# Amplicon sequencing using MinION optimized from 1D native barcoding genomic DNA

**Yiheng Hu,Benjamin Schwessinger**

## Abstract

This is a protocol optimized from Oxford Nanopore Technology 1D native barcoding genomic DNA (with EXP-NBD103 and SQK-LSK108) for sequencing amplicons of fungal marker genes (ITS, EF1a) using PCR.

Thanks to Dr. Benjamin Schwesinger for his suggestions about the protocol.

Thanks to Dr. Leon Smith (ANU, Linde lab), Prof. Wieland Meyer (USydney) and Dr. Laszlo Irinyi for their contribution of fungal samples.

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## Guidelines

This is the protocol to extend experimental details of the official MinION sequencing protocol using the native barcoding kit (EXP-NBD103) and 1D ligation kit (SQK-LSK108). I adapted this kit for amplicon sequencing of fungal marker genes (ribosomal RNA and Elongation factor 1a) of individual fungal species.

By now I sequenced 45 known fungal species to set up this workflow and 11 field samples containing disease infected wheat. The primer I used is listed here and I also attached the brief summary in the excel files:

| name | orientation | sequence | description | reference |
|---|---|---|---|---|
| NS3 | FWD | GCAAGTCTGGTGCCAGCAGCC | used to amplify fungal rRNA gene region for species identification, bind with small subunit | Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. Journal of Natural Products, 80(3), 756-770. |
| LR6 | REV | CGCCAGTTCTGCTTACC | | |
| EF1-983F | FWD | GCYCCYGGHCAYCGTGAYTTYAT | used to amplify fungal Elongation Factor 1 gene region for species identification | |
| EF1-2218R | REV | ATGACACCRACRGCRACRGTYTG | | |
| RPB2-5f | FWD | GAYGAYMGWGATCAYTTYGG | used to amplify fungal RNA polymerase II gene region for species identification | |
| RPB2-7CR | REV | CCCATRGCTTGYTTRCCCAT | | |

The original protocol is here:
**https://community.nanoporetech.com/protocols/1d-native-barcoding-genomi/v/nbe_9006_v103_revo_21dec2016** (May need an account of ONT).

In general, the majority of steps are the same but I reasonably scaled down a little for some steps to save some reagents (optimized for using 12 barcodes, so can be scaled up if using fewer barcodes). Also, I attached an Excel file with calculations that will help proceed the corresponding steps.

Please let me know your feedback and suggestions. I will super appreciate your help! my email address is: yiheng.hu@anu.edu.au

## Before start

prepare PCR reaction for sequencing. I tried different DNA polymerases including the NEB LongAmp suggested by ONT, NEB Q5 and Promega GoTaq. I found Q5 always produce the best amount of products consistently. LongAmp is with good quality but the yield is too low. GoTaq produces as good amount as Q5 but Q5 is with higher accuracy based on previous experiences.

With Q5 polymerase, 30 cycles of 100ul reaction will provide you more than enough PCR products (following manufactures protocol). I used 1 volume of magnetic beads (AMPure, Beckman) to clean up the PCR reaction. but for short fragments, I think column based should also work fine. Also, I tested some homemade megnetic beads for DNA purification and they yield the same DNA compared to AMPure Beads. I have not got the protocol for making our beads out yet, but Protocol.io must have similar ones which is worth to test using your sample.

I included the beads clean up step for PCR reaction in this protocol.

## Materials

- Native Barcoding Kit 1D (EXP-NBD103) by Contributed by users
- Ligation Sequencing Kit 1D (SQK-LSK108) by Contributed by users
- Library Loading Bead Kit (EXP-LLB001) by Contributed by users
- Thermal Cycler at 20oC and 60oC by Contributed by users
- Microcentrifuge by Contributed by users
- Vortex mixer by Contributed by users
- 96 well Megnetic rack or equivalent for Stripe PCR tubes by Contributed by users

