Overview of the protocol

Direct cDNA Sequencing V14 with SQK-LSK114 features

This protocol is recommended for users who:

- Are interested in exploring novel RNA biology.
- Are looking for splice variant and fusion transcript analysis.
- Do not wish to use PCR.
- Wish to preserve quantitative information in samples likely to be impacted by PCR bias.
- Would like full-length cDNA strands.
- Want to achieve median raw read accuracy of Q20+ (99%) and above.
- Want to optimise their sequencing experiment for output.

Introduction to the Direct cDNA sequencing protocol

This protocol describes how to carry out sequencing of cDNA using a reverse transcription and stand-switching method and the Ligation Sequencing Kit V14 (SQK-LSK114).

This protocol requires the use of three oligo primers to be ordered from a third-party (e.g. IDT) :

Oligo	Sequence (5' to 3')
VN Primer	/5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN
Strand-switching Primer	TTTCTGTTGGTGCTGATATTGCTmGmGmG
PR2 Primer	/5Phos/TTTCTGTTGGTGCTGATATTGC

Note: mG = 2' O-Methyl RNA bases.

- The VN Primer will anchor to the RNA Poly(A)+ tail and prime the first strand synthesis.
- The Strand-switching Primer will anneal to the non-template nucleotides (C’s) of the novel cDNA strand generated from the first strand synthesis, enabling strand switching.
- Following RNA degradation, the PR2 Primer will prime the second strand synthesis of the cDNA sample.

Using this strand-switching method allows for high yields of cDNA library generation from RNA, while also selecting for full-length transcripts.

Steps in the sequencing workflow:

Prepare for your experiment

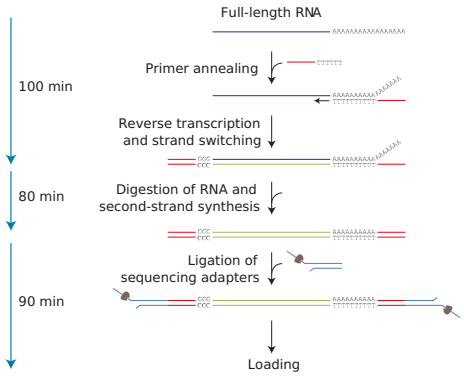
You will need to:

- Order the three oligo primers from a third-party
- Extract your RNA, and check its length, quantity and purity. Alternatively, you can start with already-prepared cDNA.**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:

- Using the strand-switching protocol, prepare full-length cDNAs from Poly(A)+ RNA
- Ligate sequencing adapters to the cDNA
- Prime the flow cell, and load your cDNA 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
- **(Optional):** Start the EPI2ME software and select a workflow for further analysis, e.g. Fastq yeast transcriptome analysis

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials	• 100 ng Poly(A)+ RNA OR 1 µg of total RNA • Ligation Sequencing Kit V14 (SQK-LSK114)
Consumables	• User-supplied VN Primer, 2 µM • User-supplied Strand-Switching Primer, 10 µM • User-supplied PR2 Primer, 10 µM • NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB,

E7180S or E7180L).

Alternatively, you can use the NEBNext® products below:

- NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
- NEBNext Quick Ligation Module (NEB, E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- 10 mM dNTP solution (e.g. NEB N0447)
- LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286)
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- Ice bucket with ice
- Timer
- Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf cat # 022510509)
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

Optional equipment

- Qubit fluorometer (or equivalent for QC check)

For this protocol, you will need 100 ng Poly(A)+ RNA or 1 µg of total RNA.

If using alternative cDNA preparation methods, start the protocol with 70–200 fmol of pre-prepared cDNA at the cDNA repair and end-prep step.

This protocol requires primer oligos to be ordered separately:

Oligo	Sequence (5' to 3')	Purity recommended	Dilution required
VN Primer	/5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTVN	HPLC	2 µM

Oligo	Sequence (5' to 3')	Purity recommended	Dilution required
Strand-switching Primer	TTTCTGTTGGTGCTGATATTGCTmGmGmG	HPLC	10 µM
PR2 Primer	/5Phos/TTTCTGTTGGTGCTGATATTGC	HPLC	10 µM

Note: mG = 2' O-Methyl RNA bases.

