正交验证组实验记录

Date: 8.1

1. Use 10μ ddH2O to dilute 2018kit part

   Plate 6-9M　T7-RFP

   Plate 3-8M　T7-sfGFP-Terminator

   Plate 3-12I　T7-GFP-Terminator

2. Pick 1μ part liquid transform DH5α and BL21

   Tomorrow observe transformation result

Date: 8.2

1. Observe transformation result: All plates have no colony, transformation fail.
2. Three part (6-9M, 3-8M, 3-12I) transform DH5α and BL21 again.

   Tomorrow observe transformation result

Date: 8.3

1. Observe transformation, Result: All DH5α transformation succeed; BL21 has only RFP transformation succeeded (and the colony is red), sfGFP and GFP transformation fail.
2. Pick the successful transform result colony, culture in LB medium for 12h
3. Culture the bacterium in LB medium, result extract plasmid, agarose gel electrophoresis for verification.

   Agarose gel electrophoresis, result is consistent with, but because plasmid is superhelix, we can't verify it.

4. Extracted plasmid sequenced, verify part

   Wait for tomorrow sequencing result

5. Store RFP in BL21 glycerin mixture

Date: 8.4

Wait for sequencing result

Date: 8.5

1. Observe sequencing result:

SfGFP sequencing result is consistent with the plasmid map, but GFP suggests a point but mutation (wiki says the part potentially has point mutation.

2. Extracted sfGFP and GFP plasmid transform BL21

   Tomorrow observe transformation result

Date: 8.6

1. Observe transformation result: Transformation result is ok, and the colony is nice.
2. Transformation, pick the result colony and culture in LB medium.
3. Because RFP part has no terminator, which needs to be added a new terminator.

   Use enzyme digestion ligation, cleave from Biobrick Suffix, which needs to add enzyme

digestion site (SpeI and PstI) at the frank of the terminator, design primer .

Date: 8.7

1. Observe sfGFP and GFP culture in LB medium result and plates are exposed under ultra violet, observing fluorescence.

The plates are not green, but the liquid culture is green under ultra violet.

2. Continue designing primers, and submit primers.

3. Pick culture in LB medium result. Exposed under ultra violet and we find that the two tubes which have the lightest fluorescence. Store sfGFP and GFP BL21 glycerin mixture

Date: 8.8

1. PCR terminator from RFP part, and add enzyme digestion sites

Reaction system: 50μl

2x super pcr mix 25μ

Templates (sfGFP plasmid) 1μ

Primer1 2μ

Primer2 2μ

ddH2O 20μ

Add a no template (use ddH2O to replace it) NC

Procedure :

98℃

98℃ 35cycle

52-64℃ grads 35cycle

72℃ 5s 35cycle

72℃

2. Agarose gel electrophoresis (2%) verification. There is a lane at the length of about 200bp, but target PCR result is 155bp, Therefore, we think the result is wrong.

Date: 8.9

1. PCR again, the system is the same as that of yesterday.

2. Agarose gel electrophoresis , gel extract .

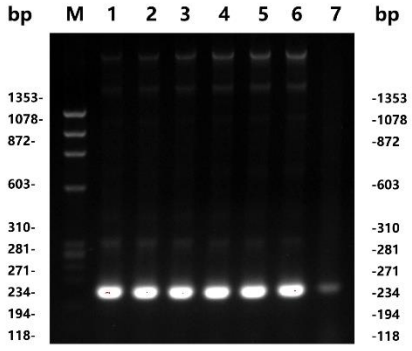


Figure . Electrophoresis result of the terminator PCR result | lane 1-6 are the results of terminator PCR result, while lane 7 is NC. We can see that NC has a weak bond.

3. Gel extract result sequenced.

Date: 8.10

1. 10min enzyme digestion test

   Reaction system: 20μ

10x Qcut green buffer	2μ
DNA (RFP plasmid)	2μ
SpeI	1μ
PstI	1μ
ddH2O	14μ

   37℃ 10min, 4℃ hold

   NC (no template)

2. Agarose gel electrophoresis verification: All digested.

3. 50μ system10min enzyme digestion

10x Qcut green buffer	5μ
DNA (RFPplasmid)	5μ
SpeI	1μ
PstI	1μ
ddH2O	38μ

   NC (no template)

4. enzyme digestion result agarose gel electrophoresis , gel extract

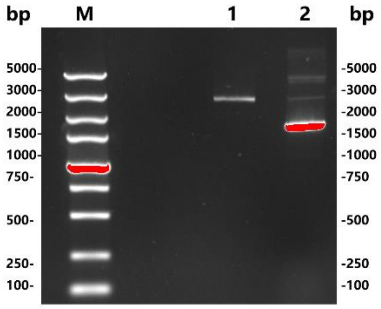


Figure . Electrophoresis result of the digested pSB1C3 backbone and sgGFP plasmid | lane 1 is the digested backbone, while lane 2 is the plasmid (is not digested). The lighted bond of lane 2 is the supercoiled plasmid ,while the bond which is near to the bond of lane 1 is the linear plasmid.

Date: 8.11

1. pcr terminator

system: 50μ

2x super pcr mix	25μ
Template (sfGFP plasmid)	1μ
Primer1	2μ
Primer2	2μ
ddH2O	20μ

DNA template is diluted to 1/10 with elusion buffer.

Procedure :

98℃	3min
98℃	30s 35cycle
52℃	30s 35cycle
72℃	5s 35cycle
72℃	2min
4℃	hold

2. PCR result agarose gel electrophoresis。

Sample sequence: φ marker, 25μ Ex, 25μ Ex, 25μ NC, 25μ NC

Result: Experiment group and NC group both have lanes, so it's possibly that the some reagents are polluted.

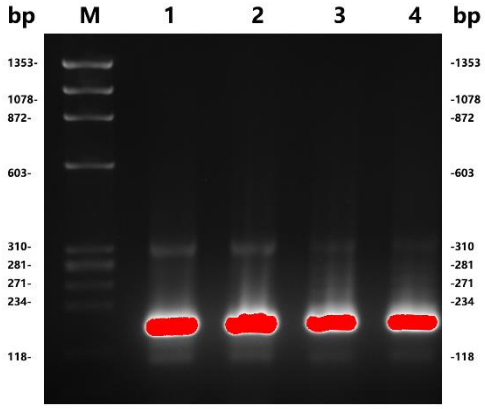


Figure . Electrophoresis result of the terminator PCR result | lane 1-3 are the results of terminator PCR result, while lane 4 is NC. The result is absolutely not correct.

3. Once more PCR, but change the annealing temperature to 54℃, set two experiment group (one use diluted template, one use original template), three negative control (one has no template, and one has no primer, one has no enzyme).

