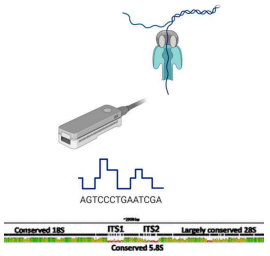
Mar 10, 2024

Sequencing fungal metabarcode with PCR primer based barcoding and Nanopore



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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:

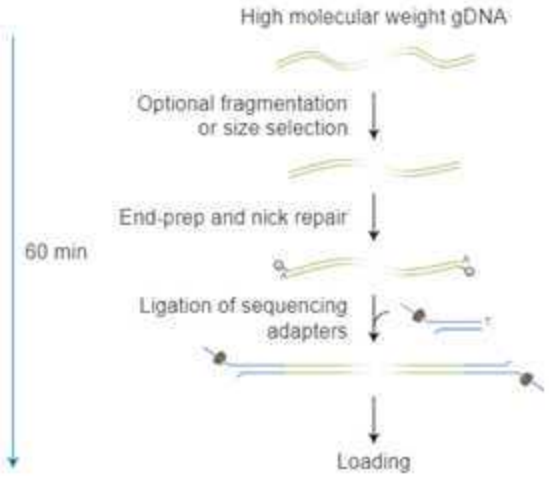
	A	B
	Treatment group 1	Unknown infective agent
	Treatment group 2	Unknown infective agent
	Treatment group 3	Unknown infective agent
	Treatment group 4	Unknown infective agent
	Treatment group 5	Uninfected control

This specific protocol describes the molecular biology and a step-by-step guide for PCR barcoding of the Internal Transcribed Spacer, ITS using primers that contain barcodes, followed by ligation based amplicon sequencing with Nanopore. The initial steps of repeated PCR with high-fidelity polymerase and purification of the PCR amplicons has been performed by your demonstrator due to time limitations. We selected DNA samples of three research groups in week 5 so that we have three replicates per treatment group. This gives us 15 PCR reactions plus 1 negative control. We skip the extraction control for this practical mini-research project. This is step 1 of the protocol.

One group will perform the library preparation on the PCR barcoded and equimolar pooled amplicons. Most of the class need not be present for this library preparation [Step 2] but will join in for loading the final library and starting sequencing runs. We will also explain more about the theory during the lab.

This protocol is applicable for week 6.

Conceptual overview:



Overview of amplicon library prep we will not perform fragmentation and size selection.

- Repeat PCR reactions on the samples of three research groups using primers containing barcodes in them (see below).
- Clean and pool amplicons at equimolar ratios.
- End-prep the pooled amplicons to make them ready to ligate sequencing adapters.
- Ligate sequencing adaptars with DNA ligase.
- Make amplicon library ready for loading.
- Prepare for loading onto the flowcell.
- Prime flowcell to make it ready for loading.
- Load library.
- Start of sequencing run.
- Basecall during or after the sequencing run.

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 :).

Primers used:

We used ITS primers that directly include DNA barcodes as follows, where $N_{15\text{-}16}$ represents a unique barcode sequence

ITSFor (a.k.a. ITS1-F_KYO2_BARCODE): 5'-$N_{15\text{-}16}$-TAGAGGAASTAAAAGTCGTAA-3'
ITSRev (a.k.a.LR6_BARCODE): 5'-$N_{15\text{-}16}$-CGCCAGTTCTGCTTACC-3'

References:

- Ohta, Nishi, Hirota and Matsuo, 2023, 'DNA metabarcoding workflow utilising nanopore long-read sequencing and consensus generation for rapid identification of fungal taxa with high phylogenetic resolution, BioRxiv, doi: https://doi.org/10.1101/2023.04.14.536971
- Hebert, P.D.N., Floyd, R., Jafarpour, S., Prosser, S.W.J., 2023. Barcode 100K Specimens: In a Single Nanopore Run, BioRxiv, doi: https://doi.org/10.1101/2023.11.29.569282

Attachments

Ligation sequencing ... 111KB

ligation-sequencing-... 3.2MB

Image Attribution

The logo image was generated with biorender.

Guidelines

This protocol describes how to carry out native barcoding of PCR amplicons that already contain barcodes using the Ligation Sequencing Kit (SQK-RBKLSK114). The PCR barcoding allows for 10-100,000 barcoded samples to be sequenced in one reaction. We will barcode 16 PCR amplicons

This approach is recommended for users who:
- wish to multiplex 10 to 10,000 samples to reduce sequencing cost per sample
- want to avoid costly (time and money) native barcoding reactions and two step PCR barcoding reactions
- want to conserve amplicon length

As always you need to bring a lab notebook, a printed version of this protocol, and a pen to record your adventures in the lab.

