9223 ENZYME SUBSTRATE COLIFORM TEST*

9223 A. Introduction

The enzyme substrate test utilizes hydrolyzable substrates for the simultaneous detection of total coliform bacteria and *Escherichia coli* enzymes. When the enzyme technique is used, the total coliform group is defined as all bacteria possessing the enzyme β-D-galactosidase, which cleaves the chromogenic substrate, resulting in release of the chromogen. *Escherichia coli* are defined as bacteria giving a positive total coliform response and possessing the enzyme β-glucuronidase, which cleaves a fluorogenic substrate, resulting in the release of the fluorogen. The test can be used in a multiple-tube, multi-well, or a presence-absence (single 100-mL sample) format.

1. Principle

a. Total coliform bacteria: Chromogenic substrates, such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG) or chlorophenol red-β-D-galactopyranoside (CPRG), are used to detect the enzyme β-D-galactosidase, which is produced by total coliform bacteria. The β-D-galactosidase enzyme hydrolyzes the substrate and produces a color change, which indicates a positive test for total coliforms at 18 and 24 h (ONPG) or 24 h (CPRG) without additional procedures. Noncoliform bacteria, such as *Aeromonas, Flavobacterium,* 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 10^4 colony-forming units (CFU)/mL (10^6 CFU/100 mL) are present.

b. Escherichia coli: A fluorogenic substrate, such as 4-methylumbelliferyl-β-D-glucuronide (MUG), is used to detect the enzyme β-glucuronidase, which is produced by *E. coli.* The β-glucuronidase enzyme hydrolyzes the substrate and produces a fluorescent product when viewed under long-wavelength (365-nm) ultraviolet (UV) light. The presence of fluorescence indicates a positive test for *E. coli.* Some strains of *Shigella* and *Salmonella* spp. also may produce a positive fluorescence response. Because *Shigella* and *Salmonella* spp. are overt human pathogens, this is not considered a detriment for testing the sanitary quality of water.

2. Applications

The enzyme substrate coliform test is recommended for the analysis of drinking and source water samples. Formulations also are available for the analysis of marine waters. Initially, laboratories planning to use this procedure should conduct parallel quantitative testing (including seasonal variations) with one of the standard coliform tests to assess the effectiveness of the test for the specific water type being analyzed and to determine the comparability of the two techniques. This is particularly important when testing source waters.

Water samples containing humic or other material may be colored. If there is background color, compare inoculated tubes to a control tube containing only water sample. In certain waters, high calcium salt content can cause precipitation but this should not affect the reaction.

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.

* Approved by Standard Methods Committee, 2004.
Joint Task Group: Carol J. Palmer (chair), Terry C. Covert, Robert E. Grant, Nancy H. Hall, Eugene W. Rice, Bruce M. Roll, Helena M. Solo-Gabriele.

9223 B. Enzyme Substrate Test

1. Substrate Media

Formulations are available commercially* in premeasured packets for presence-absence or quantification† and disposable tubes for the multiple-tube procedure.* The need for good quality assurance and uniformity requires the use of a commercial substrate medium. Avoid prolonged exposure of the substrate to direct sunlight. Store media according to directions and use before expiration date. Discard colored media.

2. Procedure

a. Multiple-tube procedure: Select the appropriate number of tubes per sample with predispensed media for the multiple-tube test and label. Follow manufacturer's instructions for preparing serial dilutions for various formulations. Aseptically add 10 mL sample to each tube, cap tightly, and mix vigorously to dissolve. The mixture remains colorless with ONPG-based tests and turns yellow with the CPRG format. Some particles may remain undissolved throughout the test; this will not affect test performance. Incubate at 35 ± 0.5°C for period specified by substrate manufacturer.

The procedure also can be performed by adding appropriate amounts of the substrate media to the sample, mixing thoroughly, and dispensing into five 20-mL or ten 10-mL sterile tubes. Incubate as stated for multiple-tube procedure.

* Colilert® and Colilert 18® and Colisure™ for multi-tube, P/A, and tray formats available from IDEXX Laboratories, Inc., Westbrook, ME.
† Quanti-Tray® or Quanti-Tray®/2000, available from IDEXX Laboratories, Inc., Westbrook, ME.

TABLE 9223:I. COLOR CHANGES FOR VARIOUS MEDIA

Substrate	Total Coliform Positive	*E. coli* Positive	Negative Result
ONPG-MUG	Yellow	Blue fluorescence	Colorless/no fluorescence
CPRG-MUG	Red or magenta	Blue fluorescence	Yellow/no fluorescence

b. Multi-well procedure: The multi-well procedure is performed with sterilized disposable packets. Add enzyme substrate to a 100-mL sample in a container, shake vigorously, and pour into tray. The tray sealer dispenses the sample into the wells and seals the package. Incubate at 35 ± 0.5°C for period specified by substrate manufacturer. The MPN value is obtained from the table provided by the manufacturer.

c. Presence-absence procedure (P/A): Aseptically add preweighed enzyme medium to 100-mL sample in a sterile, transparent, nonfluorescent borosilicate glass or equivalent bottle or container. Optionally, add the enzyme substrate to a 100-mL sample in a sterile nonfluorescent container that is purchased commercially. Aseptically cap and mix thoroughly to dissolve. Incubate as specified in manufacturer's instructions.

3. Interpretation

a. Total coliform bacteria: After the minimum proper incubation period, examine tubes or containers for the appropriate color change (Table 9223:I). ONPG is hydrolyzed by the bacterial enzyme to yield a yellow color. CPRG is hydrolyzed by the bacterial enzyme to yield a red or magenta color. If the color response is not uniform throughout the sample, mix by inversion before reading. Read manufacturer's instructions for interpretation guidelines. Some manufacturers suggest comparing sample against a color comparator available through the manufacturer. Samples are negative for total coliforms if no color is observed in ONPG tests or if the tube is yellow when CPRG is used. If a chromogenic response is questionable after 18 or 24 h for ONPG, incubate up to an additional 4 h. If response is negative after 24 h for CPRG, incubate up to an additional 24 h. If the chromogen intensifies, the sample is total-coliform positive; if it does not, the sample is negative.

b. Escherichia coli: Examine positive total coliform tubes or containers for fluorescence using a long-wavelength (365-nm) ultraviolet lamp (6-W bulb). Compare each tube against the reference comparator available from a commercial source of the substrate. The presence of fluorescence is a positive test for *E. coli*. If fluorescence is questionable, incubate for an additional 4 h for ONPG tests and up to an additional 24 h for CPRG tests; intensified fluorescence is a positive test result.

4. Reporting

If performing an MPN procedure, calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes as described in Section 9221C. If using the presence-absence procedure, report results as total coliform and *E. coli* present or absent in 100-mL sample.

5. Quality Control

Test each lot of media purchased for performance by inoculation with three control bacteria: *Escherichia coli,* a total coliform other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform. Also add a sterile water control. If the sterile water control exhibits faint fluorescence or faint positive coliform result, discard and use a new batch of substrate. Avoid using a heavy inoculum. If *Pseudomonas* is used as the representative noncoliform, select a nonfluorescent species. Incubate these controls at 35 ± 0.5°C as indicated above. Read and record results. Other quality-control guidelines are included in Section 9020.

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