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

Before start checklist

Materials

- [ ] 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample
- [ ] cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24)

Consumables

- [ ] MinION and GridION Flow Cell
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] T4 DNA Ligase 2M U/ml (NEB, M0202M)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- [ ] Lambda Exonuclease (NEB, Cat # M0262L)
- [ ] Thermolabile Exonuclease I (NEB, cat # M0568)
- [ ] USER (Uracil-Specific Excision Reagent) Enzyme (NEB, cat # M5505L)
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0752)
- [ ] LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- [ ] Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- [ ] Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
- [ ] Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 0.2 ml thin-walled PCR tubes

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)
- [ ] Agilent Bioanalyzer (or equivalent)
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription and strand-switching	
Check your flow cell.	

Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice.

- ☐ cDNA RT Adapter (CRTA): thaw at RT, briefly spin down, mix well by pipetting
- ☐ Annealing Buffer (AB): thaw at RT, briefly spin down, mix well by pipetting
- ☐ Short Fragment Buffer (SFB): thaw at RT, briefly spin down, mix well by pipetting
- ☐ RT Primer (RTP): thaw at RT, briefly spin down, mix well by pipetting
- ☐ Strand Switching Primer II (SSPII): thaw at RT, briefly spin down, mix well by pipetting
- ☐ NEBNext® Quick Ligation Reaction Buffer: thaw at RT, briefly spin down, mix by vortexing
- ☐ T4 DNA Ligase 2M U/ml: not frozen, briefly spin down, mix well by pipetting
- ☐ RNaseOUT: not frozen, briefly spin down, mix well by pipetting
- ☐ Lambda Exonuclease: not frozen, briefly spin down, mix well by pipetting
- ☐ Uracil-Specific Excision Reagent (USER): not frozen, briefly spin down, mix well by pipetting
- ☐ 10 mM dNTP solution: thaw at RT, 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

IMPORTANT

☐ It is important that the NEBNext Quick Ligation Reaction Buffer is mixed well by vortexing.

For each sample, prepare the RNA in Nuclease-free water:

- ☐ Transfer 10 ng Poly(A)+ RNA, or 500 ng total RNA into a 0.2 ml thin-walled PCR tube
- ☐ Adjust the volume to up to 10 µl with Nuclease-free water
- ☐ Mix by flicking the tube to avoid unwanted shearing
- ☐ Spin down briefly in a microfuge

Prepare the following in a 0.2 ml PCR tube per sample:

- ☐ 10 µl RNA
- ☐ 1 µl cDNA RT Adapter (CRTA)
- ☐ 1 µl Annealing Buffer (AB)

☐ Mix gently by flicking the tubes, and spin down.

☐ Incubate the reactions in the thermal cycler at 60°C for 5 mins, then cool for 5 minutes at RT.

To each of the 0.2 ml PCR tubes containing you RNA sample(s), add the following:

- ☐ 12 µl RNA sample (from previous step)
- ☐ 3.6 µl NEBNext® Quick Ligation Reaction Buffer
- ☐ 1.4 µl T4 DNA Ligase 2M U/ml
- ☐ 1 µl RNaseOUT

