Molecular Experiment Record

SYSU-China 2019

2019.5.18

Streak E.coli containing pL7Ae and pL7AeMUT onto the LB plate. After 14 hours, pick a single colony and patch it to a new plate.

2019.5.19

1. Colony PCR

Primers: L7Ae-F-KpnI and L7Ae-R-BamHI primers

Enzyme: Taq

2. Detection by agarose gel electrophoresis: negative.

Analysis: Primer design error, the restriction enzyme cutting site is in the 3' end of the primer

2019.5.26

1. Cultivate the bacteria to be ready for extracting pL7Ae and pL7AeMUT:

Escherichia coli containing pL7Ae and pL7AeMUT was inoculated in 3L Amp+ LB medium at 37 °C overnight.

2. Screen positive bacteria:

Streak E.coli containing pL7Ae and pL7AeMUT on the LB plate. After 14 hours, pick a single colony and patch it to a new plate. After 12 hours of incubation, the patch plate was stored at 4 ° C for detection.

2019.5.27

1. Extraction of plasmid: Plasmid pL7Ae and pL7AeMUT were extracted using Omega E.Z.N.A Plasmid Mini Kit.

2. DNA concentration determination: L7Ae (69.1 ng / μL) ; L7AeMUT (54.3 ng / μL)

2019.5.31

1. PCR amplification:

L7Ae and L7AeMUT were amplified by PCR using Phata enzyme and BamHI/XhoI enzyme cutting site and Myc tag were introduced. The primers are as follows:

L7Ae-Fw-BamHI: 5'-AAGGATCCATCATATGCGGCCGCTTATGTACGTGAGATTTGAGG-3’

L7Ae-Re-Myc-XhoI:5'-CTCGAGTTAATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCGAAGGGCCCTCTAGACTCCTTCTGAAGGCCTTTAATCTT-3’

2. Identification by agarose gel electrophoresis: the amount of product should be 440 bp, but the band is too wide, so consider increasing the annealing temperature and re-PCR

2019.6.1

1. PCR amplification under improved conditions: Amplify L7Ae and L7AeMUT using the above primers, and increase the annealing temperature to 58 °C for higher amplification specificity.

2. Agarose gel electrophoresis: higher band specificity was shown.

3. Gel extraction:

The target product was extracted with omega Gel Extraction Kit to obtain L7Ae-Myc and L7AeMUT-Myc DNA fragments. Store at 4 ° C.

2019.6.2

1.DNA concentration detection: UV spectrophotometer detects the concentration of extracted L7Ae-Myc and L7AeMUT-Myc fragments:

L7Ae-Myc: 169 ng/μL; L7AeMUT-Myc: 116 ng/μL

2. Digestion: double enzyme digestion of pcDNA3.1, L7Ae-Myc, L7AeMUT-Myc. The systems are as follows (unit μL):

pcDNA3.1	10	Gene	10
BamHI-HF	1	BamHI-HF	1
XhoI	1	XhoI	1
Cutsmart	5	Cutsmart	5
ddH2O	37	ddH2O	37
Total	50	Total	50

React for two hours at room temperature. Heat in a 70 °C water bath for 10 min to inactivate enzyme.

2. Separation of the digested product by agarose gel electrophoresis:

Only the pcDNA3.1 product was seen, and no L7Ae-Myc and L7AeMUT-Myc fragments were found, which were suspected to be lost during the gel recovery process.

3. Re-PCR amplification

The L7Ae and L7AeMUT were re-amplified using primers L7Ae-Fw-BamHI and L7Ae-Re-Myc-XhoI, and the annealing temperature was set to 57 ° C to increase the amplification specificity. The system is as follows (unit μL):

Gene	1
Fw Primer	2
Re Primer	2
Phata	25
ddH2O	20
Total	50

4. Detection of recovered product by agarose gel electrophoresis

Products: L7Ae-Myc and L7AeMUT-Myc both showed the target band

5. Gel extraction:

The target product was extracted with omega Gel Extraction Kit to obtain L7Ae-Myc (GSF) and L7AeMUT-Myc (GSF) DNA fragments, which were stored at 4 ° C.