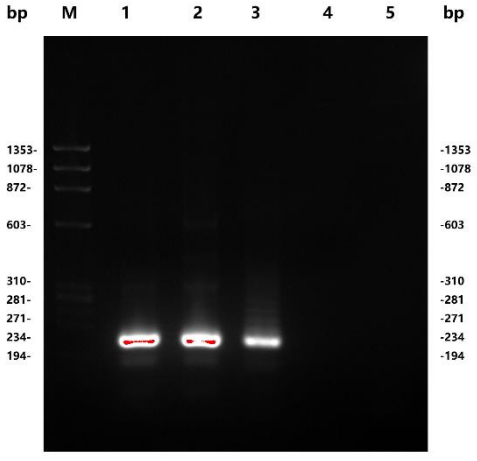


Figure . Electrophoresis result of the terminator PCR result | lane 1-2 are the experiment group result, while lane 3 is NC without template. Lane 4-5 are other two NCs. The result shows that the primers can PCR into a bond which is similar to the target bond.

4. PCR result SpeI and PstI enzyme digestion 10min, 50μsystem

Ter_pcr cut Ter_pcr_nc cut Ter_nc_nc cut

Experiment group pcr no template nc Ex no DNANC

5. enzyme digestion result

6. ligation

20μsystem:

T4 DNA ligation buffer 2μ

T4 DNA ligase 1μ

backbone 4μ

segment (enzyme digestion reaction)13μ

16℃ 8h ligation reaction

Date: 8.12

1. ligation result transforms into DH5α, culture for 14h

Result:

RFP-ter ex-ex pcr result enzyme digestion + RFP backbone enzyme digestion –> no colony

RFP-ter pcr NC-expcr no template NC enzyme digestion + RFP backbone enzyme digestion -> no colony

RFP-ter pcr NC-NC pcr no template NC enzyme digestion no pcr result NC RFP backbone enzyme digestion -> has colony

RFP-ter NC -> has colony

NC blank plate -> no colony

Date: 8.13

1. verify C+ plates

Half is added in C+ E.coli, while half is added in A+ E.coli.

2. agarose gel electrophoresis to check whether the primers are polluted.

, Result: primer has no lanes which are similar to the template, suggests that the primers have no problems.

3. Once again pcr sfGFP terminator, Using McLab enzyme.

system: 50μ

2x Master Mix25μ

Primer1 2μ

Primer2 2μ

Template(1μ sgGFP plasmid is diluted to 10μ) 1μ

ddH2O 20μ

Procedure :

98℃ 2min

98℃ 10s 35cycle

52℃ 15s 35cycle

72℃ 5s 35cycle

72℃ 5min

4℃ hold

4. agarose gel electrophoresis (2%):

Result:

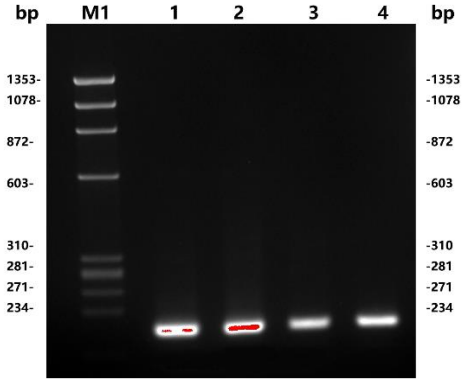


Figure . Electrophoresis result of the terminator PCR result | lane 1-2 are the experiment group result, while lane 3 is NC without template. Lane 4-5 are other two NCs. The result shows that the primers can PCR into a bond which is similar to the target bond.

5. gel extract
6. gel extract result: 1μ sequenced
7. gel extract result enzyme digestion(50μ system, SpeI and PstI)
8. enzyme digestion result perification.

Date: 8.14

1. Check C+ plate: C+ region has colonies, and A+ region has a little colonies. It suggests that the C+ plate half loses function.
2. RFP plasmid digestion, with SpeI and PstI enzyme.
3. Enzyme digestion result agarose gel electrophoresis

Sample sequence: DL5000 marker enzyme digestion result 1 enzyme digestion result 2 enzyme digestion result 3 RFP plasmid

Result: normal

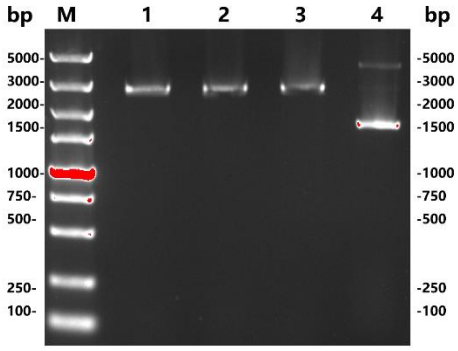


Figure . Electrophoresis result of the RFP plasmid digestion result | lane 1-3 are the digested RFP plasmid, while lane 4 is the RFP plasmid which is not digested.

4. Gel extract

5. Gel extract result Viscosity measurement

pcr + enzyme digestion result : 6.

enzyme digestion backbone 4.4ng/μl

last-time enzyme digestion backbone 2.

Date: 8.15

1. Plasmid extraction: sfGFP (Terminator) + RFP (backbone)

Viscosity measurement

2. PCR: terminator (200bp)

PCR system: 2x 50μl PrimerSTAR

template	3μ
Primers	1μ each
HS	0.5μl
Buffer	10μl
dNTP	4μ
ddH2O	31.5μl

NC 10μl

HS	0.1μl
Buffer	2μl
Primers	each 0.5μl
dNTP	0.8μ
ddH2O	5.1μ

PCR Procedure :

98℃		3min
	98℃	10s
30c	55℃	5s/15s
	72℃	15s
72℃		5min
4℃		hold

3. Agarose gel electrophoresis , gel extract

2% agarose gel

Sample sequence: φmarkerTer14 Ter10 Ter5 new5 old15 new15

(Ter14 is the newly extracted plasmid, Ter10 is the previous one, new5 represents the new primers anneal 5s)

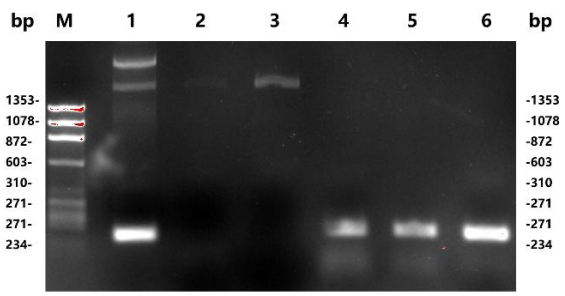


Figure . Electrophoresis result of the terminator PCR result | Lane 1 is the newly extracted plasmid PCR result, lane 2 is the previous one, lane 3 is the new plasmid annealed 5s, lane 4 is the new primers anneal 5s, lane5 is the old plasmid annealed 15s, lane 6 is the new plasmid annealed 15s.

