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

Before start checklist

Materials

- [ ] 1 µg (or 100-200 fmol) gDNA
- [ ] 1.5-3 µg (or 150-300 fmol) high molecular weight genomic DNA if using R10.3 flow cells
- [ ] OR 100+ ng high molecular weight genomic DNA if performing DNA fragmentation
- [ ] Ligation Sequencing Kit XL (SQK-LSK109-XL)
- [ ] Flow Cell Priming Kit XL (EXP-FLP002-XL)

Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- [ ] OR 0.2 ml thin-walled PCR tubes
- [ ] 15 or 50 ml Falcon tubes

Equipment

- [ ] Magnetic rack suitable for 96-well PCR plates, e.g. DynaMag™-96 Side Skirted Magnet (Thermo Fisher, cat # 12027)
- [ ] OR magnetic separator suitable for 0.2 ml PCR tube strips, e.g. DynaMag™-PCR Magnet (Thermo Fisher, #492025) or DynaMag™-96 Side Magnet (Thermo Fisher, #12331D)
- [ ] Microfuge
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Multichannel pipettes suitable for dispensing 2–20 µl and 20–200 µl, and tips
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipetting troughs
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
DNA repair and end-prep	
Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice. ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to RT and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate. Note: It is important the buffers are mixed well by vortexing. ☐ The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing.	
IMPORTANT ☐ Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix.	
Prepare the DNA in Nuclease-free water per sample: ☐ For R9.4.1 flow cells, transfer 1 µg (or 100-200 fmol) genomic DNA into a separate well of a 96-well plate or a 0.2 ml PCR tube strip ☐ For R10.3 flow cells, transfer 1.5-3 µg (or 150-300 fmol) genomic DNA into a separate well of a 96-well plate or a 0.2 ml PCR tube strip ☐ Adjust the volume to 47 µl with Nuclease-free water ☐ Mix thoroughly by pipetting up and down, or by flicking the tube ☐ If necessary, seal and spin down briefly in an appropriate centrifuge To each sample, add the following: ☐ 1 µl DNA CS ☐ 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 ☐ Mix well by gently pipetting the entire volume within each well/tube up and down 10 times, or by flicking the tubes, and spin down. ☐ Seal the plate, or close the tube lids. ☐ Using a thermal cycler, incubate the samples at 20°C for 5 minutes and 65°C for 5 mins. AMPure XP bead clean-up ☐ Resuspend the AMPure XP beads by vortexing and transfer to a pipetting trough. Ensure that the volume transferred is enough for 60 µl to be added to each DNA sample, with an excess to allow for dead volume within the pipetting trough.	
IMPORTANT ☐ Resuspend and transfer the beads to the pipetting trough immediately before use to ensure beads do not settle.	
☐ Keep the DNA samples in their original wells/PCR tubes. Add 60 µl of resuspended AMPure XP beads to each sample and mix by pipetting at least 100 µl up and down ten times. Retain any unused beads. ☐ Incubate for 5 minutes at RT. ☐ Prepare fresh 70% ethanol in Nuclease-free water and pour into a pipetting trough. Allow enough for 500 µl per sample, with an excess to allow for dead volume within the pipetting trough. After the bead washing steps, discard any unused ethanol. ☐ Pellet the beads on a magnet for at least 2 minutes, or until the supernatant is clear. Keep the plate/tube strip on the magnet and pipette off the supernatant.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Keeping the plate/tube strip on the magnet, wash each pellet of beads with 200 µl of the freshly-prepared 70% ethanol without disturbing the pellets. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Seal the plate, or close the tube lids. Spin down and place the plate/tube strip back on the magnet. Pipette off any residual ethanol. ☐ Pour Nuclease-free water into a pipetting trough. Allow enough for 61 µl per sample, with an excess to allow for dead volume within the pipetting trough. ☐ Remove the plate/tube strip from the magnetic rack and resuspend each pellet in 61 µl Nuclease-free water from the pipetting trough. Pipette the entire volume up and down ten times). ☐ Seal the plate or close the tube lids, and incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 min. ☐ Remove and retain 61 µl of each eluate in a separate, clean well/tube within a 96-well PCR plate or PCR tube strip. Dispose of the pelleted beads.	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the samples 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-XL.	
☐ Spin down the Adapter Mix (AMX) and Quick T4 Ligase, 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, and place on ice.	
IMPORTANT Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally. ☐ To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB) ☐ To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)	
☐ To enrich for DNA fragments of 3 kb or longer, thaw one bottle of Long Fragment Buffer (LFB) at RT, mix by vortexing, and place on ice. ☐ To retain DNA fragments of all sizes, thaw one bottle of Short Fragment Buffer (SFB) at RT, mix by vortexing, and place on ice.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
