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Pollen metabarcoding V.3

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Molecular Biogeography...

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Protocol status: Working
Corrected the amount of primers in the 2nd PCR reaction

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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.

Materials

MATERIALS

- Q5 Hot Start High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #**M0493S
- Water, nuclease free
- dNTP mix (25 mM of each)
- Phire Plant Direct PCR Kit **Thermo Fisher Scientific Catalog #**F130WH

Troubleshooting

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

Pollen extraction

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

Safety information

Add blank sample at this step (= "isolation blank")
http://null

2 Evaporate water in speedvac.

3 Add 5 µl of the Phire Plant Direct sample buffer.

4 Spin max speed for 2 min.

1st PCR

5 Prepare the mix:

- 14 µL molecular grade water
- 25 µL Phire Plant Direct PCR mix
- 5 µL ITS2-S2F primer
- 5 µL ITS2-4R primer

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")

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

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

Protocol

NAME

AMPure purification protocol

CREATED BY

Tomasz Suchan

Preview

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

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

8.3 Incubate 5 minutes.

8.4 Place on the magnetic rack.

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

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

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

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!

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)

8.10 Remove from the magnetic rack.

8.11 Resuspend by pipetting or vortexing.

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

8.13 Place on the magnetic rack.

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

9 Prepare the mix:

- 4.82 µL molecular grade water
- 2 µL Q5 reaction buffer
- 0.08 µL dNTPs (25 mM each)
- 0.1 µL Q5 Hot Start polymerase

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

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

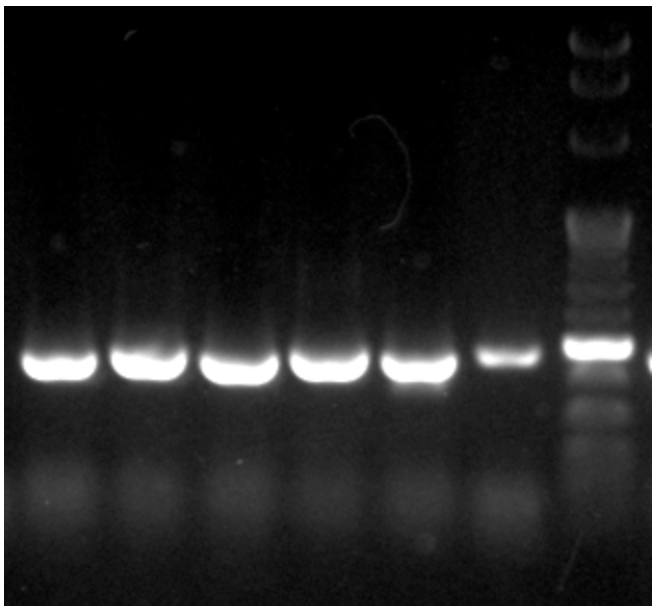
Safety information

Use different pair of indexed primers for each sample.

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.

13 Check profiles on a gel.

Expected result



Pooling

14 Pool all the samples together.

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

Protocol

NAME

AMPure purification protocol

CREATED BY

Tomasz Suchan

Preview

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

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

15.3 Incubate 5 minutes.

15.4 Place on the magnetic rack.

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

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

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

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!

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)

15.10 Remove from the magnetic rack.

15.11 Resuspend by pipetting or vortexing.

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

15.13 Place on the magnetic rack.

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

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.