North Wind

# Poultry-Specific Biomarker Quantitative PCR Analytical Summary

November 3, 2007

## Overview:

The objective of this project was to quantify the number of poultry-specific *Brevibacteria* biomarker gene copies contained in water, soil, and/or litter samples using quantitative polymerase chain reaction (qPCR). Table 1 describes the sample matrix and the condition of the samples upon arrival to the analytical laboratory.

**Table 1.** Description of samples and volume or mass filtered for DNA extraction.

| Sample ID | Matrix/ Date Sampled | Condition Received/Observations | Volume Filtered (mL) or Mass Extracted (g) |
|---|---|---|---|
| FAC03-7-6-06 | Litter/7-6-06 | Cold/sealed bag | 0.35 g |
| FAC09-8-31-06 | Litter/8-31-06 | Cold/sealed bag | 0.22 g |
| FAC02-6-21-06 | Litter/6-21-06 | Cold/sealed bag | 0.24 g |
| FAC08-8-15-06 | Litter/8-15-06 | Cold/sealed bag | 0.26 g |
| FAC04-7-12-06 | Litter/7-12-06 | Cold/sealed bag | 0.26 g |
| FAC010-9-22-06 | Litter/9-22-06 | Cold/sealed bag | 0.2 g |
| FAC07-8-3-06 | Litter/8-3-06 | Cold/sealed bag | 0.39 g |
| FAC05-7-13-06 | Litter/7-13-06 | Cold/sealed bag | 0.35 g |
| FAC06-7-20-06 | Litter/7-20-06 | Cold/sealed bag | 0.27 g |
| LAL6A2-6-14-06 | Soil/6-14-06 | Cold/sealed bag | 0.24 g |
| LAL6D2-6-15-06 | Soil/6-15-06 | Cold/sealed bag | 0.24 g |
| LAL6B2-6-14-06 | Soil/6-14-06 | Cold/sealed bag | 0.23 g |
| LAL13C2Q-7-7-06 | Soil/7-7-06 | Cold/sealed bag | 0.24 g |
| LAL14C2-7-10-06 | Soil/7-10-06 | Cold/sealed bag | 0.58 g |
| LAL14C2Q-7-10-06 | Soil/7-10-06 | Cold/sealed bag | 0.51 g |
| LAL13C2-7-7-06 | Soil/7-7-06 | Cold/sealed bag | 0.35 g |
| LAL16C2-7-18-06 | Soil/7-18-06 | Cold/sealed bag | 0.35 g |
| LAL5C2-6-12-06 | Soil/6-12-06 | Cold/sealed bag | 0.25 g |
| LAL8B2-6-21-06 | Soil/6-21-06 | Cold/sealed bag | 0.26 g |
| LAL14B2-7-10-06 | Soil/7-10-06 | Cold/sealed bag | 0.58 g |
| LAL9D2-6-22-06 | Soil/6-22-06 | Cold/sealed bag | 0.25 g |
| LAL7B2-6-20-06 | Soil/6-20-06 | Cold/sealed bag | 0.25 g |
| LAL13A2-7-6-06 | Soil/7-6-06 | Cold/sealed bag | 0.27 g |
| LAL17C2-7-18-06 | Soil/7-18-06 | Cold/sealed bag | 0.71 g |
| LAL17C2Q-7-18-06 | Soil/7-18-06 | Cold/sealed bag | 0.62 g |
| LAL14D2-7-10-06 | Soil/7-10-06 | Cold/sealed bag | 0.63 g |
| LAL8A2-6-19-06 | Soil/6-19-06 | Cold/sealed bag | 0.7 g |



