

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

Version: NBE_9169_v114_revE_15Sep2022
Last update: 06/01/2023



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

Equipment

- ☐ Hula mixer (gentle rotator mixer)
- ☐ Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, #11766427)
- ☐ Microfuge
- ☐ Magnetic rack suitable for 0.2 ml thin-walled PCR tubes or 96-well plates
- ☐ 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

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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<p>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.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Thaw all reagents on ice. <input type="checkbox"/> 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. <input type="checkbox"/> Always spin down tubes before opening for the first time each day. <input type="checkbox"/> 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. <input type="checkbox"/> The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing. 	
<ul style="list-style-type: none"> <input type="checkbox"/> 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. <p>In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), prepare your DNA samples:</p> <ul style="list-style-type: none"> <input type="checkbox"/> For >4 barcodes, aliquot 400 ng per sample <input type="checkbox"/> For ≤4 barcodes, aliquot 1000 ng per sample <ul style="list-style-type: none"> <input type="checkbox"/> Make up each sample to 11 µl using Nuclease-free water. Mix gently by pipetting and spin down. <p>Combine the following components per tube/well:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 11 µl DNA sample <input type="checkbox"/> 1 µl Diluted DNA Control Sample (DCS) <input type="checkbox"/> 0.875 µl NEBNext FFPE DNA Repair Buffer <input type="checkbox"/> 0.875 µl Ultra II End-prep Reaction Buffer <input type="checkbox"/> 0.75 µl Ultra II End-prep Enzyme Mix <input type="checkbox"/> 0.5 µl NEBNext FFPE DNA Repair Mix <ul style="list-style-type: none"> <input type="checkbox"/> Ensure the components are thoroughly mixed by pipetting. Close the tubes (or seal the plate) and spin down in a centrifuge. <input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. <input type="checkbox"/> Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Resuspend the AMPure XP beads (AXP) by vortexing. <input type="checkbox"/> Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. 	

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<input type="checkbox"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Dispose of the pelleted beads	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
Take forward an equimolar mass of samples to be barcoded and pooled forward into the native barcode ligation step. However, at this point it is also possible to store the sample 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: <input type="checkbox"/> Thaw the reagents at RT. <input type="checkbox"/> Spin down the reagent tubes for 5 seconds. <input type="checkbox"/> Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. <input type="checkbox"/> Thaw the EDTA at RT, mix by vortexing, spin down and place on ice. <input type="checkbox"/> 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. <input type="checkbox"/> 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: <input type="checkbox"/> 7.5 µl End-prepped DNA <input type="checkbox"/> 2.5 µl Native Barcode (NB01-24) <input type="checkbox"/> 10 µl Blunt/TA Ligase Master Mix <input type="checkbox"/> Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly. <input type="checkbox"/> Incubate for 20 minutes at RT. <input type="checkbox"/> Add 2 µl of EDTA to each well and mix thoroughly by pipetting and spin down briefly.	

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<input type="checkbox"/> Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Add AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting for a 0.4X clean. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Prepare 2 ml of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water by gently flicking. <input type="checkbox"/> Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. <input type="checkbox"/> Pellet the beads on a magnetic rack until the eluate is clear and colourless. <input type="checkbox"/> 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, at this point it is also possible to store the sample at 4°C overnight.	
Adapter ligation and clean-up	
IMPORTANT <input type="checkbox"/> The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.	
Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice: <input type="checkbox"/> Thaw the reagents at RT. <input type="checkbox"/> Spin down the reagent tubes for 5 seconds. <input type="checkbox"/> Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.	
IMPORTANT <input type="checkbox"/> Do not vortex the Quick T4 DNA Ligase.	

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<input type="checkbox"/> Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice. <input type="checkbox"/> Thaw the Elution Buffer (EB) at RT, mix by vortexing, 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. <input type="checkbox"/> To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB) <input type="checkbox"/> To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)	
<input type="checkbox"/> Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. In a 1.5 ml Eppendorf LoBind tube, mix in the following order: <input type="checkbox"/> 30 µl Pooled barcoded sample <input type="checkbox"/> 5 µl Native Adapter (NA) <input type="checkbox"/> 10 µl NEBNext Quick Ligation Reaction Buffer (5X) <input type="checkbox"/> 5 µl Quick T4 DNA Ligase <input type="checkbox"/> Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly. <input type="checkbox"/> Incubate the reaction for 20 minutes at RT.	
IMPORTANT <input type="checkbox"/> 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.	
<input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant. <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> Spin down and place the tube back on the magnet. Pipette off any residual supernatant. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB). <input type="checkbox"/> 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. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.	

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<input type="checkbox"/> 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.	
<input type="checkbox"/> Make up the library to 12 µl at 10-20 fmol.	
IMPORTANT <input type="checkbox"/> We recommend loading 10 - 20 fmol of this final prepared library onto the R10.4.1 flow cell.	
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 <input type="checkbox"/> Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
Using the Library Solution <input type="checkbox"/> Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at RT. Mix by vortexing and spin down.	
IMPORTANT <input type="checkbox"/> 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.	
IMPORTANT <input type="checkbox"/> We do not recommend using recombinant BSA.	
To prepare the flow cell priming mix with BSA, add the following reagents directly to the tube of Flow Cell Flush (FCF), and mix by inverting the tube and pipette mix at RT: <input type="checkbox"/> 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml <input type="checkbox"/> 30 µl Flow Cell Tether (FCT) <input type="checkbox"/> 1,205 µl Final total volume in Flow Cell Flush (FCF) tube <input type="checkbox"/> 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. <input type="checkbox"/> Slide the priming port cover clockwise to open the priming port.	
IMPORTANT <input type="checkbox"/> 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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<p>After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set a P1000 pipette to 200 µl <input type="checkbox"/> Insert the tip into the priming port <input type="checkbox"/> 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 <p>Note: Visually check that there is continuous buffer from the priming port across the sensor array.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below. <input type="checkbox"/> Thoroughly mix the contents of the Library Beads (LIB) by pipetting. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 µl Sequencing Buffer (SB) <input type="checkbox"/> 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using <input type="checkbox"/> 12 µl DNA library <p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible. <input type="checkbox"/> Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. <input type="checkbox"/> Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION or GridION device lid. 	
<p>Ending the experiment</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR <input type="checkbox"/> Follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	