# Overview of the protocol

**Ultra-Long DNA Sequencing Kit features:**

This kit is recommended for users who:

- want to reliably generate ultra-long read length N50s >50 kb, with yields of 10-20+ GB on MinION
- sequence long reads from extracted UHMW DNA

**Introduction to the Ultra-Long DNA Sequencing Kit protocol (SQK-ULK001)**

This protocol describes the step-by-step instructions to complete sequencing of ultra-high molecular weight (UHMW) gDNA using the Ultra-Long DNA Sequencing Kit (SQK-ULK001). We have extracted the UHMW gDNA using the appropriate Circulomics Nanobind Big DNA Kit and sample-specific Circulomics UHMW DNA Extraction protocol. However, customers can use other extraction kits before completing library preparation using the Ultra-Long DNA Sequencing Kit (SQK-ULK001). The Circulomics Nanobind UL Library Prep Kit (NB-900-601-01) is used for library clean-up before sequencing.
Per reaction, there is enough library generated for multiple consecutive loads onto a flow cell to increase output. To load a library three times on a flow cell, a flow cell wash is required to recover channels.

**Steps in the Sequencing workflow:**

**Prepare for your experiment**

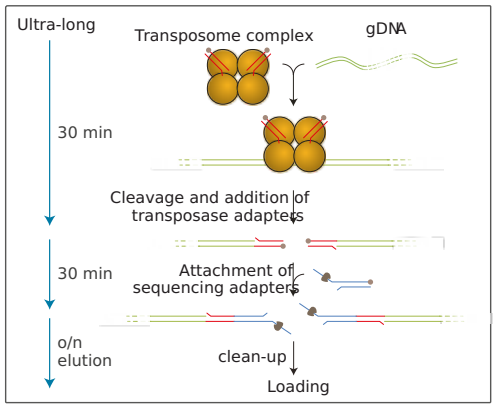
You will need to:

- Extract your UHMW gDNA
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- If not already installed, download the software for acquiring and analysing your data
- Check your flow cell(s) to ensure it has enough pores for a good sequencing run

**Libray preparation**

You will need to:

- Tagment your DNA using a diluted fragmentation mix
- Attach Rapid Adapters to the DNA ends
- Wash the DNA library using the Circulomics Nanobind disk and long fragment buffer to remove free adapter and short DNA fragments
- Prime the flow cell and load your DNA library into the flow cell




### Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads
- Start the EPI2ME software and select a workflow for further analysis (this step is optional)

### Flow cell loading and flushing

The Ultra-Long DNA Sequencing Kit (SQK-ULK001) protocol generates viscous DNA which can affect flow cell loading. We have modified the flow cell loading steps to take account for this. Please take care and follow the steps carefully to avoid damaging the flow cell.

To increase output, we recommend loading an ultra-long library three times per flow cell. A flow cell wash using the Flow Cell Wash Kit (EXP-WSH004) is required between each subsequent library load to recover channels. To run a second library straight away, please follow the modified method in this protocol: To run another library of ultra-long DNA on a MinION/GridION flow cell straight away.

**IMPORTANT**

**Compatibility of this protocol**

This protocol should only be used in combination with:

- Ultra-Long DNA Sequencing Kit (SQK-ULK001)
- Flow Cell Wash Kit (EXP-WSH004). We do **not** recommend using EXP-WSH003
- R9.4.1 flow cell (FLO-MIN106D)

# Equipment and consumables

| | |
|---|---|
| **Materials** | • 750 µl of extracted UHMW gDNA in Circulomics EB+<br>• Ultra-Long DNA Sequencing Kit (SQK-ULK001)<br>• Circulomics Nanobind Big DNA Kit (e.g. CBB, Plant or Tissue)<br>• Circulomics Nanobind UL Library Prep Kit (NB-900-601-01) |
| **Consumables** | • 1.5 ml Eppendorf DNA LoBind tubes |

| | |
|---|---|
| **Equipment** | • Magnetic separator, suitable for 1.5 ml Eppendorf tubes<br>• Thermal cycler or heat block at 75°C<br>• Vortex mixer<br>• Microfuge<br>• P1000 pipette and wide-bore pipette tips<br>• P200 pipette and wide-bore pipette tips<br>• P200 pipette and tips<br>• Ice bucket with ice<br>• Timer |
| **Optional Equipment** | • Qubit fluorometer (or equivalent for QC check) |

**For this protocol, you will need to extract 750 µl of UHMW gDNA in Circulomics EB+ before starting the library preparation.**

Customers can use any method to extract UHMW gDNA. However, we have used the appropriate Circulomics Nanobind Big DNA Kit, Ciculomics Nanobind UL Library Prep Kit (NB-900-601-01), and Circulomics UHMW DNA extraction protocol for the appropriate input material. Check their website for new protocols and sample types.

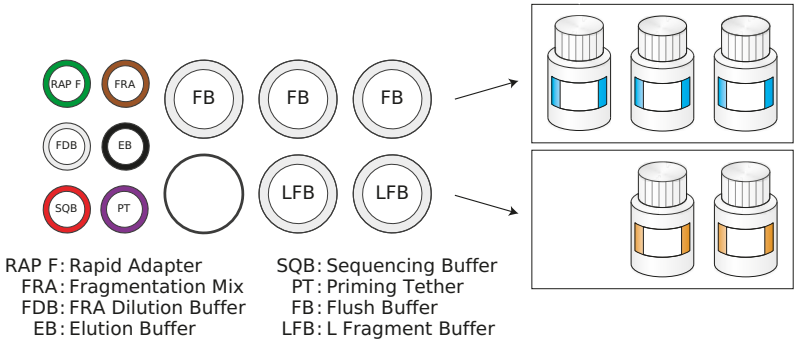
**Input materials:**

- Gram-negative bacterial cells: e.g. *E. coli*
  - Take 1 ml of *E. coli* cells grown to OD600=0.6
  - Circulomics Nanobind UHMW DNA Extraction - Gram Negative Protocol
- Mammalian cells: e.g. human line cells GM24385
  - Pellet 6 million human cell line cells
  - Circulomics Nanobind UHMW DNA Extraction - Cultured Mammalian Cells Protocol
- Human whole blood
  - Use 1 ml fresh or 2 ml frozen blood
  - Circulomics Nanobind UHMW DNA Extraction - 1.5 ml Whole Blood Protocol

Other input materials require further optimisation.

