

ATP Study Report of Modified Colitag™
ATP Case No. D05-0035

September 21, 2009

TECHNICAL SUPPORT CENTER
OFFICE OF GROUND WATER AND DRINKING WATER
U. S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

Background

Introduction:

The information and data supplied here were generated to demonstrate the comparability of the modified version of Colitag™ to the reference methods Standard Methods 9221(SM9221) B and SM9222 G which are EPA-approved method for compliance monitoring of total coliforms and *E. coli* as required under the National Primary Drinking Water Regulations.

Original Colitag™ is for use in accordance with the Code of Federal Regulations at 40 CFR 141.21(f)(3) for total coliforms, and 40 CFR 141.21(f)(6) for *E. coli* and is US EPA approved for compliance monitoring of public water systems as required by the Total Coliform Rule. This document describes the Alternative Test Procedure (ATP) study design and data that were used in the decision process for evaluation of modified Colitag™.

Both the original Colitag and modified Colitag are manufactured by CPI International (CPI).

Data for this study report were generated in accordance with CPI's study plan of November 14, 2007. This study plan was approved by EPA's Office of Ground Water and Drinking Water's Technical Support Center as compliant to the guidance issued in EPA's Microbiological Alternate Test Procedure Protocol for Drinking Water, Ambient Water and Wastewater - version 2004.

Justification

The modified version of Colitag™ was designed to reduce matrix interference through a growth inhibitor for *Aeromonas* and *Pseudomonas* bacteria. The new method was also designed to improve laboratory productivity and convenience with an expanded results reading window (16-48 hours incubation at 35° C). Modified Colitag™ may also decrease laboratory costs though a longer shelf life than the currently approved version.

Method Summary

Modified Colitag™ is a “selective and differential media for the simultaneous determination of the presence or absence or enumeration of total coliforms and *E. coli* in drinking water,” according to CPI. Complete directions for use for the modified Colitag™ are available from CPI International, located at 5580 Skylane Blvd., Santa Rosa, CA, 95403, phone: (800) 878-7654, fax: (707) 545-7901, website: www.cpiinternational.com.

Modified Colitag™ is a one-step, ready-to-use, dehydrated media for analysis of water samples. For presence/absence testing using modified Colitag™, one packet of medium is mixed with a 100-mL sample of water and incubated at 35° C for 16 to 48 hours. Modified Colitag™ must be pre-warmed for incubations of 16- 22 hours. The pre-warming step consists of pre-incubating the samples for 7-10 minutes in a 44.5° ± 0.2° C water bath, after which the rest of the incubation is performed at 35° ±0.5° C.

After the incubation period, if yellow color is observed, total coliforms are deemed present. If blue fluorescence is observed under 365 nm ultraviolet (UV) light, *E. coli* are deemed present.

Modified Colitag™ is based on the detection of two enzymes, β-glucuronidase and β-galactosidase, which are characteristic of *E. coli* and the total coliform group respectively. For detection of β-galactosidase, modified Colitag™ utilize the chromogenic substrate, ortho-nitrophenyl-β-D-galactopyranoside(ONPG). Upon hydrolysis of ONPG by β-galactosidase, a distinctly yellow-colored compound, ortho-nitrophenol, is released indicating the presence of total coliforms. For detection of β-glucuronidase, original and modified Colitag™ utilize the fluorogenic enzyme substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG). Upon hydrolysis of MUG by β-glucuronidase, 4-

methylumbelliferone is released, a compound which fluoresces when exposed to ultraviolet light. The β glucuronidase enzyme and observation of this fluorescence differentiates this organism from other members of the total coliform group.

Study Objectives and Design

Main Objectives

The main objective of the ATP study was to demonstrate the comparability of a modified version of Colitag™ to an EPA approved reference method for compliance monitoring of total coliforms and *E. coli* as required under National Primary Drinking Water Regulations. CPI International, Inc. conducted the ATP study to support their request for nationwide approval from the US EPA for modified Colitag™ to support compliance monitoring.

Data Quality Objectives

CPI's objective for the study was to provide EPA with data compliant to the approved study plan. Specifically, the third-party lab performed replicate side-by-side analyses on the chlorinated spiked drinking water samples, using SM9221 B (presumptive lauryl tryptose broth (LTB) with BGLB confirmation for total coliforms) and SM9222 G (presumptive LTB with EC-MUG confirmation for *E. coli*), to provide a basis of comparison for the modified Colitag™ method.

