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# Nanopore Sequencing of Environmental Microbial Metagenomes and Metatranscriptomes Using the Native Barcoding Kit 24 V14

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**We use this protocol and it's working**

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## Abstract

This protocol presents a streamlined workflow for **Nanopore sequencing of environmental biofilm metagenomes and metatranscriptomes** using the **Native Barcoding Kit 24 V14**. It details the DNA/RNA extraction, rRNA depletion, polyadenylation, cDNA synthesis, barcoding, and library preparation steps, optimized for long-read sequencing. The workflow incorporates magnetic bead-based purification and barcode ligation to enable multiplexed sequencing on an Oxford Nanopore Technologies (ONT) platform. Designed to maximize DNA/cDNA yield, minimize purification losses, and ensure high-quality sequencing output, this method is well-suited for studying microbial communities in environments, including deep-sea hydrothermal vents and other extreme habitats.

## Attachments

| _r2002_zymobiomics_d... | ligation-sequencing-... | ligation-sequencing-... | highprep_rna_elite_p... | ribopool-protocol.pd... | Ampure XP beads.pdf |
|---|---|---|---|---|---|
| 351KB | 1.3MB | 1.6MB | 54KB | 2.2MB | 565KB |

## Guidelines

Using the **Native Barcoding Kit 24 V14**

**Key Considerations**

- **Sample Quality**: Ensure proper collection and storage of environmental samples to prevent degradation.
- **RNA Handling**: Use RNase-free reagents and clean the workspace with RNase AWAY to avoid contamination.
- **Minimizing Sample Loss**: Be mindful of purification steps, as RNA losses can be significant.
- **Bead-Based Purification**: Magnetic bead clean-ups must be performed carefully to maximize yield.
- **Barcode Selection**: Choose unique barcodes (NB01–24) for multiplexed sequencing.
- **Library Preparation**: Follow ONT guidelines for adapter ligation and sequencing setup.

## Materials

- ZymoBIOMICS DNA/RNA Miniprep Kit R2002 (Zymo Research)
- DNase I E1010 (Zymo Research)
- AMPure XP Beads (Beckman Coulter)
- HighPrep RNA Elite RC-90005 (MagBio)
- Pan-Prokaryote riboPOOL Kit (siTOOLs Biotech)
- Qubit RNA HS Assay Kit (Thermo Fisher)
- Qubit RNA IQ Assay Kit (Thermo Fisher)
- dNTP Mix (10 mM each) R0193 (Thermo Fisher)
- RNase Cocktail Enzyme Mix AM2286 (Thermo Fisher)
- RNaseOUT Recombinant Ribonuclease Inhibitor 10777019 (Thermo Fisher)
- ATP Solution (10 mM) PV3227 (Thermo Fisher)
- Maxima Reverse Transcriptase (200 U/µL) EP0741 (Thermo Fisher)
- UltraPure BSA (50 mg/mL) (Thermo Fisher)
- VN Primer (2 µM) (/5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN)
- Strand-switching Primer (10 µM) (TTTCTGTTGGTGCTGATATTGCTmGmGmG)
- PR2 Primer (10 µM) (/5Phos/TTTCTGTTGGTGCTGATATTGC)
- E. coli Poly(A) Polymerase M0276S (New England Biolabs)
- Blunt/TA Ligase Master Mix M0367S (New England Biolabs)
- NEBNext Quick Ligation Reaction Buffer B6058S (New England Biolabs)
- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing E7180S (New England Biolabs)
- Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies)
- ONT Flow Cell R10.4.1 (Oxford Nanopore Technologies)

## Safety warnings

The loss of RNA from extraction to cDNA synthesis is very high (~98.11%). Start with **≥1000 ng of total RNA per sample** to retain enough cDNA for sequencing.

**Safety**

- Use PPE when handling chemicals and biological samples.
- Follow the latest version of protocols on **The Nanopore Community** for the best library prep and sequencing results.

## Before start

Clean the workbench and all the equipment with **RNase AWAY®** before starting any work.

## DNA/RNA extraction using ZymoBIOMICS DNA/RNA Miniprep Kit

33m 30s

1 In a **ZR BashingBead Lysis Tube (0.1 & 0.5 mm)**, add up to 250 mg of biofilm sample and 750 µL DNA/RNA Shield. Ensuring a total volume of approximately 1000 µL per tube. 1000 µL

5m

2 Close the tube caps tightly. Homogenize the sample using a Homogenizer Bead Mill or FastPrep-24 at a speed of 6 m/s for two rounds, each lasting 1 minute. Allow the sample tubes to cool On ice for 5 minutes between rounds.

