Overview of the protocol

IMPORTANT

This is an Early Access product

For more information about our Early Access programmes, please see this article on product release phases.

Ligation Sequencing Kit features

This kit is recommended for users who:

- Would like to achieve raw read sequencing modal accuracy of Q20+ (99%) or above
- Require control over read length

IMPORTANT

Kit 12 chemistry device and informatics info sheet

The Kit 12 chemistry is a new development from Oxford Nanopore Technologies, the data acquisition, basecalling and analysis will require a different set of tools to the typical sequencing run. This is decribed in more detail in the Kit 12 chemistry device and informatics info sheet. We strongly recommend that you read it before proceeding with Kit 12 chemistry sequencing experiments.

Introduction to the Ligation Sequencing Kit (SQK-LSK112) protocol

This protocol describes how to carry out sequencing of amplicon sample using the Ligation Sequencing Kit (SQK-LSK112). It is 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:

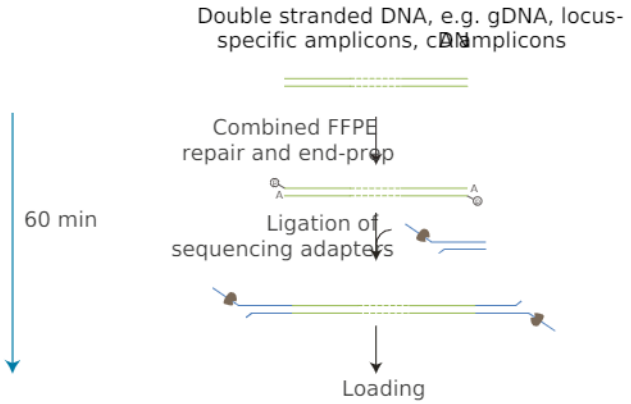
- Extract your DNA, and check its length, quantity and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:

- 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 and convert it into basecalled reads for R10.4 flow cells. For other flow cell types, basecalling is done offline at the end of the sequencing run.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (SQK-LSK112)
- Control Expansion (EXP-CTL001)
- R10.4 flow cells (FLO-MIN112)
- R9.4.1 flow cells (FLO-MIN106D)
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials

- 1 µg (or 100-200 fmol) amplicon DNA
- Ligation Sequencing Kit (SQK-LSK112)

Consumables

- 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

- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit

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
- Qubit fluorometer (or equivalent for QC check)

Optional Equipment

- Agilent Bioanalyzer (or equivalent)
- Eppendorf 5424 centrifuge (or equivalent)

IMPORTANT

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

For this protocol, you will need 1 µg (or 100-200 fmol) amplicon DNA.

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.

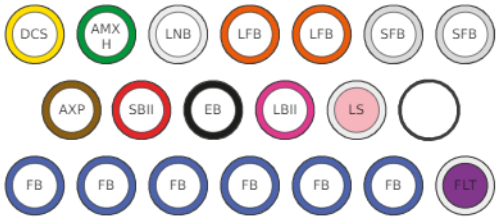
Chemical contaminants

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-LSK112) contents



DCS : DNA Control Strand
AMX H : Adapter Mix H
LNB : Ligation Buffer
LFB : L Fragment Buffer
SFB : S Fragment Buffer
AXP : AMPure XP Beads
SBII : Sequencing Buffer II
EB : Elution Buffer
LBII : Loading Beads II
LS : Loading Solution
FB : Flush Buffer
FLT : Flush Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	35
Adapter Mix H	AMX H	Green	2	40
AMPure XP Beads	AXP	Amber	1	1,200
Ligation Buffer	LNB	Clear	1	200
L Fragment Buffer	LFB	Orange	2	1,800
S Fragment Buffer	SFB	Clear	2	1,800
Sequencing Buffer II	SBII	Red	1	500
Elution Buffer	EB	Black	1	1,200
Loading Beads II	LBII	Pink	1	360
Loading Solution	LS	White cap, pink sticker on label	1	400
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

Note: This Product Contains AMPure XP Reagent Manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

Computer requirements and software

MinION Mk1C IT requirements

The MinION Mk1C contains fully-integrated compute and screen, removing the need for any accessories to generate and analyse nanopore data. Read more in the MinION Mk1C IT requirements document.

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.

IMPORTANT

Kit 12 chemistry device and informatics info sheet

The Kit 12 chemistry is a new development from Oxford Nanopore Technologies, the data acquisition, basecalling and analysis will require a different set of tools to the typical sequencing run. This is decribed in more detail in the Kit 12 chemistry device and informatics info sheet. We strongly recommend that you read it before proceeding with Kit 12 chemistry sequencing experiments.

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
- DNA CS
- AMPure XP Beads (AXP)

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)
- Freshly prepared 70% ethanol in nuclease-free water
- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit

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
- Qubit fluorometer (or equivalent for QC check)

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

2 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.

IMPORTANT

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

IMPORTANT

It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing.

Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.

3 Prepare the DNA in nuclease-free water

- Transfer 1 µg (or 100-200 fmol) amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 49 µl with nuclease-free water
- Mix thoroughly by pipetting up and down, or by flicking the tube
- Spin down briefly in a microfuge

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

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA CS	1 µl
DNA	47 µl
NEBNext FFPE DNA Repair Buffer	3.5 µl
NEBNext FFPE DNA Repair Mix	2 µl
Ultra II End-prep reaction buffer	3.5 µl
Ultra II End-prep enzyme mix	3 µl
Total	**60 µl**

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

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

7 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

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

9 **Add 60 µl of resuspended the AMPure XP Beads (AXP) 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, for at least 1 minute.**

18 **Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Quantify 1 µl of eluted sample using a Qubit fluorometer.

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

Adapter ligation and clean-up

Version: ACDE_9142_v112_revE_01Dec2021

~30 minutes

Materials

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

Consumables

- NEBNext Quick Ligation Module (E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (ThermoFisher Q32856)
- Qubit dsDNA HS Assay Kit

Equipment

- Magnetic rack
- Microfuge
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Qubit fluorometer (or equivalent for QC check)

IMPORTANT

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

1 **Spin down the Adapter Mix H (AMX H) 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:**

Between each addition, pipette mix 10-20 times.

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

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

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

9 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

10 **Add 40 µl of resuspended AMPure XP Beads (AXP) 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, for at least 1 minute.**

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

Quantify 1 µl of eluted sample using a Qubit fluorometer.

END OF STEP

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

IMPORTANT

We recommend loading 5-10 fmol of this final prepared library onto your flow cells.

Loading more than 20 fmol of DNA can reduce the rate of duplex read capture. Dilute the library in Elution Buffer if required.

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.

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-AUX002), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer II (SBII) and Loading Beads II (LBII), required for loading the libraries onto flow cells.

Priming and loading the SpotON flow cell

~10 minutes

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

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

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

- P10 pipette and tips

IMPORTANT

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

TIP

Priming and loading a MinION flow cell

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

Using the Loading Solution

We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water. Note: some customers have noticed that viscous libraries can be loaded more easily when not using Loading Beads.

1 **Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.**

2 **Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at room temperature.**

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

For kits supplied with 20 ml bottles of Flush Buffer (FB), add 30 µl of thawed and mixed Flush Tether (FLT) to 1.17 ml of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.

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

5 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 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
   Note: Visually check that there is continuous buffer from the priming port across the sensor array.

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. During this time, prepare the library for loading by following the steps below.

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

IMPORTANT

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

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

Reagent	Volume
Sequencing Buffer II (SBII)	37.5 µl
Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

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

10 Complete the flow cell priming:

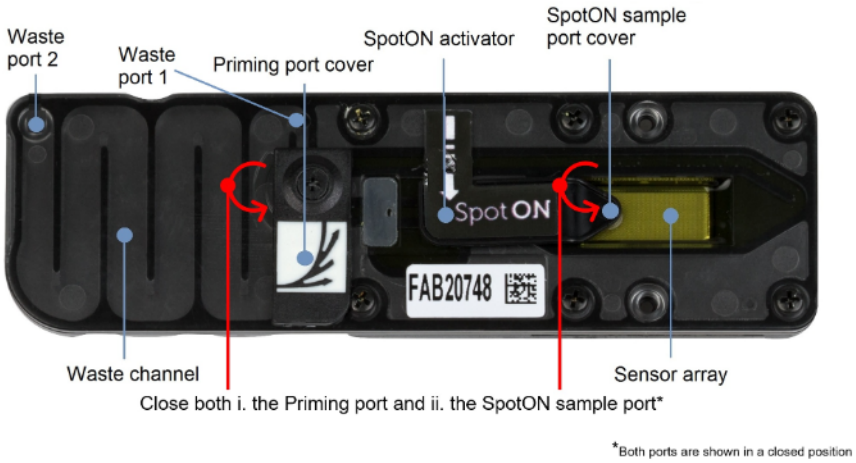
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

Note: Load the library as soon as possible after this step.

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

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

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



Data acquisition and basecalling

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 GridION device

Follow the instructions in the GridION user manual.

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

Follow the instructions in the MinION Mk1C user manual.

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

Follow the instructions in the MinIT protocol.

5. 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.

IMPORTANT

Kit 12 chemistry device and informatics info sheet

The Kit 12 chemistry is a new development from Oxford Nanopore Technologies, the data acquisition, basecalling and analysis will require a different set of tools to the typical sequencing run. This is decribed in more detail in the Kit 12 chemistry device and informatics info sheet. We strongly recommend that you read it before proceeding with Kit 12 chemistry sequencing experiments.

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.

TIP

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

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.

