Preparation of Samples for qPCR Analysis

1. SCOPE AND APPLICATION

1.1. This method describes the preparation of surface water samples by membrane filtration and weighing of solid (soil, sediment, biological solids) samples for qPCR assays.

1.2. This procedure is used for samples with LIMS test ID of PCR-HF183, PCR-HF183B, PCR-HF183S, PUR-HFI83, PMA-HF183, PCR-HUMM2, PCR-HUMM2B, PCR-HUMM2S, PUR-HUMM2, PCR-GULL2, PCR-GULL2B, PCR-GULL2S, PUR-GULL2, PCR-BACR, PCR-BACRB, PCR-BACRS, PUR-BACR, PCR-COWM2, PCR-COWM2B, PCR-COWM2S, PUR-COWM2, PCR-DG3, PCR-DG3B, PCR-DG3S, PUR-DG3, PCR-GFD, PCR-GFDB,PCR-GFDS and PUR-GFD.

2. SUMMARY OF METHOD

2.1. A water sample is passed through a membrane filter. The filter containing the bacteria is agitated in a centrifuge tube containing zirconium silica beads using a bead beater to extract the DNA from the bacterial cells into a buffer solution. The clarified extract may also be purified using a purification kit. The extract is analyzed with qPCR amplification and detection in a thermocycler system using a primer/probe assay.

2.2. Solid samples are weighed on a balance and extracted using a purification kit. The extract is analyzed with qPCR amplification and detection in a thermocycler system using a primer/probe assay.

2.3. Interferences

2.3.1. Colloidal or suspended particulate materials in water can clog the filter and prevent filtration of a sufficient amount of sample. Some substances, including humic and tannic acids, can act as inhibitors 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. Forceps

3.4. Dispersion beads, 0.1 mm, zirconium silica

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3.5. Extraction tubes, 2 mL screw cap with o-ring seal

3.5.1. Weigh out 0.3 ± 0.01 g of dispersion beads into each extraction tube. Seal tube tightly, making sure the O-ring is seated properly. Autoclave @ 121°C for 39 min (15 psi). Record in LIMS Autoclave Log.

3.6. Centrifuge tubes, 1.5 mL

3.7. Polycarbonate membrane filter, 47 mm diameter, 0.45 µm pore size

3.8. Glass fiber filter, 47 mm diameter, AP20

3.9. Pipette pump with sterile disposable 50 mL tips

3.10. Sterile, Magnetic Funnels with 47 mm bases, 500 mL

3.11. Vacuum Filtration Manifold

3.12. Sterile, wash/rinse bottle

3.13. Sterile, clean spatulas

3.14. Qiagen DNeasy PowerSoil® Pro Kit

3.14.1. PowerBead Pro Tubes

3.15. Autoclave (S/N 031761851)

3.15.1. Calibrate and use per SOP TA-6.18.

3.16. Mettler Precision Balance Model XA105DU (S/N B314195659)

3.17. PCR#8, Thermo Revco Freezer (S/N 1127018801220603), maintained at -80ºC ± 10ºC, Room B246

3.18. UV Sterilizer unit

4. REAGENTS AND STANDARDS

4.1. Deionized (DI) Water

4.2. Molecular Biology grade water. Corning Molecular Biology Grade Water tested to USP Sterile Water (Cat.#MT46000CV)

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

4.4. Phosphate Buffered Saline (PBS) – Prepare per SOP PCR-3.0.

4.5. Ethanol 80%

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5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5

5.2. Refer to SOP BB-36 for calibration and use of the balance.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1. See DEP SOP FS 1000 for sample collection and preservation.

6.2. Refer to SOP PCR-5.0 for Sample custody and labels.

7. SAMPLE FILTRATION

7.1. Clean work area counters. Spray surface of work area with 10% bleach solution. Leave the bleach solution for at least 15 minutes on the work surface. Spray and wipe down with sterile DI water **before and after** filtration.

7.1.1. To prevent rust, spray metal surfaces with 80% ethanol instead of DI water and wipe dry.

7.2. Turn on UV sterilizer and let warm up for at least 10 minutes. Place forceps in UV sterilizer for at least 2 minutes.

7.3. Unwrap 3-6 funnels and bases depending on number of samples. Place bases with funnels in vacuum manifold. Place Extra funnels and bases in the UV sterilizer, or keep them wrapped in foil until ready for use Use a new set of funnels and bases **daily**.

