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Comprehensive Workflow for Extracting High-Quality Bacterial DNA from Milk Samples

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We use this protocol and it's working

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Abstract

This protocol provides a comprehensive workflow for extracting high-quality bacterial DNA from milk samples. Milk is a notoriously difficult matrix for DNA extraction due to high concentrations of inhibitors like fats and proteins (casein). Standard kit protocols often fail because they are not designed to break down this complex matrix effectively. This procedure combines two powerful methods: a robust manual pre-treatment using Cetyltrimethylammonium bromide (CTAB) to effectively lyse bacterial cells and remove the bulk of milk's inhibitors, and an automated purification step using the RBC Biosciences MagCore® Bacterial Kit to efficiently bind, wash, and elute the DNA using magnetic-particle technology. To ensure the most accurate representation of the milk microbiome, this protocol includes an advanced option to co-process the milk fat fraction, where certain bacterial species are known to reside. By preparing a clean, inhibitor-free lysate manually, you can then use the MagCore® system for the final purification, ensuring higher yield and purity.

Guidelines

Understanding the Protocol's Purpose

This protocol is specifically designed to overcome the challenges of extracting bacterial DNA from milk, a sample type known for high levels of inhibitors like fats and proteins. It combines a robust manual pre-treatment to clean the sample with an automated system for final DNA purification, aiming for higher yield and purity than standard kits alone.

Key Decisions During the Protocol

You will need to make a critical choice after the initial centrifugation of the milk:

- **Option A (Standard Protocol):** Choose this for routine analysis. You will only process the cell pellet and discard the fat layer. This is a faster approach.
- **Option B (Comprehensive Protocol):** Choose this if you need the most complete representation of the milk's microbial community. This method co-processes the cell pellet and the fat fraction, as some bacteria reside in the fat. Note that this option requires an additional reagent, Tween-20, for the washing step.

Critical Steps & Handling Instructions

Failure to perform these steps correctly can lead to poor results or protocol failure.

1. **Initial Pellet Separation:** After centrifuging the milk, be careful when removing the top fat layer and the liquid supernatant to avoid disturbing the cell pellet at the bottom.
2. **Casein Dissolution:** Do not skip the EDTA incubation step. This is important for breaking down the milk's protein matrix, which can trap bacteria and inhibit downstream reactions. The suspension should become clearer, indicating the step was successful.
3. **Lysis Steps:**

- The **Lysozyme step is essential** because milk samples often contain Gram-positive bacteria with tough cell walls that need to be broken down.
- During the **CTAB Lysis** incubation at 65°C, make sure to invert the tube every 20-30 minutes to ensure thorough mixing and complete cell lysis.

4. Lysate Clarification (Most Critical Step):

- Before loading your sample into the MagCore® system, you **must** centrifuge the lysate at high speed to pellet insoluble debris.
- **Only transfer the clear supernatant** to the MagCore® cartridge. Transferring any of the pelleted debris can interfere with the magnetic beads and cause the automated run to fail.

General Best Practices

Sample Volume: Use a larger starting volume of milk (up to 40 mL) if you expect the bacterial count to be low.
Sterility: Use sterile tubes, tips, and reagents (like PBS) to avoid contaminating your sample with outside DNA.
Storage: Once the protocol is complete, store your purified DNA at -20°C or colder for long-term stability.

Materials

Reagents:
- Raw or pasteurized milk
- CTAB Lysis Buffer (see preparation below)
- Phosphate-Buffered Saline (PBS) or Tris-EDTA (TE) Buffer (pH 8.0)
- 50-100 mM EDTA solution (pH 8.0)
- Tween-20 (for comprehensive protocol option)
- Nuclease-free water
- Phenol
-Chloroform
-Isopropanol

From RBC MagCore® Bacterial DNA Kit (Cat.No. MBB-01/MBB-02):
- Pre-filled reagent cartridges
- Lysozyme and Lysozyme Reaction Buffer
- Proteinase K and PK Storage Buffer

Equipment:
- High-speed refrigerated centrifuge
- 50 mL and 1.5/2.0 mL microcentrifuge tubes
- Water bath or heating block set to 37°C and 65°C
- Vortex mixer
- Micropipettes and sterile tips
- Sterile swabs or spatulas
- RBC Biosciences MagCore® Instrument

Safety warnings

Phenol and chloroform are highly toxic and corrosive. Perform this step in a fume hood with appropriate personal protective equipment (gloves, lab coat, eye protection).

