Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24)

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Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials

- [ ] Native Barcoding Kit 24 V14 (SQK-NBD114.24)
- [ ] 400 ng gDNA per sample if using >4 barcodes
- [ ] OR 1000 ng gDNA per sample if using ≤4 barcodes

Consumables

- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 2 ml Eppendorf DNA LoBind tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
- [ ] (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Microfuge
- [ ] Magnetic rack
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Eppendorf 5424 centrifuge (or equivalent)
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000, Multichannel

INSTRUCTIONS	NOTES/OBSERVATIONS
DNA repair and end-prep	
☐ Thaw the AMPure XP Beads (AXP) and DNA Control Sample (DCS) at RT and mix by vortexing. Keep the beads at RT and store the DNA Control Sample (DCS) on ice.	

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INSTRUCTIONS	NOTES/OBSERVATIONS

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.

- ☐ Thaw all reagents on ice.
- ☐ 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.
- ☐ Always spin down tubes before opening for the first time each day.
- ☐ The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to RT 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.
- ☐ The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.

IMPORTANT

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

IMPORTANT

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

☐ 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.

In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), prepare your DNA samples:

- ☐ For >4 barcodes, aliquot 400 ng per sample
- ☐ For ≤4 barcodes, aliquot 1000 ng per sample

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

Combine the following components per tube/well:

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

☐ Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.

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

☐ Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

☐ Resuspend the AMPure XP beads (AXP) by vortexing.

☐ Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.

☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Prepare sufficient fresh 80% ethanol in Nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess. ☐ Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant. ☐ 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. ☐ Repeat the previous step. ☐ Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking. ☐ Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl Nuclease-free water. Spin down and incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Dispose of the pelleted beads	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
Take forward an equimolar mass of each sample to be barcoded forward into the native barcode ligation step. However, you may store the samples at 4°C overnight.	
Native barcode ligation	
Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. ☐ Thaw the EDTA at RT and mix by vortexing. Then spin down and place on ice. ☐ Thaw the Native Barcodes (NB01-24) required for your number of samples at RT. Individually mix the barcodes by pipetting, spin down, and place them on ice. ☐ Select a unique barcode for each sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment. In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well: ☐ 7.5 µl End-prepped DNA ☐ 2.5 µl Native Barcode (NB01-24) ☐ 10 µl Blunt/TA Ligase Master Mix ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate for 20 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly. ☐ 2 µl For clear cap EDTA ☐ 4 µl For blue cap EDTA	
☐ Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.	
☐ Resuspend the AMPure XP Beads (AXP) by vortexing.	
☐ Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting.	
☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.	
☐ Prepare 2 ml of fresh 80% ethanol in Nuclease-free water.	
☐ Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.	
☐ Keep the tube on the magnetic rack and wash the beads with 700 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.	
☐ Repeat the previous step.	
☐ Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.	
☐ Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water by gently flicking.	
☐ Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.	
☐ Pellet the beads on a magnetic rack until the eluate is clear and colourless.	
☐ 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.	
Adapter ligation and clean-up	
IMPORTANT ☐ The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.	

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INSTRUCTIONS	NOTES/OBSERVATIONS

Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:

- ☐ Thaw the reagents at RT.
- ☐ Spin down the reagent tubes for 5 seconds.
- ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
  Note: Do NOT vortex the Quick T4 DNA Ligase.

IMPORTANT

- ☐ Do not vortex the Quick T4 DNA Ligase.

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

☐ Thaw the Elution Buffer (EB) at RT 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)

☐ Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT and mix by vortexing. Then spin down and place on ice.

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

- ☐ 30 µl Pooled barcoded sample
- ☐ 5 µl Native Adapter (NA)
- ☐ 10 µl NEBNext Quick Ligation Reaction Buffer (5X)
- ☐ 5 µl Quick T4 DNA Ligase

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

☐ Incubate the reaction for 20 minutes at RT.

IMPORTANT

- ☐ 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.

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

☐ Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.

☐ Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

☐ 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.

☐ Repeat the previous step.

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. ☐ Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB). ☐ 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. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Depending on your required data output, prepare your final library to 35-50 fmol for high output of simplex data, or 10-20 fmol for duplex data, in 12 µl of Elution Buffer (EB).	
The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.	
Priming and loading the SpotON flow cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT 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.	
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 RT. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml ☐ 30 µl Flow Cell Tether (FCT) ☐ 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. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, 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. ☐ 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. ☐ 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.	
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using ☐ 12 µl DNA library Complete the flow cell priming: ☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible. ☐ Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ 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. ☐ 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.	
Place the light shield onto the flow cell, as follows: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ 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.	
Close the device lid and set up a sequencing run on MinKNOW.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Flow cell reuse and returns	
☐ 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. ☐ Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.	
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.	