Ligation sequencing V14 - Direct cDNA sequencing (SQK-LSK114)

Version: DCS_9187_v114_revG_19Apr2023
Last update: 07/03/2024

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Before start checklist

Materials

- [ ] 100 ng Poly(A)+ RNA OR 1 µg of total RNA
- [ ] Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables

- [ ] User-supplied VN Primer, 2 µM
- [ ] User-supplied Strand-Switching Primer, 10 µM
- [ ] User-supplied PR2 Primer, 10 µM
- [ ] 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)
- [ ] 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
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- [ ] RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286)
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g

Equipment

- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf cat # 022510509)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

Invitrogen™ UltraPure™ BSA 50 mg/ml,

AM2616)

INSTRUCTIONS	NOTES/OBSERVATIONS

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INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription and strand-switching	
IMPORTANT ☐ If you have already prepared your cDNA, use 70–200 fmol cDNA (~70–200 ng if your sample is 1.5 kb) and start from the cDNA repair and end-prep step.	
Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice. ☐ User-supplied VN Primer diluted to 2 µM: thaw at RT, briefly spin down, mix well by pipetting ☐ User-supplied Strand-Switching Primer diluted to 10 µM: thaw at RT, briefly spin down, mix well by pipetting ☐ 10 mM dNTP solution: thaw at RT, briefly spin down, mix well by pipetting ☐ RNaseOUT: not frozen, briefly spin down, mix well by pipetting ☐ Maxima H Minus Reverse Transcriptase: not frozen, briefly spin down, mix well by pipetting ☐ Maxima H Minus 5x RT Buffer: thaw at RT, briefly spin down, mix by vortexing Prepare the RNA in Nuclease-free water ☐ Transfer 100 ng Poly(A)+ RNA or 1 µg of total RNA into a 0.2 ml PCR tube ☐ Adjust the volume to up to 7.5 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge Prepare the following reaction in the 0.2 ml PCR tube containing the prepared RNA input: ☐ 7.5 µl RNA input (100 ng Poly(A)+ RNA or 1 µg of total RNA) from step above ☐ 2.5 µl VN Primer diluted to 2 µM ☐ 1 µl 10 mM dNTPs ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate at 65°C for 5 minutes and then snap cool on a pre-chilled freezer block for 1 minute. In a separate tube, mix together the following: ☐ 4 µl 5x RT Buffer ☐ 1 µl RNaseOUT ☐ 1 µl Nuclease-free water ☐ 2 µl Strand-Switching Primer diluted to 10 µM ☐ Mix gently by flicking the tube, and spin down. ☐ Add the 8 µl of strand-switching reagents (prepared in steps 6-7) to the 11 µl of snap-cooled mRNA (from steps 2-5). Mix by flicking the tube and spin down. ☐ Incubate at 42°C for 2 minutes in the thermal cycler. ☐ Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl. ☐ Mix gently by flicking the tube, and spin down.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Incubate using the following protocol using a thermal cycler: ☐ Reverse transcription and strand-switching 90 mins @ 42°C (1 cycle) ☐ Heat inactivation 5 mins @ 85°C (1 cycle) ☐ Hold @ 4°C	
RNA degradation and second strand synthesis	
Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice. ☐ User-supplied PR2 Primer diluted to 10 µM: thaw at RT, briefly spin down, mix well by pipetting ☐ RNase Cocktail Enzyme Mix: not frozen, briefly spin down, mix well by pipetting ☐ LongAmp Taq 2X Master Mix: thaw at RT, briefly spin down, mix well by pipetting ☐ Thaw the AMPure XP Beads (AXP) at RT and mix by vortexing. Keep the beads at RT. ☐ Add 1 µl RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286) to the reverse transcription reaction. ☐ Incubate the reaction for 10 minutes at 37° C in a thermal cycler. ☐ Resuspend the AMPure XP beads (AXP) by vortexing. ☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Add 17 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. ☐ 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. ☐ 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. ☐ 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 20 µl Nuclease-free water. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the following reaction in a 0.2 ml thin-walled PCR tube: ☐ 25 µl 2x LongAmp Taq Master Mix ☐ 2 µl PR2 Primer diluted to 10 µM ☐ 20 µl Reverse-transcribed sample from above ☐ 3 µl Nuclease-free water Incubate using the following protocol: ☐ Denaturation 1 mins @ 94 °C (1 cycle) ☐ Annealing 1 mins @ 50 °C (1 cycle) ☐ Extension 15 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ Resuspend the AMPure XP beads (AXP) by vortexing. ☐ Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ 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. ☐ 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. ☐ 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. ☐ 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 21 µl Nuclease-free water. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Analyse 1 µl of the strand-switched DNA for size, quantity and quality using an Agilent Bioanalyzer and Qubit fluorometer (or equivalent).	
Take forward the full volume of your sample into the cDNA repair and end-prep stage of the protocol.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
cDNA repair and end-prep	
IMPORTANT ☐ If you have prepared your own cDNA instead of performing reverse transcription using the method outlined in this protocol, start this step with 70–200 fmol cDNA (~70–200 ng if your sample is 1.5 kb) in 20 µl Nuclease-free water.	
Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice: ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep Buffer may have a little precipitate. Allow the mixture to come to RT and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate. Combine the following reagents in a 0.2 ml PCR tube: ☐ 20 µl cDNA sample ☐ 30 µl Nuclease-free water ☐ 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 pellet in 61 µl Nuclease-free water. Incubate for 2 minutes at RT.	

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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 60 µl of repaired and end-prepped cDNA 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 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.	
IMPORTANT ☐ Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.	
☐ 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 cDNA 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 (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. ☐ Wash the beads by adding 250 µl of 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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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB). IMPORTANT ☐ We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 flow cell.	
The prepared library is used for loading 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. ☐ Slide the flow cell priming port cover clockwise to open the priming port.	

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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 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.

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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.	