



Designation: D 4300 – 01

Standard Test Methods for Ability of Adhesive Films to Support or Resist the Growth of Fungi¹

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This standard has been approved for use by agencies of the Department of Defense.

1. Scope*

1.1 These test methods test the ability of adhesive films to inhibit or support the growth of selected fungal species growing on agar plates by providing means of testing the films on two agar substrates, one which promotes microbial growth, and one which does not.

1.2 These test methods are not appropriate for all adhesives. The activity of certain biocides may not be demonstrated by these test methods as a result of irreversible reaction with some of the medium constituents.

NOTE 1—As an example, quaternary ammonium compounds are inactivated by agar.

1.3 A test method is included for use with low-viscosity adhesives along with an alternative method for use with mastic-type adhesives. Also, a method approved by the government is given.

1.4 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

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 and health practices and determine the applicability of regulatory limitations prior to use.* These test methods are designed to be used by persons trained in correct microbiological techniques. Specific precautionary statements are given in Section 7 and in 14.3.2.

2. Referenced Documents

2.1 *ASTM Standards:*²

D 907 Terminology of Adhesives

D 1286 Methods of Test for Effect of Mold Contamination on Permanence of Adhesive Preparations and Adhesive Bonds³

G 21 Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi

2.2 *TAPPI Method:*

T487 Fungus Resistance for Paper and Paperboard⁴

3. Terminology

3.1 *Definitions*—Many terms in this test method are defined in Terminology D 907.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *adhesive preparation, n*—the adhesive as packaged for distribution, storage, and use.

3.2.2 *adhesive film, n*—the small portion of the adhesive preparation, as prepared for use by the consumer, either with additives or as received, which is cast on a substrate, cured 24 h, and represents the glue line.

3.2.2.1 *Discussion*—For purposes of these test methods the *adhesive film* is the thin layer of adhesive spread on either the 21-mm fiberglass disk as described in 14.2, or the adhesive layer 3 mm thick which is cast on the tile squares as described in 15.1.

3.2.3 *zone of inhibition, n*—the area on an inoculated agar plate surrounding the adhesive-coated disk or tile, showing a reduced fungal growth or an absence thereof.

3.3 *Abbreviations:*

3.3.1 *PDA*—potato dextrose agar.

3.3.2 *MSA*—mineral salts agar.

3.3.3 *ZI*—zone of inhibition.

4. Significance and Use

4.1 These test methods are designed to be used to determine the susceptibility of the adhesive film to biodegradation and whether the adhesive will carry into the bond line sufficient anti-fungal properties to prevent growth of fungi frequently present on the gluing equipment, on adherends, or in the adhesive as applied.

¹ These test methods are under the jurisdiction of ASTM Committee D14 on Adhesives and are the direct responsibility of Subcommittee D14.30 on Wood Adhesives.

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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 Withdrawn.

⁴ Available from TAPPI, P. O. Box 105113, Atlanta, GA 30348.

*A Summary of Changes section appears at the end of this standard.

4.2 Potato dextrose agar (PDA) provides a complete medium for the growth of fungi, while mineral salts agar (MSA) lacks a carbohydrate source and provides a less favorable medium. Use of PDA tests the adhesive film for its ability to resist the growth of fungi on its surface as well as its ability to repel a copious growth of fungi on the adjacent agar surface. Use of MSA tests the adhesive film primarily for its ability to resist the growth of fungi on its surface. When it is used, there is a reduced possibility that the growth from the agar will be mis-read as coming from the adhesive film, since fungal growth on the adjacent agar will be scant.

NOTE 2—The method given here using the MSA is based on Practice G 21, adapted to be used with adhesives. Requirements to meet the approval of government specifications are the use of the MSA described in 10.2, and a mixed species of fungi described in 8.2 for the inoculum.

4.3 The results obtained when using the procedures given in this method apply only to the species used for the testing. The test species listed in Section 8 are frequently used by laboratories to test for antifungal properties, but they are not the only ones which could be used. Selection of the fungal species to test against requires informed judgment by the testing laboratory or by the party requesting the tests. These methods are especially useful when species that have been isolated from contaminated adhesives are used as the test species (see Section 8) to aid in the selection of more effective fungicides.

4.4 The efficacy of some biocides may change in storage due to the chemical and thermal environment to which they are subjected as components of certain adhesives. These test methods are not appropriate for determining the effect of fungal contamination on adhesives under water-soaking conditions, because they are not designed to cover the possibility of water-soluble biocides leaching out of the bond line.

4.5 These test methods are dependent upon the physiological action of living microorganisms under a reported set of conditions. Conclusions about the resistance of the test adhesive to fungal attack can be drawn by comparing the results to simultaneously run controls of known resistance. See X5.2 for statements regarding test repeatability.

