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ONT Post-PCR Pooling & Purification for Fungal Barcoding

Stephen Douglas Russell[1]

[1]The Hoosier Mushroom Society

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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)

Stephen Douglas Russell 2022. ONT Post-PCR Pooling & Purification for Fungal Barcoding. **protocols.io** https://protocols.io/view/ont-post-pcr-pooling-amp-purification-for-fungal-b-b9qkr5uw

fungi, PCR, ONT, nanopore, minion, magnetic beads, purification

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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)

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.

8 mL 100% ethanol
2 mL molecular water

PCR Pooling

4 Using a 10uL multichannel pipette, transfer **2 µL** or **3 µL** of PCR product from each row of your 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 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 5 plates (see total volumes below in next step).

5 Using a 200uL or 1000uL pipette, 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:
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)

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.

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

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 250uL of beads.

Mix thoroughly by pipetting up and down 6-8 times.

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

10 Place sample tube on the magnetic separator for **00:03:00** or until the solution clears. 3m
Beads should now be on the side of the tube.

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

13 Remove ethanol by pipetting and discard.

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.

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 **00:03:00** , or until the solution is clear. 3m

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 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.

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.

If not, the **100 µL** of molecular water added above should put you near the right DNA concentration with room for a final adjustment.

You are looking to be around 1ug DNA per 50uL water as an end goal.

Promega Wizard Extraction - results in ~63 ng/uL
X-Amp extraction of dried tissues - results in ~72 ng/uL
X-Amp extraction of fresh tissues - results in ~73 ng/uL

Combination of all three resulted in ~69 ng/uL. There are 1000ng in a ug. 1000/69 = 14.5uL to get to 1ug.

So for the final end product:

14.5uL of the resulting DNA solution combined with 35.5uL of water for the next step. (1ug DNA per 50uL water),