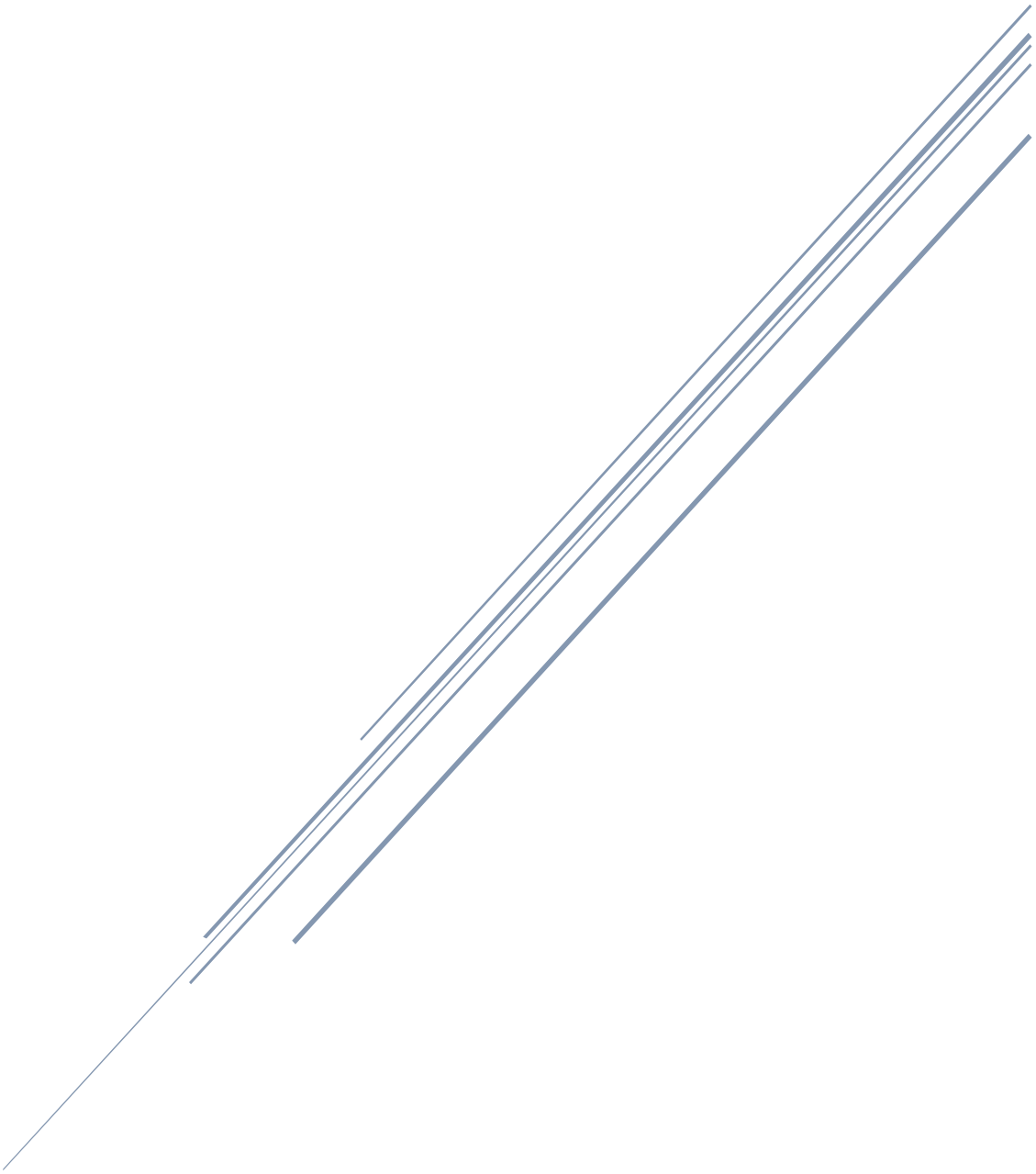
PROTOCOL

iGEM-HUST China



Protocols do not have completely standard operations; they only have universal ones. Different experiments may face different situations. When conducting them, it is essential to understand the principle behind each step. This way, one can shift from "using" the protocol to "modifying" it, and optimize the operation of the experiment based on the specific circumstances of their own experiment to achieve the best results.

LB (Luria-Bertani)

Component	Amount
NaCl	10g/L
Tryptone	10g/L
Yeast Extract	5g/L
Agar(solid)	20g/L

If antibiotics need to be added, after sterilization, when the temperature of the culture medium drops to room temperature, add kanamycin antibiotics at a concentration of 1/1000 and chloramphenicol (Chl) at a ratio of 1/1000.

When preparing solid culture media, add 1% agar. After aliquoting, add AGAR again

2. Thermal shock transformation of E.coli

Take 100 μl of ice-melted competent large intestine.

Add 2 μl of the plasmid directly extracted from the bacterial liquid or all the plasmids obtained through homologous recombination/enzymatic ligation. Be careful not to touch the bottom of the EP tube with your hand. gently tap and mix after adding. (The receptive state is very fragile) Mix well. Let it stand on ice for 5 minutes.

