Native Barcoding of amplicons (96 well plates)

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

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ABSTRACT

The following protocol is for the preparation of amplicons for sequencing using the Oxford Nanopore Native barcoding kit version 14 with 96 barcodes (SQK-NBD114.96). When using this kit, you must ensure to use the appropriate flow cell (FLO-MIN114, R10.4.1) otherwise you will face difficulty when starting the run and sequencing results will not be accurate.

We provide estimates of ng of material required for this protocol when amplifying the VP1 region of polio, the capsid region (PanEV) or the whole genome (PanPV). If you have amplicons of a different size to these, you can calculate the ng required using an online calculator such as this: Weight to Molar Quantity (for nucleic acids) (bioline.com)

GUIDELINES

If you have long fragments, reduce pipetting (and potential shearing) by carefully flicking or inverting tubes or plates to mix reactions and spinning down in a centrifuge.

MATERIALS

- Ultrapure Distilled, Nuclease Free Water **Contributed by users**
- Agencourt AmPure XP beads **Contributed by users** **Catalog #A63880**
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #E6056S**
- NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs** **Catalog #E7546L**
- Native barcoding kit (96) **Oxford Nanopore Technologies** **Catalog #SQK-NBD114.96**
- NEB Blunt/TA Ligase Master Mix **Contributed by users** **Catalog #M0367**
- Ultrapure BSA **Ambion** **Catalog #AM2616**
- Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies** **Catalog #FLO-MIN114**

BEFORE START INSTRUCTIONS

Prepare about 40mL of fresh 80% Ethanol before starting. This should be enough for the whole protocol.

Preparing input DNA

1 Allow AMPure beads to warm to room temperature then add 0.8x the PCR reaction mix volume to the reaction and pipette gently to mix.

I.e. for a 25uL reaction add 15uL of resuspended beads

1.1 Incubate on a rotator at room temperature for 5 minutes

1.2 Spin down and place on a magnetic rack until clear and colourless, then pipette off the supernatant.

1.3 Whilst on the magnet wash the beads with 100uL of 80% ethanol, remove ethanol, and repeat.

1.4 Spin down then place the tube back on the magnet and remove any residual ethanol. Allow to air dry for about 30s

1.5 Remove the tube from the magnetic rack and resuspend the pellet in 20uL nuclease free water and incubate at room temperature for 2 minutes.

1.6 Pellet the beads on a magnet and retain 20uL (or as much as possible) of each sample in a fresh 96-well plate.

2 Quantify the amplified DNA with a Qubit Broad Range dsDNA kit.

2.1 In brief, create a master mix of 200 µl (199 µl buffer, 1 µl Qubit reagent) for each sample + 2 standards + 10 %. For the two standards, add 190 µl master mix to 10 µl of standard, and for samples add 198 µl master mix to 2 µl sample. Vortex each sample or standard once they are added to the master mix and incubate for 2 minutes before quantifying.

2.2 *Important- be sure to select the broad range kit on the Qubit*
Record the concentration of DNA for each sample

3 Transfer 200 fmol of DNA per sample into a clean 96-well plate and adjust volume to 12.5 µl with nuclease free water. Mix gently by pipetting.
200 fmol = ~155ng VP1, ~584ng PanEV, ~974ng PanPV

4 Once aliquoted, spin down briefly in a plate centrifuge.

End-prep & dA-tailing

5 Prepare the following reaction mix per sample plus 10 %:

	A	B
	Reagent	Volume (µl)
	Ultra II End-prep reaction buffer	1.75
	Ultra II End-prep enzyme mix	0.75

6 Add 2.5 µl of the reaction mix to each sample.

7 Mix well by pipetting, and spin down in a plate centrifuge.

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

Ligation of Barcodes and pooling

9 Select a unique native barcode for each sample in the run and defrost at room temperature, and place them on ice.

10 Get AMPure XP beads out and warm to room temperature. Mix the AMpure XP beads by vortexing before use

11 In each required well of a 96 well plate, add:

	A	B
	Reagent	Volume (µl)

	A	B
	End-prepped DNA	3.75
	Native barcode	1.25
	Blunt/TA Ligase Master Mix	5

12 Mix gently by pipetting, seal the plate and spin down.

13 Incubate plate for 20 minutes at room temperature

14 Add 1ul EDTA to each well, mix thoroughly by pipetting and spin down

15 Pool all barcoded samples into a single 1.5ml tube

16 Prepare the AMPure XP beads for use; resuspend by vortexing.

17 Clean and concentrate the pool by performing an AMPureXP bead clean using a volume of beads 0.4x the total sample pool volume.

