PROTOCOL

Ligation sequencing gDNA (Q-SQK-LSK109) VQ_GDL_revB_24Feb2023

- This protocol uses genomic DNA
- High yield
- Library preparation time ~60 minutes
- Fragmentation optional
- No PCR

For Research Use Only

FOR RESEARCH USE ONLY

Contents

Introduction to the protocol

Library preparation

Sequencing and data analysis

1. Overview of the protocol

Ligation Sequencing Kit features

This kit is recommended for users who:

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

Introduction to the Ligation Sequencing Kit

The Ligation Sequencing Kit is designed to prepare genomic, amplicon, and cDNA, with or without barcoding, for sequencing on Oxford Nanopore devices to produce 1D reads. This kit can be used to prepare libraries from whole genome amplified DNA, starting with as little as 10 pg genomic DNA.

The kit contains an adapter, which must be ligated onto end-repaired and A-tailed fragments. This adapter is loaded with the motor protein that translocates DNA through the nanopore. The workflow for nanopore sequencing consists of steps for template preparation, and then steps required for adapter ligation.

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

Steps in the sequencing workflow

Prepare for your experiment

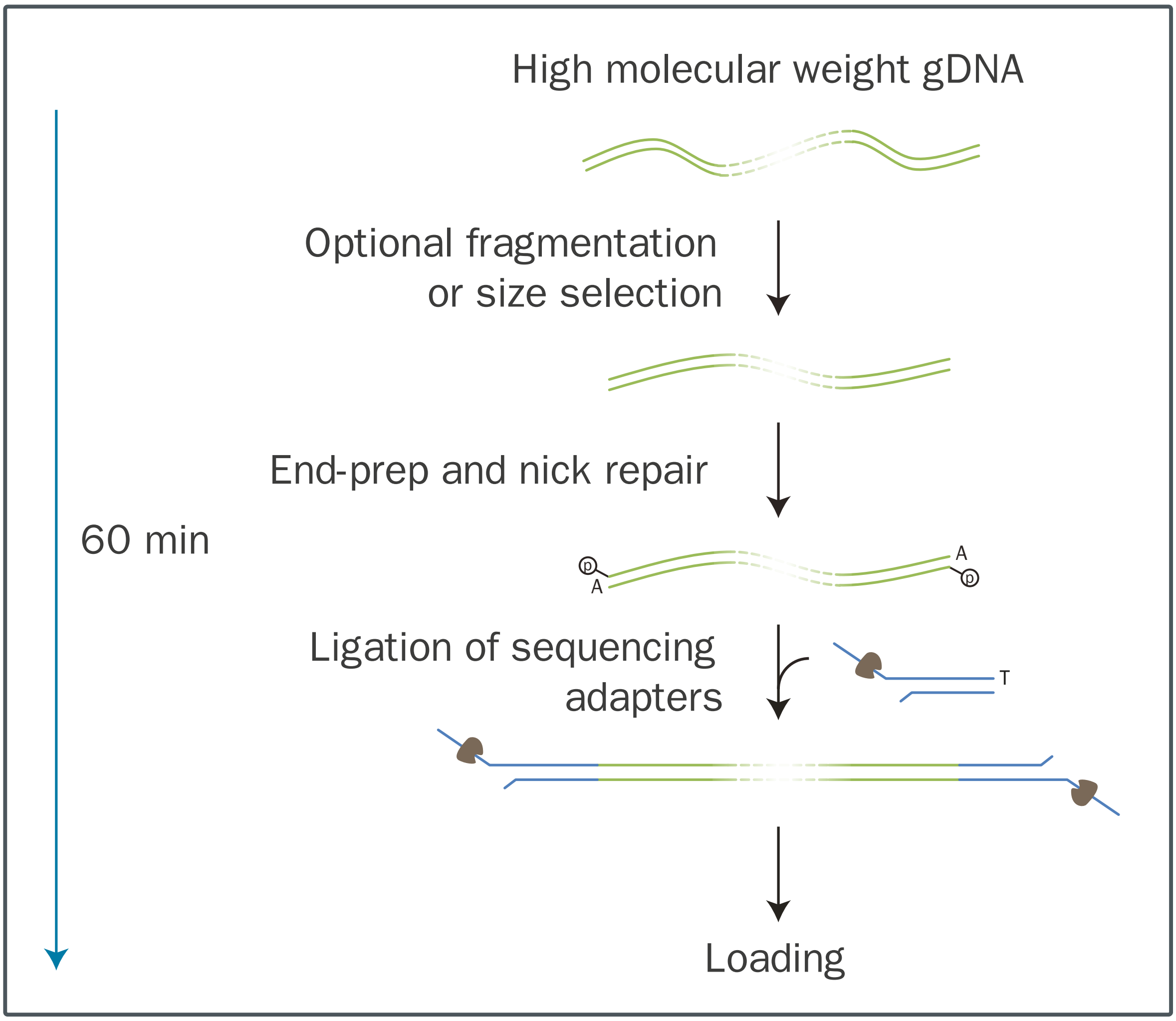
You will need to:

- Extract your DNA and check its length, quantity, and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment, and third-party reagents

Library preparation

You will need to:

- Repair nicks in 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 an assay using the sequencing software, which will collect raw data from the device and convert it into basecalled reads.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (Q-SQK-LSK109)
- Control Expansion (Q-EXP-CTL001)
- Q-FLO-MIN106D flow cells

2. Equipment and consumables

Materials	1 µg (or 100-200 fmol) gDNA OR 100+ ng high molecular weight genomic DNA if performing DNA fragmentation Ligation Sequencing Kit (Q-SQK-LSK109) Flow Cell Priming Kit (Q-EXP-FLP002) SpotON Flow Cell (Q-FLO-MIN106D)
Consumables	Agencourt AMPure XP beads (Beckman Coulter, A63881) NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below: NEBNext FFPE Repair Mix (NEB, M6630) 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
Equipment	Hula mixer (gentle rotator mixer) Magnetic rack, 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
Optional equipment	Agilent Bioanalyzer (or equivalent) Qubit fluorometer (or equivalent for QC check) Eppendorf 5424 centrifuge (or equivalent)

For this protocol, you will need 1 µg (or 100-200 fmol) high molecular weight genomic DNA.

Although 1 µg (or 100-200 fmol) gDNA is recommended, users can start with lower input quantities (down to 100 ng) if performing DNA fragmentation to increase the number of DNA molecules in the sample, or if amplifying the sample by PCR.

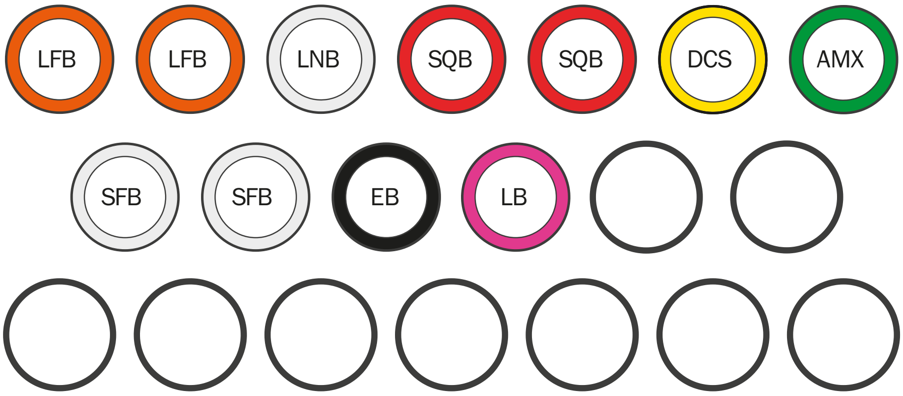
Input DNA

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 **Q system input DNA QC** protocol.

