# Direct cDNA sequencing - native barcoding (SQK-DCS109 with EXP-NBD104 and EXP-NBD114)

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

## Before start checklist

### Materials

- [ ] 100 ng PolyA+ RNA, or 70-200 ng already-prepared cDNA
- [ ] Direct cDNA Sequencing Kit (SQK-DCS109)
- [ ] Flow Cell Priming Kit (EXP-FLP002)
- [ ] Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples
- [ ] Adapter Mix II Expansion (EXP-AMII001)

### Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] NEBNext End repair / dA-tailing Module (E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (M0367)
- [ ] 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
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- [ ] RiboShredder (Epicentre, cat # RS12500), or RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286)

### 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
- [ ] Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf cat # 022510509)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

| INSTRUCTIONS | NOTES/OBSERVATIONS |
|---|---|
| **Reverse transcription and strand-switching** | |
| IMPORTANT<br>☐ If you have already prepared your cDNA, use 70-200 ng cDNA and start from the End-prep step. | |

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| Prepare the RNA in Nuclease-free water<br>☐ Transfer 100 ng PolyA+ RNA into a 1.5 ml Eppendorf DNA LoBind tube<br>☐ Adjust the volume to up to 7.5 µl with Nuclease-free water<br>☐ Mix by flicking the tube to avoid unwanted shearing<br>☐ Spin down briefly in a microfuge<br><br>Prepare the following reaction in a 0.2 ml PCR tube:<br>☐ x µl poly A+ RNA, 100 ng<br>☐ 2.5 µl VNP<br>☐ 1 µl 10 mM dNTPs<br>☐ 7.5-x µl RNase-free water<br><br>☐ Mix gently by flicking the tube, and spin down.<br><br>☐ Incubate at 65°C for 5 minutes and then snap cool on a pre-chilled freezer block for 1 minute.<br><br>In a separate tube, mix together the following:<br>☐ 4 µl 5x RT Buffer<br>☐ 1 µl RNaseOUT<br>☐ 1 µl Nuclease-free water<br>☐ 2 µl Strand-Switching Primer (SSP)<br><br>☐ Mix gently by flicking the tube, and spin down.<br><br>☐ Add the strand-switching buffer to the snap-cooled, annealed mRNA, mix by flicking the tube and spin down.<br><br>☐ Incubate at 42°C for 2 minutes in the thermal cycler.<br><br>☐ Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl.<br><br>☐ Mix gently by flicking the tube, and spin down.<br><br>Incubate using the following protocol using a thermal cycler:<br>☐ Reverse transcription and strand-switching 90 mins @ 42°C (1 cycle)<br>☐ Heat inactivation 5 mins @ 85°C (1 cycle)<br>☐ Hold @ 4°C | |
| **RNA degradation and second strand synthesis** | |
| ☐ Add 1 µl RiboShredder or RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286) to the reverse transcription reaction.<br><br>☐ Incubate the reaction for 10 minutes at 37° C in a thermal cycler.<br><br>☐ Resuspend the AMPure XP beads by vortexing.<br><br>☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.<br><br>☐ Add 17 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. | |

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| ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.<br><br>☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.<br><br>☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.<br><br>☐ 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.<br><br>☐ Repeat the previous step.<br><br>☐ 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.<br><br>☐ Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water.<br><br>☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.<br><br>☐ Pellet the beads on the magnet until the eluate is clear and colourless.<br><br>☐ Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.<br><br>Prepare the following reaction in a 0.2 ml thin-walled PCR tube:<br>☐ 25 µl 2x LongAmp Taq Master Mix<br>☐ 2 µl PR2 Primer, 10 µM<br>☐ 20 µl Reverse-transcribed sample from above<br>☐ 3 µl Nuclease-free water<br><br>Incubate using the following protocol:<br>☐ 94 °C<br>- Time: 1 mins<br>- No. of cycles: 1<br>☐ 50 °C<br>- Time: 1 mins<br>- No. of cycles: 1<br>☐ 65 °C<br>- Time: 15 mins<br>- No. of cycles: 1<br>☐ 4 °C<br>- Time: ∞<br><br>☐ Resuspend the AMPure XP beads by vortexing.<br><br>☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.<br><br>☐ Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.<br><br>☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.<br><br>☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. | |

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| ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.<br>☐ 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.<br>☐ Repeat the previous step.<br>☐ 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.<br>☐ Remove the tube from the magnetic rack and resuspend pellet in 21 µl Nuclease-free water.<br>☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.<br>☐ Pellet the beads on the magnet until the eluate is clear and colourless.<br>☐ Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.<br>☐ Analyse 1 µl of the strand-switched DNA for size, quantity and quality. | |
| **End-prep** | |
| IMPORTANT<br>☐ If you have prepared your own cDNA instead of performing reverse transcription using the Direct cDNA Sequencing Kit, please start this step with 70-200 ng cDNA in 20 µl Nuclease-free water. | |
| Perform end repair and dA-tailing of the cDNA sample as follows:<br>☐ 20 µl cDNA sample<br>☐ 30 µl Nuclease-free water<br>☐ 7 µl Ultra II End-prep reaction buffer<br>☐ 3 µl Ultra II End-prep enzyme mix<br>☐ Mix gently by pipetting and spin down.<br>☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.<br>☐ Resuspend the AMPure XP beads by vortexing.<br>☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.<br>☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting.<br>☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.<br>☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.<br>☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.<br>☐ 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. | |

