7.5

1.preparation getting started

Preparing LB broth and LB medium

Preparing antibiotics (streptomycin-50mg/ml, ampicillin-50mg/ml)

2.Overnight culture

Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing either pGEX or pCDFDuet-1 plasmid (3×replicates of each transformation)

Incubate at 37℃ with shaking (250rpm) overnight.

7.6

1.Plasmid Isolation

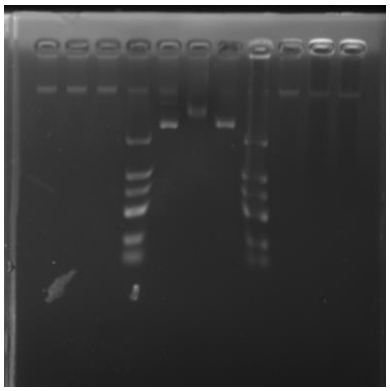
An overnight starter culture of E. coli DH5α harboring plasmid pGEX or pCDFDuet-1 was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

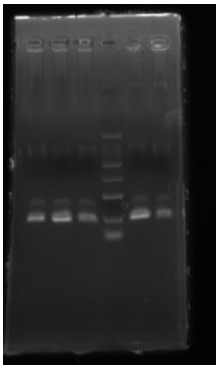
name	concentration (ng/ul)
pGEX	76.5
pCDFDuet-1	104.4

2.PCR amplification of cas1/2

According NCBI website, cas1 and cas2 proteins are extremely conservative among E.coli, thus we used DH5α colony as template to clone cas1/2 gene. We also added restriction site in our primers for future cloning on pCDF.

step	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing		30S
extension	68℃	13S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing		30S
extension	68℃	13S
Final extension	68℃	5min
Incubation	4℃	





3. Agarose-Gel electrophoresis
The fourth lane is 2000bp DNA ladder, others are products from PCR amplification. Let it run at 120V, 400mA for 20 minutes. Sadly, none of them were with the right band length.
(都是假图)

7.7
Nice and easy.

7.8
1.Overnight culture
Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli each containing pCP20(cm) and pIJ773(amp) and pIJ790(cm)
Incubate at 30℃ with shaking (250rpm) overnight.

7.9
1.Plasmid Isolation
An overnight starter culture of E. coli DH5α harboring plasmid pCP20(cm) and pIJ773(amp) and pIJ790(cm) was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pCP20	44.8
pIJ773	76.9
pIJ790	54.7

2.Creat Bacterial Glycerol Stock
Overnight culture of bw25113 harboring pIJ790 is
https://www.addgene.org/protocols/create-glycerol-stock/

3. PCR amplification of cas1/2

Since the previous PCR amplification failed, we designed a new set of primers to try for the second time. We used DH5α colony as template to clone cas1/2 gene and tested 3 different annealing temperatures.

step	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing		30S
extension	68℃	13S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	63/65/68℃	30S
extension	68℃	13S
Final extension	68℃	5min
Incubation	4℃	

4.Agarose-Gel electrophoresis

Sadly, none of them were with the right band length.

7.10

1. PCR amplification of cas1/2

We kept trying to clone cas1/2 from DH5α genome. This time the bacteria colony

step	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing		30S
extension	68℃	13S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	63/65/68℃	30S
extension	68℃	13S
Final extension	68℃	5min
Incubation	4℃	

7.11

Nice and easy.

7.12

1.Bacteria recovery and stock preparation
We happily received the gift from Columbia university, that is DH5α containing pRec or pTrig plasmid in stab.
Inoculate 10 mL of LB medium with antibiotics with colony of E. coli containing either pRec or pTrig plasmid. Incubate at 37℃ with shaking (250rpm) overnight.
Dilute 1:100 in 5ml LB with antibiotic and grow 3-4 hours at 37 C with shaking (250rpm). Streak bacteria in exponential stage in LB plate and make glycerol stock accordingly.

7.13
1.Plasmid Isolation
An overnight starter culture of E. coli DH5α harboring plasmid pRec or pTrig was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30µL of pre-warmed MilliQ.

name	concentration (ng/ul)
pRec	103.3
pTrig	78.5

2.plate streaking

7.14
Nice and easy.

7.15
1.preparation for electroporation
L-Arabinose solution 1M：Dissolve XXXmg L-Arabinose in 8ml MilliQ water. Pass through 0.2mm filter membrane to split into several 1.5ml eppendof tubes.
Apramycin 50mg/ml：Dissolve 400mg L-Arabinose in 8ml MilliQ water. Pass through 0.2mm filter membrane to split into several 1.5ml eppendof tubes. Use this apramycin antibiotic in its 50ug/ul final concentration.

8.6
1.Tansformation
Co-transformation of pTrig and pRec plasmids into BL21(DE3). However, no colony can be found the next day. We suppose the plasmids didn't survive the power failure of the refrigerator, so we cultured and isolated new plasmids.

8.8

1.Tansformation

Co-transformation of pTrig and pRec plasmids into BL21(DE3).

This time, we used freshly prepared plasmids.

8.9

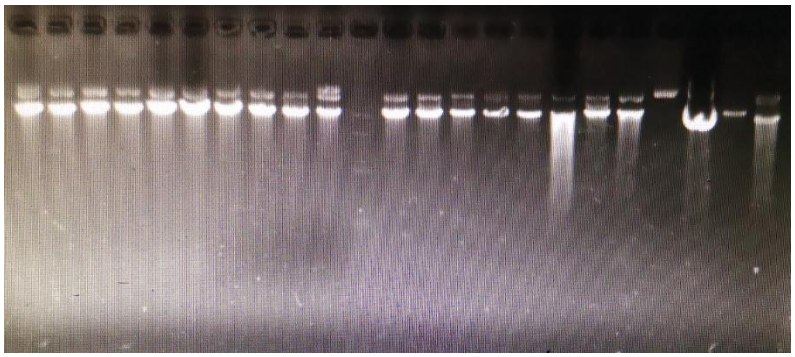
1.overnight culture

Pick 18 transformants from yesterday's cotransformation. Inoculate 5 mL of LB medium with kanamycin and chloramphenicol with single colony of freshly grown E. coli containing both pTrig and pRec. Incubate at 37℃ with shaking (250rpm) overnight.

8.10

1.Endonuclease check on cotransformation.

We isolated plasmid from 18 colonies, and cut them with corresponding unique endonuclease.



Number 9 and 11 are picked out for further experiment.

8.11

1.qPCR analysis for pTrig copy number

Dilute 1:100 Overnight culture in 5ml LB+kan/cm and grow 3-4 hours at 37 C in 15ml snap cap tube in a rotator. Add inducer IPTG 1mM to culture at early logarithmic stage and let it grow for more than 6 hours.

Perform qPCR analysis according to protocol. Results adjusted to OD600 bellow.

