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

Version 2.0

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1.0 Scope and Application

1.1 Modified Colitag™ (commercially available as "Colitag™") is a selective and differential ready-to-use, dehydrated medium for the simultaneous detection of total coliform bacteria and *Escherichia coli* in water.

~~1.2~~ Modified Colitag™ is approved by the U.S. EPA for compliance sample analyses as required by the Revised Total Coliform Rule.[1,2] This method can be used as a presence-absence (PA) test or for quantification of bacteria through several most probable number (MPN) formats: multiple tube fermentation, MPNPlate™ and MPNTray™. Although this method may be used in quantitative formats, it is important to note that EPA has not evaluated the effectiveness of the enumerative formats.

1.3 Modified Colitag™ is capable of detecting 1 CFU of total coliforms or *E. coli* in a 100 mL water sample in 16 to 48 hours and does not require further confirmation or verification steps.

2.0 Summary of Method

2.1 Modified Colitag™ is a ready-to-use, dehydrated medium for the simultaneous determination of the presence or absence or enumeration of total coliforms and *E. coli* in water. For drinking water testing, Modified Colitag™ is EPA approved for presence-absence testing, although enumerative formats may be used. When using Modified Colitag™, the contents of one packet of medium are mixed with a 100 mL water sample and incubated at 35±0.5°C for 16 to 48 hours. If results are intended to be read before 24 hours incubation, samples must be prewarmed in a water bath for 20 minutes at 35°C or alternatively, 7-10 minutes at 44.5°C.

2.2 Modified Colitag™ detects total coliforms and *E. coli* through the identification of two enzymes, ß-galactosidase and β-glucuronidase, respectively. If total coliform bacteria are present in the sample, β-galactosidase, an enzyme produced by total coliform bacteria, will hydrolyze the chromogenic indicator ortho-nitrophenyl-β-D-galactopyranoside (ONPG) to release a yellow-colored compound. If *E. coli* are present in the sample, the enzyme β-glucuronidase, produced by *E. coli* cells, will hydrolyze the fluorogenic indicator 4-methylumbelliferyl-β-D-glucuronide (MUG) to release a compound that fluoresces when exposed to longwave ultraviolet light. *E. coli* possess both enzymes and therefore the sample will have both yellow color and exhibit fluorescence when *E. coli* is present.

2.3 For enumerating *E. coli* and other total coliforms, Modified Colitag™ may be used in several formats (multiple tube MPN, MPNPlate™, and MPNTray™), as long as the total sample volume is 100 mL.

2.3.1 For the multiple tube formats, the contents of one packet of Modified Colitag™ medium is dissolved into a 100 mL water sample. The sample is then dispensed into five tubes with 20 mL or ten tubes with 10 mL volumes in each tube. *Standard Methods for the Examination of Water and Wastewater* 9221C[3], Table 9221:II and Table 9221:III, is used to determine the MPN results.

2.3.2 The MPNPlate™ is a one-piece unit designed to replace the use of individual tubes when performing the multiple tube fermentation test. The device is a clear, plastic

vessel comprised of five 10 mL, five 1.0 mL, and five 0.1 mL wells. The plate also contains an additional 16th overflow well that allows it to accommodate the extra volume for testing 100 mL water sample, as required for drinking water regulations. Based on the combination of positive wells, the MPN of total coliforms and *E. coli* is determined from Standard Methods 9221C, Table 9221:IV.

2.3.3 To use the MPNTray™ with Modified Colitag™, the contents of one packet medium are first dissolved into a 100 mL water sample and then poured into the MPNTray™, and the tray is fed into the automatic sealer. Following incubation at 35±0.5°C for 16-48 hours, ONPG and MUG reactions are read for each well against the MPNTray comparator as described in Section 9.4. Based on the combination of positive wells, the MPN of total coliforms and *E. coli* can be determined from an MPN table provided by the manufacturer.

3.0 Definitions

The definitions below are specific to this method but conform to common usage as much as possible.

3.1 **Total Coliform Bacteria:** Gram negative bacteria belonging to the family *Enterobacteriaceae* that possess the β-D-galactosidase enzyme capable of hydrolyzing the ONPG substrate in the presence of inhibitors during incubation at 35±0.5°C.

3.2 ***E. coli* (*Escherichia coli*):** Gram-negative bacterium that is a member of the total coliform group of bacteria, which also possesses a β-glucuronidase enzyme that is capable of hydrolyzing the MUG substrate after incubation at 35±0.5°C.

3.3 **Laboratory Information Management System (LIMS):** A software system to support a laboratory's operations, such as tracking samples and data, monitoring workflow, ensuring compliance with standards, and other customizable requirements.

3.4 **4-Methylumbelliferyl-ß-D-Glucuronide (MUG):** A chemical substrate which can be hydrolyzed by the ß-glucuronidase enzyme present in *E. coli*. When hydrolyzed, MUG produces 4-methylumbelliferone, which fluoresces under 365-nm UV light.

