Notebook for off-target

Clone

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 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 pCDFDuet-1 was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed double distilled water.

name	concentration
pCDFDuet-1	82.2ng/µL

7.7

Nice and easy.

7.8

7.9 设计引物啦

7.10

1.chemical transformation

We received P58-pML2-EGFP-P2A-ZF21-16-KRAB from Fudan 2017 iGEM team.

2ul of circular plasmid is mixed with 50ul competent DH5α strain. Finally, the cells are spread on LB plates containing 50ug/ml ampicillin and grow overnight at 37°C.

7.11

1.Overnight culture

Inoculate 5 mL of LB medium with antibiotics with a single colony of freshly grown E. coli containing pCDBD. Incubate at 37℃ with shaking (250rpm) overnight.

7.12

1.Plasmid Isolation

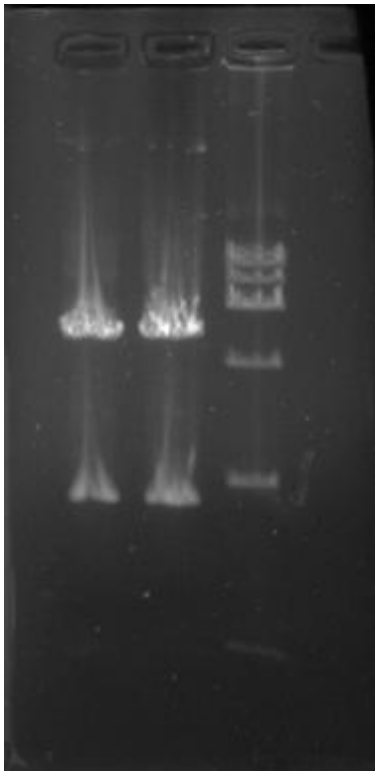
An overnight starter culture of E. coli DH5α harboring plasmid P58-pML2-EGFP-P2A-ZF21-16-KRAB was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30μL of pre-warmed double distilled water.

name	concentration
P58-pML2-EGFP-P2A-ZF21-16-KRAB	79.2ng/μL

2.pcr cloning

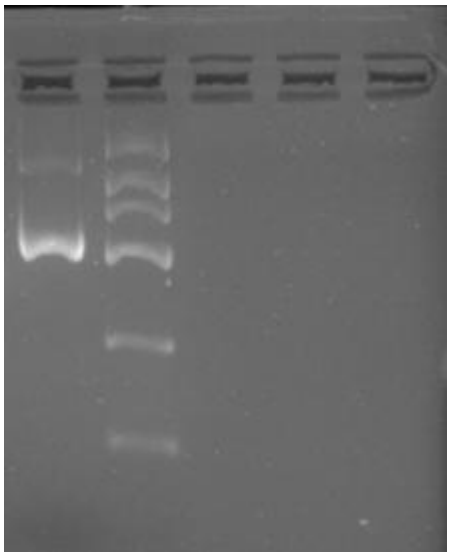
In order to get DNA fragment of DBD protein,we used P58-pML2-EGFP-P2A-ZF21-16-KRAB as the template of PCR.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	51/53/55℃	30S
extension	68℃	25S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	25S
Final extension	68℃	5min
Incubation	4℃	



7.13

1. To get a fusion protein composed of DBD protein and C-luciferase protein,we need a contact part for both of the proteins.We devised another reverse primer for the DNA fragment of DBD protein.
2. After the PCR,we purify the product from the TBE-agrose gel.



3.Bacteria recovery and stock preparation

We happily received the gift from Peking university, that is DH5α containing Pndcas9 or pcdcas9 plasmid in stab.

Inoculate 10 mL of LB medium with antibiotics with colony of E. coli containing either Pndcas9 or pcdcas9 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.

7.14

1.Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring plasmid pcdcas9 and pndcas9 were subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30μL of pre-warmed double distilled water.

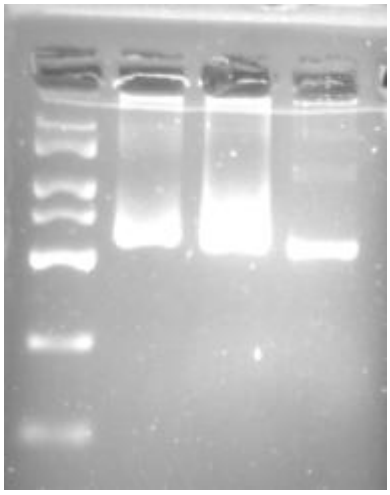
name	concentration
pcdcas9	137.3ng/μL
pndcas9	92.5ng/μL

7.15

1.C-luciferase PCR

We tried for the first time to get DNA fragment for C-luciferase protein.

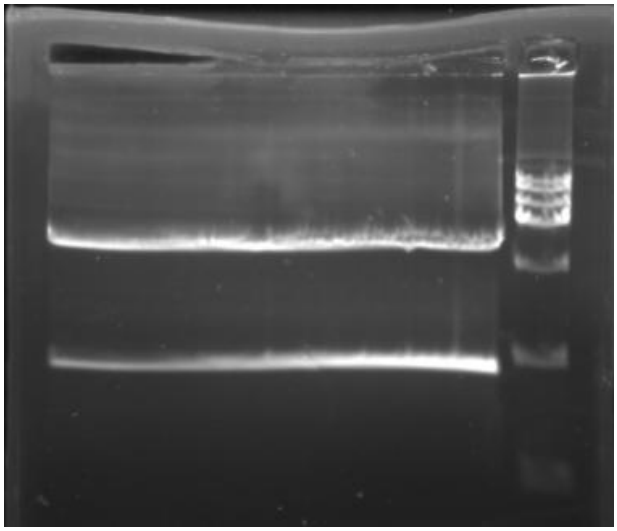
step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55℃	30S
extension	68℃	20S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	20S
Final extension	68℃	5min
Incubation	4℃	



It seemed successful at glance of the gel,so we purified the product.

2.N-luciferase PCR

We tried for the first time to get DNA fragment for N-luciferase protein.



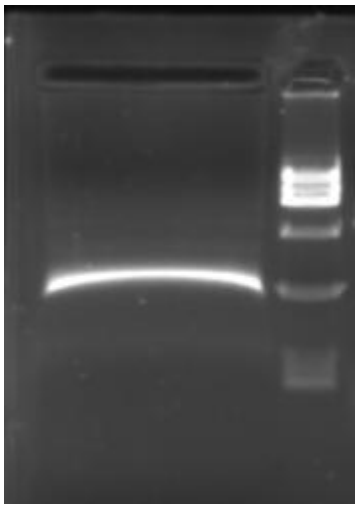
It was bad result,for neither of the band was correct.

3. overlapping PCR

To get the fusion protein of C-luciferase and DBD protein,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55/57/59℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	30S

Final extension	68℃	5min
Incubation	4℃	



The result was good.We purified the DNA fragment out of the gel.

7.16

1. Enzyme digestion of DNA of C-luciferase-DBD fusion protein and pCDFDuet-1

composition	Cluciferase-DBD DNA	pCDFDuet-1
DNA	11μL	9.5μL
10×Fast digestion buffer	5μL	5μL
Not I	1.25μL	1.25μL
Nde I	1.25μL	1.25μL
Double distilled water	31.5μL	33μL

We digested the DNA for 3 hours.

2. Purification of the backbone and the fragment

We used the PCR recycle kit and purified the digested backbone and fragment.

3. Enzyme ligation of C-luciferase-DBD fusion protein DNA and pCDFDuet-1

We ligated the DNA fragment and pCDFDuet-1 with T4 ligase.

composition	Volume
Cluciferase-DBD DNA	11μL
pCDFDuet-1	5μL
T4 ligase	1.25μL
5×T4 ligase buffer	4μL
Double distilled water	31.5μL

4. Transformation of DH5α with pCDFDuet-C-luciferase-DBD

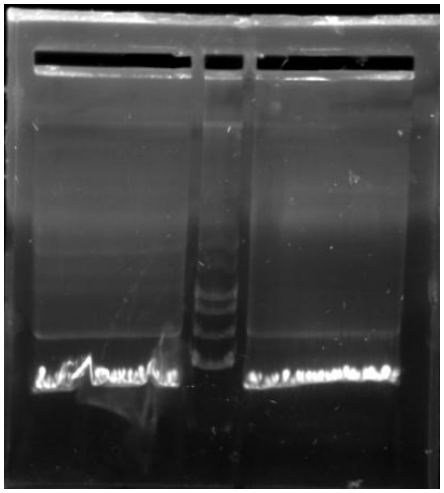
We transformed DH5α with the ligation products.

7.17

1. Exploration of NdCas9 PCR

We tried another set of conditions NdCas9 PCR.

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	52℃	30S
extension	68℃	180S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	62/64/68℃	30S
extension	68℃	180S
Final extension	68℃	5min
Incubation	4℃	



And we failed.

2. We designed new primers for NdCas9.
3. Colony PCR

We tested the transformed colonies on the LB-agar culture with antibiotics by colony PCR mix,but we got no positive results.

7.18

1.Enzyme ligation of C-luciferase-DBD fusion protein DNA and pCDFDuet-1

We ligated the DNA fragment and pCDFDuet-1 with T4 ligase.We tried two kinds of ratio of fragment and backbone.

Composition(3:1)	Volume
Cluciferase-DBD DNA	9.8μL
pCDFDuet-1	7.2μL
T4 ligase	2.5μL
10×T4 ligase buffer	2.5μL
Double distilled water	3μL

Composition(2:1)	Volume
Cluciferase-DBD DNA	6.6μL
pCDFDuet-1	7.2μL
T4 ligase	2.5μL
10×T4 ligase buffer	2.5μL
Double distilled water	6.2μL

We ligated for 12 hours.

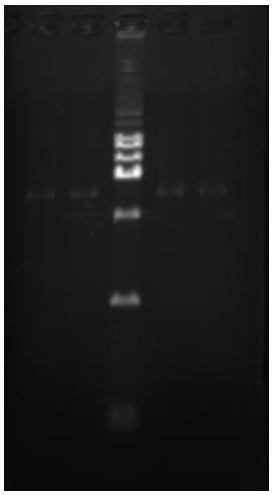
7.19

1. Attempt of conditions of new primers for NdCas9

We tried conditions of new primers for NdCas9.

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	51℃	30S
extension	68℃	180S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	62/64/66℃	30S
extension	68℃	180S
Final extension	68℃	5min
Incubation	4℃	

2. Electrophoresis of the product of N-luciferase DNA



It was not the product we wanted.

3.Transformation of DH5α

We transformed DH5α with the ligation products.

7.20

1.Colony PCR for transformed DH5α on 7.19

We had 7 colonies on one LB-agar culture plate,but none of them were correct by colony PCR.

2.Inoculation of DH5α with PCDFDuet-1

7.21

1.Enzyme digestion of DNA of C-luciferase-DBD fusion protein and pCDFDuet-1

composition	Cluciferase-DBD DNA	pCDFDuet-1
DNA	9.8μL	12.3μL
10×Fast digestion buffer	2μL	2μL
Not I	0.5μL	0.5μL
Nde I	0.5μL	0.5μL
Double distilled water	6.2μL	4.7μL

We digested the DNA for 3 hours.

2.Purification of DNA fragment from TBE-agrose gel

We used gel extraction kit to purify the digested fragment and backbone.

3.Enzyme ligation of C-luciferase-DBD fusion protein DNA and pCDFDuet-1

We ligated the DNA fragment and pCDFDuet-1 with T4 ligase.We tried two kinds of ratio of fragment and backbone.

Composition(3:1)	Volume
Cluciferase-DBD DNA	8μL
pCDFDuet-1	5.6μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	2.3μL

We ligated for 2 hours at room temperature.

