Part I Amplification luxAB

7.9

Observed and selected *Vibrio FG-1* single colony and cultured in 2216E liquid medium overnight on the 28℃ shaker.

7.10

Extracted genomic DNA from *Vibrio FG-1*, and extracted pEvolvR plasmid from *E.coli* cultured from July 8th and measured its concentration.

7.11

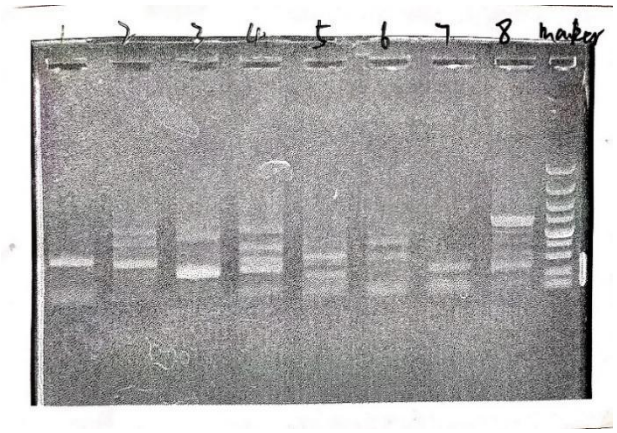
Amplified *Vibrio FG-1* by 16sRNA. Species comparison showed that it was homologous with *Vibrio Fischeri*, but we did not know its complete sequence, so we designed primers according to the sequence of *Vibrio Fischeri*.

7.12~7.17

Designed primers to amplify *lux AB* gene of *Vibrio FG-1*.

7.17

The gel diagram of *lux AB* gene amplification of *Vibrio FG-1* is as follows.No.1-7 refers to eight examples adding with the same genome.



7.18

Recoveried of DNA fragments from Agaros gel and sent it to Sangon Biotech for sequencing. Cultured pEvolvR-containing *E.coli* from July 8th in LB liquid medium.

7.19

Extracted the pEvolvR plasmid from *E.coli* which was cultured on July 18th and measured its DNA concentration.

No.	Concentration(ng/μl)	A260/A280	A260/A230
1	8.4500	1.724	0.552
2	5.4500	2.019	0.708

3	6.5500	2.079	0.682
4	4.3500	1.740	1.036

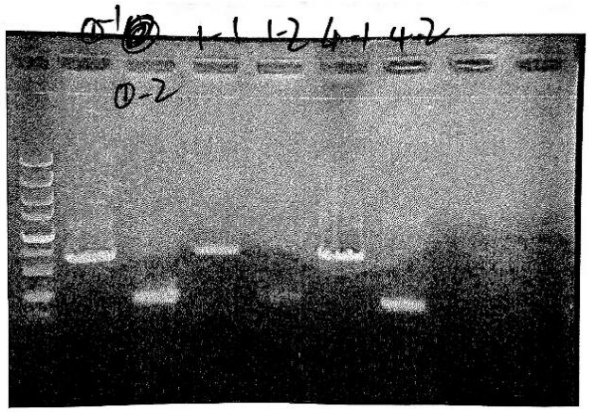
7.20

Used PCR to verify the pEvolvR plasmid.

Selected six tubes of samples, named as ①-1 and ②-2 (the above two tubes were sample No.1 of July 10th); 1-1, 1-2, 4-1, 4-2 (the above four tubes are samples No.1 and 4 of July 19th).

Primers are designed by two kinds of different sites.

Gel electrophoresis map is as follows:

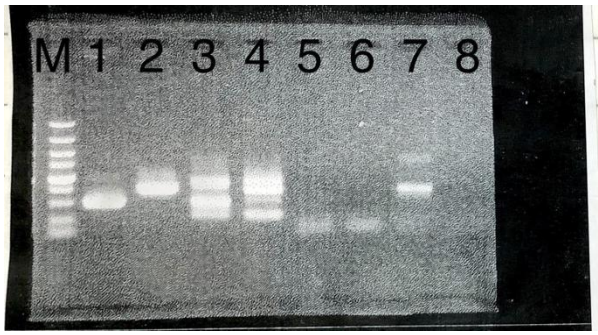


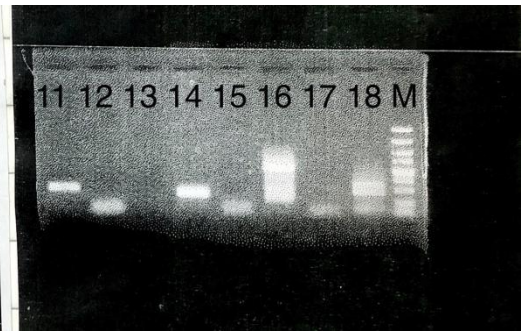
Sent the PCR product to Sangon Biotech for sequencing. The result showed that the sequence of the pEvolvR plasmid was correct.

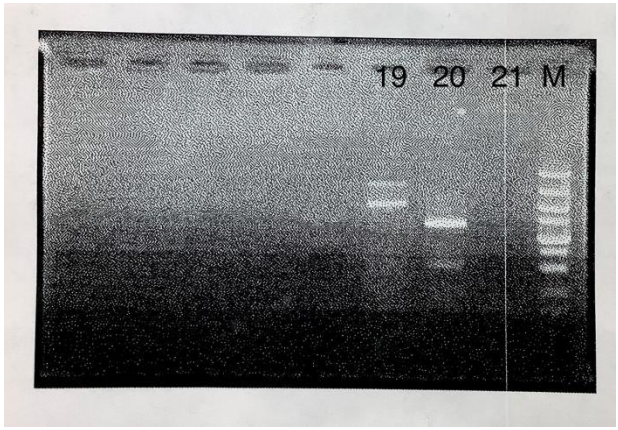
7.22

Amplified the first half sequence of *lux A* and the whole sequence of *lux B.*

We made nineteen samples in total and the Gel electrophoresis map is as follows:

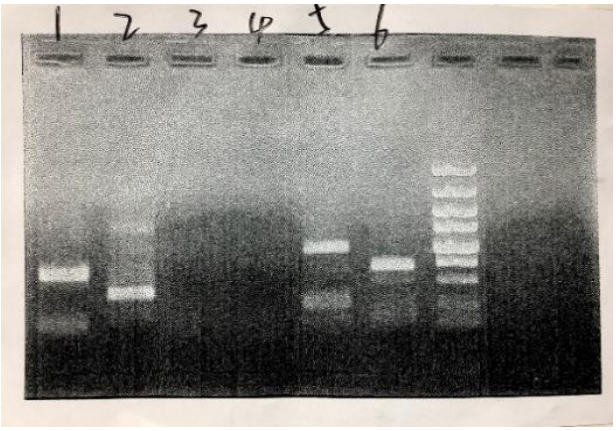






Send No.1, No.2, No.9, No.12, No.18 to Sangon Biotech for sequencing.
No.1 added with primers named as AS-1F, 488R.
No.2 added with primers named as AS-1F, 709R.
No.9 added with primers named as 587F, AF-1069R.
No.12 added with primers named as 587F, AS-967R.
No.18 added with primers named as *lux* b_1-F, *lux* b_2-R.

7.31
Amplified the sequence of *lux B*. No.1 added with primers named as 14F and 14R. No.2 added with primers named as 15F and 15R. No.3 added with primers named as 16F and 16R. No.4 added with primers named as 17F and 17R. No.5 added with primers named as 18F and 18R. No.6 added with primers named as AS-1F and 709R.
The Gel electrophoresis map is as follows:



Send No.1, No.2, No.5 to Sangon Biotech for sequencing.
The sequencing results showed overlapping peaks and too short band length, suggesting that DNA degradation was caused by the preservation of the genome at 4℃, so the experiment was repeated.