Heat at 342 ℃ for 90 seconds, then immediately take an ice bath for 2 minutes.

Add 700 μl of antibiotic-free liquid LB and incubate on a 37 ° C shaker for 20 minutes.

Centrifuge at 5000 r/min for 2 minutes, pour out some of the supernatant, resuspend and coat the plates. Incubate overnight at 37 ° C and pick the plates the next day.

The 6 plaques were incubated in 1 mL of the corresponding resistant LB medium at 37 ° C on a shaker for 4-5 hours, and the colonies were verified by pcr.

Note: This protocol needs to be modified according to the actual situation. The plasmid used in the first round of experiments is the synthetic plasmid of the company, and adding 2 μl is sufficient. For subsequent experiments, if the concentration of the recombinant plasmid is lower, it needs to be changed to 10 μL. At the same time, to increase the success rate of screening, 50 μl of competent state should be used. The quality of the sensitive state prepared by oneself for the heat shock time is relatively poor. The heat shock time is 90 seconds. The sensitive state purchased by the company should be in accordance with the company's instructions.

3. Colony PCR

Take the plates that have been coated and cultured after transformation, pick out single colonies with a pipette tip and place them in a 1.5 mL EP tube containing 1 mL of LB liquid medium. Incubate on a 37 ° C shaker for 4-5 hours, and then conduct PCR to verify whether the plasmid has been transferred into Escherichia coli.

Reagent	Table 3. PCR System (20 μL)
2×Taq Mix	10 μL
Forward Primer	1 μL
Reverse Primer	1 μL
Bacterial Liquid	1 μL
ddH_2O	7 μL

Temperature	Table 4. PCR Program Setting
95 °C	5 min
95 °C	15 sec
58 °C (Highest TM-5)	15 sec
72 °C	30 sec (10-15s/kb)
72 °C	2 min
12 °C	∞
	30 cycles

4. Place the glue tank on the glue-making plate, insert the comb, and slowly pour the agarose solution into the glue tank.

Let the gel stand at room temperature for about 30 minutes until the agarose is completely solidified. Gently pull out the comb, take out the gel bath, wipe off the broken gel at the bottom of the gel bath, and put the gel bath and gel into the electrophoresis bath.

Mix 2 μL of 5× loading buffer with 5 μL of PCR product to complete ddH2O to 10 μL. Load the mixed solution into the sample Wells of the gel, and then add 3-5 μl of Marker to the sample Wells. Electrophoresis at 120 V for 20 minutes.

After electrophoresis is completed, wear PE gloves to take out the gel and place it in the gel imaging system. Observe and take photos to determine the amplification efficiency of the PCR product and confirm whether the lengths of the amplified fragments match.

If there is no gel in the conical flask, prepare it as follows

Weigh 2 g (1/100) of aggarose powder with a balance and pour it into a 250 mL conical flask.

Measure 200 mL of TAE buffer solution with a graduated cylinder, pour it into a conical flask, mix

slightly, and then heat it in a microwave oven until the solution becomes transparent and the agarose is completely dissolved.

Take out the conical flask, add 20 μL (1/10000) of Gelred gel dye, and mix well.

4. Vaccination

Inoculate E.coli in preservation tubes/expanded shaking tubes/conical flasks at a concentration of 1/100 into LB medium and incubate overnight at 37° C and 200 rpm.

5. Large intestine conservation

Add 800 μl of 50% glycerol water solution to the seed preservation tube and sterilize by high-pressure steam. Mix well with 800 μl of bacterial liquid (1:1) and store at -20 ℃.

6. Plasmid enhancement

Add 1-5 mL of the overnight cultured bacterial liquid to an EP tube, centrifuge at 12,000 rpm for 2 minutes, and discard the supernatant.

2 Add 250 μl of solution Ⅰ (confirm that RNase has been added and store in the refrigerator at 4 ° C). Ingredients: EDTA, glucose. EDTA is a chelating agent for divalent metals. It inhibits the activity of DNase by chelating divalent metal ions, protecting the extracted DNA from degradation. Some manufacturers' kits contain glucose to prevent suspended Escherichia coli from settling rapidly. Repeatedly pipette and mix to ensure there are no bacterial blocks; otherwise, the internal bacterial blocks cannot be decomposed.

3 Add 250 μl of solution II (Ingredients:) NaOH and SDS, NaOH dissolves cells, NaOH reacts with carbon dioxide in the air, so the lid should be closed in time after each use of solution II. SDS enhances the alkalinity of sodium hydroxide and combines with proteins to precipitate. Slowly invert and rotate the test tube several times to gently mix. Let it stand for two minutes to obtain a transparent lysis product. At this step, the DNA is already in the solution. The operation must be gentle; otherwise, the strand will break. Under alkaline conditions, genomic DNA will also break. Therefore, the standing time cannot be too long; otherwise, the genomic DNA will break, and it will be difficult to separate from the plasmid DNA after breaking.