7.4. Remove forceps from UV sterilizer and place in a small beaker of 80% ethanol. Unwrap a sterile, dry rinse bottle and fill with PBS.

7.5. Use sterile forceps to place a sterile glass fiber filter and then a polycarbonate filter on the filter base and attach the funnel to the base. The vacuum may be opened to help seat the filters.

Note: Keep forceps in a small beaker of 80% ethanol during filtration process. Dip in the ethanol and shake off excess before grasping each filter. If forceps are accidentally dropped or placed on counter, replace with a new pair of sterile forceps from the UV sterilizer. Make sure no ethanol residue from the forceps touches the filter.

7.6. Shake the sample container vigorously, approximately 25 times to uniformly distribute bacteria. Carefully open container. Avoid touching the mouth of the container to prevent contamination of the sample.

7.7. Add enough PBS to saturate the filters. With a 50 mL pipette tip (**do not discard wrapping**) and pipette pump, pipette 50 mL of sample into the funnel along the

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walls. Continue adding 50 mL aliquots of sample (maximum of 200 mL) until filtration of sample slows down. Typical filtration volumes are 100-200 mL.

Note: For very turbid or muddy samples, attempt to filter a minimum of 50 ml.

7.8. When sample has filtered through, detach the pipette tip into its wrapping and place aside.

7.9. Rinse the walls of the funnel with 20-30 mL of PBS (rinse all the way around 3 times).

7.10. Record the total volume of sample filtered on the extraction tube label and in Prep Worksheet Manager.

7.11. Turn off vacuum and gently lift the funnel from the base and place it in the UV sterilizer. Aseptically fold polycarbonate filter into a cylinder (fold in half, then half again, then roll) with sample side facing inward using sterile forceps and being careful to handle only the edges.

7.12. Place the rolled polycarbonate filter into an extraction tube containing dispersion beads and cap.

7.13. Place a new polycarbonate filter and filter a second aliquot of the sample in the same manner and volume as the first aliquot. Place the second rolled filter in a 1.5 mL centrifuge tube. Record the final sample volume on the tube labels. Discard the glass fiber filter and pipette tip.

7.14. Repeat Steps 7.4.-7.9 until all samples are filtered. Make sure funnels have been UV sterilized for at least 2 minutes before starting each new sample.

7.15. Store sample filters at -80°C until analysis.

8. SOLID SAMPLE WEIGHING

8.1. Before turning on the balance, snap the weighing basket onto the grid weighing pan.

8.2. Spin down all Qiagen DNeasy Power Soil® Pro kit bead tubes, to ensure beads are at the bottom of the tube and do not interfere with sample weighing.

8.3. Place an open Qiagen DNeasy Power Soil® Pro kit bead tube into the basket. Ensure that the doors of the draft shield are all closed and press the [→T←] key to tare the balance. “0.00000 g” should be displayed.

8.4. Add a small amount of the solid sample to be weighed into the bead tube using a sterile spatula. Close the door of the draft shield to prevent drafts from skewing the weight.

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8.5. Wait for the weight to stabilize. Add more sample in small amounts at a time, to reach 0.25 ± 0.01 grams of solid.

8.6. Record the weight associated with the sample in grams (g) on the sample label.

8.7. Carefully remove the Qiagen DNeasy Power Soil® Pro kit bead tube containing the sample and cap. Place the sample label on the tube.

8.8. Repeat Steps 8.2-8.7 until all samples are weighed. Make sure spatulas have been washed with 10% bleach and triple rinsed with DI water before starting each new sample.