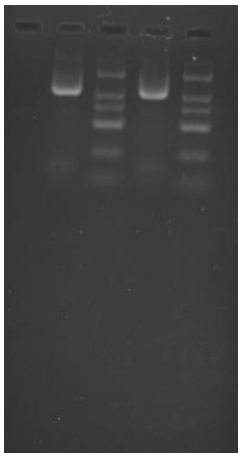
sample	Replicate 1	Replicate 2	Replicate 3
9 control	12.98	12.32	12.78
9+IPTG	6.00	6.11	6.02
11 control	13.14	13.48	12.94
11+IPTG	6.51	6,67	6.33

8.12

1.PCR amplification for cas1/2 linearized fragment

Clone linearized cas1/2 fragment with homologous sequences for Gibson assembly, using pRec as template, pREC-cas-F and pREC-cas-R as primers,

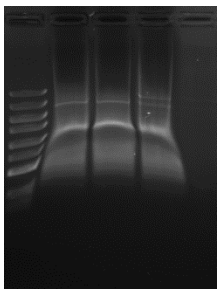
Lane1/3-product 1246bp
Lane2/4-2000bp ladder



2.PCR amplification for pTS-neon linearized fragment
Clone linearized fragment containing luxR and luxR promotor with homologous sequences for Gibson assembly, using pTS-neon as template, TS-overlap-F and TS-overlap-R as primers.

step	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing	48/50/53	30S
extension	68℃	13S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	67℃	30S
extension	68℃	13S
Final extension	68℃	5min
Incubation	4℃	

Sadly, negative result.

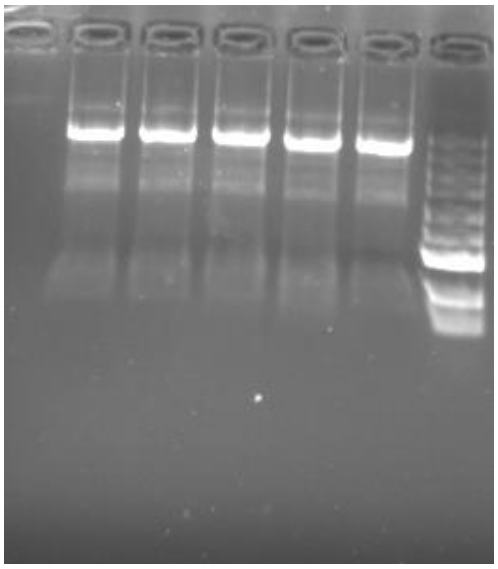


Lane1-5000bp ladder
Lane2/3/4-product from different annealing temperature.

8.14

1. PCR amplification for pTS-neon linearized fragment

Same as before, except that reaction volume changed from 50ul to 20ul.



Lane1/2/3/4/5- product 3489bp from different annealing temperature

Lane6-5000bp ladder

2. Gel purification

We let PCR product of cas1/2 and pTS-over run at 140V, 400mA for 20 minutes. Following our gel purification protocol, DNA fragments dissolved in 20ul MilliQ water are stored in -4℃.

name	Concentration (ng/ul)
Cas1/2	351.2
pTS-over	180.8

3.Gibson assembly for TS-cas

2X Mix	5ul
Cas1/2 (insert)	0.4ul
pTS-over (backbone)	0.6ul
ddH_20	4ul

Incubate this at 10ul reaction system at 50℃ for 30min.

Transform 10ul product to 50ul competent DH5α. Grow over night.

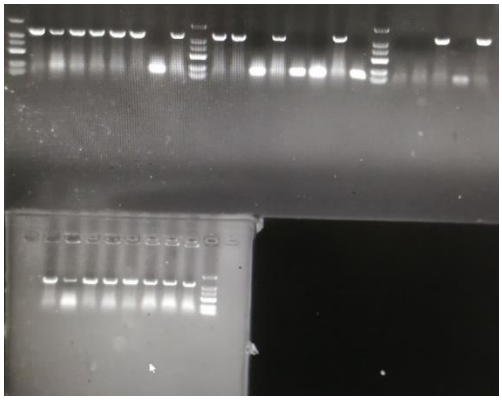
8.15

1.Colony test on pTS-cas

step	temperature	time
Initial activation	98℃	4min
Three-step cycling for 35 cycles		
denaturation	98℃	10S
Annealing	62℃	10S

extension	72℃	14S
Final extension	72℃	5min
Incubation	4℃	

Positive result from agarose gel electrophoresis is band 1246bp. We chose three of them for sequencing confirmation.



Lane1/24-different transformants from LB plate
Lane at the right-B B P B P

8.18
1.Overnight culture
The sequencing results indicate successful clone of pTS-cas.
Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing correct pTS-cas (number 7).
Incubate at 37℃ with shaking (250rpm) overnight.

8.19
1.Plasmid Isolation
An overnight starter culture of E. coli DH5α harboring plasmid pTS-cas or pCDFDuet was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pTS-cas	128.2
pCDFDuet	987.7

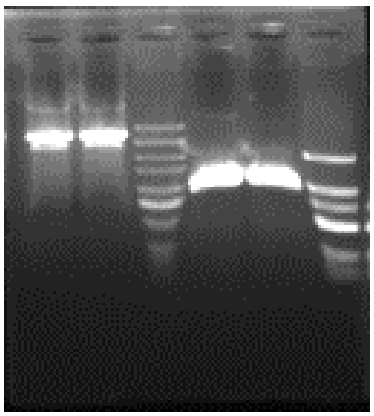
8.21
Nice and easy.

8.21

1. PCR amplification for SmR and pTS-cas linearized fragment

Clone linearized fragment containing streptomycin and pTS-cas with homologous sequences for Gibson assembly, using pCDFDuet and pTS-cas as template, smR-over-F, smR-over-R, TScas-over-F, TScas-over-R as primers.

step	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing	47/50/52	30S
extension	68℃	13S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	65℃	30S
extension	68℃	13S
Final extension	68℃	5min
Incubation	4℃	



Lane1/2-product TS-cas 3693bp
Lane3-5000bp ladder
Lane4/5-produce smR 969bp
Lane6-2000bp ladder

2. Gel purification

We let PCR product of smR and TS-cas run at 140V, 400mA for 20 minutes. Following our gel purification protocol, DNA fragments dissolved in 20ul MilliQ water are stored in -4℃.

name	Concentration (ng/ul)
smR	107.6
TS-cas	147.3

3.Gibson assembly for TS-cas

2X Mix	5ul
smR(insert)	0.8ul
TS-cas (backbone)	0.8ul
ddH20	3.4ul

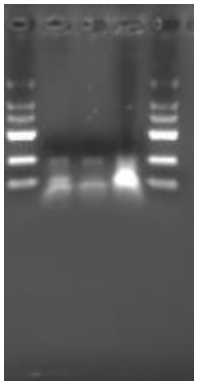
Incubate this at 10ul reaction system at 50℃ for 30min.

Transform 10ul product to 50ul competent DH5α. Grow over night.

