ITS2 metabarcoding protocol

Version 2

Forked from Pollen metabarcoding

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Molecular Biogeography Group

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working

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.

ITS2 primers used in the 1st PCR:

ITS2-4R GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNTCCTCCGCTTATTGATATGC
ITS2-S2F ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNATGCGATACTTGGTGTGAAT

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

AATGATACGGCGACCACCGAGATCTACACxxxxxxxxACACTCTTTCCCTACACGACGC
CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGTGC

PCR_F_A501: AATGATACGGCGACCACCGAGATCTACAC**TGAACCTT**ACACTCTTTCCCTACACGACGC
PCR_F_A502: AATGATACGGCGACCACCGAGATCTACAC**TGCTAAGT**ACACTCTTTCCCTACACGACGC
PCR_F_A503: AATGATACGGCGACCACCGAGATCTACAC**TGTTCTCT**ACACTCTTTCCCTACACGACGC
PCR_F_A504: AATGATACGGCGACCACCGAGATCTACAC**TAAGACAC**ACACTCTTTCCCTACACGACGC
PCR_F_A505: AATGATACGGCGACCACCGAGATCTACAC**CTAATCGA**ACACTCTTTCCCTACACGACGC
PCR_F_A506: AATGATACGGCGACCACCGAGATCTACAC**CTAGAACA**ACACTCTTTCCCTACACGACGC
PCR_F_A507: AATGATACGGCGACCACCGAGATCTACAC**TAAGTTCC**ACACTCTTTCCCTACACGACGC
PCR_F_A508: AATGATACGGCGACCACCGAGATCTACAC**TAGACCTA**ACACTCTTTCCCTACACGACGC
PCR_F_D501: AATGATACGGCGACCACCGAGATCTACAC**TATAGCCT**ACACTCTTTCCCTACACGACGC

PCR_F_D502: AATGATACGGCGACCACCGAGATCTACAC**ATAGAGGC**ACACTCTTTCCCTACACGACGC
PCR_F_D503: AATGATACGGCGACCACCGAGATCTACAC**CCTATCCT**ACACTCTTTCCCTACACGACGC
PCR_F_D504: AATGATACGGCGACCACCGAGATCTACAC**GGCTCTGA**ACACTCTTTCCCTACACGACGC
PCR_F_D505: AATGATACGGCGACCACCGAGATCTACAC**AGGCGAAG**ACACTCTTTCCCTACACGACGC
PCR_F_D506: AATGATACGGCGACCACCGAGATCTACAC**TAATCTTA**ACACTCTTTCCCTACACGACGC
PCR_F_D507: AATGATACGGCGACCACCGAGATCTACAC**CAGGACGT**ACACTCTTTCCCTACACGACGC
PCR_F_D508: AATGATACGGCGACCACCGAGATCTACAC**GTACTGAC**ACACTCTTTCCCTACACGACGC

PCR_R_A701: CAAGCAGAAGACGGCATACGAGAT**GTCGTGAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A702: CAAGCAGAAGACGGCATACGAGAT**ACCACTGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A703: CAAGCAGAAGACGGCATACGAGAT**TGGATCTG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A704: CAAGCAGAAGACGGCATACGAGAT**CCGTTTGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A705: CAAGCAGAAGACGGCATACGAGAT**TGCTGGGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A706: CAAGCAGAAGACGGCATACGAGAT**GAGGGGTT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A707: CAAGCAGAAGACGGCATACGAGAT**AGGTTGGG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A708: CAAGCAGAAGACGGCATACGAGAT**GTGTGGTG**GTGACTGGAGTTCAGACGTGTGC

PCR_R_A709: CAAGCAGAAGACGGCATACGAGAT**TGGGTTTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A710: CAAGCAGAAGACGGCATACGAGAT**TGGTCACA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A711: CAAGCAGAAGACGGCATACGAGAT**TTGACCCT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A712: CAAGCAGAAGACGGCATACGAGAT**CCACTCCT**GTGACTGGAGTTCAGACGTGTGC

