

Rapid PCR Barcoding Kit (SQK-RPB004)

Version: RPB_9059_v1_revA_08Mar2018
 Last update: 08/03/2018



Flow Cell Number:

DNA Samples:

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> Rapid PCR Barcoding Kit (SQK-RPB004)	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Microfuge
<input type="checkbox"/> Flow Cell Priming Kit (EXP-FLP001)	<input type="checkbox"/> 0.2 ml thin-walled PCR tubes	<input type="checkbox"/> Timer
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, cat # AM9937)	<input type="checkbox"/> Thermal cycler
	<input type="checkbox"/> Agencourt AMPure XP beads	<input type="checkbox"/> Pipettes P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> LongAmp Taq 2X Master Mix (e.g. NEB M0287)	
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	
	<input type="checkbox"/> 10 mM Tris-HCl pH 8.0 with 50 mM NaCl	
	<input type="checkbox"/> Pipette tips P2, P10, P20, P100, P200, P1000	

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. When the connection name appears under the Local tab, click Connect.</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 Remote and click Connect.</p> <p><input type="checkbox"/> Plug a MinION and Flow Cell into the remote computer; the connection IDs will be displayed under MinION Connection and Flowcell Connection.</p> <p>Enter the SampleID and FlowcellID being used, and click Submit.</p> <p><input type="checkbox"/> Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Select the Platform QC script under Choose Operation, and start the script using the Execute button.</p> <p><input type="checkbox"/> Check the number of active pores available for the experiment, reported in the message panel or in notifications when the check is complete.</p>	
Flow cell check complete.	


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<p>Library preparation</p> <p>Thaw and prepare the reagents as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Barcodes (RLB 01-12) at RT <input type="checkbox"/> Fragmentation Mix (FRM) on ice <input type="checkbox"/> Rapid Adapter (RAP) on ice <p>In a 0.2 ml thin-walled PCR tube, mix the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 3 µl 1-5 ng template DNA <input type="checkbox"/> 1 µl FRM <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> In a thermal cycler, incubate the tube at 30° C for 1 minute and then at 80° C for 1 minute. Briefly put the tube on ice to cool it down.</p> <p>Set up a PCR reaction as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 20 µl Nuclease-free water <input type="checkbox"/> 4 µl Tagmented DNA <input type="checkbox"/> 1 µl RLB (01-12A) <input type="checkbox"/> 25 µl LongAmp Taq 2X master mix <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p>Amplify using the following cycling conditions:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Initial denaturation 3 mins @ 95 °C (1 cycle) <input type="checkbox"/> Denaturation 15 secs @ 95 °C (14 cycles) <input type="checkbox"/> Annealing 15 secs @ 56 °C (14 cycles) <input type="checkbox"/> Extension 6 mins @ 65 °C (14 cycles) <input type="checkbox"/> Final extension 6 mins @ 65 °C (1 cycle) <input type="checkbox"/> Hold @ 4 °C <p><input type="checkbox"/> Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.</p> <p><input type="checkbox"/> Prepare the AMPure XP beads for use; resuspend by vortexing.</p> <p><input type="checkbox"/> Add 30 µl of resuspended AMPure 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"/> Prepare 500 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><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.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><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.</p>	

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<p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. Incubate for 2 minutes at RT.</p> <p><input type="checkbox"/> Pellet beads on magnet until the eluate is clear and colourless.</p> <p>Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube</p> <p><input type="checkbox"/> Dispose of the pelleted beads</p>	
<p>Quantify 1 µl of eluted sample using a Qubit fluorometer.</p>	
<p><input type="checkbox"/> Pool all barcoded libraries in the desired ratios to a total of 50-100 fmoles in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.</p> <p><input type="checkbox"/> Add 1 µl of RAP to the barcoded DNA.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Incubate the reaction for 5 minutes at RT.</p>	
<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><input type="checkbox"/> Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at RT before placing the tubes on ice as soon as thawing is complete.</p> <p><input type="checkbox"/> Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, spin down and return to ice.</p> <p><input type="checkbox"/> Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.</p> <p><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>	
<p>IMPORTANT</p> <p><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>	
<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> <p><input type="checkbox"/> Set a P1000 pipette to 200 µl</p> <p><input type="checkbox"/> Insert the tip into the priming port</p> <p><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</p> <p><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.</p> <p><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> <p><input type="checkbox"/> Thoroughly mix the contents of the SQB and LB tubes by pipetting.</p>	

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<p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 34 µl Sequencing Buffer (SQB) <input type="checkbox"/> 25.5 µl Loading Beads (LB), mixed immediately before use <input type="checkbox"/> 4.5 µl Nuclease-free water <input type="checkbox"/> 11 µl DNA library 	
<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>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"/> Wait for the MinKNOW GUI to open <input type="checkbox"/> Select the local MinION, and click Connect. <p>Enter the SampleID and FlowcellID being used, and click Submit.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <p>Select the appropriate protocol script</p> <ul style="list-style-type: none"> <input type="checkbox"/> Experiment type: Choose Sequencing Run under "Choose Operation" <input type="checkbox"/> Flow Cell product code: Choose the Flow Cell type under "Flow cell product code" <input type="checkbox"/> Sequencing kit: Choose SQK-RPB004 under Sequencing Kit <input type="checkbox"/> Choose whether or not live basecalling is enabled <input type="checkbox"/> The most appropriate script will appear in the drop-down menu. <ul style="list-style-type: none"> <input type="checkbox"/> Start the script using the Execute button at the bottom of the Connections page. <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 <input type="checkbox"/> Monitor messages in the Message panel in the MinKNOW GUI 	

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<p>The basecalled read files are stored in : \data\reads</p>	
<p>Progression of MinKNOW protocol script</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 Platform QC</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 Platform QC, restart the experiment on the Connection page. There is no need to load any additional library after restart. <ul style="list-style-type: none"> <input type="checkbox"/> Check the heatsink temperature is approximately 34° C. <input type="checkbox"/> Monitor the development of the read length histogram. <input type="checkbox"/> Check pore occupancy by looking at the panel at the top of the Status or Physical Layout views. <input type="checkbox"/> Monitor the pore occupancy 	
<p>Onward analysis of MinKNOW basecalled data</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 FASTQ barcoding workflow. <p>Select the workflow parameters.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Select the quality score cut-off (this defaults to 7 unless changed) <input type="checkbox"/> Select "Yes" in answer to "Detect barcode?" <input type="checkbox"/> If you are working with human data, please tick "Yes" in answer to "Is the data you are about to upload a whole or partial human genome?", and confirm that you have consent from the subject to upload the data. <ul style="list-style-type: none"> <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 Metrichor 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 	
<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. 	

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Prepare the flow cell for re-use or return to Oxford Nanopore.	
<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.	