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We are still developing and optimizing this protocol

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Native Barcoding

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ABSTRACT

This protocol describes how to carry out native barcoding of genomic DNA using the Native Barcoding Kit 24 (SQK-NBD112.24). There are 24 unique barcodes available, allowing the user to pool up to 24 different samples in one sequencing experiment. It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

GUIDELINES

This protocol describes how to carry out native barcoding of genomic DNA using the Native Barcoding Kit 24 (SQK-NBD112.24). There are 24 unique barcodes available, allowing the user to pool up to 24 different samples in one sequencing experiment. It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

Native Barcoding Kit 24 features
This kit is recommended for users who:

- wish to multiplex up to 24 samples to reduce price per sample
- need a PCR-free method of multiplexing to preserve additional information such as base modifications
- want to optimise their sequencing experiment for throughput
- require control over read length
- would like to utilise upstream processes such as size selection or whole genome amplification

The Native Barcoding Expansion kit (EXP-NBD112) is available to provide enough reagents for 12 reactions depending on how barcodes are used.

For common issues and concerns regarding the protocol and suggestions to resolve the same, go through this following document, which is also available at the end of the protocol.
Issues while conducting the protocol

MATERIALS

Materials

- Native Barcoding Kit 24 (SQK-NBD112.24)
- 400 ng gDNA per sample for >4 barcodes
- 1000 ng gDNA per sample for ≤4 barcodes

Consumables

- NEB Blunt/TA Ligase Master Mix (M0367)
- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S or E7180L)
- Alternatively to the NEBNext® Companion Module and the NEBNext® Quick Ligation Reaction Buffer, you can use the three NEBNext® products below:

1. NEBNext FFPE Repair Mix (M6630)
2. NEBNext Ultra II End repair/dA-tailing Module (E7546)
3. NEBNext Quick Ligation Module (E6056)

- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- Hula mixer (gentle rotator mixer)
- Microfuge Magnetic rack
- Vortex mixer
- Thermal cycler
- Multichannel pipette
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Ice bucket with ice Timer

Optional Equipment

- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)

SAFETY WARNINGS

This protocol should only be used in combination with:

- Native Barcoding Kit 24 (SQK-NBD112.24)
- R10.4 flow cells (FLO-MIN112) R9.4.1 flow cells (FLO-MIN106)
- Flow Cell Wash Kit (EXP-WSH004)
- Native Barcoding Expansion Kit (EXP-NBD112)

The Kit 12 chemistry runs at 30°C on nanopore sequencing devices. This is several degrees cooler than other chemistries. While the protocol was initially developed on GridION and PromethION, we also support its use on MinION Mk1C, as the MinION Mk1C device's temperature control allows the flow cell to be maintained at 30°C for the duration of the run. However, we cannot guarantee the same level of temperature control on the MinION Mk1B. Therefore, if you are running Kit 12 chemistry on the MinION Mk1B, ensure that the ambient temperature does not exceed 23°C.

Adapter Mix II H (AMII H) used in this kit and protocol is not interchangeable with other sequencing adapters. This kit and protocol is only compatible with Adapter Mix II H (AMII H). If more sequencing adapter is required, theNative Barcoding Expansion (EXP-NBD112) is available for additional reagents. Adapter Mix II H (AMII H) is a new sequencing adapter for Kit 12 chemistry and is loaded with an updated sequencing enzyme with improved accuracies and has a higher capture rate to lower flow cell loading amounts. It also contains the fuel fix technology to enable longer runs without the need for fuel addition during a run. Therefore, sequencing adapters from other kits are not compatible with this kit or protocol.

BEFORE START INSTRUCTIONS

Read about the kit, the system requirements and other general guidelines regarding samples and reagents from this document in detail. Please go through this thoroughly before beginning.

Native barcoding kit Information and guidelines

Preparation Before Starting

2h 20m

1 Prepare for your experiment

1.1 Extract your DNA, and check its length, quantity and purity. The quality checks performed during the protocol are essential in ensuring experimental success.

