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

Before start checklist

Materials

- [ ] 16S Barcoding Kit (SQK-RAB204)
- [ ] 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
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl

Equipment

- [ ] Microfuge
- [ ] Timer
- [ ] Thermal cycler
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
Prepare the DNA in Nuclease-free water. ☐ Transfer 10 ng genomic DNA into a DNA LoBind tube ☐ Adjust the volume to 10 µl with Nuclease-free water ☐ Mix thoroughly 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 ☐ Input mass, as measured by Qubit - 10 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.	

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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 the 16S Barcodes at RT, mix by pipetting up and down, and spin down briefly. Keep the barcodes on ice until ready to use. In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 14 µl Nuclease-free water ☐ 10 µl Input DNA (10 ng) ☐ 1 µl 16S Barcode ☐ 25 µl LongAmp Taq 2X master mix ☐ Mix gently by flicking the tube, and spin down. Amplify using the following cycling conditions: ☐ Initial denaturation 1 min @ 95 °C (1 cycle) ☐ Denaturation 20 secs @ 95 °C (25 cycles) ☐ Annealing 30 secs @ 55 °C (25 cycles) ☐ Extension 2 mins @ 65 °C (25 cycles) ☐ Final extension 5 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 30 µl of resuspended AMPure XP beads to the reaction and mix by pipetting. ☐ 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. ☐ 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. ☐ Repeat the previous step.	

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☐ 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. ☐ 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. ☐ Pellet beads on 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	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ 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. ☐ Add 1 µl of RAP to the 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.	
IMPORTANT ☐ Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol.	
☐ 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.	

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☐ 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. 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 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-RAB204 under Sequencing Kit ☐ Choose whether or not live basecalling is enabled ☐ The most appropriate script will appear in the drop-down menu. 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	

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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	
End of the sequencing protocol script.	
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 16S workflow. ☐ 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.	
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	

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☐ Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.	