

## Total Coliform/E. Coli – Presence/Absence SM9223B (18 Hours)

Reference: **SM 9223 B, Presence/Absence Chromogenic Substrate Test (Colilert)**. Standard Methods for the Examination of Water and Wastewater. APHA-AWWA-WEF. Standard Methods Online.

### 1. Scope and Application

**Matrices:** The chromogenic substrate coliform test is recommended for the analysis of drinking and clean source water samples.

**Definitions:** Refer to Alpha Analytical Quality Manual.

The chromogenic substrate coliform test utilizes hydrolyzable chromogenic substrates for the detection of enzymes of coliform bacteria. When the chromogenic technique is used the group is defined as all bacteria possessing the enzyme  $\beta$ -D-galactosidase and capable of cleaving the chromogenic substrate, resulting in release of the chromogen. Unlike lactose fermentation methods that permit growth of many aerobic organisms and eliminate or suppress some noncoliforms with inhibitory chemicals, this technique provides nutrients that are more selective and specific for coliform growth. Production of valid results requires strict adherence to quality control procedures.

In the original Colilert method (ie. The 24-28 hour method), the chromogenic substrates, such as ortho-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), are used to detect the enzyme  $\beta$ -D-galactosidase, which is produced by total coliform bacteria. The  $\beta$ -D-galactosidase enzyme hydrolyzes the substrate and produces a color change, which indicates and substantiates a positive test within 24 to 28 hours without additional procedures. Non-coliform bacteria, such as species of the genera *Aeromonas* and *Pseudomonas*, that produce small amounts of the enzyme  $\beta$ -D-galactosidase, are suppressed and will not produce a positive response within 28 hours unless more than  $10^4$  colony-forming units (CFU)/mL ( $10^6$  CFU/100 mL) are present.

However, in the Colilert-18 method, which is based on the patented DST (Defined Substrate Technology) procedure developed by the manufacturer, Idexx, the presence of total coliform only/and E.coli can be determined more quickly (18-22 hours). The total coliforms metabolize the nutrient-indicator, ONPG (described earlier), causing the sample to turn yellow. If E.coli is also present, this will metabolize the nutrient-indicator, MUG, causing the sample to fluoresce. The procedure is the same as the original Colilert method, except with a different reagent and takes  $\geq 25\%$  less time to determine a result.

The data report packages present the documentation of any method modification related to the samples tested. Depending upon the nature of the modification and the extent of intended use, the laboratory may be required to demonstrate that the modifications will produce equivalent results for the matrix. Approval of all method modifications is by one or more of the following laboratory personnel before performing the modification: Area Supervisor, Department Supervisor, Laboratory Director, or Quality Assurance Officer.

This method is restricted to use by or under the supervision of analysts experienced in microbiological analysis and in the interpretation of microbiological data. Each analyst must demonstrate the ability to generate acceptable results with this method by performing an initial demonstration of capability.

## 2. Summary of Method

For the Colilert-18 method: 100 mL of sample is mixed with chromogenic substrate (reagent specifically for Colilert-18 method), warmed in 44.5°C waterbath for 7-10 minutes, then incubated 18-22 hours at 35 ± 0.5°C. Yellow color indicates a positive result for total coliform. If positive for total coliform, the sample is inspected under UV light at 366nm. If the sample fluoresces, it is positive for E. Coli.

### 2.1 Method Modifications from Reference

None.

## 3. Reporting Limits

Results are reported as Total Coliform present or absent in 100mL sample and E. Coli present or absent in 100mL sample.

Results are reported individually for Total Coliform and for E.Coli as follows:

Presence or Positive results are defined as 1 or more cfu/100ml of sample ("cfu" stands for colony forming units").

Absence or Negative results are defined as <1 cfu/100ml of sample.

## 4. Interferences

### 4.1 Instrumental

Improper incubator temperature may inhibit Total Coliform growth.

