani) medium:

LB medium is mainly used for the basic experimental operation of Escherichia coli. And protein induces activation of the strain prior to expression. Trypsin 10 g, yeast extract 5 g, NaCl 10 g per 1000 mL of solution contains.

5. SOC medium:

SOC is mainly used for the recovery step in the construction of strains with low transformation efficiency. specific

The formula was as follows. 20 g of tryptone, 5 g of yeast extract and 0.5 g of NaCl were added to 950 mL of double distilled water. After the solute is completely dissolved, 10 mL of 0.25 M KCl solution is added. Thereafter, the pH was adjusted to 7.0 with a 5 M NaOH solution and the solution was made up to 1000 mL. After autoclaving, 5 mL of autoclaved 2 M MgCl2 solution was added, and a 0.22 μm water filter was used to filter 20 mL of 1 M glucose solution.

6. Solid medium:

When making a solid plate medium, first configure the liquid according to the liquid medium formula.

The medium was then added to agar 15 g/L. After shaking and mixing, the medium is placed in a sterilizing pot for high temperature and high pressure sterilization. After the sterilization is completed, leave it at room temperature for a while. When the temperature of the medium is

cooled to about 60 °C, add the required antibiotics and shake the container to mix well. Pour 10-20 mL of the medium into each 90 mM dish and allow to cool and solidify. The prepared solid medium can be sealed and stored in a 4 ℃ ice box in the dark, valid for about half a month. Before the next use, preheat the refrigerated plate in the incubator and pour off the condensate on the plate before applying the plate.

Preparation of competent state

1) Pick a single colony from the desired E. coli strain plate, inoculate it in 5 mL of LB liquid medium, and incubate at 37 ° C with shaking (200 rpm) overnight.
2) Take 1 mL of bacterial solution in the morning of the next day and transfer to 100 mL of LB liquid medium. Incubate at 37 °C for 2 to 3 h (OD600 should be between 0.4 and 0.5).
3) Place the bacterial solution in an ice bath for 30 min.
4) Place the bacterial solution in a 100 mL centrifuge tube, centrifuge at 4 °C for 10 min (4200 rpm), discard the supernatant, and invert to allow the culture to run out.
5) Suspend the cells with 10 mL of ice-cold 0.1 M CaCl2 solution (slightly vortex to suspend) and immediately incubate in an ice bath for 30 min.
6) Centrifuge at 4 °C for 10 min (4200 rpm), discard the supernatant, add 3 mL of ice-cold 0.1 M CaCl2 15% glycerol solution, gently shake it by hand, place it on ice, and dispense into a centrifuge tube every 100 μL.

Synthetic primer

Table 1 Primers used in the experience

primer	sequence
1-F-backbone-atoD	TTTGGGCTAACAGGAGGAATTAACCATGAAAACAAAATTGATGACATTAC
1-R-atoD-atoA	GCATCCATTATATCTCCTTCTCGAGTTATTTGCTCTCCTGTGAAAC
2-F-atoD-atoA	GCAAATAACTCGAGAAGGAGATATAATGGATGCGAAACAACGTATTG
2-R-atoA-adhE2	TTTGTAACTTTCATTATATCTCCTTTCATAAATCACCCCGTTGCG
3-F-atoA-adhE2	AACGGGGTGATTTATGAAAGGAGATATAATGAAAGTTACAAATCAAAAAGAAC
3-R-adhE2-backbone	CGAATTCACCACTAGTACCAGATCTTTAAAATGATTTTATATAGATATCCTTAAGTTC
4-F-adhE2-backbone	GGATATCTATATAAAATCATTTTAAAGATCTGGTACTAGTGGTGAATTC
4-R-backbone-atoD	GTAATGTCATCAATTTTGTTTTCATGGTTAATTCCTCCTGTTAGC

Table 2 Primers used in the experience

primer	sequence
5-F-Backbone-MazF	caaaattaacgtactgattgggtagAGATCTGGTACTAGTGGTGAATTC
5-R-Backbone-MazF	tatcgggtacgtatcggcttaccatGGTTAATTCCTCCTGTTAGC
6-F-MazF-Backbone	tttgggctaacaggaggaattaaccATGGTAAGCCGATACGTACC
6-R-MazF-Backbone	cgaattcaccactagtaccagatctCTACCCAATCAGTACGTTAATTTTG
1-F-EGFP	GCCCCATGGTACCATGGTGAGCAAGGGCGAG
1-R-EGFP	GCCGTACCTGCTCGACATGTTCATTGTGCCCCACG
2-F-lacI	GGTTACGTAAAACCAGTAACGTTATACGATGTCGCAGA
2-R-lacI	GGCTGACCTTTCGCCCGTCACTGCATGCCCG
7-F-pRB1a-Backbone-hns synth	gtacaagtaacacggggtgactagtGGTGAATTCGGTGAGCTCGG
7-R-pRB1a-Backbone-hns synth	tgacaagaaaatattcgcctctagaTTGGTAACGAATCAGACAATTGACG
8-F-pRB1a-hns synth	CGTCAATTGTCTGATTCGTTACCAATCTAGAGGCGAATATTTTCTTGTC
8-R-pRB1a-hns synth	GCAGACCGAGCTCACCGAATTCACCACTAGTCACCCCGTGTTACTTG
9-F-pRB1a-Backbone-proU-EGFP	gcaccaccaccaccaccactaacacggggtgAGATCTGGTACTAGTGGTGAATTC
9-R-pRB1a-Backbone-proU-EGFP	gagtatcagtgtagatcaccacaaaTTGGTAACGAATCAGACAATTG
10-F-pRB1a-proU-EGFP	cgtcaattgtctgattcgttaccaaTTTGTGGTGATCTACACTGATACTCTGTTG
10-R-pRB1a-proU-EGFP	cgaattcaccactagtaccagatctCACCCCGTGTTAGTGGTGGTG
11-F-pRB1a-Backbone-lacUV5-EGFP	gcaccaccaccaccaccactaacacggggtgAGATCTGGTACTAGTGGTGAATTC
11-R-pRB1a-Backbone-lacUV5-EGFP	Tagtatttctcctctttctctagtacattatacgagccggaagcataaagtgtaaa TTGGTAACGAATCAGACAATTG
12-F-pRB1a-lacUV5-EGFP	Cgtcaattgtctgattcgttaccaatttacactttatgcttccggctcgtataatg TACTAGAGAAAGAGGAGAAATACTAGGGTACC
12-R-pRB1a-lacUV5-EGFP	cgaattcaccactagtaccagatctCACCCCGTGTTAGTGGTGGTG
13-F-Backbone-Agaa-NCS	ctccatacccgtttttgggctaacTACTAGAGAGGCAAACAAACACG
13-R-Agaa-NCS	tctttctctagtaTCACTTCATCAACCAGGGCG
14-F-NCS-Agaa	gttgatgaagtgaTACTAGAGAAAGAGGAGAAATAC
14-R-NCS-EGFP	tctttctctagtaTTATTGAACTTTGCTACAATCG
15-F-EGFP-NCS	caaagttcaataaTACTAGAGAAAGAGGAGAAATACTAGATATACATATGG
15-R-EGFP-Backbone-NCS	cttctgcgttctgatttaatctgtaTTAGTGGTGGTGGTGGTGGTG
16-F-Backbone-Agaa	ccgtttttgggctaactactagagAGGCAAACAAACACGAACAAAAAG
16-R-Agaa-EGFP	tctttctctagtaTCACTTCATCAACCAGGGCG
17-F-EGFP-Agaa	gttgatgaagtgaTACTAGAGAAAGAGGAGAAATACTAGATATACATATGG
17-R-EGFP-Backbone	cttctgcgttctgatttaatctgtaTTAGTGGTGGTGGTGGTGGTG

Table 3 Primers used in the experience

primer	sequence
1-F-PSB1C3-Aro10	GCTCTAGAATGGCACCTGTTACAATTGAAA
1-R-PSB1C3-Aro10	GGACTAGTCTATTTTTTATTTCTTTTAAGTGCCG
2-F-PSB1C3-PAR	GCTCTAGAATGTCCAACAAAGTCGTGTGTGT
2-R-PSB1C3-PAR	GGACTAGTTTAGAACGACACAATTGATTTTTCTT
3-F-PSB1C3-TYRB	GCTCTAGAATGTTTCAAAAAGTTGACGCCTAC
3-R-PSB1C3-TYRB	GGACTAGTTTACATCACCGCAGCAAACG
4-F-PSB1C3-LDH-58.2	GCTCTAGAATGGGTAAGAAGAAGAGAACTAAAATT
4-R-PSB1C3-LDH-59.4	GGACTAGTTTAGCCAACCTTAACTGGAGTTTC
5-F-PSB1C3-ROCG	GCTCTAGAATGTCAGCAAAGCAAGTCTCGAA
5-R-PSB1C3-ROCG	GGACTAGTTTAGACCCATCCGCGGAAA

Table 4 Primers used in the experience

primer	sequence
Mut1-F-D-LDH-EcoR1	CAAGGAGTTCCCAGACGCACGTTTA
Mut1-R-D-LDH-EcoR1	GTGCGTCTGGGAACTCCTTGCCTT
Mut2-F-rocG-EcoR1	GAGTTCGATTCTCCGGGCTTTATTA
Mut2-R-rocG-EcoR1	CGGAGAATCGAACTCCCGCAG
Mut3-F-tyrB-Pst1	GGTGGCTGCAATGCTGAATGACG
Mut3-R-tyrB-Pst1	TCAGCATTGCAGCCACCACCTG
Mut4-F-Aro10-Pst1	TATATTTTTGTTCCGGCAGATTTTG
Mut4-R-Aro10-Pst1	AATCTGCCGGAACAAAAATATAACC
Mut5-F-Aro10-Spe1	ACAAGATTACCAGTTGGATATATTC
Mut5-R-Aro10-Spe1	ATATATCCAACTGGTAATCTTGTTG
Mut6-F-Aro10-EcoR1	CTTAAGAACTCAAACAAAAGAAGCG
Mut6-R-Aro10-EcoR1	TTTGTTTGAGTTCTTAAGCTCCTCC
Mut7-F-AdhE2-EcoR1	AATCAATATTAGTTATGAACTCAATATAC
Mut7-R-AdhE2-EcoR1	TGAGTTCATAACTAATATTGATTGTTC
Mut8-F-AdhE2-Pst1	CTACAATTGCTGCGGCAAAATTAATTTTAGATG
Mut8-R-AdhE2-Pst1	TTTTGCCGCAGCAATTGTAGATTTTTTTGC

Site-directed mutation

Design Site-directed mutation primer

Table 4 Primers used in the experience

primer	sequence
Mut1-F-D-LDH-EcoR1	CAAGGAGTTCCCAGACGCACGTTTA
Mut1-R-D-LDH-EcoR1	GTGCGTCTGGGAACTCCTTGCCTT
Mut2-F-rocG-EcoR1	GAGTTCGATTCTCCGGGCTTTATTA
Mut2-R-rocG-EcoR1	CGGAGAATCGAACTCCCGCAG
Mut3-F-tyrB-Pst1	GGTGGCTGCAATGCTGAATGACG
Mut3-R-tyrB-Pst1	TCAGCATTGCAGCCACCACCTG
Mut4-F-Aro10-Pst1	TATATTTTTGTTCCGGCAGATTTTG
Mut4-R-Aro10-Pst1	AATCTGCCGGAACAAAAATATAACC
Mut5-F-Aro10-Spe1	ACAAGATTACCAGTTGGATATATTC
Mut5-R-Aro10-Spe1	ATATATCCAACTGGTAATCTTGTTG
Mut6-F-Aro10-EcoR1	CTTAAGAACTCAAACAAAAGAAGCG
Mut6-R-Aro10-EcoR1	TTTGTTTGAGTTCTTAAGCTCCTCC
Mut7-F-AdhE2-EcoR1	AATCAATATTAGTTATGAACTCAATATAC
Mut7-R-AdhE2-EcoR1	TGAGTTCATAACTAATATTGATTGTTC
Mut8-F-AdhE2-Pst1	CTACAATTGCTGCGGCAAAATTAATTTTAGATG
Mut8-R-AdhE2-Pst1	TTTTGCCGCAGCAATTGTAGATTTTTTTGC

Overlap extension PCR

The primers were annealed to the template strand and the 2X transstart fastpfu PCR supermix was used to synthesize the mutant strand.