10m

3 Centrifuge at 16000 x g, 00:00:30

30s

4 Carefully transfer the entire supernatant into a 1.5 mL Eppendorf DNA LoBind tube, ensuring that the pellet remains undisturbed.

2m

5 Add an equal volume of **DNA/RNA Lysis Buffer** to the supernatant at a 1:1 ratio (e.g., 400 µL of Lysis Buffer for 400 µL of sample). Mix thoroughly by vortexing, then briefly spin down.

2m

6 Transfer the sample into a **Spin-Away Filter** (yellow) placed in a **Collection Tube**. Centrifuge at 16000 x g, 00:00:30. Carefully transfer the **flow-through** to a clean 1.5 mL Eppendorf DNA LoBind tube for **RNA** collection, and retain the filter for DNA purification.

30s

7 Repeat the previous step until the entire mixture from **Step 5** has been filtered.

10m

STEP CASE

## Metatranscriptomic sequencing 153 steps

## RNA extraction

55m 30s

8 Add an equal volume of absolute ethanol to the flow-through and mix thoroughly by pipetting.

3m

9 Transfer the mix into a **Zymo-Spin IIICG Column** (green) placed in a **Collection Tube**. Centrifuge at 16000 rpm, 00:00:30 . Discard the flow-through. If necessary, reload the column and repeat the centrifugation. 30s

9.1 **DNase I Treatment in-column** 30s

Add 400 µL DNA/RNA Wash Buffer to the column. Centrifuge at 16000 x g, 00:00:30 . Discard the flow-through

9.2 Prepare the following mix in a clean Eppendorf tube, adjusting the quantities based on the number of columns you use. 2m

| | Reaction mix | Volume |
|---|---|---|
| | DNase I reconstituted (1U/uL) | 5 µL |
| | DNA digestion buffer | 75 µL |
| | **Total volume per reaction** | **80 µL** |

Mix by gentle inversion, then briefly spin down. **Do not vortex**.

9.3 Add 80 µL DNase I reaction Mix (from step 9.2) directly into the column matrix. Incubate at Room temperature for 00:15:00 15m

10 Add 400 µL DNA/RNA Prep Buffer to the column. Centrifuge at 16000 rpm, 00:00:30 . Discard the flow-through 30s

11 Add 700 µL DNA/RNA Wash Buffer to the column. Centrifuge at 16000 rpm, 00:00:30 . Discard the flow-through 30s

12 Add 400 µL DNA/RNA Wash Buffer to the column. Centrifuge at 16000 x g, 00:02:00 . Carefully transfer the column into a new nuclease-free Eppendorf tube. 2m

13 Add 100 µL ZymoBIOMIC DNase/Rnase-Free Water directly to the column matrix. Incubate at Room temperature for 00:05:00 . Centrifuge at 5m 30s

16000 x g, 00:00:30 to elute RNA from the column.

Note

Alternatively, for highly concentrated RNA, use ≥50 mL elution

14 Place a **Zymo-Spin III-HRC Filter** in a Collection Tube. Add 600 µL ZymoBIOMICS HRC Prep Solution. Centrifuge at 8000 x g, 00:03:00. 3m

15 Transfer the eluted RNA from **Step 13** into a prepared **Zymo-Spin III-HRC Filter** (**Step 14**) placed in a nuclease-free Eppendorf DNA LoBind tube. Centrifuge at 16000 x g, 00:03:00. 3m

Note

The filtered RNA can be used immediately or stored frozen at -80 °C.
Quantify 1 µL of the eluted sample using Qubit fluorometer (Qubit RNA HS).

## Total RNA Purification using HighPrep RNA Elite Beads 49m 30s

16 Thoroughly shake the **HighPrep RNA Elit**e reagent before use. 1m

Note

Allow the **HighPrep RNA Elite** reagent to reach Room temperature for at least 00:30:00 before use.

