Material:

Fermentation medium:
Glycerate 40 g/L
Tryptone 20 g/L
Yeast extract 10 g/L
$(NH_4)_2SO_4$ 10 g/L
Amino-free yeast nitrogen source (YNB) 3.4 g/L
Potassium phosphate buffer: KH_2PO_4 8.34 g/L
K_2HPO_4 0.87 g/L

LB Liquid medium: 1% Tryptone, 0.5% Yeast extract, 1% NaCl
LB Solid medium: 1% Tryptone, 0.5% Yeast extract, 1% NaCl, 2% Agar

YPD Liquid medium: 2% Glucose, 2% Tryptone, 1% Yeast extract
YPD Solid medium: 2% Glucose, 2% Tryptone, 1% Yeast extract, 2% Agar

G418 10mL(100g/L):
One gram of G418 powder was dissolved in 10 ml of ddH20 (which can be completely dissolved by ultrasonic waves), and sterilized by passing through a 0.22 μm filter.

Methods:

1.Plasmid Extraction：AXYGEN AxyPrep Plasmid Miniprep Kit

2.Agarose gel electrophoresis:
1% agarose is added to 1 × TBE and heated in microwave for about 1 min to fully dissolve the agarose. Let it cool for a while, add a small amount of ExRed nucleic acid dye μL, mix them thoroughlyand pour the contents into the rubber plate. The gel is verified and a single band appeared, indicating that the plasmid is linearized. Recycled gel (35min), 40μL per well, 4μL 10× Loading Buffer μL μL
Electrophoresis: 125V 30min

3.Gel extraction: OMEGA Gel Extraction Kit

4. Preparation of competent cells:
Preparation of *Pichia pastoris* competent cells:
1) 150μL Pichia cultures were added into a flask containing 50 mL liquid YPD medium, incubating in the oscillating incubator at 200 rpm, 30 ℃. Harvest the cells at an OD_{600} of 0.6-0.8.
2) Initially, incubate the cultures on ice for about 15 min. Then, centrifuge the cooled cells at 5,000 g for 10 min at 4. ℃ Save the yeast pellet and discard

the supernatant. Re-suspend the cell pellet in 50 ml pre-cooled ddH_2O.
3) Centrifuge as above, resuspend the cell pellet in 25 mL pre-cooled ddH_2O.
4) Centrifuge as above, resuspend the cells pellet in 2 mL pre-cooled 1 M sorbitol.
5) Centrifuge as above, resuspend the cells pellet in 100 μL pre-cooled 1 M sorbitol.
150μL Pichia seed solution was added into a flask containing 50 mL liquid YPD medium, cultivating at 30℃ with shaking at 200 rpm . Harvest the cells at an OD_{600} of 0.6-0.8.
Initially, place the culture on ice for about 15 min. Then, centrifuge the cooled cells at 4 ℃, 5,000 g for 10 min . Save the yeast pellet and discard the supernatant. Re-suspend the cell pellet in 50 ml pre-cooled ddH_2O.
Centrifuge as above, resuspend the cell pellet in 25 mL pre-cooled ddH_2O.
Centrifuge as above, resuspend the cells pellet in 2 mL pre-cooled 1 M sorbitol.
Centrifuge as above, resuspend the cells pellet in 100 μL pre-cooled 1 M sorbitol.

5. Transformation:

The electrotransfer of competent yeast:
1) 60 μL of the competent cells were mixed with 10-15 μL of linearized DNA and transferred to a pre-cooled 0.2 cm electroporation cuvette.
2) Incubate it on ice for 5min
3) Electric shock （Gene Pulser Xcell Electroporation System Bio-Rad）
Conditions:

Pulse type	C(μF)	PC(ohm)	V	Cuvette(cm)	Cell vol(μL)
Exponential decay	25	200	2000	0.2	40

[Instructions for using electroporation cuvette] Firstly, immerse them in 75% ethanol, then in 100% ethanol, after being washed, place them in a clean bench to dry, and pre-cool them on ice before using. To prevent a short circuit, make sure the bottom is dry and clean before placing it into the electroporator.
4) Add 1 mL of pre-cooled 1 M sorbitol to the electric shock cup immediately afer the electric shock and transfer the contents to a sterilized centrifuge tube.
5) Incubate the centrifuge tube in the constant-temperature incubator at 30 ℃ for 2 h.
6) These yeasts will be coated on a plate to obtain single colony.7）The grown single colonies were picked from the plates, added to a test tube containing YPD liquid medium, and cultured in a oscillating incubator.

