

Construction of GFP reporter pathway of three promoters and their characterization in *Saccharomyces cerevisiae* BY4741

1. Cloning of promoter and reporter gene *gfp* by PCR amplification

Table 1 Primers used in this experiment

Primers	Sequence
Fig1-promoter-F	TTCCTGCAGCCCGGGGGATCCCTGGTGCTTCCTCTTTGGGA
Fig1-promoter-R	TAATTCTTCACCTTTAGACATTCTGGGCATACGCTTGGTAAA
Prm1-promoter-F	TTCCTGCAGCCCGGGGGATCCCTCCTATGCTGTTTACAAGGTCTATC
Prm1-promoter-R	TAATTCTTCACCTTTAGACATATCATCAACGTTCAAAATTCGAAAG
Fus2-promoter-F	TTCCTGCAGCCCGGGGGATCCAGGTAGAGGTGCCATCAGTT
Fus2-promoter-R	TAATTCTTCACCTTTAGACATCGCAAGGGGTTTTCTTGTCTTT
GFP-primer-F	ATGTCTAAAGGTGAAGAATTATTCCT
GFP-primer-R	ATGGTGGTAGCTGTGGGTTG
CYC1-Primer-F	CAACCCACAGCTACCAACCATATCCGCTCTAACCGAAAAGGA
CYC1-Primer-R	CGCTCTAGAACTAGTGGATCCGGCCTTTTTACGGTTCCTGG
M13-F	GTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGAC

* Red indicates homology arms.

Extract BY4741 genome with Yeast Bacteria Genomic DNA Kit (ZOMANBIO).

Use the genome as the template to amplify *gfp* by PCR amplification.

Use piGEM2020001 plasmid as the template, to obtain the CYC1 terminator by PCR amplifications.

Use BamH I to digest pRS415 plasmid to obtain linearized vector. Finally, connect each promoter (*pprm1*, *pfig1*, *pfus2* respectively), *gfp*, and CYC1 through One Step Cloning to obtain the fusion fragment.

2. Transformation of *E. coli* DH5α

3. Colony PCR verification

Select several single colonies and dissolve them in 10uL ddH₂O respectively.

Take out 1uL as the substrate of PCR. Then agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

4. Plasmid PCR verification and sequencing

Colony PCR verifies that the corresponding 9uL bacterial solution is connected to 5mL LB (Amp⁺) and cultured overnight. Then the plasmid is extracted and is used as the template for plasmid PCR. After agar gel electrophoresis verification, correct samples are sent to be sequenced (Genewiz). The successfully constructed recombinant vector is named as pRS415*Fig1-GFP-CYC1, pRS415*Prm1-GFP-CYC1, pRS415*Fus2-GFP-CYC1.

5. Construction of recombinant yeasts

The successfully constructed plasmid was extracted from *E. coli* DH5α and transformed into *Saccharomyces cerevisiae* BY4741 through electro transformation.

6. Colony PCR verification

Pick out yeast transformants from the SC (Leu⁻) auxotrophic plate after

electroporation, prepare a small amount of yeast liquid. Use primers M13-F and M13-R for amplification verification.

7. Genomic PCR verification

The PCR products are electrophoresed on a 1% agarose gel for 25 minutes, 120 v, and the gel imaging system is used for observation and photography.

The PCR amplification products were then sent to the company for sequencing.

8. Pheromone induced characterization

Single colonies of *Saccharomyces cerevisiae* BY4741 containing the recombinant plasmid pRS415-Prm1-GFP-CYC1/ pRS415-Fig1-GFP-CYC1/ pRS415-Fus2-GFP-CYC1 were picked from the plate and inoculated into 7mL SC liquid medium, incubating at 30°C, 220rpm for 20 hours.

Dilute the culture to OD₆₀₀ = 0.1 with fresh SC (complete) medium and incubate at 30°C for 10 hours until OD₆₀₀ = 0.6. Then add α pheromone (final concentration 0uM, 10uM, 25uM) to induce for 1.5-2 hours, then centrifuge at 8000rpm for one minute to collect the bacteria. Wash once with 4°C pre-cooled PBS and centrifuge at 8000rpm for one minute. Centrifuge at 8000 rpm for 1 min to remove the supernatant. Then add 500ul PBS to resuspend and wash the cells.