6. DNA concentration detection: UV spectrophotometer detects the extracted L7Ae-Myc (GSF) and L7AeMUT-Myc (GSF) fragments:

L7Ae-Myc (GSF): 160 ng/μL; L7AeMUT-Myc (GSF): 110 ng/μL

7. Colony PCR

Use the 5.26 preserved patch plate, pick L7Ae and L7AeMUT positive for colony PCR amplification, using primers:

L7Ae-Text-Fw: ATGTACGTGAGATTTGAGGTTC

L7Ae-Text-Re: TTACTTCTGAAGGCCTTTAATCTT

8. Agarose gel electrophoresis test:

All positive

9. Amplify positive clones to be ready for preservation

L7Ae and L7AeMUT positive clones were inoculated to 5 mL of Amp+ (1000x) LB liquid medium, and shaken at 37 ° C overnight.

2019.6.3

1. Conservation of DH5α-L7Ae and DH5α-L7AeMUT

2 tubes of DH5α-L7Ae and DH5α-L7AeMUT were stored in 15% glycerol and placed at -80 °C

2. The overnight digestion product was collected and inactivated in a water bath at 70 ° C for 10 min. Only the pcDNA3.0 band could be seen. No gene fragment band.

3. Improve digestion condition

According to NEB protocol, the enzyme should be digested for 5-15 minutes. Considering the lower enzyme digestion efficiency of XhoI, the enzyme digestion was carried out in the same system for 25 minutes at room temperature and 5 minutes at 65 °C.

4. Column extract product

Directly extract the digested products L7Ae-Myc-BamHI-XhoI and L7AeMUT-Myc-BamHI-XhoI (undetermined concentration) using Omega Gel Extraction Kit

5. T4 ligase connection

The T4 ligase was used to link the gene to the vector. Since the concentration was not determined, the following system did not conform to the gene: vector >3:1 recommendation.

The system is as follows (unit μL):

ddH_2O	9
gene-BamHI-XhoI	5
pcDNA3.1-BamHI-XhoI	3
10x T4 ligase Buffer	2
T4 ligase	1
Total	20

After 1 h incubation at room temperature, the reaction was terminated by inactivation at 65 ° C for 5 min. Product stored at 4 ° C

2019.6.4

1. Column extract product

Extract the enzymatically linked products pcDNA3.1-L7Ae-Myc-BamHI-XhoI and

pcDNA3.1-L7AeMUT-Myc using Omega Gel Extraction Kit

2. Escherichia coli DH5α plasmid transformation

Two tubes of DH5α competent were taken and the ligated plasmids pcDNA3.1-L7Ae-Myc-BamHI-XhoI and pcDNA3.1-L7AeMUT-Myc were transformed, respectively. The system is as follows:

DH5α competent state	50μL
Target plasmid	5μL

Mix and incubate for 30 min on ice. Heat shock at 42 °C for 70 s.

After adding 1L LB medium, resuscitate at 37 ° C for 1 h

Centrifuge at 10000xg for 2min, discard the supernatant, add 200ml and resuspend

The suspension was coated with Amp+ resistant LB plate and cultured at 37 °C.

2019.6.5

No positive colonies after E. coli transformation

2019.6.8

1. Improved enzyme digestion conditions

Re-cut and adjust the digestion time to 15 min.

Reset the enzyme system and increase the amount of carrier added to 5μl.

37 ° C enzyme ligation for 1 h.

2. Improved transformation conditions

The enzyme-linked product was directly added to the DH5α competent state without column purification, and the enzyme-linked product was added to 10 μl.

Ice incubation time extended to 1h.

Add ampicillin to LB liquid medium during resuscitation.