The DMT enzyme degrades non-mutant plasmid templates (methylated plasmid templates) and DMT competent cells in vitro to degrade non-mutant plasmid templates (methylated plasmid templates), thereby efficiently screening mutant clones.

Table 1 The composition of PCR

component	volume
Template	Variable
Forward Primer(10μM) Reverse	1
Reverse Primer(10μM)	1
5xTransStart® FastPfu DNA Polymerase	1
dNTP (2.5 mM)	4
5xTransStart® FastPfu Buffer	10
DdH2O	Add to 50ul

For the amplification of DNA of interest for the construction of plasmids, the PCR reaction solution is usually prepared in 100 μL and dispensed into 2 PCR tubes, each tube being 50 μL. After the end of the PCR, all of the reaction solution was recovered for the next digestion test. The primers synthesized by the company are dry powder. It needs to be centrifuged at high speed first, then add a certain amount of sterile double distilled water according to the instructions and dilute to 10μM.

Table 2 PCR procedure

Number of cycle	Temperature	Time
1 cycle	95℃	10min
	95℃	20s
30cycles	Tm-5	20s
	72	2kb/min
1 cycle	72	10min

Electrophoresis detection

1. According to the size range of the separated DNA molecules, weigh the appropriate amount of agarose powder, put it into an Erlenmeyer flask, and add an appropriate amount of 0.5×TBE running buffer. The microwave is then heated to complete dissolution and the solution is clear. Shake well and get the glue. After cooling to about 60 ° C, an appropriate amount of ethidium bromide was added to the gum to a concentration of 0.5 μg / ml.
2. Take the plexiglass plastic plate groove, seal the scotch tape along the glue groove, and add a small amount of glue to seal the gap between the tape and the glue groove.
3. Place the glue tank horizontally, insert the comb at one end, and slowly pour the glue that has been cooled to about 60 °C in the tank to form a uniform level of rubber surface.
4. After the gel is solidified, carefully pull up the comb, tear off the transparent tape, and place the cathode section at the end of the sample hole into the electrophoresis tank.
5. Add 0.5×TAE running buffer to the tank until the liquid surface covers the rubber surface.
6. Carefully mix the sample to be tested on the clean glass slide with the following amount and add it to the sample well of the gel with a pipette.

1μl loading buffer (6×) + 5μl DNA sample to be tested

Acquisition of gene sequences

Primers were designed to amplify the gene encoding the 1908 bp phenylpyruvate decarboxylase (aro10). The amplified sequence was identical to the aro10 gene sequence of S. cerevisiae S288C, numbered Gene ID: 851987 on NCBI.

Primers were designed to amplify the gene encoding the 1194 bp aromatic amino acid aminotransferase (tyrB). The amplified sequence was identical to the tyrB gene sequence of E. coli str. K-12 substr. MG1655, numbered Gene ID: 948563 on NCBI.

primer	Primer sequence	remark
		Psb1c3-aro10
		Psb1c3-tyrB
		Psb1c3-PAR

Table 2.2 Primers used in this experience.

Amplification of the gene fragment of interest with amplified XbaI and SpeI cleavage sites.
The psb1c3 and amplified fragments were digested with XbaI and SpeI.

The vector and the fragment were separately ligated using T4 ligase.

Transform competent cells and culture overnight at 37 °C, pick up the colonies on the plate, perform colony PCR verification, screen the positive clones and extract the plasmid for enzyme digestion.

Construction of expression plasmid

Primers were designed on the nebbuilder to amplify the ARO10, tyrb and par sequences with Gibson ends.

primer	Primer sequence	remark
		Psb1c3-J23100-2PE

Table 2.2 Primers used in this experience.

A total volume of 10 µL of the digested Psb1c3 vector and ARO10, par, TyrB fragments were added to 10 µL of Gibson Buffer using the Gibson ligation method. Incubate at 50 °C for 1 h.

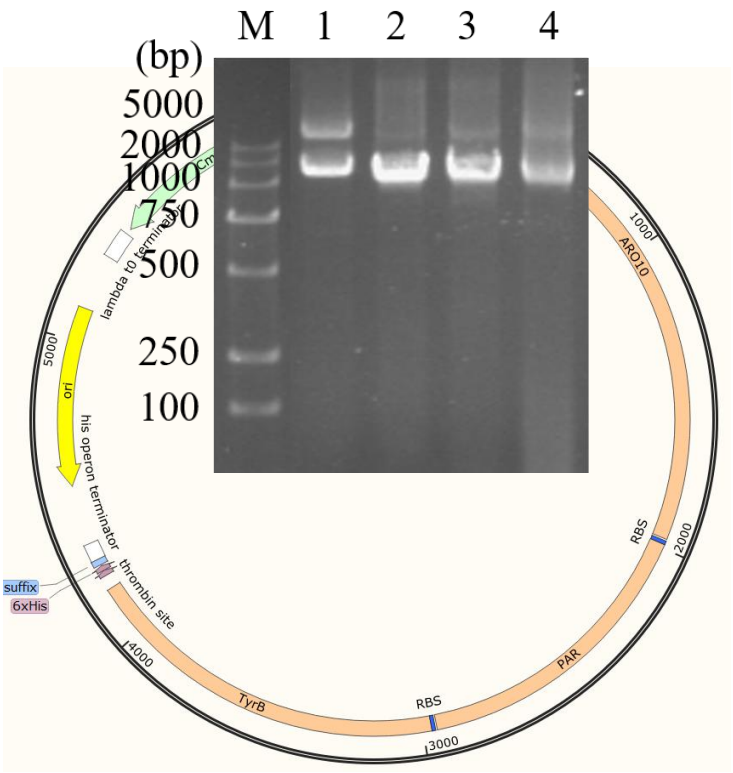
Transform competent cells and culture overnight at 37 °C, pick up the colonies on the plate, perform colony PCR verification, screen the positive clones and extract the plasmid for enzyme digestion.

Nucleic acid electrophoresis buffer (50xTAE Buffer): Tris Base 242.0 g, Na2EDTA·2H2O 37.2, g, 57.1 mL of acetic acid, and stirred well by adding 800 mL of double distilled water. Finally, the solution was made up to 1 L with double distilled water and stored at room temperature. Use 50

times diluted with double distilled water before use.

Nucleic Acid Electrophoresis Agarose Gel (Agarose Gel): Dilute the nucleic acid running buffer to a suitable multiple to obtain lxTAE Buffer, which will be used to prepare agarose gel and DNA electrophoresis. Weigh 1 g of agarose gel powder in a triangular flask, pour 100 mL of lxTAE Buffer, and dissolve in a microwave oven. Wait until it cools to about 60 °C. Pour the solution into the glue tank slowly and insert a comb with the appropriate pore size and pore volume to avoid the generation of bubbles. After standing at room temperature for half an hour, the agarose gel was completely solidified, placed in an electrophoresis tank, and completely submerged the gel by adding l xTAE Buffer, and the comb was carefully pulled out.

Fig. 00 The analysis of LDH, rocG, TyrB, Aro10 by Nucleic Acid Electrophoresis



M: Marker Lane 1: The mutantion of Aro10 Lane 2: The mutantion of TyrB Lane 3: The mutantion of rocG Lane 4: The mutantion of LDH

Fig. 2.4 Construction of expression vector psb1c3-J23100-2pe

Detecting 2-PE by HPLC

We purchased L-Phe, PPA, Pald, 2-PE standards. By consulting the 2-PE detection method reported in the literature, combined with laboratory instruments and columns. A method for detecting 2-PE by HPLC was developed. The test conditions are as follows:

Agilent 1260 High Performance Liquid Chromatography System;

Column: Waters X-Bridge C-18 3.5 μm 4.6*150

mM Column, column temperature on both sides 25 °C; mobile phase: water: acetonitrile = 70:30, flow rate 0.5 mL / min,; diode array detector, detection wavelength 214 nm, detection time 12

min.

Test results hplc

Fig. 2.1 Elution time and peak area of 2-PE

The green fluorescent protein (GFP) was used as a control, and the cells showed green fluorescence after the induction, indicating that the induction process was normal.

SDS-PAGE electrophoresis

Preparation of SDS-PAGE Protein Electrophoresis Buffer: The following components were weighed, Tris Base 15.1 g, Glycine 94.0 g, sodium dodecyl sulfate (SDS) 5.0 g, dissolved in 800 mL of double distilled water.

Preparation of SDS-PAGE protein loading buffer: 1 M Tris-HCl (pH 6.8) 1.25 mL, SDS 0.5g, bromophenol blue 25 mg, glycerol 2.5 mL, fully dissolved in sterile double distilled water, and dilute to 5 mL . 25 μL of β-mercaptoethanol was added to each tube before use, and the refrigerator was chilled at 4 °C.

Preparation of SDS-PAGE protein electrophoresis staining solution: Coomassie Brilliant Blue R-250 l g, isopropanol 250 mL, glacial acetic acid 100 mL, double distilled water 650 mL, mix well, remove the particulate matter with filter paper, and store at room temperature. SDS-PAGE protein electrophoresis decolorization solution: Add the following components to a 1 L volumetric flask, 150 mL of ethanol, 100 mL of acetic acid, and 750 mL of double distilled water. Mix well and store at room temperature.

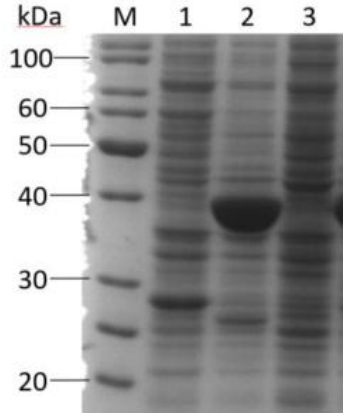


Fig. 2.3 The analysis of TyrB， Aro10 by SDS-PAGE

M: Marker Lane 1: GFP/BW Lane 2: TyrB/BW Lane 3: Aro10/BW

Lane 1 has a distinct band around 28 kDa, which is a green fluorescent protein band. Lanes 2 and 3 are electrophoresis results of E. coli expressing TyrB and Aro10, respectively. The Aro10 protein is expressed in a low amount, and its protein position is almost indistinguishable in the supernatant. It is concluded that the Aro10 protein expression is low and most of the proteins are not correctly folded in the form of inclusion bodies.

