

Microbial Source Tracking and its Applications to the Northern Gulf of Mexico

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Objectives:

1. Use Library Independent Methods to monitor human pollution in Coastal Waters of the Northern Gulf.
2. Select one method for additional research from three candidate methods

SOPs Components:

- Testing of > 1 PCR units by >1 technician
- Determine specificity, sensitivity of each method
- Share protocols among PI and stakeholder laboratories
- Compare results with EI counts
- Select one method for extensive testing

M. Smithii Protocol: Collect 500ml water and place on ice for transport to lab. Filter 500ml through a 3µm (47mm) cellulose acetate filter (Pall Gelman [#66387]; VWR Cat #28149-634) into a sterile filter flask. Collect filtrate in a sterile bottle. Filter filtrate onto a 0.2µm (47mm) Supor-200 filter (Pall Gelman [#66234]; VWR catalog # 28147-979). Place filter in a 100ml beaker (sterile) with 5-8ml sterile PBS; Stir (with sterile magnetic stir bar on top of filter) for 5-10 minutes. Collect sample in 1mL microcentrifuge tubes (sterile). Centrifuge at 12,000 x g for 15 min. Combine pellets and centrifuge for 15 min (12,000 x g). Remove supernatant and resuspend in 65µl PCR water and freeze at -20°C until ready for DNA extraction. Extract DNA using QBiogene Fast DNA Spin Kit for Soil (order from Fisher). Measure DNA concentration. Use 10 and 20 ng of DNA for the PCR reaction listed below. **PCR Reaction and Thermal Cycler Protocols:** 20µL PCR reaction: 0.5 µM primers (order from IDT) MniF-342f 5'-CAGAAAACCCAGTGAAGAG-3'; MniF-363r 5'-ACGTAAAGGCACTGAAAAACC-3' 1X PCR Buffer (Comes with the Taq Polymerase); 0.1% BSA, 0.2 mM dNTP (New England Biolabs, Cat #N0446S), 1U Taq Polymerase (ThermoPol Taq: New England Biolabs, Cat #M0267L), 10 and 20 ng template DNA. **Thermal Cycler Protocol:** 92°C for 2 min, 30 cycles of: 92°C for 1 min, 55.1°C for 30; 72°C for 1 min, Final extension at 72°C for 6 min.



Candidate PCR-based methods for the detection of human fecal pollution include:

Bacteroides-Prevotella (Bernhard and Field 2000)

Methanobrevibacter smithii (Ufnar et al. 2006),

Human polyomaviruses (McQuaig et al. 2006).

Round robin testing: Possible collaborations include Charleston NOAA laboratories, the AOML laboratory; MS and FL Departments of Environmental Quality or Marine Resources; regional/private (ex. Bonner Analytical) labs with appropriate equipment and personnel will receive the SOP and extracted DNA from known positive and negative samples from the PI laboratories so that their personnel can become familiar with the assays.

Human Bacteroides Protocols: Filter 500 ml environmental water samples through bacteriological filters (0.45 µm, 47 mm) to collect bacteria; if filter clogs replace with fresh membrane. Filters with bacteria impinged on them will be vortex-mixed for 1 min and soaked overnight at -20°C in lysis buffer. Extract DNA using MoBio UltraClean Fecal DNA Isolation Kit (order from MoBio Cat #12811). Measure DNA concentration. Use 2-5 ng of DNA for the PCR reaction listed below. **PCR of template DNA for Bacteroides and Domain Bacteria.** PCR reactions are performed 50 µl PCR reaction: 0.2 µM primers (order from Sigma-Genosys); 1X PCR Buffer with MgCl₂ and 3.0 mM MgCl₂ (Comes with the Taq Polymerase); 0.2 mM dNTP (Roche Diagnostics), 0.05 U/µl Taq Polymerase (Roche Diagnostics), 2-5 ng template DNA. Amplifications are performed with an initial step at 95°C for 8 min, followed by 25 cycles (35 cycles for B.t.) of 95°C for 30sec, annealing temp (Table 1) for 30 sec (45sec for B.t.), 72°C for 90sec (60sec for H183f), and final extension at 72°C for 6 min. Primer set/Forward sequence 5' – 3'/Reverse sequence 5' – 3'/Annealing Temp(°C) time (sec)a)27F/1492RAGAGTTTGATCMTGGCTCAGGTTACCTTGTTACGACTT45/30b)Bac32F/Bac708RAACGCTAGCTACAGGCTTCAATCGGAGTTCTTCGTG45/30c)B.t.F/B.t.R AACAGGTGGAAGCTGCGGAAGCCTCCAACCGCATCAA57/45 d)H183F/Bac708RATCATGAGTTACATGTCCGCAATCGGAGTCTTTCGTG- 59/30 a) Universal primer set for Domain Bacteria (Lane, 1991). b) Primers for *Bacteroides* spp. c) Primers for *Bacteroides thetaiotaomicron*, d) Primers for human-specific *Bacteroides*.

PCR amplification is done twice to enhance sensitivity

Human Polyomaviruses protocol: Raise pH of water sample to 9.5 to give proteins a negative charge and promote charge-charge repulsion between viruses and particles; Pre-filter water samples with 47-mm pre-filters (Millipore Reinforced; Fisher Scientific, Pittsburgh, PA, Cat. No. RW0304700); Keep filtrate and adjust pH to 3.5 to give viral capsids a net positive charge; Pass filtrate through a 0.45 µm nitrocellulose filter, which has a net negative charge so the viruses bind to the filters via electrostatic interactions; Elute viruses from the filters with 2 ml beef extract (pH 9.3); Perform DNA extraction on eluate, using QIAamp Blood Midi Kit (Qiagen, Inc., Valencia, CA); Use extracted DNA as template for nested-PCR: Each PCR reaction contains 45 µL of Platinum® Blue PCR SuperMix, 200 nM of each primer, and 4 µL of DNA template. The final reaction volume is adjusted to 50 µL using reagent grade water.; Thermocycler program: initial denaturation at 94°C for 2 min, followed by 45 cycles of: 94°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec, then a final elongation at 72°C for 2 min. The nested protocol is run under the same reaction conditions as above, with 1 µL of the first reaction used as the template. Target DNA band is 172bp

