



MassDEP

**Massachusetts Department of Environmental Protection
Bureau of Water Resources
Watershed Planning Program**

**Analytical Quantification of
Escherichia coli and Enterococci Bacteria
in Ambient Surface Waters Using an Enzyme Substrate Test
(Standard Methods 9223b)**

**January 2025 – January 2027
(rev. 3/21/25)
CN 198.2**

Prepared by:	<u><i>Suzanne H. Flint</i></u>	Date:	<u>7/15/25</u>
	<i>Suzanne Flint, Environmental Analyst</i>		
Approved by:	<u><i>Jasper Sha</i></u>	Date:	<u>7/17/25</u>
	<i>Jasper Sha, QA Officer</i>		
Approved by:	<u><i>Sherwon De Leon</i></u>	Date:	<u>7/15/25</u>
	<i>Sherwon De Leon, Monitoring Section Chief</i>		

**Analytical Quantification of *Escherichia coli* AND Enterococci Bacteria
in Ambient Surface Waters Using an Enzyme Substrate Test
(STANDARD METHODS 9223b)**

Contents

LIST OF REVISIONS	3
1.0 SCOPE & APPLICATION	3
2.0 SUMMARY OF METHODS.....	3
3.0 DEFINITIONS.....	4
4.0 INTERFERENCES	4
5.0 LAB SAFETY.....	5
6.0 EQUIPMENT AND SUPPLIES	5
7.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE.....	6
8.0 QUALITY CONTROL	6
9.0 CALIBRATION AND STANDARDIZATION	8
10.0 ANALYSIS PROCEDURE	8
11.0 PREVENTATIVE MAINTENANCE	11
12.0 DATA INTERPRETATION, ANALYSIS AND VALIDATION	11
13.0 METHOD PERFORMANCE	13
14.0 POLLUTION PREVENTION	13
15.0 WASTE MANAGEMENT	13
16.0 REFERENCES.....	13
17.0 APPENDICES	13

LIST OF REVISIONS

Rev. #	Date	Description of Revision(s)	Page #s	Initials
0	4/2004	Original draft	19	
1	6/4/2004	Mainly Section 10 changes, misc. minor revisions	19	
2	7/2006	Minor, misc changes throughout	---	
3	9/2006	Added dilution requirement for marine Enterolert samples	5, 12	
4	2/2007	Clarified the applicability of different IDEXX reagents for waters of varying conductivities/salinities	4-5, 10-11	
5	3/2009	Revised media-batch and sample batch QC	8,9	
6	4/2023	Revisions throughout document; change "DWM" to "WPP", update formatting and header pages	--	SF
7	4/2025	Minor updates; added Appendix G – Colilert Analysis QuickGuide	Appendix	SF

1.0 SCOPE & APPLICATION

This SOP describes the detection and enumeration of total coliform, *Escherichia coli* (*E. coli*) and Enterococci bacteria in surface water samples using the enzyme substrate test, as detailed in **Standard Methods 9223B**. These methods use hydrolysable substrates for the detection of target bacteria.

The commercially available supplies developed by IDEXX Laboratories, Inc. were chosen to perform this procedure. The IDEXX "Colilert" and "Colilert-18" methods are employed to detect and enumerate both total coliform and *E. coli* bacteria. The IDEXX "Enterolert" method is used to detect and enumerate Enterococci (fecal streptococci) bacteria. These defined enzyme substrate methods were included in an EPA Final Rule for Guidelines Establishing Test Procedures for the Analysis of Pollutants; Analytical Methods for Biological Pollutants in Ambient Water (July 2003).

This SOP is applicable for samples collected by the MassDEP Watershed Planning Program (WPP) and analyzed by WPP staff at WPP's offices/laboratory in Worcester, MA. The main target analytes for WPP waterbody assessments, TMDL determinations and bacteria source tracking are *E. coli* and Enterococci (total coliform is of lesser importance).

2.0 SUMMARY OF METHODS

Because pathogenic (disease-causing) organisms are difficult to isolate and identify, fecal coliform and Enterococci bacteria are used to indicate the potential presence of pathogens in water. Coliform and fecal streptococci bacteria are widely distributed in nature and are present in the intestines of warm-blooded animals, including humans. Their presence in surface waters may indicate human or animal fecal contamination. They can be relatively easily identified and enumerated.

Colilert (-24) and Colilert-18:

The Colilert reagent is added to a 100-mL volume of freshwater sample, the sample is poured into a multi-well tray, the tray sealed and then incubated for 24 hours (Colilert) at $35^{\circ} \pm 0.5^{\circ}\text{C}$. The tray is then checked for color (total coliform) and fluorescent (*E.coli*) reactions. The most probable number (MPN) technique utilizing a multiple

well system format is used to determine the number of total coliform and *E. coli* per unit volume (100 mL.). The Colilert-18 reagent provides results in only 18-22 hours.

Colilert (-24) and Colilert-18 are US EPA-approved for drinking water and source waters. Unlike Colilert (-24) (mainly freshwater use), Colilert-18 can also be used in salt water, but for *E. coli* only--- not total coliform.

NOTE: do at least a 1:10 dilution on Colilert-18 samples if they've been collected in salt/brackish water.

The method detection limit (MDL) for total coliform and *E. coli* using Colilert (-24) and Colilert-18 is a MPN of 1 colony forming unit (CFU) per 100 mL.

The total coliform group is defined as all bacteria possessing the enzyme B-D-galactosidase. The Colilert reagent contains the nutritive ortho-nitrophenyl-B-D-galactopyranoside (ONPG), which is used to detect the enzyme B-D-galactosidase of total coliforms. The hydrolyzation of the ONPG by the enzyme produces a color change (positive=yellow) at 35.0 ° +/- 0.5° C after an incubation period of 24 to 28 hours. The Colilert method also simultaneously detects the presence of *E. coli* through the hydrolyzation of the fluorogenic, nutritive substrate, 4-methyl-umbelliferyl-B-D-glucuronide (MUG) by the *E. coli* enzyme B-glucuronidase. This reaction produces a fluorescent product detectable when viewed under a long-wavelength (365-nm) ultraviolet (UV) light. Non-coliform bacteria cannot metabolize the indicator nutrients.

