Ligation sequencing V14 - low input by PCR (SQK-LSK114 with EXP-PCA001)

Version: LWP_9183_v114_revK_07Mar2023
Last update: 21/08/2024

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

Before start checklist

Materials

- [ ] 100 ng of sheared genomic DNA or amplicon DNA
- [ ] Ligation Sequencing Kit V14 (SQK-LSK114)
- [ ] PCR Expansion (EXP-PCA001)

Consumables

- [ ] MinION and GridION Flow Cell
- [ ] LongAmp Hot Start Taq 2X Master Mix (NEB, M0533)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] Salt-T4® DNA Ligase (NEB, M0467)
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] Agencourt AMPure XP beads (Beckman Coulter, A63881)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- [ ] MinION or GridION device
- [ ] MinION and GridION Flow Cell Light Shield
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
Check your flow cell.	
Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice: ☐ Thaw all reagents on ice. ☐ Ensure the reagents are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The NEBNext Ultra II End Prep Reaction Buffer may contain a white precipitate. If this occurs, allow the mixture(s) to come to RT and pipette the buffer several times to break up the precipitate, followed by a quick vortex to mix.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the DNA in Nuclease-free water ☐ Transfer 100 ng genomic DNA into a 0.2 ml thin-walled PCR tube ☐ Adjust the volume to 48 µl with Nuclease-free water ☐ Mix thoroughly by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 48 µl 100 ng DNA ☐ 7 µl Ultra II End-prep Reaction Buffer ☐ 3 µl Ultra II End-prep Enzyme Mix ☐ 2 µl Nuclease-free water ☐ Mix by pipetting and briefly spin 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 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. ☐ 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 sample from the magnet and resuspend the pellet in 16 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 16 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Take forward the repaired and end-prepped DNA into the next step. However, at this point it is also possible to store the sample at 4°C overnight.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
PCR adapters ligation and amplification	
Add the reagents as follows in the order below: ☐ 15 µl End-prepped DNA ☐ 10 µl PCR Adapter (PCA) ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix by pipetting and briefly spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads for a 0.4X clean and mix by pipetting up and down ten times. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. ☐ 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 25 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 the eluate once it is clear and colourless. Transfer the eluted sample to a fresh 0.2 ml PCR tube. ☐ Dispose of the pelleted beads	
IMPORTANT ☐ If using amplicon input, start your second round of PCR from this point. Ensure the sample has undergone a round of PCR with tailed primers.	
☐ Quantify 1 µl of adapted DNA using a Qubit fluorometer. Prepare the PCA adapted DNA in Nuclease-free water: ☐ Transfer 20 ng genomic DNA into a 0.2 ml thin-walled PCR tube. ☐ Adjust the volume to 48 µl with Nuclease-free water. ☐ Mix thoroughly by flicking the tube. ☐ Spin down briefly in a microfuge.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Set up the adapted DNA PCR as follows: ☐ 46 µl Nuclease-free water ☐ 2 µl Primer Mix (PRM) ☐ 2 µl 10 ng/µl adapter ligated template ☐ 50 µl HotStart LongAmp Taq 2x master mix ☐ Mix gently by flicking the tube, and spin down. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 94 °C (1 cycle) ☐ Denaturation 15 secs @ 94 °C (15-18 (a) cycles) ☐ Annealing 15 secs @ 56 °C (15-18 (a) cycles) ☐ Extension 6 mins (b) @ 65 °C (15-18 (a) cycles) ☐ Final extension 10 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ 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. ☐ Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. ☐ Keep the samples on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellets. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the 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 pellet in 52 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 52 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of adapted DNA using a Qubit fluorometer.	
Take forward the sample into the next step. However, at this point, it is also possible to store the sample at 4°C overnight.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice: ☐ Thaw all reagents on ice. ☐ Ensure the reagents are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The NEBNext Ultra II End Prep Reaction Buffer may contain a white precipitate. If this occurs, allow the mixture(s) to come to RT and pipette the buffer several times to break up the precipitate, followed by a quick vortex to mix. Prepare the PCR amplified DNA in Nuclease-free water ☐ Transfer 1 ug genomic DNA into a 0.2 ml thin-walled PCR tube. ☐ Adjust the volume to 50 µl with Nuclease-free water. ☐ Mix thoroughly by flicking the tube to avoid unwanted shearing. ☐ Spin down briefly in a microfuge. In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 50 µl DNA ☐ 7 µl Ultra II End-prep Reaction Buffer ☐ 3 µl Ultra II End-prep Enzyme Mix ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. ☐ Resuspend the AMPure XP Beads (AXP) by vortexing. ☐ Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 61 µl Nuclease-free water. Incubate for 2 minutes at RT.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4°C overnight.	
Adapter ligation and clean-up	
IMPORTANT ☐ Although third-party ligase products may be supplied with their 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 Salt-T4® DNA Ligase, and place on ice. ☐ Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing. ☐ Thaw the Elution Buffer (EB) at RT and mix by vortexing. Then spin down and place on ice.	
IMPORTANT Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally. ☐ To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB) ☐ To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)	
☐ Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at RT and mix by vortexing. Then spin down and place on ice. In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order: ☐ 60 µl DNA sample from the previous step ☐ 5 µl Ligation Adapter (LA) ☐ 25 µl Ligation Buffer (LNB) ☐ 10 µl Salt-T4® DNA Ligase ☐ Thoroughly mix the reaction by gently pipetting and briefly spinning down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP Beads (AXP) by vortexing. ☐ Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at RT. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
Depending on your DNA library fragment size, prepare your final library in 12 µl of Elution Buffer (EB). ☐ 100 fmol Very short (<1 kb) ☐ 35–50 fmol Short (1-10 kb) ☐ 300 ng Long (>10 kb)	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Priming and loading the MinION and GridION Flow Cell	
IMPORTANT ☐ Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice.	
IMPORTANT ☐ For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.	
To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at RT. ☐ 1,170 µl Flow Cell Flush (FCF) ☐ 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml ☐ 30 µl Flow Cell Tether (FCT)	
☐ Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Slide the flow cell priming port cover clockwise to open the priming port.	
IMPORTANT ☐ Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.	
After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip Note: Visually check that there is continuous buffer from the priming port across the sensor array. ☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below. ☐ Thoroughly mix the contents of the Library Beads (LIB) by pipetting.	
IMPORTANT ☐ The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SB) ☐ 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using ☐ 12 µl DNA library Complete the flow cell priming: ☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible. ☐ Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
Place the light shield onto the flow cell, as follows: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Close the device lid and set up a sequencing run on MinKNOW.	
Flow cell reuse and returns	
☐ After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C. ☐ Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT ☐ If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.	