

## Introduction to the protocol

Version: ACDE\_9064\_v109\_revS\_14Aug2019

# Overview of the protocol

### Ligation Sequencing Kit features

This kit is recommended for users who:

- o want to optimise their sequencing experiment for throughput
- o would like to utilise upstream processes such as size selection, whole genome amplification, or enrichment for long reads

### Introduction to the Ligation Sequencing protocol for amplicons

This protocol describes how to carry out sequencing of an amplicon sample using the Ligation Sequencing Kit (SQK-LSK109). It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

### Steps in the sequencing workflow:

#### Prepare for your experiment

You will need to:

- Extract your DNA, and check its length, quantity and purity.

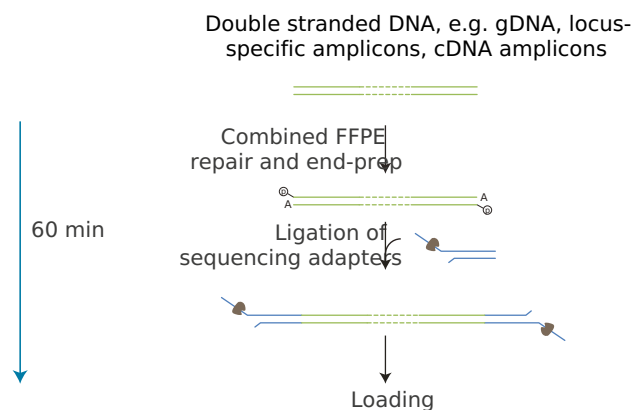
#### The quality checks performed during the protocol are essential in ensuring experimental success.

- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell to ensure it has enough pores for a good sequencing run

#### Library preparation

You will need to:

- Amplify your DNA sample
- Repair the DNA, and prepare the DNA ends for adapter attachment
- Attach sequencing adapters supplied in the kit to the DNA ends
- 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

## Equipment and consumables

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

### IMPORTANT

#### Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (SQK-LSK109)
- Control Expansion (EXP-CTL001)
- FLO-MIN106D (R9.4.1) flow cells (FLO-MIN107 not advised)
- FLO-MIN111 (R10.3) flow cells
- Flow Cell Wash Kit (EXP-WSH004)

## Equipment and consumables

### Materials

- 100-200 fmol amplicon DNA
- 150-300 fmol amplicon DNA if using R10.3 flow cells
- Ligation Sequencing Kit (SQK-LSK109)
- Flow Cell Priming Kit (EXP-FLP002)

### Consumables

- Agencourt AMPure XP beads
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:
- NEBNext FFPE Repair Mix (M6630)
- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- 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

### Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- 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

## Equipment and consumables

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### Optional Equipment

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

**For this protocol, you will need 1 µg (or 100-200 fmol) amplicon DNA for R9.4.1 flow cells, or 1.5-3 µg (or 150-300 fmol) for R10.3 flow cells.**

### Input DNA

#### How to QC your input DNA

It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

For instructions on how to perform quality control of your DNA sample, please read the [Input DNA/RNA QC protocol](#).

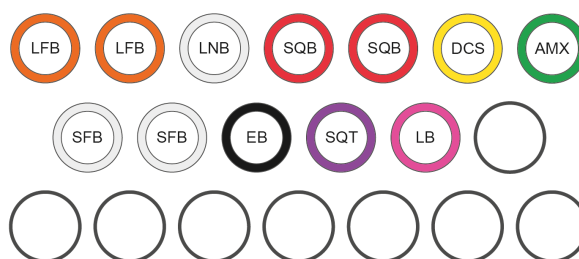
#### Chemical contaminants

Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the [Contaminants page](#) of the Community.

### NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing

For customers new to nanopore sequencing, we recommend buying the [NEBNext® Companion Module](#) for Oxford Nanopore Technologies® Ligation Sequencing (catalogue number E7180S), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

### Ligation Sequencing Kit (SQK-LSK109) contents



LFB : L fragment buffer      SFB : S fragment buffer  
 LNB : Ligation buffer      EB : Elution buffer  
 SQB : Sequencing buffer      SQT : Sequencing tether  
 DCS : DNA control strand      LB : Loading beads  
 AMX : Adapter mix

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	50
Adapter Mix	AMX	Green	1	40
Ligation Buffer	LNB	Clear	1	200
L Fragment Buffer	LFB	White cap, orange stripe on label	2	1,800

## Computer requirements and software

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Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
S Fragment Buffer	SFB	Grey	2	1,800
Sequencing Buffer	SQB	Red	2	300
Elution Buffer	EB	Black	1	200
Loading Beads	LB	Pink	1	360
Sequencing Tether	SQT	Purple	1	10

### Flow Cell Priming Kit (EXP-FLP002) contents



FLB : Flush buffer

FLT : Flush tether

Name	Acronym	Cap colour	No. of vial	Fill volume per vial (µl)
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

## Computer requirements and software

### MinION Mk1B IT requirements

Unless you are using a MinIT device, sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the [MinION 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](#).

## Library preparation

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### 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

## DNA repair and end-prep

~35 minutes

### Materials

- 100-200 fmol amplicon DNA
- 150-300 fmol amplicon DNA if using R10.3 flow cells
- DNA CS

### Consumables

- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- NEBNext FFPE DNA Repair Mix (M6630)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease-free water

### Equipment

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

# Amplicons by Ligation (SQK-LSK109)

## Library preparation

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### 1 Prepare the DNA in nuclease-free water.

- Transfer 100-200 fmol amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube (start with 150-300 fmol if sequencing on R10.3 flow cells)
- Adjust the volume to 47  $\mu$ l with nuclease-free water
- Mix thoroughly by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

### 2 Thaw DNA CS (DCS) at room temperature, spin down, mix by pipetting, and place on ice.

### 3 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.

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

Reagent	Volume
DNA CS	1 $\mu$ l
DNA	47 $\mu$ l
NEBNext FFPE DNA Repair Buffer	3.5 $\mu$ l
NEBNext FFPE DNA Repair Mix	2 $\mu$ l
Ultra II End-prep reaction buffer	3.5 $\mu$ l
Ultra II End-prep enzyme mix	3 $\mu$ l
<b>Total</b>	<b>60 <math>\mu</math>l</b>

### 5 Mix gently by flicking the tube, and spin down.

### 6 Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

#### IMPORTANT

##### AMPure XP bead clean-up

It is recommended that the repaired/end-prepped DNA sample is subjected to the following clean-up with AMPure XP beads. This clean-up can be omitted for simplicity and to reduce library preparation time. However, it has been observed that omission of this clean-up can: reduce subsequent adapter ligation efficiency, increase the prevalence of chimeric reads, and lead to an increase in pores being unavailable for sequencing. If omitting the clean-up step, proceed to the next section.

### 7 Resuspend the AMPure XP beads by vortexing.

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

# Amplicons by Ligation (SQK-LSK109)

## Adapter ligation and clean-up

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- 9 Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube.
- 10 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
- 11 Prepare 500 µl of fresh 70% ethanol in nuclease-free water.
- 12 Spin down the sample and pellet on a magnet until eluate is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.
- 13 Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 14 Repeat the previous step.
- 15 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
- 16 Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease-free water. Incubate for 2 minutes at room temperature.
- 17 Pellet the beads on a magnet until the eluate is clear and colourless.
- 18 Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

### END OF STEP

Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4° C overnight.

## Adapter ligation and clean-up

~30 minutes

### Materials

- Adapter Mix (AMX)
- Ligation Buffer (LNB)
- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- Elution Buffer from the Oxford Nanopore kit (EB)

### Consumables

- NEBNext Quick Ligation Module (E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- Agencourt AMPure XP beads

## Adapter ligation and clean-up

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### Equipment

- Magnetic rack
- Microfuge
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

### IMPORTANT

Although the recommended 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.