Enterolert:

Similarly, the Enterolert reagent is added to a 100-mL volume of fresh or marine water (diluted 10X) sample, the sample is poured into a multi-well tray, the tray sealed and then incubated for 24 hours at 41° ± 0.5°C. The tray is then checked for blue fluorescent wells indicating enterococci presence. An MPN table is used to determine the most probable number of Enterococci per 100 mL. The MDL for Enterococci is also a MPN of 1 CFU/100 mL.

The Enterococci, a sub-group of the fecal streptococci, contain the enzyme B-glucosidase. The Enterolert reagent contains 4-methyl-umbelliferyl-B-D-glucoside, which reacts with the enterococci-produced enzyme B-glucosidase to produce a fluorescent blue color.

Enterolert is most applicable for marine/brackish water samples (using the required minimal 10X dilution) but can also be used for fresh water (Note: the Massachusetts freshwater surface water quality standard is based on *E. coli* (Colilert), not Enterococcus). Enterolert is approved by the US EPA as a method for enterococci detection in ambient waters, including fresh, marine or estuarine surface water. It is also an ASTM-approved method (#D 6503-99).

[For QuickGuide: see appendix – SOP QuickGuide for Bacterial Analysis Using Colilert]

3.0 DEFINITIONS

RESERVED.

4.0 INTERFERENCES

Colilert and Colilert-18:

Non-coliform bacteria, particularly *Aeromonas*, and *Pseudomonas* species, may produce small amounts of the enzyme β-D-galactosidase, but are suppressed and generally will not produce a positive response within the incubation time unless more than 104 colony-forming units (CFU)/mL are present.

Serratia species may turn the medium yellow after 24 hours of incubation, but the yellow color is typically brighter than that represented by the color comparator.

Some strains of *Shigella* species may produce a positive fluorescence response. This is not considered a detriment for testing the sanitary quality of water due to the pathogenic nature of *Shigella*.

Some water samples containing humic material may have an innate color. If a water sample has some background color, compare inoculated Colilert® sample to a control blank of the same sample.

Incubation beyond 28 hours may yield a false positive Colilert result, due to cessation of suppression of non-coliform heterotrophic bacteria. The same is true beyond 22 hours for Colilert-18.

Presence of free chlorine in the sample may result in a transient blue color upon addition of the Colilert reagent. To avoid this, all bacteria samples should be taken using sample bottles containing sodium thiosulfate.

Dilute brackish/marine water samples with sterile fresh water at least ten-fold for Colilert-18 testing.

Enterolert: Dilute marine water samples with sterile fresh water at least ten-fold, per IDEXX Enterolert procedure.

5.0 LAB SAFETY

All applicable elements of WPP's Lab Safety Plan must be adhered to at all times.

Samples (and positive controls) may contain organisms that are pathogenic to humans, and often the analyst works with water samples of different ranges of contamination. Handle all samples and cultures as if they are infectious. All precautions are to be taken to minimize exposure. These include the use personal protective equipment (lab coats, safety glasses, and protective gloves), keeping the lab work area clean and organized, working at a reasonable pace and using good judgment at all times.

It is recommended that all bacteria lab personnel be immunized against the hepatitis A and B viruses.

All lab personnel must receive on-the-job laboratory safety training.

The sealer device is a burn hazard if not properly used and maintained. See 11.1.

Safety Data Sheets (SDS) for all chemicals used in this SOP are included in Room 229 and in Room 228.

Adherence to the waste storage and disposal procedures in Section 15 shall be maintained at all times.

Additional safety issues with regard to performance of this SOP shall be addressed by WPP's Lab Safety Officer and/or senior staff on an as needed basis.

NOTE: *Pseudomonas* sp. and *Klebsiella pneumoniae* used for media batch QC (see page 8-9) are categorized as Class 2 bio-safety hazards, whereas *Enterococcus faecium* and *E. coli* are categorized Class 1. While Class 2 organisms can be used in this SOP, their use presents greater potential risk to analysts should an accident occur, if poor lab techniques were used or if safety protocols were not followed.

6.0 EQUIPMENT AND SUPPLIES

The following lab equipment and supplies are needed to employ the multi-well tray Colilert and Enterolert methods. It is assumed that the necessary field supplies, such as sterile, 120-ml sample bottles containing sodium thiosulfate, will be available for use in the field.

Colilert and Colilert-18:

- Lab disinfectant and paper towels
- Disposable plastic gloves and lab coats
- Bench sheets
- Quanti-tray sealer
- Quanti-tray 2000 97-well sample trays
- Quanti-tray 2000 tray rubber inserts
- Colilert reagent [Store in dark at 2-30 deg. C; use within 12 months of manufacture] and Colilert-18 reagent [Store in dark at 2-25 deg. C; use within 12 months of manufacture].

