Ligation sequencing V14 - PCR barcoding (SQK-LSK114 with EXP-PBC001 or EXP-PBC096)

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

Before start checklist

Materials

- [ ] <100–200 fmol of each DNA sample to be barcoded in 45 µl
- [ ] OR <100–200 fmol first-round PCR product (with tailed primers) per sample
- [ ] PCR Barcoding Expansion 1-12 (EXP-PBC001)
- [ ] PCR Barcoding Expansion 1-96 (EXP-PBC096)
- [ ] Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables

- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Magnetic rack
- [ ] Timer
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips Multichannel, P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the DNA in Nuclease-free water. ☐ Transfer <100-200 fmol 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 Set up the end-repair reaction as follows for each library: ☐ 45 µl <100-200 fmol DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 5 µl Nuclease-free water ☐ Mix by pipetting and briefly spin down. ☐ Using a thermal cycler, incubate for 5 minutes at 20 °C and 5 minutes at 65 °C. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Incubate at RT for 5 minutes. ☐ Prepare sufficient fresh 80% ethanol in Nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess. ☐ Spin down the samples and pellet on a magnet until supernatant is clear and colourless. Keep the samples on the magnet, and pipette off the supernatant. ☐ Keep the samples 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 samples back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the samples from the magnet and resuspend each pellet in 16 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 tube or plate well. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.	
Take forward the end-prepped DNA into the next step. However, at this point it is also possible to store the sample at 4°C overnight.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Ligation of Barcode Adapter	
Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. ☐ Spin down the Barcode Adapter (BCA), pipette mix and place on ice. Add the reagents in the order given below, into fresh 0.2 ml PCR tubes or 96-well plate: ☐ 15 µl End-prepped DNA ☐ 10 µl Barcode Adapter ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix by pipetting and briefly spin down. ☐ Incubate the samples for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads to each sample for a 0.4X clean and mix by pipetting up and down ten times. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare sufficient fresh 80% ethanol in Nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep the samples 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. ☐ Place the samples back on the magnet. Pipette off any residual 80% ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the samples 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 and retain the eluate once it is clear and colourless. Transfer each eluted sample to a fresh 0.2 ml PCR tube or plate. ☐ Dispose of the pelleted beads. ☐ Quantify 1 µl of the adapter ligated DNA using a Qubit fluorometer.	
Take forward the adapter ligated samples into the Barcoding PCR step. However, at this point it is also possible to store the sample at 4°C overnight.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Barcoding PCR	
Please note, this protocol is written for a template input of 100–200 fmol with PCR Barcodes (BC01-96) used at a final concentration of 0.2 µM. However, the input mass and the number of PCR cycles may be adjusted as appropriate depending on the requirements of the experiment. ☐ Thaw the PCR Barcodes (BC01-96) required for your number of samples at RT. Individually mix the barcodes by pipetting, spin down, and place on ice.	
IMPORTANT ☐ If using amplicon samples, ensure the samples have undergone a round of PCR with tailed primers before commencing with the protocol.	
Prepare the samples in Nuclease-free water: ☐ Transfer 100-200 fmol of each sample to a clear 0.2 ml PCR tube or plate For 1–12 samples: Adjust the volume to 48 µl with Nuclease-free water For 13–96 samples: Adjust the volume to 24 µl with Nuclease-free water ☐ For 1–12 samples: Adjust the volume to 48 µl with Nuclease-free water ☐ For 13–96 samples: Adjust the volume to 24 µl with Nuclease-free water ☐ Mix thoroughly by flicking the tube or plate to avoid unwanted shearing ☐ Spin down briefly in a microfuge ☐ Select a unique barcode for each sample to be run together on the same flow cell. Set up a barcoding PCR reaction as follows for each library in fresh 0.2 ml PCR tubes or plate. ☐ PCR Barcode (one of BC1-BC96, at 10 µM) - Volume per sample for using 1–12 barcodes: 2 µl - Volume per sample for using 13 barcodes or more: 1 µl ☐ Adapter ligated DNA or amplicons with tailed primers - Volume per sample for using 1–12 barcodes: 48 µl - Volume per sample for using 13 barcodes or more: 24 µl ☐ LongAmp Taq 2X master mix - Volume per sample for using 1–12 barcodes: 50 µl - Volume per sample for using 13 barcodes or more: 25 µl ☐ Mix by pipetting and briefly spin down. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (12-15 (b) cycles) ☐ Annealing 15 secs (a) @ 62 °C (a) (12-15 (b) cycles) ☐ Extension dependent on length of target fragment (d) @ 65 °C (c) (12-15 (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
Add 0.4X volume of resuspended AMPure XP Beads to each reaction and mix by flicking the tube. ☐ AMPure XP Beads - Volume for 100 µl samples: 40 µl - Volume for 50 µl samples: 20 µl ☐ Incubate at RT for 5 minutes. ☐ Prepare sufficient fresh 80% ethanol in Nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess. ☐ Place on a magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Keep the samples on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellets. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the samples back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking. ☐ Remove the samples from the magnetic rack and resuspend each pellet in 25 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnetic rack until the eluate is clear and colourless. ☐ Remove and retain 25 µl of each eluate into clean 0.2 ml PCR tubes or plate. ☐ Quantify the barcoded library using a Qubit fluorometer 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 49 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 the AMPure XP Beads (AXP) and DNA Control Sample (DCS) at RT and mix by vortexing. Keep the beads at RT and store the DNA Control Sample (DCS) 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 ☐ 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. ☐ 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. ☐ 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.	
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 also possible to store the sample at 4°C overnight.	
Adapter ligation and clean-up	
IMPORTANT ☐ Although the recommended third-party ligase is supplied with its own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. Note: Do NOT vortex the Quick T4 DNA Ligase. ☐ Spin down the Ligation Adapter (LA) 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)	
☐ Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT and mix by vortexing. Then 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 Ligation Adapter (LA) ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads (AXP) by vortexing. ☐ Add 40 µl of resuspended AMPure XP Beads (AXP) 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
☐ Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB).	
IMPORTANT ☐ We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 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 MinION and GridION Flow Cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice.	
IMPORTANT ☐ For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.	
To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at RT. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml ☐ 30 µl Flow Cell Tether (FCT) ☐ Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact. ☐ 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 Library Beads (LIB) by pipetting.	
IMPORTANT ☐ The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), 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.	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	