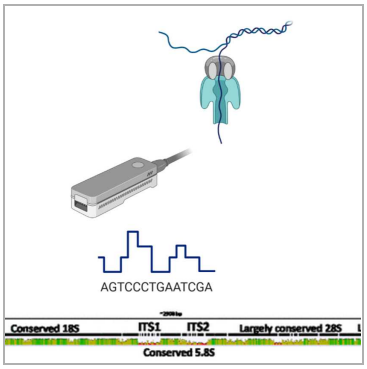

MAR 19, 2023

Sequencing fungal and bacterial metabarcodes with native barcoding and Nanopore

Forked from Native Barcoding

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ABSTRACT

This protocols is part of the ANU Biosecurity mini-research project #1 "Plant Pathogen Diagnostics: Visuals, subcultures, and genomics".

You will be provided four pots of 3-4 week old wheat plants that have been infected with different wheat pathogens. Each pot has been infected with one major pathogen. You will not know which pot has been infected with which pathogen. However, you will be provided a compendium of 10-15 wheat pathogens that will guide you to identify the infective agent for each treatment group. The fifth treatment group will be uninfected wheat plants which will be clearly identified. You can use treatment group #5 as negative control for your experiments.

In total, each group will obtain five pots each:

	A	B
	Treatment group 1	Unknown infective agent
	Treatment group 2	Unknown infective agent
	Treatment group 3	Unknown infective agent
	Treatment group 4	Unknown infective agent
	Treatment group 5	Uninfected control

This specific protocol describes the molecular biology and a step-by-step guide for native barcoding and Nanopore sequencing of the metabarcode regions for fungi (Internal Transcribed Spacer, ITS) and bacteria (16S locus). Most of the steps will have been conducted by your demonstrators due to time limitations. During class we will have the final library ready to load.

This protocol is applicable for week 5.

Protocol Citation: Carlos Goller, Benjamin Schwessinger, Carly Sjogren 2023. Sequencing fungal and bacterial metabarcodes with native barcoding and Nanopore. **protocols.io** https://protocols.io/view/sequencing-fungal-and-bacterial-metabarcodes-with-cpdrvi56

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

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PROTOCOL integer ID: 76945

Conceptual overview:

1. Clean up of PCR products with magnetic beads to remove buffer, enzyme, and primers.
2. Measure of PCR product concentration after clean up.
3. Start of library preparation; end-prep to repair the ends of each amplicon for ligation of barcodes including clean up with magnetic beads.
4. Barcode ligation. Each PCR amplicon from each group will receive a unique DNA barcode. This barcode can be used to demultiplex each sample after the sequencing run while being able to run up to 96 samples within the same reaction.
5. Clean up with magnetic beads to remove buffer, enzyme, and non-ligated barcodes.
6. Pooling of all barcoded amplicons.
7. Ligation of sequencing adapters.
8. Clean up with magnetic beads to remove buffer, enzyme, and non-ligated sequencing adapters.
9. Final library preparation for loading.
10. Flow-cell priming to be ready to load.
11. Library loading.
12. Start of sequencing run.

Demonstrators will perform step 1-8. In class we will perform step 9-12 for two libraries. One library is multiplexed to contain all ITS amplicons from class, and the other library contains all 16S amplicons from class.

You can cite this protocol in the methods section of your report as for all other protocols. No need to write it all up again :).

GUIDELINES

This protocol describes how to carry out native barcoding of amplicons using the Native Barcoding Kit 96 (SQK-NBD112.96). There are 96 unique barcodes available, allowing the user to pool up to 96 different samples in one sequencing experiment.

Native Barcoding Kit features

This kit is recommended for users who:

- wish to multiplex up to 96 samples to reduce sequencing cost per sample
- need a PCR-free method of multiplexing
- require control over read length
- would like to utilise upstream processes such as size selection or whole genome amplification

You must have read, understood, and follow the health and safety instructions provided in the "Overview Mini-Research Project #1 BIOL3106/6106" provided on Wattle (ANU learning portal).

You must have signed and returned one copy of the "Student Safety Declaration Form For Practical Class Work" before starting any laboratory work.

You must have read and understood the Hazard Sheets (Risk assessment) of all chemicals listed bellow in the "Safety Warnings" section. These Hazard Sheets are provided on Wattle as part of the "Overview Mini-Research Project #1 BIOL3106/6106" document.

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 does not require any hazardous substances.

