9223 ENZYME SUBSTRATE COLIFORM TEST*

9223 A. Introduction

Enzyme substrate tests use hydrolyzable chromogenic and fluorogenic substrates to simultaneously detect enzymes produced by total coliforms and *Escherichia coli* (*E. coli*). In this method, total coliform bacteria produce the enzyme β-D-galactosidase, which cleaves the chromogenic substrate in the medium to release chromogen. Most *E. coli* strains produce the enzyme β-glucuronidase, which cleaves a fluorogenic substrate in the medium to release fluorogen. The release of chromogen indicates that coliform bacteria are present, and the release of fluorogen indicates that *E. coli* are present.

Multiple-tube, multi-well, or presence–absence (single 100-mL sample) formats are available for use with these enzyme substrate tests.

1. Principle

a. Total coliform bacteria: Colilert®, Colilert-18®, and Colisure® media use the chromogenic substrates ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and chlorophenol red-β-D-galactopyranoside (CPRG), respectively, to detect the enzyme β-D-galactosidase, which is produced by total coliform bacteria. The β-D-galactosidase enzyme hydrolyzes the chromogenic substrate that produces a color change, thereby indicating the presence of total coliforms without additional procedures.

Although non-coliform bacteria (e.g., *Aeromonas, Flavobacterium,* and *Pseudomonas* species) may produce small amounts of the enzyme β-D-galactosidase, the growth of these organisms is suppressed so they generally will not produce a false-positive result unless $>10^6$ CFU/100 mL are present.

b. Escherichia coli: The fluorogenic substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG) is used to detect the enzyme β-D-glucuronidase, which is produced by most strains of *E. coli.* The β-D-glucuronidase enzyme hydrolyzes the fluorogenic substrate that produces bluish fluorescence when viewed under long-wavelength (365–366 nm) ultraviolet (UV) light. Together, the color change (due to β-D-galactosidase) and the fluorescence (due to β-D-glucuronidase) indicate that a sample contains *E. coli.*

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 change its color because they lack β-D-galactosidase. Such samples would be considered negative for *E. coli.*

2. Applications

These enzyme substrate coliform tests are recommended for the analysis of drinking water, source water, groundwater, and wastewater samples. If a laboratory has not used this method before, it is desirable to conduct parallel testing (including seasonal variations) with the existing method to assess site-specific effectiveness and to compare results. The results of many method-performance studies are available in the literature and the rates of false-positive and -negative results differ among various media. Users should carefully select the medium and procedure that best fits their needs. See Section 9020B.11 for guidance on validating new methods.

Water samples containing humic or other material may be colored. If there is a natural background color, note what it is. If the water is yellow enough to be misinterpreted as a weak positive after incubation, use a medium that does not turn yellow (e.g., Colisure). Some waters' high calcium-salt content can cause precipitation, but this should not affect the reaction. In samples with excessive chlorine, a blue flash may be seen while adding Colilert or Colilert-18 media. If this occurs, consider sample invalid and discontinue testing.

Do not use the enzyme substrate test to verify presumptive coliform cultures or membrane-filter colonies, because the substrate may be overloaded by the heavy inoculum of weak β-D-galactosidase-producing noncoliforms, causing false-positive results.

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9223 B. Enzyme Substrate Test

1. Samples

Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. When collecting chlorinated water samples, use sodium thiosulfate as described in Section 9060A.2. Follow the quality control (QC) guidelines for sample bottles described in Section 9020B.5*d*. Adhere to sample holding times and conditions as described in Section 9060B or required by regulations. Take care to ensure that samples are held at the appropriate temperature and analyzed as soon as possible after sample collection because failure to do so could compromise results. Ensure that samples meet laboratory-acceptance criteria upon receipt.

2. Quality Control

Method users must adhere to the quality assurance (QA)/QC guidelines in Section 9020, including, but not limited to, analytical QC (Section 9020B.9), instrumentation/equipment (Sections 9020B.4 and 9030B), and supplies (Section 9020B.5). Refer to Table 9020:I for key QC procedures.

