Ligation sequencing amplicons - native barcoding (SQK-LSK109 with EXP-NBD196)

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

Before start checklist

Materials

- [ ] 200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded
- [ ] Native Barcoding Expansion 96 (EXP-NBD196)
- [ ] Ligation Sequencing Kit (SQK-LSK109)
- [ ] Flow Cell Priming Kit (EXP-FLP002)
- [ ] Adapter Mix II Expansion (EXP-AMII001)

Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microfuge
- [ ] Magnetic rack
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000, Multichannel

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
Prepare the 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 Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep 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.	
IMPORTANT ☐ Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.	
IMPORTANT ☐ It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.	
☐ In a clean 96-well plate, aliquot 200 fmol (130 ng for 1 kb amplicons) of DNA per sample. ☐ Make up each sample per well to 12.5 µl using Nuclease-free water.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Add the following components to each well: ☐ 1.75 µl Ultra II End-prep reaction buffer ☐ 0.75 µl Ultra II End-prep enzyme mix ☐ Mix well 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.	
Take forward the end-prepped DNA 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: ☐ 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 native barcodes at RT. Use one barcode per sample. Individually mix the barcodes by pipetting, spin down, and place them on ice. ☐ Thaw the tube of Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. ☐ Select a unique barcode for every sample to be run. In a new 96-well plate, add the reagents in the order given below per well: ☐ 3 µl Nuclease-free water ☐ 0.75 µl End-prepped DNA ☐ 1.25 µl Native Barcode ☐ 5 µl Blunt/TA Ligase Master Mix ☐ Mix contents thoroughly by pipetting and spin down briefly. ☐ Using a thermal cycler, incubate at 20°C for 20 mins and at 65°C for 10 mins. ☐ Pool the barcoded library together and carry forward 480 µl of the library. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 192 µl of resuspended AMPure XP beads to the 480 µl of pooled reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet the beads on a magnet for 5 minutes. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant. ☐ Wash the beads by adding 700 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard.	

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☐ Repeat the previous step. ☐ Keep the tube on the magnet and wash the beads with 100 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ 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. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet 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 - recovery aim 2 ng/µl.	
Take forward the pooled samples into the next step. However, at this point it is also possible to store the sample at 4°C overnight.	
Adapter ligation and clean-up	
Adapter Mix II Expansion use 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.	
☐ Thaw the Elution Buffer (EB) at RT and mix by vortexing. Then spin down and place on ice. ☐ Spin down the Adapter Mix II (AMII), pipette mix and place on ice. Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition. ☐ 30 µl Pooled barcoded sample ☐ 5 µl Adapter Mix II (AMII) ☐ 10 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 5 µl Quick T4 DNA Ligase ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	

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IMPORTANT ☐ The next clean-up step uses Short Fragment Buffer (SFB) and not 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.	
☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads 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 the beads on a magnet for 5 minutes. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant. ☐ Wash the beads by adding 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ 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. ☐ Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at RT. ☐ 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.	
IMPORTANT ☐ We recommend loading ~15 ng of final prepared library onto a 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	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing, and spin down at RT. ☐ To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at RT. ☐ Open the MinION device lid and slide the flow cell under the clip. ☐ Slide the flow cell priming port cover clockwise to open the priming port.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
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 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. ☐ 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 Loading Beads (LB) by pipetting.	
IMPORTANT ☐ The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	
In a new tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SQB) ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☐ 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, close the priming port and replace the MinION device lid.	
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.	