4.Transformation of DH5α

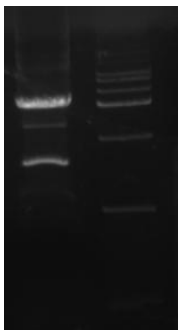
We transformed DH5α with the ligation products.

7.22-7.23

Nice and easy.

7.24

1. Redo the PCR of NdCas9 DNA on 7.19



Concerning the length of product,this was exactly what we wanted.

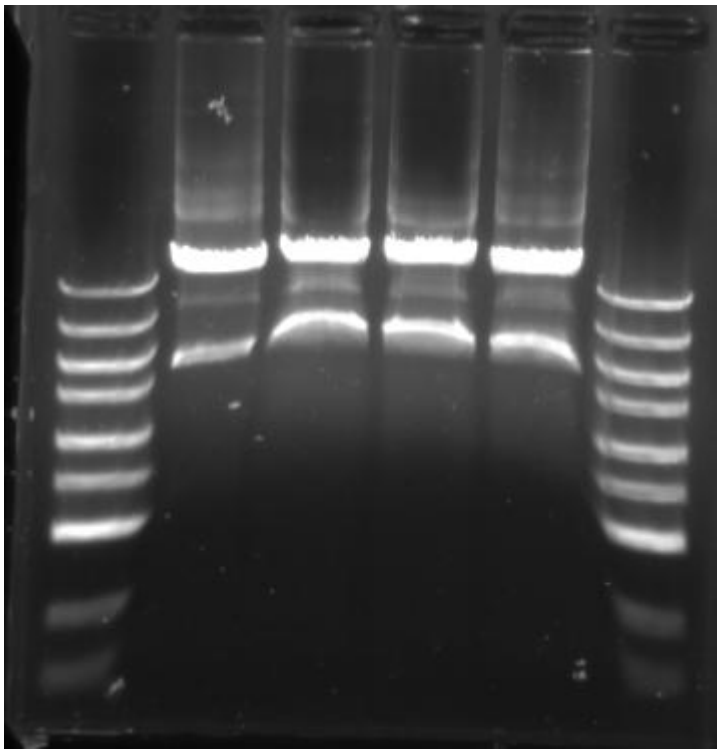
2.Conservation of bacteria DH5α with sgRNA and DH5α with pUC57 harboring DBD binding site and target for NdCas9 spaced by 5-45 base pairs

We kept the bacteria on LB-agar culture plates with different antibiotics,and isolated single colonies.

7.25

1. PCR of NdCas9 DNA

The PCR of NdCas9 on 7.24 seemed a success,so we aimed to get more PCR product.



2. Inoculation of DH5α

We inoculated the colonies on the LB-agar culture plate mentioned in note of 7.24 to liquid LB culture for more plasmids.

3.Culturing bacteria from the selection plate

We chose 5 colonies out of the plate of transformation on 7.21,and cultured it in LB liquid culture for extraction of plasmid.

7.26

1.Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring different kinds of plasmids was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed double distilled water.

name	concentration
pUC57-15bp	105.3ng/µL
pUC57-35bp	87.6ng/µL
pUC57-sgRNA	77.2ng/µL

2.overlapping PCR

To get the fusion protein of C-luciferase and DBD protein,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	30S
Final extention	68℃	5min
Incubation	4℃	

3.Purification of the product of PCR

The PCR product was correct at glance of electrophoresis result,and we purified it with gel extraction kit.

4.Plasmid isolation

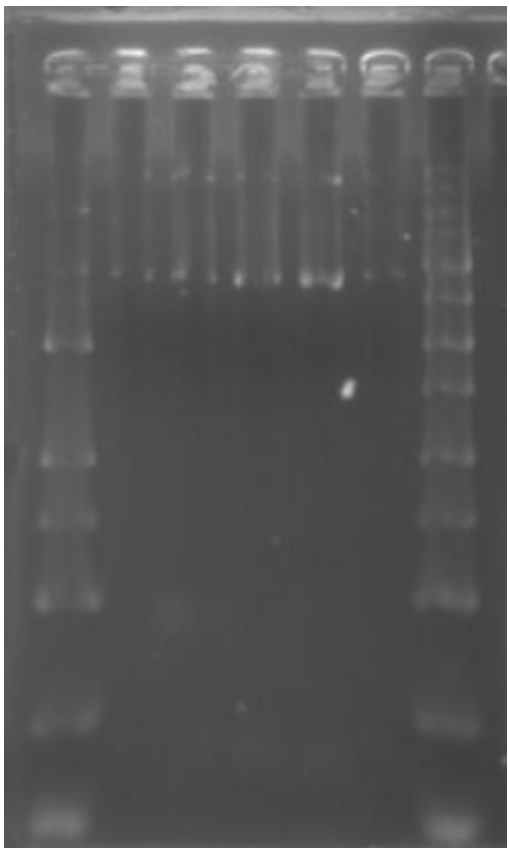
We isolated plasmid out of the culture mentioned on 7.25.

name	concentration
pCDFDuet-CD(For check)#1	67.9ng/µL
pCDFDuet-CD(For check)#2	76.1ng/µL
pCDFDuet-CD(For check)#3	92.8ng/µL
pCDFDuet-CD(For check)#4	58.3.9ng/µL
pCDFDuet-CD(For check)#5	72.7ng/µL

5.Enzyme digestion of plasmid

To check whether the plasmid from the transformed bacteria was successfully ligated,we

digested them with Not Ⅰ and Nde Ⅰ.



It seemed successful,so the colonies could be used to produce more plasmid harboring C-luciferase-DBD.

7.27

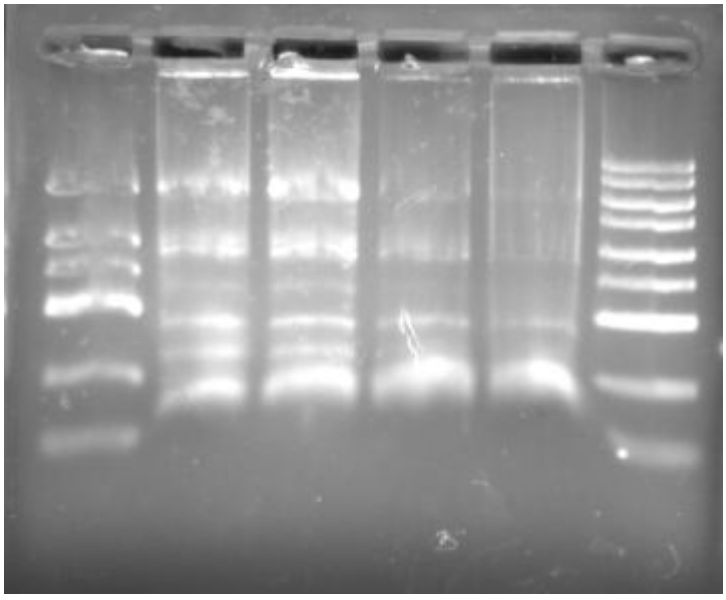
1.PCR for DNA fragment of sgRNA

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	41℃	30S
extension	68℃	28S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	28S
Final extention	68℃	5min
Incubation	4℃	

The product of PCR was correct.

3. Overlapping PCR for C-luciferase-DBD-sgRNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	53℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	61℃	30S
extension	68℃	62S
Final extention	68℃	5min
Incubation	4℃	



We got two bands at about 2kbp.That was the desired product.We planned to improve the conditions of the PCR.

7.28

1.Inoculation of DH5α

We inoculated the colonies on the LB-agar culture plate of pUC57-sgRNA and pUC57-5bp,pUC57-15bp ,pUC57-25bp,pUC57-35bp,pUC57-45bp mentioned in note of 7.24 to liquid LB culture for more plasmids.

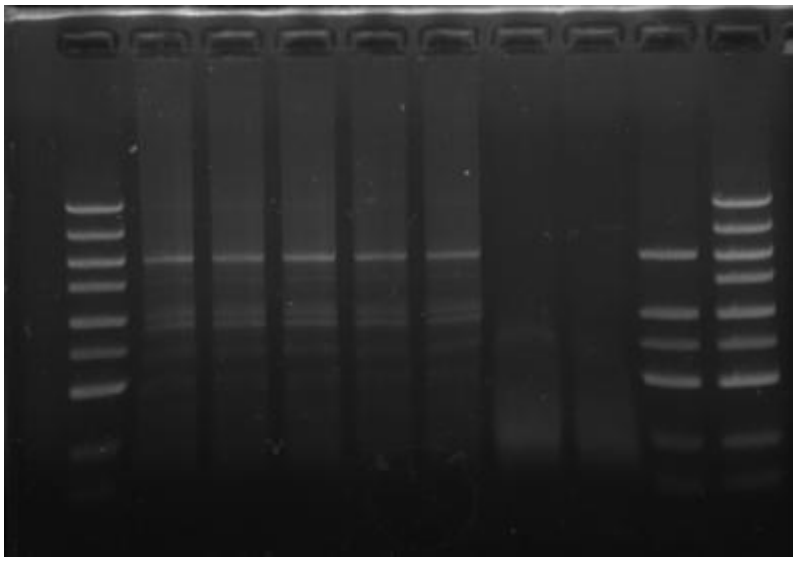
name	concentration
pUC57-5bp	62.5ng/μL
pUC57-15bp	79.4ng/μL

pUC57-25bp	73.8ng/μL
pUC57-35bp	102.2ng/μL
pUC57-45bp	90.2ng/μL
pUC57-sgRNA	82.8ng/μL

7.29

1. Overlapping PCR for C-luciferase-DBD-sgRNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	53℃	30S
extension	68℃	40S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



2. Purification of PCR product from TBE-agrose gel

We used gel extraction kit to purify the product.

3. Enzyme digestion of DNA of C-luciferase-DBD-sgRNA and pCDFDuet-1

composition	Cluciferase-DBD-sgRNA DNA	pCDFDuet-1
DNA	5.9μL	9.8μL

10×Fast digestion buffer	2μL	2μL
Not I	1μL	0.5μL
Kpn I	1μL	0.5μL
Double distilled water	20.1μL	6.2μL

We digested the DNA for 3 hours.

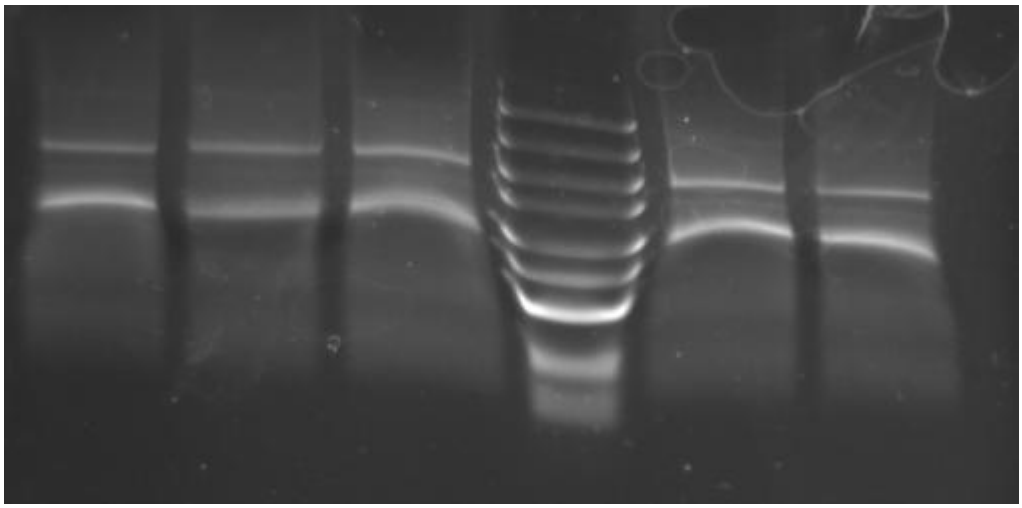
4. Purification of enzyme digestion product

We used the PCR product purification kit to purify the digested DNA.However,the concentration of the product was low.We did not do ligation experiment because of the low concentration.

