

Rapid sequencing gDNA - low input PCR barcoding (SQK-PBK004)

Version: PBK\_9073\_v1\_revS\_14Aug2019  
 Last update: 18/05/2023

Flow Cell Number: .....

DNA Samples: .....

Before start checklist		
Materials	Consumables	Equipment
<input type="checkbox"/> 100 ng high molecular weight genomic DNA	<input type="checkbox"/> Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)	<input type="checkbox"/> Hula mixer (gentle rotator mixer)
<input type="checkbox"/> PCR Barcoding Kit (SQK-PBK004)	<input type="checkbox"/> NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)	<input type="checkbox"/> Magnetic rack, suitable for 1.5 ml Eppendorf tubes
<input type="checkbox"/> Flow Cell Priming Kit (EXP-FLP002)	<input type="checkbox"/> NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)	<input type="checkbox"/> Microfuge
	<input type="checkbox"/> Covaris g-TUBE	<input type="checkbox"/> Vortex mixer
	<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"/> Thermal cycler
	<input type="checkbox"/> Freshly prepared 70% ethanol in nuclease-free water	<input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
	<input type="checkbox"/> LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)	
	<input type="checkbox"/> 10 mM Tris-HCl pH 8.0 with 50 mM NaCl	
	<input type="checkbox"/> (optional) Exonuclease I (NEB, M0293)	

INSTRUCTIONS	NOTES/OBSERVATIONS
<p><b>DNA fragmentation</b></p> <p><b>OPTIONAL</b></p> <p>Prepare the DNA in Nuclease-free water.</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Transfer 100 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube</li> <li><input type="checkbox"/> Adjust the volume to 50 µl with Nuclease-free water</li> <li><input type="checkbox"/> Mix thoroughly by gently pipetting the entire volume up and down 10 times</li> <li><input type="checkbox"/> Spin down briefly in a microfuge</li> </ul> <p><input type="checkbox"/> Transfer 100 ng genomic DNA in 50 µl to the Covaris g-TUBE.</p> <p>Spin the g-TUBE for 1 minute at RT (Eppendorf 5424; 6000 rpm for 8 kb fragments).</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Spin the g-TUBE for 1 minute</li> <li><input type="checkbox"/> Remove and check all the DNA has passed through the g-TUBE</li> <li><input type="checkbox"/> If DNA remains in the upper chamber, spin again for 1 minute at the same speed</li> </ul>	

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<p>Invert the g-TUBE and spin again for 1 minute to collect the fragmented DNA.</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Remove g-TUBE, invert the tube and replace into the centrifuge</li> <li><input type="checkbox"/> Spin the g-TUBE for 1 minute</li> <li><input type="checkbox"/> Remove and check the DNA has passed into the lower chamber</li> <li><input type="checkbox"/> If DNA remains in the upper chamber, spin again for 1 minute</li> <li><input type="checkbox"/> Remove g-TUBE</li> </ul> <p><input type="checkbox"/> Transfer the 50 µl fragmented DNA to a clean 1.5 ml Eppendorf DNA LoBind tube.</p>	
<p>100 ng fragmented DNA in 50 µl is taken into the next step.</p>	
<p><b>End-prep</b></p> <p>Prepare the NEBNext Ultra II End Repair/dA-Tailing Module reagents according to the 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 reagent tube to ensure they are well mixed.</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 and FFPE DNA Repair 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 several seconds to ensure the reagent is thoroughly mixed.</li> <li><input type="checkbox"/> The FFPE DNA repair buffer may have a yellow tinge and is fine to use if yellow.</li> </ul> <p>Mix the following reagents in a 0.2 ml thin-walled PCR tube:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 50 µl 100 ng fragmented 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> <ul style="list-style-type: none"> <li><input type="checkbox"/> Mix well by gently pipetting the entire volume within the tube up and down 10 times.</li> <li><input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.</li> <li><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</li> <li><input type="checkbox"/> Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.</li> <li><input type="checkbox"/> Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting.</li> <li><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</li> <li><input type="checkbox"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.</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"/> 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.</li> <li><input type="checkbox"/> Repeat the previous step.</li> </ul>	