The extraction protocol specifically extracts UHMW gDNA to generate ultra-long reads with high N50 and longest reads of 4+ Mb using the Ultra-Long DNA Sequencing Kit (SQK-ULK001) and our sequencing devices.

**Ultra-Long DNA Sequencing Kit (SQK-ULK001) contents**




| Name | Acronym | Cap colour | Number of vials | Fill volume (µl) |
|---|---|---|---|---|
| Rapid Adapter | RAP-F | Green | 1 | 30 |
| Fragmentation Mix | FRA | Brown | 1 | 36 |
| FRA Dilution Buffer | FDB | Clear | 1 | 1464 |
| Elution Buffer | EB | Black | 1 | 1350 |
| Sequencing Buffer | SQB | Red | 1 | 1350 |
| Priming Tether | PT | Purple | 1 | 540 |
| Flush Buffer | FB | White cap, light blue stripe on label | 1 | 7020 |
| L Fragment Buffer | LFB | White cap, orange stripe on label | 2 | 6000 |

# Computer requirements and software

### GridION IT requirements

The GridION device contains all the hardware required to control up to five flow cells and acquire the data. The device is further enhanced with high performance GPU technology for real-time basecalling. Read more in the GridION IT requirements document.

### Software for nanopore sequencing

## MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data in real time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and basecall/demultiplex data after a sequencing run has completed.

### MinKNOW use

For instructions on how to run the MinKNOW software, please refer to the relevant section in the MinKNOW protocol.

## EPI2ME (optional)

The EPI2ME cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You will use the EPI2ME platform *only* if you would like further analysis of your data post-basecalling.

### EPI2ME installation and use

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform protocol.

### Guppy (optional)

The Guppy command-line software can be used for basecalling and demultiplexing reads by barcode instead of MinKNOW. You can use it if you would like to re-analyse old data, or integrate basecalling into your analysis pipeline.

### Guppy installation and use

If you would like to use the Guppy software, please refer to the Guppy protocol.

### Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION/GridION/PromethION flow cells, or within four weeks of purchasing for Flongle flow cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

| Flow cell | Minimum number of active pores covered by warranty |
|---|---|
| Flongle Flow Cell | 50 |
| MinION/GridION Flow Cell | 800 |
| PromethION Flow Cell | 5000 |

# Tagmentation

~60 minutes

**Materials**

- 750 µl of extracted UHMW gDNA in Circulomics EB+
- Rapid Adapter-F (RAP-F)
- Fragmentation Mix (FRA)
- FRA Dilution Buffer (FDB)

**Consumables**

- 1.5 ml Eppendorf DNA LoBind tubes

**Equipment**

- Thermal cycler or heat block at 75°C
- Vortex mixer
- Microfuge
- P20 pipette and tips
- P200 pipette and wide-bore pipette tips
- P1000 pipette and wide-bore pipette tips

### Best practice for handling UHMW gDNA

When mixing, We recommend using wide-bore pipette tips to mix the full volume of a sample to ensure thorough mixing whilst minimising mechanical shearing of long fragments.

To preserve longer DNA, mix slower and more gently. Vortexing on low speeds may also be used at the expense of very long fragments.

For further information, please refer to the troubleshooting section.

**Before starting the library preparation, extract 750 µl of UHMW gDNA in Circulomics EB+.**

1 **Thaw the Fragmentation Mix (FRA), FRA Dilution Buffer (FDB) and Rapid Adapter-F (RAP-F). Spin down briefly using a microfuge and keep on ice.**

2 **In a 1.5 ml Eppendorf DNA LoBind tube, make up the diluted FRA:**

| Reagents | Volume |
|---|---|
| Fragmentation Mix (FRA) | 6 µl |
| FRA dilution buffer (FDB) | 244 µl |
| **Total** | **250 µl** |

3 **Mix the diluted FRA by vortexing.**

4 **Add 250 µl of diluted FRA to the extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution.**

5 **Immediately vortex the reaction for 5 seconds at the lowest setting able to generate a gentle vortex in the fluid.**

Visually check the reagents are thoroughly mixed. If necessary, complete the mixing by gentle pipetting with a wide-bore pipette tip.

It is important to immediately mix the diluted FRA with the DNA thoroughly. However, longer reads are obtained without vortexing and using only gentle pipette mixing.

6 **Incubate the reaction as follows:**

| Temperature | Time |
|---|---|
| Room temperature | 5 minutes |
| 75°C | 5 minutes |
| Room temperature | Cool to room temperature for a **minimum** of 10 minutes |

**Note:** The reaction must be cooled to room temperature before adding RAP F to prevent denaturing the enzyme.

7 **Add 5 µl of RAP-F with a regular pipette tip. Use a P1000 wide-bore tip to pipette mix. Visually check to ensure the reaction is thoroughly mixed. Inversion can be used to aid mixing.**

8 **Incubate for 30 minutes at room temperature.**

# Clean-up

~540 minutes

| | |
|---|---|
| **Materials** | • Long Fragment Buffer (LFB)<br>• Elution Buffer from the Oxford Nanopore kit (EB)<br>• Circulomics NAF Binding Buffer<br>• Circulomics Nanobind Disk |
| **Consumables** | • 1.5 ml Eppendorf DNA LoBind tubes |
| **Equipment** | • Magnetic separator, suitable for 1.5 ml Eppendorf tubes<br>• Microfuge<br>• P20 pipette and tips<br>• P200 pipette and wide-bore pipette tips<br>• P1000 pipette and wide-bore pipette tips |

1 **Thaw one tube of Long Fragment Buffer (LFB) and Elution Buffer (EB) at room temperature, mix by vortexing, spin down and store at room temperature.**

2 **Add a single Circulomics Nanobind disk to the DNA library and invert to ensure both sides of the disk are wetted.**

3 **Add 500 µl Circulomics NAF Binding Buffer.**

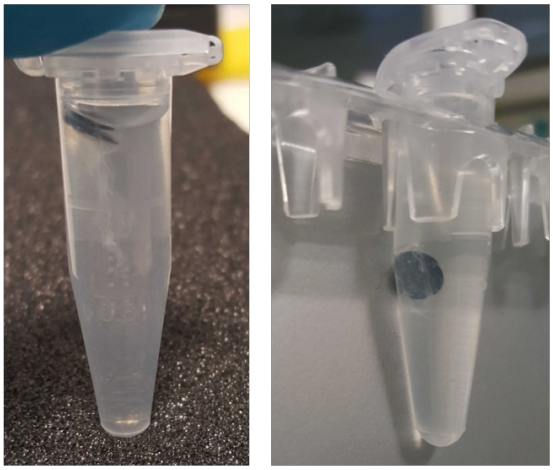
4 **Gently invert the tube 20-30 times to mix until all reagents are thoroughly mixed.**

