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

Before start checklist

Materials

- [ ] 4 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 200 ng total RNA
- [ ] PCR-cDNA Barcoding Kit (SQK-PCB111.24)

Consumables

- [ ] Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Lambda Exonuclease (NEB, Cat # M0262L)
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- [ ] 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)
- [ ] USER (Uracil-Specific Excision Reagent) Enzyme (NEB, cat # M5505L)
- [ ] Exonuclease I (NEB, Cat # M0293)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] 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)
- [ ] Agilent Bioanalyzer (or equivalent)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS

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INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription and strand-switching	
Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice. ☐ cDNA RT Adapter (CRTA): thaw at RT, briefly spin down, mix well by pipetting ☐ Annealing Buffer (AB): thaw at RT, briefly spin down, mix well by pipetting ☐ Short Fragment Buffer (SFB): thaw at RT, briefly spin down, mix well by pipetting ☐ RT Primer (RTP): 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 ☐ NEBNext® Quick Ligation Reaction Buffer: thaw at RT, briefly spin down, mix by vortexing ☐ T4 DNA Ligase 2M U/ml: not frozen, briefly spin down, mix well by pipetting ☐ RNaseOUT: not frozen, briefly spin down, mix well by pipetting ☐ Lambda Exonuclease: not frozen, briefly spin down, mix well by pipetting ☐ Uracil-Specific Excision Reagent (USER): 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	
IMPORTANT ☐ It is important that the NEBNext Quick Ligation Reaction Buffer is mixed well by vortexing.	
For each sample, 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 up to 10 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge Prepare the following in a 0.2 ml PCR tube per sample: ☐ 10 µl RNA ☐ 1 µl cDNA RT Adapter (CRTA) ☐ 1 µl Annealing Buffer (AB) ☐ Mix gently by flicking the tubes, and spin down. ☐ Incubate the reactions in the thermal cycler at 60°C for 5 mins, then cool for 10 minutes at RT. To each of the 0.2 ml PCR tubes containing you RNA sample(s), add the following: ☐ 12 µl RNA sample (from previous step) ☐ 3.6 µl NEBNext® Quick Ligation Reaction Buffer ☐ 1.4 µl T4 DNA Ligase 2M U/ml ☐ 1 µl RNaseOUT ☐ Ensure the components are thoroughly mixed by flicking the tubes and spin down. ☐ Incubate for 10 minutes at RT.	

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To each of the 0.2 ml PCR tubes, add the following: ☐ 18 µl RNA sample (from previous step) ☐ 1 µl Lambda Exonuclease ☐ 1 µl USER (Uracil-Specific Excision Reagent) ☐ Ensure the components are thoroughly mixed by flicking the tubes and spin down. ☐ Incubate for 15 minutes at 37°C in the thermal cycler. ☐ Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes. ☐ Resuspend the RNase-free XP beads by vortexing. ☐ Add 36 µl of resuspended RNase-free XP beads to each reaction and mix gently by flicking the tubes. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant. ☐ Keep the tubes on the magnet and wash the beads with 100 µl of Short Fragment Buffer (SFB) without disturbing the pellet. Remove the SFB using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tubes back on the magnet. Pipette off any residual buffer. Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of Nuclease-free water. ☐ Incubate at RT for 10 minutes. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample. To each of the 0.2 ml PCR tubes, add the following: ☐ 12 µl Eluted sample (from previous step) ☐ 1 µl RT Primer (RTP) ☐ 1 µl dNTPs (10 mM) ☐ Ensure the components are thoroughly mixed by flicking the tubes and spin down. ☐ Incubate the reaction for 15 minutes at RT. To each of the 0.2 ml PCR tubes, add the following: ☐ 14 µl RT primed sample (from previous step) ☐ 4.5 µl Maxima H Minus 5x RT Buffer ☐ 1 µl RNaseOUT ☐ 2 µl Strand Switching Primer II (SSPII)	

