

Flow Cell Number:

DNA Samples:

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> 100 ng PolyA+ RNA	<input type="checkbox"/> Agencourt AMPure XP beads	<input type="checkbox"/> Hula mixer (gentle rotator mixer)
<input type="checkbox"/> Direct cDNA Sequencing Kit (SQK-DCS109)	<input type="checkbox"/> NEBNext End repair / dA-tailing Module (E7546)	<input type="checkbox"/> Magnetic separator, suitable for 1.5 ml Eppendorf tubes
	<input type="checkbox"/> NEB Blunt/TA Ligase Master Mix (M0367)	<input type="checkbox"/> Microfuge
	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Vortex mixer
	<input type="checkbox"/> 0.2 ml thin-walled PCR tubes	<input type="checkbox"/> Thermal cycler
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, cat # AM9937)	<input type="checkbox"/> Ice bucket with ice
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	<input type="checkbox"/> Timer
	<input type="checkbox"/> 10 mM dNTP solution (e.g. NEB N0447)	<input type="checkbox"/> Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf 022510509)
	<input type="checkbox"/> LongAmp Taq 2X Master Mix (e.g. NEB M0287)	<input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)	
	<input type="checkbox"/> RNaseOUT™, 40 U/µl (Life Technologies, 10777019)	
	<input type="checkbox"/> RiboShredder (Epicentre, RS12500), or RNase Cocktail Enzyme Mix (ThermoFisher, AM2286)	
INSTRUCTIONS		NOTES/OBSERVATIONS
Preparing input RNA		
Prepare the RNA in Nuclease-free water <input type="checkbox"/> Transfer 100 ng PolyA+ RNA into a DNA LoBind tube <input type="checkbox"/> Adjust the volume to up to 7.5 µl with Nuclease-free water <input type="checkbox"/> Mix by flicking the tube to avoid unwanted shearing <input type="checkbox"/> Spin down briefly in a microfuge <input type="checkbox"/> Record the quality, quantity and size of the input RNA.		

Direct cDNA Sequencing (SQK-DCS109)

Version: DCS_9090_v109_revB_04Feb2019
 Last update: 05/02/2019



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INSTRUCTIONS	NOTES/OBSERVATIONS
<p>IMPORTANT</p> <p>Criteria for input RNA</p> <ul style="list-style-type: none"> <input type="checkbox"/> Average fragment size: ~2 kb <input type="checkbox"/> Input mass, as measured by Qubit RNA HS assay: 100 ng <input type="checkbox"/> A 260:280 ratio of ~2.0 <input type="checkbox"/> A 260:230 ratio of 2.0-2.2 <input type="checkbox"/> No detergents or surfactants in the buffer 	
<p>Check your flow cell</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set up the MinION, flow cell and host computer <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> <ul style="list-style-type: none"> <input type="checkbox"/> If running a MinION on the same host computer, plug the MinION into the computer. <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. <input type="checkbox"/> Choose the flow cell type from the selector box. Then mark the flow cell as "Selected". Note: if you are using flow cells from your Starter Pack, please select FLO-MIN106. <p>Click "Check flow cells" at the bottom of the screen.</p> <ul style="list-style-type: none"> <input type="checkbox"/> R9.4.1 FLO-MIN106 <input type="checkbox"/> R9.5.1 FLO-MIN107 <ul style="list-style-type: none"> <input type="checkbox"/> Click "Start test". <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>Flow cell check complete.</p>	
<p>Reverse transcription and strand-switching</p> <p>Prepare the following reaction in a 0.2 ml PCR tube:</p> <ul style="list-style-type: none"> <input type="checkbox"/> x µl poly A+ RNA, 100 ng <input type="checkbox"/> 2.5 µl VNP <input type="checkbox"/> 1 µl 10 mM dNTPs <input type="checkbox"/> 7.5-x µl RNase-free water <ul style="list-style-type: none"> <input type="checkbox"/> Mix gently by flicking the tube, and spin down. <input type="checkbox"/> Incubate at 65° C for 5 minutes and then snap cool on a pre-chilled freezer block. <p>In a separate tube, mix together the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 4 µl 5x RT Buffer <input type="checkbox"/> 1 µl RNaseOUT <input type="checkbox"/> 1 µl Nuclease-free water <input type="checkbox"/> 2 µl Strand-Switching Primer (SSP) 	

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<p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Add the strand-switching buffer to the snap-cooled, annealed mRNA, mix by flicking the tube and spin down.</p> <p><input type="checkbox"/> Incubate at 42° C for 2 minutes.</p> <p><input type="checkbox"/> Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p>Incubate using the following protocol:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Reverse transcription and strand-switching 90 mins @ 42° C (1 cycle) <input type="checkbox"/> Heat inactivation 5 mins @ 85° C (1 cycle) <input type="checkbox"/> Hold @ 4° C 	
<p>RNA degradation and second strand synthesis</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> Add 1 µl RiboShredder or RNase Cocktail Enzyme Mix (ThermoFisher, AM2286) to the reverse transcription reaction. <input type="checkbox"/> Incubate the reaction for 10 minutes at 37° C. <input type="checkbox"/> Prepare the AMPure XP beads for use; resuspend by vortexing. <input type="checkbox"/> Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. <input type="checkbox"/> Add 17 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. <input type="checkbox"/> Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Pellet beads on magnet until the eluate is clear and colourless. <input type="checkbox"/> Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	