- 1 Spin down the Adapter Mix (AMX) and Quick T4 Ligase, and place on ice.
- 2 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
- 3 Thaw the Elution Buffer (EB) at room temperature, mix by vortexing, spin down and place on ice.

### IMPORTANT

Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.

- 4 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.
- 5 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.
- 6 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Buffer (LNB)	25 µl
NEBNext Quick T4 DNA Ligase	10 µl
Adapter Mix (AMX)	5 µl
<b>Total</b>	<b>100 µl</b>

# Amplicons by Ligation (SQK-LSK109)

## Adapter ligation and clean-up

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**7 Mix gently by flicking the tube, and spin down.**

**8 Incubate the reaction for 10 minutes at room temperature.**

### IMPORTANT

**If you have omitted the AMPure purification step after DNA repair and end-prep, do not incubate the reaction for longer than 10 minutes.**

**9 Resuspend the AMPure XP beads by vortexing.**

**10 Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.**

**11 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.**

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

**13 Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 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.**

**14 Repeat the previous step.**

**15 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

**16 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.**

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

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

Dispose of the pelleted beads

### Optional Action

Quantify 1 µl of eluted sample using a Qubit fluorometer.

## Priming and loading the SpotON flow cell

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### IMPORTANT

**We recommend loading the final prepared library onto a flow cell following one of our recommendations depending on the flow cell type:**

- R9.4.1 flow cells, load 5-50 fmol
- R10.3 flow cells, load 25-75 fmol

Loading more than the maximal recommended amount of DNA can have a detrimental effect on output as higher quantities of DNA results in a larger number of ligated DNA ends with loaded motor protein. This depletes fuel in the Sequencing Buffer, regardless of whether or not the DNA fragments are being sequenced. This leads to fuel depletion and speed drop-off early in the sequencing run. Dilute the libraries in Elution Buffer if required.

If you are using the Flongle for sample prep development, we recommend loading 3-20 fmol instead.

### END OF STEP

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

### TIP

#### Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short term** storage or repeated use, for example, re-loading 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.

For further information, please refer to the [DNA library stability Know-How document](#).

#### Optional Action

If quantities allow, the library may be diluted in Elution Buffer (EB) for splitting across multiple flow cells.

Additional buffer for doing this can be found in the Sequencing Auxiliary Vials expansion (EXP-AUX001), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SQB) and Loading Beads (LB), required for loading the libraries onto flow cells.

## Priming and loading the SpotON flow cell

~10 minutes

### Materials

- Flow Cell Priming Kit (EXP-FLP002)
- Loading Beads (LB)
- Sequencing Buffer (SQB)

### Consumables

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

### Equipment

- MiniCN Mk1B
- SpotCN Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips

## Priming and loading the SpotON flow cell

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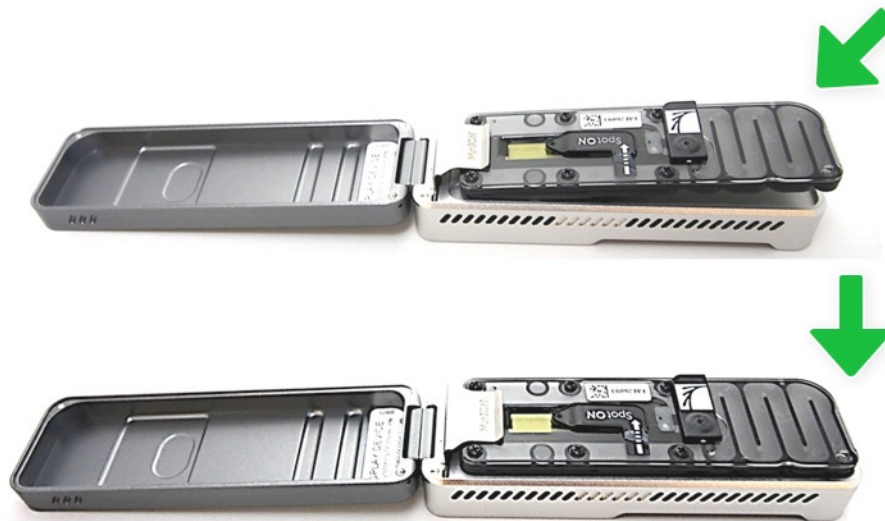
- P10 pipette and tips

### IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

- 1 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.
- 2 Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by vortexing and spin down at room temperature.
- 3 Open the MinION Mk1B lid and slide the flow cell under the clip.

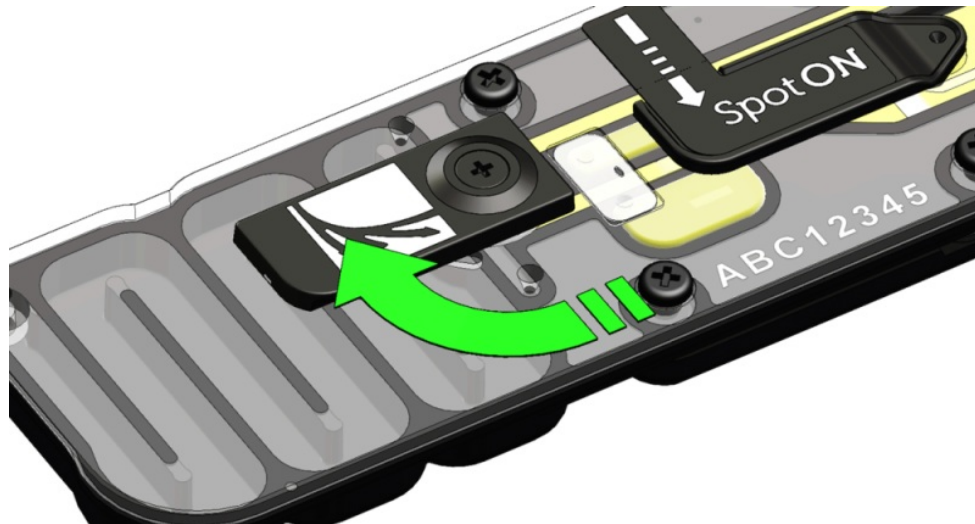
Press down firmly on the flow cell to ensure correct thermal and electrical contact.



## Priming and loading the SpotON flow cell

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- 4 Slide the priming port cover clockwise to open the priming port.



### How to prime and load the SpotON Flow Cell

**Priming and loading:** The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.

Take care to avoid introducing any air during pipetting.

#### IMPORTANT

**Take care when drawing back buffer from the flow cell. Do not remove more than 20-30  $\mu$ l, 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  $\mu$ l):

1. Set a P1000 pipette to 200  $\mu$ l
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230  $\mu$ l, 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 To prepare the flow cell priming mix, add 30  $\mu$ l 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.
- 7 Load 800  $\mu$ l of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.
- 8 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

## Priming and loading the SpotON flow cell

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### IMPORTANT

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

### TIP

#### Using the Loading Beads

Demo of how to use the Loading Beads.

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

Reagent	Volume
Sequencing Buffer (SQB)	37.5 $\mu$ l
Loading Beads (LB), mixed immediately before use	25.5 $\mu$ l
DNA library	12 $\mu$ l
<b>Total</b>	<b>75 <math>\mu</math>l</b>