- Enterolert reagent, if performing Enterococci analysis. [Store in dark at 2-30 deg. C]
- Sterile lab sample bottles for raw and diluted samples. Preferably, these bottles should be high clarity (to see end points clearly), graduated for 100 ml. (accurate to within 2.5% or approx. 2-3 ml), and be non-fluorescing. IDEXX vessels are preferred.
- Sterile pipettes (1, and 10 sizes)
- Sterile water (non-buffered) or deionized water from WPP's reagent water system. Autoclaved sterile water may be available from WES in 120-250 ml bottles or purchased sterile water from IDEXX. If sterile water is bottled by WES/WPP, use within 1-2 months. If purchased from IDEXX in sealed, 100 ml bottles, shelf life is 2 years. Store at 2-8 deg. C or in cabinet.
- Anti-foam solution (as needed; usually used with P/A test) [Store in cabinet]
- Positive (*E. coli*, *Enterococcus faecium*) and negative (*Pseudomonas*, other) control cultures (for QC). Difco Bactrol discs are acceptable. [Store at 2-8° C. Discs have 1 year shelf life]
- Two-shelf, bench-top, mechanical-convection incubators (5-65 deg. C; +/- 1.5 deg. C uniformity; +/- 0.5 deg. C stability) with thermometer. If also running Enterolert, two incubators (at each temperature) are preferred.
- NIST-certified (or traceable) thermometer
- UV viewing cabinet with 365 nm UV lamp (6 watt)
- Colilert color comparator (optional). NOTE: The color comparator is the lowest color and fluorescence level at which a result can be considered positive. A typical positive result is much more intense.
- MPN tables
- Labeled, plastic bio-waste disposal bags, step-on can and labeled 32 gallon can with cover for temporary waste storage.
- Autoclave (to disinfect bio-waste in bags). This function to be performed by WES on an as-needed basis.

7.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

For a detailed description of field sample collection for bacteria, refer to WPP's field sampling SOP.

Briefly, new, plastic, disposable, sterile, pre-labeled, 120-mL sample bottles (usually containing a sodium thiosulfate tablet/powder for chlorine neutralization) are used to collect surface water bacteria samples. Examples include Corning locking "flip-top" bottles and IDEXX clear, polystyrene "vessels" (preferred).

Samples are placed in a cooler half-filled with wet ice soon as possible after collection. Samples (at 4 °C) are delivered to the lab within 6 hours from collection with the sample tracking/chain-of-custody form filled out by the collector. At the lab, sample analysis must be initiated as soon as possible and within 8 hours of collection and two hours of receipt.

8.0 QUALITY CONTROL

Media Lot-Specific Quality Control: Media lot QC samples are run to verify that the media do not provide false positives or false negatives. Each lot is tested using Quanti-cult (see Appendix D) cultures or lyophilized microorganism pellets as follows:

Colilert and Colilert-18:

1. Negative Control: sterile or deionized (DI) water from WPP reagent water system (Barnstad).
2. Negative Control (optional): sterile water inoculated with non-fluorescing *Pseudomonas* sp. or *Enterococcus faecium* (result should be negative for both total coliform & *E. coli*) or with *Klebsiella*

pneumoniae, *Enterobacter aerogenes* or *Enterobacter cloacae* (result should be positive for total coliform & negative for *E. coli*)

3. Positive Control: sterile or DI water inoculated with *E. coli* (result should be positive for both total coliform & *E. coli*) control cultures.

Enterolert:

1. Negative Control: sterile water alone
2. Negative Control (optional): sterile water inoculated with non-fluorescent *Serratia marcescens* (gram -) and *Aerococcus viridans* (gram +), or *E. coli* (gram -)
3. Positive Control: sterile water inoculated with fluorescent *Enterococcus faecium*.

Upon receipt, check reagent packages integrity for proper seal, tears, and lack of moisture. When using, inspect appearance of Colilert reagent; it should appear dry, free-flowing, and white to off-white in color.

Each lot of media can be checked before use with the UV light to detect auto-fluorescence. If any faint fluorescence is observed, then it should be recorded, and the media discarded, and replaced. If any media causes the sample to fluoresce prior to incubation, the lot should be discarded and replaced.

Sample Batch Specific QC: For each lab batch, run a minimum of one lab blank and one lab duplicate (@ approx. 10% of total samples), and one positive control.

All analysts should participate in training including inter-analyst comparisons of readings to confirm acceptable precision among different readers. This is especially important for the *E. coli* fluorogenic endpoint.

Reagent Water Testing:

Use of WPP reagent water (Barnstead deionization system) for bacterial analyses (dilutions, lab blanks) requires periodic testing to ensure adequacy. Test results are compared against criteria identified in Table 1 (excerpted from Standard Methods, Table 9020 II.)

Table 1: WPP Reagent Water Quality

Parameter	Criteria for Microbiological Analyses	Results
Specific Conductance	< 2 uS/cm	1 uS/cm
pH	Approx. 6-7 is generally preferred	6-7 (for "new" DI H ₂ O; if allowed to equilibrate to air, pH drops below 6)
Metals (total) for Cd, Cr, Cu, Ni, Pb and Zn	< 50 ug/l	For trip and field blanks: All WPP reagent water samples < MDL (.02-1 ug/l), except Cu which showed some "hits" from .5-4 ug/l)
Metals (total, combined)	< 100 ug/l	Total metals in WPP reagent water samples (mostly < MDL) were estimated to be <10 ug/l.
Kjeldahl Nitrogen (TKN)	< 100 ug/l	Invariably less than the method detection limit of 20 ug/l for ambient field blanks
Total Residual Chlorine	< 10 ug/l	WPP's Barnstead DI water system has a dedicated carbon adsorption cartridge to adsorb chlorine and is designed based on feedwater quality to meet Type I reagent water specifications (assuming system maintenance and feedwater consistency).
Fecal coliform	NA	Rarely exceeding method detection limits (e.g., <6, <2) for ambient field blanks
<i>E. coli</i>	NA	Rarely exceeding method detection limits (e.g., <6, <2) for ambient field blanks
HPC	< 100,000 CFU/100 mL.	---

Specific conductivity and pH are measured weekly. Results of WPP's DI water used for field blanks for routine nutrient, metals (Ca, Mg, Na), DOC, and *E. coli* sampling are checked regularly. In general, results indicate that the

WPP Barnstead deionization system, when operating normally (e.g., resistance of about 18.2, maintained correctly, etc.), provides sufficient purity to perform bacterial analyses.

9.0 CALIBRATION AND STANDARDIZATION

Sample Containers: No testing of sample containers used to measure sample volume is planned. Containers are assumed to meet accuracy limits of $< \pm 2.5\%$.

