This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

Designation: E3364 − 22

Standard Test Method for Evaluating the Performance of Antimicrobials in or on Polymeric Porous and Nonporous Materials Against Staining by *Streptomyces* species (A Pink Stain Organism)[1]

This standard is issued under the fixed designation E3364; 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.

INTRODUCTION

When certain bacteria and mold species grow on the surface of non-rigid, flexible or "plasticized" polymers, metabolites such as pigments in the case of certain bacteria and melanin (dark stains from fungal growth) cause undesirable stains on the polymer surface. These stains may persist even after the surface growth is removed. This test method is used to determine the performance of antimicrobial agents used in or on synthetic polymeric porous and non-porous materials against staining by the Actinomycete, *Streptomyces* species. This organism has been chosen as an indicator organism, although other organisms have been known to cause undesirable staining in polymeric porous and non-porous materials.

1. Scope

1.1 This test method is intended to assess susceptibility of polymer materials, as well as products that may directly contact the treated polymer, to staining by the Actinomycete *Streptomyces* species.

1.2 This test method is also suitable for evaluating dark-pigmented test samples since the bacterial growth inhibition can be assessed.

1.3 Familiarity with microbiological techniques is required. This test method should not be used by persons without at least basic microbiological training.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D3273 Test Method for Resistance to Growth of Mold on the Surface of Interior Coatings in an Environmental Chamber

D3274 Test Method for Evaluating Degree of Surface Disfigurement of Paint Films by Fungal or Algal Growth, or Soil and Dirt Accumulation

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E1428 Test Method for Evaluating the Performance of Antimicrobials in or on Polymeric Solids Against Staining by *Streptomyce species* (A Pink Stain Organism)

3. Terminology

3.1 *Defintions:*

3.1.1 For definitions of terms used in this method, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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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.

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3.2.1 *microbially induced staining, n*—undesirable pigmentation or disfiguration of an object due to surface colonization by certain microorganisms. Both bacteria and fungi produce metabolic pigments that can result in surface stains on susceptible objects.

3.2.2 *pink stain organism, n*—an Actinomycete such as *Streptomyces* species ATCC 25607 (deposited as *Streptoverticillium reticulum*) that is capable of metabolically producing a light rose to orange-colored discoloring pigment.

4. Summary of Test Method

4.1 Test specimens are challenged with slurry inoculated with *Streptomyces* species and incubated. After incubation, test specimens are visually rated for microbial growth, cleaned, and rated visually by percentage of sample area stained.

5. Significance and Use

5.1 Methods such as D3273 Standard Test Method for Resistance to Growth of Mold on the Surface of Interior Coatings in an Environmental Chamber and D3274 Standard Test Method for Evaluating the Degree of Surface Disfigurement of Paint Films by Fungal or Algal Growth or Soil or Dirt Accumulation provide means for assessing mold and algal staining on paints. The Test Method E1428 Evaluating the Performance of Antimicrobials in or on Polymeric Solids Against Staining by *Streptomyces* species (A Pink Stain Organism) is used for solid polymeric materials, but is not appropriate for all antimicrobial technologies.

5.2 This test method provides a technique for evaluating antimicrobials in or on polymeric materials against staining by *Streptomyces* species and should assist in the prediction of performance of treated articles under actual field conditions.

6. Interferences

6.1 An interference may be caused by contamination of plates and agar by unwanted organisms that settle on samples from the environment. Sample bioburden may out-grow the *Streptomyces* sp. inoculum, subsequently blocking direct contact with the test specimen or preventing production of pink pigments.

6.2 Darkly pigmented samples may mask observation of the pink stain.

6.3 Biocide performance may be affected if material is conditioned. Examples of conditioning include, but are not limited to: exposure to leaching, weathering, heat treatment, or addition of fire or flame retardant.

7. Apparatus

7.1 *Petri dishes*, Height and diameter appropriate to sample thickness and diameter.

NOTE 1—Pre-sterilized and disposable plastic petri dishes are available from most laboratory supply houses.

7.2 *Cotton swabs*, sterile.

7.3 *Incubator*—capable of maintaining a temperature of 29 °C ± 1 °C.

7.4 *Autoclave*.

7.5 *Sterilizer*, suitable for the substrate to be tested, that is, 70 % isopropanol (optional).

7.6 *Parafilm*, sealable plastic bags or equivalent to prevent moisture loss.

7.7 *Spreader*, sterile.

7.8 *Waterbath*, capable of maintaining water at 47 °C ± 2 °C.

7.9 *Pipette*, capable of pipetting 2.0 mL.

8. Reagents and Materials

8.1 A modified ISP-2 agar is used in performing the testing. Prepare the medium used for testing according to the following directions and maintain at 45 °C to keep it molten.

8.1.1 Dissolve in 1 L of water the designated amount of the following:

Yeast extract	4g
Malt extract	10g
Dextrose	4g
Agar	4g

8.2 Phosphate buffered saline (PBS) prepared according to manufacturer's instructions.

The most common composition of PBS (1X)		
Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na_2HPO_4	10	1.42
KH_2PO_4	1.8	0.24

8.3 Cycloheximide of 95 % purity as fungal antibiotic for slurry media (optional).

8.4 *Inoculum Species—Streptomyces* species ATCC 25607 (deposited as *Streptoverticillium reticulum*)— Maintain stock cultures on yeast malt extract agar. The stock should be subcultured every three months and stored at approximately 3 °C to 10 °C in plastic bags or covered with Parafilm.

