Source Molecular
Leader in Microbial Source Tracking

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Preliminary Interpretation of Human Fecal Pollution ID™ Results

Detection and quantification of the fecal Human gene biomarker for Human fecal contamination by real-time quantitative Polymerase Chain Reaction (qPCR) DNA analytical technology

Submitter: ESA
Date Received: December 11, 2014
Date Reported: December 31, 2014

SM #	Client #	Approximate Contribution of Human Fecal Pollution in Water Sample	Comment
SM-4L11013	Station #1	Negative	Negative for 2 human fecal biomarkers
SM-4L11014	Station #2	Negative	Negative for 2 human fecal biomarkers
SM-4L11015	Station #3	Negative	Negative for 2 human fecal biomarkers
SM-4L11016	Station #4	Negative	Negative for 2 human fecal biomarkers
SM-4L11017	Station #5	Negative	Negative for 2 human fecal biomarkers
SM-4L11018	Station #1	Negative	Negative for 2 human fecal biomarkers
SM-4L11019	Station #2	Negative	Negative for 2 human fecal biomarkers
SM-4L11020	Station #3	Negative	Negative for 2 human fecal biomarkers
SM-4L11021	Station #4	Negative	Negative for 2 human fecal biomarkers
SM-4L11022	Station #5	Negative	Negative for 2 human fecal biomarkers

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Human Fecal Pollution ID™ Quantification

Detection and quantification of the fecal Human gene biomarker for Human fecal contamination by real-time quantitative Polymerase Chain Reaction (qPCR) DNA analytical technology

Submitter: ESA
Date Received: December 11, 2014
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SM #	Client #	Analysis Requested	Species	General Marker Quantified*	Human Specific Marker Quantified*	DNA Analytical Results
SM-4L11013	Station #1	Human Bacteroidetes ID 1	Dorei	1.29E+04	ND**	Absent
SM-4L11014	Station #2	Human Bacteroidetes ID 1	Dorei	1.40E+04	ND**	Absent
SM-4L11015	Station #3	Human Bacteroidetes ID 1	Dorei	1.69E+04	ND**	Absent
SM-4L11016	Station #4	Human Bacteroidetes ID 1	Dorei	5.70E+03	ND**	Absent
SM-4L11017	Station #5	Human Bacteroidetes ID 1	Dorei	5.35E+03	ND**	Absent
SM-4L11018	Station #1	Human Bacteroidetes ID 2	EPA	1.29E+04	ND**	Absent
SM-4L11019	Station #2	Human Bacteroidetes ID 2	EPA	1.40E+04	ND**	Absent
SM-4L11020	Station #3	Human Bacteroidetes ID 2	EPA	1.69E+04	ND**	Absent
SM-4L11021	Station #4	Human Bacteroidetes ID 2	EPA	5.70E+03	ND**	Absent
SM-4L11022	Station #5	Human Bacteroidetes ID 2	EPA	5.35E+03	ND**	Absent

*Numbers reported as copy numbers per 100 mL of water
**Non-detect

Laboratory Comments

Submitter: ESA

Report Date: December 31, 2014

Negative Results

In sample(s) classified as negative, the human-associated Bacteroidetes gene biomarker(s) was either not detected in test replicates, one replicate was detected at a cycle threshold greater than 35 and the other was not, or one replicate was detected at a cycle threshold less than 35 and the other was not after repeated analysis. It is important to note that a negative result does not mean that the sample does not definitely have human fecal contamination. Only repeated sampling (both during wet and dry sampling events) will enable you to draw more definitive conclusions as to the contributor(s) of fecal pollution.

In order to strengthen the result, a negative sample should be analyzed further for human fecal contamination with other DNA analytical tests. A list of human fecal ID tests can be found at **www.sourcemolecular.com/human**.

