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

Before start checklist

Materials

- [ ] 15 ml of whole blood
- [ ] Ligation Sequencing Kit XL V14 (SQK-LSK114-XL)
- [ ] Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114)
- [ ] Flow Cell Wash Kit XL (EXP-WSH004-XL)

Consumables

- [ ] PromethION Flow Cell (FLO-PRO114M)
- [ ] Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Monarch® HMW DNA Extraction Kit for Tissue (NEB, T3060)
- [ ] Puregene Blood Kit (QIAGEN, 158023)
- [ ] T4 DNA Ligase 400,000 U/ml (NEB M0202S/L)
- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
- [ ] NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] NEBNext FFPE DNA Repair Mix (NEB, cat # M6630)
- [ ] RBC Lysis Solution (QIAGEN, 158904)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] 5 M NaCl (Sigma, 71386)
- [ ] PEG 8000, 50% w/v (Rigaku Reagents, cat # 25322-68-3)
- [ ] TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Fisher scientific, 10224683)
- [ ] 0.5 M EDTA, pH 8 (Thermo Scientific, R1021)
- [ ] Percoll, 1.135 g/ml (Cytiva, 17-0891-01)
- [ ] (Optional) Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, 20-139)
- [ ] ECOSURF EH-9 (Dow, 64366-70-7)
- [ ] Fetal Bovine Serum (FBS) (Gibco™, A3840401)

Equipment

- [ ] PromethION 24/48 device
- [ ] P200 pipette and tips
- [ ] Pasteur pipettes
- [ ] Thermal cycler or heat block
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack
- [ ] Vortex mixer
- [ ] Temperature-controlled centrifuge
- [ ] Microfuge
- [ ] Ice bucket with ice
- [ ] Thermomixer
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Class I hood with active charcoal filter
- [ ] -80°C freezer storage
- [ ] Pipettes and pipette tips P20, P10, P100, P1000

