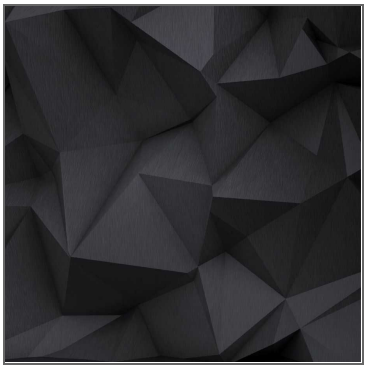

ONT Post-PCR Pooling & Purification for Fungal Barcoding V.4

Stephen Douglas Russell[1]

[1]The Hoosier Mushroom Society

The Hoosier Mushroom Society

Stephen Douglas Russell

VERSION 4

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OPEN ACCESS

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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Protocol status: Working

We use this protocol and it's working

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PROTOCOL integer ID: 79729

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

MATERIALS

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. Promega $2,000; Got mine on Ebay new for $900 shipped)

Quantifluor dsDNA System (optional - Promega) $115

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.

4 mL 100% ethanol

1 mL molecular water

4 Optional: Create Quantifluor Working Solution and Standard for this run, if you are quantifying your DNA using a Promega Quantus or Invitrogen Qubit device. 5m 5s

1. In a 1.5mL tube, combine 798 µL of 1X TE Buffer and 2 µL of Quantiflor dsDNA Dye. This working solution will be stable for the next two hours.

2. Add 200 µL of this working solution into four 0.5mL tubes.

3. Add 2 µL of the DNA standard to one of the tubes. Label it "S" for standard. Vortex it for 00:00:05. Incubate it for 00:05:00 in the dark. Recalibrate the standard on your Quantus/Qubit. Use one of the other tubes you just made as the blank.

4. Store the three blank tubes in the same dark area as the standard until you need to use them in this protocol.

PCR Pooling

5 The ultimate goal with this step is to get a standard volume of each PCR reaction (2uL - 3uL) that are in cells of 96 well plates, into a single 1.5mL eppi tube. We will first transfer PCR product from the 96-well plates into an 8-strip, and then combine each cell of the 8 strip into the final 1.5 eppi tube.

Note: If the PCR product in your 96 well plates has evaporated, particularly along the edges, then you may need to make modifications to your thermocycler, such as placing a layer of silicon baking mat material over the plate during the thermocycler run.

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. More recently I have been using a single set of 8 tips across all 10 plates.

6 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)
10 plates - 960 samples - 1440 µL (1.5uL per cell)
10 plates - 960 samples - 1920 µL (2uL per cell; what I normally use into a 2.0mL screw top)

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

PCR Bead Cleanup

22m

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

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.)

8 Vortex or shake beads thoroughly for 00:00:10 to suspend them in the solution. 10s

9 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.

10 Incubate for 00:05:00 at room temperature. 5m

11 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

Note: if you are using green Taq, then the liquid will not be completely clear at this point. It will still be green, but you should be able to see the beads on the side of the tube. No green should be visible at any step after this wash step.

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

13 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.

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

15 Repeat the ethanol wash one time. go to step #13

16 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.

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

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

18 Incubate for 00:02:00 at room temperature. 2m

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

20 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

21 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

22 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.

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)

These numbers are just for a reference. The numbers could be different depending on the type of tissue being used, extraction method being used, and the PCR program employed.

Further trials showed a typical final concentration of 86 - 108 ng/uL with 5-10 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 in the final sample.

In a new 1.5uL eppi tube combine 11.1uL of the resulting diluted DNA solution combined with 38.9uL (=50 minus 11.1) 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.

If you can quantify, just use your final concentration in the calculation.
1000/108 = 9.3uL template
50 - 9.3 = 40.7
Final in a new 1.5uL eppi tube: 9.3uL template + 40.7uL of water.