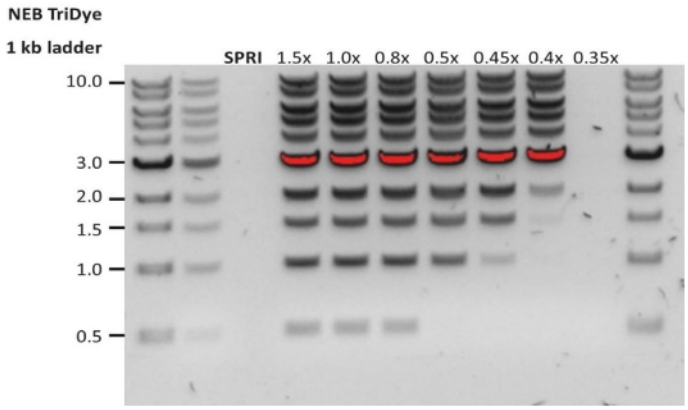
We also have an FAQ section available on the Nanopore Community Support section.

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.

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low RNA integrity (RNA integrity number <9.5 RIN, or the rRNA band is shown as a smear on the gel)	The RNA degraded during extraction	Try a different RNA extraction method). For more info on RIN, please see the RNA Integrity Number Know-how piece.
RNA has a shorter than expected fragment length	The RNA degraded during extraction	Try a different RNA extraction method). For more info on RIN, please see the RNA Integrity Number Know-how piece. 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
Low recovery	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.
Low recovery	DNA fragments are shorter than expected	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. 
Low recovery after end-prep	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 VolTRAX run terminated in the middle of the library prep

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

The VolTRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
The VolTRAX software shows an inaccurate amount of reagents loaded	Pipette tips do not fit the VolTRAX 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 VolTRAX cartridge. Rainin 20 µl is the most suitable.

Observation	Possible cause	Comments and actions
The VolTRAX software shows an inaccurate amount of reagents loaded	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 VolTRAX software before loading.

Issues during the sequencing run

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

We also have an FAQ section available on the Nanopore Community Support section.

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.

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

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	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 this video.
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	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).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	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 Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

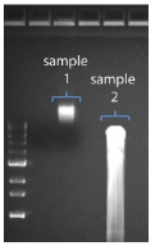
MinKNOW script failed

Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

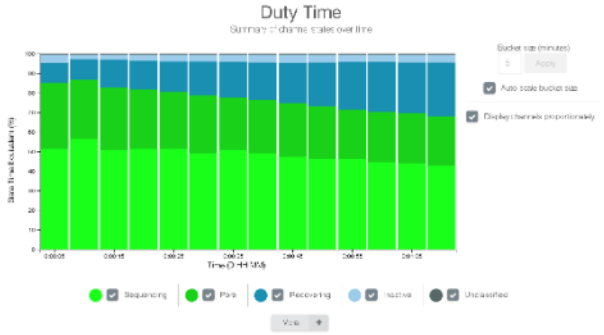
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	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 Promega Biomath Calculator, choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	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-LSK110 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.
Pore occupancy close to 0	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.
Pore occupancy close to 0	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
Shorter than expected read length	Unwanted fragmentation of DNA sample	Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep. 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented. 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	Contaminants are present in the sample	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: 1. A nuclease flush can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.  The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)	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 Priming and loading your flow cell video for best practice
Large proportion of inactive pores	Certain compounds co-purified with DNA	Known compounds, include polysaccharides, typically associate with plant genomic DNA. 1. Please refer to the Plant leaf DNA extraction method. 2. Clean-up using the QIAGEN PowerClean Pro kit. 3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive pores	Contaminants are present in the sample	The effects of contaminants are shown in the Contaminants 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
Reduction in sequencing speed and q-score later into the run	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 MinKNOW protocol. In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

Observation	Possible cause	Comments and actions
Temperature fluctuation	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
MinKNOW shows "Failed to reach target temperature" (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 this FAQ for more information on MinION Mk 1B temperature control.

Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
No input .fast5 was found or basecalled	*input_path* did not point to the .fast5 file location	The *--input_path* 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.
No input .fast5 was found or basecalled	The .fast5 files were in a subfolder at the *input_path* location	To allow Guppy to look into subfolders, add the *--recursive* flag to the command

Guppy – no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
No Pass or Fail folders were generated after basecalling	The *--qscore_filtering* flag was not included in the command	The *--qscore_filtering* 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
Unusually slow processing on a GPU computer	The *--device* flag wasn't included in the command	The *--device* 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 *--device cuda:0 cuda:1*, when 2 GPUs are specified to use by the Guppy command.

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

Observation	Possible cause	Comments and actions
The MinKNOW interface is not shown in the web browser	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 in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
The MinKNOW interface is not shown in the web browser	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	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:  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.
The MinKNOW interface is not shown in the web browser	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
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the following AWS IP ranges. Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionaly, 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 Software Downloads page. Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitvise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, *dpkg -l | grep minit*, to find out the version of the MinIT software and *sudo apt update* if an update is needed. If the issue still persists, please contact Technical Services with details of the error.