The induced engineering bacteria 6 OD cells were resuspended in 200 μL of the transformation solution, and the final concentration of the transformation liquid was 30 OD. Placed at 30 ° C, 220 rpm shaker, start the whole cell catalysis of 2-PE synthesis reaction. Samples were taken at 3 h, 6 h, 9 h, and 12 h, respectively, and the samples were subjected to HPLC. Results With the overexpression of each gene, the synthesis ability of 2-PE was gradually improved. In the case of co-expression of three genes, about 7.7 mM of 2-PE can be synthesized.

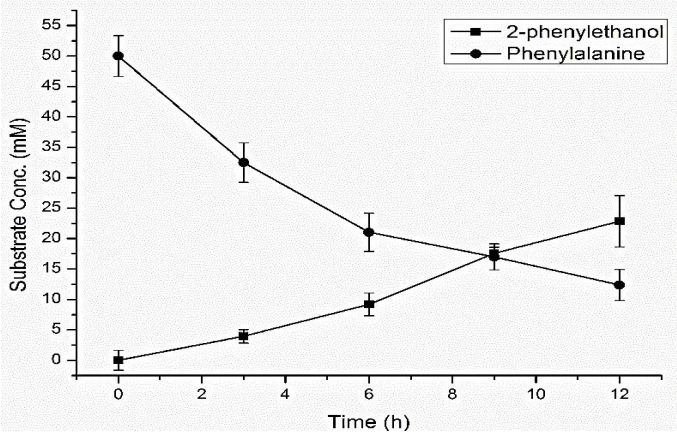


Fig. 1 Time curve of substrates depletion and production of 2-PE

At the beginning of the reaction, the substrate L-Phe was consumed very quickly, and after 12 h of reaction, the synthesis of 2-PE entered the plateau. From the HPLC results, the accumulation of 2-PE did not change much, and an unknown peak appeared at 3.5 min, which was increasing with time.

Junru

Week1: 7/7-14/7

Knock out Kana resistance of TnaA-/- deletion strain.

7/7

The TnaA-/- deletion strain was streaked in LB solid medium plates at 37° for 12 h.

8/7

Make TnaA-/- competent cells.

Protocol:

1. Pick up the activated single colonies into 5 mL of LB medium and incubate at 37 ° C, 220 rpm to logarithmic growth phase（OD600=0.4 - 0.6）.（OD value is detected every two hours）
2. In an aseptic ultra-clean bench, transfer 1.4 mL of cells in logarithmic growth phase to a sterile 1.5 mL centrifuge tube. Return to ice for 15min.
3. Centrifuge for 1 min at 4 ° C, 5000 rpm.
4. In an aseptic ultra-clean bench, discard the supernatant, and pour the centrifuge tube into the filter paper to blot out the remaining residue, and spray the suspension with 1 mL of ice-cold 0.1 M CaCl2 solution, ice bath 30 Min.
5. Centrifuge for 1 min at 4 ° C, 5000 rpm.
6. Repeat step 4, pour the supernatant, add 100 uL of pre-cooled 0.1 M CaCl2 solution, gently squirt and resuspend the cells, and ice bath for 30 min.
7. Centrifuge for 1 min at 4 ° C, 5000 rpm.
8. In an aseptic ultra-clean bench, pour the supernatant, add 100 uL of ice-cold 0.1M CaCl2-15% glycerol buffer solution, gently resuspend the cells, and store in a refrigerator at -80 °C.

9/7

Transform Pcp20 Plasmid DNA into TnaA-/- deletion strain, Plate 100ul on to the Amp antibiotic plate.

Protocol:

1. Take 100ul of competent TnaA-/- cells. Defrost cells on ice.
2. Add 10ul of Pcp20 plasmid DNA. Vortex and put on ice for 30min.
3. Heat shock at 42*C in a waterbath for 90secs. Return to ice for 2min.
4. Add 1mL of SOC medium. 37 ° C, 120 rpm shock recovery for 1 h.
5. The resuscitated cells were centrifuged at 3000 rpm for 5 min, the supernatant was aspirated, and 1-200 uL was left, and applied to an LB solid medium plate containing ampicillin, and cultured overnight at 37 °C.

10/7

In the Amp antibiotic plate , pick 8 single colonies (labeled 1-16) and incubate in 5 ml LB liquid medium containing Amp. 37°, 220rpm, shaking culture for 12h.
Streak inoculate into the Amp antibiotic plate,.
Result: Only 5 medium(labeled 1 , 5 , 6 , 9 , and 11)did not show single colonies (Kana resistance has been removed by PCP20 plasmid).

11/7
Remove PCP20 plasmid.
Single colonies that successfully removed kana resistance were picked into 5 ml LB liquid medium, placed in a 43° shaker for 12 h (removal of PCP20 plasmid), and then transferred to an anti-LB solid medium and cultured at 37° 220 rpm overnight.

12/7
One single colony was set in Amp liquid medium in each of the five LB plates to verify whether the Pcp20 plasmid was completely removed.
As a result, Only 2 LB plates(labeled 6 and 9)were grown.
Single colonies of No. 6 and No. 9 in the LB plate were picked into 5 ml LB liquid medium containing Kana (to verify whether Kana was completely removed), and cultured in 5 ml of LB liquid medium (for amplification)

13/7
Colony PCR verified that Kana resistance was completely removed

15ul system：

2xPCR Mix	7.5ul
Bacterial fluid	2.0ul
10 μM For. Primer	0.5ul
10 μM Ror. Primer	0.5ul
dd water	4.5ul

PCR Protocol ：

Initiation	95°，5min

Denaturation	95°，35s
Annealing	51°，35s
Extension	72°，1min
	30 cycles
Final Extension	72°，10min

Result：No banding, proves that Kana resistance is completely removed.
culture preservation：。300 ul of 50% glycerol and 700 ul of bacterial liquid were separately added to the preservation tube, and the sealing film was sealed and stored in a -20° refrigerator.

Week2:14/7-20/7

Build arac-MazF gene circuit

14/7
Obtaining the MazF toxin protein gene fragment from *E.coli* BW25113

15ul system(MazF)：

For. Primer ：tttgggctaacaggaggaattaaccATGGTAAGCCGATACGTACC

Ror. Primer：cgaattcaccactagtaccagatctCTACCCAATCAGTACGTTAATTTTG

2xPCR Mix	7.5ul
Genome	0.5ul
10 μM For. Primer	0.5ul
10 μM Ror. Primer	0.5ul
dd water	6.0ul

15ul system(backbone)

For. Primer：caaaattaacgtactgattgggtagAGATCTGGTACTAGTGGTGAATTC

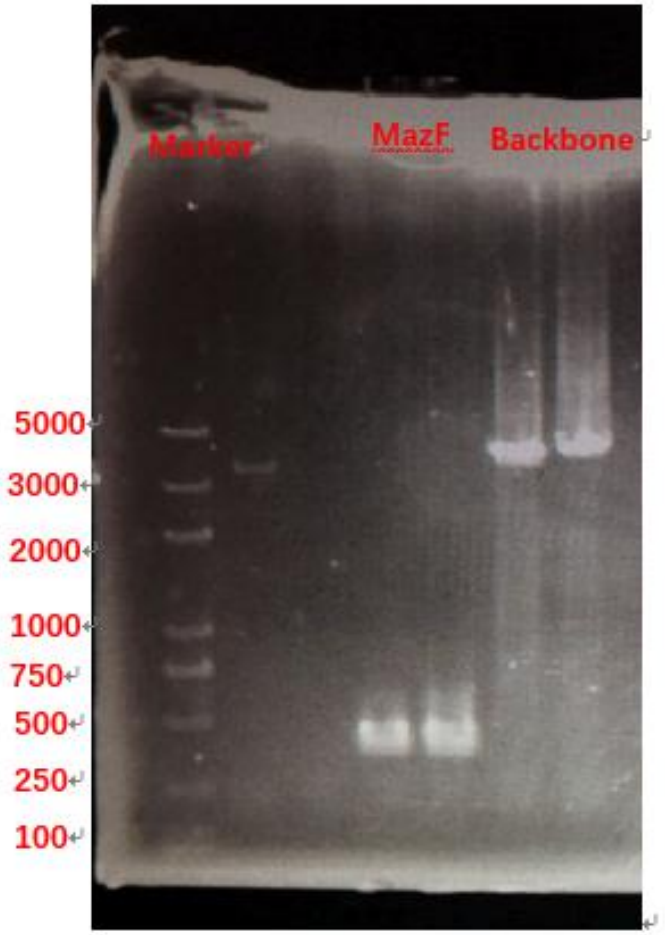
Ror. Primer：tatcgggtacgtatcggcttaccatGGTTAATTCCTCCTGTTAGC

2xPCR Mix	7.5ul
PEA32	0.5ul
10 μM For. Primer	0.5ul
10 μM Ror. Primer	0.5ul
dd water	6.0ul

PCR Protocol ：

Initiation	95°，5min
Denaturation	95°，35s
Annealing	61°，35s
Extension	72°，1min
	30 cycles
Final Extension	72°，10min

Result:



The PCR products were run on a 1% Agarose gel for 40 minutes at 100V. Each well was loaded with 4 μl of the PCR product.

Result:

Both MazF and plasmid backbones have bands of normal size, but the bands are too bright, and when using high-fidelity enzymes, consider reducing the amount of sample to 3 ul.