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North Wind

| Sample ID | Matrix/ Date Sampled | Condition Received/Observations | Volume Filtered (mL) or Mass Extracted (g) |
|---|---|---|---|
| LAL8D2-6-20-06 | Soil/6-20-06 | Cold/sealed bag | 0.26 g |
| LAL17A2-7-10-06 | Soil/7-10-06 | Cold/sealed bag | 0.68 g |
| LAL7D2-6-29-06 | Soil/6-29-06 | Cold/sealed bag | 0.24 g |
| LAL9A2-6-22-06 | Soil/6-22-06 | Cold/sealed bag | 0.26 g |
| LAL5A2-6-13-06 | Soil/6-13-06 | Cold/sealed bag | 0.35 g |
| LAL7C2-6-19-06 | Soil/6-19-06 | Cold/sealed bag | 0.25 g |
| LAL9B2-6-22-06 | Soil/6-22-06 | Cold/sealed bag | 0.25 g |
| LAL13D2-7-6-06 | Soil/7-6-06 | Cold/sealed bag | 0.24 g |
| LAL7A2-6-20-06 | Soil/6-20-06 | Cold/sealed bag | 0.24 g |
| LAL16D2-7-18-06 | Soil/7-18-06 | Cold/sealed bag | 0.52 g |
| LAL5B2-6-12-06 | Soil/6-12-06 | Cold/sealed bag | 0.25 g |
| LAL12A2Q-7-6-06 | Soil/7-6-06 | Cold/sealed bag | 0.27 g |
| LAL12D2-7-7-06 | Soil/7-7-06 | Cold/sealed bag | 0.24 g |
| LAL16-SP2-7-18-06 | Water/7-18-06 | Cold/bottle intact | 100 mL |
| EOF-SPREAD-010-5-9-06 | Water/5-9-06 | Cold/bottle intact | 40 mL |
| EOF-SPREAD-17A-01-5-1-06 | Water/5-1-06 | Cold/bottle intact | 30 mL |
| EOF-SPREAD-023-6-18-06 | Water/6-18-06 | Cold/bottle intact | 25 mL |
| EOF-SPREAD-073B-6-18-06 | Water/6-18-06 | Cold/bottle intact | 10 mL |
| EOF-SPREAD-064-5-4-06 | Water/5-4-06 | Cold/bottle intact | 50 mL |
| EOF-SPREAD-53E-01-4-29-06 | Water/4-29-06 | Cold/bottle intact | 30 mL |
| EOF-SPREAD-60-01-4-29-06 | Water/4-29-06 | Cold/bottle intact | 50 mL |
| SPREAD-023-4-25-06 | Water/4-25-06 | Cold/bottle intact | 40 mL |
| EOF-1-6-17-06 | Water/6-17-06 | Cold/bottle intact | 100 mL |
| EOF-SPREAD-053G-5-4-06 | Water/5-4-06 | Cold/bottle intact | 100 mL |
| EOF-SPREAD-048-5-9-06 | Water/5-9-06 | Cold/bottle intact | 100 mL |
| SPREAD-029-4-25-06 | Water/4-25-06 | Cold/bottle intact | 100 mL |
| SPREAD-036-4-25-06 | Water/4-25-06 | Cold/bottle intact | 100 mL |
| EOF-SPREAD-071-5-9-06 | Water/5-9-06 | Cold/bottle intact | 150 mL |
| EOF-SPREAD-065-5-4-06 | Water/5-4-06 | Cold/bottle intact | 100 mL |
| EOF-Q2-6-17-06 | Water/6-17-06 | Cold/bottle intact | 50 mL |
| EOF-26-6-8-05 | Water/6-8-05 | Cold/bottle intact | 500 ml |
| EOF-17-6-8-05 | Water/6-8-05 | Cold/bottle intact | 500 mL |
| EOF-222-4-13-07 | Water/4-13-07 | Cold/bottle intact | 20 mL |
| GPGW-18A-6-26-07 | Water/6-26-07 | Cold/bottle intact | 20 mL |
| GPGW-20-6-11-30-06 | Water/11-30-06 | Cold/bottle intact | 50 mL |
| GPGW-48-7-12-1-06 | Water/12-1-06 | Cold/bottle intact | 50 mL |
| GPGW-10-4-11-30-06 | Water/11-30-06 | Cold/bottle intact | 50 mL |

