Protocol

TPR_China

Basic Molecular Cloning:

Plasmid extraction

1. Prepare 1mL of bacteria solution in 1.5ml Eppendorf tube, label with date, operator name, plasmid name (if specific gene fragment is present, the name should also be recorded), and centrifuge at 12000rpm for 1 min, discarding the supernatant.
2. Add 150μL of Buffer P1 and vortex to blend until no bulk precipitation visible.
3. Add 150μL of Buffer P2 and gently invert 6~8 times to mix. At this point the solution should be observed as pink in color.
4. Immediately add 350μL of Buffer P5 within 2 min and gently invert 6~8 times. At this time the color of solution should change from pink to yellow with flocculent precipitation.
5. Centrifuge at 12000rpm for 5min to separate plasmid and impurities.
6. Apply the supernatants from step 5 to a CP3 spin column and centrifuge 12000rpm for 1 min, discard the liquid in the collection tube.
7. Wash the CP3 spin column by adding 300μL of Buffer PWT. Let stand for 2 min and centrifuge at 12000 rpm for 1 min, discarding the liquid in the collection tube.
8. Put back CP3 spin column and repeat step 7 without adding Buffer PWT. Discard the ethanol impurities.
9. Obtain CP3 spin column and place it in a clean Eppendorf tube with the lid open to let the residual ethanol impurities evaporate.
10. Pipette 50μL of water on the film of CPR. Centrifuge at 12000rpm for 3min and discard the CP3 spin column. At this time plasmid can be collected in the Eppendorf tube.
11. Obtain the OD value of the collected product using Microplate reader and note the value on the body of the tube. Place it in the -20 ˚C refrigerator.

PCR

High fidelity PCR

1. Set up a PCR system in a PCR tube on ice (see Reaction System Component Table for further details).
2. Blend the content of the system using a pipette or a vortex oscillator. Use microcentrifuge to eliminate bubbles and draw the reaction solution to the bottom of the PCR tube.

Place the PCR tube into a thermo cycler, close the lid, and set the program as needed (the annealing temperature was 58 °C).

4. Purify the products (see PCR product purification for further details), or carry out gel electrophoresis to obtain an image using gel imaging machine (see Gel electrophoresis for further details). Decide whether to recycle as needed (see Gel extraction for further details).

Reaction System Component Table (High fidelity PCR using Q5High fidelity PCR using Q5)

Reaction System Component	Volume of Addition
2X Q5 Mix	25 μL
Primer F	1 μL
Primer R	1 μL
DNA Template	0.25 μL
ddH2O	22.75 μL
Total Volume	50 μL

Reaction System Component Table (High fidelity PCR using FastPfu)

Reaction System Component	Volume of Addition
5X TransStart FastPfu Buffer	10 μL
Primer F	2 μL
Primer R	2 μL
DNA Template	1 μL (<20ng)
2.5 mM dNTPs	4 μL
ddH2O	32 μL
Total Volume	50 μL

Colony PCR (Using Taq)

1. Set up a PCR system in a PCR tube on ice (see Reaction System Component Table (Colony PCR) for further details).
2. Blend the content of the system using a pipette or a vortex oscillator. Use microcentrifuge to eliminate bubbles and draw the reaction solution to the bottom of the PCR tube.
3. Prepare fresh antibiotic-containing LB solid medium plates, and label the needed information on the plates and PCR tubes.
4. The single colonies on the transformed plate were picked with a sterile toothpick on a super clean table. First draw a line on the LB plate as the copy of the original colony, and dip it into the PCR tube with corresponding number. Turn the toothpick, remove it, and discard it in the waste liquid cylinder.
5. Place the PCR tube into the thermo cycler and set the program as needed (the annealing temperature was 58 °C, elongation time was 90s).
6. 30min before PCR ends, make a piece of agarose gel for DNA recycling (see Agarose gel preparation for further details)

7. Carry out gel electrophoresis (see Gel electrophoresis for further details) to detect positive clones. Decide whether to recycle as needed (see Gel extraction for further details).