### 10 Complete the flow cell priming:

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

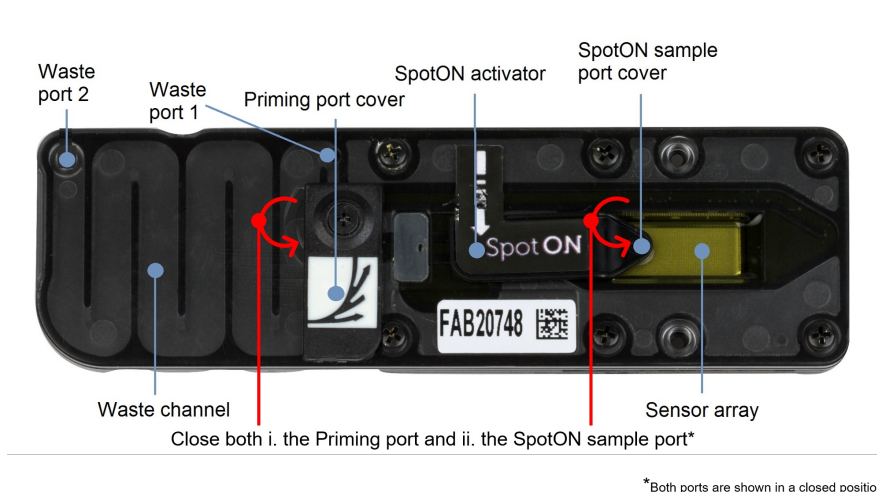
### 11 Mix the prepared library gently by pipetting up and down just prior to loading.

### 12 Add 75 $\mu$ l 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.

## Sequencing and data analysis

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- 13 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.**



# 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"

## Downstream analysis

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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 data analysis" step.

### 2. EPI2ME Labs tutorials and workflows

For more in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME Labs, which are available in the [EPI2ME Labs](#) section of the Community. The platform provides a vehicle where workflows deposited in GitHub by our Research and Applications teams can be showcased with descriptive texts, functional bioinformatics code and example data.

### 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 [Bioinformatics section of the Resource centre](#). 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.

Troubleshooting

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**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 DNA/RNA extraction and 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](mailto:support@nanoporetech.com)) or via [LiveChat](#) in the [Nanopore Community](#).

**Low sample quality**

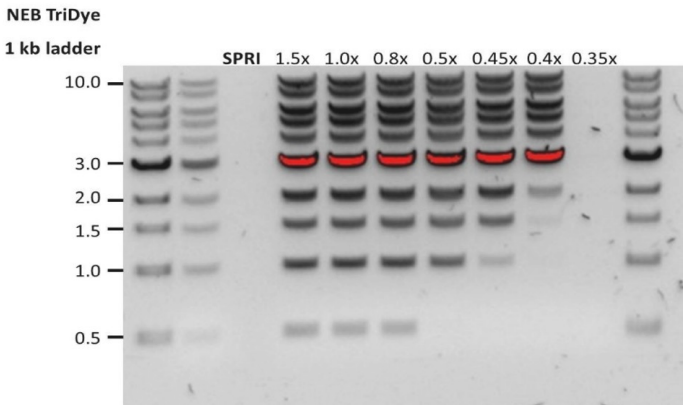
Observation	Possible cause	Comments and actions
<b>Low DNA purity (Nanodrop reading for DNA OD 260/280 is &lt;1.8 and OD 260/230 is &lt;2.0–2.2)</b>	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the <a href="#">Contaminants</a> Know-how piece. Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.  Consider performing an additional SPRI clean-up step.
<b>Low RNA integrity (RNA integrity number &lt;9.5 RIN, or the rRNA band is shown as a smear on the gel)</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .
<b>RNA has a shorter than expected fragment length</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .  We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

**Low DNA recovery after AMPure bead clean-up**

Observation	Possible cause	Comments and actions
<b>Low recovery</b>	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.  2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.

**Troubleshooting**

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Observation	Possible cause	Comments and actions
<b>Low recovery</b>	DNA fragments are shorter than expected	<p>The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use.</p> 
<b>Low recovery after end-prep</b>	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

**The VoITRAX run terminated in the middle of the library prep**

Observation	Possible cause	Comments and actions
<p><b>The green light was switched off</b></p> <p>or</p> <p><b>An adapter was used to connect the VoITRAX USB-C cable to the computer</b></p>	Insufficient power supply to the VoITRAX	The green LED signals that 3 A are being supplied to the device. This is the requirement for the full capabilities of the VoITRAX V2 device. Please use computers that meet the requirements listed on the <a href="#">VoITRAX V2 protocol</a> .

