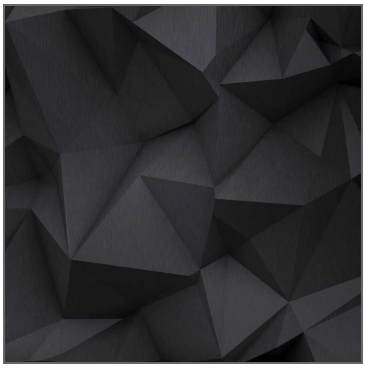

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Protocol status: Working
We use this protocol and it's working

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FUNDIS ONT V14 Nanopore Adapter Ligation for Fungal DNA Barcoding Flongle 10.4.1

Forked from ONT V14 Nanopore Adapter Ligation for Fungal DNA Barcoding

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The Hoosier Mushroom Society

Harte Singer
FUNDIS

ABSTRACT

This process will take your A-tailed library and add the nanopore adapters. Simply combine several chemicals for a single reaction and do a bead cleanup.

Tested with:
Flowcells: Flongle 10.4.1
Ligation Kit: V14 - LSK114

Time required: ~45 minutes

Adapted from dx.doi.org/10.17504/protocols.io.dm6gpb5zdlzp/v5

PROTOCOL integer ID: 98166

Keywords: nanopore, fungi, flongle, fmol, library preparation

MATERIALS

Reagents

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

: $694.43 per 6 reactions ($115.74 per MinION run; $57.87 per Flongle run)

NEBNext Quick Ligation Module **New England Biolabs Catalog #**E6056S :

$361.00 per 20 reactions ($18.05 per MinION run; $9.03 per Flongle run)
*note: This kit has two components. We use one - NEBNext Quick T4 DNA Ligase. NEB checking on whether the single one is available for purchase. Samples of this kit should be available from NEB.

HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #**AC-60005 :

$117.88 per 50 mL. $0.047 per rxn.
Note: Most magnetic beads from most vendors can be used with the same protocol.

Total per Flongle run (1/2 rxns): $66.95
Total per MinION run: $133.84
Total per sample (Flongle: 480 samples): $0.139
Total per sample (Flongle: 960 samples): $0.07

Consumables

Eppendorf DNA LoBind 1.5mL tubes
10uL pipette tips
100-200uL pipette tips

Equipment

PCR tube rack
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

In 2 steps

NEBNext Quick Ligation Module **New England Biolabs Catalog #**E6056S Step 1

Adapter Ligation Flongle 10.4.1

1 You should already have your reagents on ice from the dA tailing step, but if not you will need the NEBNext Quick Ligation Module enzyme and from the ONT kit you will need 1 tube of SFB, and LA, LNB, EB, and AXP. Spin down the NEB enzyme and immediately place on ice. Thaw AXP and allow to come up to room temperature, do not place on ice. Thaw other reagents at room temperature, flick-mix, spin down, and keep on ice.

LNB, LA, AXP, SFB, EB -
Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114
Quick T4 Ligase - NEBNext Quick Ligation Module **New England Biolabs Catalog #**E6056S

2 In a 1.5 mL DNA LoBind tube, mix in the following order:

Between each addition, pipette mix 10-20 times.

	Reagent	Volume
	dA Tailed DNA	30 µl
	Ligation Buffer (LNB)	12.5 µl
	NEBNext Quick T4 DNA Ligase	5 µl
	Ligation Adapter (LA)	2.5 µl
	Total	50 µl

3 Spin down with a mini centrifuge for 00:00:02 . 2s

4 Incubate the reaction for 00:10:00 at room temperature. This is a good time to do a flow cell check and get your computer set up. 10m

5 Resuspend AMPure XP (AXP) magnetic bead stock by vortexing.

6 Add 20 µL of resuspended beads to the reaction and mix by flicking the tube.

7 Incubate for 00:05:00 at room temperature, ONT suggests using a Hula mixer, you can just gently flick the tube a few times. 5m

8 Spin down the sample for 00:00:02 and pellet on a magnet for 00:02:00 . 2m 2s

Keep the tube on the magnet, and pipette off the supernatant.

9 Wash the beads by adding 125 µL of Short Fragment Buffer (SFB). Flick the beads vigorously to resuspend (they will only partially resuspend), spin down for 00:00:02 , then return the tube to the magnetic rack for 00:02:00 and allow the beads to pellet. Remove the supernatant using a pipette and discard. 2m 2s

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

10 Repeat the previous step.

11 Spin down for 00:00:02 and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. 2s

12 Remove the tube from the magnetic rack and resuspend the pellet in 7 µL Elution Buffer (EB). Incubate 10m for 00:10:00 at room temperature. Use this time to put away all of the reagents from the previous steps and set up your reagents for the final library mixing step. Use the glass vials that come with the Flongle kit, you will need FCF (Flow Cell Flush) SB (Sequencing Buffer) LIB (Library Beads). You will also need the FLT (Flow Cell Tether) From the main Ligation Sequencing kit. Make sure you have your Qubit reagents ready for quantification.

13 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

14 Remove and retain 7 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Store on ice until you are ready to load in your flowcell.

Quantification

15 Quantify 2 µL of your ligated library using the Qubit fluorimeter

It is recommend loading 5 fmol to 10 fmol of this final prepared library onto your flow cells. Loading more than 20 fmol of DNA can reduce the rate of duplex read capture. ONT recommends that you dilute the library in Elution Buffer if required, however we use molecular grade water instead. We dilute the entire 5uL of library, however you may dilute a smaller amount if you choose.

https://www.promega.com/resources/tools/biomath/

For 900bp length DNA (what our ITS1F-4 rxns appear to average, with adapters), we are looking for:
10 fmol - 20 fmol = 6ng - 12ng of DNA in 5uL of final dilute library.

For a 22 ng/uL sample

22ng/uL * 5uL = 110ng DNA in sample of 5uL elution buffer.

How much additional molecular water to have 5uL needed for the next step give us correct amount of DNA?

C1V1 = C2V2, rearrange C1V1/C2 = V2

(110ng * 5uL) / 10ng(17 fmol DNA) = 55uL total volume

Bring initial volume (5uL) up to 55uL = add 50uL molecular water

So at 22ng/uL quantification, add an additional 50uL of molecular water to have right concentration to use 5uL for the next step with Flongle.