5 **Wait 5-10 minutes. The DNA should start to become visible as a precipitate forming around the disk.**

6 **Invert the tube again 5-10 times to ensure all DNA has precipitated and is tightly bound to the Nanobind Disk.**

7 **Place the tube on a magnetic rack to aspirate and discard the supernatant with a P200 pipette. Take care not to disturb the DNA precipitated onto the Nanobind disk.**

**Handling DNA bound to the Nanobind disk**



**Left:** When DNA binds to the Nanobind disk, it will be visible as a fluffy white precipitate and may hang from the disk. Take extra care to not aspirate or disrupt the DNA.
**Right:** Before aspirating the supernatant, push the nanobind disk higher up the tube with a pipette tip on the magnetic rack to avoid inadvertently aspirating any DNA.

8 **Wash the Nanobind disk by gently adding 1000 µl of Long Fragment Buffer (LFB) and invert. Keep the tube on the magnetic rack and incubate for 5 minutes at room temperature. Aspirate and discard the supernatant, taking care not to disturb the DNA precipitate.**

It may be necessary to move the DNA precipitate away from the bottom of the tube with a pipette tip for the supernatant to be aspirated.

9 **Repeat the previous step and remove any supernatant in the lid of the Eppendorf tube.**

10 **Add 225 µl of Elution Buffer (EB) to elute the DNA.**

11 **Incubate the library overnight at room temperature, for a minimum of 12 hours. Gently aspirate and dispense the eluate over the Nanobind disk at regular intervals with a wide-bore pipette tip to aid elution.**

For efficient elution of the DNA from the disk during incubation, ensure the Nanobind disk is fully submerged in the Elution Buffer (EB).

12 **Transfer 225 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube after the overnight incubation with a wide-bore tip. The DNA should be viscous.**

13 **Spin the tube containing the Nanobind disk on a centrifuge at 10,000 x g for 10-15 seconds and transfer any additional liquid that comes off the disk to the previous eluate. Repeat as necessary until all DNA is removed using a standard P200 pipette.**

Remove as much eluate as possible from the Nanobind disk before continuing.

14 **Gently mix the eluate containing the DNA library five times with a wide-bore pipette tip.**

15 **Incubate for 2 hours at room temperature. The library should be very viscous.**

**END OF STEP**

**The prepared library is used for loading into the flow cell. Store at room temperature to allow DNA to homogenise before loading.**

Store the library at 4°C if sequencing is not carried out the same day as library preparation.

# Priming and loading the SpotON flow cell for GridION

~10 minutes

**Materials**
- Priming Tether (PT)
- Flush Buffer (FB)
- Sequencing Buffer (SQB)

**Consumables**
- 1.5 ml Eppendorf DNA LoBind tubes
- MinICN Flow Cell

**Equipment**
- GridICN device
- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips

1 **Thaw the Sequencing Buffer (SQB), Priming Tether (PT) and one tube of Flush Buffer (FB) at room temperature. Mix by vortexing and spin down.**

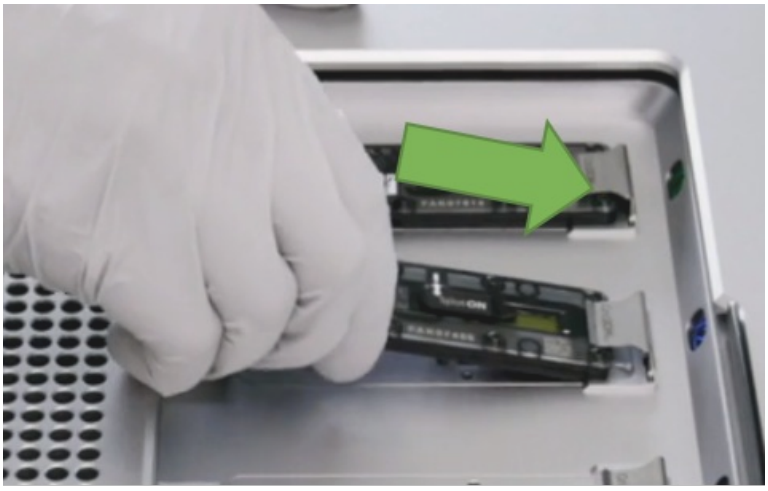
2 **In a new tube, prepare the DNA library for loading as follows and gently mix with a wide-bore tip:**

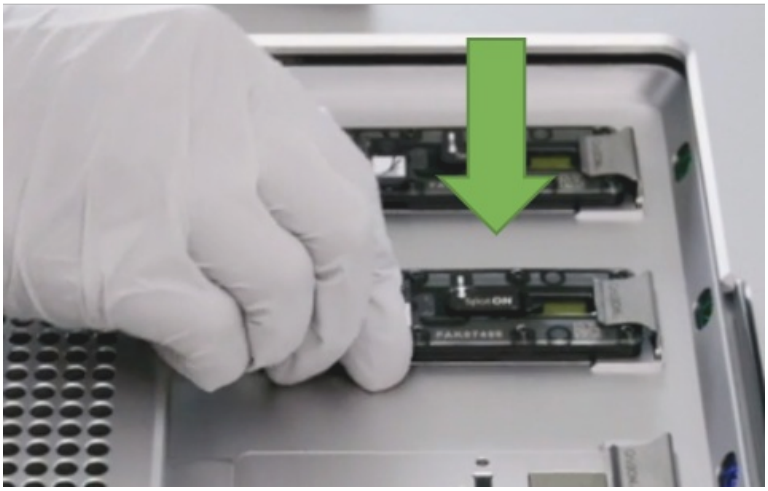
| Reagent | Volume |
|---|---|
| Sequencing Buffer (SQB) | 37.5 µl |
| DNA library | 37.5 µl |
| **Total** | **75 µl** |

3 **Wait 30 minutes and gently mix with a wide-bore tip. Visually inspect to ensure the sample is homogenous.**

4 **Slide open the GridION lid and insert the flow cell.**

Press down firmly on the flow cell to ensure correct thermal and electrical contact.