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
- Pure DNA

Consumables
- R10.4.1 MinION flowcell **Flow Cell (R10.4.1) (nanoporetech.com)**
- SQK-LSK114 **Ligation Sequencing Kit V14 (nanoporetech.com)**
- Primers as described.
- Q5 High-Fidelity Polymerase
- NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- NEBNext Quick Ligation Module (NEB, E6056)
- Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- magnetic beads (AMPure or equivalent) https://www.beckman.com/reagents/genomic/cleanup-and-size-selection/pcr
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit Assay Tubes (ThermoFisher)
- Qubit dsDNA BR Assay Kit (ThermoFisher)

Equipment
- Hula mixer (gentle rotator mixer)
- Microfuge Magnetic rack
- Vortex mixer
- Thermal cycler/PCR machine
- 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
- Qubit fluorometer (or equivalent for QC check)
- Computer with the latest version of MinKNOW installed.
- MinION Nanopore sequencer

Troubleshooting

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.

Preparation of input DNA

1 This first step has been performed by a demonstrator using DNA from the groups we selected in week 5. We selected three research groups so that we have three replicates per treatment group. This gives us 15 PCR reactions plus 1 negative control. We skip the extraction control for this practical mini-research project.

We repeated the PCR like here **PCR reaction of marker regions (a.k.a metabarcodes) (protocols.io)** for these samples with the two following adjustments:

1. We used proof-reading high fidelity polymerase like Q5 for the PCR reaction.
2. We used ITS primers that directly include DNA barcodes as follows, where $N_{15\text{-}16}$ represents a unique barcode sequence

ITSFor (a.k.a. ITS1-F_KYO2_BARCODE): 5'-$N_{15\text{-}16}$-TAGAGGAASTAAAAGTCGTAA-3'
ITSRev (a.k.a.LR6_BARCODE): 5'-$N_{15\text{-}16}$-CGCCAGTTCTGCTTACC-3'

The PCR products were checked by agarose gel electrophoresis. These confirmed PCR products were then treated as follows.

1.1 Purify your PCR amplicons with magnetic beads (e.g. AMPure) by adding an equal volume of magnetic beads to the PCR reaction.

For example if your PCR reaction was 50 ul add 50 ul of beads.

1.2 Incubate while rotating the tube for 10 minutes at room temperature

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

1.4 Keep the tube on the magnet and wash the beads with 200 ul of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

1.5 Repeat the previous step 1.4.

1.6 Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 1 - 2 mins, but do not dry the pellet to the point of cracking.

1.7 Remove the tube from the magnetic rack and resuspend the pellet in 20 ul of nuclease free water.

1.8 Incubate for 10 minutes.

1.9 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

1.10 Remove and retain the full volume of eluate into a clean 200ul PCR tube.

1.11 Quantify 1ul of purified amplicon using Qubit BR as described in previous protocols. Note down the concentration.

1.12 Now we need to combine the 16 purified PCR amplicons [3 reps * 5 treatment groups + 1 negative PCR control] at equal molar ratios. We need about 400 ng DNA in total as input for the library prep as this equates to ~ 0.2 pmol when assuming an average long-ITS PCR amplicon length of 3000 bp.

This implies that we need to combine 25 ng per purified PCR amplicon and make it up to 50 ul total. These 50 ul contain our PCR amplicons in equal molar ratios and are the staring point for our library preparations.

To note is that we expect very low amounts for the negative PCR control and that it is best add as much as possible up to 25 ng to the DNA amplicon pool.

Native amplicon library preparation

2 These steps will be performed by a single group we selected in week 5.

2.1 Program the thermal cycler: 20°C for 5 minutes and 65°C for 5 minutes. Ramp it up and keep it at 20°C so we can start the program immediately.

2.2 In a 0.2 ml thin-walled PCR tube, mix the following:

	Input Material	Volume [ul]
	Pooled purified PCR amplicons (~400 ng)	50
	Ultra II End-prep Reaction Buffer	7
	Ultra II End-prep Enzyme Mix	3
	Total	60

Between each addition, pipette mix 10-20 times.