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☐ Mix gently by flicking the tubes, and spin down. ☐ Incubate at 42°C for 2 minutes in the thermal cycler. ☐ Add 1 µl of Maxima H Minus Reverse Transcriptase to each tube. The total volume will be 22.5 µl per tube. ☐ Mix gently by flicking the tubes, 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 ☐ This kit enables multiplexing of up to 24 samples. The default method allows you to perform one 25 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 as per the cDNA-PCR Sequencing Kit protocol (SQK-PCS111). These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.	
Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice. ☐ Barcode Primers (BP01 - BP24): thaw at RT, briefly spin down, mix well by pipetting ☐ Elution Buffer (EB): thaw at RT, briefly spin down, mix well by pipetting ☐ LongAmp Hot Start Taq 2X Master Mix: thaw at RT, briefly spin down, mix well by pipetting ☐ Exonuclease I: not frozen, briefly spin down, mix well by pipetting ☐ Spin down the reverse-transcribed RNA samples. ☐ Prepare a separate 0.2 ml PCR tube for each sample and add 5 µl of reverse-transcribed RNA per tube.	
IMPORTANT ☐ Only 5 µl of the reverse-transcribed sample is to be taken forward. Do NOT use all the 22.5 µl of the reverse transcription reaction in a single PCR reaction.	
In each of the 0.2 ml PCR tubes containing reverse-transcribed RNA sample, prepare the following reaction at RT: ☐ 5 µl Reverse-transcribed sample (from previous step) ☐ 0.75 µl Unique Barcode Primer (BP01-24) ☐ 6.75 µl Nuclease-free water ☐ 12.5 µl 2x LongAmp Hot Start Taq Master Mix ☐ Mix gently by pipetting.	

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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 reactions at 37°C for 15 minutes, followed by 80°C for 15 minutes in the thermal cycler.

☐ Transfer each sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

☐ Resuspend the AMPure XP beads by vortexing.

☐ Add 20 µl of resuspended AMPure XP beads to each 1.5 ml Eppendorf DNA LoBind tube.

☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

☐ Prepare 5 ml of fresh 70% ethanol in Nuclease-free water.

☐ Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.

☐ Keep the tubes 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 tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking.

☐ Remove the tubes from the magnetic rack and resuspend each 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.

Remove and retain 12 µl of each eluate into a separate 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.

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INSTRUCTIONS	NOTES/OBSERVATIONS
Pool together equimolar samples of the amplified cDNA barcoded samples to a total of 15 – 25 fmols and make the volume up to 23 µl in Elution Buffer (EB). ☐ Please check the Mass to Molarity table in the protocol	
Adapter addition	
IMPORTANT ☐ The 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. ☐ Thaw the RAP Dilution Buffer (RDB) or Adapter Buffer (ADB) at RT, spin down briefly using a microfuge and mix by pipetting before storing on ice. In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute Rapid Adapter T (RAP T): ☐ 1.2 µl Rapid Adapter T (RAP T) ☐ 6.8 µl RAP Dilution Buffer (RDB) or Adapter Buffer (ADB) ☐ Mix well by pipetting and spin down. ☐ Add 1 µl of the diluted Rapid Adapter T (RAP T) to the amplified cDNA library, making the final volume up 24 µl. ☐ Mix well by pipetting and spin down. ☐ Incubate the reaction for 5 minutes at RT.	
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	
Using the Loading Solution ☐ Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and Flush Buffer (FB) at RT before mixing the reagents by vortexing, and spin down the SBII and FLT 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.	
IMPORTANT ☐ After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.	
For PromethION 2 Solo, load the flow cell(s) as follows: ☐ Place the flow cell flat on the metal plate. ☐ Slide the flow cell into the docking port until the gold pins or green board cannot be seen.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
For the PromethION 24/48, load the flow cell(s) into the docking ports: ☐ Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. ☐ Press down firmly onto the flow cell and ensure the latch engages and clicks into place.	
IMPORTANT ☐ Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.	
☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol. ☐ 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: ☐ 75 µl Sequencing Buffer II (SBII) ☐ 51 µl Loading Beads II (LBII) thoroughly mixed before use, or Loading Solution (LS), if using ☐ 24 µl DNA library ☐ Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Using a P1000, insert the pipette tip into the inlet port and add 150 µl of library. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Close the PromethION lid when ready to start 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.	