8.22

1.Colony test on pTScas-sm

step	temperature	time
Initial activation	98℃	4min
Three-step cycling for 35 cycles		
denaturation	98℃	10S
Annealing	63℃	10S
extension	72℃	10S
Final extension	72℃	5min
Incubation	4℃	



Lane1/5-2000bp ladder

Lane2/3/4-different transformants from LB plate

Positive result from agarose gel electrophoresis should be band 939bp. Though no convincing positive results, we still send them for sequencing.

8.26

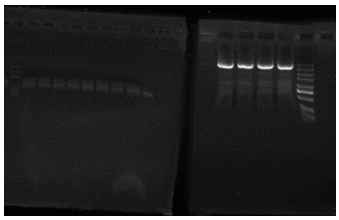
1. PCR amplification for luxR promotor and pTrig linearized fragment

Clone linearized fragment containing luxR promotor and pTrig without lac promotor with homologous sequences for Gibson assembly, using pTS-Neon and pTrig as template, Ts-Pr-F, Ts-Pr-R, pTrig-F, pTrig-R as primers.

Step for pTS-Neon	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing	48/50/53	30S
extension	68℃	5S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	65℃	30S

extension	68℃	5S
Final extension	68℃	5min
Incubation	4℃	

Step for pTrig	temperature	time
Initial activation	98℃	4min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	30S
Annealing	51/53	30S
extension	68℃	120S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	30S
Annealing	65℃	30S
extension	68℃	120S
Final extension	68℃	5min
Incubation	4℃	



Left: luxR promotor 236bp
Right: pTrig without lac promotor 3753bp

2. Gel purification
We let PCR product of luxR promotor and pTrig without lac promotor run at 140V, 400mA for 20 minutes. Following our gel purification protocol, DNA fragments dissolved in 20ul MilliQ water are stored in -4℃.

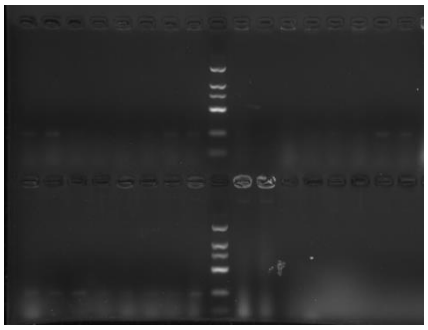
name	Concentration (ng/ul)
luxR promotor	88.6
pTrig without lac	155.3

3.Gibson assembly for pLux-rep

2X Mix	5ul
luxR promotor	0.5ul
pTrig without lacI	0.7ul
ddH20	3.8ul

Incubate this at 10ul reaction system at 50℃ for 30min.
Transform 10ul product to 50ul competent DH5α. Grow over night.
8.27
1.Colony test on pLux-rep

step	temperature	time
Initial activation	98℃	4min
Three-step cycling for 35 cycles		
denaturation	98℃	10S
Annealing	67℃	10S
extension	72℃	5S
Final extension	72℃	5min
Incubation	4℃	



According to DNA electrophoresis, light bands around 250bp are regarded as successful transformants pLux-rep, for the last two are positive control. Six colonies are picked out for sequencing confirmation.

8.28

1. PCR amplification for SmR and pTS-cas linearized fragment

The sequencing result from was weird.

Clone linearized fragment containing streptomycin and pTS-cas with homologous sequences for Gibson assembly, using pCDFDuet and pTS-cas as template, smR-over-F, smR-over-R, TScas-over-F, TScas-over-R as primers.

Repeat as before.

2. Gel purification

We let PCR product of smR and TS-cas run at 140V, 400mA for 20 minutes. Following our gel purification protocol, DNA fragments dissolved in 20ul MilliQ water are stored in -4℃.

name	Concentration (ng/ul)
smR	136.6
TS-cas	171.1

3.Gibson assembly for TS-cas

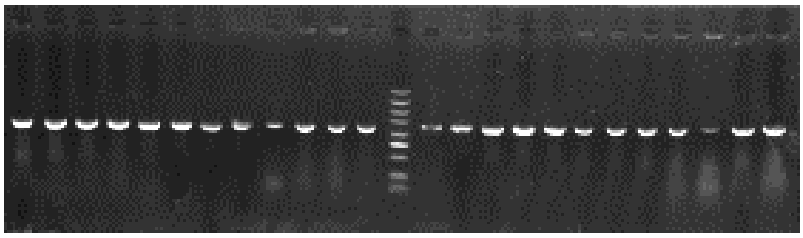
We performed a repeated Gibson assembly using the same SmR fragment and TScas fragment with a change of their molar ratio.

	3:1	5:1
2X Mix	5ul	5ul
smR(insert)	0.7ul	1ul
TS-cas (backbone)	0.6ul	0.6ul

ddH20	3.7ul	3,4ul

Incubate this at 10ul reaction system at 50℃ for 30min.
Transform 10ul product to 50ul competent DH5α. Grow over night.

8.29
1.Colony test on pLux-cas (pTScas-sm)
Primers targeting cas1/2 and SmR both showed positive results.



Lane1/12-different transformants from LB plate test on smR
Lane13-5000bp ladder
Lane14/25-corresponding transformants from LB plate test on cas1/2

Six colonies are picked out for sequencing confirmation. From the results below, we can tell the construction of pLux-cas is successful.

☐	4	su4	4_check-RiboJ	检测通过		46	1007	通过
☐	4	su4	4_check-sm	检测通过		45	990	通过
☐	5	su5	5_check-RiboJ	检测通过		45	1005	通过
☐	5	su5	5_check-sm	检测通过		45	990	通过
☐	6	su6	6_check-RiboJ	检测通过		45	1013	通过
☐	6	su6	6_check-sm	检测通过		45	973	通过

2. Overnight culture
The sequencing results indicate successful clone of pTScas-sm.
Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing correct pTScas-sm (number 6).
Incubate at 37℃ with shaking (250rpm) overnight.

8.30
1.Plasmid Isolation
An overnight starter culture of E. coli DH5α harboring plasmid pTScas-sm was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pTScas-sm	103.6

8.31
1.Bacteria recovery and stock preparation

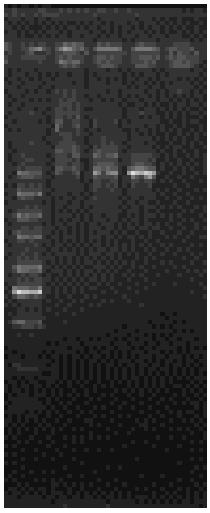
We happily received the synthetic plasmids containing RSRL-1T/2A and DH5α harboring these plasmid in stab.
Inoculate 10 mL of LB medium with antibiotics with colony of E. coli containing 1T/2A pRead. Incubate at 37℃ with shaking (250rpm) overnight.
Dilute 1:100 in 5ml LB with antibiotic and grow 3-4 hours at 37 C with shaking (250rpm). Streak bacteria in exponential stage in LB plate and make glycerol stock accordingly.

2.Tansformation
Co-transformation of pTrig and pRec plasmids into BL21(DE3).

