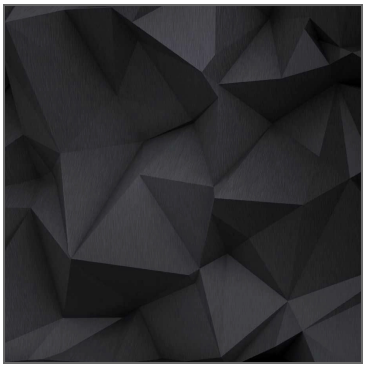

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Protocol status: Working
We use this protocol and it's working

Created: Apr 10, 2024

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FUNDIS version ONT dA-tailing for Fungal Barcoding

Forked from ONT dA-tailing for Fungal Barcoding

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The Hoosier Mushroom Society

Harte Singer
FUNDIS

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

Adapted from dx.doi.org/10.17504/protocols.io.yxmvmnze9g3p/v3

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-60005 :

$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)

PROTOCOL MATERIALS

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

Step 1

End repair/A-tailing

13m 12s

1 Put a 1.5mL aliquot of fresh molecular water on a heat block at 55 °C. This will be used after the cleanup step towards the end of this protocol. 2s

Turn on your PCR thermal cycler so that the heated lid begins to come up to temp.

Take out the materials for library prep from the freezer. Flick mix and then spin down both NEB enzymes and immediately place on ice. Thaw the NEB Next dA tailing buffer at room temperature and vortex to **completely dissolve precipitate**, then spin down. Thaw the AXP beads from the kit at room temperature and *do not* place on ice.

Do not vortex enzymes!. Thaw remaining reagents at room temperature, flick mix, then then spin down and *place on ice*.

You will need AXP, LA, LNB, EB, and SFB from nanopore library kit.
0 µL
Spin all reagents down for 00:00:02 **before opening and keep everything on ice except the Ultra II End-prep reaction buffer and the AXP.**

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

2 Mix your amplicon DNA pool thoroughly with a pipette (pipette up and down 5 times). Briefly spin down for 2s 00:00:02.

3 In a 0.2mL thin wall, sterile, nuclease-free PCR 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.

Dilute 300 ng of your purified PCR pool up to 25 µL using Molecular Grade Water. The ONT protocol suggests using 50-100fmol, which is about 20-40ng however experience shows that this will produce a poor quality run and I have used anywhere from 100-400ng of DNA with good results.

	Component	Volume
	Amplicon DNA	100-400ng in 25uL Molecular Grade H2O
	Ultra II End-prep reaction buffer	3.5uL
	Ultra II end-prep enzyme mix	1.5uL
	Total Volume	30uL

Ultra II end-prep enzyme mix	1.5uL
Total	30uL

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

4 Spin down the tube in a PCR tube centrifuge for 00:00:02 . 2s

5 Incubate in a thermocycler using the following program. *Make sure that the cycler has been on for long enough for the lid to pre-heat:*

20 °C for 5 minutes
65 °C for 5 minutes
4 °C Hold

6 Spin down the tube for 00:00:02 in a PCR tube centrifuge. 2s

7 Transfer the entire 30 µL reaction to a new 1.5mL LoBind tube.

8 Resuspend AXP (magnetic beads) in solution by vortexing. Add 30 µL (Flongle) of AXP to the reaction (1X bead cleanup) and mix gently by pipetting up and down.

9 Incubate at room temperature for 00:05:00 . The ONT protocol calls for using a Hula mixer, I simply gently flick the tube a few times during this time. 5m

10 Spin down the tube in a mini centrifuge for 00:00:02 2s

11 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

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 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

14 Remove ethanol by pipetting and discard.

15 Repeat the ethanol wash one time.

16 Spin down for 00:00:02 and place the tube back on the magnet. Use a p10 or p20 to pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. 2s

17 Remove the tube from the magnet and add 30 µL of molecular grade 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.

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 A-tailed DNA template.

21 It is recommended to go directly into adapter ligation, however you may take a break and leave the A-tailed library at 4 °C for up to 24 hours.