Modified Colitag™ Test Method for the Simultaneous Detection of *E. coli* and other Total Coliforms in Water

(ATP D05-0035)

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Modified Colitag™ Test Detection of *E. coli* and other Total Coliforms in Water as required in National Primary Drinking Water Regulations

1.0 Scope and Application

1.1 Modified Colitag™ is a selective and differential medium for the simultaneous determination of the presence or absence or enumeration of *E. coli* and other total coliforms in water.[1,2,3] Modified Colitag™ is for use in the Environmental Protection Agency's survey and monitoring programs under the Safe Drinking Water Act. This method is for use in accordance with the National Primary Drinking Water Regulations at 40 CFR Part 141.[4]

1.2. This method allows for the detection of coliform bacteria and/or *E. coli* from 16 - 48 hours and does not require further confirmation or verification steps.

1.3 Modified Colitag™ is US EPA approved for compliance monitoring of public water systems as required by the Total Coliform Rule. This method can be used as a Presence/Absence test or for quantification of bacteria in a most probable number format (MPN) format.

1.4 Modified Colitag™ is capable of detecting 1 colony forming unit (CFU) of chlorine-injured *E. coli* or other coliform bacteria per 100-mL of water sample. There is no upper limit of detection in the MPN format as dilutions are used to detect and enumerate bacteria.

2.0 Summary of Method

2.1 Modified Colitag™ is a one-step, ready-to-use, dehydrated medium for analysis of water samples. For presence/absence testing, one packet of medium is mixed with a 100-mL sample of water and incubated at 35±0.5°C for 16 to 48 hours. If results are intended to be read before 22 hours incubation, samples must be pre-warmed for 7-10 minutes in a 44.5 ±0.2°C water bath. If yellow color is observed, coliforms are present. If blue fluorescence is observed under 365 nm ultraviolet (UV) light, *E. coli* are present. Complete directions for use are available from CPI International, 5580 Skylane Blvd., Santa Rosa, CA, 95403, (800) 878-7654, http://www.colitag.com/colitag-instructions.pdf.

2.2 For enumeration of *E. coli* and other coliforms, Modified Colitag™ is suitable in a variety of MPN formats. In one format, a 100-mL of water sample would be mixed with one packet of Modified Colitag™ and divided into ten 10-mL volumes before incubation. Based on the number of positive tubes, an MPN value for this number of tubes can be looked up in Table 9221:III of Section 9221C Estimation of Bacterial Density in the Standard Methods for the Examination of Water and Wastewater.[5] Other most probable number formats suitable with Modified Colitag™ can be found in the same reference and further information is available from CPI International, 5580 Skylane Blvd., Santa Rosa, CA, 95403, (800) 878-7654, www.colitag.com.

2.3 Modified Colitag™ is based on the detection of two enzymes, ß-glucuronidase and ß-galactosidase, which are characteristic of *E. coli* and the coliform group respectively.[5,6] For detection of ß-galactosidase, which is an enzyme indicative of the coliform group, Modified Colitag™ utilizes the chromogenic substrate, ortho-nitrophenyl-ß-D-galactopyranoside (ONPG). Upon hydrolysis of ONPG by ß-galactosidase, a distinctly yellow-colored compound, ortho-nitrophenol, is released indicating the presence of coliforms. For detection of ß-glucuronidase, which is the enzyme specific to *E. coli*, Modified Colitag™ utilizes the fluorogenic enzyme substrate, 4-methylumbelliferyl-ß-D-glucuronide (MUG). Upon hydrolysis of MUG by ß-glucuronidase, 4-methylumbelliferone is released, a compound which fluoresces when exposed to ultraviolet light. The ß-glucuronidase enzyme is specific to *E. coli* and observation of this fluorescence differentiates this organism from other members of the coliform group.

2.4 Quality of the detection process is assured through resuscitative components in the medium that promote recovery of chlorine-injured *E. coli* and other coliforms.