15/7

50 ul system（backbone）:

2xSuper Mix	25ul
PEA32	0.5ul

10 μM For. Primer	1.0ul
10 μM Ror. Primer	1.0ul
dd water	22.5ul

PCR Protocol ：

Initiation	94°，5min
Denaturation	94°，30s
Annealing	61°，30s
Extension	72°，3min
	30 cycles
Final Extension	72°，10min

50ul system (MazF):

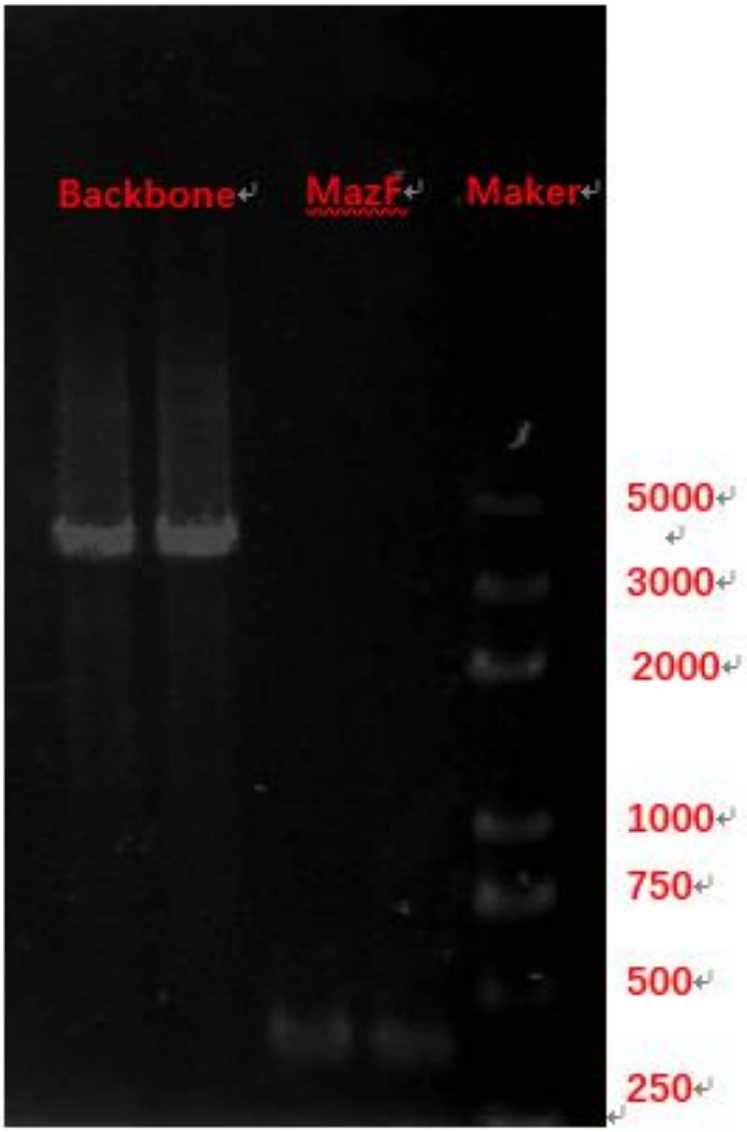
2xSuper Mix	25ul
Genome	0.5ul
10 μM For. Primer	1.0ul
10 μM Ror. Primer	1.0ul

dd water	22.5ul

PCR Protocol ：

Initiation	94°，5min
Denaturation	94°，30s
Annealing	61°，30s
Extension	72°，30s
Final Extension	72°，10min

Result:



Both MazF and plasmid backbones have bands of normal size.

Cyclepure protocol：

1. 40 ul of PCR product to a new 1.5 mL centrifuge tube and add 5 volumes of Buffer CP.
2. Mix by vortexing and collect all the mixed liquid

3. All the above mixture was transferred to a HiBind® DNA adsorption column, centrifuged at 10,000 rpm for 1 min at room temperature, and the waste liquid was discarded.
4. 500 μl of DNA Wash Buffer was added to the HiBind® DNA adsorption column, centrifuged at 10,000 rpm for 1 min at room temperature, and the waste liquid was discarded.
5. Repeat step 4 three times.
6. Place the HiBind® DNA adsorption column at 10,000 rpm for 2 min.
7. The HiBind® DNA adsorption column was transferred to a new 1.5 ml centrifuge tube, 50 μl of ddwater was added to the HiBind® DNA adsorption column, allowed to stand for 2 min, and then centrifuged at 1000 rpm for 1 min at room temperature.

Result:

MazF: 22.761ng/ul

Backbone:107.337ng/ul

17/7

The MazF and PEA32 backbones were connection by Gibsion.

Protocol:

The reaction system of Gibson

The reaction system of Gibson	
MazF fragment and PEA32 plasmid backbone	The plasmid and the gene fragment are in a molar ratio of 1:3. Total volume are 4 μL
Gibson system	6 μL
Reaction conditions	Reaction at 50 °C for 1 h

1. When the Gibson junction was left for 5 min, take out the prepared 100 uL *E. coli* BW25113 competent from the -80 degree refrigerator and left to stand in the ice .
2. The Gibson-linked product was added to the competent cell and gently mixed, ice-bathed for 30 min, and allowed to stand.
3. Heat shock at 42*C in a waterbath for 90secs. Return to ice for 2min.
4. Add 1ml of SOC medium. 37 ° C, 120 rpm shock recovery for 1 h.

5. The resuscitated cells were centrifuged at 3000 rpm for 5 min, the supernatant was aspirated, and 1-200 uL was left, and plated on a LB solid medium plate containing streptomycin and cultured overnight at 37 °C.

18/7

The monoclonal recombinants grown on the streptomycin-resistant plates were transferred to 5 mL of liquid LB medium containing streptomycin resistance, and cultured at 37 ° C, 220 rpm for about 10 h.

19/7

Colony PCR was performed to verify that the MazF connection was successful.

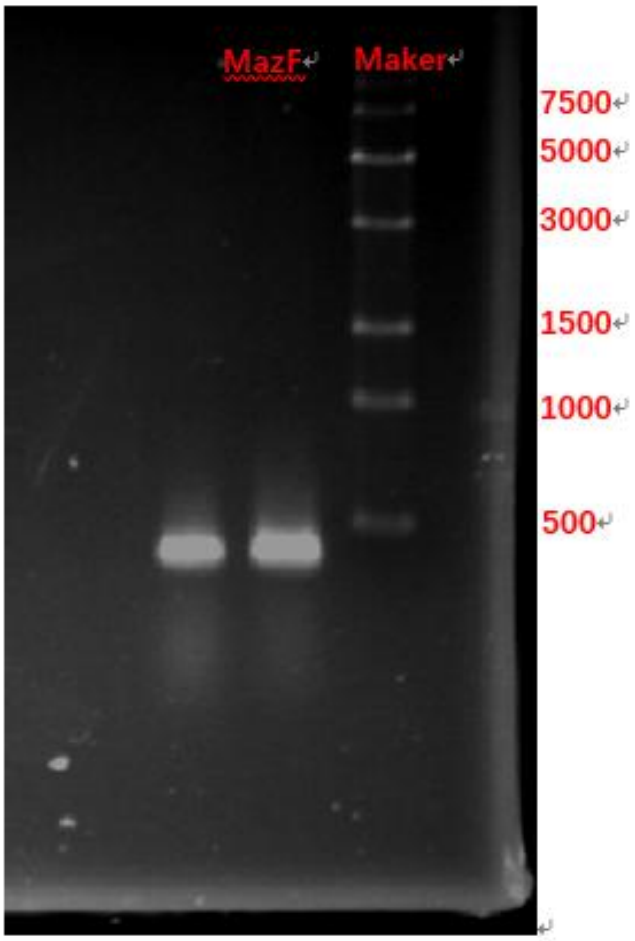
15ul system:

2xPCR Mix	7.5ul
Genome	0.5ul
10 μM For. Primer	0.5ul
10 μM Ror. Primer	0.5ul
dd water	6.0ul

PCR Protocol ：

Initiation	95°，5min
Denaturation	95°，35s
Annealing	61°，35s
Extension	72°，30s
	30cycles
Final Extension	72°，10min

Result:



Gibsion connection succeeded.

Week3 21/7-27/7

Different concentrations of arabinose concentration induction were used to verify the function of MazF.

21/7

1. The monoclonal recombinants grown on the streptomycin-resistant plates were transferred to 5 mL of liquid

LB medium containing streptomycin resistance, and cultured at 37 ° C,220 rpm, until the OD value reaches 0.6.

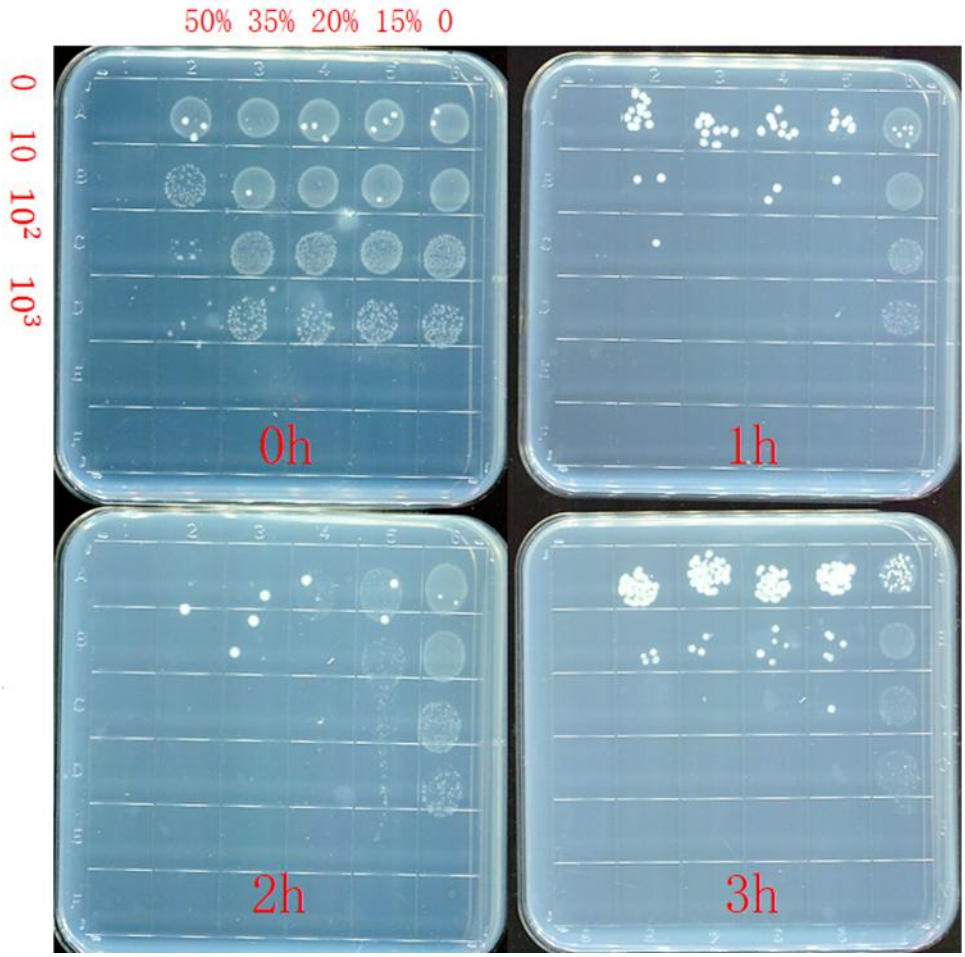
2. Add 500 ul of bacteria to each of the 5 1.5 mL centrifuge tubes.
3. Control group: no arabinose induction

   Experimental group: 5ul of arabinose was added separately, the concentration was 15% 20% 35% 50%

   Mixing thoroughly.
4. Add 5 ul of bacteria solution to the square Str antibiotic plate.