7.31

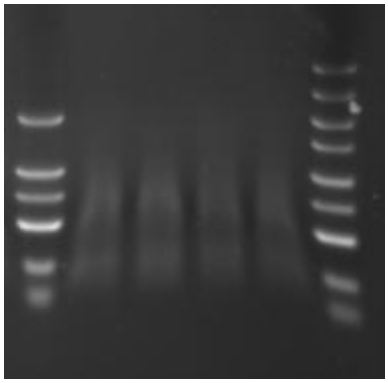
1. Attempt of improvement of overlapping PCR of C-luciferase-DBD-sgRNA DNA

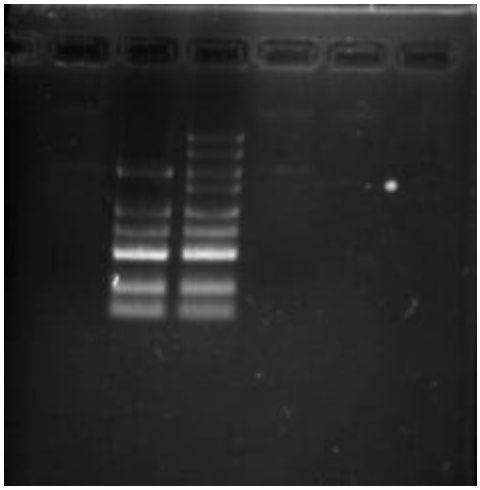
step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	53℃	30S
extension	68℃	40S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	55/59/61/63/65℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



That was not what we wanted.

We Tried for two more times.





And we failed.

8.1

1.Enzyme digestion of DNA of C-luciferase-DBD-sgRNA and pCDFDuet-1

composition	Cluciferase-DBD-sgRNA DNA	pCDFDuet-1
DNA	27μL	14μL
10×Fast digestion buffer	2μL	2μL
Not Ⅰ	1μL	1μL
Kpn Ⅰ	1μL	1μL
Double distilled water	/	2μL

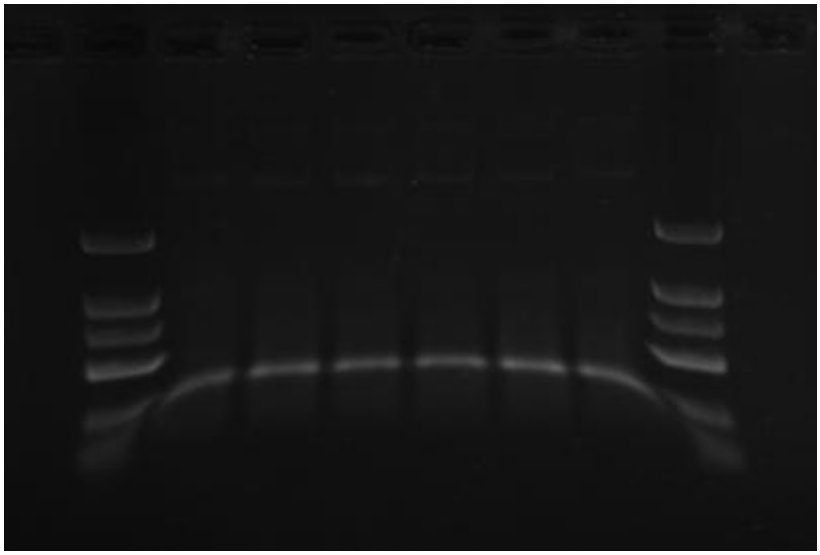
2. PCR product purification

We used the recycle kit to purify the digested product.

3. The trial of overlapping PCR for C-luciferase-DBD-sgRNA DNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S

Annealing	53℃	30S
extension	68℃	40S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	54/57/60/63/65℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



And it was a failure.

8.2

1. PCR primer design

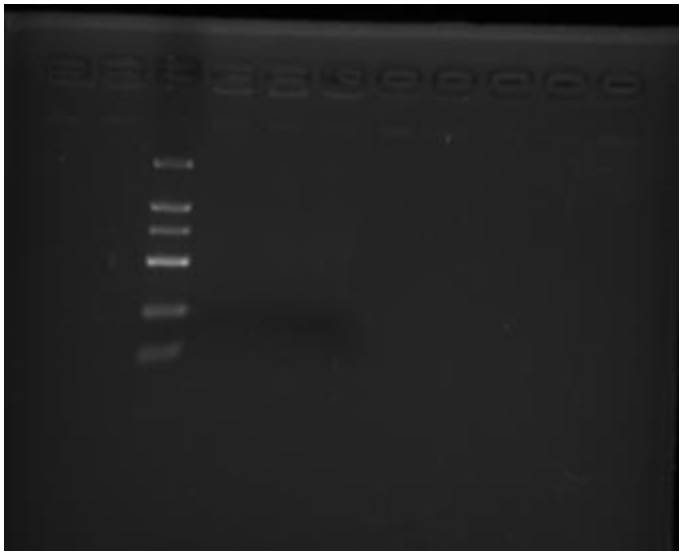
In order that we could detect the protein product,we aimed to bring Flag tag to the C-terminal of C-luciferase-DBD protein.We designed new PCR primer for the tag.

8.3

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	40/44/48/52/56℃	30S
extension	68℃	7S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	56℃	30S
extension	68℃	7S

Final extention	68℃	5min
Incubation	4℃	

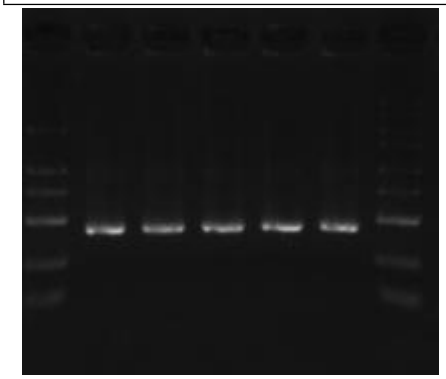
1.Condition test of PCR for DNA of DBD protein with Flag tag



And we failed.This might be the lack of ingredients,or the bad condition.

2.Second condition test of PCR for DNA of DBD protein with Flag tag

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	36/40/44/48/52℃	30S
extension	68℃	7S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	56℃	30S
extension	68℃	7S
Final extention	68℃	5min
Incubation	4℃	



This time,it was good.

2. Purification of DNA of DBD protein with Flag tag

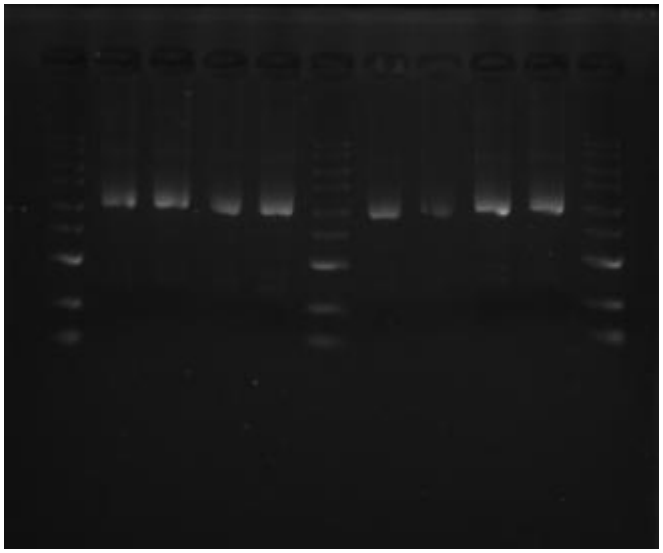
We used the recycle kit to purify the PCR product.

8.4

1. Overlapping PCR

To get the fusion protein of C-luciferase and DBD protein with Flag tag,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	35S
Final extention	68℃	5min
Incubation	4℃	



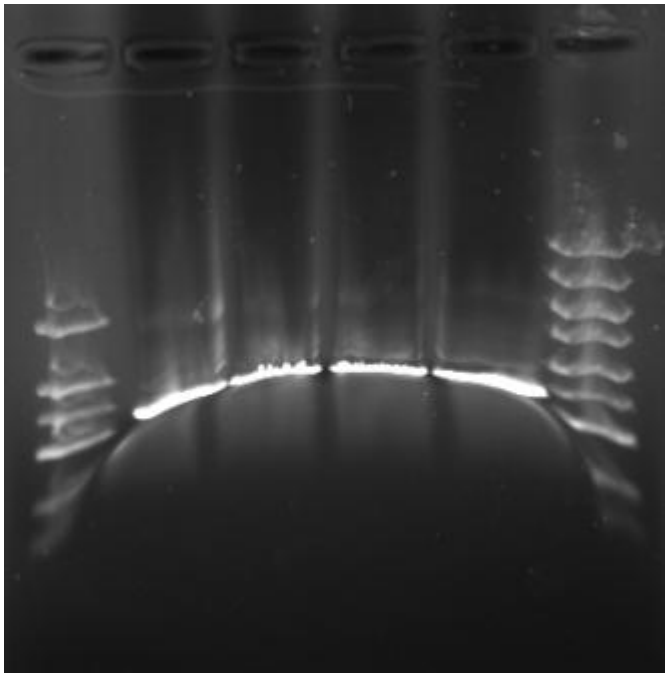
The result was good.

2. PCR product purification

We used gel extraction kit to purify the PCR product.

3.Attempt of overlapping PCR of C-luciferase-DBD(with tag)-sgRNA DNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	44/47/50/53℃	30S
extension	68℃	40S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



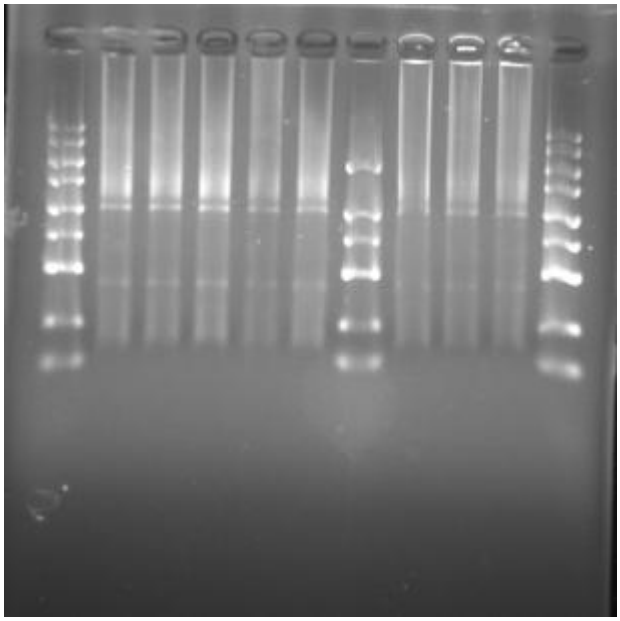
And we failed.

