BGL

Double Enzyme Digestion

Date: 8.9

1. PCR BGL from plasmid pVC57- BGL.

Volume: 50μL

MCLAB Mix	25μL
Primer F	2μL
Primer R	2μL
pVC57- BGL	2μL
ddH2O	19μL

Program:

1	98℃	2min
2	98℃	10s
3	60℃	15s
4	72℃	30s
5	Go to 2	×30
6	72℃	5min
7	4℃	-----

2. Agarose gel electrophoresis.

Result: As expected.

3. Double enzyme digestion.

Volume: 20μL

QC 10×buffer	2μL	QC 10× buffer	2μL
BGL	12μL	pET21a	16μL
QC XhoI	1μL	QC XhoI	1μL
QC NdeI	1μL	QC NdeI	1μL
ddH2O	4μL	ddH2O	0μL

37℃，30min

Date: 8.10

1. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

2. Gel extraction purification.

Date: 8.11

1. Ligation.

Volume: 25 μL

T4 buffer	2.5 μL
BGL	13 μL
pET21	7.5 μL
T4 ligase	2 μL

4℃, overnight

Date: 8.12

1. Agarose gel electrophoresis of ligation.

Result: As expected.

2. Transformation

Add 20 μL of ligation product into 100 μL DH5 α competent cells.

Heat shock for 75s at 42℃.

Overnight culture.

Result: We didn't get any colony, the transformation was failed.

3. PCR BGL from plasmid pVC57- BGL.

Volume: 50 μL

MCLAB Mix	25 μL
Primer F	2 μL
Primer R	2 μL
pVC57- BGL	2 μL
ddH2O	19 μL

Program:

1	98℃	2min
2	98℃	10s

3	60℃	15s
4	72℃	30s
5	Go to 2	×30
6	72℃	5min
7	4℃	-----

4. Agarose gel electrophoresis.

Result: As expected.

Date: 8.13

1. Double enzyme digestion.

Volume: 20 μL

QC 10×buffer	2 μL	QC 10× buffer	2 μL
BGL	12 μL	pET21a	16 μL
QC XhoI	1 μL	QC XhoI	1 μL
QC NdeI	1 μL	QC NdeI	1 μL
ddH2O	4 μL	ddH2O	0 μL

37℃，30min

2. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

3. Gel extraction purification.

4. Ligation.

Volume: 25 μL

T4 buffer	2.5 μL
BGL	13 μL
pET21	7.5 μL
T4 ligase	2 μL

22℃, 2.5h

5. Agarose gel electrophoresis of ligation.

Result: As expected.

6. Transformation

Add 10 μL of ligation product into 100 μ DH5 α competent cells.

Add 10 μL of ligation product into 100 μ BL 21 competent cells.

Heat shock for 80s at 42℃.

Overnight culture.

Result: We got some colonies on the plates.

Date: 8.14

1. Gel extraction purification of pET21a.

2. Double enzyme digestion.

Volume: 20 μL, 16 μ

QC 10×buffer	2 μL	QC 10× buffer	2 μL
BGL	12 μL	pET21a	12 μL
QC XhoI	1 μL	QC XhoI	1 μL
QC NdeI	1 μL	QC NdeI	1 μL
ddH2O	4 μL	ddH2O	0 μL

37℃，30min

3. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

4. Gel extraction purification.

5. Ligation.

（1）Volume: 30 μL

T4 buffer	3 μL
BGL	10 μL
pET21	10 μL
T4 ligase	2 μL
ddH2O	5 μL

22℃，2.5h

（2）Volume: 30 μL

T4 buffer	3 μL
BGL	13 μL
pET21	7.5 μL
T4 ligase	2 μL
ddH2O	4.5 μL

22℃，2.5h

（3）Volume: 30 μL

T4 buffer	3 μL
BGL	5 μL
pET21	10 μL
T4 ligase	2 μL
ddH2O	10 μL

22℃，2.5h

6. Agarose gel electrophoresis of ligation.

Result: We got some expected product of ligation, but the concentration was too low.