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9. Test Specimens

9.1 From each test unit (Note 2), cut triplicate 5.0 cm. diameter discs or 5.0 cm. squares. If the test unit is of different construction on each side triplicate specimens of each side, three face up and three face down, shall be tested.

NOTE 2—A test unit may be in the form of foam, vinyl, porous films, coated fabrics, or any other polymeric material, as well as any product that may directly contact them.

9.2 A test unit containing no biocide (Blank) should be included as a positive stain control. If a blank test unit is unavailable, the inoculum slurry plate should be considered as positive control and viability of the test at the same time.

9.3 Heavily soiled specimens should not be used. For stain comparison, a non-inoculated test unit should be kept until the end of the experiment.

9.4 If sanitization of test specimens is considered necessary, samples can be sanitized by spraying with 70 % alcohol and allowing to air dry thoroughly.

10. Procedure

10.1 *Slurry inoculum:*

TABLE 1 Degree of Bacterial Growth

Bacterial Growth CFU/sample	Rating
no growth	0
1-10 CFU	1
11-50 CFU	2
51-100 CFU	3
>100-No distinct colonies	4

TABLE 2 Degree of Stain Rating

Observed Stain on Specimens	Rating
No Stain	0
Trace of Stain (less than 10 % coverage)	1
Slight Stain (11 % to 30 % coverage)	2
Moderate Stain (31 % to 50 % coverage)	3
Heavy Stain (51 % to complete coverage)	4

10.1.1 Prepare subcultures to be used in the slurry inoculation by passing a cotton swab for 3 passes of 2.54 cm across the surface of a stock plate, rotate the swab and repeat until four sides have been passed over the stock plate. Immerse the swab in a 10.0 mL suspension of PBS. The amount of culture transferred should be sufficient to give a light pink coloration to the PBS. Incubate the inoculated PBS at 29 °C ± 1 °C for 3 to 5 days, yielding a robust bacterial growth phase with mycelia, aerial mycelia and spores. Vortex the PBS tube to re-suspend the microorganism's right before slurry preparation.

10.1.2 If fungal contamination is a concern add 0.5 g of cycloheximide in 100.0 mL of sterile deionized water, then sterilize either via filtration or autoclave.

10.1.3 Prepare a final slurry inoculum containing 90 % of melted modified ISP-2 agar from 8.1.1 and maintained at 47 °C ± 2 °C, 10 % of the vortexed PBS subculture from 10.1.1, and 2.0 % of the cycloheximide solution (if desired) from 10.1.2 in sterile deionized water.

NOTE 3—The subculture may be run through a tissue grinder to grind up large particles prior to adding the subculture to the inoculum slurry.

NOTE 4—Dilution plate counts of the inoculum slurry may be performed to enumerate the CFU/mL in the slurry at the time of inoculation.

10.2 *Sample inoculation:*

10.2.1 Place samples from 9.1 into empty Petri dishes.

10.2.2 Gently mix the slurry inoculum from 10.1, and carefully pipette 2.0 mL of the slurry over the top of each test specimen.

10.2.3 Spread the slurry with a sterile spreader, loop, or the pipet tip used for inoculation. Ensure the slurry on top of the samples will not touch the Petri dish lid. Any large colonies visible in the slurry should be removed using a sterile pipet.

10.2.4 A viability control of the inoculum is required to validate the results of this test. For the viability control add 2.0 mL of slurry to an empty Petri dish.

10.3 *Incubation*—Cover the inoculated sample test plates and viability control, wrap the plate edges with Parafilm or place them in a plastic bag, or equivalent, to maintain the moisture and incubate at 29 °C ± 1 °C and at least 80 % RH for 14 days.

10.4 *Post Incubation:*

10.4.1 Observe the growth of *Streptomyces* species in the slurry of controls and viability inoculum plates, using Table 1 as a guide. If desired, test specimens may also be scored for bacterial growth.

10.4.2 Remove the slurry with a loop or spatula and rinse the specimens with water until the slurry is completely removed (about 10 seconds). Squeeze the samples or dry with absorbent paper.

10.4.3 Observe the degree of staining on the samples by comparing with the inoculated, untreated control and the untested sample. To have a better perspective, look at the test specimen perpendicularly and within an angle of 45°.

10.4.4 Record pink staining (if any) or similar range of color (yellow to brown) on the top of the inoculated surface. Estimate the degree of staining by the amount of sample surface stained rather than the intensity of the color. However, pigmented samples that show a color change associated with pink (that is, a blue pigmented sample changing to purple) should be rated according to degree of bacterial growth in accordance to Table 1. Estimate the degree of staining and rate in accordance with Table 2.

11. Interpretation of Results

11.1 If contamination is apparent on the test plate, limiting the growth of the test inoculum or preventing production of pigment, the test is invalid.

11.2 The viability control should yield a stain rating of 4 according to Table 2, or the test is invalid.

12. Report

12.1 Report the visual rating of bacterial growth and stain on the topside of the samples in accordance with Table 1 and Table 2.

NOTE 5—CFU/mL of the inoculum may be reported but is not required.

12.2 Report the presence of contaminants as described in 6.1.

TABLE 3 Bacterial Growth (numeric rating)

Material	Number of Laboratories	Average[A]	Repeatability Standard Deviation	Reproducibility Standard Deviation	Repeatability Limit	Reproducibility Limit
	n	$\bar{x}$	s_r	s_R	r	R
Material A	6	2.6	0.5	1.1	1.5	3.0
Material B	6	1.1	0.6	0.8	1.7	2.3

[A]The average of the laboratories' calculated averages