

Ligation sequencing amplicons - PCR barcoding (SQK-LSK112 with EXP-PBC096)

Version: PBA96\_9152\_v112\_revH\_09Feb2022  
 Last update: 15/03/2023

Flow Cell Number: .....

DNA Samples: .....

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> 100-200 fmol first-round PCR product in 24 µl (with tailed primers, see full protocol for further details)	<input type="checkbox"/> Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)	<input type="checkbox"/> Hula mixer (gentle rotator mixer)
<input type="checkbox"/> PCR Barcoding Expansion 1-96 (EXP-PBC096)	<input type="checkbox"/> NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:	<input type="checkbox"/> Magnetic rack, suitable for 1.5 ml Eppendorf tubes
<input type="checkbox"/> Ligation Sequencing Kit (SQK-LSK112)	<input type="checkbox"/> NEBNext FFPE Repair Mix (NEB, M6630)	<input type="checkbox"/> Microfuge
	<input type="checkbox"/> NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)	<input type="checkbox"/> Vortex mixer
	<input type="checkbox"/> NEBNext Quick Ligation Module (NEB, E6056)	<input type="checkbox"/> Thermal cycler
	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Ice bucket with ice
	<input type="checkbox"/> 0.2 ml thin-walled PCR tubes	<input type="checkbox"/> Timer
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, AM9937)	<input type="checkbox"/> Pipettes and pipette tips Multichannel, P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	
	<input type="checkbox"/> LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)	
	<input type="checkbox"/> 0.2 ml 96-well PCR plate	

INSTRUCTIONS	NOTES/OBSERVATIONS
<p><b>Barcoding PCR</b></p> <p>Prepare the DNA in Nuclease-free water.</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Transfer &lt;100-200 fmol of each DNA sample to be barcoded into a 1.5 ml Eppendorf DNA LoBind tube</li> <li><input type="checkbox"/> Adjust the volume to 24 µl with Nuclease-free water</li> <li><input type="checkbox"/> Mix thoroughly by flicking the tube to avoid unwanted shearing</li> <li><input type="checkbox"/> Spin down briefly in a microfuge</li> </ul> <p>In a 0.2 ml 96 well plate, set up a barcoding PCR reaction as follows for each library:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 1 µl PCR Barcode (one of BC1-BC96, at 10 µM)</li> <li><input type="checkbox"/> 24 µl &lt;100-200 fmol first-round PCR product</li> <li><input type="checkbox"/> 25 µl LongAmp Taq 2x master mix</li> </ul>	

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<p><input type="checkbox"/> Mix by pipetting.</p> <p><input type="checkbox"/> Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner.</p> <p>Amplify using the following cycling conditions:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Initial denaturation 3 mins @ 95 °C (1 cycle)</li> <li><input type="checkbox"/> Denaturation 15 secs @ 95 °C (12-15 (b) cycles)</li> <li><input type="checkbox"/> Annealing 15 secs (a) @ 62 °C (a) (12-15 (b) cycles)</li> <li><input type="checkbox"/> Extension dependent on length of target fragment (d) @ 65 °C (c) (12-15 (b) cycles)</li> <li><input type="checkbox"/> Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle)</li> <li><input type="checkbox"/> Hold @ 4 °C</li> </ul> <p><input type="checkbox"/> Purify the barcoded DNA using standard methods which are suitable for the fragment size.</p> <p><input type="checkbox"/> Quantify the barcoded library using standard techniques, and pool all barcoded libraries in the desired ratios in a 1.5 ml DNA LoBind Eppendorf tube.</p> <p><input type="checkbox"/> Prepare 1 µg of pooled barcoded libraries in 49 µl Nuclease-free water.</p>	
<p>This pooled library is now ready to be end-repaired and adapted for sequencing. However, at this point it is also possible to store the sample at 4°C overnight.</p>	
<p><b>End-prep</b></p>	
<p><input type="checkbox"/> Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice.</p> <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"> <li><input type="checkbox"/> Thaw all reagents on ice.</li> <li><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.</li> <li><input type="checkbox"/> Always spin down tubes before opening for the first time each day.</li> <li><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.</li> </ul> <p>In a 0.2 ml thin-walled PCR tube, mix the following:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 1 µl DNA Control Sample (DCS)</li> <li><input type="checkbox"/> 49 µl DNA</li> <li><input type="checkbox"/> 7 µl Ultra II End-prep Reaction Buffer</li> <li><input type="checkbox"/> 3 µl Ultra II End-prep Enzyme Mix</li> </ul> <p><input type="checkbox"/> Ensure the components are thoroughly mixed by pipetting.</p> <p><input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.</p>	

