



## Standard Test Method for Isolation and Enumeration of *Pseudomonas aeruginosa* from Water

This standard is issued under the fixed designation D 5246; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon ( $\epsilon$ ) indicates an editorial change since the last revision or reapproval.

### 1. Scope

1.1 This test method covers the isolation and enumeration of *Pseudomonas aeruginosa* (*P. aeruginosa*) from surface waters; recreational waters; ground water, water supplies; especially rural nonchlorinated sources; waste water; and saline waters. The detection limit of this test method is one microorganism per 100 mL.

1.2 This test method was used successfully with reagent water and it is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 10.

### 2. Referenced Documents

#### 2.1 ASTM Standards:

- D 1129 Terminology Relating to Water<sup>1</sup>
- D 1193 Specification for Reagent Water<sup>1</sup>
- D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water<sup>1</sup>
- D 3370 Practices for Sampling Water from Closed Conduits<sup>1</sup>

### 3. Terminology

#### 3.1 Definitions:

3.1.1 For definitions of terms used in this test method, refer to Terminology D 1129.

#### 3.2 Definitions of Terms Specific to This Standard:

3.2.1 *Pseudomonas aeruginosa*—an aerobic, motile, gram negative rod that produces fluorescent pigments and pyocyanin. It is oxidase and caseinase positive, is able to grow at 42°C, is relatively resistant to many antibiotics, and may utilize acetamide.

3.2.2 *refrigeration*—storage at 2 to 8°C.

### 4. Summary of Test Method

4.1 A water sample is passed through a 0.45 mm or equivalent membrane filter. The filter carrying the retained organisms is placed on a selective medium (M-PA-C)<sup>2</sup> and is incubated at 41.5 ± 0.5°C for 48 to 72 h. The resulting pink-brown to black colonies of *Pseudomonas aeruginosa* are counted and reported per 100 mL of the sample. Colonies may be verified on skim milk agar.

### 5. Significance and Use

5.1 *Pseudomonas aeruginosa* is an opportunistic pathogen, and has been linked as the causative agent of numerous infections that may be transmitted through a contaminated water supply to a susceptible host. In addition to its direct pathogenicity, the association of *P. aeruginosa* with human fecal waste indicates that where elevated levels of *P. aeruginosa* are found, a serious health hazard may exist due to the presence of other pathogens.

5.2 The membrane filtration procedure described is a rapid and reliable test method of detecting *P. aeruginosa* in water.

### 6. Interferences

6.1 For certain samples, bacterial cells may have been exposed to adverse environmental factors that lower their probability for survival and growth on a membrane filter medium. This effect may be pronounced in this test method due to the presence of antibiotics and the elevated incubation temperature.

6.2 The selection of an appropriate dilution volume is essential. Too small a dilution volume may fail to detect any *P. aeruginosa* organisms, while too large a volume may cause an overabundance of colonies that would interfere with an accurate count.

6.3 Chemicals or a combination of chemicals in certain samples can have a toxic effect upon *P. aeruginosa* when concentrated.

<sup>1</sup> Annual Book of ASTM Standards, Vol 11.01.

<sup>2</sup> Available from BBL Microbiological Systems, Division of Becton Dickinson and Co., Cockeysville, MD 21030. Other suppliers may be utilized if equivalent.

6.4 Turbidity in samples may clog filter or effect color detection of organisms that develop on the filter.

6.5 Water samples containing residual chlorine can be detrimental to *P. aeruginosa*. Utilize the procedure defined in Practices D 3370 to address chlorinated water samples.

## 7. Apparatus

7.1 *Top-Loading Balance*, sensitive to 0.1 g.

7.2 *pH Meter and Surface pH Electrode*.

7.3 *Incubator*, capable of maintaining temperature of  $41.5 \pm 0.5^\circ\text{C}$  and  $35 \pm 0.5^\circ\text{C}$ .

7.4 *Stereoscopic Microscope*, with a cool white fluorescent light.

7.5 *Colony Counter*.

7.6 *Containers*, with lids (for incubating test petri dishes containing membrane filters under high humidity).

7.7 *Long-Wave Ultraviolet Light*.

7.8 *Autoclave*, or other sterilizing equipment.