PCR_R_D701: CAAGCAGAAGACGGCATACGAGAT**CGAGTAAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D702: CAAGCAGAAGACGGCATACGAGAT**TCTCCGGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D703: CAAGCAGAAGACGGCATACGAGAT**AATGAGCG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D704: CAAGCAGAAGACGGCATACGAGAT**GGAATCTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D705: CAAGCAGAAGACGGCATACGAGAT**TTCTGAAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D706: CAAGCAGAAGACGGCATACGAGAT**ACGAATTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D707: CAAGCAGAAGACGGCATACGAGAT**AGCTTCAG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D708: CAAGCAGAAGACGGCATACGAGAT**GCGCATTA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D709: CAAGCAGAAGACGGCATACGAGAT**CATAGCCG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D710: CAAGCAGAAGACGGCATACGAGAT**TTCGCGGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D711: CAAGCAGAAGACGGCATACGAGAT**GCGCGAGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D712: CAAGCAGAAGACGGCATACGAGAT**CTATCGCT**GTGACTGGAGTTCAGACGTGTGC

MATERIALS

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

BEFORE STARTING

Prepare 5 µM primer solutions.

1st PCR

1 Prepare the mix:

- **11.64 µl molecular grade water**
- **4 µl Q5 polymerase buffer**
- **1 µl ITS2-S2F primer**
- **1 µl ITS2-4R primer**
- **0.16 µl dNTPs (25 mM each)**
- **0.2 µl Q5 Hot Start polymerase**

2 Add 2 µl of the sample to 18 µl of the mix. Use water instead of the sample for the blanks.

SAFETY INFORMATION

Add one blank sample at this step (= "PCR blank") and also use the blank from the DNA extraction step (= "extraction blank").

3 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

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

PROTOCOL

AMPure purification protocol

by Tomasz Suchan,
W. Szafer Institute of Botany, Polish Academy of Sciences

PREVIEW START EXPERIMENT

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

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

4.3 Incubate 5 minutes.

4.4 Place on the magnetic rack.

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

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

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

4.8 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!

4.9 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)

4.10 Remove from the magnetic rack.

4.11 Resuspend by pipetting or vortexing.

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

4.13 Place on the magnetic rack.

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

2nd PCR

5 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

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

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

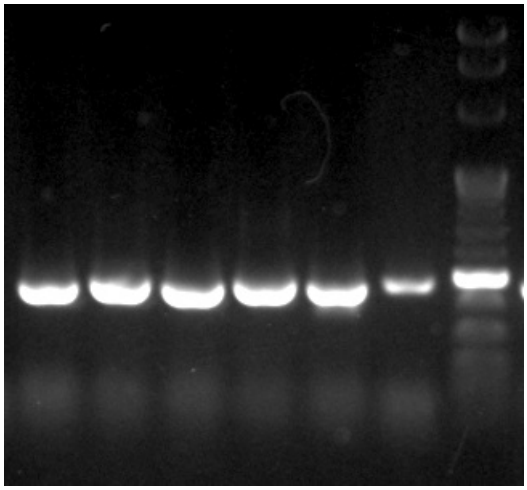
SAFETY INFORMATION

Use different pair of indexed primers for each sample.

8 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.

9 Check profiles on a gel.

EXPECTED RESULT



Pooling

10 Pool all the samples together.

NOTE

The sample concentrations can be normalized at this step, depending on the experimental questions. Purify the samples separately using AMPure, quantify and pool in the equimolar proportions. Alternatively you can use bead-based normalization, e.g: doi: 10.1186/1471-2164-15-645.

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

PROTOCOL

AMPure purification protocol

by Tomasz Suchan,
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PREVIEW START EXPERIMENT

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

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

11.3 Incubate 5 minutes.

11.4 Place on the magnetic rack.

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

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

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

11.8 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!

11.9 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)

11.10 Remove from the magnetic rack.

11.11 Resuspend by pipetting or vortexing.

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

11.13 Place on the magnetic rack.

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

12 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.

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