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Before start checklist		
Materials	Consumables	Equipment
<1 μg (or <100–200 fmol) high molecular weight genomic DNA	☐ Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)	Hula mixer (gentle rotator mixer)
Ligation Sequencing Kit (SQK-LSK109)	NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:	Magnetic rack, suitable for 1.5 ml Eppendorf tubes
PCR Expansion (EXP-PCA001)	NEBNext FFPE Repair Mix (NEB, M6630)	Microfuge
Flow Cell Priming Kit (EXP-FLP002)	NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)	☐ Vortex mixer
	NEBNext Quick Ligation Module (NEB, E6056)	Thermal cycler
	LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)	lce bucket with ice
	NEB Blunt/TA Ligase Master Mix (NEB, M0367)	☐ Timer
	1.5 ml Eppendorf DNA LoBind tubes	Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
	0.2 ml thin-walled PCR tubes	
	Nuclease-free water (e.g. ThermoFisher, AM9937)	
	Freshly prepared 70% ethanol in nuclease-free water	
	10 mM Tris-HCl pH 8.5	
INSTRUCTIONS		NOTES/OBSERVATIONS
End-prep		
Prepare the DNA in Nuclease-free water Transfer <1 µg (or <100-200 fmol) genomic D Adjust the volume to 48 µl with Nuclease-free Mix thoroughly by flicking the tube to avoid un Spin down briefly in a microfuge		

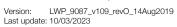
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INSTRUCTIONS	NOTES/OBSERVATIONS
In a 0.2 ml thin-walled PCR tube, mix the following: 48 μl <1 μg DNA 7 μl Ultra II End-prep Reaction Buffer 3 μl Ultra II End-prep Enzyme Mix 2 μl Nuclease-free water	
☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down.	
☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.	
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.	
$\hfill \Box$ Add 60 μI 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.	
\square 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.	
$\hfill\square$ 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 next step. However, at this point it is also possible to store the sample at 4°C overnight.	
PCR adapters ligation and amplification	
Add the reagents as follows in the order below: 30 µl End-prepped DNA 20 µl PCR Adapter (PCA) 50 µl Blunt/TA Ligase Master Mix	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down.	
☐ Incubate the reaction for 10 minutes at RT.	
Resuspend the AMPure XP beads by vortexing.	
$\hfill \Box$ Add 40 μI 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.	
☐ 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.	
Given 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 26 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 26 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 μl of adapted DNA using a Qubit fluorometer.	
☐ Dilute the library to a concentration of 10 ng/µl with Nuclease-free water or 10 mM Tris-HCl pH 8.5.	
Set up the adapted DNA PCR as follows: 46 µl Nuclease-free water 2 µl Primer Mix (PRM) 2 µl 10 ng/µl adapter ligated template 50 µl LongAmp Taq 2x master mix	
☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down.	
Amplify using the following cycling conditions: Initial denaturation 3 mins @ 95 °C (1 cycle) Denaturation 15 secs @ 95 °C (15-18 (b) cycles) Annealing 15 secs (a) @ 62 °C (a) (15-18 (b) cycles) Extension dependent on length of target fragment (d) @ 65 °C (c) (15-18 (b) cycles) Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle) Hold @ 4 °C	
Resuspend the AMPure XP beads by vortexing.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
In a 0.2 ml thin-walled PCR tube, mix the following: 47 µl DNA from the previous step 1 µl DNA CS (optional) 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	
\square 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.	
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	

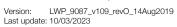
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INSTRUCTIONS	NOTES/OBSERVATIONS
Adapter ligation and clean-up	
IMPORTANT	
Although the recommended third-party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.	
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 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)	
☐ 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 (AMX)	
\square 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.	

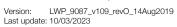
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INSTRUCTIONS	NOTES/OBSERVATIONS
Repeat the previous step.	
Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.	
Remove the tube from 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.	
IMPORTANT We recommend loading 5-50 fmol of the final prepared library onto a 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.	
Priming and loading the SpotON flow cell	
 □ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), 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 device lid and slide the flow cell under the clip.	
 □ Open the MinION device lid and slide the flow cell under the clip. □ Slide the flow cell priming port cover clockwise to open the priming port. 	

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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.	
☐ Thoroughly mix the contents of the Loading Beads (LB) by pipetting.	
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 new tube, prepare the library for loading as follows:	
☐ 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 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, close the priming port and replace the MinION device lid.	
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.	

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