Protocol

- **Agarose-gel electrophoresis**
  1. Prepare a 0.8 % agarose-gel: Dissolve the agarose in 1X TAE by boiling and add goldview (5 µl per 100 ml agarose solution).
     For short DNA fragments, gels with higher agarose percentage should be used.
  2. Pour the gel and let it curdle
  3. Add 1ul 6X blue loading dye to sample(5ul)
  4. Place the gel into the tank and add 1X TAE buffer so that the gel is fully covered.
  5. Pipet sample and marker into the pockets.
  6. Run the gel at 180 V for 20 min.
  7. Image the gel using ultraviolet light. If necessary, cut out bands.
- **PCR**
  1. For a 50 µL reaction：
  2. In a PCR tube on ice, combine 1-10 ng of template DNA, 2.50 µL of 10 µM forward primer, 2.50 µL of 10 µM reverse primer to a PCR tube on ice, 10 µL of 5× PrimeSTAR Buffer, 4 µL dNTP mixture 0.5 µL PrimeSTAR HS DNA Polymerase and sterile water up to 50 µL.
  3. Gently mix the reaction
  4. Collect the liquid to the bottom of the PCR tube by centrifuging briefly
  5. Transfer the PCR tube from ice to a PCR machine preheated to 98°C to begin thermocycling
  6. Thermocycling:
  7. The PCR machine should be set to run the following steps:

step	Temperature(℃)	Time
Initial denaturation	98	5 minutes
30 cycles	98(denaturation) 55-60(annealing) 72(extension)	10 seconds 10 seconds 1 minute per kb
Final extension	72	5 minutes
Hold	4	indefinitely

- **Preparation of fungal protoplasts**

  Materials:

  1/4 SDAY(liquid):3g Sucrose,0.9g Yeast Extract,0.45g Tryptone,0.609g $MgCl_2 \cdot 6H_2O$

  TPS1: 0.6M Sucrose,5mM Na2HPO4, 45Mm KH_2PO4

  TPC(1L):Tris 1.21g,$CaCl_2$ 1.11g,Sucrose 205g,

  1. Prepare moderate amounts of fungus grown in 1/4 SDAY broth
  2. Filter broth by two-layer filter cloth then washes hyphae by TPSI.
  3. Weigh the hyphae, then collect hyphae into the sterile conical flask,
  4. Add 15~20 mL TPS1 with the cellulose and snailase(a total of 3g) dissolved.
  5. Incubate for 2~4h at 37℃ on a shaker, then observe the extent of digestion under the microscope
  6. Filter mixture by four-layer filter cloth, then wash the filter cloth with TPS1, gather follow-through in a 50 mL centrifuge tube.
     P.S.protoplasts are in the follow-through

7. Centrifuge at 3200 rpm for 10 minutes. Then discard the supernatant.
8. Wash precipitation with 20~30 TPS1, centrifuge at 3200rpm for 10 minutes.
9. Add 20~30 mL TPC,centrifuge at 3200rpm for 10 minutes.Discard the supernatant.
10. Repeat step 9, not discard all supernatant but remain 1 mL to resuspend sediment
11. Divide the mixture into five portions,200 μL per serving.
12. Store at -20℃

- Gel Extraction
  Performed according to the E.Z.N.A Gel Extraction Kit
- PCR Purification
  Performed according to the E.Z.N.A. Cycle Pure Kit
- Fungal DNA Extraction
  Performed according to the E.Z.N.A. SP Fungal DNA Mini Kit
- Plasmid Extraction
  Performed according to the E.Z.N.A. Plasmid DNA Mini Kit
- Extraction of total RNA
  1. Pulverize hyphae in liquid nitrogen, collect two spoons of powder into 1.5ml EP microcentrifuge tube.
  2. Add 1ml RNAiso Plus(TAKARA), then homogenate
  3. Sit at room temperature for 5 minutes
  4. Centrifuge at 4℃ and 12,000g for 5 minutes
  5. Add 200μL chloroform, mix thoroughly
  6. Centrifuge at 4℃ and 12,000g for 15 minutes
  7. Transfer supernatant into new 1.5 mL microcentrifuge tube
  8. Add 1ml isopropanol, sit at room temperature for 10 minutes
  9. Centrifuge at 4℃ and 12,000g for 10 minutes
  10. Add 1mL 75% ethanol to wash the precipitate
  11. Centrifuge at 4℃ and 7,500g for 5 minutes
  12. Discard the supernatant
  13. Open the cap, let sit at room temperature until ethanol volatile completely
  14. Dissolve precipitate with moderate RNAase-free H_2O
- **reverse transcription**
  Performed according to TAKARA PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time)
- **qPCR**

  Preparation of reaction system:

  1. In a PCR tube on ice, combine 1μL cDNA, 0.5 μL of 10 μM forward primer, 0.50 μL of 10 μM reverse primer to a PCR tube on ice, 10 μL of 2× PLUS SYBR Real-Time PCR Premixture, RNAase-free water up to 20 μL.