You need to wear safety equipment at all times including lab coats, gloves, and safety goggles when handling chemicals and biological agents. While the major biological agents used for the wheat infection are pathogens commonly found in Australia, you must treat them as they were infective agents of general concern. Treat them with care. Do not remove them from the laboratory. Do not spread them via clothing. Use a dedicated notebook and pen to make notes during the mini-research project. Do not put anything into your mouth while in the laboratory. Wash your hands each time you leave the laboratory.

BEFORE START INSTRUCTIONS

You must study the protocol carefully before you start. If anything is unclear post questions directly here on protocols.io.

This protocol is mostly for reference purposes and we will demonstrate all steps during practical session in class.

Preparation Before Starting

2h 20m

1 Prepare for your experiment (done bye the demonstrators for you).

1.1 Purify your PCR amplicons with AMPure beads or similar and measure concentrations (Step 1+2 in Abstract Overview).

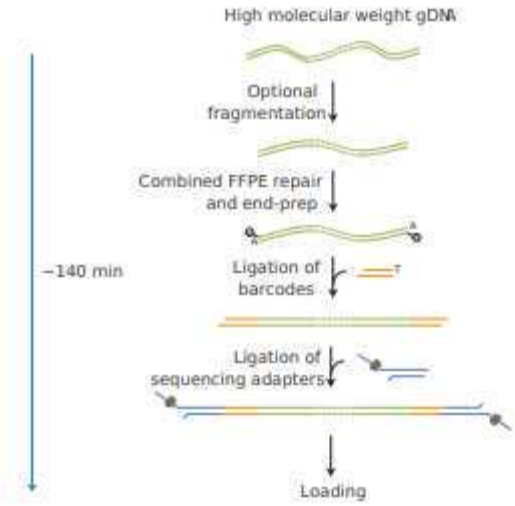
1.2 Ensure you have your sequencing kit, the correct equipment and third-party reagents.

1.3 Download the software for acquiring and analysing your data including MiniKNOW.

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 (Step 3 in Abstract Overview).

2.2 Ligate Native barcodes supplied in the kit to the DNA ends (Step 4+5 in Abstract Overview)..

2.3 Ligate sequencing adapters supplied in the kit to the DNA ends (Step 6-8 in Abstract Overview)..

2.4 Prime the flow cell, and load your DNA library into the flow cell (Step 9-11 in Abstract Overview)..

3 Sequencing (Step 12 in Abstract Overview).

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

DNA repair and end-prep (Step 3 in Abstract Overview) 20m

4 **Materials**

25-200 ng amplicon DNA per sample for >12 barcodes.

- AMPure XP Beads (AXP)

Consumables

- 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 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 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.

Safety information

It is important that the 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 25-200 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

	A	B
	Reagent	Volume
	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
	H20	0.875 ul
	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 Combine 12 ul of sample and 3ul of reaction master mix for 15 ul reactions. 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 (Step 4-6 in Abstract Overview) 1h

25 **Materials**

- AMPure XP Beads (AXP)
- Native Barcodes (NB01-96)
- 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 96 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.

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 0.7X volume clean up.

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. Incubate for 10 mins.

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

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

45 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 (Step 7-8 in Abstract Overvie.. 50m

46 **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

47 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.

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

49 To deplete un-ligated sequencing adapters, thaw one tube of Short Fragment Buffer (SFB) at Room temperature, mix by vortexing, spin down and place on ice.

50 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
	Total	50 µl

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

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

Safety information

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

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

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

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

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

57 Wash the beads by adding either 125 µL 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

58 Repeat the previous step.

59 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.

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

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

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

63 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 20-50ng for this amplicon length of this final prepared library onto the flow cell.

> **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 at 4°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 (Step 9-11 in Abst... 10m

64 **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

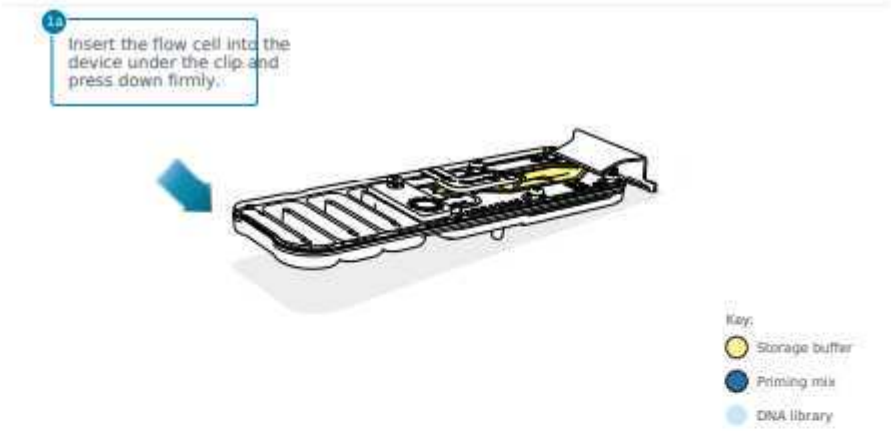
65 We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