### 4.2 Parameters

- 4.2.1 One interference common in this analysis is the negative interference due to the presence of bacteriacidal concentrations of chlorine or other halogen. This interference is countered in the sampling and preservation step by using sodium thiosulfate at a great enough concentration to counter the chlorine.
- 4.2.2 Another interference is the positive interference due to the use of non-sterile sample containers or the use of non-aseptic sampling technique allowing the contamination of the sample. This interference is countered by ensuring the use of properly sterilized containers. Non-sterile sampling technique is countered via education of the samplers.
- 4.2.3 Water samples containing humic or other material may be colored. If there is background color, compare inoculated vessels to a control vessel containing only water sample (Refer to Section 10.3.1).
- 4.2.4 Strict attention must be paid to the length of the incubation period. False positives may result if incubation exceeds 22 hours for the Colilert-18 method.

## 5. Health and Safety

The toxicity or carcinogenicity of each reagent and standard used in this method is not fully established; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. A reference file of material safety data sheets is available to all personnel

involved in the chemical analysis. Additional references to laboratory safety are available in the Chemical Hygiene Plan.

All personnel handling environmental samples known to contain or to have been in contact with municipal waste must follow safety practices for handling known disease causative agents.

## **6. Sample Collection, Preservation, Shipping and Handling**

### **6.1 Sample Collection**

**6.1.1** Samples for microbiological examination are collected in pre-sterilized specimen cups.

**6.1.2** The volume of sample collected should be sufficient to carry out all tests required, preferably no less than 100mL.

**6.1.2.1** If less than 100ml volume is submitted, then notify the client first to see if they are okay with doing dilutions, which will increase mdl/rdl information.

If the client cannot be reached before hold time is exceeded, set samples as needed.

Narrate any required dilutions necessary for limited sample volume.

**6.1.3** All sample containers must also contain at least one (1) inch of air space for adequate mixing of the sample prior to analysis.

### **6.2 Sample Preservation**

For the collection of samples having residual chlorine, pre-sterilized specimen cups containing sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) are used.  $\text{Na}_2\text{S}_2\text{O}_3$  is added to neutralize any residual halogen and prevent continuation of bacteriacidal action during sample transit. The analysis will therefore indicate more accurately the true microbial content of the water at the time of sampling.

### **6.3 Sample Shipping**

No specific requirements.

### **6.4 Sample Handling**

**6.4.1** Start microbial examination of water sample promptly after collection to avoid unpredictable changes. If samples cannot be processed within 1 hour after collection, use an iced cooler for storage during transport to the Laboratory.

**6.4.2** Refrigerate samples upon receipt in the laboratory at a temperature below 4°C, but do not freeze.

**6.4.3** Initiate analysis as soon as possible after collection to minimize changes in bacterial population. Analysis must be performed within 30 hours of collection for drinking water matrices, and within 8 hours of collection for non-drinking water matrices.

## 7. Equipment and Supplies

**7.1 Incubator:** 35 ±0.5°C

**7.2 UV Lamp:** Wavelength 366nm

**7.3 100mL Vessels:** Sterile, non-fluorescent, containing sodium thiosulfate. Volume is verified per lot, and recorded on the Filter Funnel and Colilert Container Form 18026.

**7.4 Auto-clavable Bottles:** 500mL, with caps.

**7.5 Autoclave Tape**

**7.6 Autoclave:** For use at 121 °C, 20psi

**7.7 Residual Cl- Strips:** 0.05mg/L Residual Chlorine sensitivity

**7.8 pH Paper Strips:** Range 0 – 14 pH units

**7.9 10ml Sterile dilution vials,** VWR cat. # 10018-740

**7.10 100ml Sterile specimen cups,** VWR cat # 470173-818 (or similar)

**7.11 Permanent markers (ie. Sharpies),** black ink

**7.12 Water Bath:** Circulating water bath with a stable temperature of 44.5 ± 0.2°C.

## 8. Reagents and Standards

### 8.1 Substrate

Colilert-18 hour method reagent, single doses for 100mL samples. Each lot of substrate is checked for viability using the organisms as defined in Section 8.5.

If results are not as indicated in Section 8.5, do not use the lot of Colilert Substrate.

Run a second test of the same lot of Colilert Substrate to confirm, and if the results are still failing manufacturer's criteria, then DO NOT USE, and contact the manufacturer.