4. Add 350 μl of solutionⅢ (Ingredients: Potassium acetate, acetic acid, SDS, when encountering potassium ions, forms insoluble PDS, generating a large amount of flocculent precipitate. Since the genomic DNA chain is very long, it is very easy to be carried by these precipitates. However, if the genomic DNA breaks into short chains, it will be difficult to precipitate. Acetic acid is used to neutralize the sodium hydroxide in the previous step. Immediately invert and mix well.

Centrifuge at 12,000 rpm for 10 minutes.

Carefully draw the supernatant (700 μL) into the purification column. It is better to draw a little less supernatant than to draw up the precipitate. 12,000 rpm for 1 minute, repeat once, and discard the filtrate.

7 Add 500 μl of HBC washing Buffer, 12,000 rpm for 1 minute, and discard the filtrate.

Add 700 μl of DNA washing Buffer (pre-added with 80% ethanol) to clean the purification column, rpm at 12,000 for 1 minute, discard the filtrate, and repeat once. Two cleaning solutions are used to remove impurities such as small molecule proteins and inorganic ions.

Centrifuge the 9 empty columns at 12,000 rpm for 2 minutes

Dry the incubator until it is free of ethanol (15 minutes). These two steps remove ethanol; otherwise, it will have a significant impact on the subsequent elution efficiency.

Place the purification column into a clean 1.5 ml EP tube, add 30 to 50 μl of ddH2O to the column, let it stand for 2 minutes, and then centrifuge at 12,000 rpm for 1 to 2 minutes.

Repeat the elution with the above-mentioned eluent, but do not let it stand. The first stand is to allow the eluent to cover the silica gel membrane to de-column the DNA.

7. High-fidelity PCR amplification fragments

Reagent	High-Fidelity PCR Amplification Fragment
2 ×Phanta Max Master Mix	10 ul
Forward Primer	1 ul
Reverse Primer	1 ul
Template DNA	1 ul
ddH_2O	7 ul
Template-Primer Correspondence: See Primer Table	
PCR System	
95 °C	30 sec
95 °C	15 sec
58 °C (Highest TM-5)	15 sec
72 °C	1.5 min(30s/kb)
72 °C	2 min
12 °C	∞
	30 cycles
Taq enzyme: 10s/kb; Phanta enzyme: 30s/kb	

The correct linear fragments were purified by electrophoresis (for specific steps, see 3. ④-⑦).

8. Glue Recovery (Purification

Cut the gel as thin as possible. Add 300-350 μL of BD Buffer and water bath sol at 55-60 ℃.

During this period, reverse the EP tube 2-3 times to ensure the gel is completely melted.

Add 100 μL of AC buffer to the purification column, let it stand for 1 minute, and centrifuge at 12,000 rpm for 1 minute

Briefly centrifuge to collect the dissolved gel solution, then transfer the liquid to a purification column at 12,000 RPM for 1 minute (note to balance).

Discard the liquid, add 300 μl of BD Buffer, 12,000 RPM for 1 minute, and discard the filtrate

5 Add 700 μl GW wash Buffer, 12,000 RPM for 1 min, and discard the filtrate

Repeat the previous step

Idle at 12,000 rpm for 2 minutes

Discard the collection tube, open the column cover and dry it in a 37℃ incubator for 15 to 20 minutes

Place the purification column on a 1.5 ml EP tube, add 50 μl of ddH2O to the membrane center, and let it stand for 2 minutes

10 12000 rpm 1 min

11 inhalation columns, 12000 rpm for 1 minute

12 Abandoned Pillar

9. Double enzymatic digestion

Reagent	Double Enzyme Digestion System
Fragment Template	60 μl
10×Buffer	8 μl
Enzyme 1	2 μl
Enzyme 2	2 μl
ddH_2O	8 μl
Total	80 μl

37 ℃ water bath for 20 minutes. (SS/FD rapid enzyme water bath for 20 minutes, q.ut rapid enzyme water bath for 10 minutes)

Subsequently, all 100 μL of the reaction solution was used for gel recovery.

10. Enzymatic linkage

Component	Ligation System
Fragment	1 μl
Vector	4 μl
T4 ligation mix	5 μl

React at 16 ℃ for 30 minutes.

11. Vazyme homologous recombination reaction system

Reagent	Vazyme Homologous Recombination System
Exnase II	2 μl
5×CE buffer	4 μl
Fragment	200 ng (about 4 μl)
Vector	50-200 ng (about 1 μl)
ddH_2O	Make up to 20 μl

React at 50 ℃ for 20 minutes and then place on ice for 5 minutes after the reaction.

12. YESEN homologous recombination system

Reagent	YESEN Homologous Recombination System
2×Hieff clone enzyme premit	5 μl
Vector	1 μl
Fragment	4 μl

React on ice at 50 ℃ for 5 minutes, and then leave on ice for 5 minutes after the reactiopin.

Reagent

1. 10× YNB: Weigh 67 g of YNB, add ultrapure water to a total volume of 500 mL, sterilize by filtration, and store at low temperature.