Reaction System Component Table (Colony PCR)

Reaction System Component	Volume of Addition
2X Taq PCR Mix	10 μL
Primer F	1 μL
Primer R	1 μL
ddH2O	8 μL
Total Volume	20 μL

Agarose gel preparation

1. Place an adhesive film onto the horizontal working table and place the rubber sheet into the corresponding film. Note that there should be no air or debris between the bottom of the adhesive film and the rubber sheet. Then insert the sample comb while ensuring that the bottom edge of the comb tooth does not touch the adhesive film (otherwise it will lead to hole leakage).
2. Weigh 1.0g agarose in a triangulated flask and add 100ml of 1xTAE buffer to prepare 1%(w/v) agarose.
3. Put the solution into a microwave oven, and heat on high heat for 0.5-1min, during whichobserve and shake (it will boil several times in the process) until the liquid is clear and uniform.
4. Add 10μL SYBR Safe DNA Gel Stain (10000x) into the heated solution, shake until fully mixed, and carefully pour the solution into the gel tray (avoid bubbles during the process). Allow it to solidify at room temperature for 20-60 minutes.

Gel electrophoresis

1. When the agarose gel completely solidifies, pull out the comb with both hands carefully, creating separate spot-holes without breaking the gel.
2. Put the gel together with the gel tray into the electrophoresis tank. Add 1xTAE buffer until it fully immerses the gel surface (with water level 2-3mm above the gel). Gently move the gel and gel sheet to remove bubbles from the punctured pores.
3. Slowly add the sample into the sample hole with a pipette, taking care not to spill to adjacent holes. Samples are generally taken at 5 μL.
4. Add a suitable DNA Marker separately into a point-sample hole as a control (3 μL for small hole, 5 μL for medium hole, and 10 μL for large hole).
5. Set the program as needed (the general voltage is set as 120V and the duration is set at 20min).

Gel extraction

1. Run electrophoresis for 20min at 120V.
2. Obtain gel image using gel imaging system.
3. Prepare and label a 1.5mL centrifuge tube.
4. Cut the gel and place the wanted fragment in the centrifuge tube.
5. Weigh the gel to obtain its mass and calculate its volume.
6. Add equal volume of PC solution into the tube, mix thoroughly using a vortex oscillator.
7. Heat up the centrifuge tube for 10 minutes on a dry bath set to 50˚C, gently shaking the tube from time to time to ensure that the gel block is fully dissolved.
8. Put the adsorbent column CB2 into the collection tube. Balance the Spin column with 500μL Buffer BL and centrifuge at 12,000 rmp for 1min, separating the filtrate.
9. Add the filtrate obtained from the previous step into the adsorption column and placed at room temperature for 2min. Centrifuge the column for 30~60s at 12,000 rmp.
10. Add a 600μL bleach solution PW (plus ethanol) into the column and centrifuge at 12,000 rmp for 30~60s. Discard the rinse solution. Repeat this step again.
11. Put the adsorbent column back into the collection tube and centrifuge at 12,000 rmp for 2min to remove as much of the rinse solution as possible.
12. Place the adsorption column into a clean, labelled 1.5mL EP tube and place it at room temperature for 1-3min to allow the ethanol to evaporate.
13. Add 40μL of ddH2O onto the silicone membrane in the middle of the adsorption column, and leave the solution at room temperature for 1min. DNA solution can be collected after centrifugation at 12,000 rmp for 1min.
14. Obtain the product's OD value using Microplate reader and note the value on the body of the tube. Place it in the -20 ˚C refrigerator.

PCR product purification

1. Transfer the PCR reaction solution or enzymatic reaction solution to a clean 1.5mL centrifuge tube, add 3 times the volume of Buffer B3, and mix well.
2. Transfer all the mixture into the adsorption column and centrifuge it at 8,000 × g for 30s. Discard the liquid in the collecting tube and place the adsorbent column in the same collecting tube.
3. Add 500 μL Wash Solution to the column and centrifuge it at 9,000 × g for 30 s. Discardthe liquid in the collecting tube and place the adsorbent column in the same collecting tube.
4. Repeat step 3 once.
5. Put the empty adsorption column and the collection tube into the centrifuge, and centrifuge it at 9,000 × g for 1 min.

6. Add 15~40 μL Elution Buffer to the center of the adsorption membrane and let it stand for 1-2 min at room temperature. Then centrifuge it for 1 min at 9,000 × g. Obtain the filtrate DNA product and place at -20°C to preserve or use in subsequent tests.