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Ensure the components are thoroughly mixed by pipetting the contents of the tubes 10 times and spin down. ☐ Incubate for 10 minutes at RT. To each of the 0.2 ml PCR tubes, add the following: ☐ 18 µl RNA sample (from previous step) ☐ 1 µl Lambda Exonuclease ☐ 1 µl USER (Uracil-Specific Excision Reagent) ☐ Ensure the components are thoroughly mixed by flicking the tubes and spin down. ☐ Incubate for 5 minutes at 37°C in the thermal cycler. ☐ Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes. ☐ Resuspend the RNase-free XP beads by vortexing. ☐ Add 36 µl of resuspended RNase-free XP beads to each reaction and mix gently by flicking the tubes. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant. Keep the tubes on the magnet and wash the beads with 100 µl of Short Fragment Buffer (SFB) as follows: ☐ Wash the beads with 100 µl of Short Fragment Buffer (SFB). ☐ Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and to form a pellet. ☐ Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again. ☐ Without disturbing the pellet, remove the Short Fragment Buffer (SFB) using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tubes back on the magnet. Pipette off any residual buffer. Briefly 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 each pellet in 12 µl of Nuclease-free water. ☐ Incubate at RT for 10 minutes. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample. To each of the 0.2 ml PCR tubes, add the following: ☐ 12 µl Eluted sample (from previous step) ☐ 1 µl RT Primer (RTP) ☐ 1 µl dNTPs (10 mM)	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Ensure the components are thoroughly mixed by flicking the tubes and spin down. ☐ Incubate the reaction for 5 minutes at RT. To each of the 0.2 ml PCR tubes, add the following: ☐ 14 µl RT primed sample (from previous step) ☐ 4.5 µl Maxima H Minus 5x RT Buffer ☐ 1 µl RNaseOUT ☐ 2 µl Strand Switching Primer II (SSPII) ☐ Mix gently by flicking the tubes, and spin down. ☐ Incubate at 42°C for 2 minutes in the thermal cycler. ☐ Add 1 µl of Maxima H Minus Reverse Transcriptase to each tube. The total volume will be 22.5 µl per tube. ☐ Mix gently by flicking the tubes, and spin down. Incubate using the following protocol using a thermal cycler: ☐ Reverse transcription and strand-switching 30 mins @ 42°C (1 cycle) ☐ Heat inactivation 5 mins @ 85°C (1 cycle) ☐ Hold @ 4°C	
Take your samples forward into the next step. However, at this point it is also possible to store the sample at -20°C overnight.	
Selecting for full-length transcripts by PCR	
IMPORTANT ☐ This kit enables multiplexing of up to 24 samples. The default method allows you to perform one 25 µl PCR reaction per sample. If multiplexing two or three samples, however, two separate PCR reactions per sample should be performed; if running just one sample, four separate PCR reactions should be performed as per the PCR-cDNA Sequencing Kit V14 (SQK-PCS114) protocol. These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.	
Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice. ☐ Barcode Primers (BP01 - BP24): thaw at RT, briefly spin down, mix well by pipetting ☐ Elution Buffer (EB): thaw at RT, briefly spin down, mix well by pipetting ☐ LongAmp Hot Start Taq 2X Master Mix: thaw at RT, briefly spin down, mix well by pipetting ☐ Thermolabile Exonuclease I: not frozen, briefly spin down, mix well by pipetting ☐ Spin down the reverse-transcribed RNA samples. ☐ Prepare a separate 0.2 ml PCR tube for each sample and add 5 µl of reverse-transcribed RNA per tube.	
IMPORTANT ☐ Only 5 µl of the reverse-transcribed sample is to be taken forward. Do NOT use all the 22.5 µl of the reverse transcription reaction in a single PCR reaction.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In each of the 0.2 ml PCR tubes containing reverse-transcribed RNA sample, prepare the following reaction at RT: ☐ 5 µl Reverse-transcribed sample (from previous step) ☐ 0.75 µl Unique Barcode Primer (BP01-24) ☐ 6.75 µl Nuclease-free water ☐ 12.5 µl 2x LongAmp Hot Start Taq Master Mix ☐ Mix gently by pipetting. Amplify using the following cycling conditions. ☐ Initial denaturation 30 secs @ 95°C (1 cycle) ☐ Denaturation 15 secs @ 95°C (10-18* cycles) ☐ Annealing 15 secs @ 62°C (10-18* cycles) ☐ Extension 60 secs per kb @ 65°C (10-18* cycles) ☐ Final extension 6 mins @ 65°C (1 cycle) ☐ Hold @ 4°C ☐ Add 1 µl Thermolabile Exonuclease I directly to each PCR tube. Mix by flicking the tube and briefly spin down. ☐ Incubate the reaction at 37°C for 5 minutes, followed by 80°C for 2 minutes in the thermal cycler. ☐ Transfer each sample to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 18 µl of resuspended AMPure XP beads to each 1.5 ml Eppendorf DNA LoBind tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 5 ml of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant. ☐ Keep the tubes on the magnet and wash the beads with 100 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ 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 pellets to the point of cracking. ☐ Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of Elution Buffer (EB). ☐ Incubate at RT for 10 minutes. ☐ Pellet the beads on the magnet until the eluate is clear and colourless.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Remove and retain 12 µl of each eluate into a separate clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the cDNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ For each sample, analyse 1 µl of the amplified cDNA for size, quantity and quality using a Qubit fluorometer and Agilent Bioanalyzer (or equivalent) for a QC check.	
IMPORTANT ☐ Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.	
Pool together equimolar samples of the amplified cDNA barcoded samples to a total of 50 fmols and make the volume up to 11 µl in Elution Buffer (EB). ☐ Please check the Mass to Molarity table in the protocol	
Adapter addition	
IMPORTANT ☐ The Rapid Adapter (RA) used in this kit and protocol is not interchangeable with other sequencing adapters.	
Thaw the kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below: In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix: ☐ 1.5 µl Rapid Adapter (RA) ☐ 3.5 µl Adapter Buffer (ADB) ☐ Add 1 µl of the diluted Rapid Adapter (RA) to the amplified cDNA library, making the total volume 12 µl. ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 5 minutes at RT. ☐ Spin down briefly.	
The prepared library is used for loading onto the flow cell. Store the library on ice 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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 the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix 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) ☐ 1,205 µl Final total volume in tube ☐ 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.	
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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
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.	