

PCR barcoding genomic DNA (SQK-LSK109)

Version: PBGE12_9066_v109_revO_14Aug2019
 Last update: 20/03/2020



Flow Cell Number:

DNA Samples:

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> <1 µg of each DNA sample to be barcoded in 45 µl	<input type="checkbox"/> NEB Blunt/TA Ligase Master Mix (M0367)	<input type="checkbox"/> Hula mixer (gentle rotator mixer)
<input type="checkbox"/> PCR Barcoding Expansion 1-12 (EXP-PBC001)	<input type="checkbox"/> Agencourt AMPure XP beads	<input type="checkbox"/> Magnetic separator, suitable for 1.5 ml Eppendorf tubes
<input type="checkbox"/> Ligation Sequencing Kit (SQK-LSK109)	<input type="checkbox"/> NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:	<input type="checkbox"/> Microfuge
<input type="checkbox"/> Flow Cell Priming Kit (EXP-FLP002)	<input type="checkbox"/> NEBNext FFPE Repair Mix (M6630)	<input type="checkbox"/> Vortex mixer
	<input type="checkbox"/> NEBNext Ultra II End repair/dA-tailing Module (E7546)	<input type="checkbox"/> Thermal cycler
	<input type="checkbox"/> NEBNext Quick Ligation Module (E6056)	<input type="checkbox"/> Ice bucket with ice
	<input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes	<input type="checkbox"/> Timer
	<input type="checkbox"/> 0.2 ml thin-walled PCR tubes	<input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, cat # AM9937)	
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	
	<input type="checkbox"/> LongAmp Taq 2X Master Mix (e.g. NEB M0287)	
INSTRUCTIONS		NOTES/OBSERVATIONS
End-prep		
<p>Prepare the DNA in Nuclease-free water.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Transfer <1 µg DNA into a 1.5 ml Eppendorf DNA LoBind tube <input type="checkbox"/> Adjust the volume to 45 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>Using a separate 0.2 ml thin-walled PCR tube for each DNA sample to be barcoded, set up the end-repair / dA-tailing reactions as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 45 µl <1 µg DNA <input type="checkbox"/> 7 µl Ultra II End-prep reaction buffer <input type="checkbox"/> 3 µl Ultra II End-prep enzyme mix <input type="checkbox"/> 5 µl Nuclease-free water 		

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<ul style="list-style-type: none"> <input type="checkbox"/> Mix by pipetting. <input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins. <input type="checkbox"/> Transfer each sample to a separate 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Resuspend the AMPure XP beads by vortexing. <input type="checkbox"/> Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare sufficient fresh 70% ethanol in Nuclease-free water. <input type="checkbox"/> Place on magnetic rack, allow beads to pellet and pipette off supernatant. <input type="checkbox"/> Keep the tube on the magnet and wash the beads with 180 µl of freshly-prepared 70% 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 tubes 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. <input type="checkbox"/> Remove the tubes 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. <input type="checkbox"/> Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 0.2 ml thin-walled PCR tube. <input type="checkbox"/> Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng. 	
<p>Take forward approximately 700 ng of end-prepped DNA in 30 µl Nuclease-free water into adapter ligation.</p>	
<p>Ligation of Barcode Adapter</p>	
<p>Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 30 µl End prep DNA <input type="checkbox"/> 20 µl Barcode Adapter <input type="checkbox"/> 50 µl Blunt/TA Ligase Master Mix <ul style="list-style-type: none"> <input type="checkbox"/> Mix gently by flicking the tube, and spin down. <input type="checkbox"/> Incubate the reaction for 10 minutes at RT. <input type="checkbox"/> Resuspend the AMPure XP beads by vortexing. <input type="checkbox"/> Add 40 µl of resuspended AMPure XP beads 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"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. 	

