Flongle

Oxford Nanopore Technologies

Ligation sequencing gDNA - Native Barcoding Kit 96 V14 (SQK-NBD114.96)

Version: NBE_9171_v114_revS_02Jul2025

Last update: 7/2/2025

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

Checklist: DNA repair and end-prep

Materials

- [ ] 400 ng gDNA per barcode
- [ ] DNA Control Sample (DCS)

Consumables

- [ ] NEBNext® FFPE DNA Repair Mix (NEB, M6630)
- [ ] NEBNext® Ultra II End Repair/dA-tailing Module (NEB, E7546)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, 0030129504) with heat seals
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)

Equipment

- [ ] P1000 pipette and tips
- [ ] P200 pipette and tips
- [ ] P100 pipette and tips
- [ ] P20 pipette and tips
- [ ] P10 pipette and tips
- [ ] P2 pipette and tips
- [ ] Multichannel pipette and tips
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Microfuge
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific,11766427)
- [ ] Ice bucket with ice

DNA repair and end-prep

For samples containing long gDNA fragments, we recommend using wide-bore pipette tips for the mixing steps to preserve the DNA length.

1. Thaw the DNA Control Sample (DCS) at room temperature, mix by vortexing, and place on ice.

Notes / Observations

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

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 Reaction 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.

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

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

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

3 **Dilute your DNA Control Sample (DCS) by adding 105 µl Elution Buffer (EB) directly to one DCS tube. Mix gently by pipetting and spin down.**

One tube of diluted DNA Control Sample (DCS) is enough for 140 samples. Excess can be stored at -20°C.

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.

4. In a clean 96-well plate, aliquot 400 ng DNA per sample.

5. Make up each sample to 11 µl using nuclease-free water. Mix gently by pipetting and spin down.

6. Combine the following components per well:

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA sample	11 µl
Diluted DNA Control Sample (DCS)	1 µl
NEBNext FFPE DNA Repair Buffer	0.875 µl
Ultra II End-prep Reaction Buffer	0.875 µl
Ultra II End-prep Enzyme Mix	0.75 µl
NEBNext FFPE DNA Repair Mix	0.5 µl
Total	**15 µl**

We recommend making up a mastermix of the End Prep and DNA Repair reagents for the total number of samples and adding 3 µl to each well.

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

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

Take forward the end-prepped DNA into the native barcode ligation step.

If users want to pause the library preparation here, we recommend cleaning up your sample with 1X AMPure XP Beads (AXP) and eluting in nuclease-free water before storing at 4°C.

Please note, extra AMPure XP Beads (AXP) will be required for this optional step.

Checklist: Native barcode ligation

Materials

- [ ] Native Barcodes (NB01-NB96)
- [ ] AMPure XP Beads (AXP)
- [ ] EDTA (EDTA)
- [ ] Short Fragment Buffer (SFB)

Consumables

- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, 0030129504) with heat seals
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)

Equipment

- [ ] Magnetic separation rack
- [ ] Vortex mixer
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microfuge
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Multichannel pipette and tips
- [ ] P1000 pipette and tips
- [ ] P200 pipette and tips
- [ ] P100 pipette and tips
- [ ] P20 pipette and tips
- [ ] P10 pipette and tips
- [ ] P2 pipette and tips
- [ ] Qubit™ fluorometer (or equivalent for QC check)

Native barcode ligation

1. **Prepare the NEB Blunt/TA Ligase Master Mix and NEBNext Quick Ligation Module according to the manufacturers instructions, and place on ice.**
   - Thaw the reagents at room temperature.
   - Spin down the reagent tubes for 5 seconds.
   - Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
2. **Thaw the AMPure XP Beads (AXP) at room temperature and mix by vortexing. Keep the beads at room temperature.**
3. **Thaw the EDTA 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. Place on ice.**

Notes / Observations

5. Thaw the Native Barcodes (NB01-96) required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place them on ice.

6. Select a unique barcode for every sample to be run together on the same flow cell. Up to 96 samples can be barcoded and combined in one experiment.