3.0 Definitions

3.1 The definition and purposes below are specific to this method but conform to common usage as much as possible.

Hydrolyzable Substrate: A chemical substrate (i.e. ONPG, MUG) that is capable of being hydrolyzed by bacteria such as coliforms and *E. coli*.

Chromogenic Enzyme Substrate: A substrate which can be hydrolyzed by an enzyme releasing a colored (or chromogenic) compound.

Fluorogenic Enzyme Substrate: A substrate which can be hydrolyzed by an enzyme releasing a fluorescent compound.

Ortho-nitrophenyl-ß-D-galactopyranoside (ONPG): A chemical substrate which is hydrolyzed by ß-D-galactosidase activity in coliforms. When a drinking water sample containing coliform bacteria is added to Modified Colitag™ and subjected to incubation, enzymatic hydrolysis of ONPG causes production of ortho-nitrophenol, a yellow colored compound indicating the presence of coliforms.

4-Methylumbelliferyl-ß-D-glucuronide (MUG): A chemical substrate which is hydrolyzed by ß-glucuronidase activity in *E. coli*. When a drinking water sample containing *E. coli* bacteria is added to Modified Colitag™ and subjected to incubation, enzymatic hydrolysis causes production of
4-methylumbelliferon, which is a compound that fluoresces when exposed to long wavelength ultra violet light (365 nm). Fluorescence by 4-methylumbelliferone indicates the presence of *E. coli*.

Proton Gradient Resuscitation: Modified Colitag™ utilizes a patented method of resuscitating chlorine-injured bacteria, whereby a slightly acidic medium allows injured coliforms to more easily maintain a proton gradient across the cell membrane[3].

Coliform bacteria: Bacteria, which hydrolyze ONPG at 35±0.5 degrees C after 24 ± 2 h of incubation.

***E. coli*:** Bacteria, which hydrolyze MUG at 35±0.5 degrees C after 24 ± 2 h of incubation.

4.0 Interferences

4.1 **Chemical/Physical:** There are no known chemical interferences that would be found in drinking water or source water that would inhibit or interfere with the development of color or the production of fluorescence when one uses Modified Colitag™ medium. If a water sample has background color prior to analyses, a control blank (same water sample not inoculated with Modified Colitag™ medium) should be run and used as the control blank.

4.2 **Biological:** Heterotrophic bacteria greater than 10^4/ml can yield positive coliform reactions[7].

5.0 Safety

5.1 None of the components used in Modified Colitag™ are listed as a carcinogen or suspected carcinogen. Reference should be made to the

Material Safety Data Sheet provided with the product for specific information.

5.2 The analyst should follow the recommended safety guidelines described in *Standard Methods for the Examination of Water and Waste Water* (American Public Health Association 2005), 21st edition, section 1090).[8]

5.3 When working with Modified Colitag™ it is important to know and practice normal safety procedures for working in a microbiology laboratory. Routine biosafety procedures should be followed when handling this medium and related samples.

6.0 Instrumentation, Equipment and Supplies

6.1 Incubator or water bath set to 35±0.5°C

6.2 Circulating water bath set to 44.5 ±0.2°C

6.3 Long wavelength 6-watt UV light (365 nanometers)

6.4 Non-fluorescing sterile glass or plastic sample collection vessels (120-mL or larger bottles or bags) with thiosulfate. All glassware and plastic vessels used for determining the presence of coliform bacteria or *E. coli* should be handled according to recommended guidelines.[5] All glassware and containers should be washed with a suitable laboratory detergent, rinsed thoroughly with tap water followed by rinsing with reagent water[9] and sterilized by autoclaving at 121°C for 15 minutes before use. Certified sterile plastic disposable coliform sample vessels with 100-mL fill line, thiosulfate tablet, and tamper seals are available through a variety of commercial suppliers and CPI International. Sterile Coli-Test bags are available through Nasco or CPI International.

