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

Before start checklist

Materials

- [ ] 750 µl of extracted UHMW gDNA in Circulomics EB+
- [ ] Ultra-Long DNA Sequencing Kit (SQK-ULK001)
- [ ] Circulomics Nanobind Big DNA Kit (e.g. CBB, Plant or Tissue)
- [ ] Circulomics Nanobind UL Library Prep Kit (NB-900-601-01)

Consumables

- [ ] 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- [ ] Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- [ ] Thermal cycler or heat block at 75°C
- [ ] Vortex mixer
- [ ] Microfuge
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P200

INSTRUCTIONS	NOTES/OBSERVATIONS
Tagmentation	
Before starting the library preparation, extract 750 µl of UHMW gDNA in Circulomics EB+. ☐ Thaw the Fragmentation Mix (FRA), FRA Dilution Buffer (FDB) and Rapid Adapter-F (RAP-F). Spin down briefly using a microfuge and keep on ice. In a 1.5 ml Eppendorf DNA LoBind tube, make up the diluted FRA: ☐ 6 µl Fragmentation Mix (FRA) ☐ 244 µl FRA dilution buffer (FDB) ☐ Mix the diluted FRA by vortexing. ☐ Add 250 µl of diluted FRA to the extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution. ☐ Immediately vortex the reaction for 5 seconds at the lowest setting able to generate a gentle vortex in the fluid. Incubate the reaction as follows: ☐ 5 minutes Room temperature ☐ 5 minutes 75°C ☐ Cool to RT for a minimum of 10 minutes Room temperature ☐ Add 5 µl of RAP-F with a regular pipette tip. Use a P1000 wide-bore tip to pipette mix. Visually check to ensure the reaction is thoroughly mixed. Inversion can be used to aid mixing. ☐ Incubate for 30 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Clean-up	
☐ Thaw one tube of Long Fragment Buffer (LFB) and Elution Buffer (EB) at RT, mix by vortexing, spin down and store at RT. ☐ Add a single Circulomics Nanobind disk to the DNA library and invert to ensure both sides of the disk are wetted. ☐ Add 500 µl Circulomics NAF Binding Buffer. ☐ Gently invert the tube 20-30 times to mix until all reagents are thoroughly mixed. ☐ Wait 5-10 minutes. The DNA should start to become visible as a precipitate forming around the disk. ☐ Invert the tube again 5-10 times to ensure all DNA has precipitated and is tightly bound to the Nanobind Disk. ☐ Place the tube on a magnetic rack to aspirate and discard the supernatant with a P200 pipette. Take care not to disturb the DNA precipitated onto the Nanobind disk. ☐ Wash the Nanobind disk by gently adding 1000 µl of Long Fragment Buffer (LFB) and invert. Keep the tube on the magnetic rack and incubate for 5 minutes at RT. Aspirate and discard the supernatant, taking care not to disturb the DNA precipitate. ☐ Repeat the previous step and remove any supernatant in the lid of the Eppendorf tube. ☐ Add 225 µl of Elution Buffer (EB) to elute the DNA. ☐ Incubate the library overnight at RT, for a minimum of 12 hours. Gently aspirate and dispense the eluate over the Nanobind disk at regular intervals with a wide-bore pipette tip to aid elution. ☐ Transfer 225 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube after the overnight incubation with a wide-bore tip. The DNA should be viscous. ☐ Spin the tube containing the Nanobind disk on a centrifuge at 10,000 x g for 10-15 seconds and transfer any additional liquid that comes off the disk to the previous eluate. Repeat as necessary until all DNA is removed using a standard P200 pipette. ☐ Gently mix the eluate containing the DNA library five times with a wide-bore pipette tip. ☐ Incubate for 2 hours at RT. The library should be very viscous.	
The prepared library is used for loading into the flow cell. Store at RT to allow DNA to homogenise before loading.	
Priming and loading the SpotON flow cell for GridION	
☐ Thaw the Sequencing Buffer (SQB), Priming Tether (PT) and one tube of Flush Buffer (FB) at RT. Mix by vortexing and spin down. In a new tube, prepare the DNA library for loading as follows and gently mix with a wide-bore tip: ☐ 37.5 µl Sequencing Buffer (SQB) ☐ 37.5 µl DNA library	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Wait 30 minutes and gently mix with a wide-bore tip. Visually inspect to ensure the sample is homogenous. ☐ Slide open the GridION lid and insert the flow cell. ☐ 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 Prepare the flow cell priming mix in a 1.5 ml Eppendorf tube and mix by vortexing at RT. ☐ 30 µl Priming Tether (PT) ☐ 1170 µl Flush Buffer (FB) ☐ 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. 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. ☐ Thoroughly mix the DNA library and ensure the SpotON port and Priming port covers of the flow cell are open in preparation for loading. ☐ Load the DNA library dropwise onto the SpotON port until 75 µl has been loaded. Ensure each drop flows into the port before adding the next. ☐ Cover Waste port 2 and the Priming port with clean, gloved fingers. ☐ Using a fully depressed P200 pipette, insert the tip in Waste port 1 whilst Waste port 2 and the Priming port are covered. ☐ Very slowly aspirate to pull the DNA library into the SpotON sample port. Closely watch the DNA library on the SportON port and completely remove the pipette as soon as the library starts to be pulled into the port. ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the GridION lid.	
IMPORTANT ☐ When setting up your run parameters in MinKNOW, under Run Options, set the time between mux scans to 6 hours from the default 1.5 hours before starting the sequencing run.	
We recommend loading an ultra-long DNA library three times per flow cell to increase output.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Reloading ultra-long DNA library on a MinION/GridION flow cell	
To run a second library of ultra-long DNA straight after flushing a flow cell, we recommend removing all fluid from the waste channel after each priming step, as outlined below. ☐ Mix the Sequencing Buffer (SQB), Priming Tether (PT) and Flush Buffer (FB) tubes by vortexing and spin down at RT. In a new tube, prepare the DNA library for loading as follows and gently mix with a wide-bore tip: ☐ 37.5 µl Sequencing Buffer (SQB) ☐ 37.5 µl DNA library ☐ Wait 30 minutes and gently mix with a wide-bore tip. Visually inspect to ensure the sample is homogenous. ☐ Slide the priming port cover of the flow cell 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 Prepare the flow cell priming mix in a 1.5 ml Eppendorf tube and mix by vortexing at RT. ☐ 30 µl Priming Tether (PT) ☐ 1170 µl Flush Buffer (FB) ☐ 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. ☐ Close the priming port cover and ensure the SpotON sample port cover is closed. ☐ Using a P1000, remove all fluid from the waste channel through Waste port 1. As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area. ☐ Slide open the priming port and load 200 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. ☐ Close the priming port and use a P1000 to remove all fluid from the waste channel through Waste Port 1. ☐ Thoroughly mix the DNA library and ensure the SpotON port and Priming port covers of the flow cell are open in preparation for loading. ☐ Load the DNA library dropwise onto the SpotON port until 75 µl has been loaded. Ensure each drop flows into the SpotON port before adding the next. ☐ Cover Waste port 2 and the Priming port with clean, gloved fingers.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Using a fully depressed P200 pipette, insert the tip in Waste port 1 whilst Waste port 2 and the Priming port are covered. ☐ Very slowly aspirate to pull the DNA library into the SpotON sample port. Closely watch the DNA library on the SportON port and completely remove the pipette as soon as the library starts to be pulled into the port. ☐ 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. ☐ Once the flow cell is reloaded, resume the sequencing run on MinKNOW and trigger a mux scan.	
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.	