17.1 Add the AMPureXP beads, pipette to mix then incubate for 10 minutes at room temperature on rotator mixer

17.2 Spin down the sample then place on a magnet, allow beads to pellet until the eluate is clear and colourless then remove the superantant

17.3 Still on the magnet, wash the beads with 700uL of 80% Ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard, then repeat.

17.4 Spin down the tube, place back on the magnet then remove any residual ethanol and allow the pellet to dry for about 30 seconds

17.5 Resuspend the pellet in 35uL of nuclease free water by gently flicking the tube.

17.6 Incubate the tube for 10 minutes at 37 degrees, flicking the tube every 2-3 minutes to aid DNA elution

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

18.1 Remove and retain 35µL of eluate (the library) in a clean 1.5mL Eppendorf DNA LoBind tube

Adapter Ligation

19 Thaw and prepare the Kit reagents as follows: Thaw NEB Quick Ligation Reaction Buffer (5X), Elution Buffer (EB), and either one tube of Long Fragment Buffer (LFB) for enriching fragment >3kb or one tube of Short Fragment Buffer (SFB) for keeping all fragment sizes at room temperature, mix by vortexing, spin down and place on ice. Spin down Native Adapter (NA) and NEB Quick T4 Ligase, mix by pipetting and place on ice.

20 To the 30uL of pooled barcoded DNA sample, add the following reagents:

	A	B
	Native Adapter (NA)	5uL
	NEBNext Quick LIgation Reaction Buffer (5x)	10uL
	NEB Quick T4 Ligase	5uL

21 Mix the reaction by flicking the tube then spinning down briefly

22 Incubate the reaction for 20 minutes at room temperature.

23 Resuspend AMPure XP beads by vortexing then add 20uL to the ligation reaction, mix by pipetting.

23.1 Incubate for 10 minutes at room temperature on a rotator

23.2 Spin down the sample then place on a magnetic rack to allow beads to pellet, then pipette off the supernatant

23.3 Wash the beads by adding 125uL of the LFB or SFB, flick the tube to resuspend the beads, spin down then place back on the magnet to pellet the beads. Pipette off the supernatant and repeat step

23.4 Spin down the tube and place back on the magnet. Pipette off any residual buffer and air dry for about 30 seconds.

23.5 Remove the tube from the magnet and add 15uL of Elution Buffer (EB) then fick the tube gently to resuspend the pellet

23.6 Incubate at 37 degrees for 10minutes, gently flicking the tube every 2-3 minutes to encourage DNA elution.

23.7 Spin down then place the tube on the magnet. Remove and retain 15uL of the eluate containing the DNA library into a clean 1.5ml tube. Discard the pelleted beads.

24 Quantify 1uL of your DNA library using the Qubit or tapestation

25 Transfer 20fmol of your library into a 0.2ml PCR tube and make up to 12uL using elution buffer. If you need to dilute the library for easier pipetting, you can dilute it in elution buffer.
20fmol = ~16ng VP1, ~59ng PanEV, ~98ng PanPV

Priming and loading the MinION Flowcell

26 Thaw the Sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature then place on ice.

Mix the SB, FCF, and FCT by vortexing, spin down, and return to ice.
Spin down the LIB once thawed then return to ice.

27 To create the priming mix, add 30µL of FCT and 5uL of BSA (50mg/ml) to the tube of FCF then mix by pipetting to create the priming mix

28 Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible. After opening the priming port, check for any bubbles under the cover. Draw back a small volume to remove any bubbles (a few µLs). Visually check that there is continuous buffer from the priming port across the sensor array.

29 Using a P1000 pipette, slowly load 800µL of the priming mix into the flow cell via the priming

port.

Leave a small amount of liquid in the end of the pipette tip to ensure you do not introduce air into the flowcell.

Leave for 5 minutes.

30 Mix the contents of the LIB tube by pipetting just before adding to the following library mix in a 1.5ml tube:

	A	B
	Reagent	Volume (µL)
	DNA library	12
	Sequencing buffer (SB)	37.5
	Library beads (LIB)	25.5

31 Complete the flowcell priming by opening the SpotOn port cover and carefully loading 200µL of the priming mix into the **priming port.** As before, leave a small amount of liquid in the bottom of the tip to avoid the introduction of air bubbles.

When adding the priming mix, you may see a small amount of liquid come up through the SpotOn port. If you do, pause and allow the liquid to flow back into the flowcell before continuing putting through the priming mix.

32 Mix the prepared library mix gently by pipetting.

Add the library mix to the flowcell via the SpotOn port in a dropwise fashion, allowing each drop to flow into the flowcell before adding the next.

33 Replace the SpotOn port cover and close the priming port, then replace the lid of your sequencing device.

34 Open the ONT MinKNOW software and follow the steps to set up and start your sequencing run.

In the Start section, select start run and follow the prompts to select the kit used, set the run time, and set basecalling and demultiplexing.