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

Before start checklist

Materials

- [ ] 10 pg high molecular weight genomic DNA
- [ ] Ligation Sequencing Kit (SQK-LSK112)
- [ ] Qiagen REPLI-g Midi Kit

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)
- [ ] Covaris g-TUBE
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] T7 Endonuclease I (NEB, cat # M0302)
- [ ] TE buffer: 10 mM Tris (pH 8.0), 0.1 mM EDTA
- [ ] PEG 8000, 50% w/v (Rigaku Reagents, cat # 25322-68-3)
- [ ] 0.5 M EDTA, pH 8 (Thermo Scientific, R1021)
- [ ] 5 M NaCl (Sigma, 71386)
- [ ] 1 M Tris-HCl pH 8.0 (Thermo Scientific, cat # 15893661)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Heating block at 37°C capable of taking 1.5 ml tubes
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS

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INSTRUCTIONS	NOTES/OBSERVATIONS
Whole genome amplification	

Prepare the DNA in Nuclease-free water.

- ☐ Transfer 10 pg genomic DNA into a DNA LoBind tube
- ☐ Adjust the volume to 5 µl with Nuclease-free water
- ☐ Mix thoroughly by inversion avoiding unwanted shearing
- ☐ Spin down briefly in a microfuge

Reconstitute the DLB buffer and Stop Solution from the Qiagen REPLI-g Midi kit as follows:

- ☐ 9 µl DLB buffer
- ☐ 32 µl Nuclease-free water
- ☐ 12 µl Stop Solution
- ☐ 68 µl Nuclease-free water

In a clean 1.5 ml Eppendorf DNA LoBind tube, mix the following:

- ☐ 5 µl Input gDNA, 10 pg
- ☐ 5 µl Reconstituted DLB buffer

☐ Incubate the reaction for 3 minutes at RT.

☐ Add 10 µl of reconstituted Stop Buffer to the reaction and mix by pipetting.

In a clean 1.5 ml Eppendorf DNA LoBind tube, mix the following:

- ☐ 29 µl REPLI-g Midi Reaction Buffer
- ☐ 1 µl REPLI-g Midi DNA Polymerase

☐ Add the REPLI-g Midi polymerase mastermix to the DNA reaction, and mix by pipetting.

☐ Transfer the sample to a clean 0.2 ml PCR tube, and incubate for 16 hours at 30° C and 3 minutes at 65° C using the thermal cycler.

☐ Resuspend the AMPure XP beads by vortexing.

☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

☐ Add 90 µl of resuspended AMPure XP beads to the amplification 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 when clear and colourless.

☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

☐ 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.

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☐ Remove the tube from the magnetic rack and resuspend pellet in 100 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 100 µl of eluate in a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
In a clean 0.2 ml PCR tube, mix the reagents in the following order: ☐ x µl 1.5 µg of amplified DNA ☐ 3 µl NEBuffer 2 ☐ 1.5 µl T7 Endonuclease I ☐ 25.5-x µl Nuclease-free water ☐ Incubate the reaction for 15 minutes at 37° C. ☐ Resuspend the AMPure XP beads by vortexing. Prepare the Custom buffer in a clean 2 ml Eppendorf DNA LoBind tube: ☐ 20 µl 1 M Tris-HCl ☐ 4 µl 0.5 M EDTA pH 8 ☐ 640 µl 5 M NaCl ☐ 440 µl PEG 8000 ☐ 888 µl Nuclease-free water ☐ Transfer thoroughly mixed Agencourt AMPure XP beads into two 1.5 ml Eppendorf DNA LoBind tubes, so that each contains 1 ml. ☐ Pellet the beads on a magnet. Keeping the tube on the magnet, pipette off the supernatant. ☐ Wash the beads with 1 ml of Nuclease-free water by resuspending the pellet. Return the tube to the magnetic rack, allow beads to pellet, remove the water using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual water. ☐ Pool the two bead pellets together by resuspending them in 200 µl of Custom buffer. Then transfer the beads into the remaining Custom buffer. ☐ Make up the amplified DNA sample to a total volume of 50 µl in TE buffer, pH 8. ☐ Add 35 µl of the custom bead suspension with beads to the DNA sample, and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. This step may be extended to 20 minutes if more efficient ligation is desired. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.	

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☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol 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 49 µl Nuclease-free water. Incubate for 1 minute at 50°C, and then for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 49 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of DNA using a Qubit fluorometer - recovery aim ~700 ng.	
Take forward approximately 700 ng of DNA in 48 µl into the DNA repair and end-prep step. However, at this point it is also possible to store the sample at 4°C overnight.	
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.	
IMPORTANT ☐ Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix.	
IMPORTANT ☐ It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing.	
In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 48 µl DNA ☐ 3.5 µl NEBNext FFPE DNA Repair Buffer ☐ 3.5 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 2 µl NEBNext FFPE DNA Repair Mix ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.	

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IMPORTANT ☐ AMPure XP bead clean-up	
☐ Resuspend the AMPure XP beads 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 until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 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, for at least 1 minute. ☐ Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of 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 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 H (AMX H) is higher when using the Ligation Buffer (LNB) supplied within the Ligation Sequencing Kit.	
☐ Spin down the Adapter Mix H (AMX H) 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 and mix by vortexing. Then spin down and place on ice.	

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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 tube of Long Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. ☐ To retain DNA fragments of all sizes, thaw one tube of Short 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 H (AMX H) ☐ Ensure the components are thoroughly mixed by pipetting, 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, do not incubate the reaction for longer than 10 minutes.	
☐ Resuspend the AMPure XP beads 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 when clear and colourless. ☐ Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, 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 the pellet in 15 µl Elution Buffer (EB). Spin down and 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, for at least 1 minute. ☐ Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
IMPORTANT ☐ We recommend loading 5-10 fmol of this final prepared library onto your flow cells.	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Priming and loading the SpotON flow cell	
IMPORTANT ☐ The Kit 12 chemistry runs at 30°C on nanopore sequencing devices. This is several degrees cooler than other chemistries. While the protocol was initially developed on GridION and PromethION, we also support its use on MinION Mk1C, as the MinION Mk1C device's temperature control allows the flow cell to be maintained at 30°C for the duration of the run. However, we cannot guarantee the same level of temperature control on the MinION Mk1B. Therefore, if you are running Kit 12 chemistry on the MinION Mk1B, ensure that the ambient temperature does not exceed 23°C.	
Using the Loading Solution ☐ Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing and spin down at RT. ☐ To 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 vortexing at RT. ☐ Open the MinION lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact. ☐ 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. ☐ 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. ☐ Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.	

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IMPORTANT ☐ The Loading Beads II (LBII) 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 II (SBII) ☐ 25.5 µl Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using ☐ 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 library 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.	