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

Before start checklist

Materials

- [ ] 100 ng PolyA+ RNA
- [ ] Direct cDNA Sequencing Kit (SQK-DCS109)
- [ ] PromethION Flow Cell Priming Kit (EXP-FLP001.PRO.6)

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
Preparing input RNA	
Prepare the RNA in Nuclease-free water ☐ Transfer 100 ng PolyA+ RNA into a 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 ☐ Record the quality, quantity and size of the input RNA.	

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IMPORTANT Criteria for input RNA ☐ Average fragment size: ~2 kb ☐ Input mass, as measured by Qubit RNA HS assay: 100 ng ☐ A 260:280 ratio of ~2.0 ☐ A 260:230 ratio of 2.0-2.2 ☐ No detergents or surfactants in the buffer	
Check your flow cell	
☐ Click on the Operation wheel icon on the Desktop to load the GUI. ☐ Load the flow cells to be run into the docking ports of the PromethION.	
IMPORTANT ☐ Wait 10 minutes after loading the flow cells into the PromethION before initiating any experiments or flow cell checks.	
☐ On the home screen of the GUI select the flow cells to be run. ☐ Select the flow cell type from the flow cell drop down. ☐ Click "Check flow cells" at the bottom left of the screen ☐ A screen will appear confirming your selections. Click "Start test" to begin the flow cell check	
When Platform QC completes the message bar will indicate the number of pores found on each flow cell	
Reverse transcription and strand-switching	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf 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. ☐ Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% 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	

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Incubate using the following protocol: ☐ 94 °C 1 mins 1 ☐ 50 °C 1 mins 1 ☐ 65 °C 15 mins 1 ☐ 4 °C ∞ ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf 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 on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% 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. ☐ 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	
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 minutes and 65° C for 5 mins. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.	

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☐ 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 on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% 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.	
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	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 ABB Buffer (ABB) to the beads. Close the tube lid, and resuspend the beads by pipetting. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. ☐ Repeat the previous step. ☐ 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.	
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 into 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 PromethION Flush Buffer (PFB) at RT before placing the tubes on ice as soon as thawing is complete. ☐ Mix the Sequencing Buffer (SQB) and PromethION Flush Buffer (PFB) tubes by vortexing, spin down and return to ice. ☐ Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice. ☐ Prepare the flow cell priming mix: add 46 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed PromethION Flush Buffer (PFB), and mix by pipetting up and down. ☐ Load the flow cell(s) into the docking ports within the PromethION	

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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 ☐ After opening the valve, 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): ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the inlet 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 ☐ Using a P1000 pipette load 500 µl of the Priming Mix flush 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 SQB and LB tubes by pipetting. Prepare the library for loading in a single 1.5 ml Eppendorf DNA LoBind 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 ☐ If no further flow cells are to be loaded, close the PromethION lid ☐ Wait 10 minutes after loading the flow cells into the PromethION before initiating any experiments	
Starting a sequencing run	
☐ Click on the "Operation" wheel icon on the Desktop to load the GUI. IMPORTANT ☐ Wait 10 minutes after loading the flow cells into the PromethION before initiating any experiments or flow cell checks. ☐ On the home screen of the GUI select the flow cells to be run. ☐ Select the flow cell type from the flow cell drop down. ☐ Click "New experiment" at the bottom left of the screen ☐ On the New experiment popup screen select the running parameters for your experiment from the individual tabs IMPORTANT ☐ Please do NOT deselect .fast5 in the Output tab. The Dogfish software only passes data to the basecaller after a certain number of files have been written out. Deselecting .fast5 files will result in no basecall data being produced. IMPORTANT ☐ Currently basecalling in MinKNOW (through Dogfish) will ignore the options on the new experiment settings screen	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Once all options have been chosen, click "Start run".	
The home screen will re-appear with your chosen flow cells displaying a progress bar for progression of the sequencing script	
The format and location of your data will depend on the options chosen in the new experiment settings screen:	
Completing a run	
Close down the GUI and power off the PromethION device.	
☐ Close the GUI window using the close x. ☐ To turn off the PromethION select "Shut Down" from the power options at the top right of the screen. ☐ Once the screen goes blank, turn off the sequencing module via the switch at the rear of the device. ☐ Turn off both Sequencing and Compute Power Supply Units with their respective switches on the back of the units.	
Handling flow cells post-experiment	
If not run for the full 64 hours, flow cells can be stored for subsequent flow cell checks and sequencing experiments. If the flow cell is ready for returning to Oxford Nanopore, please follow the returns and packing instructions. Flongle flow cells are single-use consumables. Once sequencing with a biological sample has occured and the experiment has finished, dispose of the flow cell via a biological waste procedure.	