GridION

Oxford Nanopore Technologies

Ligation sequencing DNA V14 (SQK-LSK114)

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Kit batch number ..... **Flow cell number** ..... **DNA Samples** .....

Checklist: DNA repair and end-prep

Materials

- [ ] 1 µg (or 100-200 fmol) gDNA
- [ ] DNA Control Sample (DCS)
- [ ] AMPure XP Beads (AXP)

Consumables

- [ ] NEBNext® FFPE DNA Repair Mix (M6630) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
- [ ] NEBNext® FFPE DNA Repair Buffer v2 (E7363) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
- [ ] NEBNext® Ultra II End Prep Enzyme Mix (E7646) from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 0.2 ml thin-walled PCR tubes

Equipment

- [ ] P1000 pipette and tips
- [ ] P100 pipette and tips
- [ ] P10 pipette and tips
- [ ] Microfuge
- [ ] Thermal cycler
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic separation rack
- [ ] Ice bucket with ice
- [ ] Qubit™ fluorometer (or equivalent for QC check)

- [ ] 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 (catalogue number 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 instructions in the MinKNOW protocol 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 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
Ultra II End-prep Enzyme Mix	3 µl
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 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 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 µl 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 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.

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

- [ ] Ligation Adapter (LA)
- [ ] Ligation Buffer (LNB)
- [ ] Long Fragment Buffer (LFB)
- [ ] Short Fragment Buffer (SFB)
- [ ] AMPure XP Beads (AXP)
- [ ] Elution Buffer (EB)

Consumables

- [ ] Salt-T4® DNA Ligase (NEB, M0467)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- [ ] Magnetic separation 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)

Adapter ligation and clean-up

We recommend using the Salt-T4® DNA Ligase (NEB, M0467).

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 (catalogue number E7672S or E7672L).

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.

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.

1. Spin down the Ligation Adapter (LA) and Salt-T4® DNA 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.

Notes / Observations

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 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.

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 Depending on your DNA library fragment size, prepare your final library in 12 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 MinION and GridION Flow Cell

Materials	Consumables	Equipment
☐ Flow Cell Flush (FCF)	☐ MinION/GridION Flow Cell	☐ MinION or GridION device
☐ Flow Cell Tether (FCT)	☐ Nuclease-free water (e.g. ThermoFisher, AM9937)	☐ MinION/GridION Flow Cell Light Shields
☐ Library Solution (LIS)	☐ Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)	☐ P1000 pipette and tips
☐ Library Beads (LIB)	☐ 1.5 ml Eppendorf DNA LoBind tubes	☐ P100 pipette and tips
☐ Sequencing Buffer (SB)		☐ P20 pipette and tips
		☐ P10 pipette and tips

Priming and loading the MinION and GridION Flow Cell	Notes / Observations

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

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.

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 priming port cover to ensure correct thermal and electrical contact.

> **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.

> **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.

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.

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.

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

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

When a flow cell is inserted into the MinION Mk1D, the device lid will sit on top of the flow cell, leaving a small gap around the sides. This is normal and has no impact on the performance of the device.

Please refer to this FAQ regarding the device lid.

Checklist: Flow cell reuse and returns

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

Flow cell reuse and returns	Notes / Observations

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.