17 Transfer the total RNA sample into a clean 1.5 mL Eppendorf tube. 1m

18 Add the appropriate volume of **HighPrep RNA Elite** reagent based on the volume of your total RNA sample. 2m

| | Volume of RNA (µL) | Volume of HighPrep RNA Elite 1.8X (µL) |
|---|---|---|
| | 10 | 18 |
| | 50 | 90 |
| | 100 | 180 |

19 Mix thoroughly by pipetting up and down 6-8 times. 1m

20 Incubate on a Hula mixer for 00:05:00 at Room temperature . 5m

21 Place the mixture on a magnetic separation device for 00:15:00 or until the solution becomes clear. 15m

22 While keeping the Eppendorf tube **on the magnet**, carefully pipette off and discard the supernatant. 1m

23 With the tube **on the magnet**, add 200 µL 70% ethanol to the Eppendorf tube. 30s
Incubate at Room temperature for 00:00:30

24 While keeping the Eppendorf tube **on the magnet**, carefully pipette off and discard the supernatant. 1m

Note

If the pellet is disturbed, allow the beads to settle again before removing the ethanol.

25 Repeat steps 23-24 for **three** 70% ethanol **washes**. 5m

26 Dry the beads by incubating the Eppendorf tube **on the magnetic separation device** at Room temperature for up to 00:10:00 . 10m

Note

Ensure that all traces of ethanol are completely removed. However, avoid overdrying the beads, as a cracked dry pellet may reduce the yield.

27 Remove the tube from the magnetic separation device. Add 40 µL elution buffer (e.g., nuclease-free water) to the reaction tube and mix by pipetting up and down. 1m

Note

Prewarming the nuclease-free water to 55 °C can help increase the yield.

28 Incubate the tube on a Hula mixer for 00:02:00 at Room temperature . 2m

29 Briefly spin down the tube. Place it back on the magnetic separation device and wait for 00:03:00 or until the beads have completely cleared from the solution. 3m

30 Transfer the eluate (cleared supernatant) to a clean 1.5 mL Eppendorf tube for storage or subsequent applications. 1m

Note

Quantify 1 µL of the eluted sample using Qubit fluorometer (Qubit RNA HS).

## rRNA depletion using riboPOOL Kit 59m 30s

31 Add the following reagent to a 1.5 mL Eppendorf tube 2m

| | Reagent | Volume |
|---|---|---|
| | Hybridization Buffer (HB) | 5 µL |
| | Resuspended RP | 1 µL |
| | RNA sample (containing 100ng - 5 ug of total RNA) | 14 µL |
| | RNaseOUT™ | 0.5 µL |
| | **Total** | **20.5 µL** |

32 Vortex thoroughly and briefly spin down. 30s

33 Incubate at 68 °C for 00:10:00 to denature RNA. 10m

34 Allow the sample to cool gradually from 68 °C to 37 °C for optimal hybridisation. 15m

Note

To achieve this, turn off the heating block and allow the temperature to naturally decrease to 37°C. If using temperature-controlled ramping, cool down at a rate of 3°C per minute.

35 Resuspend the **streptavidin-coated magnetic beads (SMB)** by carefully vortexing the tube at medium speed. 30s

36 Transfer 90 µL of bead suspension per sample into a fresh tube. 30s

Note

For batch washing of beads for multiple samples, aliquot the bead suspension into a single tube: use up to 540 µL for 6 samples or 1080 µL for 12 samples.

37 Place the tube containing **SMB beads** on a magnetic rack and wait for 00:01:00 . 1m

38 Remove and discard the supernatant carefully. 1m

39 Add 80 µL of **Depletion Buffer (DB)** per sample (e.g., 480 µL for 6 samples and 960 µL for 12 samples). Agitate the tube well to fully resuspend the beads. 1m

40 Repeat **Steps 37-39**. 3m

41 Briefly centrifuge the tube containing 20.5 µL of hybridized riboPOOL and total RNA (from **Step 34**). 30s

42 Pipette 80 µL of the prepared beads (from **Step 40**) into the tube containing hybridised riboPOOL-RNA solution. Agitate the tube thoroughly to ensure proper resuspension. 1m

43 Incubate the tube at 37 °C for 00:15:00 , followed by 50 °C for 00:05:00 in a thermal cycler. 20m

44 Briefly spin down to collect any droplets. 30s

45 Place on the magnet for 00:02:00 . Then, carefully transfer the supernatant to a new 1.5 mL Eppendorf tube. 2m

46 Place the tube on the magnet for 00:01:00 to remove any trace amounts of beads (optional).
Carefully transfer the supernatant to a new tube. 1m

Note

At this stage, the elution should primarily contain **mRNA**. The RNA concentration is expected to decrease by approximately **90%** from the initial amount due to the depletion of rRNA from the total RNA sample.

## mRNA Purification using HighPrep RNA Elite

50m

47 go to step #16 Follow the same protocol to **purify total RNA** precisely (from **Step 16** to **Step 30**). The volume of elution buffer (**Step 27**) can be adjusted to 30 µL to achieve a higher RNA concentration.