3.5 **Modified Colitag™:** An EPA approved medium for the determination of total coliforms and *E. coli* in drinking water; sold under the commercial name, "Colitag™".

3.6 **Most Probable Number (MPN):** A statistical estimate of the mean density of organisms in a sample derived from certain probability formulas. These formulas may be found in Standard Methods 9221C "*Estimation of Bacterial Density*". Results are reported in terms of the Most Probable Number of organisms per 100 mL (MPN/100 mL).

3.7 **Multiple Tube Fermentation (MTF) Technique:** A three stage procedure in which the results are statistically expressed in terms of the Most Probable Number of organisms.

3.8 **Ortho-Nitrophenyl-ß-D-Galactopyranoside (ONPG):** A chemical substrate which is hydrolyzed by the ß-D-galactosidase enzyme. The β-galactosidase enzyme is present in total coliform bacteria. When hydrolyzed, ONPG produces ortho-nitrophenol, a yellow-colored compound indicating the presence of total coliform bacteria.

4.0 Interferences

4.1 Modified Colitag™ contains an inhibitor to limit the growth of potentially interfering organisms. At concentrations of 10^6 per 100 mL sample or higher, however, some *Pseudomonas* species may cause fluorescent reactions and some *Aeromonas* species may cause positive ONPG reactions.

4.2 Large numbers of some bacteria or strains of bacteria (e.g. some strains of *Shigella* and *Salmonella* spp.) may cause a sample to fluoresce but will not have a yellow color change (because they lack β-D-galactosidase). Such samples would be considered negative for *E. coli*.

4.3 Water samples containing humic or other material may be colored. If there is a natural background color, note what it is. Such samples may require a control blank of the same water sample for results interpretation. This medium should not be used if the water is yellow-hued and a positive result cannot be ascertained.

4.4 Some waters' high calcium-salt content can cause precipitation, but this should not affect the reaction.

4.5 In samples with excessive chlorine, the sample may turn dark grey or black upon addition of the medium. If this occurs, sample should be invalidated, and a new sample collected.

4.6 If the samples plus medium exhibit an inappropriate color change before incubation, it should be discarded and collect a new sample.

4.7 If the water sample is turbid but not yellow following the recommended incubation times, the sample should be invalidated, and a new sample collected and tested.

5.0 Safety

5.1 Materials contained in the plate, sealing film, and MPNTray™ are inert and non-toxic.

5.2 When using the MPNPlate™, the sample should be poured into the larger (10 mL) wells first, which minimizes the potential risk of splashing.

5.3 None of the components used in Modified Colitag™ are listed as - carcinogens or suspected carcinogens. Refer to the Colitag™ Safety Data Sheet for specific information.

5.4 This method description does not address all safety issues associated with the use of Modified Colitag™ or performing microbiological analyses. It is important to know and practice normal safety procedures for working in a microbiology laboratory. Aseptic technique and routine biosafety practices should be followed when handling samples. The laboratory is responsible for maintaining a safe work environment and a current file of OSHA regulations regarding the safe handling of the chemicals used for analyses. A reference file of safety data sheets (SDSs) should be available to all laboratory personnel.

5.5 Analysts should wear UV protective safety glasses when operating a UV lamp.

5.6 For further information on safe microbiological practices, consult *Biosafety in Microbiological and Biomedical Laboratories* (BMBL, 5th edition), Centers for Disease Control and Prevention (CDC).

6.0 Equipment and Supplies

6.1 Modified Colitag™ Media (commercially available under the name "Colitag™"), Neogen Corp., Lansing, MI.

6.2 Incubator that can maintain a temperature of 35±0.5°C for the duration of the incubation period.

6.3 6-Watt, 365-nm UV lamp.

6.4 Sterile sample collection vessel with 100 mL fill line (120 mL or larger bottles) with thiosulfate if chlorine residual is present in the sample. Colitag™ vessel with thiosulfate, or equivalent, Neogen Corp.

6.5 Gable covered, circulating water bath able to hold temperatures of 35±0.5°C or 44.5±0.2°C.

6.6 MPNPlate™, Neogen Corp.

6.7 MPNPlate™ Sealing Film, Neogen Corp.

6.8 MPNPlate™ Sealing Tool, Neogen Corp.

6.9 97 or 51 well MPNTray™, Neogen Corp., or equivalent.

6.10 MPNTray™ Sealer, Neogen, Corp. or equivalent.

6.11 MPNTray™ rubber insert – 97 or 51-well rubber insert, both from Neogen Corp., or equivalent.

6.12 Modified Colitag™ Comparator for PA and for 51 and/or 97 well MPNTray™ Comparator, Neogen Corp.

7.0 Reagents and Standards

7.1 Modified Colitag™ is provided as a pre-measured, dehydrated medium. The contents of one packet dissolve directly into a 100 mL water sample for either PA or MPN testing. Modified Colitag™ is stable when stored at -20 to 30°C (preferably 4 to 7° C) away from light and moisture. The expiration date and lot number are indicated on each unit.