Before start

1. Reagent Preparation:
- The **CTAB Lysis Buffer** must be prepared according to the recipe. Crucially, warm it to 65°C before use to ensure all components are fully dissolved.
- The **Lysozyme solution** must be prepared fresh right before you need it for the lysis step.
2. Equipment Setup:
- Ensure your refrigerated centrifuge can reach the required speeds (e.g., 10,000 x g).
- Have two water baths or heating blocks ready, one set to 37°C (for lysozyme) and another to 65°C (for CTAB lysis).

Buffer Preparation

1h

1 **CTAB Lysis Buffer (2% w/v):** 1h

1.1 To prepare 100 mL, dissolve the following in nuclease-free water: 2 g CTAB (Cetyltrimethylammonium bromide), 8.18 g NaCl (for [M] 1.4 Molarity (M) final concentration), 10 mL of 1 M Tris-HCl (for 100 mM final concentration, pH 8), 4 mL of [M] 0.5 Molarity (M) EDTA (for [M] 20 millimolar (mM) final concentration, pH 8).

1.2 Adjust the final volume to 100 mL with nuclease-free water.

1.3 Warm to 65 °C before use to ensure the CTAB is fully dissolved.

Part 1: Manual Pre-Treatment – Sample & Inhibitor Removal

57m

2 **Sample Collection & Centrifugation:** 10m

2.1 Pour 10-40 mL of milk into a 50 mL centrifuge tube. A larger starting volume is recommended for low-biomass samples.

2.2 Centrifuge at 10.000 x g, 4°C, 00:15:00 to pellet bacterial and somatic cells. 15m

3 **Separating Milk Fractions:** 2m
After centrifugation, three layers will be visible: a solid fat layer on top, the liquid skim milk, and a cell pellet at the bottom.
Choose one of the following two paths:

3.1 Option A (Standard Protocol - Pellet Only): For routine analysis, carefully remove and discard the top fat layer and the middle liquid supernatant, leaving only the cell pellet. Proceed to Step 4.

3.2 Option B (Comprehensive Protocol - Pellet + Fat Fraction): For the most accurate representation of the entire milk microbiome, it is important to process the fat fraction, as

some bacterial species preferentially associate with it. Use a sterile spatula to carefully transfer the top fat layer into a new, clean 50 mL centrifuge tube. Aspirate and discard the middle liquid supernatant from the original tube, leaving the cell pellet. Resuspend the cell pellet in 1-2 mL of sterile PBS and transfer this suspension into the tube containing the fat fraction. This combines the two key fractions for co-extraction. Proceed to Step 4.

4 **Pellet Washing & Detergent Wash:** 5m

4.1 If you chose Option A: Resuspend the pellet in 5-10 mL of sterile PBS.

4.2 If you chose Option B: Resuspend the combined fat and pellet mixture in 5-10 mL of sterile PBS **containing 0.1% Tween-20** to help dissolve residual fat and release associated bacteria. Vortex thoroughly. Centrifuge at 10.000 x g, 00:10:00 . Discard the supernatant. Perform one additional wash on the pellet using plain PBS to remove residual detergent. Centrifuge and discard the supernatant. 10m

5 **Casein Dissolution (Recommended):**

5.1 To break down the protein matrix and release trapped bacteria, resuspend the washed pellet in 1 mL of [M] 50-100 millimolar (mM) EDTA solution (pH 8).

5.2 Incubate at room temperature for 00:10:00 . The milky suspension should become clearer as casein micelles dissolve. 10m

5.3 Centrifuge at > 12.000 x g, 00:05:00 to pellet the cells. Carefully discard the supernatant. 5m

Part 2: Manual Pre-Treatment – Lysis 2h 40m

6 **Gram-Positive Lysis (Lysozyme)**: This step is essential for milk samples, which are likely to contain Gram-positive bacteria with tough cell walls.

6.1 Prepare Lysozyme Solution: Freshly prepare a [M] 20 mg/mL Lysozyme solution by dissolving Lysozyme powder in the Lysozyme Reaction Buffer provided with the kit.

