Week1 (3.1-3.7): pYGRa1-mCherry plasmid construction and function validation

(A) pYGRa1-mCherry plasmid construction:

1. Amplification of vectors by PCR

Target vector fragments were obtained by PCR with the following RCR system:

PCR system (50μL)	
2×Mix	25ul
mCherry-F	2ul
mCherry-R	2ul
Template	50ng
DDW	20ul

PCR		
98° C	5min	
98° C	30s	×25
59° C	30s	
72° C	90s	
72° C	5min	
25° C	∞	

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 2900 bp. We got the correct target fragment.

2.(1) Amplification of Orf4 fragment by PCR

The target fragments were obtained by PCR. The RCR system was as follows:

PCR system (50μL)	
2×Mix	25ul
ORF4-F	2ul
ORF4-R	2ul
template	50ng
DDW	20ul

PCR		
98° C	5min	
98° C	30s	×25
59° C	30s	
72° C	35s	
72° C	5min	
25° C	∞	

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 1118 bp. we got the correct target fragment.

2.(2) Amplification of PA1 fragment by PCR

The target fragments were obtained by PCR. The RCR system was as follows:

PCR		
98° C	5min	
98° C	30s	×25
59° C	30s	
72° C	15s	
72° C	5min	
25° C	∞	

PCR system (50μL)	
2×Mix	25ul
PA1-F	2ul
PA1-R	2ul
template	50ng
DDW	20ul

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 407 bp. we got the correct target fragment and the sample was cut and recovered.

2.(3) Amplification of PA2 fragment by PCR

The target fragments were obtained by PCR, and the RCR system was as follows:

PCR system (50μL)	
2×Mix	25ul
PA2-F	2ul
PA2-R	2ul
template	50ng
DDW	20ul

PCR		
98° C	5min	
98° C	30s	×25
58° C	30s	
72° C	10s	
72° C	5min	
25° C	∞	

The polymerase chain reaction product was detected by agarose gel electrophoresis and the correct length of the target fragment was obtained as 340 bp. we got the correct target fragment.

3.Cutting glue recycling

4. Connecting the four fragments using the Gibsion assembly method

Gibson (C116) ligation was used to obtain pYGRa1-mCherry plasmid, mCherry 2900bp, Orf4 1118bp, PA1 407bp, PA2 340bp

Gibson system	
0.02 x 2900bp	ng
0.04 x 1118bp	ng
0.04 x 407bp	ng
0.04 x 340bp	ng
2×c115 Mix	5ul
DDW	To 10ul

Gibson.	
50° C	30min
4° C	∞

5. Chemical transformation

Using the chemical method, the ligation product was transformed into DH5α receptor cells and then spread on LB plates containing Amp antibiotics and incubated overnight at 37°C.

6. Monoclonal inoculation

After overnight incubation at 37°C, only 1 colony grew on the plate. The single clone was inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

7. Extract plasmid, send for sequencing

The plasmid was extracted and sequenced. pYGRa1-mCherry sequencing results were correct.

(B) Validation of pYGRa1-mCherry function

1. Conversion

The correctly sequenced plasmid was transfected into BW25113 sensory cells and then spread on LB plates containing Amp resistance and incubated at 37°C overnight. Expected resistant single colonies grew on the plates, and single colonies were subsequently selected for plasmid extraction and functional verification.

2. Pick monoclonal inoculation

The monoclone was inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

3. Induction

Three sets of parallel experiments were done for each system, induced in a shaker at 30℃ for 18h,200ul of each tube was taken into a 96-well plate, and the fluorescence and OD values were measured in an enzyme lab, the two systems were as follows:

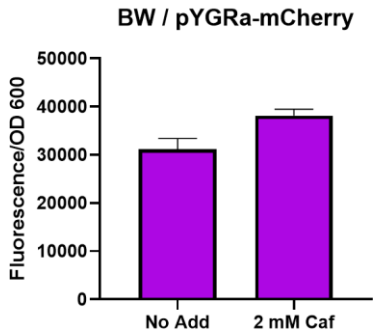
Induction of system1	
LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul

Induction of system2

LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul
Caffeine	2mM

4. Measure fluorescence and OD values

The results were as follows: strong fluorescence was found without caffeine, and the Orf4 pre-promoter should be too weak.