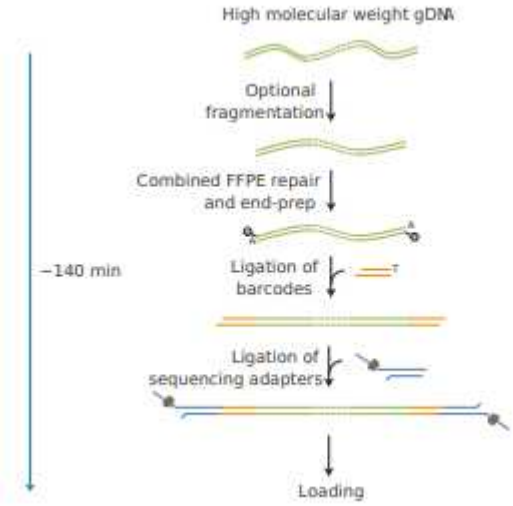
1.2 Ensure you have your sequencing kit, the correct equipment and third-party reagents

1.3 Download the software for acquiring and analysing your data

1.4 Check your flow cell to ensure it has enough pores for a good sequencing run

2 Prepare your library

2h 20m



2.1 Repair the DNA, and prepare the DNA ends for adapter attachment

2.2 Ligate Native barcodes supplied in the kit to the DNA ends

2.3 Ligate sequencing adapters supplied in the kit to the DNA ends

2.4 Prime the flow cell, and load your DNA library into the flow cell

3 Sequencing

3.1 Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads

3.2 Demultiplex barcoded reads in MinKNOW or the Guppy basecalling, choosing the SQK-NBD112.24 kit option

3.3 Start the EPI2ME software and select a workflow for further analysis (this step is optional)

Safety information

We do not recommend mixing barcoded libraries with non-barcoded libraries prior to sequencing.

Safety information

By default, the protocol contains no DNA fragmentation step, however in some cases it may be advantageous to fragment your sample. For example, when working with lower amounts of input gDNA (100 ng–500 ng), fragmentation will increase the number of DNA molecules and therefore increase throughput. Instructions are available in the DNA Fragmentation section of Extraction methods. There are options available for size-selecting your DNA sample to enrich for long fragments - instructions are available in the Size Selection section of Extraction methods.

DNA repair and end-prep

20m

4 **Materials**

400 ng gDNA per sample for >4 barcodes 1000 ng gDNA per sample for ≤4 barcodes

- AMPure XP Beads (AXP)

Consumables

- NEBNext FFPE DNA Repair Mix (M6630)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Thermal cycler at 20°C and 65°C
- Ice bucket with ice
- Microfuge Hula mixer (gentle rotator mixer)
- Magnetic rack

5 Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End repair / dA-tailing Module reagents in accordance with manufacturer’s instructions, and place on ice.

Note

For optimal perfomance, NEB recommend the following:
1. Thaw all reagents on ice.
2. Flick and/or invert reagent tube to ensure they are well mixed.
3. Always spin down tubes before opening for the first time each day.
4. The Ultra II End prep buffer and FFPE DNA Repair buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for several seconds to ensure the reagent is thoroughly mixed.
5. The FFPE DNA repair buffer may have a yellow tinge and is fine to use if yellow.

Safety information

Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix.

Safety information

It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing.
Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.

6 Thaw the AMPure XP Beads (AXP) at room temperature and mix by vortexing.

7 In clean 0.2 ml thin-walled PCR tubes, prepare your DNA sample:
- For >4 barcodes, aliquot 400 ng per sample
- For ≤4 barcodes, aliquot 1000 ng per sample

8 Make up each sample to 12 µL using nuclease-free water. Mix gently by pipetting and spin down.

9 Combine the following components per sample:
Between each addition, pipette mix 10 - 20 times.

9.1

	A	B
	Reagent	Volume
	NEBNext FFPE DNA Repair Buffer	0.875 µl
	Ultra II End-prep reaction buffer	0.875 µl
	Ultra II End-prep enzyme mix	0.75 µl
	NEBNext FFPE DNA Repair Mix	0.50 µl
	Total	3 µl

Note

It is recommended to make up a mastermix for the total number of samples and add 3 µl to each individual sample.