9.6
Start fluctuation test. See Protocol for details.

Part II The construction of pTarget and pEvolvR by AQUA

8.7
Prepared *E.coli* competent cells and *Vibrio FG-1* competent cells.
The method of preparing competent cells in our project is as follows:
1) DH5α was inoculated on LB solid medium and incubated overnight at 37℃ incubator.
2) Selected single colonies from the LB solid medium and inoculated into 5 test tubes containing 5mL LB liquid medium for overnight culture at 37℃ incubator.
3) Inoculated 500ul the overnight cultured microbial into a conical flask containing 100mL LB liquid medium at 37℃ shaker, 2000rpm for 3h. When the OD600 value reached between 0.3 and 0.4, terminated the culture, and the conical flask was buried in an ice box for 10min for cooling
4) Taked ten 1.5ml EP tubes which were injected with 1.5ml cooling culture solution, then placed them on ice.

5) Centrifuged these ten tubes at 0 ℃, 4000rpm for 5min.
6) Discard supernatant. Place the ten tubes on ice and add refrigerated 500μL 50mM's $CaCl_2$ solution to each tube, carefully and slowly blow the precipitation of tubes until they are suspended again.
7) Centrifuged these ten tubes at 0 ℃, 4000rpm for 5min.
8) Discard supernatant. Place the ten tubes on ice and add refrigerated 500μL 50mM's $CaCl_2$ solution to each tube, carefully and slowly blow the precipitation of tubes until they are suspended again. Placed the cell suspension on ice for 30 min.
9) Centrifuged these ten tubes at 0 ℃, 4000rpm for 5min.
10) Discard supernatant. Place the ten tubes on ice and add refrigerated 500μL 50mM's $CaCl_2$ solution to each tube, carefully and slowly blow the precipitation of tubes until they are suspended again. Placed the cell suspension on ice for 30 min.
11) Centrifuged these ten tubes at 0 ℃, 4000rpm for 5min.
12) Discard supernatant. Place the ten tubes on ice and add refrigerated 80μL 50mM $CaCl_2$ solution containing 15% glycerol to each tube, carefully and slowly blow the precipitation of tubes until they are suspended again. Placed the cell suspension on ice for 1h.
13) Boxed the cells which were on ice quickly and placed in -80℃ refrigerator.
Test the transformation efficiency of competent cells.

8.8
Validation test of competent cells: Transformed the plasmid RiboG into the cells. Plasmid RiboG owns the ampicillin resistance, which provided by the lab. The bacterium was coated on the resistant LB solid medium of ampicillin to verify whether the transfer was successful.

8.10
Indicated the extracted *Vibrio FG-1* genome. The reaction system is below.

The reaction system of PCR

Composition of reaction system	sample volume（μL）
Genomic DNA	2.5
sense primer	1.25
anti-sense primer	1.25
ddH_20	20
2✖Taq PCR MasterMix	25
total	50

PCR reaction conditions:
1) Pre-denaturation at 94℃ for 3min;
2) Denaturation at 94℃ for 30 s;
3) Annealing the primers at 55℃ for 30 s;
4) The primer extended at 72℃ for 1min;
5) Repeat steps B -D 35 times;
6) Continue at 72℃ for 5 min.

8.21

Pre-experiment for AQUA: Primers were designed and PCR was performed on plasmid psB1C3-J04450 , which was divided into three adjacent DNA fragments(named as A,B,C) containing homologous arms.

The reaction system of PCR

Composition of reaction system	sample volume（μL）
Genomic DNA	2
sense primer	1
anti-sense primer	1
10x Buffer	5
Taq Polymerase	1
dNTPS	4
ddH_20	36
total	50

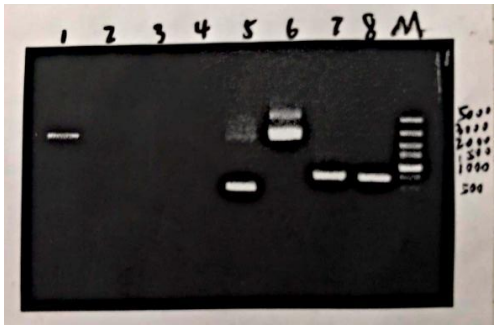
PCR reaction conditions:

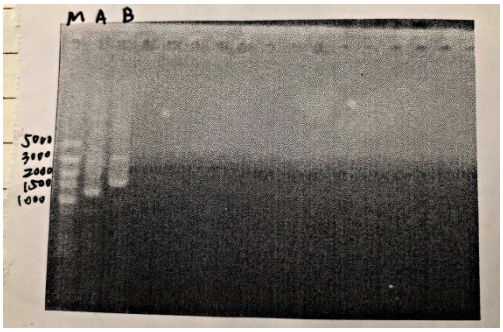
1) Pre-denaturation at 94℃ for 3min;
2) Denaturation at 94℃ for 30 s;
3) Annealing the primers at 55℃ for 30 s;
4) The primer extended at 72℃ for 1min;
5) Repeat steps B -D 35 times;
6) Continue at 72℃ for 5 min.

*Lux*AB was also amplified from FG-1 genome.

We got the expected the fragment of *lux*AB and fragments of the plasmid psb1c3-j0445 from the results.

The results were below：





We purified the fragments and detected the concentration.

Fragments	Concentration(ng/ul)
*lux*AB	25.25
A(psb1c3-j0445)	37.80
B(psb1c3-j0445)	30.60
C(psb1c3-j0445)	29.20

8.22

AQUA：

1) Added the fragment to the EP tube in a 1:1:1 ratio.
2) Placed the tube at room temperature for 1h.

3) The DNA mixture was converted into *E.coli* cells by chemical conversion.
4) Placed the tube on ice for 10min.
5) Heat at 42℃ for 45s.
6) Placed the tube on ice again for 2min.
7) Added 300ul LB liquid medium.
8) At 37℃, centrifuged at 700rpm for 1h.
9) Coated on LB solid medium containing chloramphenicol resistance for overnight culture.

8.24
Observed the colonies, chose the single colonies that expressed rfp for PCR in order to verify the success of AQUA assembly. It is a pity that until now we haven't succeeded.

8.29
Amplified the sequence of *lux AB*.

8.30
Amplified *p15A* and *AmpR* fragments.

8.31
Amplified *lux AB + promoter*. Recovery of these DNA fragments from Agaros gel.

9.1
AmpR and *p15A* connect to the homologous arm.
Purified PCR product and constructed plasmid.
Lux AB+ promoter, *AmpR+homo* and *p15A+homo* were sent to Sangon Biotech for sequencing.

9.2~9.17
Primers were designed for PCR of the pEvolvR plasmid, which was divided into adjacent DNA fragments containing homologous arms. AQUA method was used for recombination, but the effect seems not to be optimistic so far.

9.4
AQUA was prepared by mixing *Lux AB+ promoter*, *p15A+homo* and *AmpR+homo* with a molar ratio of 1:1:1, And use water to supplement 10ul of the total system. Reaction at room temperature for one hour.

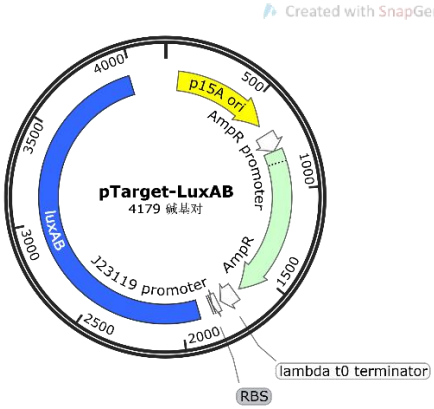
The reaction system of AQUA

Composition of reaction system	Sample volume(ul)	the sample concentration
Lux AB	0.8	15.500
P15A	0.4	33.750
AmpR	0.4	28.850
ddH_2O	8.4	
total	10	

Mixed 25ul *DH5α* and 5ul AQUA mixture, and then ice bath for 10min, 42℃ water bath for 45s to

hot shock, finally ice bath for 2min.
Added 250ul LB liquid medium and cultured the *DH5α* at 37℃ shaker, 200rpm, 1h.
Coated the bacterial solution on LB resistant medium (*AmpR* resistant) and cultured for 16h.

9.5
Selected single colonies on the plate for colony PCR to verify whether the three sequences were successfully connected.
The primers used were *lux-F* and *p15A-R; p15A -F* and *AmpR - R*.