9.1
Nice and easy

9.2
1. Try Gibson assembly for pLux-rep for the second time
PCR amplification for luxR promotor and pTrig linearized fragment.
Gel purification of luxR promotor and pTrig without lac.
This time the molecular ration for insert to backbone is around 7:1. Transform 10ul assembly product to 50ul competent DH5α. Grow over night.

2. Endonuclease check on pTrig and pRec in BL21



All three are successful cotransformant, for they show 2 bands with correct length.

3.Making chemical competent cells for pRead transformation.
We chose the number 2 transformant as chassis to make chemical competent cells. 400ul competent cells harboring pTrig and pRec was made from 10ml bacteria solution.
Use 100ul for pRead transformation. Freeze the left at -80℃.

4.Test on positive EGFP expression
Inoculate 5 mL of LB medium with antibiotics with 6 colonies of BL21 harboring original pGEX-

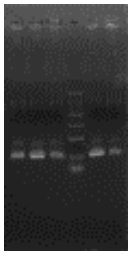
egfp. Incubate at 37℃ with shaking (250rpm) overnight.
Transfer 1% overnight bacterial culture in Erlenmeyer flask.
Incubate for 2h to reach early exponential stage (OD600 is 0.2-0.3).
Add IPTG to final concentration of 1mM and grow at 37℃ for 6 hours.

However, none of them show observable florescence. And for the following days we have tried many other positive control strains, different induction protocol.

2Acontrol	829845
2Acontrol诱导	851890
EGFP-DH5	818602
EGFP-DH5诱导	815489
EGFP-BL21-3诱导	896455
EGFP-BL21-5诱导	952596
无关菌	805383
LB	630062

9.3
1.Colony test on pLux-rep

step	temperature	time
Initial activation	98℃	4min
Three-step cycling for 35 cycles		
denaturation	98℃	10S
Annealing	67℃	10S
extension	72℃	5S
Final extension	72℃	5min
Incubation	4℃	



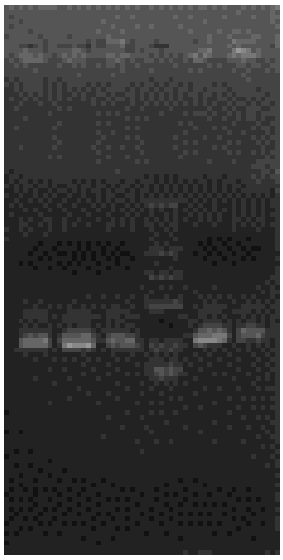
Lane1/2/3/5/6-different colonies
Lane4-2000bp ladder
According to DNA electrophoresis, light bands around 250bp are regarded as successful transformants pLux-rep. All five colonies are picked out for sequencing confirmation.

9.3

1.Colony test on pLux-rep

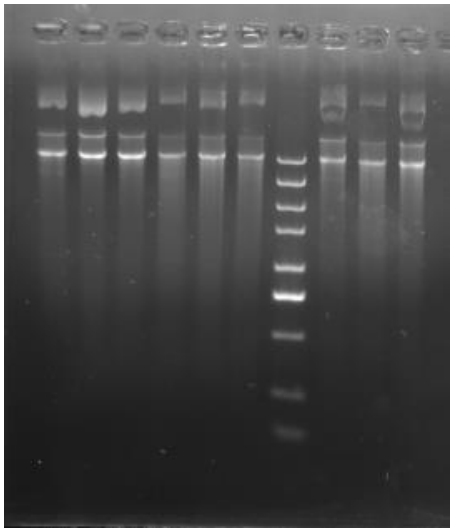
step	temperature	time

Initial activation	98℃	4min
Three-step cycling for 35 cycles		
denaturation	98℃	10S
Annealing	67℃	10S
extension	72℃	5S
Final extension	72℃	5min
Incubation	4℃	



Lane1/2/3/5/6-different colonies
Lane4-2000bp ladder
According to DNA electrophoresis, light bands around 250bp are regarded as successful transformants pLux-rep. All five colonies are picked out for sequencing confirmation. Also inoculate them for overnight culture.

2. Endonuclease check on pTrig and pRec and pRead in BL21



Lane1/4/8-endonuclease targeting pTrig for three differenct transformant

Lane2/5/9- endonuclease targeting pRec for three differenct transformant
Lane3/6/10- endonuclease targeting pRead for three differenct transformant
Lane7-5000bp ladder
All three are successful cotransformant, for they show 3 bands with correct length.

3.Steak the three successful cotransformant onto LB plate and keep them safe.

4.qPCR analysis for pTrig copy number
Dilute 1:100 Overnight culture in 20ml LB+kan/cm and grow 1-2 hours at 37 C in in 100ml conical flask in a rotator. Add inducer IPTG 1mM to culture at early logarithmic stage and let it grow for 8 hours.
Take samples every 1 hour.

	1	2	3	4	5	6	7	8	9	10	11	12
A	19.58	19.94	19.19	26.79	25.62	26.92	13.83	13.86	13.61	17.8	17.49	17.42
B	18.32	18.28	18.27	23.98	26.71	25.72	13.61	13.62	13.65	17.59	17.69	17.7
C	18.77	18.63	18.86	29.16	27.67	27.48	13.16	13.03	13.03	17.48	17.61	17.79
D	24.92	25.78	25.77	24.83	26.34	26.25	18	17.89	17.97	18.06	18.06	18.02
E	27.06	25.02	27.12	24.67	26.16	26.55	17.95	17.95	17.91	18.01	17.83	17.79
F	26.97	23.95	26.11	23.16	25.91	25.69	17.72	16.02	17.5	17.48	17.48	17.6
G	16.07	16.15	16.18	16.54	16.65	16.56	17.91	17.72	23.71	17.9	17.89	18.02
H	17.49	17.35	17.3	/	/	/	17.24	17.52	17.3	**17.93**	**17.93**	**17.92**

△△Ct	①	-7.4850	-10.5550	-9.2183	-9.4417
	②	-4.6367	-7.7833	-8.4367	-7.8900
	③	-7.0334	-11.8300	-9.9500	-8.7534
		3h	4.5h	5.5h	6.5h
质粒扩增倍数 (2^(-△△Ct))		179	1504	596	695
		25	220	346	237
		131	3641	989	432

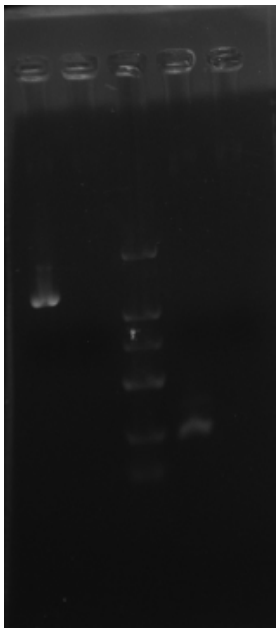
9.4
1.Plasmid isolation and Cotransformation
We got the correct sequencing result of pLux-rep.
An overnight starter culture of E. coli DH5α harboring plasmid pLux-rep number 3 was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pLux-rep	67.4

Co-transform pLux-rep and pLux-cas plasmids into BL21(DE3).