2. Gently mix the reaction
3. Collect the liquid to the bottom of the PCR tube by centrifuging briefly
4. Transfer the PCR tube from ice to a PCR machine preheated to 98°C to begin thermocycling
5. Thermocycling:
6. The PCR machine should be set to run the following steps:

step	Temperature(℃)	Time
Initial denaturation	94	2 minutes
40 cycles	94(denaturation) 60(annealing) 72(extension)	15 seconds 15 seconds 30 seconds
Melting curve: the default settings of the machine		

- **BCA assay**

Performed according to Betotime BCA kit

1. Preparation of protein standards
   Prepare BSA solution at the concentration of 0.5 mg/mL
2. Preparation of working agent
   According to the sample capacity, prepare BCA working agent (agent A: agent B=50:1), mix thoroughly
3. A measure of protein concentration
   a. Add 0,1,2,4,8,12,16,20 μL protein standard into 96-well plate,then add standard dilution to 20μL
   b. Add the appropriate volume of sample to the sample wells of 96-well plate
      If the volume of the sample is less than 20μL, add the standard solution to 20μL.
   c. Add 200μL working agent to ezch well, sit at 37℃ for 30 minutes
   d. Determine the OD value at A562
   e. According to the standard curve and the volume of the sample to calculate protein concentration

- **Protein purification**

Performed according to Betotime His-tag Protein Purification Kit

1. Collect 1 mL bacteria solution ,centrifuge at 10,000g for 1 minute,then discard the supernatant.
2. Add 100 μL nondenaturing lysis buffer, resuspend the precipitate, vortex gently
3. Add lysozyme to 1mg/mL and mix gently, avoid bubbles.
4. Centrifuge at 15,000g for 10 minutes, reserve 100 μL supernatant for subsequent testing, then collect the rest of supernatant into a new 1.5 mL microtube
5. Add 20 μL 50% BeyoGold His-tag Purification Resin, put on 4℃ shaker for 30 minutes to make resin combine with protein fully.
6. Centrifuge at 4℃ and 1,000g for 10 seconds to deposit the gel. Reserve 20 μLsupernatant for further use.Discard the rest of the supernatant
7. Add 40 μL nondenatured clearing solution to resuspend, centrifuge at 1,000g for 10 seconds. Reserve 20 μL supernatant for further use. Discard the rest of supernatant.

8. Repeat step 7
9. Add 20 μL nondenatured eluent to resuspend resin, centrifuge at 4℃ and 1,000g for 10 seconds, collect the supernatant which has target protein in.
10. Repeat step 9 twice. Collect 60 μL elute in sum.

- **Protein concentration**

1. Add protein solution into ultrafiltration device(Millipore),centrifuge at 5,000 rpm for 8 minutes
2. Collect the liquid above, and discard the flow-through

- **SDS-PAGE**

1. Collect 10mL hypha grown in 1/4 SDAY broth
2. Add appropriate acid-washed beads, then FastPrep at the speed of 5.0m/s for 20 seconds.
3. Centrifuge the lysate at 4℃ and 5,000 rpm for 8 minutes. Protein ultrafiltration is necessary if the protein concentration is low.
4. Collect the supernatant into a new 1.5 ml microtube.
5. Heat at 95℃ for 10 minutes to denature the protein
6. Electrophoresed on SDS-PAGE.80 V for 20 minutes, then 120 V until the band go to the bottom

- **Coomassie blue staining**

1. Add appropriate commassie solution to the SDS-PAGE
2. Shake at the room temperature for 30 minutes
3. Remove the dye, then wash the gel with sterilized water

- **Western Blot**

1. SDS-PAGE performed according to protocol talked before
2. Membrane transfer:

(1)Prepare sufficient transfer buffer to fill the transfer tank, plus an additional 200 mL to equilibrate the gel and membrane, and wet the filter paper.

(2)Remove the gel from its cassette; trim away any stacking gel and wells.

(3)Immerse the gel in transfer buffer for 10 to 30 minutes.

(4)Soak filter papers in transfer buffer for at least 30 seconds.