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<p><input type="checkbox"/> Spin down the sample and pellet on a magnet. 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 70% 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 70% ethanol. Briefly allow to dry.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 25 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.</p> <p>Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube</p> <p><input type="checkbox"/> Dispose of the pelleted beads</p> <p><input type="checkbox"/> Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.</p> <p><input type="checkbox"/> Dilute the library to a concentration of 10 ng/µl with Nuclease-free water or 10 mM Tris-HCl pH 8.5.</p>	
<p>Barcoding PCR</p>	
<p>Set up a barcoding PCR reaction as follows for each library:</p> <p><input type="checkbox"/> 2 µl PCR Barcode (one of BC01-BC12, at 10 µM)</p> <p><input type="checkbox"/> 2 µl 10 ng/µl adapter ligated template</p> <p><input type="checkbox"/> 50 µl LongAmp Taq 2x master mix</p> <p><input type="checkbox"/> 46 µl Nuclease-free water</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p>Amplify using the following cycling conditions:</p> <p><input type="checkbox"/> Initial denaturation 3 mins @ 95 °C (1 cycle)</p> <p><input type="checkbox"/> Denaturation 15 secs @ 95 °C (12-15 (b) cycles)</p> <p><input type="checkbox"/> Annealing 15 secs (a) @ 62 °C (a) (12-15 (b) cycles)</p> <p><input type="checkbox"/> Extension dependent on length of target fragment (d) @ 65 °C (c) (12-15 (b) cycles)</p> <p><input type="checkbox"/> Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle)</p> <p><input type="checkbox"/> Hold @ 4 °C</p> <p><input type="checkbox"/> Transfer each sample to a separate 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</p> <p><input type="checkbox"/> Add 60 µl of resuspended AMPure XP beads to each 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 sufficient fresh 70% ethanol in Nuclease-free water.</p>	

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<p><input type="checkbox"/> Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes 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 70% 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 tubes 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 tubes from the magnetic rack and resuspend each pellet in 10 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.</p> <p>Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube</p> <p><input type="checkbox"/> Dispose of the pelleted beads</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 47 µl Nuclease-free water.</p>	
<p>This pooled library is now ready to be end-repaired and adapted for nanopore sequencing.</p>	
<p>DNA repair and end-prep</p>	
<p><input type="checkbox"/> Thaw DNA CS (DCS) at RT, spin down, mix by pipetting, and place on ice.</p> <p><input type="checkbox"/> 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.</p> <p>In a 0.2 ml thin-walled PCR tube, mix the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 1 µl DNA CS <input type="checkbox"/> 47 µl DNA <input type="checkbox"/> 3.5 µl NEBNext FFPE DNA Repair Buffer <input type="checkbox"/> 2 µl NEBNext FFPE DNA Repair Mix <input type="checkbox"/> 3.5 µl Ultra II End-prep reaction buffer <input type="checkbox"/> 3 µl Ultra II End-prep enzyme mix <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> AMPure XP bead clean-up</p>	
<p><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</p> <p><input type="checkbox"/> Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.</p>	

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<ul style="list-style-type: none"> <input type="checkbox"/> Add 60 µl of resuspended AMPure XP beads 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 70% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet until eluate 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 70% 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. <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. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless. <input type="checkbox"/> Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	
<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>Adapter ligation and clean-up</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Although the recommended 3rd 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. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Spin down Adapter Mix (AMX) 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> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<ul style="list-style-type: none"> <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. <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. <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"/> 60 µl DNA sample from the previous step <input type="checkbox"/> 25 µl Ligation Buffer (LNB) <input type="checkbox"/> 10 µl NEBNext Quick T4 DNA Ligase <input type="checkbox"/> 5 µl Adapter Mix (AMX) 	

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<ul style="list-style-type: none"> <input type="checkbox"/> Mix gently by flicking the tube, and spin down. <input type="checkbox"/> Incubate the reaction for 10 minutes at RT. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <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. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Resuspend the AMPure XP beads by vortexing. <input type="checkbox"/> Add 40 µl of resuspended AMPure XP beads 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. <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. <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 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. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless. <input type="checkbox"/> Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Quantify 1 µl of eluted sample using a Qubit fluorometer. 	
<p>The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> We recommend loading 5–50 fmol of this final prepared library onto R9.4.1 flow cells. For R10.3 flow cells, we recommend loading 25-75 fmol. 	
<p>Priming and loading the SpotON flow cell</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before placing the tubes on ice as soon as thawing is complete. 	

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<ul style="list-style-type: none"> <input type="checkbox"/> Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down then return to ice. <input type="checkbox"/> Open the MinION Mk1B lid and slide the flow cell under the clip. <input type="checkbox"/> Slide the priming port cover clockwise to open the priming port. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <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 (a few μl):</p> <ul style="list-style-type: none"> <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, or until you can see a small volume of buffer entering the pipette tip <ul style="list-style-type: none"> <input type="checkbox"/> 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. <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 5 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 (LB) by pipetting. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	
<p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 μl Sequencing Buffer (SQB) <input type="checkbox"/> 25.5 μl Loading Beads (LB), mixed immediately before use <input type="checkbox"/> 12 μl DNA library <p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible. <input type="checkbox"/> Load 200 μl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> Add 75 μl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. <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 Mk1B lid. 	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Ending the experiment	
<input type="checkbox"/> After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR <input type="checkbox"/> Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT <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.	