Study Design

The design for the modified-Colitag ATP study was based on EPA's Microbiological Alternate Test Procedure Protocol for Drinking Water, Ambient Water and Wastewater, version 2004. Ten geographically dispersed waste waters were used as sources of total coliform bacteria and *E. coli* for the experiments. The total coliform bacteria or *E. coli* were enumerated in the samples and exposed to chlorine to cause an approximately 2 to 4 log reduction in bacterial numbers due to chlorine stress. The bacteria were then diluted if necessary so that approximately 1 to 10 bacteria were added to the test media. Ideally, the inoculation of the stressed total coliforms or *E. coli* to the test media would result in half of the replicate cultures being positive for total coliforms or *E. coli* and half negative. This distribution of positive and negative replicate cultures indicates that very low numbers of total coliforms or *E. coli* have been inoculated into the test media. Since it is quite difficult to achieve an inoculation level where exactly half of the replicate cultures are positive and half are negative, the ATP protocol uses a guideline of a 25% to 75% split (positive or negative). The stressed total coliforms or *E. coli* were inoculated into a drinking water sample. For this ATP study two different total coliform and *E. coli* methods were used. Modified Colitag™ and SM9221 B were compared for total coliform measurement; and modified Colitag and SM 9221 F/SM9222G were compared for *E. coli* measurement. Twenty replicate 100 mL cultures of modified Colitag™ and twenty of SM 9221 B were prepared from the drinking water inoculated with stressed total coliforms/*E. coli*. The twenty modified Colitag™ cultures were incubated at 35° C. Each of the modified Colitag™ culture vessels was checked for the presence of total coliforms and *E. coli* at 16, 18, 24 and 48 hours. The twenty replicate SM9221 B cultures were incubated and tested for total coliforms or *E. coli* as specified in Standard Methods for the Examination of Water and Wastewater. Sensitivity was determined by comparing the number of positive modified Colitag™ vessels with the number of positive vessels from the SM9221 B and SM9222 G tests. Specificity was determined by testing at least 100 positive and 100 negative modified cultures for total coliforms and *E. coli* with SM9221 B and SM9222 G, respectively. Statistical tests, sensitivity and specificity were calculated as specified in Section 8 of the 2004 ATP Protocol.

Study Implementation

Study Management

AEMTEK, Inc. was the laboratory responsible for performing the subject study. CPI International coordinated approval of the study plan with EPA, sourcing the sample donors and submission of the study report. AEMTEK Inc, was the sole entity responsible for testing procedures and data generation.

Type of Study

The modified Colitag™ method was compared to Standard Methods 9221 B and 9222 G to provide side-by-side comparison data.

Study Schedule

Collection of data for the information presented in this study report occurred over a period of several months in 2008. For each test run the schedule was dictated by the required steps of the CPI's approved protocol, beginning with sample collection. The first range finding analysis for each sample was performed within 30 hours of collection, with subsequent chlorination and recovery testing performed as quickly as possible. The actual timeline of a test run varied, depending on the results of the chlorination and whether dilutions produced a 25% to 75% split on the first attempt. For test runs where the 25% to 75% split in results was not initially achieved, dilutions were repeated on the original spiked sample, in accordance with EPA's guidance, or the sample was rechlorinated in cases where too many coliforms were eradicated to fulfill the 25% to 75% split.

Matrix and spiking source

The test matrix was finished drinking water, exposed to UV light for 48 hours to produce oxidant-free, reductant-free (OFRF) water samples. The spiking sources were non-chlorinated wastewater samples from wastewater treatment plants. Wastewater samples were acquired from dispersed geographic locations around the United States, according to the guidance of EPA's Microbiological Alternate Test Procedure Protocol for Drinking Water, Ambient Water and Wastewater, Version 2004.

Preliminary testing:

Preliminary testing runs using the approved study plan were initiated in March of 2008, after sourcing AEMTEK as a qualified independent research laboratory. During these trials, wastewater samples were collected from dispersed geographic locations as spiking sources to support the evaluation of modified Colitag™. Two runs were performed to evaluate the sensitivity and specificity of Colitag™ with varying incubation times - 16h, 18h, 24h and 48 hours. Data collected by AEMTEK demonstrated good sensitivity of the method with 18 to 48 hour incubations, but generally less sensitivity at 16h. Given the preliminary results, AEMTEK implemented a 7-10 minute pre-warming step prior to incubation, which notably improved the results at 16 hours. Subsequently, AEMTEK executed complete test runs using the pre-warming step for modified Colitag™ and recorded data for modified Colitag™ after 16h, 18h, 24h, and 48 hours of incubation time.

Numbers and Types of Analyses:

11 samples were collected for use as spiking sources of *E. coli* and total coliforms in drinking water samples for modified Colitag™ comparability testing.

Sample Collection Distribution and Storage:

Samples were collected at donor wastewater facilities using the procedures in CPI's approved study plan, section 1.2. Samples were chilled immediately after collection and shipped packed with frozen gel packs to CPI's independent ATP testing lab by FedEx priority delivery along with a chain of custody form. The temperature was recorded upon receipt to ensure that the sample was between 1-10 °C. The one exception to this was the sample from Oakland, CA, which was locally sourced. This sample was

collected and immediately packed with frozen gel packs and transported to the lab for analyses within 30 minutes of collection. The sample did not yet reach a temperature under 10° C before it arrived at the laboratory, due to the short transport time. Initial range finding analysis for all samples was initiated within 30 hours of collection.