Date: 8.17

1. Plasmid extraction, Viscosity measurement
2. Enzyme digestion backbone , terminator

enzyme digestion system 20μl

backbone	5μl	terminator	10μl
Green buffer	2μl		2μl
SpeI	1μl		1μl
PstI	1μl		1μl
ddH2O	11μ		6μl

enzyme digestion37℃backbone 30min terminator 60min

3. Ligation

Ligation system: 20μl

10x T4 buffer	2μl	
backbone	2μl	
Terminator	1μl	
T4 ligase	1μl	Takara
ddH2O	14μ	

16℃ hold, overnight

segment: 150bp 6.2ng 150/2700*112ng = 6.2ng

Backbone: 2700bp 112ng

Date: 8.18

1. PCR verifies positive colony

1-8

mRFP-F	mRFP-F	terminator-F
terminator-R	mRFP-R	terminator-R

2. Agarose gel electrophoresis

mRFP-F + terminator-R –> 1-8 all none

mRFP-F + mRFP-R ->1-8 all none

terminator-F + terminator-R -> 1-8

Date: 8.19

1. PCR sfGFP-terminator

50μl

Mix	25μl
Plasmid	1μ/0μl

Primer 2μ/1μ each
ddH2O 20μ/22μ/23μ
total 3 tubes

2. Agarose gel electrophoresis , gel extract
3. Enzyme digestion
4. Transform sgRNA-generator

Date: 8.20

1. Enzyme digestion sfGFP-terminator and T7-RFP Plasmid
20μl
Mix 2μ
Q.SpeI 1μ
Q.PstI 1μ
DNA 16μ
37℃ 1h
2. T7-RFP enzyme digestion result agarose gel electrophoresis , gel extract
3. Calculate ter-cut and RFP-cut 的 gel extract result viscosity
QDL2000, RFP-cut, ter-cut
14.18ng/ml,96ng/ml
4. RFP-cut Dephosphorylation
RFP-cut 17μ
Cutsmart Buffer 2μ
rSAP 1μ

37℃ 3min + 65℃ 5min
5. New primer pcr sfGFP backbone +terminator
Tm: ~57 49-57℃ grads
system: 10μl
Master Mix 5μ
Template 1μ
Primer1 0.5μ
Primer2 0.5μ
ddH2O 3μ
6. Ligation
20μl
Ligase buffer 2μ
Vector 10μ
Terminator 5μ
Ligase 1μ
ddH2O 2μ

16℃ overnight

Date: 8.21

1. Transform(RFP-terminator)ligation result
   10μl
2. PCR, T7-GFP-rrnB + T7-terminator
   PCR solution: 50μl
   mix(Mclab) 25μ
   Plasmid 3μ
   Primers 1μ each
   ddH2O 20μ

   NC(No template)

PCR procedure

98℃		3min
30c	98℃	5s
	52℃	25s
	72℃	1min10s
72℃		5min
4℃		hold

   PCR result: NC no lane

3. Gel extract
4. sgRNA generator added in the plates with IPTG, XGal and C+

Date: 8.22

1. ligation result colony pcr
   (NC has colony!)
   NC Pick 4: (1)mRFP-F+Ter-R (2)Ter-F+Ter-R
   Positive colony pick 8: (1)mRFP-F+Ter-R (2)Ter-F+Ter-R
   NC: mRFP-F+Ter-R Ter-F+Ter-R
   PC: Ter-F+Ter-R(sfGFP-terminator)
2. Agarose gel electrophoresis
   27 sample + 2 marker + φmarker -> 30 sample hole
   Result: choose No.7 and 8 to make further culture, PC has lane, NC(Ter) has lane, NC no lane
3. sgRNA annealing(primer-10μM)

sgRNA-F	5μ
sgRNA-R	5μ
10x T4 ligase buffer	2μ
ddH2O	8μ

4. DNA backbone linearize (sgRNA-generator)

BasI	0.2μ
DNA	10μ
10x buffer	2μ
ddH2O	7.8μ

5. sgRNA, sgRNA generator ligation(20ng/μl)

Backbone: 20ng-50ng(5μ)

sgRNA: 5:1 nuclear ratio over vector (dilute 170 multiple, use 1-2μ)

10x T4 ligase bubffer: 1μ

T4 ligase: 0.2μ

ddH2O:

6. PCR-mRFP(hm-pcr)

PCR: 50μl NC: 10μl

Mix	25μ
Plasmid	3μ
Primers	1μ each
ddH2O	20μ

PCR procedure

98℃		5min
	98℃	10s
30c	57℃	15s
	72℃	45s
72℃		5min
4℃		hold

7. agarose gel electrophoresis

Result: Lane all have correct length, but NC has lanes!!!

8. PCR T7-GFP-rrnB-T7-terminator

PCR: 50μl

PCR procedure

98℃		5min
	98℃	10s
30c	52℃	25s
	72℃	1min10s
72℃		5min
4℃		hold

9. agarose gel electrophoresis

Result: lanes are all correct and NC has no lanes.

10. PCR-lacZ-stop codon:

PCRsystem: 50μl		NC: 10μl
Plasmid	3μl	0μ
Primers	1μ each	0.5μ each
Mix	25μ	5μ
ddH2O	20μ	4μ

PCR procedure

98℃ 5min
98℃ 10s
30s 59℃ 15s
72℃ 30s
72℃ 5min
4℃ hold

Date: 8.23

1. Plasmid extraction

(1)mRFP-terminator ->Sequencing

(2)sgRNA-generator ->Viscosity Detection

2. enzyme digestion Stop codon-lacZ(insert) and pSB1C3 Plasmid(backbone)(EcoRI and SpeI)

system: 20μl

Green buffer	2μ
EcoRI	1μ
SpeI	1μ
Insert/backbone	14μ
ddH2O	2μ

37℃ 1h

3. agarose gel electrophoresis , gel extract

lane length: backbone 2000bp

Insert 450bp

4. Gel extract resultViscosity measurement

5. ligation between stop codon-lacZ and pSB1C3 (1:4 mol ratio)

system: 20μ

10x T4 buffer	2μ
Backbone	5μ
Insertion	4μ
T4 ligase	1μ
ddH2O	8μ

16℃ overnight

Date: 8.24

1. Transform ligation result into DH5α, on the plates added with IPTG,C+ and Xgal

Result: No colony.

2. Check the sequencing result of mRFP-terminator

Date: 8.26

1. sgRNA-primer annealing

sgRNA-F	5μ
sgRNA-R	5μ

10x T4 buffer 2μ
ddH2O 8μ

95℃ 5min, and stop heating, leaving it cooled to room temperature.

2. enzyme digestion(sgRNA-generator)

system: 20μ

BasI	0.2μ
DNA	10μ
10x buffer	2μ
ddH2O	7.8μ

3. ligation between sgRNA and sgRNA-generator

(First, dilute the sgRNA primer annealing result to 1/11.

system: 10μ

10x T4 buffer	1μ
Backbone	2μ
sgRNA-anneal	0.5μ
T4 ligase	1μ
ddH2O	5.5μ

16℃ overnight

4. Ligation between lacZ and pSB1C3 (Previous experiment failed).

system: 20μ

10x T4 buffer	2μ
Backbone	5μ
Insertion	4μ
T4 ligase	1μ
ddH2O	8μ

16℃ overnight

Date: 8.27

1. Transform the ligation result of last day.

Date: 8.28

1. Check the transform result.

Result: Only sgRNA3 and sgRNA4 succeed.

