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 10⁷ colony-forming units (CFU)/mL (10⁶ 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.

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.

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2. Procedure

   a. Multiple-tube procedure: Select the appropriate number of tubes per sample with predisposed 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.
TABLE 9223:I. COLOR CHANGES FOR VARIOUS MEDIA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total Coliform</th>
<th>E. coli</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>ONPG-MUG</td>
<td>Yellow</td>
<td>Blue</td>
</tr>
<tr>
<td>CPRG-MUG</td>
<td>Red or magenta</td>
<td>Blue</td>
</tr>
</tbody>
</table>

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.

6. Bibliography


