

Flow Cell Number:

DNA Samples:

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> Direct RNA Sequencing Kit (SQK-RNA002) <input type="checkbox"/> Flow Cell Priming Kit (EXP-FLP001)	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes <input type="checkbox"/> 0.2 ml thin-walled PCR tubes <input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, cat # AM9937) <input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water <input type="checkbox"/> SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044) <input type="checkbox"/> 10 mM dNTP solution (e.g. NEB N0447) <input type="checkbox"/> Concentrated T4 DNA Ligase 2M U/ml (NEB M0202) <input type="checkbox"/> NEBNext® Quick Ligation Reaction Buffer (NEB B6058) <input type="checkbox"/> Agencourt RNAClean XP beads <input type="checkbox"/> Qubit RNA HS Assay Kit (ThermoFisher Q32852) <input type="checkbox"/> Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)	<input type="checkbox"/> Hula mixer (gentle rotator mixer) <input type="checkbox"/> Magnetic separator, suitable for 1.5 ml Eppendorf tubes <input type="checkbox"/> Microfuge <input type="checkbox"/> Vortex mixer <input type="checkbox"/> Ice bucket with ice <input type="checkbox"/> Timer <input type="checkbox"/> Thermal cycler <input type="checkbox"/> Qubit fluorometer (or equivalent for QC check) <input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
INSTRUCTIONS		NOTES/OBSERVATIONS
<p>Preparing input RNA</p> <p>Prepare the RNA in Nuclease-free water.</p> <input type="checkbox"/> Transfer 500 ng RNA into a DNA LoBind tube <input type="checkbox"/> Adjust the volume to 9 µl with Nuclease-free water <input type="checkbox"/> Mix thoroughly by inversion <input type="checkbox"/> Spin down briefly in a microfuge <input type="checkbox"/> Record the quality, quantity and size of the input RNA.		
<p>IMPORTANT</p> <p>Criteria for input RNA</p> <input type="checkbox"/> Average fragment size: >500 bp <input type="checkbox"/> Input mass, as measured by Qubit RNA HS assay: 500 ng <input type="checkbox"/> No detergents or surfactants in the buffer		

Direct RNA sequencing (SQK-RNA002)

Version: DRS_9080_v2_revA_22Nov2018
 Last update: 28/11/2018



Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Check your flow cell</p> <p><input type="checkbox"/> Set up the MinION, flow cell and host computer</p> <p>Once successfully plugged in, you will see a light and hear the fan.</p> <p>Open the MinkNOW GUI from the desktop icon and establish a local or remote connection.</p> <p><input type="checkbox"/> If running a MinION on the same host computer, plug the MinION into the computer.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Choose the flow cell type from the selector box. Then mark the flow cell as "Selected":</p> <p>Click "Check flow cells" at the bottom of the screen.</p> <p><input type="checkbox"/> R9.4.1 FLO-MIN106</p> <p><input type="checkbox"/> R9.5.1 FLO-MIN107</p> <p><input type="checkbox"/> Click "Start test".</p> <p><input type="checkbox"/> Check the number of active pores available for the experiment, reported in the System History panel when the check is complete.</p>	
<p>Flow cell check complete.</p>	
<p>Library preparation</p> <p>In a 0.2 ml thin-walled PCR tube, mix the reagents in the following order:</p> <p><input type="checkbox"/> 3.0 µl NEBNext Quick Ligation Reaction Buffer</p> <p><input type="checkbox"/> 9.0 µl RNA</p> <p><input type="checkbox"/> 0.5 µl RNA CS</p> <p><input type="checkbox"/> 1.0 µl RT Adapter (RTA)</p> <p><input type="checkbox"/> 1.5 µl T4 DNA Ligase</p> <p><input type="checkbox"/> Mix by pipetting and spin down.</p> <p><input type="checkbox"/> Incubate the reaction for 10 minutes at RT.</p> <p>Mix the following reagents together to make the reverse transcription master mix:</p> <p><input type="checkbox"/> 9.0 µl Nuclease-free water</p> <p><input type="checkbox"/> 2.0 µl 10 mM dNTPs</p> <p><input type="checkbox"/> 8.0 µl 5x first-strand buffer</p> <p><input type="checkbox"/> 4.0 µl 0.1 M DTT</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Add 2 µl of SuperScript III reverse transcriptase to the reaction and mix by pipetting.</p> <p><input type="checkbox"/> 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.</p>	

Direct RNA sequencing (SQK-RNA002)

