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We use this protocol and it's working

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ONT dA-tailing for Fungal Barcoding V.3

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ABSTRACT

This protocol is for dA-tailing, which is an enzymatic method for adding a non-templated nucleotide to the 3' end of a blunt, double-stranded DNA molecule. In other words, 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 with three chemicals, cleanup the product with beads.

Time required: ~45 minutes

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

Keywords: oxford, minion, flongle, a-tailing, nanopore, fungi, fungal

MATERIALS

Reagents

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

$283.00 per 24 reactions

Molecular Water **IBI Scientific Catalog #IB42130** (or any molecular water)

HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #AC-6000**

: $117.88 per 50 mL. $0.047 per rxn. (any bead cleanup will work)

Total per Flongle run (1/2 rxns): $5.95
Total per MinION run: $11.85
Total per 96 samples: $0.061
Total per sample (Flongle: 480 samples): $0.012
Total per sample (Flongle: 672 samples): $0.0089
Total per sample (Flongle: 960 samples): $0.0061

Consumables

Eppendorf DNA LoBind 1.5mL tubes
0.2mL PCR tubes (Amazon): $12.83
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. This will be used after the cleanup step towards the end of this protocol.

Thaw the NEB End-prep Reaction Buffer and NEB End-prep Enzyme Mix at room temperature. (Won't take long.)

Also if you are continuing on with adapter ligation (next protocol) once this step is done, it would be good to set the chems for next step on ice so they can begin to thaw as well.

This includes LA, Quick T4 Ligase, LNB, EB, and SFB. (if using V14 chemistry)

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 V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

(DNA CS consists of a standard DNA sequence that can be used to provide quality control for sequencing and alignment. Generally unnecessary for DNA barcoding efforts with hundreds of repeats of a single amplicon. I usually skip it)

3 Mix your amplicon DNA pool thoroughly with a pipette (pipette up and down 5 times). 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 R10.4.1. At this concentration you can use the volumes described below.

Component	**R10.4.1 Flongle Volume**	**R10.4.1 MinION 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 (Flongle) - 60 µL (MinION) of beads to the reaction (1X bead cleanup) 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 available. I typically do not use one; just let it sit in a tube rack.) 5m

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

13 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

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 for 00:00:05 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. 5s

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 00:02:00 or until the solution is clear. 2m

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 typically have a concentration of 11-33 ng/uL at this point and used it at this level for the next step. No dilutions or adjustments.

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.