Upon receipt at the lab, samples were immediately transferred to a refrigerator, regulated at 1- 4° C. Refrigeration was monitored daily with a temperature-recording instrument calibrated yearly in reference to a NIST thermometer traceable to NIST and conforming to NIST specifications. The spiking suspension was stored at refrigeration temperatures throughout the experiment except for short periods when being used, such as during the initial chlorination procedures.

Spiking Enumeration:

To determine the initial concentrations of both total coliforms and *E. coli* in the original sewage effluent spiking suspension, MPN analyses were performed in accordance with the recommendations of the Standard Methods for the Examination of Water and Wastewater, 21st Edition, Sections 9221 B and 9222 G, Multiple-Tube Fermentation Technique for Members of the Coliform Group and *E. coli*, respectively. Lauryl tryptose broth (LTB) was used in the presumptive portion of the multiple tube test and brilliant green lactose bile broth and EC-MUG were used for the confirmed total coliform and *E. coli* testing phases, respectively.

Sample and Dilution Waters:

For comparability testing, a single finished drinking water source was used for analyses and was collected from an on-site tap at the lab location. The service line was cleared by maintaining a steady water flow for at least two minutes. The drinking water was dechlorinated through exposure to UV light for 48 hours at ambient temperatures to produce an oxidant-free, reductant-free (OFRF) drinking water sample. The same source of OFRF drinking water was used to make any dilutions needed to reach the required one to ten CFU/100-mL of chlorine-stressed total coliforms and *E. coli*.

Concentrations of total coliforms and *E. coli* were significantly dissimilar for a given wastewater sample, so that separate dilutions were required to acquire one to ten MPN of target organism(s) per 100-mL replicate. On some occasions the post-chlorination range find was not an exact predictor of target microbe responses for the subsequent testing day. In these cases, the 25% to 75% required split in positive/negative responses was not realized. When this occurred the laboratory returned to the original sample as recommended in section 7.2.1.3 of the EPA Microbiological ATP protocol wherever possible. In the event that the sample was over-chlorinated, killing off the total coliform or *E. coli* populations, the laboratory returned to the original sample and performed the chlorination a second time.

Data Reporting and Validation

Quality Assurance/Quality Control:

QA/QC procedures were followed in this study per the study plan, EPA's ATP guidance document, Standard Methods, and the QA/QC guidelines for certified drinking water laboratories.

Control cultures:

For each lot of medium, analytical procedures were checked by testing with known positive and negative control cultures as noted below:

Control	Control strain
<i>E. coli</i>	<i>E. coli</i> ATCC 25922
Total coliform	<i>Klebsiella pneumoniae</i> ATCC 13883 or <i>Citrobacter freundii</i> ATCC 8090
Non-coliform	<i>Salmonella enterica</i> serovar <i>typhimurium</i> or <i>Pseudomonas aeruginosa</i> ATCC 10145

Control Blanks: Control Blanks were used for each test run with both the test and reference methods. Blanks consisted of a 0.1 ml aliquot of the oxidant-free, reductant-free drinking water used as the diluent for the test and MPN procedure.

Results

Total Coliform Data

Total coliform recovery data for the 11 finished drinking water samples spiked with chlorinated spiking suspensions are shown in Table 1 below. Procedures for sample collection, chlorination of spiking suspensions and range finding analyses for log reduction calculations were performed according to the ATP study plan.

Table 1 summarizes the positive total coliform test results for modified ColitagTM incubated for 16, 18, 24 and 48 hours. Positive total coliform results for test replicates incubated for 24 hours in modified ColitagTM and reference method SM9221 B (LTB with BGLB confirmation) are also summarized in Table 1. The summations of positive results are shown at the bottom of each column. Modified ColitagTM produced 101, 115, 126 and 137 positive results for 16h, 18h, 24, and 48h incubations, respectively, compared to 118 positive results for SM9221 B.

Table 1. Total Coliform Positives

Sample	Modified Colitag				SM 9221 B
	16 h	18h	24h	48h	LTB/BGLB
Fort Wayne, IN	6	9	11	12	13
Phoenix, AZ	10	11	13	13	13
Boulder, CO	11	12	13	14	12
Salem, OR	8	9	9	9	9
Oakland, CA	12	13	13	13	14
Vancouver, WA	15	15	15	15	14
Houston, TX	7	8	10	11	12
Queen Ann, MD	5	6	8	11	10
Casselberry, FL	7	8	8	11	5
Henderson, NV	15	15	15	16	10
Tullahoma, TN	5	9	11	12	6
Sum	101	115	126	137	118
% of Total Samples	45.9%	52.3%	57.3%	62.3%	53.6%
% Compared to SM9221 B	85.6%	97.5%	106.8%	116.1%	100.0%

Tables 2 and 3 (Total Coliform Data Summary) summarizes the confirmed positive and negative results for coliforms in modified Colitag™ at 16, 18, 24 and 48 hours. The reference method data are shown at the far right. More than 10 samples were needed to satisfy the minimum requirement for providing at least 400 positive and 400 negative specificity results as described in EPA's ATP guidance document and according to the approved study protocol. Specificity confirmations from 11 sources runs are included.