North Wind

| Sample ID | Matrix/ Date Sampled | Condition Received/Observations | Volume Filtered (mL) or Mass Extracted (g) |
|---|---|---|---|
| GPGW-40-6-27-07 | Water/6-27-07 | Cold/bottle intact | 15 mL |
| HFS22-EVENTA-5-10-06 | Water/5-10-06 | Cold/bottle intact | 150 mL |
| HFS04-BF2-01-8-1-06 | Water/8-1-06 | Cold/bottle intact | 500 mL |

**Methods:**

***DNA Extraction.*** For soil and/or litter samples, DNA was extracted from 0.25 g of soil or litter using the FastDNA®SPIN® Kit for soil protocol. For surface water shipped to the laboratory, between 100 and 1,000 mL of groundwater was filtered through a Supor-200, 0.2 µm filter. The filters were frozen at -80°C and then shattered. Next, each sample tube was amended with 2 mL of DNA-free water, vortexed vigorously for 15 minutes, and the liquid volume was partitioned into DNA extraction tubes. DNA extractions were performed using the FastDNA®SPIN® Kit for soil according to the manufacturer's instructions. All DNA extractions were cleaned using an ethanol precipitation method. Community DNA was eluted in nuclease-free water (50 µL) and stored at -20°C.

***Amplification of Bacteria.*** The PCR was used to amplify nearly full-length 16S rDNA genes from *Bacteria*. Each 25-µL PCR reaction included 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM each 8F forward and 907R reverse primer, 1 u/50uL Taq DNA polymerase, 0.2 mM dNTP, 1 µL template DNA, and 20 µL molecular-grade water. Amplification was performed on a MJ Research Peltier Gradient thermocycler using the following regime: 94°C (5 min) followed by 30 cycles of 94°C (1 min), 53.5°C (1 min), and 72°C (1 min, 50 sec). The reaction was finished with an additional 7 minutes at 72°C. PCR products were examined by ultraviolet (UV) light in a 1% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions.

***Sepharose Cleanup.*** Any sample not amplifying in the PCR was processed through a Sepharose CL-4B (Sigma-Aldrich) size exclusion gel chromatography cleanup. Briefly, the micro-bio spin columns (Bio-Rad) were packed with sterile Sepharose CL-4B and washed with Tris-HCl buffer (pH 8). The sample was added to the packed gel column and eluted by spinning in a micro-centrifuge.

***Detection of a Poultry Specific Brevibacteria Biomarker.*** The qPCR methods for assessing the 16S rRNA gene are very sensitive in detecting specific DNA fragments. The detection limit for the methods used is approximately 6 gene copies per µL of the DNA extraction. Biomarker DNA was cloned into a plasmid and was used as the source of the quantitative standards used in the analysis. Plasmid DNA containing the target 16S rRNA gene from the poultry-specific *Brevibacteria* biomarker was purified and quantified fluorometrically. Based on the known size of the plasmid and insert, DNA concentrations were converted to insert copy numbers. A dilution series spanning seven orders of magnitude was generated using known concentrations of each plasmid. Amplification and detection of the DNA was performed using the MJ Chromo-4 System. The acceptance criterion for the standard curve is a linear $R^2$ value of greater than 0.995.

To determine qPCR results, sample DNA diluted to a final concentration of 15 ng/5 µL DNA was combined with following reagents to reach a final concentration of 1X SYBR Green Master Mix and 0.5 µM 157F and 727R primer and water to reach 20 µL and 5 µL, respectively, of diluted sample DNA. Amplification was performed on the MJ Research PTC-2004 thermocylcer using the following regime: 50°C (2 min), 95°C (15 min), 40 cycles of 95°C (30 sec), 60°C (1 min), plate read and 50°C (5 min). The melting curve was determined using the following protocol: heat from 60°C to 90°C, by 0.3°C increments, holding for 5 seconds before reading the fluorescence of the samples. Nested qPCR results were determined by purifying the PCR products using the QIAquick PCR Purification Kit, as per the manufacturer's protocol, and then running the purified samples through qPCR, as described above.