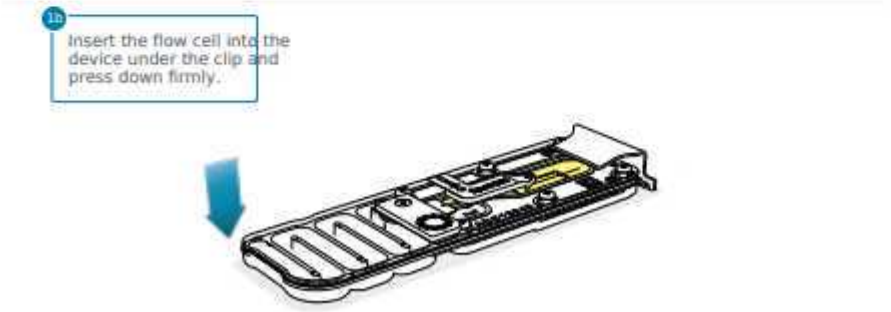
We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water

66 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

67 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 .

68 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





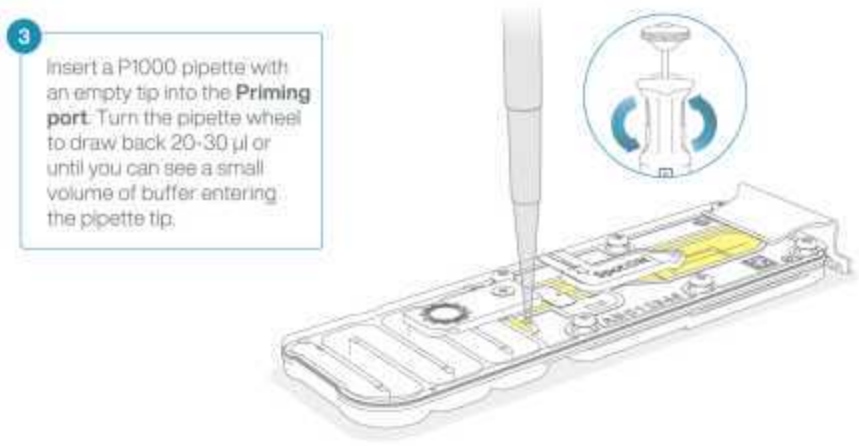
69 Complete a flow cell check to assess the number of pores available before loading the library. This step can be omitted if the flow cell has been checked previously.

70 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.

71 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):



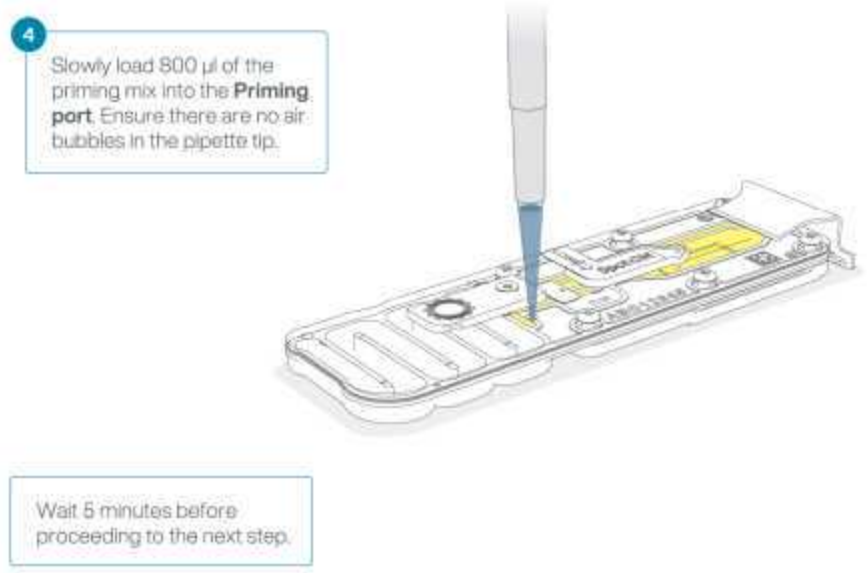
71.1 Set a P1000 pipette to 200 µl

71.2 Insert the tip into the priming port

71.3 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.