Transform DH5α:
1） Take the competent DH5α from the -80 ° C refrigerator and thaw it on ice.
2） Add 10 μL of recombinant product to 100 μL of competent cells, mix gently by pipe wall (do not shake and mix), then incubate it on ice for 30 min.
3） After heat-shocking for 48 sec in 42 ℃ water bath, set them on ice immediately for 2-3 min.

4） Add 900 μL SOC or LB medium (without antibiotics), shaking at 37℃, 200-250 rpm for 1 h.

5） Preheat the corresponding resistant LB plate solid medium in a 37℃ constant-temperature incubator.

6) Centrifuge at 5000 rpm for 5 min, discard 900 μL of supernatant. Suspend the bacteria in the remaining medium and gently spread them on a plate containing the correct resistance with a sterilized coating bar.

7) Place the plate in a 37℃ incubator for 12-16 h.

Transform BL21:

1) Take the competent BL21 from the -80 ° C refrigerator and thaw it on ice.

2) Add the DNA fragments to be transformed into 100 μL competent cells, mix the walls of the tube (do not shake and mix), and incubate them on ice for 30 min.

3) Heat-shocking for 42 sec in a 42℃ water bath, then immediately set it on ice for 2 min. Do not shake the tube.

4) Add 900 μL SOC or LB medium (without antibiotics), shaking at 37℃, 200-250 rpm for 1 h.

5) 2500g, centrifuge for 3min, discard 900μL of supernatant, suspend the bacteria body with the remaining medium, and evenly coat on the LB solid plate containing the corresponding antibiotics。

6) Incubate the plate in a 37 ℃ incubator for 10 min. After the bacteria solution is completely absorbed, invert the plate and incubate it overnight.

6.PCR:

50μL system (2 × Rapid Taq Master Mix):

2 × Rapid Taq Master Mix 25.0 μl

Primer1 (10 μM) 2.0 μl

Primer2 (10 μM) 2.0 μl

Template DNA* x μl

Add ddH_2O to 20μL

*For a 50 μL reaction system, the recommended input amount of template is as follows:

Template type	Template start amount
Genomic DNA of animals and plants	0.1-1 μg
Genomic DNA of E. coli	10-100 ng
cDNA	1-5 μl (< 1/10 of the reaction volume)
Plasmid DNA	0.1-10 ng
λDNA	0.5-10 ng

Program:

Loop step	Temperature	Time	Number of cycles

Pre-denatured	95℃	3 min	
Changing nature	95℃	15 sec	×30-35 cycles
Annealing	60℃	15 sec	
extend	72℃	15sec	
Extend completely	72℃	5 min	

50 μL system （Phanta Max Super-Fidelity）:

2 × Phanta Max Master Mix 25 μL

Upstream primer (10 μM) 2 μL

Downstream primer (10 μM) 2 μL

Template DNA x μL

Add ddH_2O to 50 μL

The optimal reaction concentrations for different templates are different. The following table shows the recommended template usage for the 50 μL system:

Template type	Template start amount
Genomic DNA	50-400 ng
Plasmid or viral DNA	10 pg-30 ng
cDNA	1-5 μL (Not more than 1/10 of the total volume of the PCR reaction)

Program:

Loop step	Temperature	Time	Number of cycles
Pre-denatured	95℃	30 sec/3 min	
Changing nature	95℃	15 sec	×25-35 cycles
Annealing	56-72℃	15 sec	
extend	72℃	30-60sec	
Extend completely	72℃	5min	

7. Enzyme digestion （The restriction enzymes used were purchased from Thermo）

Thermo Scientific FastDigest enzyme	Reaction temperature	Lambda, 1 μg/20 μL	Plasmid DNA, 1 μg/20 μL	PCR product, ~0.2 μg/30 μL	Genomic DNA, 1 μg/10 μL	bp from end of DNA required for complete digestion	Thermal inactivation	Incubation time (in hours) without star activity
FastDigest BamHI	37°C	5	5	5	5	2	80°C, 5 min	1
FastDigest Xba Ⅰ	37°C	5	5	5	10	2	65°C, 20 min	16
FastDigest EcoRI	37°C	5	5	20	5	2	80°C, 5min	0.5

BamHⅠ 20μL system:
Plasmid(1000ng) 4μL
10×FastDigest Buffer 2μL
Enzyme (BamH Ⅰ) 1μL
Add ddH_2O to 20μL
Incubate at 37 ℃ for 3 h
×16