Week2 (3.8-3.14): construction and functional validation of pYGRa2-mCherry plasmid

(A) Construction of pYGRa2-mCherry plasmid:

The ORF4 front region was removed and the Pfrm promoter was added before Orf4 to substantially repress mCherry expression.

1. Amplification of vectors by PCR

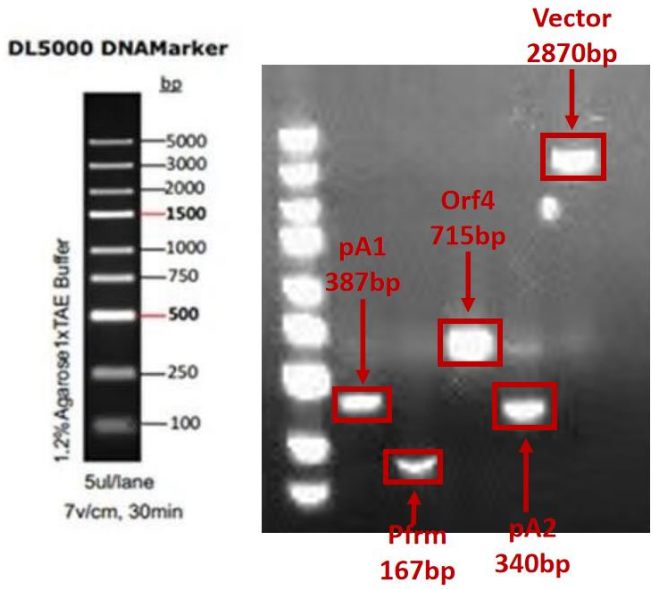
The target vector fragments were obtained by PCR, the corresponding primers and annealing temperature were changed, and the remaining operations were the same as those in the first week of the experiment.

2. Amplification of Orf4, pfrm, PA1 and PA2 fragments by PCR

The fragments were obtained by PCR, replacing the corresponding primers and annealing temperatures, and the rest of the procedure was the same as in the first week of the experiment.

3.Cutting glue recycling

The correct bands obtained were cut and recovered, and the PCR products were detected by agarose gel electrophoresis as follows:



4. Linking five fragments using the Gibsion assembly method

The pYGRa2-mCherry plasmid was obtained using Gibson (C116) ligation
mCherry 2870bp, Orf4 715bp, pfrm167bp,PA1 387bp,PA2 340bp

Gibson system	
0.02 x 2870bp	ng
0.04 x 715bp	ng
0.04 x 167bp	ng
0.04 x 387bp	ng
0.04 x 340bp	ng
2×c116 Mix	5ul
DDW	to10ul

Gibson.	
50° C	30min
4° C	∞

5. Chemical transformation

Same chemical transformation operations as in Week 1

6. Colony PCR

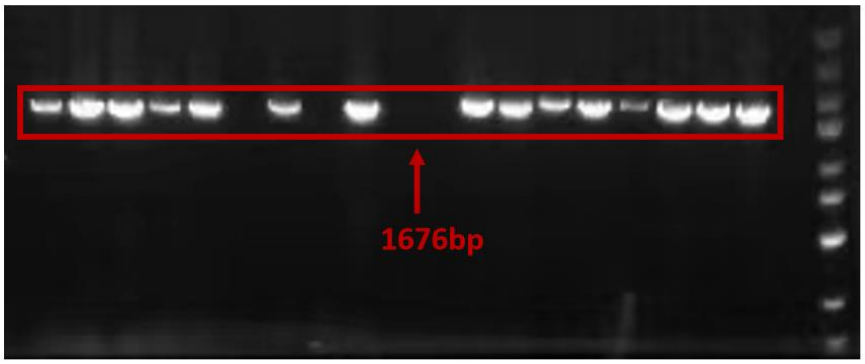
After 12 hours of incubation at 37° C, 20 colonies were selected on the plate and the colony PCR system was as follows:

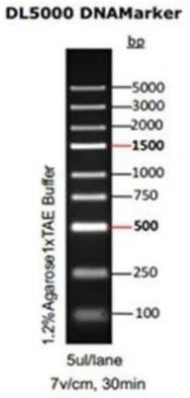
PCR system (10μL)

2×Hieff	25ul
CE-F	2ul
CE-R	2ul
DDW	4.2ul

PCR		
98° C	5min	
98° C	30s	×25
57° C	30s	
72° C	50s	
72° C	5min	
25° C	∞	

The PCR products were examined by agarose gel electrophoresis with the following results:



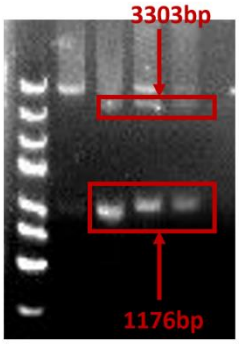


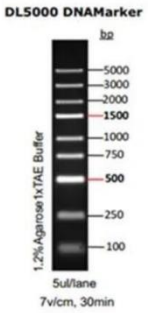
7. Inoculation with monoclonal

Each of the four monoclones was selected and inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

8. Plasmid extraction + digestion verification

Single enzyme cleavage was verified using BglII, and the cleavage products were detected by agarose gel electrophoresis. The target bands were 3303bp, 1176bp. the results are plotted below:





9. pYGRa2-mCherry sequencing result is correct.

(B) Functional validation of pYGRa2-mCherry

1. Transformation

The correctly sequenced pYGRa2-mCherry plasmid was transfected into BW25113 receptor cells and then spread on LB plates containing Amp antibiotics and incubated inverted at 37°C overnight. Expected resistant single colonies grew on the plates, and single colonies were subsequently selected for plasmid extraction and functional verification.

2. Pick monoclonal inoculation

Selected monoclones were inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

3. Induction

Comparative induction was done for GRA1 and GRA2, 3 sets of parallel experiments were done for each system, and the induction was carried out in a shaker at 30℃ for 18h, 200ul was taken from each tube into a 96-well plate, and fluorescence and OD values were measured, the systems were as follows：

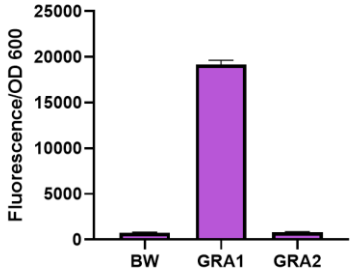
Induction of system1	
LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul
Caffeine	2mM

Induction of system2

LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul
Caffeine	2mM

4. Measure fluorescence and OD values

RESULTS: The addition of Pfrm promoter before Orf4 significantly inhibited the expression of mCherry, but the inhibition could not be lifted even after the addition of caffeine. This may be due to the fact that Pfrm is too strong.



Week3 (3.15-3.21): construction and functional validation of pYGRa3-mCherry, PYGRa4-mCherry plasmids

Two approaches to weaken Orf4 expression were tried separately:

(A) pYGRa3-mCherry plasmid construction

We attempted to weaken the expression of Orf4, mimic the endogenous formaldehyde transcriptional regulator in E. coli, and use the ndmA promoter to initiate Orf4 and fluorescent proteins to achieve "self-regulation".

1. Amplification of vectors by PCR

The ORF4 front region was removed and the Pfrm promoter was added before Orf4 to substantially repress mCherry expression.
The target vector fragments were obtained by PCR, the corresponding primers and annealing temperature were changed, and the remaining operations were the same as those in the first week of the experiment.

2. Amplification of Orf4, pndmA fragments by PCR

The fragments were obtained by PCR, replacing the corresponding primers and annealing temperatures, and the rest of the procedure was the same as in the first week of the experiment.

3.Cutting glue recycling

Recover the correct strip by cutting the glue.

4. Connecting three fragments using Gibsion assembly method

The pYGRa3-mCherry plasmid was obtained using Gibson (C116) ligation, vector 2979bp, Orf4 663bp, pndmA 727bp

Gibson system	
0.02 x 2979bp	ng
0.04 x 663bp	ng
0.04 x 727bp	ng
2×c116 Mix	5ul
DDW	to10ul