Note

Quantify 1 µL of the eluted RNA using Qubit fluorometer (Qubit RNA HS)
Qualify 1 µL of the eluted RNA using either Bioanalyzer RNA 6000 Pico Kit (Agilent) or Qubit fluorometer (Qubit RNA IQ)

## Polyadenylating RNA with *E. coli* poly(A) polymerase

39m 30s

48 In a 1.5 mL Eppendorf tube, set up a 3′ polyadenylation reaction as follows

2m

| | Reagent | Volume |
|---|---|---|
| | Non-polyadenylated RNA (from step 47) | X µL (e.g., 10 µL) |
| | 10X E. coli Poly(A) Polymerase Reaction Buffer | 2 µL |
| | ATP (10mM) | 2 µL |
| | E. coli Poly(A) Polymerase | 1 µL |
| | Nuclease-free water | 14.5 µL - X µL (e.g., 14.5 - 10 = 4.5 µL) |
| | RNaseOUT™ | 0.5 µL |
| | **Total** | **20 µL** |

Gently agitate by pipetting up and down. **Do not vortex**.

49 Incubate reaction at 37 °C for 00:10:00 . 10m

50 Stop the reaction by adding 5 µL of [M] 50 millimolar (mM) EDTA (to achieve a final concentration of [M] 10 millimolar (mM) EDTA ). The final reaction volume will be 25 µL . 30s

51 Add 45 µL of resuspended **HighPrep RNA Elite Beads** to the reaction tube. 30s

52 Incubate on a Hula mixer for 00:05:00 at Room temperature 5m

53 Spin down the sample, then pellet it using a magnetic separation device. **Keep the tube on the magnet**, carefully pipette off and discard the supernatant. 5m

54 Keep the tube **on the magnet** and wash the beads with 200 µL of freshly prepared 70% ethanol. Gently rotate the tube 180° twice in the rack to ensure thorough washing of the pelleted beads. Carefully pipette off and discard the supernatant. 2m

55 Repeat **Step 54** to perform a total of two washes. 2m

56 Briefly spin down the tube and place it back **on the magnet**. Carefully pipette off any residual ethanol. Allow the beads to dry for 00:00:30 , ensuring the pellet does not over-dry or crack. 30s

57 Remove the tube from the magnetic rack and resuspend the pellet in 12 µL of nuclease-free water. Incubate On ice for 00:05:00 . 5m

58 Place the tube **on the magnet** until the beads pellet and the eluate becomes clear and colourless. 5m

59 Carefully remove and retain 12 µL of the eluate containing the 3′-polyadenylated RNA in a clean 1.5 mL Eppendorf tube. 2m

Note

Quantify 1 µL of the eluted sample using Qubit fluorometer (Qubit RNA HS)

## Reverse transcription and strand-switching

2h 4m

60 Prepare the following reaction in the 0.2 mL PCR tube: 2m

| | Reagent | Volume |
|---|---|---|
| | RNA (100 ng Poly(A)+ RNA) | 7.5 µL |
| | VN Primer diluted to 2 uM | 2.5 µL |
| | 10 mM dNTPs | 1 µL |
| | **Total** | **11 µL** |

61 Mix gently by flicking the tube, then briefly spin down. 30s

62 Incubate at 65 °C for 00:05:00 and then snap cool on a pre-chilled freezer block for 00:01:00 6m

63 In a separate tube, mix the following: 2m

| | Reagent | Volume |
|---|---|---|
| | 5x RT Buffer | 4 µL |
| | RNase OUT | 1 µL |
| | Nuclease-free water | 1 µL |
| | Strand-switching Primer diluted to 10 uL | 2 µL |
| | **Total** | **8 µL** |

63.1 Mix gently by flicking the tube, and spin down. 30s

64 Add 8 µL of the **strand-switching reagents** (prepared in **Step 63**) to the 11 µL of snap-cooled mRNA (from **Step 62**). Mix gently by flicking the tube, then briefly spin down. 1m

65 Incubate at 42 °C for 00:02:00 in the thermal cycler. 2m

66 Add 1 µL of **Maxima H Minus Reverse Transcriptase**, bringing the total volume to 20 µL .

67 Mix gently by flicking the tube, and spin down.

68 Incubate using the following protocol using a thermal cycler: 1h 50m

| | Cycle step | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| | Reverse transcription and strand-switching | 42°C | 90 mins | 1 |
| | Heat inactivation | 85°C | 5 mins | 1 |
| | Hold | 4°C | ∞ | |