***QA/QC Requirements.*** To determine if and where potential contamination or interference occurred during sample processing, positives and reagent blanks or negatives and matrix spikes of the PCR and qPCR samples were prepared. A positive control consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. A matrix spike consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. Negative controls consisted of water-only blanks for the PCR and qPCR procedure. The qPCR reactions were run in triplicate for each sample to determine the reproducibility of the method.

North Wind

## Results:

The samples arrived at the lab in good condition at 4°C with ice still in the cooler. The samples were filtered in the lab, and the filters were immediately placed in a -80°C freezer and stored until the DNA extraction was performed. Following DNA extraction, the samples were first subjected to polymerase chain reaction (PCR) using universal bacterial probes in order to verify amplifiable DNA was present in the sample. In addition, for the 16S rRNA gene, a "nested" qPCR approach was applied in which the universal bacterial PCR-amplified DNA is used as the template in a qPCR reaction. Although the results from the nested qPCR cannot be quantified per se, they can be used to lower the detect limit for the qPCR in order to determine if the poultry-specific *Brevibacteria* biomarker gene is present at concentrations lower than the method detect limit (MDL) using the groundwater DNA extractions. The results of these studies are presented in Table 2. The DNA extraction negative control and all PCR negative controls did not amplify any product. In addition, all calibration control checks were within acceptable values.

Sample LAL16C2-7-18-06 results were reported previously (September 17, 2007). This sample was inhibited in the prior analysis as reported in September. This sample was sepharose cleaned an additional time and reanalyzed per the standard operating procedure (SOP). All inhibition was removed in the second cleanup and the *Brevibacteria* biomarker was detected in the qPCR analysis. The *Brevibacteria* biomarker was identified in 81% of the samples and was quantifiable in 39% of the samples analyzed.

**Table 2.** Results of Poultry Specific Biomarker analyses for samples.

| Sample ID | Matrix | DNA (ng/L or ng/g) | qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)[a] | | | qPCR Matrix Spike Amplified?[b] | Nested qPCR Amplified?[c] | Biomarker Melt Peak Identified? | Other Melt Peaks Observed? |
|---|---|---|---|---|---|---|---|---|---|
| FAC03-7-6-06 | Litter | 21.3 | 1.03E+09 | ± | 8.00E+07 | Yes | N/A | Yes | No |
| FAC09-8-31-06 | Litter | 170.1 | 7.57E+08 | ± | 1.55E+08 | Yes | N/A | Yes | No |
| FAC02-6-21-06 | Litter | 51.9 | 4.13E+08 | ± | 1.78E+07 | Yes | N/A | Yes | No |
| FAC08-8-15-06 | Litter | 154.0 | 1.47E+09 | ± | 2.25E+08 | Yes | N/A | Yes | No |
| FAC04-7-12-06 | Litter | 6.8 | 1.67E+08 | ± | 2.98E+07 | Yes | N/A | Yes | No |
| FAC010-9-22-06 | Litter | 120.1 | 2.04E+09 | ± | 4.14E+08 | Yes | N/A | Yes | No |
| FAC07-8-3-06 | Litter | 98.1 | 2.49E+09 | ± | 9.54E+07 | Yes | N/A | Yes | No |
| FAC05-7-13-06 | Litter | 76.6 | 1.47E+09 | ± | 1.93E+08 | Yes | N/A | Yes | No |
| FAC06-7-20-06 | Litter | 57.1 | 4.46E+08 | ± | 7.34E+07 | Yes | N/A | Yes | No |
| LAL6A2-6-14-06 | Soil | 10.5 | 1.55E+04 | ± | 2.57E+03 | Yes | N/A | Yes | No |

