VERSION 4

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We use this protocol and it's working

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# ONT V14 Nanopore Adapter Ligation for Fungal DNA Barcoding V.4

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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 or MinION 10.4.1
Ligation Kit: V14 - LSK114

Time required: ~45 minutes

**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-6000**

: $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)

## Adapter Ligation

1 Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice.

LA -

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

Quick T4 Ligase -

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

2 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.

LNB -

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

3 Thaw the Elution Buffer (EB) at room temperature, mix by vortexing, spin down and place on ice.

EB -

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

4 Thaw one tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.

SFB -

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

5 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:

Between each addition, pipette mix 10-20 times.

| **Reagent** | **10.4.1 Flongle Volume** | **10.4.1 MinION Volume** |
|---|---|---|
| DNA sample from the previous step | 30 µl | 60 µl |
| Ligation Buffer (LNB) | 12.5 µl | 25 µl |
| NEBNext Quick T4 DNA Ligase | 5 µl | 10 µl |
| Ligation Adapter (LA) | 2.5 µl | 5 µl |
| Total | 50 µl | 100 µ |

6 Spin down with a mini centrifuge for 00:00:05 . 5s

7 Incubate the reaction for 00:10:00 at room temperature. 10m

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

9 Add 20 µL (Flongle) or 40 µL (MinION) of resuspended beads to the reaction and mix by flicking the tube.

10 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. (or just place in a tube rack without the mixer) 5m

11 Spin down the sample for 00:00:05 and pellet on a magnet for 00:02:00. 2m 5s

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

12 Wash the beads by adding 125 µL (Flongle) or 250 µL (MinION) of Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down for 00:00:05, 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 5s

Note: flicking the tube does not seem to fully resuspend the beads. Just flick 10 times or so and spin down.

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

13 Repeat the previous step. go to step #12

14 Spin down for 00:00:05 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. 5s

15 Remove the tube from the magnetic rack and resuspend the pellet in 7 µL Elution Buffer (EB). Incubate for 00:10:00 at room temperature. 10m

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

17 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

18 If you have access to a Quantus or Qubit fluorimeter, now is a good time to quantify 2uL of DNA in your sample.

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. Dilute the library in Elution Buffer if required.

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 = .006ug - .012ug of DNA.

For a 22 ng/uL sample (Quantus quantification):

22ng 1ug
-------- * -------- = 0.022ug/uL (22/1000=0.022)
1uL 1000ng

0.022ug/uL * 5uL (elution buffer; or x7 if you did not quantify using 2uL)

= 0.11 ug DNA in sample of 5uL elution buffer.

*Note: the 0.11 in the calculations below will change based on your individual DNA amount.

**Also note: The ONT protocol suggests using additional EB in order to make concentration adjustments. As there is not a lot of the reagent in the standard packets, and it is not possible to buy more individually, I have been using molecular water for this step with no ill effect.

**Flongle 10.4.1**
How much additional molecular water to have 5uL needed for the next step give us correct amount of DNA?

0.11ug / xuL = 0.010ug (17 fmol DNA)

x = 11uL * 5uL = 55uL - 5uL (or 7 if you did not quanitfy) = 50uL

Overall summary: ([DNA Concentration] / 1000 * 5 * 100 * 5) - 5 = [Amount of H2O to add]

So at 0.022ug/uL quantification, add an additional 50uL of molecular water to have right concentration to use 5uL for the next step with Flongle.

11ng/uL sample comes out to adding an additional 22.5 uL of molecular water.
31ng/uL sample comes out to adding an additional 72.5 uL of molecular water.

I would use 50 uL of extra molecular water if you are not able to quantify your sample.