9.5
1.Colony test on pLux-rep and pLux-cas
There are only two colonies on LB plate. Protocols are indicated as before.

But the results were unsatsfying.



Lane1/2-check colonies for cas
Lane4/5-check colonies for plux
Lane3-2000bp ladder

2.Overnight culture
Inoculate 20mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing pLux-rep plasmid.
Incubate at 37℃ with shaking (250rpm) overnight.

3.Test on positive EGFP expression
Inoculate 5 mL of LB medium with antibiotics with 6 colonies of BL21 harboring original pGEX-egfp. Incubate at 37℃ with shaking (250rpm) overnight.
Transfer 1% overnight bacterial culture in Erlenmeyer flask.
Incubate for 2h to reach early exponential stage (OD600 is 0.2-0.3).
Add IPTG to final concentration of 0.5mM and grow at 20℃ overnight.

Finally, we have the right protocol for IPTG induction on Tac promotor to express EGFP.

9.6
1.Plasmid Isolation
An overnight culture of E. coli DH5α harboring either pLux-rep or pLux-cas was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pLux-rep	112.9

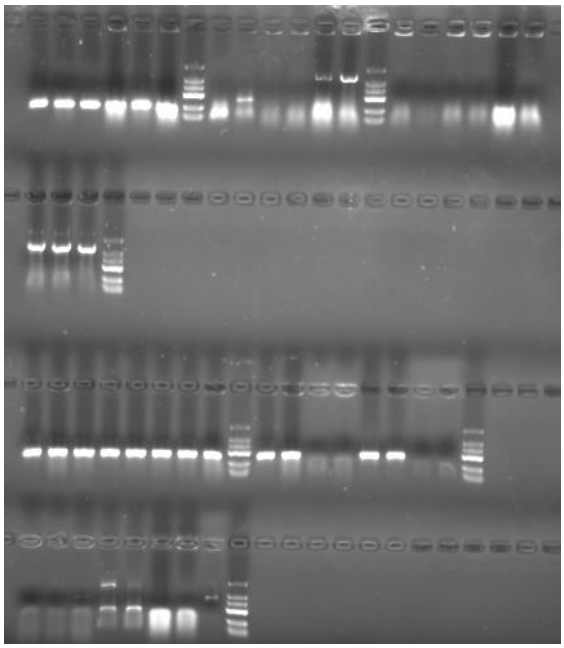
pLux-cas 132.1

2.Transformation
Co-transformation of pLux-rep and pLux-cas plasmids into BL21(DE3).
Compared to the first time, the plasmid concentration was much higher.

9.7
1.Colony test on pLux-rep and pLux-cas
Similar as before, primers targeting cas1/2 and pluxR both showed negative results.

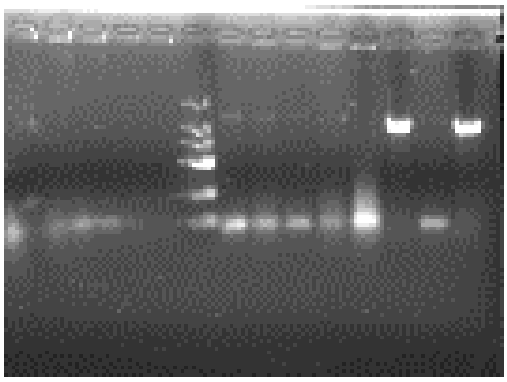
9.10
1.Colony test again
Since the induction using tetracycline and IPTG didn't work as expected. We thought maybe pTrig, pRec or pRead plasmid was lost during reproduction. So, we checked it using colony PCR.

‘



Up: repL
Low: EGFP

Primers targeting repL and EGFP showed positive results, suggesting the existence pTrig and pRead plasmids. Primers targeting cas1/2 showed negative results.



Lane1/2/3/4/5-different colonies
Lane6/8-positive control

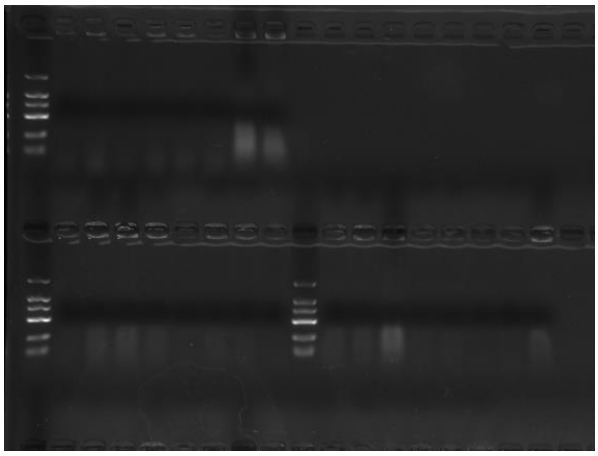
2.Overnight culture
Inoculate 5 mL of LB medium with antibiotics with 3 positive colonies of BL21 containing both pTrig and pRead plasmids.
Incubate at 37℃ with shaking (250rpm) overnight.

9.11
1. Making chemical competent cells for pRec transformation.
200ul competent cells harboring pTrig and pRead were made from 10ml bacteria solution.
Use 50ul each for pRec transformation. Freeze the left at -80℃.

9.12
1.Transformation　医学院的啦
Transformation of pLux-rep plasmid into BL21(DE3).
Transformation of pLux-cas plasmid into BL21(DE3).

2.Antibiotic selection on transformant
pRec plasmid has tet resistant gene, so 5 colonies grown on LB plate containing kan/cm/amp were picked out.
Later, add tetracycline to final concentration of 100 ng/μL and incubate overnight at 20℃.

9.13
1.Colony test on pLux-rep and pLux-cas



Sadly, no positive result.

9.14

1. Again pRec transformation.

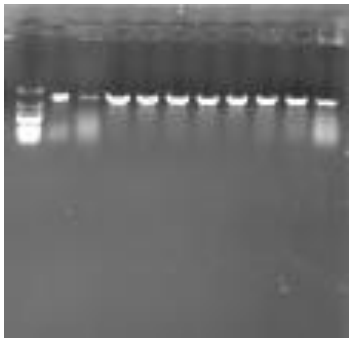
No transformant survived after the addition of tetracycline for some reason. So, we tried transformation again.

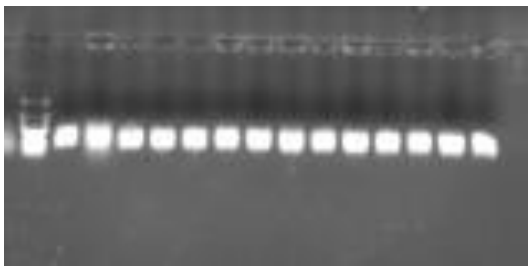
Use 50ul competent cells harboring pTrig and pRead for pRec transformation.

9.15

1.Colony test

To be sure, we checked three plasmids. Primers targeting repL and cas1/2 and EGFP showed positive results, suggesting the existence of three wanted plasmids.





Up-cas1/2

Low-repL and EGFP

9.18

1.Overnight culture

Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing either pLux-trig or pLux-cas plasmid.