2. Culture in LB medium

sgRNA4	(1)(2)
sgRNA3	(1)(2)(3)

Date: 8.29

1. colony PCR

NC has colonies we should PCR these colonies altogether.

system:

Culture 1μ
Primers 0.5μ each
Mix 5μ
ddH2O 3μ

primers: sgRNA4 (sgRNA4-F,VR) sgRNA3 (sgRNA3F,VR)
NC (sgRNA4-F,VR) (sgRNA3F,VR)

Procedure:
98℃ 5min
98℃ 10s
30c 54℃ 15s
72℃ 20s
72℃ 5min
4℃ hold

2. agarose gel electrophoresis
Φmarker. Positive colony has lanes about 200bp.
3. culture in LB
4. lacZ-stop codon PCR

system: 50μl		NC	10μ
sgRNA generator	3μ		0μ
primers	1μ each		0.5μ each
mix (Mclab)	25μ		5μ
ddH2O	20μ		4μ

primers:
primer1 Tm 63.2
primer2 Tm 65.5

PCR procedure:
98℃ 5min
98℃ 10s
30c 59℃ 15s
72℃ 30s
72℃ 5min
4℃ hold

5. agarose gel electrophoresis, gel extract.
φ marker + DL2000
target lane: 450bp
sample sequence: DL2000 lacZ lacZ NC φmarker

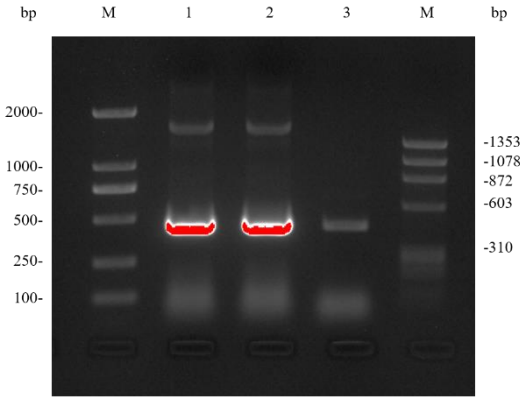


Figure. Electrophoresis result of gel extraction product.
From line 1 to line 3 are LacZ 1, LacZ 2, NC, respectively

Date: 8.30

1. Colony PCR

System:

bacterium solution(μL)	1
primers(μL)	0.5 for each
mix(μL)	5
ddH_2O(μL)	3

2. Gel electrophoresis of colony PCR product

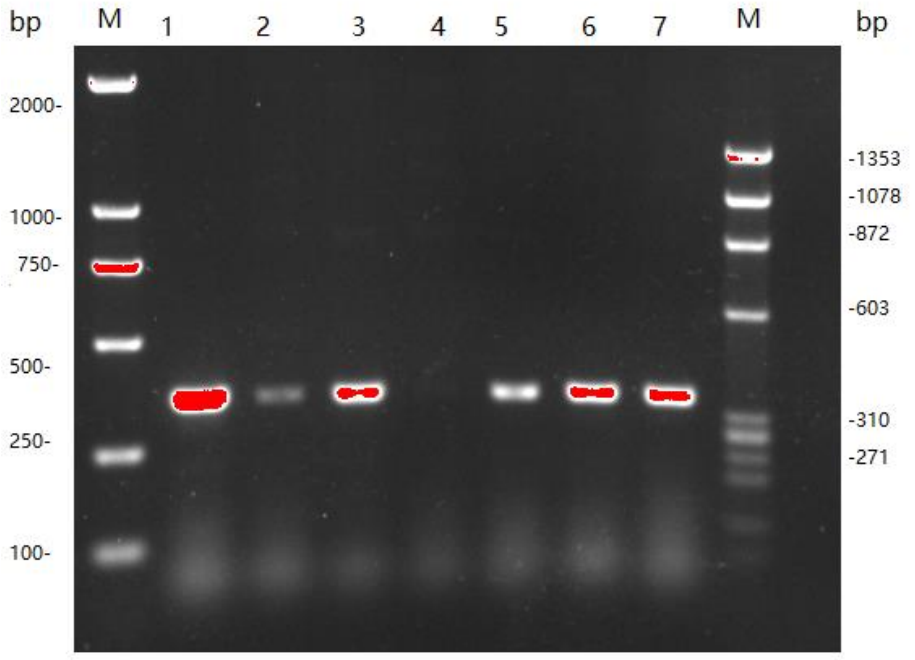


Figure. Electrophoresis result of the colony PCR product. Line1-7 are results of bacteria solution from different colonies. We get positive result in line 1, 2, 3, 5, 6, 7.

Date: 9.1

1. Plasmid PCR for verification

We use three primers:

sgRNA3-F + VR

sgRNA4-F + VR

VF2 + VR

2. agarose gel electrophoresis, gel extract.

Marker sg3-1 sg3-VF2 sg4-1 sg4-VF2 NC-sg3 NC-sg4 NC-VF2 Marker

(sg3-1 represents sgRNA3 sample with sgRNA3-F and VR primers)

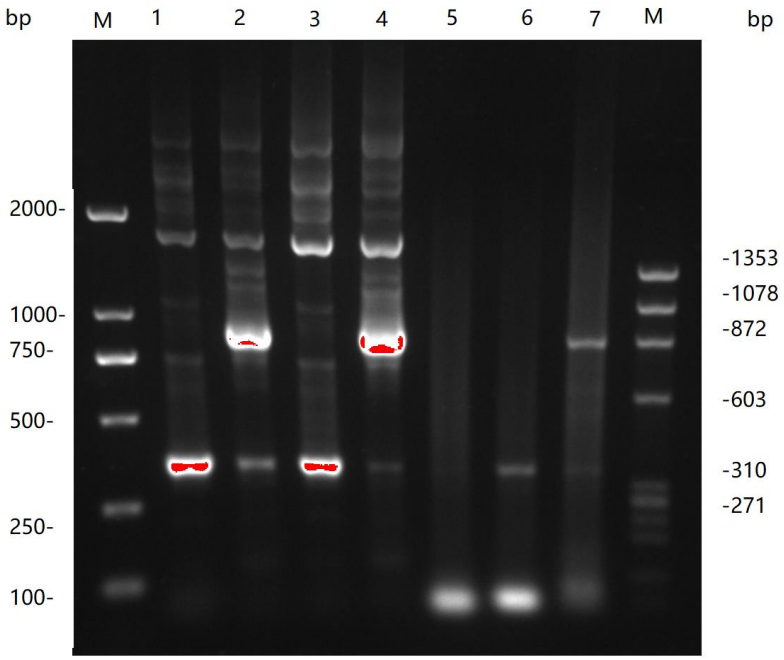


Figure. Electrophoresis result of the Plasmid PCR product. We use three primers: sgRNA3-F + VR, sgRNA4-F + VR and VF2 + VR. From Line 1-line 7 are sg3-1, sg3-VF2, sg4-1, sg4-VF2, NC-sg3, NC-sg4, NC-VF2.

Date: 9.4

1. Restriction enzyme digestion of sgRNA generator plasmid

Date: 9.8

1. Basic circuit1 arrangement

pTet-gRNA4 ligation

(1)pTet F/R PCR each other to form the whole segment with part of the gRNA4 sequence..

