一、 SV40（Enhancer）

1、PCR clone

1.1、PCR try

PCR trial system ：

SV40+Rlu plasmid：	1μL
SV40（王皓 up） ：	0.25μL
SV40（王皓 down）：	0.25μL
H2O：	3.5μL
Mix：	5μL
total：	10μL

Set three temperature gradients：

① 95℃：5min→ 94℃：30s→ 48℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

② 95℃：5min→ 94℃：30s→ 55℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

③ 95℃：5min→ 94℃：30s→ 58℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

After the PCR,run agarose gel electrophoresis.If the light band was in the 419bp position, indicating that PCR was successful, you could directly enter the step 1.2. If not you should try again with changing the conditions,such as renatural temperature.

1.2、PCR amplification

PCR amplification system：

SV40+Rlu plasmid：	1μL
SV40（王皓 up）：	0. 5μL
SV40（王皓 down）：	0. 5μL
H2O：	13μL
Mix：	15μL
total：	30μL

① The system was formulated with 5 tubes, that was, 30ul*5.

② Temperature setting should be the same with the temperature in step 1.1.

③ After PCR, if the bright band was in the position of 419bp, indicated the PCR was successful, and next,should purify the PCR product.

④ Made marks: PCR SV40 purification + date, and entered step 2.

2、Enzyme digestion

SV40 PCR purification product：	3μL
EcoR1：	1μL
Bcu1(Spe1)：	4μL
Buffer R：	1μL
H2O：	1μL
total：	10μL

① The enzyme digestion system was formulated with 10 tubes, which were digested with 10 L*10.

② Enzyme digestion condition: 37 C, 3h above (digestion overnight is best , also can digest all day.It is good for ligation overnight by T4 ligase)

③ After enzyme digestion, the system was merged 10 tubes into one tube, and purified after running agarose gel electrophoresis,which showed the bright band in 419bp.

④ Made marks:The SV40（ES)digestion and purification + date, and than entered step 3.

3、Ligation

Ligated SV40 and Rlu with pSB1C3:

SV40（ES）：	3μL
Rlu（XP）：	3μL
PSB1C3（EP）：	1μL
T4 ligase：	1μL
10*T4 buffer：	2μL
Total:	10μL

① The ligation system needed to be formulated with 2 tubes, and each tube was 10 ul/

② Ligation condition: 16, 3h (best ligation overnight).

③ After the ligation, could directly enter step 4.

4、Transformation

① Packed a box of ice. Took a tube of competent cells(100ul) from -80 degrees refrigerator and placed in the ice to melt (can not be placed at room temperature, nor a violent shock, otherwise the competent cells would be dead).

② When competent cells melted completely(about 10min), added all the ligation product (10 ul) the competent cells, and gently blew. (not a violent shock, can not come out of the ice box, it is necessary to carry on the ice.)

③ Placed 30min on ice.

④ Set the competent cells(110ul) in sponge and placed in 42℃ water to bath 90s (not too long), immediately removed and placed in the ice, ice bath for five minutes.

⑤ Lighted alcohol burner in the super clean bench, added 890ul LB to competent cells (the super clean bench before using should be disinfected by ultraviolet,the LB before using and covering are required to the long time firing.)

⑥ Put the competent cells which added LB on the table concentrator (set in the sponge) ,150rpm shaking an hour(table password is: 3).

⑦ An hour later,took out the competent cells,and centrifugated with 12000rpm 3min.

⑧ Took a chloromycetin(Chl) LB Plating. Removed 900ul LB of competent cells,which was centrifugated, leaving 100ul competent cells,and blew 100ul competent cells with transfer liquid gun,and than added it to the Chl LB Plating. Used glass spreader (before using,needed to be cool after alcohol burner firing,about 5min) to flat the bacterial liquid gently on the LB Plating. Sealed the sealing film, and finally made good marks: hUPll+AckRS+ date, placed in a 37℃ constant temperature

incubator for 2 days .

Two days later,entered step 5.

5、Picking bacterial colonies and Culture

① Took ten 1.5ml EP tubes from the lunch box in the super clean bench, numbered 1-10, each EP tube added in 1ml LB liquid medium and 1ul Chl. Picked 10 bacterial colonies and respectively put into the 10 EP tubes.

② Set the 10 EP tubes into the sponge, put it in the table concentratort, shaking with 220rpm,until the medium became turbid.

③ Entered step 6.

6、PCR verification

① Put the 10 EP tubes of step 5 in the centrifuge to centrifugate in 12000rpm 3min.

② Preparation of the following PCR system:

SV40（up）：	0.25μL
SV40（down）：	0.25μL
Rlu（up）：	0.25μL
Rlu（down）：	0.25μL
H2O：	4μL
Mix：	5μL
total：	10μL

③ The PCR system needed to be formulated with 10 tubes, and numbered them 1-10.

