Designation: D 5246 – 92 (Reapproved 1998)

An American National Standard

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 (ε) 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[1]

D 1193 Specification for Reagent Water[1]

D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[1]

D 3370 Practices for Sampling Water from Closed Conduits[1]

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)[2] 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.

[1] *Annual Book of ASTM Standards*, Vol 11.01.

[2] 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 ± 0.5°C and 35 ± 0.5°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 ±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.[3] 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 (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*[2]—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*[2] *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.

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*[4]—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.

[3] 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."

[4] 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.

12. Preparation of Apparatus

12.1 *Washing and Cleaning*—Thoroughly clean all glassware and filtration equipment, using a suitable detergent in hot water. After rinsing first in hot tap water and then in water, thoroughly dry the equipment prior to sterilization.

12.2 *Sterilization*— Follow standard microbiological laboratory practices for preparing glassware and filtration equipment prior to sterilization. Autoclave the apparatus at 121°C for 20 to 30 min or at 132°C for 5 min. Sterilization times should be increased in cases where large loads are sterilized at one time.

13. Procedure

13.1 *Membrane Filtration*:

13.1.1 Using alcohol-flamed forceps, aseptically remove the presterilized membrane filter from its package and place it, grid side up, on the base of the membrane filter unit. Connect the filtration flask and vacuum trap to a vacuum source.

13.1.2 If *P. aeruginosa* levels are high the sample must be diluted to obtain measurable levels. This is accomplished by serially diluting a known quantity of the sample (for example, 1.0 mL) through a series of known volumes (for example, 99 mL) of sterile buffered water until the desired bacterial density is obtained.

13.1.3 Thoroughly mix the sample prior to filtration.

13.1.4 Pour appropriate volumes (for example, 100 to 200 mL for natural waters, 500 mL for swimming pools) of the sample or sample dilution into the filter funnel. If smaller volumes (for example, 1 to 10 mL) of the sample are to be filtered, add 20 to 30 mL of sterile buffered water to the filter funnel before adding the sample to evenly disperse cells. Apply vacuum, filter the contents of the funnel, and then rinse the funnel three times with 20 to 30 mL of sterile buffered water. Shut off the vacuum when all the liquid has been filtered. Remove the funnel and, with sterile forceps, carefully remove the membrane filter from the base.

13.1.5 Place the membrane on the M-PA-C[2] medium by holding the filter at an angle of 45° and carefully rolling it onto the agar surface. Ensure that there is no air trapped between the membrane and the surface of the medium. If an air bubble is observed, raise the membrane and repeat the procedure.

13.2 *Incubation*:

13.2.1 Within 30 min after filtration, invert the petri dishes and place them in a humidified incubator at 41.5 ± 0.5°C. To provide a humid atmosphere, place the dishes in plastic containers lined with moistened paper towels and sealed with a lid.

13.2.2 Incubate 48 ± 2 h.

13.3 *Counting Colonies*:

13.3.1 Typical *P. aeruginosa* colonies are flat and are pink-brown to black in color. The colony usually has a dark center with lighter colored edges. Slowly developing *P. aeruginosa* colonies may be almost clean with a small dark center.

13.3.2 Count bacterial colonies using a stereoscopic microscope with 10× magnification. The cool white fluorescent light is set up to provide the best illumination.

13.3.3 The density range for accurate counting should be 20 to 80 *P. aeruginosa* colonies and not more than 150 total colonies.

14. Interpretation of Results

14.1 Pick a well isolated colony with a sterile loop or needle and streak onto a soybean casein digest agar plate to obtain isolated colonies. Incubate for 24 to 48 h at 35 ± 0.5°C.

14.2 Aseptically transfer a typical colony from TSA onto a skim milk agar plate, prepared as described in 9.2 and incubate at 35 ± 0.5°C for 24 to 48 h. Clearing of the medium (caseinase production), production of a diffusible blue-green pyocyanin pigment, and a diffusible yellow-green fluorescent pigment constitutes a positive result. Observe production of the fluorescent pigment in a darkened room shining long-wave (366-nm) ultraviolet light source on the plate.

14.3 Adjust counts based on percentage of confirmed colonies.

15. Report

15.1 Report counts as number of organisms per 100 mL. If precise counts are required, verify all colonies. As a quality control check, verify at least ten colonies per plate.

16. Precision and Bias

16.1 Because microbiological samples are not stable, it is not possible to distribute environmental samples to multiple laboratories and establish precision and bias for common environmental samples.

16.2 In the collaborative study, each of seven laboratories was asked to collect two environmental water samples for analyses, expected to contain *Pseudomonas aeruginosa*. A freeze-dried reference sample was shipped to all laboratories for analyses at two dilution levels, for a total of four samples. Three replicate analyses were performed on each sample dilution. Then colonies were picked from each countable plate (20 to 80 colonies) and verified to determine final counts. All seven laboratories reported data for the freeze-dried samples, but one laboratory did not report any data for environmental samples and another laboratory only reported data for only one environmental sample.

16.3 The data analysis was performed utilizing the procedures defined in Practice D 2777.

16.4 Arithmetic means, single operator precision (S_o) and relative standard deviations were calculated for each environmental sample at each laboratory. Since the reference culture was common to all laboratories, the grand mean, pooled $S_o$and relative standard deviations were calculated for each laboratory, and the grand mean, overall precision, S_t, and the overall relative standard deviation were calculated for the study. These data and statistical estimates are reported in Table 1. The means and standard deviations are stated in terms of colony forming units.

17. Keywords

17.1 enumeration; membrane filtration; *Pseudomonas aeruginosa*

TABLE 1 Round Robin Data

Data from Environmental Water Samples Supplied by Each Laboratory [A]						
Laboratory	Sample 1			Sample 2		
	X	S_o	RSD,%	X	S_o	RSD, %
2	8.3	0.58	6.98	26.5 [B]	4.95 [B]	18.7
3	5.0	1.73	34.6	6.3	0.58	9.20
4	12.7	4.73	37.2	29.0	7.0	24.1
5	26.0	5.57	21.4	44.3	5.03	11.4
6	51.7	3.51	6.78	...	...	...
7	8.3	2.52	30.4	30.7	6.43	20.9
Data from Reference Sample Supplied to Each Laboratory [A]						
Laboratory	2.1 mL Dilution			8 mL Dilution		
	X	S_o	RSD,%	X	S_o	RSD,%
1	7.67	0.58	7.56	32.0	2.00	6.25
2	23.33	2.08	8.92	74.0 [B]	8.49 [B]	11.5
3	25.33	4.62	18.24	92.33	3.06	3.31
4	19.0	3.00	15.8	71.33	9.07	12.7
5	16.67	3.21	19.26	53.33	1.53	2.87
6	20.0	4.36	21.8	68.67	10.02	14.6
7	13.0	4.36	33.5	62.67	2.31	3.69
Grand Mean	17.86	...	...	64.45	...	...
S_o pooled	...	3.45	19.32	...	6.07	9.42
S_t	6.68	...	37.4	19.41	...	30.0

[A] Triplicate plates.
[B] Duplicate plates.