(2)Use primers to PCR backbone with part of the gRNA4 sequence.

(3)Gibson Assembly

2. Basic circuit2arrangement

T7-gRNA1-PamGFP change promoter to form pLac-gRNA1-PamGFP

(1)pLac primers annealing

(2)backbone PCR with additional BsaI cut site

(3)enzyme digestion backbone

(4)ligation between two segments.

3. pTet-F/R Primer-interact-PCR

system: 50μl

PrimerSTAR HS	1μl	1μ
Primers	1μ each	2μ each
dNTP	4μ	4μ
buffer	10μ	10μ
ddH2O	33μ	31μ

PCR procedure

98℃		3min
	98℃	10s
30c	60.2℃	5s
	72℃	15s
72℃		5min
4℃		hold

Date: 9.9

1. Plasmid extraction sgRNA Pam-GFP
2. Agarose gel electrophoresis

Marker Pam-GFP1 Pam-GFP2 Pam-GFP3 Pam-GFP4 Marker sgRNA1 sgRNA2 sgRNA3 sgRNA4

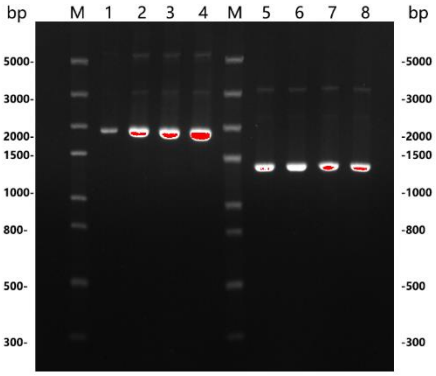


Figure . Electrophoresis result of pT7-PamGFP plasmid and sgRNA1-sgRNA4 plasmid | M represents the trans5k marker, lane 1-4 are pT7-PamGFP sample 1-4, while lane 5-8 are sgRNA1-sgRNA4 sample.

.

3. Viscosity measurement

PamGFP2	47.1ng/μl
Pam-GFP4	43.0ng/μl
sgRNA1	25.1ng/μl
sgRNA2	24.1 ng/μl

4. enzyme digestion

(1)Pam-GFP 20μl

XBal	1μ
PstI	1μ
10x buffer	2μ

DNA 16μ
(37℃ 1h)
(2)sgRNA 25μl
SpeI 1μ
PstI 1μ
10x buffer 2.5μ
DNA 20.5μ
(37℃ 1h)

5. Dephosphorylation
DNA 17μ
CutSmart buffer 2μ
rSAP 1μ
(37℃ 30min + 65℃ 5min)

6. ligation
system: 20μl
T4 DNA ligase 1μ
T4 buffer 2μ
Pam-GFP 8μ
gRNA1 9μ

Date: 9.10

1. Backbone: 79/5 = 15.8 ng/μl (2200) 50ng
2200/50 = 100/x => x = 50/2200 = 2.27 ng * 10 = 22.7ng (1:10)
pTet x 1: 502ng/μl Dilute 20 multiple
pTet x 2: 5.1.1ng/μl Dilute 20 multiple

Homologous recombination 20μl
4/9 mix (no T4 ligase) 15μ/tube
Backbone 3.5μl
pTet-insert 1μ
ddH2O 0.5μ
(50℃ 25min/20min)

2. Transform homologous recombination result

Date: 9.11

1. colony PCR using pTet forward primer and VR

System:

bacterium solution(μL)	1
primers(μL)	0.5 for each
mix(μL)	5
ddH_2O(μL)	3

Procedure:

Initial activation step	5 min	98°C
Denaturation	10 s	98°C

Annealing	15 s	54°C
Extension	20 s	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

2. Transform the PCR product into trans5α on C^{+} plates

Date: 9.12

1. PCR of gRNA generator backbone

System:

	Experiment group	Negative control
GXL(μL)	1	0.5
Primers(μL)	1 for each	0.5 for each
dNTP(μL)	4	0.8
Buffer(μL)	10	2
Plasmid(μL)	3	0
ddH_2O(μL)	30	5.7

Procedure

Initial activation step	3 min	98°C
Denaturation	10 s	98°C
Annealing	2 min	72°C
Extension	5 min	72℃
Number of cycles	30 cycles	72°C
End of PCR cycling	Indefinite	4°C

2. Primer-interact-PCR

System:

	Experiment group	Negative control
HS(μL)	1	0.5
Primers(μL)	1 for each	0
dNTP(μL)	4	0.8
Buffer(μL)	10	2
ddH_2O(μL)	33	6.7

Procedure

Initial activation step	3 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	60.2°C
Extension	15 s	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

3. Gel electrophoresis

1） measure the concentration of backbone PCR product, then perform gel extraction

2) measure the concentration of Primer-interact-PCR 产物,

4. Gibson Assembly

System：

2/3 Mix(μL)	15
Backbone(μL)	3.5
pTet(μL)	1
ddH_2O(μL)	0.5

Hold at 50℃ for 35 min

Date: 9.13

1. pTetPrimer-interact-PCR

System:

Mix(μL)	25
Primer(μL)	1 for each
ddH_2O(μL)	23

Procedure

Initial activation step	3 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	65°C
Extension	15 s	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

2. Gibson Assembly

First dilute the pTet primers 20 times with ddH_2O

2/3 Mix(μL)	15
Backbone(μL)	3.5
Diluted pTet(μL)	1
ddH_2O(μL)	0.5

Hold at 50℃ for 35 min

Date: 9.14

1. Restriction enzyme digestion

PAM-GFP

XBaI(μL)	1
PstI(μL)	1
Buffer	2
DNA	16

sgRNA1

speI(μL)	1
PstI(μL)	1
Buffer(μL)	2.5
DNA(μL)	20.5

Hold at 37℃ for 1 hour

2. Gel electrophoresis，胶回收

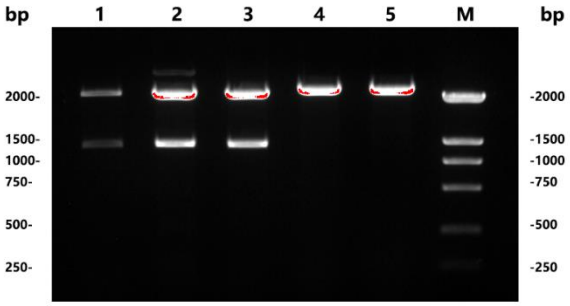


Figure . Electrophoresis result of the enzyme digestion of Pam-GFP plasmid using XBaII and PstI, and sgRNA1 generator plasmid using SpeI and PstI | lane 1-3 are the results of PamGFP digestion, while lane 4-5 are the results of sgRNA1 digestion.