2019.6.9

1. 4 plates produce positive

Analysis: It can be seen that the previous unsuccessful conversion problem is not in the plasmid, but in the conversion process and conditional issues.

Patch positives expanded for 5h.

2. Identification of positives by primer L7Ae-Text-Fw and L7Ae-Text-Re colony PCR

The test was fully positive and the plasmid was successfully constructed.

Inoculate and overnight shaking.

2019.6.10

1. Endotoxin-free extract plasmid endo-pcDNA3.1-L7AeMUT and endo-pcDNA3.1-L7Ae

2. Mr. He Lei provided E.coli containing the pcDNA-EGFP plasmid. Inoculate it in LB to prepare for extracting plasmids.

2019.6.14

1. DNA concentration detection

UV spectrophotometer was used to detect endo-pcDNA3.1-L7AeMUT and endo-pcDNA3.1-L7Ae

concentrations:

endo-pcDNA3.1-L7AeMUT: 264.35 ng/μL; endo-pcDNA3.1-L7Ae: 209.35 ng/μL

2019.6.24

1. Plasmid extraction

pTK295, pEF1α_reTA3, pTRE_EGFP2, pAdeasy: extract plasmid (endotoxin-free)

pShuttle, pAdTrack, pAVV-Ubc, LSBr5and3: extract plasmid (not endotoxin-free)

2019.6.29

1. Gel extraction of 1xKt-EGFP and 2xKt-EGFP

2. UV spectrophotometer to measure concentration

1xKt-EGFP: 43.6 ng/uL ; 2xKt-EGFP: 64.3ng/uL

3. Inoculate DH5α-pcDNA3.1 in LB medium. Shake overnight.

2019.6.30

1. Extract plasmid pcDNA3.1

Extract the plasmid pcDNA3.1 from the endotoxin-free plasmid extract kit and measure the concentration by UV spectrophotometer.

2. Double enzyme digestion (unit μL):

Gene	10
BamHI-HF	1
XbaI	1
Cutsmart	2
ddH_2O	6
Total	20

37 ° C, 25 min incubation. 65 ° C water bath 10 min to inactivate the enzyme.

3. Separation of the digested product by agarose gel electrophoresis:

1xKt-EGFP, 2xKt-EGFP, and pcDNA3.1 target band was seen. Gel extraction to separate the digested product.

4. Measuring concentration:

The concentration of 1xKt-EGFP, 2xKt-EGFP, and pcDNA3.1 was measured by an ultraviolet spectrophotometer.

5. Enzyme ligation (unit μL):

Gene	10
Plasmid	2
T4 ligase	1
2x T4 buffer	2
ddH2O	5
Total	20

Incubated at 37 ° C for 1 h.

6. Transformation of DH5α

7.1

1. Inoculate DH5α-pcDNA3.1-1xKt-EGFP, DH5α-pcDNA3.12xKt-EGFP each for two tubes, and

shake at 37 ° C for 1d.

7.2

1. Preservation pf DH5α-pcDNA3.1-1xKt-EGFP, DH5α-pcDNA3.12xKt-EGFP

DH5α-pcDNA3.1-1xKt-EGFP, DH5α-pcDNA3.12xKt-EGFP was stored at -80 °C

2. Plasmid extraction

pcDNA3.1-1xKt-EGFP and pcDNA3.12xKt-EGFP, each for one tube.

7.13

Configure 40ml doxycycline (100μg/mg)

Enzyme digestion: double digestion of pcDNA3.1, rtTA3, rtTA3-HA

Plasmid system (μl):	
pcDNA3.1	6
BamHI-HF	1
XbaI	1
Cutsmart	5
ddH_2O	37
Total	50
Gene fragment system (μl):	
pcDNA3.1	10
BamHI-HF	1
XbaI	1
Cutsmart	5
ddH_2O	33
Total	50

(incubated at 37 ° C for 15 min, heated in a 70 ° C water bath for 10 min to inactivate enzyme activity)

7.17

1. Detection of products by agarose gel electrophoresis

Electrophoresis result shows that the digested products are in the correct position.

