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

Before start checklist

Materials

- [ ] ~400 ng plasmid DNA per sample
- [ ] Rapid Barcoding Sequencing Kit (SQK-RBK004)
- [ ] 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, AM9937)
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)

Equipment

- [ ] Ice bucket with ice
- [ ] Microfuge
- [ ] Timer
- [ ] Thermal cycler or heat blocks
- [ ] Pipettes and pipette tips P2, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
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 Prepare the DNA in Nuclease-free water. ☐ 1000 ng/µl 19 µl @ 1 µl (20 µl cycles) ☐ 800 ng/µl 28 µl @ 2 µl (30 µl cycles) ☐ 600 ng/µl 41 µl @ 4 µl (45 µl cycles) ☐ 200 ng/µl 22 µl @ 8 µl (30 µl cycles) ☐ 100 ng/µl 6 µl @ 7 µl (13 µl cycles) ☐ 90 ng/µl 11 µl @ 16 µl (27 µl cycles) ☐ 70 ng/µl 3 µl @ 10 µl (13 µl cycles) ☐ <53 ng/µl 0 µl @ 7.5 µl (7.5 µl cycles) 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) ☐ Pipette mix and spin down	

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☐ 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 into a single 1.5 ml Eppendorf DNA LoBind tube, noting the total volume.	
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 7-17. 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 18.	
☐ Resuspend the AMPure XP beads by vortexing. ☐ To the entire pooled barcoded sample from Step 6, 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. ☐ 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 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 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, for at least 1 minute. Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Dispose of the pelleted beads ☐ Add 1 µl of RAP to 10 µl of barcoded DNA. ☐ Pipette mix 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.	
Priming and loading the SpotON Flow Cell	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing, and spin down at RT.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Open the MinION device 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: ☐ 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 Loading Beads (LB) tubes by vortexing. 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 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
Ending the experiment	
☐ 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.	