2.3 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

2.4 Incubate the tubes at 20°C for 5 minutes and 65°C for 5 minutes using the PCR machine we already set up. Once done, briefly put the tubes on ice to cool.

2.5 Resuspend the magentic beads for DNA binding by vortexing.

2.6 Add an equal volume of 60 ul of magnetic beads to End-prep reaction.

2.7 Incubate while rotating the tube with your hand for 5 minutes at room temperature

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

A demonstrator will help you with this step.

2.9 Keep the tube on the magnet and wash the beads with 200ul of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

2.10 Repeat the previous step 2.9.

2.11 Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 1 - 2 mins, but do not dry the pellet to the point of cracking

2.12 Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease free water.

2.13 Incubate for 3 minutes while warming the tube with your fingers.

2.14 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

2.15 Remove and retain the full volume of eluate into a clean 1.5ml Eppendorf PCR tube.

2.16 Now we will add the sequence adapters to the End-prepped PCR amplicons

	Input Material	Volume [ul]
	End-prepped PCR amplicons	60
	Ligation Buffer (LNB)	25
	NEBNext Quick T4 DNA Ligase	10
	Ligation Adapter (LA)	5
	Total	100

Between each addition, pipette mix 10-20 times.

2.17 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

2.18 Incubate the reaction for 10 minutes at room temperature.

2.19 Add 55 µl of resuspended magnetic beads to the reaction and mix by flicking the tube.

2.20 Incubate while rotating the tube with your hand for 5 minutes at room temperature

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

A demonstrator will help you with this step.

2.22 Wash the beads by adding 250 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.

2.23 Repeat the previous step 2.22.

2.24 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at room temperature.

2.25 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

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

2.27 Quantify 1ul of purified amplicon using Qubit BR as described in previous protocols. Note down the concentration and calculate the volume you need to obtain 20 fmol assuming an average amplicon length of ~ 3000 bp.

Remember:

n = c*V with n being the amount in ng, c the concentration in ng/ul, and V the volume in ul.

and use the following online calculator.

Biomath Calculators | DNA Calculator | Vector Insert Ratio (promega.com.au)

2.28 Make up 20 fmol of final library in 12 ul of Elution Buffer.

2.29 Add 37.5 uL of Sequencing Buffer (SB) and 25.5uL of Library Beads (LIB) to the sample.

Note – the library beads settle on the bottom of the tube very quickly. Make sure to mix well before pipetting.

3 We now have our library ready to load and will wait for the rest of the class to come in.

Flowcell preparation for sequencing aka priming

4 These steps will be performed by one group member while the rest of the class is watching.

If you are not familiar with MinION flowcells check out this explanation video here **Community - Flow cell introduction (nanoporetech.com)**.

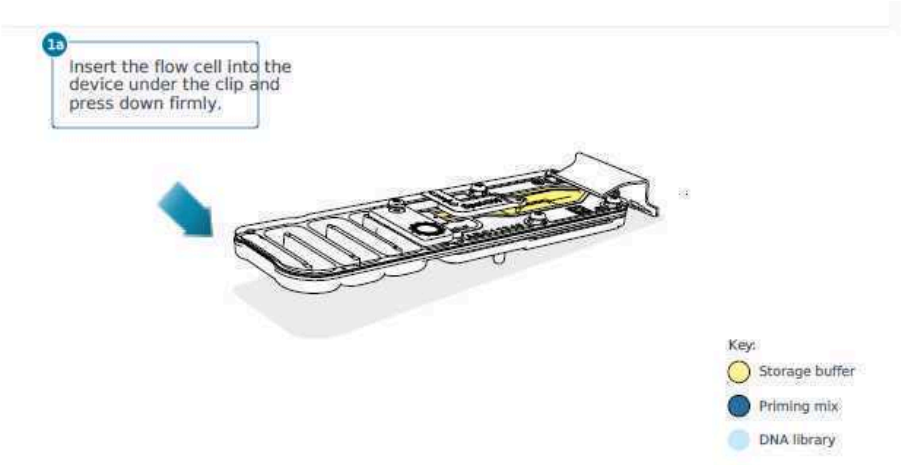
This is a useful video for flowcell priming and loading **Community - Priming and loading your flow cell (nanoporetech.com)**.