2. 500× B (Biotin Solution) : Dissolve 0.04 g of biotin in 200 mL of deionized water, sterilize by filtration, and store at low temperature in the dark.

3. 50% (m/v) Glucose Solution : Weigh 25 g of anhydrous glucose, add ultrapure water to a total volume of 50 mL, and sterilize by steam at 117 °C for 20–25 min.

4. 20× Ura (Uracil Solution) : Dissolve 89.6 mg of uracil in 200 mL of deionized water, sterilize by filtration, and store at low temperature in the dark.

5. 40× Leu (Leucine Solution) : Dissolve 2.1 g of leucine in 200 mL of deionized water, sterilize by filtration, and store at low temperature in the dark.

6. 50× TAE Buffer : Add 28.55 mL of glacial acetic acid, 18.61 g of disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$), and 121 g of Tris base to ultrapure water, then adjust the total volume to 500 mL with ultrapure water.

7. 10× TE Buffer : Accurately weigh 0.29 g of ethylenediaminetetraacetic acid (EDTA) and 1.21 g of Tris base, dissolve in 90 mL of ultrapure water, adjust the pH to 8.0, and bring the total volume to 100 mL with ultrapure water. Dilute 10-fold with ultrapure water to obtain 1× TE Buffer.

8. Tris-Glycine Protein Electrophoresis Buffer : Dissolve 0.8 g of sodium dodecyl sulfate (SDS), 15 g of glycine, and 4.8 g of Tris base in 800 mL of double-distilled water. Prepare fresh before use.

9. PEG 4000 Solution : In a clean bench, weigh 500 g of polyethylene glycol 4000 and dissolve in sterile deionized water to a total volume of 1 L.

10. 2% (m/v) PVA Solution : Add 10 g of polyvinyl alcohol (PVA) to 450 mL of ultrapure water, heat and stir on an asbestos gauze until fully dissolved, filter the solution, and adjust the total volume to 500 mL with ultrapure water.

11. Lithium Acetate (2 M) : Add 66 g of anhydrous lithium acetate to 400 mL of ultrapure water, adjust the pH to 6.0, bring the total volume to 500 mL, sterilize by steam, and store at 4 °C (low temperature).

12. Lithium Acetate (0.1 M) : Dilute the 2 M lithium acetate solution 20-fold with ultrapure water.

13. DTT (1 M) : Dissolve 5 g of dithiothreitol (DTT) in deionized water to a total volume of 32.5 mL, sterilize by filtration, and store at low temperature in the dark.

14. Tris-HCl Buffer (0.05 M) : Dissolve 5.05 g of Tris base in 450 mL of double-distilled water, adjust the pH to 8.0, and bring the total volume to 500 mL with double-distilled water.

15. NaOH (0.05 M) : Dissolve 1 g of granular sodium hydroxide (NaOH) in 400 mL of ultrapure water, then adjust the total volume to 500 mL with ultrapure water.

16.Olive Oil Emulsion : Mix 2% PVA solution and olive oil at a volume ratio (v/v) of 3:1, emulsify at 5000 rpm for 5–10 min, and repeat the emulsification process 2–3 times.

17. 0.5% (m/v) Phenolphthalein Solution : Weigh 2 g of phenolphthalein powder, dissolve in anhydrous ethanol to a total volume of 400 mL, and store at room temperature in the dark.

18. Potassium Phosphate Buffer (1 M) : Prepare 1 M dipotassium hydrogen phosphate (K_2HPO_4) solution and 1 M potassium dihydrogen phosphate (KH_2PO_4) solution separately. Mix the two solutions according to *Molecular Cloning: A Laboratory Manual* to obtain 1 M potassium phosphate buffers with different pH values.

19. Triton/SDS Cell Lysis Buffer : Mix 2.5 mL of 10× TE Buffer, 2.5 g of SDS, 1.45 g of NaCl, and 5 mL of Triton X-100, then add ultrapure water to a total volume of 250 mL. Store at room temperature.

20. Coomassie Brilliant Blue G-250 Solution : Dissolve 50 mg of Coomassie Brilliant Blue G-250, 25 mL of absolute ethanol, and 50 mL of 85% phosphoric acid in ultrapure water, adjust the total volume to 500 mL with ultrapure water, let stand overnight in the dark, and then filter.

21. Protein Gel Destaining Solution : Mix glacial acetic acid, absolute methanol, and ultrapure water at a volume ratio (v/v/v) of 1:5:4.

22. Protein Gel Staining Solution : Dissolve 0.6 g of Coomassie Brilliant Blue R-250 in 250 mL of protein gel destaining solution, mix thoroughly, and store at room temperature.

23. Amp (100 mg/mL, Ampicillin Solution) : Dissolve 1 g of ampicillin in ultrapure water to a total volume of 10 mL, sterilize by filtration, and store at low temperature in the dark.

23. Kan (100 mg/mL, Ampicillin Solution) : Dissolve 1 g of kanamycin in ultrapure water to a total volume of 10 mL, sterilize by filtration, and store at low temperature in the dark.