3. Dephosphorylation

System:

dephosphorylation product	25
Cutsmart buffer(μL)	3
rSAP(μL)	1.5

Procedure:

37℃	30 min
65℃	5 min

4. Colony PCR of yesterday's ligation product

Select 4 colonies from each plate

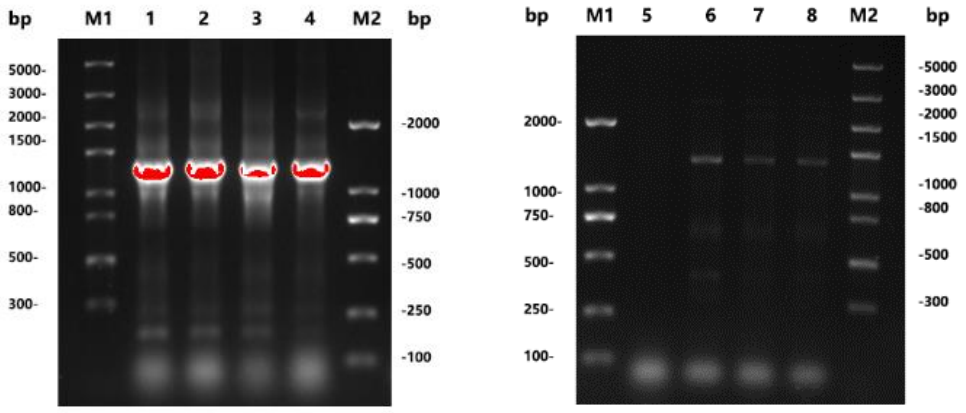
System:

bacterium solution(μL)	1
primers(μL)	0.5 for each
mix(μL)	5
ddH_2O(μL)	3

Procedure:

Initial activation step	5 min	98°C
Denaturation	10 s	98°C
Annealing	15 s	52°C
Extension	39 s	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

5. Gel electrophoresis of colony PCR product
Order of samples



(a) (b)

Figure . Electrophoresis result of colony PCR in the last step | **(a)** 4 colony in plate1 PCR with primer VF2 and VR. **(b)** 4 colony in plate 2 PCR with primer VF2 and VR.

6. Ligation

PAM-GFP(μL)	11
gRNA 1(μL)	6
10x T4 ligase buffer(μL)	2
T4 ligase(μL)	1

7. culture number 1, 2, 4 of colony PCR product

Date: 9.15
1. Colony PCR
Colony number 2 has the correct band

2. Gel electrophoresis of colony PCR product

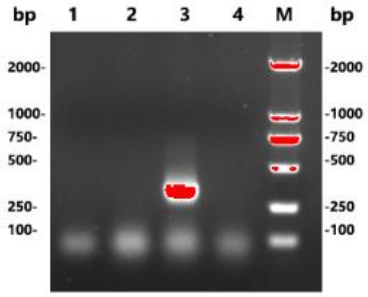


Figure . Electrophoresis result of colony PCR in the last step (pTet-gRNA4-pSB1C3) | lane 1 is NC, while lane 2-4 are the colony sample 1-4. From the result, we can see that sample 2 (lane 3) has target bond.

3. Culture colony 2 in a 50 mL EP tube with 5 mL LB added C+

4. Plasmid spin and sequencing

Date: 9.16

1. the result of sequencing has no signal

2. Activate glycerol stocks with T7-PAM-GFP plasmids

3. Plasmid spin and gel electrophoresis

There is a signal in correct length

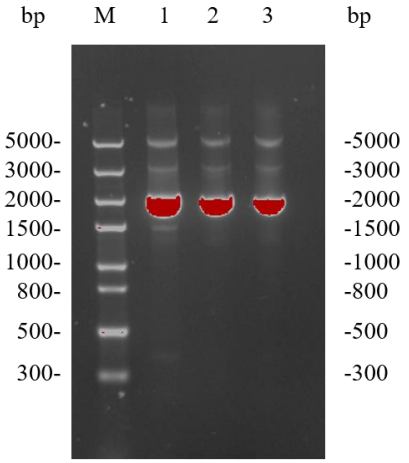


Figure. Electrophoresis result of plasmid spin product. From line 1 to line 3 are T7_pamGFP_gRNA1-plasmid 1, T7_pamGFP_gRNA1-plasmid 2, T7_pamGFP_gRNA1-plasmid 3, respectively

Date: 9.17

1. Extract T7-PAM-GFP plasmids and sequencing

2. PCR the backbone with pTet

System:

5x PrimeSTAR DXL buffer(μL)	1
Primers(μL)	1 for each
dNTP mixture(μL)	4
template(μL)	1
PrimeSTAR GXL DNA polymerase(μL)	1
ddH_2O(μL)	32

Procedure

Initial activation step	2 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	60/65°C
Extension	2 min 12s	68℃
Number of cycles	30 cycles	
Extension	5 min	68℃

End of PCR cycling	Indefinite	4°C

3. Gel electrophoresis of gRNA generator backbone with pTet added at the terminal.

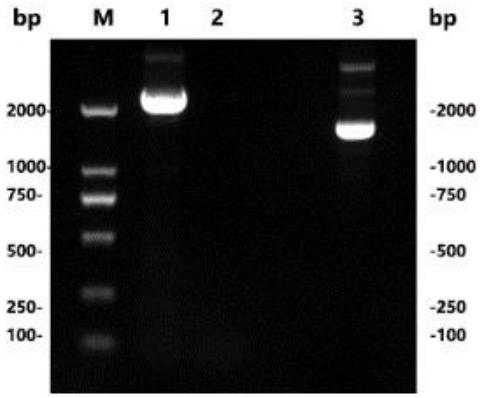


Figure . Electrophoresis result of gRNA generator PCR backbone with pTet sequence added at the terminal. | lane 1 is the PCR result, and lane 2 is PCR NC, while lane 3 is the plasmid which is the PCR template.

Date: 9.18

1. pTet primers-interact-PCR

System:

5x PrimeSTAR GXL buffer(μL)	10
Primers(μL)	1 for each
dNTP mixture(μL)	4
PrimeSTAR GXL DNA polymerase(μL)	1
ddH_2O(μL)	33

Procedure

Initial activation step	3 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	65°C
Extension	15 s	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

2. PCR of sgRNA generator backbone

System:

5x PrimeSTAR GXL buffer(μL)	10
Primers(μL)	1 for each
dNTP mixture(μL)	4
PrimeSTAR GXL DNA polymerase(μL)	1
Template	3
ddH_2O(μL)	30

Procedure

Initial activation step	3 min	98°C
Denaturation	10 s	98°C
Annealing	10 s	60°C
Extension	2 min	72℃
Number of cycles	30 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