**The VoITRAX software shows an inaccurate amount of reagents loaded**

Observation	Possible cause	Comments and actions
<b>The VoITRAX software shows an inaccurate amount of reagents loaded</b>	Pipette tips do not fit the VoITRAX cartridge ports	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VoITRAX cartridge. Rainin 20 µl is the most suitable.
<b>The VoITRAX software shows an inaccurate amount of reagents loaded</b>	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VoITRAX software before loading.

Issues during the sequencing run

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# Issues during the sequencing run

**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](mailto:support@nanoporetech.com)) or via [LiveChat](#) in the [Nanopore Community](#).

## Fewer pores at the start of sequencing than after Flow Cell Check

Observation	Possible cause	Comments and actions
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in <a href="#">this video</a> .
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the <a href="#">Contaminants Know-how piece</a> . Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.

## MinKNOW script failed

Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Script failed"</b>		Restart the computer and then restart MinKNOW. If the issue persists, please collect the <a href="#">MinKNOW log files</a> and contact Technical Support.

## Pore occupancy below 40%

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Observation	Possible cause	Comments and actions
<b>Pore occupancy &lt;40%</b>	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION flow cell. Please quantify the library before loading and calculate mols using tools like the <a href="#">Promega Biomath Calculator</a> , choosing "dsDNA: µg to pmol"
<b>Pore occupancy close to 0</b>	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK109 kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
<b>Pore occupancy close to 0</b>	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
<b>Pore occupancy close to 0</b>	No tether on the flow cell	Tethers are adding during flow cell priming (FLT tube). Make sure FLT was added to FB before priming.

**Shorter than expected read length**

Observation	Possible cause	Comments and actions
<b>Shorter than expected read length</b>	Unwanted fragmentation of DNA sample	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> <li>Please review the <a href="#">Extraction Methods</a> in the Nanopore Community for best practice for extraction.</li> <li>Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.</li> </ol>  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> <li>During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.</li> </ol>

**Large proportion of recovering pores**

Observation	Possible cause	Comments and actions
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Issues during the sequencing run

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Observation	Possible cause	Comments and actions
<b>Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)</b>	Contaminants are present in the sample	<p>Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases:</p> <ol style="list-style-type: none"> <li>1. A <a href="#">nuclease flush</a> can be performed, or</li> <li>2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.</li> </ol> <div style="text-align: center;"> <p><b>Duty Time</b> Summary of channel states over time</p> <p>Bucket size (minutes): 5 Apply</p> <p><input checked="" type="checkbox"/> Auto scale bucket size</p> <p><input checked="" type="checkbox"/> Display channels proportionately</p> <p>Legend: <input checked="" type="checkbox"/> Sequencing <input checked="" type="checkbox"/> Pore <input checked="" type="checkbox"/> Recovering <input checked="" type="checkbox"/> Inactive <input checked="" type="checkbox"/> Unclassified</p> <p>More +</p> </div> <p>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment</p>

**Large proportion of inactive pores**

Observation	Possible cause	Comments and actions
<b>Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)</b>	Air bubbles have been introduced into the flow cell	Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the <a href="#">Priming and loading your flow cell</a> video for best practice
<b>Large proportion of inactive pores</b>	Certain compounds co-purified with DNA	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none"> <li>1. Please refer to the <a href="#">Plant leaf DNA extraction method</a>.</li> <li>2. Clean-up using the QIAGEN PowerClean Pro kit.</li> <li>3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.</li> </ol>
<b>Large proportion of inactive pores</b>	Contaminants are present in the sample	The effects of contaminants are shown in the <a href="#">Contaminants</a> Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

**Reduction in sequencing speed and q-score later into the run**

Observation	Possible cause	Comments and actions
<b>Reduction in sequencing speed and q-score later into the run</b>	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–50 fmol of library is recommended).	Add more fuel to the flow cell by following the instructions in the <a href="#">MinKNOW protocol</a> . In future experiments, load lower amounts of library to the flow cell.