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INSTRUCTIONS	NOTES/OBSERVATIONS
Custom SPRI bead preparation for the Pore-C experiment	
Custom SPRI bead suspension Prepare a custom buffer in a 2 ml Eppendorf DNA LoBind tube as follows for use in step 7. ☐ Tris-HCl, 1 M - Final: 10 mM - Volume: 20 µl ☐ EDTA, pH 8, 0.5 M - Final: 1 mM - Volume: 4 µl ☐ NaCl, 5 M - Final: 1.6 M - Volume: 640 µl ☐ PEG 8000, 50% (w/v) - Final: 11% (w/v) - Volume: 440 µl ☐ Nuclease-free water - Final: - - Volume: 888 µl ☐ Transfer 1 ml of resuspended Agencourt AMPure XP beads into two 2 ml Eppendorf DNA LoBind tubes, so that each tube contains 1 ml. ☐ Place the tubes on a magnetic rack to pellet the beads until the solution is clear and colourless. Pipette off and discard the supernatant. ☐ Remove the tubes from the magnet and resuspend the pellets with 1 ml of Nuclease-free water. Pellet the beads on the magnet until supernatant is clear and colourless and pipette off the supernatant. ☐ Repeat the previous step. ☐ Spin down and place the tubes back on the magnet to pipette off any residual water. ☐ Resuspend both tubes of pelleted beads in 200 µl of custom buffer and then pool both tubes into a single tube to a total of 400 µl. ☐ Transfer the remaining custom buffer into the tube containing the pooled beads.	
Store the beads at 4°C. Before use, bring the suspension to RT.	
Whole blood sample preparation for the Pore-C experiment	
PBMC sample preparation for Pore-C DNA extraction	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare three solutions in preparation for PBMC isolation: ☐ 50 ml 10X PBS ☐ 10 ml FBS ☐ 440 ml Nuclease-free water ☐ 10 ml 10X PBS ☐ 60 ml Percoll ☐ 30 ml Nuclease-free water ☐ 1,600 µl FBS ☐ 400 µl DMSO ☐ Allow the whole blood sample to warm to RT and then dilute with equal volume of RT 1X PBS supplemented with 2% FBS. Transfer the diluted blood to a 50 ml centrifuge tube. ☐ Centrifuge at 800 x g at 20°C for 10 minutes with the brake off to prevent remixing of the separated fractions. ☐ After centrifugation, the whole blood should have separated into the plasma, buffy coat and red blood cells. Check the turbidity of the plasma layer (the top layer). If it is not clear, centrifuge at 800 x g at 20°C for a further 10 minutes with the brake off. ☐ Using a Pasteur pipette, remove as much of the plasma layer as possible without disturbing the layer of buffy coat. Gently remove the buffy coat layer, taking care to draw as little of the red blood cell layer as possible. Transfer the recovered buffy coat to a fresh 50 ml centrifuge tube. ☐ Make up the recovered buffy coat sample to 25 ml of 1X PBS supplemented with 2% FBS. ☐ Aliquot 20 ml of 1X PBS supplemented with 60% Percol in a fresh 50 ml centrifuge tube. ☐ Using a fresh Pasteur pipette, very gently layer the diluted buffy coat sample over the Percol layer at a 45° angle. ☐ Centrifuge at 350 x g at 20°C for 40 minutes with slow acceleration and with the brake off. ☐ Check the turbidity of the plasma layer and the formation of the PMBCs layer. If the plasma layer is not clear or the PBMC layer is not well defined, continue to centrifuge at 350 x g at 20°C for a further 20 minutes using slow acceleration with the brake off. ☐ Using a Pasteur pipette, remove as much of the plasma layer as possible without disturbing the layer of PMBCs, then gently remove the layer of PBMCs. It is acceptable to draw plasma with the layer of PBMCs; however, take care to draw as little of the Percol layer as possible. ☐ Transfer the recovered PBMCs to a fresh 50 ml centrifuge tube. ☐ Resuspend the recovered PBMCs in 50 ml of RT 1X PBS supplemented with 2% FBS. ☐ Centrifuge at 350 x g at 20°C for 15 minutes with the brake on. ☐ Aspirate and discard the supernatant. Gently resuspend the PBMCs in 25 ml of RT 1X PBS supplemented with 2% FBS. Centrifuge at 350 x g at 20°C for 15 minutes with the brake on. ☐ Repeat the previous step.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Aspirate and discard the supernatant. Gently resuspend the PBMCs in another 25 ml of RT 1X PBS supplemented with 2% FBS. ☐ Centrifuge at 200 x g at 20°C for 10 minutes with the brake on. ☐ Assuming every 1 ml of whole blood originally used will yield approximately 1.5 million PBMCs, resuspend cells to approximately 10 million PBMCs/ml in RT 1X PBS supplemented with 2% FBS. ☐ Transfer an aliquot of approximately 10 million PBMCs total to a fresh 2 ml Eppendorf DNA LoBind tube. ☐ Cool on ice for 5 minutes.	
Take forward approximately 10 million PBMCs into the Pore-C experiment. Store the cells at 4°C until the experiment can begin.	
Whole blood sample gDNA extraction for the Duplex experiment	
Whole blood gDNA extraction for the Duplex experiment Perform cell separation and lysis according to the QIAGEN Puregene Handbook for 3 ml of blood (pages 19–20, steps 1–7): ☐ Dispense 9 ml of RBC Lysis Solution into a 15 ml Falcon tube. ☐ Add 3 ml of whole blood and mix by inverting 10 times. ☐ Incubate for 5 minutes at RT. Invert at least once during the incubation. ☐ Centrifuge for 2 minutes at 2000 x g to pellet the white blood cells. ☐ Carefully discard the supernatant by pipetting or pouring, leaving approximately 200 µl of the residual liquid and the white blood cell pellet. ☐ Vortex the tube vigorously to resuspend the pellet in the residual liquid. Vortexing greatly facilitates cell lysis in the next step. ☐ Add 3 ml of Cell Lysis Solution and pipette mix to lyse the cells or vortex for 10 seconds. ☐ Incubate the samples at 37°C for 30 minutes. If the sample is not homogenous, gently invert the tubes and extend the incubation for another 30 minutes. Purify the lysate according to the QIAGEN Puregene Handbook for 3 ml of blood (pages 20–21, steps 8–17): ☐ Add 15 µl of RNase A Solution and mix by inverting 25 times. Incubate for 15 minutes at 37°C. Then incubate for 3 minutes on ice to quickly cool the sample. ☐ Add 1 ml of Protein Precipitation Solution and vortex vigorously for 20 seconds at high speed. ☐ Centrifuge for 5 minutes at 2000 x g. The precipitated proteins should form a tight brown pellet. If the protein pellet is not tight, incubate on ice for 5 minutes and repeat the centrifugation. ☐ Pipette 3 ml of isopropanol into a clean 15 ml Falcon tube and add the supernatant from the previous step by pouring carefully. Be sure that the protein pellet is not dislodged during pouring. ☐ Mix by inverting 50 times until the DNA is visible as threads or a clump. ☐ Centrifuge for 3 minutes at 2000 x g. The DNA may be visible as a small white pellet. ☐ Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbant paper, taking care that the pellet remains in the tube. ☐ Add 3 ml of 80% ethanol and invert several times to wash the DNA pellet. ☐ Centrifuge for 1 minute at 2000 x g. ☐ Carefully discard the supernatant. Drain the tube on a clean piece of absorbant paper, taking care that the pellet remains in the tube. Dry the pellet for 5-10 minutes. The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet as the DNA will be difficult to dissolve.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ To maximize the DNA yield, we recommend that the elution is performed for 2 hours at 50°C, using 150 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), occasionally mixing the tube contents by gentle inversion.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Dilute your DNA sample to 60 ng/µl in a final volume of 50 µl of TE buffer at pH 8. ☐ Add 0.7X (35 µl) of RT custom SPRI bead suspension to your DNA sample, and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Spin down briefly and pellet on a magnet until the 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 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 40 µl of TE buffer. Incubate for 1 minute at 50°C, and then for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 40 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of size-selected DNA using a Qubit fluorometer.	
Take forward 1 µg of extracted DNA into the Duplex experiment. Store at 4°C until the experiment can begin.	
Whole blood sample preparation for the Ultra-long DNA experiment	
PBMC sample preparation for the Ultra-long DNA experiment Prepare 10 ml of 1X PBS in Nuclease-free water, as follows: ☐ 1 ml 10X PBS ☐ 9 ml Nuclease-free water ☐ Add 4.8 ml (3X the volume) of RBC Lysis Solution to 1.6 ml of whole blood in a 15 ml Falcon tube. ☐ Gently invert the tube 10 times to mix. ☐ Incubate for 5 minutes at RT and gently invert twice during the incubation. ☐ Centrifuge at 2000 x g for 2 minutes at 4°C to pellet the white blood cells. ☐ Discard the supernatant by pouring. There will be ~200 µl supernatant remaining in the tube.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Resuspend the cells in the residual supernatant by gently flicking the tube. ☐ Make up the volume to 1.6 ml with 1X PBS. ☐ Repeat steps 1-7 twice more to complete three washes in total. ☐ After the final spin, remove the entire supernatant by pouring and aspirating any remaining supernatant. ☐ Resuspend the cell pellet in 40 µl 1X PBS. There will be approximately 6 million cells in the suspension.	
Take forward 6 million PBMCs forward into the Ultra-Long DNA experiment. Store the pellet at 4°C until the experiment can begin.	
Day 1: Pore-C experiment	
Day 1: Pore-C experiment overview Thaw the NlaIII restriction enzyme and CutSmart Buffer in accordance with the manufacturer's instructions and place on ice. ☐ Thaw both reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the NlaIII restriction enzyme. ☐ Spin down tubes before opening for the first time each day. Prepare 1 ml of 1% SDS in Nuclease-free water, as follows: ☐ 100 µl 10% SDS ☐ 900 µl Nuclease-free water Prepare 10 ml of 10% (v/v) ECOSURF™ EH-9 in Nuclease-free water, as follows: ☐ Weigh out 1 g of ECOSURF™ EH-9. ☐ Transfer to a fresh 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 9 ml of Nuclease-free water. ☐ Gently pipette mix with a wide-bore pipette tip until the solution is homogenous. ☐ Prepare 1 ml of 2.5 M glycine filtered through a 0.2 µm filter and store at RT. ☐ Prepare 200 ml filtered 1X PBS and chill at 4°C. ☐ Pre-cool a centrifuge to 4°C.	