8.9. Turn the balance off and clean any spilled substances from the balance or surrounding area.

8.10. Store samples at -80°C until analysis.

9. SAMPLE ANALYSIS

9.1. Refer to SOP PCR-1.0 for analysis of samples with LIMS ID: PCR-HF183, PCR-HF183S, PCR-HF183B, and PUR-HF183.

9.2. Refer to SOP-PCR-1.1 for analysis of samples with LIMS ID: PMA-HF183.

9.3. Refer to SOP PCR-1.2 for analysis of samples with LIMS ID: PCR-GULL2, PCR-GULL2B, PCR-GULL2S, and PUR-GULL2.

9.4. Refer to SOP PCR-1.3 for analysis of samples with LIMS ID: PCR-GFD, PCR-GFDB, PCR-GFDS, and PUR-GFD.

9.5. Refer to SOP PCR-1.4 for analysis of samples with LIMS ID: PCR-BACR, PCR-BACRB, PCR-BACRS, and PUR-BACR.

9.6. Refer to SOP PCR-1.5 for analysis of samples with LIMS ID: PCR-DG3, PCR-DG3B, PCR-DG3S, and PUR-DG3.

9.7. Refer to SOP PCR-1.6 for analysis of samples with LIMS ID: PCR-COWM2, PCR-COWM2B, PCR-COWM2S, and PUR-COWM2.

9.8. Refer to SOP-PCR-1.7 for analysis of samples with LIMS ID: PCR-HUMM2, PCR-HUMM2B, PCR-HUMM2S, PUR-HUMM2.

10. DATA ARCHIVAL

10.1. Refer to SOP PCR-5.0 for worksheet instructions.

11. QUALITY CONTROL

11.1. Method Blank (MB) – labeled MBLK_#

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11.1.1. Analyze a method blank for each batch of samples filtered (maximum of 13 per batch). Filter 50 mL of sterile PBS at the beginning of each sample batch. Extract and analyze with the same batch of samples.

11.2. Replicate Sample

11.2.1. Analyze at least one replicate per filtration batch (maximum of 13 per batch). Filter and analyze replicate in the same manner as original sample and within the same batch.

11.2.2. Analyze at least one replicate per weighing batch (maximum of 13 per batch). Purify and analyze replicate in the same manner as original sample and within the same batch.

12. SAFETY/HAZARDOUS WASTE MANAGEMENT

12.1. For sample filtration/weighing, wear safety glasses, gloves, and a clean lab coat.

12.2. When sample filtration is complete, discard remaining sample in the sink and place sample containers in a trash bag. When trash bag is full, label with the date range of the samples and leave bag in the hallway outside of the microbiology room for pickup by the Receiving section. After report certification, sample containers are discarded by the Receiving section.

12.3. Refer to the Laboratory Health and Safety Manual for Hazardous Waste Management.

12.4. Read MSDS information for reagent handling and disposal.

13. REFERENCES

13.1. DEP Laboratory Quality Manual

13.2. Health and Safety Plan

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

13.4. SOP PCR 1.1, *PMA-qPCR for Quantitative Differentiation of Live Human-associated Bacteroides*

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

13.6. SOP PCR 1.3, *Bird specific Helicobacter GFD marker with quantitative Polymerase Chain Reaction (qPCR) Assay*

13.7. SOP PCR 1.4, *Ruminant-associatedBacteroidetes BacR marker with quantitative Polymerase Chain Reaction (qPCR) Assay*

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13.8. SOP PCR 1.5, *Canine-associated Bacteroidales DG3 marker by quantitative Polymerase Chain Reaction* (qPCR) *Assay*

13.9. SOP PCR 1.6, *Cow-associated CowM2 marker by quantitative Polymerase Chain Reaction* (qPCR) *Assay*

13.10. SOP PCR 1.7, *Human-associated HumM2 marker by a quantitative Polymerase Chain Reaction (qPCR) Assay*

13.11. SOP BB-36, *Calibration, Use and Maintenance of Mettler Toledo XA105DU Balance*

13.12. SOP PCR-3.0, *Preparation of Reagents for quantitative Polymerase Chain Reaction (qPCR)*

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

13.14. DEP SOP TA-6.18 *AMSCO Renaissance Series 3021 Autoclave Calibration and Use*

13.15. DEP SOP FS 1000, *General Sampling*

Appendix of Changes

06/08/2016	Added LIMS Analysis codes for GULL2 marker and method SOP reference.
07/14/2017	Added updated LIMS Analysis codes and method SOP reference.
07/06/2018	Added LIMS Analysis codes for DG3 marker and method SOP reference.
06/20/2019	Added information for HUMM2 and CowM2 markers
01/26/2021	Changed storage from PCR#5 Thermo Fridge/Freezer to PCR#6 UXF Freezer
01/27/2022	Specified that the filtered volume must be the same when filtering the same sample. Updated lab manual link.
01/12/2023	Updated freezer information from PCR#6 Thermo UXF Freezer to PCR#8 Thermo Revco Freezer. Added cleaning procedure for metal surfaces.

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