The summations of positive and negative results are shown at the bottom of each column along with the relative percent of total samples compared to SM9221 B. Modified Colitag™ at 16h, 18h, 24h and 48 hours produced 85.6%, 97.5%, 106.8%, and 116.1% total coliform positives, relative to SM9221 B.

Table 2. Total Coliform Data Summary – Positives

Sample	Modified Colitag 16h	Confirmed true +	Diff. (false+)	Modified Colitag 18h	Confirmed true +	Diff. (false +)	Modified Colitag 24h	Confirmed true +	Diff. (false+)	Modified Colitag 48h	Confirmed true +	Diff. (False+)	SM9221B
Ft. Wayne, IN	6	6	0	9	9	0	11	11	0	12	12	0	13
Phoenix, AZ	10	10	0	11	11	0	13	13	0	13	13	0	13
Boulder, CO	11	11	0	12	12	0	13	13	0	14	14	0	12
Salem, OR	8	8	0	9	9	0	9	9	0	9	9	0	9
Oakland, CA	12	12	0	13	13	0	13	13	0	13	13	0	14
Vancouver, WA	15	15	0	15	15	0	15	15	0	15	15	0	14
Houston, TX	7	7	0	8	8	0	10	10	0	11	11	0	12
Queen Ann, MD	5	5	0	6	6	0	8	8	0	11	11	0	10
Cassleberry, FL	7	7	0	8	8	0	8	8	0	11	10	1	5
Henderson, NV	15	9	6	15	9	6	15	11	4	16	12	4	10
Tullahoma, TN	5	5	0	9	8	1	11	10	1	12	10	2	6

Sums 101 95 6 115 108 7 126 121 5 137 130 7 118

% of total

Samples 45.9% 52.3% 57.3% 62.3% 53.6%

Compared to

SM9221B 85.6% 97.5% 106.8% 116.1% 100.0%

Table 3. Total Coliform Data Summary – Negatives

Sample	Modified Colitag 16h	Confirmed true - 16h	Diff. (false-) 16h	Modified Colitag 18h	Confirmed true - 18h	Diff. (false -) 18h	Modified Colitag 24h	Confirmed true - 24h	Diff. (false-) 24h	Modified Colitag 48h	Confirmed true - 48h	Diff. (False-) 48h
Ft. Wayne, IN	14	13	1	11	10	1	9	9	0	8	8	0
Phoenix, AZ	10	9	1	9	7	2	7	6	1	7	5	2
Boulder, CO	9	9	0	8	8	0	7	7	0	6	6	0
Salem, OR	12	11	1	11	11	0	11	11	0	11	11	0
Oakland, CA	8	6	2	7	7	0	7	7	0	7	7	0
Vancouver, WA	5	5	0	5	5	0	5	5	0	5	5	0
Houston, TX	13	12	1	12	10	2	10	9	1	9	9	0
Queen Ann, MD	15	14	1	14	12	2	12	9	3	9	9	0
Cassleberry, FL	13	13	0	12	12	0	12	12	0	9	9	0
Henderson, NV	5	5	0	5	5	0	5	5	0	4	4	0
Tullahoma, TN	15	14	1	11	10	1	9	9	0	8	8	0
Sums	119	111	8	105	97	8	94	89	5	83	81	2

Table 4. Calculations for total coliform sensitivity, specificity, false-positive and false-negative rates are shown at the bottom of Table 4 Coliform Data Summary . Modified Colitag™ demonstrated a sensitivity rate of 92.2%, 93.1%, 96.0% and 98.5%, relative to SM9221 B. Modified Colitag™ demonstrated a specificity rate of 94.9%, 93.3%, 94.7% and 92.0%, relative to SM9221 B. Modified Colitag™ demonstrated a false-negative rate of 7.8%, 6.9%, 4.0% and 1.5% relative to SM9221 B. Modified Colitag™ demonstrated a false-positive rate of 5.1%, 6.7%, 5.3% and 8.0%, relative to SM9221 B. Overall agreement was 94.5% (see formula for overall agreement in Table 4).

