

Ligation sequencing DNA V14 (SQK-LSK114)

Version: GDE_9161_v114_revAC_24Sep2025

Last update: 9/24/2025

Kit batch number Flow cell number DNA Samples

Checklist: DNA repair and end-prep

Materials	Consumables	Equipment
<input type="checkbox"/> 1 µg (or 100-200 fmol) gDNA	<input type="checkbox"/> NEBNext® FFPE DNA Repair Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)	<input type="checkbox"/> P1000 pipette and tips
<input type="checkbox"/> DNA Control Sample (DCS)	<input type="checkbox"/> NEBNext® FFPE DNA Repair Buffer v2 from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)	<input type="checkbox"/> P100 pipette and tips
<input type="checkbox"/> AMPure XP Beads (AXP)	<input type="checkbox"/> NEBNext® Ultra II End Prep Enzyme Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)	<input type="checkbox"/> P10 pipette and tips
	<input type="checkbox"/> Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)	<input type="checkbox"/> Microfuge
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher,	<input type="checkbox"/> Thermal cycler
		<input type="checkbox"/> Hula mixer (gentle rotator mixer)
		<input type="checkbox"/> Magnetic separation rack
		<input type="checkbox"/> Ice bucket with ice
		<input type="checkbox"/> Qubit™ fluorometer (or equivalent for QC check)

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- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes

DNA repair and end-prep

Notes / Observations

We recommend using the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

The previous version, NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is also compatible, but the recommended v2 module offers more efficient dA-tailing and ligation.

Check your flow cell

We recommend performing a flow cell check before starting your library prep to ensure you have a flow cell with sufficient pores for a good sequencing run.

See the [flow cell check document](#) for more information.

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

We recommend using the DNA Control Sample (DCS) in your library prep for troubleshooting purposes. However, you can omit this step and make up the extra 1 µl with your sample DNA.

- 2 Prepare the NEB 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. Vortex the FFPE DNA Repair Buffer v2 to ensure it is well mixed.
Note: This buffer may contain a white precipitate. If this occurs, allow the mixture to come to room temperature and pipette the buffer several times to break up the precipitate, followed by a quick vortex to mix.
5. The FFPE DNA Repair Buffer v2 may have a yellow tinge and is fine to use if yellow.

3 Prepare the DNA in nuclease-free water:

1. Transfer 1 µg (or 100-200 fmol) input DNA into a 1.5 ml Eppendorf DNA LoBind tube.
2. Adjust the volume to 47 µl with nuclease-free water.
3. Mix thoroughly by pipetting up and down, or by flicking the tube.
4. 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 from the previous step	47 µl
DNA CS (optional)	1 µl
NEBNext FFPE DNA Repair Buffer v2	7 µl
NEBNext FFPE DNA Repair Mix	2 µl

Reagent	Volume
Ultra II End-prep Enzyme Mix	3 μ l
Total	60 μl

- 5 Thoroughly mix the reaction by gently pipetting and briefly spinning down.
- 6 Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. Then cool down to between 4°C and 20°C on the thermal cycler or place the samples on ice.
- 7 Resuspend the AMPure XP Beads (AXP) by vortexing.
- 8 Spin down and transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.
- 9 Add 60 μ l 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 Freshly prepare 500 μ l of 80% ethanol in nuclease-free water.
- 12 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.
- 13 Keep the tube on the magnet and wash the beads with 200 μ l of freshly prepared 80% 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 μ l nuclease-free water by gently pipetting up and down or by flicking the tube. 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 μ l of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify 1 μ l of eluted sample using a Qubit fluorometer.

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.

