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Bacterial Isolate Genomic DNA Isolation - ONT NO-MISS

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We use this protocol and it's working

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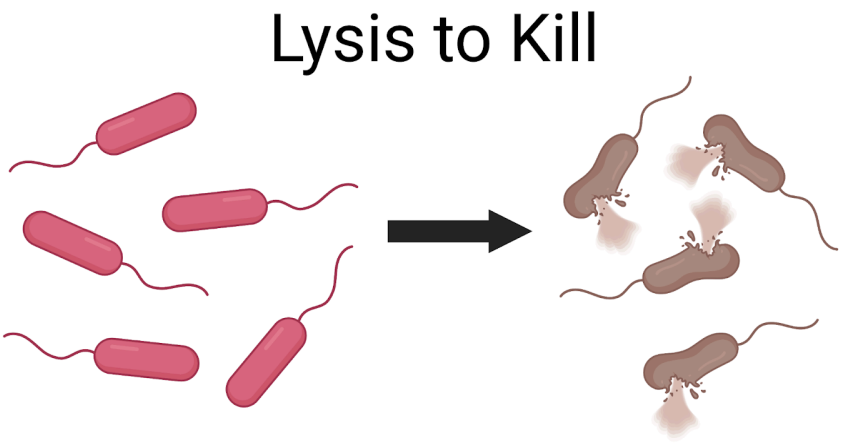
Disclaimer

This protocol was adapted from the ONT NO-MISS protocol (February 2025) and modified for teaching lab sessions.

Abstract

Overview and Goals

Your bacterial isolate has been grown on agar plates. We now need to lyse open the bacteria and isolate only the genomic DNA for sequencing. We need to lyse the bacteria with **enzymes** and a lysis buffer for this. We will then purify the genomic DNA. We will use a modification of the NEB Monarch Spin Column Kit extraction kit. This protocol has been recommended by Oxford Nanopore Technologies (ONT), the company that makes the sequencer we will use for sequencing our bacterial genomes.



Lysis of bacteria. Image created with BioRender.com

On the left are healthy living bacteria (An example is Bacillus). On the right, the bacteria are lysed open, which results in cell death. Consider this question as you read the protocol: How will we facilitate **bacterial lysis**? Images of Bacilli werecreated using BioRender.com.

Learning Objectives

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

- Lab safety and proper personal protective equipment (**PPE**)
- Proper use of a centrifuge and pipettors
- **Isolation and purification of genomic DNA**

Image Attribution

Icon from The Noun Project and recolored by Carlos C. Goller
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Guidelines

All plastics and cultures will be treated as biohazards. Cultures will be treated with 10% (final) bleach overnight. Plastic tubes, pipettes, and spin columns used for the extraction process will be disposed of in the biohazard containers.

Materials

Supplies

We will need several supplies for this lab, including enzymes.

Materials

- 200 µl liquid overnight culture (~1 × 10^8 – 1 × 10^9 cfu/ml) or 1/8 of a 10 µl loop of colonies from a plate

Consumables

- Lysozyme human (Sigma, L1667)
- Trizma® hydrochloride solution (Sigma, T2819)
- 5 M sodium chloride solution (Sigma, S6546)
- SDS at 10% v/v (Sigma, 71736)
- Phosphate Buffered Saline (PBS), pH 7.4 (Thermo Fisher, 10010023)
- TE buffer (Sigma, 8890-100ML)
- Proteinase K (NEB, P8200AAVIAL) from the Monarch Spin gDNA Extraction Kit
- Monarch® RNase A (NEB, T3018-1) from the Monarch Spin gDNA Extraction Kit
- CTAB buffer (Promega, MC1411)
- Monarch® gDNA Elution Buffer (NEB, T3016-1) from the Monarch Spin gDNA Extraction Kit
- Monarch® gDNA Binding Buffer (NEB, T3014-1) from the Monarch Spin gDNA Extraction Kit
- Monarch® Spin Collection Tubes (NEB, T2118-1) from the Monarch Spin gDNA Extraction Kit
- Monarch® Spin Columns S2C and Tubes (NEB, T3017-1) from the Monarch Spin gDNA Extraction Kit
- Monarch® gDNA Wash Buffer (NEB, T3015-1) from the Monarch Spin gDNA Extraction Kit
- Qubit 1x dsDNA HS Assay Kit (ThermoFisher, Q33230)
- Nuclease-free water (e.g., ThermoFisher, AM9937)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 1.5 ml Eppendorf DNA LoBind tubes
- 5 ml Eppendorf DNA LoBind tubes