Results are recorded on Form 18023.

### 8.2 Color Comparator

Obtained from the same manufacturer as the substrate reagent for Colilert-18.

### 8.3 Sterile DI

Dispense DI into 500mL autoclavable bottles and cap loosely. Place a clean strip of autoclave tape on each bottle. Label each bottle with a batch number and autoclave for 30 minutes at 121°C and 20 psi.

Once bottles have cooled, tighten the caps and store in the refrigerator (1 – 4 °C) until ready to use.

Prior to use, however, the following positive/negative check must be completed for each batch of sterile DI. This will confirm that the DI is indeed sterile and ready for use.

- ♦ Take one pre-made, 50mL glass bottle of sterile TSB (2x) (Section 8.4). Label it as the positive/negative check for the particular batch number of DI to be checked.
- ♦ Transfer 50mL of the sterile DI into the TSB (2x) using a sterile pipet.
- ♦ Incubate the bottle at  $35 \pm 0.5^{\circ}\text{C}$  for 24 hours.
- ♦ Examine bottle for turbidity. If turbidity is present, the DI is not sterile and the entire batch of DI is discarded. If the TSB (2x) remains clear, incubate the bottle for another 24 hours. If turbidity is present after 48 hours, the DI is not sterile and the entire batch is discarded. If the TSB (2x) remains clear after 48 hours, this indicates that there is no growth and the DI batch is sterile and ready for use.

Note these results in the Microbiology Dilution Water logbook (Form No.: 18012).

#### **8.4 Tryptic Soy Broth (TSB) x2 conc, glass bottle:**

Commercially prepared media from Lakewood Biochemical Co. Inc, Catalog #B40162.

Store at room temperature. Expires upon manufacturer's specified date.

Take 1 bottle of TSB to run sterility check for each Lot of the media.. No media should be used until it has been checked. This is also used for the sterility check on Sterile DI (Sec 8.3, container checks (Sec 7.3 and 7.10), prior to use in the lab.

Incubate the bottle of TSB at  $35 \pm 0.5^{\circ}\text{C}$  for  $48 \pm 2$  hours.

Examine bottles for turbidity. If the liquid is not clear, then there is a problem that needs to be corrected. The problem could lie in several different areas:

- 8.4.1** The TSB sterility check should be reset into a new bottle, and if the results pass, then the media is acceptable for use. If the results fail again, then discard that batch of media and use a new batch.

#### **8.5 Control Bacteria Test Kit by IDEXX for Coliform & E. Coli IDEXX PART # 98-29000-00, Purchased and stored in freezer until use.**

- 8.5.1** This kit contains E. Coli (EC), Klebsiella pneumoniae (KP), and Pseudomonas aeruginosa (PA). Follow instructions below to rehydrate and incubate. Use to verify each new lot of Colilert-18 Substrate (Section 8.1). Record results in the Colilert Reagent Check Log (Form 18023). Expected results will be:

**EC = Positive** for total coliform and **positive** for E. Coli.

**KP = Positive** for total coliform and **negative** for E. Coli.

**PA = Negative** for total coliform and **negative** for E. Coli.

**Blank** = Sterile DI set with each batch of reagent checks.

##### **8.5.2 Culture (Micro-organism) Preparation**

**Remove the Organism Kit from the freezer. Allow to warm to room temperature.**

Prepare one 100ml colilert vessel for each organism listed above, plus one vessel for the Blank. Fill each vessel with 100ml sterile DI.

Once warmed, open each vial, aseptically transfer the colored disc within to the coordinating labeled vessel and cap.

Swirl each vessel, then let sit for approximately 15 minutes, or until the disc is entirely dissolved.

Once dissolved, invert the vessel ten times to mix, then incubate for 22 hours per the method.

Record the final results in the laboratory notebook.

If any organisms do not pass the criteria with the reagent, the test must be rerun with a new organism kit, and the tested reagent lot must not be used in the lab until verified.

If the reagent lot fails a second test, it should be removed from the lab, not used, and a new lot of reagent should be purchased to test.