   At the same time, in order to better observe the results, the control group and the experimental group were separately diluted to 10 10^2 10^3, and 5ul of the bacterial solution was also added to the square Str antibiotic plate.
5. After adding the bacterial liquid to the LB plate, the 1.5 ml centrifuge tube containing the bacterial liquid was further placed in a shaker at 37 °,220 rpm for 1 hour.
6. Repeat step 5 until the arabinose action time reaches 3 hours

Result:

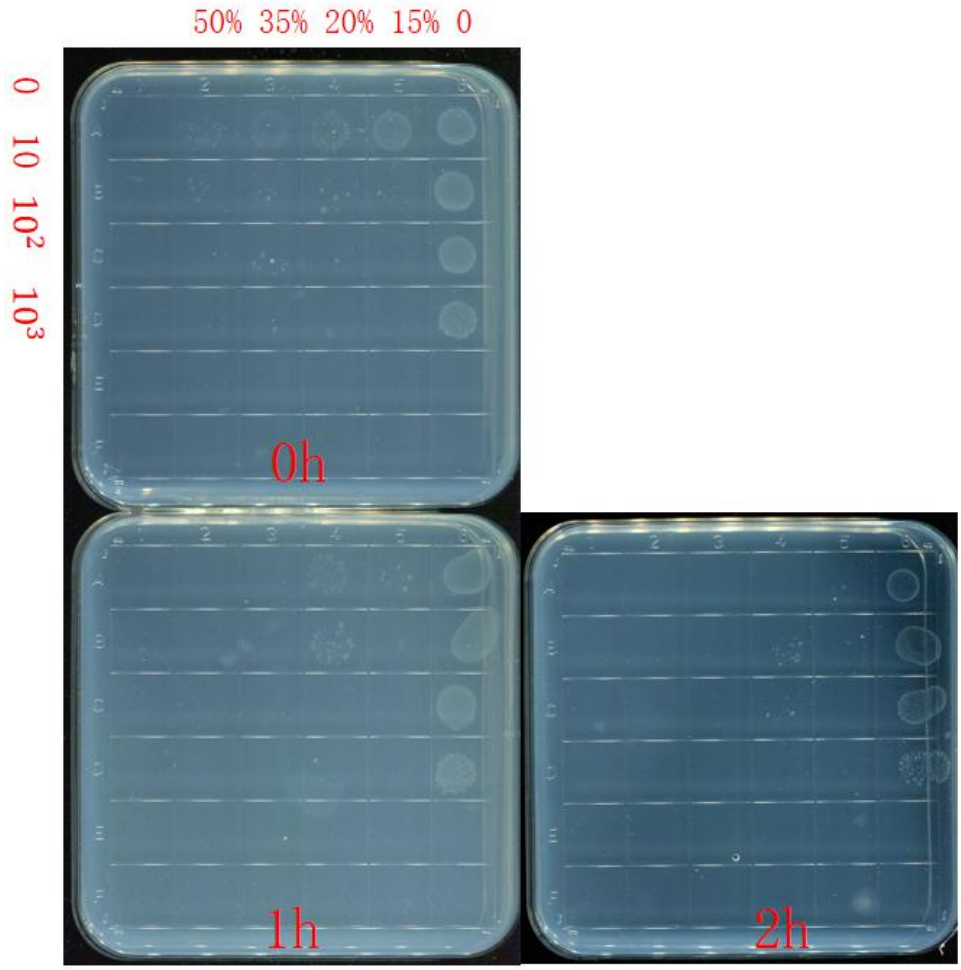


The operation process was infected with other bacteria, and the results of the experiment could not judge the function of MazF.

22/7

Repeat the experiment done yesterday and pay attention to the aseptic operation during the plating process.

Result:



According to the experimental results, when 50% arabinose was added, the bacteria were almost killed immediately. After adding arabinose for 1 hour, 35% and 50% arabinose-induced bacteria were also killed. After 2 hours. 15% arabinose-induced bacteria were also killed.At the sametime， the 20% arabinose-induced bacteria showed an abnormal reaction, considering that 20% of the arabinose may have been infected with the bacteria during the preparation process.

Week4: 28/7-3/8

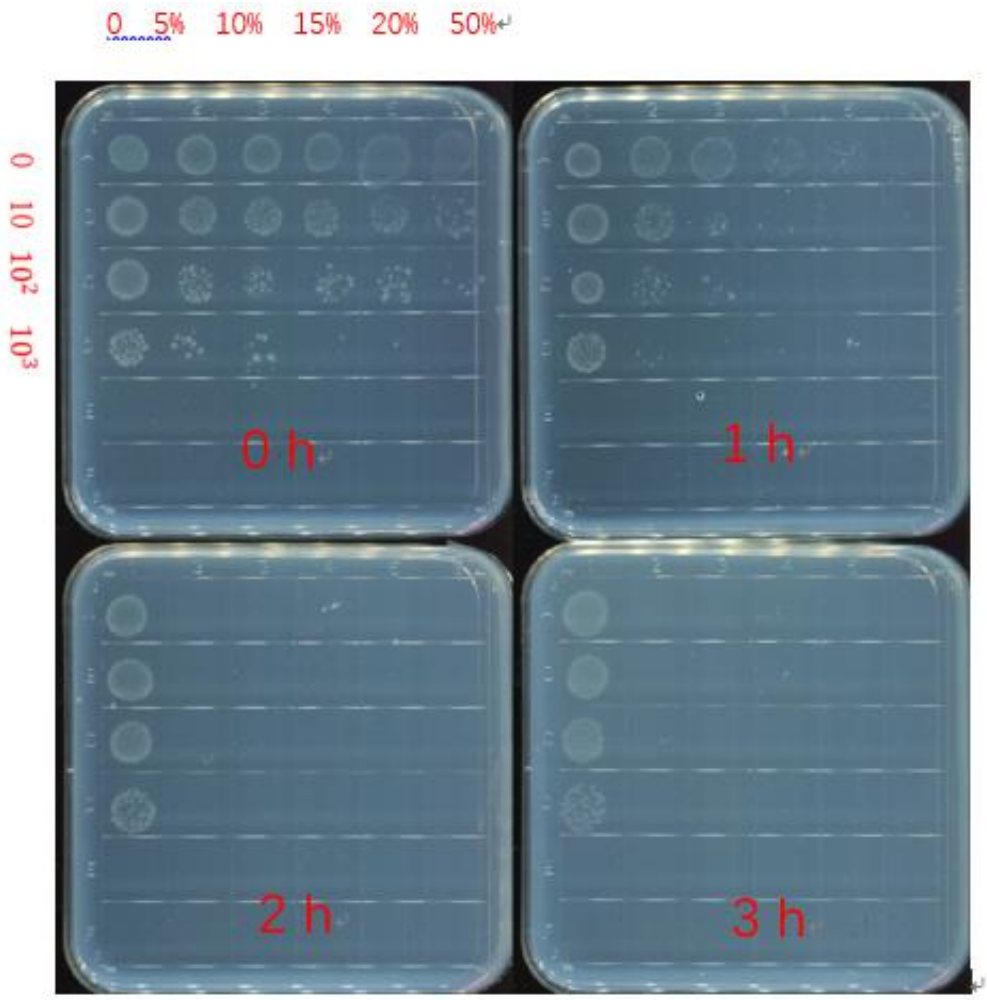
1/8

To revalidate the effect of 20% arabinose and provide more useful data to the modeling group, reconstitute 5% 10% 15% 20% 50% arabinose concentration.

2/8

Use the 5% 10% 15% 20% 50% arabinose concentration to verify the function of MazF once again.

Result:



From the experimental results, the 50% concentration of arabinose-induced MazF suicide was the best, and the bacteria were killed after adding the different concentrations of arabinose to the bacterial solution for 2 hours.

HANRONG'S

NOTEBOOK

Week 1 07.09~07.15

07.09 Make calibration 1: OD 600 Reference point - LUDOX

Experiment material

1ml LUDOX CL-X
ddH20
96 well plate,
black with clear flat bottom preferred

Protocol

1. Add 100 µl LUDOX into wells A1, B1, C1, D1
2. Add 100 µl of dd H2O into wells A2, B2, C2, D2
3. Measure absorbance at 600 nm of all samples in the measurement mode you plan to use for cell measurements
4.Record the data in the table below
5. Import data into Excel sheet provided

Result

	LUDOX CL-X	H2O
Replicate 1	0.0480	0.0316
Replicate 2	0.0593	0.0309
Replicate 3	0.0482	0.0312
Replicate 4	0.0560	0.0315
Arith. Mean	0.0529	0.0313
Corrected Abs600	0.0216	
Reference OD600	0.0630	
OD600/Abs600	2.9200	

07.10 (1) make Calibration 2: Particle Standard Curve - Microsphere

Experiment material

300 µL Silica beads - Microsphere suspension
ddH20
96 well plate, black with clear flat bottom preferred

Protocol

1.Obtain the tube labeled “Silica Beads” from the InterLab test kit and vortex 4 vigorously for 30 seconds.
2. Immediately pipet 96 µL microspheres into a 1.5 mL eppendorf tube
3. Add 904 µL of ddH2O to the microspheres
4. Vortex well.
5.Prepare the serial dilution of Microspheres
6.Measure Abs600 of all samples in instrument and record the data

Result

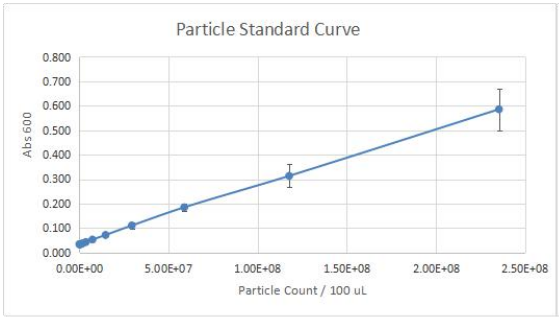


Figure 1. Particle standard curve

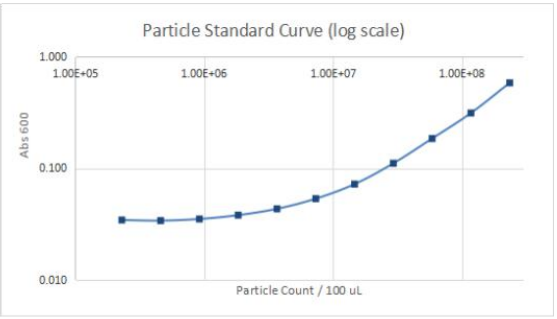


Figure 2. Particle standard curve(log scale)

(2) Calibration 3: Fluorescence standard curve - Fluorescein

Experiment material

Fluorescein

10ml 1xPBS pH 7.4-7.6

96 well plate, black with clear flat bottom

Protocol

1.Spin down fluorescein kit tube to make sure pellet is at the bottom of tube.