Equipment

- Eppendorf 5424 centrifuge (or equivalent)
- Eppendorf Thermomixer
- Vortex mixer
- P1000 pipette and filter tips
- P200 pipette and filter tips
- P100 pipette and filter tips
- P20 pipette and filter tips
- P10 and P2.5 pipette and filter tips

Safety warnings

Dispose of biohazard in the appropriate biohazard bins.

Before start

Review the complete protocol before beginning. Several of the steps in this procedure are time and/or temperature-sensitive, so it’s important that you know what to expect and how to manage your time. There are also several types of tubes with specific names that need to be used for specific steps of the protocol. They are shown in the illustration below to help you keep track of these materials.

Growth of Isolates

1 A colony of each of your isolates will be used to start a liquid culture in **Tryptic Soy Broth (TSB)**.

2 Grow liquid cultures with shaking at 30 °C for 16-18 hours.

Bacterial Lysis 32m

3 For general bacterial inputs, reconstitute the **lysozyme in TE buffer to 10 mg/ml.** We recommend preparing enzymes freshly on the day as enzymatic activity reduces over time once reconstituted.

4 In a fresh 1.5 ml Eppendorf DNA LoBind tube, centrifuge 1 mL (~1 × 10^8 – 10^9 cfu/ml) of cell culture or 1/8 of a 10 µl loop of colonies from a plate at 12.000 x g, 20°C, 00:01:00 . 1m

5 Remove the supernatant by pipetting and resuspend the pellet in 1 mL of PBS.

6 Centrifuge at 12.000 x g, 20°C, 00:01:00 . 1m

7 Depending on your input, remove the supernatant and resuspend as follows:

7.1 **For most bacterial inputs:** Remove the supernatant and resuspend the pellet in 100 µL TE buffer with 10 µL lysozyme. **This is the protocol we will follow.**

7.2 **For staphylococcal inputs:** Remove the supernatant and resuspend the pellet in 100 µL Staphylococcal Lysis Buffer (SLB) with 10 µL achromopeptidase. Note: It is important to remove as much PBS as possible without disturbing the pellet, as the buffer has an inhibitory effect on achromopeptidase activity.

8 Incubate at 37 °C for 10 minutes in a thermomixer at 500 RPM. 500 rpm, 37°C, 00:10:00 Use Thermomixer . If a thermomixer is unavailable, the

reaction can be incubated stationary and agitated by gently flicking for 10 seconds every two minutes.

9 Combine the following reagents with your sample in the 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing.

	A	B
	Reagent	Volume
	Proteinase K	10 µl
	RNase A	3 µl
	Total (including your sample)	113 µl

Proteinase K and RNaseA treatment

10 Vortex mixing your sample before adding the CTAB buffer allows the enzymes to fully mix with the bacterial cells as the mixture can **become viscous**.

11 Add 200 µL of CTAB buffer and **mix by vortexing**.

12 Extractions performed using the CTAB buffer improves sequencing health and output compared to using NEB Lysis Buffer from the Monarch Spin gDNA Extraction Kit. 30m

Expected result

IMPORTANT: The full 30-minute incubation must be completed to ensure the proteinase and RNase activity occurs, regardless of turbidity.

00:30:00

13 Incubate at 56 °C for 30 minutes in a thermomixer at 1000 RPM:

1000 rpm, 56°C, 00:30:00 Use Thermomixer .

Note

Depending on the bacterial input, the solution may become fully transparent or may remain hazy.

INFO: The Monarch Spin gDNA Extraction Kit is used for gDNA extraction. The remainder of this method is written following the Genomic DNA Binding and Elution protocol with our recommended alterations.

