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

Before start checklist

Materials

- [ ] 50-100 fmol of amplicon DNA
- [ ] Ligation Sequencing Kit V14 (SQK-LSK114)
- [ ] Flongle Sequencing Expansion (EXP-FSE002)

Consumables

- [ ] Flongle Flow Cell
- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] 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
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

Equipment

- [ ] Flongle adapter
- [ ] MinION or GridION device
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] 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
End-prep	
Check your flow cell.	
IMPORTANT ☐ Flow cell deterioration/saturation	
IMPORTANT ☐ Flongle Sequencing Expansion (EXP-FSE002)	
IMPORTANT ☐ Optional fragmentation and size selection	
☐ Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the 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 Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep 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.	
IMPORTANT ☐ Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.	
IMPORTANT ☐ It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.	
Prepare the amplicon DNA in Nuclease-free water: ☐ Transfer 50-100 fmol of amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube ☐ Adjust the volume to 24.5 µl with Nuclease-free water ☐ Mix thoroughly by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge	
In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 0.5 µl DCS ☐ 24.5 µl DNA ☐ 3.5 µl Ultra II End-prep Reaction Buffer ☐ 1.5 µl Ultra II End-prep Enzyme Mix	
☐ Thoroughly mix the reaction by gently pipetting and briefly spinning 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 30 µl of resuspended 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Remove the tube from the magnetic rack and resuspend the 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, for at least 1 minute. ☐ Remove and retain 31 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.	
☐ 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.	
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)	
☐ Thaw either Long Fragment Buffer (LFB) or 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: ☐ 30 µl DNA sample from the previous step ☐ 12.5 µl Ligation Buffer (LNB) ☐ 5 µl NEBNext Quick T4 DNA Ligase ☐ 2.5 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 20 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or 125 µl Short 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 7 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, for at least 1 minute. ☐ Remove and retain 7 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 your final library to 5-10 fmol in 5 µl of Elution Buffer (EB).	
IMPORTANT ☐ We recommend loading 5-10 fmol of this final prepared library onto the R10.4.1 flow cell.	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Loading the Flongle Flow Cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-FLG114).	
IMPORTANT ☐ Flongle Sequencing Expansion (EXP-FSE002)	
IMPORTANT ☐ Do NOT touch the reverse side of the Flongle flow cell array or the contact pads on the Flongle adapter. ALWAYS wear gloves when handling Flongle flow cells and adapters to avoid damage to the flow cell or adapter.	
The diagram below shows the components of the Flongle flow cell: ☐ 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. ☐ In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flow Cell Flush (FCF) with 3 µl of Flow Cell Tether (FCT) and mix by pipetting. ☐ Place the Flongle adapter into the MinION or one of the five GridION positions.	
IMPORTANT ☐ The adapter needs to be plugged into your device, and the device should be plugged in and powered on before inserting the Flongle flow cell.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click. Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed, as follows: ☐ Lift up the seal tab: ☐ Pull the seal tab to open access to the sample port: ☐ Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid: ☐ To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the 120 µl of priming fluid into the Flongle flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.	
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.	
Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows: ☐ 15 µl Sequencing Buffer (SB) ☐ 10 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using. ☐ 5 µl DNA library ☐ To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously. Seal the Flongle flow cell using the adhesive on the seal tab, as follows: ☐ Stick the transparent adhesive tape to the sample port. ☐ Replace the top (Wheel icon section) of the seal tab to its original position.	
Close the device lid and set up a sequencing run on MinKNOW.	