2.Prepare 10x fluorescein stock solution (100 μM) by resuspending fluorescein in 1 mL of 1xPBS

3.Dilute the 10x fluorescein stock solution with 1xPBS to make a 1x fluorescein solution with concentration 10 μM: 100 μL of 10x fluorescein stock into 900 μL 1x PBS

4.Prepare the serial dilutions of fluorescein

5.Measure fluorescence of all samples in instrument and record the data

Result

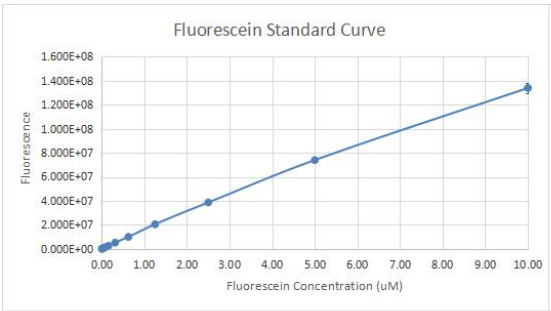


Figure 3. Fluorescein standard curve

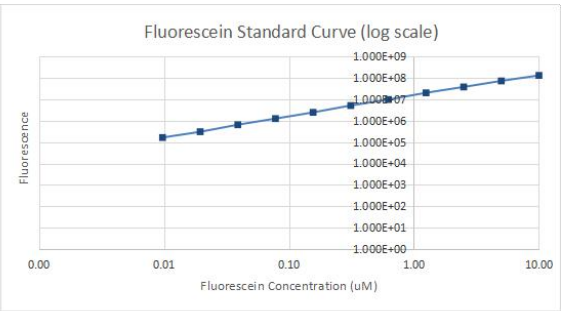


Figure 4. Fluorescein standard curve(log scale)

07.13 Make competent Cells

Experiment material

Escherichia coli strain DH5 α

LB (Luria Bertani) media

1.5 ml eppendorf tubes
Ice bucket with ice
Micropipettes and tips
$Cacl_2$ solution and glycerin

Protocol

1.Grow the culture to an OD600 = 0.4 – 0.6.
2.Put eppendorf tubes on ice. Split the culture into falcon tubes and incubate on ice for 15 min.
3.Centrifuge for 1 min at 5000 rpm and 4℃.Remove supernatant.
4.Resuspend cells in chilled $Cacl_2$ solution.Incubate on ice for 30 min.
5.Centrifuge for 1 min at 5000 rpm and 4℃.Remove supernatant.
6.Repeat step 4~5
7.Resuspend cells in chilled $Cacl_2$ -glycerin solution， store at -80℃

Result

Eighteen competent cells were made.

07.14 Transform Escherichia coli DH5α with plasmids

Experiment material

Resuspended DNA to be transformed
Competent Cells
1.5mL Microtubes
SOC Media
Petri plates w/ LB agar and Chloramphenicol
Ice & ice bucket
42°C water bath
37°C incubator
Pipettes and Tips

Protocol

1.Resuspend DNA in selected wells in the Distribution Kit with 10µl dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
2.Thaw competent cells on ice
3.Pipette 2µl of resuspended DNA intocompetent cells
4.Close 1.5ml tubes, incubate on ice for 30min
5.Heat shock tubes at 42°C for 45 sec
6.Incubate on ice for 5min
7.Pipette 800µl SOC media to each transformation
8.Incubate at 37°C for 1 hours, shaking at 120rpm
9.Spin down cells at 5000r/min for 1mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates
10.Incubate transformations overnight (14-18hr) at 37°C
11.Pick single colonies

07.15 Culture

Pick 2 colonies from each of the transformation plates and inoculate in 5-10 mL LB medium + Chloramphenicol. Grow the cells overnight (16-18 hours) at 37°C and 220 rpm.

Week 2 07.16~07.22

07.16 Cell growth, sampling, and assay

Experiment material

LB (Luria Bertani) media

Chloramphenicol

Falcon tube

Incubator at 37°C

1.5 ml eppendorf tubesIce bucket with ice

Micropipettes and tips

96 well plate, black with clear flat bottom preferred

Protocol

1. Make a 1:10 dilution of each overnight culture in LB+Chloramphenicol (0.5mL of culture into 4.5mL of LB+Chlor)
2. Measure Abs600 of these 1:10 diluted cultures
3. Record the data
4. Dilute the cultures further to a target Abs600 of 0.02 in a final volume of 12 ml LB medium + Chloramphenicol in 50 mL falcon tube.
5. Take 500 µL samples of the diluted cultures at 0 hours into 1.5 ml eppendorf tubes, prior to incubation. Place the samples on ice.
6. Incubate the remainder of the cultures at 37°C and 220 rpm for 6 hours.
7. Take 500 µL samples of the cultures at 6 hours of incubation into 1.5 ml eppendorf tubes. Place samples on ice.
8. measure samples (Abs600 and fluorescence measurement). Record data.

07.18~19 Repeated the experiment

Result

Fluorescence Raw Readings:

Hour 0:	Neg. Contr	Pos. Contr	Device 1	Device 2	Device 3	Device 4	Device 5	Device 6	LB + Chlor (blank)
Colony 1, Replicate 1	234635	321865	1482733	517560	238025	1240218	492124	329310	195892
Colony 1, Replicate 2	214236	322412	1519604	466269	242088	1532315	475383	337312	182358
Colony 1, Replicate 3	223401	322925	1638620	475101	236508	1308734	469898	321226	176741
Colony 1, Replicate 4	218682	312401	1583346	476307	239755	1283264	498938	321860	172938
Colony 2, Replicate 1	226387	299181	1472902	452770	229634	1298023	476724	335084	184159
Colony 2, Replicate 2	220005	309884	1612050	521951	235373	1408997	478673	327878	184160
Colony 2, Replicate 3	216405	312220	1616992	467469	234433	1242882	474318	361680	184161
Colony 2, Replicate 4	209968	308432	1635977	465022	250262	1198754	501682	355738	184162

Hour 6:	Neg. Contr	Pos. Contr	Device 1	Device 2	Device 3	Device 4	Device 5	Device 6	LB + Chlor (blank)
Colony 1, Replicate 1	209204	1096095	13682992	1793230	329628	13379232	3567988	1035020	230423
Colony 1, Replicate 2	216758	1095063	13642016	1804197	302621	13403197	4554169	993452	236406
Colony 1, Replicate 3	225901	1143036	13874274	1520515	296961	13910366	3978624	993469	223297
Colony 1, Replicate 4	219396	1140826	13922005	1771940	288825	13488009	3362810	855977	220167
Colony 2, Replicate 1	233065	1187847	12227292	1889448	300796	13017417	4496309	902118	287825
Colony 2, Replicate 2	214514	1123114	13409129	1799720	291232	13480249	3947810	901828	263927
Colony 2, Replicate 3	209431	1125588	13750141	1790238	292263	13674885	3885465	862416	219973
Colony 2, Replicate 4	211654	1219960	13511328	1913346	292125	13882210	4204558	893472	243625

Abs600 Raw Readings:

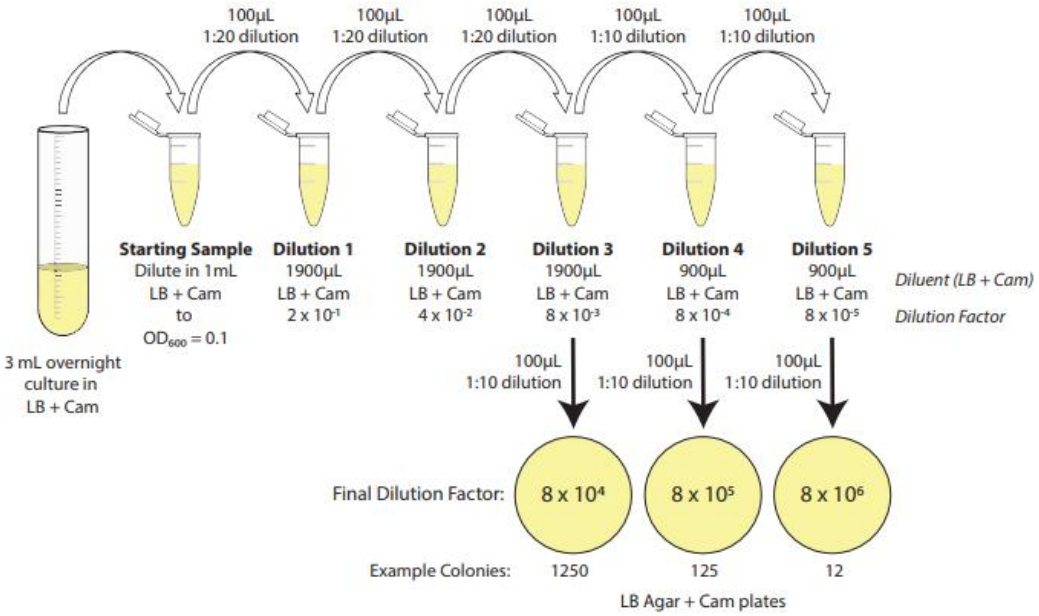
Hour 0:	Neg. Contr	Pos. Contr	Device 1	Device 2	Device 3	Device 4	Device 5	Device 6	LB + Chlor (blank)
Colony 1, Replicate	0.0644	0.0629	0.0594	0.0647	0.0637	0.0586	0.0586	0.062	0.0386
Colony 1, Replicate	0.0642	0.0625	0.0593	0.0642	0.0649	0.0575	0.0582	0.0636	0.0381
Colony 1, Replicate	0.064	0.0643	0.0611	0.0648	0.0632	0.0567	0.0579	0.062	0.0372
Colony 1, Replicate	0.0649	0.0631	0.0594	0.0652	0.0632	0.0569	0.0582	0.0648	0.0369
Colony 2, Replicate	0.0633	0.0632	0.0607	0.064	0.0632	0.058	0.0572	0.0644	0.0373
Colony 2, Replicate	0.0631	0.0634	0.0613	0.0656	0.0622	0.0577	0.0596	0.0638	0.0385
Colony 2, Replicate	0.0647	0.0628	0.0578	0.0647	0.0634	0.056	0.0588	0.0663	0.0383
Colony 2, Replicate	0.0649	0.0627	0.0584	0.0646	0.063	0.0574	0.0587	0.0658	0.0382

Hour 6:	Neg. Contr	Pos. Contr	Device 1	Device 2	Device 3	Device 4	Device 5	Device 6	LB + Chlor (blank)
Colony 1, Replicate	0.3237	0.3762	0.2807	0.3218	0.4024	0.3388	0.3967	0.3615	0.0356
Colony 1, Replicate	0.3592	0.3777	0.3234	0.3273	0.3982	0.3817	0.4437	0.3463	0.0355
Colony 1, Replicate	0.3533	0.3844	0.3181	0.2895	0.3917	0.368	0.4199	0.3409	0.0365
Colony 1, Replicate	0.3444	0.3801	0.3159	0.3297	0.4048	0.3685	0.4428	0.3116	0.0345
Colony 2, Replicate	0.3472	0.3916	0.3205	0.3479	0.3887	0.3693	0.4333	0.3476	0.0361
Colony 2, Replicate	0.3437	0.3833	0.3425	0.3335	0.4202	0.3713	0.439	0.3335	0.0357
Colony 2, Replicate	0.3302	0.3849	0.3292	0.3321	0.3979	0.3667	0.4312	0.316	0.0372
Colony 2, Replicate	0.3333	0.4069	0.3325	0.3474	0.4092	0.3602	0.4346	0.3232	0.0354

Table 2. cell measured data

07.20~07.21 Counting colony-forming units (CFUs)

Protocol



Result

For example, you would enter the following information: 1 x 10^8 CFU/mL.

