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

Before start checklist

Materials

- [ ] PCR Sequencing Kit (SQK-PSK004)
- [ ] Flow Cell Priming Kit (EXP-FLP001)

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 rack
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Thermal cycler
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
Prepare the DNA in Nuclease-free water. ☐ Transfer 100 ng 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 ☐ Record the quality, quantity and size of the DNA.	
IMPORTANT Criteria for input DNA ☐ Purity as measured using Nanodrop - OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2 ☐ Input mass, as measured by Qubit - 100 ng ☐ No detergents or surfactants in the buffer	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Check your flow cell	
☐ Set up the MinION, flow cell and host computer Once successfully plugged in, you will see a light and hear the fan. Open the MinKNOW GUI from the desktop icon and establish a local or remote connection. ☐ If running a MinION on the same host computer, plug the MinION into the computer. ☐ If running a MinION on a remote computer, first enter the name or IP address of the remote host under Connect to a remote computer (if running from the Connection page), or Connections (if running from the homepage) and click Connect. ☐ Choose the flow cell type from the selector box. Then mark the flow cell as "Selected": Click "Check flow cells" at the bottom of the screen. ☐ R9.4.1 FLO-MIN106 ☐ R9.5.1 FLO-MIN107 ☐ Click "Start test". ☐ Check the number of active pores available for the experiment, reported in the System History panel when the check is complete.	
Flow cell check complete.	
DNA fragmentation	
OPTIONAL ☐ 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 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	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 1.5 ml DNA LoBind Eppendorf 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 beads on 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. ☐ Repeat the previous step.	
PCR adapter ligation and amplification	
Thaw and prepare the kit reagents as follows: ☐ PCR Adapter (PCA) at RT ☐ Whole Genome Primers (WGP) at RT ☐ Blunt/TA ligation Master Mix on ice ☐ LongAmp Taq 2x Master Mix on ice	

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INSTRUCTIONS	NOTES/OBSERVATIONS

Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.

- ☐ Mix the contents of each tube by flicking
- ☐ Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate)
- ☐ Spin down briefly before accurately pipetting the contents into the reaction

Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:

- ☐ 15 µl End-prepped DNA
- ☐ 10 µl PCR Adapters (PCA)
- ☐ 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 beads on 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
- ☐ Whole Genome Primers (WGP) 1 µl
- ☐ LongAmp Taq 2x Master Mix 25 µl

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix gently by flicking the tube, and spin down. 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 6 mins @ 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 beads on 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. ☐ Make up 50-100 fmol of PCR product to 10 µl in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.	
Rapid Adapter ligation	
☐ Spin down the Rapid Adapter (RAP) tube, and place it on ice. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
The prepared library is used for loading into the MinION 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 (FLB) at RT before placing the tubes on ice as soon as thawing is complete. ☐ Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) 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.	
☐ 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 µl risks damaging the pores in the array.	
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 (FLB), 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ 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.	
Starting a sequencing run	
☐ Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI. ☐ If your MinION was disconnected from the computer, plug it back in. ☐ Choose the flow cell type from the selector box. Then mark the flow cell as "Selected". ☐ Click the "New Experiment" button at the bottom left of the GUI. Select the appropriate protocol script ☐ Experiment type: Choose Sequencing Run under "Choose Operation" ☐ Flow Cell product code: Choose the Flow Cell type under "Flow cell product code" ☐ Sequencing kit: Choose SQK-PSK004 under Sequencing Kit ☐ Choose whether or not live basecalling is enabled ☐ The most appropriate script will appear in the drop-down menu. ☐ Click "Begin Experiment". Allow the script to run to completion. ☐ The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen ☐ Monitor messages in the Message panel in the MinKNOW GUI The basecalled read files are stored in :\data\reads	
Progression of MinKNOW protocol script	
The running experiment screen Experiment summary information Check the number of active pores reported in the MUX scan are similar (within 10-15%) to those reported at the end of the Platform QC ☐ If there is a significant reduction in the numbers, restart MinKNOW. ☐ If the numbers are still significantly different, close down the host computer and reboot. ☐ When the numbers are similar to those reported at the end of the Platform QC, restart the experiment on the Connection page. There is no need to load any additional library after restart. ☐ Stopping the experiment is achieved by clicking "Stop experiment" button at the top of the screen. ☐ Check the temperature is approximately 34° C.	

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Check pore occupancy in the channel panel at the top of the experimental view. ☐ A good library will be indicated by a higher proportion of light green channels in Sequencing than are in Pore. The combination of Sequencing and Pore indicates the number of active pores at any point in time. A low proportion of Sequencing channels will reduce the throughput of the run. ☐ Recovering indicates channels that may become available for sequencing again. A high proportion of this may indicate additional clean up steps are required during your library preparation. ☐ Inactive indicates channels that are no longer available for sequencing. A high proportion of these as soon as the run begins may indicate an osmotic imbalance. ☐ Unclassified are channels that have not yet been assigned one of the above classifications ☐ Monitor the pore occupancy Duty time plots ☐ Monitor the development of the read length histogram. Trace viewer	
Further analysis with EPI2ME (optional)	
OPTIONAL ☐ Open the Desktop Agent using the desktop shortcut. ☐ Click on the New Workflow tab in the Desktop Agent and select the workflow to be used in the analysis. Select the workflow parameters. ☐ Select the quality score cut-off (this defaults to 7 unless changed) ☐ Select whether the reads should be demultiplexed by barcode ☐ If you are working with human data, please tick "Yes" in answer to "Is the data you are about to upload a whole or partial human genome?", and confirm that you have consent from the subject to upload the data. ☐ Check the correct settings are selected in the Desktop Agent. ☐ Click "Start Run" to start data analysis. ☐ Follow the progression of upload and download of read files in the Desktop Agent. Click on VIEW REPORT. ☐ Click on VIEW REPORT to navigate to the EPI2ME website, this can be done at any point during data exchange ☐ Return to the Desktop Agent to see progression of the exchange ☐ When the upload and download numbers are the same, the data exchange is complete. The processed reads will be in downloads folder in the selected location on the host computer.	
Close down MinKNOW and the Desktop Agent	
☐ Quit Desktop Agent using the close x. ☐ Quit MinKNOW by closing down the web GUI.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Disconnect the MinION.	
Prepare the flow cell for re-use or return to Oxford Nanopore.	
☐ If you would like to reuse the flow cell, follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR ☐ Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.	