Incubate at 37℃ with shaking (250rpm) overnight.

9.19

1.Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring plasmid pLux-trig or pLux-cas was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pLux-trig	54.5
pLux-cas	89.1

2.Transformation

Transformation of pLux-rep plasmid into BL21(DE3).

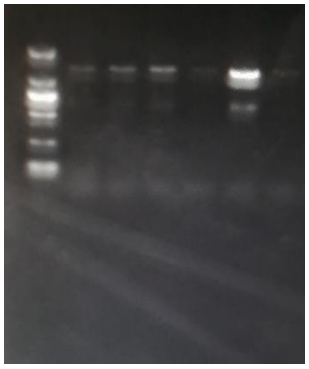
Transformation of pLux-cas plasmid into BL21(DE3).

Cotransformation of pLux-rep and pLux-cas plasmids into BL21(DE3).

9.20

1.Colony test on pLux-cas

Plates from last night's transformation did not look so well with pTrig. So, we only tested pLux-cas.



Lane 1/2/3/4/5/6 -different colonies

Number 5 was picked out to grow overnight for the following transformation.

9.21

1. Making chemical competent cells for transformation.

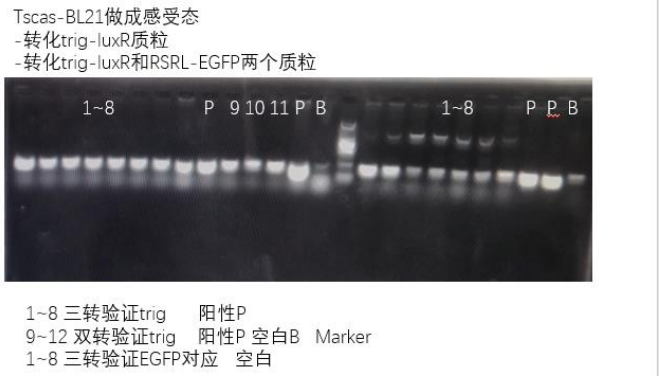
900ul competent cells harboring pLux-cas were made from 20ml bacteria solution.

Use 100ul for pLux-trig transformation and 100ul for pLux-trig plus pRead cotransformation.

Freeze the left at -80℃.

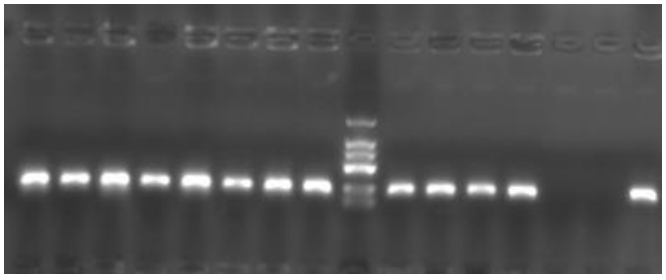
2.Dealing with microplate reader
Throughout several days, data collected from microplate reader were strange.

9.22
1.Colony test on pLux-trig and pRead

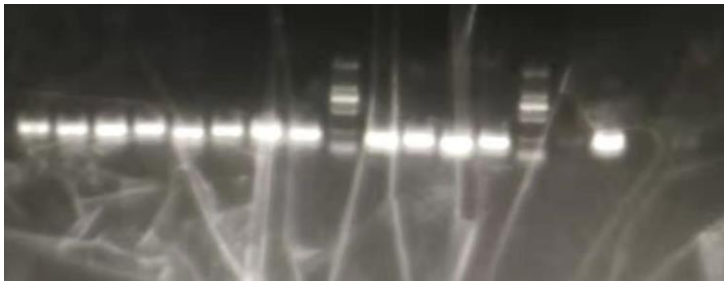


Luckily, most of them were successful transformants harbouring pLux-cas and pLux-trig and pRead plasmids.
Number 11 here is picked out as the successful transformants harbouring pLux-cas and pLux-trig plasmids and used for pLux-trig's response to AHL induction.
It's overnight culture is well kept.

9.23
1.Colony test on pLux-trig and pRead
Last time, negative control showed a light band, so we used bacteria solution as template and tried another colony PCR.



Lane1''12-different colonies
Lane13/14-blank
Lane15-positive control



Lane1''12-different colonies
Lane13/15/16-blank
Lane14-positive control

Now, we have got twelve successful transformants harbouring pLux-cas and pLux-trig and pRead plasmids.

2.Preparation for qPCR analysis

9.24
1.qPCR analysis for pLux-trig copy number
Dilute 1:100 Overnight culture in 20ml LB+kan/sm and grow 1-2 hours at 37℃ in 100ml conical flask in a rotator. Add inducer AHL 10^{-8}M or no inducer to culture at early logarithmic stage and let it grow at 37℃.
Take samples every 30 minutes for 9 hours.

取样次数	1	2	3	4	5	6
诱导时间（h）	0	0.5	1	1.5	2	2.5
AHL -8						
Genome	22.6	23	22.75	22.1	21.98	22.25
Target	16.31	16.36	16.64	16.6	16.5	16.49
绝对	6.29	6.64	6.11	5.5	5.48	5.76
未诱导						
G	22.37	22.3	21.9	21.51	19.92	18.75
T	16.5	16.64	16.43	16.66	16.51	16.5
绝对	5.87	5.66	5.47	4.85	3.41	2.25
ΔΔCT	0.42	0.98	0.64	0.65	2.07	3.51
扩增倍数	1.34	1.97	1.56	1.57	4.20	11.39

7	8	9	10	11	12
3	3.5	4	4.5	5	5.5
23.16	23.19	21.89	22.18	20.58	20.25
14.58	14.57	14.56	14.74	14.12	14.03
8.58	8.62	7.33	7.44	6.46	6.22
18.38	19.02	17.65	17.35	18.32	18.5
14.64	14.58	14.63	14.74	14.6	14.6
3.74	4.44	3.02	2.61	3.72	3.9
4.84	4.18	4.31	4.83	2.74	2.32
28.64	18.13	19.84	28.44	6.68	4.99

13	14	15	16	17	18
6	6.5	7	7.5	8	8.5
20.49	22.33	21.21	22.64	20.53	20.69
13.44	14	13.51	13.94	13.35	13.38
7.05	8.33	7.7	8.7	7.18	7.31
19.29	18.16	18.29	18.05	17.4	17.64
14.14	14.13	14.2	14.34	14.09	14.04
5.15	4.03	4.09	3.71	3.31	3.6
1.9	4.3	3.61	4.99	3.87	3.71
3.73	19.70	12.21	31.78	14.62	13.09

9.26

1.qPCR analysis for pLux-trig copy number

Dilute 1:100 Overnight culture in 20ml LB+kan/sm and grow 1-2 hours at 37℃ in 100ml conical flask in a rotator. Add inducer AHL 10^{-7}M or 10^{-9}M to culture at early logarithmic stage and let it grow at 37℃.

Take samples every 30 minutes for 9 hours.