24. LB Liquid Medium : Add 2 g of tryptone, 2 g of NaCl, and 1 g of yeast extract to ultrapure water, adjust the total volume to 200 mL, sterilize by steam, and store at room temperature.

25. LB-Amp Medium : Supplement LB liquid medium with ampicillin to a final concentration of 100 μg/mL.

26. LB-Kan Medium : Supplement LB liquid medium with kanamycin (Kan) to a final concentration of 100 μg/mL.

27. YPD Medium : Add 4 g of tryptone and 2 g of yeast extract to ultrapure water, adjust the total volume to 192 mL, sterilize by steam, and store at room temperature. Before use, add 8 mL of 50% glucose solution.

28. BMSY Medium : Mix 5 mL of 1 M potassium phosphate buffer (pH 6.5), 1 g of tryptone, 2.5 g of sorbitol, and 0.5 g of yeast extract, add ultrapure water to a total volume of 45 mL, sterilize, and store at room temperature. Before use, add 5 mL of 10× YNB and 200 μL of 500× B in a clean bench. For carbon source optimization experiments, replace sorbitol with an equal amount of sucrose, glucose, or glycerol.

29. MD Medium : Mix 180 mL of sterile deionized water, 20 mL of 10× YNB, 400 μL of 500× B, and 8 mL of 50% glucose solution. Store at 4 °C (low temperature).

30. MD-Leu Medium : Supplement 5 mL of MD medium with 150 μL of 40× Leu solution.

31. MD-Ura Medium : Supplement 5 mL of MD medium with 300 μL of 20× Ura solution.

32. MO-Leu-Ura Medium : Sterilize 80 mL of ultrapure water by autoclaving, cool to 70 °C, then add 10 mL of 10× YNB, 200 μL of 500× B, 2 mL of oleic acid, 300 μL of 20× Ura, and 150 μL of 40× Leu. Aliquot into 5 mL portions before use.

33. Tris-HCl buffer (0.05 M): Dissolve 5.05 g of Tris base in 450 mL of double-distilled water, adjust the pH to 8.0, and then bring the total volume to 500 mL with double-distilled water.

34.Nile Red Staining Solution (1 mg/mL in DMSO): Prepare 1 mM stock solution with Nile Red in anhydrous DMSO, then dilute 1:1000 with HHBS/buffer (pH 7.0) to make working solution. Adjust concentration refer to literatures.

Small-scale extraction of plasmids

Refer to the Plasmid Mini kit I instruction manual of Omega Company. The specific steps are as follows:

1. Add 1-2 mL of the overnight cultured bacterial liquid to 2 mL EP tubes, centrifuge at 12,000 rpm for 2 minutes, and discard the supernatant.

2. Add 250 μL of Solution I (pre-added RNase A) and resuspend the bacterial cells.

3. Add 250 μL of Solution II (preheat to clear before use if there are flocs), gently invert 4-5 times to mix well, and let it stand at room temperature for 2 minutes.

4. Add 350 μL of Solution III and immediately mix until a white flocculent precipitate is formed. Centrifuge at 12,000 rpm for 10 minutes.
5. Transfer the supernatant to the adsorption column, centrifuge at 12,000 rpm for 1 minute, and discard the waste liquid in the collection tube.
6. Add 500 μL of Buffer HB, centrifuge at 12,000 rpm for 1 minute, and discard the waste liquid in the collection tube.
7. Add 700 μL of DNA Wash Buffer (pre-added with anhydrous ethanol), centrifuge at 12,000 rpm for 1 minute, discard the waste liquid in the collection tube, and repeat the operation once.
8. Centrifuge the empty column at 12,000 rpm for 2 minutes. Leave the adsorption column open for 2 to 5 minutes and then place it in a 1.5 mL new EP tube.
9. Add 50-100 μL of sterile water to the center of the adsorption column, let it stand at room temperature for 2 minutes, centrifuge at 12,000 rpm for 1-2 minutes, and then add the plasmid collection solution back to the adsorption column. Repeat the operation once.

Gel Recovery and Purification

Refer to the instruction manual of Omega's Gel Extraction Kit. The specific steps are as follows:

1. Electrophorese the DNA sample in 0.5-1% (m/v) agarose gel for 10-20 minutes. Under ultraviolet light, accurately cut the fragment at the target DNA band and place it in a 1.5 mL EP tube. Add 300-350 μL of Binding Buffer XP2.
2. Incubate in a water bath at 55-60 °C for 7-10 minutes, during which invert the EP tube 2-3 times to completely melt the gel.
3. After a brief centrifugation, transfer all the contents to an adsorption column, centrifuge at 12,000 rpm for 1 minute, and discard the waste liquid in the collection tube.
4. Add 300 μL of Binding Buffer XP2, centrifuge at 12,000 rpm for 1 minute, and discard the waste liquid in the collection tube.
5. Add 750 μL of SPW Washing Buffer (with anhydrous ethanol pre-added), centrifuge at 12,000 rpm for 1 minute, remove the waste liquid in the collection tube, and repeat this operation once.
6. Centrifuge the empty column at 12,000 rpm for 2 minutes. Place the adsorption column with

the opening exposed for 2-5 minutes, then put it into a new 1.5 mL EP tube.