10 Mix well by pipetting and spin down in a centrifuge.

11 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

12 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

13 Resuspend the AMPure XP Beads (AXP) by vortexing.

14 Add 15 µL of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.

15 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at Room temperature . 5m

16 Prepare 500 µL of fresh 70% ethanol in nuclease-free water.

17 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.

18 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Wait for the beads to migrate towards the magnet and form a pellet. Remove the ethanol using a pipette and discard.

19 Repeat the previous step.

20 Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 00:00:30 . 30s

Safety information

Do not dry the pellet to the point of cracking.

21 Remove the tube from the magnetic rack and resuspend the pellet in 10 µL nuclease-free water. Spin down and incubate for 00:02:00 at Room temperature . 2m

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

23 Remove and retain 10 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

> **Note**
>
> Quantify 1 µl of each eluted sample using a Qubit fluorometer.

24 Take forward equimolar mass of samples to be barcoded and pooled forward into the native barcode step. However, at this point it is also possible to store the sample at 4 °C overnight.

Native Barcode Ligation 1h

25

Materials

- AMPure XP Beads (AXP)
- Native Barcodes (NB01-24)
- EDTA (EDTA)

Consumables

- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- NEB Blunt/TA Ligase Master Mix (M0367)
- 1.5 ml Eppendorf DNA LoBind tubes
- 2 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- Magnetic rack
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Microfuge

- Thermal cycler
- Ice bucket with ice
- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

26 Prepare third party reagents in accordance with manufacturer's instructions, and place on ice.

27 Thaw the native barcodes at Room temperature . Use one barcode per sample. Individually mix the barcodes by pipetting, spin down, and place them on ice.

28 Select a unique barcode for every sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment.

29 In clean 0.2 ml thin-walled PCR tubes, add the reagents in the following order per sample: Between each addition, pipette mix 10 - 20 times.

	A	B
	Reagent	Volume
	End-prepped DNA	7.5 µl
	Native barcode	2.5 µl
	Blunt/TA Ligase Master Mix	10 µl
	Total	20 µl

30 Ensure the components are thoroughly mixed by pipetting and spin down briefly.

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

32 Add 2 µL of EDTA to each tube and mix thoroughly by pipetting and spin down briefly.

> **Note**
>
> EDTA is added at this step to stop the reaction.

33 Pool the barcoded samples in a clean 1.5 ml Eppendorf DNA LoBind tube.

> **Note**
>
> We expect ~20 µl per sample.

	A	B	C	D
		X6 Samples	X12 Samples	X24 Samples
	Total volume	120 µl	240 µl	480 µl

34 Resuspend the AMPure XP Beads (AXP) by vortexing.

35 Add AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting for a 0.4X clean.

	A	B	C	D	E
		Volume per sample	x6 samples	x12 samples	x24 samples
	Volume of AXP	8 µl	48 µl	96 µl	192 µl

36 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at Room temperature . 10m

37 Prepare 2 mL of fresh 70% ethanol in nuclease-free water.

38 Spin down the sample and pellet on a magnet for 00:05:00 . Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant. 5m

39 Keep the tube on the magnetic rack and wash the beads with 700 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

40 Repeat the previous step.

41 Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 . 30s

> **Safety information**
>
> Do not dry the pellet to the point of cracking.

42 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water.

43 Incubate for 00:10:00 at 37 °C . Periodically agitate the sample by gently flicking to encourage DNA elution. 10m

44 Pellet the beads on a magnetic rack until the eluate is clear and colourless.

45 Remove and retain 35 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

46 Take forward the barcoded DNA library to the adapter ligation and clean-up step. However, at this point it is also possible to store the sample at 4 °C Overnight .

Adapter ligation and clean-up

50m

47

Materials

- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB) Elution Buffer from the Oxford Nanopore kit (EB)
- Adapter Mix II H (AMII H)
- AMPure XP Beads (AXP)

Consumables

- Quick T4 DNA Ligase in NEBNext® Quick Ligation Module (NEB E6056)
- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- Microfuge
- Magnetic rack
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Thermal cycler
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Ice bucket with ice

48 Thaw the Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at Room temperature, mix by vortexing, spin down and place on ice. Check the contents of each tube are clear of any precipitate.