Before using each lot of new medium, verify its performance via positive and negative control organisms. To conduct culture controls, inoculate medium with three control bacteria: *E. coli*, a total coliform strain other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform (see Table 9020:VI). An uninoculated negative control should also be analyzed. In addition, test me-

dium and vessels (bottles, multi-well trays, tubes) to confirm sterility and lack of autofluorescence.

3. Substrate Media

Colilert, Colilert-18, and Colisure media are available commercially* in premeasured packets for presence–absence testing or in disposable tubes for use in a multiple-tube format. The Quanti-Tray® and Quanti-Tray/2000* are multi-well formats that may be used with the premeasured packets to quantitate the coliform bacteria present in a sample.

Store media according to directions and use before expiration date. Avoid prolonged exposure of media to direct sunlight. Discard media that have changed color, appearance, and/or texture (media are hygroscopic and will clump and darken if exposed to moisture).

4. Procedure

Begin analysis by mixing the sample properly to promote even distribution of bacteria. For proper mixing to occur, samples should have ≥1-in. headspace and be shaken vigorously for 7 s (back and forth 1 ft approximately 25 times).

Failure to properly mix sample can lead to erroneous results, as bacteria are known to clump together and are therefore not homogeneously distributed throughout sample. For instance, most probable number (MPN) results are based on a Poisson (random) distribution of cells in the sample; failure to properly mix sample before analysis will result in an MPN value that underestimates actual bacterial density. Removing a portion of sample without proper mixing—such as when performing presence–absence analyses with a single bottle (one bottle used to both collect and analyze sample)—may result in false negative results if the target organisms were clumped together and removed from the bottle without being homogenized.

If the bottle lacks enough headspace for adequate mixing, pour sample into a larger sterile vessel so it can be mixed properly. Measure out desired sample volume and proceed with analysis.

For each medium or format used, tests should be placed in the incubator within 30 min after medium is added to sample. No matter which format is used, all media must be incubated at 35 ± 0.5°C. Colilert medium must be incubated for ≥24 h, Colilert-18 medium must be incubated for ≥18 h, and Colisure medium must be incubated for ≥24 h.

The coliform tests described here have been developed to obtain optimal bacterial growth at the indicated incubation temperatures. Failure to maintain this temperature throughout incubation could result in false negative results, especially with the shorter incubation times for Colilert-18. To ensure that samples are at proper temperature for the entire incubation period, laboratories should pre-warm samples after adding medium but before placing them in the incubator.

To pre-warm a test sample, place it in a 35 ± 0.5°C water bath for 20 min or in a 44.5 ± 0.2°C waterbath for 7 to 10 min to bring it to incubation temperature. The laboratory may need to conduct load studies to determine how long samples need to be incubated for effective pre-warming (depends on number of samples being incubated). Pre-warming is unnecessary if the Quanti-Tray format is used.

a. Presence–absence procedure (P/A): Aseptically add contents of packet containing premeasured medium to a 100-mL sample in a sterile, transparent, non-fluorescent borosilicate glass or equivalent bottle or container. Aseptically cap and shake vigorously to dissolve medium. Some medium may remain undissolved, but this will not affect test performance.

b. Multiple-tube procedure:

1) Multiple-tube procedure using a 5- or 10-tube MPN test—A 5-tube series (20 mL sample per tube) or 10-tube series (10 mL sample per tube) can be used when bacteria levels are anticipated to be fairly low or a fixed 100-mL sample volume must be analyzed (e.g., for regulatory compliance).

Add a premeasured packet of medium to a well-mixed 100-mL water sample in a container and shake vigorously to dissolve medium. 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 5 sterile tubes or 10 mL into each of 10 sterile tubes, cap tightly, and mix vigorously to dissolve medium. If using 10 tubes already containing premeasured medium (available from manufacturer), aseptically dispense 10 mL sample into each tube.

Some medium particles may remain undissolved; this will not affect test performance.

After incubation, refer to Tables 9221:II and III to determine the MPN of total coliforms and *E. coli* present.

2) Multiple-tube procedure using 15-tube MPN test—A 15-tube test typically involves three serial dilutions of a sample, with each dilution inoculated into 5 tubes. Typically, 5 tubes contain undiluted sample, 5 contain a 1:10 dilution, and 5 contain a 1:100 dilution.

