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

Before start checklist

Materials

- [ ] Input RNA
- [ ] 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)

Consumables

- [ ] Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- [ ] Random Primer Mix (NEB, S1330S)
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] SuperScript IV reverse transcriptase, 5X RT buffer and 100 mM DTT (ThermoFisher Scientific, 18090010)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, 10777019)
- [ ] COVID-19 primers (lab-ready at 100 µM, IDT)
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] Agencourt AMPure XP beads
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- [ ] NEBNext Ultra II End repair / dA-tailing Module (E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (M0367)
- [ ] NEBNext Quick Ligation Module (E6056)
- [ ] DNA 12000 Kit & Reagents - optional (Agilent Technologies)
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic separator, 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

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INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription	
IMPORTANT ☐ Keep the RNA sample on ice as much as possible to prevent nucleolytic degradation, which may affect sensitivity.	
In a clean pre-PCR hood, mix together the following components in a 0.2 ml PCR tube on ice or in a PCR cool rack such as the Eppendorf PCR-Cooler: ☐ 1 µl 60 µM random hexamers and anchored polyT(23) ☐ 1 µl 10 mM dNTPs ☐ 11 µl RNA sample ☐ Mix gently by flicking the tube, and spin down. Return the tube to ice. ☐ Preheat the thermal cycler to 65°C, with a heated lid at 105°C. ☐ Incubate the reaction in the thermal cycler at 65°C for 5 mins. ☐ Immediately snap-cool the sample by placing on ice for >1 min. More consistent cooling may be achieved using a PCR tube cool block such as the Eppendorf PCR-Cooler. In a clean 1.5 ml Eppendorf DNA LoBind tube, mix together the following reagents in a clean pre-PCR hood: ☐ 4 µl 5X SuperScript IV buffer ☐ 1 µl 100 mM DTT ☐ 1 µl RNaseOUT RNase Inhibitor ☐ 1 µl Superscript IV Reverse Transcriptase ☐ After the RNA sample has cooled for >1 min, add 7 µl of the above master mix to the sample in the pre-PCR hood. ☐ Mix gently by flicking the tube, and spin down. Return the tube to ice. ☐ Preheat the thermal cycler to 42°C, with a heated lid at 105°C. Incubate the sample in the thermal cycler using the following program: ☐ 50 min 42°C ☐ 10 min 70°C ☐ Hold 4°C	
While the reverse transcription reaction is running, prepare the primer pools as described in the next section.	
PCR and clean-up	
IMPORTANT ☐ We recommend ordering the required primers from IDT in a lab-ready format at 100 µM. However, if primers have been ordered lyophilised, they should be resuspended in water or low-EDTA TE buffer to a final concentration of 100 µM.	
IMPORTANT ☐ We recommend handling the primer stocks and derivatives in a clean pre-PCR hood.	

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☐ Add 5 µl of each primer from pool A to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool. ☐ Add 5 µl of each primer from pool B to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool. ☐ Dilute each 100 µM stock 1 in 10 with Nuclease-free water to form a working stock of each pool at 10 µM. In a clean pre-PCR hood, set up two individual reactions using primer pool A and primer pool B in clean 0.2 ml PCR tubes: ☐ Reverse-transcribed sample from above 2.5 µl 2.5 µl ☐ Q5® Hot Start High-Fidelity 2X Master Mix 12.5 µl 12.5 µl ☐ Primer pool at 10 µM (A or B) 3.7 µl 3.7 µl ☐ Nuclease-free water 6.3 µl 6.3 µl	
IMPORTANT ☐ Carry forward the negative control from the reverse transcription reaction to monitor cross-contamination events.	
☐ Mix gently by flicking the tubes, and spin down. Incubate using the following program, with the heated lid set to 105°C: ☐ Initial denaturation 30 sec @ 98°C (1 cycle) ☐ Denaturation 15 sec @ 98°C (25–35 cycles) ☐ Annealing and extension 5 min @ 65°C (25–25 cycles) ☐ Hold @ 4°C	
IMPORTANT ☐ If available, a clean post-PCR hood should be used for all steps that involve handling amplified material. Decontamination with UV and or DNAzap between sample batches is recommended.	
☐ Combine the 25 µl reaction from pool A and the 25 µl reaction from pool B into a new 1.5 ml Eppendorf DNA LoBind tube; one tube per sample. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 50 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. ☐ 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 mins. 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 80% ethanol without disturbing the pellet. Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step.	

