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

Before start checklist

Materials

- [ ] Rapid Barcoding Sequencing Kit (SQK-RBK004)
- [ ] 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)
- [ ] Agencourt AMPure XP beads (optional)
- [ ] Freshly-prepared 70% ethanol in nuclease-free water (optional)
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (optional)

Equipment

- [ ] Ice bucket with ice
- [ ] Microfuge
- [ ] Timer
- [ ] Thermal cycler at 30° C and 80° C
- [ ] Pipettes and pipette tips P2, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
Prepare the DNA in Nuclease-free water. ☐ Transfer ~400 ng genomic DNA into a DNA LoBind tube ☐ Adjust the volume to 7.5 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge ☐ Record the quality, quantity and size of the DNA.	
IMPORTANT Criteria for input DNA ☐ Purity as measured using Nanodrop - OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2 ☐ Average fragment size, as measured by pulse-field, or low percentage agarose gel analysis >30 kb ☐ Input mass, as measured by Qubit - ~400 ng ☐ No detergents or surfactants in the buffer	
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. When the connection name appears under the Local tab, click Connect. ☐ If running a MinION on a remote computer, first enter the name or IP address of the remote host under Remote and click Connect. ☐ Plug a MinION and Flow Cell into the remote computer; the connection IDs will be displayed under MinION Connection and Flowcell Connection.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Enter the SampleID and FlowcellID being used, and click Submit. ☐ Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears. ☐ 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. ☐ 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. ☐ Select the Platform QC script under Choose Operation, and start the script using the Execute button. ☐ Check the number of active pores available for the experiment, reported in the message panel or in notifications when the check is complete.	
Flow cell check complete.	
Library preparation	
Thaw kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below: ☐ Fragmentation Mix RB01-12: not frozen, briefly spin down, mix well by pipetting ☐ Rapid Adapter (RAP): not frozen, briefly spin down, mix well by pipetting ☐ Sequencing Buffer (SQB): thaw at RT, briefly spin down, mix well by pipetting* ☐ Loading Beads (LB): thaw at RT, briefly spin down, mix by pipetting or vortexing immediately before use ☐ Flush Buffer (FLB) - 1 tube: thaw at RT, briefly spin down, mix well by pipetting* ☐ Flush Tether (FLT): thaw at RT, briefly spin down, mix well by pipetting In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 7.5 µl 400 ng template DNA ☐ 2.5 µl Fragmentation Mix RB01-12 (one for each sample) ☐ Mix gently by flicking the tube, and spin down. ☐ 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. ☐ Pool all barcoded samples in your desired ratio, noting the total volume.	RB 01
IMPORTANT ☐ If barcoding four or more samples, increased throughput can be achieved through cleaning up and concentrating the pooled material using AMPure XP beads as outlined in Steps 6-15. Otherwise, for a more rapid sample preparation, transfer 10 µl of pooled sample from Step 5 into a clean 1.5 ml Eppendorf DNA LoBind tube, and proceed directly to Step 16.	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ To the entire pooled barcoded sample from Step 5, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads End of optional steps. ☐ Add 1 µl of RAP to 10 µl barcoded DNA. ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 5 minutes at RT.	
The prepared library is used for loading into the MinION flow cell. Store the library on ice until ready to load.	
Priming and loading the SpotON Flow Cell	
☐ 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. ☐ Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, spin down and return to ice. ☐ Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice. ☐ 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.	
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. ☐ Thoroughly mix the contents of the SQB and LB tubes by pipetting.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a new tube, prepare the library for loading as follows: ☐ 34 µl Sequencing Buffer (SQB) ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☐ 4.5 µl Nuclease-free water ☐ 11 µl DNA library	
IMPORTANT ☐ 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.	
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. ☐ Wait for the MinKNOW GUI to open ☐ Select the local MinION, and click Connect. Enter the SampleID and FlowcellID being used, and click Submit. ☐ Once a MinION and Flow Cell are connected, a Label Experiment dialogue box appears. ☐ 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. ☐ 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. Select the appropriate protocol script. ☐ Experiment type: Choose Sequencing Run under "Choose Operation" ☐ Flow Cell product code: Choose the Flow Cell type under "Flow cell product code" ☐ Sequencing kit: Choose SQK-RBK004 under Sequencing Kit ☐ Choose whether or not live basecalling is enabled ☐ The most appropriate script will appear in the drop-down menu. ☐ Start the script using the Execute button at the bottom of the Connections page. Allow the script to run to completion. ☐ The MinKNOW Experiment page will indicate the progression of the script ☐ Monitor messages in the Message panel in the MinKNOW GUI	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
The basecalled read files are stored in :\data\reads	
Progression of MinKNOW protocol script	
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 ☐ 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 Platform QC, restart the experiment on the Connection page. There is no need to load any additional library after restart. ☐ Check the heatsink temperature is approximately 34° C. ☐ Monitor the development of the read length histogram. ☐ Check pore occupancy by looking at the panel at the top of the Status or Physical Layout views. ☐ Monitor the pore occupancy	
Onward analysis of MinKNOW basecalled data	
☐ Open the Desktop Agent using the desktop shortcut. ☐ Click on the New Workflow tab in the Desktop Agent and select the FASTQ barcoding workflow. Select the workflow parameters. ☐ Select the quality score cut-off (this defaults to 7 unless changed) ☐ Select "Yes" in answer to "Detect barcode?" ☐ 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. ☐ Check the correct settings are selected in the Desktop Agent. ☐ Click "Start Run" to start data analysis. ☐ Follow the progression of upload and download of read files in the Desktop Agent. Click on VIEW REPORT. ☐ Click on VIEW REPORT to navigate to the Metrichor website, this can be done at any point during data exchange ☐ Return to the Desktop Agent to see progression of the exchange	
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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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.	