The green fluorescence intensities of 10,000 cells were quantitatively measured through flow cytometer.

Construction of the *Ste5ΔN-CTM* strain

1.Design PCR primers

Table 2 Primers used in this experiment

Primers	Primers
Ste5_N-F	CTTGGTACCGAGCTCGGATCCATGTCAAAGGCAGACGTTCTGTG
Ste5_N-R	CATTTTTAGATCTTTCCACCATATATAATCCATATGGAGGGGACAGTT
CTM -F	TGGTGGAAAGATCTAAAAATGAGAATGTG
CTM -R	GCGGCCGTTACTAGTGGATCCTTATTAGCTGAAATGGACGACGATAGG
PYES2-F	AAACCCCGGATCGGACTACT
PYES2-R	GGCGTGAATGTAAGCGTGAC

*The red part indicates homology arms.

2.Construction of PYES2-Ste5ΔN-CTM plasmid

Obtain *Ste5ΔN* by PCR amplification using *pprm1*-GFP-CYC1 plasmid as the template and Ste5_N-F and Ste5_N-R as primers. Obtain CTM by PCR amplification using *pprm1*-GFP-CYC1 plasmid as template and CTM-F and CTM-R as primers. Use BamH I to digest pRS415 plasmid to obtain linearized vector. Finally, connect *Ste5ΔN*, CTM and linearized pRS415

through One Step Cloning to obtain the fusion fragment. Transformation of *E. coli* DH5 α .

3.Colony PCR verification

Select several single colonies and dissolve them in 10uL ddH₂O respectively. Take out 1uL as the substrate of PCR. Then agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

4.Plasmid PCR verification and sequencing

Inoculate the corresponding 9uL bacterial solution which colony PCR verifies corrected to 5mL LB (Amp⁺) medium and cultured overnight. Extract the plasmid with AxyPrep Plasmid Miniprep Kit (AXYGEN). Use it as template for PCR and perform agar gel electrophoresis to evaluate the length of the obtained fragment. Deliver the correct samples for sequencing (Genewiz). The recombinant plasmid is named as PYES2-Ste5 Δ N-CTM.

5.Construction of recombinant yeasts

The successfully constructed plasmid was extracted from *E. coli* and transformed into the strain *ste5 Δ -loxp-1* through electro transformation.

6.Colony PCR verification

Pick out yeast transformants from the SC (Ura⁻) auxotrophic plate after electroporation and expand culture in another SC (Ura⁻) auxotrophic plate. Pick a small part of monoclonal colony into 10uL ddH₂O and water bath at 95°C for 10 minutes. Then take out 1uL as the template of colony PCR (primers: PYES2-F, PYES-R). Agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

7.Genomic PCR verification and sequencing

Inoculate the corresponding monoclonal colony which was verified correct to 5mL SC (Ura⁻) auxotrophic medium and cultured for 24 hours. Extract the genome with Yeast Bacteria Genomic DNA Kit (ZOMANBIO), and use it as the template for PCR. After agar gel electrophoresis verification, correct samples are sent to be sequenced (Genewiz).

Improvement and characterization of *pprm1*

1. Design PCR primers

Table 3 Primers used in this experiment

Primers	Sequence
GFPv2-F	TTCCTGCAGCCCGGGGGATCCATGTCTAAAGGTGAAGAATTATTCAC TGG
CYC1-terminatorv 2-R	CCGCTCTAGAACTAGTGGATCGGCCTTTTTACGGTTCCTGG
Prm1-promoter-F	TTCCTGCAGCCCGGGGGATCCCTCCTATGCTGTTTACAAGGTCTATC
PRM1-PRE-R	GCAGAGTCCGGGTAATACATATGAAACAGTATTGAAACAGTATTGAA ACAGAAGTGCGTCACATATTAATTTTAACTTATAAC

Prm1-promoter-R	AGTGAATAATTCTTCACCTTTAGACATATCATCAACGTTACAAATTCTG AAAG
PRM1_PRE-F	GCAGAGTCCGGGTAATACATATGTTTCAATACTGTTTCAATACTGTTTC AATACTGTTTCAATACTGTTTCAATACTGTTTCAG
PRM1_PREv2-F	GCAGAGTCCGGGTAATACATATGAAACAGTATTGAAACAGTATTGAAA CAGAAGTGCGTCACATATTAATTTTAACTTATAAC
M13-F	GTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGAC

*The red part indicates homology arms.