Table 4. Total Coliform Data Summary -Confirmations

Incubation Time (hrs)	16	18	24	48	Combined
Sensitivity	92.2%	93.1%	96.0%	98.5%	95.2%
Specificity	94.9%	93.3%	94.7%	92.0%	93.8%
False +	5.1%	6.7%	5.3%	8.0%	6.2%
False -	7.8%	6.9%	4.0%	1.5%	4.8%

Overall agreement- 94.4%

Modified Colitag Data Combined Over All Time Points

Sample type	Verified +	Verified -	Total
Positive samples	454	25	479
Negative samples	23	378	401
Totals	477	403	880

Calculations

Sensitivity= $[\text{TP}/(\text{TP} + \text{FN})] * 100\%$
 Specificity= $[\text{TN}/(\text{TN} + \text{FP})] * 100\%$
 False + = 1- Specificity
 False - = 1- Sensitivity
 Overall Agreement = $[(\text{TP} + \text{TN})/\text{TS}] * 100\%$

TP= true positives
 TN= true negative
 TS= total samples
 FP= false positives
 FN= false negatives

***E. coli* Data**

Table 5 (*E. coli* Positives) summarizes the positive *E. coli* test results for Colitag™ incubated for 16, 18, 24 and 48 hours according to the manufacturer's directions. SM9222 G (EC-MUG confirmation of positives transferred from LTB) results are summarized in Table 5. The summations of positive results are shown at the bottom of each column. Modified Colitag™ produced 101, 114, 121 and 133 positive results for 16h, 18h, 24, and 48h incubations, respectively, compared to 112 positive results for SM9222G.

Table 5. *E. coli* Positives

Sample	Modified Colitag 16 h	Modified Colitag 18 h	Modified Colitag 24 h	Modified Colitag 48 h	SM9222 G EC-MUG
Ft. Wayne, IN	5	6	6	9	7
Phoenix, AZ	10	15	15	16	12
Boulder, CO	9	10	12	12	10
Salem, OR	14	14	14	14	12
Oakland, CA	8	9	9	9	8
Vancouver, WA	10	11	12	13	11
Houston, TX	10	11	13	15	10
Queen Ann, MD	10	11	12	15	11
Cassleberry, FL	7	8	8	8	6
Henderson, NV	9	10	10	10	14
Tullahoma, TN	9	9	10	12	11

Sums	101	114	121	133	112
% of Total Samples	45.9%	51.8%	55.0%	60.5%	50.9%
Compared to SM9222 G	90.2%	101.8%	108.0%	118.8%	100.0%

Tables 6 and 7. (*E. coli* Data Summary)- These tables summarize the confirmed positive and negative results for *E. coli* in modified ColitagTM at 16, 18, 24 and 48 hours. The reference method SM9222 G data is shown at the far right. More than 10 samples were needed to provide at least 400 positive and 400 negative specificity results, per EPA's ATP guidance document and according to the approved study protocol. Specificity confirmations from 11 sources are included. The summations of positive and negative results are shown at the bottom of each column of Tables 6 and 7 along with the relative percent of total samples compared to SM9222 G. Modified ColitagTM at 16h, 18h, 24h and 48 hours produced 90.2%, 101.8%, 108.0%, and 118.8% of *E. coli* positives, relatively, compared to SM9222 G.

Table 6. *E. coli* Summary- Positives

Sample	Modified Confirmed Diff.			Modified Confirmed Diff.			Modified Confirmed Diff.			Modified Confirmed Diff.			SM9222 G
	Colitag 16h	true +	(false+)	Colitag 18h	true +	(false+)	Colitag 24h	true +	(false+)	Colitag 48h	true +	(False+)	
Ft. Wayne, IN	5	4	1	6	3	3	6	6	0	9	6	3	7
Phoenix, AZ	10	10	0	15	14	1	15	14	1	16	14	2	12
Boulder, CO	9	9	0	10	10	0	12	12	0	12	12	0	10
Salem, OR	14	14	0	14	14	0	14	14	0	14	14	0	12
Oakland, CA	8	8	0	9	9	0	9	9	0	9	9	0	8
Vancouver, WA	10	10	0	11	11	0	12	12	0	13	13	0	11
Houston, TX	10	10	0	11	11	0	13	13	0	15	15	0	10
Queen Ann, MD	10	10	0	11	11	0	12	12	0	15	14	1	11
Cassleberry, FL	7	7	0	8	8	0	8	8	0	8	8	0	6
Henderson, NV	9	9		10	10	0	10	10	0	10	10	0	14
Tullahoma, TN	9	8	1	9	8	1	10	10	0	12	10	2	11