7.0 Reagents and Standards

7.1 Modified Colitag™ product for P/A or MPN testing is provided as ready-to-use, pre-measured dehydrated media. One packet dissolves directly into a 100-mL sample for presence/absence testing or is dissolved with 100-mL sterile reagent water to make 100-mL of single strength medium. The medium is stable when stored at 4 to 30 degrees Centigrade away from light. The expiration date and lot number are indicated on each unit. Modified Colitag™ dehydrated medium has a shelf life of at least 18 months from the date of manufacture under proper storage conditions.

7.2 Sodium thiosulfate ($Na_2S_2O_3$) is used to dechlorinate drinking water samples.

7.3 A reagent blank (one Modified Colitag™ test packet mixed with 100-mL of sterile distilled water) may be incubated along with the test samples as a negative control standard. A Modified Colitag™ color comparator may be used as a positive control standard. If the sample has yellow color equal or greater than the comparator, coliforms are present. If the fluorescence of the sample is equal or greater than the fluorescence of the comparator then *E. coli* are present in the sample.

8.0 Sample Collection, Dechlorination, Preservation, Shipment and Storage

8.1 Water Sample Collection: Sampling procedures are described in the USEPA Manual for the Certification of Laboratories Analyzing Drinking Water and the Standard Methods for the Examination of Water and Wastewater, part 9000[5].

8.1.1 Water samples should be collected in sterile, plastic or glass leakproof containers and sufficient sodium thiosulfate to neutralize chlorine at the time of collection. For drinking water, 0.1-mL of a 3% solution of $Na_2S_2O_3$ in a 120-mL bottle will neutralize up to 5 mg/L of residual chlorine.

8.1.2 Storage temperature and handling conditions: Samples should be tested as soon as possible after collection and should be placed on ice or refrigerator at a temperature of 1-10^{o}C during transit to the laboratory. Use insulated containers to transport sample vessels to maintain proper storage temperatures. Sample vessels should be packed so they do not become immersed should the ice melt during transit or storage.

8.1.3 Drinking water samples should be analyzed within 30 h of collection.

9.0 Quality Control (QC):

Each lot of Modified Colitag™ should be tested for quality. Four presence/absence packs or single strength MPN tubes will be needed; the first as a blank (uninoculated), and the others to be inoculated with a representative positive and negative cultures. For additional guidance or clarity regarding QC, refer to *Standard Methods*, Sections 9223 B and 9020 A. The QC results for a particular lot are valid until the expiration date is realized.

9.1 Incubate the individual tubes or bottles containing Modified Colitag™ medium with a loopful of growth from an 18-24 hour broth culture of the following: *E. coli* (ATCC 25922 or a characterized MUG positive strain), *Klebsiella pneumoniae* (ATCC 13883 or another characterized non-

fluorescing coliform species), *Salmonella* subsp. *enterica* serovar *Typhimurium* (ATCC 14028 or another characterized non-coliform). Include an uninoculated tube or bottle of Modified Colitag™ as an additional control.

9.2 Incubate QC samples at 35±0.5°C for 16-48 hours. If results are intended to be read before 22 hours incubation, samples must be pre-warmed for 7-10 minutes in a 44.5 ±0.2°C water bath. Observe for the following reactions:

Modified Colitag™ Control Organism Reactions — 24 hours

Organism	Color	Fluorescence
E. coli	Yellow	Positive
Klebsiella species	Yellow	Negative
Salmonella subsp. *enterica serovar Typhimurium*	Colorless	Negative
None	Clear	Negative

9.3 For additional quality control testing, positive Modified Colitag™ may be inoculated into Brilliant Green Lactose Bile, EC-MUG or other media used for confirmation.

10.0 Calibration and Standardization

10.1 Check incubation temperatures twice daily (morning and afternoon) to ensure proper temperature regulation.

10.2 Temperature measurement devices should be calibrated annually against a NIST-certified thermometer or one traceable to NIST.