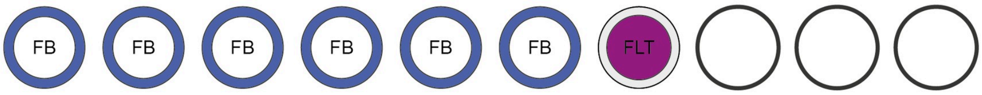
Ligation Sequencing Kit (Q-SQK-LSK109) contents



LFB : L Fragment Buffer
LNB : Ligation Buffer
SQB : Sequencing Buffer
DCS : DNA Control Strand

SFB : S Fragment Buffer
EB : Elution Buffer
LB : Loading Beads
AMX : Adapter Mix

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



FLB : Flush buffer
FLT : Flush tether

3. Computer requirements and software

GridION IT requirements

The GridION device contains all the hardware required to control up to five sequencing experiments simultaneously and acquire the data. The device is further enhanced with high performance GPU technology for real-time basecalling. Read more in the **GridION Q IT Requirements** document in the Q system channel.

The sequencing software

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

For instructions on how to run the sequencing software on the GridION, please refer to the **Q-Line sequencing software user guide**.

4. DNA repair and end-prep

Materials	gDNA in 47 µl nuclease-free water DNA Control Sample (DCS)
Consumables	0.2 ml thin-walled PCR tubes 1.5 ml Eppendorf DNA LoBind tubes Nuclease-free water (e.g. ThermoFisher, AM9937) NEBNext FFPE DNA Repair Mix (NEB, M6630) NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546) Agencourt AMPure XP beads (Beckman Coulter™, A63881) Freshly prepared 80% ethanol in nuclease-free water
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

1 Program a thermal cycler to run at 20°C for 5 minutes and 65°C for 5 mins.

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

3 Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

For optimal performance, 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 FFPE DNA Repair Mix or 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 and FFPE DNA Repair 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.
   Note: It is important the buffers are mixed well by vortexing.
5. The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.

4 Prepare the DNA in nuclease-free water

- Transfer 1 µg (or 100-200 fmol) genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 47 µl with nuclease-free water
- Mix thoroughly by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

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

Reagent	Volume
DNA from the previous step	47 µl
DNA CS	1 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**

6. **Ensure the components are thoroughly mixed by pipetting, and spin down.**

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

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

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

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

10. **Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube.**

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

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

13 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.

14 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.

15 Repeat the previous step.

16 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.

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

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

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

✓ **CHECKPOINT**

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.

5. Adapter ligation and clean-up

Materials	Adapter Mix (AMX) Ligation Buffer (LNB) Long Fragment Buffer (LFB) Short Fragment Buffer (SFB) Elution Buffer (EB) from the Ligation Sequencing Kit
Consumables	NEBNext Quick Ligation Module (NEB, E6056) 1.5 ml Eppendorf DNA LoBind tubes Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Equipment	Magnetic rack Microfuge Vortex mixer P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

IMPORTANT

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

1. **Spin down the Adapter Mix (AMX) and Quick T4 Ligase, and place on ice.**

2. **Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.**

3. **Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then 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.

- To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)
- To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

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 (AMX)	5 µl
Total	**100 µl**

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

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

IMPORTANT

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

9. Resuspend the AMPure XP beads by vortexing.

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

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

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

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

✓ **CHECKPOINT**

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 or at 4°C until ready to load.

! **IMPORTANT**

We recommend loading 5–50 fmol of this final prepared library onto a flow cell. Loading more than 50 fmol of DNA can have a detrimental effect on throughput. Dilute the library in Elution Buffer if required.

6. Priming and loading the MinION Flow Cell

Materials	Flush Tether (FLT) Flush Buffer (FB) Sequencing Buffer (SQB) Loading Beads (LB)
Consumables	1.5 ml Eppendorf DNA LoBind tubes Nuclease-free water (e.g. ThermoFisher, AM9937)
Equipment	MinION Flow Cell P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