#### **8.6 Nutrient Broth, 500mL bottle by Teknova (product cat #N1260) 0.8% Nutrient Broth (8g/L formulation) Purchased commercially prepared: VWR, cat # 76062-100**

Store in the refrigerator at 1-4 °C. Use as needed, and allow to warm to room temperature before use. Expires upon manufacturer's specified date.

To check Nutrient Broth prior to use: Pour 30ml of broth into one sterile bacteria cup with volume line markings. Incubate 35 ±0.5°C for 48 ±2 hours; remove and check for turbidity/growth. If broth remained clear, then okay for use.

### **9. Quality Control**

The laboratory must maintain records to document the quality of data that is generated. Ongoing data quality checks are compared with established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

#### **9.1 Blank (Negative Check)**

Use 100mL of sterile DI in a 100mL sterile, non-fluorescent vessel with one dose of Colilert or Colilert-18 reagent. Analyze one Blank with each batch of ten or less samples. Blank results are expected to be Negative.

##### **Corrective Action:**

When the negative Blank check fails and is positive, the analyst must locate and correct the source of the problem. Notify the Laboratory Manager and/or the Department Supervisor immediately. Due to short hold-time involved, reanalysis would not be valid. Repeated failure confirms a general problem with the measurement system or analytical technique of the analyst.

- If the Blank is positive and all associated samples within the batch are negative, then the batch can be reported with a narrative, and no client contact is necessary.
- However, if the Blank is positive and any or all associated samples are positive, then the client must be contacted before any results (with appropriate narration) can be reported.

#### **9.2 Laboratory Duplicate**

A Duplicate is analyzed with each batch of ten or less samples for each microbiology analysis.

When the Duplicate sample matches the native sample, the results are both recorded in the Microbiology Logbook, and the native result is reported.

#### **Corrective Action:**

If the Duplicate sample result does not match the native sample result, then the results are still noted in the Microbiology Logbook, and the native result is still reported according to Laboratory Reporting Rules. But any differences would be communicated with Client Services immediately upon completion of the analysis, so that the client can be notified.

### **9.3 Method-specific Quality Control Samples**

None

### **9.4 Method Sequence**

- ◆ Sample Preparation:
  1. Blank
  2. Samples
  3. Duplicate (if sample volume permits)
- ◆ Check samples for Free Chlorine and pH.
- ◆ Colilert-18: Samples into water for 7-10 minutes prior to incubator.
- ◆ Any samples determined to be positive for CI must be re-checked after removal from the waterbath. The result (still positive or now negative) must be recorded in the logbook.
- ◆ Sample incubation at 35 ±0.5°C for 18-22 hours (Colilert-18).
- ◆ Sample inspection for yellow color change.
- ◆ Compare sample color to Color Comparator:
  1. If color is greater than or equal to the Comparator, the sample is positive for Total Coliform.
  2. If color is less than the comparator, the sample is negative for Total Coliform and E. Coli.
- ◆ Total Coliform positive samples are subjected to UV light.
  1. If sample fluoresces, it is positive for E. Coli.
  2. If sample does not fluoresce, it is negative for E. Coli.
- ◆ Report ANY positive results to Client Services Department **immediately after determination of results.**
  1. Client is notified within 24-hours

## **10. Procedure**

### **10.1 Equipment Set-up**

**10.1.1 Laboratory Notebook:** The lab notebook must contain the following information:

- ◆ Date/ Time/ Analyst
- ◆ Name of Test
- ◆ Sample Numbers
- ◆ Blank per 10 samples or less
- ◆ Duplicate per 10 samples or less (as volume permits)
- ◆ Sample dilutions

- ◆ pH
- ◆ Cl +/-
- ◆ Reagent lot # and expiration
- ◆ Sterile DI lot # and expiration
- ◆ Date/ Time/ Analyst: to water
- ◆ Date/ Time/ Analyst: to incubator
- ◆ Date/ Time/ Analyst: out of incubator
- ◆ Comments

**10.1.2** An analytical batch for microbiology analysis is defined as a group of up to ten samples processed as a unit. If the number of samples in a group to be analyzed is greater than ten, each group of ten or fewer samples is handled as a separate batch.

