Human sweet receptor T1R2-T1R3 heterologous expression

1. Human sweet receptor T1R2-T1R3 gene synthesis

1.1 sequence seeking

Through paper[1] we know that human sweet receptor T1R2-T1R3 can be stabilized in a heterodimer shape and stay active in the body.

We searched gene from web site NCBI and successfully found it, ID: 80834, ID: 83756.

[1] Dubois G E. Molecular mechanism of sweetness sensation[J]. Physiology & Behavior, 2016, 164(Pt B):453.

1.2 oligo design

1.2.1 codon optimization

Because we wanted to express our target protein in yeast, for better expression, we used the software Snapgene to optimize the sequence.

1.2.2 oligo design

After discussed with our advisors, they provided an economical and efficient approach to synthesis target gene, that was to use oligo design software OLIGO.

First we designed 90 primers for each sweet receptor, each primer had 15bp overlap region with the adjacent one. Next we divided 90 primers into 3 groups, named block A, B and C, and mixed the primers of each group and made the final concentration to 10μm. We successfully got 6 desire fragments after PCR and gel electrophoresis.

Building block:

5X fastPfu buffer	5μl
H_2O	16.5μl
dNTPs mix	2μl
fastPfu	0.5μl
primer mix	1μl
total	25μl

PCR process：

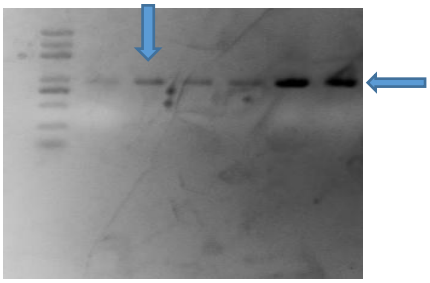
94℃ 5min，

（94℃ 30sec，55℃ 30sec，72℃ 1min）30 cycles

72℃ 7min，

16℃ remain

T1R2 block A B C



1.3 gene synthesis

1.3.1 OE-PCR

We used building blocks as template and got the correct sequence of T1R2 and T1R3 by OE-PCR.

OE-PCR:

5X fastPfu buffer	5μl
H_2O	15.5μl
dNTPs mix	2μl
T1R2-1 -F	0.5μl
T1R2-90-R	0.5μl
fastPfu	0.5μl
building block mix	1μl
total	25μl

PCR process：

94°C 5min，

（94°C 30sec，58°C 30sec，72°C 3min）30 cycles

72°C 7min，

16°C remain

5X fastPfu buffer	5μl
H_2O	15.5μl
dNTPs mix	2μl
T1R3-1 -F	0.5μl
T1R3-90-R	0.5μl
fastPfu	0.5μl
building block mix	1μl
total	25μl

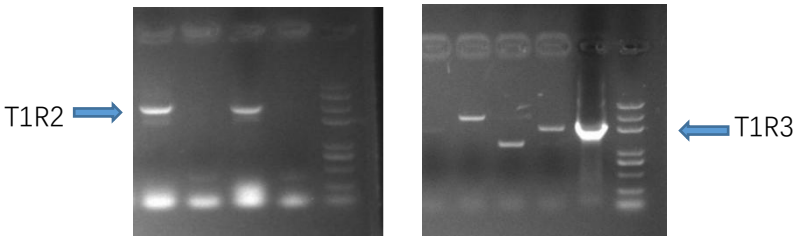
PCR process：

94°C 5min，

（94°C 30sec，56°C 30sec，72°C 3min）30 cycles

72°C 7min，

16℃ remain



1.3.2 Gibson assembly

We used building blocks as template and got the correct sequence of T1R3 by Gibson assembly.

1.4 troubleshooting

The reason we use Gibson assembly to synthesis T1R3 is that it has many sequence similar regions, they disturb the primer and template pairing thus reduced pairing property as well as PCR efficiency. We use Gibson assembly to avoid this problem.