Note: Please ensure your primer oligos are ordered at HPLC purity level for optimal results.
If ordering from IDT, the primer oligos will need to be ordered at a minimum scale of 100 nmole to enable HPLC purification.

Input RNA

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

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

For further information on using RNA as input, please read the links below.

- Polyadenylation of non-poly(A) transcripts using E. coli poly(A) polymerase
- RNA contaminants
- RNA stability
- RNA Integrity Number (RIN)
- Enrichment of polyadenylated RNA molecules

These documents can also be found in the DNA/RNA Handling page.

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 or E7180L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

Please note, for this protocol, NEBNext FFPE DNA Repair Mix and NEBNext FFPE DNA Repair Buffer are not required.

Third-party reagents

We have validated and recommend the use of all the third-party reagents used in this protocol. Alternatives have not been tested by Oxford Nanopore Technologies.

For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

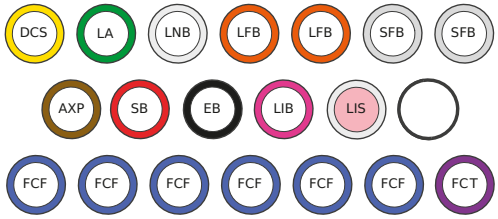
IMPORTANT

We strongly recommend using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit V14 rather than the third-party ligase buffer supplied in the NEBNext Quick Ligation Module to ensure high ligation efficiency of the Ligation Adapter (LA).

Ligation Sequencing Kit V14 (SQK-LSK114) contents

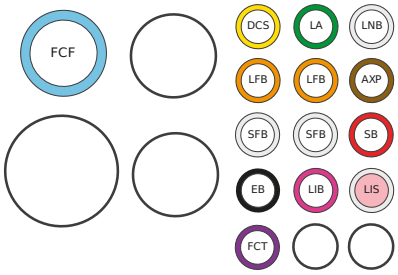
Note: We are in the process of reformatting our kits with single-use tubes into a bottle format.

Single-use tubes format:



DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

Bottle format:



DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

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.

Note: The DNA Control Sample (DCS) is a 3.6 kb standard amplicon mapping the 3' end of the Lambda genome.

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

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 Mk1B IT Requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence, basecall and demultiplex if your samples were barcoded.

For instructions on how to run the MinKNOW software, please refer to 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.

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform 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 or within four weeks of purchasing 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

Reverse transcription and strand-switching

~100 minutes

Materials

- 100 ng Poly(A)+ RNA OR 1 µg of total RNA

Consumables

- User-supplied VN Primer, 2 µM
- User-supplied Strand-Switching Primer, 10 µM
- 10 mM dNTP solution (e.g. NEB cat # N0447)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 0.2 ml thin-walled PCR tubes
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)

- RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)

Equipment

- Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf cat # 022510509)
- Microfuge
- 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

IMPORTANT

If you have already prepared your cDNA, use 70–200 fmol cDNA (~70–200 ng if your sample is 1.5 kb) and start from the cDNA repair and end-prep step.

1 Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
User-supplied VN Primer diluted to 2 µM	✓	✓	✓
User-supplied Strand-Switching Primer diluted to 10 µM	✓	✓	✓
10 mM dNTP solution	✓	✓	✓
RNaseOUT	Not frozen	✓	✓
Maxima H Minus Reverse Transcriptase	Not frozen	✓	✓
Maxima H Minus 5x RT Buffer	✓	✓	Mix by vortexing

2 Prepare the RNA in nuclease-free water

- Transfer 100 ng Poly(A)+ RNA or 1 µg of total RNA into a 0.2 ml PCR tube
- Adjust the volume to up to 7.5 µl with nuclease-free water
- Mix by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

3 **Prepare the following reaction in the 0.2 ml PCR tube containing the prepared RNA input:**

Reagent	Volume
RNA input (100 ng Poly(A)+ RNA or 1 µg of total RNA) from step above	7.5 µl
VN Primer diluted to 2 µM	2.5 µl
10 mM dNTPs	1 µl
Total volume	**11 µl**

