Ligation sequencing amplicons - dual barcoding (SQK-LSK109 with EXP-NBD104, EXP-NBD114, and EXP-PBC096)

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Before start checklist

Materials

- [ ] 100–200 fmol of each DNA sample to be barcoded in 45 µl
- [ ] PCR Barcoding Expansion 1-96 (EXP-PBC096)
- [ ] 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™ cat # 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
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack suitable for 96-well PCR plates, e.g. DynaMag™-96 Side Skirted Magnet (Thermo Fisher, cat # 12027)
- [ ] Magnetic rack
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips Multichannel, P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	

Prepare the DNA in Nuclease-free water.

- [ ] Transfer 100–200 fmol DNA for each sample to be barcoded into a separate 1.5 ml Eppendorf DNA LoBind tube
- [ ] Adjust the volume to 45 µl with Nuclease-free water
- [ ] Mix thoroughly by flicking the tube to avoid unwanted shearing
- [ ] Spin down briefly in a microfuge

In a 0.2 ml 96 well PCR plate, set up the end-repair / dA-tailing reactions as follows:

- [ ] 45 µl DNA sample
- [ ] 7 µl Ultra II End-prep reaction buffer
- [ ] 3 µl Ultra II End-prep enzyme mix
- [ ] 5 µl Nuclease-free water

- [ ] Mix by pipetting.

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Seal the plate with adhesive film or PCR strip caps, spin down in a centrifuge and incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep the tube on the magnet and wash the beads with 180 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim 70–140 fmol.	
Take forward approximately 70–140 fmol of end-prepped DNA in 30 µl Nuclease-free water into adapter ligation.	
Ligation of Barcode Adapter	
Add the reagents to a fresh 96-well plate, in the order given below: ☐ 30 µl End-prepped DNA ☐ 20 µl Barcode Adapter ☐ 50 µl Blunt/TA Ligase Master Mix ☐ Mix by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to each sample and mix by pipetting up and down ten times.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep the tube on the magnet and wash the beads with 180 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 25 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer. ☐ Dilute the library to 20–30 fmol with Nuclease-free water or 10 mM Tris-HCl pH 8.5.	
Barcoding PCR	
Set up a barcoding PCR reaction as follows for each library: ☐ 1 µl PCR Barcode (one of BC1-BC96, at 10 µM) ☐ 2 µl Adapter-ligated DNA ☐ 25 µl LongAmp Taq 2x master mix ☐ 22 µl Nuclease-free water The amount of input DNA may need to be adjusted depending on application. For example, for sequencing human or larger genomes, we recommend putting ~50 ng DNA into a PCR reaction. For amplicons or smaller genomes, the 20 ng stated above is sufficient. If the amount of input material is altered, the number of PCR cycles may need to be adjusted to produce the same yield. ☐ Mix by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (15-18 (b) cycles) ☐ Annealing 15 secs (a) @ 62 °C (a) (15-18 (b) cycles) ☐ Extension dependent on length of target fragment (d) @ 65 °C (c) (15-18 (b) cycles) ☐ Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle) ☐ Hold @ 4 °C	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 35 µl of of resuspended AMPure XP beads to each sample and mix by pipetting the entire combined volume up and down 10 times. ☐ Incubate for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Pellet the beads on a magnet for at least 2 min, or until the supernatant is clear. Keep the plate on the magnet and pipette off the supernatant. ☐ Wash each pellet of beads by adding 200 µl of freshly-prepared 70% ethanol. Resuspend each pellet thoroughly by pipetting the entire volume of buffer up and down ten times. Return the plate to the magnetic rack and allow the beads to pellet until the supernatant is clear. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Seal the plate. Spin down and place the plate back on the magnet. Pipette off any residual supernatant. ☐ Remove the plate from the magnetic rack and resuspend each pellet in 21 µl Nuclease-free water, pipetting the entire volume up and down 10 times. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 21 µl of each eluate into a well of a clean 96-well plate. ☐ Quantify 1 µl of each barcoded DNA sample using a Qubit fluorometer. ☐ Using the DNA mass (calculated using the Qubit fluorometer) and size distribution (calculated using a gel or Agilent Bioanalyzer), pool equimolar quantities of barcoded amplicons in batches of 96, ensuring that every sample within a given pool has a unique barcode.	
IMPORTANT ☐ Note that after the subsequent end-prep step, you will need 100–200 fmol of DNA for each pool of samples to take into the native barcode ligation step.	
End-prep	
Mix the following reagents in a separate 0.2 ml thin-walled PCR tube for each pool of samples: ☐ 20 µl Pool of barcoded DNA samples ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 30 µl Nuclease-free water ☐ Mix by pipetting. ☐ Using a thermal cycler, incubate at 20° C for 15 minutes and 65° C for 5 mins. ☐ Transfer each sample to a separate 1.5 ml Eppendorf DNA LoBind tube.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Keep the tube on the magnet and wash the beads with 180 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ 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 23.5 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 23.5 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.	
Take forward 100–200 fmol of end-prepped DNA in 22.5 µl into native barcode ligation.	
Native barcode ligation	
☐ 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. Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 22.5 µl End-prepped DNA ☐ 2.5 µl Native Barcode ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix well by pipetting and spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads to the reaction and mix by gently 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 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 pellet in 21 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 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Analyse 1 µl of sample using the Agilent Bioanalyzer. Determine the average amplicon size from this data, and use this to calculate the input sample volume for the next step. ☐ Pool equimolar amounts of each barcoded sample into a 1.5 ml Eppendord DNA LoBind tube, ensuring that sufficient sample is combined to produce a pooled sample of 0.2 pmol 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.	
Adapter ligation and clean-up	
Adapter Mix II Expansion use 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 the Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at RT, mix by vortexing, spin down and place on ice. Check the contents of each tube are clear of any precipitate. ☐ Spin down the T4 Ligase and the Adapter Mix II (AMII), and place on ice. ☐ To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. ☐ To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 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. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	