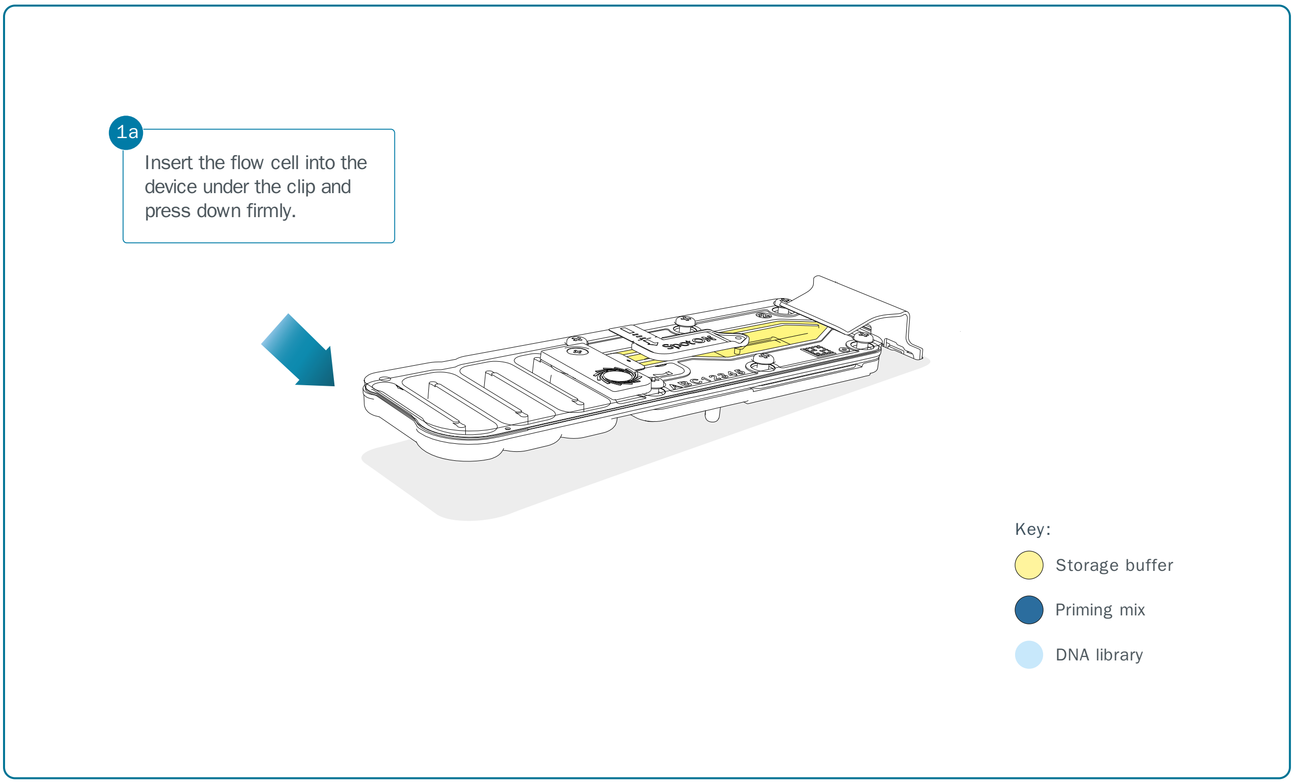
① **Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before mixing the reagents by**

vortexing, and spin down at room temperature.