Use this technique when a water sample may contain higher bacteria levels and there is no requirement to analyze a fixed volume (e.g., when analyzing nonpotable waters). The number of tubes and sample volumes selected depend on the quality and characteristics of the water to be examined. To preclude any unwanted interaction with the medium, use only sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water) to prepare dilutions.

When working with diluted samples, best laboratory practice is to ensure that all tubes are in place and labeled before analysis begins. Additionally, use clean, sterile pipets to pipet each dilution because bacterial carryover from dirty pipets will make test results inaccurate.

a) Using disposable tubes containing premeasured medium (available from manufacturer)

i) Preparing sample for the undiluted series—Aseptically pipet 10 mL of well-mixed sample into each of 5 tubes containing predispensed medium. Cap tubes and mix vigorously to dissolve medium.

ii) Preparing 1:10 dilution—Aseptically pipet 10 mL of well-mixed sample into a sterile vessel containing 90 mL of sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water). Mix well. Aseptically pipet 10 mL of this dilution into each of 5 tubes containing pre-dispensed medium. Cap tubes and mix vigorously to dissolve medium.

iii) Preparing 1:100 dilution—Aseptically pipet 10 mL of well-mixed sample from the 1:10 dilution into a sterile vessel containing 90 mL of sterile, non-buffered, oxidant-free water

* Available from IDEXX Laboratories, Inc., Westbrook, ME.

(e.g., deionized or distilled water). Mix well. Aseptically pipet 10 mL of this dilution into each of 5 tubes containing pre-dispensed medium. Cap tubes and mix vigorously to dissolve medium.

b) Using packets of premeasured medium

i) Preparing sample for the undiluted series—Add one packet of premeasured medium to a sterile vessel containing 100 mL of well-mixed sample, and mix vigorously to dissolve medium. Aseptically pipet 10 mL of sample/medium mixture into each of 5 sterile, non-fluorescing tubes.

ii) Preparing 1:10 and 1:100 dilutions—Add one packet of premeasured medium to 100 mL sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water) in a sterile container, and mix vigorously to dissolve medium. Aseptically pipet 9 mL of prepared medium into 10 sterile, non-fluorescing tubes. This preparation of enzyme substrate medium must be completed ≤1 h of adding sample to prepared medium.

iii) Inoculating tubes for 1:10 dilution—Aseptically pipet 1 mL of well-mixed sample into each of 5 tubes containing 9 mL of prepared medium. Cap and mix well.

iv) Inoculating tubes for 1:100 dilution—Pipet 10 mL of well-mixed sample into a vessel containing 90 mL sterile, non-buffered, oxidant-free water (e.g., deionized or distilled water). Close and mix well to dissolve medium. Aseptically pipet 1.0 mL of this diluted sample into 5 tubes containing 9 mL of prepared medium. Cap and mix well.

For any additional dilutions needed, continue with the dilution process as described above.

After incubation, use Table 9221:IV to determine the MPN for both total coliforms and *E.coli*. If further dilutions were performed, the MPN value must be multiplied by the dilution factor to obtain the proper quantitative results.

c. Multi-well procedure: This procedure is performed with sterilized disposable multi-well trays [either the Quanti-Tray (51 well) or Quanti-Tray/2000]. Aseptically add premeasured medium from packet to a 100-mL water sample in a container and shake vigorously to dissolve medium. To open Quanti-Tray, use one hand to hold unit upright (with the well side facing the palm) and squeeze the upper part of the tray so it bends toward the palm. Gently pull foil tab to separate foil from tray, being careful not to touch the inside of either foil or tray. Add reagent-water sample mixture directly into tray, avoiding contact with foil tab. Gently tap the small wells (Quanti-Tray/2000) 2 to 3 times to release any air bubbles that may be trapped. Allow foam to settle, although some foam is acceptable. Place tray into the appropriate rubber insert with the well (plastic) side facing down, and feed it into the Quanti-Tray sealer. The sealer disperses the sample into the wells and seals the package.

5. Interpretation

a. Total coliform bacteria: The bacterial enzyme β-D-galactosidase hydrolyzes ONPG (Colilert and Colilert-18) to yield a yellow color and hydrolyzes CPRG (Colisure) to yield a red or magenta color. After the minimum incubation period, examine for the appropriate color change (Table 9223:I). If color response is not uniform throughout sample, mix by inversion before reading.