## RNA degradation and second-strand synthesis 1h 36m 30s

69 Add 1 µL of **RNase Cocktail Enzyme Mix** to the reverse transcription reaction (end product from **Step 68**). 1m

70 Incubate the reaction for 00:10:00 at 37 °C in the thermal cycler. 10m

71 Resuspend **the AMPure XP beads (AXP)** by vortexing thoroughly. 1m

72 Transfer the sample to a clean 1.5 mL Eppendorf DNA LoBind tube. 1m

73 Add 17 µL of resuspended **AMPure XP beads (AXP)** to the reaction and mix gently by flicking the tube. 1m

74 Incubate on a Hula mixer for 00:05:00 at Room temperature. 5m

75 Spin down the sample, then pellet it using a magnetic separation device until the supernatant is clear and colourless. Keep the tube **on the magnetic** device and carefully pipette off the supernatant. 5m

76 Keep the tubes on the magnetic device and wash the beads with 200 µL of freshly prepared 80% ethanol, ensuring the pellet remains undisturbed. Carefully remove and discard the ethanol using a pipette. 2m

Note

If the pellet is disturbed, allow the beads to re-pellet before removing the ethanol.

77 Repeat **Step 76** for a total of two washes. 2m

78 Spin down the tube and place it back on the magnetic device. Carefully pipette off any residual ethanol. Allow the beads to dry for approximately 00:00:30, ensuring the pellet does not over-dry or crack. 30s

79 Remove the tube from the magnetic rack and resuspend the pellet in 20 µL nuclease-free water. 1m

80 Incubate on a Hula mixer for 00:10:00 at Room temperature 10m

81 Briefly spin down the tube, then pellet the beads on the magnet until the eluate is clear and colourless, for at least 00:01:00 1m

82 Carefully remove and retain 20 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube. 1m

83 Prepare the following reaction in a 0.2 mL thin-walled PCR tube: 3m

| | Reagent | Volume |
|---|---|---|
| | 2x LongAmp Taq Master Mix | 25 µl |
| | PR2 Primer diluted to 10 µM | 2 µl |
| | Reverse-transcribed sample from above (Step 82) | 20 µl |
| | Nuclease-free water | 3 µl |
| | **Total** | **50 µl** |

Mix gently by flicking the tube

84 Incubate using the following protocol in a thermal cycler: 20m

| | Cycle step | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| | Denaturation | 94 °C | 1 min | 1 |
| | Annealing | 50 °C | 1 min | 1 |
| | Extension | 65 °C | 15 mins | 1 |
| | Hold | 4 °C | ∞ | |

85 Resuspend **the AMPure XP beads (AXP)** by vortexing thoroughly. 1m

86 Transfer the sample (end product of **Step 84**) to a clean 1.5 mL Eppendorf DNA LoBind tube. 1m

87 Add 40 µL of resuspended **AMPure XP beads (AXP)** to the reaction and mix gently by flicking the tube. 1m

88 Incubate on a Hula mixer for 00:05:00 at Room temperature . 5m

89 Spin down the sample, then pellet it using a magnetic separation device until the supernatant is clear and colourless. Keep the tube **on the magnetic** device and carefully pipette off the supernatant. 5m

90 Keep the tubes **on the magnetic device** and wash the beads with 200 µL of freshly prepared 80% ethanol, ensuring the pellet remains undisturbed. Carefully remove and discard the ethanol using a pipette. 2m

> Note
>
> If the pellet is disturbed, allow the beads to re-pellet before removing the ethanol.

91 Repeat **Step 90** for a total of two washes. 2m

92 Spin down the tube and place it back **on the magnetic device**. Carefully pipette off any residual ethanol. Allow the beads to dry for approximately 00:00:30, ensuring the pellet does not over-dry or crack. 1m

93 Remove the tube from the magnetic rack and resuspend the pellet in 21 µL nuclease-free water. 1m

94 Incubate on a Hula mixer for 00:10:00 at Room temperature. 10m

95 Briefly spin down the tube, then pellet the beads on the magnet until the eluate is clear and colourless, for at least 00:01:00. 1m

96 Carefully remove and retain 21 µL of eluate in a clean 1.5 mL Eppendorf DNA LoBind tube. 2m

> Note
>
> Quantify 1 µL of the eluted sample using Qubit fluorometer (Qubit DNA HS)

## cDNA repair and end-prep 34m 30s

97 Combine the following reagents in a 0.2 m PCR tube: 3m

| | Reagent | Volume |
|---|---|---|
| | cDNA sample (Step 96) | 20 µl |
| | Nuclease-free water | 30 µl |
| | Ultra II End-prep reaction buffer | 7 µl |
| | Ultra II End-prep enzyme mix | 3 µl |
| | **Total** | **60 µl** |

Note

Ensure the reaction is thoroughly mixed by gently pipetting up and down, then briefly spin down.