49 Spin down the Quick T4 Ligase and the Adapter Mix II H (AMII H), and place on ice.

50 To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at Room temperature, mix by vortexing, spin down and place on ice.

51 To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at Room temperature, mix by vortexing, spin down and place on ice.

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

> **Note**
>
> Between each addition, pipette mix 10 - 20 times.

	A	B
	Reagent	Volume
	Pooled barcoded sample	30 µl
	Adapter Mix II H (AMII H)	5 µl
	NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
	Quick T4 DNA Ligase	5 µl

	A	B
	Total	50 µl

53 Ensure the components are thoroughly mixed by pipetting and spin down briefly.

54 Incubate the reaction for 00:20:00 at Room temperature . 20m

Safety information

The next clean-up step uses Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) rather than 70% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.

55 Resuspend the AMPure XP Beads (AXP) by vortexing.

56 Add 20 µL of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

57 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at Room temperature 10m

58 Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

59 Wash the beads by adding either 125 µL Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard

60 Repeat the previous step.

61 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~ 00:00:30 30s

Safety information

Do not dry the pellet to the point of cracking.

62 Remove the tube from the magnetic rack and resuspend the pellet in 15 µL of Elution Buffer (EB).

63 Spin down and incubate for 00:10:00 at 37 °C . Periodically agitate the sample by gently flicking to encourage DNA elution 10m

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

65 Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Safety information

We recommend loading 5 - 10 fmol of this final prepared library onto the flow cell. Calculate the mass which corresponds to 5 - 10 fmol and if required, make up the final volume to 12 µl with Elution Buffer (EB). Loading more than 20 fmol of DNA can reduce the rate of duplex read capture. The loading concentration is the same across both R9.4.1 and R10.4 flow cells.

Note

The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.

We recommend storing libraries in Eppendorf DNA LoBind tubes at4°C for short term storage or repeated use, for example, reloading flow cells between washes. For single use and long term storage of more than 3 months, we recommend storing libraries at-80°C in Eppendorf DNA LoBind tubes.

Priming and loading the SpotON flow cell

10m

66 **Materials**

- Flush Buffer (FB)
- Flush Tether (FLT)
- Loading Beads II (LBII)
- Sequencing Buffer II (SBII)
- Loading Solution (LS)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

- MinION
- SpotON Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

67 We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

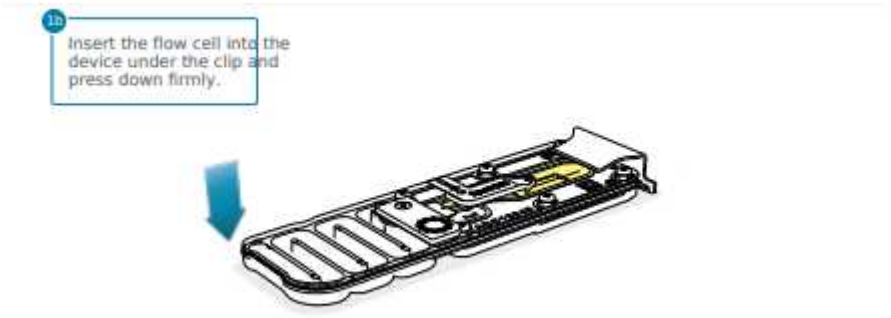
We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for

most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water

68 Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at Room temperature before mixing the reagents by vortexing and spin down at Room temperature

