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ONT Q20+ Adapter Ligation for Fungal DNA Barcoding

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protocol .

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

Time required: ~45 minutes

Stephen Douglas Russell 2022. ONT Q20+ Adapter Ligation for Fungal DNA Barcoding. **protocols.io** https://protocols.io/view/ont-q20-adapter-ligation-for-fungal-dna-barcoding-b9qrr5v6

nanopore, fungi, flongle, fmol, library preparation

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Reagents

Ligation Sequencing Kit (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112** In 5 steps : $694.43 per 6 reactions

NEBNext Quick Ligation Module **New England Biolabs Catalog #E6056S** Step 1 : $361.00 per 20 reactions
*note: This kit has two components. We use one. NEB checking on whether the single one is available for purchase. Samples of this kit should be available to start.

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): $66.95
Total per MinION run: $133.89
Total per 96 samples: $13.38
Total per sample (Flongle: 480 samples): $0.139

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 Adapter Mix H (AMX H) and Quick T4 Ligase, and place on ice.

AMX H - Ligation Sequencing Kit (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112**

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 (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112**

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

EB - Lig
Ligation Sequencing Kit (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112**

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 (Q20) **Oxford Nanopore Technologies Catalog #SQK-LSK112**

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

Between each addition, pipette mix 10-20 times.

Reagent	Flongle Volume	R10.3 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
Adapter Mix H (AMX H)	2.5 µl	5 µl
Total	50 µl	100 µ

6 Spin down with a mini centrifuge for 5 seconds.

7 Incubate the reaction for 10 minutes at room temperature.

8 Resuspend magnetic bead stock by vortexing.

9 Add **20 µL** (Flongle) or **40 µL** (R10.3) 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. 5m

11 Spin down the sample for **00:00:05** and pellet on a magnet for **00:03:00** . Keep the tube on the magnet, and pipette off the supernatant. 3m 5s

12 Wash the beads by adding 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down for **00:00:05** , then return the tube to the magnetic rack for **00:03:00** and allow the beads to pellet. Remove the supernatant using a pipette and discard. 3m 5s

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

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

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 15 µl Elution Buffer (EB). Spin down for **00:00:05** and incubate for **00:10:00** at room temperature. 10m 5s

16 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

17 Remove and retain 15 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 1uL 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), we are looking for
Flongle: 5 fmol - 20 fmol = .003ug - .012ug of DNA.
R9.4.1: 5 - 50 fmol = .003ug - .029ug of DNA.
R10.3: 25 - 75 fmol = .015ug - .044ug of DNA.

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

22ng 1ug
-------- * -------- = 0.022ug/uL * 15uL (elution buffer) = 0.33 ug DNA in sample of 15uL elution buffer
1uL 1000ng

*Note: the 0.33 in the calculations below will change based on your individual DNA amount.

Flongle
How much additional EB to have 5.5uL needed for the next step give us correct amount of DNA?
0.33ug / xuL = 0.010ug (17 fmol DNA) x = 33uL x 5.5uL = 181.5uL - 15uL = 166.5uL

So at 0.022ug/uL quantification, add an additional 166.5uL of elution buffer to have right concentration to use 5.5uL for the next step with Flongle.

MinION R9.4.1
0.33ug / xuL = 0.025ug (42 fmol DNA) x = 13.2uL x 11uL = 145.2uL - 15uL = 130uL elution buffer addition.

MinION R10.3
0.33ug / xuL = 0.04ug (67 fmol DNA) x = 8.25uL x 11uL = 90.75uL - 15uL = 75uL elution buffer addition.