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

Before start checklist

Materials

- [ ] Ultra-Long DNA Sequencing Kit (SQK-ULK001)
- [ ] Monarch® HMW DNA Extraction Kit for Tissue (New England Biolabs, T3060)

Consumables

- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 2 ml Eppendorf DNA LoBind tubes
- [ ] 5 ml Eppendorf DNA LoBind tubes
- [ ] 15 ml Falcon tubes
- [ ] 10 mM Tris.HCl, 1 mM EDTA pH 9
- [ ] Triton X-100 0.5% v/v
- [ ] Spermine tetrahydrochloride (Sigma-Aldrich, cat # 85605 1-G)
- [ ] Nuclease-free water (e.g. ThermoFisher, cat #AM9937)
- [ ] Isopropanol, 100% (Fisher, 10723124)
- [ ] Ethanol, 100% (Fisher, 16606002)
- [ ] Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Thermal cycler or heat block
- [ ] Vortex mixer
- [ ] Microfuge
- [ ] Qubit fluorometer (or equivalent)
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P200

INSTRUCTIONS	NOTES/OBSERVATIONS
Isolation of PBMCs from whole blood	
PBMC sample preparation for the Ultra-long DNA experiment ☐ Add 4.8 ml (3X the volume) of RBC Lysis Solution to 1.6 ml of whole blood in a 15 ml Falcon tube. ☐ Gently invert the tube 10 times to mix. ☐ Incubate for 5 minutes at RT and gently invert twice during the incubation. ☐ Centrifuge at 2000 x g for 2 minutes at 4°C to pellet the white blood cells. ☐ Discard the supernatant by pouring. There will be ~200 µl supernatant remaining in the tube. ☐ Resuspend the cells in the residual supernatant by gently flicking the tube.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Make up the volume to 1.6 ml with 1X PBS. ☐ Repeat steps 1-7 twice more to complete three washes in total. ☐ After the final spin, remove the entire supernatant by pouring and aspirating any remaining supernatant. ☐ Resuspend the cell pellet in 40 µl 1X PBS. There will be approximately 6 million cells in the suspension.	
Take the cell pellet forward into the "uHMW gDNA extraction" step.	
Preparation of tissue samples for gDNA extraction	
Prepare the Cell Suspension Buffer (CSB) as follows: ☐ Tris.HCl, pH 8 0.05 M @ 1 M (50 ml cycles) ☐ EDTA 0.1 M @ 0.5 M (200 ml cycles) ☐ Sucrose 0.35 M @ 2.5 M (140 ml cycles) ☐ Nuclease-free water - @ - (610 ml cycles) ☐ Add 1 g of the frozen tissue sample to a weighing boat. ☐ Using the scalpel, slice the tissue into thin strips and then dice the sample. ☐ Transfer the tissue sample to a fresh 50 ml Falcon tube. ☐ Add 10 ml of the Cell Suspension Buffer (CSB) into the 50 ml Falcon tube. Using the QIAGEN TissueRuptor, gently homogenise the tissue sample. ☐ Insert the probe and pulse at minimum speed for one second. Stir the homogenate between each pulse. ☐ Repeat this five times.	
IMPORTANT ☐ During homogenisation, only apply as much force as is required to gently break up the tissue. Excessive force will damage the nuclei and make them difficult to quantify. It is not a problem if there is intact material remaining at the end of this step, as it will be re-processed in later steps.	
☐ Assemble the pluriStrainer apparatus with a 200 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions. ☐ Pass the full volume of the tissue sample homogenate through the 200 µm PluriStrainer®. ☐ Disassemble the pluriStrainer® apparatus according to the manufacturer's instructions, setting aside the strained homogenate in the 50 ml Falcon tube for later use. Repeat the homogenisation process on any intact tissue caught by the pluriStrainer®: ☐ Transfer any intact tissue caught by the 200 µm pluriStrainer® into a fresh 50 ml Falcon tube by inverting the strainer and tapping out the intact tissue. Tip: A spatula can be used to help remove the intact tissue from the strainer. ☐ Add 10 ml of the Cell Suspension Buffer (CSB) into the 50 ml Falcon tube. ☐ Repeat steps 6-10 two more times to perform a total of three rounds of tissue homogenisation.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Combine the contents of the 50 ml Falcon tube with the original strained homogenate set aside in step 10.	
The combined volume of 200 µm strained homogenate is ready for further processing.	
Strain the 200 µm strained homogenate through the 100 µm pluriStrainer®: ☐ Assemble the pluriStrainer apparatus with a 100 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions. ☐ Pass the full volume of the 200 µm strained homogenate through the 100 µm PluriStrainer®. Tip: The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer. ☐ Disassemble the pluriStrainer® and retain the 100 µm strained homogenate in the 50 ml Falcon tube. Strain the 100 µm strained homogenate through the 50 µm pluriStrainer®: ☐ Assemble the pluriStrainer apparatus with a 50 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions. ☐ Pass the full volume of the 100 µm strained homogenate through the 50 µm PluriStrainer®. Tip: The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer. ☐ Disassemble the pluriStrainer® and retain the 50 µm strained homogenate in the 50 ml Falcon tube. Strain the 50 µm strained homogenate through the 30 µm pluriStrainer®: ☐ Assemble the pluriStrainer apparatus with a 30 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions. ☐ Pass the full volume of the 50 µm strained homogenate through the 30 µm PluriStrainer®. Tip: The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer. ☐ Disassemble the pluriStrainer® and retain the 30 µm strained homogenate in the 50 ml Falcon tube. ☐ Determine the concentration of the nuclei in the purified homogenate using a fluorescent microscope and a stain appropriate for the nuclei in the sample. ☐ Take forward a volume corresponding to 6 million nuclei and add this to a 5 ml Eppendorf DNA LoBind tube. ☐ Centrifuge the 5 ml Eppendorf tube at 16,000 x g for five minutes to pellet the nuclei/cells. ☐ Pipette off all the supernatant and discard, taking care not to disturb the pellet. ☐ Add 40 µl of PBS to the 5 ml Eppendorf DNA LoBind tube. ☐ Thoroughly mix the tube by repeatedly flicking. Ensure the pellet breaks up and no clumps remain in the nuclei/cell suspension.	
Take the nuclei/cell suspension forward into the "uHMW gDNA extraction" step.	
uHMW gDNA extraction	
☐ Add 6 million cells resuspended in 40 µl PBS to a fresh 5 ml tube. Cells can be isolated from cell culture, PBMCs from blood, or tissue according to the above methods.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ In a separate 2 ml Eppendorf DNA LoBind tube, mix 1.8 ml of Monarch HMW gDNA Tissue Lysis Buffer and 60 µl Proteinase K.	
☐ Add 1.8 ml of pre-mixed lysis buffer/proteinase K to the resuspended cells.	
☐ Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip.	
☐ Incubate at 56°C for 10 minutes.	
☐ Add 15 µl of RNase A.	
☐ Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip.	
☐ Incubate at 56°C for 10 minutes.	
☐ Add 900 µl of Protein Separation Solution and mix using a Hula Mixer (rotator mixer) for 4 minutes rotating at 5 rpm.	
☐ Centrifuge the reaction at 16,000 x g for 10 minutes at 4°C to separate the protein from the DNA.	
☐ Carefully aspirate the upper phase containing the DNA and transfer to a fresh 5 ml tube without disturbing the phase below.	
☐ Add three beads to the collected DNA phase.	
☐ Add 2 ml of isopropanol to the tube and mix using a Hula Mixer (rotator mixer) for 10 minutes rotating at 5 rpm.	
☐ Allow the tubes to rest at RT for 1 minute without rotating.	
☐ Remove 1.5 ml of liquid from the top of the 5 ml tube by aspiration, being careful not to aspirate any DNA that has formed around the beads at the bottom of the tube.	
☐ Add 1.5 ml of isopropanol to the tube to further condense the DNA around the beads and mix using a Hula Mixer (rotator mixer) for 5 minutes rotating at 5 rpm.	
☐ Aspirate the isopropanol from the tube, being careful not to aspirate the DNA that is bound to the glass beads. Also remove any isopropanol remaining in the lid of the tube.	
☐ Add 1 ml Wash Buffer and mix using a Hula Mixer (rotator mixer) for 5 minutes rotating at 5 rpm. DNA will bind tightly around the beads at this step.	
☐ Remove the Wash Buffer by aspiration, being careful not to aspirate the DNA that is bound to the beads. Also remove any Wash Buffer remaining in the lid of the tube.	
☐ Add 1 ml of Wash Buffer and mix using a Hula Mixer (rotator mixer) for 5 minutes rotating at 5 rpm.	
☐ Remove the Wash Buffer by aspirating, being careful not to aspirate the DNA that is bound to the beads. Also remove any Wash Buffer remaining in the lid of the tube.	
☐ Transfer the beads to a bead retainer with an empty collection tube beneath.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Collect the residual Wash Buffer by performing a quick soft spin (<1 second) to remove any remaining Wash Buffer from the beads.	
IMPORTANT ☐ Do NOT use the elution buffer in the NEB Monarch® HMW DNA Extraction Kit for Tissue.	
☐ Immediately transfer the beads from the bead retainer into a new 2 ml tube containing 560 µl of EEB (prepared previously).	
IMPORTANT ☐ Beads should be transferred immediately to ensure that they do not over-dry, which could lead to increased solubilisation times.	
☐ Incubate for 10 minutes at 56°C.	
☐ Separate the EEB from the beads by pouring into a new bead retainer with a collection tube beneath, and spin at 1000 x g for 1 minute.	
☐ Add an additional 200 µl of EEB to the collection tube to bring the total elution volume to 760 µl.	
☐ Transfer the sample to a fresh 2 ml Eppendorf DNA LoBind tube and discard the beads and bead retainer.	
☐ Incubate for 10 minutes at 56°C.	
☐ Gently mix 10 times using a P1000 pipette and wide-bore pipette tip.	
Take forward the resuspended DNA into the quantification step.	
gDNA quantification	
OPTIONAL Quantification of uHMW gDNA	
☐ Use a regular P200 pipette tip to aspirate 10 µl of gDNA.	
☐ Dispense the aspirated gDNA into a fresh 2 ml Eppendorf DNA LoBind tube.	
☐ Add a glass bead to the isolated 10 µl and vortex aggressively for 1 minute to shear all the gDNA.	
☐ Separate the liquid from the bead using a microfuge, bead retainer and collection tube.	
Quantify the sample using a Qubit fluorometer. The expected yield is 30-40 µg of DNA.	
Take forward 750 µl DNA into the library preparation process.	
Tagmentation	
☐ 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.	FRA