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<ul style="list-style-type: none"> <li><input type="checkbox"/> Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.</li> <li><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 16 µl Nuclease-free water. Incubate for 2 minutes at RT.</li> <li><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless.</li> <li><input type="checkbox"/> Remove and retain 16 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</li> <li><input type="checkbox"/> Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.</li> </ul>	
<p><b>PCR adapters ligation and amplification</b></p>	
<ul style="list-style-type: none"> <li><input type="checkbox"/> Thaw the Blunt/TA Ligase Master Mix, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Check for any precipitate (if any is visible, continue to mix) and place on ice.</li> <li><input type="checkbox"/> Thaw the Barcode Adapter (BCA), spin down and mix by pipetting the entire volume within the tube up and down 10 times. Place on ice.</li> </ul> <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"> <li><input type="checkbox"/> 15 µl End-prepped DNA</li> <li><input type="checkbox"/> 10 µl Barcode Adapters (BCA)</li> <li><input type="checkbox"/> 25 µl Blunt/TA Ligase Master Mix</li> </ul> <ul style="list-style-type: none"> <li><input type="checkbox"/> Mix well by gently pipetting the entire volume within the tube up and down 10 times.</li> <li><input type="checkbox"/> Incubate the reaction for 10 minutes at RT.</li> <li><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</li> <li><input type="checkbox"/> Add 20 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.</li> <li><input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</li> <li><input type="checkbox"/> Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.</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"/> 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.</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 ethanol. 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 pellet in 21 µl Nuclease-free water. Incubate for 2 minutes at RT.</li> <li><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless.</li> </ul>	

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<p>Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube</li> <li><input type="checkbox"/> Dispose of the pelleted beads</li> </ul> <p><input type="checkbox"/> Quantify 1 µl of adapted DNA using a Qubit fluorometer.</p> <p><input type="checkbox"/> Calculate how much DNA to take forward into the PCR step for a final DNA concentration of 0.2 ng/µl in a 50 µl reaction.</p> <p><input type="checkbox"/> Thaw the LongAmp® Hot Start Taq 2X Master Mix at RT, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Place on ice.</p> <p><input type="checkbox"/> Thaw the required Barcode Primers (BP01-12) at RT, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Place on ice.</p> <p>Set up the adapted DNA PCR as follows:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Adapter ligated DNA, diluted                     <ul style="list-style-type: none"> <li>- Volume: x µl</li> <li>- Final concentration in 50 µl: 0.2 ng/µl</li> </ul> </li> <li><input type="checkbox"/> Nuclease-free water                     <ul style="list-style-type: none"> <li>- Volume: 24-x µl</li> </ul> </li> <li><input type="checkbox"/> Barcode Primers (BP 01-12, at 10 µM)                     <ul style="list-style-type: none"> <li>- Volume: 1 µl</li> </ul> </li> <li><input type="checkbox"/> LongAmp® Hot Start Taq 2x Master Mix                     <ul style="list-style-type: none"> <li>- Volume: 25 µl</li> </ul> </li> </ul> <p><input type="checkbox"/> Mix well by gently pipetting the entire volume within the tube up and down 10 times.</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 (14 (b) cycles)</li> <li><input type="checkbox"/> Annealing 15 secs (a) @ 56 °C (a) (14 (b) cycles)</li> <li><input type="checkbox"/> Extension 50 secs/kb @ 65 °C (c) (14 (b) cycles)</li> <li><input type="checkbox"/> Final extension 6 mins @ 65 °C (1 cycle)</li> <li><input type="checkbox"/> Hold @ 4 °C</li> </ul> <p><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</p> <p><input type="checkbox"/> Add 30 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.</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 70% ethanol in Nuclease-free water.</p> <p><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.</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>	

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<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 pellet in 10 µl of 10 mM Tris.HCl pH 8.0 with 50 mM NaCl. 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"/> Dispose of the pelleted beads</p> <p><input type="checkbox"/> Quantify 1 µl of adapted DNA using a Qubit fluorometer.</p> <p><input type="checkbox"/> Pool all barcoded libraries in the desired ratios to a total of 50-100 fmoles in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.</p>	
<p><b>Rapid adapter ligation</b></p>	
<p><input type="checkbox"/> Add 1 µl RAP to the 10 µl amplified DNA library.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Incubate the reaction for 5 minutes at RT.</p>	
<p>The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.</p>	
<p><b>Priming and loading the SpotON flow cell</b></p>	
<p><input type="checkbox"/> 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.</p> <p><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 at RT.</p> <p><input type="checkbox"/> Open the MinION device lid and slide the flow cell under the clip.</p> <p><input type="checkbox"/> Slide the priming port cover clockwise to open the priming port.</p>	
<p><b>IMPORTANT</b></p> <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>	

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<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> <ul style="list-style-type: none"> <li><input type="checkbox"/> Set a P1000 pipette to 200 µl</li> <li><input type="checkbox"/> Insert the tip into the priming port</li> <li><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</li> </ul> <p>Note: Visually check that there is continuous buffer from the priming port across the sensor array.</p> <ul style="list-style-type: none"> <li><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.</li> <li><input type="checkbox"/> Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.</li> </ul>	
<p><b>IMPORTANT</b></p> <ul style="list-style-type: none"> <li><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.</li> </ul>	
<p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 34 µl Sequencing Buffer (SQB)</li> <li><input type="checkbox"/> 25.5 µl Loading Beads (LB), mixed immediately before use</li> <li><input type="checkbox"/> 4.5 µl Nuclease-free water</li> <li><input type="checkbox"/> 11 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> </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> <ul style="list-style-type: none"> <li><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.</li> </ul>	