72 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



73 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.

74 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
	DNA library 50 ng total	12 µl
	Total	75 µl

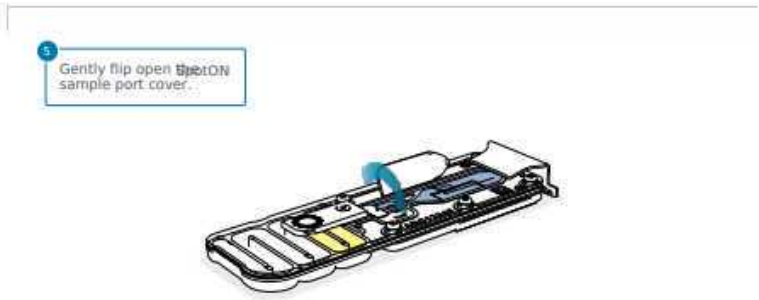
Note

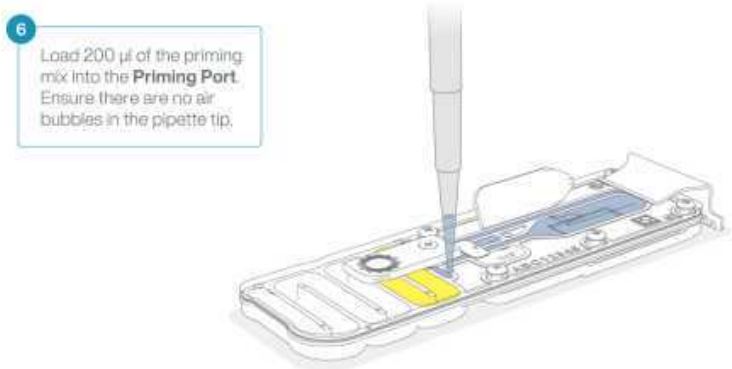
Load the library onto the flow cell immediately after adding the Sequencing Buffer II (SBII) and Loading Beads II (LBII) because the fuel in the buffer will start to be consumed by the adapter.

75 Complete the flow cell priming:

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

75.2 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





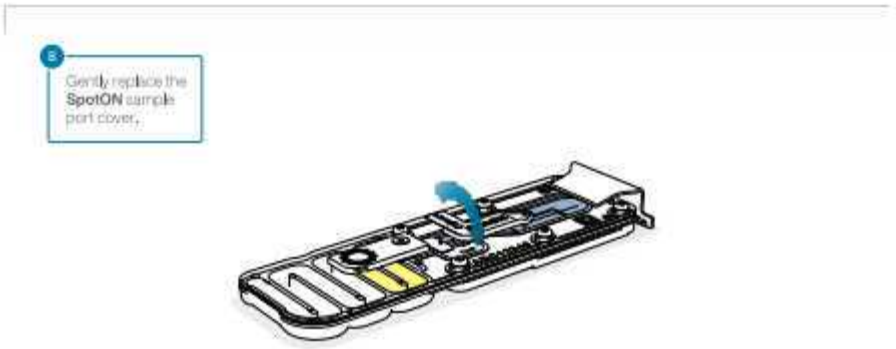
Note

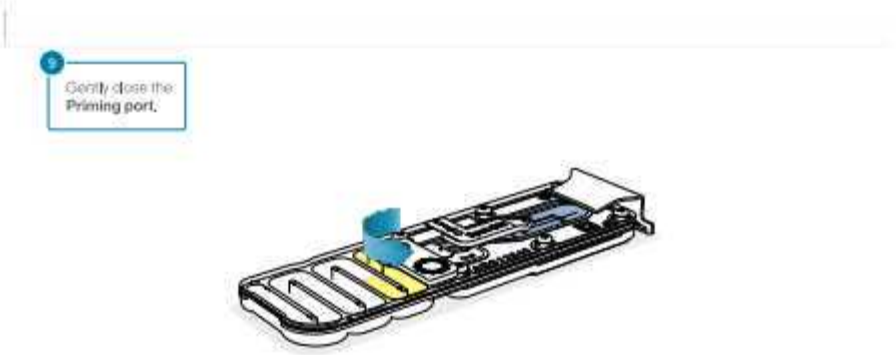
Load the library as soon as possible after this step

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

77 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.

78 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 (Step 12 in Abstract Over...

79 For information on starting a sequencing run in MinKNOW (with a slight different library prep) check out this video https://www.youtube.com/watch?v=oCOixfewXo4

Ending the Experiment

80

> **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 80 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

81 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.