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

Before start checklist

Materials

- [ ] <1 µg of each DNA sample to be barcoded in 45 µl
- [ ] PCR Barcoding Expansion Pack 1-96 (EXP-PBC096)
- [ ] Ligation Sequencing Kit (SQK-LSK109)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] Agencourt AMPure XP beads
- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:
- [ ] NEBNext FFPE Repair Mix (M6630)
- [ ] NEBNext End repair / dA-tailing Module (E7546)
- [ ] NEBNext Quick Ligation Module (E6056)
- [ ] 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)

Equipment

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
- [ ] 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 - <1 µg, or 100-200 fmol for short-fragment libraries - [ ] No detergents or surfactants in the buffer	
Prepare the DNA in Nuclease-free water. - [ ] Transfer <1 µg DNA into a DNA LoBind tube - [ ] Adjust the volume to 45 µl with Nuclease-free water - [ ] Mix thoroughly by inversion avoiding unwanted shearing - [ ] Spin down briefly in a microfuge	

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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. ☐ Plug the MinION into the computer. ☐ Choose the flow cell type from the selector box. Then check the "Available" box. Note: if you are using flow cells from your Starter Pack, please select FLO-MIN106. 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.	
End-prep	
In a 0.2 ml 96 well PCR plate, set up the end-repair / dA-tailing reactions as follows: ☐ 45 µl <1 µg DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 5 µl Nuclease-free water ☐ Mix by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps, spin down in a centrifuge 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. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep on magnet, wash beads with 180 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.	
Take forward approximately 700 ng of end-prepped DNA in 30 µl into adapter ligation.	
Ligation of Barcode Adapter	
Add the reagents to a fresh 96 well plate, in the order given below: ☐ 30 µl End prep DNA ☐ 20 µl Barcode Adapter ☐ 50 µl Blunt/TA Ligase Master Mix ☐ Mix by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. ☐ Incubate the reaction for 10 minutes at RT. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep on magnet, wash beads with 180 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 25 µl Nuclease-free water. Incubate for 2 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer. ☐ Dilute the library to a concentration of 10 ng/µl with Nuclease-free water or 10 mM Tris-HCl pH 8.5	
Barcoding PCR	
Set up a barcoding PCR reaction as follows for each library: ☐ 1 µl PCR Barcode (one of BC1-BC96) ☐ 2 µl 10 ng/µl adapter ligated template ☐ 25 µl LongAmp Taq 2x master mix ☐ 22 µl Nuclease-free water ☐ Mix by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (15-18 (b) cycles) ☐ Annealing 15 secs (a) @ 62 °C (a) (15-18 (b) cycles) ☐ Extension dependent on length of target fragment (d) @ 65 °C (c) (15-18 (b) cycles) ☐ Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ Purify the barcoded DNA using standard methods which are suitable for the fragment size. ☐ Quantify the barcoded library using standard techniques, and pool all barcoded libraries in the desired ratios in a 1.5 ml DNA LoBind Eppendorf tube. ☐ Prepare 1 µg of pooled barcoded libraries in 47 µl Nuclease-free water.	
This pooled library is now ready to be end-repaired and adapted for nanopore sequencing.	
DNA repair and end-prep	
☐ Thaw DNA CS (DCS) at RT, spin down, mix by pipetting, and place on ice. ☐ Prepare the NEBNext FFPE DNA Repair Mix and NEBNext End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice. In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 1 µl DNA CS ☐ 47 µl DNA ☐ 3.5 µl NEBNext FFPE DNA Repair Buffer ☐ 2 µl NEBNext FFPE DNA Repair Mix ☐ 3.5 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix gently by flicking the tube, and spin down. ☐ Using a thermal cycler, incubate at 20° C for 5 minutes and 65° C for 5 mins.	
IMPORTANT ☐ AMPure XP bead clean-up	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the DNA 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 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 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 the pellet in 61 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 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4° C overnight.	
Adapter ligation and clean-up	
IMPORTANT ☐ Although the recommended 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within SQK-LSK109.	
☐ Spin down Adapter Mix (AMX) and T4 Ligase from the NEBNext Quick Ligation Module (E6056), and place on ice. ☐ Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing. ☐ Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice.	
IMPORTANT ☐ Depending on the wash buffer used in this section, the clean-up step after adapter ligation is designed to either enrich for long DNA fragments, or purify all fragments equally.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ To enrich for DNA fragments of 3 kb or longer, thaw one tube of L Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. ☐ To retain DNA fragments shorter than 3 kb (by purifying fragments of all sizes), thaw one tube of S Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order: ☐ 60 µl DNA sample from the previous step ☐ 25 µl Ligation Buffer (LNB) ☐ 10 µl NEBNext Quick T4 DNA Ligase ☐ 5 µl Adapter Mix (AMX) ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	
IMPORTANT ☐ If you have omitted the AMPure purification step after DNA repair and end-prep, incubating the ligation reaction for longer than 10 minutes is not recommended.	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☐ Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl S Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. 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 15 µl Elution Buffer (EB). Incubate for 10 minutes at RT. For high molecular weight DNA, incubating at 37° C can improve the recovery of long fragments. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 15 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 eluted sample using a Qubit fluorometer.	
The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Recommendation on library loading	
Priming and loading the SpotON flow cell	
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.	
☐ 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. ☐ 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 (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 Loading Beads (LB) by pipetting.	
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.	
In a new tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SQB) ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☐ 12 µl DNA library 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 check the "Available" box. ☐ Click the "New Experiment" button at the bottom left of the GUI. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs. ☐ Fast - This model is able to keep up with a full experiment on a GridION or MinIT. It implements a lite version of the Flip-flop algorithm. This delivers the same level of accuracy as the previous default transducer models, which have been deployed prior to the introduction of Flip-flop. ☐ HAC - The High accuracy (HAC) Flip-flop model provides a higher consensus/raw read accuracy than the 'Fast' model. It contains a more computationally intense Flip-flop architecture that can deliver higher accuracy using the same data produced by nanopore sequencing. It is currently 5-8 times slower than the fast model so users should ensure their data transfer, disk-space and device utilisation is scaled appropriatley for this. ☐ Click "Start run". 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 Basecalled read files	
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 Flow Cell Check ☐ 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 Flow Cell Check, 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 run" button at the top of the screen. Data acquisition will stop, but the software will continue basecalling unless the user clicks the "Stop basecalling" button.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Check that the temperature has reached 34° C. 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 ☐ Monitor the disk space Duty time plots ☐ Monitor the development of the read length histogram. Cumulative output ☐ the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters ☐ MinKNOW 19.05 has several new interpretations and graphics of the data obtained from a sequencing run. Temperature and Bias voltage graph Translocation speed and Qscore graphs Cumulative output of multiple flow cells - experiment view on PromethION Trace viewer	
Onward analysis of MinKNOW basecalled data	
☐ Open the Desktop Agent using the desktop shortcut. ☐ Click on the New Workflow tab in the Desktop Agent and select the FASTQ barcoding workflow. Select the workflow parameters. ☐ Select the quality score cut-off ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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	
Close down MinKNOW and the Desktop Agent	
☐ Quit Desktop Agent using the close x. ☐ Quit MinKNOW by closing down the web GUI. ☐ 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.	