14 Preheat the Monarch® gDNA Elution Buffer to 60 °C for later use.

Note

IMPORTANT: The ratio of binding buffer to lysate (2:1) is important for the column binding efficiency.

14.1 We recommend taking through 200 µL of lysate for use with 400 µL of binding buffer per sample.

14.2 To run more samples from the same isolate, the volumes can be increased in relation to the 2:1 ratio of buffer to lysate.

15 Combine the following reagents in a clean 1.5 ml Eppendorf DNA LoBind tube:

	A	B
	Reagent	Volume
	Lysate from the previous step	200 µl
	Monarch gDNA Binding Buffer	400 µl
	Total	600 µl

Monarch gDNA Binding Mixture

16 Ensure the reaction is thoroughly mixed by **pipetting (gently!) until the liquid is homogenous.**

gDNA Binding and Elution

8m

17 Thorough mixing is essential to ensure the DNA binds properly to the column and to homogenise the CTAB and Monarch® gDNA Binding Buffer which solidify on initial contact.

18 Transfer the reaction to a Monarch® Spin Column pre-inserted into a Monarch® collection tube, without touching the upper column area.

19 Close the tube and centrifuge for 1.000 x g, 20°C, 00:03:00 to bind the gDNA and then for 1 minute at maximum speed (>12,000 x g) 16000 rpm, 20°C, 00:01:00 , 1 min at maximum speed to clear the membrane to clear the membrane.

4m

20 Do not empty or remove the collection tube from the centrifuge when changing settings.

21 Remove the gDNA spin column from the collection tube and discard the tube with the flow-through.

22 Transfer the gDNA spin column to a new collection tube and add 500 µL Monarch® gDNA Wash Buffer.

IMPORTANT: Do NOT vortex.

23 Close the collection tube and invert multiple times until the wash buffer reaches the cap.

24 Immediately centrifuge the collection tube for 1 minute at maximum speed (>12,000 x g). 16000 rpm, 20°C, 00:01:00 , Maximum speed Remove the gDNA spin column from the collection tube to discard the flow-through.

1m

25 Re-insert the gDNA spin column into the collection tube and add 500 µL gDNA wash buffer and close the tube.

26 Immediately centrifuge the collection tube for 1 minute at maximum speed (>12,000 x g) 16000 rpm, 20°C, 00:01:00 , Maximum speed . Remove the gDNA spin column from the collection tube to discard the flow-through.

1m

27 After centrifugation, ensure there is no liquid in the gDNA spin column above or below the silica membrane. If there is liquid, repeat the centrifugation until all liquid is removed.

IMPORTANT: Preheating the Monarch® gDNA Elution Buffer to 60 °C is essential for maximum recovery of larger gDNA fragments.

28 Place the gDNA spin column in a DNase-free 1.5 ml microfuge tube and add 100 µL of preheated Monarch® gDNA Elution Buffer. Close the tube and incubate at room temperature for 00:01:00 . 1m

TIP: If extractions are not yielding high enough concentrations of DNA for 200 ng per sample, less gDNA elution buffer can be run through the column to increase the concentration.

Note

Note: Elution of less than 100 µL results in lower total yield, and elution of less than 50 µL is not recommended.

29 Centrifuge for 1 minute at maximum speed (>12,000 x g) to elute the gDNA into the 1.5 ml microfuge tube. 16000 rpm, 20°C, 00:01:00 , Maximum speed 1m

30 The gDNA spin column can be discarded.

31 **CHECKPOINT:** Quantify 1 µL the eluted sample using a **Qubit fluorometer.** Expected yield is ~15-20 ng/µl per sample with a **DNA Integrity Number (DIN) of 9.**

32 **END OF STEP:** Take forward 200 ng per sample into the library preparation step of the **Nanopore-only Microbial Isolate Sequencing Solution (NO-MISS) - Rapid Barcoding Kit V14** (SQK-RBK114.24 or SQK-RBK114.96) end-to-end protocol. We recommend storing the DNA frozen (-30 to -15°C or -90 to -65°C). We will freeze the DNA and use it for quantification in the following lab sessions before sequencing.

Acknowledgements

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