69 To prepare the flow cell priming mix, add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at Room temperature .

70 Open the MinION lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact

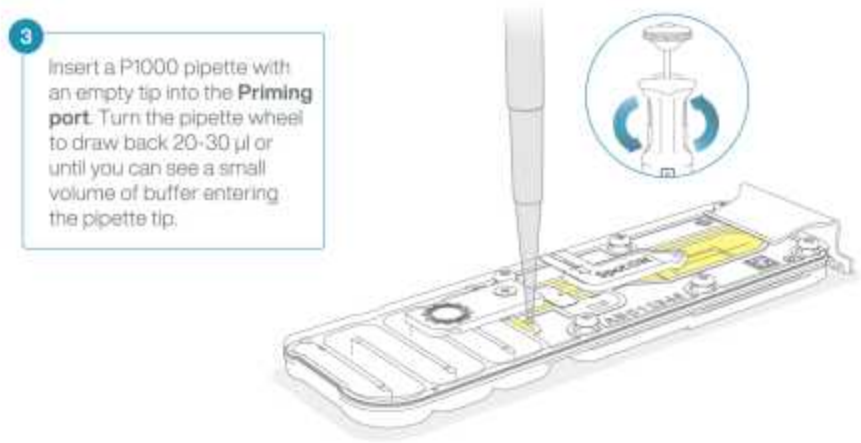


71 Complete a flow cell check to assess the number of pores available before loading the library. This step can be omitted if the flow cell has been checked previously.

72 Slide the priming port cover clockwise to open the priming port.

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

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



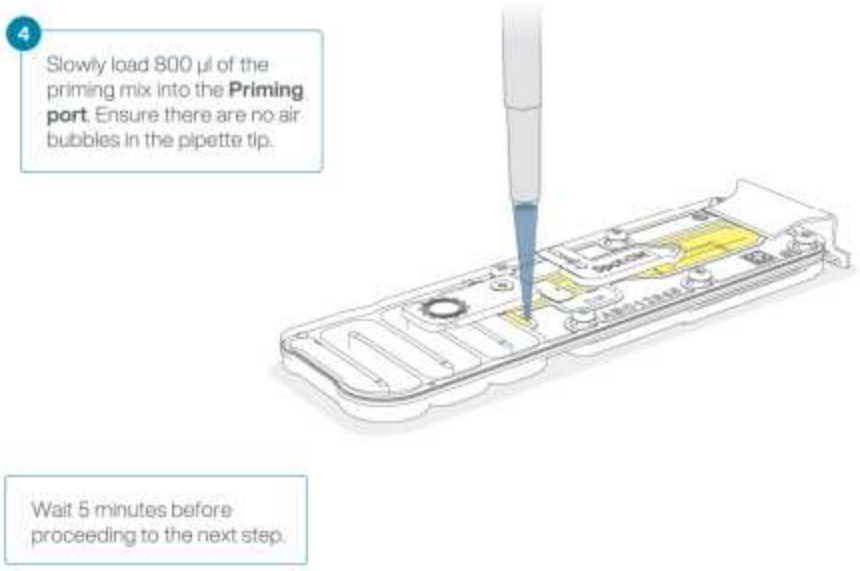
73.1 Set a P1000 pipette to 200 µl

73.2 Insert the tip into the priming port

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

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

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



75 Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.

Safety information

The Loading Beads II (LBII) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

76 In a new tube, prepare the library for loading as follows:

	A	B
	Reagent	
	Sequencing Buffer II (SBII)	37.5 µl
	Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using	25.5 µl