11.0 Procedure

11.1 Test Procedure for the Presence/Absence method

11.1.1 Aseptically add Modified Colitag™ medium from one packet to a 100-mL water sample in a sterile nonfluorescing, leakproof, container.

11.1.2 Shake to begin dissolution.

11.1.3 Incubate bottles at 35±0.5°C for 16-48 hours. If results are intended to be read before 22 hours incubation, samples must be pre-warmed for 7-10 minutes in a 44.5 ±0.2°C water bath.

11.1.4 Read results (refer to section 11.3, below).

11.2 Test procedure for the MPN method: Refer to Section 9221C of the *Standard Methods for the Examination of Water and Wastewater:*

11.2.1 Method A:

11.2.1.1 Select the appropriate number of tubes per sample for the MPN test (10 tubes x 10-mL or 5 tubes x 20-mL).

11.2.1.2 Mix 100-mL of a water sample with one packet of Modified Colitag™ medium in a sterile vessel. Shake to dissolve medium.

11.2.1.3 Aseptically dispense the sample into the tubes.

11.2.1.4 Incubate tubes at 35± 0.5 ºC for 16-48 hours.

11.2.1.5 Read results (refer to section 11.3, below) and report as the most probable number (MPN)/100-mL using values from Table 9221:III, Section 9221 C in the *Standard Methods for the Examination of Water and Wastewater*.

11.2.2 Method B:

11.2.2.1 Dissolve Modified Colitag™ in sterile distilled water to make up desired quantity of medium. One packet contains sufficient contents to make 100-mL of single strength medium, or 50-mL of double strength medium.

11.2.2.2 Perform serial dilutions and inoculations according to the MPN method described in The Standard Methods for the Examination of Water and Wastewater, 21st Ed. Section 9221C, Estimation of Bacterial Density.

11.3 Sample Interpretation

11.3.1 Visually check each bottle, bag or tube for a yellow color. If the sample (P/A bottles or bags or MPN tubes) are yellow, coliform bacteria are present.

11.3.2 The absence of yellow color in the sample after 16-48 hours incubation indicates the sample is negative for coliform bacteria.

11.3.3 If samples are yellow, examine for fluorescence using a long wavelength 365 nm UV lamp in a darkened environment. If a bright blue fluorescence is present, the sample is confirmed for the

presence of *E. coli.* As with the ONPG test, known positive and negative cultures may be run parallel to the unknown sample, or the sample may be compared to a Colitag™ comparator (description included in this section).

11.3.4 The absence of fluorescence in the sample under longwave UV light after 16-48 hours incubation indicates the sample is negative for *E. coli* bacteria.

11.3.5 For quality control, the test sample may be compared to any of the following:

- Negative control – either sterile water with CPI Modified Colitag™ and or a negative control culture with Modified Colitag™, incubated at 35±0.5°C for 16-48 hours (see Table 9.2 in the quality control section).
- Positive control – Coliform-positive, MUG-positive organism with Modified Colitag™, incubated at 35±0.5°C for 16-48 hours (see Table 9.2 in the quality control section).
- Color Comparator -- Colitag™ comparator transferred to a similar test vessel- if the sample is equal or darker yellow than the Colitag™ comparator, the sample is confirmed for the presence of total coliforms. If the sample fluoresces with equal or greater intensity than the comparator, the sample is confirmed for the presence of *E. coli*. The color comparator should be stored protected from light when not in use.

11.3.6 Use Modified Colitag™ medium on or before the printed expire date. For optimal performance, it is recommended that the powdered medium be stored in cool (preferably 4-7 degrees C), dry conditions, protected from light and moisture.

12.0 Data Analysis, Calculation, Interpretation and Reporting Results

12.1 Follow the same interpretation directions from 11.3 above.

12.1.1 Presence/Absence: Report results as presence/absence of total coliforms/*E. coli* per 100-mL. No further data analysis or calculation is required.

