Version 2

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ONT Post-PCR Pooling & Purification for Fungal Barcoding V.2

In 1 collection

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ABSTRACT

Overview: The goals of this protocol are to pool your PCR product into a single 1.5 mL tube and to purify that product using magnetic beads.

Time required: ~45 minutes (mostly waiting)

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COLLECTIONS

ONT DNA Barcoding Fungal Amplicons w/ MinION & Flongle

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ONT DNA Barcoding Fungal Amplicons w/ MinION & Flongle

MATERIALS TEXT

Reagents:

Molecular Water **IBI**

Scientific Catalog #IB42130 (cost in extraction step)

Ethanol **IBI**

Scientific Catalog #IB15721 80%: $56.18 per 1L

HighPrep™ PCR Clean-up System **MagBio Genomics**

Inc. Catalog #AC-60005

$117.88 per 5mL

Lab Consumables:

0.2mL PCR tube strips - 8 cell
DNA LoBind 1.5 mL tubes - Eppendorf
1000uL pipette tips (Amazon): $13.28
10uL pipette tips
15mL tubes (Amazon): $17.99

Equipment:

1000uL pipette (Amazon): $32.39
10uL multichannel pipette
Magnetic bead separator for 1.5mL eppi tubes (Ebay): $59.00
Tip disposal bucket
Gel electrophoresis system (miniPCR): $300
Heat block (Amazon): $179.99
Quantus/Qubit Fluorometer (optional)

Preparation 22m

1 Bring magnetic beads to room temp. (Should be stored in the fridge)

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2 Heat a 1.5uL tube of molecular water to **55 °C** in the heat block. ~1000uL should be sufficient in the tube. This step is optional but is helpful if a heat block is available.

3 Create a fresh batch of 80% ethanol. You will be using **2 mL** in this protocol. You will be using more later, so make extra. A 15mL tube is one potential type of vessel.

4 mL 100% ethanol

1 mL molecular water

PCR Pooling

4 Using a 10uL multichannel pipette, transfer **2 µL** or **3 µL** of PCR product from each row of each 96 well plate of PCR amplicons into the corresponding cells of a new eight tube strip. (Ex - If you are transferring 3 plates of amplications, at the conclusion, there should be **108 µL** of product in each cell of the eight tubes in the strip [12 cells x 3uL x 3 plates].)

Use **3 µL** from each PCR reaction if you are combining three plates; **2 µL** if you are combining more than four plates (see total volumes below in next step). The primary goal here is to use the maximum amount that will still fit into a single 1.5 eppi tube once they are all combined.

I will typically use filtered tips here, but use the same tips for each strip for a given plate. Using a new set of tips between plates. Remember, we will ultimately be combining all of the PCR product into a single library, so not much concern about cross-contamination at this point.

5 Using a 200uL or 1000uL pipette, with filter tips, transfer the PCR pools from each of the eight tubes of the strip into a new 1.5mL LoBind eppi tube.

Final 1.5mL tube volumes (as a reference):

3 plates - 288 samples - **864 µL** (3uL per cell)

4 plates - 384 samples - **768 µL** (2uL per cell) or **1.15 mL** (3uL per cell)

5 plates - 480 samples - **960 µL** (2uL per cell)

7 plates - 672 samples - **1344 µL** (2uL per cell)

Mix the tube by turning it upside down 3-5 times.

PCR Bead Cleanup 22m

6 Subsample **500 µL** of the amplicon pool to a new 1.5mL LoBind eppi tube.

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Optional: Retain the tube with the original product. You will run a gel electrophoresis comparison between this product and the purified product.

(In order to reduce the amount of cleaning beads used, it is possible to reduce the amount of PCR product subsampled here, such as only **250 µL** of amplicon pool. Keep in mind this will change the quantified DNA concentrations reported at the end of each protocol.)

7 Vortex or shake beads thoroughly to suspend them in the solution.

8 Add 0.5X ratio of magnetic beads to the 1.5mL tube containing the pooled amplicons. Ex - for 250uL subsampled pool, add **125 µL** of beads. For 500mL amplicon pool, add **250 µL** of beads.

Mix thoroughly by pipetting up and down 10 times.

9 Incubate for **00:05:00** at room temperature. 5m

10 Spin down tube for **00:00:05** . Place sample tube on the magnetic separator for **00:02:00** or until the solution clears. Beads should now be on the side of the tube. 2m 5s

11 With the tube still on the magnet, remove the liquid from the tube and discard. Be sure not to disturb the beads.

12 With the tube still on the magnet, add **1000 µL** of 80% ethanol to the tube and let sit for **00:02:00** . Try to minimize disturbance of the beads. Fill gently with liquid stream from the pipette tip on opposite side of the beads. 2m

I will typically leave the pipette tip on the pipettor until the time is up, and remove the ethanol with the same tip.

13 Remove ethanol by pipetting and discard. I will typically discard the tip with the fluid still in it.

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14 Repeat the ethanol wash one time. **go to step #12**

15 Dry by incubating the tube for 10-15 minutes at room temperature. Ensure all of the ethanol has evaporated from the tube.

If there is much visible ethanol in the tube, you can remove from the magnet, spin down for 10 seconds, put the tube back on the magnet, and remove the excess with a pipette tip. If there is visible ethanol, but not enough to suck up in a tip, you can move it around the side of the tube with clean tip. This will help it evaporate faster.

16 Remove the tube from the magnet and add **100 µL** of **55 °C** molecular water. Pipette up and down five times to mix until the pellet is fully suspended.

The DNA will now be released from the beads and suspended in the water.

17 Incubate for **00:02:00** at room temperature. 2m

18 Place the tube back on the magnet for **00:02:00** , or until the solution is clear. 2m

19 Transfer the water containing the DNA to a new 1.5mL LoBind eppi tube.

You should now have your pooled and purified DNA template.

Gel Electrophoresis Validation

20 Optional: Run a gel with a lane of the original product and a lane of the purified DNA template side by side.

Perform another purification if the primer band is still visible. (Have never needed to do this.)

Quantification

21 If you have access to a Quantus/Qubit fluorometer, now is a good time to quantify the resulting amount of DNA in your purified sample.

You are looking to be around 1ug DNA per 50uL water as an end goal. Each plate of end-product contains approximately the following amount of DNA with this protocol, assuming a 500uL subsample was taken near the beginning of this protocol.

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Assuming 100uL of water added:
Promega Wizard Extraction - results in ~63 ng/uL (6,300ng)
X-Amp extraction of dried tissues - results in ~72 ng/uL (7,200 ng)
X-Amp extraction of fresh tissues - results in ~73 ng/uL (7,300 ng)

Combination of all three resulted in ~69 ng/uL (when they each cleaned individually at the outset and were individually diluted with their own 100uL of water at the end). These numbers are just for a reference. The numbers will be different depending on the extraction method being used, total number of plates combined, and the amount of product that is subsampled.

Further trials showed a typical final concentration of 86 - 95 ng/uL with 5-7 plates of PCR product combined of X-amp extractions from dried tissue.

So for the final end product:

There are 1000ng in a ug. 1000/90 = 11.1uL to get to 1ug.

11.1uL of the resulting diluted DNA solution combined with 38.9uL of water for the next step. (1ug DNA per 50uL water).

This is what I would utilize if you do not have the ability to accurately quantify DNA.

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