

Ligation sequencing amplicons V14 (SQK-LSK114)

Version: ACDE_9163_v114_revS_29Jun2022
Last update: 07/03/2024



Flow Cell Number:

DNA Samples:

Before start checklist

Materials

- ☐ 100-200 fmol of amplicon DNA
- ☐ Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables

- ☐ MinION and GridION Flow Cell
- ☐ 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)
- ☐ Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- ☐ (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g. Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- ☐ Freshly prepared 80% ethanol in nuclease-free water
- ☐ Nuclease-free water (e.g. ThermoFisher, AM9937)
- ☐ 1.5 ml Eppendorf DNA LoBind tubes
- ☐ 0.2 ml thin-walled PCR tubes
- ☐ Qubit™ Assay Tubes (Invitrogen, Q32856)

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.	
<div><input type="checkbox"/> Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice.</div> <div>Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice:</div> <div><input type="checkbox"/> Thaw all reagents on ice.</div> <div><input type="checkbox"/> Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix.</div> <div><input type="checkbox"/> Always spin down tubes before opening for the first time each day.</div> <div><input type="checkbox"/> The Ultra II End Prep Buffer may have a little precipitate. Allow the mixture to come to RT and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.</div>	

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<p>IMPORTANT</p> <p><input type="checkbox"/> Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.</p>	
<p>Prepare the DNA in Nuclease-free water:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Transfer 100-200 fmol of amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube <input type="checkbox"/> Adjust the volume to 49 µl with Nuclease-free water <input type="checkbox"/> Mix thoroughly by pipetting up and down, or by flicking the tube <input type="checkbox"/> Spin down briefly in a microfuge <p>In a 0.2 ml thin-walled PCR tube, mix the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 1 µl DNA CS <input type="checkbox"/> 49 µl DNA <input type="checkbox"/> 7 µl Ultra II End-prep Reaction Buffer <input type="checkbox"/> 3 µl Ultra II End-prep Enzyme Mix <ul style="list-style-type: none"> <input type="checkbox"/> Thoroughly mix the reaction by gently pipetting and briefly spinning down. <input type="checkbox"/> Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. <input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> Remove the tube from the magnetic rack and resuspend the pellet in 61 µl Nuclease-free water. Incubate for 2 minutes at RT. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> 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.	

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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 <input type="checkbox"/> Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.	
IMPORTANT <input type="checkbox"/> 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.	
<input type="checkbox"/> Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB) <input type="checkbox"/> To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)	
<input type="checkbox"/> 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: <ul style="list-style-type: none"> <input type="checkbox"/> 60 µl DNA sample from the previous step <input type="checkbox"/> 25 µl Ligation Buffer (LNB) <input type="checkbox"/> 10 µl NEBNext Quick T4 DNA Ligase <input type="checkbox"/> 5 µl Ligation Adapter (LA) <input type="checkbox"/> Thoroughly mix the reaction by gently pipetting and briefly spinning down. <input type="checkbox"/> Incubate the reaction for 10 minutes at RT. <input type="checkbox"/> Resuspend the AMPure XP Beads (AXP) by vortexing. <input type="checkbox"/> Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. <input type="checkbox"/> 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.	

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<input type="checkbox"/> Repeat the previous step. <input type="checkbox"/> 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. <input type="checkbox"/> 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. <input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> 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.	
<input type="checkbox"/> Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB).	
IMPORTANT <input type="checkbox"/> We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 flow cell.	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Priming and loading the MinION and GridION Flow Cell	
IMPORTANT <input type="checkbox"/> Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).	
<input type="checkbox"/> 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 <input type="checkbox"/> 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. <input type="checkbox"/> 1,170 µl Flow Cell Flush (FCF) <input type="checkbox"/> 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml <input type="checkbox"/> 30 µl Flow Cell Tether (FCT) <input type="checkbox"/> 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. <input type="checkbox"/> Slide the flow cell priming port cover clockwise to open the priming port.	

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<p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> <p>After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set a P1000 pipette to 200 µl <input type="checkbox"/> Insert the tip into the priming port <input type="checkbox"/> 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 <p>Note: Visually check that there is continuous buffer from the priming port across the sensor array.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Thoroughly mix the contents of the Library Beads (LIB) by pipetting.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> <p>In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 µl Sequencing Buffer (SB) <input type="checkbox"/> 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using <input type="checkbox"/> 12 µl DNA library <p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible. <input type="checkbox"/> Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> 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. <input type="checkbox"/> Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port. 	
<p>IMPORTANT</p> <p><input type="checkbox"/> Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.</p> <p>Place the light shield onto the flow cell, as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. <input type="checkbox"/> 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. 	
<p>Close the device lid and set up a sequencing run on MinKNOW.</p>	

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Flow cell reuse and returns	
<input type="checkbox"/> 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.	
<input type="checkbox"/> Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT <input type="checkbox"/> 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.	