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ONT dA-tailing for Fungal Barcoding

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protocol .

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Tailing is an enzymatic method for adding a non-templated nucleotide to the 3' end of a blunt, double-stranded DNA molecule. This puts A-chains on the end of our PCR product, creating a site for the ligation adapter to attach to. Simple process - create a reaction, PCR bead cleanup.

Time required: ~45 minutes

Stephen Douglas Russell 2022. ONT dA-tailing for Fungal Barcoding. **protocols.io** https://protocols.io/view/ont-da-tailing-for-fungal-barcoding-b9qqr5vw

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Reagents

NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

Molecular Water **IBI Scientific Catalog #IB42130**

HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #AC-60005** : $117.88 per 50 mL. $0.047 per rxn.

Total per Flongle run (1/2 rxns):
Total per MinION run:
Total per 96 samples:
Total per sample (Flongle: 480 samples):

Consumables

Eppendorf DNA LoBind 1.5mL tubes
0.2mL PCR tubes (USA Scientific): $
10uL pipette tips
100-200uL pipette tips

Equipment

Vortex mixer
Mini centrifuge
PCR cleanup magnet
10uL Pipette
100uL Pipette
Hula mixer (Ebay): $200.00 (optional)
Quantus or Qubit Fluorometer (optional)

End repair/A-tailing

1 Put a 1.5mL tube of molecular water on a heat block at **55 °C** .

2 Optional: Thaw DNA CS at room temperature, spin down for **00:00:05** , mix by pipetting, and place on ice. 5s

DNA CS -
Ligation Sequencing Kit (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112**

(DNA CS consists of a standard DNA sequence that can be used to provide quality control for sequencing and alignment.)

3 Mix your amplicon DNA pool throroughly with a pipette. Briefly spin down for **00:00:05** . 5s

4 Important: Vortex the Ultra II End-prep Reaction Buffer for 30 seconds.

(Do not vortex the End-prep Enzyme Mix)

5 In a 0.2mL thin wall, sterile, nuclease-free tube, combine the following in order. Mix each reagent together after it is added by gently pipetting the entire volume up and down 10-20 times for each addition.

Ideal amplicon DNA concentration is 0.5ng per 50mL for Flongle or 1ug DNA per 50uL for R10.3. At this concentration you can use the volumes described below.

Component	Flongle Volume	R10.3 Flowcell Volume
DNA CS (optional)	0.5uL	1uL
Amplicon DNA	24.5uL (0.5ng)	49uL (1ng)
Ultra II End-prep reaction buffer	3.5uL	7uL
Ultra II end-prep enzyme mix	1.5uL	3uL
Total	30uL	60uL

The NEB protocol this is based on can be found here.

6 Spin down the tube in a mini centrifuge for **00:00:05** . 5s

7 Incubate in a thermocycler using the following program:

20 °C for 5 minutes

65 °C for 5 minutes

4 °C Hold

8 Spin down the tube for 5 seconds in a mini centrifuge.

9 Transfer the entire **30 µL** / **60 µL** reaction to a new 1.5mL LoBind eppi tube.

10 Resuspend magnetic beads in solution by vortexing. Add **30 µL** - **60 µL** of beads to the reaction (1X bead clean) and mix gently by pipetting up and down.

11 Incubate at room temperature for **00:05:00** . (Can put the tube in a rotator [hula] mixer if one is 5m

available.)

12 Spin down the tube in a mini centrifuge for 5 -10 seconds.

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

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

15 With the tube still on the magnet, add **200 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

16 Remove ethanol by pipetting and discard.

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

18 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

19 Remove the tube from the magnet and add **31 µL** for Flongle or **61 µL** of molecular water for MinION. 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.

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

21 Place the tube back on the magnet for **00:03:00** or until the solution is clear. 3m

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

You should now have your A-tailed DNA template.

DNA Quantification

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

If not, the **31 µL** / **61 µL** of molecular water added above should put you in the ballpark of the right DNA concentration.

I have a concentration of 17ng/uL at this point and used it at this level for the next step.

24 It is possible to break and store the sample at 4C overnight if needed. It would be ideal to continue on to adapter ligation at this time.