7.2 Three types of standards may be used with Modified Colitag™ PA and MPNTray™ formats: 1) a reagent blank consisting of one Modified Colitag™ test packet mixed with 100 mL of sterile distilled or deionized water; 2) positive and negative bacterial control strains; or 3) a color/fluorescence comparator. NOTE: comparator used must be in the same format as is used for analyses. Suggested bacterial control strains are listed in Table

1 in Section 9.4.2 of this document.

7.3 Dehydrated media should be stored in a cool, dry location, protected from light and discarded by manufacturer's expiration date. Exposure to certain environmental conditions (i.e. heat and humidity) may cause the Modified Colitag™ medium to be unsuitable for use. Each lot of medium must be checked before use to ensure that it is free flowing and not discolored. The medium can be checked by inverting a slim-stick packet back and forth at least once prior to opening. The powder will fall back and forth from one end of the packet to the other if it is free flowing and not caked. Any caked or discolored medium should be discarded.

8.0 Sample Collection, Dechlorination, Shipment and Storage

8.1 Recommended sampling and dechlorination procedures are described in Part 9060 of *Standard Methods for the Examination of Water and Wastewater* as well as any applicable regulatory requirements.

8.2 Water samples should be collected in sterile, plastic, or glass leak-proof containers. The sample bottle must have at least 120 mL capacity at least a 1-inch headspace. It is inappropriate for a portion of the sample to be poured to waste (without proper homogenization) to meet the required sample volume as this could result in erroneous results.

8.3 If chlorinated water is tested, sufficient sodium thiosulfate ($Na_2S_2O_3$) must be used to neutralize any residual chlorine in the water sample. 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.

8.4 Sample shipping times and temperatures must be followed according to the applicable regulatory requirements. Samples should be tested as soon as possible after collection and should be placed on ice, frozen gel packs, or under refrigeration to maintain a temperature of 1 - 10°C (without freezing) 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.

9.0 Quality Control

9.1 The laboratory is responsible for adhering to appropriate quality assurance (QA) and quality control (QC) measures. All QA/QC described in the *Manual for the Certification of Laboratories Analyzing Drinking Water*[4] should be followed. There are also additional QA/QC measures as described in Standard Methods 9020.

9.2 Aseptic technique is required for accurate results. Introduction of contamination to the water sample, sample vessels, MPNPlate™, the portion of the sealing film that comes into contact with the wells, or into the MPNTrays™ can alter results.

9.3 Quality control testing should be performed on Modified Colitag™ media and accessories for testing.

9.3.1 Modified Colitag™ media should be QC tested with blank, positive, and negative control organisms, once per quarter.

9.3.2 In addition, sterility checks should be conducted on each new lot of media. Dissolve a packet of medium in sterile water in a vessel and incubate at 35±0.5°C for 48 h. After the incubation, the medium should not exhibit turbidity or color change. Media that does not pass sterility QC testing should not be used.

9.3.3 Each lot of medium should be checked before use with a 365-366 nm UV light with a 6-watt bulb. To conduct this test, a packet of medium is dissolved in sterile water in a non-fluorescing vessel. If the medium exhibits fluorescence, the medium should not be used.

9.3.4 The pH of Modified Colitag™ should be verified, once per lot. The pH of Modified Colitag™ should be 6.6-7.0.

9.3.5 MPNplates™, MPNTrays™, and any other sample vessels should be tested for sterility and autofluorescence, once per lot. Plates, trays, and vessels that do not pass QC testing should not be used. See the *Manual for the Certification of Laboratories Analyzing Drinking Water*,[4] "Chapter V Critical Elements for Microbiology" for more information about how to perform these tests.

9.3.6 The volume for any device used for measuring sample volumes (i.e., sample bottles, pipettes, etc.) must be verified to be accurate within 2.5% tolerance, once per lot.

9.3.7 If a tray sealer is used, it should be checked monthly by adding a dye (e.g. bromocresol purple) to the water. If dye is observed outside the wells, it is indicative that proper sealing is not occuring. Either perform maintenance or use another sealer.

9.3.8 If MPNPlates™ are used, sealing should be checked, once per lot, by adding a dye (e.g. bromocresol purple) to the water. If dye is observed outside the wells, check that sealing technique is consistent with the steps described in 11.7 of this document. If the procedural steps are being performed correctly and leakage is still observed, contact the manufacturer for a new pack of sealing film. MPNPlate™ sealing film should be discarded by the printed expiration date.

9.3.9 Long wave (365 nm) UV light bulbs used for reading fluorescence generally last thousands of hours on average but will eventually have diminished intensity, making the detection of faint fluorescence difficult. It is important that the lightbulb be replaced on a routine basis, with the timing based on usage. The glass surface of the UV lamp used for fluorescence readings should be kept clean and may be wiped with a soft cloth moistened with ethanol.

9.3.10 Corrective actions should include rejection of data produced with out-of-specification materials and reagents or whenever QC controls demonstrate unexpected reactions.