	1	2	3	4	5	6
-7						
G	22.66	22.96	22.39	22.35	22.91	22.77
T	15.21	15.41	15.4	15.36	15.31	15.24
绝对	7.45	7.55	6.99	6.99	7.6	7.53
相比不诱导	1.58	1.89	1.52	2.14	4.19	5.28
浓度-9	22.37	22.19	21.95	22.02	20.98	19.96
	15.24	15.46	15.4	15.59	17.89	15.32
绝对	7.13	6.73	6.55	6.43	3.09	4.64
相比不诱导	5.87	5.66	5.47	4.85	3.41	2.25

	7	8	9	10	11	12
-7						
G	23.06	24	22.01	22.01	21.02	20.99
T	14.86	14.88	14.85	14.69	14.66	14.63
绝对	8.2	9.12	7.16	7.32	6.36	6.36
相比不诱导	4.46	4.68	4.14	4.71	2.64	2.46
浓度-9	20.28	19.15	18.68	18.25	17.57	18.31
	15.06	14.85	14.81	14.84	14.26	14.34
绝对	5.22	4.3	3.87	3.41	3.31	3.97
相比不诱导	1.48	-0.14	0.85	0.8	-0.41	0.07

	13	14	15	16	17	18
G	20.87	21.15	20.37	20.22	19.69	20.77
T	15.95	15.99	15.35	15.21	15.21	16.23
绝对	4.92	5.16	5.02	5.01	4.48	4.54
	-0.23	1.13	0.93	1.3	1.17	0.94
G	17.22	17.22	17.32	17.13	17.33	18.28
T	15.08	14.92	14.77	14.51	14.58	15.55
绝对	2.14	2.3	2.55	2.62	2.75	2.73
	-3.01	-1.73	-1.54	-1.09	-0.56	-0.87

9.28

1.qPCR analysis for pLux-trig copy number

Dilute 1:100 Overnight culture in 20ml LB+kan/sm and grow 1-2 hours at 37℃ in 100ml conical flask in a rotator. Add inducer AHL 10^{-6}M or 10^{-8}M to culture at early logarithmic stage and let it grow at 37℃.

Take samples every 30 minutes for 9 hours.

	1	2	3	4	5	6
-6						
	29.33	29.26	29.25	28.48	29.38	29.22
	28.37	29.56	29.14	28	28.62	27.97
绝对	0.96	-0.3	0.11	0.48	0.76	1.25
	0					
-8						
	29.31	28.28	28.07	27.25	28.6	27.84
	28.33	27.9	27.56	24.7	26.52	26.34
绝对	0.98	0.38	0.51	2.55	2.08	1.5

	7	8	9	10	11	12
-6						
	26.53	26.69	23.25	24.77	23.99	23.82
	25.34	24.85	22.64	22.5	25.85	22.38
绝对	1.19	1.84	0.61	2.27	-1.86	1.44
-8						
	24.48	22.77	19.55	19.38	21.76	21.04
	20.34	15.97	15.82	17.12	16.69	15.32
绝对	4.14	6.8	3.73	2.26	5.07	5.72

	13	14	15	16	17	18
	27.28	27.2	27.42	28.32	28.21	27.31
	24.39	24.2	24.49	25.07	23.99	23.87
绝对	2.89	3	2.93	3.25	4.22	3.44
	23.25	24.84	23.21	24.91	26.43	24.34
	17.2	18.77	17.12	17.99	19.6	17.86
绝对	6.05	6.07	6.09	6.92	6.83	6.48

9.30

1.Dilute bacteria culture to get single colony

To analysis spacer acquisition.

10.1

1.qPCR analysis for pLux-trig copy number

Dilute 1:100 Overnight culture in 20ml LB+kan/sm and in 20ml LB+kan/sm/amp, which contains three plasmid, and grow 1-2 hours at 37℃ in 100ml conical flask in a rotator. Add inducer AHL 10^{-7}M to each culture at early logarithmic stage and let it grow at 37℃.

Take samples every 30 minutes for 9 hours.

补图!!!

2.Dilute bacteria culture to get single colony

Make serial dilution 10-6, 10-7, 10-8 of bacteria culture.

However, there were no colony grown on LB plate.

10.2

1.Overnight culture

Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing pRec.

Incubate at 37℃ with shaking (250rpm) overnight.

2.SDS-PAGE analysis for cas1-cas2 expression

Two successful transformants harbouring pLux-cas and pLux-trig and pRead plasmids, positive control (successful transformant which exhibit EGFP after tet induction) and negative control (BL21) are grow overnight at 30℃ with 10^{-7}M AHL or 100ug/mL tetracycline.

Centrifuge at 4000rpm for 20min at 4℃ and discard the supernatant.

Wash them with bacteria lysis buffer (50 mM Tris + 500mM NaCl) and perform several rounds of ultrasonication.

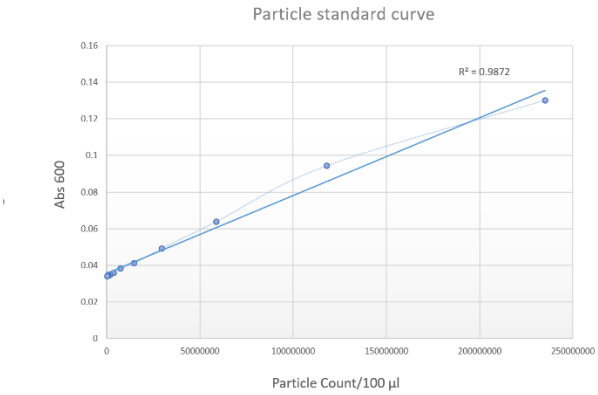
Centrifuge at 4000rpm for 30min at 4℃ and take the supernatant as our sample.

Make 15% SDS-PAGE gel. Load 5 ul marker or 20 ul protein sample each lane.

Run at 80V until samples enter the separation gel in gel running buffer (19.3 mM Glycine, 2.5 mM Tris base, 0.1% SDS), and then run at 120V. Electrophoresis is complete when the dye front migrates about 2 mm from the bottom of the gel.

Stain with Coomassie brilliant blue for 1 h, and then destain in destain buffer (50% H2O, 20% AcOH, 30% methanol) for 1h.

3.Measurement OD600.



10.3

1.Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring plasmid pTScas-sm was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed MilliQ.

name	Concentration (ng/ul)
pRec	94.6

2.Transformation

Cotransformation of pRec and pRSRL-EGFP into BL21.

10.4

1.Colony test on pRec and pRSRL-EGFP

Several colonies were inoculated in 5ml LB with antibiotics and grew overnight for further experiments.

2.Induction

Successful transformants harbouring pLux-cas, pLux-trig and pRead plasmids were subjected to induction protocol with different concentrations of AHL.

Sadly, we didn't see florescence after centrifuging.