8.5

1. Attempt of overlapping PCR of C-luciferase-DBD(with tag)-sgRNA DNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S

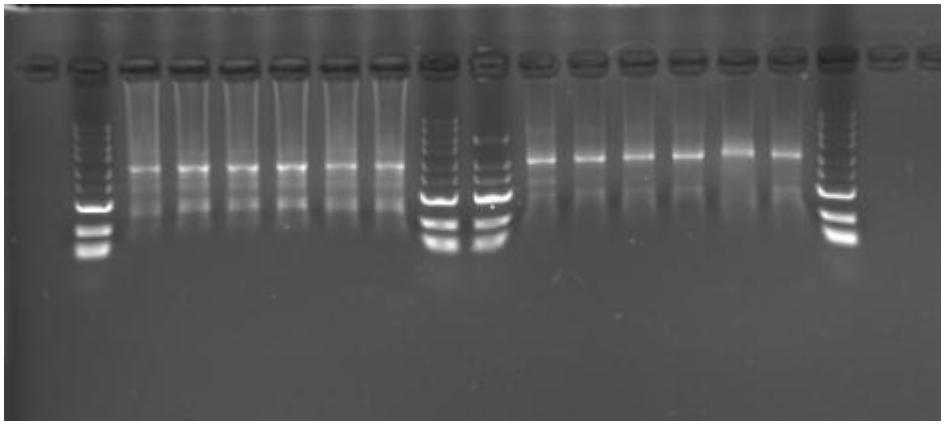
Annealing	47/53℃	30S
extension	68℃	40S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



And we failed.

2. Another attempt of overlapping PCR of C-luciferase-DBD(with tag)-sgRNA DNA

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	47/50/53/56℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



And we failed again.

8.6

1.ligation of backbone and two fragments

We had failed for times in overlapping PCR for C-luciferase-DBD-sgRNA,so we planned to try ligation of two fragments and the backbone.

composition	Volume
Cluciferase-DBD DNA	5.5μL
pCDFDuet-1	2.4μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	3.4μL
sgRNA DNA	4.6μL

2.Transformation of DH5α

We transformed the ligation product to DH5α.

8.7

1.Culturing bacteria from the selection plate

We chose 5 colonies out of the plate of transformation on 8.6,and cultured it in LB liquid culture for extraction of plasmid.

8.8

1.Plasmid Isolation

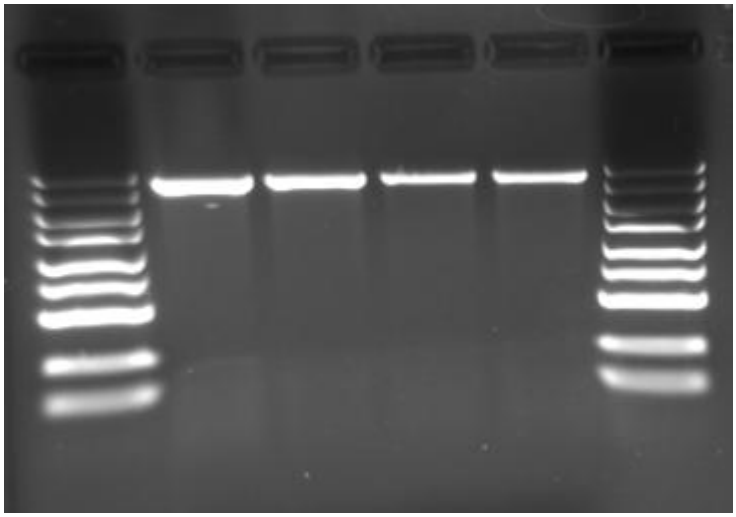
An overnight starter culture of E. coli DH5α harboring plasmid pCDFDuet-1 with C-D-S was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 μL of pre-warmed double distilled water.

name	concentration
pCDFDuet-1 with C-D-S #1 for test	83.6ng/μL
pCDFDuet-1 with C-D-S #2 for test	78.9ng/μL
pCDFDuet-1 with C-D-S #3 for test	90.3ng/μL
pCDFDuet-1 with C-D-S #4 for test	76.5ng/μL

2.Enzyme digestion of pCDFDuet-1 with C-D-S

composition	pCDFDuet-1 with C-D-S
DNA	16μL
10×Fast digestion buffer	2μL
Kpn I	1μL
Nde I	1μL
Double distilled water	/

We digested the DNA for 4 hours.



All negative,with no digested small fragment.

3.ligation of backbone and two fragments

We tried ligation of two fragments and the backbone.

composition	Volume
Cluciferase-DBD DNA	5.5μL
pCDFDuet-1	3.5μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	1.9μL
sgRNA DNA	5μL

4.Transformation of DH5α

We transformed DH5α with the ligation products.

8.9

1..Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring plasmid pCDFDuet-1 with C-D-S was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 μL of pre-warmed double distilled water.

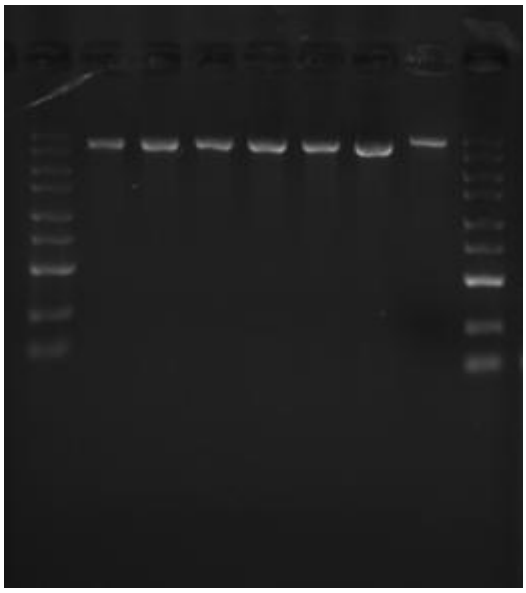
name	concentration
pCDFDuet-1 with C-D-S #1 for test	76.2ng/μL
pCDFDuet-1 with C-D-S #2 for test	82.3ng/μL
pCDFDuet-1 with C-D-S #3 for test	70.9ng/μL

pCDFDuet-1 with C-D-S #4 for test	102.4ng/μL
pCDFDuet-1 with C-D-S #5 for test	92.5ng/μL
pCDFDuet-1 with C-D-S #6 for test	88.7ng/μL

2.Enzyme digestion of pCDFDuet-1 with C-D-S

composition	pCDFDuet-1 with C-D-S
DNA	16μL
10×Fast digestion buffer	2μL
Kpn I	1μL
Nde I	1μL
Double distilled water	/

We digested the DNA for 4 hours.



There were no small fragment after digestion.All negative.

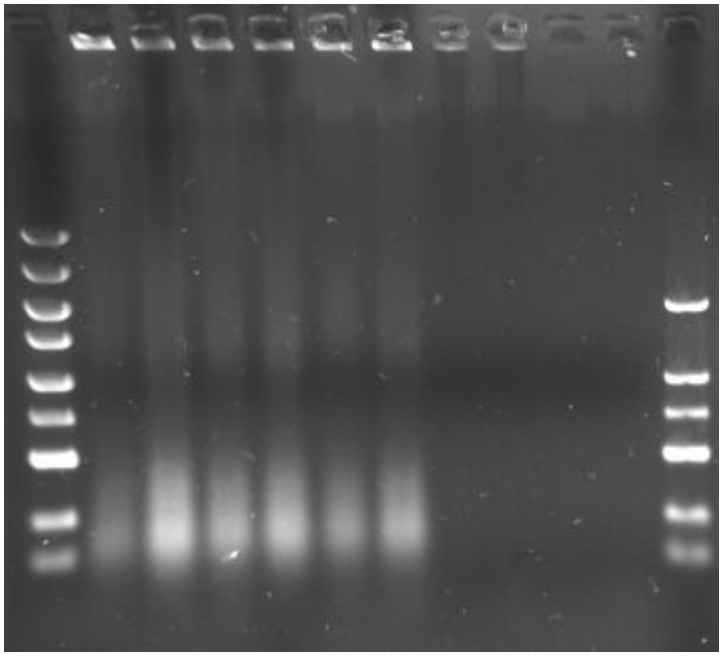
8.10

1.colony PCR

We wanted to know if there were any positive colonies on the LB-agar plate of transformed DH5α on 8.8.We used colony PCR to test another 6 colonies by sgRNA primers.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S

Final extention	72℃	2min
Incubation	4℃	

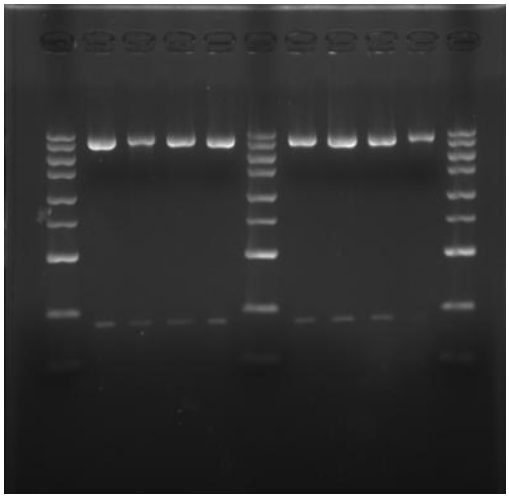


No negative result.

2.Enzyme digestion of pCDFDuet-1 with C-D-S

We supposed that on 8.9,there was no small fragment after digestion because the enzyme was expired.We changed another combination of digestion enzyme and digested the same batch of plasmid.

composition	pCDFDuet-1 with C-D-S
DNA	16μL
10×Fast digestion buffer	2μL
Kpn I	1μL
Sal I	1μL
Double distilled water	/



There were small fragments,but the length was not correct.The correct length was about 1000 base pairs.There were no positive results in the batch.

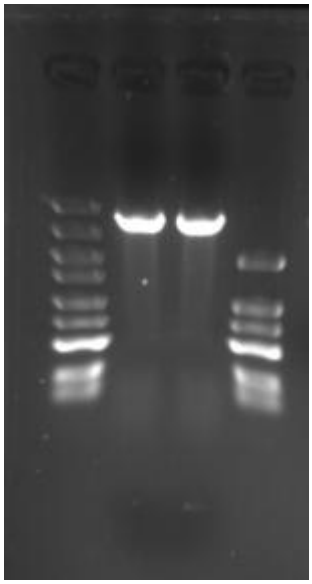
8.11

1.Enzyme digestion of pCDFDuet-1 and sgRNA

We thought that it was too difficult to ligate the digested backbone and the two fragements.So,we had to do digestion-ligation-seletion for two times.

This time,we first ligate sgRNA part and the pCDFDuet-1.

composition	pCDFDuet-1	sgRNA
DNA	12μL	4.5μL
10×Fast digestion buffer	2μL	2μL
Kpn I	1μL	1μL
Nde I	1μL	1μL
Double distilled water	4μL	21.5μL



2.DNA purification

We purify the digested plasmid and fragment with gel extraction kit and recycle kit.

3.Enzyme ligation of sgRNA DNA and pCDFDuet-1

We ligated the DNA fragment and pCDFDuet-1 with T4 ligase.

composition	Volume
sgRNA DNA	7.8μL
pCDFDuet-1	3.6μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	4.5μL

4.Transformation of DH5α

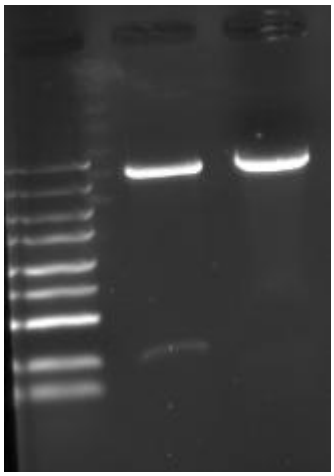
We transformed DH5α with the ligation products.

8.12

1.Enzyme digestion of pCDFDuet-1, sgRNA and C-luciferase-DBD DNA

We planned to do the ligation of sgRNA-plasmid and C-D-plasmid at the same time

composition	pCDFDuet-1(BglⅡ, XhoⅠ)	sgRNA(BglⅡ, XhoⅠ)	pCDFDuet-1 (NotⅠ, KpnⅠ)	Cluc-DBD(NotⅠ, KpnⅠ)
DNA	12μL	13.6μL	12μL	16μL
10×Fast digestion buffer	2μL	2μL	2μL	2μL
Enzyme1	1μL	1μL	1μL	1μL
Enzyme2	1μL	1μL	1μL	1μL
Double distilled water	4μL	2.4μL	4μL	/



2.DNA purification

We purify the digested plasmid and fragment with gel extraction kit and recycle kit.

