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

Before start checklist

Materials

- [ ] 1 ng PolyA+ RNA (or ~50 ng total RNA)
- [ ] PCR-cDNA Barcoding kit (SQK-PCB109)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] 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)
- [ ] Exonuclease I (NEB, Cat # M0293)

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)
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription and strand-switching	
Prepare the following reaction in a 0.2 ml PCR tube: ☐ x µl 1 ng PolyA+ RNA (or ~50 ng total RNA) ☐ 1 µl VN Primers (VNP), at 2 µM ☐ 1 µl 10 mM dNTPs ☐ 9-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 for 1 minute. 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, at 10 µM)	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 in the thermal cycler. ☐ 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 using a thermal cycler: ☐ Reverse transcription and strand-switching 90 mins @ 42°C (1 cycle) ☐ Heat inactivation 5 mins @ 85°C (1 cycle) ☐ Hold @ 4°C	
Selecting for full-length transcripts by PCR and barcoding samples	
The PCR steps outlined below adds barcodes to each reverse transcribed RNA (cDNA) sample. The Barcode Primers provided in the PCR-cDNA Barcoding kit (SQK-PCB109) are used to barcode/multiplex up to 12 individual samples on a single flow cell.	
IMPORTANT ☐ Each PCR reaction uses 5 µl of reverse transcribed RNA sample (out of a total of 20 µl). Therefore, sufficient material is available to perform up to four PCR reactions per sample. Do NOT, however, use all 20 µl of reverse transcribed RNA (cDNA) in a single PCR reaction.	
IMPORTANT ☐ This kit enables multiplexing of up to 12 samples. The default method allows you to perform one 50 µl PCR reaction per sample. If multiplexing two or three samples, however, two separate PCR reactions per sample should be performed; if running just one sample, four separate PCR reactions should be performed. These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.	
It is recommended that any remaining reverse transcription reaction is retained to allow for further PCR reactions if greater yield is required. For each sample (up to 12), prepare the following reaction at RT: ☐ 5 µl Reverse-transcribed RNA sample ☐ 1.5 µl Barcode Primers (BP01-BP12) ☐ 18.5 µl Nuclease-free water ☐ 25 µl 2x LongAmp Taq Master Mix Amplify using the following cycling conditions: ☐ Initial denaturation 30 secs @ 95° C (1 cycle) ☐ Denaturation 15 secs @ 95° C (11-18* cycles) ☐ Annealing 15 secs @ 62° C (11-18* cycles) ☐ Extension 50 secs per kb @ 65° C (11-18* cycles) ☐ Final extension 6 mins @ 65° C (1 cycle) ☐ Hold @ 4° C	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Add 1 µl Exonuclease I directly to each PCR tube. Mix by pipetting. ☐ Incubate the reaction at 37°C for 15 minutes, followed by 80°C for 15 minutes in the thermal cycler. ☐ Pool any PCR reactions containing the same barcoded sample in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 0.8X equivalents of resuspended AMPure XP beads to the 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 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 12 µl of Elution Buffer (EB). ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Pellet the beads on the magnet until the eluate is clear and colourless. Remove and retain 12 µl of eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Dispose of the pelleted beads ☐ Analyse 1 µl of the amplified cDNA for size, quantity and quality.	
IMPORTANT ☐ Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.	
In a 1.5 ml Eppendorf DNA LoBind tube, pool together a total of 100 fmol of the amplified cDNA barcoded samples to a final volume of 11 µl in Elution Buffer (EB). ☐ Please check the Mass to Molarity table in the protocol	
Adapter addition	
☐ Add 1 µl of Rapid Adapter (RAP) to the amplified cDNA library. ☐ Mix well by pipetting and spin down. ☐ Incubate the reaction for 5 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down briefly.	
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. ☐ 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. ☐ Open the MinION Mk1B lid and slide the flow cell under the clip. ☐ Slide the 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 and close the priming port.	
IMPORTANT ☐ 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: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ 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. ☐ 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.	