North Wind

| Sample ID | Matrix | DNA (ng/L or ng/g) | qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)[a] | | | qPCR Matrix Spike Amplified?[b] | Nested qPCR Amplified?[c] | Biomarker Melt Peak Identified? | Other Melt Peaks Observed? |
|---|---|---|---|---|---|---|---|---|---|
| LAL6D2-6-15-06 | Soil | 2.1 | 4.98E+03 | ± | 1.88E+02 | Yes | N/A | Yes | No |
| LAL6B2-6-14-06 | Soil | 12.4 | Present | | | Yes | Yes | Yes | No |
| LAL13C2Q-7-7-06 | Soil | 27.3 | Present | | | Yes | Yes | Yes | No |
| LAL14C2-7-10-06 | Soil | 20.3 | Present | | | Yes | Yes | Yes | No |
| LAL14C2Q-7-10-06 | Soil | 77.3 | Present | | | Yes | Yes | Yes | No |
| LAL13C2-7-7-06 | Soil | 3.4 | Present | | | Yes | Yes | Yes | No |
| LAL16C2-7-18-06 | Soil | 9.5 | 1.42E+04 | ± | 1.97E+03 | Yes | N/A | Yes | No |
| LAL5C2-6-12-06 | Soil | 40.3 | Present | | | Yes | Yes | Yes | No |
| LAL8B2-6-21-06 | Soil | 27.7 | 0.00E+00 | | | Yes | No | N/A | N/A |
| LAL14B2-7-10-06 | Soil | 91.7 | Present | | | Yes | Yes | Yes | No |
| LAL9D2-6-22-06 | Soil | 13.0 | Present | | | Yes | Yes | Yes | No |
| LAL7B2-6-20-06 | Soil | 47.4 | Present | | | Yes | Yes | Yes | No |
| LAL13A2-7-6-06 | Soil | 38.6 | Present | | | Yes | Yes | Yes | No |
| LAL17C2-7-18-06 | Soil | 17.3 | Present | | | Yes | Yes | Yes | No |
| LAL17C2Q-7-18-06 | Soil | 29.9 | Present | | | Yes | Yes | Yes | No |
| LAL14D2-7-10-06 | Soil | 28.1 | Present | | | Yes | Yes | Yes | No |
| LAL8A2-6-19-06 | Soil | 22.3 | 7.00E+03 | ± | 4.43E+02 | Yes | N/A | Yes | No |
| LAL8D2-6-20-06 | Soil | 38.4 | 0.00E+00 | | | Yes | No | N/A | N/A |
| LAL17A2-7-10-06 | Soil | 21.4 | Present | | | Yes | Yes | Yes | No |
| LAL7D2-6-29-06 | Soil | 20.6 | Present | | | Yes | Yes | Yes | No |
| LAL9A2-6-22-06 | Soil | 29.2 | Present | | | Yes | Yes | Yes | No |
| LAL5A2-6-13-06 | Soil | 35.9 | Present | | | Yes | Yes | Yes | No |
| LAL7C2-6-19-06 | Soil | 41.8 | Present | | | Yes | Yes | Yes | No |
| LAL9B2-6-22-06 | Soil | 61.4 | Present | | | Yes | Yes | Yes | No |
| LAL13D2-7-6-06 | Soil | 34.6 | Present | | | Yes | Yes | Yes | No |