3. Quantify the two PCR products and perform gel extraction on sgRNA generator's PCR product.

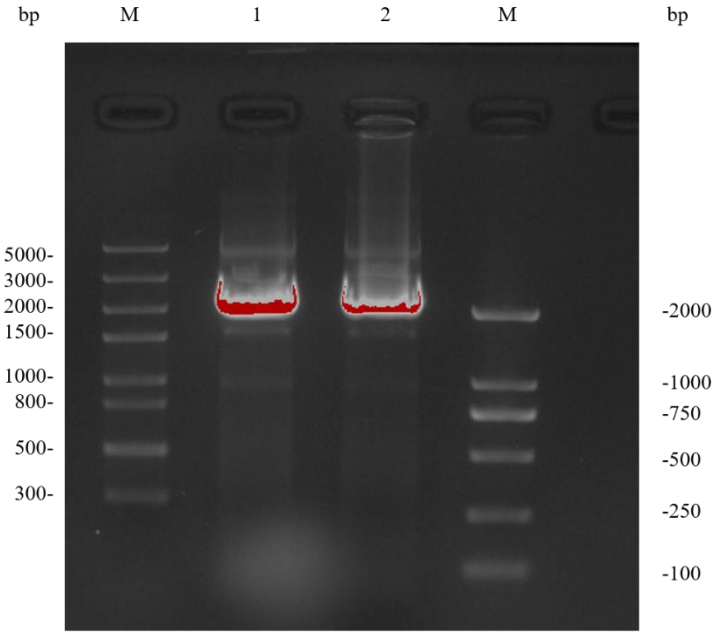


Figure. Electrophoresis result of gel extraction product. From line 1 to line 2 are gRNA generator backbone 1, gRNA generator backbone 2 respectively

5. Gibson Assembly

2/3 Mix(μL)	15
Backbone(μL)	3.5
Diluted pTet(μL)	1
ddH_2O(μL)	0.5

Hold at 50℃ for 35 min

Date: 9.20

1. Colony PCR for pTet-gRNA4 using vf2 and VR

System:

bacterium solution(μL)	1
primers(μL)	0.5 for each
mix(μL)	5

ddH_2O(μL)	3

2.Gel electrophoresis of colony PCR product

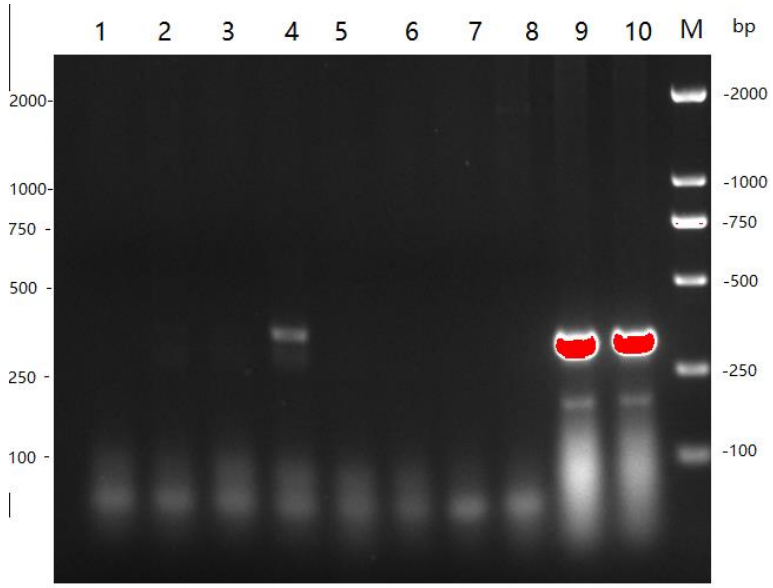


Figure. Electrophoresis result of the colony PCR product. Line1-10 are results of bacteria solution from different colonies. We get positive result in line 9 and line 10.

Date: 9.22

1. BL21(DE3) electro-competent cells preparation
2. Electro-transformation

Date: 9.27

1.PCR for BsaI-gRNA1-PAM-GFP-BsaI backbone

Sysem

	Experiment group	Negative control
GXL(μL)	1	0.5
Primers(μL)	1 for each	0.5 for each
dNTP(μL)	4	0.8
Buffer(μL)	10	2
Plasmid(μL)	3	0
ddH_2O(μL)	30	5.7

Procedure

Initial activation step	2 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	67°C
Extension	2 min 12s	72℃
Number of cycles	35 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

2. Gel electrophoresis and gel extraction of the PCR product

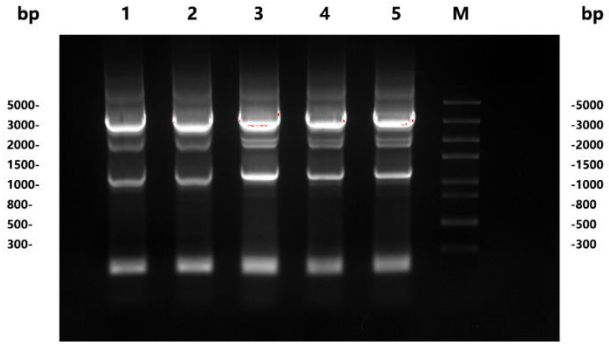


Figure . Electrophoresis result of BsaI-gRNA1-PAM-GFP-BsaI PCR backbone. | lane 1-5 is the PCR result. The bonds are about 3000bp.

3. Concentration determination of Gel extraction product

Result:26.46 ng/μL

4. Primer annealing for pLac fragment

System:

Primers(μL)	5 for each
10x T4 ligase buffer(μL)	2
ddH2O(μL)	8

5. Restriction enzyme digestion of backbone

System

BsaI(μL)	0.5
Backbone(μL)	15
10xBuffer(μL)	2.5
dd H2O	7
total(μL)	25

Procedure

1h30min	37°C

6. Adsorption column extraction of restriction enzyme digestion product

Date: 9.28

1. Ligation

Backbone(μL)	19.55
Plac fragment(μL) (200 times dilution)	0.45
10x T4 ligase buffer(μL)	2.5
T4 ligase(μL)	2.5

Procedure

5h	16°C

2. Transfer the ligation product into trans5α on C^{+} plates to culture at 37°C overnight

Date: 9.29

1. PCR for BsaI-gRNA1-PAM-GFP-BsaI backbone

Sysem

	Experiment group	Negative control
GXL(μL)	1	0.5
Primers(μL)	1 for each	0.5 for each
dNTP(μL)	4	0.8
Buffer(μL)	10	2
Plasmid(μL)	3	0
ddH_2O(μL)	30	5.7

Procedure

Initial activation step	2 min	98°C
Denaturation	10 s	98°C
Annealing	5 s	67°C
Extension	2 min 12s	72℃
Number of cycles	35 cycles	
Extension	5 min	72℃
End of PCR cycling	Indefinite	4°C

2. Gel extraction of the PCR product

3. Concentration determination of Gel extraction product

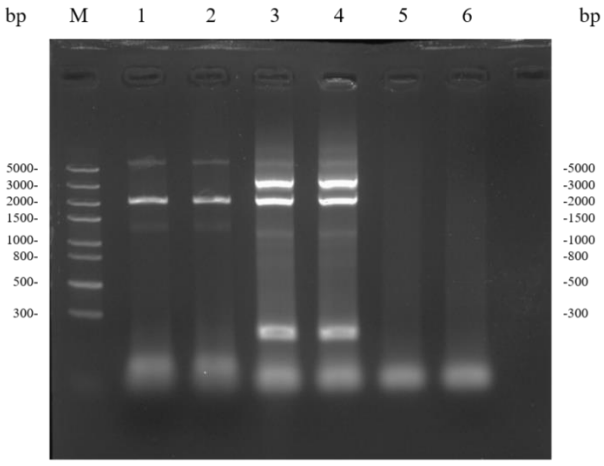


Figure. Electrophoresis result of gel extraction product. From line 1 to line 6 are T7-gRNA 1, T7-gRNA 1, PAM-GFP 1, PAM-GFP 2, NC 1, NC 2 respectively

