

Flow Cell Number:

DNA Samples:

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

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

Version: DCS_9187_v114_revH_19Apr2023
 Last update: 18/04/2024

Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Reverse transcription and strand-switching</p> <p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> <p>Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.</p> <ul style="list-style-type: none"> <input type="checkbox"/> User-supplied VN Primer diluted to 2 μM: thaw at RT, briefly spin down, mix well by pipetting <input type="checkbox"/> User-supplied Strand-Switching Primer diluted to 10 μM: thaw at RT, briefly spin down, mix well by pipetting <input type="checkbox"/> 10 mM dNTP solution: thaw at RT, briefly spin down, mix well by pipetting <input type="checkbox"/> RNaseOUT: not frozen, briefly spin down, mix well by pipetting <input type="checkbox"/> Maxima H Minus Reverse Transcriptase: not frozen, briefly spin down, mix well by pipetting <input type="checkbox"/> Maxima H Minus 5x RT Buffer: thaw at RT, briefly spin down, mix by vortexing <p>Prepare the RNA in Nuclease-free water</p> <ul style="list-style-type: none"> <input type="checkbox"/> Transfer 100 ng Poly(A)+ RNA or 1 μg of total RNA into a 0.2 ml PCR tube <input type="checkbox"/> Adjust the volume to up to 7.5 μl with Nuclease-free water <input type="checkbox"/> Mix by flicking the tube to avoid unwanted shearing <input type="checkbox"/> Spin down briefly in a microfuge <p>Prepare the following reaction in the 0.2 ml PCR tube containing the prepared RNA input:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 7.5 μl RNA input (100 ng Poly(A)+ RNA or 1 μg of total RNA) from step above <input type="checkbox"/> 2.5 μl VN Primer diluted to 2 μM <input type="checkbox"/> 1 μl 10 mM dNTPs <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Incubate at 65°C for 5 minutes and then snap cool on a pre-chilled freezer block for 1 minute.</p> <p>In a separate tube, mix together the following:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 4 μl 5x RT Buffer <input type="checkbox"/> 1 μl RNaseOUT <input type="checkbox"/> 1 μl Nuclease-free water <input type="checkbox"/> 2 μl Strand-Switching Primer diluted to 10 μM <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Incubate at 42°C for 2 minutes in the thermal cycler.</p> <p><input type="checkbox"/> Add 1 μl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 μl.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p>	

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

Version: DCS_9187_v114_revH_19Apr2023
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DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Incubate using the following protocol using a thermal cycler:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Reverse transcription and strand-switching 90 mins @ 42°C (1 cycle) <input type="checkbox"/> Heat inactivation 5 mins @ 85°C (1 cycle) <input type="checkbox"/> Hold @ 4°C 	
<p>RNA degradation and second strand synthesis</p>	
<p>Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.</p> <ul style="list-style-type: none"> <input type="checkbox"/> User-supplied PR2 Primer diluted to 10 µM: thaw at RT, briefly spin down, mix well by pipetting <input type="checkbox"/> RNase Cocktail Enzyme Mix: not frozen, briefly spin down, mix well by pipetting <input type="checkbox"/> LongAmp Taq 2X Master Mix: thaw at RT, briefly spin down, mix well by pipetting <input type="checkbox"/> Thaw the AMPure XP Beads (AXP) at RT and mix by vortexing. Keep the beads at RT. <input type="checkbox"/> Add 1 µl RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286) to the reverse transcription reaction. <input type="checkbox"/> Incubate the reaction for 10 minutes at 37° C in a thermal cycler. <input type="checkbox"/> Resuspend the AMPure XP beads (AXP) by vortexing. <input type="checkbox"/> Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. <input type="checkbox"/> Add 17 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"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. <input type="checkbox"/> 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. <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 pellet in 20 µl Nuclease-free water. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	

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

Version: DCS_9187_v114_revH_19Apr2023
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Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>Prepare the following reaction in a 0.2 ml thin-walled PCR tube:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 25 µl 2x LongAmp Taq Master Mix <input type="checkbox"/> 2 µl PR2 Primer diluted to 10 µM <input type="checkbox"/> 20 µl Reverse-transcribed sample from above <input type="checkbox"/> 3 µl Nuclease-free water <p>Incubate using the following protocol:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Denaturation 1 mins @ 94 °C (1 cycle) <input type="checkbox"/> Annealing 1 mins @ 50 °C (1 cycle) <input type="checkbox"/> Extension 15 mins @ 65 °C (1 cycle) <input type="checkbox"/> Hold @ 4 °C <ul style="list-style-type: none"> <input type="checkbox"/> Resuspend the AMPure XP beads (AXP) by vortexing. <input type="checkbox"/> Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube. <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"/> Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. <input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. <input type="checkbox"/> 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. <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 pellet in 21 µl Nuclease-free water. <input type="checkbox"/> Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. <input type="checkbox"/> Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colourless, for at least 1 minute. <input type="checkbox"/> Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. 	
<p>Analyse 1 µl of the strand-switched DNA for size, quantity and quality using an Agilent Bioanalyzer and Qubit fluorometer (or equivalent).</p>	
<p>Take forward the full volume of your sample into the cDNA repair and end-prep stage of the protocol.</p>	

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

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Flow Cell Number:

DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<p>cDNA repair and end-prep</p> <p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> <p>Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Thaw all reagents on ice. <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. <input type="checkbox"/> Always spin down tubes before opening for the first time each day. <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. <p>Combine the following reagents in a 0.2 ml PCR tube:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 20 µl cDNA sample <input type="checkbox"/> 30 µl Nuclease-free water <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 pellet in 61 µl Nuclease-free water. Incubate for 2 minutes at RT. 	

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

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DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<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.	
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	
<p>IMPORTANT</p> <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.	
<p>IMPORTANT</p> <input type="checkbox"/> Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.	
<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. <input type="checkbox"/> 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: <ul style="list-style-type: none"> <input type="checkbox"/> 60 µl cDNA 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 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.	

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

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DNA Samples:

INSTRUCTIONS	NOTES/OBSERVATIONS
<ul style="list-style-type: none"> <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. <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. 	
<p>Quantify 1 µl of eluted sample using a Qubit fluorometer.</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> Prepare 35-50 fmol of your final library to 12 µl with Elution Buffer (EB). 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> We recommend loading 35-50 fmol of this final prepared library onto the R10.4.1 flow cell. 	
<p>The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.</p>	
<p>Priming and loading the MinION and GridION Flow Cell</p>	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114). 	
<ul style="list-style-type: none"> <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. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <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. 	
<p>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.</p> <ul style="list-style-type: none"> <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) <ul style="list-style-type: none"> <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. <ul style="list-style-type: none"> <input type="checkbox"/> Slide the flow cell priming port cover clockwise to open the priming port. 	

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INSTRUCTIONS	NOTES/OBSERVATIONS
<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. 	

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



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INSTRUCTIONS	NOTES/OBSERVATIONS
Close the device lid and set up a sequencing run on MinKNOW.	
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.	