Please note: Only use one barcode per sample.

7. In a new 96-well plate, add the reagents in the following order per well mixing well by pipetting between each addition:

Reagent	Volume
Nuclease-free water	3 µl
End-prepped DNA	0.75 µl
Native Barcode (NB01-96)	1.25 µl
Blunt/TA Ligase Master Mix	5 µl
Total	**10 µl**

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

9. Incubate for 20 minutes at room temperature.

10. Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly.

Note: Ensure you follow the instructions for the cap colour of your EDTA tube.

EDTA cap colour	Volume per well
For clear cap EDTA	1 µl
For blue cap EDTA	2 µl

EDTA is added at this step to stop the reaction.

11. Pool the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

Note: Ensure you follow the instructions for the cap colour of your EDTA tube.

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
Total volume for preps using	11 µl	264 µl	528 µl	1,056 µl

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
clear cap EDTA				
Total volume for preps using **blue cap EDTA**	12 µl	288 µl	576 µl	1,152 µl

We recommend checking the base of your tubes/plate are all the same volume before pooling and after to ensure all the liquid has been taken forward.

12 Resuspend the AMPure XP Beads (AXP) by vortexing.

13 Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting.

Note: Ensure you follow the instructions for the cap colour of your EDTA tube.

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
Volume of AXP for preps using **clear cap EDTA**	4 µl	106 µl	211 µl	422 µl
Volume of AXP for preps using **blue cap EDTA**	5 µl	115 µl	230 µl	461 µl

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

For optimal results we recommend using Short Fragment Buffer (SFB) for the clean-up steps following native barcoding.

Our development teams have determined that using the **Short Fragment Buffer (SFB) instead of ethanol** for the post-barcoding washes removes excess barcode more efficiently. This translates to improved barcode classification rates and reduced physical barcode cross-talk.

New batches of the native barcoding kits will contain sufficient Short Fragment Buffer (SFB) to follow the updated method. If you have an older format of the native barcoding kit with a lower volume of Short Fragment Buffer (SFB), you may require additional reagents available through the SFB Expansion (EXP-SFB001).

Please note, the old method using 80% ethanol is still compatible with this method. If you wish to continue using 80% ethanol for your post-barcoding wash, please follow the steps below:

- Prepare sufficient fresh 80% ethanol in nuclease-free water for your washes.
- Use the freshly prepared 80% ethanol in place of the Short Fragment Buffer (SFB) for the wash steps below.

15 Spin down the sample and pellet on a magnet for 5 minutes. Keep the plate on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.

16 Keep the tube on the magnetic rack and wash the beads with 700 µl of Short Fragment Buffer (SFB) without disturbing the pellet. Remove the buffer using a pipette and discard.

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

17 Repeat the previous step.

18 Spin down and place the tube back on the magnetic rack. Pipette off any residual buffer.

19 Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nuclease-free water by gently flicking.

20 Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.

21 Pellet the beads on a magnetic rack until the eluate is clear and colourless.

22 Remove and retain 35 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 barcoded DNA library to the adapter ligation and clean-up step. However, you may store the sample at 4°C overnight.

Checklist: Adapter ligation and clean-up

Materials

- [ ] Long Fragment Buffer (LFB)
- [ ] Short Fragment Buffer (SFB)
- [ ] Elution Buffer (EB)
- [ ] Native Adapter (NA)
- [ ] AMPure XP Beads (AXP)

Consumables

- [ ] NEBNext® Quick Ligation Module (NEB, E6056)
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)

Equipment

- [ ] Microfuge
- [ ] Magnetic separation rack
- [ ] Vortex mixer
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Thermal cycler
- [ ] P1000 pipette and tips
- [ ] P200 pipette and tips
- [ ] P100 pipette and tips
- [ ] P20 pipette and tips
- [ ] P10 pipette and tips
- [ ] Ice bucket with ice
- [ ] Qubit™ fluorometer (or equivalent for QC check)

Adapter ligation and clean-up

The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.

Check your flow cell.

