Ligation sequencing DNA V14 - dual barcoding (SQK-NBD114.24 with 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 1-96 (EXP-PBC096)
- [ ] Native Barcoding Kit 24 V14 (SQK-NBD114.24)

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)
- [ ] 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)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml,

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microfuge
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Magnetic rack
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000, Multichannel

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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 - recovery aim <100–200 fmol.	
Take forward each end-prepped DNA sample in 15 µl Nuclease-free water into adapter ligation. However, at this point, it is also possible to store the sample at 4°C overnight.	

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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 processed in the PCR barcoded pool. Set up a barcoding PCR reaction as follows for each library in fresh 0.2 ml PCR tubes or a 0.2 ml 96-well PCR 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 - 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 samples 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 10 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 10 µl of each eluate into clean 0.2 ml PCR tubes or a clean PCR plate. ☐ Dispose of the pelleted beads ☐ Quantify the PCR barcoded samples using a Qubit fluorometer and pool all barcoded samples in the desired ratios into a 1.5 ml DNA LoBind Eppendorf tube for each PCR barcoded sample pool.	
IMPORTANT ☐ Each PCR barcoded sample pool should contain 1–96 unique barcodes.	
☐ Prepare each pooled PCR barcoded sample pool to 200 fmol (130 ng for 1 kb amplicons) in separate tubes and make the volume up to 12.5 µl Nuclease-free water.	
The pooled barcoded libraries are now ready to be end-prepped and undergo secondary barcoding. However, at this point it is also possible to store the libraries at 4°C overnight.	
End-prep	
☐ Thaw the AMPure XP Beads (AXP) at RT and mix by vortexing. Keep the beads at RT until use. 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
IMPORTANT ☐ Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.	
IMPORTANT ☐ It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.	
☐ In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), prepare 200 fmol (130 ng for 1 kb amplicons) of each PCR barcoded sample pool.	
☐ Make up each PCR barcoded sample pool to 12.5 µl using Nuclease-free water. Mix gently by pipetting and spin down.	
Combine the following components per tube/well: ☐ 12.5 µl PCR barcoded sample pool ☐ 1.75 µl Ultra II End-prep Reaction Buffer ☐ 0.75 µl Ultra II End-prep Enzyme Mix	
☐ Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.	
☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.	
☐ Transfer each PCR barcoded sample pool into a clean 1.5 ml Eppendorf DNA LoBind tube.	
☐ Resuspend the AMPure XP beads (AXP) by vortexing.	
☐ Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.	
☐ 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 PCR barcoded sample pool, with some excess.	
☐ 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.	
☐ Keep the tubes 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.	
☐ Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.	
☐ Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl Nuclease-free water. Spin down and incubate for 2 minutes at RT.	
☐ Pellet the beads on a magnet until the eluate is clear and colourless.	
Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Dispose of the pelleted beads	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Quantify 1 µl of each eluted end-prepped PCR barcoded sample pool using a Qubit fluorometer.	
IMPORTANT ☐ You will have up to 24 separate end-prepped PCR barcoded sample pools to take forward into secondary barcoding via native barcode ligation.	
Take forward an equimolar mass of each of the end-prepped PCR barcoded sample pools to undergo secondary barcoding in the native barcode ligation step. However, at this point it is also possible to store the PCR barcoded sample pools at 4°C overnight.	
Native barcode ligation	
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. ☐ Thaw the EDTA at RT and mix by vortexing. Then spin down and place on ice. ☐ Thaw the Native Barcodes (NB01-24) required for your number of PCR barcoded sample pools at RT. Individually mix the barcodes by pipetting, spin down, and place them on ice. ☐ Select a unique barcode for each PCR barcoded sample pool to be run together on the same flow cell. Up to 24 PCR barcoded sample pools can be barcoded and combined in one experiment. In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well: ☐ 7.5 µl End-prepped DNA (PCR barcoded sample pools) ☐ 2.5 µl Native Barcode (NB01-24) ☐ 10 µl Blunt/TA Ligase Master Mix ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate for 20 minutes at RT. Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly. ☐ 2 µl For clear cap EDTA ☐ 4 µl For blue cap EDTA ☐ Pool all the native barcoded sample pools in a 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP Beads (AXP) by vortexing. ☐ Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Prepare 2 ml of fresh 80% ethanol in Nuclease-free water.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant. ☐ Keep the tube on the magnetic rack and wash the beads with 700 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 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 tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water by gently flicking. ☐ Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. ☐ Pellet the beads on a magnetic rack until the eluate is clear and colourless. ☐ Remove and retain 35 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 dual barcoded DNA library to the adapter ligation and clean-up step. However, at this point it is also possible to store the library at 4°C overnight.	
Adapter ligation and clean-up	
IMPORTANT ☐ The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.	
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.	
IMPORTANT ☐ Do not vortex the Quick T4 DNA Ligase.	
☐ Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice. ☐ 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)	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 LoBind tube, mix in the following order: ☐ 30 µl Pooled barcoded libraries ☐ 5 µl Native Adapter (NA) ☐ 10 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 5 µl Quick T4 DNA Ligase ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 20 minutes at RT.	
IMPORTANT ☐ The next clean-up step uses Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) rather than 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.	
☐ Resuspend the AMPure XP Beads (AXP) by vortexing. ☐ Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant. ☐ Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or 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. ☐ Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB). ☐ Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. ☐ 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 onto the flow cell. Store the library on ice until ready to load.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Priming and loading the SpotON 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.	
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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
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.	