2. Recovery of the digested product by adsorption column

Recover the digested products BamHI-rtTA3-HA-XbaI, BamHI-rtTA3-XbaI and pcDNA3.1 products (undetermined concentration) using Omega Gel Extraction Kit.

7.19-7.30

Construct pTRE-L7Ae, pTRE-L7AeMUT, pTRE-2kt-egf, pcDNA-rtta3 and pcDNA-rtTa-HA

Electrophoresis verification was performed after chemical transformation, the results were all false positive.

7.31

1. The above 5 plasmids were retransformed.

2. Customize miRNA mimics and inhibitor.

8.1-8.4

Only pTRE-2Kt-EGFP, pcDNA-rtTA3, pcDNA-rtTA3-HA had positive colonies

Only pcDNA-rtTA3-HA could show target band in colony PCR.
pTRE-EBFP2 is cultured in shaking tube for extraction of plasmid DNA

8.5
1. Enzyme digestion: pTRE, L7Ae, 2Kt-EGFP.
2. Ligate overnight.

8.7
Extraction of plasmid DNA and enzyme digestion.

8.9
1. The DNA sequencing result of pcDNA3.1-rtTA3-HA is negative.
2. Enzyme digestion and ligation of pcDNA-rtTA3, pcDNA-rtTA-HA are performed again, stored at 4℃ for transformation tomorrow
3. pTRE-EBFP and hEF1α-rtTA3 are cultured in shaking tube. Plan to extract plasmid with endo-free kit tomorrow

8.10
1. Chemical transform DH5α with pcDNA-rtTA3, pcDNA-rtTA3-HA separately, coat plate and culture at 37℃
2. Prepare Dox solution 0.1mg/ml, 1mg/ml 40ml each, refrigerate and store
3. Preserve+ Extraction of plasmid (endo-free): hEF1α-rtTA, pTRE-EBFP
4. Cultivation of hEF1α-rtTA failed so no plasmid is extracted
5. The concentration of pTRE-EBFP is 18ng/μl

8.11
1. hEF1α-rtTA (Ampicillin) is cultured shakily with 5ml LB
2. Discard the hEF1α-rtTA preserved yesterday

8.12
1. Preserve hEF1α-rtTA
2. hEF1α-rtTA is cultured shakily with 5.5ml LB
3. Culture pcDNA-rtTA3 in shaking tube
4. Cultivate pcDNA-rtTA3 by streaking
5. Culture pTRE-EBFP with 4ml LB

8.13
1. Extraction of plasmid (endo-free): hEF1α-rtTA, pTRE-EBFP
2. Cultivate pcDNA-rtTA3 by streaking
3. PCR

Reaction system (ul):

pTRE:

pTRE-EBFP	1
pTRE_Vector_PrimerF	2
pTRE_Vector_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

L7Ae:

pL7Ae	1
L7Ae_Insert_PrimerF	2
L7Ae_Insert_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

2Kt EGFP:

pTX295-Kt-EGFP	1
2Kt EGFP_Insert_F	2
2Kt EGFP_Insert_R	2
Phanta	25
ddH_2O	20
Total	50

Extension: 3min10s

4. Verify by 1% agarose electrophoresis

All are positive

5. Gibson assembly

L7Ae:

L7Ae	20 ng
pTRE	127ng
5x CE II Buffer	4 μl
Exnase II	2 μl
ddH_2O	to 20 μl

2KtEGFP:

2KtEGFP	33ng
pTRE	127ng
5x CE II Buffer	4 μl
Exnase II	2 μl
ddH_2O	to 20 μl

React at 37℃ for 30min

6. Chemical transform DH5α

pTRE-L7Ae and pTRE-2Kt-EGFP are transferred to DH5α, and plate with Amp. are applied