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

5 **Incubate at 65°C for 5 minutes and then snap cool on a pre-chilled freezer block for 1 minute.**

6 **In a separate tube, mix together the following:**

Reagent	Volume
5x RT Buffer	4 µl
RNaseOUT	1 µl
Nuclease-free water	1 µl
Strand-Switching Primer diluted to 10 µM	2 µl
Total	**8 µl**

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

8 **Add the 8 µl of strand-switching reagents (prepared in steps 6-7) to the 11 µl of snap-cooled mRNA (from steps 2-5). Mix by flicking the tube and spin down.**

9 **Incubate at 42°C for 2 minutes in the thermal cycler.**

10 **Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl.**

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

12 Incubate using the following protocol using a thermal cycler:

Cycle step	Temperature	Time	No. of cycles
Reverse transcription and strand-switching	42°C	90 mins	1
Heat inactivation	85°C	5 mins	1
Hold	4°C	∞	

RNA degradation and second strand synthesis

~80 minutes

Materials

- AMPure XP Beads (AXP)

Consumables

- User-supplied PR2 Primer, 10 µM
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- LongAmp Taq 2X Master Mix (e.g. NEB cat # M0287)
- RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286)
- Freshly prepared 80% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Thermal cycler
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- Ice bucket with ice
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

Optional equipment

- DNA QC equipment, e.g. Qubit fluorometer, NanoDrop spectrophotometer, Agilent Bioanalyzer or Tapestation, Agilent FEMTO Pulse

1 **Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.**

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
User-supplied PR2 Primer diluted to 10 µM	✓	✓	✓
RNase Cocktail Enzyme Mix	Not frozen	✓	✓
LongAmp Taq 2X Master Mix	✓	✓	✓

2 **Thaw the AMPure XP Beads (AXP) at room temperature and mix by vortexing. Keep the beads at room temperature.**

3 **Add 1 µl RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286) to the reverse transcription reaction.**

4 **Incubate the reaction for 10 minutes at 37° C in a thermal cycler.**

5 **Resuspend the AMPure XP beads (AXP) by vortexing.**

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

7 **Add 17 µl of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube.**

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

9 **Prepare 500 µl of fresh 80% ethanol in nuclease-free water.**

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

11 **Keep the tubes on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

12 **Repeat the previous step.**

13 **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.**

14 **Remove the tube from the magnetic rack and resuspend pellet in 20 µl nuclease-free water.**

15 **Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.**

16 **Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute.**

17 **Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

18 **Prepare the following reaction in a 0.2 ml thin-walled PCR tube:**

Reagent	Volume
2x LongAmp Taq Master Mix	25 µl
PR2 Primer diluted to 10 µM	2 µl
Reverse-transcribed sample from above	20 µl
Nuclease-free water	3 µl
Total	**50 µl**

19 **Incubate using the following protocol:**

Cycle step	Temperature	Time	No. of cycles
Denaturation	94 °C	1 mins	1
Annealing	50 °C	1 mins	1
Extension	65 °C	15 mins	1
Hold	4 °C	∞	

20 **Resuspend the AMPure XP beads (AXP) by vortexing.**

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

22 **Add 40 µl of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube.**

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

24 **Prepare 500 µl of fresh 80% ethanol in nuclease-free water.**

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

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

If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

27 Repeat the previous step.

28 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.

29 Remove the tube from the magnetic rack and resuspend pellet in 21 µl nuclease-free water.

30 Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

31 Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute.

32 Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Analyse 1 µl of the strand-switched DNA for size, quantity and quality using an Agilent Bioanalyzer and Qubit fluorometer (or equivalent).

END OF STEP

Take forward the full volume of your sample into the cDNA repair and end-prep stage of the protocol.