④ Sucked up about 1ul bacterial precipitation to add to the corresponding PCR tubes with the small transfer liquid gun in the super clean bench,and PCR at the optimum temperature of step1.1.

⑤ Took 5ul from each of 10 tubes and verified by running agarose electrophoresis. If there were bright bands at 419bp（SV40）and 936bp（Rlu）, needed to transfer the bacteria to a Erlenmeyer flask to take a large amount of culture(adding about 20ml LB, and the addition 20ul of chloromycetin (Chl)) .

⑥ If there was no corresponding bright brand,did again from step 5 to step 6.

⑦ Extracted plasmid, and verified by single and double enzyme digestion.

⑧ Prepared the following enzyme digestion system:

Single enzyme digestion:

plasmid：	5μl
Pst1：	1μl
10*Buffer O:	2μl
H2O：	12μl
total：	20μl

double enzyme digestion:

plasmid：	5μl
EcoR1：	1μl
Pst1：	1μl
10*Buffer O:	2μl
H2O：	11μl
total：	20μl

⑨ If the single and double digestion could cut out the corresponding size of the bright band, it indicated the BioBrick was successfully constructed.

二、 TERT+tRNA（455bp+ 72bp）

Because tRNA only has 72bp, we directly amplified TERT and tRNA by PCR.

1、PCR clone

1.1、PCR try

PCR trial system：

TERT+tRNA plasmid：	1μL
TERT（王皓 up）：	0.25μL
tRNA（王皓 down）：	0.25μL
H2O：	3.5μL
Mix：	5μL
total：	10μL

Set three temperature gradients：

① 95℃：5min→ 94℃：30s→ 48℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

② 95℃：5min→ 94℃：30s→ 55℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

③ 95℃：5min→ 94℃：30s→ 58℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

After the PCR, run agarose gel electrophoresis.If the light band was in the 527bp position, indicating that PCR was successful, you can directly enter the step 1.2. If not you should try again with changing the PCR conditions,such as renatural temperature.

1.2、PCR amplification

PCR amplification system：

TERT+tRNA plasmid:	1μL
TERT（王皓 up）：	0. 5μL
tRNA（王皓 down）：	0. 5μL
H2O：	13μL
Mix：	15μL
total：	30μL

① After PCR, if the bright band was in the position of 527bp,indicated the PCR was successful, and the next step should purify the PCR product.

② Made marks: TERT+tRNA PCR purification + date, and enter step 2.

2、Enzyme digestion

TERT+tRNA plasmid：	5μL
EcoR1：	1μL
Pst1：	1μL
10*Buffer O:	2μL
H2O：	11μL
total：	20μL

① The enzyme digestion system was formulated with 10 tubes, which were digested with 10 L*10.

② Enzyme digestion condition: 37 C, 3h above (digestion overnight is best , also can digest all day.It is good for ligation overnight by T4 ligase)

③ After enzyme digestion, the system was merged 10 tubes into one tube, and purified after running agarose gel electrophoresis,which showed the bright band in 527bp.

④ Made marks:TERT+tRNA（EP）digestion and purification+ date, and than entered step 3.

3、Ligation

Ligated TERT+tRNA with pSB1C3:

TERT+tRNA（EP）：	3μL
PSB1C3（EP）：	1μL
T4 ligase：	1μL
10*T4 buffer：	2μL
H2O：	1μL
Total:	10μL

① The ligation system needed to be formulated with 5 tubes, and each tube was 10 ul.

② Ligation condition: 16, 3h (best ligation overnight).

③ After the ligation, could directly enter step 4.

4、Transformation

The operation is the same as above.

5、Picking bacterial colonies and Culture

The operation is the same as above.

6、Bacterial colonies PCR verification

① Put the 10 EP tubes of step 5 in the centrifuge to centrifugate in 12000rpm 3min.

② Preparation of the following PCR system:

TERT（up）：	0.25μL
tRNA（down）：	0.25μL
H2O：	4.5μL
Mix：	5μL
total：	10μL

③ The PCR system needed to be formulated with 10 tubes, and numbered them 1-10.

④ Sucked up about 1ul bacterial precipitation to add to the corresponding PCR tubes with the small transfer liquid gun in the super clean bench,and PCR at the optimum temperature of step1.1.

⑤ Took 5ul from each of 10 tubes and verified by running agarose electrophoresis. If there was a bright band at 527bp（TERT+tRNA）, needed to transfer the bacteria to a Erlenmeyer flask to take a large amount of culture(adding about 20ml LB, and the addition 20ul of chloromycetin (Chl)) .

⑥ If there was no corresponding bright brand,did again from step 5 to step 6.

⑦ Extracted plasmid, and verified by single and double enzyme digestion.