BBa_I20270 Culture 1, Dilution Replicate 1	1.56 x 10^8
BBa_I20270 Culture 1, Dilution Replicate 2	1.408 x 10^8
BBa_I20270 Culture 1, Dilution Replicate 3	1.68 x 10^8
BBa_I20270 Culture 2, Dilution Replicate 1	1.51 x 10^8
BBa_I20270 Culture 2, Dilution Replicate 2	1.55 x 10^8
BBa_I20270 Culture 2, Dilution Replicate 3	1.608 x 10^8
BBa_R0040 Culture 1, Dilution Replicate 1	0.928 x 10^8
BBa_R0040 Culture 1, Dilution Replicate 2	1.072 x 10^8
BBa_R0040 Culture 1, Dilution Replicate 3	1.12 x 10^8
BBa_R0040 Culture 2, Dilution Replicate 1	1.32 x 10^8
BBa_R0040 Culture 2, Dilution Replicate 2	1.048 x 10^8
BBa_R0040 Culture 2, Dilution Replicate 3	0.976 x 10^8

Week 4　07.29~08.04

07.30 Making a standard curve of PLA

Experiment material

mobile phase	A：water	B：methyl alcohol	C:water+0.05% formic acid
sample	PLA (gradient dilution)		
column	Waters XBridge C-18 3.5 μm column (2.1*150 mm)		
HPLC	Prominence-i LC-2030 3d		

Protocol

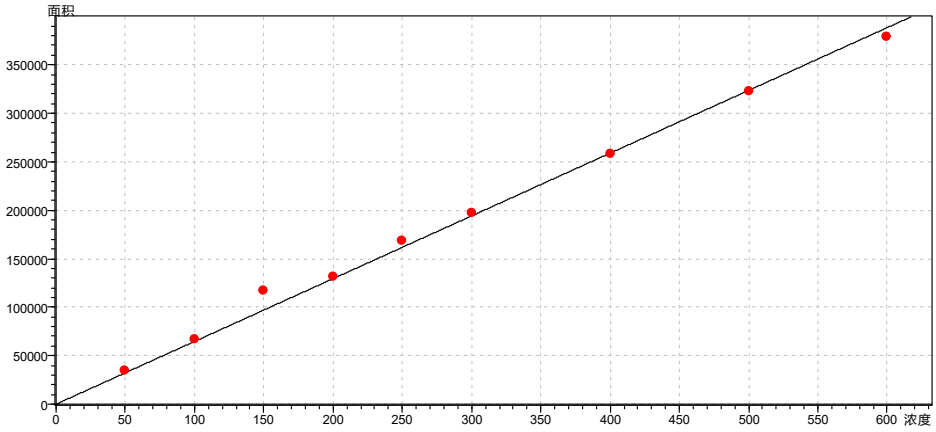
1. Prepare the sample of PLA(50,100,150,200,250,300,400,500,600mM)
2. Prepare mobile phase and ultrasonic cleaning for 30 min

The analysis was performed at 40℃ with a mobile phase comprising 45% methyl alcohol, 55% water+0.05% formic acid at a flow rate of 0.2 mL/min and the analytes were detected at OD254 nm.

concrete program

Wash column (before the injection of samples)	1.100% methyl alcohol, 30min 2.gradient elution to 15% methyl alcohol, 30min 3.15% methyl alcohol, 10min
Elution sample	gradient elute:45% methyl alcohol, 55% water+0.05% formic acid
Wash column (after the injection of samples)	1.15% methyl alcohol, 30min 2.gradient elution to 100% methyl alcohol, 30min 3.100%methyl alcohol, 10min

Result



Week 5　08.05~08.11

08.06　Making a standard curve of 2-PE

Experiment material

mobile phase	A：water	C：acetonitrile
sample	2-PE (50,100,200,300,400,500uM/L)	
column	Waters XBridge C-18 3.5 μm column (2.1*150 mm)	
HPLC	Prominence-i LC-2030 3d	

Protocol

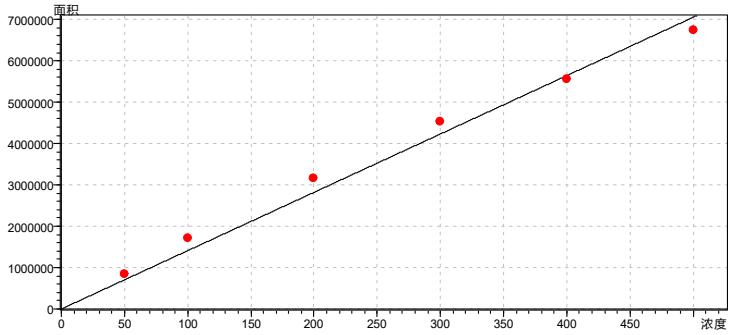
1. Make 2-PE stock solutions
   Add 1.2uLof 2-PE to the 998.8uL ddH2O, Mix it by pipetting up and down
2. Prepare the serial dilution of 2-PE
   Dilute the storage solution to 50,100,200,300,400,500uM/L
3. Filter the serial dilution of 2-PE
4. Prepare mobile phase and ultrasonic cleaning for 30 min
5. The analysis was performed at 25 ℃ with a mobile phase comprising 30% acetonitrile in water at a flow rate of 0.2 mL/min and the analytes were detected at OD214 nm.

concrete program

Wash column (before the injection of samples)	1.100% acetonitrile, 30min 2.gradient elution to 30% acetonitrile, 30min 3.30% acetonitrile, 10min
Elution sample	30% acetonitrile isocratic elution
Wash column (after the injection of samples)	1.10% acetonitrile, 30min 2.gradient elution to 100% acetonitrile, 30min 3.100% acetonitrile, 10min

Result

The accuracy is not enough and needs to do it once again



08.10 Measure the production of PLA (Modeling)

experiment material

mobile phase	A：water	B：methyl alcohol	C:water+0.05% formic acid
sample	PLA (from modeling team)		
column	Waters XBridge C-18 3.5 μm column (2.1*150 mm)		
HPLC	Prominence-i LC-2030 3d		

Protocol

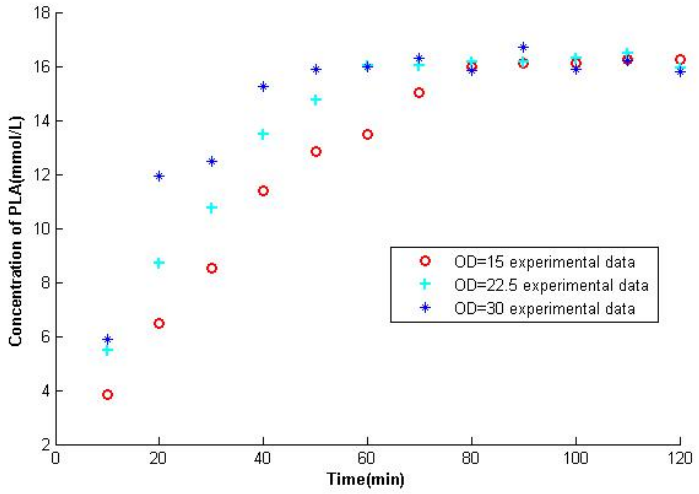
1. Prepare the sample of PLA
2. Prepare mobile phase and ultrasonic cleaning for 30 min

The analysis was performed at 40℃ with a mobile phase comprising 45% methyl alcohol, 55% water+0.05% formic acid at a flow rate of 0.2 mL/min and the analytes were detected at OD254 nm.

concrete program

Wash column (before the injection of samples)	1.100% methyl alcohol, 30min 2.gradient elution to 15% methyl alcohol, 30min 3.15% methyl alcohol, 10min
Elution sample	gradient elute:45% methyl alcohol, 55% water+0.05% formic acid
Wash column (after the injection of samples)	1.15% methyl alcohol, 30min 2.gradient elution to 100% methyl alcohol, 30min 3.100%methyl alcohol, 10min

Result



Week 6　08.12~08.18

site-directed mutation

08.12 **Transform**

Experiment material

Mutation5 plasmid
Competent Cells
1.5mL Microtubes
SOC Media
Ice & ice bucket
42°C water bath
37°C incubator
Pipettes and Tips

Protocol

1.Thaw competent cells on ice
2.Pipette 2µl of plasmid into competent cells
3.Close 1.5ml tubes, incubate on ice for 30min
4.Heat shock tubes at 42°C for 90 sec
5.Incubate on ice for 5min
6.Pipette 800µl SOC media to each transformation
7.Incubate at 37°C for 1 hours, shaking at 120rpm
8.Spin down cells at 5000r/min for 1mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates
9.Incubate transformations overnight (14-18hr) at 37°C

Result

Single colonies grow on the plate.

08.13 culture

Pick two single colonies and incubate at 37°C.

08.14 plasmids extracted

protocol

1. Add 1ml culture into Eppendorf tube.
2. Centrifuge at 10,000 x g for 1 minute at room temperature.
3. Decant or aspirate and discard the culture media.
4. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.
5. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate.
6. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.

7. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes. A compact white pellet will form.
8. Insert a Column into a 2 mL Collection Tube.
9. Transfer the cleared supernatant into the column.
10. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse the collection tube.
11. Add 500 µL HBC Buffer.
12. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse collection tube.
13. Add 700 µL DNA Wash Buffer.
14. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse the collection tube.
15. Centrifuge the for 2 minutes at maximum speed to dry the column matrix.
16. Transfer the Column to a clean 1.5 mL microcentrifuge tube.
17. Add 30 µL sterile deionized water directly to the center of the column membrane.
18. Let sit at room temperature for 1 minute.
19. Centrifuge at maximum speed for 1 minute.
20. Store DNA at -20°C.

Result

①Plasmid concentration: 12.173 ng/ul
260/230: 0.9
260/280: 1.52
②Plasmid concentration: 6.688 ng/ul
260/230: 0.91
260/280: 2.00
③Plasmid concentration: 9.03 ng/ul
260/230: 1.04
260/280: 2.21

Failed to extract plasmid, need to do it again.

08.14 Transform and culture

Transform Mutation5 plasmid, incubate transformations overnight (14-18hr) at 37°C.
Pick 2 colonies from the transformation plates and inoculate in 5mL LB medium + streptomycin. Grow the cells overnight (16 hours) at 37°C and 220 rpm.

08.16 Plasmids extracted

Failed to extract plasmid, need to do it again.

Week 7 08.19~08.26

site-directed mutation

08.21 PCR

50ul PCR system：

Component	Volume
Plasmid	10ng
Forward primer	1ul
Reverse primer	1ul
5×fastpfu Fly Buffer	10ul
2.5 mM dNTPs	4ul
fastpfu Fly DNA Polymerase	1ul
ddH_2O	32ul
Total volume	50ul

PCR Protocol:

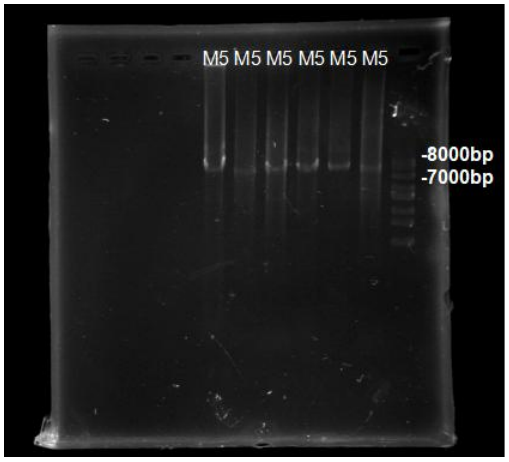
Initiation	94℃, 5min
Denaturation	94℃, 20sec
Annealing	55℃, 20sec
Extension	72℃, 3.5min
	25 cycles
Final Extension	72℃, 10min

08.22 DMT enzyme digestion

Add 1ul DMT enzyme to PCR products, mix by pipetting.

