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

Before start checklist

Materials

- [ ] 300 ng of poly(A) tailed RNA or 1 µg of total RNA in 8 µl
- [ ] Direct RNA Sequencing Kit (SQK-RNA004)

Consumables

- [ ] SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, cat # 18080044)
- [ ] 10 mM dNTP solution (e.g. NEB, cat # N0447)
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- [ ] RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
- [ ] Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] 1.5 ml Eppendorf DNA LoBind 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
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Thermal cycler
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Eppendorf 5424 centrifuge (or equivalent)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Library preparation	
Prepare the NEBNext Quick Ligation Reaction Buffer and T4 DNA Ligase according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. Note: Do NOT vortex the T4 DNA Ligase.	

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INSTRUCTIONS	NOTES/OBSERVATIONS

IMPORTANT

- [ ] We do not recommend using the Quick T4 Ligase for this protocol. We have found that the T4 DNA Ligase (2M U/ml - NEB M0202T/M) works better. It needs to be used in combination with the Quick Ligation Reaction Buffer (NEB B6058).

- [ ] Spin down the RT Adapter (RTA), RNA CS (RCS) (if using), and RNA Ligation Adapter (RLA), pipette mix and place on ice.

- [ ] Thaw the Wash Buffer (WSB) and RNA Elution Buffer (REB) at RT and mix by vortexing. Then spin down and place on ice.

Prepare the RNA in Nuclease-free water:

- [ ] Transfer 300 ng of poly(A) tailed RNA or 1 µg of total RNA into a 0.2 ml thin-walled PCR tube.
- [ ] Adjust the volume to 8 µl with Nuclease-free water.
- [ ] Mix thoroughly by flicking the tube to avoid unwanted shearing.
- [ ] Spin down briefly in a microfuge.

In the same 0.2 ml thin-walled PCR tube, combine the reagents in the following order:

- [ ] 8 µl RNA
- [ ] 3 µl NEBNext Quick Ligation Reaction Buffer
- [ ] 0.5 µl RNA CS (RCS)
- [ ] 1 µl RNaseOUT™
- [ ] 1 µl RT Adapter (RTA)
- [ ] 1.5 µl T4 DNA Ligase

- [ ] Mix by pipetting and spin down.

- [ ] Incubate the reaction for 10 minutes at RT.

In a clean 1.5 ml DNA LoBind Eppendorf tube, combine the following reagents together to make the reverse transcription master mix:

- [ ] 9 µl Nuclease-free water
- [ ] 2 µl 10 mM dNTPs
- [ ] 8 µl 5X First-strand buffer
- [ ] 4 µl DTT

- [ ] Transfer the reverse transcriptase master mix to the 0.2 ml PCR tube containing your adapter-ligated RNA and mix by pipetting.

- [ ] Add 2 µl of SuperScript III Reverse Transcriptase to the reaction and mix by pipetting.

- [ ] Place the tube in a thermal cycler and incubate at 50°C for 50 minutes, then 70°C for 10 minutes, and bring the sample to 4°C before proceeding to the next step.

- [ ] Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

- [ ] Resuspend the stock of Agencourt RNAClean XP beads by vortexing.

- [ ] Add 72 µl of resuspended Agencourt RNAClean XP beads to the reverse transcription reaction and mix by pipetting.

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 200 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 when clear and colourless. Keep the tube on magnet until the supernatant is clear and colourless before washing the beads with 150 µl of freshly prepared 70% ethanol, as described below: ☐ Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and to form a pellet. ☐ Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again. ☐ Carefully remove the 70% ethanol using a pipette and discard. ☐ Spin down and place the tube back on the magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off any residual ethanol. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 23 µl Nuclease-free water. Incubate for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 23 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. In the same 1.5 ml Eppendorf DNA LoBind tube, combine the reagents in the following order: ☐ 23 µl RT-RNA sample ☐ 8 µl NEBNext Quick Ligation Reaction Buffer ☐ 6 µl RNA Ligation Adapter (RLA) ☐ 3 µl T4 DNA Ligase ☐ Mix by pipetting. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the stock of Agencourt RNAClean XP beads by vortexing. ☐ Add 16 µl of resuspended Agencourt RNAClean XP beads to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet for 5 minutes, and pipette off the supernatant when clear and colourless. ☐ Add 150 µl of the Wash Buffer (WSB) to the beads. Close the tube lid and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow the beads to pellet for 5 minutes and pipette off the supernatant when clear and colourless. ☐ Repeat the previous step. ☐ Spin down the tube and replace onto the magnetic rack until the beads have pelleted to pipette off any remaining Wash Buffer (WSB).	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Remove the tube from the magnetic rack and resuspend the pellet in 13 µl RNA Elution Buffer (REB) by the gently flicking the tube. Incubate for 10 minutes at RT. ☐ Pellet the beads on a magnet for 5 minutes until the eluate is clear and colourless. ☐ Remove and retain 13 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay.	
The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.	
IMPORTANT ☐ The RNA library must be sequenced immediately and cannot be stored for later use.	
Priming and loading the SpotON flow cell	
IMPORTANT ☐ Please note, this kit is only compatible with RNA flow cells (FLO-MIN004RA).	
☐ Thaw the Sequencing Buffer (SB), Library Solution (LIS), RNA Flush Tether (RFT) and Flow Cell Flush (FCF) at RT. Mix by vortexing and spin down. To prepare the flow cell priming mix in a clean 1.5 ml Eppendorf DNA LoBind tube, combine the following reagents. Mix by vortexing and spin down at RT. ☐ 30 µl RNA Flush Tether (RFT) ☐ 1,170 µl Flow Cell Flush (FCF) ☐ Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact. ☐ Slide the flow cell 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Solution (LIS) ☐ 12 µl RNA 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.	
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.	