2. Construction of pRS415-GFP-CYC1 plasmid

Obtain GFP-CYC1 by PCR amplification using *pprm1*-GFP-CYC1 plasmid as the template and GFPv2-F and CYC1-terminatorv2-R as primers. Use BamH I to digest pRS415 plasmid to obtain linearized vector. Finally, connect GFP-CYC1 and linearized pRS415 through One Step Cloning to obtain the fusion fragment. Transformation of *E. coli* DH5 α .

3. Colony PCR verification

Select several single colonies and dissolve them in 10uL ddH₂O respectively. Take out 1uL as the substrate of PCR. Then agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

4. Plasmid PCR verification and sequencing

Inoculate the corresponding 9uL bacterial solution which colony PCR verifies corrected to 5mL LB (Amp⁺) medium and cultured overnight. Extract the plasmid with AxyPrep Plasmid Miniprep Kit (AXYGEN). Use it as template for PCR and perform agar gel electrophoresis to evaluate the length of the obtained fragment. Deliver the correct samples for sequencing (Genewiz). The recombinant plasmid is named as *pRS415-GFP-CYC1*.

5. Construction of pRS415-6×PRE-*pprm1*-GFP-CYC1 and pRS415-3×PRE_R-*pprm1*-GFP-CYC1 plasmid

Obtain Fragment1 by PCR amplification using *pprm1*-GFP-CYC1 plasmid as the template and Prm1-promoterv2-F and PRM1_PRE-R as primers. Obtain Fragment2 by PCR amplification using *pprm1*-GFP-CYC1 plasmid as template and PRM1_PRE-F and Prm1-promoter-R as primers. Obtain Fragment3 by PCR amplification using *pprm1*-GFP-CYC1 plasmid as template and PRM1_PREv2-F and Prm1-promoter-R as primers. Use BamH I to digest pRS415-GFP-CYC1 plasmid to obtain linearized vector. Finally, connect Fragment1, Fragment2 and pRS415-GFP-CYC1 linearized plasmid vector through One Step Cloning to obtain the fusion fragment pRS415-6×PRE-*pprm1*-GFP-CYC1. Connect Fragment1, Fragment3 and pRS415-GFP-CYC1 linearized plasmid vector through One Step Cloning to obtain the fusion fragment pRS415-3×PRE_R-*pprm1*-GFP-CYC1.

Transformation of *E. coli* DH5 α .

6. Colony PCR verification

Select several single colonies and dissolve them in 10uL ddH₂O. Take out 1uL

as the template of PCR. Then agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

7. Plasmid PCR verification and sequencing

Inoculate the corresponding 9uL bacterial solution which was verified correct to 5mL LB (Amp⁺) and cultured overnight. Extract the plasmid and use it as the template for PCR. After agar gel electrophoresis verification, correct samples are sent to be sequenced (Genewiz). The recombinant plasmids are named as pRS415-6×PRE-pprm1-GFP-CYC1 and pRS415-3×PRE_R-pprm1-GFP-CYC1.

8. Construction of recombinant yeasts

The successfully constructed plasmid was extracted from *E. coli* and transformed into *Saccharomyces cerevisiae* BY4741 through electro transformation.

9. Colony PCR verification

Pick out yeast transformants from the SC (Leu⁻) auxotrophic plate after electroporation and expand culture in another SC (Leu⁻) auxotrophic plate. Pick a small part of monoclonal colony into 10uL ddH₂O and water bath at 95°C for 10 minutes. Then take out 1uL as the template of colony PCR (primers: M13-F, M13-R). Agar gel electrophoresis is performed to evaluate the length of the obtained fragment.

10. Genomic PCR verification and sequencing

Inoculate the corresponding monoclonal colony which was verified correct to 5mL SC (Leu⁻) auxotrophic medium and cultured for 24 hours. Extract the genome with Yeast Bacteria Genomic DNA Kit (ZOMANBIO), and use it as the template for PCR. After agar gel electrophoresis verification, correct samples are sent to be sequenced (Genewiz).