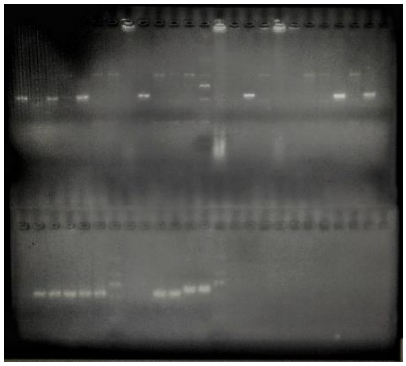
10.7

1.Dilute bacteria culture to get single colony

Make serial dilution 10-4, 10-5, 10-6 of bacteria culture.
This time we had colonies grown on LB plate.

2.Induction
We tried this again. Successful transformants harbouring pLux-cas, pLux-trig and pRead plasmids were subjected to induction protocol with different concentrations of AHL.
Still, we didn't see any florescence after centrifuging. Based on SDS-PAGE results, cas1-cas2 complex should be successfully expressed. We didn't come up with proper explanation.

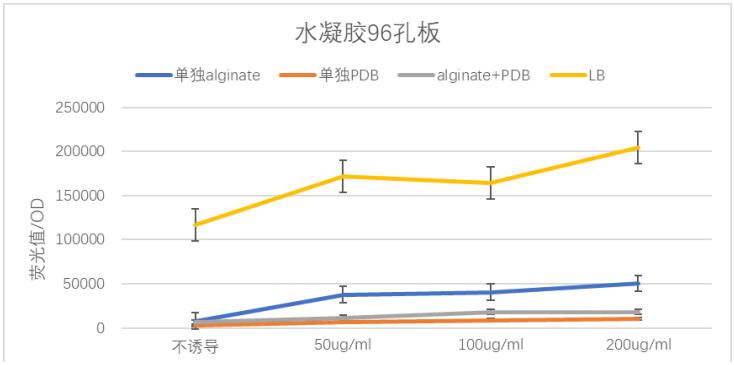
10.8
1.PCR test on spacer acquisition



We used spacer-F-seq and spacer-R-seq targeting two ends of RSRL array to see the length change after spacer adaptation mediated by cas1-cas2 complex.
The last four were two negative and two positive controls, differing by 61nt. Though the colonies are from the successfully induced bacteria, none of them showed positive results.

10.10
1.Test on hydrogel media
We happily received PDB from Professor Feng and hurried on our test.
This time we tested 4 different types of culture media, including 5mg/ml alginate cross linked with CaCl2, 5mg/ml alginate cross linked PDB, 5mg/ml PDB and LB broth, along with 3 different concentrations of tetracycline.

	四种四环素浓度	0	1	2	3		0	1	2	3
						左右最初菌液量不同				
OD	单独alginate	0.21	0.12	0.12	0.12		0.05	0.02	0.03	0.01
florescence		1717.70	4637.70	5007.70	6304.70		644.70	2111.70	1697.70	1966.70
	单独PDB	0.23	0.26	0.24	0.24		0.29	0.29	0.22	0.22
		664.70	1835.70	2139.70	2484.70		510.70	1316.70	1151.70	1578.70
	alginate+PDB	0.09	0.11	0.08	0.53		0.09	0.18	0.10	0.53
		586.70	1206.70	1456.70	1596.70		573.70	1844.70	1926.70	1166.70
	LB	0.30	0.24	0.26	0.21		0.29	0.23	0.20	0.01
		34659.70	41254.70	41866.70	42280.70		32286.70	41231.70	40759.70	39773.70
	florescence/OD									
	单独alginate	7995	37903	40694	50577		13472	84957	62517	271045
	单独PDB	2893	6976	8811	10526		1777	4573	5282	7161
	alginate+PDB	6529	11421	17905	2985		6590	10161	18916	2211
	LB	116913	171854	164147	204298		109537	175562	204559	7714062



Culture and induction in LB broth seemed to work just fine.

Three new types of media also exhibit the increase of florescence. However, we couldn't draw any conclusion due to the limitation on sample numbers.

10.11

1.Test on hydrogel media

Based on previous data, we tested 5mg/ml alginate cross linked with CaCl2 or 5mg/ml alginate cross linked PDB according to Culture and induction in hydrogel panel protocol.

Centrifuge the bacteria culture in its early exponential stage at 4000rpm for 3min.

Discard supernatant and resuspend it with 5mg/ml alginate at reach OD 0.15~0.2.

Seed 100ul alginate mixed with alginate into 96 well plate.

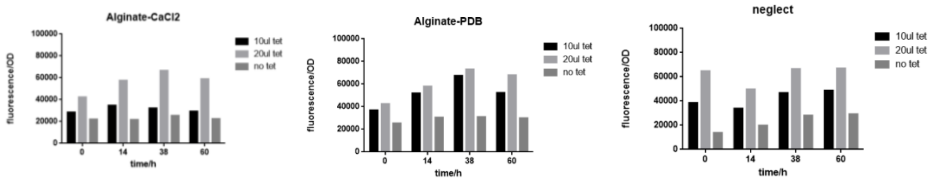
Add 40ul cross-linking agent（0.025M CaCl2 or 1.25mg/ml PDB）to each well.

Add 10ul or 20ul tetracycline of 2mg/ml to each well.

Measure OD600 and florescence under microplate reader at time intervals.

	10ul tet				20ul tet			不诱导		
荧光/OD	19397.85	26625.74	32833.54	34666.03	34996.18	36446.01	55515.48	22370.38	25335.49	18371.42
0h 开始诱导										
	34916.08	34055.89	41002	37114	41515.57	41017.76	44605.33	25347.3	25658.88	24838.01
	34566.86	45946.4	39426.42	33488.19	52402.4	52214.62	89518.4	15826.73	12607.94	12980.87
	10ul tet				20ul tet			不诱导		
荧光/OD	24004.31	34015.96	37234.58	43283.99	43853.12	41779.07	86957.46	22673.23	23212.32	19239.39
14h										
	48673.95	52131.99	53479.28	53071.67	59573.41	56155.54	58223.11	31474.46	30636.2	29135.75
	31723.72	36957.94	33338.28	33001.16	43090.09	43204.73	62758.83	22397.74	18196	18808.15

	10ul tet				20ul tet			不诱导		
荧光/OD	25519.66	31193.19	30535.07	41186.41	44822.87	54657.83	100151.4	22671.57	28355.05	25034.86
38h										
	63016.29	69248.54	69222.03	68378.66	76039.19	68856.64	74349.95	31540.68	31224.8	30128.62
	43660.46	50504.3	46438.22	46182.83	58567.82	58079.63	82557.36	32992.04	25639.85	25437.06
	10ul tet				20ul tet			不诱导		
荧光/OD	24998.33	30014.36	21330.91	40667.41	29735.43	55877.13	91328.03	14400.9	30636.22	22291.34
60h										
	46872.52	55178.09	55131.18	52095.87	71292.31	63386.64	69241.23	32379.32	28765.91	28457.45
	45761.97	53323.51	47754.88	47456.71	60011.66	58691.95	81899.77	34060.27	27110.44	26361.35



The volume of tetracycline didn't affect the results too much and alginate cross-linked by CaCl2 or PDB were practical media for bacteria culture. The third method.