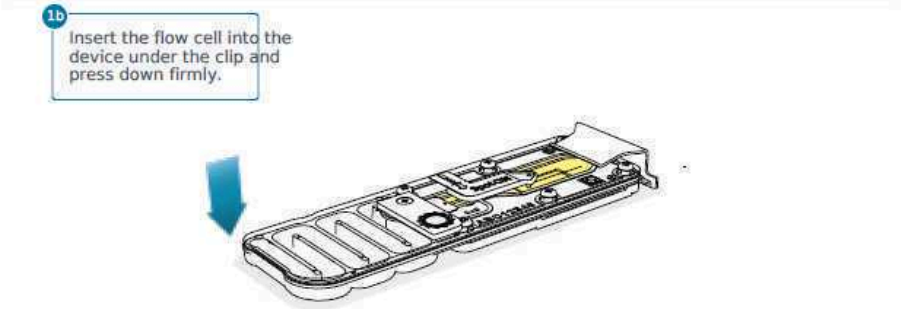
4.1 Perform a flowcell check as shown here **Community - MinKNOW: Flow cell check (nanoporetech.com)**

4.2 To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at room temperature.

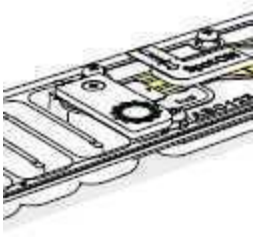
	Reagents	Volume [ul]
	Flow Cell Flush (FCF)	1170
	Bovine Serum Albumin (BSA) at 50 mg/ml	5
	Flow Cell Tether (FCT)	30
	Total volume	1205

4.3 Open the MinION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact. This step might have been already performed if you did the flow cell check.

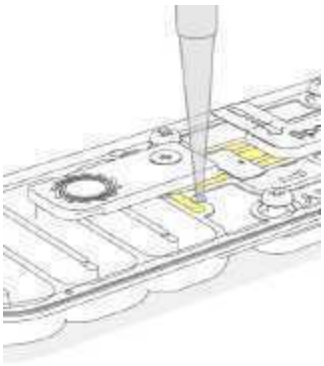




4.4 Slide the flow cell priming port cover clockwise to open the priming port. The priming port cover has the Nanopore logo.



Closed priming port cover.



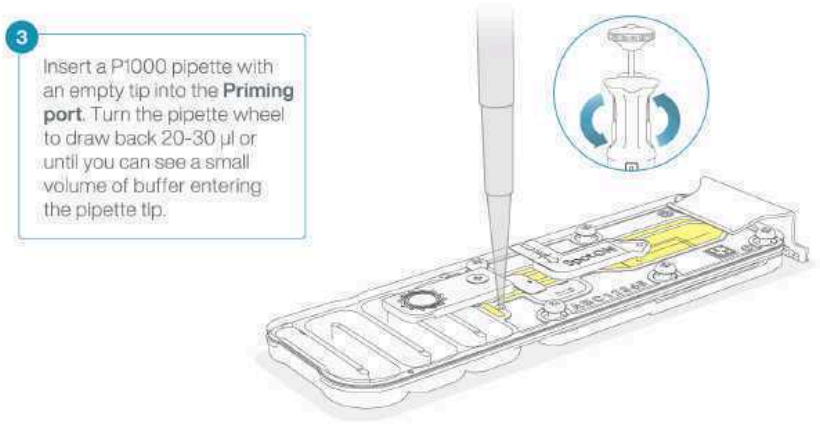
Open priming port cover with pipette entering the priming port.

4.5 IMPORTANT
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.

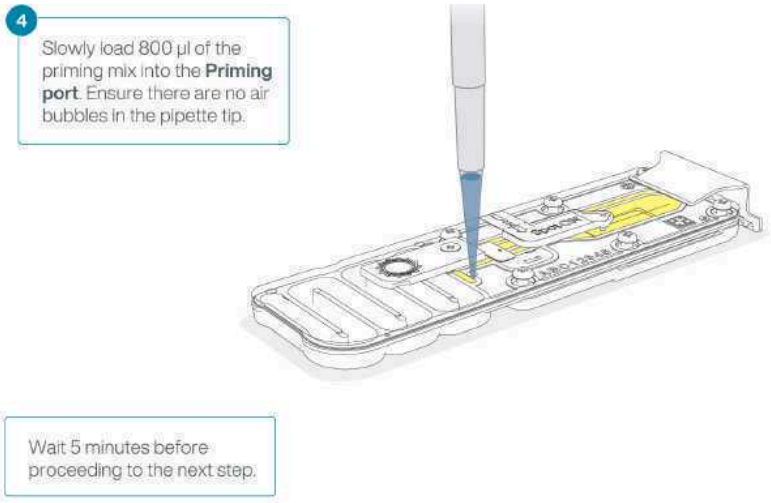
4.6 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
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.