98 Using a thermal cycler, incubate at 20 °C for 00:05:00, followed by 65 °C for 00:05:00. 10m

99 Resuspend the **AMPure XP Beads (AXP)** by vortexing thoroughly. 1m

100 Transfer the sample (**Step 98**) to a clean 1.5 mL Eppendorf DNA LoBind tube. 1m

101 Add 60 µL of resuspended **AMPure XP Beads (AXP)** to the end-prep reaction (**Step 100**) and mix gently by flicking the tube. 2m

102 Incubate on a Hula mixer for 00:05:00 at Room temperature. 5m

103 Spin down the sample, then pellet it using a magnetic separation device until the supernatant is clear and colourless. Keep the tube **on the magnetic** device and carefully pipette off the supernatant. 5m

104 Keep the tubes **on the magnetic device** and wash the beads with 200 µL of freshly prepared 80% ethanol, ensuring the pellet remains undisturbed. Carefully remove and discard the ethanol using a pipette. 2m

Note

If the pellet is disturbed, allow the beads to re-pellet before removing the ethanol.

105 Repeat **Step 104** for a total of two washes 2m

106 Spin down the tube and place it back **on the magnetic device**. Carefully pipette off any residual ethanol. Allow the beads to dry for approximately 00:00:30, ensuring the pellet does not over-dry or crack. 30s

107 Remove the tube from the magnetic rack and resuspend the pellet in 20 µL nuclease-free water. Incubate for 00:02:00 at room temperature. 2m

108 Pellet the beads on a magnetic rack until the eluate is clear and colourless, for at least 00:01:00 1m

109 Remove and retain 20 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

Note

Quantify 1 µL of the eluted sample using a Qubit fluorometer (Qubit DNA HS)
At this point, cDNA can be stored at -20 °C for up to a month or at -80 °C for longer storage.

## Native barcode ligation 1h 6m 30s

110 For each sample to be run together on the same flow cell, select a unique barcode from the **Native Barcodes (NB01-24)**. 1m

111 In clean 0.2 mL PCR tubes, add the reagents in the following order per tube: 2m

| | Reagent | Volume |
|---|---|---|
| | End-prepped DNA (from Step 109) | 7.5 µl |

| | Reagent | Volume |
|---|---|---|
| | Native Barcode (NB01-24) | 2.5 µl |
| | Blunt/TA Ligase Master Mix | 10 µl |
| | **Total** | **20 µl** |

Note

Thoroughly mix the reaction by gently pipetting up and down, then briefly spin down.

112 Incubate for 00:20:00 at Room temperature . 20m

113 Add the appropriate volume of EDTA to each tube, selecting the correct version based on the bottle cap colour. Mix thoroughly by pipetting, then briefly spin down. 2m

| | EDTA cap colour | Volume per tube |
|---|---|---|
| | For clear cap EDTA | 2 µl |
| | For blue cap EDTA | 4 µl |

EDTA is added at this step to stop the reaction.

Note

The concentration of EDTA may vary depending on the version of your Nanopore reaction kit, as detailed below.

- The EDTA vials with a **clear cap** contain 0.5M EDTA (old version concentration).
- The EDTA vials with a **blue cap** contain 0.25M EDTA (new version concentration).

The new EDTA concentration is indicated in all batch numbers formatted as NBD1424.**20**.0001 and NBD1424.**20**.0002 (or higher). The old format was used in batch numbers following the format NBD1424.**10**.0001.

114 **Pool** all the barcoded samples in a 1.5 mL Eppendorf DNA LoBind tube. 2m

| | - | Volume per sample | For 6 samples | For 12 samples | For 24 samples |
|---|---|---|---|---|---|
| | Total volume of preps using clear cap EDTA | 22 µl | 132 µl | 264 µl | 528 µl |
| | Total volume of preps using blue cap EDTA | 24 µl | 144 µl | 288 µl | 576 µl |

Note

**IMPORTANT:**
Ensure that the liquid volumes at the base of all tubes or wells in the plate are consistent before and after pooling to confirm that all liquid has been transferred correctly.

