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)

Consumables

- [ ] Agencourt AMPure XP beads
- [ ] NEBNext End repair / dA-tailing Module (E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (M0367)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # 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, 10777019)
- [ ] RiboShredder (Epicentre, RS12500), or RNase Cocktail Enzyme Mix (ThermoFisher, AM2286)

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription and strand-switching	
IMPORTANT ☐ If you have already prepared your cDNA, use 70-200 ng cDNA and start from the End-prep step.	
Prepare the RNA in Nuclease-free water ☐ Transfer 100 ng PolyA+ RNA into a 1.5 ml Eppendorf DNA LoBind tube ☐ Adjust the volume to up to 7.5 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge	

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

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 20 µl Nuclease-free water. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Pellet beads on magnet until the eluate is clear and colourless. ☐ Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Prepare the following reaction in a 0.2 ml thin-walled PCR tube: ☐ 25 µl 2x LongAmp Taq Master Mix ☐ 2 µl PR2 Primer (PR2) ☐ 20 µl Reverse-transcribed sample from above ☐ 3 µl Nuclease-free water Incubate using the following protocol: ☐ 94 °C 1 mins 1 ☐ 50 °C 1 mins 1 ☐ 65 °C 15 mins 1 ☐ 4 °C ∞ ☐ Resuspend the AMPure XP beads by vortexing. ☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 40 µl of resuspended AMPure XP beads to the 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. 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 pellet in 21 µl Nuclease-free water. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.	

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☐ Pellet beads on 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. ☐ Analyse 1 µl of the strand-switched DNA for size, quantity and quality.	
End-prep	
IMPORTANT ☐ 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 fragmented DNA as follows: ☐ 20 µl cDNA sample ☐ 30 µl Nuclease-free water ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Mix gently by pipetting and spin down. ☐ Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep 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. ☐ 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 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.	
Take forward 30 µl of end-prepped cDNA into adapter ligation.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Adapter ligation	
Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction. ☐ Mix the contents of each tube by flicking ☐ Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate) ☐ Spin down briefly before accurately pipetting the contents in the reaction Taking the end-prepped DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition. ☐ 30 µl End-prepped DNA ☐ 5 µl Adapter Mix ☐ 50 µl Blunt/TA Ligation Master Mix ☐ 15 µl Nuclease-free water ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	
Adapted and tethered DNA library.	
AMPure XP bead binding	
☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Place on magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Add 200 µl of Wash Buffer (WSB) to the beads. Resuspend the beads by pipetting up and down. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. ☐ 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 25 µl of Elution Buffer (EB). ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 25 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of eluted cDNA using a Qubit fluorometer - recovery aim ~60 fmol.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT Please be aware that the flow cell's pore occupancy could be compromised when loading lower amounts of cDNA. Please use the table below as a guide: ☐ Please check the Mass to Molarity table in the protocol	
The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.	
Priming and loading the flow cell	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT. ☐ Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes 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 1 tube of thawed and mixed Flush Buffer (FB), and mix by vortexing. ☐ Load the flow cell(s) into the docking ports within the PromethION. Prime the flow cell using the following steps, taking care to avoid the introduction of air bubbles. ☐ Turn the valve to expose the inlet port (Port 1) ☐ A small tract of air will be visible beyond the inlet port. Draw back a small volume to remove any air bubbles (a few µls): a. Set a P1000 pipette to 200 µl b. Insert the tip into the inlet port c. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip ☐ Using a P1000 pipette, flush 500 µl of the Priming Mix into the inlet port of the flow cell, avoiding the introduction of air bubbles ☐ Wait five minutes. During this time you can prepare your library for loading, as described in the next steps ☐ Repeat the priming step with another 500 µl flush ☐ Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing. In a new tube, prepare the library for loading as follows: ☐ 75 µl SQB ☐ 51 µl LB ☐ 24 µl DNA library Load your sample ☐ Load 150 µl of your sample through the inlet port ☐ Close the valve to seal the inlet port and close the PromethION lid when ready ☐ Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	