7. Pick colonies from the plate we cultured yesterday.

DH5α: 5

BL21:3

Overnight culture.

8. Colony PCR.

Volume: 24 μL

T5 Super Mix	12 μL
Primer F	1 μL
Primer R	1 μL
Colony	1
ddH2O	10 μL

Program:

1	98℃	3min
2	98℃	10s
3	60℃	10s
4	72℃	45s

5	Go to 2	×30
6	72℃	2min
7	4℃	-----

9. Agarose gel electrophoresis.

Result: DH5α 1, BL21 1 had expected strips, other samples didn't.

Date:8.15

1. Colony PCR.

Volume: 24μL

T5 Super Mix	12μL
Primer F	1μL
Primer R	1μL
Colony	1
ddH2O	10μL

Program:

1	98℃	3min
2	98℃	10s
3	60℃	10s
4	72℃	45s
5	Go to 2	×30
6	72℃	2min
7	4℃	-----

2. Agarose gel electrophoresis.

Result: All of the samples were false positive.

3. Extract plasmids from DH5α-BGL.

4. Agarose gel electrophoresis of plasmids.

Result: All of the false positive samples had empty vectors (pET21a).

5. Overnight culture of pET21a.

Date: 8.16

1. Extract plasmids pET21a.

2. Double enzyme digestion of pET21a.

Volume: 20 μL

QC 10× buffer	2 μL
pET21a	16 μL
QC XhoI	1 μL
QC NdeI	1 μL
ddH2O	0 μL

37℃，30min

3. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

4. Gel extraction purification.

5. Ligation.

（1）Volume: 30 μL

T4 buffer	3 μL
BGL	10 μL
pET21	10 μL
T4 ligase	2 μL
ddH2O	5 μL

22℃，2.5h

（2）Volume: 30 μL

T4 buffer	3 μL
BGL	13 μL
pET21	7.5 μL
T4 ligase	2 μL
ddH2O	4.5 μL

22℃，2.5h

（3）Volume: 30 μL

T4 buffer	3 μL
BGL	5 μL

pET21	10 μL
T4 ligase	2 μL
ddH2O	10 μL

22℃，2.5h

6. Agarose gel electrophoresis of ligation.

Result: We got expected products of ligation from each group, but the concentration was too low.

Date: 8.17

1. Gel extraction purification of BGL PCR product.

The result of agarose gel electrophoresis of BGL PCR showed more than one strips, so we need to purify the PCR product.

2. Double enzyme digestion of BGL.

Volume: 20 μL

QC 10×buffer	2 μL
BGL	12 μL
QC XhoI	1 μL
QC NdeI	1 μL
ddH2O	4 μL

37℃，30min

3. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

4. Gel extraction purification.

5. Ligation.

（1）Volume: 30 μL

T4 buffer	3 μL
BGL	10 μL
pET21	10 μL

T4 ligase	2 μL
ddH2O	5 μL

22℃，6h

（2）Volume: 30 μL

T4 buffer	3 μL
BGL	13 μL
pET21	7.5 μL
T4 ligase	2 μL
ddH2O	4.5 μL

22℃，6h

Date:8.18

1. Agarose gel electrophoresis of ligation.

Result: We got expected products of ligation from each group, but the concentration was too low. The result of reaction system (1) was better. And the clearest strips were about 1000bp, so we inferred that they might be homo-dimers of pET21a.

2. Ligation.

Volume: 20 μL

Label	1	2	3
10× NEB buffer	2 μL	2 μL	2 μL
BGL	10 μL	14 μL	12 μL
pET21	3 μL	3 μL	5 μL
NEB T4	1 μL	1 μL	1 μL
ddH2O	4 μL	0 μL	0 μL

Room temperature, >2h

3. Agarose gel electrophoresis of ligation.

Result: All the strips were about 1000bp, they might be homo-dimers of pET21a.

4. Transformation

Add 20 μL of 8.16 and 8.17 ligation products into 100 μL DH5α competent cells.

