Microbial Source Tracking Assays Using Methanogen Indicators

Step 1: Collect water samples, concentrate bacteria and extra DNA. See Sample Preparation below.

Step 2: Determine whether fecal indicator of interest is present by PCR. See PCR Protocols below.

Sewage-specific indicator:

Target organism: *Methanobrevibacter smithii*
Gene target: *nifH*, encodes nitrogenase reductase
Primer name: Mnif
Reference: Ufnar, J.A., S.Y. Wang, J. Christiansen, H. Yampara-Iquise, C. Carson and R.D. Ellender. 2006. Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. J. Applied Microbiol. 101:44-52.

Swine-specific fecal indicator:

Target organism: methanogens
Gene target: *mcrA,* encodes α subunit of methyl coenzyme M reductase
Primer name: P23-2
Reference: Ufnar, J.A., D.F. Ufnar, S.Y. Wang and R.D. Ellender. 2007. Development of a swine-specific fecal pollution marker based on host differences in methanogen *mcrA* genes. App. Environ. Microbiol. 73:5209-5217.

Domesticated ruminant-specific fecal indicator:

Target organism: *Methanobrevibacter ruminantium*
Gene target: *nifH,* encodes nitrogenase reductase
Primer name: Mrnif
Reference: Ufnar, J.A., S.Y. Wang, D.F. Ufnar and R.D. Ellender. 2007. *Methanobrevibacter ruminantium* as an indicator of domesticated ruminant fecal pollution in surface waters. App. Environ. Microbiol. (Accepted for publication 8-30-2007).

Sample Preparation:

1. Collect 500 mL water, place on ice and transport to lab.
2. Remove particulate matter with 3 µm filter. Filter 500 mL water sample through a 3 µm (47 mm) cellulose acetate filter (Pall Gelman #66387; VWR Cat #28149-634). Collect filtrate in sterile flask
3. Concentrate bacteria with 0.2 µm filter. Filter filtrate from previous step using a 0.2 µm (47 mm) Supor-200 filter (Pall Gelman #66234; VWR catalog # 28147-979).
4. Place filter in a 100 mL sterile beaker with 5-8 mL sterile PBS and stir using a sterile magnetic stir bar on top of the filter for 5-10 min.
5. Collect resuspended cells in centrifuge tube and pellet cells by centrifugation.
6. Discard supernatant and resuspend cells in 65 µL sterile water. The sample may be stored at -20°C or processes immediately for DNA extraction.
7. Extract DNA using MO BIO PowerSoil Kit and quantify DNA concentration.
8. Perform PCR using 10 and 20 ng of DNA.

PCR Protocols:

Sewage-specific indicator:

Target organism: *Methanobrevibacter smithii*
Gene target: *nifH*, encodes nitrogenase reductase
Primer name: Mnif
Reference: Ufnar, J.A., S.Y. Wang, J. Christiansen, H. Yampara-Iquise, C. Carson and R.D. Ellender. 2006. Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. J. Applied Microbiol. 101:44-52.

PCR:

	Stock Conc	Volume	Final Conc
Water		10.8 uL	
10X Buffer	10X	2 uL	1X
BSA	1 %	2 uL	0.1%
dNTPs	2 mM each	2 uL	0.2 mM each
Template DNA*		1 uL	
Primers	5 uM	2 uL	0.5 uM
Thermal stable DNA pol	5 U/uL	0.2 uL	1 U
Total		20 uL	

*Template DNA: 10- 20 ng per reaction

Primers: Mnif-342f 5'-AACAGAAAACCCAGTGAAGAG-3'
Mnif-363r 5'-ACGTAAAGGCACTGAAAAACC-3'

Amplification protocol:

Denaturation: 92°C for 2 min
Amplification, 30 cycles:
92°C for 1 min
55.1°C for 30 sec
72°C for 1 min
Final extension:72°C for 6 min

PCR Protocols:

Swine-specific fecal indicator:

Target organism: methanogens

Gene target: *mcrA,* encodes α subunit of methyl coenzyme M reductase

Primer name: P23-2

Reference: Ufnar, J.A., D.F. Ufnar, S.Y. Wang and R.D. Ellender. 2007. Development of a swine-specific fecal pollution marker based on host differences in methanogen *mcrA* genes. App. Environ. Microbiol. 73:5209-5217.

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Template DNA*		1 uL	
Primers	5 uM	2 uL	0.5 uM
Thermal stable DNA pol	5 U/uL	0.2 uL	1 U
Total		20 uL	

*Template DNA: 10 -50 ng per reaction

Primers:

P23-2f-5'-TCTGCGACACCGGTAGCCATTGA-3
P23-2r-5'-ATACACTGGCGACATTCTTGAGGATTAC-3'

Amplification protocol:

Denaturation: 92°C for 2 min
Amplification, 30 cycles:
92°C for 30 sec
60°C for 15 sec
72°C for 30 sec
Final extension:72°C for 6 min

An Internal Amplification Control (IAC) can be included to guard against false negatives. Use 10^{-9} µM in the reaction. The sequence of the IAC is 5'- GAGACCGCAG TCTGCGACAC CGGTAGCCAT TGAACCTGCG ATACCGGTTG CTGCTGCTGC AACTGTTGCC TTGTCCTTGA TGTAGTCGAT TGCATAGTAT GTGTAATCCT CAAGAATGTC GCCAGTGTAT GCTGCTGTAG -3' It can be custom synthesized by oligo vendors such as IDT, Inc.

PCR Protocols:

Domesticated ruminant-specific fecal indicator:

Target organism: *Methanobrevibacter ruminantium*
Gene target: *nifH,* encodes nitrogenase reductase
Primer name: Mrnif
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Template DNA*		1 uL	
Primers	5 uM	2 uL	0.5 uM
Thermal stable DNA pol	5 U/uL	0.2 uL	1 U
Total		20 uL	

*Template DNA: 10 - 20 ng per reaction

Primers:

Mrnif-f-5'-AATATTGCAGCAGCTTACAGTGAA-3'
Mrnif-r-5'-TGAAAATCCTCCGCAGACC-3'

Amplification protocol:

Denaturation: 92°C for 2 min
Amplification, 30 cycles:
92°C for 30 sec
60°C for 15 sec
72°C for 30 sec
Final extension:72°C for 6 min

An Internal Amplification Control (IAC) can be included to guard against false negatives. Use 10^{-9} µM in the reaction. The sequence of the IAC is 5'- TACAGTAGCT AATATTGCAG CAGCTTACAG TGAAGACAAT AAGAAAGTCA TGGTTATTGG CTGCCTTGAA GAGGAATTTG ATGTAATCTT ATATGATGTT CTTGGAGATG TGGTCTGCGG AGGATTTTCA GTTCCTCTAA – 3'. It can be custom synthesized by oligo vendors such as IDT, Inc.