We recommend performing a flow cell check before starting adapter ligation and clean-up 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. **Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:**

   1. Thaw the reagents at room temperature.

   2. Spin down the reagent tubes for 5 seconds.

   3. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. **Note:** Do NOT vortex the Quick T4

Notes / Observations

DNA Ligase.

The NEBNext Quick Ligation Reaction Buffer (5x) 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 several seconds to ensure the reagent is thoroughly mixed.

Do not vortex the Quick T4 DNA Ligase.

2. Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice.

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. Place on ice.

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

Between each addition, pipette mix 10 - 20 times.

Reagent	Volume
Pooled barcoded sample	30 µl
Native Adapter (NA)	5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
Quick T4 DNA Ligase	5 µl
Total	**50 µl**

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

7. Incubate the reaction for 20 minutes at room temperature.

The next clean-up step uses Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) rather than 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.

8. Resuspend the AMPure XP Beads (AXP) by vortexing.
9. Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.
10. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.
11. Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.
12. Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or 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.
15. Remove the tube from the magnetic rack and resuspend pellet in 7 µl Elution Buffer (EB).
16. Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.
17. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.
18. Remove and retain 7 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.**

19. Prepare your final library to 5-10 fmol in 5 µl of Elution Buffer (EB).

If required, we recommend using a mass to mol calculator such as the NEB calculator.

We recommend loading 5-10 fmol of this final prepared library onto the R10.4.1 flow cell.

Following standard input recommendations, the protocol should produce enough final library (adapter DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load onto a second flow cell. Loading more than 10 fmol can have a detrimental effect on output. Dilute the library in EB or nuclease-free water to a final volume of 5 µl.

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

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.

Checklist: Flongle Flow Cell loading

Materials	Consumables	Equipment
☐ Flongle Sequencing Expansion (EXP-FSE002)	☐ Flongle Flow Cell	☐ Flongle adapter
☐ Flow Cell Tether (FCT)	☐ 1.5 ml Eppendorf DNA LoBind tubes	☐ MinION or GridION device
		☐ P200 pipette and tips
		☐ P20 pipette and tips
		☐ P10 pipette and tips

Flongle Flow Cell loading

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-FLG114).

Flongle Sequencing Expansion (EXP-FSE002)

To load a library onto your Flongle Flow Cell, you will need to use the following:

Flongle Sequencing Expansion (EXP-FSE002) components

- Sequencing Buffer (SB)
- Flow Cell Flush (FCF)
- Library Beads (LIB) or Library Solution (LIS)

Sequencing Kit components

- Flow Cell Tether (FCT)

Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

Do NOT touch the reverse side of the Flongle flow cell array or the contact pads on the Flongle adapter. ALWAYS wear gloves when handling Flongle flow cells and adapters to avoid damage to the flow cell or adapter.

Notes / Observations

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. In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flow Cell Flush (FCF) with 3 µl of Flow Cell Tether (FCT) and mix by pipetting.
3. Place the Flongle adapter into the MinION or one of the five GridION positions.

The adapter should sit evenly and flat on the MinION Mk1B or GridION platform. This ensures the flow cell assembly is flat during the next stage.

The adapter needs to be plugged into your device, and the device should be plugged in and powered on before inserting the Flongle flow cell.

4 **Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click.**

The flow cell should sit evenly and flat inside the adapter, to avoid any bubbles forming inside the fluidic compartments.

5 **Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed, as follows:**

1. Lift up the seal tab:

2. Pull the seal tab to open access to the sample port:

3. Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid:

6 **To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the 120 µl of priming fluid into the Flongle flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.**

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.

7 **Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows:**

Reagents	Volume
Sequencing Buffer (SB)	15 µl

Reagents	Volume
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using.	10 µl
DNA library	5 µl
Total	**30 µl**

8 To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.

9 Seal the Flongle flow cell using the adhesive on the seal tab, as follows:

1. Stick the transparent adhesive tape to the sample port.

2. Replace the top (Wheel icon section) of the seal tab to its original position.

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.