② **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.**

③ **Slide open the GridION lid and insert the flow cell.**

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



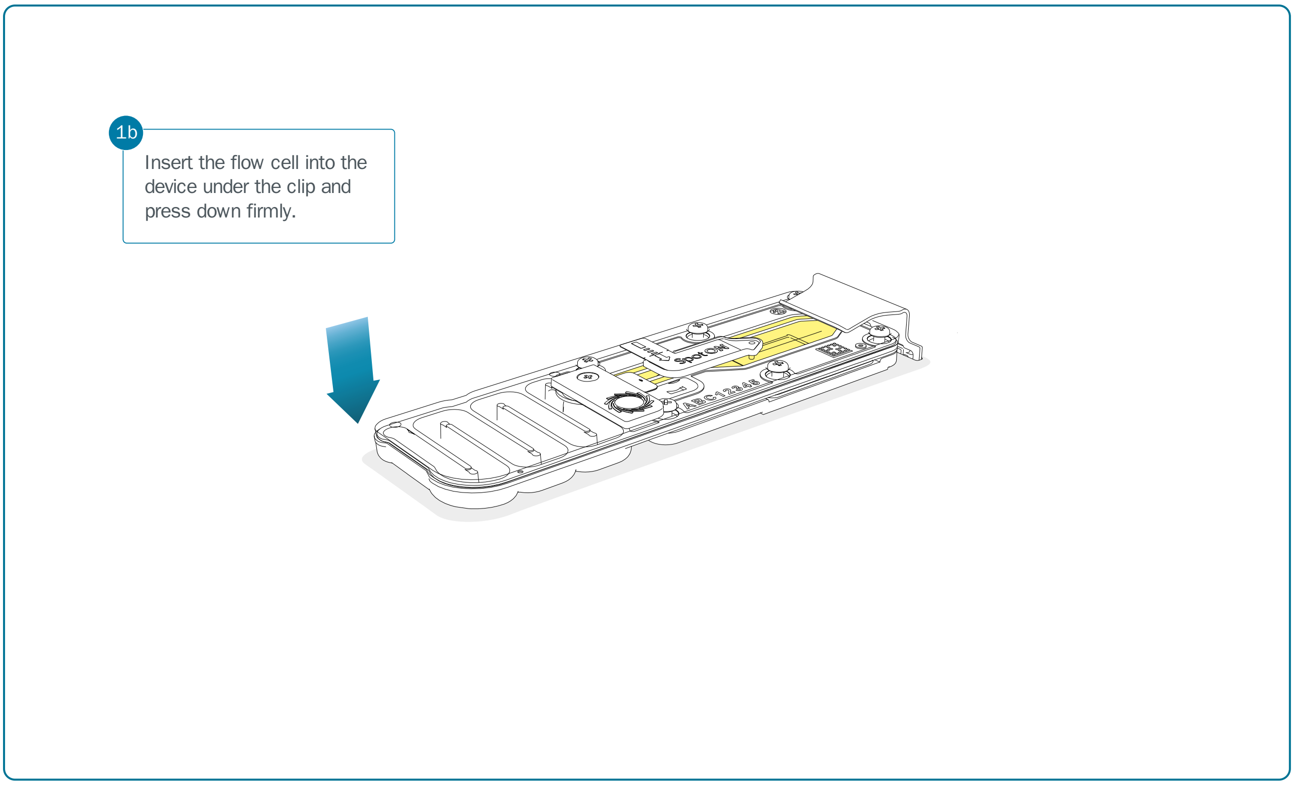
1a Insert the flow cell into the device under the clip and press down firmly.

Key:

🟡 Storage buffer

🔵 Priming mix

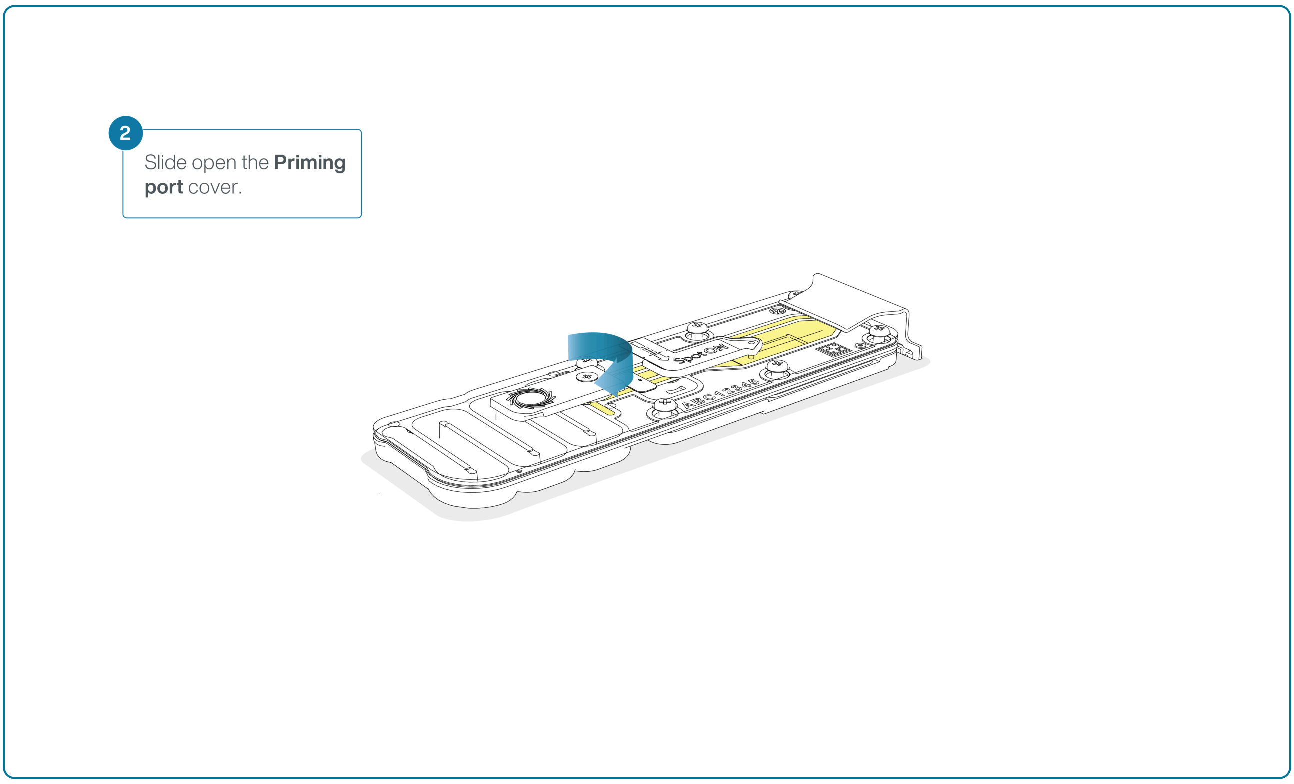
○ DNA library



1b

Insert the flow cell into the device under the clip and press down firmly.

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



2

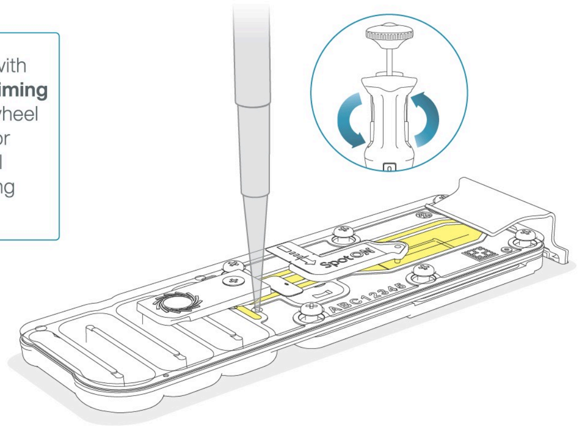
Slide open the **Priming port** cover.

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.

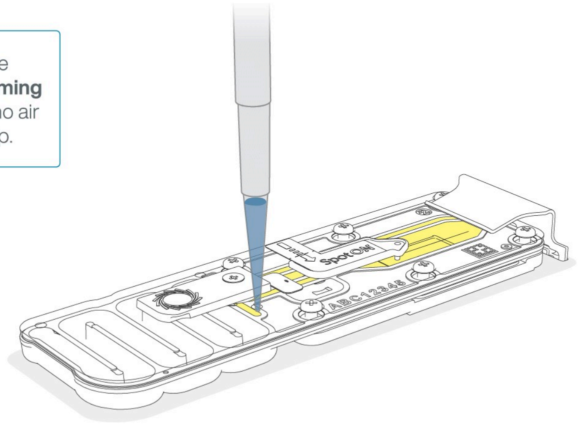


3 Insert a P1000 pipette with an empty tip into the **Priming port**. Turn the pipette wheel to draw back 20-30 µl or until you can see a small volume of buffer entering the pipette tip.

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.

4

Slowly load 800 µl of the priming mix into the **Priming port**. Ensure there are no air bubbles in the pipette tip.



Wait 5 minutes before proceeding to the next step.