Sums 101 99 2 114 109 5 121 120 1 133 125 8 112

% of total
 Samples 45.9% 51.8% 55.0% 60.5% 50.9%

Compared
 to
 SM9222 G 90.2% 101.8% 108.0% 118.8% 100.0%

Table 7. *E. coli* Summary- Negatives

Sample	Modified Colitag 16h			Confirmed true + (false+)			Diff. Colitag 18h			Confirmed true + (false +)			Diff. Colitag 24h			Confirmed true + (False+)			Diff. Colitag 48h		
	Colitag	true +	(false+)	Colitag	true +	(false +)	Colitag	true +	(false+)	Colitag	true +	(False+)	Colitag	true +	(False+)	Colitag	true +	(False+)			
Ft. Wayne, IN	15	14	1	14	12	2	14	13	1	11	10	1									
Phoenix, AZ	10	9	1	5	5	0	5	5	0	4	4	0									
Boulder, CO	11	10	1	10	9	1	8	8	0	8	8	0									
Salem, OR	6	6	0	6	6	0	6	6	0	6	6	0									
Oakland, CA	12	11	1	11	11	0	11	11	0	11	11	0									
Vancouver, WA	10	10	0	9	9	0	8	8	0	7	7	0									
Houston, TX	10	10	0	9	7	2	7	6	1	5	5	0									
Queen Ann, MD	10	9	1	9	7	2	8	7	1	5	5	0									
Cassleberry, FL	13	12	1	12	12	0	12	12	0	12	12	0									
Henderson, NV	11	11	0	10	10	0	10	9	1	10	8	2									
Tullahoma, TN	11	11	0	11	11	0	10	10	0	8	8	0									
Sums	119	113	6	106	99	7	99	95	4	87	84	3									

Table 8. Calculations for *E. coli* sensitivity, specificity, false-positive and false-negative rates are shown in Table 8 (*E. coli* Data Summary – Confirmations). Modified Colitag™ demonstrated a sensitivity rate of 94.3%, 94.0%, 96.8%, and 97.7% relatively, compared to SM9222 G. Modified Colitag™ demonstrated a specificity rate of 98.3%, 95.2%, 99.0% and 91.3%, relatively, compared to SM9222 G. Modified Colitag™ demonstrated a false-negative rate of 5.7%, 6.0%, 3.2% and 2.3%, relatively, compared to SM9222 G. Modified Colitag™ demonstrated a false-positive rate of 1.7%, 4.8%, 1.0 and 8.7% relatively, compared to SM9222 G. Overall agreement was 95.9%.

Table 8. *E. coli* Data Summary- Confirmations

Incubation Time (hrs)	16	18	24	48	Combined
Sensitivity	94.3%	94.0%	96.8%	97.7%	95.8%
Specificity	98.3%	95.2%	99.0%	91.3%	96.1%
False +	1.7%	4.8%	1.0%	8.7%	3.9%
False -	5.7%	6.0%	3.2%	2.3%	4.2%

Overall agreement- 95.9%

Modified Colitag Data Combined Over All Time Points

Sample type	Verified +	Verified -	Total
Positive samples	453	16	469
Negative samples	20	391	411
Totals	473	407	880

Calculations

Sensitivity= $[\text{TP}/(\text{TP} + \text{FN})] * 100\%$
Specificity= $\text{TN}/(\text{TN} + \text{FP}) * 100\%$
False + = 1- Specificity
False - = 1- Sensitivity
Overall Agreement = $[(\text{TP} + \text{TN})/\text{TS}] * 100\%$

TP= true positives
TN= true negative
TS= total samples
FP= false positives
FN= false negatives

Data Analysis and Discussion

Comparability data for 200 replicate analyses at each incubation period (16, 18, 24 and 48 hours) using modified ColitagTM were generated for both total coliforms and *E. coli*. For the analyses, 11 wastewater samples were collected from geographically diverse locations across the United States and used as spiking samples for the finished drinking water analyses. Chlorination procedures of spiking sources, comparability testing, confirmatory tests and other analyses were performed according to CPI's approved study plan of November 14, 2007.

Total Coliform Results Positives

The proposed method produced a total of 101, 115, 126 and 137 positive total coliform results, compared to 118 positives for the reference method SM9221B (LTB/BGLB). Results are summarized in Table 1. If one assumes the reference method SM9221B to detect 100% of the total coliforms, the proposed method detected 85.6%, 97.5%, 106.8% and 116.1% of total coliforms, for the incubation times of 16h, 18h, 24h and 48 h, respectively.

Total Coliform Results Negatives

The proposed method produced a total of 119, 105, 94, and 83 negative total coliform results compared to 102 negatives for the reference method SM9221B (LTB/BGLB). Results are summarized in Table 7. Confirmations: The frequency of confirmed positive and negative total coliform results for each sample and incubation time for modified ColitagTM are shown in Table 2. Summations of total positive and negative results as well as confirmed positive and negative results are shown. Modified ColitagTM demonstrated high specificity for the detection of total coliforms using the reference method SM9221 B (LTB/BGLB). Specificity rates for the proposed method at 16h, 18h, 24h and 48h were 94.9%, 93.3%, 94.7% and 92.0%,

Statistical Analyses

AEMTEK performed the Chi Squared test for independence both separately for each run and over all the samples; comparing the proposed method for each incubation time period against the Standard Reference Method of 9221 B. Using the chi squared test, the method should be independent of the presence/absence test result if it is similar to the reference method. When analyzed separately, 10 of the 11 sets of replicate data demonstrated independence when comparing modified ColitagTM at 16h incubation to SM9221 B for the detection of total coliforms. At 18h and 24h, all 11 samples demonstrated independence for each incubation time period. At 48 hours, ten of eleven samples demonstrated independence. Data are summarized in Tables 9 through 12.

