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

Before start checklist

Materials

- [ ] 100 ng high molecular weight genomic DNA
- [ ] PCR Barcoding Kit (SQK-PBK004)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] Agencourt AMPure XP beads
- [ ] NEBNext End repair / dA-tailing Module (E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (M0367)
- [ ] Covaris g-TUBE
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl
- [ ] (optional) Exonuclease I (NEB, M0293)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Thermal cycler
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
DNA fragmentation	

OPTIONAL

Prepare the DNA in Nuclease-free water.

- [ ] Transfer 100 ng genomic DNA into a DNA LoBind tube
- [ ] Adjust the volume to 50 µl with Nuclease-free water
- [ ] Mix thoroughly by inversion avoiding unwanted shearing
- [ ] Spin down briefly in a microfuge

- [ ] Transfer 100 ng genomic DNA in 50 µl to the Covaris g-TUBE.

Spin the g-TUBE for 1 minute at RT (Eppendorf 5424; 6000 rpm for 8 kb fragments).

- [ ] Spin the g-TUBE for 1 minute
- [ ] Remove and check all the DNA has passed through the g-TUBE
- [ ] If DNA remains in the upper chamber, spin again for 1 minute at the same speed

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INSTRUCTIONS	NOTES/OBSERVATIONS
Invert the g-TUBE and spin again for 1 minute to collect the fragmented DNA. ☐ Remove g-TUBE, invert the tube and replace into the centrifuge ☐ Spin the g-TUBE for 1 minute ☐ Remove and check the DNA has passed into the lower chamber ☐ If DNA remains in the upper chamber, spin again for 1 minute ☐ Remove g-TUBE ☐ Transfer the 50 µl fragmented DNA to a clean 1.5 ml Eppendorf DNA LoBind tube.	
100 ng fragmented DNA in 50 µl is taken into the next step.	
End-prep	
Perform end repair and dA-tailing of fragmented DNA as follows: ☐ 50 µl 100 ng fragmented DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Mix gently by flicking the tube, and spin down. ☐ Transfer the sample to a 0.2 ml PCR tube, and incubate for 5 minutes at 20° C and 5 minutes at 65° C using the thermal cycler. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep 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. ☐ 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 16 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 16 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
PCR adapters ligation and amplification	
Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 15 µl End-prepped DNA ☐ 10 µl Barcode Adapters (BCA) ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 20 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. ☐ 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 21 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 21 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 adapted DNA using a Qubit fluorometer. ☐ Calculate how much DNA to take forward into the PCR step for a final DNA concentration of 0.2 ng/µl in a 50 µl reaction. Set up the adapted DNA PCR as follows: ☐ Adapter ligated DNA, diluted x µl 0.2 ng/µl ☐ Nuclease-free water 24-x µl ☐ Barcode Primers (LWB 01-12, at 10 µM) 1 µl ☐ LongAmp Taq 2x Master Mix 25 µl ☐ Mix gently by flicking the tube, and spin down.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (14 (b) cycles) ☐ Annealing 15 secs (a) @ 56 °C (a) (14 (b) cycles) ☐ Extension 50 secs/kb @ 65 °C (c) (14 (b) cycles) ☐ Final extension 6 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ 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. ☐ 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 adapted DNA 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.	
Rapid adapter ligation	
☐ Add 1 µl RAP to the 10 µl amplified DNA library. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 placing the tubes on ice as soon as thawing is complete. ☐ Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) 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. It is provided in the kit for potential future product compatibility.	
☐ 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 µls risks a loss of sequencing channels.	
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 (FB), 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. 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
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.	