9.4 Post- incubation samples may be compared against a reagent blank, negative, and positive bacterial strain controls, and a comparator.

9.4.1 Reagent blank – Sterile water combined with Modified Colitag™ incubated in parallel with samples or used immediately. The blank should be prepared and used in the same test format as the sample.

9.4.2 When using positive and negative bacteria controls, the strains listed in Table 1are recommended for use. Other control organisms that have been confirmed to appropriately react with ONPG and/or MUG may also be used.

Table 1. Modified Colitag™ Recommended Control Organisms

Organism	Control Type	Expected Color Response When Using Modified Colitag™	Expected Fluorescence Response When Using Modified Colitag™
Escherichia coli ATCC 25922	Positive control for *E. coli*	Yellow	Positive
Klebsiella pneumoniae ATCC 13883 *or Citrobacter freundii ATCC 8090*	Positive control for total coliforms	Yellow	Negative
Pseudomonas aeruginosa ATCC 10145 *or Salmonella enterica ATCC* 700720	Negative control for total coliforms and *E. coli*	No change in color	Negative

9.4.3 A comparator represents a sample that is weakly positive for ONPG and MUG and may be used for results determinations with both the PA and enumerative formats.

9.4.4 If using Colitag™ in a PA format, use the PA comparator directly.

9.4.5 If using Colitag™ in an MPNTray™ format, use the MPNTray comparator directly.

9.4.6 If using Colitag™ in an MPN tube format, a tube comparator should be generated in the same volume as the MPN tubes. Pour the same volume of comparator solution (from a Colitag P/A comparator) into the same type of tube being used for analyses.

9.4.7 If using Colitag™ in the MPNPlate format, generate an MPNPlate™ comparator by aseptically pouring an entire Colitag P/A comparator into a plate and seal the plate.

9.4.8 The comparator must be discarded by the expiration date.

10.0 Calibration and Standardization

10.1 Equipment used in conjunction with analyses performed using the plates or trays should be monitored and calibrated as usual according to laboratory QA and QC measures given in the latest edition of the *Manual for the Certification of Laboratories Analyzing Drinking Water* and *Standard Methods* 9020.[4]

11.0 Procedure

11.1 Sample bottle must have at least a 1-inch headspace to allow for proper homogenization. If the bottle lacks enough headspace for adequate mixing, the sample is poured into a larger sterile vessel so it can be mixed properly. Measure out desired sample volume and proceed with analysis.

11.2 Before removing any portion of the sample, the sample must be properly homogenized by shaking vigorously for 7 s (back and forth in a 1 ft arc approximately 25 times). Failure to properly mix sample can lead to erroneous results, as bacteria are known to clump together and are therefore not evenly distributed throughout sample. This shaking technique can be integrated into the mixing process to dissolve the medium, but the shaking should be performed directly prior to dispensing the sample. The medium may need one to two minutes to fully dissolve in the water sample.

11.3 No matter the format used, all tests are incubated at 35±0.5°C for 16-48 hours. If results are intended to be read before 24 hours of incubation have elapsed, samples must be prewarmed at 35°C for 20 minutes or alternatively for 7-10 minutes at 44.5°C in a circulating water bath. The water level should be above the upper level of the medium. Depending on the incubator used and the sample load, it may be necessary to prewarm all samples so that the incubator temperature does not drop significantly and take a long period of time to rebound after the incubator is opened or cold samples are added. Failure to maintain the proper temperature throughout incubation period could result in false-negative results, especially with the shorter incubation times. To ensure that samples are at proper temperature for the entire incubation period, laboratories should prewarm samples after adding medium but before placing them in the incubator. Prewarming is not required if the MPNTray™ format is used.

11.4 No matter the format used, all tests should be placed into the incubator within 30 minutes once the medium is added to the sample.

11.5 Test Procedure for the Presence-Absence Method

11.5.1 Aseptically add the contents of one packet of pre-measured Modified Colitag™ medium to a 100 mL water sample. Replace cap on bottle and shake vigorously to dissolve medium. It may take one to two minutes for the medium to dissolve. Some small particles of medium may remain undissolved which will not affect method performance.

11.5.2 Incubate at 35±0.5°C for 16 – 48 hours. Read results (refer to section 12.0, below).

11.6 Test Procedure for the Multiple Tube MPN Procedures

11.6.1 A 5-tube series (20 mL sample per tube) or 10-tube series (10 mL sample per tube) can be used.

11.6.2 Aseptically add one packet of pre-measured Modified Colitag™ medium to 100 mL of water sample in a sterile vessel. Shake vigorously to dissolve medium.

11.6.3 Arrange tubes in rows of 5 or 10 in a test tube rack, and label each set of tubes. Aseptically dispense 20 mL sample into each of the five tubes containing the prepared medium if using the five tube test or add 10 mL into each of ten tubes containing the medium if using the ten tube test. Cap and mix using gentle swirling.