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☐ Spin down and place the tubes 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 30 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 30 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of eluted sample using a Qubit fluorometer. ☐ Store any unused amplified material at -20°C for use in later experiments. Expected results	
End-prep	
IMPORTANT ☐ For optimal efficiency of the end-prep reaction, use ~0.2 pmol of cDNA from the previous step.	
IMPORTANT ☐ We recommended carrying the RT negative control through this step until sequencing.	
☐ Determine the volume of the cleaned-up PCR reaction that yields 0.2 pmol of DNA. ☐ Prepare the NEBNext Ultra II End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice. In a 0.2 ml thin-walled PCR tube, mix in the following order: ☐ x µl cDNA ☐ 50-x µl Nuclease-free water ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Mix gently by flicking the tube, and spin down. ☐ Using a thermal cycler, incubate at 20°C for 10 minutes and 65°C for 10 mins, with a heated lid set to 105°C. ☐ 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 10 minutes at RT. ☐ Prepare 300 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant.	

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☐ Keep the tube on the magnet and wash the beads with 100 µl of freshly-prepared 80% ethanol without disturbing the pellet. Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a 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 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 eluted sample using a Qubit fluorometer.	
Take forward the end-prepped DNA into the native barcode ligation step.	
Native barcode ligation	
IMPORTANT ☐ To monitor cross-contamination events, we recommend that the RT negative control is carried through this process and a barcode is used to sequence this control. ☐ Thaw the Native Barcodes at RT, enough for one barcode per sample. Individually mix the barcodes by pipetting, 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 contents thoroughly by pipetting and spin down briefly. ☐ 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 10 minutes at RT. ☐ Prepare 300 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant.	

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☐ Keep the tube on the magnet and wash the beads with 100 µl of freshly-prepared 80% ethanol without disturbing the pellet. Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a 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 15 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 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ 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 to a final quantity of 200 fmol, and make the total volume up to 65 µl with Nuclease-free water.	
Adapter ligation and clean-up	
☐ Thaw Elution Buffer (EB), Short Fragment Buffer (SFB) and NEBNext Quick Ligation Reaction Buffer (5x) at RT, mix by vortexing, spin down and place on ice. Check the contents or each tube are clear of any precipitate. ☐ Spin down the T4 Ligase and the Adapter Mix II (AMII), 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 Pooled barcoded sample ☐ 5 µl Adapter Mix II (AMII) ☐ 20 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 10 µl Quick T4 DNA Ligase ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT. IMPORTANT ☐ The next clean-up step uses SFB (Short Fragment Buffer) and not 80% ethanol to wash the beads. The use of ethanol will significantly damage the sequencing reaction. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 80 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.	

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☐ Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant. ☐ Wash the beads by adding either 140 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, 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 pellet in 14 µl Elution Buffer (EB). ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 14 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ Quantify 1 µl of eluted sample using a Qubit fluorometer.	
IMPORTANT ☐ We recommend loading ~50 fmol (100 fmol for R10.3 flow cells) of this final prepared library onto the flow cell. Loading more than 50 fmol can have a detrimental effect on throughput. Dilute the library in EB if required.	
The prepared library is used for loading into the MinION Mk 1B flow cell. Store the library on ice until ready to load.	
Priming and loading the SpotON flow cell	
IMPORTANT ☐ Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before placing the tubes on ice as soon as thawing is complete. ☐ Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down then return to ice. ☐ Open the MinION Mk 1B lid and slide the flow cell under the clip. ☐ Slide the priming port cover clockwise to open the priming port. How to prime and load the SpotON Flow Cell	
IMPORTANT ☐ Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µls, and make sure that the array of pores is covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.	

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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 µls): ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip ☐ 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. ☐ 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. ☐ 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 via the 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 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. ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk 1B lid.	
Ending the experiment	
☐ 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 ☐ Follow the returns procedure by washing 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.	