Restrict Enzyme Digestion & Ligation

Digestion：

1. Set up the system as shown below and carry out the process on ice.
2. Thoroughly mix with vortex oscillator and place in a dry bath at 37˚C for 1h

CutSmart Buffer：3μL

Enzyme 1：1μL

Enzyme 2：1μL

ddH2O up：to30μL

Ligation：

1. Set up a system as shown below, all operations on ice .
2. Set PCR program: 50˚C 20min, 12˚C forever

10x T4 ligase Buffer：1.2μL

RD DNA sample 60 ng

T4 ligase：1μL

ddH2O：up to 12μL

Gibson Assembly

1. Using 5μL smart mix to construct a Gibson assembly system
2. Set PCR program as: 50˚C 20min, 12˚C forever

Competent cell transformation

1. Mix plasmid solution with 50μL of competent cells, place the mixture on ice bath over 30 min to allow thorough contact between DNA and cells, enabling DNA to enter the cell wall.
2. Heat shock at 42°C for 90s (Cells swell at high temperature, allow thorough contact of the cell membrane and cell wall, enabling DNA to pass through the cell membrane into the cytoplasm).
3. Place on ice bath for 5min (Cell shrinks at low temperature, dragging DNA on the cell wall into the cell).
4. Pipette 200μL of nutrient broth into the tube (operate inside a laminar flow hood), put the bacteria into an incubator at 37°C for 1h30min.
5. Spread the bacteria on plates and observe after 16h~20h

Basic Microbiology Experiment:

High pressure steam sterilization (autoclaving)

Steam sterilization (autoclaving) is generally carried out in 121°C (250°F) and 14.5 psi (1 bar) for 20 minutes.

Broth culture & agar culture

The main form of cell culture we have used is agar plating. Bacterial solution is firstly added onto the prepared LB agar plate, and spreaders are used to evenly coat the plate with bacterial solution. The agar plates are then covered with caps and sealed with Parafilm sheets. Finally, the agar plates are inverted and placed into a 37˚C incubator overnight.

Cryopreservation

Bacteria liquid and 50% glycerin are added in 1:1 volume ratio into the bacteria preservation tube and stored in the refrigerator at -80°C.

Basic Reagents and Medium:

Luria-Bertani (LB) medium

LB medium(liquid)：

Tryptone 10g/L

Yeast extract 5g/L

Nacl 10g/L

Mix them and then add ddH2O until it reaches 1 liter.

121°C steam sterilization for 20min.

LB medium(solid)：

Tryptone 10g/L

Yeast extract 5g/L

Nacl 10g/L

Agar power 15g/L

Mix them and then add ddH2O until it reaches 1 liter.

121°C steam sterilization for 20min.

Antibiotic

Process:

1. 10g of solid ampicillin is weighed in a beaker using an electronic balance.

2. The measured solid is dissolved with 100 mL sterilized deionized water in an ultra-clean table (note the operation near the flame), the solution is stirred until completely dissolved.

3. Sterilized by filter membrane, the product is aliquoted into Eppendorf tubes.

4. The solution is stored in refrigerator at -20°C for later use.

Kanamycin

Process:

1. 5g of solid kanamycin is weighed in a beaker using an electronic balance.

2. The measured solid is dissolved with 100 mL sterilized deionized water in an ultra-clean table (note the operation near the flame), the solution is stirred until completely dissolved.

3. Sterilized by filter membrane, the product is aliquoted into Eppendorf tubes.

4. The solution is stored in refrigerator at -20°C for later use.

Inducer

IPTG:

Deionized water

Eppendorf tube (sterilized)

Small beaker 100ml

Glass rod

Injector

Filtration membrane

Solid IPTG

PAN

o-Cresol

4-Methylsalicylic acid

2-Hydroxyphenylacetic Acid

m-Toluic acid

Process：

1. 1mol of solid Inducer is weighed in a beaker using an electronic balance.

2. The measured solid is dissolved with 100 mL sterilized deionized water in an ultra-clean table (note the operation near the flame), the solution is stirred until completely dissolved.

3. Sterilized by filter membrane, the product is aliquoted into Eppendorf tubes.

4. The solution is stored in refrigerator at -20°C for later use.

PBS buffer

0.05% Tween-20 pH7.4 Phosphate Buffered Saline:

KH2PO4 0.24g

Na2HPO4 1.44g

NaCl 8g

KCl 0.2g

The solids mentioned above are weighed and added into a flask.

800mL deionized water is added to the mixture; the solution is stirred thoroughly for all solute to dissolve.

Concentrated hydrochloric acid is then added to adjust pH to 7.4, and the volume is set to 1L.

After high temperature and high pressure sterilization, the solution is stored in refrigerator at 4 °C for later use.