11.6.4 Incubate at 35±0.5°C for 16 – 48 hours.

11.6.5 Read results (refer to section 12.0, below) and record as the most probable number (MPN) of total coliforms and *E. coli* per 100 mL using the appropriate MPN table from *Standard Methods for the Examination of Water and Wastewater* 9221C, Table 9221:II "MPN Index and 95% Confidence Limits for all Combinations of Positive and Negative Results When Five 20-mL Portions are Used" or Table 9221:III "MPN Index and 95% Confidence Limits for all Combinations of Positive and Negative Results When Ten 10-mL Portions are Used".

11.7 Test Procedure for the MPNPlate™ Method (Figures 1 & 2)

11.7.1 Aseptically add the contents of one packet of premeasured Modified Colitag™ medium into a 100 mL water sample in a sterile vessel. Shake to dissolve medium and to evenly disperse bacteria in the sample to ensure accurate MPN results. Medium may take one to two minutes to dissolve. Some small particles of medium may remain undissolved which will not affect method performance.

11.7.2 Open the sterile packaging and remove the MPNPlate™ taking care not to touch or contaminate the top surface of the plate.

11.7.3 Pour the medium-sample mixture over the top of the plate, filling the large wells first and making sure to completely fill all 15 wells. The remainder of the sample can be poured directly into the largest 16th overflow well or anywhere over the top of the plate (the extra volume will automatically flow into the 16th (or overflow) well which is joined with an overflow channel). If a well has bubbles or is not full, use a sterile pipet to remove the bubbles or aseptically transfer some sample from the overflow well to fill it but avoid splashing.

11.7.4 Remove one of the release liner side tabs from a piece of the sterile sealing film by folding along the score line to help separate the pieces.

11.7.5 Align the score line to the top edge of the plate and affix the film to the end of the plate while allowing the main portion of the film to fall back away from the surface of the plate. Do not allow the film to fall forward across the open wells.

11.7.6 Remove the large middle piece of release liner from the sealing film taking care not to touch the portion of the sterile adhesive surface that will come into contact with the wells or overflow channel. The user may twist the edge of the film near the score line or push the film towards the plate to help lift the backing.

11.7.7 Holding the second end tab, brace the plate and pull the film straight into sealing position over the top of the plate. Do not pull the film hard enough to cause wrinkles to form. Use the sealing tool to seal the wells by rolling across the film from the first tab towards the opposite end. Any excess sample on the surface of the plate will automatically be pushed into the overflow channel that leads to the largest well.

11.7.8 Remove the last piece of release liner from the end tab and secure the rest of the sealing film to the end of the plate.

11.7.9 Fold and attach all edges and corners of the film to completely seal the plate.

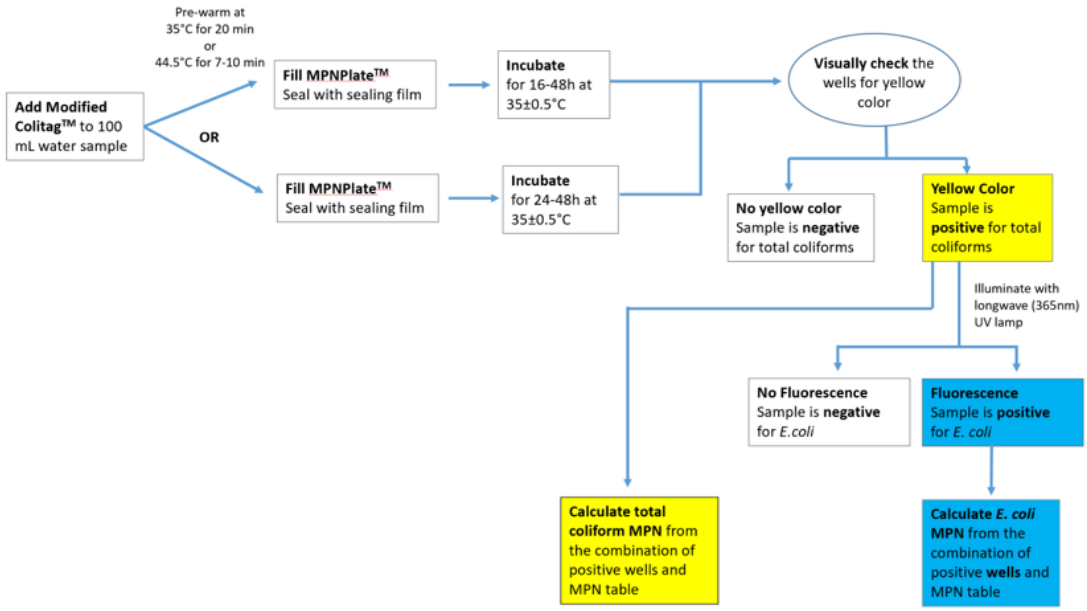
11.7.10 Place unit in incubator. Side cut-out vents facilitate air circulation around the wells so there is no limit on how high plates may be stacked for efficient use of space in the incubator. Plates should be placed in the incubator with adequate space so that air can circulate around them to allow for consistent and accurate incubation temperature. Incubate at 35±0.5°C for 16 to 48 hours.