- Megnetic rack for Eppendorf tubes (1.5ml - 2ml) by Contributed by users
- Heating block at 37oC or equivalent waterbath by Contributed by users
- Pipettes P2, P10, P20, P200, P1000 and their corresponding tips by Contributed by users
- Multichannel pipette P1-10, P200 and their corresponding tips by Contributed by users
- NEBNext End repair / dA-tailing Module (E7546) by Contributed by users
- NEB Blunt/TA Ligase Master Mix (M0367) by Contributed by users
- Agencourt AMPure XP beads by Contributed by users
- Freshly prepared 70% ethanol in nuclease free water by Contributed by users
- 1.5ml Eppendorf DNA LoBind tubes by Contributed by users
- 0.2 ml stripe PCR tubes by Contributed by users
- 0.5 ml thin wall PCR tubes (for Qubit) by Contributed by users
- nuclease free water by Contributed by users
- NEB Next Quick Ligation Module by Contributed by users
- Troughs (I use ThermoFisher ones) by Contributed by users
- Invitrogen Quibit or equivalent (We use Promega Quantus Fluorometer) and its corresponding reagents by Contributed by users

## Protocol

Clean up PCR reaction

**Step 1.**

Add 1 volume (100ul) of resuspended beads into each PCR reactions and mix by flicking the tubes

REAGENTS
- Agencourt AMPure XP beads by Contributed by users

**Step 2.**

Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

**Step 3.**

Prepare 20ml of fresh 70% ethanol in nuclease-free water.

**Step 4.**

Spin down the sample and pellet on a magnet. Keep the tube on the 96 well magnet rack, and pipette off the supernatant.

**Step 5.**

Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

**Step 6.**

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 -5 mins

**Step 7.**

Remove the tube from the magnetic rack and resuspend pellet in 20 µl nuclease-free water. Incubate for 2 minutes at room temperature.

**Step 8.**

Pellet beads on magnet until the eluate is clear and colourless.

**Step 9.**

Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 0.08pmol.

After quantification, record the concentration into the calculation Excel file.

REAGENTS

✓ Invitrogen Quibit or equivalent (We use Promega Quantus Fluorometer) and its corresponding reagents by Contributed by users

NOTES

**Yiheng Hu** 09 Jan 2018

Prepare more Qubit working sollution for future measurement based on the number of samples.

**Step 10.**

pipette out 15 ul of eluate into new PCR tubes

NOTES

**Yiheng Hu** 09 Jan 2018

Clearly labelled clean PCR products can be stored in 4°C for one week.

**Step 11.**

Based on the calculation, pipette out the corresponding volume of each PCR products into a new tube and dilute into 25ul.

End-prep

**Step 12.**

Perform end-repair / dA-tailing of fragmented DNA as follows:

| Reagents | Volume |
|---|---|
| 0.12 pmol PCR products in water | 25 |
| Ultra II End-prep reaction buffer | 3.5 |
| Ultra II End-prep enzyme mix | 1.5 |
| Total | 30 |

prepare End-prep buffer and enzyme into master mix first and then add into each 0.2 ul PCR tubes.

REAGENTS
- NEBNext End repair / dA-tailing Module (E7546) by Contributed by users

**Step 13.**

Mix gently by flicking the tube, and spin down

**Step 14.**

Incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler.

**Step 15.**

Add 1 colume (30 µl) of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube.

REAGENTS
- Agencourt AMPure XP beads by Contributed by users

NOTES
**Yiheng Hu** 09 Jan 2018

Vortext the beads before adding

**Step 16.**

Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

**Step 17.**

Spin down the sample and pellet on a 96 well magnet rack. Keep the tube on the magnet, and pipette off the supernatant individually

**Step 18.**

Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

**Step 19.**

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 -5 mins at room temperature.

**Step 20.**

Remove the tube from the magnetic rack and resuspend pellet in 15 µl nuclease-free water by flicking the tubes. Incubate for 2 minutes at room temperature.

NOTES

**Yiheng Hu** 09 Jan 2018

Sometime if it is too dried the megnetic beads will not be fully resuspended. Try incubate longer time but carry on, it would not affect too much.

**Step 21.**

Pellet beads on magnet until the eluate is clear and colourless.

**Step 22.**

Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim: 50% of input.

After quantification, record the concentration into the calculation Excel file to calculate the volume needed for next step.