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<p>Prepare the following reaction in a 0.2 ml thin-walled PCR tube:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 25 µl 2x LongAmp Taq Master Mix <input type="checkbox"/> 2 µl PR2 Primer (PR2) <input type="checkbox"/> 20 µl Reverse-transcribed sample from above <input type="checkbox"/> 3 µl Nuclease-free water <p>Incubate using the following protocol:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 94 °C 1 mins 1 <input type="checkbox"/> 50 °C 1 mins 1 <input type="checkbox"/> 65 °C 15 mins 1 <input type="checkbox"/> 4 °C ∞ <ul style="list-style-type: none"> <input type="checkbox"/> Prepare the AMPure XP beads for use; resuspend by vortexing. <input type="checkbox"/> Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. <input type="checkbox"/> Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. <input type="checkbox"/> Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 21 µl Nuclease-free water. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Pellet beads on 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. <input type="checkbox"/> Analyse 1 µl of the strand-switched DNA for size, quantity and quality. 	
<p>End-prep</p>	
<p>Perform end repair and dA-tailing of fragmented DNA as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 20 µl cDNA sample <input type="checkbox"/> 30 µl Nuclease-free water <input type="checkbox"/> 7 µl Ultra II End-prep reaction buffer <input type="checkbox"/> 3 µl Ultra II End-prep enzyme mix <ul style="list-style-type: none"> <input type="checkbox"/> Mix gently by pipetting and spin down. 	

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<ul style="list-style-type: none"> <input type="checkbox"/> Using a thermal cycler, incubate at 20° C for 5 minutes and 65° C for 5 mins. <input type="checkbox"/> Prepare the AMPure XP beads for use; resuspend by vortexing. <input type="checkbox"/> Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. <input type="checkbox"/> Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. <input type="checkbox"/> Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 30 µl Nuclease-free water. Incubate for 2 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 30 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	
<p>Take forward 30 µl of end-prepped cDNA into adapter ligation.</p>	
<p>Adapter ligation</p>	
<p>Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Mix the contents of each tube by flicking <input type="checkbox"/> Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate) <input type="checkbox"/> Spin down briefly before accurately pipetting the contents in the reaction <p>Taking the end-prepped DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.</p> <ul style="list-style-type: none"> <input type="checkbox"/> 30 µl End-prepped DNA <input type="checkbox"/> 5 µl Adapter Mix <input type="checkbox"/> 50 µl Blunt/TA Ligation Master Mix <input type="checkbox"/> 15 µl Nuclease-free water <ul style="list-style-type: none"> <input type="checkbox"/> Mix gently by flicking the tube, and spin down. <input type="checkbox"/> Incubate the reaction for 10 minutes at RT. 	
<p>Adapted and tethered DNA library.</p>	

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Version: DCS_9090_v109_revB_04Feb2019
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<p>AMPure XP bead binding</p> <ul style="list-style-type: none"> <input type="checkbox"/> Prepare the AMPure XP beads for use; resuspend by vortexing. <input type="checkbox"/> Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Place on magnetic rack, allow beads to pellet and pipette off supernatant. <input type="checkbox"/> Add 200 µl of ABB Buffer (ABB) to the beads. Close the tube lid, and resuspend the beads by pipetting. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 13 µl of Elution Buffer (EB). <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) 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 13 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Quantify 1 µl of eluted cDNA using a Qubit fluorometer - recovery aim ~60 ng. 	
<p>The prepared library is used for loading into the MinION flow cell. Store the library on ice until ready to load.</p>	
<p>Priming and loading the SpotON flow cell</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of PromethION Flush Buffer (PFB) at RT before placing the tubes on ice as soon as thawing is complete. <input type="checkbox"/> Mix the Sequencing Buffer (SQB) and PromethION Flush Buffer (PFB) tubes 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"/> Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming 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 	

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<ul style="list-style-type: none"> <input type="checkbox"/> Prepare the flow cell priming mix: add 46 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed PromethION Flush Buffer (PFB), 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. <input type="checkbox"/> Thoroughly mix the contents of the Loading Beads (LB) by pipetting. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use. 	
<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 Sequencing Buffer (SQB) <input type="checkbox"/> 25.5 µl Loading Beads (LB), mixed immediately before use <input type="checkbox"/> 12 µl DNA library <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 <input type="checkbox"/> Click "Start run". 	

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<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>Read file location</p>	
<p>Progression of MinKNOW protocol script</p>	
<p>The running experiment screen</p> <p>Experiment summary information</p> <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 style="padding-left: 20px;">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>	

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<p>Further analysis with EPI2ME (optional)</p> <p>OPTIONAL</p> <ul style="list-style-type: none"> <input type="checkbox"/> Open the Desktop Agent using the desktop shortcut. <input type="checkbox"/> Click on the New Workflow tab in the Desktop Agent and select the workflow to be used in the analysis. <input type="checkbox"/> Select the workflow parameters. <input type="checkbox"/> Check the correct settings are selected in the Desktop Agent. <input type="checkbox"/> Click "Start Run" to start data analysis. <input type="checkbox"/> Follow the progression of upload and download of read files in the Desktop Agent. <p>Click on VIEW REPORT.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Click on VIEW REPORT to navigate to the EPI2ME website, this can be done at any point during data exchange <input type="checkbox"/> Return to the Desktop Agent to see progression of the exchange <ul style="list-style-type: none"> <input type="checkbox"/> When the upload and download numbers are the same, the data exchange is complete. The processed reads will be in downloads folder in the selected location on the host computer. 	
<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. 	