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Human Bacteroidetes "Quantification" ID™

Detection and Quantification of the Fecal *Bacteroidetes* Human Gene Biomarker for Human Fecal Contamination by Real-Time Quantitative Polymerase Chain Reaction (qPCR) DNA Analytical Technology

Submitter: Raintree Lake Property Owners Association
Submitter #: Ward North
Source Molecular #: SM 10866
Samples Received: September 27, 2005
Date Reported: October 04, 2005

SM #	Client #	Human Fecal *Bacteroidetes* Quantified[*,7]	DNA Analytical Results
SM 10866	Ward North	BDL ‡	Negative (No Human Gene Biomarker Detected)

*** Number given is the copy number of the human *Bacteroides* 16S RNA marker per liter of water - see DNA Analytical Method Explanation, Reference 7 and Appendix A.**
‡ Below Detection Limit. Detection limit is <1000 copy no./ml of DNA extract. See also Human Bacteroidetes ID results.

Laboratory Comments

The submitted water sample was filtered for fecal *Bacteroidetes.* Afterwards, the filter was eluted in a buffer. The buffer was centrifuged and DNA was extracted from the resultant pellet. qPCR (i.e.: real-time quantitative PCR) targeting the fecal *Bacteroidetes* human gene biomaker was performed on the DNA extract. Fecal *Bacteroidetes* are found in abundant amounts in feces of warm-blooded animals. They are considered a good indicator of recent fecal pollution because they are strict anaerobes (i.e. they do not survive long outside the host organism).

All reagents, chemicals and apparatuses were verified and inspected beforehand to ensure that no false negatives or positives could be generated. In that regards, positive and negative controls were run to attest the integrity of the analysis. All inspections and controls tested negative for possible extraneous contaminates, including PCR inhibitors.

The result for sample Ward North (Our Ref: SM 10866) was below the detection limits of the real-time qPCR assay. It was therefore classified as negative for the fecal *Bacteroidetes* human gene biomarker. It is important to note that a negative result does not mean that the sample does not definitely have human contamination. In order to strengthen the result, a negative sample should be analyzed further for human fecal contamination with other DNA analytical tests such as the Human Enterococcus ID™ and Human Fecal Virus ID™ services.

DNA Analytical Method Explanation

The water sample was filtered through a 0.45 micron membrane filter. The filter was placed in a separate 50-ml disposable centrifuge tube containing 5 ml of lysis buffer (20 mM EDTA, 400 mM NaCl, 750 mM sucrose, 50 mM Tris; pH 9).[2] DNA extraction was prepared using the Qiagen DNA extraction kit, as per manufacturers instructions. Five micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions.

The copy number of the *Bacteroides* human marker was determined using the primer sequences described by Seurinck et al.[7] Amplifications were run on an ABI Prism 7700 using 25ul final reaction volumes and reaction component concentrations as detailed in reference 7. Applied Biosystems SyBr Green PCR Master Mix was used for preparing the human marker assays. Thermal cycling parameters were 2 min at 50 deg.C, 10 min at 95 deg.C followed by 40 cycles of 30 s at 95 deg.C, 50 deg. C for 1 min and 60 deg. C for 1 min. All assays were run in triplicate. Absolute quantification was achieved by generating standard curves from serial dilutions of synthesized final amplicon target sequence.

DNA Analytical Theory Explanation

The phylum *Bacteroidetes* is composed of three large groups of bacteria with the best-known category being *Bacteroidaceae*. This family of gram-negative bacteria is found primarily in the intestinal tracts and mucous membranes of warm-blooded animals and is sometimes considered pathogenic.

Comprising *Bacteroidaceae* are the genus *Bacteroides* and *Prevotella*. The latter genus was originally classified within the former (i.e. *Bacteroides*), but since the 1990's it has been classified in a separate genus because of new chemical and biochemical findings. *Bacteroides* and *Prevotella* are gram-negative, anaerobic, rod-shaped bacteria that inhabitant of the oral, respiratory, intestinal, and urogenital cavities of humans, animals, and insects. They are sometimes pathogenic.

Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[1] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems. This is a particularly strong reference point when trying to determine recent outbreaks in fecal pollution. They are also more abundant in feces of warm-blooded animals than *E. coli* and *Enterococci*. Furthermore, these latter two organisms are facultative anaerobes and as such they can be problematic for monitoring purposes since it has been shown that they are able to proliferate in soil, sand and sediments.

