CRUDE LYSATE EXTRACTION METHOD FOR qPCR ANALYSIS

1. SCOPE AND APPLICATION

1.1. This method describes a procedure for genomic DNA (gDNA) extraction from environmental water samples that have been prepared by membrane filtration.

2. SUMMARY OF METHOD

2.1. After membrane filtration, environmental samples are added to bead tubes and placed in a bead beater for rapid and thorough homogenization. Cell lysis occurs by mechanical shearing in Salmon DNA extraction buffer. The extract is clarified by centrifugation and can be analyzed with qPCR amplification and detection in a thermocycler system using a primer/probe assay.

2.2. Interferences: Some substances, including humic and tannic acids, can act as inhibitors of PCR amplification and reduce efficiency by either binding to the DNA polymerase or interfering with the annealing and extension cycles. Sample purification or dilution can help alleviate the effects of inhibitors.

3. EQUIPMENT AND SUPPLIES

3.1. Equipment and Supplies –

Equipment/Supply Name	Notes
Gloves, Latex or Nitriles	N/A
Tube Racks	N/A
Centrifuge Tubes, 1.5 mL	N/A
Brady FreezerBondz Laboratory Labels	Cat#19-140-586
Forceps	N/A
Micropipettes	Use per SOP PCR-2.4
Sterile PCR Grade Filter Tips	
CBS Optimizer PCR Workstation #2	S/N 007632
Fisher Mini Vortexer	N/A
Eppendorf Centrifuge 5418	S/N 5418FR825055, S/N 5418AG715992
Fisher Scientific™ Bead Mill 24 Homogenizer	Property #152281
PCR#9, Thermo Refrigerator	S/N TR1562211012Maintained at 4ºC ± 2ºC, Room B246A
PCR #5 Thermo Fridge/Freezer	S/N 0121174701121218. Maintained at -20ºC ± 2ºC, Room B246
Polycarbonate Membrane Filter, 47 mm diameter, 0.45 µm pore size	Fisher Cat # HTTP04700
Glass Fiber Filter, 47 mm Diameter	Fisher Cat #AP2004700
Or Equivalent	

4. REAGENTS AND STANDARDS

4.1. Reagents and Standards –

Reagent/Standard Name	Notes
Deionized (DI) Water	N/A

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Salmon DNA Extraction Buffer	Prepare per SOP PCR-3.1 Section 8.
HFG2GD, BRD3, CowM2, and HumM2 Extraction Controls	Prepare per SOP PCR-3.2, PCR-3.3, PCR-3.4, PCR-3.5, PCR-3.6, PCR-3.7, and PCR-3.8.
Bleach solution, 10% (v/v)	Prepare per SOP BG-05
80% Ethanol	Prepare per SOP IZ-09.

5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5.

6. SAMPLE PREPARATION

6.1. Prepare samples per SOP PCR-4.0. Sample labels can be printed and applied to tubes the day before extraction. The tubes can be placed in a sample block with a cover.

6.2. Clean work area counters before and after use. Spray surface of work area with 10% bleach solution and wipe down after 15 minutes. Spray and wipe down with sterile DI water.

6.3. Place two pairs of forceps in PCR Workstation #2 or the UV box at the filtration station. Turn on the UV light and allow to warm up for 15 minutes. Leave forceps for at least 2 additional minutes to ensure sterility.

6.4. For each sample and control bead tube (Method Blank, Extraction Blank, Extraction Controls), set up two sets of 1.5 mL centrifuge tubes in the tiered tube racks.

6.4.1. Use colored tubes for one set and label the top of the sample tubes with the last 3 numbers of the sample ID. Write last 4 digits of sample ID if last 3 digits match another sample. Label the Extraction Control tubes as EC along with the assay name, the Extraction Blank tube as EB, and the Method Blank tube as MB.

6.4.2. Use clear tubes for the second set. Print labels and place them on the top and side of clear 1.5 mL centrifuge tubes. See SOP PCR-5.0 Section 5.2. for instructions on printing labels for extraction.

6.5. <u>Setting up the Extraction Blank and Extraction Controls</u>:

6.5.1. A vacuum manifold and sterile funnel base can be used to fold a sterile filter. Dip forceps in 80% ethanol and shake to remove excess. Place glass fiber filter onto the funnel base. Dip forceps in 80% ethanol and shake to remove excess. Place a sterile polycarbonate filter on top of the glass fiber filter and turn on the vacuum. Using two forceps, fold the filter in quarters and place the filter into a bead tube. Dispose of glass fiber filter.

6.5.2. Repeat for each EC and EB tube and extract along with the samples in the batch.

6.6. Retrieve the Extraction Control standards from freezer storage and allow to thaw. Mix each extraction control spike by tapping with a finger and centrifuge briefly to coalesce any drops.

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6.7. Add 5 µl of HFG2GD, BRD3, CowM2, or HumM2 to the corresponding Extraction Control tubes. Be sure to spike directly onto the filter.

6.8. Add 590 µL of Salmon DNA extraction buffer into each sample and MB bead tubes.

6.9. Add 595 µL of Salmon DNA extraction buffer to each of the EC bead tubes.

6.10. Add 600 µL of Salmon DNA extraction buffer to the EB bead tube.

6.11. Make sure the bead tubes are tightly sealed and vortex briefly. Place the tubes in the bead beater and homogenize for 45s at speed 4 in the Bead Mill 24 (Program 01).