Version: DRS_9080_v2_revA_22Nov2018
 Last update: 28/11/2018



Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p><input type="checkbox"/> Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.</p> <p><input type="checkbox"/> Resuspend the stock of Agencourt RNAClean XP beads by vortexing.</p> <p><input type="checkbox"/> Add 72 µl of resuspended RNAClean XP beads to the reverse transcription reaction and mix by pipetting.</p> <p><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</p> <p><input type="checkbox"/> Prepare 200 µl of fresh 70% ethanol in Nuclease-free water.</p> <p><input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.</p> <p>Keep the tube on magnet, and wash the beads with 150 µl of freshly prepared 70% ethanol without disturbing the pellet as described below.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet.</p> <p><input type="checkbox"/> Remove the 70% ethanol using a pipette, and discard.</p> <p><input type="checkbox"/> Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water. Incubate for 5 minutes at RT.</p> <p><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless.</p> <p><input type="checkbox"/> Pipette 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p>In a clean 1.5 ml Eppendorf DNA LoBind tube, mix the reagents in the following order:</p> <p><input type="checkbox"/> 20.0 µl Reverse-transcribed RNA from the "Reverse Transcription" step</p> <p><input type="checkbox"/> 8.0 µl NEBNext Quick Ligation Reaction Buffer</p> <p><input type="checkbox"/> 6.0 µl RNA Adapter (RMX)</p> <p><input type="checkbox"/> 3.0 µl Nuclease-free water</p> <p><input type="checkbox"/> 3.0 µl T4 DNA Ligase</p> <p><input type="checkbox"/> Mix by pipetting.</p> <p><input type="checkbox"/> Incubate the reaction for 10 minutes at RT.</p> <p><input type="checkbox"/> Resuspend the stock of Agencourt RNAClean XP beads by vortexing.</p> <p><input type="checkbox"/> Add 40 µl of resuspended RNAClean XP beads to the adapter ligation reaction and mix by pipetting.</p> <p><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</p> <p><input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Repeat the previous step.</p>	

Direct RNA sequencing (SQK-RNA002)

Version: DRS_9080_v2_revA_22Nov2018
 Last update: 28/11/2018



Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<ul style="list-style-type: none"> <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer. Incubate for 10 minutes at RT. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless. <input type="checkbox"/> Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	
<p>Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay - recovery aim ~200 ng.</p>	
<p>The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.</p>	
<p>Priming and loading the SpotON flow cell</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> Mix the RNA Running Buffer (RRB) and Flush Buffer (FLB) tubes thoroughly by vortexing, spin down and return to ice. <input type="checkbox"/> Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice. <input type="checkbox"/> Flip back the MinION lid and slide the sample port cover clockwise to that the sample port is visible. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<p>After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set a P1000 pipette to 200 µl <input type="checkbox"/> Insert the tip into the priming port <input type="checkbox"/> Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip <ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> 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. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Thoroughly mix the contents of the RRB tube by vortexing or pipetting, and spin down briefly. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Take 20 µl of the prepared RNA library and mix it with 17.5 µl of Nuclease-free water. <p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 µl RRB <input type="checkbox"/> 37.5 µl RNA library in Nuclease-free water 	

Direct RNA sequencing (SQK-RNA002)

Version: DRS_9080_v2_revA_22Nov2018
 Last update: 28/11/2018



Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible. <input type="checkbox"/> 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. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> 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. <input type="checkbox"/> Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid. 	
<p>Starting a sequencing run</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> Double-click the MinKNOW icon located on the desktop to open the MinKNOW GUI. <input type="checkbox"/> If your MinION was disconnected from the computer, plug it back in. <input type="checkbox"/> Choose the flow cell type from the selector box. Then mark the flow cell as "Selected". <input type="checkbox"/> Click the "New Experiment" button at the bottom left of the GUI. <p>On the New experiment popup screen, select the running parameters for your experiment from the individual tabs.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Output settings - FASTQ: The number of basecalls that MinKNOW will write in a single file. By default this is set to 4000 <input type="checkbox"/> Output settings - FAST5: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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'. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Click "Start run". <p>Allow the script to run to completion.</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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 <input type="checkbox"/> Monitor messages in the Message panel in the MinKNOW GUI <p>The basecalled read files are stored in :\\data\\reads</p>	
<p>Progression of MinKNOW protocol script</p>	
<p>The running experiment screen</p> <p>Experiment summary information</p>	

Direct RNA sequencing (SQK-RNA002)

Version: DRS_9080_v2_revA_22Nov2018
 Last update: 28/11/2018



Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>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</p> <ul style="list-style-type: none"> <input type="checkbox"/> If there is a significant reduction in the numbers, restart MinKNOW. <input type="checkbox"/> If the numbers are still significantly different, close down the host computer and reboot. <input type="checkbox"/> 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. <input type="checkbox"/> Stopping the experiment is achieved by clicking "Stop run" button at the top of the screen. <p>Data acquisition will stop, but the software will continue basecalling unless the user clicks the "Stop basecalling" button.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Check that the temperature has reached 34° C. <p>Check pore occupancy in the channel panel at the top of the experimental view.</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Unclassified are channels that have not yet been assigned one of the above classifications <ul style="list-style-type: none"> <input type="checkbox"/> Monitor the pore occupancy <p>Duty time plots</p> <ul style="list-style-type: none"> <input type="checkbox"/> Monitor the development of the read length histogram. <p>Cumulative throughput</p> <ul style="list-style-type: none"> <input type="checkbox"/> the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters <p>Trace viewer</p>	
<p>Close down MinKNOW and the Desktop Agent</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> Quit Desktop Agent using the close x. <input type="checkbox"/> Quit MinKNOW by closing down the web GUI. <input type="checkbox"/> Disconnect the MinION. 	
<p>Prepare the flow cell for re-use or return to Oxford Nanopore.</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> 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 <input type="checkbox"/> Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore. 	