Incubator: The incubator has an internal temperature monitoring device and maintains a temperature of $35^{\circ} \pm 0.5^{\circ}\text{C}$ for *E. coli*. Monitor temperatures both in air and “totally immersed” in water using total immersion incubator thermometers—should be the same.

Because pre-heated, air type incubators may not bring water sample(s) to the specified incubation temperature of 35°C quickly, false negative results could result. Therefore, the time it takes for water samples (or a set of 100-ml, water samples, depending on normal use) to reach 35°C must be accounted for and should be known to ensure that the specified incubation period occurs (typ. >24 hrs.). See also 10.25, 10.29 discussing additional incubation periods. Incubator temperature should be recorded at the start and end of each incubation.

10.0 ANALYSIS PROCEDURE (assumes reagent lot has been tested and passed; see 8.1)

Coordinate analytical work with WPP field survey staff regarding the timing of sample delivery. The maximum number of trays allowed in a WPP-Worcester per incubator at one time is about 60. For each WPP survey, one primary analyst and one backup analyst will be scheduled. **NOTE:** Depending on timing, available reagents and logistics, decide on which reagent will be used (Colilert (24 hour) or Colilert-18).

1. Turn on the Quanti-Tray sealer to allow preheating (usually takes around 10 minutes). Sealer is ready to use when green light is on.
2. Prepare all work areas by cleaning with lab disinfectant.
3. Fill out lab bench sheet (in binder) with required information, in anticipation of sample delivery.
4. Check the incubator to ensure it is ON, maintaining the proper temperature and has sufficient space for sample trays when they are ready.
5. Prepare counter work area with the necessary dilution bottles, reagents, trays, pipettes, etc., leaving sufficient space to prepare samples for incubation.
6. When samples arrive, transfer custody of the samples by removing them from the ice and checking off each sample on the COC form. When all samples have been transferred, sign the COC form and record the time. Review the COC form and discuss with sample delivery staff for information regarding samples that are suspected of containing a high MPN value (indicating the possible need for dilution).
7. Mark the bench sheet with date, sample ID, lab identification number, date and time of collection, date and time of analysis, lab QC sample information, start/end times of incubation, and the analyst's name. It is at this point that the dilution scheme for each sample is identified.
8. Use a Sharpie pen to mark any sample bottles with “HI” suspected of having high counts.
9. With a Sharpie (so as not to tear the paper on the back of the Quanti-Tray) gently mark the Quanti-Trays with the lab identification number of each sample.
10. For each sample (including dilutions), invert sample bottle 20-25 times to ensure complete mixing of the sample. DO NOT TOUCH THE INSIDES OF CONTAINERS OR SAMPLE WATER.
11. The preferred order for sample analysis is to start with the “oldest” samples first and proceed in the order in which they were collected. Lab duplicates, QC samples, and dilutions can be done at any time during the analysis. Lab blanks should be done last.

12. **NO DILUTION SAMPLES:** If no dilutions are to be performed on the sample, mix sample (as above). If over the 100-ml line, pour off to meet the 100 ml meniscus, or use a sterile 1-10 ml pipette to pipette off to the 100 ml line. Cap sample and proceed to step 15.
13. **1:10 ML DILUTION SAMPLES:** (*e.g., for marine/brackish Colilert-18 samples; known or suspected "HOT" samples to be quantified at a level > 2420 org./100 ml.*). Pour 100 mL of sterile DI water into a new, sterile IDEXX vessel to the 100-ml line; pipette off as needed to 100 mL using a sterile 10 ml pipette. Using the same pipette, pipette off 10 mL to achieve 90 mL of sterile water. Then use the same pipette to pipette 10 ml of the original, mixed sample into the 90 mL of sterile water. Cap and mix as above. See Table 2 below for dilution summary.
14. **1:100 ML DILUTION SAMPLES:** (*e.g., for marine/brackish Colilert-18 samples; for known or suspected "VERY HOT" samples to be quantified at a level >> 2420 org./100 ml.*). Pour 100 mL of sterile DI water into a new, sterile IDEXX vessel to the 100-ml line (and pipette off as needed to 100 mL using a sterile 1 ml pipette.). Now, using the same pipette, pipette off 1 ml to achieve 99 mL. Then use the same pipette to pipette 1 ml. of the original, mixed sample into the 99 mL of sterile water. Cap and mix as above. See Table 2 below for dilution summary.
15. Tap each reagent packet before opening to ensure complete dispensing. Open Colilert reagent packet and add entire contents to the 100-ml sample (or diluted sample). Cap and invert the bottle > 25 times until all the reagent is dissolved. Let any foam settle. Some particles may remain; these may dissolve during incubation.
16. After complete mixing, dispense sample into the Quanti-Tray. Use one hand to hold the Quanti-Tray upright with the well side facing the palm. Squeeze upper part of the Quanti-Tray so that it bends towards the palm. Open the Quanti-Tray by pulling the foil tab away from the well side. Avoid touching the inside of the foil or tray. (**NOTE:** If Quanti-Tray is believed to have been contaminated at this point, discard the contaminated Quanti-tray and set up a new one, if possible, and note in bench sheet). Pour the Colilert-treated sample directly into the Quanti-Tray while avoiding contact with the foil tab. Tap the small wells to release any air bubbles.
17. Place the sample-filled Quanti-Tray onto the rubber insert tray of the Quanti-Tray Sealer with well side (plastic) of the Quanti-Tray facing down to fit into the insert.
18. Seal the Quanti-Tray by moving the rubber tray into the sealer. The sealer will automatically take the rubber tray to dispense the sample into the wells and seal the Quanti-Tray. Remove the Quanti-Tray, assuring that it is fully sealed. (**NOTE:** If the Sealer stops in the middle of processing a Quanti-Tray sample, use the black button (with the arrows) on the front of the Sealer to reverse the direction of the Quanti-Tray in the Sealer. Allow the green light on the Sealer to go on before attempting to seal the sample again.) If the sealer does not easily "accept" the rubber tray, lift up the end of the tray slightly while continuing to push it into the sealer, so that the leading edge clears the opening of the sealer.
19. **Reagent Lot QC samples (WPP QA Analyst only).** Run the following *E. coli* QC samples for each new lot of reagents when reagents are first received: 1) **Negative Control: DI water.** 2) **Positive Control.** Prepare a positive QC sample by pouring 100 mL into an IDEXX vessel (as above), then adding an *E. coli* pellet according to manufacturer's directions, then adding Colilert reagent. Cap/tray/seal/incubate. Confirm correct analytical endpoints.
20. **Batch QC samples (Lab analysts).** For each lab batch of samples, prepare a positive CONTROL: Pour 100 ml DI water into an IDEXX vessel, add the lyophilized *E. coli* pellet. Prepare **LAB BLANK:** Pour 100 mL of sterile, deionized water into an IDEXX vessel. Prepare **LAB DUPLICATE:** Using 250-ml sample: Mix sample as above and pour two 100-mL aliquots into two separate IDEXX vessels. Or using a 120 ml. sample: Mix sample (each time) and pipette two 10 mL aliquots into two separate IDEXX vessels containing 90 mL of sterile, deionized water. Multiply results by 10. Provided 100 mL is left in the sample container, run "raw", 100 ml (undiluted) sample also. Run the samples as usual.

