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

Before start checklist

Materials

- [ ] 4 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 200 ng total RNA
- [ ] cDNA-PCR Sequencing Kit (SQK-PCS111)
- [ ] Custom-ordered sequence-specific primer

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 Hot Start Taq 2X Master Mix (NEB, M0533S)
- [ ] 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)
- [ ] Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)

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
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] 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	
Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice. ☐ Custom-ordered sequence-specific primer: thaw at RT, briefly spin down, mix well by pipetting ☐ Strand Switching Primer II (SSPII): thaw at RT, briefly spin down, mix well by pipetting ☐ RNaseOUT: not frozen, briefly spin down, mix well by pipetting ☐ 10 mM dNTP solution: thaw at RT, briefly spin down, mix well by pipetting ☐ Maxima H Minus Reverse Transcriptase: not frozen, briefly spin down, mix well by pipetting ☐ Maxima H Minus 5x RT Buffer: thaw at RT, briefly spin down, mix by vortexing	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the RNA in Nuclease-free water. ☐ Transfer 4 ng Poly(A)+ RNA, or 200 ng total RNA into a 1.5 ml Eppendorf DNA LoBind tube ☐ Adjust the volume to up to 9 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge Prepare the following reaction in a 0.2 ml PCR tube: ☐ 9 µl RNA ☐ 1 µl Custom-ordered sequence-specific primer, diluted to 2 µM ☐ 1 µl 10 mM dNTPs ☐ 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. To the same 0.2 ml PCR tube, add the following: ☐ 4 µl Maxima H Minus 5x RT Buffer ☐ 1 µl RNaseOUT ☐ 1 µl Nuclease-free water ☐ 2 µl Strand Switching Primer II (SSPII) ☐ Mix gently 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	
Take your samples forward into the next step. However, at this point it is also possible to store the sample at -20°C overnight.	
Selecting for full-length transcripts by PCR	
IMPORTANT ☐ The 22.5 µl of reverse-transcribed sample is used to make 4x 50 µl PCR reactions which will be pooled at a later stage, with 5 µl of reverse-transcribed sample in each PCR reaction. Do NOT use all 22.5 µl of the reverse transcription reaction in a single PCR reaction.	
☐ Thaw the cDNA Primer (cPRM), Elution Buffer (EB). LongAmp Hot Start Taq 2X Master Mix and Exonuclease I at RT, spin down and pipette mix. Store the reagents on ice. ☐ Spin down the reverse-transcribed RNA sample.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Prepare four fresh 0.2 ml PCR tubes and add 5 µl of reverse-transcribed sample per tube. In each of the 0.2 ml PCR tubes containing the reverse-transcribed sample, prepare the following reaction at RT: ☐ 5 µl Reverse-transcribed sample (from previous step) ☐ 1.5 µl cDNA Primer (cPRM) ☐ 18.5 µl Nuclease-free water ☐ 25 µl 2x LongAmp Hot Start Taq Master Mix ☐ Mix gently by pipetting. Amplify using the following cycling conditions. ☐ Initial denaturation 30 secs @ 95°C (1 cycle) ☐ Denaturation 15 secs @ 95°C (10-18* cycles) ☐ Annealing 15 secs @ 62°C (10-18* cycles) ☐ Extension 60 secs per kb @ 65°C (10-18* cycles) ☐ Final extension 6 mins @ 65°C (1 cycle) ☐ Hold @ 4°C ☐ 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 the four PCR reactions (total 204 µl) in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 160 µl of resuspended AMPure XP beads to the reaction. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 1 ml 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 500 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 12 µl of Elution Buffer (EB). ☐ Incubate at RT for 10 minutes. ☐ Pellet the beads on the magnet until the eluate is clear and colourless.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Remove and retain 12 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the cDNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ For each sample, analyse 1 µl of the amplified cDNA for size, quantity and quality using a Qubit fluorometer and Agilent Bioanalyzer (or equivalent) for a QC check.	
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.	
Take forward 15-25 fmol of amplified cDNA and make the volume up to 11 µl in Elution Buffer (EB). ☐ Please check the Mass to Molarity table in the protocol	
Adapter addition	
IMPORTANT ☐ Rapid Adapter T (RAP T) used in this kit and protocol is not interchangeable with other sequencing adapters.	
☐ Spin down the Rapid Adapter T (RAP T) and place on ice. ☐ Add 1 µl of Rapid Adapter T (RAP T) to the amplified cDNA library. ☐ Mix well by pipetting and spin down. ☐ Incubate the reaction for 5 minutes at RT. ☐ 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	
Using the Loading Solution ☐ Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents 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 the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at RT. ☐ Open the MinION device lid and slide the flow cell under the clip. ☐ Slide the flow cell priming port cover clockwise to open the priming port.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 II (LBII) by pipetting.	
IMPORTANT ☐ The Loading Beads II (LBII) 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 II (SBII) ☐ 25.5 µl Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using ☐ 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. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	