(5)Prepare the membrane:

a. let the PVDF membrane in methanol for 15 seconds. The membrane should uniformly change from
opaque to semi-transparent.

b. Carefully place the membrane in Milli-Q® water and soak for 2 minutes.

c. Carefully place the membrane in transfer buffer and let equilibrate for at least 5 minutes.

3. Transfer Stack Assembly:

(1) Open the cassette holder.

(2) Place a foam (fiber) pad on one side of the cassette.

(3)Place one sheet of filter paper on top of the pad.

(4)Place the gel on top of the filter paper.

(5)Place the membrane on top of the gel.

(6)Place a second sheet of filter paper on top of the stack.

(7)Place second foam pad on top of the filter paper.

(8)Close the cassette holder.

4. Method for Protein Transfer

(1) Place the cassette holder in the transfer tank so that the gel side of the cassette holder is facing the cathode (–) and the membrane side is facing the anode (+).

(2) Add adequate buffer into the tank to cover the cassette holder.

(3) Insert the black cathode lead (–) into the cathode jack and the red anode lead (+) into the anode jack on the transfer unit.

(4) Connect the anode lead and cathode lead to their corresponding power outputs.

(5) Turn on the system for 1 to 2 hours at 6 to 8 V/cm inter-electrode distance. Follow the tank manufacturer's guidelines, for optimization details.

(6) After the transfer is complete, remove the cassette holder from the tank.

(7) Using forceps, carefully disassemble the transfer stack.

4.Wash the PVDF membrane by TBST for 5 minutes

5.Blocking： put the membrane in the blocking liquid, shake at room temperature for 1h.

6. Primary antibody hybridization: prepare a primary antibody dilution solution in a suitable ratio with blocking solution; incubate the membrane with the antibody dilution at room temperature for 1 h, or incubate at 4 ° C overnight;

7.Wash PVDF membrane for 5 minutes

8.Repeat step 7 twice

9. Secondary antibody hybridization: prepare a secondary antibody dilution solution in a suitable ratio with blocking solution; and incubate the membrane and antibody dilution solution at room temperature for 1 hour;

10. Wash PVDF membrane for 8 minutes

11.Repeat step 10 twice

12. Development of PVDF membrane

- **Culturing medium recipe**

  LB Broth Formulation per one liter

  10g Peptone

  5g Yeast Extract

  5g Sodium Chloride

- **Chemical transformation of *Metarhizium anisopliae*<1>**

We transferred PBC plasmid into M.anisopliae CQ128 by $CaCl_2$-PEG, and transformants were screened by G418.

Materials:

STC buffer:20% sucrose,10mM Tris-HCl(pH8.0),50mM $CaCl_2$, dissolved in sterile water

TB3(100ml):3g Yeast Extract,3g Casamino Acids,20% sucrose,dissolved in sterile water

PDAY(100ml):2g glucose,0.5g Yeast Extract,1.8g Agar,dissolved in potato infusion.

7. Thaw 200 μL protoplasts of M.Anasopliae on the ice, then transfer them into 15 ml centrifuge tube.
8. Add 30 μL plasmid into a centrifuge tube, then pipet up and down gently to mix thoroughly.
9. Sit at room temperature for 20 minutes
10. Add 1.25 ml 40% PTC and invert several times to obtain a homogeneous mixture
11. Sit at room temperature for 20 minutes
12. Add 5ml TB3 culture solution with chloramphenicol (30μg/ml)
13. Incubate overnight at room temperature and 200 rpm/min
14. Centrifuge mixture at room temperature and 4000 rpm/min, then discard the supernatant.
15. Add 200 μL STC to resuspend
16. Add 10 ml TB3 medium containing 0.7% low melting point agarose into centrifuge tube,then add G418(420μg/ml) and chloramphenicol (30ug/ml).Gently rotate the tube to mix thoroughly. Pour 9 cm plates with 10 ml mixture to form the bottom culture medium
17. The next day, pour 10 ml TB3 containing 0.7% low melting point agarose and G418(420μg/ml) above the bottom culture medium.
18. Incubate 2-3days at 27℃, then pick a single colony and inoculate it on the PDAY with G418(420μg/ml)

- **Chemical Transformation of *E.Coli***

1. Thaw 100 μL DH5α competent cells on ice.
2. Pipet plasmid into competent cells and mix them thoroughly.
3. Incubate on ice for half an hour.
4. Heat shock at 42 ℃ for 90 seconds
5. Incubate on ice for 1 minute
6. Add 1 ml LB solution and incubate for 1h at 37℃ on a shaker.
7. Add 50 μL of the transformed cells to the selection plate.

