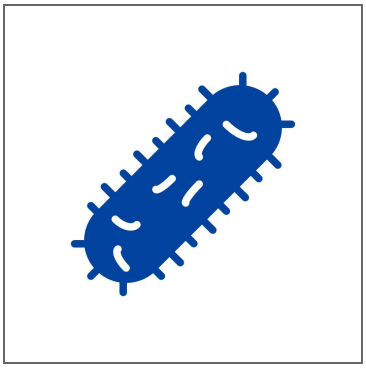

VERSION 2

MAR 13, 2024

OPEN ACCESS

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Protocol status: In development
We are still developing and optimizing this protocol

Rapid Sequencing gDNA Barcoding RBK2004 V.2

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DISCLAIMER

Please note that this protocol is now being replaced by V14 kits and protocols.

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PROTOCOL integer ID: 96673

Keywords: sequencing, gDNA, Barcoding, Nanopore

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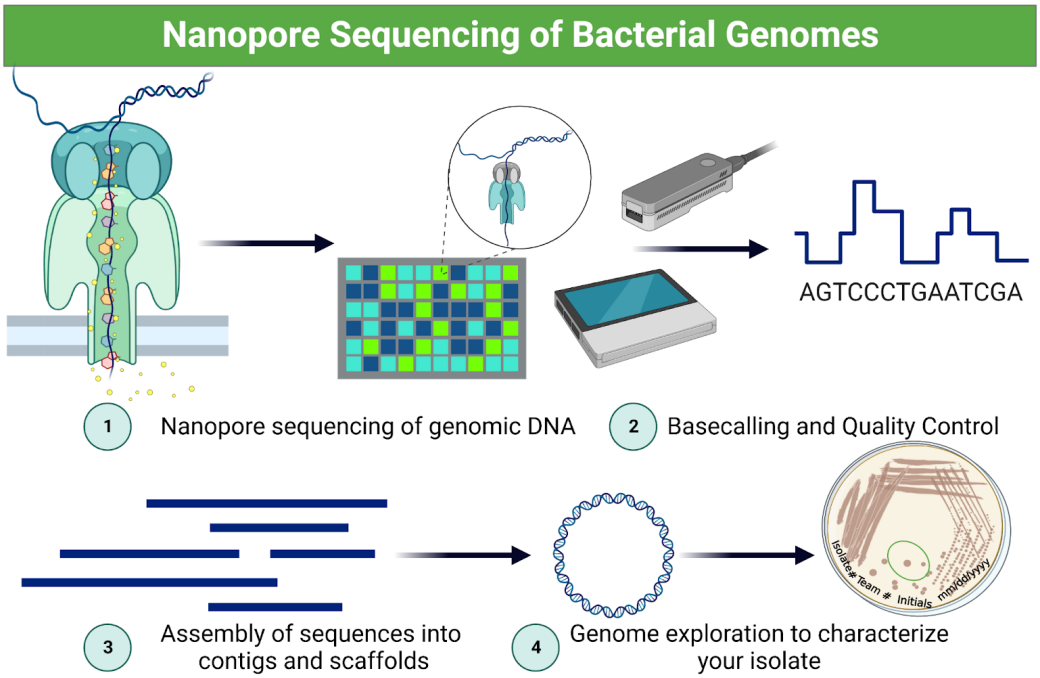
NC State Biotechnology Program
Grant ID: NC State

ABSTRACT

This protocol has been adapted from Oxford Nanopore Technologies.

You have isolated, purified, and quantified DNA from your bacterial isolate. You have also worked with your bacterium and grown in the presence of different carbon sources and pH levels to characterize its properties using Biolog GEN3 plates. Now, we can sequence the DNA of your isolate using long-read sequencing and Oxford Nanopore Technologies (ONT) MinION. With the genetic sequences we obtain, we will attempt to assemble the genome of this organism and explore its genomic potential!



