Rapid sequencing V14 - Amplicon sequencing (SQK-RBK114.24 or SQK-RBK114.96)

Version: RAA_9198_v114_revH_29Nov2023
Last update: 29/04/2024

Flow Cell Number: ....

DNA Samples: ....

Before start checklist

Materials

☐ 50 ng amplicon DNA per sample (500 bp – 5 kb amplicon size)

☐ Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) OR Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)

Consumables

☐ MinION and GridION Flow Cell

☐ Agencourt AMPure XP beads (Beckman Coulter™, A63881) (or equivalent for DNA purification)

☐ Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

☐ 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)

☐ Qubit™ Assay Tubes (Invitrogen, Q32856)

☐ 1.5 ml Eppendorf DNA LoBind tubes

☐ 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate

☐ 2 ml Eppendorf DNA LoBind tubes

Equipment

☐ MinION or GridION device

☐ MinION and GridION Flow Cell Light Shield

☐ Hula mixer (gentle rotator mixer)

☐ Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)

☐ Microfuge

☐ Vortex mixer

☐ Thermal cycler or heat blocks

☐ Qubit fluorometer (or equivalent for QC check)

☐ Magnetic rack

☐ Ice bucket with ice

☐ Timer

☐ Pipettes and pipette tips Multichannel, P2, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Library preparation	
Check your flow cell.	
IMPORTANT ☐ PCR clean-up of amplicon DNA samples	
☐ Program the thermal cycler: 30°C for 2 minutes, then 80°C for 2 minutes.	

INSTRUCTIONS	NOTES/OBSERVATIONS

Thaw kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

- ☐ Rapid Barcodes (RB01-24) or Rapid Barcode Plate (RB01-96): not frozen, briefly spin down, mix well by pipetting
- ☐ Rapid Adapter (RA): not frozen, briefly spin down, mix well by pipetting
- ☐ AMPure XP Beads (AXP): thaw at RT, briefly spin down, mix by pipetting or vortexing immediately before use
- ☐ Elution Buffer (EB): thaw at RT, briefly spin down, mix well by pipetting
- ☐ Adapter Buffer (ADB): thaw at RT, briefly spin down, mix by vortexing

Prepare the DNA in Nuclease-free water, as follows. Approximately 50 ng of amplicon DNA is required in 9 µl of volume for each sample for barcoding.

- ☐ 100 ng/µl 34 µl @ 2 µl (36 µl cycles)
- ☐ 90 ng/µl 31 µl @ 2 µl (33 µl cycles)
- ☐ 80 ng/µl 27 µl @ 2 µl (29 µl cycles)
- ☐ 70 ng/µl 35 µl @ 3 µl (38 µl cycles)
- ☐ 60 ng/µl 20 µl @ 2 µl (22 µl cycles)
- ☐ 50 ng/µl 16 µl @ 2 µl (18 µl cycles)
- ☐ 40 ng/µl 31 µl @ 5 µl (36 µl cycles)
- ☐ 30 ng/µl 22 µl @ 5 µl (27 µl cycles)
- ☐ 20 ng/µl 13 µl @ 5 µl (18 µl cycles)
- ☐ 10 ng/µl 8 µl @ 10 µl (18 µl cycles)
- ☐ <5.56 ng/µl 0 µl @ 9 µl (9 µl cycles)

☐ Select a unique barcode for every sample to be run together on the same flow cell. Up to 96 samples can be barcoded and combined in one experiment.

In 0.2 ml thin-walled PCR tubes or plate, mix the following reagents. The Rapid Barcodes can be transferred using a multichannel pipette:

- ☐ 9 µl 50 ng template DNA
- ☐ 1 µl Rapid Barcodes (RB01-96, one for each sample)

☐ Ensure the components are thoroughly mixed by pipetting and spin down briefly.

☐ Incubate the tubes or plate at 30°C for 2 minutes and then at 80°C for 2 minutes. Briefly put the tubes or plate on ice to cool.

☐ Spin down the tubes or plate to collect the liquid at the bottom.

☐ Pool all the barcoded samples into a clean 1.5 ml Eppendorf DNA LoBind tube, noting the total volume.

☐ Resuspend the AMPure XP beads (AXP) by vortexing.

INSTRUCTIONS	NOTES/OBSERVATIONS
To the entire pooled barcoded sample, add an equal volume of resuspended AMPure XP Beads (AXP) and mix by flicking the tube. ☐ Volume of AXP - Volume per sample: 10 µl - For 12 samples: 120 µl - For 24 samples: 240 µl - For 48 samples: 480 µl - For 96 samples: 960 µl ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Prepare at least 3 ml 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 tube on the magnet and wash the beads with 1.5 ml of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Briefly 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 15 µl Elution Buffer (EB). 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 into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
☐ Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube. 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 barcoded DNA. ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 5 minutes at RT.	
The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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.

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.

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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.	
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.	
Downstream analysis using EPI2ME	
Post-basecalling analysis	
IMPORTANT ☐ The wf-amplicon workflow is optimised for 500 bp – 5 kb amplicons.	
☐ Open the EPI2ME app using the desktop shortcut.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Navigate to the workflow downloads page. Click on the wf-amplicon workflow to download and confirm to install. ☐ Navigate to the Workflows tab and click on wf-amplicon. ☐ Click on "Run this workflow" to open the launch wizard. ☐ Set up your run by selecting your sequencing data in the "Input Options". ☐ To speed up the analysis, you can set "Downsampling size" to 500. ☐ For the remaining parameter options we recommend keeping the default settings. ☐ Click "Launch workflow". ☐ Once the workflow finishes, a report will be produced. Amplicon workflow report	