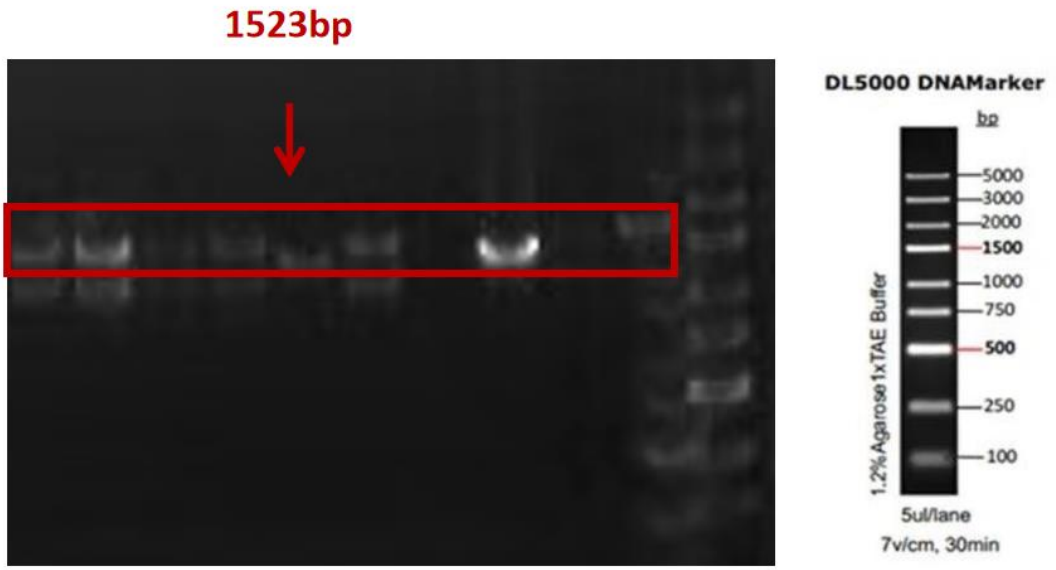
Gibson.	
50° C	30min
4° C	∞

5. Chemical transformation

Same chemical transformation operations as in Week 1

6. Colony PCR

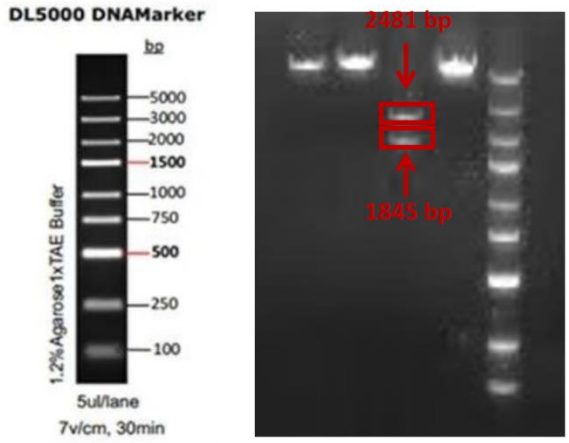
Positive clones were selected and inoculated into liquid medium, and after 12 hours of incubation at 37°C, 10 colonies were selected on the plate and colony PCR was performed, and the PCR products were detected by agarose gel electrophoresis, and the results were as follows:



Positive monoclones were selected and inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

7. pYGRa3-mCherry plasmid was extracted and verified by digestion.

Single enzyme cleavage was verified using BglII, and the cleavage products were detected by agarose gel electrophoresis. The target bands were 1845bp and 2481bp. The results are shown below:



8. pYGRa3-mCherry sequencing result is correct

(B) Construction of PYGRa4-mCherry plasmid

Orf4 was initiated using a medium-strength Anderson promoter, p101

1. Amplification of vectors by PCR

The target vector fragments were obtained by PCR, the corresponding primers and annealing temperature were changed, and the remaining operations were the same as those in the first week of the experiment.

2. Amplification of Orf4, pndmA, P101 fragments by PCR

The fragments were obtained by PCR, replacing the corresponding primers and annealing temperatures, and the rest of the procedure was the same as in the first week of the experiment.

3.Cutting glue recycling

Recover the correct strip by cutting the glue.

4. Connecting the four fragments using the Gibsion assembly method

The pYGRa4-mCherry plasmid was obtained using Gibson (C116) ligation. mCherry 2970bp, Orf4 695bp, pndmA 707bp, P101 124

Gibson system	
0.02 x 2970bp	ng
0.04×695bp	ng
0.04×707bp	ng
0.04 x 124	ng
2×c116 Mix	5ul
DDW	to10ul