Heat shock for 90s at 42℃.

Overnight culture.

Result: We got colonies on every plate.

Date: 8.19

1. Colony PCR.

Volume: 24 μL

T5 Super Mix	12 μL
Primer F	1 μL
Primer R	1 μL
Colony	1
ddH2O	10 μL

Program:

1	98℃	3min
2	98℃	10s
3	60℃	10s
4	72℃	45s
5	Go to 2	×30
6	72℃	2min
7	4℃	-----

2. Agarose gel electrophoresis.

Result: No.3 and No.12 had expected strips, other samples didn't.

3. Double enzyme digestion of BGL and pET21a.

Volume: 20 μL

QC 10×buffer	2 μL	QC 10× buffer	2 μL
BGL	12 μL	pET21a	16 μL
QC XhoI	1 μL	QC XhoI	1 μL
QC NdeI	1 μL	QC NdeI	1 μL
ddH2O	4 μL	ddH2O	0 μL

37℃，1h

4. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

5. Gel extraction purification.

6. Ligation.

Volume: 30 μL

T4 buffer	3 μL
BGL	10 μL
pET21	10 μL
T4 ligase	2 μL
ddH2O	5 μL

16℃，overnight

Date: 8.20

1. Extract plasmids from No.3 and No.12.

2. Double enzyme digestion validation.

Volume: 10 μL

QC 10×buffer	1 μL
Sample(3,12)	5 μL
QC XhoI	1 μL
QC NdeI	1 μL
ddH2O	2 μL

37℃，30min

3. Agarose gel electrophoresis of double enzyme digestion validation.

Result: No.3 sample had expected strips, yet No.12 didn't. It seemed like we got a successful recombinant plasmid pET21a-BGL.

4. Send plasmids of No.3 and No.12 for gene sequencing.

Result: Plasmid of No.12 was empty vector pET21a. Plasmid of No.3 was recombinant plasmid but the inserted gene was not what we want—it was FMO, not BGL. It was because of the sundry bacteria contamination.

Date:8.21

1. Overnight culture of pET21a.

Date: 8.22

1. Extract plasmids pET21a.

Date: 8.23

1. PCR BGL from plasmid pVC57- BGL.

Volume: 50 μL

MCLAB Mix	25 μL
Primer F	2 μL
Primer R	2 μL
pVC57- BGL	2 μL
ddH2O	19 μL

Program:

1	98℃	2min
2	98℃	10s
3	60℃	15s
4	72℃	30s
5	Go to 2	×30
6	72℃	5min
7	4℃	-----

2. Double enzyme digestion of pET21a.

Volume: 20 μL

QC 10× buffer	2 μL
pET21a	16 μL
QC XhoI	1 μL
QC NdeI	1 μL
ddH2O	0 μL

37℃，2h

3. Gel extraction purification of BGL PCR product.

The result of agarose gel electrophoresis of BGL PCR showed more than one strips, so we need to purify the PCR product.

4. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

5. Gel extraction purification.

6. Send the product of gel extraction purification of BGL PCR product for gene sequencing.

Result: PCR product was BGL , so the plasmid pET21a-FMO we had got was truly because of the sundry bacteria contamination.

Date: 8.25

1. Double enzyme digestion of BGL.

Volume: 20 μL

QC 10×buffer	2 μL
BGL	12 μL
QC XhoI	1 μL
QC NdeI	1 μL
ddH2O	4 μL

37℃，2h

2. Agarose gel electrophoresis of double enzyme digestion.

Result: As expected.