2. adding tags to track T1R2-T1R3 gene expression

2.1 fluorescent protein cloning

The idea was to add different color proteins to each sweet receptor, so that we can know whether the heterodimer was successfully expressed and located at the certain position using fluorescent microscope. We checked the part library, got blue, yellow and green color proteins. After transformation, we successfully got target genes through PCR.AAA

During the primer designing, we added another 20bp of N-terminal of each sweet receptor as the overlap region.

Primer	Sequence
BamHI-BFP-t1r2-F	CGGGATCCATGAGTGTGATCGCTAAACAAATG
BamHI-BFP-t1r2-R	GTCTTAGCTCTTGGACCCATaccttgaaaatataaattttcTTATTAGGCGACCACAGGTTTGCGTG
BamHI-YFP-t1r2-F	CGGGATCCATGTCTTATTCAAAGCATGG
BamHI-YFP-t1r2-R	GTCTTAGCTCTTGGACCCATaccttgaaaatataaattttcTTATTATTTAACCTTCAAAGGGTT
BamHI-GFP-t1r2-F	CGGGATCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
BamHI-GFP-t1r2-R	GTCTTAGCTCTTGGACCCATaccttgaaaatataaattttcGTGGTGGTGGTGGTGGTG
NotI-Blue-t1r3-F	ATAAGAATGCGGCCGCATGAGTGTGATCGCTAAACAAATG
NotI-Blue-t1r3-R	AAAACAGCTGGACCCAACATaccttgaaaatataaattttcTTATTAGGCGACCACAGGTTTGCGTG
NotI-yellow-t1r3-F	ATAAGAATGCGGCCGCATGTCTTATTCAAAGCATGG
NotI-yellow-t1r3-R	AAAACAGCTGGACCCAACATaccttgaaaatataaattttcTTATTATTTAACCTTCAAAGGGTT

5X fastPfu buffer	5μl
H_2O	16μl
dNTPs mix	2μl
primer-F	0.5μl

primer-R	0.5μl
fastPfu	0.5μl
Template	0.05μl
Total	25μl

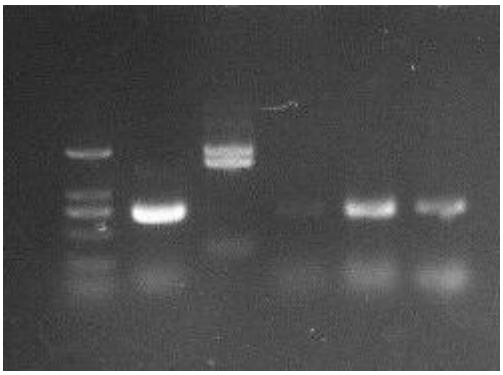
PCR process：

94℃ 5min，

（94℃ 30sec，60℃ 30sec，72℃ 40s）30 cycles

72℃ 7min，

16℃ remain



700bp color protein

2.2 fluorescent tags link to T1R2-T1R3

Though OE-PCR we can link fluorescent tags to each sweet receptor, the overlap region had been added when cloning color proteins.

Primer	Sequence
T1R3-speI-R	GGACTAGTTTCGTGCTTACCTTGGTTACCGGTG
T1R2-SalI-R	TTCCGCGGCCGCTATGGCCGACGTCGACGCGTCTTCTCATGGTGTAACCTTG

OE-PCR:

5X fastPfu buffer	5μl
H_2O	16μl
dNTPs mix	2μl
primer-F	0.5μl
primer-R	0.5μl
fastPfu	0.5μl
Template	0.5μl
total	25μl

