Ligation sequencing gDNA - Multiplex Ligation Sequencing Kit V14 XL (SQK-MLK114.96-XL)

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

Before start checklist

Materials

- [ ] Multiplex Ligation Sequencing Kit V14 XL (SQK-MLK114.96-XL)
- [ ] 1000 ng gDNA per sample

Consumables

- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 2 ml Eppendorf DNA LoBind tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

Equipment

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

INSTRUCTIONS	NOTES/OBSERVATIONS
DNA repair and end-prep	
- [ ] Thaw the AMPure XP Beads (AXP) at RT and mix by vortexing. Keep the beads at RT. 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix.	
☐ In clean 0.2 ml thin-walled PCR tubes, aliquot 1000 ng per sample.	
☐ Make up each sample to 12 µl using Nuclease-free water. Mix gently by pipetting and spin down.	
Combine the following components per sample: ☐ 12 µl DNA sample ☐ 0.875 µl NEBNext FFPE DNA Repair Buffer ☐ 0.875 µl Ultra II End-prep reaction buffer ☐ 0.75 µl Ultra II End-prep enzyme mix ☐ 0.50 µl NEBNext FFPE DNA Repair Mix	
☐ Ensure the components are thoroughly mixed by pipetting and spin down briefly.	
☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.	
☐ Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tube.	
☐ Resuspend the AMPure XP beads (AXP) by vortexing.	
☐ Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.	
☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.	
☐ Prepare 500 µl of 80% ethanol in Nuclease-free water per sample.	
☐ Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.	
☐ Keep the tubes on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.	
☐ Repeat the previous step.	
☐ Briefly spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.	
☐ Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl Nuclease-free water. Spin down and incubate for 2 minutes at RT.	
☐ Pellet the beads on a magnet until the eluate is clear and colourless.	
☐ Remove and retain 10 µl of eluate for each sample into clean 1.5 ml Eppendorf DNA LoBind tubes, individually.	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
☐ Keeping your samples separate, sandardise them to an equimolar mass.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Take forward the equimolar samples in 7.5 µl to be barcoded and pooled in the native barcode ligation step. However, at this point it is also possible to store the sample at 4°C overnight.	
Native barcode ligation	
Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. ☐ Thaw the EDTA at RT and mix by vortexing. Then spin down and place on ice. ☐ Thaw the native barcodes at RT. Use one barcode per sample. Individually mix the barcodes by pipetting, spin down, and place them on ice. Select a unique barcode for each sample to be run in a group: ☐ For two samples on one flow cell, select two unique barcodes, one for each sample. ☐ For three samples on two flow cells, select three unique barcodes, one for each sample. In clean 1.5 ml Eppendorf DNA LoBind tubes, add the reagents in the following order per sample: ☐ 7.5 µl End-prepped DNA ☐ 2.5 µl Native barcode ☐ 10 µl Blunt/TA Ligase Master Mix ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate for 20 minutes at RT. ☐ Add 4 µl of EDTA to each tube and mix thoroughly by pipetting and spin down briefly. ☐ Pool each group of two or three uniquely barcoded samples in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Ensure the beads are at RT and resuspend the AMPure XP beads (AXP) by vortexing. Add AMPure XP Beads (AXP) to the pooled reaction in a 0.4X ratio and mix by pipetting. The volume for AMPure XP Beads (AXP) will vary depending on the number of barcoded samples in the pool: ☐ For two barcoded samples add 19 µl of AMPure XP Beads (AXP) ☐ For three barcoded samples add 29 µl of AMPure XP Beads (AXP) ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Prepare 1 ml of fresh 80% ethanol in Nuclease-free water per barcoded sample pool. ☐ Spin down the sample for 5 seconds and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant. ☐ Keep the tube on the magentic rack 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water by gently flicking. ☐ Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. ☐ Spin down the tube for 5 seconds and pellet the beads on a magnetic rack until the eluate is clear and colourless. ☐ Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
Take forward the barcoded DNA library to the adapter ligation and clean-up step. However, you may store the sample at 4°C overnight.	
Adapter ligation and clean-up	
IMPORTANT ☐ The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.	
Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. Note: Do NOT vortex the Quick T4 DNA Ligase.	
IMPORTANT ☐ Do not vortex the Quick T4 DNA Ligase.	
☐ Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice. ☐ Thaw the Elution Buffer (EB) at RT and mix by vortexing. Then spin down and place on ice. ☐ To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. In a 1.5 ml Eppendorf LoBind tube, mix in the following order: ☐ 30 µl Pooled barcoded sample ☐ 5 µl Native Adapter (NA) ☐ 10 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 5 µl Quick T4 DNA Ligase ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 10 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ The next clean-up step uses Long Fragment Buffer (LFB) rather than 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.	
☐ Resuspend the AMPure XP Beads (AXP) by vortexing. ☐ Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant. ☐ Wash the beads by adding 125 µl Long Fragment Buffer (LFB). 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 pellet in 35 µl Elution Buffer (EB). ☐ Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 35 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 each library to 32 µl at 50 fmol, using Elution Buffer (EB). ☐ For two samples on one flow cell, make one DNA library from your eluate. ☐ For three samples on two flow cells, make two DNA libraries from your eluate.	
IMPORTANT ☐ Where possible, we recommend loading ~50 fmol of this final prepared library onto the R10.4.1 flow cell for our Multiplex Ligation Sequencing V14 protocol.	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Priming and loading multiple flow cells on a PromethION	
IMPORTANT ☐ This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).	
☐ 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 before storing on ice.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ Scale up reagent volumes as needed.	
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 PromethION 2 Solo, load the flow cell(s) as follows: ☐ Place the flow cell flat on the metal plate. ☐ Slide the flow cell into the docking port until the gold pins or green board cannot be seen. 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.	
☐ If not already completed, perform a flow cell check on all flow cells. ☐ 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.	

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 ☐ 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 and close the PromethION lid when ready.	
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. ☐ For multiple flow cell washing, use the same experiment name and identifying sample IDs for all runs to enable all flow cells to be paused simultaneously.	
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.	