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INSTRUCTIONS	NOTES/OBSERVATIONS

Prepare the formaldehyde solution as follows:

- ☐ Transfer 10 ml of 1X PBS into a 50 ml Falcon tube.
  Note: Using a 15 ml Falcon tube is not recommended.
- ☐ Inside a class I hood, with double gloves, add 291 µl of 36.5% formaldehyde to the 10 ml 1X PBS aliquot to a final concentration of 1% formaldehyde in ~10.3 ml.
- ☐ Mix by gentle inversion, and open the tube to allow gases to escape, then close the tube.
- ☐ Check that no formaldehyde residue has remained on the gloves, Falcon tube, or pipette.
- ☐ Remove the outer gloves and discard them in a biohazard bag in the hood.
- ☐ Remove the 1% formaldehyde 1X PBS solution from the hood.
- ☐ Store the tube with formaldehyde inside a zip lock bag at 4°C prior to use.

Prepare the PBMCs as follows:

- ☐ Take approximately 10 million PBMCs and briefly homogenise the suspension by gently pipetting with a wide-bore pipette tip.
- ☐ Transfer the cell suspension to a 50 ml centrifuge tube.
- ☐ Rinse the original tube with a further 1 ml of chilled 1X PBS into the 50 ml centrifuge tube.
- ☐ Bring the volume of the resuspended PBMCs to 10 ml in chilled 1X PBS.
- ☐ Proceed with the Pore-C experiment using approximately 10 million PBMCs as input.

☐ Centrifuge the sample at 300 x g at 4°C for 5 minutes.

☐ Aspirate and discard the supernatant, then add 10 ml of chilled 1X PBS to the pellet. Resuspend the pellet by gently pipetting up and down using a wide-bore pipette tip.

☐ Centrifuge the sample at 300 x g at 4°C for 5 minutes.

☐ Check the 2.5 M glycine solution has not precipitated before crosslinking the sample. Dissolve precipitate with heat and vortexing if required.

☐ Inside a class I hood, with double gloves, aspirate and discard the supernatant.

☐ Add 1 ml of the previously prepared 1% formaldehyde solution 1X PBS to the pellet. Resuspend the pellet by gently pipetting up and down using a wide-bore pipette tip.

☐ Once resuspended, add a further 9 ml of the 1% formaldehyde solution in 1X PBS. Mix gently by pipetting up and down, using a wide-bore pipette tip.

☐ Incubate at RT for exactly 10 minutes to crosslink the sample. The incubated solution should be mixed by gentle agitation every few minutes.

IMPORTANT

☐ We do not recommend extending incubation times as it may have a detrimental impact on the efficiency of de-crosslinking the DNA later in the protocol.

☐ Inside the hood with double gloves, quench the formaldehyde by adding 527 µl of 2.5 M glycine to the sample suspension for a final concentration of 1% w/v glycine (125 mM) in ~10.5 ml. Mix gently by pipetting up and down, using a wide-bore pipette tip.

☐ Incubate at RT for 5 minutes, then chill on ice for a further 10 minutes with regular, gentle agitation.

☐ Centrifuge the crosslinked sample suspension at 300 x g at 4°C for 5 minutes.