3. Gel extraction purification.

4. Dephosphorylation of pET21a.

Volume: 20 μL

Smart buffer	2 μL
pET21	10 μL
rSAP	1 μL
ddH2O	7 μL

37℃, 30min

65℃, 10min

5. Ligation.

Label	1	2	3	4
T4 buffer	5 μL	5 μL	4 μL	2.5 μL
pET21(after dephosphorylation)	20 μL	20 μL	20 μL	10 μL
BGL	20 μL	20 μL	10 μL	10 μL
T4 ligase	3 μL	3 μL	3 μL	2 μL
ddH2O	2 μL	2 μL	3 μL	0.5 μL
Volume	50 μL	50 μL	40 μL	25 μL

Label	5	6
T4 buffer	3 μL	2.5 μL
pET21(before dephosphorylation)	10 μL	10 μL
BGL	10 μL	10 μL
T4 ligase	2 μL	2 μL
ddH2O	5 μL	0.5 μL
Volume	30 μL	25 μL

16℃，overnight

Date: 8.26

1. Agarose gel electrophoresis of ligation.

Result: No.1-4 did not have any product of ligation, No.5-6 had some expected product of ligation, but the concentration was too low. And the rate of homo-dimers of pET21a was still high.

2. Pick 28 colonies from the plate we used on 8.18.

3. Colony PCR.

Volume: 24 μL

T5 Super Mix	12 μL
Primer F	1 μL
Primer R	1 μL
Colony	1
ddH2O	10 μL

Program:

1	98℃	3min
2	98℃	10s
3	60℃	10s
4	72℃	45s
5	Go to 2	×30
6	72℃	2min
7	4℃	-----

4. Agarose gel electrophoresis.

Result: No.1, No.7 and No.14 didn't have expected strips, other samples did.

5. Overnight culture.

No.2-6, No. 8-13, No. 15-18

Date: 8.27

1. Colony PCR.

Volume: 24 μL

T5 Super Mix	12 μL
Primer F	1 μL
Primer R	1 μL
Colony	1
ddH2O	10 μL

Program:

1	98℃	3min
2	98℃	10s
3	60℃	10s
4	72℃	45s
5	Go to 2	×30
6	72℃	2min
7	4℃	-----

2. Agarose gel electrophoresis.

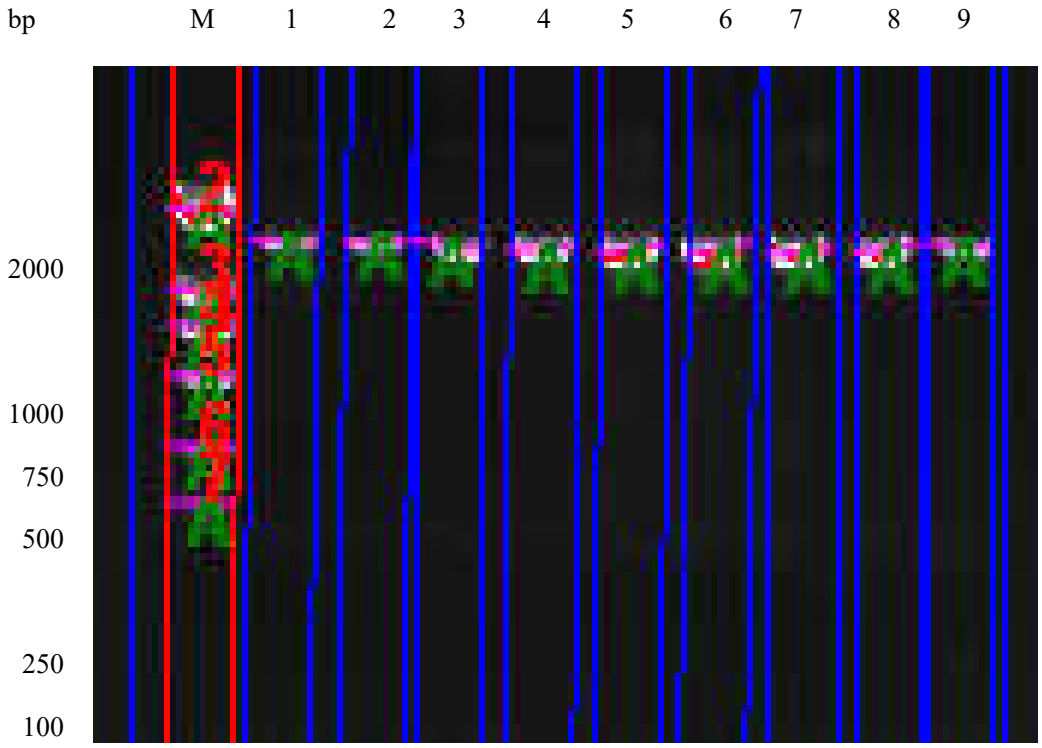
Result: All of the samples were false positive.