With regard to sample ID #4 that did not demonstrate independence at 16 hours, the additive chi squared test can increase the power of the statistical analyses to examine the significance of the outlying chi squared value, taking into account all the data, The additive chi squared test calculation is shown in the table 9 below as additive X^2 .

The additive chi squared test, with an additive X^2 value of 15.044 with a critical X^2 of 19.68 with 11 degrees of freedom demonstrates that the proposed method is similar in performance to the reference method.

Table 9. Total coliforms- 16 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9221 B		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	6	14	13	7	4.912	0.027
Phoenix, AZ	10	10	13	7	0.921	0.337
Boulder, CO	11	9	12	8	0.102	0.749
Salem, OR	8	12	9	11	0.102	0.749
Oakland, CA	12	8	14	6	0.440	0.507
Vancouver, WA	15	5	14	6	0.125	0.723
Houston, TX	7	13	12	8	2.506	0.113
Queen Ann, MD	5	15	10	10	2.667	0.102
Cassleberry, FL	7	13	5	15	0.476	0.490
Henderson, NV	15	5	10	10	2.667	0.102
Tullahoma, TN	5	15	6	14	0.125	0.723
Totals	101	119	118	102	2.627	0.105
Additive χ^2					15.044	0.1805

Table 10. Total coliforms- 18 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9221B		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	9	11	13	7	1.616	0.204
Phoenix, AZ	11	9	13	7	0.417	0.519
Boulder, CO	12	8	12	8	0.000	1.000
Salem, OR	9	11	9	11	0.000	1.000
Oakland, CA	13	7	14	6	0.114	0.736
Vancouver, WA	15	5	14	6	0.125	0.723
Houston, TX	8	12	12	8	1.600	0.206
Queen Ann, MD	6	14	10	10	1.667	0.197
Cassleberry, FL	8	12	5	15	1.026	0.311
Henderson, NV	15	5	10	10	2.667	0.102
Tullahoma, TN	9	11	6	14	0.960	0.327
Totals	115	105	118	102	0.082	0.774
Additive χ^2					10.191	0.5133

Table 11. Total coliforms- 24 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
 df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9221B		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	11	9	13	7	0.417	0.519
Phoenix, AZ	13	7	13	7	0.000	1.000
Boulder, CO	13	7	12	8	0.107	0.744
Salem, OR	9	11	9	11	0.000	1.000
Oakland, CA	13	7	14	6	0.114	0.736
Vancouver, WA	15	5	14	6	0.125	0.723
Houston, TX	10	10	12	8	0.404	0.525
Queen Ann, MD	8	12	10	10	0.404	0.525
Cassleberry, FL	8	12	5	15	1.026	0.311
Henderson, NV	15	5	10	10	2.667	0.102
Tullahoma, TN	11	9	6	14	2.558	0.110
Totals	126	94	118	102	0.589	0.443
Additive χ^2					7.821	0.7292

Table 12. Total coliforms- 48 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
 df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9221B		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	12	8	13	7	0.107	0.744
Phoenix, AZ	13	7	13	7	0.000	1.000
Boulder, CO	14	6	12	8	0.440	0.507
Salem, OR	9	11	9	11	0.000	1.000
Oakland, CA	13	7	14	6	0.114	0.736
Vancouver, WA	15	5	14	6	0.125	0.723
Houston, TX	11	9	12	8	0.102	0.749
Queen Ann, MD	11	9	10	10	0.100	0.752
Cassleberry, FL	11	9	5	15	3.750	0.053
Henderson, NV	16	4	10	10	3.956	0.047
Tullahoma, TN	12	8	6	14	3.636	0.057
Totals	137	83	118	102	3.367	0.067
Additive χ^2					12.331	0.3393

***E. coli* Results Positives:**

The proposed method produced a total of 101, 114, 121 and 133 positive *E. coli* results compared to 112 positives for the reference method of SM9221 B (LTB/BGLB). Results are summarized in Table 6. The proposed method detected 90.2%, 101.8%, 108.0% and 118.8% of *E. coli*, relative to the reference method of SM9221 B, for the incubation times of 16h, 18h, 24h and 48h, respectively.

***E. coli* Results Negatives:**

The proposed method produced a total of 119, 106, 99, and 87 negative *E. coli* results compared to 108 negatives for the reference method SM9221 B (LTB/BGLB). Results are summarized in Table 7 along with the sums for negative results.

