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

Before start checklist

Materials

- [ ] 50 pg high molecular weight genomic DNA
- [ ] Ligation Sequencing Kit V14 (SQK-LSK114)
- [ ] REPLI-g® Single Cell Kit (QIAGEN, cat # 150343)

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
- [ ] 2 ml Eppendorf DNA LoBind tubes
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% 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)
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

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
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS

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INSTRUCTIONS	NOTES/OBSERVATIONS
Whole genome amplification	
☐ Thaw the REPLI-g sc DNA Polymerase on ice, mix well by pipetting and spin down. Store on ice until ready to use. Prepare the DNA in Nuclease-free water. ☐ Transfer 50 pg genomic DNA into a clean 0.2 ml thin-walled PCR tube. ☐ Adjust the volume to 4 µl with Nuclease-free water. ☐ Mix thoroughly by inversion and gently flicking to avoiding unwanted shearing. ☐ Spin down briefly in a microfuge. Reconstitute the Buffer DLB from the QIAGEN REPLI-g Single Cell kit as follows: ☐ Add 500 µl of Nuclease-free water to the Buffer DLB tube. ☐ Thoroughly mix by vortexing and briefly spin down. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare sufficient Buffer D2 for the total number of reactions required as follows: ☐ DTT, 1M 3 µl @ 1 µl (6 µl cycles) ☐ Reconstituted Buffer DLB 33 µl @ 11 µl (66 µl cycles) ☐ Add 3 µl of prepared Buffer D2 to the gDNA input sample in the 0.2 ml thin-walled PCR tube. ☐ Mix gently by flicking the tube and spin down. ☐ Incubate the reaction at 65°C for 10 minutes. ☐ Add 3 µl of of Stop Solution to the denatured DNA sample tube. Mix by flicking the tube, briefly spin down and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube placed on ice, prepare the master mix as follows: ☐ 9 µl Nuclease-free water ☐ 29 µl REPLI-g sc Reaction Buffer ☐ 2 µl REPLI-g sc DNA Polymerase ☐ Mix thoroughly by pipetting and briefly spin down before storing the master mix on ice. Combine the following reagents in the same 0.2 ml thin-walled PCR tube containing the sample: ☐ 10 µl Denatured DNA sample (from previous step) ☐ 40 µl Prepared master mix ☐ Mix gently by flicking the tube and spin down. ☐ Incubate the reaction for 2 hours at 30°C and 3 minutes at 65°C using a thermal cycler. ☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 90 µl of resuspended AMPure XP beads to the amplification reaction and mix by pipetting.	

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☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of 80% 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 80% 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. ☐ 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 the eluted sample using a Qubit fluorometer.	
Prepare your amplified DNA sample as follows: ☐ Transfer 1.5 µg of amplified DNA into a clean 0.2 ml thin-walled PCR tube. ☐ Adjust the volume to 24 µl with Nuclease-free water. ☐ Mix thoroughly by inversion and gently flicking to avoiding unwanted shearing. ☐ Spin down briefly in a microfuge. Prepare the following reaction in the 0.2 ml thin-walled PCR tube containing the sample by adding the reagents in the following order: ☐ 24 µl 1.5 µg of amplified DNA (from previous step) ☐ 3 µl NEBuffer 2 ☐ 3 µl T7 Endonuclease I ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 60 minutes at 37°C. Prepare the Custom buffer with beads as follows: ☐ 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 Prepare the amplified DNA sample as follows: ☐ Transfer the 30 µl of amplified DNA sample into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Adjust the volume to 50 µl with TE buffer, pH 8. ☐ Mix thoroughly by inversion and gently flicking to avoiding unwanted shearing. ☐ Spin down briefly in a microfuge.	

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☐ Add 35 µl of the Custom buffer 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 a slightly higher DNA recovery yield is desired. ☐ Prepare 500 µl of 80% 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 80% 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 48 µl Amplified 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 ☐ 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. ☐ Resuspend the AMPure XP beads (AXP) by vortexing. ☐ Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 60 µl of resuspended the AMPure XP Beads (AXP) 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 80% 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 80% 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 third-party ligase is supplied with its own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down the Ligation Adapter (LA) 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. ☐ Thaw the Short Fragment Buffer (SFB) at RT and mix by vortexing. Then 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 Ligation Adapter (LA) ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads (AXP) by vortexing. ☐ Add 40 µl of resuspended AMPure XP Beads (AXP) 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 250 µl of 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.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB). IMPORTANT ☐ We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 flow cell.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 MinION and GridION Flow Cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice.	
IMPORTANT ☐ For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.	
To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at RT. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml ☐ 30 µl Flow Cell Tether (FCT)	
☐ Open the MinION or GridION device 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 Library Beads (LIB) by pipetting.	
IMPORTANT ☐ The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), 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.	