- **Transformation of *Bacillus subtilis***

1. Select a small clone on the plate, inoculate into 3ml LB medium, incubate at 37 °C for 12 hours, dilute the bacterial solution to OD=1, add xylose with a final concentration of 1% at 37 °C for 2 h, and dispense 200 ul per tube.
2. Add 1 ug of plasmid to each competent state, incubate at 37 ° C for 1.5 h,
3. Add 50 μL of the transformed cells to the selection plate, evenly coated

- **HsbA macro verification protocol**
    1. Remove the six legs of dead cockroaches and place them in clean Petri dishes 1 and 2 on average. That is three legs in each petri dish.
    2. Put the culture medium full of *Metarhizium anisopliae 128* spores upside down on petri dish 1, and tap the culture medium to spread the spore powder on cockroaches' legs in petri dish 1. In the same way, the spores of *Metarhizium anisopliae* HsbA transformant are spread on the legs of cockroaches in petri dish 2 and then left them alone for 16 hours.
    3. After 16 hours, take out and place the cockroaches' legs which are in the petri dish 1 on the TM3000 scanning electron microscope for observation, then look for an area that is easy to identify and has a proper number of spores attached to take a photo record.
    4. Remove the cockroaches' legs and rinse the observed area on the cockroach legs with 200ul ddH2O. Then suck the water to be dry and put them back into the scanning electron microscope for observation.
    5. Find the area recorded at the first observation again, observe and compare whether there is any change in the position and number of spores in the area. If changes, *Metarhizium anisopliae 128* spores adhesion is not strong.
    6. At the same time, *Metarhizium anisopliae* HsbA transformant spores are treated in the same way. Take out and place the cockroaches' legs in petri dish 2 on the TM3000 scanning electron microscope to observe, then find an area that is easy to identify and has a proper number of spores attached to take a photo record.
    7. Remove the cockroaches' legs and rinse the observed area on the cockroach legs with 200ul ddH2O. Then suck the water to be dry and put them back into the scanning electron microscope for observation.
    8. Find the area recorded at the last observation again, observe and compare whether there is any change in the position and number of spores in the area. If there is no change, it indicates that the spore adhesion ability of the HsbA transformant spores is strong.

- **The Bbchit's activity and macro verification protocol**

DNS colorimetric method for measuring enzyme activity

1. prepare 1 mg/mL glucose standard solution, take 16 18 mm×180 mm test tubes, add DNS 1.5 mL to each tube, then set 1 control and 5 glucose gradients.
2. Add 2.0 mL of distilled water to the control tube, add 0.2, 0.3, 0.4, 0.5, 0.6 mL glucose standard solution to the 5 gradient tubes, and make up the volume with distilled water to make the volume of each tube mixture 3.5 mL (per 3 replicates, except for the control), mix, boil in boiling water for 5 min, immediately after cooling, cool to room temperature with cold water, then add 4.5 mL of distilled water to each tube and shake well.
3. The concentration of glucose in each tube after dilution was 0, 0.0250, 0.0375, 0.050, 0.0625, and 0.0750 mg/mL. The absorbance (OD) was measured from the low to the high glucose concentration at 540 nm, and the glucose standard curve was plotted with the glucose concentration (mg/mL) as the abscissa and the OD value as the ordinate.
4. Wild-type and transformed strains were simultaneously cultured on two plates. When the colonies were expanded, around colony was taken from the edge of the colony with a puncher, and 100 ml of Capek medium was added, and cultured at 28 ° C, 150 rpm/min, respectively.
5. On the 3, 5, 7, 9 and 12 days, 0.5 ml of the bacterial solution was filtered through a 0.22 μm filter membrane. The filtered 0.5 ml crude enzyme solution was mixed with 0.5 ml of 1% chitin colloid, incubated at 37 ° C for 60 min, then added with 0.5 ml of DNS developer, boiled in water for 10 min, mixed and 1 ml added to the cuvette, and split. The absorbance was measured at a wavelength of 540 nm. The enzyme activity was calculated using the standard curve formula U=(A540+0.03279)/2.202 and plotted.

Congo Red transparent ring method

1. The Czapek chitin solid medium (chitin is the only carbon and nitrogen source) was prepared, and the plate was cooled and stained with 0.1% Congo red solution for 2 hours and washed with sterile water to remove the floating color.
2. Wild-type and transformed Metarhizium were separately plated and placed in a 28 ° C incubator for seven days to obtain colonies and transparent circles.
3. The size of the transparent circle is the diameter of the chitin transparent ring (R2) and colonies. The ratio of the diameter (R1), expressed as R2/R1.