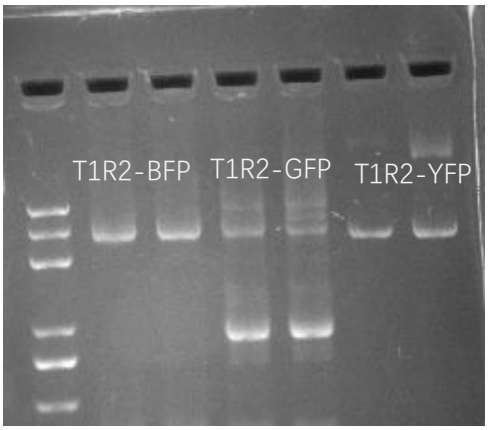
PCR process：

94℃ 5min，

（94℃ 30sec，58℃ 30sec，72℃ 3min）30 cycles

72℃ 7min，

16℃ remain



2.3 epitope tags link to T1R2-T1R3

The idea was to add different epitope tags to each sweet receptor, so that through immunofluorescence technology can we know whether the heterodimer was successfully expressed and located at the certain position. Though OE-PCR we can link epitope tags to each sweet receptor, the overlap region had been added when cloning epitope tags.

Primer	Sequence
BamHI-HIS-T1R2-F	CGGGATTCATGCATCATCATCATCATCATATGGGTCCAAGAGCTAAGACCATCTCTTC
BamHI-FLAG-T1R2-F	CGGGATTCATGGATTACAAGGATGACGACGATAAGATGGGTCCAAGAGCTAAGACCATCTCTTC
BamHI-MYC-T1R2-F	CGGGATTCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATGGGTCCAAGAGCTAAGACCATCTCTTC
NOTI-HIS-T1R3-F	ATAAGAATGCGGCCGCATGCATCATCATCATCATCATATGTTGGGTCCAGCTGTTTTGGG
NOTI-FLAG-T1R3-F	ATAAGAATGCGGCCGCATGGATTACAAGGATGACGACGATAAGATGTTGGGTCCAGCTGTTTTGGG
NOTI-MYC-T1R3-F	ATAAGAATGCGGCCGCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATGTTGGGTCCAGCTGTTTTGGG

5X fastPfu buffer	5μl
H_2O	16μl
dNTPs mix	2μl
primer-F	0.5μl
primer-R	0.5μl
fastPfu	0.5μl
Template	0.5μl
total	25μl

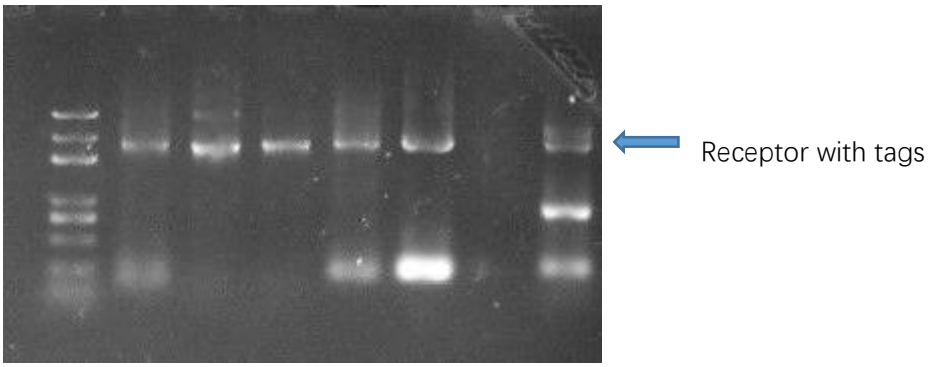
PCR process：

94℃ 5min，

（94℃ 30sec，58℃ 30sec，72℃ 3min）30 cycles

72℃ 7min，

16℃ remain



3. heterologous expression in *Saccharomyces cerevisiae*

3.1 shuttle vector construction

In order to express the human receptor, we chose *Saccharomyces cerevisiae* strain *CEN.PK2-1C* as our host and the shuttle vector, pESC-Ura, to express T1R2-T1R3. We selected two restrict sites for two receptor genes respectively, using PCR to add *BamHI* and *SalI* to the T1R2 fragment and *SpeI* and *NotI* to T1R3. And the fragments with fluorescent tags or other tags also linked with restrict sites through PCR. The PCR was performed as previous.