**5 Slide the priming port cover clockwise to open the priming port.**


**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.**

**6 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):**

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 µl, or until you can see a small volume of buffer entering the pipette tip

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

**7 Prepare the flow cell priming mix in a 1.5 ml Eppendorf tube and mix by vortexing at room temperature.**

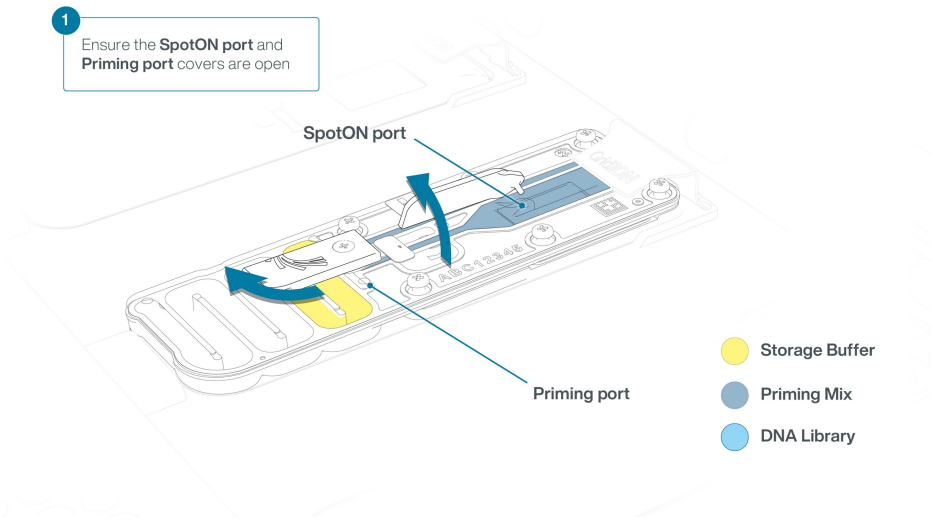
| Reagent | Volume |
|---|---|
| Priming Tether (PT) | 30 µl |
| Flush Buffer (FB) | 1170 µl |
| **Total** | **1200 µl** |

**8 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.**

**9 Complete the flow cell priming:**

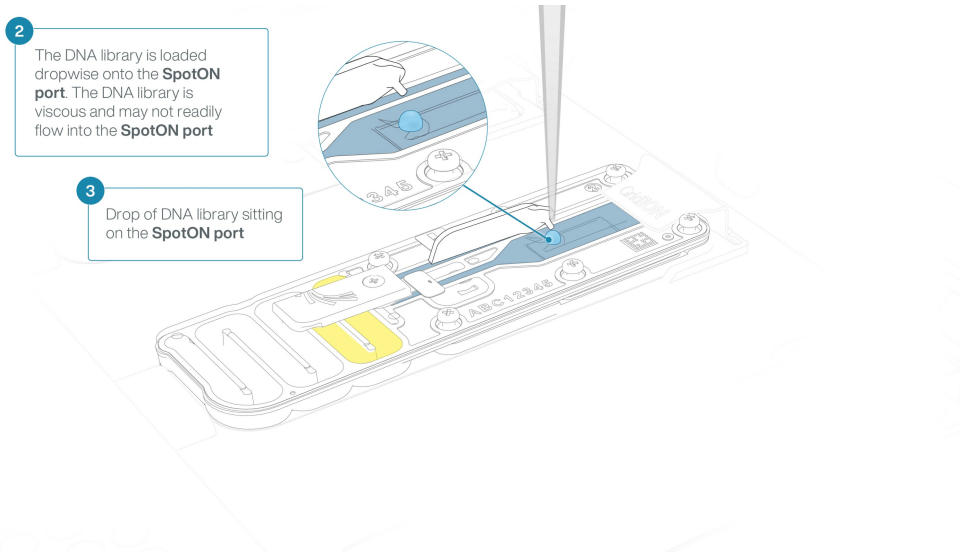
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

**10 Thoroughly mix the DNA library and ensure the SpotON port and Priming port covers of the flow cell are open in preparation for loading.**

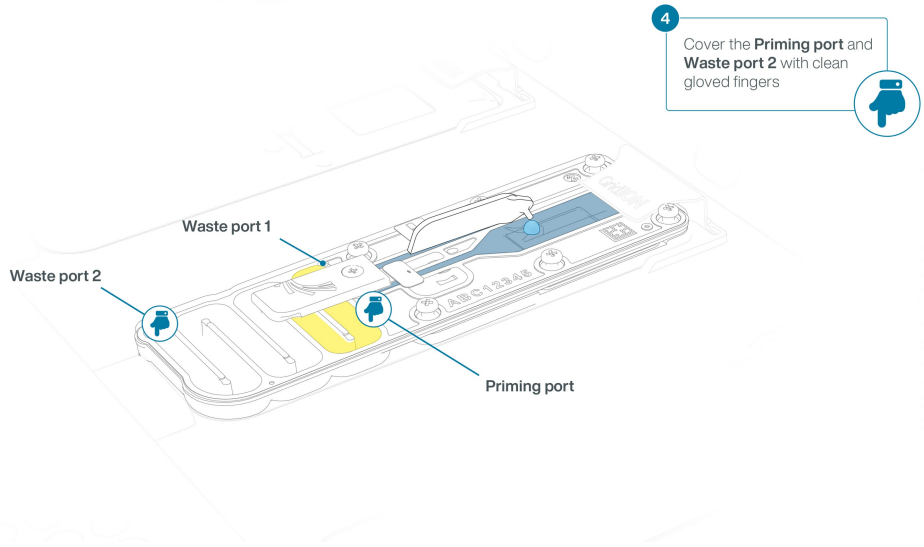



**11 Load the DNA library dropwise onto the SpotON port until 75 µl has been loaded. Ensure each drop flows into the port before adding the next.**