11. Pheromone induced characterization

Single colonies of *Saccharomyces cerevisiae* BY4741 containing the recombinant plasmid

pRS415-6×PRE-pprm1-GFP-CYC1/pRS415-3×PRE_R-pprm1-GFP-CYC1 were picked from the plate and inoculated into 7ml SC liquid medium. Incubate at 30°C, 220rpm for 20 hours. Dilute the solution into 50mL fresh SC (complete) medium till OD = 0.1. Culture for three hours at 30 °C, 220rpm. Then add α pheromone (final concentration 0uM, 1uM, 5uM, 10uM, 25uM) to induce for 1.5 hours. Centrifuge at 12,000rpm for two minutes to collect the bacteria. Resuspend and wash the sediment once with 4°C pre-cooled PBS and centrifuge at 12,000rpm for two minutes to remove the supernatant. Then add 500ul PBS to resuspend the cells. Take 200μL solution and spot it on a 96-well plate. The green fluorescence intensities of 10,000 cells were quantitatively measured through flow cytometer.

The CRISPR-Cas9 system was used for gene knockout

1) Construction of recombinant plasmid pCas9-gSTE5, pCas9-gFUS1, pCas9-gFUS2 and plasmid pCas9-gPRM1

The sequences of gRNA-STE5, gRNA-FUS1, gRNA-FUS2, and gRNA-PRM1 were synthesized by GenScript. The SNR52 promoter with a gRNA scaffold was added to the plasmid.

The plasmid was first cut open by fast digest enzyme-Not I which from Vazyme, and then the recombinant plasmid with gRNA was constructed by one-step cloning.

2) Transformation of DH5α *E. coli*

Transformation Using Heat Shock

1. Take competent *E. coli* cells from -80°C freezer. Keep tubes on ice.
2. Turn on the water bath to 42°C .
3. Add 1-2ul of the recombinant plasmid into *E. coli* cells. Incubate on ice for 30 minutes.
4. Put tubes with recombinant plasmid and *E. coli* into a water bath at 42°C for 90 seconds.
5. Put tubes back on the ice for 2 minutes to reduce damage to the *E. coli* cells.
6. Add 0.8 ml of LB medium. Incubate tubes for 1 hour at 37°C with shaking at 200rpm.
7. Spread about 160 ul of the resulting culture on LB plates (with appropriate antibiotic added -Kanamycin). Grow overnight.
8. Pick colonies about 12-16 hours later.

3) Cultivate in LB

1. Pick a single colony into 5ml of LB medium (with appropriate antibiotic-Kanamycin added).
2. Inoculate the culture overnight at 37°C for 10-12 hours with shaking at 200rpm.

4) Plasmid extraction

The Plasmid Extraction Kit of AXYGEN in this paper was used to extract plasmid.

5) Plasmid PCR and Preserving strains with glycerol

We chose to conduct Sanger sequencing. The sequencing service is provided by GENEWIZ. After conducting Sanger sequencing, we preserved these recombinant DH5α *E. coli* of pCas9-gSTE5, pCas9-gFUS1, pCas9-gFUS2, and plasmid pCas9-gPRM1 in 50% glycerin LB solution at -80°C according to the results of sequencing.

6) Electroporation Transformation of *S. cerevisiae*

If the sequencing results match the designed sequence, we will take some recombinant plasmid to conduct the electroporation transformation of *S. cerevisiae*.

Preparation of Competent Cells

1. Inoculate 5 mL of YPD in a test tube with an aliquot from an overnight culture of *S. cerevisiae*. The doubling time of *S. cerevisiae* is approximately two hours at 30 °C.
2. Incubate at 30 °C overnight, shaking at 220 rpm, to a density of $\sim 1 \times 10^8$ cells/mL.
3. Chill the cells in an ice water bath for 15 minutes to stop growth.
4. Decant the cells into one sterile 50 ml centrifuge bottle and pellet the cells by centrifugation at 4000rpm for 10 minutes at 4 °C.
5. Carefully pour off and discard the supernatant; place the centrifuge bottles with the cell pellets on ice.
6. Add ~ 40 ml of sterile, ice-cold water to each of the bottles and vortex to resuspend the cell pellets; bring the volume in each of the centrifuge bottles to 50 ml. Pellet the cells by centrifugation at 4000rpm for 10 min at 4 °C; pour off and discard the supernatant.
7. Wash the cells again as in step 6 with a total of 25 ml sterile, ice-cold water.
8. Resuspend the cell pellet in 2 ml of sterile, ice-cold 1 M sorbitol. Pellet the cells by centrifugation at 4000rpm for 10 min at 4 °C; pour off and discard the supernatant.
9. Resuspend the cell pellet in 0.3 ml of sterile, ice-cold 1 M sorbitol. Keep the cells on ice and use them as soon as possible for electroporation.