7. Add 30-50 μL of sterile water to the center of the adsorption column, let it stand at room temperature for 2 minutes, centrifuge at 12,000 rpm for 1-2 minutes. Add the DNA eluate to the adsorption column again and repeat this operation once.

Extraction of Yeast Genomic DNA

1. Inoculate a yeast single colony or suspension into 5 mL of YPD medium, and incubate it in a 200rpm shaker at 28 °C for 18–24 hours.

2. Take 2 mL of the suspension each tube, centrifuge at 12,000 rpm for 2 minutes, and collect the yeast cells.

3. Resuspend the cells in 1 mL of deionized water, centrifuge at 12,000 rpm for 1 minute, and discard the supernatant.
   If the concentration of the bacterial suspension is low and enrichment is required: Use 1 mL of deionized water to resuspend the cells by pipetting to prepare Suspension 1. Transfer Suspension 1 to another centrifuge tube containing the centrifuged bacterial pellet and resuspend again to achieve a 2-fold enrichment effect.

4. The cell wall of *Yarrowia lipolytica* is relatively thick. For better results, after discarding the supernatant, add 300 μL of lywallzyme to each tube and incubate at 30 °C for 30 minutes.

5. Add 200 μL of Triton/SDS bacterial lysis buffer and 200 μL of the lower phase solution of phenol/chloroform/isoamyl alcohol, then incubate in a water bath at 65 °C for 10 minutes.

6. Vigorously oscillate for 10 minutes (note: after water bath incubation, open the tube cap to release air pressure, otherwise the solution may splash out during oscillation).

7. Add 200 μL of TE buffer (pH 8.0) and quickly oscillate vigorously for 1 minute.

8. Centrifuge at 12,000 rpm for 10–15 minutes. Use a pipette to transfer the supernatant to an EP tube (a tube with a conical bottom is more conducive to DNA adsorption)

9. Add 2.5 volumes of pre-cooled absolute ethanol (stored in a -80 °C refrigerator) (approximately 800–1000 μL), gently mix well, place in a -80 °C refrigerator, and let stand for 5–10 minutes.

10. Centrifuge at maximum rpm for 5–10 minutes (temperature can be set to 4 °C) and discard the supernatant.

11. Add 1 mL of absolute ethanol for washing, centrifuge at 12,000 rpm for 1 minute, and discard the supernatant.
    Pay special attention to the placement of the EP tube in the centrifuge: orient the tube

opening toward the center of the centrifuge. In this case, DNA will be adsorbed on the side of the tube away from the centrifuge center. At this point, simply pour off the supernatant carefully in the original inclined direction, so that the liquid flows down along the side of the tube without the DNA precipitate.

12. Add 500 μL of 75% ethanol, centrifuge at maximum rpm for 1 minute, and carefully remove the supernatant with a pipette while avoiding the DNA precipitate.

13. Add 30–50 μL of ultrapure water, oscillate vigorously to dissolve the DNA attached to the tube wall into the water, and then centrifuge briefly with a microcentrifuge to allow the genomic DNA solution on the tube wall to settle to the bottom of the tube.

PCR amplification of the target fragment

1. 2 × Prime Star Mix high-fidelity polymerase was used for DNA fragment cloning, with an extension efficiency of 30 s/kb; 2 × Rapid Taq Master Mix low-fidelity polymerase was used for PCR verification of vectors and genomes, and the extension efficiency was 15 s/kb. PCR system: Premixed polymerase 25 μL, upstream and downstream primers each 1 μL, template (plasmid, genome or bacterial liquid) 1 μL, and add ultrapure water to 50 μL.

2. PCR procedure: Pre-denaturation at 95 °C for 5 to 8 minutes; Denaturation at 95 °C for 15 to 30 seconds, annealing at 50 to 55 °C for 15 to 30 seconds, elongation at 72 °C, cycle number 30 to 35. Extend at 72 °C for 7 minutes.

Restriction endonuclease digestion

1. Enzyme digestion system: 40 μL of plasmid or gel recovery product, 5 μL of each restriction endonucase, 10 μL of 10 × buffer buffer, and add ultrapure water to 100 μL.

2. The enzyme digestion temperature is the optimal reaction temperature of the endonuclease.

3. *When multiple enzymes are digested, if the optimal temperatures of the endonuclease are different, the digestion should be carried out step by step. The enzymatic digestion time is 0.5 to 1 hour. 6

Enzyme-linked or recombination ligation

1. Enzyme-linked system: 1 μL of carrier fragment, 6.5 μL of target DNA fragment, 2 × Ligation Mix or 2 × SolutionI ligase 7.5 μL. Mix well and react at 16 °C for more than

1 hour. Recombinant ligation system: 2 μL vector fragment, 6 μL target DNA fragment, 2 μL ClonExpress MultisOne Step Cloning Kit recombinase, 4 μL 5 × CE buffer, 6 μL ultrapure water.