8.15

Colony PCR: pcDNA-rtTA3, pTRE-2Kt-EGFP, pTRE-L7Ae

The result is that almost all of them succeed except L7Ae

8.15

1.PCR (unit: μ l)

pEF1α_rtTA3:	
pEF1α_rtTA3	1
rtTA3 PrimerF	2
rtTA3 PrimerR	2
Phanta	25
ddH_2O	20
Total	50

pcDNA:	
pcDNA	1
pcDNA PrimerF	2
pcDNA PrimerR	2
Phanta	25
ddH_2O	20
Total	50

2. Verify by 1% agarose electrophoresis

Both are positive

3. Ligate and purify with the kit

The concentration are low. Plan to try again tomorrow with higher use level

4. Gibson assembly

Reaction system:

L7Ae 20 ng（1.75μ l）

pTRE 127ng（4μ l）

5x CE II Buffer 4 μ l

Exnase II 2 μ l

ddH_2O to 20 μ l

5. Chemical transform

Transfer pTRE-L7Ae to DH5α, coating plate with Amp.

8.16

1. Preservation

pcDNA-rtTA3, pTRE-2kt-EGFP

2. Extraction of plasmid

pcDNA-rtTA3, pTRE-2kt-EGFP

Extraction of pcDNA-rtTA3 failed, need to be cultured again

3. Colony PCR

pTRE-L7Ae

4. Cultivation in shaking tube

The result of Colony PCR is negative. Recultivate pcDNA-rtTA3

5. Construct plasmid

1) PCR (μ l)

pTRE:

pTRE-EBFP	1
pTRE_Vector_PrimerF	2
pTRE_Vector_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

L7Ae

pL7Ae	1
L7Ae_Insert_PrimerF	2
L7Ae_Insert_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

2) Verify by 1% agarose electrophoresis

Both are positive

3) Enzyme digested by Dnp I and purified using kit

pTRE: 127.12ng/ul, L7Ae: 9.92ng/ul

4) Ligation

	Positive Control	Experimental Group	Negative Control 1	Negative Control 2
Positive	4			
L7Ae		6.7		
pTRE		3.3		3.3
GenBuilder…Master Mix	6	10	10	10
ddH2O			10	6.7
Total	20			

5) Chemical transform DH5 α

8.20

Recombine pcDNA3.1-rtTA3 by Gibbson assembly

20μ l system:

pcDNA	2 ul
diluted 4 times rtta3	1ul
Mastermix	10 ul
ddH2O	7 ul

or

pcDNA	2 ul
diluted 4 times rtta3	1ul
cebuffer	4 ul
enzyme	2 ul
ddH2O	11 ul

The recombinant plasmid are transferred into DH5α, coated with Amp, separately.

8.22

1. Colony PCR

8 colonies are selected from 2 plates and 20ul system is used.

2. Verify by agarose electrophoresis

The voltage is low and consume a long time. Marker strip was blurred and there was no strip in the swimming lane of the experimental group, or even no primer dimer. New colony should be selected for PCR again.

8.28

1. Construct plasmid

1) PCR (μ l)

rtTA3:

pEF1α-rtTA3	1
rtTA3-Insert-PrimerF	2
rtTA3-Insert-PrimerR	2
Phanta	25
ddH_2O	20
Total	50

pcDNA:

pcDNA 3.1	1
pcDNA-Vecter-PrimerF	2
pcDNA-Vecter-PrimerR	2
Phanta	25
ddH_2O	20
Total	50

Extension: 3min

2) Verify by 1% agarose electrophoresis

Both are positive but the band of pcDNA is shallow

3) Concentration

pcDNA 341ng/μl

rtTA3 291ng/μl

4) Ligation

Reaction system:

pcDNA	0.3μl
rtTA3	0.2μl
5x CE II Buffer	4 μ l
Exnase II	2 μ l
ddH_2O	up to 20μl

5) Chemical transform DH5α, cultured in shaking tube, coated with Amp. Plate.