Checklist: Adapter ligation and clean-up

Materials	Consumables	Equipment
<input type="checkbox"/> Ligation Adapter (LA)	<input type="checkbox"/> Salt-T4® DNA Ligase (NEB, M0467)	<input type="checkbox"/> Magnetic separation rack
<input type="checkbox"/> Ligation Buffer (LNB)	<input type="checkbox"/> NEBNext Quick Ligation Module (NEB, E6056)	<input type="checkbox"/> Microfuge
<input type="checkbox"/> Long Fragment Buffer (LFB)	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Vortex mixer
<input type="checkbox"/> S Fragment Buffer (SFB)	<input type="checkbox"/> Qubit™ Assay Tubes (Invitrogen, Q32856)	<input type="checkbox"/> P1000 pipette and tips
<input type="checkbox"/> AMPure XP Beads (AXP)		<input type="checkbox"/> P100 pipette and tips
<input type="checkbox"/> Elution Buffer (EB)		<input type="checkbox"/> P20 pipette and tips
		<input type="checkbox"/> P10 pipette and tips
		<input type="checkbox"/> Qubit™ fluorometer (or equivalent for QC check)

Adapter ligation and clean-up	Notes / Observations
<p>We recommend using the Salt-T4® DNA Ligase (NEB, M0467).</p> <p>Salt-T4® DNA Ligase (NEB, M0467) can be bought separately or is provided in the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L).</p> <p>The Quick T4 DNA Ligase (NEB, E6057) available in the previous version NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is also compatible, but the new recommended reagent offers more efficient end ligation.</p> <p>Although third-party ligase products may be supplied with their own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.</p>	
<p>1 Spin down the Ligation Adapter (LA) and Salt-T4® DNA Ligase, and place on ice.</p>	

- 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.

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 Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and keep at room temperature.

- 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
DNA sample from the previous step	60 µl
Ligation Adapter (LA)	5 µl
Ligation Buffer (LNB)	25 µl
Salt-T4® DNA Ligase	10 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 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.
- 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 25 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.
- 16 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.
- 17 Remove and retain 25 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 Depending on your DNA library fragment size, prepare your final library in 32 µl of Elution Buffer (EB).

Fragment library length	Flow cell loading amount
Very short (<1 kb)	100 fmol
Short (1-10 kb)	35–50 fmol
Long (>10 kb)	300 ng

Note: If the library yields are below the input recommendations, load the entire library.

If required, we recommend using a mass to mol calculator such as the [NEB calculator](#).

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

Checklist: Priming and loading the PromethION Flow Cell

Materials	Consumables	Equipment
<input type="checkbox"/> Sequencing Buffer (SB)	<input type="checkbox"/> PromethION Flow Cells	<input type="checkbox"/> PromethION device
<input type="checkbox"/> Library Beads (LIB)	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> PromethION Flow Cell Light Shield
<input type="checkbox"/> Library Solution (LIS)		<input type="checkbox"/> P1000 pipette and tips
<input type="checkbox"/> Flow Cell Tether (FCT)		<input type="checkbox"/> P200 pipette and tips
<input type="checkbox"/> Flow Cell Flush (FCF)		<input type="checkbox"/> P20 pipette and tips

Priming and loading the PromethION Flow Cell

Notes / Observations

This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).

After taking the flow cell out of the fridge, wait 20 minutes for the flow cell to reach room temperature, before inserting it into the PromethION. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.

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

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

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 μ l
Flow Cell Tether (FCT)	30 μ l
Total volume	1,200 μl

3 For PromethION 2 Solo, load the flow cell(s) as follows:

1. Place the flow cell flat on the metal plate.
2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.

4 For the PromethION 24/48, load the flow cell(s) into the docking ports:

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.

Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.

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 document](#) for more information.

5 Slide the inlet port cover clockwise to open.

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

6 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 μ l.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 μ l, or until you see a small volume of buffer entering the

pipette tip.

- 7 Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.
- 8 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

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.

- 9 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)	100 µl
Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS)	68 µl
DNA library	32 µl
Total	200 µl

Note: Library loading volume has been increased to improve array coverage.

- 10 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.
- 11 Mix the prepared library gently by pipetting up and down just prior to loading.
- 12 Load 200 µl of library into the inlet port using a P1000 pipette.
- 13 Close the valve to seal the inlet port.

For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

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.

14 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.

Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION, before initiating any experiments. This will help to increase the sequencing output.

Checklist: Flow cell reuse and returns

Materials	Consumables	Equipment
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- Flow Cell Wash Kit
(EXP-WSH004)

Flow cell reuse and returns	Notes / Observations
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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°C to +8°C. The [Flow Cell Wash Kit protocol](#) is available on the Nanopore Community.

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 send the flow cell back to Oxford Nanopore. Instructions for returning flow cells can be found [here](#).

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.