3.Enzyme ligation of sgRNA DNA and pCDFDuet-1

We ligated the DNA fragments and pCDFDuet-1 with T4 ligase.

composition	Volume
sgRNA DNA	8.6μL
pCDFDuet-1	5μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	2.3μL

composition	Volume
C-luciferase-DBD DNA	9.9μL
pCDFDuet-1	6μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	/

4.Transformation of DH5α

We transformed DH5α with the ligation products.

8.13

1.colony PCR

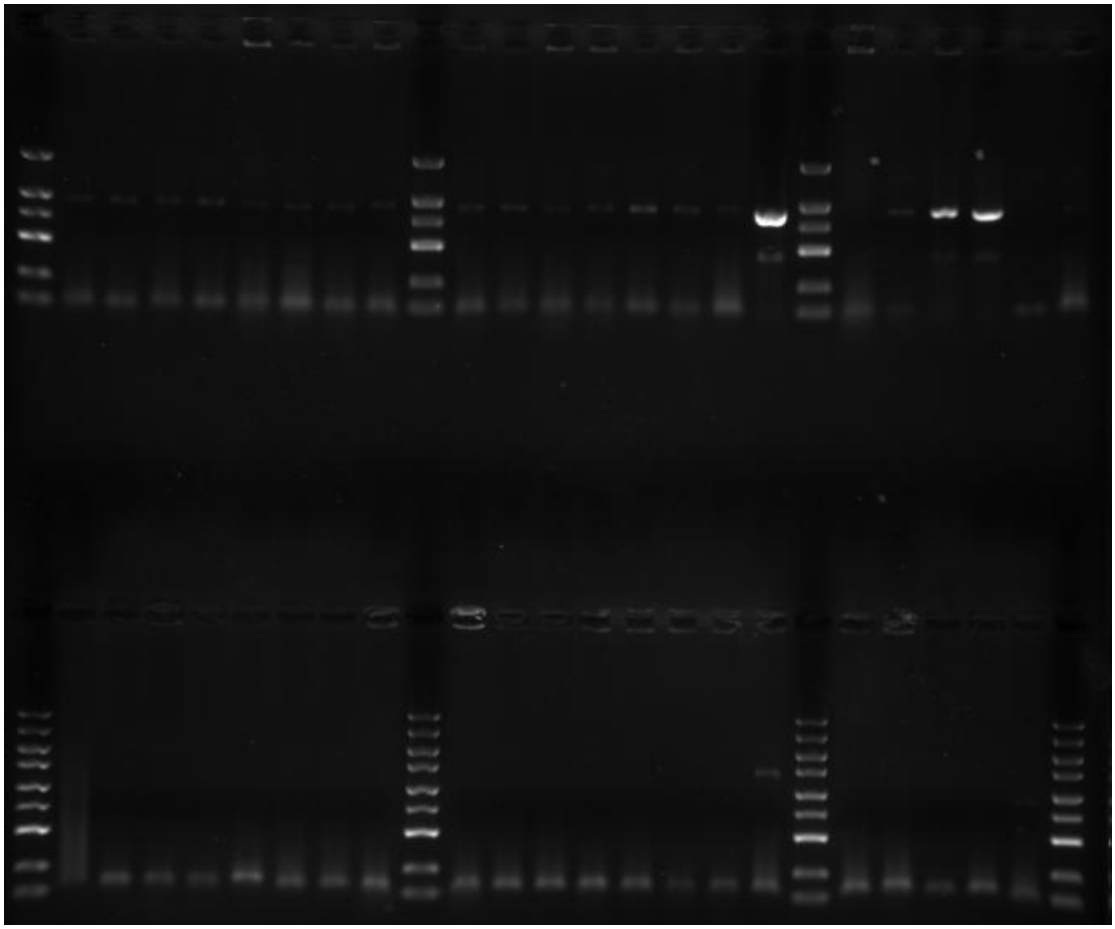
We wanted to know if there were any positive colonies on the LB-agar plate of transformed DH5α on 8.12.We used colony PCR to test the colonies by both sgRNA and DBD primers.

sgRNA primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S
Final extention	72℃	2min
Incubation	4℃	

DBD primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	53℃	10S
extension	72℃	5S
Final extention	72℃	2min
Incubation	4℃	



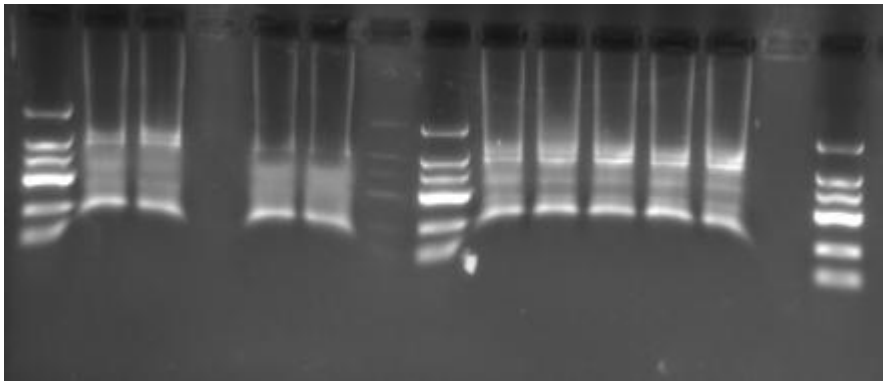
Nothing to see there.

8.14

1.Overlapping PCR for C-luciferase-DBD-sgRNA

We tried this method for one more time to try our luck.

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	53℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	65S
Final extention	68℃	5min
Incubation	4℃	



Still,there was no correct product.

8.15

1.Enzyme digestion of pCDFDuet-1, sgRNA and C-luciferase-DBD DNA

We planned to ligate sgRNA and pCDFDuet-1.

composition	pCDFDuet-1	sgRNA
DNA	12μL	16μL
10×Fast digestion buffer	2μL	2μL
Bgl II	1μL	1μL
Xho I	1μL	1μL
Double distilled water	4μL	/

2.Enzyme ligation of sgRNA DNA and pCDFDuet-1

We ligated the DNA fragments and pCDFDuet-1 with T4 ligase.

composition	Volume (fragment:plasmid=3:1)	Volume(fragment:plasmid=5:1)
sgRNA DNA	2.6μL	4.1μL
pCDFDuet-1	4.5μL	4.5μL
T4 ligase	0.1μL	0.1μL
5×T4 ligase buffer	4μL	4μL
Double distilled water	8.8μL	7.3μL

3.Enzyme ligation of NdCas9 DNA and pET28a(+)

We ligated the DNA fragment and plasmid with T4 ligase.

composition	Volume (fragment:plasmid=3:1)
NdCas9 DNA	10μL
pET28a(+)	5.3μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	0.6μL

4.Enzyme ligation of C-luciferase-DBD DNA and pCDFDuet-1

We ligated the DNA fragment and plasmid with T4 ligase.

composition	Volume (fragment:plasmid=3:1)	Volume(fragment:plasmid=5:1)
C-luciferase-DBD DNA	2.7μL	4.4μL
pCDFDuet-1	3.7μL	3.7μL
T4 ligase	0.1μL	0.1μL
5×T4 ligase buffer	4μL	4μL
Double distilled water	9.5μL	7.8μL

5.Transformation of *E.coli*

We transformed DH5α with C-luciferase-DBD-pCDFDuet-1,sgRNA-pCDFDuet-1 ligation products to DH5α,and BL21 with NdCas9-pET28a(+).

8.16

1.colony PCR

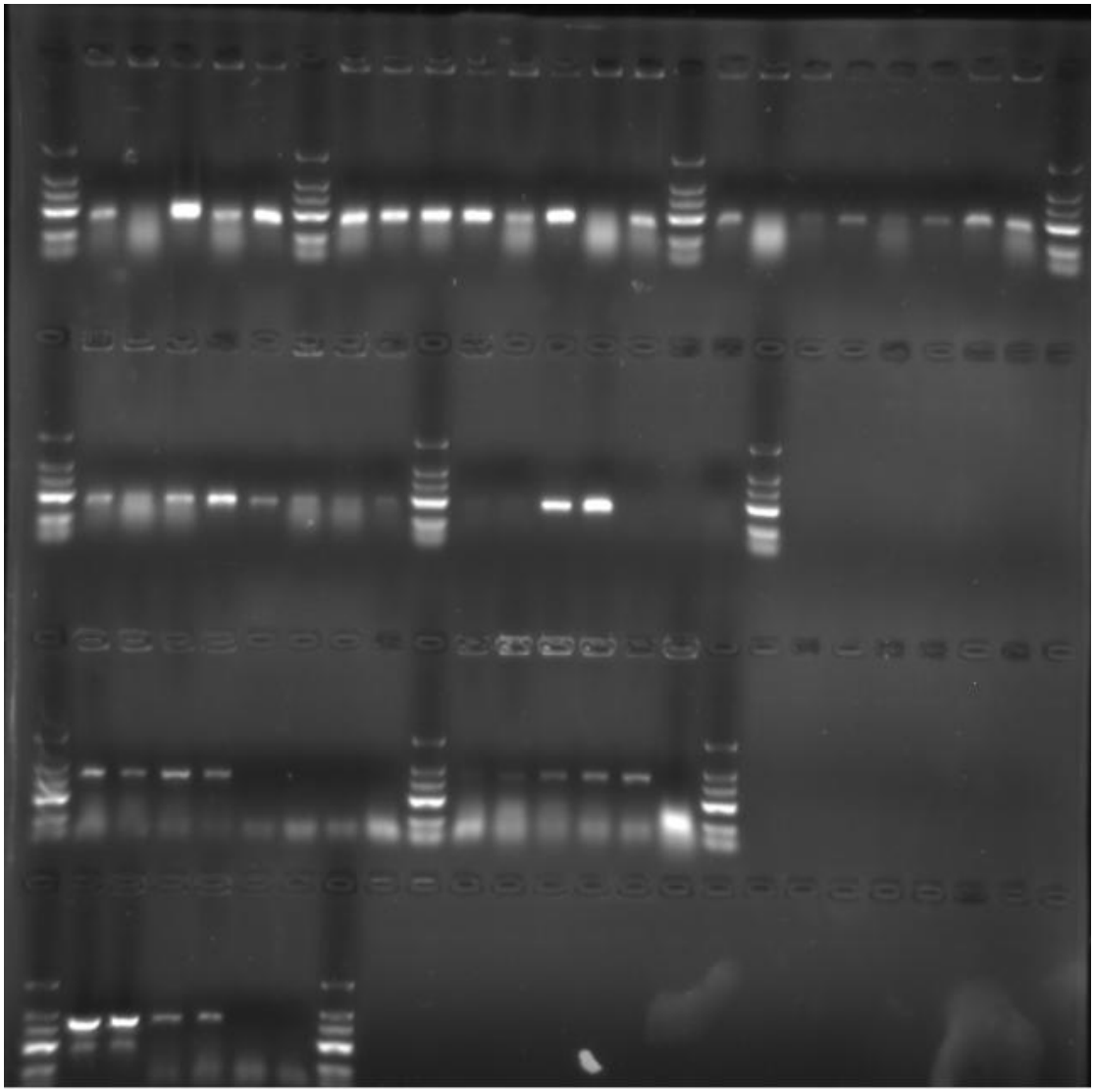
We wanted to know if there were any positive colonies on the LB-agar plate of transformed DH5α on 8.15.We used colony PCR to test colonies by sgRNA and DBD primers.

sgRNA primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S
Final extention	72℃	2min
Incubation	4℃	

DBD primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	53℃	10S
extension	72℃	5S
Final extention	72℃	2min
Incubation	4℃	



There were lots of positive results by DBD primers.
Also,there were some positive results by sgRNA primers.