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Continuing in the class I hood, aspirate and discard the supernatant. Add 10 ml of chilled 1X PBS to the tube. ☐ Centrifuge the sample at 500 x g at 4°C for 5 minutes. ☐ Continuing in the class I hood, aspirate and discard the supernatant, and add 1 ml of chilled 1X PBS to the pellet. Mix gently by pipetting up and down using a wide-bore pipette tip. ☐ Split the resuspended sample into two separate 500 µl aliquots in fresh 2 ml Eppendorf tubes. ☐ Wash the previous sample tube with a further 1 ml of 1X PBS, and split this between the two aliquots in 2 ml Eppendorf DNA LoBind tubes. ☐ Centrifuge the samples at 500 x g at 4°C for 5 minutes. Aspirate and discard the supernatant. IMPORTANT ☐ Process each crosslinked sample pellet separately. Do not pool multiple pellets into a single reaction. IMPORTANT ☐ Do not proceed any further unless it is possible to complete the remainder of this section consecutively without interruption. It is not advisable to incubate any step longer than stated in this protocol. Doing so may be detrimental to Pore-C data quality and sequencing performance. ☐ Pre-cool a microfuge to 4°C and set a thermomixer to 65°C. Prepare 600 µl of 1.5X CutSmart Buffer in Nuclease-free water as follows in a 1.5 ml Eppendorf DNA LoBind tube. Keep on ice. ☐ 510 µl Nuclease-free water ☐ 90 µl 10X CutSmart Buffer To make the permeabilisation solution, add the components below to a 1.5 ml Eppendorf DNA LoBind tube in the following order. Keep the prepared permeabilisation solution on ice at 4°C until ready to use. ☐ Tris-HCl, pH 8.0, 1 M - Final: 10 mM - Volume: 5 µl ☐ NaCl, 5 M - Final: 10 mM - Volume: 1 µl ☐ IGEPAL CA-630, 10% - Final: 0.2% - Volume: 10 µl ☐ Nuclease-free water - Final: - - Volume: 484 µl ☐ Thaw the protease inhibitor cocktail on ice and spin down. ☐ Add 50 µl of protease inhibitor cocktail to 500 µl of permeabilisation solution at 4°C.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Add 550 µl protease inhibitor cocktail-permeabilisation solution to the sample pellet. Resuspend the pellet by gently pipetting up and down, using a wide-bore pipette tip. ☐ Incubate on ice for 15 minutes and mix by regular, gentle inversion. ☐ Centrifuge the sample at 500 x g at 4°C for 10 minutes.	
IMPORTANT ☐ Following centrifugation, the pellet will be delicate. Carefully aspirate and discard as much of the supernatant as possible without disturbing the pellet	
☐ Resuspend the pellet in 200 µl of the prepared chilled 1.5X CutSmart buffer by gently pipetting up and down, using a wide-bore pipette tip. ☐ Centrifuge the sample at 500 x g at 4°C for 5 minutes. Aspirate and discard the supernatant. ☐ Resuspend the pellet in 300 µl of the prepared chilled 1.5X CutSmart buffer by gently pipetting up and down, using a wide-bore pipette tip. ☐ To denature the chromatin, add 33.5 µl 1% SDS directly to the sample suspension to a final concentration of 0.1% SDS and a total volume of 333.5 µl. Mix gently by pipetting up and down using a wide-bore pipette tip. ☐ Incubate the sample suspension in a thermomixer at 300 rpm at 65°C for 10 minutes. ☐ Remove the tube from the thermomixer and immediately put on ice. ☐ Set the thermomixer to 37°C. ☐ Add 37.5 µl of 10% (v/v) ECOSURF™ EH-9 directly to the cell suspension for a final concentration of 1% ECOSURF™ EH-9 (total volume of 371 µl). Mix gently by pipetting with a wide-bore pipette tip. ☐ Incubate the tube on ice for 10 minutes. Add the following reagents to the sample suspension and invert 3-4 times to mix. ☐ Permeabilised cells - Final: - - Volume: 371 µl ☐ NEB NlaIII, 10 U/µl - Final: 1 U/µl - Volume: 45 µl ☐ Nuclease-free water - Final: - - Volume: 34 µl ☐ Incubate the tube in a thermomixer at 37°C for 18 hours with periodic <1000 rpm rotation for <30 seconds every 15 minutes. This will prevent condensation inside the lid.	
During the Pore-C incubation, start the Duplex experiment.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Day 1: Duplex experiment	
Day 1: Duplex experiment overview ☐ Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice. 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. ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ 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 30 seconds to solubilise any precipitate. Note: It is important the buffers are mixed well by vortexing. ☐ The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow. Prepare the DNA in Nuclease-free water: ☐ Transfer 1 µg (or 100-200 fmol) input DNA into a 1.5 ml Eppendorf DNA LoBind tube. ☐ Adjust the volume to 47 µl with Nuclease-free water. ☐ Mix thoroughly by pipetting up and down, or by flicking the tube. ☐ Spin down briefly in a microfuge In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 47 µl DNA from the previous step ☐ 1 µl DNA CS (optional) ☐ 3.5 µl NEBNext FFPE DNA Repair Buffer ☐ 2 µl NEBNext FFPE DNA Repair Mix ☐ 3.5 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 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 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
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. ☐ 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. ☐ Thaw the 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 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Wash the beads by adding 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 25 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 25 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.	
☐ Make up your library to 32 µl at 10-20 fmol, using Elution Buffer (EB). IMPORTANT ☐ We recommend loading 10-20 fmol of this final prepared library onto the R10.4.1 flow cell.	
Take the 32 µl of the library forwards for loading onto the flow cell. Store on ice until ready to load.	
Day 1: Priming and loading Duplex library on the PromethION Flow Cell	
Day 1: Duplex experiment flow cell loading ☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT)	
IMPORTANT ☐ After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.	
For the PromethION 24/48, load the flow cell(s) into the docking ports: ☐ Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. ☐ Press down firmly onto the flow cell and ensure the latch engages and clicks into place.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.	
☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library ☐ Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Close the PromethION lid when ready to start a sequencing run on MinKNOW.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Day 2: Pore-C experiment	