6.2 Resuspend Pellet: Resuspend the cell pellet in 200 µL of the freshly prepared Lysozyme solution. Incubate at 37 °C for 01:00:00, mixing occasionally. 1h

7 **RNA Removal (RNase Treatment)**

7.1 After the lysozyme incubation, add 4 µL **of RNase A** to the sample mixture.

7.2 Vortex briefly to mix.

7.3 Incubate at room temperature for 1 00:10:00. 10m

8 **CTAB Lysis & Protein Digestion:**

8.1 To the lysozyme-treated sample, add 500 µL **of pre-warmed (** 65 °C **) CTAB Lysis Buffer**.

8.2 Add 20 µL **of reconstituted Proteinase K** from the RBC MagCore® kit.

8.3 Vortex briefly and incubate at **65°C for 1-2 hours**, inverting the tube every 20-30 minutes to mix. The solution will become viscous as cells lyse. 1h 30m

Part 3A: Automated Purification – MagCore® Integration 50m

9 **Lysate Clarification:**

9.1 After the 65°C incubation, centrifuge the lysate at > 13.000 x g, 00:05:00 at room temperature. This will pellet any insoluble debris that could interfere with the magnetic beads. The clear liquid supernatant contains your DNA. 5m

10 **Load Sample into MagCore® Cartridge:**

10.1 This is the critical 'hand-off' step. You will bypass the lysis step of the automated protocol. Carefully pipette the clear supernatant from Step 9 into the Lysis Buffer well (Well 11) of the RBC MagCore® pre-filled cartridge. Do not transfer any of the pelleted debris.

11 **Add Magnetic Beads:**

11.1 Using a clean pipette tip, transfer the magnetic beads from the 'Beads Mixture' well (Well 9) of the cartridge into the same well containing your lysate (Well 11).

12 **Run Automated Protocol:**

12.1 Place the prepared cartridge into the MagCore® instrument. Select and run the standard pre-programmed Bacterial DNA extraction protocol (502). The instrument will now automatically perform the DNA binding, washing, and elution steps on your pre-cleaned lysate. 45m

13 **Collect Purified DNA:**

13.1 Once the run is complete, retrieve the elution tube containing your purified bacterial DNA. Store the DNA at -20 °C or below for long-term stability.

Part 3B: Purification with Phenol:Chloroform (if the automated solution isn't available) 1h 19m

14 **SAFETY NOTE:** Phenol and chloroform are highly toxic and corrosive. Perform this step in a fume hood with appropriate personal protective equipment (gloves, lab coat, eye protection).

14.1 Cool the lysate to room temperature.

14.2 Add an equal volume (approx. 700 µL) of phenol:chloroform:isoamyl alcohol (25:24:1).

14.3 Vortex for 10 seconds to create an emulsion, then centrifuge at 14.000 x g, 00:10:00 to separate the phases. 10m

14.4 Carefully transfer the upper, clear aqueous phase (which contains the DNA) to a new sterile microcentrifuge tube. Do not disturb the white protein layer at the interface.

14.5 Repeat the phenol:chloroform extraction on the aqueous phase until the interface is clean and white, indicating that most proteins have been removed.

15 **DNA Precipitation:**

15.1 To the final aqueous phase, add 0.7 volumes of ice-cold isopropanol (e.g., if you have 500 µL of aqueous phase, add 350 µL of isopropanol).

15.2 Mix gently by inverting the tube several times. You should see white, stringy DNA precipitate out of the solution.

15.3 Incubate at -20 °C for at least 00:30:00 to maximize DNA precipitation. Optionally incubate overnight. 30m

16 **Pellet DNA:**

16.1 Centrifuge at 14.000 x g, 4°C, 00:15:00 . A small white pellet of DNA should be visible at the bottom of the tube. 15m

16.2 Carefully decant the supernatant without disturbing the pellet.

17 **Wash DNA Pellet:**

17.1 Add 500 µL of ice-cold 70% ethanol to the tube. This step washes away residual salts and CTAB.

17.2 Centrifuge at 14.000 x g, 4°C, 00:06:00 6m

17.3 Carefully decant the ethanol. Repeat the 70% ethanol wash one more time for higher purity.

18 **Dry and Resuspend DNA:**

18.1 After the final wash, remove as much ethanol as possible with a pipette.

18.2 Air-dry the pellet for 5–10 minutes at room temperature. Do not over-dry the pellet, as this can make it difficult to dissolve. 8m

18.3 Resuspend the DNA pellet in 30–50 µL of TE buffer or nuclease-free water. You may need to warm the tube to 65°C for 10 minutes to help dissolve the DNA completely. Store the purified DNA at -20°C. 10m

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