To each repaired/end-prepped DNA sample (60 µl), add the following: ☐ 25 µl Ligation Buffer (LNB) ☐ 10 µl NEBNext Quick T4 DNA Ligase ☐ 5 µl Adapter Mix (AMX) ☐ Mix well by gently pipetting the entire volume within each well/tube up and down 10 times. ☐ Incubate the reaction for 10 minutes at RT.	
IMPORTANT ☐ If you have omitted the AMPure purification step after DNA repair and end-prep, do not incubate the reaction for longer than 10 minutes.	
☐ Resuspend the AMPure XP beads by vortexing and transfer to a pipetting trough. Ensure that the volume transferred is enough for 40 µl to be added to each DNA sample, with an excess to allow for dead volume within the pipetting trough.	
IMPORTANT ☐ Resuspend and transfer the beads to the pipetting trough immediately before use to ensure beads do not settle.	
☐ Add 40 µl of resuspended AMPure XP beads to each sample and mix by pipetting the entire combined volume up and down 10 times. ☐ Incubate for 5 minutes at RT. ☐ Add sufficient Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) to a pipetting trough. Allow enough for 400 µl per sample, with an excess to allow for dead volume within the pipetting trough. Retain any unused reagent after the wash steps. ☐ Pellet the beads on a magnet for at least 2 minutes, or until the supernatant is clear. Keep the plate/tube strip on the magnet and pipette off the supernatant. ☐ Remove the plate/tube strip from the magnetic rack and wash each pellet of beads by adding either 200 µl Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Resuspend each pellet thoroughly by pipetting the entire volume of buffer up and down ten times. Fully resuspending the beads at this step ensures optimal kit performance. Return the plate/tube strip to the magnetic rack and allow the beads to pellet until the supernatant is clear. Remove the supernatant using a pipette and discard.	
IMPORTANT ☐ It is essential that beads are resuspended fully and not simply moved around the tubes through use of the magnet.	
☐ Repeat the previous step. ☐ Seal the plate, or close the tube lids. Spin down and place the plate/tube strip back on the magnet. Pipette off any residual supernatant. ☐ Add sufficient Elution Buffer (EB) to a pipetting trough. Allow enough for 15 µl per sample, with an excess to allow for dead volume within the pipetting trough. Retain any unused Elution Buffer (EB) after the elution step.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Remove the plate/tube strip from the magnetic rack and resuspend each pellet in 15 µl Elution Buffer (EB) from the pipetting trough, pipetting the entire volume up and down 10 times. ☐ Seal the plate (or close the tube lids), and incubate for 10 minutes at 37°C in a thermal cycler. Any heated lid used should be limited to 50°C. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 15 µl of each eluate in a separate, clean well/tube within a 96-well PCR plate or PCR tube strip. Dispose of the pelleted beads.	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
IMPORTANT We recommend loading the final prepared library onto a flow cell following one of our recommendations depending on the flow cell type: ☐ R9.4.1 flow cells, load 5-50 fmol ☐ R10.3 flow cells, load 25-75 fmol	
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	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and Flush Buffer (FB) at RT, before mixing the reagents by vortexing and spin down at RT. ☐ Prepare the flow cell Priming Mix: in a suitable vial, prepare a mix of Flush Buffer (FB) and Flush Tether (FLT) for priming all flow cells to be loaded. Allow 30 µl of Flush Tether (FLT) and 1.17 ml of Flush Buffer (FB) for every flow cell; no excess is required. Once combined, mix well by briefly vortexing. ☐ Open the MinION Mk1B lid and slide the flow cell under the clip. ☐ 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: ☐ 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.	

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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. During this time, prepare the library for loading by following the steps below. ☐ Meanwhile, thoroughly mix the contents of the thawed Loading Beads (LB) tube(s) by vortexing.	
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 separate 0.2 ml PCR tube for each library, prepare for loading by adding the following reagents: ☐ 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 priming port (not the SpotON sample port), avoiding the introduction of air bubbles. ☐ Mix the prepared libraries gently by pipetting up and down just prior to loading. ☐ Add 75 µl of the prepared library 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 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.	
Close the device lid and set up a sequencing run on MinKNOW.	
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.	