Rapid sequencing DNA - 16S Barcoding Kit 24 V14 (SQK-16S114.24)

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

Before start checklist

Materials

- [ ] 10 ng high molecular weight genomic DNA
- [ ] 16S Barcoding Kit 24 V14 (SQK-16S114.24)

Consumables

- [ ] MinION and GridION Flow Cell
- [ ] LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 0.2 ml thin-walled PCR tubes

Equipment

- [ ] MinION or GridION device
- [ ] MinION and GridION Flow Cell Light Shield
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips Multichannel, P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Library preparation	
Check your flow cell.	
☐ 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 at RT.	
☐ Briefly centrifuge barcodes in a microfuge to make sure the liquid is at the bottom of the tubes and place on ice.	

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INSTRUCTIONS	NOTES/OBSERVATIONS

- ☐ 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 of each genomic DNA sample into a 0.2 ml thin-walled PCR tube
- ☐ Adjust the volume to 15 µl with Nuclease-free water
- ☐ Mix thoroughly by flicking avoiding unwanted shearing
- ☐ Spin down briefly in a microfuge

In each 0.2 ml thin-walled PCR tube containing a sample to be tested, prepare the following mixture:

- ☐ 15 µl 10 ng input DNA (from previous step)
- ☐ 25 µl LongAmp Hot Start Taq 2X Master Mix

- ☐ Ensure the components are thoroughly mixed by pipetting and spin down briefly.
- ☐ 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.
- ☐ 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.
- ☐ Ensure the components are thoroughly mixed by pipetting the contents of the tubes 10 times 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

Thaw reagents at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

- ☐ Rapid Adapter (RA) □ @ Not frozen (Pipette cycles)
- ☐ Adapter Buffer (ADB) □ @ □ (Vortex or Pipette cycles)
- ☐ AMPure XP Beads (AXP) □ @ □ (Mix by vortexing immediately before use cycles)
- ☐ Elution Buffer (EB) □ @ □ (Vortex or Pipette cycles)
- ☐ EDTA (EDTA) □ @ □ (Vortex or Pipette cycles)

- ☐ Add 4 µl of EDTA to each barcoded sample, mix thorougly by pipetting and spin down briefly.
- ☐ Incubate for 5 minutes at RT.
- ☐ Quantify 1 µl of each barcoded sample using a Qubit fluorometer (or equivalent) for QC check.
- ☐ Pool all barcoded samples in equimolar ratios in a 1.5 ml Eppendorf DNA LoBind tube.
- ☐ Resuspend the AMPure XP Beads (AXP) by vortexing.

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INSTRUCTIONS	NOTES/OBSERVATIONS
To the pool of barcoded samples, add a 0.6X volume ratio of resuspended AMPure XP Beads (AXP) and mix by pipetting: ☐ Volume of AMPure XP Beads (AXP) - 37.5 µl: 22.5 µl - 75 µl: 45 µl - 150 µl: 90 µl - 300 µl: 180 µl - 600 µl: 360 µl ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 2 ml of fresh 80% ethanol in Nuclease-free water. ☐ Briefly spin down the sample and pellet on a magnetic rack until supernatant is clear and colourless. Keep the tube on the magnetic rack, and pipette off the supernatant. ☐ Keep the tube on the magnet and wash the beads with 1 ml of freshly-prepared 80% 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 the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library 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.	
☐ Transfer 50 fmol of your eluted sample into a clean 1.5 ml Eppendorf DNA LoBind tube. Make up the volume to 11 µl with Elution Buffer (EB). In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix: ☐ 1.5 µl Rapid Adapter (RA) ☐ 3.5 µl Adapter Buffer (ADB) ☐ Add 1 µl of the diluted Rapid Adapter (RA) 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 flow cell. Store the library on ice until ready to load.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Priming and loading the MinION and GridION Flow Cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice.	
IMPORTANT ☐ For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.	
To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at RT: ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml ☐ 30 µl Flow Cell Tether (FCT) ☐ 1,205 µl Final total volume in tube	
☐ Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.	
☐ Slide the flow cell 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: ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip Note: Visually check that there is continuous buffer from the priming port across the sensor array.	
☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.	
☐ Thoroughly mix the contents of the Library Beads (LIB) by pipetting.	
IMPORTANT ☐ The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using ☐ 12 µl DNA library 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 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 the prepared library 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 and close the priming port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
Place the light shield onto the flow cell, as follows: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.	
Close the device lid and set up a sequencing run on MinKNOW.	
Flow cell reuse and returns	
☐ After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C. ☐ Alternatively, follow the returns procedure to flush 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.	