- **MCL1 functional verification**

Preparation of hyphae homogenate

1. Prepare 1 mL fungus grown in 1/4 SDAY broth, put into 2 mL sterile microcentrifuge tube
2. Fastprep for 5 seconds without beads
3. Dilute the solution to OD=1

Injection of hyphae homogenate

1. 1. Put the cockroach in the culture dish, put a piece of absorbent cotton soaked with ether into the culture dish, and let it stand for 30 seconds. When the cockroach is anesthetized and loses its power, remove the cotton wool and transfer the cockroach to the new culture dish.
2. 2. Use 75% ethanol to sterilize the surface of cockroaches
3. 3.Inject hyphae homogenate through the interphase membrane of the abdomen,10 μl per cockroach
4. 4.Extract hemolymph in 0.5h,1h,2h,4h,8h,12h,24h

Extraction of cockroach hemolymph

1. Put the cockroach in the culture dish, put a piece of absorbent cotton soaked with ether into the culture dish, and let it stand for 30 seconds. When the cockroach is anesthetized and loses its power, remove the cotton wool and transfer the cockroach to the new culture dish.
2. Use 75% ethanol to sterilize the surface of cockroaches
3. Stab metacoxacoria by sterilized fine needle, then collect 10 μL hemolymph by pipette<2>
4. Transfer hemolymph into microtube filled with 90 μL anticoagulant.<3>

Observation of hemolymph

1. Add 10μL Hemolymph diluent on the glass slide, then cover the slide, sit for 2 minutes

2.Count the number of nodules under a light microscope(Criterion: more than 10 hemocytes assemble closely)

3. Meanwhile, make hemolymph smear, watch it under phase contrast microscope and take photos

- **L-Trp- concentration gradient experiment**

  To detect the limited concentration of Tryptophan that Metarhizium transformed with our L-Trp-MazF killing switch, we designed an L-Trp-concentration gradient experiment. If Metarhizium can survive in one concentration but can't in a lower concentration, or it grows at a low speed, it would be the limited concentration.

  Culture conditions:

  Medium component: solid and liquid czapek + different concentrations of L-Tryptophan.

	1	2	3	4	5	6	7	8	9	10
[L-Trp] 1mL	0.5%	0.6%	0.7%	0.08%	0.09%	0.10%	0.11%	0.12%	0.13%	0.14%
$NaNO_3$	0.02g									
K_2HPO_4	0.01g									
KCl	0.005g									
$MgSO_4$	0.005g									
$FeSO_4$	0.001g									
Sucrose	0.03g									
Geneticin	1g									
Agar	0.17g									
Ultra-pure water	9mL									

Chart1 Medium component of the killing switch (solid)

	1	2	3	4	5	6	7	8	9	10
[L-Trp] 1mL	0.5%	0.6%	0.7%	0.08%	0.09%	0.10%	0.11%	0.12%	0.13%	0.14%
$NaNO_3$	0.02g									
K_2HPO_4	0.01g									
KCl	0.005g									
$MgSO_4$	0.005g									
$FeSO_4$	0.001g									
Sucrose	0.03g									
Geneticin	1g									

Ultra-pure water	9mL

Chart1 Medium component of killing switch (liquid)

1. We successively weight and put 0.05g, 0.06g, 0.07g, 0.08g, 0.09g, 1.00g, 1.10g, 1.20g, 1.30g and 1.40g of Tryptophan in 10 tubes, added 1mL of EMSO to dissolve Tryptophan and added ultra-pure water to 10mL totally for preparation.
2. Because the FeSO4 can be oxidized while heating, we need filtration sterilization instead of wet heat sterilization
3. After sterilization, we added 1mL of different concentration of Tryptophan and 1g of Geneticin in czapek and shook. Then we poured the solid medium in 10 Petrie dishes waiting for solidification and poured the liquid medium in 15mL centrifuge tubes.
4. Pipette 5 μl of MazF transformant hyphae and place it in the center of the czapek plate and inject it into the agar interior.
5. We put our petite dishes and in 28℃ temperate box and 15mL centrifuge tubes in a shaker to cultivate.
6. We compared Metarhizium cultivated in 10 concentration of L-Trp and found the lowest concentration that Metarhizium can survive and grow healthily.

- **Cockroaches experiment**
  1. Put the cockroach in the culture dish, put a piece of absorbent cotton soaked with ether into the culture dish, and let it stand for 30 seconds. When the cockroach is anesthetized and loses its power, remove the cotton wool and transfer the cockroach to the new culture dish.
  2. Place the anesthetized cockroach abdomen facing upward, suck and pipette 10ul emulsion to its abdomen, and put it back into the petri dish.
  3. Control the room temperature to be 25 ℃, stand it, observe it once every 12 hours

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