Day 2: Pore-C experiment overview Thaw the T4 DNA Ligase and T4 DNA Ligase Reaction Buffer in accordance with the manufacturer's instructions and place on ice. ☐ Thaw both reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the T4 DNA Ligase enzyme. ☐ Spin down tubes before opening for the first time each day. Prepare 5 ml of 20% Tween-20 in nuclease free water as follows: ☐ Weigh out 1.095 g of Tween-20 and transfer to a fresh 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 4 ml of Nuclease-free water. ☐ Gently invert the tube until the solution is homogenous. ☐ Set the thermomixer to 65°C. ☐ Heat denature the restriction enzyme by incubating the sample suspension in the thermomixer at 65°C with 300 rpm rotation for 20 minutes. Allow the reaction to cool to RT. ☐ Set the thermomixer to 16°C. Set up the proximity ligation reaction according to the table below, adding reagents directly to the sample suspension in the following order. Mix gently by pipetting up and down, using a wide-bore pipette tip. ☐ Digestion reaction (from Day 1) - Final: - - Volume: 450 µl ☐ Nuclease-free water - Final: - - Volume: 395 µl ☐ T4 DNA Ligase Reaction Buffer, 10X - Final: 1X - Volume: 100 µl ☐ Recombinant albumin, 20 µg/µl - Final: 0.1 µg/µl - Volume: 5 µl ☐ T4 DNA Ligase, 400 U/µl - Final: 20 U/µl - Volume: 50 µl ☐ Incubate the sample suspension in a thermomixer at 16°C for 6 hours, with periodic <1000 rpm rotation for <30 seconds every 15 minutes. This prevents condensation inside the lid. IMPORTANT ☐ Do not extend incubation as prolonged ligation may increase trans-chromosomal contacts in the Pore-C data. ☐ Set the thermomixer to 56°C.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Add the reagents to the previous ligation reaction in the following order to make up the protein degradation reaction. Mix the sample gently by inverting the tube 3–4 times. ☐ Ligation reaction (from the Proximity Ligation) - Final: - - Volume: 1000 µl ☐ Nuclease-free water - Final: - - Volume: 300 µl ☐ Tween-20, 20% - Final: 5% - Volume: 500 µl ☐ SDS, 10% - Final: 0.5% - Volume: 100 µl ☐ Proteinase K, 20 µg/µl - Final: 1 µg/µl - Volume: 100 µl ☐ Incubate the sample suspension in a thermomixer at 56°C for 18 hours with periodic <1000 rpm rotation for <30 seconds every 15 minutes to prevent condensation inside the lid.	
IMPORTANT ☐ Incubation at 56°C compromises enzyme activity over a prolonged incubation. It is not advisable to incubate at higher temperatures as enzyme activity will reduce over time.	
During the Pore-C incubation, start the Ultra-long DNA experiment.	
Day 2: Ultra-long DNA experiment	
Day 2: Ultra-long DNA experiment overview ☐ Thaw the Extraction EB (EEB) at RT, mix by vortexing and place on ice. ☐ Add 6 million cells resuspended in 40 µl PBS to a fresh 5 ml tube. ☐ In a separate 2 ml Eppendorf DNA LoBind tube, mix 1.8 ml of Monarch HMW gDNA Tissue Lysis Buffer and 60 µl Proteinase K. ☐ Add 1.8 ml of mixed Monarch HMW gDNA Tissue Lysis Buffer and Proteinase K to the resuspended cells. ☐ Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip. ☐ Incubate the reaction at 56°C for 10 minutes. ☐ Using a regular pipette tip, add 15 µl of Monarch RNase A. ☐ Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip. ☐ Agitate the reaction at 56°C for 10 minutes on a thermomixer at 650 rpm.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Using a regular pipette tip, add 900 µl of the Monarch Protein Separation Solution to the reaction and mix using a Hula Mixer (rotator mixer) for 10 minutes, rotating at 3 rpm.	
☐ Centrifuge the reaction at 16,000 x g for 10 minutes at 4°C to separate the protein from the DNA.	
☐ Using a wide-bore pipette tip, carefully aspirate the upper phase containing the DNA and transfer to a fresh 5 ml tube without disturbing the phase below.	
☐ Add three Monarch DNA Capture Beads to the collected DNA phase.	
☐ Add 2.5 ml isopropanol to the tube and mix using a Hula Mixer (rotator mixer) for 20 minutes rotating at 3 rpm. Ensure the DNA has fully precipitated around the glass beads.	
☐ Leave the tube to stand for 1 minute, without rotating, at RT.	
☐ Aspirate the supernatant from the tube, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any supernatant remaining in the lid of the tube.	
☐ Add 2 ml of Monarch gDNA Wash Buffer to the tube containing DNA bound to the beads. Invert the tube to mix.	
☐ Aspirate the Wash Buffer, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any Wash Buffer remaining in the lid of the tube.	
☐ Add 2 ml of Monarch gDNA Wash Buffer to the tube containing the DNA bound to the beads.	
☐ Add 560 µl of Extraction EB (EEB) to a fresh 2 ml Eppendorf tube.	
☐ Aspirate the Wash Buffer, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any Wash Buffer remaining in the lid of the tube.	
☐ Transfer the beads to a Monarch Bead Retainer inserted in a Monarch Collection Tube II.	
☐ Briefly spin the tube using a microfuge to remove any remaining Wash Buffer from the beads. Dispose of the collection tube containing residual wash buffer.	
IMPORTANT ☐ Do NOT use the Monarch Elution Buffer II in the Monarch® HMW DNA Extraction Kit for Tissue.	
☐ Immediately transfer the beads from the bead retainer into the 2 ml tube containing 560 µl of Extraction EB (EEB).	
IMPORTANT ☐ Beads should be transferred immediately to ensure that they do not over-dry, which could lead to increased solubilisation times.	
☐ Incubate the tube for 10 minutes at 56°C.	
☐ Pour the eluate and beads into a clean bead retainer inserted in a collection tube. Spin the tube at 1000 x g for 1 minute to separate eluate from the beads. Dispose of beads and bead retainer.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Add 200 µl of Extraction EB (EEB) to the collection tube to bring the total elution volume to 760 µl. ☐ Transfer the eluate to a fresh 2 ml Eppendorf DNA LoBind tube. ☐ Incubate the eluate for 10 minutes at 56°C. ☐ Gently mix the eluate by slowly pipetting 10 times using a 1 ml wide-bore pipette tip. ☐ Use a regular P200 pipette tip to aspirate 10 µl of gDNA. ☐ Dispense the aspirated gDNA into a fresh 2 ml Eppendorf DNA LoBind tube. ☐ Add a Monarch DNA Capture Bead to the 10 µl of gDNA and vortex aggressively for 1 minute to shear the gDNA. ☐ Transfer the gDNA and beads into a clean Monarch Bead Retainer inserted in a Monarch Collection Tube II. Spin the tube at 1000 x g for 1 minute to separate gDNA from the beads. Dispose of beads and bead retainer. ☐ Transfer the gDNA into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify the sample using a Qubit fluorometer. The expected yield is 30-40 µg of DNA.	