Incubate them one hour at 37°C.

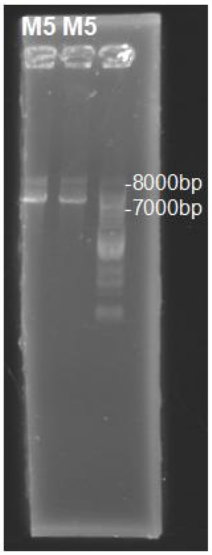
Agarose gel electrophoresis



Gel Extraction

1. Perform agarose gel electrophoresis to fractionate DNA fragments.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 mL microcentrifuge tube.
4. Add 3 volume Binding Buffer.
5. Incubate at 60℃ for 7 minutes or until the gel has completely melted. Vortex or shake the tube every 2-3 minutes.
6.Insert a Column in a 2 mL Collection Tube.
7.Centrifuge at 12,000 x g for 1 minute at room temperature. Repeat 3 times. Discard the filtrate and reuse collection tube.
8. Add 650 μL SPW Wash Buffer.
9. Centrifuge at 12,000 x g for 1 minute at room temperature. Discard the filtrate and reuse collection tube.
10. Centrifuge the empty Column for 2 minutes at maximum speed to dry the column matrix.
11. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
12. Add 30 μL deionized water directly to the center of the column membrane.
13. Let sit at room temperature for 2 minutes. Centrifuge at maximum speed for 1 minute.
14. Store DNA at -20℃.

Agarose gel electrophoresis



Plasmid concentration: 33.393 ng/ul
260/230: 0.43
260/280: 2.12

08.22 Transform and culture

Transform Mutation5 plasmid, incubate transformations overnight (14-18hr) at 37°C.
Pick 2 colonies from the transformation plates and inoculate in 5mL LB medium + streptomycin. Grow the cells overnight (16 hours) at 37°C and 220 rpm.

08.23 Plasmids extracted

Result

Plasmid concentration: 627.043 ng/ul
260/230: 2.28
260/280: 1.99

08.24 Measure the production of 2-PE

Experiment material

mobile phase	A：water	C： acetonitrile
sample	2-PE	
column	Waters XBridge C-18 3.5 μm column (2.1*150 mm)	
HPLC	Prominence-i LC-2030 3d	

Protocol

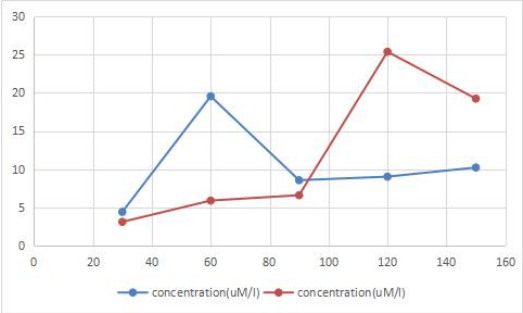
1. Prepare the sample of 2-PE
2. Prepare mobile phase and ultrasonic cleaning for 30 min

The analysis was performed at 40℃ with a mobile phase comprising 45% methyl alcohol, 55% water+0.05% formic acid at a flow rate of 0.2 mL/min and the analytes were detected at OD254 nm.

concrete program

Wash column (before the injection of samples)	1.100% acetonitrile, 30min 2.gradient elution to 30% acetonitrile, 30min 3.30% acetonitrile, 10min
Elution sample	30% acetonitrile isocratic elution
Wash column (after the injection of samples)	1.10% acetonitrile, 30min 2.gradient elution to 100% acetonitrile, 30min 3.100% acetonitrile, 10min

Result



08.25 Measure the production of PLA (Modeling)

experiment material

mobile phase	A：water	B：methyl alcohol	C:water+0.05% formic acid
sample	PLA (from modeling team)		
column	Waters XBridge C-18 3.5 μm column (2.1*150 mm)		
HPLC	Prominence-i LC-2030 3d		

Protocol

1.Prepare the sample of PLA

2.Prepare mobile phase and ultrasonic cleaning for 30 min

The analysis was performed at 40℃ with a mobile phase comprising 45% methyl alcohol, 55% water+0.05% formic acid at a flow rate of 0.2 mL/min and the analytes were detected at OD254 nm.

concrete program

Wash column (before the injection of samples)	1.100% methyl alcohol, 30min 2.gradient elution to 15% methyl alcohol, 30min 3.15% methyl alcohol, 10min
Elution sample	gradient elute:45% methyl alcohol, 55% water+0.05% formic acid
Wash column (after the injection of samples)	1.15% methyl alcohol, 30min 2.gradient elution to 100% methyl alcohol, 30min 3.100%methyl alcohol, 10min

Result

Data#	concentration (uM/L)
1	424.096
2	430.657
3	436.62
4	443.076

Week 8 09.02~09.09
site-directed mutation

09.06 PCR

50ul PCR system：

Component	Volume
Plasmid	10ng
Forward primer	1ul
Reverse primer	1ul
5×fastpfu Fly Buffer	10ul
2.5 mM dNTPs	4ul
fastpfu Fly DNA Polymerase	1ul
ddH_2O	32ul
Total volume	50ul

PCR Protocol:

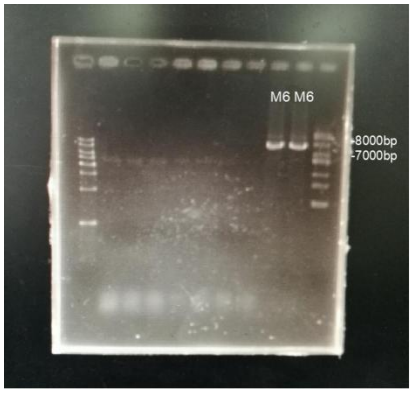
Initiation	94℃, 5min
Denaturation	94℃, 20sec
Annealing	56℃, 20sec
Extension	72℃, 3.5min
	25 cycles
Final Extension	72℃, 10min

09.07 DMT enzyme digestion

Add 1ul DMT enzyme to PCR products, mix by pipetting.

Incubate them one hour at 37°C.

Agarose gel electrophoresis



09.08 Transform and culture

Transform Mutation5 plasmid, incubate transformations overnight (14-18hr) at 37°C.
Pick 2 colonies from the transformation plates and inoculate in 5mL LB medium + streptomycin.
Grow the cells overnight (16 hours) at 37°C and 220 rpm.

09.09 Plasmids extracted

Plasmid concentration: 127.043 ng/ul

09.11 enzyme digestion analysis

15ul system:

Plasmid	1000ng
Spe Ⅰ	0.5 ul
EcoR Ⅰ	0.5 ul
ddH2O	11.4 ul
Buffer	1.5 ul

10.05 Measure the production of 2-PE again
Method as above

10.09-10.16

Failed to extract the E-3M2H from sample.

XIAOYU'S

NOTEBOOK

Week 1- 14 27.05-02.09

In this module, I mainly assist my teammates to complete the experiment.

Week15 03.09-09.09

Complete collaborative experiment

04.09

I used a five-point sampling method to sample the soil in our team's area. Then put collected soil is dried for 24h and saved.

06.09

I did detect the pH of the soil.

07.09

I did detect soluble salt content in soil

Week16 10.09-16.09

Customize our team uniforms

Week17 17.09-23.09

I planned to complete the garbage cans simulation experiment.

18.09

1. I tried to simulate the environment of two small garbage cans in the laboratory. And I add equal amounts of discarded peels to the two beakers, suck the pipettetips of the bacteria, polluted water, etc. then mix them thoroughly.

2. I chose to put a certain amount 10 mmol/l PLA in one of the beakers (this concentration is based on our modeling guidelines). The other was a beaker without a PLA for comparison. Then they were cultured in the same environment for 3 days.

21.09

I can see some differences in the growth of the bacteria in the two beakers. Continue to cultivate.

Week18 24.09-30.09

27.09

I observed the condition of the long bacteria in the two beakers again.

Result

As shown in the two beakers, the case of the beaker with PLA added is lower than the beaker without the PLA. In the figure, the growth situation of bacterium that the right beaker is lower than the beaker without PLA added on the left.

No PLA Have

Week19 01.10-07.10

To verify the function of PLA

01.10

I made an investigation to select some suitable products. After market research, we choose hand soap with jasmine fragrance, one kind of soap which is typical in market.

02.10

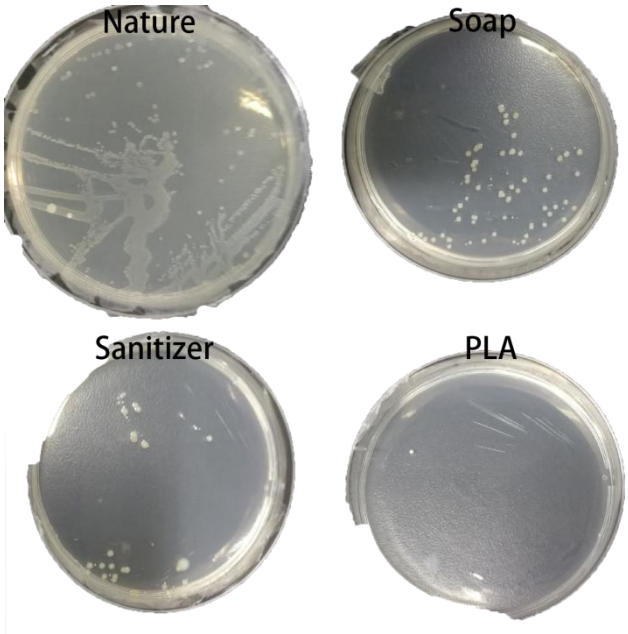
I prepared 10mmol/l of PLA and took 5 grams of two market products into 45g of sterile water to dissolution. I used100ul mixed liquid solution of the same concentration for coating on the medium and then coated 10ul of the three products. Then they were placed in a constant temperature incubator at 37°C for 12h.

03.10

I observed it.

Result

Compared with the natural growth of the bacteria, as the picture shows, it obviously shows that PLA has a more effective effect on sterilization.



Week20 08.10-14.10

09.10

I exposed the medium in air for 24 hours. Due to the cold weather, and the growth of the bacteria is not seen when exposed to the air.

11.10

Due to the cold weather, and the growth of the bacteria is not seen when exposed to the air. I added 100 ul of tap water, placed in a 37 ° C incubator for 12 hours.

12.10

Put out them and observed. It can see a little bacteria on the medium. Then I added 100 ul of three products in the medium and placed in 37°C incubator for 12 hours.

13,10

I took out them and observed again.