4. Primer annealing for pLac fragment

System:

	Experiment group
Primers(μL)	5 for each
10x T4 ligase buffer(μL)	2
ddH2O(μL)	8

Date: 9.30

1. Colony PCR using vf2 and VR

System:

bacterium solution(μL)	1
primers(μL)	0.5 for each

mix(μL)	5
ddH_2O(μL)	3

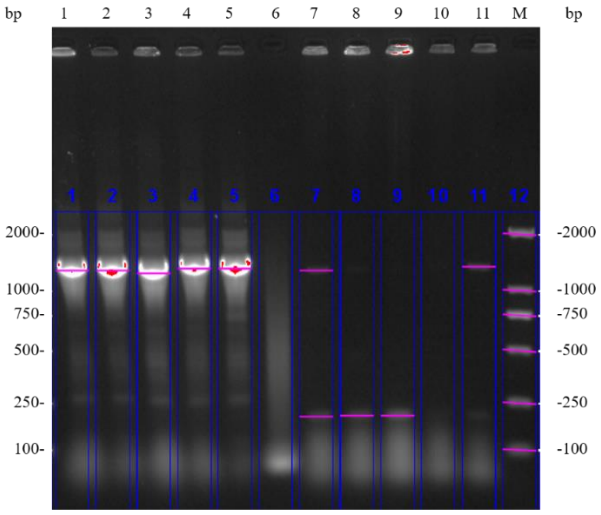


Figure. Electrophoresis result of colony PCR product.
From line 1 to line 11 are VR-VF2 1, VR-VF2 2, VR-VF2 3, VR-VF2 4, VR-VF2 5, NC, BBF-VR 1, BBF-VR 2, BBF-VR 3, BBF-VR 4, BBF-VR 5 respectively

2. Primer annealing for gRNA4 fragment

System:

Primers(μL)	2 for each
10x T4 ligase buffer(μL)	2
ddH2O(μL)	14

3. Restriction enzyme digestion of BsaI-gRNA1-PAM-GFP-BsaI backbone and sgRNA generator backbone

System

BsaI(μL)	0.5
sgRNA generator backbone(μL)	1.5
10xBuffer(μL)	2.5
ddH2O	15.5
total(μL)	25

BsaI(μL)	0.5
BsaI-gRNA1-PAM-GFP-BsaI backbone(μL)	15
10xBuffer(μL)	2.5
dd H2O	7
total(μL)	25

4. Adsorption column extraction of restriction enzyme digestion product

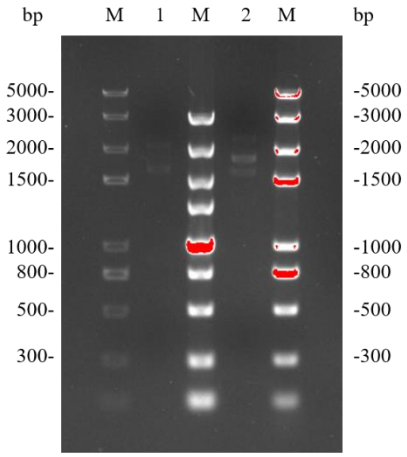


Figure. Electrophoresis result of enzyme digestion product
From line 1 to line 2 are Digestion Product 1, Digestion Product 2, NC, respectively

5. Ligation

sgRNA generator backbone(μL)	13
Plac fragment(μL) (200 times dilution)	1.7
10x T4 ligase buffer(μL)	2.5
T4 ligase(μL)	2.5
ddH2O(μL)	0.3

BsaI-gRNA1-PAM-GFP-BsaI backbone(μL)	13
Plac fragment(μL) (200 times dilution)	0.6
10x T4 ligase buffer(μL)	2.5
T4 ligase(μL)	2.5
ddH2O(μL)	1.4

Procedure

Overnight	16°C

Date: 10.5

1.Gel electrophoresis of plac-gRNA4-pam-GFP Plasmid.

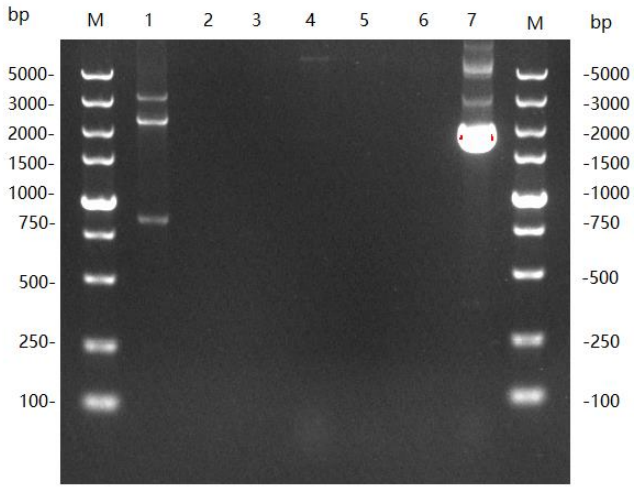


Figure.Electrophoresis result of plac-gRNA4-pam-GFP Plasmid | . Line2-6 are the results of our plasmid sample.The concentration of these sample are too low to be detected in Electrophoresis.

Date: 10.6

1. Colony PCR for plac-gRNA4 using vf2 and VR

System:

bacterium solution(μL)	1
primers(μL)	0.5 for each
mix(μL)	5
ddH_2O(μL)	3

3. Gel electrophoresis of colony PCR product

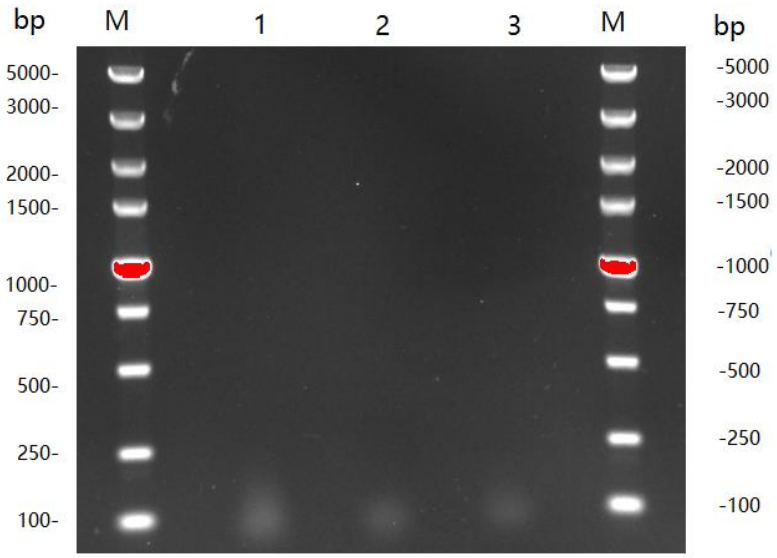


Figure. Electrophoresis result of the colony PCR product. | Line1-2 are experiment group (colony), while line 3 is a NC.