115 Resuspend the **AMPure XP Beads (AXP)** by vortexing. 1m

116 Add **AMPure XP Beads (AXP)** to the pooled reaction at a 0.4X ratio and mix thoroughly by pipetting. 1m

| | - | Volume per sample | For 6 samples | For 12 samples | For 24 samples |
|---|---|---|---|---|---|
| | Volume of AXP for preps using clear cap EDTA | 9 µl | 53 µl | 106 µl | 211 µl |
| | Volume of AXP for preps using blue cap EDTA | 10 µl | 58 µl | 115 µl | 230 µl |

117 Incubate on a Hula mixer for 00:10:00 at Room temperature 10m

118 Spin down the sample, then pellet it using a magnetic separation device until the supernatant is clear and colourless. Keep the tube **on the magnetic** device and carefully pipette off the supernatant. 5m

119 Keep the tubes **on the magnetic device** and wash the beads with 700 µL of freshly prepared 80% ethanol, ensuring the pellet remains undisturbed. Carefully remove and discard the ethanol using a pipette. 2m

Note

If the pellet is disturbed, allow the beads to re-pellet before removing the ethanol.

120 Repeat **Step 119** for a total of two washes 2m

121 Spin down the tube and place it back **on the magnetic device**. Carefully pipette off any residual ethanol. Allow the beads to dry for approximately 00:00:30, ensuring the pellet does not over-dry or crack. 30s

122 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water by gently flicking. 1m

123 Incubate for 00:10:00 at 37 °C. Every 00:02:00, gently flick the sample for 00:00:10 to promote DNA elution. 10m

124 Pellet the beads **on a magnetic rack** until the eluate is clear and colourless. 5m

125 Carefully remove and retain 35 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube. 2m

Note

Quantify 1 µL of the eluted sample using a Qubit fluorometer (Qubit DNA HS)

## Adapter ligation and clean-up

1h 8m

126 Thaw all reagents at Room temperature. Mix gently by pipetting, then briefly spin down. Place all reagents On ice. 3m

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

| | Reagent | Volume |
|---|---|---|
| | Pooled barcoded sample | 30 µL |
| | Native Adapter (NA) | 5 µL |
| | NEBNext Quick Ligation Reaction Buffer (5X) | 10 µL |
| | Quick T4 DNA Ligase | 5 µL |
| | **Total** | **50 µL** |

Note

**IMPORTANT**
The **Native Adapter (NA)** used in this kit and protocol is not interchangeable with other sequencing adapters.
Do **NOT** vortex the **Quick T4 DNA Ligase**.

127 Thoroughly mix the reaction by gently pipetting up and down, then briefly spin down. 1m

128 Incubate the reaction for 00:20:00 at Room temperature 20m

129 Resuspend the **AMPure XP Beads (AXP)** by vortexing. 1m

130 Add 20 µL of resuspended **AMPure XP Beads (AXP)** to the reaction and mix thoroughly by pipetting. 1m

131 Incubate on a Hula mixer for 00:10:00 at Room temperature 10m

132 Spin down the sample, then pellet it on the magnetic rack. Keep the tube **on the magnet** and carefully pipette off the supernatant. 5m

133 Wash the beads by adding 125 µL **Short Fragment Buffer (SFB)**. Flick the tube to resuspend the beads, then briefly spin down. Return the tube to the magnetic rack and allow the beads to pellet. Carefully remove and discard the supernatant using a pipette. 5m

> Note
>
> **IMPORTANT:**
> Use **Short Fragment Buffer (SFB)** rather than 80% ethanol to wash the beads, as ethanol can be detrimental to the sequencing reaction.

134 Repeat **Step 133** for a total of two washes. 5m

135 Spin down the tube and place it back **on the magnetic rack**. Carefully pipette off any residual supernatant. 2m

136 Remove the tube from the magnetic rack and resuspend the pellet in 15 µL of **Elution Buffer (EB)**. 2m

137 Spin down the tube and incubate for 00:10:00 at 37 °C. Every 00:02:00, gently flick the sample for 00:00:10 to promote DNA elution. 10m

138 Pellet the beads **on a magnet** until the eluate is clear and colourless, for at least 00:01:00 1m

139 Carefully remove and retain 15 µL of the eluate containing the DNA library in a clean 1.5 mL Eppendorf DNA LoBind tube. 2m

Note

Quantify 1 µL of the eluted sample using a Qubit fluorometer (Qubit DNA HS)
The prepared library is now ready for loading onto the flow cell. Store the library on ice until it is ready to be loaded.