Electro transformation of yeast

1. Pipette the DNA samples (5–100 ng in a volume of 5 μ L) to be electroporated into sterile 1.5 ml microfuge tubes. Place tubes on ice.
2. If 0.2 cm cuvettes are used, add 40 μ L of the competent cells to each DNA sample. Mix gently and incubate on ice for ~ 5 min.
3. Set the Micro Pulser to “Sc2” when using 0.2 cm cuvettes.
4. Transfer the DNA-cell samples to the appropriate electroporation cuvettes that have been chilled in ice and tap the suspension to the bottom of the tube. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.
5. Remove the cuvette from the chamber and immediately add 0.1 ml of ice-cold 1 M sorbitol to the cuvette; gently transfer the diluted cells into a sterile tube.
6. Check and record the pulse parameters. The time constant should be close to 5 milliseconds. The field strength can be calculated as actual volts (kV) / cuvette gap (cm).
7. Plate aliquots of the electroporated cells on selective agar plates. Incubate plates for 48–72 h at 30°C.

7) Validation of transformed yeast strains

1. We first picked a single colony of yeast from the transformation plate and streaked it on the plate. A plate was divided into 16 grids, and each grid was streaked with a single colony. Cultivated in a 30-degree incubator.

2. Picked single colonies.

A single colony was taken from the culture plate with a pipette (inoculation ring) and placed into a PCR tube, which contained 10ul 10um NaOH. Boiled the PCR tube in a 96-degree water bath for 30min.

3. Performed colony PCR.

Performed colony PCR. According to the gel image of the colony PCR, selected the microbial to be transferred to 5ml SC medium with G418 resistance for culture and incubated them in a 30°C shaker at 220 rpm.

4. Genome extraction.

The genome of yeast cultured overnight was extracted and verified by genomic PCR.

5. Sanger sequencing

The sequencing service is provided by GENEWIZ.

8) Preserving strains with glycerol

After conducting Sanger sequencing, we preserved these Defective yeast strains of STE5, FUS1, FUS2, and PRM1 in 50% glycerin SC solution at -80°C according to the results of sequencing.

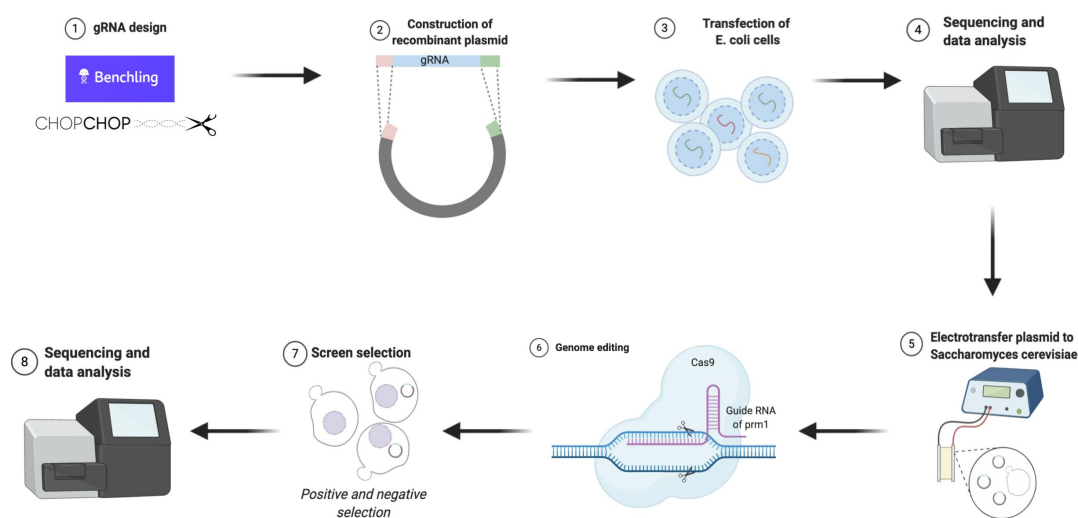


Fig. 1 The experimental process of Crispr-Cas9 system

The Cre-loxp system was used for gene knockout

1) Construction of homologous recombination fragment for knocking out the STE5 gene

The sequences of ste5-pUG6-F, ste5-pUG6-R, pUG6B-R, pUG6C-F were synthesized by GenScript. The KanMx gene fragment with two LOXP loci was obtained by PCR amplification.

