

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

Version: NBE_9169_v114_revQ_15Sep2022
Last update: 16/02/2024



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)

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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| <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 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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| <p><input type="checkbox"/> Prepare sufficient fresh 80% ethanol in Nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess.</p> <p><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.</p> <p><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.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><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.</p> <p><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.</p> <p><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless.</p> <p>Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Dispose of the pelleted beads</p> | |
| 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 | |
| <p>Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice:</p> <p><input type="checkbox"/> Thaw the reagents at RT.</p> <p><input type="checkbox"/> Spin down the reagent tubes for 5 seconds.</p> <p><input type="checkbox"/> Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.</p> <p><input type="checkbox"/> Thaw the EDTA at RT and mix by vortexing. Then spin down and place on ice.</p> <p><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.</p> <p><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.</p> <p>In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well:</p> <p><input type="checkbox"/> 7.5 µl End-prepped DNA</p> <p><input type="checkbox"/> 2.5 µl Native Barcode (NB01-24)</p> <p><input type="checkbox"/> 10 µl Blunt/TA Ligase Master Mix</p> <p><input type="checkbox"/> Thoroughly mix the reaction by gently pipetting and briefly spinning down.</p> <p><input type="checkbox"/> Incubate for 20 minutes at RT.</p> | |

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| <p>Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly.</p> <p><input type="checkbox"/> 2 µl For clear cap EDTA</p> <p><input type="checkbox"/> 4 µl For blue cap EDTA</p> <p><input type="checkbox"/> Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing.</p> <p><input type="checkbox"/> Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting.</p> <p><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.</p> <p><input type="checkbox"/> Prepare 2 ml of fresh 80% ethanol in Nuclease-free water.</p> <p><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.</p> <p><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.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><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.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water by gently flicking.</p> <p><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.</p> <p><input type="checkbox"/> Pellet the beads on a magnetic rack until the eluate is clear and colourless.</p> <p><input type="checkbox"/> Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> | |
| 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 | |
| <p>IMPORTANT</p> <p><input type="checkbox"/> The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.</p> | |

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| <p>Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:</p> <ul style="list-style-type: none"> <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. Note: Do NOT vortex the Quick T4 DNA Ligase. | |
| <p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Do not vortex the Quick T4 DNA Ligase. | |
| <ul style="list-style-type: none"> <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 and mix by vortexing. Then spin down and place on ice. | |
| <p>IMPORTANT</p> <p>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.</p> <ul style="list-style-type: none"> <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) | |
| <ul style="list-style-type: none"> <input type="checkbox"/> Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT and mix by vortexing. Then spin down and place on ice. <p>In a 1.5 ml Eppendorf LoBind tube, mix in the following order:</p> <ul style="list-style-type: none"> <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 <ul style="list-style-type: none"> <input type="checkbox"/> Thoroughly mix the reaction by gently pipetting and briefly spinning down. <input type="checkbox"/> Incubate the reaction for 20 minutes at RT. | |
| <p>IMPORTANT</p> <ul style="list-style-type: none"> <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. | |
| <ul style="list-style-type: none"> <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. | |

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| <input type="checkbox"/> 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. | |
| <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 25 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. | |
| <input type="checkbox"/> Remove and retain 25 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"/> 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 32 µl of Elution Buffer (EB). | |
| The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load. | |
| | |
| Priming and loading the PromethION Flow Cell | |
| IMPORTANT <input type="checkbox"/> This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M). | |
| <input type="checkbox"/> 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. To prepare the flow cell priming mix, combine Flow Cell Tether (FCT) and Flow Cell Flush (FCF), as directed below. Mix by vortexing at RT. <div> <input type="checkbox"/> 1,170 µl Flow Cell Flush (FCF) <input type="checkbox"/> 30 µl Flow Cell Tether (FCT) </div> | |
| IMPORTANT <input type="checkbox"/> After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. 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. | |
| For PromethION 2 Solo, load the flow cell(s) as follows: <div> <input type="checkbox"/> Place the flow cell flat on the metal plate. <input type="checkbox"/> Slide the flow cell into the docking port until the gold pins or green board cannot be seen. </div> For the PromethION 24/48, load the flow cell(s) into the docking ports: <div> <input type="checkbox"/> Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. <input type="checkbox"/> Press down firmly onto the flow cell and ensure the latch engages and clicks into place. </div> | |

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| <p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Slide the inlet port cover clockwise to open.</p> | |
| <p>IMPORTANT</p> <p><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.</p> <p>After opening the inlet port, draw back a small volume to remove any air bubbles:</p> <p><input type="checkbox"/> Set a P1000 pipette tip to 200 µl.</p> <p><input type="checkbox"/> Insert the tip into the inlet port.</p> <p><input type="checkbox"/> Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Thoroughly mix the contents of the Library Beads (LIB) by pipetting.</p> | |
| <p>IMPORTANT</p> <p><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> <p>In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:</p> <p><input type="checkbox"/> 100 µl Sequencing Buffer (SB)</p> <p><input type="checkbox"/> 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS)</p> <p><input type="checkbox"/> 32 µl DNA library</p> <p><input type="checkbox"/> Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.</p> <p><input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading.</p> <p><input type="checkbox"/> Load 200 µl of library into the inlet port using a P1000 pipette.</p> <p><input type="checkbox"/> Close the valve to seal the inlet port.</p> | |
| <p>IMPORTANT</p> <p><input type="checkbox"/> Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.</p> <p>If the light shield has been removed from the flow cell, install the light shield as follows:</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.</p> | |
| <p>Close the PromethION lid when ready to start a sequencing run on MinKNOW.</p> | |

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| Flow cell reuse and returns | |
| <input type="checkbox"/> 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. | |
| <input type="checkbox"/> Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore. | |
| IMPORTANT <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. | |