11.7.11 The MPNPlate™ should not be used with cold samples. The sample volume will expand as it warms to incubation temperature which may cause breaches in the seal and possible leakage between the wells. Leakage may affect the accuracy of the MPN count.

11.7.12 Read results, refer to section 12.0 below. When using the MPNPlate™, MPN results are obtained from the MPN table found in *SM 9221C, "Estimation of Bacterial Density*", specifically, Table 9221:IV *"MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used per Dilution (10 mL, 1.0 mL, 0.1 mL*)".

Figure 1. MPNPlate™

Figure 2. MPNPlate™ Procedural Flowchart



11.8 Test Procedure for the MPNTray™ Method (Figures 3 & 4)

This procedure is for using a sterile disposable MPNTray™ (either 51 well or 97 well) with Modified Colitag™ medium for the detection of total coliforms and *E. coli* and in water samples. Aseptically add premeasured medium from packet to a 100-mL water sample in a container and shake vigorously to dissolve medium. Medium may take one to two minutes

to dissolve. Some medium may remain undissolved which will not affect method performance.

11.8.1 Turn on automatic sealer to warm up before use. A signal light will illuminate when sealer is ready.

11.8.2 Open the sterile packaging and remove the MPNTray™.

11.8.3 Use one hand to hold the MPNTray™ upright with the well side facing the palm. Squeeze the tray so that the center bends towards the palm while pulling the foil tab to create an opening. Avoid contacting the surface area on the inside of the tray.

11.8.4 Pour the medium-sample mixture directly into the tray while avoiding contact with the foil tab. Tap the small wells of the tray to release any air bubbles that may have formed. Allow foam to settle, although some foam is acceptable.

11.8.5 Fit the tray into the rubber insert well side down. Place rubber insert and MPNTray™ onto sealer input tray oriented so the smallest wells are drawn in first. Gently feed into the sealer until the motor grabs the insert.

11.8.6 After the tray is heat sealed, it will be ejected from the sealer. Remove from the insert and place in the incubator. Trays should be placed in the incubator such that air can circulate around them to allow for consistent and accurate incubation temperature. Trays may be put into stacks up to four high. Incubate the trays at 35±0.5°C for 16 to 48 hours.

Figure 3. Image of MPNTray™ (97-well version)