4.7 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. This is best done by turning the wheel slowly and ejecting the priming buffer slowly into the priming port. Close the priming port cover during incubation. Wait for five minutes.



4.8 The flowcell is now ready for loading and sequencing.

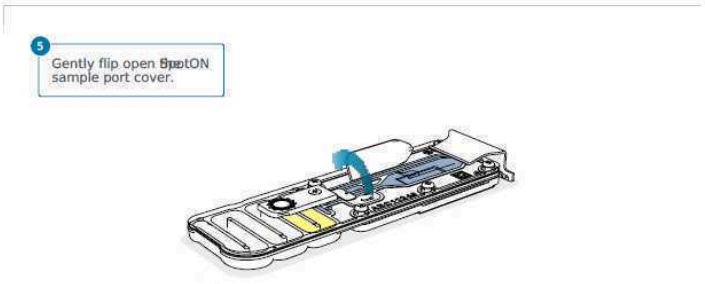
Loading of DNA library onto MinION flowcell

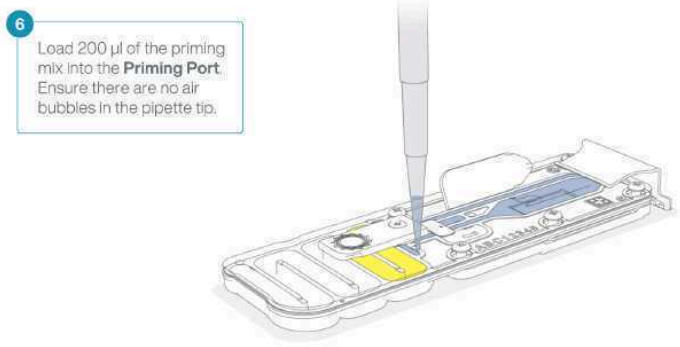
5 Now we will load the DNA library from step 3.23 to start sequencing.

This is a useful video for flowcell priming and loading **Community - Priming and loading your flow cell (nanoporetech.com)**.

5.1 IMPORTANT
The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

5.2 Complete the flow cell priming:
1. priming port cover.
2. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
3. Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

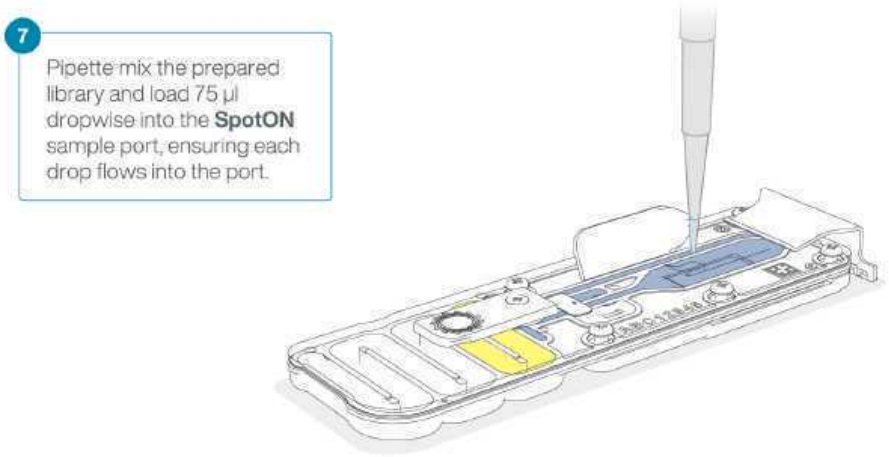




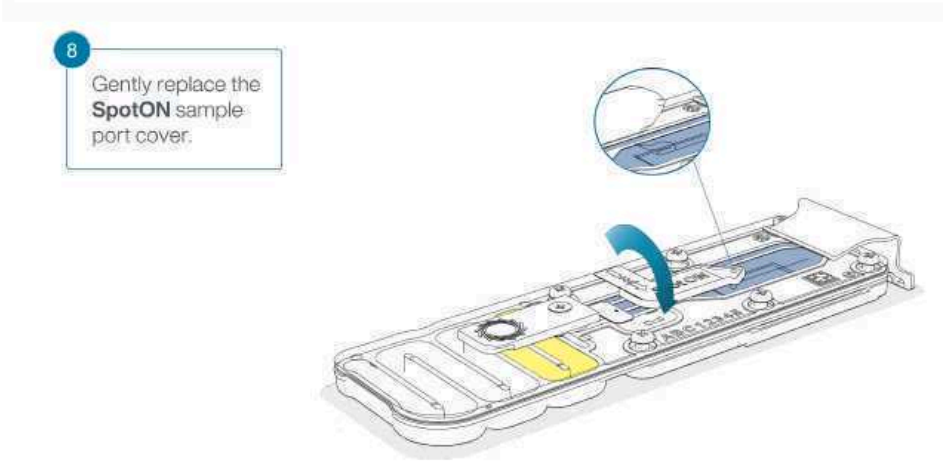
5.3 Mix the prepared library gently by pipetting up and down just prior to loading.