9.8
Since AQUA repeated several times without success, pTarget was reconnected with a seamless clone kit.

9.18
Four adjacent DNA fragments with homologous arms were amplified from the pEvolvR plasmid. Then we purified the fragments for future use.

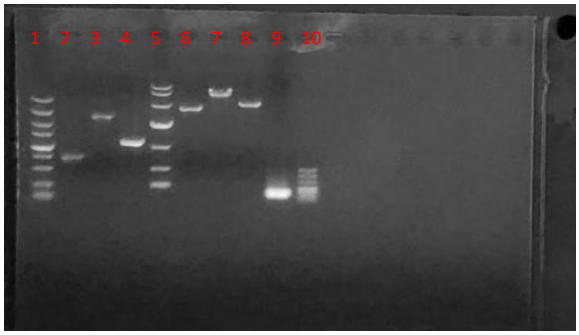
9.19
DNA fragments p15A-Homo, AmpR-Homo, *lux*AB containing promoter(abbreviated to J&*lux*AB), sgRNA with promoter(abbreviated to J&gRNA) were amplified from the pEvolvR plasmid , and then we purified the fragments for future use.

9.20
Detected the concentration

Fragments	Concentration(ng/ul)
J&*lux*AB	25.55
p15A-Homo	138.20
AmpR-Homo	104.15
J&gRNA	216.70
A(pEvolvR-enCas9-PolI3M-TBD 11597-2627)	55.95
B(pEvolvR-enCas9-PolI3M-TBD 2601-8450)	49.15
C(pEvolvR-enCas9-PolI3M-TBD 8425-11615)	34.50
D(pEvolvR-enCas9-PolI3M-TBD 11662-2627)	30.60

9.21

Verify the length of the fragments：AGEagarose gel electrophoresis



From No.1 to No.10 are DL5000, p15A-Homo, J&*luxAB* , AmpR, DL10000 Marker, C, B, D, J&gRNA and DL500 Marker.

9.22

Used seamless cloning to assemble plasmids and designed control experiments for pEvolvR and pTarget:

1) A, B, C
2) J&gRNA, B, C, D
3) The plasmid pEvolvR-enCas9-PolI3M-TBD
4) P15A-Homo
5) AmpR-Homo
6) dd H_2O
7) J&*lux*AB, P15A-Homo, AmpR-Homo

The assembly products were converted into *E.coli* competent cells and screened by coating on the resistance plate.

Part III The construction of pEvolvR by RED/ET

9.12

Prepared *Escherichia coli* competent cells.

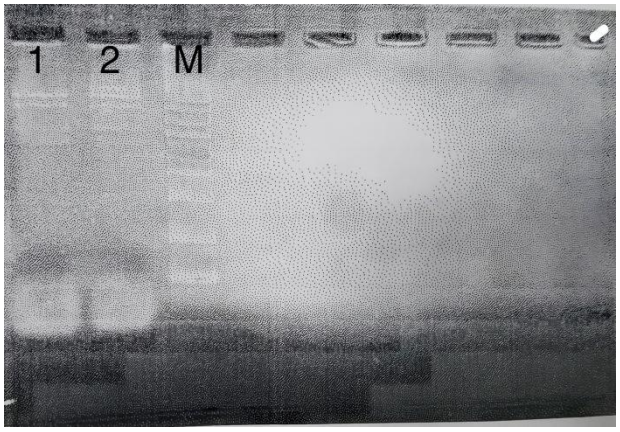
Validation test of competent cells: Transformed the plasmid RiboG into the cells. The bacterium was coated on the resistant LB solid medium of ampicillin to verify whether the transfer was successful.

9.13

Observed that the single colonies grew on the medium. It proved that *E.coli* competent cells were successfully prepared.

Obtained *E.coli* liquid containing plasmid psc101. Extracted the plasmid from the bacteria solution and did enzyme validation. The enzyme validation site was selected as EcoRI, and the enzyme validation time was to be 4 hours. No.1 and No.2 are a set of repetitions.

Electrophoresed the enzyme validation product.



9.14

Transformed the psc101 plasmid into *E.coli* competent cells.