## Priming and loading on the SpotON flow cell

27m 30s

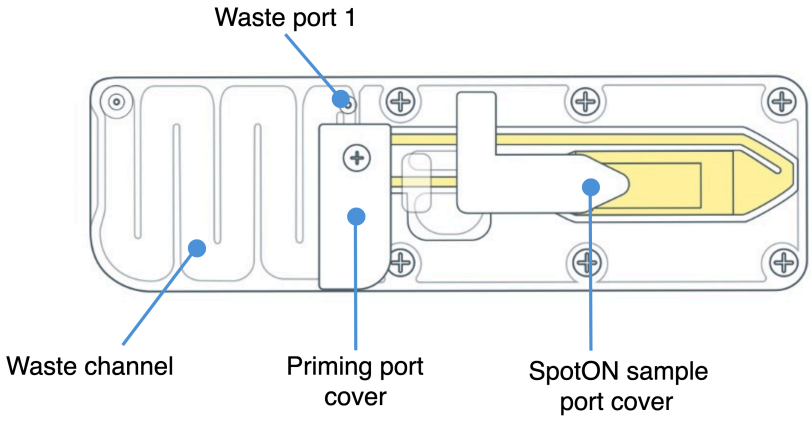
140 In a 1.5 mL Eppendorf LoBind tube, mix in the following order: 2m

| | Reagent | Volume per flow cell |
|---|---|---|
| | Flow cell flush (FCF) | 1170 µL |
| | Bovine serum albumin (BSA) at 50 mg/mL | 5 µL |
| | Flow cell tether (FCT) | 30 µL |
| | **Total** | **1205 µL** |

Note

Mix by pipetting at room temperature

141 Place the flow cell on the sequencing device and follow the manufacturer's instructions for setup and operation. 1m




MinION Flow Cell

141.1 Complete a flow cell check to assess the number of pores available before loading the library. 5m

142 Slide the flow cell priming port cover clockwise to open the priming port. 30s

143 After opening the priming port, check for a small air bubble under the cover. Drawback a small volume to remove any bubbles by following these sub-steps 30s

> Note
>
> Visually check for a continuous buffer from the priming port across the sensor array.

143.1 Set a P1000 pipette to 200µL 30s

143.2 Insert the tip into the priming port 30s

143.3 Turn the wheel until the dial shows 220-230 µL, to draw back 20-30 µL or until you can see a small volume of buffer entering the pipette tip. 30s

Note

**IMPORTANT**
Take care when drawing back thebuffer from the flow cell. **Do not** remove more than **20-30 µl**, and make sure that the array of pores is covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

144 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding introducing air bubbles. Wait for 00:05:00 . During this time, prepare the library for loading by following the steps below. 5m

145 Thoroughly mix the contents of the **Library Beads (LIB)** by pipetting. 1m

146 In a new 1.5 m Eppendorf DNA LoBind tube, prepare the library for loading as follows: 2m

| | Reagent | Volume per flow cell |
|---|---|---|
| | Sequencing Buffer (SB) | 37.5 µL |
| | Library Beads (LIB) mixed immediately before use | 25.5 µL |
| | DNA library | 12 µL |
| | **Total** | **75 µL** |

147 **Complete the flow cell priming:** 30s

147.1 Gently lift the SpotON sample port cover to make the **SpotON** sample port accessible. 30s

147.2 Load 200 µL of the priming mix into the flow cell **priming port** (not the SpotON sample port), avoiding introducing air bubbles. 30s

148 Mix the prepared library gently by pipetting up and down just before loading. 30s

149 Add 75 µL of the prepared library to the flow cell via the **SpotON sample port** in a **dropwise fashion**. Ensure each drop flows into the port before adding the next. 2m

150 Gently replace **the SpotON sample port cover**, ensuring the bung enters the SpotON port. Close the priming port. Place the light shield onto the flow cell. Close the device lid and set up a sequencing run. Start sequencing. 5m

## Protocol references

ZymoBIOMICS DNA/RNA Miniprep Kit: Microbiome DNA and RNA from any sample. Catalog no. R2002
Pan-Prokaryote riboPOOL Kit Manual v1.1 (2019)
HighPrep RNA Elite Manual v1.01. Catalog No. RC-90050
Ligation Sequencing gDNA Native Barcoding V14 SQK-NBD114.24 (2022) ONT
Ligation Sequencing v14: Direct cDNA Sequencing DCS_9187_v114 (2023) ONT
Agencourt AMPure XP Information For Use Guide PN B37419AB (2016)

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