Ligation sequencing amplicons - native barcoding (SQK-LSK109 with EXP-NBD104 and EXP-NBD114)

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

Before start checklist

Materials

- [ ] 1 µg (or 100–200 fmol) amplicon DNA for every sample to be barcoded
- [ ] Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples
- [ ] 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
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

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.

Prepare the DNA in Nuclease-free water.

- [ ] Transfer 100–200 fmol amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube for R9.4.1 flow cells
- [ ] Adjust the volume to 48 µl with Nuclease-free water
- [ ] Mix thoroughly by flicking the tube to avoid unwanted shearing
- [ ] Spin down briefly in a microfuge

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 48 µl Amplicon DNA ☐ 3.5 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.	
IMPORTANT ☐ AMPure XP bead clean-up	
☐ Resuspend the AMPure XP beads by vortexing. ☐ Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% 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 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 25 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, for at least 1 minute. ☐ Remove and retain 25 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 repaired and 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Thaw the native barcodes at RT. Use one barcode per sample. Individually mix the barcodes by pipetting, spin down, and place them on ice. ☐ Select a unique barcode for every sample to be run together on the same flow cell, from the provided 24 barcodes. Up to 24 samples can be barcoded and combined in one experiment. ☐ Dilute 100–200 fmol of each end-prepped sample to be barcoded to 22.5 µl in Nuclease-free water. Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 22.5 µl 100–200 fmol end-prepped DNA ☐ 2.5 µl Native Barcode ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix well by pipetting using wide-bore pipette tips. Alternatively, if you are concerned about preserving the integrity of very long DNA fragments, mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 50 µl of resuspended AMPure XP beads to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% 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 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 26 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 26 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.	
☐ Pool equimolar amounts of each barcoded sample into a 1.5 ml Eppendorf DNA LoBind tube, ensuring that sufficient sample is combined to produce a pooled sample of 100–200 fmol total. ☐ Quantify 1 µl of pooled and barcoded DNA using a Qubit fluorometer. ☐ Dilute 100–200 fmol pooled sample to 65 µl in Nuclease-free water.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
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. Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition. ☐ 65 µl 100–200 fmol for R9.4.1 pooled barcoded sample ☐ 5 µl Adapter Mix II (AMII) ☐ 20 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 10 µl Quick T4 DNA Ligase ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 50 µl of resuspended AMPure XP beads to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at RT. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments. ☐ 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 adapter ligated DNA using a Qubit fluorometer - recovery aim 50–100 fmol.	
IMPORTANT ☐ We recommend loading 5-50 fmol of the 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.	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	