2. Extraction of plasmid DNA

Successfully cultured BJ5183 AdEasy-pTrack with shaking tube. Extract plasmid DNA (endo-free).

The concentration of it is 317.9ng/ul.

8.30

1. DH5α-pcDNA-rtTA3 is overgrown with satellite colonies. Select 2 colonies and culture on new plates by streaking. The former plate is sealed up and store at 4℃.

8.31

1.PCR

Reaction system (ul):

pTRE:

pTRE-EBFP	1
pTRE_Vector_PrimerF	2
pTRE_Vector_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

L7Ae

pL7Ae	1
L7Ae_Insert_PrimerF	2
L7Ae_Insert_PrimerR	2
Phanta	25
ddH_2O	20
Total	50

2. Enzyme digestion with Dpn I for 1h without purification

(subsequent experiments are performed, but no results were obtained due to the lack of purification. Meanwhile, it is suspected that the mutation generated in the last sequencing was caused by the lack of purification.)

9.2

1. Colony PCR

Reaction system:

Bacteria	1μl
L7Ae-insert-Forward	1μl
L7Ae-insert-Reverse	1μl
Taq	10μl
ddH_2O	7μl
Total	20μl

Verify by agarose electrophoresis and all are positive

2. Cultivation in shaking tube

5ml LB+5μl Amp., 2 tubes and culture overnight

3. Gibbson Assembly

	Experiment group	NEG1	NEG2	POS1
L7Ae-insert	1.2μl	1.2uL	0	1uL
1pTRE-linearized	1.5uL	0	1.5 uL	1uL
5x CE II buffer	4uL	0	0	4uL
Exnase II	2uL	0	0	2uL
ddH2O	to 20uL	to 20uL	to 20uL	to 20uL

After reaction at 37℃ for 30min, ice bath was performed, and DH5α was transformed by heat shock (resting on ice for 1h). 4 Amp. plates are applied. Culture at 37℃

9.4

1. Colony PCR

1) pTRE-L7Ae-miR592

The result is positive and culture shakily with 2 tubes

2) pTRE-L7Ae

The result is positive and culture shakily with 3 tubes

9.5

1. Send 2 tubes of DH5α-pTRE-L7Ae and 1 tube of DH5α-pTRE-L7Ae-miR592 for gene sequencing

9.9

The results of gene sequencing are negative. It is suspected that the bacterial liquid is too thin to extract the plasmid.

9.10

1.Gibson assembly pTRE-L7Ae-miR

2.2% agarose purification E1A，gel extracted in about 900bp.

3.PCR amplification E1B55K.

phanta	25μl
E1B55K-R	1μl
E1B55K-F	1μl
HEK293 cDNA	1μl
ddH_20	22μl
Total	50μl

4. DH5α-L7Ae-miR592 was cultivated in Amp medium andput in 37℃ shaker.

9.11

1.Gibson assembly:pTRE-L7Ae-miR

Conditions:

miR592	10ng
L7Ae-miR	14ng
pTRE	100ng

2.PCR

pcDNA、rtTA3

template：pcDNA3.1（7.18）hEF1α-rtTA3（8.14）
3.Enzyme digested template、product purification.
4.Gibson assembly pcDNA-rtTA3
Conditions:
rtTA3 28ng
pcDNA 107ng

9.22
L7Ae-miR colony PCR: positive. Cultivate bacteria 1-5 in 37 ℃ shaker.

9.23
1.Plasmid extraction
pcDNA-rtTA3、pTRE-2kt-EGFP each for one tube (endotoxin-free).

