

Flow Cell Number:

DNA Samples:

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> 1 µg of total RNA in 8.5 µl	<input type="checkbox"/> SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, cat # 18080044)	<input type="checkbox"/> Hula mixer (gentle rotator mixer)
<input type="checkbox"/> Custom-ordered sequence targeted reverse transcription adapter	<input type="checkbox"/> 10 mM dNTP solution (e.g. NEB, cat # N0447)	<input type="checkbox"/> Magnetic rack, suitable for 1.5 ml Eppendorf tubes
<input type="checkbox"/> Direct RNA Sequencing Kit (SQK-RNA004)	<input type="checkbox"/> NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)	<input type="checkbox"/> Microfuge
	<input type="checkbox"/> T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)	<input type="checkbox"/> Vortex mixer
	<input type="checkbox"/> RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)	<input type="checkbox"/> Ice bucket with ice
	<input type="checkbox"/> Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)	<input type="checkbox"/> Timer
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, AM9937)	<input type="checkbox"/> Thermal cycler
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	<input type="checkbox"/> Qubit fluorometer (or equivalent for QC check)
	<input type="checkbox"/> 0.2 ml thin-walled PCR tubes	<input type="checkbox"/> Eppendorf 5424 centrifuge (or equivalent)
	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)	
	<input type="checkbox"/> Qubit 1x dsDNA BR Assay Kit (ThermoFisher, cat # Q33265)	
	<input type="checkbox"/> Qubit™ Assay Tubes (Invitrogen, Q32856)	
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: <ul style="list-style-type: none"> <input type="checkbox"/> Thaw the reagents at RT. <input type="checkbox"/> Spin down the reagent tubes for 5 seconds. <input type="checkbox"/> Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. Note: Do NOT vortex the T4 DNA Ligase.		

Direct RNA sequencing - sequence-specific (SQK-RNA004)

Version: DSS_9197_v4_revA_20Sep2023
 Last update: 15/11/2023



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INSTRUCTIONS	NOTES/OBSERVATIONS
<p>IMPORTANT</p> <p><input type="checkbox"/> 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).</p> <p><input type="checkbox"/> Spin down the custom-ordered sequence targeted reverse transcription adapter and RNA Ligation Adapter (RLA), pipette mix and place on ice.</p> <p><input type="checkbox"/> Thaw the Wash Buffer (WSB) and RNA Elution Buffer (REB) at RT and mix by vortexing. Then spin down and place on ice.</p> <p>Prepare the RNA in Nuclease-free water:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Transfer 1 µg of total RNA into a 0.2 ml thin-walled PCR tube. <input type="checkbox"/> Adjust the volume to 8.5 µl with Nuclease-free water. <input type="checkbox"/> Mix thoroughly by flicking the tube to avoid unwanted shearing. <input type="checkbox"/> Spin down briefly in a microfuge. <p>In the same 0.2 ml thin-walled PCR tube, combine the reagents in the following order:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 8.5 µl RNA <input type="checkbox"/> 3 µl NEBNext Quick Ligation Reaction Buffer <input type="checkbox"/> 1 µl RNaseOUT™ <input type="checkbox"/> 1 µl Custom-ordered reverse transcription adapter <input type="checkbox"/> 1.5 µl T4 DNA Ligase <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>In a clean 1.5 ml DNA LoBind Eppendorf tube, combine the following reagents together to make the reverse transcription master mix:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 9 µl Nuclease-free water <input type="checkbox"/> 2 µl 10 mM dNTPs <input type="checkbox"/> 8 µl 5X First-strand buffer <input type="checkbox"/> 4 µl DTT <p><input type="checkbox"/> Transfer the reverse transcriptase master mix to the 0.2 ml PCR tube containing your adapter-ligated RNA and 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 minutes, then 70°C for 10 minutes, and bring the sample to 4°C before proceeding to the next step.</p> <p><input type="checkbox"/> Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind 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 Agencourt 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>	

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<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 when clear and colourless.</p> <p>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:</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again.</p> <p><input type="checkbox"/> Carefully remove the 70% ethanol using a pipette and discard.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 23 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"/> Remove and retain 23 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p>In the same 1.5 ml Eppendorf DNA LoBind tube, combine the reagents in the following order:</p> <p><input type="checkbox"/> 23 µl RT-RNA sample</p> <p><input type="checkbox"/> 8 µl NEBNext Quick Ligation Reaction Buffer</p> <p><input type="checkbox"/> 6 µl RNA Ligation Adapter (RLA)</p> <p><input type="checkbox"/> 3 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 16 µl of resuspended Agencourt RNAClean XP beads to the 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 for 5 minutes, and pipette off the supernatant when clear and colourless.</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 the beads to pellet for 5 minutes and pipette off the supernatant when clear and colourless.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><input type="checkbox"/> Spin down the tube and replace onto the magnetic rack until the beads have pelleted to pipette off any remaining Wash Buffer (WSB).</p> <p><input type="checkbox"/> 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.</p>	

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<ul style="list-style-type: none"> <input type="checkbox"/> Pellet the beads on a magnet for 5 minutes until the eluate is clear and colourless. <input type="checkbox"/> Remove and retain 13 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay. 	
<p>The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> The RNA library must be sequenced immediately and cannot be stored for later use. 	
<p>Priming and loading the SpotON flow cell</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Please note, this kit is only compatible with RNA flow cells (FLO-MIN004RA). 	
<ul style="list-style-type: none"> <input type="checkbox"/> 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. <p>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.</p> <ul style="list-style-type: none"> <input type="checkbox"/> 30 µl RNA Flush Tether (RFT) <input type="checkbox"/> 1,170 µl Flow Cell Flush (FCF) <ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> Slide the flow cell priming port cover clockwise to open the priming port. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<p>After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:</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, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip <p>Note: Visually check that there is continuous buffer from the priming port across the sensor array.</p> <ul style="list-style-type: none"> <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 five minutes. During this time, prepare the library for loading by following the steps below. <p>In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 µl Sequencing Buffer (SB) <input type="checkbox"/> 25.5 µl Library Solution (LIS) <input type="checkbox"/> 12 µl RNA library 	

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<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 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 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. <input type="checkbox"/> Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output. 	
<p>Place the light shield onto the flow cell, as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. <input type="checkbox"/> 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. 	
<p>Close the device lid and set up a sequencing run on MinKNOW.</p>	
<p>Flow cell reuse and returns</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	