Primer	Sequence
BamHI-T1R2-F	CGCGGATCCATGGGTCCAAGAGCTAAGACCA
SalI-T1R2-R	ACGCGTCGACTTAGTCTCTTCTCATGGTGTAACCTTGGA
T1R3-NotI-F	GCGGCCGCTTCTAGAGATGTTGGGTCCAGCTGTTTTGGGTT
T1R3-SpeI-R	ATACTAGTATTCGTGCTTACCTTGGTTACCGGTGTTACCGTCG

The PCR production was purified and prepared the digestion mixture as follow:

10 X FastDigest Buffer	2μl
Upstream restrict enzyme	1μl
Downstream restrict enzyme	1μl
Purified PCR production	16μl

The production was inactivated by incubating at 80℃ for 20min.

The digestion for plasmid was prepared as follow:

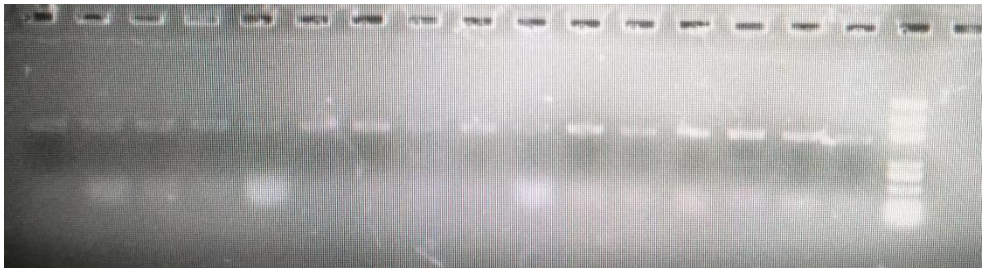
10 X FastDigest Buffer	6μl
Upstream restrict enzyme	1.5μl
Downstream restrict enzyme	1.5μl
Plasmid	30μl
Water	up to 60μl

And the digestion production was purified.

T4 ligase was used to ligate the digested DNA fragments to the multi cloning sites of plasmid pESC-Ura. After ligation, we transformed the ligation production to the *E.coli* TOP 10 then selected the positive colonies using colony-PCR.
Ligation mixture was as following.

10 X T4 ligase Buffer	1μl
DNA fragment	5.5μl
Plasmid fragment	3μl
T4 ligase	0.5μl

Bathing at 25℃ for 1 hour or at 16℃ for a night then can be performed transformation.



3.2 yeast transformation

After finishing the construction of vector with sweetness receptor in *E.coli*, the recombination plasmids was transformed into yeast, *CEN.PK2-1C* with P_{fus}-*mRFP-CYC1t*, through LiAc transformation. Because of the auxotrophic selection marker Ura in the pESC-Ura, the colony was chosen under the SD-Ura medium. Then the positive cloning can survive in this medium. In this way, we can get the reconstruction *Saccharomyces cerevisiae* successfully.

SD-Ura medium:

yeast nitrogen base without amino acids (YNB)	6.7g/L
Necessary amino acid mixture	1.3g/L
Glucose	20g/L
Leucine	0.1g/L
Tryptophan	0.04g/L
Histidine	0.02g/L

Then control the pH between 6.2 and 6.5.

3.3 fluorescence detection

3.3.1 yeast own fluorescence detection

As we all know, the membrane protein is hard to identify because the amount of them is low and it is difficult to purify. So the T1R2-T1R3 with fluorescent tags is designed to confirm the expression and location of T1R2-T1R3 in the *CEN.PK2-1C* through the immunofluorescence technology. And we tested our yeast by fluorescence plate reader in generally. We used the minimal induction medium to induce the Gal 1/10 promoter to express their downstream gene then detected the fluorescence.

Protocol for fluorescence test through fluorescence plate reader (Red fluorescence protein):

1. Incubate the yeast for 24 hours preciously using SD-Ura medium with 0.3% G418

to get the harvest the cells.