Thaw the the kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below: ☐ Fragmentation Mix (FRA) □ @ Not frozen (□ cycles) ☐ FRA dilution buffer (FDB) □ @ Not frozen (□ cycles) ☐ Rapid Adapter (RA) □ @ Not frozen (□ cycles) In a 1.5 ml Eppendorf DNA LoBind tube, dilute the Fragmentation Mix (FRA) with FRA Dilution Buffer (FDB) as follows: ☐ 6 µl Fragmentation Mix (FRA) ☐ 244 µl FRA dilution buffer (FDB) ☐ Mix the diluted Fragmentation Mix (FRA) by pipetting. ☐ Using a regular pipette tip, add 250 µl of diluted Fragmentation Mix (FRA) to the 750 µl of extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted Fragmentation Mix (FRA) to ensure an even distribution. ☐ Immediately mix the reaction by slowly pipetting 10 times with a wide-bore pipette tip. Incubate the reaction as follows: ☐ 10 minutes Room temperature ☐ 10 minutes 75°C ☐ Cool on ice for a minimum of 10 minutes On ice ☐ Add 5 µl Rapid Adapter (RA) to the reaction using a regular pipette tip.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Gently mix the reaction by slowly pipetting five times using a 1 ml wide-bore pipette tip. ☐ Incubate the reaction for 30 minutes at RT. Thaw the kit components at RT, spin down briefly using a microfuge and mix by vortexing as indicated by the table below: ☐ Precipitation buffer (PTB) ⍰ @ ⍰ (⍰ cycles) ☐ Elution Buffer (EB) ⍰ @ ⍰ (⍰ cycles) ☐ Using a regular pipette tip, add 500 µl of Precipitation Buffer (PTB) to the sample. ☐ Mix the sample by rotating on a Hula Mixer (rotator mixer) for 20 minutes at 3 rpm. ☐ Centrifuge the sample at 1000 x g for 1 minute. ☐ Using a regular pipette tip, carefully remove the supernatant from the tube, taking care not to aspirate the DNA pellet. ☐ Centrifuge the sample at 1000 x g for 1 minute. ☐ Using a regular pipette tip, carefully remove any residual supernatant from the tube, taking care not to aspirate the DNA pellet. ☐ Using a regular pipette tip, add 300 µl of Elution Buffer (EB) to the tube containing the DNA. Incubate overnight at RT, for a minimum of 12 hours.	
During the Ultra-long DNA experiment incubation, complete the first wash and reload of the Duplex experiment.	
Day 2: Washing and reloading Duplex library on the PromethION Flow Cell	
Day 2: Duplex experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip. Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. ☐ Close the inlet port and wait for 1 hour.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open.	
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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
After opening the inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Resume the sequencing run in MinKNOW to continue data acquisition.	
Day 3: Pore-C experiment	
Day 3: Pore-C experiment overview ☐ Pre-cool the centrifuge to 15°C. ☐ Place the sample on ice until cool, then transfer the entire volume to a 5 ml centrifuge tube. ☐ Rinse the original tube with a further 200 µl of Nuclease-free water and add this to the same 5 ml centrifuge tube for a total sample volume of ~2200 µl. ☐ Add an equal volume of chilled phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris.HCl pH 8.0, 1 mM EDTA, adjusting this volume as needed to match that of the sample. Mix by gently inverting the tube for 5 minutes to achieve a homogeneous emulsion. ☐ Centrifuge the aliquots at 16,000 g at 15°C for 15 minutes. ☐ Incubate the aliquots on ice for 2 minutes until the organic phase becomes cloudy; this will strengthen the integrity of the interphase layer. ☐ Transfer the aqueous phase into a fresh 5 ml centrifuge tube for each aliquot and make note of the recovered volume (expect ~2000 µl). ☐ Transfer half of the recovered aqueous phase to a second 5 ml centrifuge tube to create two equal aliquots. For each aliquot, add 0.02X of 5 M NaCl (0.1 M final) and 0.1X of 3 M sodium acetate pH 5.5 (0.3 M final), relative to the volume of the recovered aqueous phase of the aliquot. Mix by gently inverting the tube. ☐ 40 µl of 5 M NaCl ☐ 200 µl of 3 M sodium acetate ☐ For each aliquot, add 3X of 100% ethanol relative to the volume of the recovered aqueous phase. Mix by gently inverting the tubes. ☐ Precipitate at –80°C for >1 hour. ☐ Pre-cool a centrifuge to 4°C. ☐ Centrifuge the sample at 16,000 x g at 4°C for 30 minutes. ☐ Aspirate and discard the supernatant, then wash the pellets with 4 ml of 80% ethanol. ☐ Centrifuge the sample at 16,000 x g at 4°C for 5 minutes. ☐ Aspirate and discard the supernatant, then wash the pellets with 2 ml of 70% ethanol. ☐ Centrifuge the sample at 16,000 x g at 4°C for 5 minutes. ☐ Aspirate and discard the supernatant. Briefly spin down the tubes and aspirate any residual supernatant. Allow the pellets to dry for 5 minutes.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Carefully resuspend each aliquot in 75 µl of TE buffer. Incubate for 5 minutes at RT, mixing by gently inverting the tube every few minutes. ☐ Briefly spin down the tubes, then transfer and pool all aliquots together into a 1.5 ml Eppendorf DNA LoBind tube.	
Quantify DNA concentration by using the Qubit dsDNA HS Assay Kit. Ensure a 1/10 dilution is used, as the Qubit reading will be affected by high salt concentration.	
☐ Dilute your sample to 60 ng/µl in a final volume of 50 µl of TE buffer at pH 8. ☐ Add 42.5 µl (0.85X) of RT custom SPRI bead suspension and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Spin down briefly and pellet on a magnet until the 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 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 40 µl of TE buffer. Incubate for 1 minute at 50°C, and then for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 40 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
Store the sample at 4°C and complete the Ultra-long DNA experiment.	
Day 3: Ultra-long DNA experiment	
Day 3: Ultra-long DNA experiment overview ☐ Gently mix the DNA library by slowly pipetting 10 times with a wide-bore pipette tip.	
Take the DNA library forwards for loading into the flow cell. Store the library on ice until ready to load.	
Day 3: Priming and loading ultra-long DNA library on the PromethION Flow Cell	
Day 3: Ultra-long DNA experiment flow cell loading ☐ Thaw the Sequencing Buffer UL (SBU), Loading Solution UL (LSU), Flush Tether UL (FTU) and one tube of Flow Cell Flush (FCF) at RT and mix by vortexing. Then spin down and place on ice.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a new tube, prepare the DNA library for loading as follows. Use a wide-bore pipette tip for the addition of the DNA library: ☐ 100 µl Sequencing Buffer UL (SBU) ☐ 10 µl Loading Solution UL (LSU) ☐ 90 µl DNA library ☐ Gently mix the prepared DNA library by slowly pipetting ten times using a wide-bore pipette tip. ☐ Incubate at RT for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous. Prepare the flow cell priming mix in a 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing at RT. ☐ 30 µl Flush Tether UL (FTU) ☐ 1170 µl Flow Cell Flush (FCF)	
IMPORTANT ☐ After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.	
For the PromethION 24/48, load the flow cell(s) into the docking ports: ☐ Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. ☐ Press down firmly onto the flow cell and ensure the latch engages and clicks into place.	
IMPORTANT ☐ Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.	
☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. ☐ Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. ☐ Ensure the inlet port cover of the flow cell is still open in preparation for loading.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Aspirate the DNA library with a wide-bore pipette tip. Ensure there are no air bubbles in the pipette tip. Place the wide-bore pipette tip directly on the inlet port. Slowly depress the pipette to dispense the library into the inlet port. ☐ Using a P200 pipette, set the pipette to 50 µl and insert the tip into Port 2. ☐ Very slowly turn the wheel of the pipette to pull the DNA library into the inlet port. Closely watch the DNA library on the inlet port and completely remove the pipette as soon as the library starts to be pulled into the port. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Day 3: Washing and reloading Duplex library on the PromethION Flow Cell	