21. Incubate all prepared Colilert Quanti-Trays in the incubator at **35 ±0.5 °C** for 24 - 28 hours. If Colilert-18 is used, incubate for 18-22 hours at **35 ±0.5 °C**. Record the dates and times on the bench sheet. Avoid opening and closing the incubator during use in order to maintain stable proper temperature.
22. After sample trays have been placed in the incubator, thoroughly clean and disinfect the work area.
23. Record the information from the bench sheet to the electronic workbook (see Quickguide for file locations updated yearly). Save a copy ("save as") of the Ecoli_Lab_Book_Template with the new batch number as the file name.
24. After 24 hours (18 hours for Colilert-18), remove the Colilert trays from the incubator. Record the time in the bench sheet. For each tray, count the number of wells that turn yellow. Record in the lab workbook the number of positive (yellow) wells in the Quanti-Tray. Use the MPN table (Appendix A) to then record the confirmed total coliform value as MPN/100 ml sample in the lab workbook; or record the result from the electronic worksheet (that uses a lookup table).
25. To determine the *E. coli* result, expose each sample to 365 nm UV light by placing them one-by-one into the UV light viewing cabinet. If the well fluoresces, it is positive for *E. coli*. Count the number of fluorescent wells and record in the lab workbook. Use the MPN table (Appendix a) to record the confirmed E-coli value as MPN/100 mL sample in the lab notebook or record the result from the electronic worksheet. **NOTE:** *Empty wells do not affect the test interpretation as long as the entire sample is in the tray. The effect on the Most Probable Number (MPN) table is statistically insignificant. An empty or partially filled well is interpreted the same way as a full well.*
26. If the sample results are questionable after 24 hours of incubation (18 hours for Colilert-18), the sample may be incubated for up to an additional 4 hours (total of 28 hours for Colilert; 22 for Colilert-18), and rechecked for color and fluorescent reactions. If the color intensifies, the sample is total coliform positive; if it does not, the sample is negative. If an inoculated test is inadvertently incubated over 28 hours (22 for Colilert-18), the following guidelines apply: 1) lack of yellow color is a valid negative test, and 2) a yellow color after 28 hours is not valid and must be repeated.
27. Colilert and Colilert-18 results are definitive at 24-28 and 18-22 hours, respectively. Any positives for both total coliform and *E. coli* observed before the minimum time and negatives observed after the maximum time are also valid. Positive results after the maximum time are not valid.
28. Enter all results from the bench sheet into the electronic workbook; detailed directions are available in the "Read Me" tab. Final results will be automatically displayed in the blue-shaded "Total Coliform MPN" and "E coli MPN" columns.
29. After all MPN results have been recorded, review the printed and electronic workbooks to ensure that all required data and metadata have been recorded completely and accurately.
30. Place used Colilert trays and other waste in "bio-waste" bags, tie securely when only ½ full and temporarily store in the lab in the designated, secured location.
31. Periodically, coordinate with WES for the delivery of the "bio-waste" to WES for autoclave sterilization and disposal at WES.

Enterolert: (in addition to or changes from that above for Colilert)

1. Incubate all prepared Enterolert Quanti-Trays in the incubator at **41 ± 0.5 °C** for 24 - 28 hours.
2. Prepare negative and positive controls as indicated in Section 8.
3. Dilute marine (brackish to salt) water samples with sterile fresh water at least ten-fold, per IDEXX Enterolert procedure.
4. There is no fluorescent end-point comparator for Enterolert. Compare fluorescence in sample trays to negative control.

General Dilution Schemes (depending on sampling bottle used and without pre-filled dilution bottles):

Table 2: Summary of General Dilution Procedures

Sample bottle used	Flip-top locking HDPE (or other)	IDEXX vessel PS
	Procedures	
0X dilution MIX bottle	Mix, pour 100 mL into clear PS IDEXX vessel, pour off/pipette off to 100 mL if necessary, add reagent	Mix, pour off/pipette off for necessary dilutions and to 100 mL, then add reagent
10X dilution bottle	Pour 100 mL. of sterile DI into IDEXX vessel and pour off/pipette off to 100 ml. line as necessary. Pipette off 10 mL. Pipette 10 mL. of mixed sample into the 90 mL. in the IDEXX vessel. Add reagent	Pour 100 mL. of sterile DI into a new IDEXX vessel and pour off/pipette off to 100 ml. line as necessary. Pipette off 10 mL. Mix sample and pipette off 10 mL into 90 ml IDEXX vessel. Add reagent
100 X dilution bottle	Same as above for 10X but using 1 ml. Add reagent	Same as above for 10X but using 1 ml. Add reagent
* These procedures assume that the IDEXX vessel 100 ml line can be used as an accurate (+/- 2.5%) measure.		