North Wind

| Sample ID | Matrix | DNA (ng/L or ng/g) | qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)[a] | | | qPCR Matrix Spike Amplified?[b] | Nested qPCR Amplified?[c] | Biomarker Melt Peak Identified? | Other Melt Peaks Observed? |
|---|---|---|---|---|---|---|---|---|---|
| LAL7A2-6-20-06 | Soil | 31.7 | Present | | | Yes | Yes | Yes | No |
| LAL16D2-7-18-06 | Soil | 36.4 | Present | | | Yes | Yes | Yes | No |
| LAL5B2-6-12-06 | Soil | 27.3 | Present | | | Yes | Yes | Yes | No |
| LAL12A2Q-7-6-06 | Soil | 26.8 | Present | | | Yes | Yes | Yes | No |
| LAL12D2-7-7-06 | Soil | 51.4 | Present | | | Yes | Yes | Yes | No |
| LAL16-SP2-7-18-06 | Water | -1.0 | 0.00E+00 | | | Yes | No | N/A | N/A |
| EOF-SPREAD-010-5-9-06 | Water | 1.7 | 1.05E+07 | ± | 1.70E+06 | Yes | N/A | Yes | No |
| EOF-SPREAD-17A-01-5-1-06 | Water | 72.5 | 2.48E+06 | ± | 4.71E+05 | Yes | N/A | Yes | Yes |
| EOF-SPREAD-023-6-18-06 | Water | 4.3 | 1.11E+05 | ± | 2.49E+03 | Yes | N/A | Yes | No |
| EOF-SPREAD-073B-6-18-06 | Water | 133.5 | 5.56E+07 | ± | 5.25E+06 | Yes | N/A | Yes | No |
| EOF-SPREAD-064-5-4-06 | Water | 79.2 | 1.89E+06 | ± | 7.63E+04 | Yes | N/A | Yes | No |
| EOF-SPREAD-53E-01-4-29-06 | Water | 57.7 | 5.45E+07 | ± | 4.80E+06 | Yes | N/A | Yes | No |
| EOF-SPREAD-60-01-4-29-06 | Water | 431.4 | 3.90E+07 | ± | 8.26E+06 | Yes | N/A | Yes | No |
| SPREAD-023-4-25-06 | Water | 194.2 | 1.25E+06 | ± | 2.35E+05 | Yes | N/A | Yes | No |
| EOF-1-6-17-06 | Water | 2.5 | 1.15E+05 | ± | 1.80E+04 | Yes | N/A | Yes | No |
| EOF-SPREAD-053G-5-4-06 | Water | 14.5 | 0.00E+00 | | | Yes | No | N/A | N/A |
| EOF-SPREAD-048-5-9-06 | Water | 25.2 | 0.00E+00 | | | Yes | No | N/A | N/A |
| SPREAD-029-4-25-06 | Water | 56.1 | Present | | | Yes | Yes | Yes | No |
| SPREAD-036-4-25-06 | Water | 64.9 | 1.48E+05 | ± | 4.04E+04 | Yes | N/A | Yes | No |
| EOF-SPREAD-071-5-9-06 | Water | 5.2 | 3.63E+04 | ± | 8.25E+03 | Yes | N/A | Yes | No |
| EOF-SPREAD-065-5-4-06 | Water | 13.3 | 3.45E+04 | ± | 1.64E+03 | Yes | N/A | Yes | No |
| EOF-Q2-6-17-06 | Water | 2.0 | 0.00E+00 | | | Yes | No | N/A | N/A |
| EOF-26-6-8-05 | Water | 14.3 | 0.00E+00 | | | Yes | No | N/A | N/A |
| EOF-17-6-8-05 | Water | 22.7 | Present | | | Yes | Yes | Yes | No |
| EOF-222-4-13-07 | Water | 81.3 | 1.32E+05 | ± | 2.71E+04 | Yes | N/A | Yes | No |
| GPGW-18A-6-26-07 | Water | 1.1 | 0.00E+00 | | | Yes | No | N/A | N/A |

North Wind

| Sample ID | Matrix | DNA (ng/L or ng/g) | qPCR Poultry Specific Biomarker (copies/µL water or g soil or g litter)[a] | | | qPCR Matrix Spike Amplified?[b] | Nested qPCR Amplified?[c] | Biomarker Melt Peak Identified? | Other Melt Peaks Observed? |
|---|---|---|---|---|---|---|---|---|---|
| GPGW-20-6-11-30-06 | Water | 13.4 | 0.00E+00 | | | Yes | No | N/A | N/A |
| GPGW-48-7-12-1-06 | Water | 2.6 | 0.00E+00 | | | Yes | No | N/A | N/A |
| GPGW-10-4-11-30-06 | Water | 2.9 | Present | | | Yes | Yes | Yes | No |
| GPGW-40-6-27-07 | Water | -1.5 | 0.00E+00 | | | Yes | No | N/A | N/A |
| HFS22-EVENTA-5-10-06 | Water | 4.2 | 0.00E+00 | | | Yes | No | N/A | N/A |
| HFS04-BF2-01-8-1-06 | Water | 0.0 | 0.00E+00 | | | Yes | No | N/A | N/A |

[a] "Present" indicates that the biomarker was amplified, but was not quantifiable.

[b] if "no" indicates that sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration indicative of inhibition.

[c] N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because none was detected in the qPCR sample run.