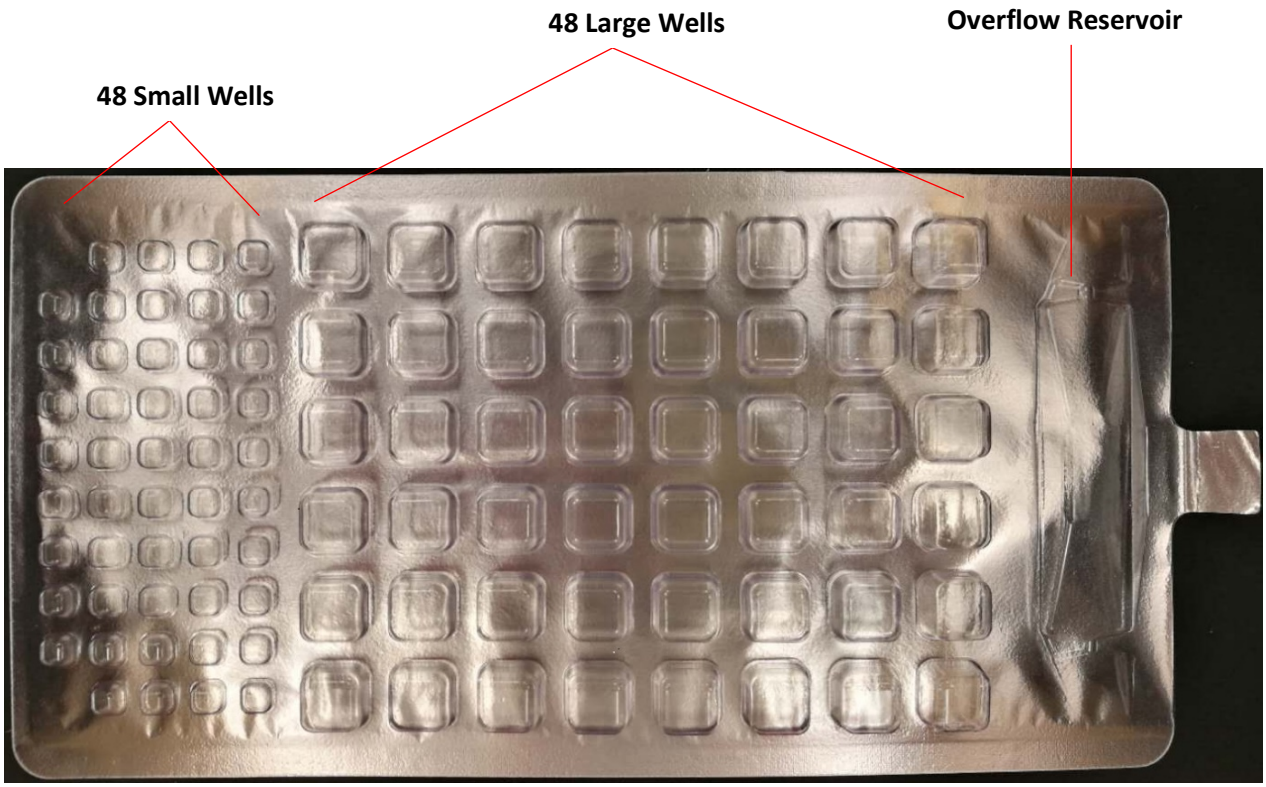
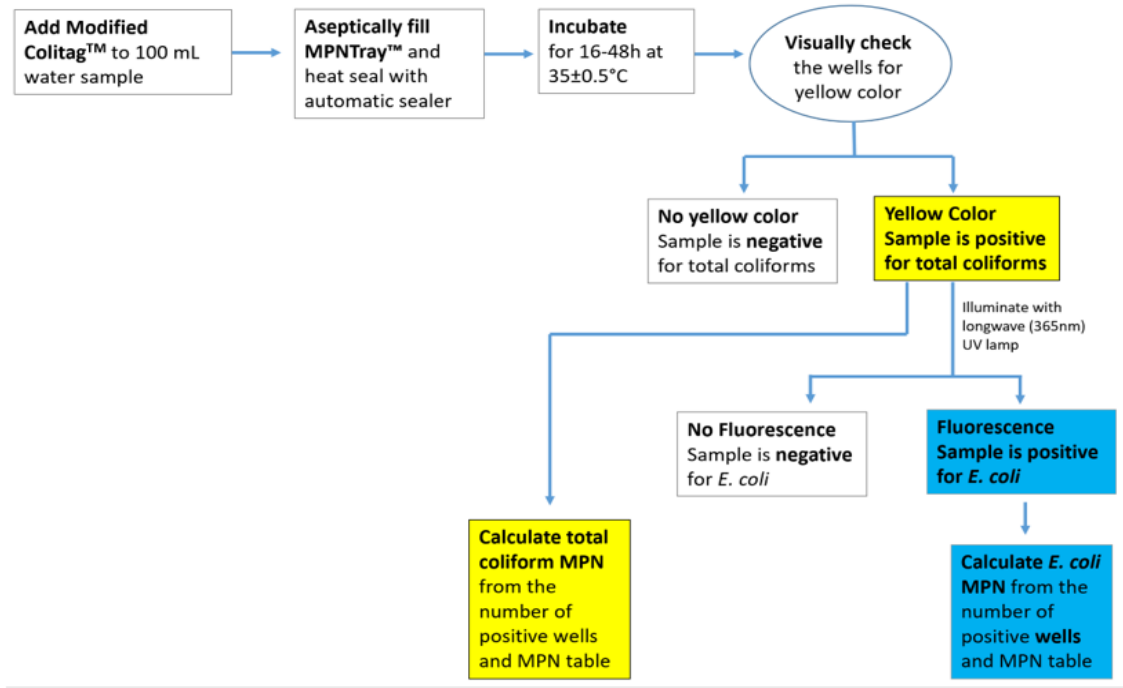


Figure 4. Flowchart of MPNTray™ Procedure



12.0 Data Analysis and Calculations

12.1 Result interpretation

After incubation, test sample(s) should be compared to a comparator. The comparator used must be in the same format as the test format used and before the manufacturer's expiration date. A blank or a positive control may also be used. Details for use of standards are described in section 9.4.2., Table 1 above.

12.1.1 **For total coliform detection**: After the incubation period, examine each sample vessel for the development of yellow color. If color response is not uniform throughout the sample, mix by inversion before reading. It may be helpful to place the test in front of a piece of white paper to discern any color change. If the yellow color is observed in the sample is: 1) as yellow as or darker yellow than the comparator, or 2) darker yellow than a blank or negative control strain, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms. However, if the chromogenic response is ambiguous (color cannot be discerned, or sample is turbid without yellow color), return sample to incubator for two to four more hours but no longer than a total incubation time of 48 h, to allow color to intensify. If the color becomes: 1) equal or darker yellow than the comparator, or 2) darker yellow than a blank or negative control within this period, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms. Colitag can be incubated for a maximum time of 48 h. After 48 h, negative test results are still considered valid, but positive results are not.