11.0 PREVENTATIVE MAINTENANCE

Sealer: Trained personnel (only) must inspect, clean and maintain the sealer according to the manufacturer's recommendations (Appendix C).

Rubber Inserts: Rinse with clean water; autoclave or clean with isopropyl alcohol or bleach.

12.0 DATA INTERPRETATION, ANALYSIS AND VALIDATION

For each sample batch analysis, draft sample and quality control data are reviewed, interpreted and validated using the following definitions and criteria.

Data Report: The lab analyst shall count the number of positive Quanti-Tray cells for each sample/tray and use the MPN table (or Excel spreadsheet) to obtain the Most Probable Number (MPN) per 100 mL. MPN results will be multiplied by the dilution factor as needed to obtain the final draft result. The lab analyst shall then generate a brief draft results lab report for separate peer review.

Dilutions: Multiply MPN value (using IDEXX MPN table) by the dilution factor used for that sample to get the sample result.

Peer Review and Preliminary Approval: A separate lab report reviewer shall be designated to evaluate the accuracy and completeness of the lab report. The reviewer shall discuss any problems, concerns and issues with the lab analyst as needed, and if all data appear OK, preliminarily approve the MPN results as DRAFT data.

Data Validation: WPP's QA Analyst shall perform more rigid and comprehensive data validation for larger data sets (e.g., annual projects), including compiled *E. coli* MPN data (by project), in relation to project-specific data quality objectives (DQOs).

Table 3: Colilert (and Colilert-18 Reactions)

Reaction	Result or interpretation
Yellow	Total Coliform positive
No color or indeterminate	Negative
Fluorescent (yellow)	<i>E. coli</i> positive
Yellow color intensity at or near the comparator color	Inconclusive; re-incubate an additional 4 hours
> 28 (22) hours, positive	Not valid
> 28 (22) hours, negative	Valid
< 24 (18) hours, positive	Valid
< 24 (18) hours, negative	Not valid

Table 4: Enterolert Reactions

Reaction	Result
Blue fluorescence in 24-28 hours	Enterococci positive
No fluorescence in 24-28 hours	Enterococci negative
Blue fluorescence < 24 hours	Enterococci positive
No fluorescence beyond 28 hours	Enterococci negative

Table 5: QC Interpretations (Colilert and Enterolert)

Quality Control Organism	Yellow	Fluorescent
<i>Pseudomonas</i> sp. (Non-fluorescent strain)	No	No
<i>Klebsiella pneumonia</i> , <i>Enterobacter aerogenes</i> or <i>Enterobacter cloacae</i>	Yes	No
<i>E. coli</i>	Yes	Yes (yellow)
<i>Enterococcus faecium</i>	---	Yes (blue)
<i>Serratia marcescens</i>	---	No

Table 6: QC Validation Criteria

Quality Control	Frequency	Acceptance Criteria (control limits)	Corrective Action
Use of expired reagents and/or QC organisms	NA	Any and all >1 year passed exp date	Qualify data (m) Censor data (m)
Positive Culture Control (<i>E. coli</i>)	5%	Positive	Retest reagent lot, replace as needed.
Negative Control (sterile water)	5%	Negative	Retest reagent lot, replace as needed
Sample storage	Every sample	Samples are analyzed within 8 hours of collection and are stored at $\leq 4^{\circ}\text{C}$ from time of collection to time of analysis	Qualify or censor data "h" (hold time issues)
Lab duplicate (precision)	Min. one per batch per analyst and ~5% of samples	+/- 50 MPN OR <30%RPD (<50 MPN); <20% (50-500 MPN); <10 % (500-5000 MPN); < 5% (>5000 MPN) for \log_{10} transformed data (this DQO can also be applied to field duplicate evaluation)	Qualify or censor data "d" (duplicate issues)
Lab blank (bias)	one per batch and ~5% of samples	< MDL	Qualify or censor data "b" (blank issues)

13.0 METHOD PERFORMANCE

The method detection limit for Colilert, Colilert-18 and Enterolert has been determined to be a MPN of 1 colony-forming unit (CFU) per sample volume or dilution tested. The WPP Lab minimum reporting limit (MRL) for data generated through use of this SOP has been chosen to be a MPN of 1 CFU per 100 mL.

Use of this method by WPP will be evaluated on a periodic basis to ensure consistent generation of quality-controlled data.

14.0 POLLUTION PREVENTION

In order to avoid or minimize waste due to non-use, the quantity of media and chemicals purchased should be based upon expected usage during its shelf life. Actual preparation volumes should reflect anticipated usage and stability.

Recyclable materials should be used whenever possible.

Generation of wastewater will be minimized. Discharge of any wastewater from sample preparation and analysis to the sanitary sewer will be minimized and does not pose a pollution potential.

All solid waste and “bio-waste” will be disposed of properly.

15.0 WASTE MANAGEMENT

All used Colilert trays will be bagged as “bio-waste” in autoclavable bags and temporarily stored in 32-gallon, plastic cans in a designated, secured location at WPP, prior to transport to the MADEP WES Lab in Lawrence, MA, for proper disposal (autoclave sterilization, plastics recycling and solid waste disposal). Bio-waste bags will be transported to WES in a covered, rigid-sided container to prevent potential spills.

WPP’s waste disposal procedures are consistent with State regulations (State Sanitary Code Title VIII, 105 CMR 480.00 and Solid Waste Regulations, 310 CMR 19.000) governing the disposal of “infectious waste”. Infectious waste is considered a “special waste”, not a regulated hazardous waste. It can be shipped to another location for treatment and disposal.

