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

Before start checklist

Materials

- [ ] 10 ng high molecular weight genomic DNA
- [ ] 16S Barcoding Kit 1-24 (SQK-16S024)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

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 Hot Start Taq 2X Master Mix (NEB, M0533S)
- [ ] 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
Library preparation	

- [ ] Take one 96-well plate containing 16S barcodes. Break one set of barcodes (1-24, or as desired) away from the plate and return the rest to storage.

IMPORTANT

- [ ] The 96-well plates are designed to break in one direction only. Strips, or multiple strips, of eight wells/barcodes can be removed from the plate at any one time.

- [ ] Thaw the desired barcodes, make sure the liquid is at the bottom of the tubes, and place on ice.
- [ ] Thaw the LongAmp Hot Start Taq 2X Master Mix, spin down briefly, mix well by pipetting and place on ice.

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

For each sample to be tested, prepare the following mixture in separate 0.2 ml thin-walled PCR tubes.

- [ ] 5 µl Nuclease-free water
- [ ] 10 µl Input DNA (10 ng)
- [ ] 25 µl LongAmp Hot Start Taq 2X Master Mix

- [ ] Mix gently by flicking the tube, and spin down.
- [ ] Using clean pipette tips, carefully pierce the foil surface of the required barcodes. Use a new tip for each barcode to avoid cross-contamination. Make a note of which barcode numbers will be run for each sample.

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☐ Using a multichannel pipette, mix the 16S barcodes by pipetting up and down 10 times. Transfer 10 µl of each 16S Barcode into respective sample-containing tubes. ☐ Mix thoroughly by pipetting up and down ten times. 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 each sample to a separate 1.5 ml DNA LoBind Eppendorf tube. Carry out steps 11-21 for each sample, before pooling the samples at step 22. ☐ Resuspend the AMPure XP beads 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 the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ 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 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	
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. For 16S amplicons of ~1500 bp, 50-100 fmoles equates to ~50-100 ng. ☐ Add 1 µl of RAP to the barcoded DNA. ☐ Mix gently by flicking the tube, and spin down.	

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☐ 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	
IMPORTANT ☐ 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.	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT. ☐ Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by vortexing and spin down at RT. ☐ Open the MinION Mk1B lid and slide the flow cell under the clip. ☐ Slide the priming port cover clockwise to open the priming port.	
IMPORTANT ☐ 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.	
After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl): ☐ 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 ☐ To 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 (FB), and mix by vortexing at RT. ☐ 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. During this time, prepare the library for loading by following the steps below. ☐ Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.	
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.	
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	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 Mk1B lid.	
Ending the experiment	
☐ After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR ☐ Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT ☐ 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.	