2. Replace the SD medium by minimal induction medium:

yeast nitrogen base without amino acids (YNB)	6.7g/L
Necessary amino acid mixture	1.3g/L
Galactose	2%
Glycerol	2%
Leucine	0.1g/L
Tryptophan	0.04g/L
Histidine	0.02g/L

3. Culture the yeast for 12 hours.
4. Sampling and test the fluorescence adsorption value (FAV) by plate reader. The fluorescence excitation wavelength is 560 nm and the fluorescence adsorption wavelength is 610nm. (The excitation/absorption wavelength of different fluorescence protein is chosen according to their characteristic excitation/adsorption.
5. Measure the OD600 of sample and calculate the single cell fluorescence (SCFAV) adsorption value according to the formula.Favorite

$$\mathrm{SCFAV} = \frac{FAV}{OD_{600}}$$

3.3.2 immunofluorescence

Protocol

1. Take out 1ml *S.cerevisiae* which have been induced by galatose in bechtop. Then harvest by centrifugation by 6000g for 5 min.
2. Remove the supernatant and add 1×PBS to wash the thallus with centrifugation of 6000g, 5 min, for 3 times.
3. Add optimum amount TBS containing 1% BSA to resuspend the thallus and adjust the OD600 to five.
4. Add 1.2ul monoclonal antibody to 200ul of cell suspension and incubate the mix at 37℃ for 2h.
5. Centrifuge the mix with 6000g for 5min and then wash the cell by 1×PBS for 3 times.
6. Wash the cell by 1×PBS containing 1% BSA for 1 time.
7. Wash the cell by 1×PBS containing 1% BSA adding 1.2 ul Alexa Floor 488 which is an IgG antibody. Incubate the mix at 37℃ for 1h
8. Wash the cell by 1×PBS for 3 times.
9. Resuspend the cell using 1.5ml 1×PBS
10. Take out 2ul cell suspension to put on microscope slide and then observe it by fluorescence microscope.

Plasmid construction by homologous recombination in yeast

1.Gene knockout

To make our yeast detect sweeteners, we replaced Ste2 receptor with Human sweet receptor T1R2-T1R3. So, first, we had to knock out *ste2* gene. To decrease other interference factor we knocked out *sst2* and *far1*.

1.1 primer design

1.2 gene synthesis

1.2.1 PCR(left arm/right arm)

5 X fast Pfu buffer	5μl
H_2O	12.5μl
dNTPs mix（buffer）	2μl
primer(F/R)	2μl
fast Pfu	0.5μl
Template	1μl
total	25μl

PCR process：

94℃ 5min

（94℃ 30sec，50℃ 30sec，72℃ 30sec）30 cycles

72℃ 7min

16℃ remain

PCR(Marker:His/Trp/Ura)

5 X fast Pfu buffer	5μl
H_2O	12.5μl
dNTPs mix(buffer)	2μl
primer(F/R)	2μl
fast Pfu	0.5μl
Template	1μl
total	25μl

PCR process：

94℃ 5min

（94℃ 30sec，55℃ 30sec，72℃ 30sec）30 cycles

72°C 7min

16°C remain

1.2.2 OE-PCR(left arm+marker+right arm)

5 X fast Pfu buffer	5μl
H_2O	12.5μl
dNTPs mix（buffer）	2μl
primer(F/R)	2μl
fast Pfu	0.5μl
Template	1μl
total	25μl

PCR process：

94°C 5min

（94°C 30sec，55°C 30sec，72°C 30sec）30 cycles

72°C 7min

16°C remain

1.3 *E.coli* transformation

To enrich our pESC-URA、 pESC-His、 pESC-Trp plasmid, we transfered the plasmid into *E.coli*.

(1). Take the competent cells (BM TOP 10) out of the Ultra-low temperature freezer.

(2). Put the competent cells in the ice for 5-10 mintues.

(3). Add 1ul of the plasmid into the competent cells and put them in the ice for 15-30 mintues.

(4). Next put the competent cells in the water bath at 42°C for 90 seconds. Then put the competent cells in the ice for 5mintues quickly. And don't remove the cells.

(5). Add 600-1000ul LB-media to the competent cells and put them into the Incubator shaker for 45-60 minutes in 37°C.

(6). Harvest by centrifugation at 4000rpm for 1min and remove 500-900ul of supernatant. Plate the rest of 100ul of E.coli onto selective plates.

