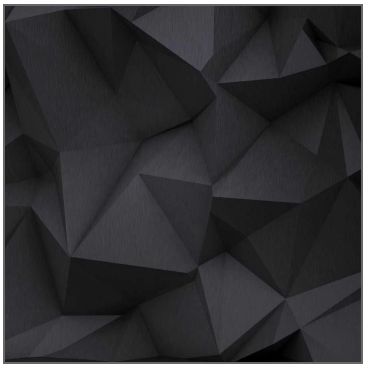

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We use this protocol and it's working

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FUNDIS ONT Post-PCR Pooling & Purification for Fungal Barcoding

Forked from ONT Post-PCR Pooling & Purification for Fungal Barcoding

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Harte Singer
FUNDIS

ABSTRACT

Pooling PCR products and purifying them.

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

PROTOCOL MATERIALS

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** Step 20

Preparation

22m

1 Bring magnetic beads to room temp. **Very Important**

2 Heat a fresh, sterile 1.5uL tube of molecular grade water or Low TE buffer to 55 °C in the heat block. 500uL should be sufficient.

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 (denatured from IBISCI works fine)
1 mL molecular water

PCR Pooling

4 We will be combining an equal volume of PCR product from each well of each plate into a single pool in a 1.5mL DNA LoBind tube.

Using a 10uL multichannel pipette with filtered tips, transfer 2 µL or 3 µL of PCR product from each row of each 96 well plate of PCR amplicons into the corresponding tubes of a new PCR 8 strip.

The PCR products are already barcoded so you can use a single row of tips for each 96 well plate.

Use 3 µL from each PCR reaction for a standard run of 5 plates

5 Using a 200uL pipette with filter tips, transfer the PCR pools from each of the eight tubes of the strip into a new 1.5mL DNA Low Bind tube.

Optional: You can make pools containing any number of samples or plates and store them separately, although I typically don't do this.

Final 1.5mL tube volumes (as a reference):

5 plates - 480 samples - 960 µL (3uL per cell)
7 plates - 672 samples - 1344 µL (2uL per cell)
10 plates - 960 samples - 1440 µL (1.5uL per cell)

Mix the tube gently by pipette mixing or inverting the tube. Don't vortex.

The pool can be stored at 4 °C for at least 1 week or at -20 °C for much longer.

PCR Bead Cleanup

22m

6 Subsample 250 µL of the amplicon pool to a new 1.5mL DNA LoBind tube.

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

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

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.

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 2m
Try to minimize disturbance of the beads. Fill gently with liquid stream from the pipette tip on opposite side of the beads.

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.

14 Repeat the ethanol wash one time.

15 Dry by incubating the tube for 10 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.

Plan ahead: This is a good time to prepare your Qubit solution and gather supplies for library prep.

16 Remove the tube from the magnet and add 45 µL of 55 °C Low TE buffer or 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.

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.

Quantification

3m 10s

20 Quantify DNA using the Qubit fluorometer. 3m 10s

Mix 995 µL of Qubit buffer with 5 µL of fluorescent dye from the Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** and vortex for 5 seconds.

Pipette 190 µL into each of 2 Qubit assay tubes and label them S1 and S2. Pipette 199 µL into each of 3 Qubit assay tubes and label them 1, 2, and 3. Store the filled tubes away from light. Do not expose the dye to light for longer than necessary.

Mix 10 µL of Standard 1 into tube S1 and with 10 µL of Standard 2 into tube S2 and vortex both tubes for 00:00:05. Add 1 µL of your purified DNA to tube 1 and vortex for 00:00:05 wait for 00:03:00 before reading the standards and then quantify the sample in tube 1.

A good cleanup should yield [M] 100 ng/µL and anything less than [M] 30 ng/µL is not good enough to proceed. Troubleshooting would include running pre and post-cleanup samples on a gel to estimate concentration. Cleanup may be repeated or if the input DNA is not great then repeat PCR entirely.

The purified DNA can be stored at -20 °C until needed.

To begin library prep:

In a single PCR tube, combine approx. 250 ng of purified DNA with enough molecular grade water to make 25 µL total volume.