9.25
1.Cultivate bacteria pTRE-L7Ae-miR for 5 tubes
Resuspend the bacteria, pick 10ul every tube for sequencing. Remain is cultivated in 5mlLB+5ulAmp, and put in shaker about 10:30.
2.Cultivate glycerin bacteria for 1 tube (miR663b+885)
Pick 10ul bacteria, use 5mlLB+5ulAmp to cultivate in shaker，put in shaker in 11:37. Remain is put in -80° C.

9.26
1.Plastimd extraction
5 tube pTRE-L7Ae-miR; 1 tube miR663B+885

9.27
1.Gibson assembly
Conditions: Amplified fragments; phanta system 50ul; 32x cycle.
template：2kt-eGFP; mir663; mir885
DpnI enzymolysis for 1h

9.28
1.Gibson assembly: pTRE-miR663b-2Kt-miR885-5P
The system is as follows:
2KtEGFP miR inserted fragment 16ng
miR 663b inserted fragment 10ng
miR 885 inserted fragment 10ng
pTRE vector 127ng
5 x CE II Buffer 4μl
Exnase II 2μl
ddH_2O to 20 μl
put in medium and cultivate in 37℃.
2. pTRE-L7Ae extraction

The concentration is too low.

10.1

1. PCR amplification: miR663, miR885-5p

The system is as follows:

template	2uL
F	2uL
R	2uL
phanta	25uL
dd H_2O	19uL
Total	50uL

2. Gibson assembly : pTRE-miR663b-2Kt-miR885-5P

3. Chemical transformation DH5α

The system is as follows:

Linked product	10uL
DH5α	50uL

42°C 45s heat-shock, put on ice 2-3min.

Add 900uL no resistant LB and cultivate at 37°C in shaker for 1h

10.3

Cultivate 5 tubes bacteria solution in shaker.

10.4

1. plasmid extraction: pTRE-miR663b-2kt-miR885

10.12

1. plasmid extraction: pTRE-miR663b-kt-miR885 tube
   plasmid extraction: pTRE-L7Ae-miR 4g tube
2. plasmid above sent to sequence
3. pTRE-E1B-55k cultivate in Amp medium.

10.14

1. Cultivate bacteria solution in shaker

pTRE-E1B-55k No.2.4.5 temporarily conserved bacteria were cultivated in shaker in 5mlLB+5ulAmp.

10.15

1. Cultivate bacteria solution in shaker

-80° C conserved pcDNA-L7Ae、pcDNA-2kt-EGFP each 50ul were cultivated in 5mlLB+5ulAmp in shaker.

10.16

1. Gibson Assembly

pTRE-miR663b-2kt-EGFP-miR885

pTRE-L7Ae-miR592
pTRE-E1A 9.9
pTRE-E1A 10.8
pTRE-E1B 55K
2. Chemical transformation.
pTRE-miR663b-2kt-EGFP-miR885（old）
pTRE-miR663b-2kt-EGFP-miR885
pTRE-L7Ae-miR592
pTRE-E1A 9.9
pTRE-E1A 10.8
pTRE-E1A（old）
pTRE-E1B 55K
3. Linearized vector pTRE
Because the pTRE-E1B 55K which was sent to sequence before has found EBFP fragment in it, which shows that the linearized vector pTRE template was not degraded completely, so we linearized again. After PCR, add 2μl DpnI enzyme. 37℃ react for 1h.
4. Extract endotoxin-free plasmids
pcDNA-L7Ae、pcDNA-2kt-EGFP

10.16
1. Colony PCR
pTRE-miR663b-2kt-EGFP-miR885（old）
pTRE-miR663b-2kt-EGFP-miR885
pTRE-L7Ae-miR592
pTRE-E1A 9.9
pTRE-E1A 10.8
pTRE-E1A（old）
pTRE-E1B 55K
pcDNA-E1A 9.9
pcDNA-E1A 10.8
2. Gibson Assembly
pTRE-miR663b-2kt-EGFP-miR885
pTRE-L7Ae-miR592
pTRE-E1A 9.9
pTRE-E1A 10.8
pTRE-E1B 55K