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

Before start checklist

Materials

- [ ] Direct RNA Sequencing Kit (SQK-RNA001)

Consumables

- [ ] 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
- [ ] SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044)
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] Concentrated T4 DNA Ligase 2M U/ml (NEB M0202)
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- [ ] Agencourt RNAClean XP beads
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- [ ] Pipette tips P2, P10, P20, P100, P200, P1000

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Thermal cycler
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Check your flow cell	
- [ ] Set up the MinION, Flow Cell and host computer Once successfully plugged in, you will see a light and hear the fan. Open the MinKNOW GUI from the desktop icon and establish a local or remote connection. - [ ] If running a MinION on the same host computer, plug the MinION into the computer. When the connection name appears under the Local tab, click Connect. - [ ] If running a MinION on a remote computer, first enter the name or IP address of the remote host under Remote and click Connect. - [ ] Plug a MinION and Flow Cell into the remote computer; the connection IDs will be displayed under MinION Connection and Flowcell Connection. Enter the SampleID and FlowcellID being used, and click Submit. - [ ] Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears. - [ ] Click into the Sample ID box and name your sample using free text in alphanumeric format only, deleting any default Sample_ID that is present. Warning: SampleID should not contain any personally identifiable information. - [ ] Click into the FlowcellID box and enter the Flow Cell ID, which is the code found on a sticker on the top side of a Flow Cell.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Select the Platform QC script under Choose Operation, and start the script using the Execute button. ☐ Check the number of active pores available for the experiment, reported in the message panel or in notifications when the check is complete.	
Flow cell check complete.	
Library preparation	
In a 0.2 ml thin-walled PCR tube, mix the reagents in the following order: ☐ 3.0 µl NEBNext Quick Ligation Reaction Buffer ☐ 9.5 µl RNA CS (RCS) ☐ 1.0 µl RT Adapter (RTA) ☐ 1.5 µl T4 DNA Ligase ☐ Mix by pipetting and spin down. ☐ Incubate the reaction for 10 minutes at RT. Mix the following reagents together to make the reverse transcription master mix: ☐ 9.0 µl Nuclease-free water ☐ 2.0 µl 10 mM dNTPs ☐ 8.0 µl 5x first-strand buffer ☐ 4.0 µl 0.1 M DTT ☐ Add the master mix to the 0.2 ml PCR tube containing the RT adapter ligated RNA from the "RT Adapter ligation" step. 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 min, then 70° C for 10 min, and bring the sample to 4° C before proceeding to the next step. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. ☐ Resuspend the stock of Agencourt RNAClean XP beads by vortexing. ☐ Add 72 µl of resuspended RNAClean XP beads to the reverse transcription reaction and mix by pipetting. ☐ 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. Keep the tube on magnet, and wash the beads with 150 µl of freshly prepared 70% ethanol without disturbing the pellet as described below. ☐ Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet. ☐ Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet. ☐ Repeat the previous step.	

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☐ Remove the 70% ethanol using a pipette, and discard. ☐ 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 for 5 minutes at RT. ☐ Pellet beads on magnet until the eluate is clear and colourless. ☐ Pipette 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. In a clean 1.5 ml Eppendorf DNA LoBind tube, mix the reagents in the following order: ☐ 20.0 µl Reverse-transcribed RNA from the "Reverse Transcription" step ☐ 8.0 µl NEBNext Quick Ligation Reaction Buffer ☐ 6.0 µl RNA Adapter (RMX) ☐ 3.0 µl Nuclease-free water ☐ 3.0 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 40 µl of resuspended RNAClean XP beads to the adapter ligation 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, and pipette off the supernatant. ☐ 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 beads to pellet and pipette off the supernatant. ☐ Repeat the previous step. ☐ Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer. Incubate 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.	
Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay - recovery aim ~200 ng.	
The reverse-transcribed and adapted RNA is now ready for loading into the MinION Flow Cell.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Priming and loading the SpotON Flow Cell	
IMPORTANT ☐ Thoroughly mix the contents of the RRB tube by vortexing or pipetting, and spin down briefly.	
☐ Flip back the MinION lid and slide the sample port cover clockwise to that the sample port is visible.	
IMPORTANT ☐ Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks damaging the pores in the array.	
After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls): ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming 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 Prepare the Flow Cell priming mix in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ 600 µl RRB ☐ 600 µl Nuclease-free water ☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.	
IMPORTANT ☐ Thoroughly mix the contents of the RRB tube by vortexing or pipetting, and spin down briefly.	
☐ Take 20 µl of the prepared RNA library and mix it with 17.5 µl of Nuclease-free water. In a new tube, prepare the library for loading as follows: ☐ 37.5 µl RRB ☐ 37.5 µl RNA library in Nuclease-free water 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 via the 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 sample 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, close the priming port and replace the MinION lid.	
Starting a sequencing run	
☐ Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI. ☐ Wait for the MinKNOW GUI to open	

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

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Enter the SampleID and FlowcellID being used, and click Submit. ☐ Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears. ☐ Click into the Sample ID box and name your sample using free text in alphanumeric format only, deleting any default Sample_ID that is present. Warning: SampleID should not contain any personally identifiable information. ☐ Click into the FlowcellID box and enter the Flow Cell ID, which is the code found on a sticker on the top side of a Flow Cell. Select the appropriate protocol script ☐ Experiment type: Choose Control Experiment under "Choose Operation" ☐ Flow Cell product code: Choose the Flow Cell type under "Flow cell product code" ☐ Sequencing kit: Choose SQK-RNA001 under Sequencing Kit ☐ Choose whether or not live basecalling is enabled ☐ The most appropriate script will appear in the drop-down menu. ☐ Start the script using the Execute button at the bottom of the Connections page. Allow the script to run to completion. ☐ The MinKNOW Experiment page will indicate the progression of the script ☐ Monitor messages in the Message panel in the MinKNOW GUI Read file location	
Onward analysis of MinKNOW basecalled data	
☐ Open the Desktop Agent using the desktop shortcut. ☐ Click on the New Workflow tab in the Desktop Agent and select the RNA Calibration Alignment workflow. ☐ Check the correct settings are selected in the Desktop Agent. ☐ Click "Start Run" to start data analysis. ☐ Follow the progression of upload and download of read files in the Desktop Agent. Click on VIEW REPORT. ☐ Click on VIEW REPORT to navigate to the Metrichor website, this can be done at any point during data exchange ☐ Return to the Desktop Agent to see progression of the exchange	
Close down MinKNOW and the Desktop Agent	
☐ Quit Desktop Agent using the close x. ☐ Quit MinKNOW by closing down the web GUI. ☐ Disconnect the MinION.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the flow cell for re-use or return to Oxford Nanopore.	
☐ If you would like to reuse the flow cell, follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR ☐ Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.	