Ligation sequencing gDNA - PCR barcoding (SQK-LSK110 with EXP-PBC096)

Version: PBGE96_9113_v110_revJ_10Nov2020
Last update: 10/03/2023

Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials

- [ ] <1 µg of each DNA sample to be barcoded in 45 µl
- [ ] PCR Barcoding Expansion 1-96 (EXP-PBC096)
- [ ] Ligation Sequencing Kit (SQK-LSK110)

Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
Prepare the DNA in Nuclease-free water. ☐ Transfer <1 µg DNA of each sample into a fresh 0.2 ml PCR tube or plate ☐ Adjust the volume to 45 µl with Nuclease-free water ☐ Mix thoroughly by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge In a 0.2 ml 96 well PCR plate, set up the end-repair / dA-tailing reactions as follows: ☐ 45 µl <1 µg DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 5 µl Nuclease-free water	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Seal the plate with adhesive film or PCR strip caps, spin down in a centrifuge and incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ 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. ☐ Repeat the previous step. ☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim >700 ng.	
Take forward approximately 700 ng of end-prepped DNA in 30 µl Nuclease-free water into adapter ligation. However, at this point, it is also possible to store the sample at 4°C overnight.	
Ligation of Barcode Adapter	
Add the reagents to a fresh 96-well plate, in the order given below: ☐ 30 µl End-prepped DNA ☐ 20 µl Barcode Adapter ☐ 50 µl Blunt/TA Ligase Master Mix ☐ Ensure the components are thoroughly mixed by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Add 40 µl of resuspended AMPure XP beads to each sample and mix by pipetting up and down ten times. ☐ Allow DNA to bind to beads for 5 minutes at RT. ☐ Prepare sufficient fresh 70% ethanol in Nuclease-free water. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ 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. ☐ Repeat the previous step. ☐ Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution. ☐ Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed. ☐ Remove the plate from the magnet and resuspend pellet in 25 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove eluate once it is clear and colourless. Transfer each eluted sample to a new 96-well PCR plate. ☐ Quantify 1 µl of end-prepped 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.	
Take forward the samples to the next step. However, at this point it is also possible to store the sample at 4°C overnight.	
Barcoding PCR	
Set up a barcoding PCR reaction as follows for each library: ☐ 1 µl PCR Barcode (one of BC1-BC96, at 10 µM) ☐ 2 µl Adapter-ligated DNA ☐ 25 µl LongAmp Taq 2x master mix ☐ 22 µl Nuclease-free water ☐ Ensure the components are thoroughly mixed by pipetting. ☐ Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner. 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	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Purify the barcoded DNA using standard methods which are suitable for the fragment size. ☐ 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. ☐ Prepare 1 µg of pooled barcoded libraries in 47 µl Nuclease-free water.	
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.	
End-prep	
☐ Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice. Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice: ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ 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. In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 1 µl DNA Control Sample (DCS) ☐ 49 µl DNA ☐ 7 µl Ultra II End-prep Reaction Buffer ☐ 3 µl Ultra II End-prep Enzyme Mix ☐ Ensure the components are thoroughly mixed by pipetting. ☐ 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 (AXP) by vortexing. ☐ Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 60 µl of resuspended the AMPure XP Beads (AXP) 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 80% ethanol in Nuclease-free water.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 80% 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.	
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	
IMPORTANT ☐ Although the recommended 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix F (AMX-F) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.	
☐ Spin down the Adapter Mix F (AMX-F) 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 F (AMX-F)	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ 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	
Using the Loading Solution ☐ 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.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Slide the flow cell priming port cover clockwise to open the priming port.	
IMPORTANT ☐ 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.	
After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ 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 Note: Visually check that there is continuous buffer from the priming port across the sensor array. ☐ 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 II (LBII) by pipetting.	
IMPORTANT ☐ 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.	
In a new tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer II (SBII) ☐ 25.5 µl Loading Beads II (LBII), mixed immediately before use, or Loading Solution (LS), if using ☐ 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 and close the priming port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
Place the light shield onto the flow cell, as follows: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.	
Close the device lid and set up a sequencing run on MinKNOW.	
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.	