Protein Hetero-Expression:

Culture growing & protein expression inducing

Pick several single colonies from a streaked plate of the expression host at 5mL of LB medium.

Let the culture grow overnight.

Inoculate with the picked colony up to 30 ml of medium until the OD600 is 0.2

Inoculate each single colony to 2 tubes, growing the culture until OD600 reaches 0.4.

Pick 1 tubes of each colony and add the inducer IPTG*: the concentration of 0.1mmol/L.

Incubate the culture for 20h at 30°C

After 20 hours of incubation, centrifuge the culture for 20 minutes at 9000rm, 4°C. Use the PBS buffer to resuspend the bacteria

*IPTG(Isopropyl ß-D-1-thiogalactopyranoside) induces the E. coli lac operon activity by binding and inhibiting the lac repressor without being degraded.

SDS-PAGE Protein Gel electrophoresis

Prepare SDS-PAGE running buffer(1X): 10mL.

Prepare gels and assemble the electrophoresis cell.

Fill the inner and outer buffer chambers with running buffer.

Fill the lower buffer chamber to the indicator mark.

Prepare samples: 20μL for each.

Heat samples for 5 minutes at 100°C.

Cover the chamber and firmly connect both the anode and the cathode. Set the voltage on the electrophoresis power supply to a constant voltage of 120 V.

Run the gel electrophoresis cell for 1.5h.

After electrophoresis is complete, turn off the power and disconnect the electrodes and remove the cover. Remove the gel by floating it off the plate into water.

Stain the SDS-PAGE with Coomassie blue dyes, then destain it.

Observe the electrophoresis results that show up in the E-gel imager.

Nitrilase Enzyme Activity:

Standard curve characterization

Prepare the (NH4)2SO4 solution: M=132 g/mol, V=5mL (Use 66mg (NH4)2SO4 and dissolve 50mLPBS into 0.01mol /L)

Five samples are filled separately with 0μL, 20μL, 40μL, 60μL, 80μL, and 100μL of (NH4)2SO4 solution.

Each sample is filled with, correspondingly, 1000μL, 980μL, 960μL, 940μL, 920μL, and 900μL of PBS, causing all 5 samples to contain 1mL of liquid in total.

Add 20μL Nessler reagent to each of the solutions above.

Then, a standard curve can be constructed with each sample's absorbance level at OD425 against the sample's NH4+ concentration.

The experimental sample's NH4+ concentration can thus be calculated from the standard concentration equation.

Mechanics of terminal enzyme activity

Collect the bacteria colony located at the bottom of the tube and add 3ml PBS. Then, use ultrasonic crusher for crushing bacteria.

100μL of each bacteria solution to diluted with 900μl PBS

Configure the solution according to the system:

PBS: 153μL,

PAN: 2μL

bacteria solution: 25μL.

Blank control is the following system:

PBS: 178μL

PAN: 2 μL.

Put the solution into a 96-well plate, incubate at 30°C for 90min, and then add 20 μL Nessler reagent into each well.

Measure the absorbance at OD425 using a micro-plate reader.

Calculate the concentration of the sample according to the standard concentration equation (using the standard curve created previously)

Aromatic Sensor Test:

Culture incubation & induction

1, Select one single colony of the engineered bacteria and put it into 5ml liquid culture medium; incubate overnight at 37˚C, 200rm.

2, Get 1 percent dilution of the bacteria in 5ml liquid culture medium, put it into a 30ml culture medium, 37°C，200rm cultivate until the OD600 reading is equal to 0.2. Get each single cell bacteria to two different cultures and cultivate under 37°C，200rm until the OD600 reading is equal to 0.4. Add the corresponding inducer in one culture and leave another one without, and cultivate for 20 hrs under 37°C.

Inducers that match up with each sensor are

DMPR sensor: o-Cresol

NAHR sensor: 4-Methylsalicylic acid

PAAX sensor: 2-Hydroxyphenylacetic Acid

XYLS sensor: m-Toluic acid

Culture harvest

After 20 hours of incubation, centrifuge the culture for 20 minutes at 9000rm, 4°C. Use the PBS buffer to resuspend the bacteria.

Micro-plate Reader

First put the culture under the blueray to see if there is any fluorescence.

Add 200μl of each bacteria culture into three wells of a 96-well plate. Meanwhile, add three wells of PBS medium for blank comparison.

Test the fluorescent intensity of the sample in a micro-plate reader, the conditions are

excitation：485 nm；

emission：515 nm；

gain：optimal.