**Temperature fluctuation**

## Issues during the sequencing run

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Observation	Possible cause	Comments and actions
<b>Temperature fluctuation</b>	The flow cell has lost contact with the device	Check that there is a heat pad covering the metal plate on the back of the flow cell. Re-insert the flow cell and press it down to make sure the connector pins are firmly in contact with the device. If the problem persists, please contact Technical Services.

### Failed to reach target temperature

Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Failed to reach target temperature"</b> (37°C for Flow Cell Check, 34°C for sequencing on MinION Mk 1B/PromethION flow cells, and 35°C for sequencing on Flongle)	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to <a href="#">this FAQ</a> for more information on MinION Mk 1B temperature control.

### Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
<b>No input .fast5 was found or basecalled</b>	<i>input_path</i> did not point to the .fast5 file location	The <i>--input_path</i> has to be followed by the full file path to the .fast5 files to be basecalled, and the location has to be accessible either locally or remotely through SSH.
<b>No input .fast5 was found or basecalled</b>	The .fast5 files were in a subfolder at the <i>input_path</i> location	To allow Guppy to look into subfolders, add the <i>--recursive</i> flag to the command

### Guppy – no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
<b>No Pass or Fail folders were generated after basecalling</b>	The <i>--qscore_filtering</i> flag was not included in the command	The <i>--qscore_filtering</i> flag enables filtering of reads into Pass and Fail folders inside the output folder, based on their strand q-score. When performing live basecalling in MinKNOW, a q-score of 7 (corresponding to a basecall accuracy of ~80%) is used to separate reads into Pass and Fail folders.

### Guppy – unusually slow processing on a GPU computer


Observation	Possible cause	Comments and actions
<b>Unusually slow processing on a GPU computer</b>	The <i>--device</i> flag wasn't included in the command	The <i>--device</i> flag specifies a GPU device to use for accelerate basecalling. If not included in the command, GPU will not be used. GPUs are counted from zero. An example is <i>--device cuda:0 cuda:1</i> , when 2 GPUs are specified to use by the Guppy command.

### MinIT – the MinKNOW interface is not shown in the web browser

## Amplicons by Ligation (SQK-LSK109)

## Issues during the sequencing run

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Observation	Possible cause	Comments and actions
<b>The MinKNOW interface is not shown in the web browser</b>	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
<b>The MinKNOW interface is not shown in the web browser</b>	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	<p>Make sure the computer or mobile device is using the MinIT Wi-Fi. It should be shown as MT-xxxxxx (x is a number) on the underside label on the MinIT:</p>  <p>Disable the Ethernet connection from the computer or mobile device as needed. If necessary, contact your IT department to determine if the MinIT Wi-Fi is blocked (MinIT generic IP: 10.42.0.1). Please white-list MinIT as needed.</p>
<b>The MinKNOW interface is not shown in the web browser</b>	The MinIT was not on the same network that the computer was connected to.	Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network.

## MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
<b>The MinIT software cannot be updated</b>	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the <a href="#">following AWS IP ranges</a> . Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
<b>The MinIT software cannot be updated</b>	The device already has the latest version of the software	Occasionally, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the <a href="#">Software Downloads page</a> . Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitwise or Putty, as described in the <a href="#">MinIT protocol</a> ) on a Windows computer or the terminal window on a Mac, run the command, <code>dpkg -f   grep minit</code> , to find out the version of the MinIT software and <code>sudo apt update</code> if an update is needed. If the issue still persists, please contact Technical Services with details of the error.