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<p><b>IMPORTANT</b></p> <p><input type="checkbox"/> AMPure XP bead clean-up</p> <p><input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing.</p> <p><input type="checkbox"/> Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.</p> <p><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</p> <p><input type="checkbox"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water.</p> <p><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.</p> <p><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.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><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.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 61 µl Nuclease-free water. Incubate for 2 minutes at RT.</p> <p><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.</p> <p><input type="checkbox"/> Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p>	
<p>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.</p>	
<p><b>Adapter ligation and clean-up</b></p>	
<p><b>IMPORTANT</b></p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Spin down the Adapter Mix H (AMX H) and Quick T4 Ligase, and place on ice.</p> <p><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.</p> <p><input type="checkbox"/> Thaw the Elution Buffer (EB) at RT and mix by vortexing. Then spin down and place on ice.</p>	

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<p><b>IMPORTANT</b></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 &gt;3 kb, or purify all fragments equally.</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)</li> <li><input type="checkbox"/> To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)</li> </ul>	
<ul style="list-style-type: none"> <li><input type="checkbox"/> 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.</li> <li><input type="checkbox"/> 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.</li> </ul> <p>In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 60 µl DNA sample from the previous step</li> <li><input type="checkbox"/> 25 µl Ligation Buffer (LNB)</li> <li><input type="checkbox"/> 10 µl NEBNext Quick T4 DNA Ligase</li> <li><input type="checkbox"/> 5 µl Adapter Mix H (AMX H)</li> </ul> <ul style="list-style-type: none"> <li><input type="checkbox"/> Thoroughly mix the reaction by gently pipetting and briefly spinning down.</li> <li><input type="checkbox"/> Incubate the reaction for 10 minutes at RT.</li> </ul>	
<p><b>IMPORTANT</b></p> <ul style="list-style-type: none"> <li><input type="checkbox"/> If you have omitted the AMPure purification step after DNA repair and end-prep, do not incubate the reaction for longer than 10 minutes.</li> </ul>	
<ul style="list-style-type: none"> <li><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</li> <li><input type="checkbox"/> Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.</li> <li><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</li> <li><input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.</li> <li><input type="checkbox"/> 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.</li> <li><input type="checkbox"/> Repeat the previous step.</li> <li><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.</li> <li><input type="checkbox"/> 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.</li> <li><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.</li> <li><input type="checkbox"/> Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.</li> </ul>	

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Quantify 1 µl of eluted sample using a Qubit fluorometer.	
<p><b>IMPORTANT</b></p> <input type="checkbox"/> 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.	
<p><b>Priming and loading the SpotON flow cell</b></p>	
<p><b>IMPORTANT</b></p> <input type="checkbox"/> 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.	
<p>Using the Loading Solution</p> <input type="checkbox"/> 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.	
<p><b>IMPORTANT</b></p> <input type="checkbox"/> 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.	
<p>After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:</p> <input type="checkbox"/> Set a P1000 pipette to 200 µl <input type="checkbox"/> Insert the tip into the priming port <input type="checkbox"/> 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	
<p>Note: Visually check that there is continuous buffer from the priming port across the sensor array.</p> <input type="checkbox"/> 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.	
<input type="checkbox"/> Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.	

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<p><b>IMPORTANT</b></p> <p><input type="checkbox"/> 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.</p>	
<p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 37.5 µl Sequencing Buffer II (SBI)</li> <li><input type="checkbox"/> 25.5 µl Loading Beads II (LBII), mixed immediately before use, or Loading Solution (LS), if using</li> <li><input type="checkbox"/> 12 µl DNA library</li> </ul> <p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible.</li> <li><input type="checkbox"/> Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.</li> <li><input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading.</li> <li><input type="checkbox"/> 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.</li> <li><input type="checkbox"/> 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.</li> </ul>	
<p><b>Flow cell reuse and returns</b></p>	
<ul style="list-style-type: none"> <li><input type="checkbox"/> 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.</li> <li><input type="checkbox"/> Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.</li> </ul>	
<p><b>IMPORTANT</b></p> <p><input type="checkbox"/> 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.</p>	