Human Fecal Reference Samples

The client is encouraged to submit samples from the surrounding wastewater facilities and/or septic systems in order to gain a better understanding of the concentration of the human-associated fecal Bacteroidetes genetic marker as well as the concentration of the general fecal Bacteroidetes genetic marker in the geographic region of interest. A more precise interpretation would be available to the client with the submittal of such baseline samples.

Result Interpretations

Quantitative results are reported along with interpretations. Interpretations are given as "negative", “trace”, "minor contributor", "important contributor", or "major contributor" based on the concentration and proportion of the genetic markers found in the water samples.

Additional Testing

A portion of all samples has been frozen and will be archived for 3 months. The client is encouraged to perform additional tests on the sample(s) for other hosts suspected of contributing to the fecal contamination. A list of available tests can be found at **www.sourcemolecular.com/tests**

DNA Analytical Method Explanation

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.

Each submitted water sample was filtered through 0.45 micron membrane filters. Each filter was placed in a separate, sterile 2ml disposable tube containing a unique mix of beads and lysis buffer. The sample was homogenized for 1min and the DNA extracted using the Generite DNA-EZ ST1 extraction kit (GeneRite, NJ), as per manufacturer's protocol.

Amplifications were run on an Applied Biosystems StepOnePlus 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: 50°C for 2 min, 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 genome copy numbers from standard curves generated from serial dilutions of Human specific and generic genomic DNA.

For quality control purposes, a positive control consisting of appropriate genomic 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.

Human Bacteroidetes ID™ Species*: B. dorei*

The **Human Bacteroidetes ID™ Species*: B. dorei*** service targets the species *Bacteroides dorei*. *B. dorei* is an anaerobe that is frequently shed from the gastrointestinal tract and isolated from human feces worldwide. It is a newly discovered species that is widely distributed in the USA.[1,2] The human-associated marker DNA sequence is located on the 16S rRNA gene of *B. dorei*.[3] The marker is the microbial source tracking (MST) marker of choice for detecting human fecal pollution due to its exceptional sensitivity and specificity. Internal validations have been conducted on hundreds of sewage, septage, human and animal host fecal samples collected from throughout the U.S and archived in the Source Molecular fecal bank. The marker has also been evaluated in both inland and coastal waters. A recent, comprehensive, multi-laboratory MST method evaluation study, exploring the performance of current MST methods, concluded the *B. dorei* qPCR assay to be the top performing human-associated assay amongst those tested. The success and consistency of this marker in numerous studies around the world[1,3,4] makes the **Human Bacteroidetes ID™ Species*: B. dorei*** service the primary service for identifying human fecal pollution at Source Molecular.

Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[5] 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*.

The Human Bacteroidetes ID™ service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[3,5,6,7,8] Furthermore, certain strains of *Bacteroidetes* have been found to be associated with humans.[3,6] As such, these bacterial strains can be used as indicators of human fecal contamination.

Accuracy of the results is possible because the method amplifies DNA into a large number of small copies of the gene biomarker of interest. This is accomplished with small pieces of DNA called primers that are complementary and specific to the unique *B. dorei* DNA sequence. Through a heating process called thermal cycling, the double stranded DNA is denatured, hybridized to the complementary primers and amplified to create many copies of the DNA fragment desired. If the primers are successful in finding a site on the DNA fragment that is specific to the *B. dorei* DNA sequence, 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 by the qPCR software. The absence of an amplification curve indicates that the *B. dorei* gene biomarker is not detected in the water sample because it is either not present or present at concentrations below the analytical detection limit.

To strengthen the validity of the results, additional tests targeting other high-ranking, human-associated *Bacteroidetes* species should be performed, such as
Human Bacteroidetes ID™ Species*: B. stercoris*,
Human Bacteroidetes ID™ Species*: B. fragilis,* and
Human Bacteroidetes ID™ Species*: B. thetaiotaomicron*.