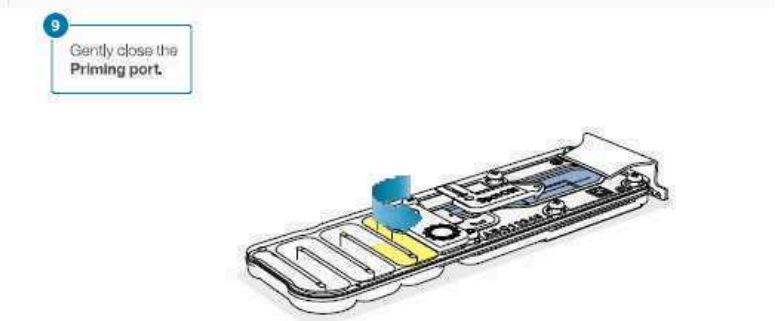
5.4 Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

A demonstrator will assist you with this step.



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



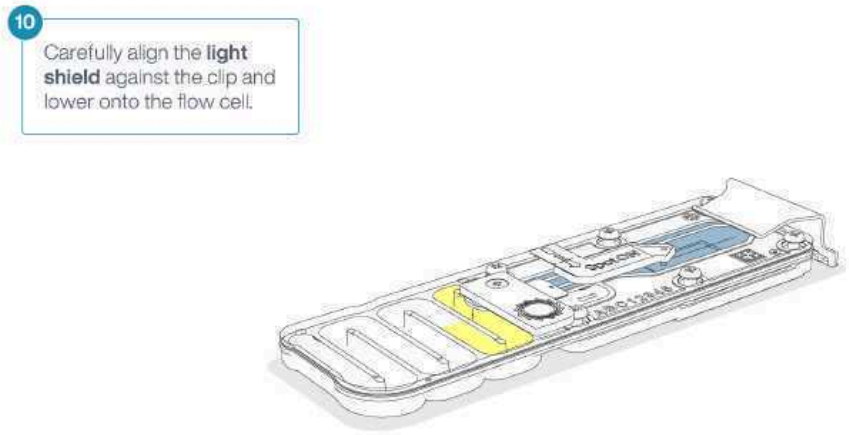


5.6 IMPORTANT
Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.
We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps.

The shield can be removed when the library has been removed from the flow cell. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

5.7 Place the light shield onto the flow cell, as follows:
1. Carefully place the leading edge of the light shield against the clip.
   Note: Do not force the light shield underneath the clip.

2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.



5.8 Now we are ready for DNA sequencing via the MinKNOW graphical user interface.

DNA sequencing and basecalling

6 The demonstrators will help setting up the sequencing runs. We will perform basecalling later on.

Here is a list of useful videos:

- Introduction to the MinKNOW graphic user interface **Community - MinKNOW: The graphical user interface (GUI) (nanoporetech.com)**
- How to set up a sequencing experiment **Community - MinKNOW: Setting up a new experiment (nanoporetech.com)**
- How to assess your run is going fine **Community - MinKNOW: Assessing your run (nanoporetech.com)**
- How MinKNOW selects the best channels **Community - MinKNOW: Mux scan and active channel selection (nanoporetech.com)**
- More about checking on if your sequencing run is going smoothly **Community - MinKNOW: Pore occupancy (nanoporetech.com)**

6.1 We will share sequencing results and initial analysis beginning of week 7.

Protocol references

This protocol is based on publicly available protocols from Oxford Nanopore Technology found here **Community - Ligation sequencing amplicons V14 (SQK-LSK114)Protocol - Ligation sequencing amplicons V14 (SQK-LSK114) (nanoporetech.com)** and the preprint here **Barcode 100K Specimens: In a Single Nanopore Run | bioRxiv**.