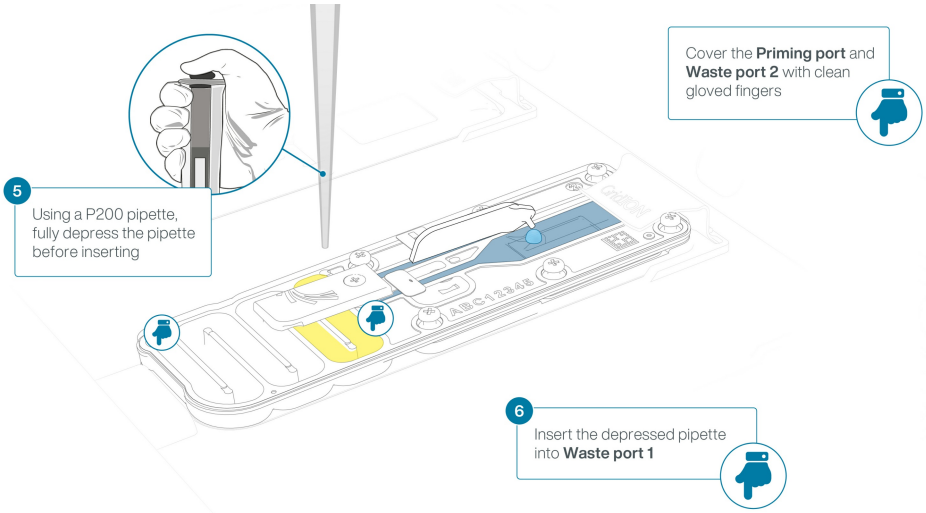
The DNA library is viscous and may not readily flow through the sample port into the flow cell, instead sitting on the SpotON port. In this case, we recommend applying negative pressure in the flow cell as explained further below.



**12 Cover Waste port 2 and the Priming port with clean, gloved fingers.**

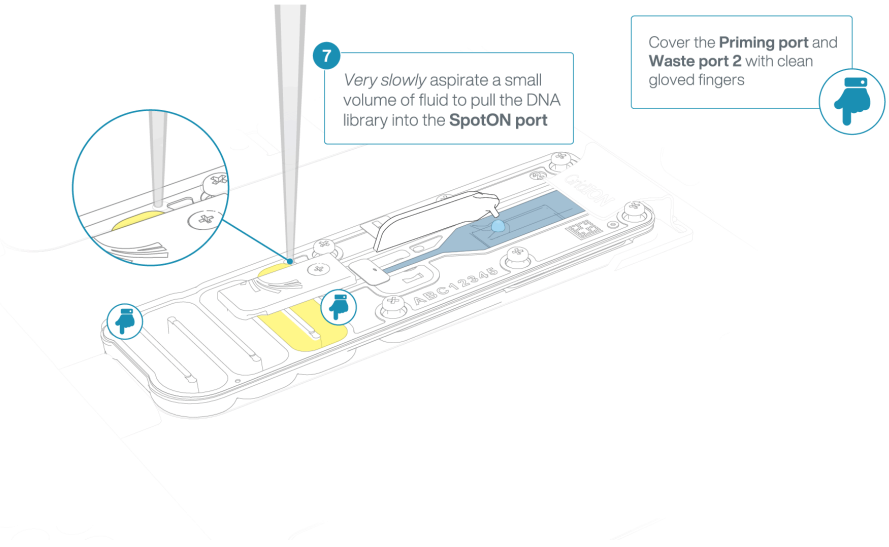



**13 Using a fully depressed P200 pipette, insert the tip in Waste port 1 whilst Waste port 2 and the Priming port are covered.**

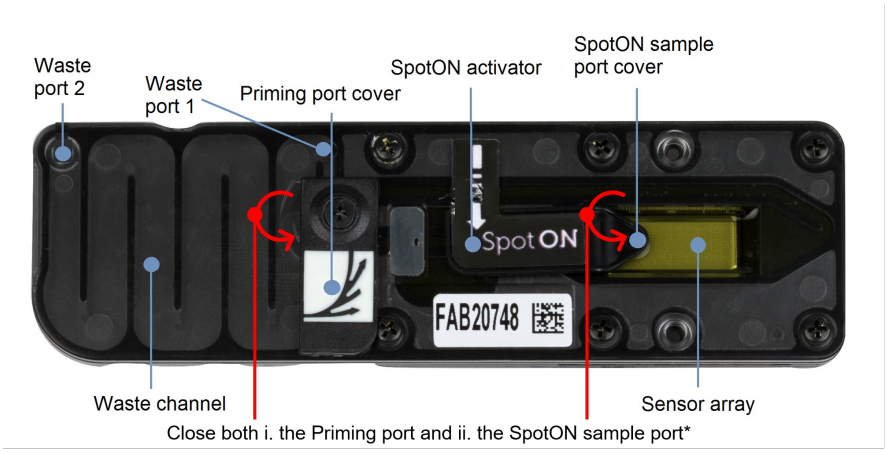


**14 Very slowly aspirate to pull the DNA library into the SpotON sample port. Closely watch the DNA library on the SportON port and completely remove the pipette as soon as the library starts to be pulled into the port.**

**Note:** Take care to not apply too much negative pressure too quickly to avoid bringing air bubbles into the flow cell. Air bubbles will cause irreversible damage to the flow cell.



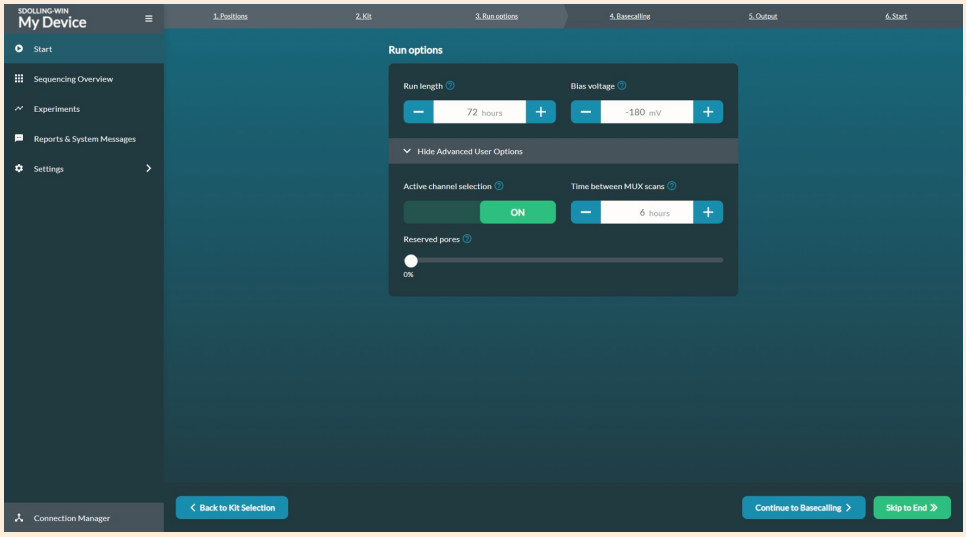

**15 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the GridION lid.**




*Both ports are shown in a closed position

**IMPORTANT**

**When setting up your run parameters in MinKNOW, under Run Options, set the time between mux scans to 6 hours from the default 1.5 hours before starting the sequencing run.**




**We recommend loading an ultra-long DNA library three times per flow cell to increase output.**

A nuclease wash using the Flow Cell Wash Kit (EXP-WSH004) is required between each subsequent library load to recover channels and maximise sequencing output.