(7). Put the LB-medium to the Incubator.

1.4 colony PCR

(1) Prepare the reaction mix in PCR tube:

ddwater	8 µl
Forward primer	1 µl
Reverse primer	1µl
2×Taq PCR Master Mix	10 µl
Total volume	20 µl

(2) Use pipettor spear to pick a single colony and put it in the PCR tube.

(3) Make sure the bacteria is in the cPCR tube.

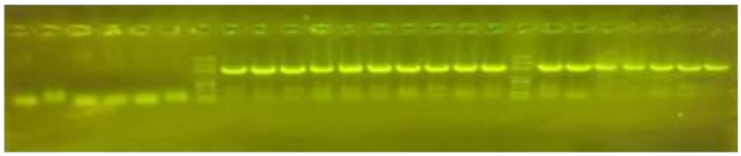
(4) Pick out the pipettor spear.

(5) PCR process:

94℃	10min
94℃	30s
55℃	30s
72℃	1.5min
72℃	7min
16℃	∞

(6) Take positive colonies into LB medium (medium according to the proportion of the latter to join the corresponding antibiotics)

(7) Keep the life of bacteria and extract plasmid



1.5 yeast transformation

(1) Pick a fresh colony of yeast from a plate and add 5ml YPD-media to the fresh colony, then incubate overnight at 30℃

(2) Inoculate the yeast into the fresh YPD at a volume ratio of 10%

(3)30℃、200rpm for 4-5h

(4) Add 1.5ul of the yeast into the centrifuge tube and centrifuge it at 4000rpm for 5min(at 5000rpm for 2min)

(5) Remove the supernatant and add 1ml ddH_2O to resuspend the cells. Then centrifuge it again.

(6) Remove the supernatant and add 1ml、100mM LiAc to resuspend the cells. Don't remove the cells for 5min.

(7) Centrifuge it at 4000rpm for 3min and remove the supernatant.

(8) Place a thawed tube of SS-DNA in a boiling water bath for 5 min and chill immediately in ice.

(9) Conversion mixture includes:

69 ul ddH_2O
5 ul DNA
10ul SS-DNA
240ul PEG
36ul 1.0M LiAc

(10) Eddy the centrifuge tube for 1min

(11) Put the tube into the incubator for 30min in 30℃.Then incubate the tube in a water bath at 42℃ for 20min.

(12) Centrifuge it at 4000rpm for 3min and remove the supernatant.

(13) Add 1ml ddH_2O to resuspend the cells and centrifuge it again.

(14) Do (13)step again.

(15) Add 100ul ddH_2O to resuspend the cells gently.

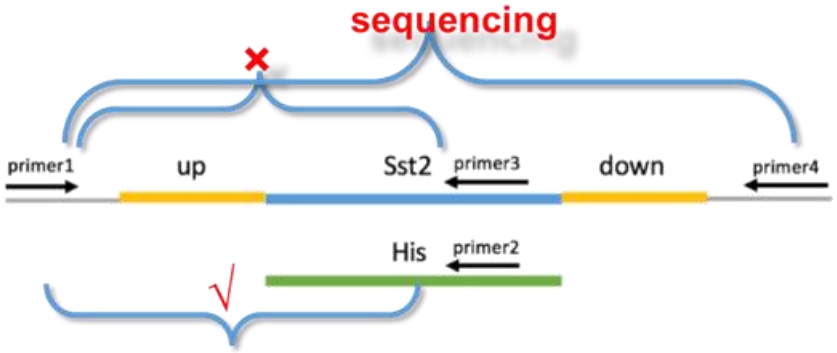
(16) Plate the cell suspension onto plates.