	A	B
	DNA library	12 µl
	Total	75 µl

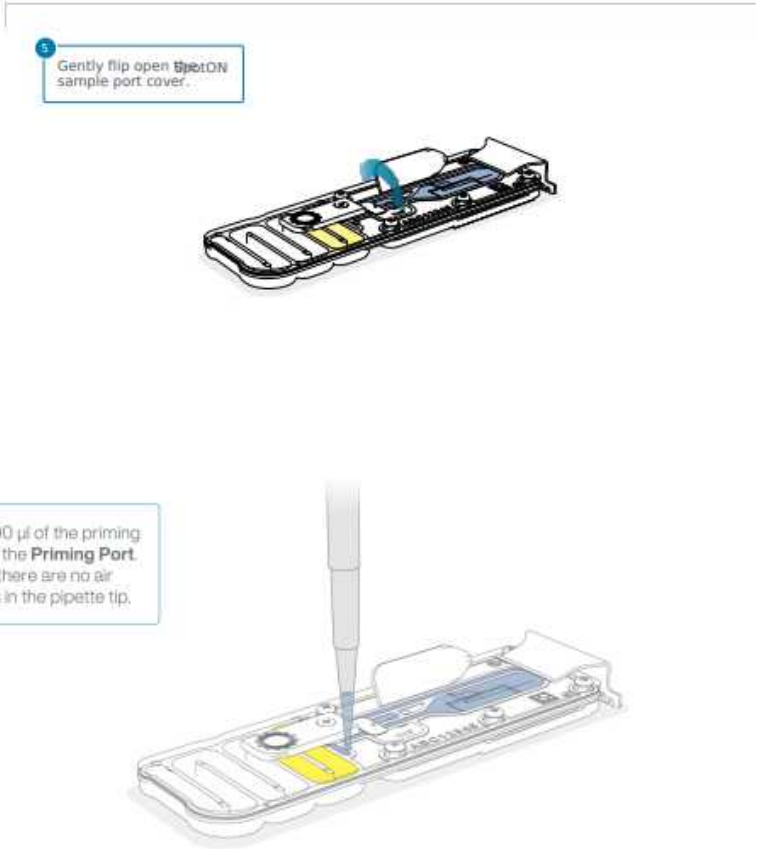
Note

Load the library onto the flow cell immediately after adding the Sequencing Buffer II (SBII) and Loading Beads II (LBII) because the fuel in the buffer will start to be consumed by the adapter.

77 Complete the flow cell priming:

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

77.2 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

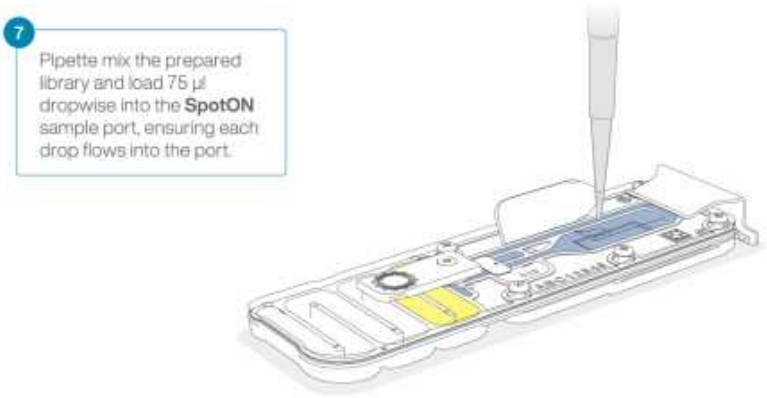


Note

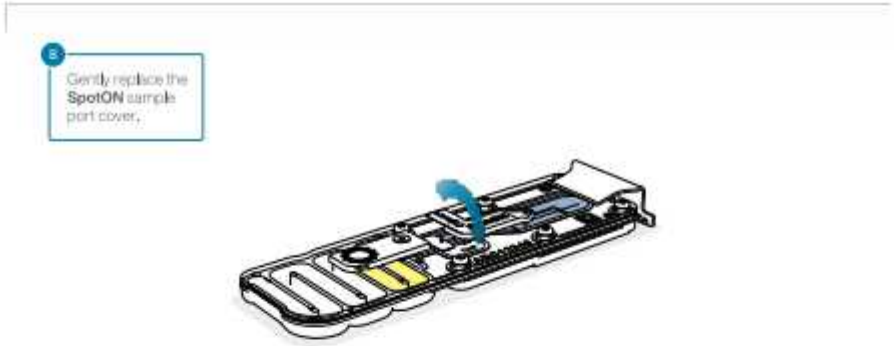
Load the library as soon as possible after this step

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

79 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.



80 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.



Data Acquisition and Basecalling

81 For information on Data Acquisition and Basecalling, go through the following document in detail
Data Acquisition and Basecalling

Ending the Experiment

82

Note

We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day

Step 82 includes a Step case.
Option 1
Option 2

step case

Option 1

After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C

83 Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore. Instructions for returning flow cells can be foundhere.

Safety information

If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.

Issues in the protocol

84 For common issues and concerns regarding the protocol and suggestions to resolve the same, go through this following document, which is also available in guidelines
Issues while conducting the protocol