For MinION/GridION flow cells, there is enough library generated for six consecutive loads per reaction, using 37.5 µl of fresh library combined with an equal volume of Sequencing Buffer (SQB) before re-loading for further sequencing.

Please follow Flushing a MinION/GridION Flow Cell in the Flow Cell Wash Kit protocol for the nuclease wash instructions. To run another library straight away, follow the modified method: Reloading ultra-long DNA library on a MinION/GridION flow cell.

# Reloading ultra-long DNA library on a MinION/GridION flow cell

**Materials**

- Priming Tether (PT)
- Flush Buffer (FB)
- Sequencing Buffer (SQB)

**Consumables**

- 1.5 ml Eppendorf DNA LoBind tubes
- MinICN Flow Cell

**Equipment**

- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips

**To run a second library of ultra-long DNA straight after flushing a flow cell, we recommend removing all fluid from the waste channel after each priming step, as outlined below.**

Follow the steps below carefully to avoid damaging your flow cell.

Once the flow cell has been primed and loaded correctly, either resume the run in MinKNOW or start a new sequencing experiment.

> **TIP**
>
> **For the best results, it is advised to adjust the starting voltage of the new experiment due to voltage drift in the course of the previous run.**
>
> The voltage adjustment scheme is described in Adjusting the starting potential for multiple runs in series.

1 **Mix the Sequencing Buffer (SQB), Priming Tether (PT) and Flush Buffer (FB) tubes by vortexing and spin down at room temperature.**

2 **In a new tube, prepare the DNA library for loading as follows and gently mix with a wide-bore tip:**

| Reagent | Volume |
|---|---|
| Sequencing Buffer (SQB) | 37.5 µl |
| DNA library | 37.5 µl |
| **Total** | **75 µl** |

3 **Wait 30 minutes and gently mix with a wide-bore tip. Visually inspect to ensure the sample is homogenous.**

4 **Slide the priming port cover of the flow cell clockwise to open the priming port.**

**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.**

**5 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):**

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 µl, or until you can see a small volume of buffer entering the pipette tip

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

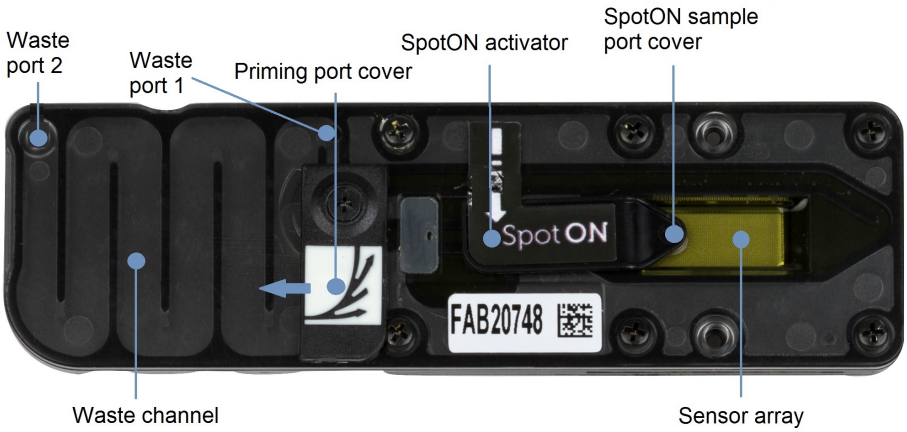
**6 Prepare the flow cell priming mix in a 1.5 ml Eppendorf tube and mix by vortexing at room temperature.**

| Reagent | Volume |
|---|---|
| Priming Tether (PT) | 30 µl |
| Flush Buffer (FB) | 1170 µl |
| **Total** | **1200 µl** |

**7 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.**

**8 Close the priming port cover and ensure the SpotON sample port cover is closed.**

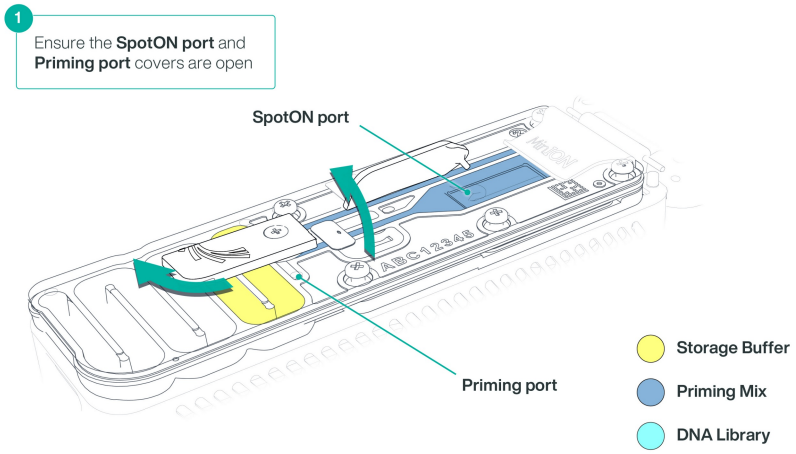
**9 Using a P1000, remove all fluid from the waste channel through Waste port 1. As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.**