Confirmations:

The frequency of confirmed positive and negative results for each sample and incubation time for modified ColitagTM are shown in Tables 6 and 7. Summations of total positive and negative results as well as confirmed positive and negative results are shown. Modified ColitagTM demonstrated high specificity for the detection of *E. coli* using the confirmatory method of SM9222 G (EC-MUG). Specificity rates for the proposed method at 16h, 18h, 24h and 48h were 98.3%, 95.2%, 99.0% and 91.3%.

Statistical Analyses:

AEMTEK performed the chi square test for independence both separately for each run and over all the samples, comparing the proposed method for each incubation time period against the reference method of SM9222 G. When analyzed separately, all eleven sets of replicate data, 16h, 18h, 24h, and 48h of incubation demonstrated independence when comparing modified ColitagTM to SM9222 G for the detection of *E. coli*. Data are summarized in Tables 13 through 16.

Table 13. *E. coli* - 16 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag™		SM9222 G		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	5	15	7	13	0.476	0.490
Phoenix, AZ	10	10	12	8	0.404	0.525
Boulder, CO	9	11	10	10	0.100	0.752
Salem, OR	14	6	12	8	0.440	0.507
Oakland, CA	8	12	8	12	0.000	1.000
Vancouver, WA	10	10	11	9	0.100	0.752
Houston, TX	10	10	10	10	0.000	1.000
Queen Ann, MD	10	10	11	9	0.100	0.752
Cassleberry, FL	7	13	6	14	0.114	0.736
Henderson, NV	9	11	14	6	2.558	0.110
Tullahoma, TN	9	11	11	9	0.400	0.527
Totals	101	119	112	108	1.101	0.294
Additive χ^2					4.692	0.9452

Table 14. *E. coli* - 18 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag™		SM9222 G		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	6	14	7	13	0.114	0.736
Phoenix, AZ	15	5	12	8	1.026	0.311
Boulder, CO	10	10	10	10	0.000	1.000
Salem, OR	14	6	12	8	0.440	0.507
Oakland, CA	9	11	8	12	0.102	0.749
Vancouver, WA	11	9	11	9	0.000	1.000
Houston, TX	11	9	10	10	0.100	0.752
Queen Ann, MD	11	9	11	9	0.000	1.000
Cassleberry, FL	8	12	6	14	0.440	0.507
Henderson, NV	10	10	14	6	1.667	0.197
Tullahoma, TN	9	11	11	9	0.440	0.527
Totals	114	106	112	108	0.036	0.849
Additive χ^2					4.288	0.9607

Table 15. *E. coli* - 24 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9222 G		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	6	14	7	13	0.114	0.736
Phoenix, AZ	15	5	12	8	0.026	0.311
Boulder, CO	12	8	10	10	0.404	0.525
Salem, OR	14	6	12	8	0.440	0.507
Oakland, CA	9	11	8	12	0.102	0.749
Vancouver, WA	12	8	11	9	0.102	0.749
Houston, TX	13	7	10	10	0.921	0.337
Queen Ann, MD	12	8	11	9	0.102	0.749
Cassleberry, FL	8	12	6	14	0.440	0.507
Henderson, NV	10	10	14	6	1.667	0.197
Tullahoma, TN	10	10	11	9	0.100	0.752
Totals	111	89	101	99	1.004	0.316
Additive χ^2					5.417	0.9093

Table 16. *E. coli* - 48 hours

Chi Squared Test for Independence- Critical Limits: df=1, $\chi^2 = 3.84$, p=0.05
df= 11, $\chi^2 = 19.68$, p=0.05

Sample	Modified Colitag TM		SM9222 G		Sources= 11	
	+	-	+	-	Chi Square	p-value
Ft. Wayne, IN	9	11	7	13	0.417	0.519
Phoenix, AZ	16	4	12	8	1.905	0.168
Boulder, CO	12	8	10	10	0.404	0.525
Salem, OR	14	6	12	8	0.440	0.507
Oakland, CA	9	11	8	12	0.102	0.749
Vancouver, WA	13	7	11	9	0.417	0.519
Houston, TX	15	5	10	10	2.667	0.102
Queen Ann, MD	15	5	11	9	1.758	0.185
Cassleberry, FL	8	12	6	14	0.440	0.507
Henderson, NV	10	10	14	6	1.667	0.197
Tullahoma, TN	12	8	11	9	0.102	0.749
Totals	133	87	112	108	4.062	0.044
Additive χ^2					10.317	0.5021

Conclusions

The results presented in this summary report show that modified Colitag™ demonstrates appropriate specificity and sensitivity in detecting low levels of highly injured total coliforms and *E. coli* in drinking water. When compared to EPA approved SM9221 B and SM9222 G, modified Colitag™ demonstrates similar sensitivity and specificity.. Chi-squared analyses to analyze independence between the proposed method and the Agency approved reference methods support these conclusions, with additional confirmation provided by the additive chi squared calculations.