12.1.2 MPN Format: Report results as the most probable number of total coliforms/*E. coli* (MPN) per 100-mL. MPN values and further

information are available in Section 9221C of the Standard Methods for Examination of Water and Wastewater, refer to tables 9221: II, III, and IV.

13.0 Method Performance Characteristics of Modified Colitag™

13.1 **Specificity**: 93.8% overall (16-48 hours incubation) when compared to SM9221B (LTB, BGLB) and 96.1% overall compared to SM9221F (EC+MUG).

13.2 **Comparability**: Modified Colitag was found to have an overall agreement of 94.5% with LTB, BGLB for total coliforms and 95.9% with EC-MUG for *E.coli* detection.

13.3 **Sensitivity**: 95.2% overall (16-48 hours incubation) when compared to SM9221B (LTB, BGLB) and 92.8% overall compared to SM9221F (EC+MUG).

14.0 Pollution Prevention

14.1 Wherever possible, it is recommended that laboratory personnel use pollution control techniques to minimize waste generation. When waste cannot be reduced at the source, recycling is recommended.

15.0 Waste Management

15.1 It is the responsibility of each laboratory to comply with all federal, state and local regulations governing waste management, particularly to hazardous waste identification rules and land disposal restrictions. In addition, it is the responsibility of each laboratory to protect the air, water and land by minimizing and controlling all release from fume hoods and bench operations. Compliance is also required with any National Pollutant Discharge Elimination System (NPDES) Permits and regulations.[10] For further information, Federal, State or local agencies should be contacted.

16.0 References

16.1 Colitag™ product insert, prepared by CPI International.

16.2 "Colitag: An Indole- and MUG-based Medium for Detecting and Recognizing Fecal Coliforms and *Escherichia coli*." Abstract with poster presentation, 1991 ASM General Meeting, Dallas TX.

16.3 "A Comparison of the Colitag Method and the Standard Methods for the Detection of Fecal Coliforms and *Escherichia coli* in Urban Creeks "WEF/CWEA Collection Systems 2002 Conference. San Francisco, CA.

16.4 Chang, G. W. and Lum, R. A., 1992. *Improved Recovery of Chlorine-Injured E. coli on Acidified Media. Colitag™ and mCT3*. Abstract with poster presentation. American Society for Microbiology General Meeting, New Orleans, LA.

16.5 United States Environmental Protection Agency. Nat*ional Primary Drinking Water Regulations, Total Coliforms (Including Fecal Coliforms and E. coli); Final Rule*. Federal Register **54** (124): 27547-27568. Washington, D. C., Office of Federal Register. June 29, 1989.

16.6 American Public Health Association, American Water Works Association, Water Environment Federation. Microbiological Examination, Part 9000. IN: *Standard Methods for the Examination of Water and Wastewater, 21st edition*.

16.7 Krieg, N. R. and J. G. Holt., eds. *Bergey's Manual of Systematic Bacteriology*, vol. 1. Baltimore, Williams and Wilkins, 1989.

16.8 Unpublished data, CPI International Inc., 5580 Skylane Blvd, Santa Rosa, Ca, 95403.

16.9 American Public Health Association, American Water Works Association, Water Environment Federation. Microbiological Examination, *Standard Methods for the Examination of Water and Wastewater, 21st edition,* Sections 1090 H and 1090 J.

16.10 American Society of Testing Materials. Specifications for Reagent Water, (Type III Grade), D1193-91. In: *Annual Book of ASTM Standards*, 1991, vol. 11.01. p. 45. Philadelphia, PA.

16.11 Code of Federal Regulations Title 40 Protection of the Environment. Chapter I Environmental Protection Agency. Part 122 EPA Administered Permit Programs: The National Pollutant Discharge Elimination System. http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=344382d789a386c880645c300a514061&rgn=div5&view=text&node=40:21.0.1.1.12&idno=40.