Use an unexpired color comparator (available from manufacturer) to ensure that Colilert and Colilert-18 test results are read accurately. The comparator used must have the same volume in the same type of container as the sample.

TABLE 9223:I. COLOR CHANGES FOR VARIOUS MEDIA

Substrate	Total Coliform Positive	*E. coli* Positive	Negative Result
Colilert® Colilert-18®	Yellow	Blue fluorescence	Colorless or color lighter than the comparator/no fluorescence
Colisure®	Red or magenta	Blue fluorescence	Yellow, pink, or orange/no fluorescence

1) Colilert—If sample color is as yellow as or darker yellow than the comparator, then it 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) after 24 h, incubate sample for up to 4 h longer to allow test color to intensify. If the color does become as yellow as or darker than that of the comparator within this period, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms.

Colilert can be incubated for ≤28 h. After 28 h, negative test results are still considered valid, but positive results are not.

2) Colilert-18—If sample color is as yellow as or darker yellow than the comparator, then it is positive for total coliforms. If not, then it is negative for total coliforms.

However, if the chromogenic response is ambiguous (color cannot be discerned) after 18 h, incubate sample for up to 4 h longer to allow the test color to intensify. If the color does become as yellow as or darker than that of the comparator within this period, then the sample is positive for total coliforms. If not, then the sample is negative for total coliforms.

Colilert-18 can be incubated for ≤22 h. After 22 h, negative test results are still considered valid, but positive results are not.

3) Colisure—If the sample has a red or magenta color, it is positive for total coliforms. If the chromogenic response is questionable (color may be orange or pink) after 24 h, incubate sample for up to 24 h longer to allow test color to intensify. If color does become red or magenta within this period, then the sample is positive for total coliforms.

Colisure tests turn yellow after medium is added; if color does not change to red or magenta after incubation, then the sample is negative for total coliforms.

Colisure can be incubated for ≤48 h. After 48 h, results are not valid.

Sometimes a sample's high calcium-salt content can cause precipitation, but this will not affect the reaction. However, if the test medium turns an inappropriate color (e.g., green or black) that interferes with test-result reading, another method must be used.

b. Escherichia coli: The fluorogenic substrate MUG is hydrolyzed by the bacterial enzyme ß-D-glucuronidase to yield a bluish fluorescence when viewed under long-wavelength (365–366 nm) UV light. The 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 minimum incubation period, examine positive total coliform tests for a bluish fluorescence; use a long-wavelength (365–366 nm) UV lamp with a 6-W bulb and hold it within 5 in. of sample in a dark environment. Use a color comparator (available

from the manufacturer) before its expiration date to ensure that test results are read accurately. The comparator used must have the same volume in the same type of container as the sample.

1) Colilert—If the sample has a bluish fluorescence equal to or greater than that of a total-coliform-positive comparator, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned) after 24 h, the sample may be incubated for up to 4 h longer to allow the fluorescence to intensify. If sample fluorescence does intensify to equal to or greater than that of the comparator within this period, then the sample is positive for *E. coli*.

If sample florescence remains less than that of the comparator after 28 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

2) Colilert-18—If the sample has a bluish fluorescence equal to or greater than that of a total-coliform-positive comparator, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned), the sample may be incubated for up to 4 h longer to allow the fluorescence to intensify. If sample fluorescence does intensify to equal to or greater than that of the comparator within this period, then the sample is positive for *E. coli*.

If sample florescence remains less than that of the comparator after 22 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

3) Colisure—If a total-coliform-positive sample fluoresces, then it is positive for *E. coli*. If the fluorescence is ambiguous (cannot be discerned), the sample should be incubated for up to 24 h longer to allow the fluorescence to intensify. If the sample clearly fluoresces within this period, then it is positive for *E. coli*.

If sample does not fluoresce after 48 h of incubation, then it is negative for *E. coli*. Samples that are negative for total coliform bacteria are also negative for *E. coli*.

6. Reporting

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

For the multiple-tube procedure, calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes, as described in Section 9221C.

For the multi-well procedure, determine the MPN from the appropriate MPN tables obtained from the tray manufacturer.

7. Bibliography

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