(17) Incubate the plates at 30℃

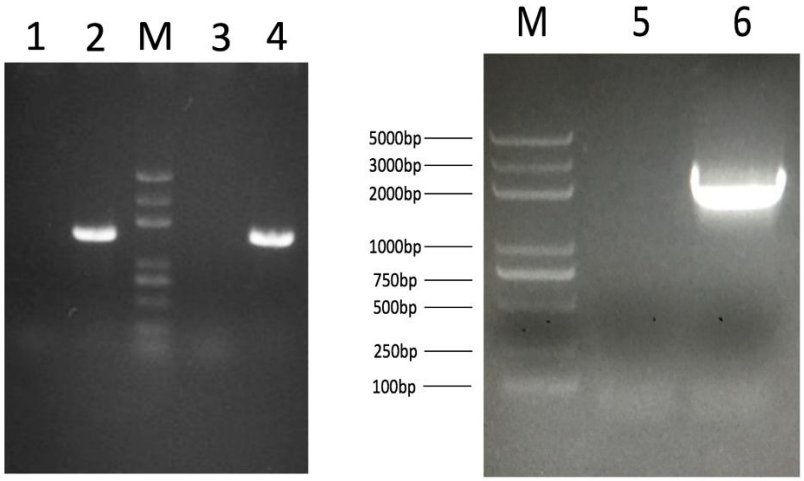
1.6 extract the yeast genome

1.7 verify the reconstructed yeast genome

To verify whether the gene was knocked out, we designed the primer 1, 2, 3 and 4 as shown in the following figure. The primer 1 and primer 4 were on the yeast genome.



We knock out the *far1* gene as shown in the following figure.



The lanes 1 and 3 are products of PCR using primer 1, 3 and *Δsst2* yeast's genome are the template.
The lanes 2 and 4 are products of PCR using primer 1, 2 and *Δsst2* yeast's genome are the template.
The lane 5 is the product of PCR using primer 1, 2 and the original yeast's genome are the template.
The lane 6 is the product of PCR using primer 1, 3 and the original yeast's genome are the template.

At the same time, we knocked out the *ste2* and *sst2* in the same way successfully.

The detection circuit

1. Construction of the P_{fus}-*mRFP-CYC1t*

1.1 sequence cloning

The detection circuit consists of a promoter P_{fus}(438bp), reporter gene *mRFP*(706bp), and a terminator *CYC1t*(229bp).

We got the sequence of P_{fus} from *Saccharomyces* genome. We got *mRFP* from our lab, and *CYC1t* from yeast genome. Each one had overlap region with the adjacent one.

1.2 OE-PCR

We used the three parts as templates, trying to connect them together by OE-PCR.

OE-PCR:

2Xbuffer	25μl
dNTP	4μl
H_2O	14.5μl
primer-F	1μl
primer-R	1μl

pFUS	1μl
RFP	1μl
CYC1t	2μl
primer star	0.5μl
total	50μl

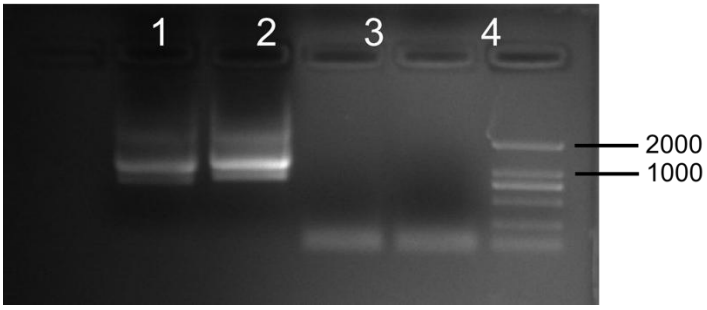
PCR process：

98℃ 1min

（98℃ 10sec，55℃ 5sec，72℃ 1min30sec）30 cycles

72℃ 7min

16℃ remain



result: As shown in the figure, the bands were about 1100bp, but the correct band should be 1400bp.

1.3 Gibson assembly

We used Gibson assembly to connect the three parts with the linear plasmid pRS42K. pRS42K is a kind of shuttle vector.