**10.1.3 Colilert Vessels:** Use one vessel per sample, another for a Blank, and another for the Duplicate (if sample volume permits). Using a permanent marker, label each vessel with the sample number for which it will be used.

## **10.2 Initial Calibration**

Not Applicable

## **10.3 Equipment Operation and Sample Processing**

**10.3.1** Shake each sample vigorously, and pour 100ml to the 100ml marked line in the labeled colilert vessel

Add one dose of Colilert-18 reagent Cap and mix thoroughly. (Reagent will not dissolve completely.)

Clients are requested to submit two containers per sample for all analyses in Microbiology.

If any submitted sample has any coloration, this could interfere with the interpretation of results after incubation.

To ensure that no coloration issues affect the final results, pour another 100mL (if sample volume permits from a second sample container) or else with whatever volume remains) into a separate Colilert vessel that does not contain the Substrate. Incubate this vessel along with the others to compare any color change that may occur.

Blank: 100mL of sterile DI with reagent pillow added.

Duplicate (if available sample)

A duplicate is analyzed per batch of 10 samples or less for each microbiology analysis

### **10.3.2 Check samples for presence of Chlorine**

The presence of chlorine is checked by using Residual Free Chlorine test strips (Section 7.8). These strips reveal if there is some level of Free Chlorine present in the sample by changing color. Compare color change (if any) with the color code on the product container. If no color change occurs, then the sample is recorded as a result of <0.05 mg/L. This test is performed after the sample has been set for analysis, (on the original sample container (not the analysis vessel)



Following manufacturer's instructions: Dip the Chlorine test strip into the original sample container and record results in the laboratory notebook: positive (color code on container) or negative (white) for Free Chlorine.

Any samples which have a reading of POSITIVE for Free Chlorine will be noted in the laboratory book, and CS will be notified.

At the beginning of the analysis, if any samples were positive for CI BEFORE the waterbath step, then they are rechecked for CI AFTER removal from the waterbath. Remove the cover of the sample vessel which needs to be rechecked: with the CI strip ready in hand, carefully drip 1 drop from the cover onto the strip and record the result (positive or negative) in the logbook. Once done, return the cover to the sample vessel and place into the incubator for the duration of the analysis.

If the sample remains positive for Free Chlorine AFTER the recheck procedure, this is considered a variance from the method. Therefore, the result is not only written into a laboratory logbook, but also submitted with the reported data, and CS is notified regarding this result.

#### 10.3.3 Check pH of Samples

The pH of the sample is checked by using the pH paper strips (Section 7.9). These strips reveal the pH by comparison to a color-coded chart. This test is performed after the sample has been set into Colilert vessels for analysis.

Dip the pH paper strip into the sample container and compare the strip color pattern to the coded chart on the box of pH paper strips. Record results in the laboratory notebook (Section 10.1.1).

#### 10.3.4 Sample Incubation

**Colilert-18 Method:** If the samples are not already at 33-38°C, the Colilert vessels are placed in a 44.5°C water bath for 7-10 minutes. Following this pre-warming step, the sample vessels are placed into the 35°C incubator for the remainder of the 18 to 22 hours.

**Record the time to water in the Micro logbook.** Then also record the time when the samples are moved from the water to the incubator. **The time that the samples are removed from the incubator is calculated as 18 to 22 hours from the time which the samples when into the water.**

After incubation, examine containers for color change. (Refer to Sec 10.3.1 for additional guidance). Yellow color is a positive reaction for Total Coliform. If the color is not uniform throughout, mix by inversion before reading. Use the color comparator as a guide to color intensity. If the color intensity is greater than or equal to that of the comparator, Total Coliforms are present. Samples are negative for Total Coliform if no color is observed, or color is less intense than the comparator.

**10.3.5 For the Colilert-18 method:** If a color response is questionable after 18 hours, incubate up to an additional 4 hours. **Do not exceed a total incubation time of 22 hours.** Record the 'Time In' and 'Time Out' of the incubator in the laboratory notebook (Section 10.1.1). If the color intensifies, the sample is positive for Total Coliform; if it does not, the sample is negative for Total Coliform and E. Coli.