2.Enzyme ligation of C-luciferase-DBD DNA and pCDFDuet-1
We ligated the DNA fragment and plasmid with T4 ligase.

composition	Volume (fragment:plasmid=3:1)	Volume(fragment:plasmid=5:1)
C-luciferase-DBD DNA	8.6μL	4.4μL
pCDFDuet-1	6μL	3.7μL
T4 ligase	0.1μL	0.1μL
5×T4 ligase buffer	4μL	4μL
Double distilled water	1.3μL	7.8μL

3.Enzyme ligation of sgRNA DNA and pCDFDuet-1
We ligated the DNA fragments and pCDFDuet-1 with T4 ligase.

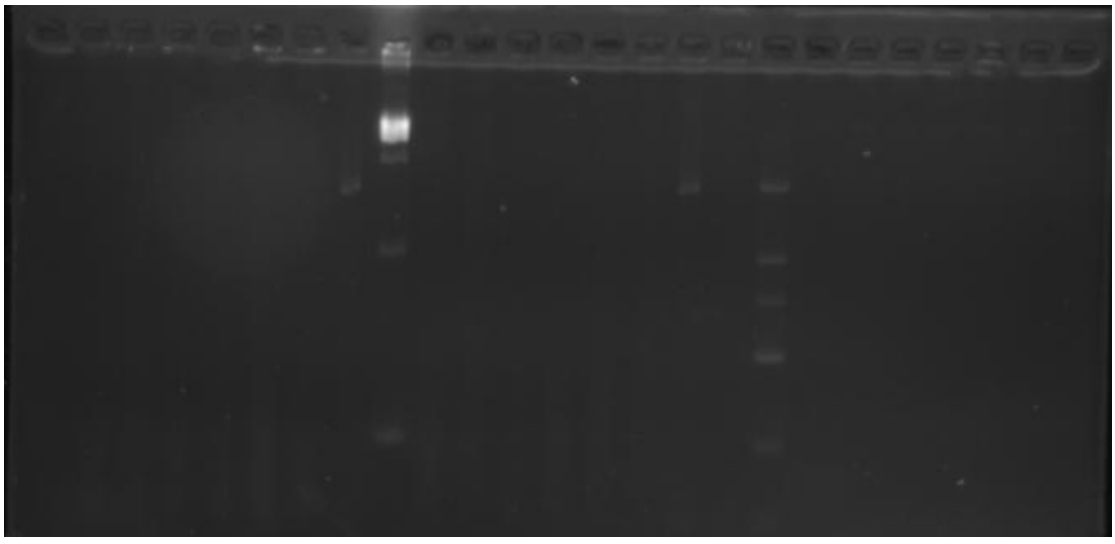
composition	Volume (fragment:plasmid=3:1)

sgRNA DNA	9.5μL
pCDFDuet-1	12μL
T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	/

5.Transformation of DH5α
We transformed DH5α with the ligation products.

8.17
1.colony PCR
We wanted to know if there were any positive colonies on the LB-agar plate of transformed BL21 on 8.15.We used colony PCR to test colonies by NdCas9 primers.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles(with primers)		
denaturation	98℃	10S
Annealing	64℃	10S
extension	72℃	56S
Final extention	72℃	2min
Incubation	4℃	



However,there were no positive results.

2.Overlapping PCR
To get the fusion protein of C-luciferase and DBD protein with Flag tag,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	35S
Final extention	68℃	5min
Incubation	4℃	

The result was good.

3. PCR product purification

We used gel extraction kit to purify the PCR product.

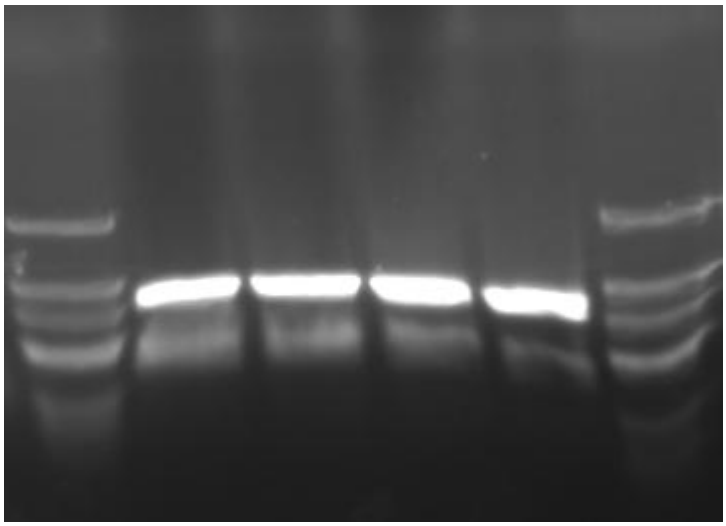
4. Primer design

For quick assembly of two fragments and one backbone,we chose gibson assembly and designed new primers for pCDFDuet-1.

8.18

1.PCR for DNA fragment of sgRNA

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	41℃	30S
extension	68℃	28S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	63℃	30S
extension	68℃	28S
Final extention	68℃	5min
Incubation	4℃	

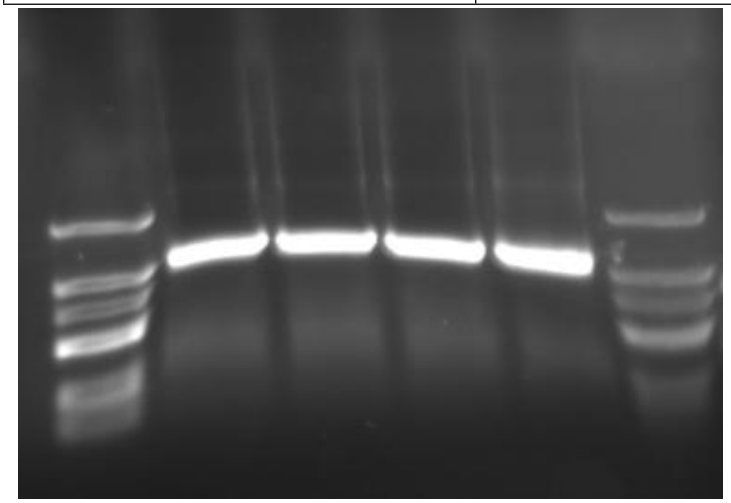


The result was good.

2.C-luciferase-DBD overlapping PCR

To get the fusion protein of C-luciferase and DBD protein,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	46℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	64℃	30S
Extension	68℃	35S
Final extention	68℃	5min
Incubation	4℃	



The result was good.

3. PCR product purification

We used gel extraction kit to purify the PCR product.

4. Gibson assembly

composition	Volume
sgRNA DNA	1.1μL
C-luciferase-DBD DNA	0.8μL
pCDFDuet-1	2.3μL
2× Assembly Mix	10μL
Double distilled water	5.8μL

We incubated the mixture at 55℃ for 30 minutes.

5.Transformation of DH5α with gibson assembly product

We transformed DH5α with the gibson assembly products.

8.19

1.colony PCR

We wanted to know if there were any positive colonies on the LB-agar plate of transformed DH5α on 8.18.We used colony PCR to test 12 colonies by sgRNA and DBD primers.

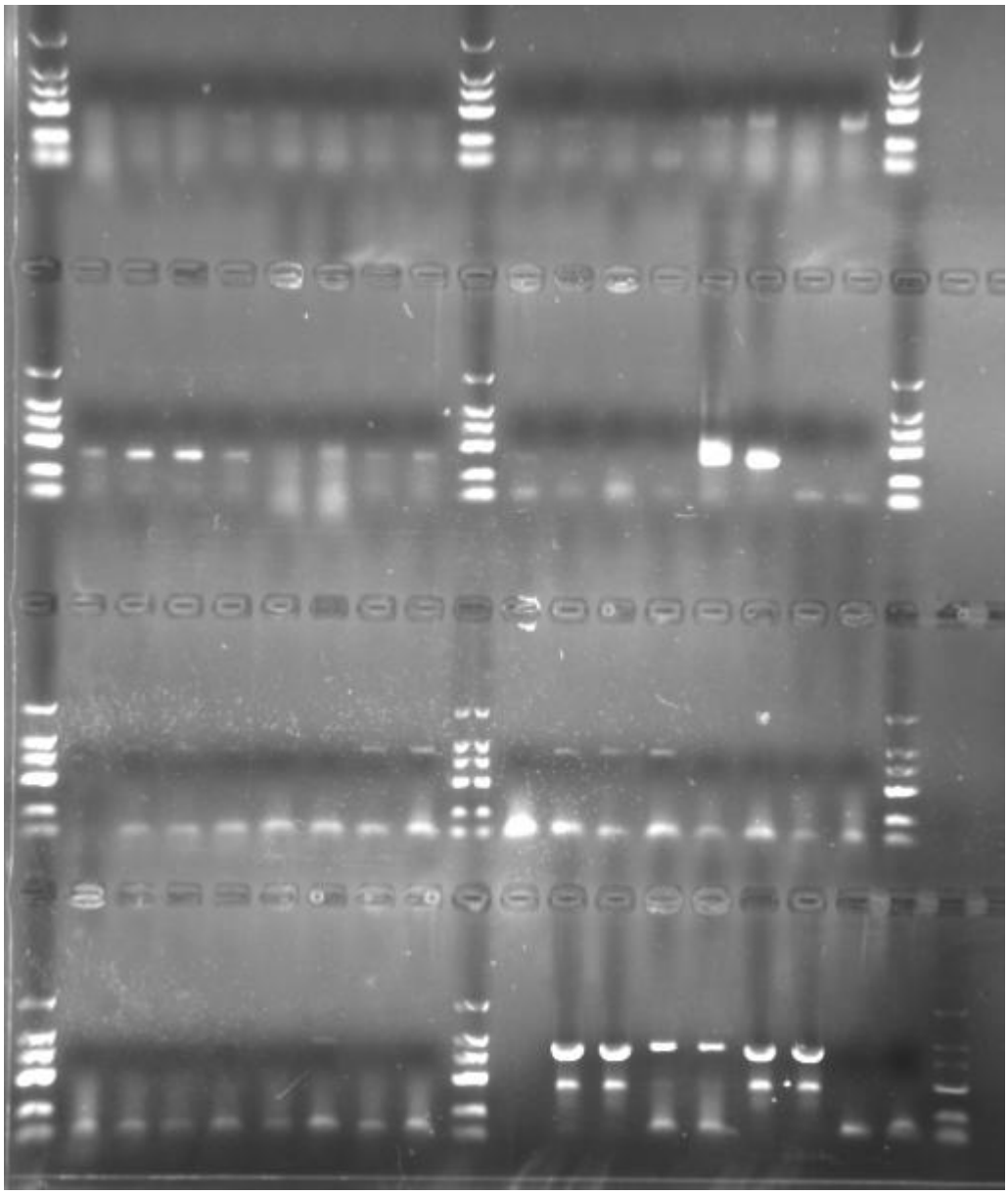
sgRNA primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles(with primers)		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S
Final extention	72℃	2min
Incubation	4℃	

DBD primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles(with primers)		
denaturation	98℃	10S
Annealing	53℃	10S
extension	72℃	5S
Final extention	72℃	2min

Incubation	4℃	

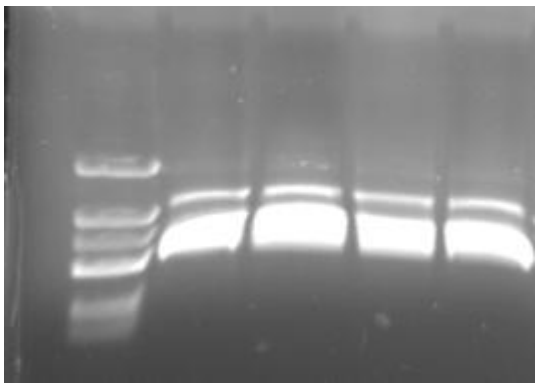


The result showed that there were no double-positive colony in this batch.