The bacterium was coated on the resistant LB solid medium of tetracycline to verify whether the transfer was successful.

9.15

Observed that the single colonies grew on the medium.

Single colonies were selected from the solid medium and cultured in the LB liquid medium.

9.16

Prepared *E.coli* competent cells with psc101 plasmids.

Validation test of competent cells: Transformed the plasmid RiboG into the cells. The bacterium was coated on the resistant LB solid medium of ampicillin to verify whether the transfer was successful.

9.17

Failed to observe the single colonies grown on the medium.

9.18

Observed that the single colonies grew on the medium.

Transformed the pEvolvR plasmid into *E.coli* competent cells with psc101 plasmids. The bacterium was coated on the resistant LB solid medium of kanamycin and chloramphenicol to verify whether the transfer was successful.

9.19

Failed to observe the single colonies grown on the medium.

9.20

Observed that the single colonies grew on the medium.

Single colonies were selected from the solid medium and cultured in the LB liquid medium of kanamycin.

9.21

Extracted the plasmid from the bacteria solution and measured the concentration of the concentration. The sample concentrations used in the following experiments are attached in the table below.

No.	Concentration(ng/μl)	A260/A280	A260/A230
1	352.65	1.970	2.475
2	432.75	1.905	2.486

Designed the primers to prove the existence of pEvolvR plasmids.

9.22

Verify the presence of the pEvolvR plasmids through PCR.

The reaction system of PCR

Composition of reaction system	sample volume（μL）
Plasmid	2.5
sense primer	1.25
anti-sense primer	1.25
ddH_20	20
2✖Taq PCR MasterMix	25
total	50

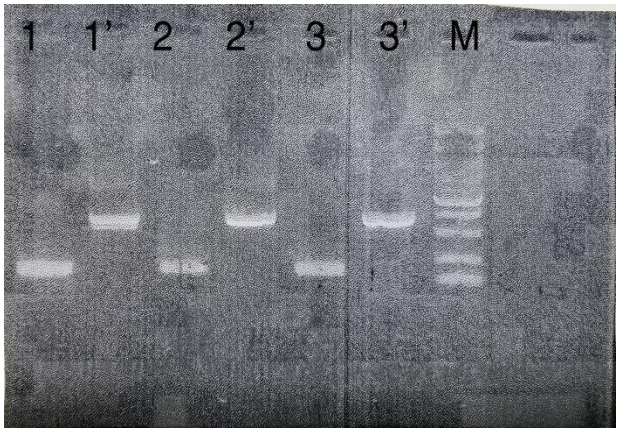
PCR reaction conditions:

1) Pre-denaturation at 94℃ for 3min;
2) Denaturation at 94℃ for 30 s;
3) Annealing the primers at 55℃ for 30 s;
4) The primer extended at 72℃ for 1min;
5) Repeat steps B -D 35 times;
6) Continue at 72℃ for 5 min.

No.1, No.2 and No.3 added with primers named as 12p EvolvR-s and 12p EvolvR-as.

No.1', No.2' and No.3' added with primers named as 13p EvolvR-s and 13p EvolvR-as.

Electrophoresed the PCR product.



9.23

Prepared *E.coli* competent cells with psc101 plasmids and pEvolvR plasmids.

Validation test of competent cells: Transformed the plasmid RiboG into the cells. The bacterium

was coated on the resistant LB solid medium of ampicillin to verify whether the transfer was successful.

9.24
Failed to observe the single colonies grown on the medium.

9.25
Observed that the single colonies grew on the medium.
Designed sgRNA. The sequences of the sgRNA attached to the homologous arms and J23115 promoter were sent to Sangon Biotech (Shanghi, China) for synthesis. The sequences of the homologous arms birthed from the pEvolvR plasmid.

9.26
Annealed the primers of the sgRNA attached to the homologous arms and J23115 promoter.

9.27
Transformed the sgRNA into *E.coli* competent cells with psc101 plasmids and pEvolvR plasmids. The bacterium was coated on the resistant LB solid medium of kanamycin and chloramphenicol to verify whether the transfer was successful.

9.28
Observed that the single colonies grew on the medium.