The MinION sequencer from Oxford Nanopore Technologies (ONT) will allow us to sequence genomic DNA using a flow cell with hundreds of nanopores. The DNA will be passed through the pores, and changes in current will be interpreted as bases (“base calling”). After performing quality control to remove low-quality sequences, we will assemble the sequences to create **contigs** and **scaffolds** with the goal of exploring the genome of your isolates. Created with BioRender.com.

After completing this lab you will gain the following lab skills:

- Lab safety and proper personal protective equipment (PPE)

- Pipetting with micropipettes
- Preparing sequencing libraries for the ONT MinION

IMAGE ATTRIBUTION

Bacteria from Smashicon from The Noun Project
https://thenounproject.com/icon/bacteria-1028252/

MATERIALS

Oxford Nanopore Technologies (ONT) Kit and Flow Cells:

- ONT Rapid Barcoding Kit (RBK004)
- ONT Flow Cell Priming Kit (EXP-FLP002)
- MinION FLO-MIN106 R9.4.1 flow cells
- Sequencing auxiliary kit EXP-AUX001

Materials

- ~400 ng high molecular weight genomic DNA
- Rapid Barcoding Sequencing Kit (SQKRBK004)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g., ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads (optional)
- Freshly prepared 70% ethanol in nuclease-free water (optional)
- 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (optional)

Equipment

- ONT MinION Mk1B and computer
- Ice bucket with ice
- Microfuge
- Timer
- Thermal cycler or heat block at 30°C and 80°C
- Pipettes and pipette tips :P2, P20, P100, P200, P1000

SAFETY WARNINGS

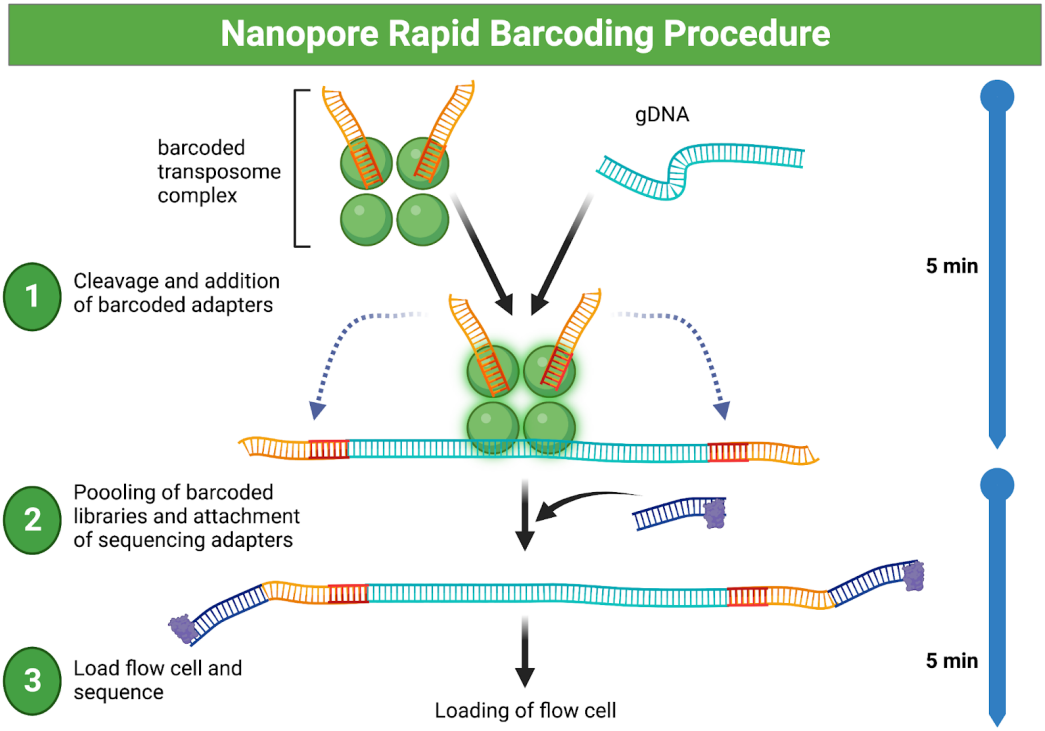
Wear PPE at all times in the lab.

