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

Before start checklist

Materials

- [ ] Direct RNA Sequencing Kit (SQK-RNA002)
- [ ] Flow Cell Priming Kit (EXP-FLP001)

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 RNA HS Assay Kit (ThermoFisher Q32852)
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Thermal cycler
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input RNA	
Prepare the RNA in Nuclease-free water. ☐ Transfer 500 ng RNA into a DNA LoBind tube ☐ Adjust the volume to 9 µl with Nuclease-free water ☐ Mix thoroughly by inversion ☐ Spin down briefly in a microfuge ☐ Record the quality, quantity and size of the input RNA.	
IMPORTANT Criteria for input RNA ☐ Average fragment size: >500 bp ☐ Input mass, as measured by Qubit RNA HS assay: 500 ng ☐ No detergents or surfactants in the buffer	

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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. ☐ If running a MinION on a remote computer, first enter the name or IP address of the remote host under Connect to a remote computer (if running from the Connection page), or Connections (if running from the homepage) and click Connect. ☐ Choose the flow cell type from the selector box. Then check the "Available" box. Note: if you are using flow cells from your Starter Pack, please select FLO-MIN106. Click "Check flow cells" at the bottom of the screen. ☐ R9.4.1 FLO-MIN106 ☐ R9.5.1 FLO-MIN107 ☐ Click "Start test". ☐ Check the number of active pores available for the experiment, reported in the System History panel 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 ☐ 9.0 µl RNA ☐ 0.5 µl RNA CS ☐ 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.	

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☐ 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. ☐ Remove the 70% ethanol using a pipette, and discard. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. ☐ Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water. Incubate for 5 minutes at RT. ☐ Pellet the beads on a 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.	

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☐ Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer. Incubate for 10 minutes at RT. ☐ Pellet the beads on a 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 flow cell.	
Priming and loading the SpotON flow cell	
☐ Thaw the RNA Running Buffer (RRB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at RT before placing the tubes on ice as soon as thawing is complete. ☐ Mix the RNA Running Buffer (RRB) and Flush Buffer (FLB) tubes thoroughly by vortexing, spin down and return to ice. ☐ Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice. ☐ Flip back the MinION lid and slide the priming port cover clockwise so that the priming 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: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB), and mix by pipetting up and down. ☐ 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	

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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. ☐ If your MinION was disconnected from the computer, plug it back in. ☐ Choose the flow cell type from the selector box. Then check the "Available" box. ☐ Click the "New Experiment" button at the bottom left of the GUI. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs. ☐ Output settings - FASTQ: The number of basecalls that MinKNOW will write in a single file. By default this is set to 4000 ☐ Output settings - FAST5: The number of files that MinKNOW will write to a single folder. By default this is set to 4000	
IMPORTANT ☐ Please note that, unlike DNA, RNA is translocated through the nanopore in the 3'-5' direction. However, the basecalling algorithms automatically flip the data, and the reads are displayed 5'-3'.	
☐ Click "Start run". Allow the script to run to completion. ☐ The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen ☐ Monitor messages in the Message panel in the MinKNOW GUI Basecalled read files	
Progression of MinKNOW protocol script	
The running experiment screen Experiment summary information	

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Check the number of active pores reported in the MUX scan are similar (within 10-15%) to those reported at the end of the Flow Cell Check ☐ If there is a significant reduction in the numbers, restart MinKNOW. ☐ If the numbers are still significantly different, close down the host computer and reboot. ☐ When the numbers are similar to those reported at the end of the Flow Cell Check, restart the experiment on the Connection page. There is no need to load any additional library after restart. ☐ Stopping the experiment is achieved by clicking "Stop run" button at the top of the screen. Data acquisition will stop, but the software will continue basecalling unless the user clicks the "Stop basecalling" button. ☐ Check that the temperature has reached 34° C. Check pore occupancy in the channel panel at the top of the experimental view. ☐ A good library will be indicated by a higher proportion of light green channels in Sequencing than are in Pore. The combination of Sequencing and Pore indicates the number of active pores at any point in time. A low proportion of Sequencing channels will reduce the throughput of the run. ☐ Recovering indicates channels that may become available for sequencing again. A high proportion of this may indicate additional clean up steps are required during your library preparation. ☐ Inactive indicates channels that are no longer available for sequencing. A high proportion of these as soon as the run begins may indicate an osmotic imbalance. ☐ Unclassified are channels that have not yet been assigned one of the above classifications ☐ Monitor the pore occupancy Duty time plots ☐ Monitor the development of the read length histogram. Cumulative throughput ☐ the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters Trace viewer	
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.	
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.	