Recovery aim for the samples after RNA degradation and second strand synthesis is 70–200 fmol (~70–200 ng if your sample is 1.5 kb).

cDNA repair and end-prep

~35 minutes

Materials

- Strand-switched cDNA in 20 µl
- 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, AM9937)
- NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

Equipment	• P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips • Thermal cycler • Microfuge • Hula mixer (gentle rotator mixer) • Magnetic rack • Ice bucket with ice
Optional equipment	• Qubit fluorometer (or equivalent for QC check)

IMPORTANT

If you have prepared your own cDNA instead of performing reverse transcription using the method outlined in this protocol, start this step with 70–200 fmol cDNA (~70–200 ng if your sample is 1.5 kb) in 20 µl nuclease-free water.

1 Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice:

For optimal perfomance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert the reagent tubes to ensure they are well mixed.

   Note: Do not vortex the Ultra II End Prep Enzyme Mix.
3. Always spin down tubes before opening for the first time each day.
4. The Ultra II End Prep Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.

2 Combine the following reagents in a 0.2 ml PCR tube:

Reagent	Volume
cDNA sample	20 µl
Nuclease-free water	30 µl
Ultra II End-prep reaction buffer	7 µl
Ultra II End-prep enzyme mix	3 µl
Total	**60 µl**

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

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

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

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

7 **Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.**

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

9 **Prepare 500 µl of fresh 80% ethanol in nuclease-free water.**

10 **Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.**

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

12 **Repeat the previous step.**

13 **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.**

14 **Remove the tube from the magnetic rack and resuspend pellet in 61 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

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

16 **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 60 µl of repaired and end-prepped cDNA 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

~45 minutes

Materials	• Ligation Adapter (LA) • Ligation Buffer (LNB) • Short Fragment Buffer (SFB) • AMPure XP Beads (AXP) • Elution Buffer (EB)
Consumables	• NEBNext® Quick Ligation Module (NEB, E6056) • 1.5 ml Eppendorf DNA LoBind tubes • Qubit™ Assay Tubes (Invitrogen, Q32856) • Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
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 third-party ligase is supplied with its own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.

IMPORTANT

Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.

1 **Spin down the Ligation Adapter (LA) 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 and mix by vortexing. Then spin down and place on ice.**

4 **Thaw the Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.**

5 **In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:**

Between each addition, pipette mix 10-20 times.

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

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

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

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

9 **Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.**

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

11 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.**

12 **Wash the beads by adding 250 µl of 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.**

Note: Take care when removing the supernatant, the viscosity of the buffer can contribute to loss of beads from the pellet.

13 **Repeat the previous step.**

14 **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.**

15 **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.**

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

17 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.

18 Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB).

IMPORTANT

We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 flow cell.

This is to ensure high pore occupancy of >95% is reached. How to calculate pore occupancy can be found here.

END OF STEP

The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C 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.

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

Depending on how many flow cells the library will be split across, more Elution Buffer (EB) than what is supplied in the kit will be required.

Priming and loading the MinION and GridION Flow Cell

~5 minutes

Materials	• Flow Cell Flush (FCF) • Flow Cell Tether (FCT) • Library Solution (LIS) • Library Beads (LIB) • Sequencing Buffer (SB)
Consumables	• MinION and GridION Flow Cell • 1.5 ml Eppendorf DNA LoBind tubes

- Nuclease-free water (e.g. ThermoFisher, AM9937)
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- MinION or GridION device
- MinION and GridION Flow Cell Light Shield
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

TIP

Priming and loading a flow cell

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

1 **Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.**

IMPORTANT

For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

2 **To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at room temperature.**

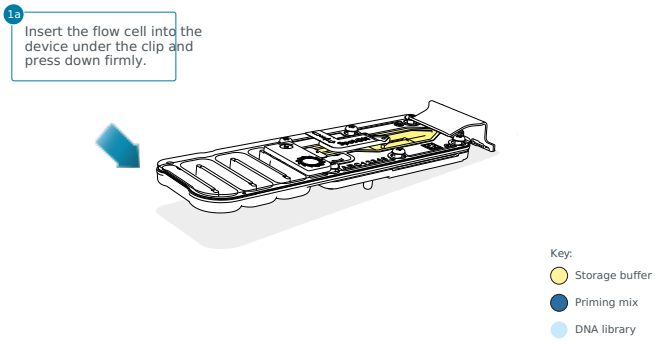
Note: We are in the process of reformatting our kits with single-use tubes into a bottle format. Please follow the instructions for your kit format.

