Designation: ~~D5246 – 92 (Reapproved 2004)~~ D5246 – 13

Standard Test Method for Isolation and Enumeration of *Pseudomonas aeruginosa* from Water[1]

This standard is issued under the fixed designation D5246; 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 (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 ~~This~~The test method covers the isolation and enumeration of *Pseudomonas ~~aeruginosa~~aeruginosa.* ~~(~~Testing~~P. aeruginosa was) 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.~~ performed on spiked reagent grade water samples.

1.2 ~~This test method was used successfully with reagent water and it is the user's~~It is the user's responsibility to ensure the validity of this ~~test~~ method for ~~waters of untested matrices.~~surface waters, ground waters, recreational waters fresh and marine), wastewaters.

1.3 The values stated in SI units are to be regarded as ~~the~~ standard. No other units of measurement are included in this 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:*[2]

D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water
D3370 Practices for Sampling Water from Closed Conduits

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this test method, refer to Terminology D1129.

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)[3] 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~~

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from BBL Microbiological Systems, Division of Becton Dickinson and Co., Cockeysville, MD 21030. Other suppliers may be utilized if equivalent.

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.

NOTE 1—Fecal waste is >95 % *E. coli* which is found in humans and warm bloodied animals.

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.

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 D3370 to address chlorinated water samples.

7. Apparatus

7.1 Equipment for collection and transport of samples to laboratory:
7.1.1 Autoclavable sample container; Use sterile, non-toxic, glass or plastic containers with a leak-proof lid. Ensure that the sample container is capable of holding a 1-L sample with ample headspace to facilitate mixing of sample by shaking prior to analysis.
7.1.2 Ice chest.
7.1.3 Ice packs.

7.2 Rinse water bottles, sterile, polypropylene or glass.

7.3 Pipettes, sterile, plastic or autoclavable glass pipettes with a 2.5 % tolerance with pipette bulbs or automatic pipette.

7.4 Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional).

7.5 Pipette container, autoclavable stainless steel, aluminum or borosilicate glass (if using glass pipettes).

7.6 Inoculation loops, at least 3 mm diameter, and needles, nichrome or platinum wire, 26 B&S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.

7.7 Graduated cylinders from 100 mL to 1 L, sterile, polypropylene or glass covered with aluminum foil or kraft paper.

7.8 Temperature monitoring device, glass, dial, or electronic thermometer graduated in 0.5°C increments, checked against a National Institute of Standards and Technology (NIST) certified thermometer or one traceable to an NIST thermometer.

7.9 *Top-Loading Balance,* sensitive to Balance, with a readability of 0.1 g.

7.10 *pH Meter and Surface pH Electrode.* pH Meter, with accuracy ±0.1 units and equipped with surface electrode.

7.11 Equipment for membrane filter procedure:
7.11.1 Petri dishes, sterile, plastic, 9 × 50 mm, with tight-fitting lids; or 15 × 60 mm, glass or plastic, with loose-fitting lids; or 15 × 100 mm.
7.11.2 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterilized. Purchased disposable plastic sterile filters can also be used.
7.11.3 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size.
7.11.4 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional).
7.11.5 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.
7.11.6 Filter flask, vacuum, usually 1 L, with appropriate tubing.
7.11.7 Flask for safety trap placed between the filter flask and the vacuum source.
7.11.8 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size.
7.11.9 Flame or electric incinerator for sterilizing metal inoculating loops and forceps.

7.12 Forceps, straight or curved, with smooth tips to handle filters.

7.13 *Incubator,* capable of maintaining Incubator, hot air or water-jacketed microbiological type to maintain a temperature of 41.5 ± 0.5°C and 3535.0 ± 0.5°C.

7.14 *Stereoscopic Microscope,* Magnification of 10–15× with a cool white fluorescent light.light or optional stereoscopic microscope.

7.15 ~~*Colony Counter.*~~ Colony counting device, mechanical, electric or hand tally (optional).

~~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.~~

7.16 ~~*Petri Dishes,* sterile, 50 by 9 or 60 by 15 mm and 100 by 15 mm.~~365 nm UV lamp.

~~7.10 *Pipets,* sterile, 1 and 10 mL, with 0.1-mL graduations and an accuracy of ±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.[4] 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 D1193.

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. This can be purchased or prepared in the laboratory. Add after stock solution (see 8.4). Add after 99 mL or volume as required based on dilution.

8.4 *Buffered Water Stock*—Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_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 ($MgCl_2$ $6H_2O$) 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 µm pore size) or equivalent.

9. Media Preparation

9.1 *M-PA-C Medium*[3]—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*[3] 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 ± 0.1. If it is not, adjust pH of the remaining solution accordingly with potassium hydroxide solution. (It is recommended this should be purchased and not prepared from individual ingredients.)

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.

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[4] *Reagent Chemicals, American Chemical Society* ~~*Specifications.*~~*Specifications,* ~~Am. Chem. Soc., Washington, D.C.~~ American Chemical Society, Washington, DC, www.chemistry.org. For suggestions on the testing of reagents not listed by the American Chemical Society, see ~~*"Analar*~~*Analar Standards* ~~*For*~~*for Laboratory* ~~*Chemicals,"*~~*Chemicals,* BDH Ltd., Poole, Dorset, ~~UK,~~U.K., and ~~the"~~the *United States* ~~*Pharmacopeia."*~~*Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD, http://www.usp.org.