7 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

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

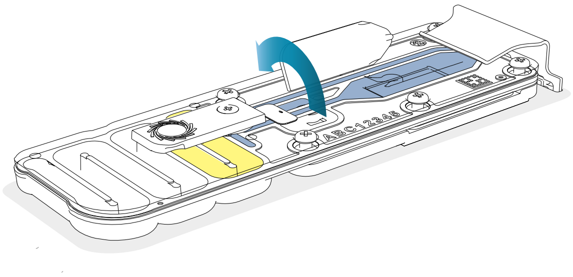
8 In a new tube, prepare the library for loading as follows:

Reagent	**Volume per flow cell**
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
Total	**75 µl**

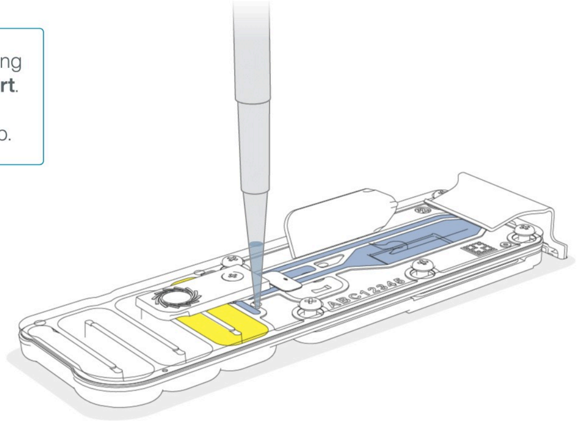
Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer (SQB) and Loading Beads (LB) because the fuel in the buffer will start to be consumed by the adapter.

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.



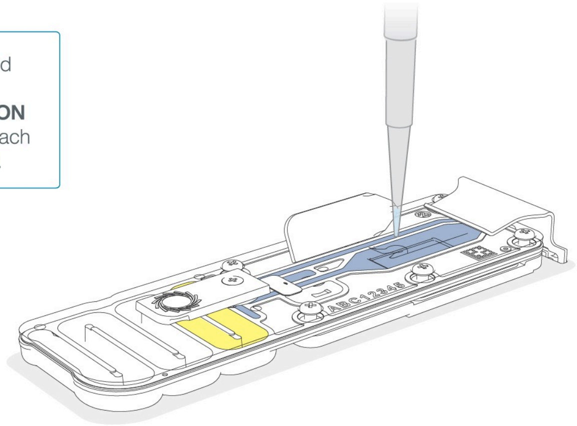
5 Gently flip open the SpotON sample port cover.



6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.

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

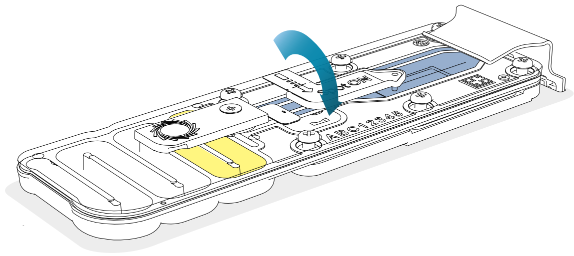
11 Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.



7

Pipette mix the prepared library and load 75 µl dropwise into the **SpotON** sample port, ensuring each drop flows into the port.

12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the flow cell priming port and close the GridION lid.

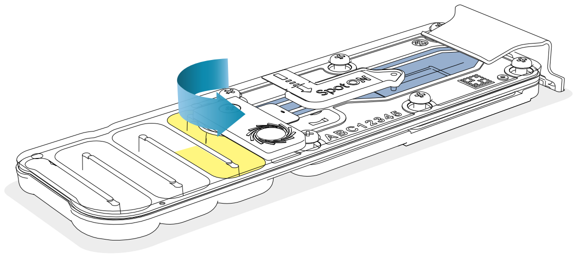


8

Gently replace the **SpotON** sample port cover.

9

Gently close the **Priming port**.



7. Data acquisition and basecalling

How to start sequencing

Follow the instructions in the **Q-Line sequencing software user guide** beginning from the "Running an assay" section.