2. After thorough mixing, it was placed in a 37 ° C water bath for 30 minutes, with the homologous recombination reaction time precisely controlled

E. coli Transformation

1. Take out E. coli competent cells *TOP10* or *TOP10F'* from the ultra-low temperature refrigerator, place them on ice, and let stand for 5-8 minutes until they thaw.
2. Add the ligation product and mix gently, then place in an ice bath for 30 minutes.
3. After heat shock at 42 °C for 60-90 seconds, place in an ice bath for 2 minutes.
4. Add 700 μL of LB medium and incubate in a shaker at 37 °C for 30-60 minutes.
5. Centrifuge at 5000 rpm for 30-60 seconds, leave approximately 100 μL of bacterial solution, spread it on the LB-Amp plate, and incubate in a 37 °C constant temperature incubator for 10-15 hours.

Detection of Lipase Activity

1. Prepare a 9 mL hydrolysis reaction system by mixing Tris - HCl buffer and olive oil emulsion at a volume ratio (v/v) of 5:4, and preheat it in a water bath shaker at 60 °C for 10 min.

2. For the experimental group, add 1 mL of appropriately diluted fermentation supernatant (diluted with Tris - HCl buffer); for the control group, add 1 mL of Tris - HCl buffer instead.

3. Incubate the reaction system with shaking in a water bath at 60 °C and 125 rpm for 10 min.

4. Immediately after the reaction, add 15 mL of 95% ethanol to terminate the hydrolysis reaction.

5. Add 50 μL of 0.5% phenolphthalein solution as an indicator.

6. Titrate the reaction mixture with 0.05 M NaOH solution.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1.Take the precast SDS-PAGE gel, remove the protective film from the bottom, and install it into the protein gel electrophoresis tank.

2.Slowly pour the electrophoresis buffer into the electrophoresis tank, let it stand for a period of time, check the tightness of the device, and remove the comb.

3.Mix 5× protein electrophoresis loading buffer with fermentation supernatant at a volume ratio of 1:4, and incubate in a metal bath at 99.9 °C for 10 min.

4.Load 20–40 μL of each sample and 10 μL of pre-stained protein molecular weight Marker.

5.Perform electrophoresis at 130–150 V for approximately 1 h. After completion, transfer the protein gel into the staining solution and boil for 1 min.

Protein Deglycosylation

1. Add 1 μL of 10× glycoprotein denaturation buffer to 9 μL of fermentation supernatant, and incubate in a boiling water bath for 10 min.

2. Take 3 μL of Endo H endoglycosidase, 10 μL of the denatured protein sample from the previous step, and 2 μL of 10× Endo H reaction buffer.

3. Supplement with ultrapure water to a total volume of 20 μL and mix thoroughly.

4. Incubate the mixture at 37 °C for 1 h.

5. Heat the reaction mixture at 65 °C for 10 min to terminate the reaction.

Determination of Protein Content

1. Dilute the BSA standard protein solution (5 mg/mL) with Tris-HCl solution to 1 mg/mL and set aside.

2. Pipette 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 μL of the 1 mg/mL BSA standard protein solution respectively into separate containers.

3. For each container, add Tris-HCl (pH 8.0) solution to bring the total volume to 1 mL.

4. Add 5 mL of Coomassie Brilliant Blue G-250 solution to each container and vortex at high speed for 60 s.

5. After standing for 2 min, measure the absorbance at 595 nm.

6. Generate a standard curve using the A595nm absorbance values of the standard samples.

7. Appropriately dilute the fermentation supernatant with Tris-HCl (pH 8.0) solution.

8. Add 5 mL of Coomassie Brilliant Blue G-250 solution to the diluted supernatant, vortex thoroughly for 60 s, and let stand for 2 min.

9. Determine the absorbance at 595 nm as described in step 5, and calculate the target protein content using the standard curve generated in step 6.

Preparation of *Yarrowia Lipolytica* Po1f competent cells

1. Put *Y.Lipolytica* into 5mL YPD liquid culture medium and inoculate at 28°C for 18-24h in 200 rpm shaker until the yeast solution becomes turbid..
2. Take 1.5 mL of the overnight cultured yeast solution, centrifuge at 5000 rpm for 1 minute, and discard the supernatant.
3. Resuspend the yeast cells in 1 mL of 1× TE buffer, centrifuge at 5000 rpm for 1 minute, and discard the supernatant.
4. Gently resuspend the cell pellet in 600 μL of 0.1 M lithium acetate buffer (pH 6.0), and incubate in a water bath at 28 °C for 1 hour.
5. Before use, subject salmon sperm DNA to a boiling water bath for 10 minutes and then place it on ice. Take 6 μL each of the linearized plasmid and salmon sperm DNA, mix them well, and set aside.
6. Centrifuge at 3000 rpm for 2 minutes to collect the yeast cells. Resuspend the cells in 40 μL of 0.1 M lithium acetate buffer, add the premixed linearized plasmid and salmon sperm DNA, mix well, and incubate in a water bath at 28 °C for 10 minutes.
7. Add 320 μL of 50% PEG 4000, 20 μL of 2 M lithium acetate (pH 6.0), and 16 μL of 1 M DTT, mix gently, and incubate in a water bath at 28 °C for 1 hour.
8. Incubate in a warm water bath at 39 °C for 10 minutes, add 600 μL of 0.1 M lithium acetate, and mix gently.
9. Centrifuge at 3000 rpm for 30–60 seconds, retain approximately 100 μL of the yeast solution, and spread it onto an appropriate selection plate. Incubate at a constant temperature of 28 °C for 2–3 days; alternatively, transfer to a small flask using the "liquid method" for subsequent cultivation.