The PCR products were purified by the AxyPrep PCR Clean-up Kit.

2) Electroporation Transformation of *S. cerevisiae*

If the gel image of the PCR results matches the designed sequence, we will take some purified PCR products of the fragment to conduct the electroporation transformation of *S. cerevisiae*.

Preparation of Electrocompetent Cells

1. Inoculate 5 ml of YPD in a test tube with an aliquot from an overnight culture of *S. cerevisiae*. The doubling time of *S. cerevisiae* is approximately 2h at 30 °C.
2. Incubate at 30 °C overnight, shaking at 220 rpm, to a density of $\sim 1 \times 10^8$ cells/ml.
3. Chill the cells in an ice water bath for 15 min to stop growth.
4. Decant the cells into one sterile 50 ml centrifuge bottle and pellet the cells by centrifugation at 4000rpm for 10 min at 4 °C.
5. Carefully pour off and discard the supernatant; place the centrifuge bottles with the cell pellets on ice.
6. Add ~ 40 ml of sterile, ice-cold water to each of the bottles and vortex to resuspend the cell pellets; bring the volume in each of the centrifuge bottles to 50 ml. Pellet the cells by centrifugation at 4000rpm for 10 min at 4 °C; pour off and discard the supernatant.
7. Wash the cells again as in step 6 with a total of 25 ml sterile, ice-cold water.
8. Resuspend the cell pellet in 2 ml of sterile, ice-cold 1 M sorbitol. Pellet the cells by centrifugation at 4000rpm for 10 min at 4 °C; pour off and discard the supernatant.
9. Resuspend the cell pellet in 0.3 ml of sterile, ice-cold 1 M sorbitol. Keep the cells on ice and use them as soon as possible for electroporation.

Electro transformation of yeast

1. Pipette the DNA samples (5–100 ng in a volume of 5 μ L) to be electroporated into sterile 1.5 ml microfuge tubes. Place tubes on ice.
2. If 0.2 cm cuvettes are used, add 40 μ L of the competent cells to each DNA sample. Mix gently and incubate on ice for ~ 5 min.
3. Set the Micro Pulser to “Sc2” when using 0.2 cm cuvettes.
4. Transfer the DNA-cell samples to the appropriate electroporation cuvettes that have been chilled in ice and tap the suspension to the bottom of the tube. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.
5. Remove the cuvette from the chamber and immediately add 0.1 ml of ice-cold 1 M sorbitol to the cuvette; gently transfer the diluted cells into a sterile tube.
6. Check and record the pulse parameters. The time constant should be close to 5 milliseconds. The field strength can be calculated as actual volts (kV) /

cuvette gap (cm).

7. Plate aliquots of the electroporated cells on selective agar plates. Incubate plates for 48–72 h at 30°C.

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A single colony was taken from the culture plate with a pipette (inoculation ring) and placed into a PCR tube, which contained 10ul 10mM NaOH. Boiled the PCR tube in a 96-degree water bath for 30min.

3. Performed colony PCR.

Performed colony PCR. According to the gel image of the colony PCR, selected the microbial to be transferred to 5ml SC medium with G418 resistance for culture and incubated them in a 30°C shaker at 220 rpm.

4. Genome extraction.

The genome of yeast cultured overnight was extracted and verified by genomic PCR.

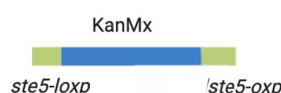
5. Sanger sequencing

The sequencing service is provided by GENEWIZ.

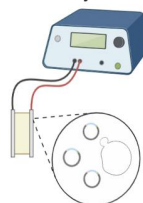
4) Preserving strains with glycerol

After conducting Sanger sequencing, we preserved these Defective yeast strains of STE5 in 50% glycerin SC solution at -80°C according to the results of sequencing.