XbaⅠ 20μL system:
Plasmid 1000ng/230ng/μL=4.3μL → 5μL
XbaⅠ 1μL
10×FastDigest Buffer 2μL
Add ddH_2O to 20μL
Incubate at 37 ℃ for 1 h

20μL Double digestion system:
EcoRⅠ 1μL
BamHⅠ 1μL
10×FastDigest Buffer 2μL
DNA 1000ng
Add ddH_2O to 20μL
Incubate at 37 ℃ for 30 min

8. Gene fragment purification and recovery: AXYGEN AxyPrep PCR Cleanup Kit

9. Extraction of yeast genome: Solarbio Yeast Genomic DNA Extraction Kit

10. One-step cloning: Vazyme ClonExpress II One Step Cloning Kit (C112)

11. Expression:

Take the bacteria solution, incubat it to the expression medium of 200mL per bottle at 1mL per bottle, culture at 30° C, 200 rpm. During the expression stage, take samples from the flask every 24 hours for microscopic examination to determine if contamination occurred. At the same time, the OD_{600} value of the fermentation broth was measured to monitor the growth of our engineered yeasts. When the value stabilizes, the expression process is considered as being over.

12. Product collection and purification:

Ultrafiltration:

1) Take appropriate amount of fermentation solution, disperse them into two 100mL centrifuge tubes, centrifuge at 12,000 rpm, 10℃ for 10 min after equilibration.

2) After centrifugation, the supernatant is poured out, and 50 μL of the supernatant is taken and marked as sample 1;

Take a certain amount of ultrapure water, resuspend the yeast, and marke it as sample 2;

The remaining supernatant is subjected to ultrafiltration (the membrane is placed in a 35 mL centrifuge tube):

(1)Add 10 mL of ultrapure water to each of the two ultrafiltration tubes, balance, 4000 rpm, and centrifuge for 20 min;

(2)Add 10 mL of sample 1 to each of the two ultrafiltration tubes,balance, 4000 rpm, centrifuge for 20 min, aspirate the liquid on the filter, and transfer to a 1.5 mL centrifuge tube;

Repeat the operation of step 2 until all the samples 1 are processed, the liquid in the 1.5 mL centrifuge tube is marked as sample 3, and the liquid in the 35 mL centrifuge tube (i.e. the supernatant after ultrafiltration) is marked as sample 4;

(3) Add 10 mL of ultrapure water to each of the two ultrafiltration tubes, level, 4000 rpm, centrifuge for 20 min (clean filter), and immerse the filter in ethanol.

Affinity chromatography：

1) Take the packed nickel column (Here, take the 1 day nickel column of Takara Company as an example.) and let the tip stand down until the blue resin completely precipitate. Then add 10ml of equilibration buffer to balance, open the lower plug, and drop the liquid. Guide to the wash container.

2) After the 10ml liquid has flowed out, plug the water outlet plug, add the processed clarified sample, seal the top cover, and slowly and repeatedly turn the column for 1 hour (or invert one hour, this process is best at 4℃).

3) Place the tip of the column vertically downwards, let the resin sink to the bottom of the column, and connect the EP tube below the tip, depending on how much the sample is applied.

4) Carefully remove the upper and lower plugs to collect the dripping liquid. If you continue to load after the collection is completed, return to the 2 process.

5) Wash the column with 10 ml of equilibration buffer and then wash the column again with 10 ml of wash buffer. (Use kit 635665, use 710 μL of eluent and 9.29 ml of equilibration buffer to prepare wash buffer)

6) Elute the column with 10 ml eluent, collect all effluent liquid and concentrate by ultrafiltration.

Sample processing:

The IL-2 fermentation broth is concentrated by ultrafiltration and washed, and then loaded. The ultrafiltration tube is 10 kDa, the speed is 4000 rpm, and the time is 15 min.
The IL-2-RFP fermentation broth is concentrated by ultrafiltration and washed, and then loaded. The ultrafiltration tube is 30 kDa, the speed is 4000 rpm, and the time is 15 min.
Balance solution formula: sodium phosphate: 19.01g, sodium chloride 17.533g, imidazole 1.362g, add water to 1L
Washing liquid formula: sodium phosphate: 19.01g, sodium chloride 17.533g, imidazole 2.723g, add water to a volume of 1L
Eluent formulation: sodium phosphate: 19.01g, sodium chloride 17.533g, imidazole 20.424g, add water to a volume of 1L
All the above buffers are adjusted pH to 7.4 with concentrated hydrochloric acid.

13.SDS-PAGE verification：Beyotime SDS-PAGE Gel Preparation Kit