3. C-luciferase DNA PCR

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	55℃	30S
extension	68℃	20S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S

extension	68℃	20S
Final extension	68℃	5min
Incubation	4℃	

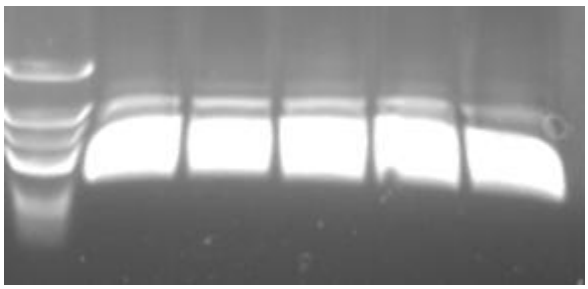


The gel was not well made,so the result seemed ambiguous.

4. DBD DNA PCR

In order to get DNA fragment of DBD protein,we used P58-pML2-EGFP-P2A-ZF21-16-KRAB as the template of PCR.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	50/52/48℃	30S
extension	68℃	16S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	16S
Final extension	68℃	5min
Incubation	4℃	



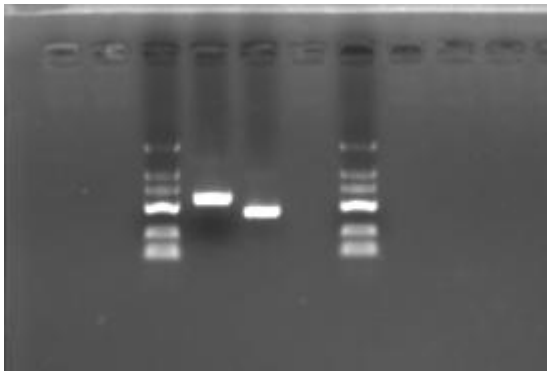
The gel was not well made,so the result seemed ambiguous.

5. Purification of PCR product

We cut the brighter parts of both of the gels,and used gel extraction kit to purify the PCR product.

6. Electrophoresis for product check

Because it was difficult to tell whether the purified PCR products of C-luciferase and DBD were correct in terms of length,we decided to check the purified products with another electrophoresis.



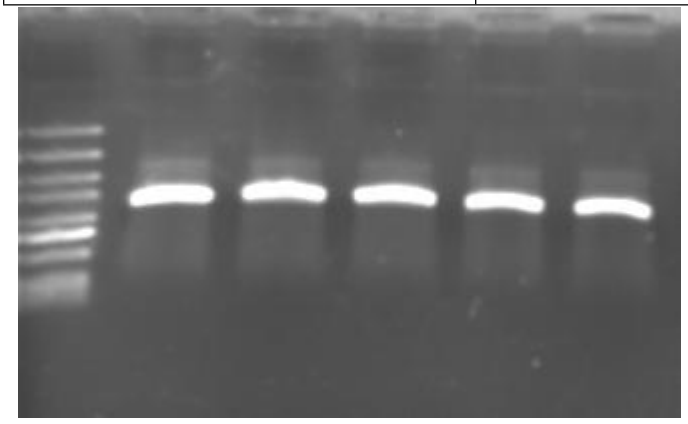
The result suggested that they were correct.

8.22

1. Overlapping PCR of DNA of C-luciferase and DBD

To get the fusion protein of C-luciferase and DBD protein,we used overlapping PCR to get the fused DNA fragment of them.

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-1 for 5 cycles		
denaturation	98℃	10S
Annealing	50/52/54℃	30S
extension	68℃	35S
Three-step cycling-2 for 30 cycles		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	35S
Final extension	68℃	5min
Incubation	4℃	



The result was good.

2. Purification of PCR product

We used gel extraction kit to purify the PCR product.

3. Enzyme digestion of DNA of C-luciferase-DBD

composition	Volume
Cluciferase-DBD DNA	6.2μL
10×Fast digestion buffer	2μL
Not I	1μL
Nde I	1μL
Double distilled water	19.8μL

We digested the DNA for 3 hours.

4. Purification of enzyme digestion product

We used PCR recycle kit to purify the digestion product.

5. Gibson assembly

composition	Volume
sgRNA DNA	1.7μL
C-luciferase-DBD DNA	1.5μL
pCDFDuet-1	1.8μL
2× Assembly Mix	10μL
Double distilled water	5μL

We incubated the mixture at 55℃ for 30 minutes.

6.Transformation of DH5α with gibson assembly product

We transformed DH5α with the gibson assembly products.

8.23

Nice and easy.

8.24

1.1.colony PCR

We wanted to know if there were any positive colonies on the LB-agar plate of transformed DH5α on 8.18.We used colony PCR to test 12 colonies by sgRNA and DBD primers.

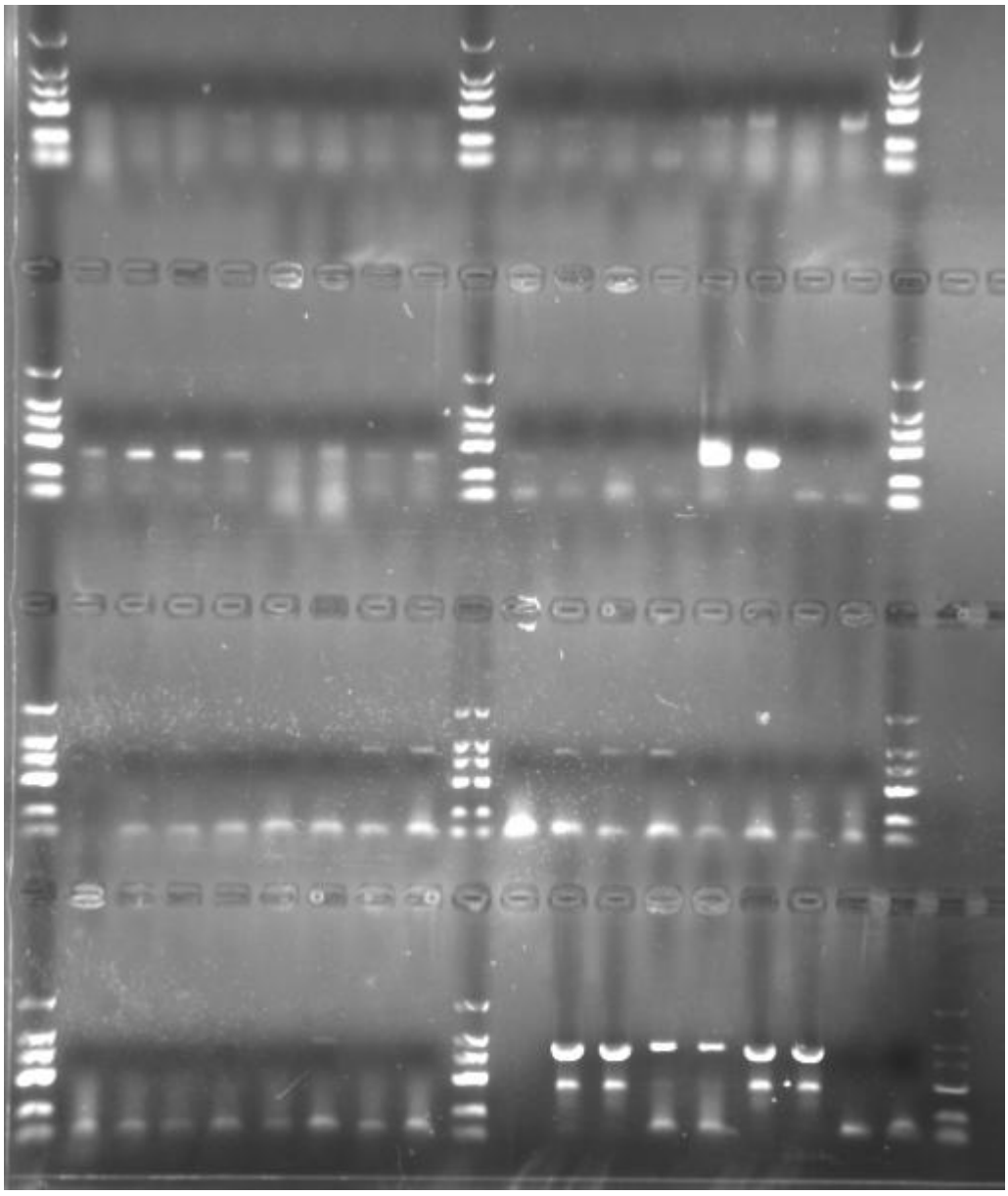
sgRNA primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S
Final extention	72℃	2min

Incubation	4℃	

DBD primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	53℃	10S
extension	72℃	5S
Final extention	72℃	2min
Incubation	4℃	

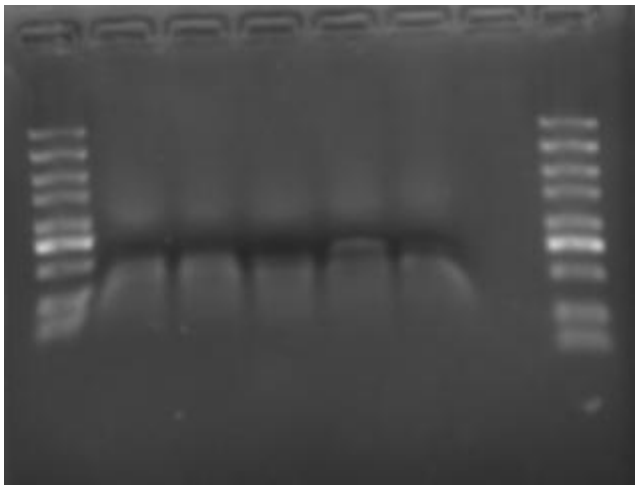


2.Inoculation of DH5α

We inoculated 5 colonies on the LB-agar culture plate mentioned in note of 8.15 to liquid LB culture for plasmid digestion to check,because we thought that colony PCR was not so accurate.

3.PCR of gibson assembly backbone pCDFDuet-1

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	50/52℃	30S
extension	68℃	75S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	67℃	30S
extension	68℃	75S
Final extention	68℃	5min
Incubation	4℃	



And we failed.