WPP waste management procedures for Colilert shall adhere to all applicable aspects of WPP’s Lab Safety Plan. This includes use of proper gloves, use of waste receipt forms when delivering waste to WES, and provisions for lab audits/inspections to ensure compliance and remedial training as necessary.

16.0 REFERENCES

Standard Methods for the Examination of Water and Wastewater, 23rd Edition. (SM 9323B) American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC

<http://www.idexx.com/>

17.0 APPENDICES

Appendix A: Lab Bench Sheet Example
Appendix B: Batch and Temperature Log
Appendix C: Waste Receipt Form
Appendix D: Colilert Analysis QuickGuide

Appendix A: Bench Sheet & EDD Example

<i>E. coli</i> Lab Book		Batch #		L25-10		Analyst Day 1	A. Gouvin-Moffat		Incubate Start Date/Time			6/30/25 13:35					
		Project		TAM C1-5 (2025)		Analyst Day 2	O. Hart		Incubation Start Temp (°C)			34.8					
									Incubate End Date/Time			7/1/25 14:31					
									Incubation End Temp (°C)			35.6					
									UV Read Date/Time			7/1/25 14:31					
Lab QC (LB, LD, QC, blank)	Lab ID Example: L25-###-##	OWMID Example: 71-####	Collection Date from COC	Collection Time from COC	Analysis Date	Analysis Time	Dilution (none = "1")	# large yellow [of 49]	# small yellow [of 48]	# large fluor. [of 49]	# small fluor. [of 48]	Sample & Batch Comments		Total Coliform (MPN)	E. coli (MPN)	Recount if: # yellow > # fluorescent	
	L25-10-01	27-0318	6/30/2025	08:32	6/30/2025	13:12	1	49	48	3	0			>2419.6	3.1		
	L25-10-02	27-0319	6/30/2025	09:20	6/30/2025	13:12	1	49	29	2	0			579.4	2		
	L25-10-03	27-0320	6/30/2025	09:20	6/30/2025	13:12	1	49	24	4	0			435.2	4.1		
	L25-10-04	27-0321	6/30/2025	09:24	6/30/2025	13:12	1	0	0	0	0			<1	<1		
	L25-10-05	27-0322	6/30/2025	10:09	6/30/2025	13:12	1	49	47	10	1			2419.6	12.1		
	L25-10-06	27-0323	6/30/2025	11:11	6/30/2025	13:12	1	48	48	43	16	Only 48 Large		1011.2	139.1		
	L25-10-07	27-0324	6/30/2025	11:43	6/30/2025	13:12	1	49	42	1	5			1299.7	6		
	L25-10-08	27-0325	6/30/2025	12:13	6/30/2025	13:12	1	49	39	1	0			1046.2	1		
LD	L25-10-09	27-0318	6/30/2025	08:32	6/30/2025	13:12	1	49	48	2	2			>2419.6	4.1		
LB	L25-10-10	-	6/30/2025	12:58	6/30/2025	13:12	1	0	0	0	0			<1	<1		
QC	L25-10-11	-	6/30/2025	12:58	6/30/2025	13:12	1	48	21	48	21			285.1	285.1		

Appendix E: Batch & Temperature Log

INCUBATOR # _____

[illegible]☐

Appendix F: Waste Receipt Form for “Bio-Waste”

WASTE RECEIPT FORM

GENERATOR _____ ID # M _____

RECEIVING FACILITY _____ ID # M _____

NAME OF WASTE DELIVERED: _____ AMOUNT: _____

SIGNATURE OF PERSON DELIVERING WASTE: _____ DATE: _____

SIGNATURE OF PERSON RECEIVING WASTE: _____ DATE: _____

GENERATOR COPY

WASTE RECEIPT FORM

GENERATOR _____ ID # M _____

RECEIVING FACILITY _____ ID # M _____

NAME OF WASTE DELIVERED: _____ AMOUNT: _____

SIGNATURE OF PERSON DELIVERING WASTE: _____ DATE: _____

SIGNATURE OF PERSON RECEIVING WASTE: _____ DATE: _____

Receiving Unit Copy

Colilert® QuickGuide

MassDEP Watershed Planning Program

(updated 03/21/25)

- 1) Check that the incubator is on and reading 35.0° C (+/-0.5°C) and record temperature on the log sheet (front of incubator). Clean all work areas with lab disinfectant. Sign chain of custody (in pocket of fridge) transferring sample custody from field crew (or fridge) to analyst.
- 2) Allow samples to warm up to near room temperature.
- 3) Turn on Quanti-Tray sealer to allow preheating (takes around 10 minutes). The switch is in the back near the plug. Sealer is ready to use when green light is on.
- 4) Fill out the bench sheet (in binder): batch number (next sequential number), sample IDs, lab sample #, and date/time collected. Add lab sample ID#s for a lab duplicate (if there is enough sample to split for a lab dup), positive control ("QC"), and lab blank ("LB").
- 5) Line up the sample bottles and equipment. Mark the back of the Quanti-Trays with the lab ID# of each sample.
- 6) Analysis order: work from the oldest samples to newest in the order they were collected. Sample analysis must be started within 8 hours of collection. QC samples (lab duplicates, QC samples) and dilutions can be done at any time during the analysis; lab blanks should be done last.
- 7) Prepare the control samples for each analysis batch:
 - a. Blank: add sterile water to an IDEXX sample bottle to the 100-ml line.
 - b. Positive control: add sterile water to an IDEXX sample bottle to the 100-ml line and add a lyophilized *E. coli* pellet to the sample. Re-cap and mix well.
 - c. Lab duplicate: using 290-ml field sample, mix sample well and pour 100-ml aliquot into a new 100-ml IDEXX bottle, and decant original bottle to the 100-ml line.
- 8) SAMPLES (without dilution): Mix the field sample (by inverting the bottle 20-25x). If necessary, pour off excess sample to the 100-ml line (bottom of meniscus). Avoid touching the inside of the bottle or lid.