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a 1.5 ml Eppendorf DNA LoBind tube, make up the diluted Fragmentation Mix (FRA): ☐ 6 µl Fragmentation Mix (FRA) ☐ 244 µl FRA dilution buffer (FDB) ☐ Mix the diluted Fragmentation Mix (FRA) by vortexing. ☐ Add 250 µl of diluted Fragmentation Mix (FRA) to the extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted Fragmentation Mix (FRA) to ensure an even distribution. ☐ Immediately mix the reaction by pipetting 10 times with a wide-bore pipette tip. 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.	
Clean-up	
☐ Add 1 ml of PPT buffer. ☐ Mix using a Hula Mixer (rotator mixer) for 5 minutes rotating at 5 rpm. ☐ Check the DNA has precipitated, forming a glassy white mass. ☐ Centrifuge at 1000 x g for 1 minute. ☐ Remove the supernatant without disturbing the DNA pellet. ☐ Centrifuge at 1000 x g for 1 minute and remove the residual supernatant without disturbing the DNA pellet. ☐ Add 225 µl of Elution Buffer (EB) and incubate overnight at RT, for a minimum of 12 hours. ☐ Gently mix 10 times using a P1000 with a wide-bore pipette tip.	
The prepared libraries are used for loading into the flow cells. Store the libraries on ice until ready to load.	
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 ☐ Incubate at RT for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Slide open the GridION lid and insert the flow cell. ☐ 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. 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 five 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 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 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
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.	
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 ☐ Incubate at RT for 30 minutes then gently mix by slowly pipetting 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: ☐ 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. 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)	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Close the priming port cover and ensure the SpotON sample port cover is closed. ☐ Remove all fluid from the waste channel through waste port 1 using a P1000 pipette. ☐ 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. ☐ 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 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. ☐ Once the flow cell is reloaded, resume the sequencing run on MinKNOW and trigger a mux scan.	
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.	