**10.3.6** Submit all Total Coliform positive samples to inspection under UV light at 366nm. The UV light must be utilized in a darkened environment (i.e. under a box). If the sample fluoresces, it is positive for E. Coli; if it does not, the sample is negative for E. Coli.

**10.3.7** Record results in the laboratory notebook.

## 10.4 Continuing Calibration

Not Applicable

## 10.5 Preventive Maintenance

Incubators are calibrated on an annual basis by an instrument service company. These records are kept on file. The stability and uniformity of temperature distribution of any incubator must be established prior to use (refer to Work Instructions WI/07-04).

Temperatures of all incubators are recorded twice daily (with a minimum of 4 hours between each reading). Adjustments are made as necessary and noted on the data logger comments screen. The QA department monitors the daily recordings by the Data Logger.

All thermometers used within the laboratory are verified on a routine basis by the QA department, and labeled with expiration information.

The UV Lamp is kept clean and free of dust.

# 11. Data Evaluation, Calculations and Reporting

## 11.1 Calculations

Record in the laboratory notebook the presence (identified as "+",) or absence (identified as "-") of Total Coliform and E. Coli, in each corresponding column.

## 11.2 Reporting Sample Results

**11.2.1** Immediate notification to clients of positive results: Every effort is made to notify the Client within a 24-hour period of any findings other than negative.

The Microbiology Manager or Microbiology analyst notifies the Client Services Department of the sample number(s), Client name, analysis, and sample results. The Client Services Department notifies the Client.

**11.2.2** Data Entry to LIMS computer system for Report Generation:

All Microbiology Logbook results are transferred to in-house templated Excel sheets for each Microbiological analysis. The results are saved and approved through multiple steps in the Laboratory's LIMS computer system before the report is generated for the client

**11.2.3** Determination of reported analysis time:

The time of analysis reported for all samples reflects the time the samples are put into the incubator/water bath, which is recorded as "Time In" in the laboratory notebooks as indicated in Section 10.1.1

Colilert-18 Method: Report as the time the samples enter the water.

# 12. Contingencies for Handling Out-of-Control Data or Unacceptable Data

Holding time exceedance, and improper preservation are noted on the nonconformance report form.

Review of Blank and Duplicate response for acceptable performance occurs for each batch of samples. Record any trends or unusual performance on a nonconformance action form.

Refer to Section 9.1 and 9.2 for details.

## 13. Method Performance

### 13.1 Detection Limit Study (DL) / Limit of Detection Study (LOD) / Limit of Quantitation (LOQ)

The laboratory follows the procedure to determine the DL, LOD, and/or LOQ as outlined in Alpha SOP ID 1732. These studies performed by the laboratory are maintained on file for review.

This is not applicable for this method.

### 13.2 Demonstration of Capability Studies

Refer to Alpha SOP ID 1739 for further information regarding IDC/DOC Generation.

#### 13.2.1 Initial (IDC)

The analyst must make an initial, one-time, demonstration of the ability to generate acceptable accuracy and precision with this method, prior to the processing of any samples.

#### 13.2.2 Continuing (DOC)

The analyst must make a continuing, annual, demonstration of the ability to generate acceptable accuracy and precision with this method.

## 14. Pollution Prevention and Waste Management

All sample waste which is generated before, during, and after analysis is collected in a temporary waste collection zone (a box lined with an autoclave bag). When this becomes full, the waste is taken to the Hazardous Waste Room adjacent to the Loading Dock, and placed in the Fiber Drum labeled Medical Waste.

Refer to Alpha's Chemical Hygiene Plan and Hazardous Waste Management and Disposal SOP for further pollution prevention and waste management information.

## 15. Referenced Documents

2121 Chemical Hygiene Plan

1732 Detection Limit (DL), Limit of Detection (LOD) & Limit of Quantitation (LOQ) SOP

1739 Demonstration of Capability (DOC) Generation SOP

1728 Hazardous Waste Management and Disposal SOP

18010 Filter Funnel and Colilert Container Form

18012 Microbiology Dilution Water Logbook

18023 Colilert Reagent Check Form

## 16. Attachments

None.