**10 Slide open the priming port and load 200 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles.**

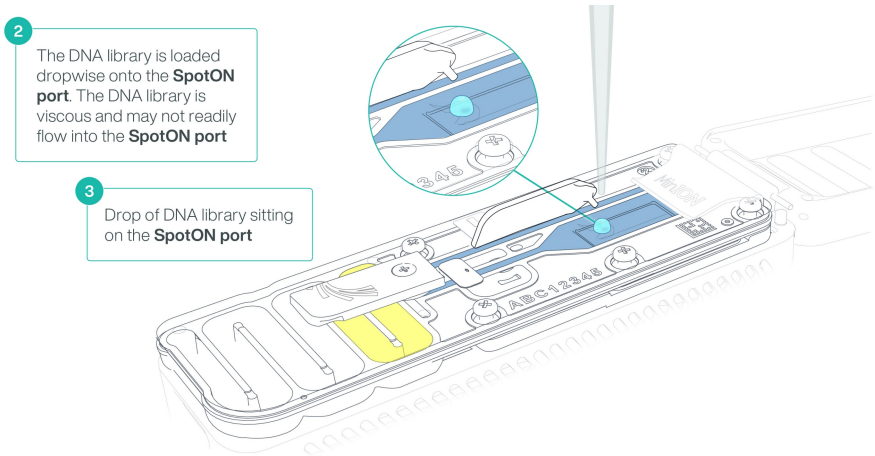
**11 Close the priming port and use a P1000 to remove all fluid from the waste channel through Waste Port 1.**

**12 Thoroughly mix the DNA library and ensure the SpotON port and Priming port covers of the flow cell are open in preparation for loading.**

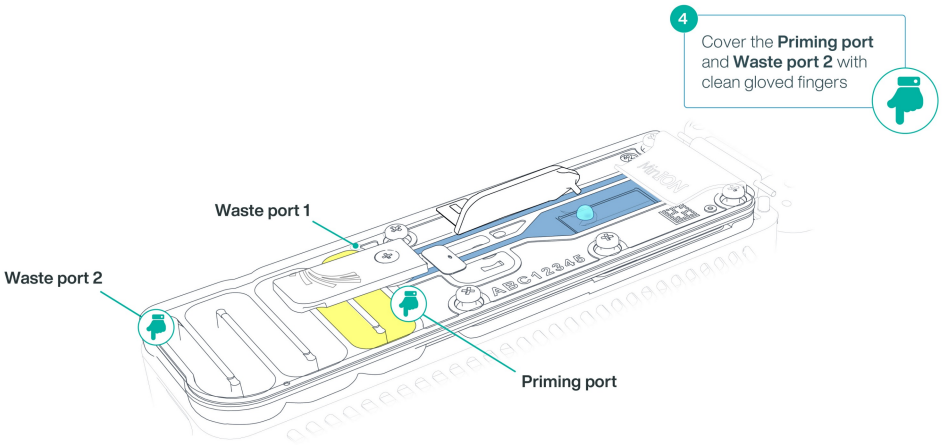



**13 Load the DNA library dropwise onto the SpotON port until 75 µl has been loaded. Ensure each drop flows into the SpotON port before adding the next.**

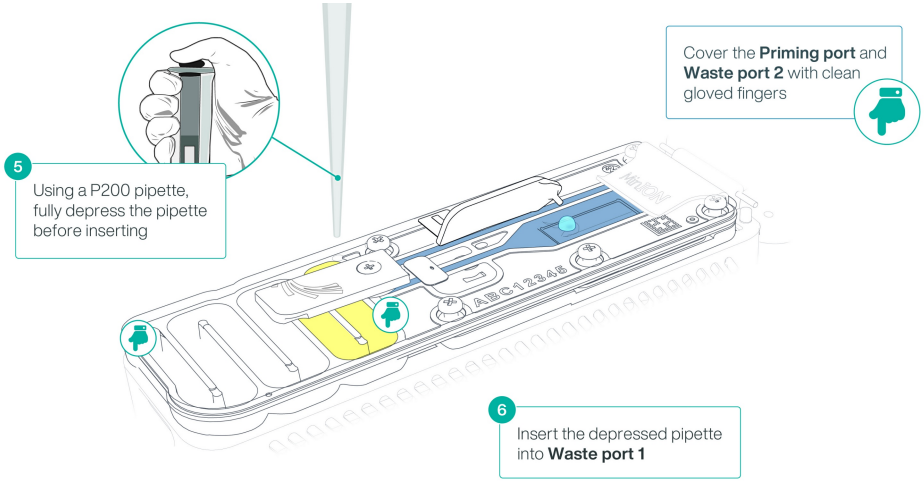
The DNA library is viscous and may not readily flow through the sample port into the flow cell. In this case, we recommend applying negative pressure in the flow cell as explained further below.



**14 Cover Waste port 2 and the Priming port with clean, gloved fingers.**

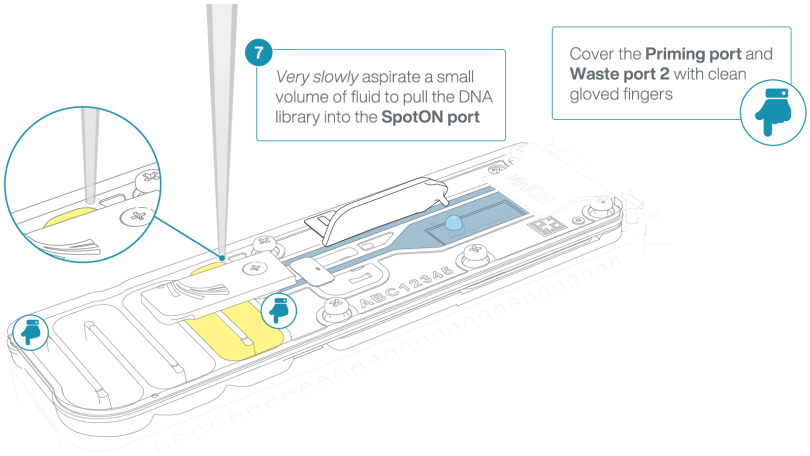



**15 Using a fully depressed P200 pipette, insert the tip in Waste port 1 whilst Waste port 2 and the Priming port are covered.**



16 **Very slowly aspirate to pull the DNA library into the SpotON sample port. Closely watch the DNA library on the SportON port and completely remove the pipette as soon as the library starts to be pulled into the port.**

**Note:** Take care to not apply too much negative pressure too quickly to avoid bringing air bubbles into the flow cell. Air bubbles will cause irreversible damage to the flow cell.