Gibson assembly:

pRS42K	0.5μl
pFUS	0.7μl
RFP	0.6μl
CYC1t	0.7μl
total	2.5μl

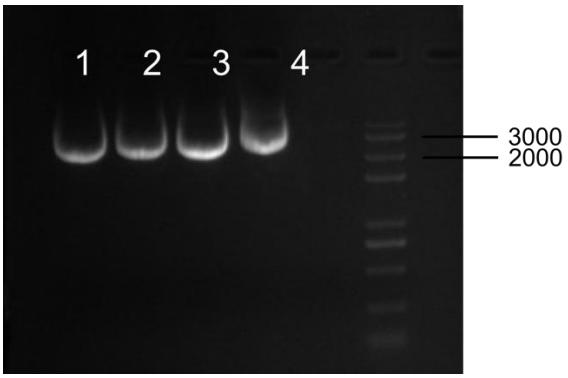
process: 50℃ 1h

2. The signal reporter device expression in *Saccharomyces cerecisiae*

2.1 E.coli transformation

We chose Top10 as host bacteria. Transformed Gibson products into the competent Top10. And plated the *E.coli* onto the ampicillin plates. Then screened positive colonies on ampicillin plates, extracted the plasmids of positive yeast colonies and sequenced them.

The recombinant plasmid is shown as the following figure.



2.2 yeast transformation

We transformed the plasmid into competent *CEN.PK2-1C*, the mating a type haploid yeast. And plated the yeast onto the G418 plates.

Then we did cPCR to verify whether the transformation was successful.

cPCR:

Taq mix	10μl
H_2O	8μl
Primer-R	1μl
Primer-F	1μl
Template	colonies
Total	20μl

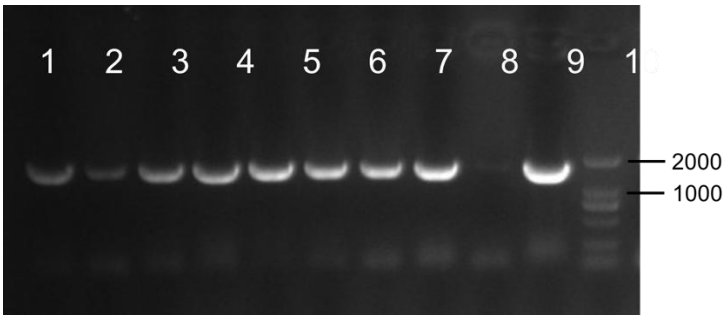
PCR process:

94℃ 5min

（94℃ 30sec，53℃ 30sec，72℃ 2min）25 cycles

72℃ 7min

16℃ remain



result: almost all the colonies are positive.

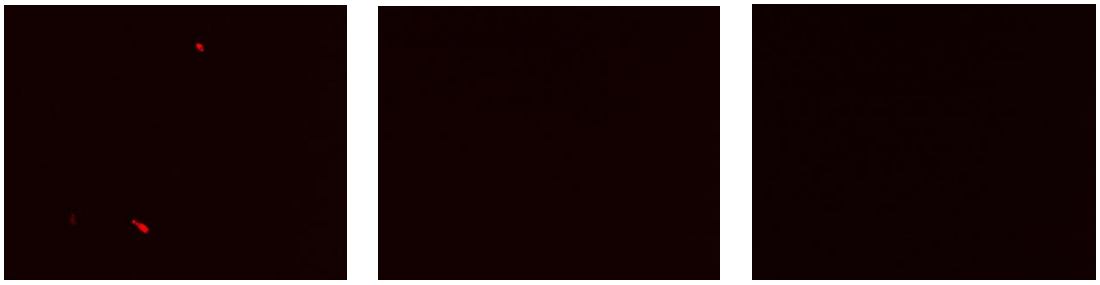
2.3 fluorescence detection

In order to find out whether the detection circuit works, we cultivated the yeast and then observed them by fluorescence microscopy.

Group A: transformated *CEN.PK2-1C*(a type) and *CEN.PK2-1D*(αtype)

Group B: transformated *CEN.PK2-1C*(a type) alone

Group C: *CEN.PK2-1D*(α type) alone



group A group B group C

After 9 hours, group A was fluorescent, and group B and group C didn’t fluoresce. The result means detection circuit worked.