7.9 *Petri Dishes*, sterile, 50 by 9 or 60 by 15 mm and 100 by 15 mm.

7.10 *Pipets*, sterile, 1 and 10 mL, with 0.1-mL graduations and an accuracy of  $\pm 5\%$ .

## 8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.<sup>3</sup> Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*— Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Type II of Specification 1193.

8.3 *Buffered Water*— Dispense 1.25 mL of buffered water stock solution and 5.0 mL magnesium chloride solution (see 8.5) and dilute to 1 L with water. Dispense in amount to provide 99 mL after sterilization.

8.4 *Buffered Water Stock*—Dissolve 34.0 g potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in 500 mL water, adjust to pH 7.2 with KOH solution (5.6 g/L) and dilute to 1 L with water.

8.5 *Magnesium Chloride Solution* (81.1 g/L)—Dissolve 81.1 g magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) in water and dilute to 1 L with water.

8.6 *Potassium Hydroxide Solution* (5.6 g/L)—Dissolve 5.6 g of potassium hydroxide (KOH) in water and dilute to 1 L with water.

8.7 *Membrane Filters*, sterile, 47 mm with grid (0.45  $\mu\text{m}$  pore size) or equivalent.

## 9. Media Preparation

9.1 *M-PA-C Medium*<sup>2</sup>—Formula per litre of water:

L-lysine	5.0 g
Sodium chloride	5.0 g
Yeast extract	2.0 g
Xylose	1.25 g
Sucrose	1.25 g
Lactose	1.25 g
Phenol red	0.08 g
Ferric ammonium citrate, anhydrous	0.80 g
Sodium thiosulfate, anhydrous	5.0 g
Agar	12.0 g
Magnesium sulfate, anhydrous	1.5 g
Kanamycin	0.008 g
Nalidixic acid	0.037 g

9.1.1 *M-PA-C Medium*<sup>2</sup> or *Equivalent*—Dissolve mixture of above items into 1 L of water, boiling for 1 min to solubilize the chemicals. Cool to 45 to 50°C before dispensing. Pour one plate of medium and measure the pH of the surface with a suitable pH electrode. The surface pH of the solidified medium should be  $7.2 \pm 0.1$ . If it is not, adjust pH of the remaining solution accordingly with potassium hydroxide solution.

9.1.2 Aseptically dispense 5 to 6 mL of media into each sterile 50 or 60 mm petri dish. This medium should be stored under refrigeration and used within one week after preparation.

9.2 *Skim Milk Agar*— Skim milk powder is high grade skim milk reduced to powder by a spraying process. Slowly add 100 g of skim milk powder to 500 mL of water and stir without heat for approximately 30 min. Prepare an agar solution by adding 15.0 g of agar to 500 mL of water and heat at 90°C for 10 to 12 min. Autoclave the solutions separately at 121°C for 12 min. Cool, with stirring, until temperature reaches 50 to 55°C. Add the skim milk solution to the agar solution, thoroughly mix, and dispense aseptically into sterile petri plates. The plates may be stored in sealed containers in the refrigerator for up to two weeks.

9.3 *Soybean Casein Digest Agar*<sup>4</sup>—Formula per litre of water:

Pancreatic digest of casein	15.0 g
Papaic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

9.3.1 *Soybean Casein Digest Agar*—Prepare the media according to manufacturer's instructions and dispense it aseptically into sterile petri dishes.

## 10. Hazards

10.1 *P. aeruginosa* is an opportunistic human pathogen; thus handle all culture material (plates, pipets, dilution tubes) using accepted microbiological techniques, including sterilization of all discarded material.

## 11. Sampling, Test Specimens, and Test Units

11.1 Collect the sample according to Practices D 3370, refrigerate, and analyze the sample within 6 h.

<sup>3</sup> Reagent Chemicals, American Chemical Society Specifications. Am. Chem. Soc., Washington, D.C. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards For Laboratory Chemicals," BDH Ltd., Poole, Dorset, UK, and the "United States Pharmacopeia."

<sup>4</sup> Difco or BBL Trypticase Soy Agar, available from BBL Microbiological Systems, Division of Becton Dickinson and Co., Cockeysville, MD 21030. Other suppliers may be utilized if equivalent.