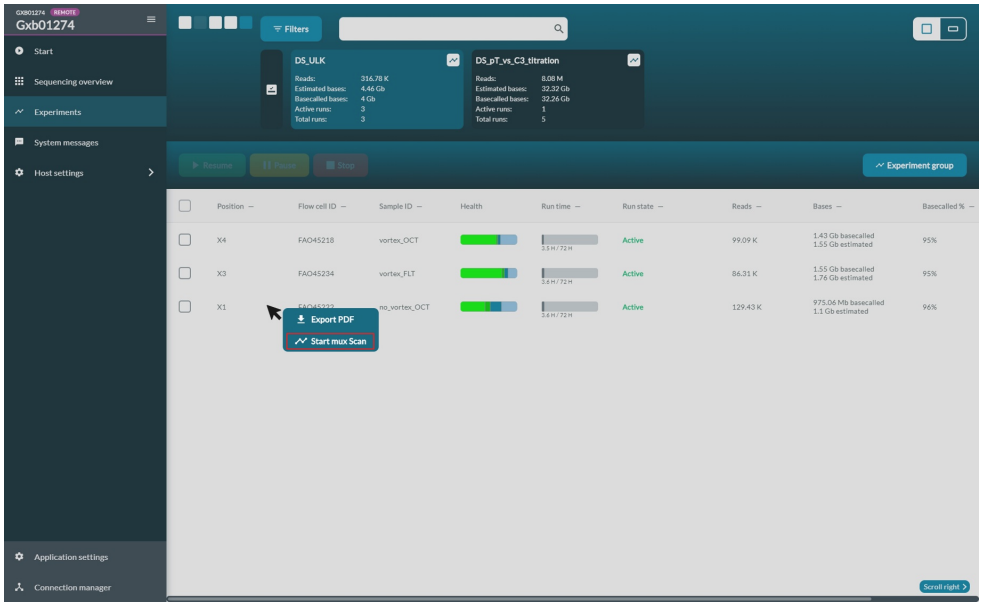

17 **Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.**

**18 Once the flow cell is reloaded, resume the sequencing run on MinKNOW and trigger a mux scan.**

To resume sequencing run, navigate to the Experiments page, select flow cell position and click 'Resume'.

To manually trigger a mux scan, right click the flow cell position and click 'Start mux scan'.

For further information, please see the MinKNOW protocol.




# Data acquisition and basecalling

### Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

### How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing:

### 1. Data acquisition and basecalling in real-time using MinKNOW on a computer

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

### 2. Data acquisition and basecalling in real-time using the MinION Mk1C device

Follow the instructions in the MinION Mk1C protocol.

### 3. Data acquisition and basecalling in real-time using the MinIT device

Follow the instructions in the MinIT protocol.

### 4. Data acquisition using MinKNOW on a computer and basecalling at a later time using Guppy

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the *Basecalling* tab to OFF.** After the sequencing experiment has completed, follow the instructions in the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

# Downstream analysis

**Post-basecalling analysis**

There are several options for further analysing your basecalled data:

### 1. EPI2ME platform

The EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of Oxford Nanopore Technologies. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment, and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results. For instructions on how to run an analysis workflow in EPI2ME, please follow the instructions in the EPI2ME protocol, beginning at the "Starting an EPI2ME workflow" step.

### 2. Bioinformatics tutorials

For more in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials, which are available in the Bioinformatics resource section of the Community. The tutorials take the user through installing and running pre-built analysis pipelines, which generate a report with the results. The tutorials are aimed at biologists who would like to analyse data without the help of a dedicated bioinformatician, and who are comfortable using the command line.

### 3. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, which are available in the Oxford Nanopore GitHub repository. The tools are aimed at advanced users, and contain instructions for how to install and run the software. They are provided as-is, with minimal support.

### 4. Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, please refer to the Community-developed data analysis tool library. Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

# Ending the experiment

**Materials**

- Flow Cell Wash Kit (EXP-WSH004)

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, OR**

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

2 **Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

**IMPORTANT**

**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 during library preparation

**Below is a list of the most commonly encountered issues, with some suggested causes and solutions.**

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

### Troubleshooting

| Observation | Comments and actions |
|---|---|
| Low throughput | 1. Vortex gently after adding FRA to break up the largest fragments.<br>3. Ensure FRA is thoroughly mixed with the gDNA.<br>2. Use less input material if the DNA library was too viscous to load onto the flow cell. |
| DNA is too viscous and will not load onto a flow cell | 1. Lower the input material to reduce the amount of gDNA going into the library preparation and reduce viscosity.<br>2. If DNA library will not load using the method outlined in this protocol, slowly pipette mix 5 times with a standard P200 pipette set to the full volume of the library and reload the flow cell. |
| Read lengths are not long enough | 1. Increase input material.<br>**Note:** Library viscosity increases with more gDNA.<br>2. Reduce volume of FRA added to avoid over-fragmentation of gDNA.<br>**Note:** We do not recommend less than 2 µl FRA.<br>3. We recommend using PFGE to check the extracted gDNA is of ultra-high molecular weight (UHMW), thus capable of generating ultra-long read lengths. |
| No sequencing output | 1. Check gDNA has been recovered in library preparation using a NanoDrop spectrophotometer.<br>2. Check viscosity of the sample. The library should be viscous if it contains UHMW gDNA in this protocol. |
| Aspirating supernatant when the DNA has precipitated | Move the Nanobind disk up the tube when on the magnetic rack and take care to not aspirate the DNA. Remove smaller volumes of supernatant incrementally to reduce the risk of aspirating the DNA. |
| Mixing | Mix slowly and carefully to prevent DNA shearing. Low vortexing can be used to mix at the expense of ultra-long reads. With vortexing, long read lengths of ~90 kb N50 can still be generated with improved outputs. |

| Observation | Comments and actions |
|---|---|
| No DNA recovered from the library preparation clean-up | If the DNA is no longer viscous or the NanoDrop reading is low, DNA may have been lost during the clean-up step of the library preparation.<br>1. Ensure UHMW DNA is used or users risk DNA loss.<br>2. Take care to not aspirate the precipitated DNA during the clean-up step. To avoid this, nudge the Nanobind disk up the side of the tube with a pipette tip when on the magnetic rack.<br>3. Ensure as much eluate as possible is removed from the Nanobind disk after the clean-up step. Spin down the tube with the Nanobind disk at 10,000 x g for 15 seconds and transfer any eluate. Complete elution requires patience and the centrifuge step is critical but should not take more than 1-2 spins. |