Liquid-Based Screening for Lithium-Transformed Positive Monoclones (po1f)

1. Inoculate 100 μL of transformed cells into 5 mL of MD-Leu medium for primary screening and enrichment of positive transformants, followed by incubation in a 200rpm shaker at 28 °C for 18–24 h.
2. Inoculate 50–100 μL of the primary screening bacterial culture into 5 mL of fresh MD-Leu medium for secondary screening, and incubate in a shaker at 28 °C for 36–48 h.
3. Inoculate 100 μL of the secondary screening bacterial culture into 5 mL of MO-Leu-Ura induction medium, and incubate in a shaker at 28 °C for 18–24 h.

4. Use an inoculating loop to streak an appropriate amount of bacterial culture onto a YPD plate. After incubation at 28 ° C for 24 – 48 h, monoclones will be obtained. Spot the transformed monoclones separately onto MD-Leu plates and YPD plates for screening.
5. Monoclones that fail to grow on MD-Leu plates but grow well on YPD plates are the correct strains with marker excision.

Verification of the Targeting Function of Signal Peptides

1. Put *Y.Lipolytica* into 5mL YPD liquid culture medium and inoculate at 28°C for 24h cultivation shaking at 200 rpm until the yeast solution becomes turbid.
2. Add Nile red stain solution (1 mg/mL in DMSO) to the yeast culture (0.1 v/v)
3. Incubate at room temperature in the dark environment for 60 minutes.
4. Centrifuge at 5000 rpm for 1 minute and discard the supernatant.
5. Resuspend cells in physiological saline. Then centrifuge at 5000 rpm for 1 minute and discard the supernatant.
6. Repeat step5 for multiple times until cells are colorless.
7. Gently resuspended in potassium phosphate buffer (pH 7.4).
8. Transfer small volume onto the microscope slide.
9. Set laser scanning confocal microscope(LSCM) to observe the yeast at 488 nm and Nile red staining at 561 nm, if the highlighted parts of the two fluorescent spots completely overlap and produce bright yellow light, it proves that the ePTS1 sequence functions as a peroxisomal targeting signal in *Y.Lipolytica*.

Protocol for Squalene Extraction and Content Determination

Squalene Extraction

1. Cell Treatment: Collect bacterial cells and freeze-dry them to constant weight.
2. Disruption and Extraction: Weigh 50 mg of freeze-dried cells, add 500 μL of methanol and 1 mL of chloroform, and grind for 2 min at 60 Hz in a high-throughput grinder. Then, incubate in a shaker at 30° C and 200 r/min for 12 h for extraction.
3. Centrifugation and Drying: Centrifuge at 8000 rpm for 5 min, transfer the supernatant to a 2 mL centrifuge tube. Dry in a metal bath at 65° C for 2 h and cool to room temperature.
4. Re-dissolution and Purification: Add 1 mL of n-hexane, vortex for 5 min. Centrifuge at 12000 rpm for 5 min, filter through a 0.22 μm organic filter membrane, collect the n-hexane phase, and store at -20° C until detection.

Squalene Content Determination (Gas Chromatography)

1. Chromatographic Conditions
    - Injector temperature: 300°C
    - Chromatographic column: Rtx-5 capillary column (30 m×0.32 mm×0.25 μm)
    - Carrier gas: High-purity nitrogen, constant flow mode, flow rate 2.0 mL/min
    - Injection mode: Splitless, injection volume 1 μL
    - Detector: Flame Ionization Detector (FID), temperature 330°C
    - Temperature program: Initial temperature 160°C (hold for 0 min), ramp to 280°C at 15°C/min (hold for 6 min)

Construction of Target Gene-Knockout Strains of Yarrowia lipolytica

1. Use the method "Preparation of Yarrowia Lipolytica Po1f competent cells" to transform the constructed knockout plasmids carrying crRNA into competent cells of Yarrowia lipolytica, and screen for positive strains.
2. Obtain single colonies of Yarrowia lipolytica by streaking on YPD plates, then pick individual colonies for expanded culture respectively.
3. Screen the strains meeting the requirements by detecting the growth status or product expression levels of each bacterial group, which contain the desired target gene mutations we need.