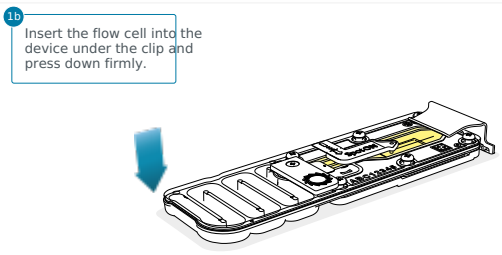
Single-use tubes format:
Add 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml and 30 µl Flow Cell Tether (FCT) directly to a tube of Flow Cell Flush (FCF).

Bottle format:
In a suitable tube for the number of flow cells, combine the following reagents:

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,205 µl**

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





Optional action
Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check instructions in the MinKNOW protocol for more information.

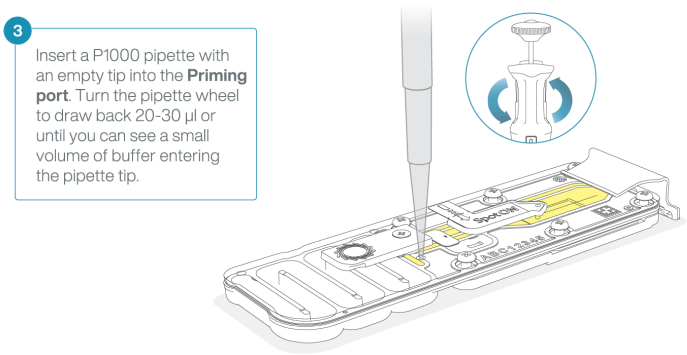
4 Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT

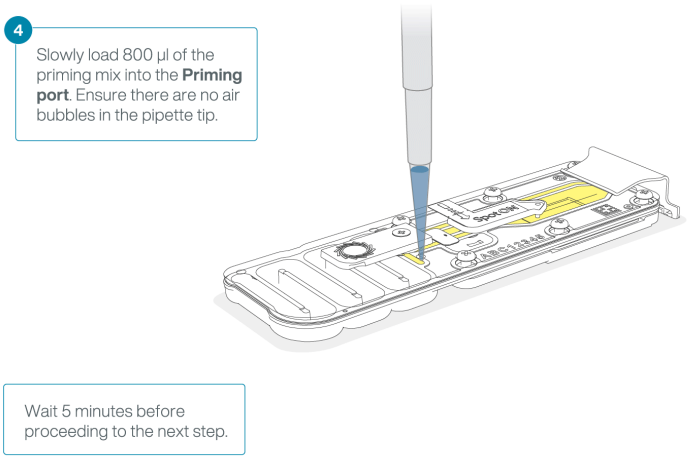
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 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.



6 **Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.**



7 **Thoroughly mix the contents of the Library Beads (LIB) by pipetting.**

IMPORTANT

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

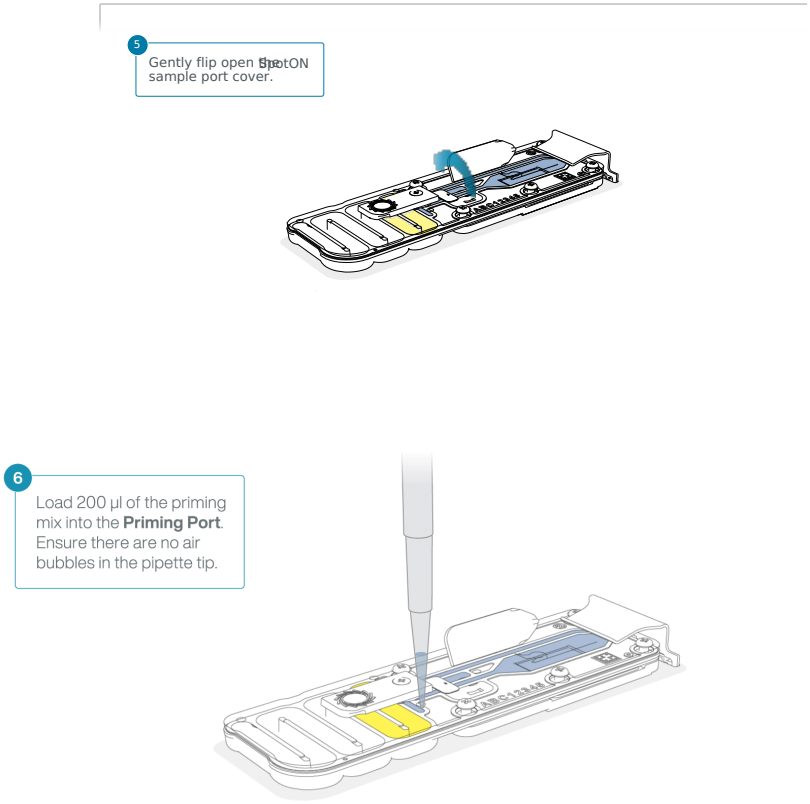
We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