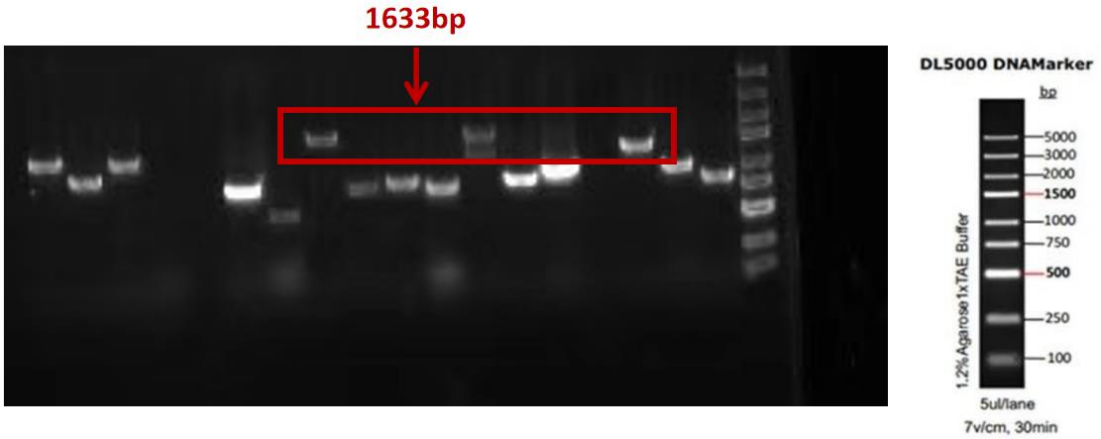
Gibson.	
50° C	30min
4° C	∞

5. Chemical transformation

Same chemical transformation operations as in Week 1

6. Colony PCR

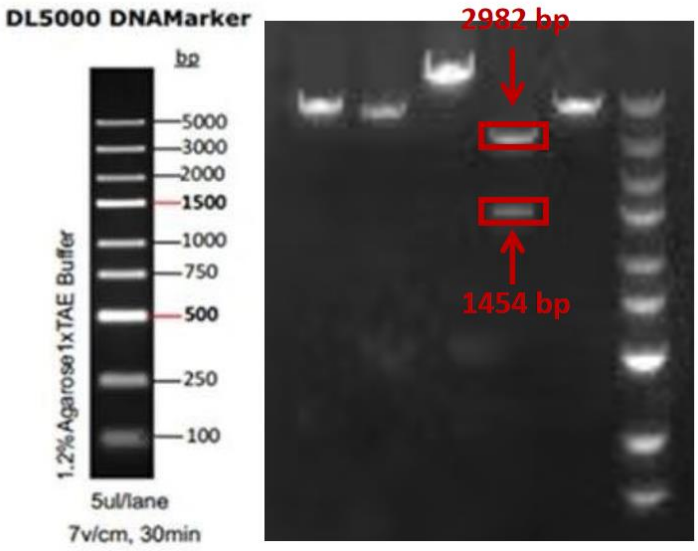
Positive clones were selected and inoculated into liquid medium, and after 12 hours of incubation at 37°C, 10 colonies were selected on the plate and colony PCR was performed, and the PCR products were detected by agarose gel electrophoresis, and the results were as follows:



Three positive monoclones were selected and inoculated into liquid medium supplemented with 1% ampicillin and incubated at 37°C for 12 hours.

7. pYGRa4-mCherry plasmid was extracted for digestion and validation.

Single enzyme cleavage was verified using EcoRI, and the cleavage product was detected by agarose gel electrophoresis. The target band was 454bp+2982bp and the resultant graph is shown below:



8. pYGRa4-mCherry sequencing result is correct

(C) Functional validation of pYGRa3-mCherry, PYGRa4-mCherry

1. Transformation

The successfully sequenced pYGRa4-mCherry and pYGRa3-mCherry were transformed into BW25113 receptor cells, respectively, for functional validation together with the two previously constructed plasmids.

2. Induction

Comparative induction was done for GRA1, GRA2, GRA3 and GRA4, and 3 sets of parallel experiments were done for each system, which were induced in a shaker at 30℃ for 18h, 200ul of each tube was taken into 96-well plates, and fluorescence and OD values were measured in an enzyme labeller, and the systems were as follows:

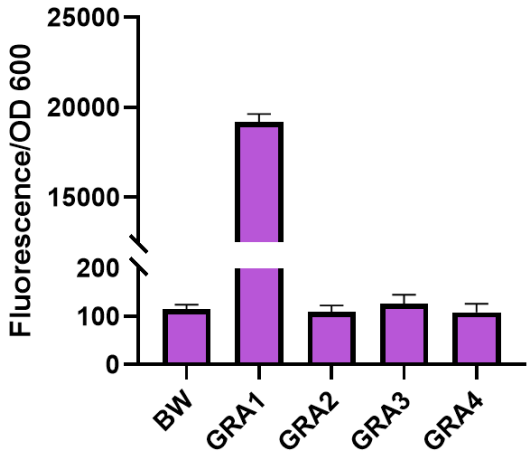
Induction of system1	
LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul

Induction of system2	
LB	5ml
Amp	2ul
Inoculum amount	5ul

IPTG	5ul
Caffeine	2mM

3.Measure fluorescence and OD values

The results were as follows: several methods used so far were ineffective, GRA1 could not repress the NdmA promoter due to its own promoter being too weak, resulting in low ORF4 expression; GRA2, GRA3, and GEA4 could not open the NdmA promoter after being repressed due to high ORF4 expression.
Afterwards it is planned to test for a weaker promoter P107.



Week4 (3.22-3.28): Construction and functional validation of pYGRa5-mCherry plasmid

(A) pYGRa5-mCherry plasmid construction

Orf4 was initiated using a moderately weak Anderson promoter p107

1.PCR amplification

Amplification of pYGRA4 fragments by PCR The target vector fragments were obtained by PCR with the following RCR system.

PCR		
98° C	5min	
98° C	30s	×25
59° C	30s	
72° C	2min	
72° C	5min	
25° C	∞	

PCR system (50μL)	
2×Mix	25ul
GRA5-F	2ul
GRA5-R	2ul
pYGRa5-mCherry	50ng
DDW	20ul

The expected product size of 4456 bp was obtained, pending subsequent analysis.

2. Product purification

The PCR products were purified and analysed by electrophoresis through 1% agarose gel. The electrophoresis ran clear bands and the corresponding positions were consistent with the expected size of 4456 bp. The purified PCR product was successfully obtained. The purity and concentration of the product were as expected and the band size was correct.

3. DpnI digestion

PCR products were digested using DpnI to remove the unmutated template plasmid.

Dpn Ⅰ system (10μL)	
DNA	200ng
Dpn Ⅰ	1ul
Cutsmart	1ul
DDw	to 10ul

Dpn Ⅰ	
37° C	2h
55° C	15min
80° C	15min
4° C	∞

The digestion reaction went smoothly and the enzyme was inactivated after treatment at 85°C. The PCR product was efficiently digested and prepared for transformation of the receptor cells in the next step.

4. Chemical transformation

The digested PCR product was transformed into DH5α receptor cells. After transformation, they were spread on LB plates containing Amp resistance and incubated at 37°C overnight. Expected resistant single colonies grew on the plates, and single colonies were subsequently selected for plasmid extraction and validation.

5. Induction

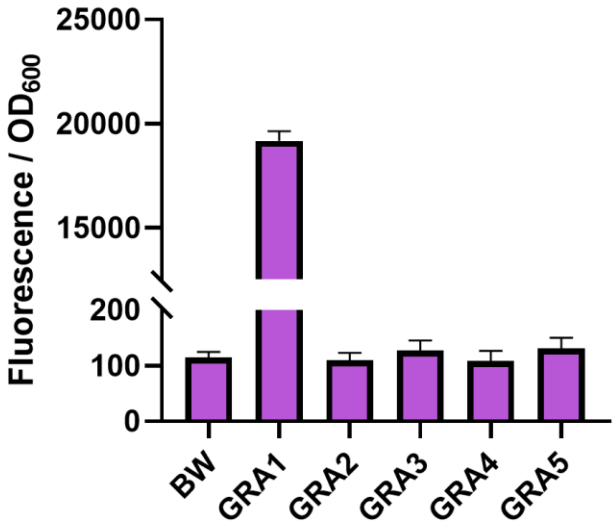
Comparative induction was done for GRA1, GRA2, GRA3, GRA4 and GRA5, and 3 sets of parallel experiments were done for each system, which were induced in a shaker at 30°C for 18h, 200ul of each tube was taken into 96-well plates, and fluorescence and OD values were measured, and the two systems were as follows:

Induction of system1	
LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul

Induction of system2	
LB	5ml
Amp	2ul
Inoculum amount	5ul
IPTG	5ul
Caffeine	2mM

6. Measure fluorescence and OD value

The results are as follows:



Several methods used so far have been ineffective, GRA1 was unable to repress the NdmA promoter due to its own promoter being too weak resulting in low ORF4 expression, GRA2, GRA3, GRA4, and GRA5 were all unable to turn on the NdmA promoter after repression due to too high ORF4 expression, and ultimately gave up on the development of the transcription factors.