BEFORE START INSTRUCTIONS

Review the "How to use a micropipette" lab we completed at the beginning of the semester. Before coming to the lab, watch this 5-minute video, How to Micropipette, that demonstrates three critical pipetting skills:

- How to hold a micropipette
- How to set a micropipette to a desired volume
- How to use the plunger and ejector

In this lab session, we will pipette small volumes of DNA and enzymes to prepare your genomic DNA for sequencing by adding adaptors for Nanopore sequencing. Once we have added adapters, we will combine all our samplesto distinguish one sample from another bioinformatically.



Created with BioRender.com

The Oxford Nanopore Technologies (ONT) Rapid Barcoding Kit will allow us to sequence the genomic DNA from several different genomic DNA samples in one flow cell by using an enzyme to cleave and add **barcoded adapter sequences** to the ends of the genomic DNA (Step 1). Next, **sequencing adapters** are added to the pooled sample (Step 2) before loading and sequencing (Step 3). Created with BioRender.com.

Library Preparation

15m

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

- Fragmentation Mix RB01-12: not frozen, briefly spin down, mix well by pipetting
- Rapid Adapter (RAP): not frozen, briefly spin down, mix well by pipetting
- Sequencing Buffer (SQB): thaw at RT, briefly spin down, mix well by pipetting*
- Loading Beads (LB): thaw at RT, briefly spin down, mix by pipetting or vortexing immediately before use
- Flush Buffer (FLB) - 1 tube: thaw at RT, briefly spin down, mix well by pipetting*
- Flush Tether (FLT): thaw at RT, briefly spin down, mix well by pipetting

2 Prepare the DNA in Nuclease-free water.

2.1 Transfer ~ 400 ng genomic DNA into a DNA LoBind tube.

2.2 Adjust the volume to 7.5 µL with Nuclease-free water.

2.3 Mix by flicking the tube to avoid unwanted shearing.

2.4 Spin down briefly in a microfuge.

3 In a 0.2 ml thin-walled PCR tube, mix the following:

- 7.5 µL 400 ng template DNA

- 2.5 µL Fragmentation Mix RB01-12 (**one** for each sample)

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

5 Incubate the tube at 30 °C for 00:01:00 and then at 80 °C for 00:01:00 . Briefly put 2m the tube On ice to cool it down.

6 Pool the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

> **Note**
>
> If barcoding four or more samples, increased throughput can be achieved through cleaning up and concentrating the pooled material using AMPure XP beads as outlined in Steps 6-17. Otherwise, for a more rapid sample preparation, transfer 10 µl of pooled sample from Step 6 into a clean 1.5 ml Eppendorf DNA LoBind tube and proceed directly to Step 18

7 Resuspend the AMPure XP beads by vortexing.

8 To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads and mix by flicking the tube.

9 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at Room temperature 5m

10 Prepare 500 µL of fresh 70% ethanol in Nuclease-free water.

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

12 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

13 Repeat the previous step.

14 Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.

15 Remove the tube from the magnetic rack and resuspend pellet in 10 µL of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 00:02:00 at Room temperature 2m

16 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 00:01:00 1m

17 Remove and retain 10 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

18 Add 1 µL of RAP to 10 µL of barcoded DNA.

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

20 Incubate the reaction for 00:05:00 at Room temperature 5m

Note

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

Instrument Setup Priming and loading the SpotON Flow Cell 5m

21 Remove a flow cell from the refrigerator and allow it to equilibrate to room temperature.

22 Connect the MinION Mk1B to the computer and carefully insert the flow cell.

23 Open MinKNOW software and run Flow Cell Check.

24 Confirm the number of active pores. A MinION flow cell should have a minimum of 800 active pores as covered by the warranty.