The Human Bacteroidetes "Quantification" ID™ service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[2,3,4,5,6] Furthermore, certain categories of *Bacteroidetes* have been shown to be predominately found in humans. Within these *Bacteroidetes*, certain strains of the *Bacteroides* and *Prevotella* genus have been found to be specific to humans.[2,3] As such, these bacterial strains can be used as indicators of human fecal contamination.

One of the advantages of the Human Bacteroidetes "Quantification" ID™ service is that the entire water is sampled and filtered for fecal *Bacteroidetes*. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates off a petri dish. This is a particular advantage for highly contaminated water systems with potential multiple sources of fecal contamination.

Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected.

Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for analysis.

Real-time quantitative PCR (qPCR) adds a variant to the PCR step by inserting of a fluorescent probe within the primer set. This fluorescent probe serves as a molecular beacon for the quantification step. During each PCR cycle, real-time quantification PCR monitors the fluorescence emitted during the reaction. This is done in "real-time" during the first PCR cycles as a way to quantify the targeted gene.

The Human Bacteroidetes "Quantification" ID™ service uses real-time quantification PCR to simultaneously confirm and quantify the human-specific HF183 *Bacteroides* 16S rRNA genetic marker.[6,7] This PCR technology avoids the cumbersome process of distinguishing DNA bands on a gel electrophoresis apparatus. The results are presented on a computer screen and printout thus avoiding ambiguities in interpretation.

This data should serve only as a preliminary indicator of the relative human pollution in the water sample. The context of the sample should be taken into account when interpreting the amount of the fecal *Bacteroidetes* human gene biomaker. The table in Appendix A of this report provides examples of the human marker in different sample contexts that are cited in the Seurinck et al paper.[7] The client is encouraged to compare the sample results with this table and to call to discuss the interpretation of the data. To strengthen the validity of the results, the Human Bacteroidetes "Quantification" ID™ service should also be combined with other DNA analytical services such as the Human Enterococcus ID™ and Human Fecal Virus ID™ services.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.

[2] Bernhard, A.E., and K.G. Field (2000a). **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Applied and Environmental Microbiology, 66: 1,587-1,594.

[3] Bernhard, A.E., and K.G. Field (2000b). **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Applied and Environmental Microbiology, 66: 4,571-4,574.

[4] Kreader, C.A. (1995). **Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution.** Applied and Environmental Microbiology, 61: 1,171-1,179.

[5] Kreader, C.A. (1998). **Persistence of PCR-detectable Bacteroides distasonis from human feces in river water.** Applied and Environmental Microbiology, 64: 4,103-4,105.

[6] Dick, Linda K., Field, Katharine G.**Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes.** Appl. Environ. Microbiol. 2004 70: 5695-5697.

[7] Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano. **Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human fecal pollution in freshwater.** Environmental Microbiology 2005 7:2 p. 249.

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Appendix A

(Fecal *Bacteroidetes* Human Gene Biomarker Comparison)

The table below is a summary of the different fecal *Bacteroidetes* human gene biomarker levels presented in different sample contexts from the Seurinck et al research note[7]. The client is encouraged to call to discuss the results and this data.

Table 1 (From Seurinck et al[7]). Real-time PCR assay results of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker in human fecal samples and raw sewage samples, collected at the same wastewater treatment plant on four consecutive days.

Sample	Human-specific *Bacteroides* markers per gram of wet feces or per liter of influent (mean ±SD)
Human 1	$1.2 \pm 0.1 \times 10^{7}$
Human 2	$3.0 \pm 0.3 \times 10^{7}$
Human 3	$1.0 \pm 0.1 \times 10^{7}$
Human 4	ND
Human 5	$7.2 \pm 1.1 \times 10^{9}$
Human 6	$8.4 \pm 0.1 \times 10^{5}$
Human 7	$2.0 \pm 0.2 \times 10^{9}$
Raw sewage 7/14	$6.8 \pm 1.2 \times 10^{9}$
Raw sewage 7/15	$5.9 \pm 0.7 \times 10^{9}$
Raw sewage 7/16	$2.5 \pm 0.3 \times 10^{10}$
Raw sewage 7/17	$3.1 \pm 0.3 \times 10^{10}$

ND – Not Detected