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Protocol status: Working
We use this protocol and it's working

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FUNDIS Setting up PCR for rDNA ITS Barcoding of Fungi using ONT Nanopore

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ABSTRACT

This protocol involves using pre-made dual-indexed master mix 96 well plates to run PCR on fungal specimens that have already had their DNA extracted, diluted and in PCR 8 strip tubes. Quality check includes using the BlueGel platform to screen for successful PCR. Adapted from dx.doi.org/10.17504/protocols.io.36wgq7qykvk5/v3

MATERIALS

Filtered 10 µL pipette tips
4" wide aluminum foil tape
Silicon baking mat - cut to fit over a 96 well plate
Sharpie marker
blueGel agarose tablets
blueGel powdered TBE buffer
p10 multichannel micropipette
p10 single channel micropipette
GeneAmp 9700 Thermocycler
PCR Tube microcentrifuge

Equipment

blueGel	NAME
MiniPCR	BRAND
QP-1500-01	SKU

Keywords: ONT, PCR, Fungi, Barcoding, Amplicon Sequencing, Nanopore, ITS

Setting up PCR

35s

1 Remove DNA extracts from the freezer and allow to thaw before beginning. **Note the plate number in your notebook** (i.e. FDS-CA TB05) Take a photo of the plate showing the labeling.

2 Once thawed, spin DNA extracts down for 00:00:02 in a benchtop PCR tube centrifuge. 2s

3 Remove dual-indexed master mix plate from the freezer and **note the index number and primer pair in your notebook** (i.e. ONT97 ITS1F ITS4) Remove the foil cover and allow to thaw for 00:00:30 30s

4 Orient yourself so that PCR Strip 1 Tube 1 aligns with well H1 on a standard 96 well plate, and pipette 0.5-1 µL from each strip into the corresponding row of the 96 well plate using a p10 multichannel pipette.

Open the PCR strip tubes carefully to avoid splashing the liquid around. Check your fingers to make sure there is no residue from the PCR tubes on them in between opening strips. Wipe any residue off with a paper towel in between opening tube strips or replace gloves as needed.

Visually inspect the pipette tips for presence of liquid at an approximately equal amount and no large debris before adding to the wells. Gently pipette mix.

Be extra careful to avoid cross-contamination from drips, splashes, aerosols etc. Gentle, intentional movements and pipetting is necessary.

Choose how much to add based on how much tissue was in the original tube and how dilute it is. In most cases I will use 0.7 µL .

5 Place a new foil seal on the plate and using a sharpie marker, ensure the seal is tightly attached to the plate. Write the run number using your initials (i.e. HSONT01) The plate number and sampling code (i.e. FDS-PL01

HS01), the primer pair (i.e. ITS1F ITS4) and the date. **Record this information in the notebook** and take a photo of the plate for future reference.

6 Visually inspect the underside of the plate to see the level of liquid in the tubes. Note any tubes that are obviously low on liquid **in the noteboook.**

7 Hold the PCR plate in the correct orientation close to your chest and in a single swift but controlled motion, extend your arm away from your chest stopping abruptly when your arm extends. This should dislodge any bubbles from the bottom of the wells of the tube. Do not do this too hard, you definitely do not want to splash the liquid.

PCR Thermal Cycler conditions for ITS Barcoding

7m 15s

8 I have experimented with many different PCR conditions with no noticeable change in amplification efficiency. Touchdown PCR is probably superior. Here are some conditions that should work. 7m 15s

95 °C 00:03:00

Followed by 33 cycles of:

95 °C 00:00:30

60 °C 00:00:30

72 °C 00:00:45

Followed by a final extension

72 °C 00:01:30

Hold

12 °C 00:01:00

The final hold simply brings the PCR product down in temperature and is not meant to actually hold the PCR product at any particular temperature. It is stable at room temperature for weeks, but should be removed from the machine and placed in the fridge in a plastic ziploc bag within 24 hours. Make sure to turn off the machine.

Electrophoresis using the blueGel system.

9 Choose 3-5 samples at random from each PCR plate to confirm successful amplification using electrophoresis. Consult the blueGel manual for support.