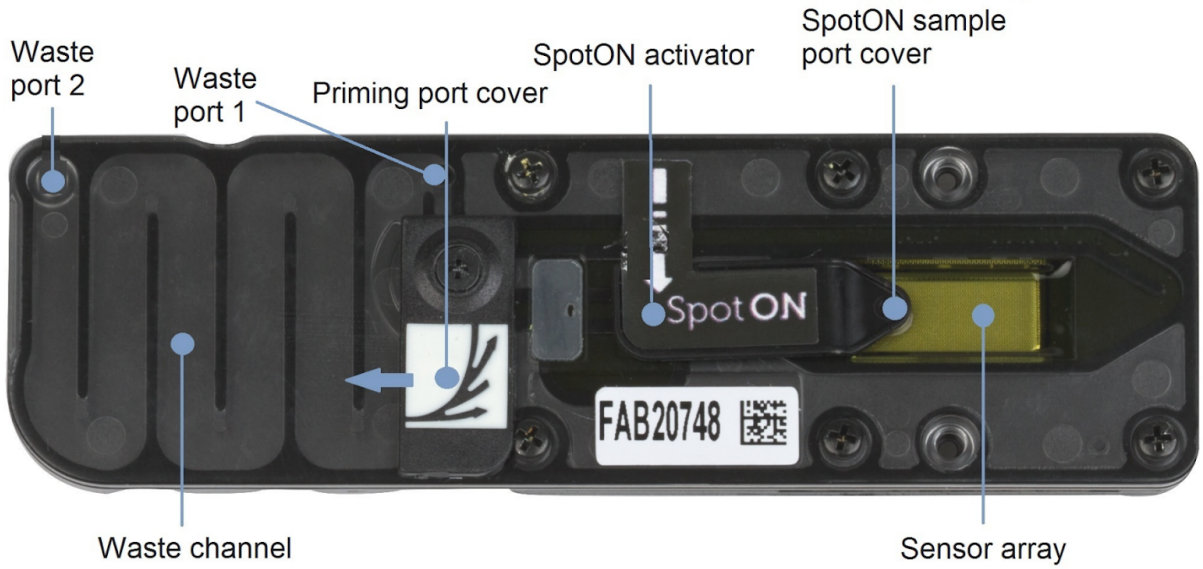


Diagram of flow cell indicating priming port and SpotON port locations for priming and library loading.

25

Safety information

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol.

Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at Room temperature before mixing the reagents by vortexing, and spin down at Room temperature .

26 To prepare the flow cell priming mix, add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at Room temperature

27 Open the MinION device lid and slide the flow cell under the clip.

28 Slide the priming port cover clockwise to open the priming port.

Safety information

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.

29 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):

29.1 Set a P1000 pipette to 200 µl.

29.2 Insert the tip into the priming port.

29.3 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

30 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 00:05:00 . During this time, prepare the library for loading by following the steps below. 5m

31 Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.

32 In a new tube, prepare the library for loading as follows:

- 34 µL Sequencing Buffer (SQB)
- 25.5 µL Loading Beads (LB), mixed immediately before use
- 4.5 µL Nuclease-free water
- 11 µL DNA library

Safety information

The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

33 Complete the flow cell priming:

33.1 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

33.2 Load 200 µL of the priming mix into the flow cell via the priming port (not the SpotON sample port), **avoiding the introduction of air bubbles.**

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

35 Add 75 µL of sample to the flow cell via the SpotON sample port in a dropwise fashion. **Ensure each drop flows into the port before adding the next.**

36 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

Ending the experiment

37

STEP CASE

Option 1 1 step

After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C

38

Note

Critical Thinking Questions for Nanopore Sequencing
1. What are some advantages of barcoding our samples to sequence together?
2. What are some disadvantages of combining all our samples and sequencing together?
3. What were the key steps in preparing the flow cell before sequencing?
4. Describe in your own words why the flow cell and Nanopore sequencing are unique and powerful resources.