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Preliminary Interpretation of Horse "Quantification" ID™ Results

Detection and Quantification of the Fecal Horse Gene Biomarker for horse fecal contamination by Real-Time Quantitative Polymerase Chain Reaction (qPCR) DNA Analytical Technology

Submitter: ABC Company
Submitter Reference: 01012011A, 01012011B, 01012011C, 01012011D, 01012011E, 01012011F
Source Molecular #'s: SM 16294, SM 16295, SM 16296, SM 16297, SM 16298, SM 16302
Date Received: October 3, 2011
Date Reported: October 11, 2011

SM #	Client #	Approximate Contribution of Horse Fecal Pollution in Water Sample	Comment
SM 16294	01012011A	Negative	Negative for the horse biomarker
SM 16295	01012011B	Negative	Negative for the horse biomarker
SM 16296	01012011C	**Major Contributor**	High levels of horse biomarker detected
SM 16297	01012011D	**Major Contributor**	High levels of horse biomarker detected
SM 16298	01012011E	**Major Contributor**	High levels of horse biomarker detected
SM 16302	01012011F	**Major Contributor**	High levels of horse biomarker detected

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Horse Bacteroidetes Quantification ID™

Detection and quantification of the fecal Bacteroidetes Horse Gene Biomarker for horse fecal contamination by Quantitative Polymerase Chain Reaction (qPCR) DNA Analytical Technology

Submitter: ABC Company
Date Received: October 3, 2011
Date Reported: October 11, 2011

SM #	Client #	Analysis Requested	General Marker Quantified*	Horse Specific Marker Quantified*	DNA Analytical Results
SM 16294	01012011A	Horse Bacteroidetes ID			Negative
SM 16295	01012011B	Horse Bacteroidetes ID			Negative
SM 16296	01012011C	Horse Bacteroidetes ID	5.97E+02	9.66E+01	**Positive**
SM 16297	01012011D	Horse Bacteroidetes ID	4.57E+03	9.66E+01	**Positive**
SM 16298	01012011E	Horse Bacteroidetes ID	5.44E+03	3.44E+02	**Positive**
SM 16302	01012011F	Horse Bacteroidetes ID	5.65E+03	1.29E+02	**Positive**

*Numbers reported as copy numbers per 100 mL of water

Laboratory Comments
Submitter: ABC Company
Report Date: October 11, 2011

Each submitted water sample was filtered for *Bacteroidetes* and the DNA was extracted and purified for DNA analysis. All reagents, chemicals and apparatus 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.

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-Summary and Evaluation of Client Results

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DNA Analytical Method Explanation

Each submitted water sample was filtered through 0.45 micron membrane filters. Each filter was placed in a separate, sterile 5ml disposable tubes containing a unique mix of beads and lysis buffer and then bead beated for 5min. DNA extraction was prepared using the MoBio Power Water DNA Isolation kit (MoBio, Carlsbad, CA), as per manufacturer's protocol

Amplifications to detect the target gene biomarker were run on an Applied Biosystems StepOne real-time thermal cycler (Applied Biosystems, Foster City, CA) in a final reaction volume of 20ul containing sample extract, forward primer, reverse primer, probe and an optimized buffer. The following thermal cycling parameters were used: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. All assays were run in duplicate. Absolute quantification was achieved by extrapolating target gene copy numbers from a standard curve generated from serial dilutions of known gene copy numbers.

For quality control purposes, a positive control consisting of horse fecal DNA and a negative control consisting of PCR-grade water, were run alongside the sample(s) to ensure a properly functioning reaction and reveal any false negatives or false positives. The accumulation of PCR product is detected and graphed in an amplification plot. If the fecal indicator organism is absent in the sample, this accumulation is not detected and the sample is considered negative. If accumulation of PCR product is detected, the sample is considered positive.

Theory Explanation of Horse Bacteroidetes "Quantification" ID™

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 Horse Bacteroidetes 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 detected in horses. Within these *Bacteroidetes*, certain strains of the *Bacteroides* and *Prevotella* genus have been found in horses.[2,6] As such, these bacterial strains can be used as indicators of horse fecal contamination.

One of the advantages of the Horse Bacteroidetes 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 and detected in real-time. The accumulation of DNA product is plotted as an amplification curve. The absence of an amplification curve would indicate that the horse *Bacteroidetes* gene biomarker is not present.

References

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[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.

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