4.PCR of gibson assembly backbone pET28a(+)

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S

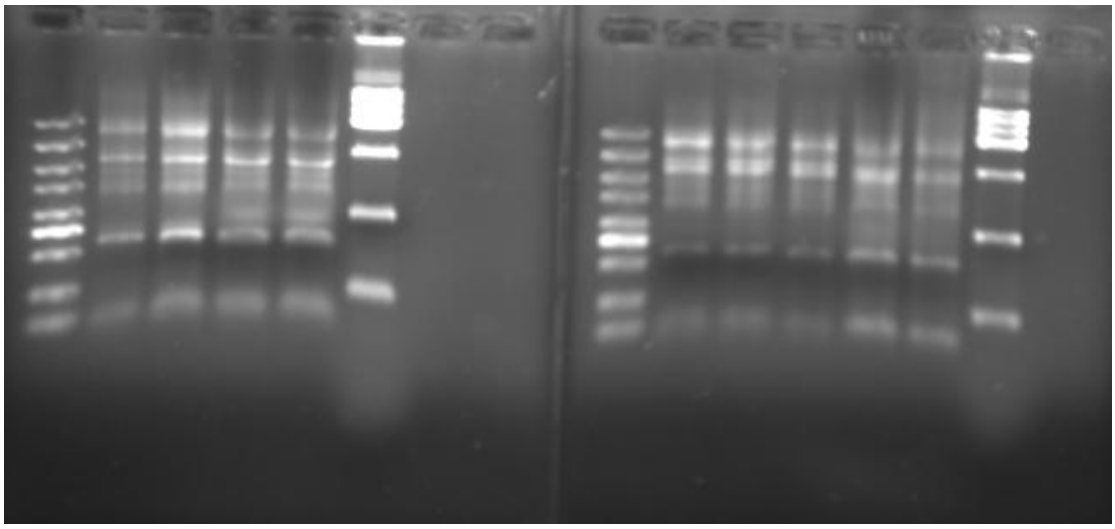
Annealing	54℃	30S
extension	68℃	170S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	170S
Final extention	68℃	5min
Incubation	4℃	

We also failed.

8.25

1.PCR of gibson assembly backbone pET28a(+)

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S
Annealing	46/48/50/52/54℃	30S
extension	68℃	170S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	170S
Final extention	68℃	5min
Incubation	4℃	



We failed.

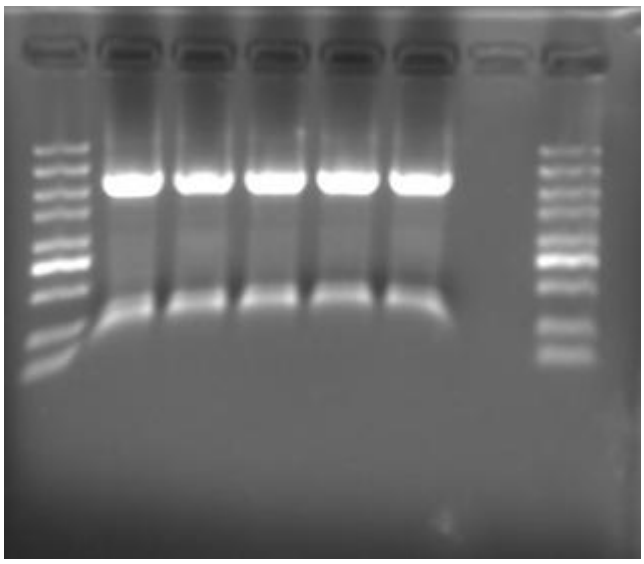
2.Plasmid Isolation

An overnight starter culture of E. coli DH5α harboring plasmid pCDFDuet-1 with sgRNA production module was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 μL of pre-warmed double distilled water.

name	concentration
pCDFDuet-1 with sgRNA #1 for test	77.3ng/μL
pCDFDuet-1 with sgRNA #2 for test	90.7ng/μL
pCDFDuet-1 with sgRNA #3 for test	78.5ng/μL
pCDFDuet-1 with sgRNA #4 for test	86.3ng/μL
pCDFDuet-1 with sgRNA #5 for test	81.8ng/μL

3.Enzyme digestion

composition	pCDFDuet-1
DNA	16μL
10×Fast digestion buffer	2μL
Bgl II	1μL
Xho I	1μL
Double distilled water	/



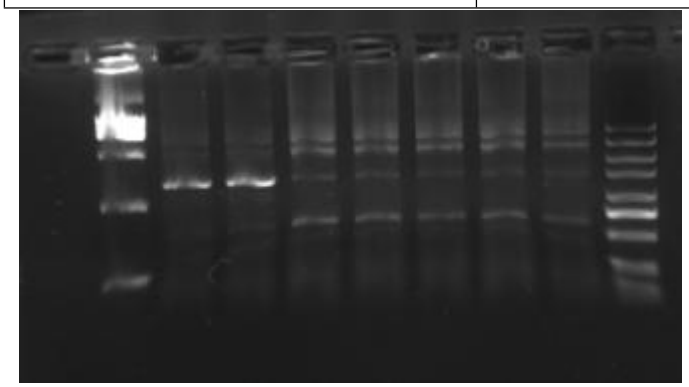
All the samples were positive,so we sent the plasmids for sequencing.

8.26

1.PCR of gibson assembly backbone pET28a(+)

step	temperature	time
Initial activation	94℃	2min
Three-step cycling-1 for 5 cycles(without primers)		
denaturation	98℃	10S

Annealing	46/48/50/52/54℃	30S
extension	68℃	170S
Three-step cycling-2 for 30 cycles(with primers)		
denaturation	98℃	10S
Annealing	68℃	30S
extension	68℃	170S
Final extention	68℃	5min
Incubation	4℃	



The PCR products were so impure.And we gave up gibson assembly of NdCas9 and pET28a(+).

8.27

1. Design of new Genetic Circuit

We decided that the whole system should consist of three plasmids.The first one is pET21a (Amp^R) with NdCas9.The second plasmid is pCDFDuet-1(Sm^R) with C-luciferase-DBD.The third one is pUC57(Kan^R) with target of NdCas9 and DBD binding site.The third plasmid was devised and was going to be synthesized by GeneScript.There are 5 versions of the third plasmid,each with a unique spacer between the target sequence and the DBD binding site.We use the 5 versions of the plasmid to test which one is the proper spacer between the two protein-binding sequences.

2.Inoculation of DH5α

We inoculated 2 colonies on the LB-agar culture plate mentioned in note of 8.22 to liquid LB culture for more plasmids.

8.28

1..Plasmid Isolation

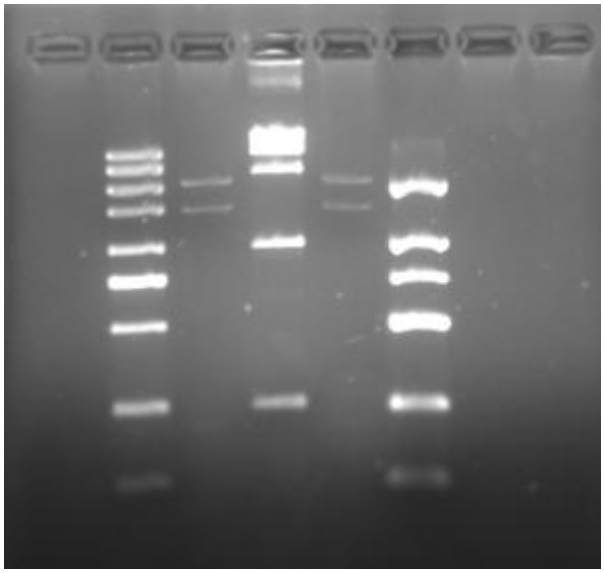
An overnight starter culture of E. coli DH5α harboring plasmid pCDFDuet-1-C-D-S was subjected to plasmid isolation following the Plasmid Isolation Protocol. Final elution was done with 30 µL of pre-warmed double distilled water.

name	concentration
pCDFDuet-1-C-D-S for test#1	62.8ng/µL
pCDFDuet-1-C-D-S for test#2	59.3ng/µL

2. Enzyme digestion

composition	pCDFDuet-1-C-D-S for test#1	pCDFDuet-1-C-D-S for test#2
DNA	16μL	16μL
10×Fast digestion buffer	2μL	2μL
Xbal I	1μL	1μL
Xho I	1μL	1μL
Double distilled water	/	/

We digested the plasmids for 4 hours.



The result was pretty good,so we sent the plasmids for sequencing.

8.29

The sequencing result was positive,both of the plasmids were successfully constructed,with C-luciferase-DBD and sgRNA modules.We named one of the plasmids “G2”.

1. Enzyme digestion

Since we devised new genetic circuit,in which the sgRNA production module was not involved in pCDFDuet-1,we must delete it from the original one.After scrutinizing the original plasmid sequence,we found that there were Nde Ⅰ digestion sites upstream and downstream the sgRNA production module.So we decided to make the cut with Nde Ⅰ and ligate the plasmid.We picked one

composition	volume
G2	16μL
10×Fast digestion buffer	2μL
Nde I	1μL
Double distilled water	1μL

We digested the plasmid for 4 hours.

2.Self-ligation of backbone

composition	Volume
G2	10μL

T4 ligase	0.1μL
5×T4 ligase buffer	4μL
Double distilled water	5.9μL

2.Transformation of DH5α
We transformed the ligation product to DH5α.

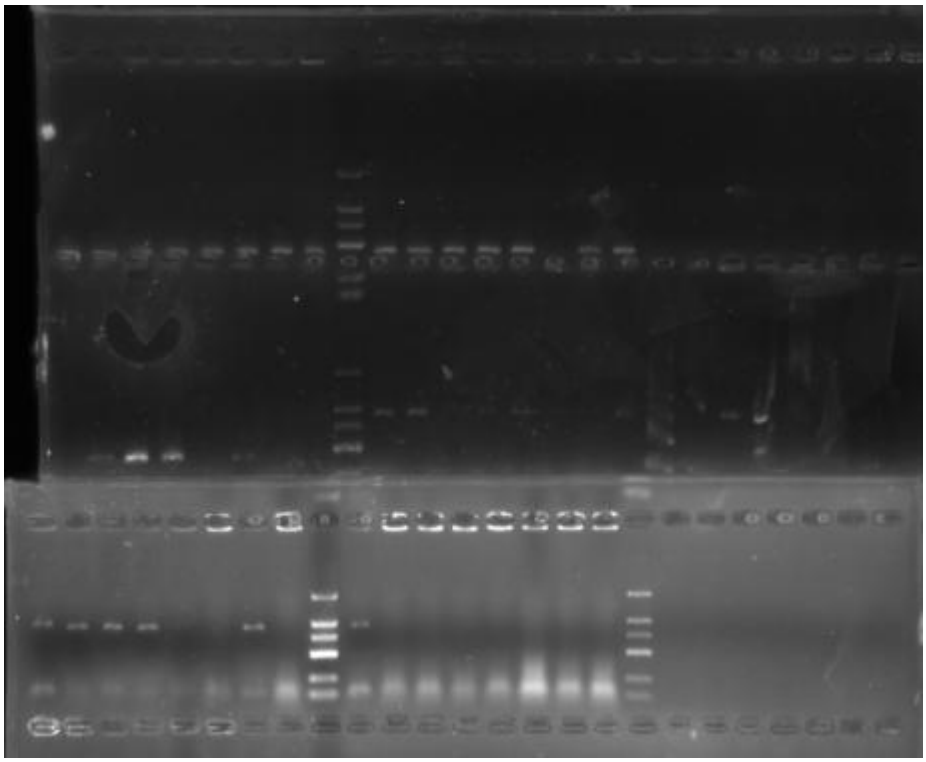
8.30
1. Colony PCR
We wanted to know if there were any positive colonies of self-ligation on the LB-agar plate of transformed DH5α on 8.29.We used colony PCR to test the colonies by sgRNA and DBD primers.

sgRNA primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	60℃	10S
extension	72℃	10S
Final extention	72℃	2min
Incubation	4℃	

DBD primer test

step	temperature	time
Initial activation	98℃	2min
Three-step cycling-2 for 35 cycles		
denaturation	98℃	10S
Annealing	53℃	10S
extension	72℃	5S
Final extention	72℃	2min
Incubation	4℃	



We set too much time for electrophoresis,so the bands of the top part(checked with DBD primer) went into the wells.But the positive results were still recognizable.The bands of the bottom part of the gel represented for the results checked with sgRNA primers.We picked colonies with DBD-positive results and sgRNA-negative results for sequencing.