REAGENTS

Invitrogen Quibit or equivalent (We use Promega Quantus Fluorometer) and its corresponding reagents by Contributed by users

**Step 23.**

Pipette corresponding volume of each End-prepped PCR products into new stripe PCR tubes, add corresponding volume of nuclease free water based on the calculation to dilute samples into 9ul

If the concentration is too low, dilute into 13.5 ul and scale up the bacoding reaction to 1.5 times.

**Step 24.**

Thaw the Native Barcodes at room temperature. Transfer all the barcode reagents into twelve 0.2ml stripe PCR tubes so multichannel can be used.

## Adding barcodes

**Step 25.**

Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:

| Reagent | Volume |
|---|---|
| <0.038pmol but as much as possible | 9 |
| Native Barcode | 1 |
| Blunt/TA Ligase Master Mix | 10 |
| Total | 20 |

REAGENTS

- NEB Blunt/TA Ligase Master Mix (M0367) by Contributed by users
- Native Barcoding Kit 1D (EXP-NBD103) by Contributed by users

**Step 26.**

Mix gently by flicking the tube, and spin down

**Step 27.**

Incubate the reaction for 10 minutes at room temperature

**Step 28.**

Add 20 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

REAGENTS

- Agencourt AMPure XP beads by Contributed by users

**Step 29.**

Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

**Step 30.**

Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

**Step 31.**

Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the

pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

**Step 32.**

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 - 5 minutes at room temperature.

**Step 33.**

Remove the tube from the magnetic rack and resuspend pellet in 10 µl nuclease-free water. Incubate for 2 minutes at room temperature

NOTES
**Yiheng Hu** 09 Jan 2018

Better not to use too much water for elution as you will need to pool 12 samples.

**Step 34.**

Pellet beads on magnet until the eluate is clear and colourless.

**Step 35.**

Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim: > 0.011pmol each

Record all the concentration in the Excel file.

**Step 36.**

Pool equimolar amounts of each barcoded sample into a DNA LoBind 1.5 ml Eppendorf tube, ensuring that sufficient sample is combined to produce a pooled sample of 0.15 pmol total.

**Step 37.**

(Optional) Quantify 1 µl of pooled and barcoded DNA using a Qubit fluorometer.

NOTES
**Yiheng Hu** 09 Jan 2018

Just for double check. I normally not perform this step.

**Step 38.**

Dilute 0.15 pmol pooled sample to 50 µl in nuclease-free water.

NOTES

**Yiheng Hu** 09 Jan 2018

Optional Action

If 0.15pmol of the pooled sample exceeds 50 µl in volume, use SpeedVac to reduce the volume and dilute again.

If there is no SpeedVac you could also use the official protocol to add 2.5X beads and eluted in 50ul of nuclease-free water.

**Step 39.**

Thaw and prepare the kit reagents as follows:

| **Contents** | On ice | Room temperature |
|---|---|---|
| Adapter Beads Binding Buffer (ABB) | | X |
| Elution Buffer (ELB) | | X |
| Barcode Adapter Mix (BAM) | X | |
| Running Buffer with Fuel Mix (RBF) | X | |
| NEBNext Quick Ligation Reaction Buffer (5X) | X | |

REAGENTS

- NEB Next Quick Ligation Module by Contributed by users
- Ligation Sequencing Kit 1D (SQK-LSK108) by Contributed by users
- Library Loading Bead Kit (EXP-LLB001) by Contributed by users

**Step 40.**

Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.

- Mix the contents of each tube by vortexing
- Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate)
- *Spin down* briefly before accurately pipetting the contents in the reaction

## Adding adapters

**Step 41.**

Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

| Reagent | Volume |
|---|---|
| 0.15pmol pooled barcoded sample | 50 µl |
| Barcode Adapter Mix (BAM) | 20 µl |
| NEBNext Quick Ligation Reaction Buffer (5X) | 20 µl |
| Quick T4 DNA Ligase | 10 µl |
| **Total** | **100 µl** |

REAGENTS

NEB Next Quick Ligation Module by Contributed by users

**Step 42.**

Incubate the reaction for 10 minutes at room temperature.

**Step 43.**

Prepare the AMPure XP beads for use; resuspend by vortexing. Prepare another 1ml of fresh 70% Ethanol. Prepare all the tubes and megnetic rack ready.