Day 3: Duplex experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet port.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. ☐ Close the inlet port and wait for 1 hour.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open the inlet 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
After opening the inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Resume the sequencing run in MinKNOW to continue data acquisition.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Day 4: Pore-C experiment	
Day 4: Pore-C experiment overview ☐ Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice. 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. ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ 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 30 seconds to solubilise any precipitate. Note: It is important the buffers are mixed well by vortexing. ☐ The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow. Prepare the DNA in Nuclease-free water: ☐ Transfer 100-200 fmol input DNA into a 1.5 ml Eppendorf DNA LoBind tube. ☐ Adjust the volume to 47 µl with Nuclease-free water. ☐ Mix thoroughly by pipetting up and down, or by flicking the tube. ☐ Spin down briefly in a microfuge In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 47 µl DNA from the previous step ☐ 1 µl DNA CS (optional) ☐ 3.5 µl NEBNext FFPE DNA Repair Buffer ☐ 2 µl NEBNext FFPE DNA Repair Mix ☐ 3.5 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 15 minutes and 65°C for 5 minutes. ☐ Resuspend the AMPure XP Beads 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 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
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. ☐ 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. ☐ Thaw the 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 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Wash the beads by adding 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 25 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 25 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.	
☐ Make up your library to 32 µl at 10-20 fmol, using Elution Buffer (EB). IMPORTANT ☐ We recommend loading 10-20 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.	
Day 4: Priming and loading Pore-C library on the PromethION Flow Cell	
Day 4: Pore-C experiment flow cell loading ☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT)	
IMPORTANT ☐ After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.	
For the PromethION 24/48, load the flow cell(s) into the docking ports: ☐ Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. ☐ Press down firmly onto the flow cell and ensure the latch engages and clicks into place.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.	
☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library ☐ Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Close the PromethION lid when ready to start a sequencing run on MinKNOW.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Day 4: Washing and reloading Duplex library on the PromethION Flow Cell	
Day 4: Duplex experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip. Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. ☐ Close the inlet port and wait for 1 hour.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
After opening the inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Resume the sequencing run in MinKNOW to continue data acquisition.	
Day 4: Washing and reloading the PromethION Flow Cell with ultra-long DNA library	
Day 4: Ultra-long DNA experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device. ☐ Ensure the inlet port is closed and remove the buffer from the waste port, using a P1000 pipette. ☐ Slide the inlet port cover clockwise to open the inlet port.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
After opening the inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip. ☐ Load 400 µl of the prepared Flow Cell Wash Mix into the flow cell via the inlet port, avoiding the introduction of air. ☐ Close the inlet port and wait for 1 hour. ☐ Ensure the inlet port is closed and remove buffer from the waste port a second time.	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ Thaw the Sequencing Buffer UL (SBU), Loading Solution UL (LSU), Flush Tether UL (FTU) and one tube of Flow Cell Flush (FCF) at RT and mix by vortexing. Then spin down and place on ice. In a new tube, prepare the DNA library for loading as follows. Use a wide-bore pipette tip for the addition of the DNA library: ☐ 100 µl Sequencing Buffer UL (SBU) ☐ 10 µl Loading Solution UL (LSU) ☐ 90 µl DNA library ☐ Gently mix the prepared DNA library by slowly pipetting ten times using a wide-bore pipette tip. ☐ Incubate at RT for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous. Prepare the flow cell priming mix in a 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing at RT. ☐ 30 µl Flush Tether UL (FTU) ☐ 1170 µl Flow Cell Flush (FCF) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. ☐ Turn the valve to close the inlet port and use a P1000 to remove all fluid from the waste channel through one of the waste ports. ☐ Slide open the inlet port and load 500 µl of the priming mix into the flow cell via the inlet port to complete a second flow cell flush, avoiding the introduction of air bubbles. ☐ Close the inlet port and use a P1000 to remove all fluid from the waste channel through a waste port again. ☐ Open the inlet port cover of the flow cell in preparation for loading. ☐ Aspirate the DNA library with a wide-bore pipette tip. Ensure there are no air bubbles in the pipette tip. Place the wide-bore pipette tip directly on the inlet port. Slowly depress the pipette to dispense the library into the inlet port. ☐ Using a P200 pipette, set the pipette to 50 µl and insert the tip into Port 2. ☐ Very slowly turn the wheel of the pipette to pull the DNA library into the inlet port. Closely watch the DNA library on the inlet port and completely remove the pipette as soon as the library starts to be pulled into the port. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Resume the sequencing run on MinKNOW to continue data acquisition.	
Day 5: Washing and reloading Pore-C library on the PromethION Flow Cell	
Day 5: Pore-C experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL)	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip. Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. Once the 5 minute incubation time is complete, carefully load the remaining 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Close the inlet port and wait for 1 hour.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Resume the sequencing run in MinKNOW to continue data acquisition.	
Day 5: Washing and reloading the PromethION Flow Cell with ultra-long DNA library	
Day 5: Ultra-long DNA experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS

After opening the inlet 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 inlet port
- ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows:

- ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix
- ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip
- ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.
- ☐ Set a timer for a 5 minute incubation.

Once the 5 minute incubation time is complete, carefully load the remaining 200 µl of the prepared flow cell wash mix into the inlet port, as follows:

- ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix
- ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip
- ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.

☐ Close the inlet port and wait for 1 hour.

IMPORTANT

☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

Remove waste buffer, as follows:

- ☐ Close the inlet port.
- ☐ Insert a P1000 pipette into a waste port and remove the waste buffer.

IMPORTANT

☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.

☐ Thaw the Sequencing Buffer UL (SBU), Loading Solution UL (LSU), Flush Tether UL (FTU) and one tube of Flow Cell Flush (FCF) at RT and mix by vortexing. Then spin down and place on ice.

In a new tube, prepare the DNA library for loading as follows. Use a wide-bore pipette tip for the addition of the DNA library:

- ☐ 100 µl Sequencing Buffer UL (SBU)
- ☐ 10 µl Loading Solution UL (LSU)
- ☐ 90 µl DNA library

☐ Gently mix the prepared DNA library by slowly pipetting ten times using a wide-bore pipette tip.

☐ Incubate at RT for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous.

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the flow cell priming mix in a 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing at RT. ☐ 30 µl Flush Tether UL (FTU) ☐ 1170 µl Flow Cell Flush (FCF) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ Turn the valve to close the inlet port and use a P1000 to remove all fluid from the waste channel through one of the waste ports. ☐ Slide open the inlet port and load 500 µl of the priming mix into the flow cell via the inlet port to complete a second flow cell flush, avoiding the introduction of air bubbles. ☐ Close the inlet port and use a P1000 to remove all fluid from the waste channel through a waste port again. ☐ Open the inlet port cover of the flow cell in preparation for loading. ☐ Aspirate the DNA library with a wide-bore pipette tip. Ensure there are no air bubbles in the pipette tip. Place the wide-bore pipette tip directly on the inlet port. Slowly depress the pipette to dispense the library into the inlet port. ☐ Using a P200 pipette, set the pipette to 50 µl and insert the tip into Port 2. ☐ Very slowly turn the wheel of the pipette to pull the DNA library into the inlet port. Closely watch the DNA library on the inlet port and completely remove the pipette as soon as the library starts to be pulled into the port. ☐ Close the valve to seal the inlet port.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Resume the sequencing run in MinKNOW to continue data acquisition.	
Day 6: Washing and reloading Pore-C library on the PromethION Flow Cell	
Day 6: Pore-C experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. Once the 5 minute incubation time is complete, carefully load the remaining 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Close the inlet port and wait for 1 hour.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Resume the sequencing run in MinKNOW to continue data acquisition.	
Day 7: Washing and reloading Pore-C library on the PromethION Flow Cell	
Day 7: Pore-C experiment flow cell washing and reloading ☐ Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube. ☐ Thaw one tube of Wash Diluent (DIL) at RT. ☐ Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix: ☐ 2 µl Wash Mix (WMX) ☐ 398 µl Wash Diluent (DIL) ☐ Mix well by pipetting, and place on ice. Do not vortex the tube. ☐ Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove waste buffer, as follows: ☐ Close the inlet port. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer. ☐ Slide the inlet port cover clockwise to open the inlet 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 inlet 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 inlet port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip. Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Set a timer for a 5 minute incubation. Once the 5 minute incubation time is complete, carefully load the remaining 200 µl of the prepared flow cell wash mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 200 µl of the flow cell wash mix ☐ Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip. ☐ Close the inlet port and wait for 1 hour.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer	
IMPORTANT ☐ The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.	
☐ 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. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 30 µl Flow Cell Tether (FCT) ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.	
☐ Close the inlet port and wait five minutes. ☐ 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: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. Slowly load 500 µl of the priming mix into the inlet port, as follows: ☐ Using a P1000 pipette, take 500 µl of the priming mix ☐ Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip ☐ Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger very slowly, leaving a small volume of buffer in the pipette tip.	
IMPORTANT ☐ It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.	
Remove the waste buffer, as follows: ☐ Ensure the inlet port is closed. ☐ Insert a P1000 pipette into a waste port and remove the waste buffer ☐ Slide the inlet port cover clockwise to open.	
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 inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Mix the prepared library gently by pipetting up and down just prior to loading.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Load 200 µl of library into the inlet port using a P1000 pipette. ☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover. ☐ Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Resume the sequencing run in MinKNOW to continue data acquisition.	
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.	