Pollen metabarcoding Version 3

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Abstract

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Guidelines

Perform reactions in small batches until you are confident that there is no cross-contamination among the samples. Including isolation blanks and PCR blanks is crucial for the quality control.

Before start

Prepare 5 µM primer solutions:

ITS2 primers used in the 1st PCR:

ITS2-4R GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNTCCTCCGCTTATTGATATGC

ITS2-S2F ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNATGCGATACTTGGTGTGAAT

Indexing primers used in the 2nd PCR (xxxxxxxx - index):

AATGATACGGCGACCACCGAGATCTACACxxxxxxxxACACTCTTTCCCTACACGACGC

CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGTGC

Materials

- Q5 Hot Start High-Fidelity DNA Polymerase - 100 units M0493S by New England Biolabs
- Water, nuclease free by Contributed by users
- dNTP mix (25 mM of each) by Contributed by users

Phire Plant Direct PCR Kit F130WH by Thermo Fisher Scientific

Protocol

Pollen extraction

Step 1.

Vortex the butterfly in 50 µl of water with 0.1% SDS.

SAFETY INFORMATION

Add blank sample at this step (= "isolation blank")

Pollen extraction

Step 2.

Evaporate water in speedvac.

Pollen extraction

Step 3.

Add 5 µl of the Phire Plant Direct sample buffer.

Pollen extraction

Step 4.

Spin max speed for 2 min.

1st PCR

Step 5.

Prepare the mix:

AMOUNT

14 µl : molecular grade water

AMOUNT

25 µl : Phire Plant Direct PCR mix

AMOUNT

5 µl : ITS2-S2F primer

AMOUNT

5 µl : ITS2-4R primer

1st PCR

Step 6.

Add 1 µl of the sample to 49 µl of the mix. Use water instead of the sample for the blanks.

SAFETY INFORMATION

Add another blank sample at this step (= "PCR blank")

1st PCR

Step 7.

Run the PCR program: initial denaturation at 98°C for 5 min; 20 cycles of denaturation at 98°C for 40 s, annealing at 49°C for 40 s and elongation at 72°C for 40 s; followed by a final extension step at 72°C for 5 min.

Purification

Step 8.

Perform AMPure purification with ratio 1x. Elute in 10 µl.

PROTOCOL

. AMPure purification protocol

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Step 8.1.
Remove beads from the fridge, equilibrate to the room temperature, mix thoroughly but do not vortex.

Step 8.2.

Add desired ratio of AMPure beads to the DNA to be purified and mix well by pipetting.

Step 8.3.
Incubate 5 minutes.

Step 8.4.
Place on the magnetic rack.

Step 8.5.
Let it stand for 5 minutes on the rack, aspirate and discard supernatant.

Step 8.6.
Add 200 µl of freshly prepared 70% EtOH, incubate 30 seconds, aspirate and discard EtOH.

Step 8.7.
Repeat the wash: add 200 µl of freshly prepared 70% EtOH, incubate 30 seconds, aspirate and discard EtOH.

Step 8.8.
Wait until the pellet dries out completely. DO NOT remove the plate/tubes from the magnetic rack, as the dried AMPure might pop out from the tubes!

Step 8.9.
Add desired volume of Tris 10 mM or water, wetting the dried AMPure pellets (add 1 µl to the final volume to avoid pipetting out the beads)

Step 8.10.
Remove from the magnetic rack.

Step 8.11.
Resuspend by pipetting or vortexing.

Step 8.12.

Incubate 10 minutes, incubating in 37°C can improve DNA yield.

Step 8.13.
Place on the magnetic rack.

Step 8.14.
Let it stand for 5 minutes, pippete out and save supernatant. The eluted DNA is in the supernatant, do not discard it!

2nd PCR

Step 9.