Prepare any dilutions needed. (Look for any notes from the field crews indicating any suspected need for dilution.)

- a. 10 ML DILUTION SAMPLES: Pour 100 mL of sterile water into a new, sterile IDEXX vessel to as close as possible to the 100-ml line. Use a sterile pipette to pipette off 10 mL for a final volume of 90 mL of sterile water. Use the same pipette to transfer 10 mL of the original, mixed sample into the 90 mL of sterile water.
 - b. 100 ML DILUTION SAMPLES: Pour 100 mL of sterile water into a new, sterile IDEXX vessel as close as possible to the 100-ml line. Then use a sterile pipette to transfer 1 mL of the original, mixed sample into the 99 mL of sterile water. Cap and mix.
- 9) Open Colilert reagent packet (snap off top) and add entire contents to each 100-ml sample. Re-cap and mix thoroughly until no more reagent particles are visible. Pour into pre-labeled tray.
 - 10) Open the Quanti-Tray by pulling the foil tab away from the well side. Squeeze upper part of the tray gently so that it bends slightly to make a bigger opening. Avoid touching the inside of the foil or tray. Pour the whole 100-ml sample into the tray. Tap the small wells to release any air bubbles.
 - 11) Seal the trays: Put the Quanti-Tray well-side down on the sealer tray. Put the tray into the sealer with beveled edge first. The sealer will automatically pull the tray in.

(NOTES: If the Sealer stops in the middle of processing a Quanti-Tray, use the button with the arrows to reverse the direction of the Quanti-Tray. If the sealer does not easily accept the tray, lift the end of the tray slightly, so that the leading edge clears the opening of the sealer.)



Incubate all prepared Colilert Quanti-Trays for **24 - 28 hours**. (If Colilert-18 is used, incubate for 18-22 hours at 35 °C.) Record the incubation start time in the workbook.

12) Thoroughly clean and disinfect the work area.

13) After 24 hours (**18 hours for Colilert-18**), remove the trays from the incubator and record the incubation end time in the lab workbook. Check the incubator that the temperature reading is still 35.0° C (+/-0.5°C); record temperature on the log sheet (front of incubator).

14) Enterococci results: For each tray, count the number of large wells (including the biggest well) that turned yellow and number of small wells that turned yellow. (Count the well even if it has a smaller amount of sample if it is yellow.) Record the number of positive (yellow) wells in the workbook.

15) *E. coli* results: expose each tray to 365-nm UV light by placing them into the UV light viewing cabinet. Count the number of large wells and the number of small wells fluorescing. Record in the workbook.

16) Save a copy ("save as") of the **Ecoli_Lab_Book_Template** from OneDrive (Monitoring/WPP Lab SOPs and Results) with the new batch number as the file name.

17) Enter all results from the bench sheet into the electronic workbook; detailed directions are available in the "Read Me" tab. Final results will be automatically displayed in the blue-shaded "Total Coliform MPN" and "E coli MPN" columns.

18) After all results have been recorded and entered into the electronic workbook, check that all required data and metadata have been recorded completely and accurately. Keep the printed worksheet in the lab binder.



NOTE: Colilert and Colilert-18 results are definitive at 24-28 and 18-22 hours, respectively. Any positives for both total coliform and *E.coli* observed before the minimum time and negatives observed after the maximum time are also valid. Positive results after the maximum time are not valid.

EXAMPLE DATA ENTRY SHEET

<i>E. coli</i> Lab Book		Batch #		L25-10		Analyst Day 1	A. Gouvin-Moffat		Incubate Start Date/Time		6/30/25 13:35				
									Incubation Start Temp (°C)		34.8				
									Incubate End Date/Time		7/1/25 14:31				
									Incubation End Temp (°C)		35.6				
		Project		TAM C1-5 (2025)		Analyst Day 2	O. Hart		UV Read Date/Time		7/1/25 14:31				
Lab QC (LB, LD, QC, blank)	Lab ID Example: L25-###-##	OWMID Example: 71-####	Collection Date from COC	Collection Time from COC	Analysis Date	Analysis Time	Dilution (note 1:1)	# large yellow [of 49]	# small yellow [of 48]	# large fluor. [of 49]	# small fluor. [of 48]	Sample & Batch Comments	Total Coliform (MPN)	E. coli (MPN)	Recount if: # yellow > # fluorescent
	L25-10-01	27-0318	6/30/2025	08:32	6/30/2025	13:12	1	49	48	3	0		>2419.6	3.1	
	L25-10-02	27-0319	6/30/2025	09:20	6/30/2025	13:12	1	49	29	2	0		579.4	2	
	L25-10-03	27-0320	6/30/2025	09:20	6/30/2025	13:12	1	49	24	4	0		435.2	4.1	
	L25-10-04	27-0321	6/30/2025	09:24	6/30/2025	13:12	1	0	0	0	0		<1	<1	
	L25-10-05	27-0322	6/30/2025	10:09	6/30/2025	13:12	1	49	47	10	1		2419.6	12.1	
	L25-10-06	27-0323	6/30/2025	11:11	6/30/2025	13:12	1	48	48	43	16	Only 48 Large	1011.2	139.1	
	L25-10-07	27-0324	6/30/2025	11:43	6/30/2025	13:12	1	49	42	1	5		1299.7	6	
	L25-10-08	27-0325	6/30/2025	12:13	6/30/2025	13:12	1	49	39	1	0		1046.2	1	
LD	L25-10-09	27-0318	6/30/2025	08:32	6/30/2025	13:12	1	49	48	2	2		>2419.6	4.1	
LB	L25-10-10	-	6/30/2025	12:58	6/30/2025	13:12	1	0	0	0	0		<1	<1	
QC	L25-10-11	-	6/30/2025	12:58	6/30/2025	13:12	1	48	21	48	21		285.1	285.1	