Construction of BGL expression vector and function verification of BGL.

7.23~8.4

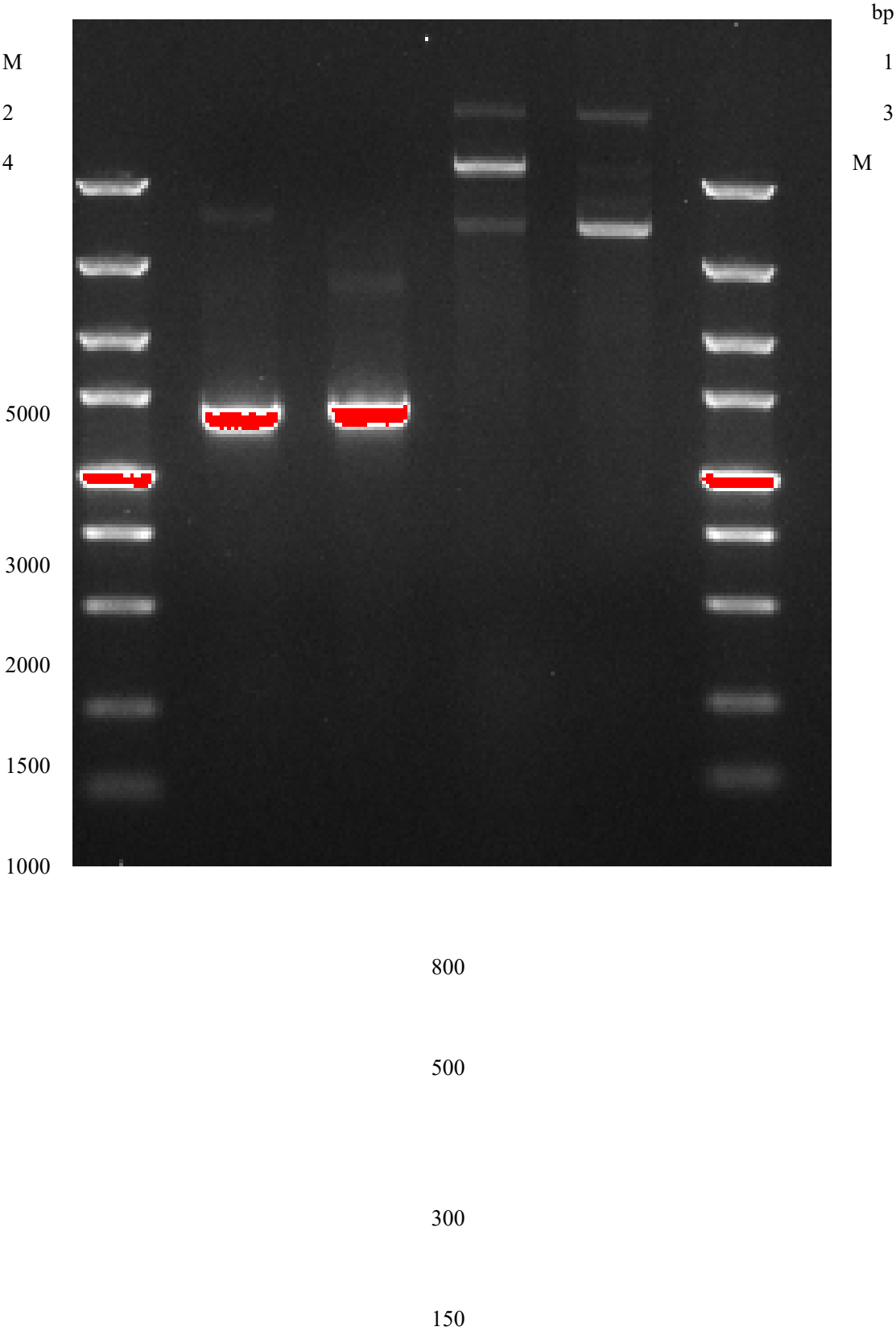
Restriction cloning of DNA fragment

After we synthesized the enzymes, BGL. We designed the primers for the BGL and the vectors pET21a(+) which contain the EcoRI recognition sites. By the process of PCR, we obtained the BGL with EcoRI site. And PCR products processed gel extraction for purification before use.



Gel extraction result for BGL PCR products. Line1 to 9 are BGL PCR products from different reaction condition。

After that, we used EcoRI to digest the PCR products and vector pET21a(+) respectively. Then we verified the size of fragments and processed gel extraction for purification.



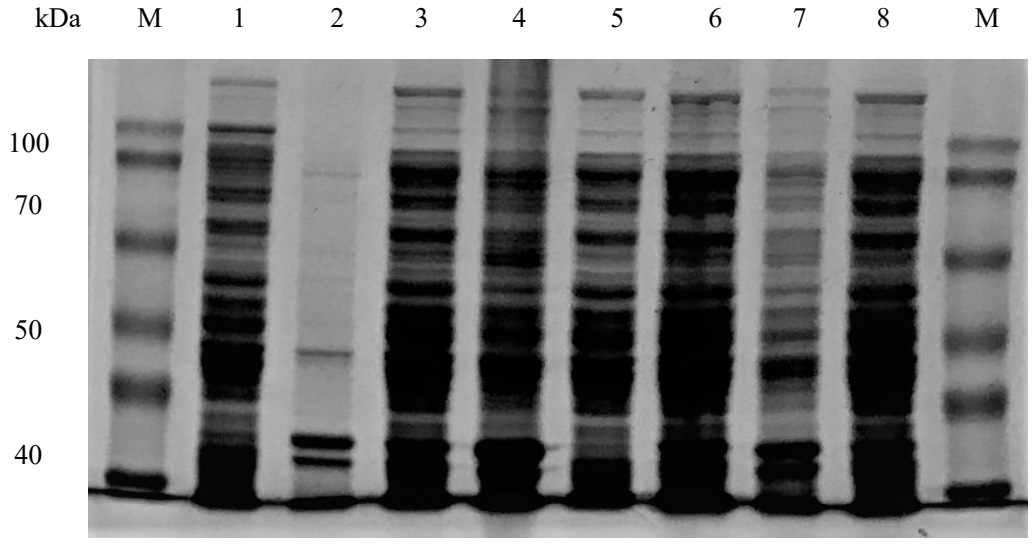
Gel extraction result for BGL and vector after enzyme digestion. From Line 1to 4 are BGL after digestion, BGL without digestion, pET21a(+) after digestion, pET21a(+) without digestion respectively.

So we got the BGL fragment and pET21a(+) backbone with complementary sticky ends. Considering the mono-restriction cloning could lead to backbone self-ligation, we did dephosphorylation for pET21a(+) backbone. In the way of ligation, the BGL inserted into the vectors.

8.5~8.6

Prove the BGL expressed in E.coli system

We transformed the recombinant plasmids of BGL into *E.coli* DH5α for amplification. And then, the amplified plasmids were transformed into *E.coli* BL21(DE3). SDS-PAGE was used to verify the expression of enzymes. Unfortunately, BGL was failed to express.



SDS-PAGE for the expression in E.coli BL21（DE3）strain of BGL enzymes(in pET21a vector). Line 1 represents the supernate of Negative control (BL21 without recombinant plasmids). Line 3,6 represent the supernate of BGL BL21 with different dilution factor(2X and 4X).Line 2 represents the precipitates of Negative control. Line 4,7 represent the precipitates of BGL BL21 with different dilution factor (2X and 4X).