⑧ Prepared the following enzyme digestion system:

Single enzyme digestion:		double enzyme digestion:	
TERT+tRNA plasmid：	5μl	TERT+tRNA plasmid：	5μl
Pst1：	1μl	EcoR1：	1μl
10*Buffer O:	2μl	Pst1：	1μl
H2O：	12μl	10*Buffer O:	2μl
		H2O：	11μl
total：	20μl	total：	20μl

⑨ If the single and double digestion could cut out the corresponding size of the bright band, it indicated the BioBrick was successfully constructed.

三、 Improved TERT+tRNA（455bp+72bp）

Directly amplified improved TERT and tRNA by PCR from the plasmid.

1、PCR clone

1.1、PCR try

PCR trial system：

高效 TERT+tRNA plasmid：	1μL
高效 TERT（王皓 up）：	0.25μL
tRNA（王皓 down）：	0.25μL
H2O：	3.5μL
Mix：	5μL
total：	10μL

Set three temperature gradients：

① 95℃：5min→ 94℃：30s→ 48℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

② 95℃：5min→ 94℃：30s→ 55℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

③ 95℃：5min→ 94℃：30s→ 58℃：30s→ 72℃：2min→ 72℃：7min→ 4℃：∞

After the PCR, run agarose gel electrophoresis.If the light band was in the 527bp position, indicating that PCR was successful, you can directly enter the step 1.2. If not you should try again with changing the PCR conditions,such as renatural temperature.

1.2、PCR amplification

PCR amplification system：

高效 TERT+tRNA plasmid：	1μL
高效 TERT(王皓 up) ：	0. 5μL
tRNA（王皓 down）：	0. 5μL
H_2O：	13μL
Mix：	15μL
total：	30μL

① The system was formulated with 5 tubes, that was, 30ul*5.

② Temperature setting should be and the same with the temperature in step 1.1.

③ After PCR, if the bright band was in the position of 527bp,indicated the PCR was successful, and next should purify the PCR product.

④ Made marks: 高效 TERT+tRNA PCR purification + date, and entered step 2.

2、Enzyme digestion

高效 TERT+tRNA plasmid：	5μL
EcoR1：	1μL
Pst1：	1μL
10*Buffer O:	2μL
H2O：	11μL
Total:	20μL

① The enzyme digestion system was formulated with 10 tubes, which were digested with 10 L*10.

② Enzyme digestion condition: 37 C, 3h above (digestion overnight is best , also can digest all day.It is good for ligation overnight by T4 ligase)

③ After enzyme digestion, the system was merged 10 tubes into one tube, and purified after running agarose gel electrophoresis,which showed the bright band in 527bp.

④ Made marks:高效 TERT+tRNA（EP）digestion and purification+ date, and than entered step 3.

3、Ligation

Ligated TERT+tRNA with pSB1C3:

高效 TERT+tRNA（EP）：	3μL
PSB1C3（EP）：	1μL
T4 ligase：	1μL
10*T4 buffer：	2μL
Total:	10μL

① The ligation system needed to be formulated with 2 tubes, and each tube was 10 ul.

② Ligation condition: 16, 3h (best ligation overnight).

③ After the ligation, should directly enter step 4.

4、Transformation

The operation is the same as above.

5、Picking bacterial colonies and Culture

The operation is the same as above.

6、Bacterial colonies PCR verification

① Put the 10 EP tubes of step 5 in the centrifuge to centrifugate in 12000rpm 3min.

② Preparation of the following PCR system:

高效 TERT（up）：	0.25μL
tRNA（down）：	0.25μL
H2O：	4.5μL
Mix：	5μL
total：	10μL

③ The PCR system needed to be formulated with 10 tubes, and numbered them 1-10.

④ Sucked up about 1ul bacterial precipitation to add to the corresponding PCR tubes with the small transfer liquid gun in the super clean bench,and PCR at the optimum temperature of step1.1.

⑤ Took 5ul from each of 10 tubes and verified by running agarose electrophoresis. If there was a bright band at 527bp（高效 TERT+tRNA）, needed to transfer the bacteria to a Erlenmeyer flask to take a large amount of culture(adding about 20ml LB, and the addition 20ul of chloromycetin (Chl)) .

⑥ If there was no corresponding bright brand,did again from step 5 to step 6.

⑦ Extracted plasmid, and verified by single and double enzyme digestion.

⑧ Prepared the following enzyme digestion system:

Single enzyme digestion:		double enzyme digestion:	
高效 TERT+tRNA plasmid：	5μl	高效 TERT+tRNA plasmid：	5μl
Pst1：	1μl	EcoR1：	1μl
10*Buffer O:	2μl	Pst1：	1μl
H2O：	12μl	10*Buffer O:	2μl
		H2O：	11μl
total：	20μl	total：	20μl

⑨ If the single and double digestion could cut out the corresponding size of the bright band, it indicated the BioBrick was successfully constructed.