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

        2.2.1. 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. Gloves, latex or nitriles

    3.2. Tube racks

    3.3. Centrifuge tubes, 1.5 mL

    3.4. Brady FreezerBondz Laboratory labels (Cat#19-140-586)

    3.5. Forceps

    3.6. Micropipettes with sterile PCR grade filter tips (use per SOP PCR-2.4.)

    3.7. CBS Optimizer PCR Workstation #2 (S/N 007632)

    3.8. Fisher Mini Vortexer

    3.9. Centrifuge

        3.9.1. Eppendorf Centrifuge 5418 (S/N 5418FR825055)

        3.9.2.Eppendorf Centrifuge 5418 (S/N) 5418AG715992)

    3.10. Bead Beater

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3.10.1. Fisher Scientific™ Bead Mill 24 Homogenizer (Property #152281)

3.11. PCR#3, Thermo Refrigerator (S/N 0121179201121219), maintained at 4ºC ± 2ºC, Room B246B

3.12. Thermo Fridge/Freezer (S/N 0121174701121218) maintained at -20ºC ± 2ºC, Room B246

4. REAGENTS AND STANDARDS

4.1. Deionized (DI) water

4.2. Salmon DNA extraction buffer - Prepare per SOP PCR-3.1 Section 8.

4.3. 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.

4.4. Bleach solution, 10% (v/v)

4.5. 80% Ethanol – Prepare per SOP IZ-09.

5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5

5.2. 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. SAMPLE PREPARATION

6.1. Prepare samples per SOP PCR-4.0.

6.2. Place two pairs of forceps in PCR Workstation #2. Turn on the UV light and leave for at least 3 minutes.

6.3. 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. Use colored tubes for one set and clear tubes for the other. On the first set of 1.5 mL centrifuge tubes (colored tubes), label the top of the sample tubes with the last 3 numbers of the sample ID. 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. Print labels and place them on the top and side of the second set of 1.5 mL centrifuge tubes (clear tubes). See SOP PCR-5.0 Section 5.2 for instructions on printing labels for extraction.

6.4. Set up Extraction Blank and Extraction Control bead tubes per Section 9. If needed, transfer sample filters to labeled bead tubes.

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6.5. Add 590 µL of Salmon DNA extraction buffer into each sample and MB bead tubes. Add 595 µL of Salmon DNA extraction buffer to each of the EC bead tubes. Add 600 µL of Salmon DNA extraction buffer to the EB bead tube.

6.6. 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).

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

6.8. With a 1000 µL micropipette, carefully transfer approximately 400 µL (minimum of 200 µL) of supernatant to the corresponding 1.5 mL colored centrifuge tube that was labeled in step 6.2. Avoid pellet debris and beads. If some pellet debris or beads carry over, it will not affect the sample at this stage. It may be necessary to push down into the filter to remove enough supernatant.

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

6.10. 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 Step 6.7.

6.11. 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 Human-Associated *Bacteroidales* 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 per batch). Using forceps sterilized by UV light, place a sterile polycarbonate membrane filter (47 mm diameter with 0.45 pore size) into a bead tube 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 per batch). Label bead tubes and centrifuge tubes for each assay. Mix each extraction control spike by tapping with a finger then centrifuge briefly. Using forceps sterilized by UV light, place a sterile polycarbonate membrane filter (47 mm diameter with 0.45 pore size) into a bead tube and then spike with 5 µl of HFG2GD, BRD3, CowM2, or HumM2 Extraction Control. Be sure to expel the 5 µL of spike directly onto the filter. Extract and analyze along with the samples.

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

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

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

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

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

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

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

11.11. DEP SOP PCR-2.4, *Calibration and Use of Mechanical Pipettes using the PCS Pipette Calibration System*

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

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

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

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

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

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

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

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

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

11.21. DEP 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

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11/13/2019 Added sample analysis reference for CowM2 and HumM2

08/19/2021 Updated gBlocks and added GFD analysis.

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