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| ☐ Repeat the previous step.<br>☐ 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.<br>☐ Remove the tube from the magnetic rack and resuspend pellet in 22.5 µl Nuclease-free water. Incubate for 2 minutes at RT.<br>☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.<br>☐ Remove and retain 22.5 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. | |
| Take forward 22.5 µl of end-prepped cDNA into barcode ligation. | |
| **Barcode ligation** | |
| Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:<br>☐ 22.5 µl End-prepped DNA<br>☐ 2.5 µl Native Barcode<br>☐ 25 µl Blunt/TA Ligase Master Mix<br>☐ Mix gently by flicking the tube, and spin down.<br>☐ Incubate the reaction for 10 minutes at RT.<br>☐ Resuspend the AMPure XP beads by vortexing.<br>☐ Add 50 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.<br>☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.<br>☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.<br>☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.<br>☐ 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.<br>☐ Repeat the previous step.<br>☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.<br>☐ Remove the tube from the magnetic rack and resuspend the pellet in 26 µl Nuclease-free water. Incubate for 2 minutes at RT.<br>☐ Pellet the beads on the magnet until the eluate is clear and colourless.<br>☐ Remove and retain 26 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. | |
| Quantify 1 µl of eluted sample using a Qubit fluorometer. | |

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| ☐ Pool the barcoded samples at the desired ratio to a final volume of 65 µl in a 1.5 ml Eppendorf DNA LoBind tube. Aim for as high a concentration as possible which does not exceed 200 fmol total. If the total volume is >65 µl, perform a 2.5x AMPure clean up and elute in 65 µl of nuclease free water. | |
| **Adapter ligation** | |
| Adapter Mix II Expansion use<br><br>☐ Thaw the Wash Buffer (WSB), Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at RT and mix by vortexing. Then spin down and place on ice. Check the contents or each tube are clear of any precipitate.<br><br>☐ Spin down the T4 Ligase and the Adapter Mix II (AMII), and place on ice.<br><br>Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.<br>☐ Check that there is no precipitate present (DTT in the Blunt/TA Master Mix, if used, can sometimes form a precipitate)<br>☐ Spin down briefly before accurately pipetting the contents into the reaction<br><br>Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.<br>☐ 65 µl 200 fmol pooled barcoded sample<br>☐ 5 µl Adapter Mix II (AMII)<br>☐ 20 µl NEBNext Quick Ligation Reaction Buffer (5X)<br>☐ 10 µl Quick T4 DNA Ligase<br><br>☐ Mix gently by flicking the tube, and spin down.<br><br>☐ Incubate the reaction for 10 minutes at RT.<br><br>☐ Resuspend the AMPure XP beads by vortexing.<br><br>☐ Add 50 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.<br><br>☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.<br><br>☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant.<br><br>☐ Add 140 µl of the Wash Buffer (WSB) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.<br><br>☐ Repeat the previous step.<br><br>☐ 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.<br><br>☐ Remove the tube from the magnetic rack and resuspend pellet in 13 µl of Elution Buffer (EB).<br><br>☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.<br><br>☐ Pellet the beads on the magnet until the eluate is clear and colourless. | |

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| ☐ Remove and retain 13 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. | |
| Quantify 1 µl of eluted sample using a Qubit fluorometer. | |
| 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 the SQB, FB and FLT at RT.<br>☐ 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.<br>☐ Open the MinION Mk1B lid and slide the flow cell under the clip.<br>☐ Slide the priming port cover clockwise to open the priming port. | |
| IMPORTANT<br>☐ 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:<br>☐ Set a P1000 pipette to 200 µl<br>☐ Insert the tip into the priming port<br>☐ 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<br>Note: Visually check that there is continuous buffer from the priming port across the sensor array.<br>☐ 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.<br>☐ Thoroughly mix the contents of the Loading Beads (LB) by pipetting. | |
| IMPORTANT<br>☐ 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:<br>☐ 37.5 µl Sequencing Buffer (SQB)<br>☐ 25.5 µl Loading Beads (LB), mixed immediately before use<br>☐ 12 µl DNA library | |

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| Complete the flow cell priming:<br>☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible.<br>☐ Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.<br><br>☐ Mix the prepared library gently by pipetting up and down just prior to loading.<br><br>☐ 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.<br><br>☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port. | |
| IMPORTANT<br>☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output. | |
| Place the light shield onto the flow cell, as follows:<br>☐ Carefully place the leading edge of the light shield against the clip.<br>Note: Do not force the light shield underneath the clip.<br>☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell. | |
| Close the device lid and set up a sequencing run on MinKNOW. | |
| **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.<br><br>☐ Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore. | |
| IMPORTANT<br>☐ 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. | |