8 **In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:**

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

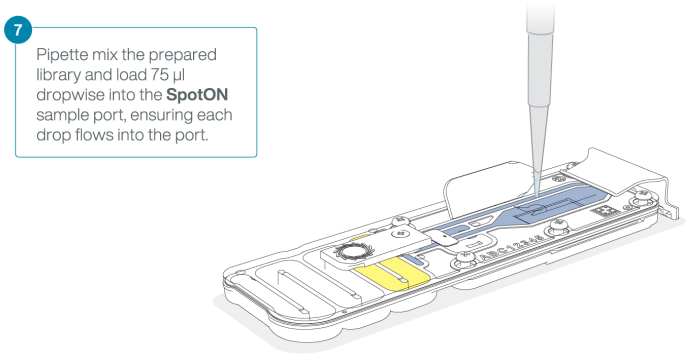
9 Complete the flow cell priming:

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 priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

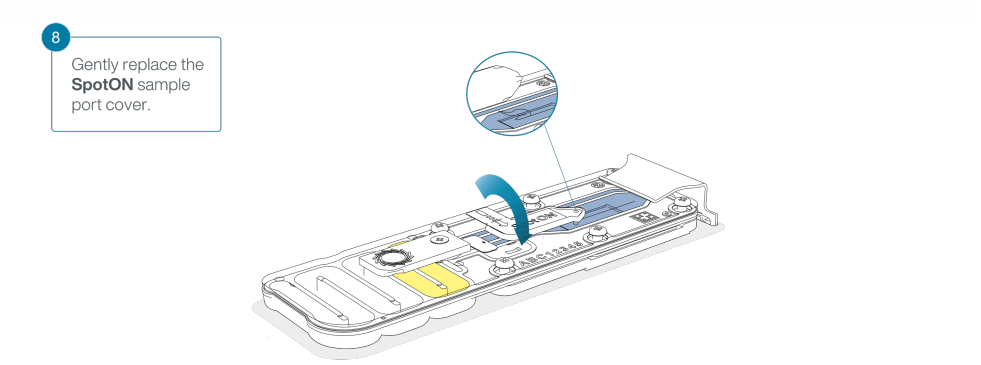


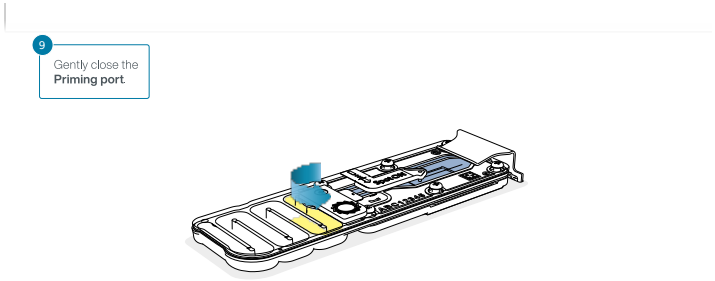
10 Mix the prepared library gently by pipetting up and down just prior to loading.