6.12. Centrifuge at 14000 X g for 5 min to pellet the debris.

6.13. Use a 1000 µL pipette to push the filter down and carefully transfer the supernatant (minimum of 200 µL) to the corresponding 1.5 mL colored centrifuge tubes that were labeled in step 6.4.1. If some pellet debris or beads carry over, it will not affect the sample at this stage.

6.14. Vortex briefly and centrifuge crude supernatant for 10 min at 14000 X g.

6.15. Carefully, without disturbing the pellet, use a 1000 µL pipette to transfer the clarified supernatant (minimum of 100 µL) to the labeled clear centrifuge tube. If any of the pellet is aspirated or pulled up into the supernatant, repeat Section 6.14. for that sample.

6.16. Refrigerate extracts at 4°C if analysis will begin within 24 hours. Otherwise, freeze extracts at -20°C until analysis.

7. SAMPLE ANALYSIS

7.1. Refer to SOP PCR-1.0 for HF183 qPCR sample analysis.

7.2. Refer to SOP PCR-1.2 for GULL2 qPCR sample analysis.

7.3. Refer to SOP PCR-1.3 for GFD qPCR sample analysis.

7.4. Refer to SOP PCR-1.4 for BacR qPCR sample analysis.

7.5. Refer to SOP PCR-1.5 for DG3 qPCR sample analysis.

7.6. Refer to SOP PCR-1.6 for CowM2 qPCR sample analysis.

7.7. Refer to SOP PCR-1.7 for HumM2 qPCR sample analysis.

8. DATA ARCHIVAL

8.1. Refer to SOP PCR-5.0 Section 5 for prep worksheet data instructions.

9. QUALITY CONTROL

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9.1. Analyze an Extraction Blank (EB) as a negative control for each set of samples extracted (maximum of 13 samples per batch) and extract and analyze along with the same batch of samples.

9.2. Analyze an Extraction Control (EC) for each applicable assay as a positive control for each set of samples extracted (maximum of 13 samples per batch).

9.3. Refer to the method SOPs for further details on required quality controls, acceptance limits, and corrective actions.

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

10.1. For sample extraction and analysis, wear safety glasses, gloves, and a clean disposable lab coat. If any substance falls on the gloves during processing, change into a new pair immediately to prevent any possible contamination of samples or controls.

10.2. Refer to the Health and Safety Plan. Read MSDS information for reagent handling and disposal.

11. REFERENCES

11.1. FDEP Laboratory Quality Manual

11.2. Health and Safety Plan

11.3. DEP SOP IZ-09, *Preparation of 80% Ethanol*

11.4. SOP PCR-1.0, *Human-Associated Bacteroidales HF183 Marker in Water by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.5. SOP PCR-1.2, *Gull-Associated GULL2 Marker by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.6. SOP PCR-1.3, *Bird specific Helicobacter GFD Marker Using a Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.7. SOP PCR-1.4, *Ruminant-associated Bacteroidetes BacR Marker by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.8. SOP PCR-1.5, *Canine-associated Bacteroidales DG3 Marker by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.9. SOP PCR-1.6, *Cow-associated CowM2 Marker by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.10. SOP PCR-1.7, *Human-associated HumM2 Marker by Quantitative Polymerase Chain Reaction (qPCR) Assay*

11.11. SOP PCR-2.4, *Calibration and Use of Mechanical Pipettes Using the Artel® PCS® 3 Pipette Calibration System*

11.12. SOP PCR-3.1, *Preparation of SKETA Assay Reagents and Standards for qPCR Analysis*

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11.13. SOP PCR-3.2, *Preparation of HF183 Assay Reagents and Standards for qPCR Analysis*

11.14. SOP PCR-3.3, *Preparation of Gull2 Assay Reagents and Standards for qPCR Analysis*

11.15. SOP PCR-3.4, *Preparation of GFD Assay Reagents and Standards for qPCR Analysis*

11.16. SOP PCR-3.5, *Preparation of BacR Assay Reagents and Standards for qPCR Analysis*

11.17. SOP PCR-3.6, *Preparation of DG3 Assay Reagents and Standards for qPCR Analysis*

11.18. SOP PCR-3.7, *Preparation of CowM2 Assay Reagents and Standards for qPCR Analysis*

11.19. SOP PCR-3.8, *Preparation of HumM2 Assay Reagents and Standards for qPCR Analysis*

11.20. SOP PCR-4.0, *Preparation of Samples for qPCR Analysis*

11.21. SOP PCR-5.0, *Sample Custody, Labels, and Worksheet Instructions for Molecular Biology Samples*

Appendix of Changes

11/27/2017	Added new bead beater and BacR analysis. Updated hyperlinks.
11/21/2018	Added DG3 analysis. Updated hyperlink
11/13/2019	Added sample analysis reference for CowM2 and HumM2
08/19/2021	Updated gBlocks and added GFD analysis.
02/14/2024	Reformatted SOP and clarified extraction procedure in Section 6. Added use of the vacuum funnel to fold filters for extraction controls and blanks.

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