**Step 44.**

Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by flicking.

NOTES

**Yiheng Hu** 09 Jan 2018

Sometimes I did not have enough pore occupancy when I ran the flowcell and so one thing I suspected is that I lost too much in this step. Therefore I tried 50ul once and it did result in a better pore occupancy. It is not necessarily sure that the better result is from the 10ul more beads but I just noted here for you to have an additional idea.

**Step 45.**

Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature. Take out the flowcell from the fridge to let it recover to room temperature.

During this time, prepare the priming mix as follows in a 2 ml Eppendorf tube:

| Content | Volume |
|---|---|
| Nuclease-free water | 576 |
| RPF | 624 |
| **Total** | 1200 |

**Step 46.**

Place on magnetic rack, allow beads to pellet and pipette off supernatant.

**Step 47.**

Add 140 µl of the Adapter Bead Binding buffer to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. Repeat.

NOTES
**Yiheng Hu** 09 Jan 2018

Sometimes the beads will not come down from the tube walls easily, just flick harder.

In the second step, be very careful when pipetting out the remaining ABB. Before pipetting, spin the tube once again, try to pipette everything out. The remaining beads do not need to be dried.

**Step 48.**

Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer by flicking. Incubate for 10 minutes at room temperature.

NOTES
**Yiheng Hu** 09 Jan 2018

If experienced, this time can be utilized to do the priming and prepare the library for loading. Check content in step 52 and 53.

**Step 49.**

Pellet beads on magnet until the eluate is clear and colourless, Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Store on ice.

## Priming flowcell

**Step 50.**

Take out the flowcell from the box, flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

**Step 51.**

After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):

Set a P1000 pipette to 200 µl;

Insert the tip into the priming port;

Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

Visually check that there is continuous buffer from the priming port across the sensor array.

NOTES

**Yiheng Hu** 09 Jan 2018

**Important: Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by** buffer **at all times. Removing more than 20-30 µl risks damaging the pores in the array.**

**Step 52.**

Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

Pipette 830 ul of the priming mix, before putting the tip on the priming port, slightly drop a little of the liquid to cover the port and attach the tip to the port. Slowly pipette the priming mix out but DO NOT pipette out everything. Remaining the last few (20ul) of priming mix in the tip and take off the tip straightway. This is to make sure no air goes into the port.

There is a video demo showed how to load a flowcell, check it before the first time doing it. It may slightly different from what I wrote above, so just find a way that suites you the best. The only key is to avoid air bubble.

1. straight copied from ONT official protocol.
2. another one in the extenal link

LINK:
https://www.youtube.com/watch?v=CC11Jlydqrc

## Loading DNA library

**Step 53.**

Thoroughly mix the contents of the RBF and LLB tubes by pipetting, Prepare the library for loading as follows:

| Reagent | Volume |
|---|---|
| RBF | 35.0 µl |
| LLB | 25.5 µl |
| Nuclease-free water | 2.5 µl |
| DNA library | 12 µl |
| **Total** | **75.0 µl** |

**Step 54.**

Complete the flow cell priming:

1.Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

2. Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. The same idea, pipette 230ul of the priming mix and be careful as the previous priming step.

NOTES
**Yiheng Hu** 09 Jan 2018

Here you can see the liquid come up and down from the SpotON sample port.

**Step 55.**

Mix the prepared library gently by pipetting up and down just prior to loading.

**Step 56.**

Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

NOTES
**Yiheng Hu** 09 Jan 2018

DO NOT touch the port using the tip.

**Step 57.**

After the last drop was absorbed, quickly replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

NOTES
**Yiheng Hu** 09 Jan 2018

This step is better to perform immediately after the drops are absorbed to avoid air from the area of bung to be pressed down when replace the SpotON port cover.

**Step 58.**

Congrats! All done! Now you can double chek everthing is selected corectly and press the excute bttom .

NOTES
**Yiheng Hu** 09 Jan 2018

Kit is SQK-LSK108

I normally do not do live basecalling.

I also note down how many pores avalaible after mux scan, and compare that with the initial

platform QC.

## Warnings

There will be a usage of Ethanols so make sure you have the safety paperwork for it.