12.1.2 **For *E. coli* detection:** A yellow color change (indicating β-D-galactosidase is active) and fluorescence (indicating β-D-glucuronidase is active) together show that *E. coli* is present. After the incubation period, examine positive total coliform tests for a bluish fluorescence using a long-wavelength (365–366 nm) UV lamp with a 6-W bulb. Hold light within 5 in. of sample in a dark environment to observe fluorescence. Use comparator to ensure that test results are read accurately. If at the end of the incubation period, the fluorescence is: 1) lighter than the comparator, or 2) equal to the blank or negative control, the sample is considered negative. If for any reason the fluorogenic response is ambiguous (fluorescence cannot be easily discerned), the sample should be inverted at least two to four times to mix and returned to the incubator for additional incubation time. The sample should be re-incubated, but no longer than a total incubation time of 48 h to allow fluorescence to intensify. If the fluorescence becomes: 1) equal or more intense than the comparator, or 2) more intense than a blank or negative control within this period, then the sample is positive for *E. coli*. After 48 h, negative test results are still considered valid, but positive results are not.

12.2 Reporting

12.2.1 **For the presence or absence procedure:** report results as total coliforms and *E. coli* present or absent in a 100-mL sample.

12.2.2 **For the multiple tube procedure:** calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes, as described in Standard Methods 9221C, using the table appropriate for the number of tubes used.

12.2.3 **For the MPNPlate™ procedure:** calculate the MPN value for total coliforms and *E. coli* from the number of positive wells using the table provided by the manufacturer, or Table 9221:IV from Standard Methods 9221C*, "MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used per Dilution (10 mL, 1.0 mL, 0.1 mL*)".
If MPN results do not appear in the table, MPN values may also be determined using the probability formulas described in Standard Methods 9221C. *Estimation of Bacterial Density, Subsection 2.*

12.2.4 **For the MPNTray™ procedure**: to find the MPN value for total coliforms and *E. coli*, record the combination of positive wells. Use this number to determine the MPN value of total coliform bacteria and *E. coli* with the MPN chart provided by the manufacturer. If it is discovered that one or more wells are empty, that well should be counted as negative.

13.0 Method Performance

13.1 Modified Colitag™ medium has been determined to be equally effective as Standard Method 9221B (LTB/BGLB) for total coliforms and 9221F (EC-MUG) for *E. coli* detection.

13.2 Precision of the multiple tube MPN format is discussed in Standard Methods 9221C, "Multiple Tube Fermentation Technique for Members of the Coliform Group." Most probable number point values and confidence intervals are provided in the MPN tables under subsection 2.

When the MPNPlate™ or MPNTrays™ are used with Modified Colitag™, the false-positive and false-negative rates are determined by the medium and not the devices. Performance characteristics for Modified Colitag™ are as follows:

13.2.1 Overall false-positive rate for coliforms (combined incubation times 16 through 48 hours): 6.2%

13.2.2 Overall false-negative rate for coliforms (combined incubation times 16 through 48 hours): 4.8%

13.2.3 Overall false-positive rate for *E. coli* (combined incubation times 16 through 48 hours): 3.9%

13.2.4 Overall false-negative rate for *E. coli* (combined incubation times 16 through 48 hours): 4.2%

13.2.5 Detailed false-positive and false-negative data are included in Appendix 1.

14.0 Pollution Prevention

The MPNPlate™ may be recyclable (polystyrene, plastic #6). All samples need to be sterilized and rinsed prior to recycling. Refer to local recycling programs and regulations governing waste disposal.

15.0 Waste Management

It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by controlling all release from operations.

16.0 References

1. Title 40 – Protection of the Environment, Chapter 1 – Environmental Protection Agency, Subchapter D – Water Programs, Part 141 – Drinking Water Programs, Subpart C – Monitoring and Analytical Requirements, Appendix A – Alternative Testing Methods Approved for Analyses Under the Safe Drinking Water Act. Available online: https://www.ecfr.gov/cgi-bin/text-idx?tpl=/ecfrbrowse/Title40/40cfr141_main_02.tpl

2. United States Environmental Protection Agency, National Primary Drinking Water Regulations: Revisions to the Total Coliform Rule; Final Rule (2013). Federal Register 78(30): 10270- 10365. Available online: https://www.epa.gov/dwreginfo/revised-total-coliform-rule-and-total-coliform-rule

3. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, Water Environment Federation, 1015 Fifteenth Street, NW, Washington, D.C.: https://www.standardmethods.org/

4. Manual for the Certification of Laboratories Analyzing Drinking Water, Criteria and Procedures Quality Assurance, Fifth Edition, United States Environmental Protection Agency, 815-R-05-004, January 2005. Available online: http://www.epa.gov/ogwdw/methods/pdfs/manual_labcertification.pdf

17.0 Appendix 1. Sensitivity and Specificity Rates for Modified Colitag™

Reference: Colitag™ EPA ATP Study Report D05-0035, 2009

Sensitivity = [TP/TP +FN]*100%
Specificity = TN/(TN+FP)]*100%
False positive = 1-Specificity
False negative = 1-Sensitivity

Overall Agreement = [(TP+TN)/TS]*100%

TP = true positives
TN = true negatives
FP = false positives
FN = false negatives

TS = total samples

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

Coliform Data Summary

Incubation Time (h)	**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%

***E. coli* Data Summary**

Incubation Time (h)	**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

END