[1]Boehm, A., Fuhrman, J., Mrse, R., Grant, S. **Tiered approach for identification of a human fecal pollution source at a recreational beach: case study at Avalon Bay, Catalina Island, California**. Environ Sci Technol. 2003 37**:** 673–680.
[2]Bakir, M., Sakamoto, M., Kitahara, M., Matsumoto, M., Benno, Y. **Bacteroides dorei sp. nov., isolated from human faeces**. Int. J. Syst. Evol. Microbiol. 2006 56: 1639–1641.
[3] Bernhard, A., Field, K. **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Appl. Environ. Microbiol. 2000b 66: 4571-4574.
[4]Ahmed, w., Masters, N., Toze, S. **Consistency in the host specificity and host sensitivity of the Bacteroides HF183 marker for sewage pollution tracking. Lett. Appl. Microbiol. 2012 55: 283-289.**
[5] Scott, T., Rose, J., Jenkins, T., Farrah, S., Lukasik, J. **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. 2002 68: 5796-5803.
[6] Bernhard, A., Field, K. **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Appl. Environ. Microbiol. 2000a 66: 1587-1594.
[7] Fogarty, L., Voytek, M. **A Comparison of Bacteroides-Prevotella 16S rRNA Genetic Markers for Fecal Samples from Different Animal Species.** Appl. Environ. Microbiol. 2005 71: 5999-6007.
[8] Dick, L., Bernhard, A., Brodeur, T., Santo Domingo, J., *et al*. **Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic**

Human Bacteroidetes ID™: EPA Developed Assay

The **Human Bacteroidetes ID™: EPA Developed Assay** service targets a functional gene biomarker in *Bacteroidales*-like anaerobic bacteria that is present in high concentrations in the human gut. The U.S Environmental Protection Agency (U.S. EPA) was the first to target the biomarker using quantitative Polymerase Chain Reaction (qPCR) technology in order to detect ground and surface waters impacted by human fecal pollution.[1] Since it's development, the assay has been used succesfully around the U.S to identify fecal pollution originating from human sources, such as sewage and septage wastewaters.

The U.S. EPA Developed assay has been shown to be highly associated with human fecal pollution. It has successfully been validated in multiple nationwide studies using at least 300 individual reference fecal material from 22 different animal species known to commonly contaminate environmental waters.[1,2] A reported 99.2% specificity to human fecal material makes this one of the leading assays to confirm the presence of fecal contamination that is of human origin.[1] The *Bacteroidales*-like bacteria is widely distributed. It was detected in 100% of hundreds of sewage and human reference fecal samples collected from more than 20 human populations, making it highly sensitive. Internal validations have also been conducted on hundreds of wastewater, human and animal host fecal samples archived in the Source Molecular fecal bank.

Fecal anaerobic bacteria are considered for several reasons an interesting alternative to more traditional fecal indicator organisms such as *E. coli* and *Enterococci*.[3] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems.[3] 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*.

The **Human Bacteroidetes ID™: EPA Developed Assay** service is designed around the principle that fecal *Bacteroidales*-like bacteria are found in large quantities in feces of warm-blooded animals.[4,5] Furthermore, certain strains have been shown to be associated with humans.[4,5] As such, these bacterial strains can be used as indicators of human fecal contamination. An advantage of the Human Bacteroidetes ID™ service is that the entire portion of water sampled is filtered to concentrate bacteria. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates. This is an advantage for highly contaminated water systems with potential multiple sources of fecal contamination.

Accuracy of the results is possible because the method amplifies DNA into a large number of copies of the gene biomarker of interest. This is accomplished with small pieces of DNA called primers that are complementary and specific to the gene biomarker. Through a heating process called thermal cycling, the double stranded DNA is denatured, hybridized to the complementary primers and amplified to create many copies of the DNA fragment. If the primers are successful in finding a site on the DNA fragment that is specific to the human-associated biomarker, 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 by qPCR software. The absence of an amplification curve indicates that the gene biomarker is not detectable in the water sample either because it is not present or present at concentrations below the analytical detection limit.

