

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 device - flow cell and adapter
- 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, cat # E7546)
- NEBNext Quick Ligation Module (NEB, cat # 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

- Hula mixer (gentle rotator mixer)
- Magnetic separator, 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****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.

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Thaw all reagents on ice. <input type="checkbox"/> Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. <input type="checkbox"/> Always spin down tubes before opening for the first time each day. <input type="checkbox"/> 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. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Do not vortex the NEBNext Ultra II End Prep Enzyme Mix. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing. 	
<p>Prepare the amplicon DNA in Nuclease-free water:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Transfer 50-100 fmol of amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube <input type="checkbox"/> Adjust the volume to 24.5 µl with Nuclease-free water <input type="checkbox"/> Mix thoroughly by flicking the tube to avoid unwanted shearing <input type="checkbox"/> Spin down briefly in a microfuge <p>In a 0.2 ml thin-walled PCR tube, mix the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 0.5 µl DCS <input type="checkbox"/> 24.5 µl DNA <input type="checkbox"/> 3.5 µl Ultra II End-prep Reaction Buffer <input type="checkbox"/> 1.5 µl Ultra II End-prep Enzyme Mix <ul style="list-style-type: none"> <input type="checkbox"/> Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly. <input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. <input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Add 30 µl of resuspended AMPure XP beads (AXP) to the end-prep reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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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<ul style="list-style-type: none"> <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> 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	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice. <input type="checkbox"/> 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. <input type="checkbox"/> Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice. 	
<p>IMPORTANT</p> <p>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.</p> <ul style="list-style-type: none"> <input type="checkbox"/> To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB) <input type="checkbox"/> To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB) 	
<ul style="list-style-type: none"> <input type="checkbox"/> Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. <p>In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 30 µl DNA sample from the previous step <input type="checkbox"/> 12.5 µl Ligation Buffer (LNB) <input type="checkbox"/> 5 µl NEBNext Quick T4 DNA Ligase <input type="checkbox"/> 2.5 µl Ligation Adapter (LA) <ul style="list-style-type: none"> <input type="checkbox"/> Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly. <input type="checkbox"/> Incubate the reaction for 10 minutes at RT. <input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Add 20 µl of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. 	

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<ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> 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.	
<ul style="list-style-type: none"> <input type="checkbox"/> Make up your library to 5 µl at 5-10 fmol. 	
IMPORTANT <ul style="list-style-type: none"> <input type="checkbox"/> 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 until ready to load.	
Loading the Flongle flow cell	
IMPORTANT <ul style="list-style-type: none"> <input type="checkbox"/> Flongle Sequencing Expansion (EXP-FSE002) 	
IMPORTANT <ul style="list-style-type: none"> <input type="checkbox"/> 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:	
<ul style="list-style-type: none"> <input type="checkbox"/> Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at RT. Mix by vortexing and spin down. <input type="checkbox"/> 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. <input type="checkbox"/> Place the Flongle adapter into the MinION or one of the five GridION positions. 	
IMPORTANT <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<input type="checkbox"/> Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click.	

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<p>Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed, as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Lift up the seal tab: <input type="checkbox"/> Pull the seal tab to open access to the sample port: <input type="checkbox"/> Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid: <input type="checkbox"/> 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 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. <p>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:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 15 µl Sequencing Buffer (SB) <input type="checkbox"/> 10 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using. LIS can be used instead of LIB when preparing libraries with the Ligation Sequencing Kit V14 (SQK-LSK114) <input type="checkbox"/> 5 µl DNA library <input type="checkbox"/> 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. <p>Seal the Flongle flow cell using the adhesive on the seal tab, as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Stick the transparent adhesive tape to the sample port. <input type="checkbox"/> Replace the top (Wheel icon section) of the seal tab to its original position. <input type="checkbox"/> Replace the sequencing platform lid. 	