Prepare the mix:

AMOUNT
4.82 µl : molecular grade water
AMOUNT
2 µl : Q5 reaction buffer
AMOUNT
0.08 µl : dNTPs (25 mM each)
AMOUNT
0.1 µl : Q5 Hot Start polymerase

2nd PCR

Step 10.

Add 1 µl of the template to 7 µl of the mix.

2nd PCR

Step 11.

Add 1 µl of each 5 uM primer (forward and reverse).

SAFETY INFORMATION
Use different pair of indexed primers for each sample.

2nd PCR

Step 12.

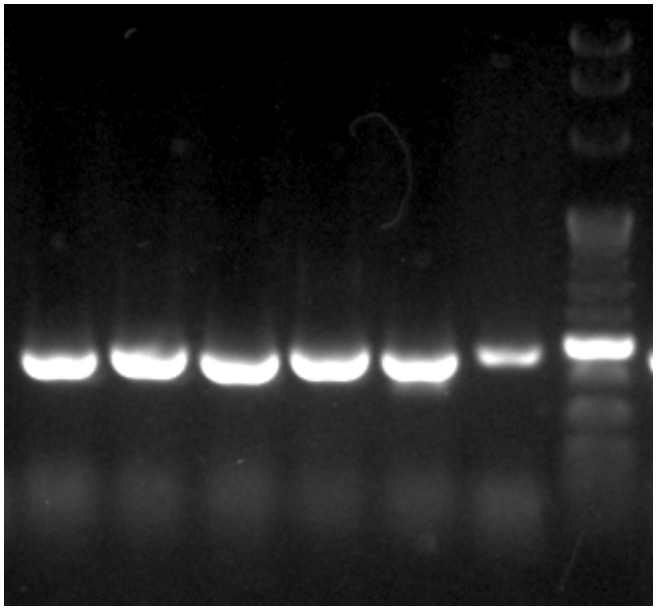
Run the PCR program: 30 s denaturation at 98°C; 12 cycles of denaturation at 98 °C for 10 s, combined annealing and extension at 72°C for 30 s (shuttle PCR); the final extension at 72°C for 5 min.

2nd PCR

Step 13.

Check profiles on a gel.

EXPECTED RESULTS



Pooling

Step 14.

Pool all the samples together.

Pooling

Step 15.

Perform AMPure purification with ratio 1x. Elute in 100 µl.

PROTOCOL

. AMPure purification protocol

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Step 15.1.

Remove beads from the fridge, equilibrate to the room temperature, mix thoroughly but do not vortex.

Step 15.2.

Add desired ratio of AMPure beads to the DNA to be purified and mix well by pipetting.

Step 15.3.

Incubate 5 minutes.

Step 15.4.

Place on the magnetic rack.

Step 15.5.

Let it stand for 5 minutes on the rack, aspirate and discard supernatant.

Step 15.6.

Add 200 µl of freshly prepared 70% EtOH, incubate 30 seconds, aspirate and discard EtOH.

Step 15.7.

Repeat the wash: add 200 µl of freshly prepared 70% EtOH, incubate 30 seconds, aspirate and discard EtOH.

Step 15.8.
Wait until the pellet dries out completely. DO NOT remove the plate/tubes from the magnetic rack, as the dried AMPure might pop out from the tubes!

Step 15.9.
Add desired volume of Tris 10 mM or water, wetting the dried AMPure pellets (add 1 µl to the final volume to avoid pipetting out the beads)

Step 15.10.
Remove from the magnetic rack.

Step 15.11.
Resuspend by pipetting or vortexing.

Step 15.12.

Incubate 10 minutes, incubating in 37°C can improve DNA yield.

Step 15.13.
Place on the magnetic rack.

Step 15.14.
Let it stand for 5 minutes, pippete out and save supernatant. The eluted DNA is in the supernatant, do not discard it!

Pooling

Step 16.

Check the concentration using Qubit and the profile using Tapestation/Fragment Analyzer. Calculate molarity from that and proceed to the sequencing. Add 15% PhiX to the sequencing run.