To strengthen the validity of the results, additional tests targeting other high-ranking, human-associated *Bacteroidetes* species should be performed, such as
Human Bacteroidetes ID™ Species*: B. dorei*,
Human Bacteroidetes ID™ Species*: B. fragilis,* and
Human Bacteroidetes ID™ Species*: B. stercoris*

[1] Shanks, O., Kelty, C., Sivaganesan, M., Varma, M. and Haugland, R. **Quantitative PCR for Genetic Markers of Human Fecal Pollution**. Appl. Environ. Microbiol. 2009 75: 5507-5513.
[2] Layton, B., Cao, Y., Ebentier, D., Hanley, K., Ballesté, E., Brandão, J., *et al*. **Performance of Human Fecal Anaerobe-Associated PCR-Based Assays in a Multi-Laboratory Method Evaluation Study.** Water Research. 2013 In Press.
[3] Scott, T., Rose, J., Jenkins, T., Farrah, S. and Lukasik, J. **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. 2002 68: 5796-5803.
[4] Bernhard, A., Field, K. **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Appl. Environ. Microbiol. 2000a 66: 1587-1594.
[5] Bernhard, A., Field, K. **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Appl. Environ. Microbiol. 2000b 66: 4571-4574.

General Bacteroidetes ID™

The **General Bacteroidetes ID™** service is designed around the principle that general fecal *Bacteroidetes* are commonly found in the gastrointestinal tract and feces of Humans and warm-blooded animals worldwide.[1,2,3,4] Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*. Since they are strict anaerobes, they are unlikely to persist for extended periods of time in oxygenated environments. Their presence in water systems is therefore indicative of recent fecal contamination.[1,2] 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*. [1,2] In addition, a strong positive correlation has been observed between qPCR-measured *Bacteroidetes* and swimming-associated gastrointestinal illness rates (GIIR).[5] As such, testing for general *Bacteroidetes* can supplement routine *E. coli* and *Enterococci* membrane filtration culture methods and enhance water quality testing results.

The **General Bacteroidetes ID™** service adapts the qPCR assay from the U.S. EPA Method B for the detection and quantification of the16S ribosomal RNA (16S rRNA) target gene from all known Bacteroidales in water.[6]

Accuracy of the results is possible because the method amplifies DNA into a large number of small copies of the gene biomarker of interest. This is accomplished with small pieces of DNA called primers that are complementary and specific to the general *Bacteroidetes* 16S rRNA DNA sequence. Through a heating process called thermal cycling, the double stranded DNA is denatured, hybridized to the complementary primers and amplified to create many copies of the DNA fragment desired. If the primers are successful in finding a site on the DNA fragment that is specific to the *Bacteroidetes* DNA sequence, 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 by the qPCR software. The absence of an amplification curve indicates that the *Bacteroidetes* gene biomarker is not detected in the water sample because it is either not present or present at concentrations below the analytical detection limit. Once general *Bacteroidetes* is found to be present in the water sample, additional tests may be used to determine the specific sources of the fecal pollution. Please call **(786) 220-0379** or visit **www.sourcemolecular.com** for a list of available tests.

[1] Scott, T., Rose, J., Jenkins, T., Farrah, S., Lukasik, J. **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. 2002 68: 5796-5803.
[2]Dick, L. K., Field, K. 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.
[3] Bernhard, A., Field, K. **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Appl. Environ. Microbiol. 2000b 66: 4571-4574.
[4]Dick, L., Bernhard, A., Brodeur, T., Santo Domingo, J., *et al*. **Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic Markers for Fecal Source Identification.** Appl. Environ. Microbiol. 2005 71: 3184-3191.
[5]Siefring, S., Varma, M., Atikovic, E., Wymer, L., Haugland, R. A. **Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems**. J. of Water And Health. 2008 06.2 225-237.
[6]United States Environmental Protection Agency. **Method B: Bacteroidales in Water by Quantitative Polymerase Chain Reaction (qPCR) Assay.** 2010