Oxford Nanopore Technologies

MINION | GRIDION | FLONGLE | PROMETHION

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

Overview

Before beginning, we strongly recommend that first-time users read and review the complete online protocol on the Nanopore Community which can be accessed via the QR code. The online protocol provides the full instructions, additional commentary, and considerations for various steps, as well as guidance on input amounts, DNA handling, and sequencing. **This reference card is designed for experienced users.**

1. Library preparation (~45 minutes)

1 Minimum Rapid Barcode use requirements

For optimal output, we currently do not recommend using fewer than 4 barcodes. If you wish to multiplex less than 4 samples, please ensure you split your sample(s) across barcodes so a minimum of 4 barcodes are run:

- For 1 sample, run your sample across 4 barcodes (e.g. RB01-RB04 using 200ng of sample A per barcode)
- For 2 samples, run each sample across two barcodes. (e.g. RB01-RB02 for sample A and RB03-RB04 for sample B)
- For 3 samples, run two samples individually and one across 2 barcodes. (e.g. RB01 and RB02 for sample A and B respectively, and RB03-RB04 for sample C)

Please note that the required sample input for **each barcode** is 200 ng gDNA.

Alternatively, you might want to explore our Rapid Sequencing Kit V14 (SQK-RAD114) for sequencing individual or small sets of samples.

2 Check your flow cell.

We recommend performing a flow cell check before starting your library prep to ensure you have a flow cell with enough pores for a good sequencing run.

See the flow cell check instructions in the MinKNOW protocol for more information.

3 Program the thermal cycler: 30°C for 2 minutes, then 80°C for 2 minutes.

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

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Rapid Barcodes (RB01-24 or RB01-96))	Not frozen	✓	✓
Rapid Adapter (RA)	Not frozen	✓	✓
AMPure XP Beads (AXP)	✓	✓	Mix by pipetting or vortexing immediately before use
Elution Buffer (EB)	✓	✓	✓
Adapter Buffer (ADB)	✓	✓	Mix by vortexing

5 **Prepare the DNA in nuclease-free water.**

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with nuclease-free water.
3. Pipette mix the content of the tubes 10-15 times to avoid unwanted shearing.
4. Spin down briefly in a microfuge.

6 **In the 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind, mix the following:**

Reagent	Volume per sample
Template DNA (200 ng from previous step)	10 µl
Rapid Barcodes (RB01-24 or RB01-96, one for each sample)	1.5 µl
Total	**11.5 µl**

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

8 **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.**

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

10 **Pool all barcoded samples in a clean 2 ml Eppendorf DNA LoBind tube, noting the total volume.**

	Volume per sample	For 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Total volume	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,104 µl

11 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

12 **Ensure you have sufficient capacity in your reaction tube for all the reagents.**

Limit the volume taken forward of pooled barcoded sample to 1,000 µl (i.e. half the capacity of the 2 ml Eppendorf DNA LoBind tube) to ensure feasibility of the next step.

13 **Add an equal volume of resuspended AMPure XP Beads (AXP) to the entire pooled barcoded sample, and mix by flicking the tube.**

.	Volume per sample	For 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Volume of AMPure XP Beads (AXP) added	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,000 µl

14 **Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.**

15 **Prepare at least 2 ml of fresh 80% ethanol in nuclease-free water.**

16 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**

17 **Keep the tube on the magnet and wash the beads with 1 ml of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

18 **Repeat the previous step.**

19 **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.**

20 **Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB) per 24 barcodes used.**

.	For 24 barcodes	For 48 barcodes	For 72 barcodes	For 96 barcodes
Volume of Elution Buffer (EB)	15 µl	30 µl	45 µl	60 µl

21 Incubate for 10 minutes at room temperature.

22 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

23 Remove and retain the full volume 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

24 Quantify 1 µl of eluted sample using a Qubit fluorometer.

25 Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

26 In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

Reagent	Volume
Rapid Adapter (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5 µl**

27 Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.

28 Mix gently by flicking the tube, and spin down.

29 Incubate the reaction for 5 minutes at room temperature.

Tip: While this incubation step is taking place you can proceed to the Flow Cell priming and loading section of the protocol.

30 The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.

2. Priming and loading the MinION and GridION Flow Cell (~10 minutes)

1 Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

2 Priming and loading a flow cell

We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

3 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

4 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.

Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

5 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 room temperature:

Reagents	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Final total volume in tube	**1,205 µl**

6 Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the priming port cover to ensure correct thermal and electrical contact.

7 Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check instructions in the MinKNOW protocol for more information.

8. **Slide the flow cell priming port cover clockwise to open the priming port.**

9. **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.**

10. **After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:**

    1. Set a P1000 pipette to 200 µl
    2. Insert the tip into the priming port
    3. 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.

11. **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.**

12. **Thoroughly mix the contents of the Library Beads (LIB) by pipetting.**

13. **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.**

We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

14. **In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:**

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

15. **Complete the flow cell priming:**

    1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
    2. Load **200 µl** of the priming mix into the flow cell priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

16. **Mix the prepared library gently by pipetting up and down just prior to loading.**

17. **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.**

18. **Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.**

19. **Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.**

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

20. **Place the light shield onto the flow cell, as follows:**

    1. Carefully place the leading edge of the light shield against the clip. **Note:** Do not force the light shield underneath the clip.

    2. 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.

21 The MinION Flow Cell Light Shield is not secured to the flow cell and careful handling is required after installation.

22 Close the device lid and set up a sequencing run on MinKNOW.

3. Flow cell reuse and returns

1 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°C to +8°C.

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

2 We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day.

3 Alternatively, follow the returns procedure to send the flow cell back to Oxford Nanopore.

Instructions for returning flow cells can be found here.

4 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.