① BP reaction



② Electrotransfer plasmid to *Saccharomyces cerevisiae*



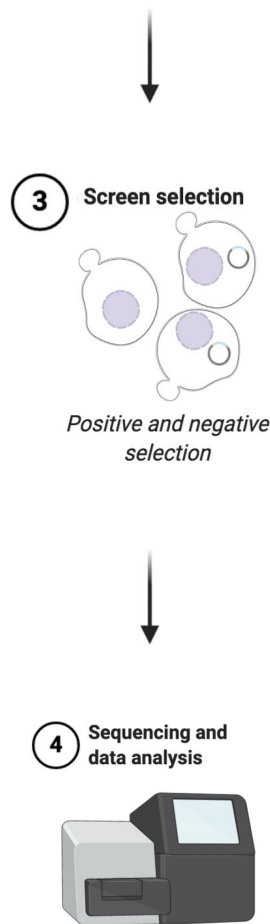


Fig. 2 The experimental process of loxp system

Production of amajLime protein and eforRed protein

1) Construction of plasmid pET28a(+)-amajLime and plasmid pET28a(+)-eforRed

Plasmid pET28a(+)-amajLime and plasmid pET28a(+)-eforRed were synthesized by GenScript. The sequence of amajLime and eforRed were optimized for *Saccharomyces cerevisiae* and a 6xHis tag for protein purification was a label that's already on the plasmid.

2) Transformation of DH5α *E. coli* and BL21 *E. coli*

Transformation Using Heat Shock

1. Take competent *E. coli* cells from -80°C freezer. Keep tubes on ice.
2. Turn on the water bath to 42°C.
3. Add 1-2ul of circular DNA into *E. coli* cells. Incubate on ice for 30 min.
4. Put tubes with DNA and *E. coli* into a water bath at 42°C for 90 seconds.
5. Put tubes back on the ice for 2 minutes to reduce damage to the *E. coli* cells.
6. Add 0.8 ml of LB medium. Incubate tubes for 1 hour at 37°C with shaking at 200rpm.
7. Spread about 160 ul of the resulting culture on LB plates (with appropriate

antibiotic added -Kanamycin.). Grow overnight.

8. Pick colonies about 12-16 hours later.

3) Cultivate in LB

1. Pick a single colony into 5ml of LB medium (with appropriate antibiotic-Kanamycin added).

2. Inoculate the culture overnight at 37°C for 10-12 hours with shaking at 200rpm.

4) Plasmid extraction

The Plasmid Extraction Kit of ZOMANBIO in this paper was used to extract plasmid.

5) Plasmid PCR and Preserving strains with glycerol

We chose to do Plasmid PCR. We used primers designed to copy the sequences of amajLime and eforRed. After the reaction, we gave the PCR solution electrophoresis to judge the result of transformation. If it appears to the right band, we preserved these recombinant DH5α *E. coli* and BL21 *E. coli* of amajLime and eforRed in 50% glycerin LB solution at -80°C.

6) Fermentation

If the sequencing results match the designed sequence, we will take some recombinant BL21 *E. coli* of amajLime and eforRed from the cryotube and add them to the 5ml LB liquid medium. After 12 hours of incubation, we transformed all 5ml of cultures into fermentation medium and incubated them in an oscillating incubator at 220 rpm, 37 degrees. We recorded the OD600 after 1 hour when the value became about 0.6. Then, we added IPTG to the fermentation medium for protein induction culture at 220 rpm, 16 degrees for 24 hours. Finally, we stored the fermentation solution at 4°C, waiting for product collection and purification.

7) Product collection and purification

We dispersed the fermentation solution to get supernatant. We gave the supernatant ultrafiltration. Since our produced proteins-amajLime and eforRed are about 26.8kDa and 26.7kDa, we chose two 10kDa filters to ultrafilter our samples. As a result, we can get our proteins in the solution above the membrane. We did the affinity chromatography with a packed nickel column on the ultrafiltered samples to obtain the further purified products. After each step of single processing, we used SDS-PAGE to detect the effect of extraction and purification of the extracted and residual samples.

8) Protein detection

We perform SDS-PAGE gel electrophoresis on the amajLime protein and eforRed protein to detect the protein purification effect and observe the protein

expression. Next, we did the Time of Flight Mass Spectrometer for the amajLime protein samples and eforRed protein samples. We used the BCA protein assay to determine the concentration of the amajLime protein and eforRed protein. Finally, we applied the full wavelength measurement on the amajLime protein and eforRed protein.