SOURCE MOLECULAR CORPORATION

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Bird Bacteroidetes "Quantification" ID™

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

Submitter: XYZ Municipal Beach
Submitter #'s: 675, 676, 677 and 678
Source Molecular #'s: SM 0625, SM 0626, SM 0627 and SM 0628
Samples Received: January 3rd, 2011
Date Reported: January 10th, 2011

SM #	Client #	Total Fecal *Bacteroidetes* Quantified*	Bird Fecal *Bacteroidetes* Quantified*	DNA Analytical Results
SM 0625	675	5.85×10^{10}	BDL ‡	Negative ‡
SM 0626	676	7.50×10^{9}	6.55×10^{5}	Bird Gene Biomarker Detected
SM 0627	677	4.66×10^{9}	BDL ‡	Negative ‡
SM 0628	678	2.54×10^{10}	BDL ‡	Negative ‡

*** Number given is the copy number of the bird *Bacteroidetes* biomarker per liter of water.**
‡ Below Detection Limit. Detection limit is < 2,000 copy number per liter of water.

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Laboratory Comments
Submitter: XYZ Municipal Beach
Report Date: October 23, 2007

The submitted water samples were filtered for fecal *Bacteroidetes*. Afterwards, the filters were eluted in a buffer. The buffer was centrifuged and DNA was extracted from the resultant pellet. qPCR (i.e.: real-time quantitative PCR) targeting total fecal *Bacteroidetes* and the fecal *Bacteroidetes* bird 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 regard, 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 results for samples 675 (Our Ref: SM 0625), 677 (Our Ref: SM 0627) and 678 (Our Ref: SM 0628) were below the detection limits of the real-time qPCR assay. They were therefore classified as negative for the fecal *Bacteroidetes* bird gene biomarker. It is important to note that a negative result does not mean that the sample does not definitely have bird contamination. In order to strengthen the result, a negative sample should be analyzed further for bird fecal contamination with other DNA analytical tests such as the Bird Enterococcus ID™ service. On the other hand, one can infer the presence of mammalian sources of fecal pollution since generic forms of fecal *Bacteroidetes* were found present in the negative samples.

Preliminary Interpretation of Positive Result

Sample 676 (Our Ref: SM 0626) tested positive for the fecal *Bacteroidetes* bird gene biomarker suggesting that bird fecal contamination is present in this water sample. Using real-time quantitative PCR DNA analytical technology (qPCR), the fecal *Bacteroidetes* with the bird gene marker was quantified and compared to the total fecal *Bacteroidetes* population. The fecal *Bacteroidetes* with the bird gene marker gave a ratio of 0.009% of the total fecal *Bacteroidetes* population. It is important to take into account the context of the sample when interpreting the percentage provided.

Our preliminary interpretation suggests that bird fecal sources of contamination are a minor component of the positive sample. Using our internal ratios (i.e. bird fecal *Bacteroidetes* / total fecal *Bacteroidetes*), the bird fecal pollution would seem to be less than 1% of the overall fecal pollution of the sample. The client is encouraged nonetheless to submit additional samples from this site both during wet and dry events to get a better understanding of the bird fecal pollution contribution. Furthermore, a baseline of bird fecal samples from the surrounding area of study would help gain a better understanding of the percentage of the bird marker present within the geographic region. A more precise interpretation would be available to the client with the submittal of such baseline samples. The client is also encouraged to conduct other DNA analytical tests such as the service mentioned above to further confirm the positive result.

DNA Analytical Method Explanation

Water samples (100 ml each) were filtered through 0.45 micron membrane filters. The filters were placed in separate 15-ml disposable centrifuge tubes containing 2 ml of lysis buffer. DNA extraction was prepared using a Qiagen DNA extraction kit, as per manufacturer's instructions. Two micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions.

The copy number of the Bacteroidetes bird marker was determined using variations of primer sequences described research literature.[6,7] Amplifications were run on an Applied Biosystems StepOne thermal cycler. The final reaction volume (20ul) contained 2.0ul of sample extract, 900nM of forward and reverse primers and 1X Applied Biosystems SyBr Green PCR Master Mix. 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 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 classfied 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 Bird 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 birds. Within these *Bacteroidetes*, certain strains of the *Bacteroidetes* have been found to be specific to birds. As such, these bacterial strains can be used as indicators of bird fecal contamination.

One of the advantages of the Bird 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 Bird Bacteroidetes "Quantification" ID™ service uses real-time quantification PCR to simultaneously confirm and quantify total fecal *Bacteroidetes* and the bird-specific *Bacteroidetes* 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.

Once each targeted gene is quantified, a relative percentage can be calculated. As such, it has been hypothesized that relative levels of bird pollution can be interpreted by the proportion of the bird gene biomaker found in fecal *Bacteroidetes* relative to the total population of fecal *Bacteroidetes* in the water sample.[6,7] Nonetheless this data should serve only as a preliminary indicator of relative bird pollution in the water sample. Furthermore, the context of the sample should be taken into account when interpreting the relative percentage provided. To strengthen the validity of the results, the Bird Bacteroidetes "Quantification" ID™ service should also be combined with other DNA analytical services such as the Bird Enterococcus ID™ service.

[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] Wood, Jacqueline, Scott, Karen P., Newbold, C. James, Flint, Harry J. **Estimation of the Relative Abundance of Different Bacteroides and Prevotella Ribotypes in Gut Samples by Restriction Enzyme Profiling of PCR-Amplified 16S rRNA Gene Sequences** Appl. Environ. Microbiol. 1998 64: 3683-3689.

[4] Dick, Linda K., Simonich, Michael T, Field, Katharine G. **Microplate Subtractive Hybridization To Enrich for Bacteroidales Genetic Markers for Fecal Source Identification** Appl. Environ. Microbiol. 2005 71: 3179-3183.

[5] Lamendella, Regina, Santo Domingo, Jorge W., Kelty, Catherine, Oerther, Daniel B. **Occurrence of bifidobacteria in feces and environmental waters** Appl. Environ. Microbiol. 2007 0: AEM.01221-07.

[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] Dick, Linda K., Bernhard, Anne E., Brodeur, Timothy J., Santo Domingo, Jorge W., Simpson, Joyce M., Walters, Sarah P., Field, Katharine G. **Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic Markers for Fecal Source Identification** Appl. Environ. Microbiol. 2005 71: 3184-3191.

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