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



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





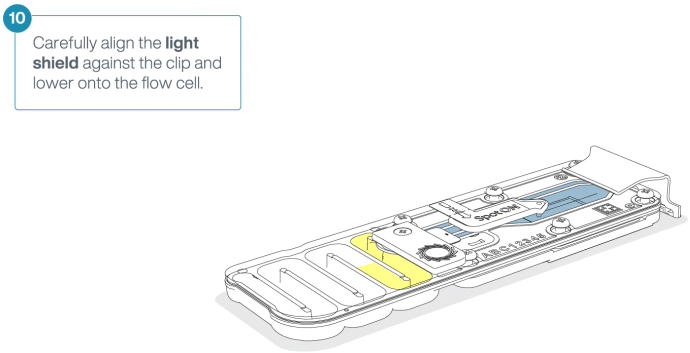
IMPORTANT

Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

13 Place the light shield onto the flow cell, as follows:

1. Carefully place the leading edge of the light shield against the clip.
   Note: Do not force the light shield underneath the clip.
2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.



CAUTION

The MinION Flow Cell Light Shield is not secured to the flow cell and careful handling is required after installation.

END OF STEP

Close the device lid and set up a sequencing run on MinKNOW.

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. There are multiple 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 PromethION device

Follow the instructions in the PromethION user manual or the PromethION 2 Solo user manual.

5. Data acquisition using MinKNOW on a computer and basecalling at a later time using MinKNOW

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 Post-run analysis section of the MinKNOW protocol.

Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME workflows

For 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.

2. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, that 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.

3. 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.

Flow cell reuse and returns

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 Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C.**

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 **Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be foundhere.

Note: All flow cells must be flushed with deionised water before returning the product.

> **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.

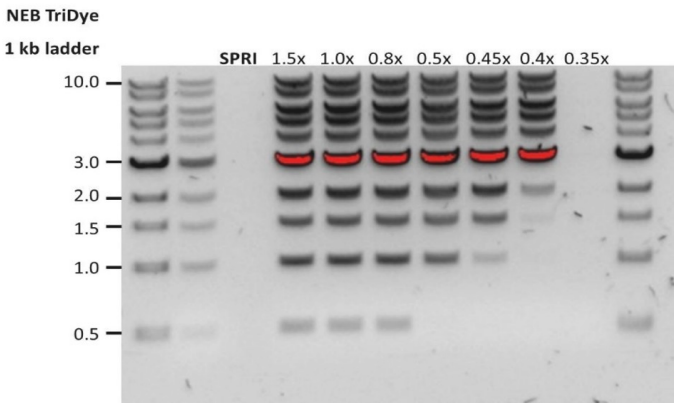
We also have an FAQ section available on theNanopore Community Support section.

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 document. 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 document. Further information can be found in the DNA/RNA Handling page.
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 document. Further information can be found in the DNA/RNA Handling page. 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.

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

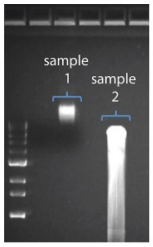
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

Pore occupancy below 40%

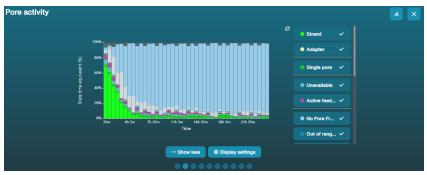
Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure you load the recommended amount of good quality library in the relevant library prep protocol onto your flow cell. Please quantify the library before loading and calculate mols using 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 sequencing 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/FCT tube). Make sure FLT/FCT was added to FB/FCF before priming.

Shorter than expected read length

Observation	Possible cause	Comments and actions

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 unavailable pores

Observation	Possible cause	Comments and actions
Large proportion of unavailable pores (shown as blue in the channels panel and pore activity plot)  The pore activity plot above shows an increasing proportion of "unavailable" pores over time.	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 "sequencing pore". If the portion of unavailable pores stays large or increases: 1. A nuclease flush using the Flow Cell Wash Kit (EXP-WSH004) can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores (shown as light blue in the channels panel and pore activity 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

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable 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/unavailable 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	For Kit 9 chemistry (e.g. SQK-LSK109), fast fuel consumption is typically seen when the flow cell is overloaded with library (please see the appropriate protocol for your DNA library to see the recommendation).	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"	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.