5. Apparatus

5.1 In addition to the standard equipment found in any fully equipped microbiological laboratory, items from the following list are needed for various tests. Not all items are needed for each test.

5.1.1 *Chromist Laboratory Spray Unit*.⁵

5.1.2 *Constant Temperature Chamber*, capable of being maintained at $35 \pm 0.5^\circ\text{C}$ ($95 \pm 1^\circ\text{F}$) or $25 \pm 0.5^\circ\text{C}$ ($77 \pm 1^\circ\text{F}$), or two chambers if needed simultaneously.

5.1.3 *Filter Disk, Glass Microfibre, 934-AM*, diameter-21 mm.⁶

5.1.4 *Filter Disk, Sterile Whatman No. 1*.⁶

5.1.5 *Filter Paper Assay Disk*, 1.5 cm diameter, sterile. Schleicher and Schnell, Inc., or the equivalent, has been found satisfactory for this purpose.⁶

5.1.6 *Glass Rods*, 305 mm in length having a diameter of 6.3 mm.

5.1.7 *Glove Bag*, 68 cm in length and width, 38 cm in height.⁷

5.1.8 *Hemocytometer Levy Counting Chamber*, cell depth-0.1 mm, Newbauer rulings.⁶

5.1.9 *Hood, Laminar-Flow Type, Class II Type I*.⁸

5.1.10 *Jar, Screw Cap*, round, approximately 1 L (1 qt, mason type).

5.1.11 *Pipet, Pasteur*.⁶

5.1.12 *Petri Dishes*, sterile, disposable, top-diameter of 150-mm, bottom-height of 15-mm.

5.1.13 *Refrigerator*, capable of maintaining $4 \pm 1^\circ\text{C}$ ($39 \pm 2^\circ\text{F}$).

5.1.14 *Teflon Paper or Grid*, pressure sensitive overlay, coated with TFE-fluorocarbon (PTFE), vinyl sheet backing, to be used at up to 93°C (200°F).⁹

6. Materials

6.1 *Potato Dextrose Agar*, Difco or equivalent.

6.2 *Sterile Deionized or Distilled Water*.

6.3 *Disinfectant Solution*—Amphyll, Alcide, or comparable product.

6.4 *Materials for Mineral Salts Agar*. (See list in 10.2.1.)

6.5 *Sorbitan mono-oleate polyoxyethylene*.¹⁰

7. Precautions

7.1 Assign laboratory personnel trained in correct microbiological techniques to run these tests. These test methods employ live cultures of fungi, some of which are capable of causing disease or allergic reaction in some humans. Use proper microbiological procedures in order to prevent contamination of the cultures or of the work area. Disinfect and sterilize in an approved manner all spills and all equipment coming into contact with the cultures. Also sterilize in an approved manner all cultures and contaminated disposable equipment before discarding. See 1.5 and 14.3.2.

7.2 In addition to other precautions, the use of a Class II, Type I containment hood is highly recommended for all procedures that would cause formation of fungal aerosols. This type of laminar flow hood prevents the spread of fungal spores throughout the laboratory and inhalation of spores by the operator. The hoods should be monitored by a biological safety officer or a health physicist if they are to be used with hazardous agents. Refer to the operating manual supplied by the manufacturer for detailed information. This warning applies specifically to the use of the Chromist laboratory spray unit listed in 5.1.1 and in the instructions in 14.3.2.

8. Test Species of Fungi¹¹

⁷ Available from Instruments for Research and Industry, 108 Franklin Ave., Cheltenham, PA, or most laboratory supply houses.

⁸ The Biogard Hood or similar equipment is available from laboratory supply houses.

⁹ Gelman Sciences, or most laboratory supply houses.

¹⁰ Available commercially as Tween 80.

¹¹ Cultures may be purchased from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

⁵ Available from Gelman Sciences, Ann Arbor, MI.

⁶ Available from laboratory supply houses.

8.1 Cultures of one or more of the following species are suggested for use when PDA is the medium:

	ATCC No.
8.1.1 <i>Aspergillus niger</i>	9642
8.1.2 <i>Aspergillus flavus</i>	9643
8.1.3 <i>Penicillium pinophilum</i>	9644 (See X1.1.6)
8.1.4 <i>Phanerochaete chrysosporium</i>	24725
8.1.5 <i>Aureobasidium pullulans</i> Var.— <i>melanigenum</i>	15233

NOTE 3—The choice of test organisms is often made from the fungal species listed above. Information on these and other species is given in Appendix X1.

8.2 Cultures of the following species are used for the government requirements described in Section 16, using MSA:

	ATCC No.
8.2.1 <i>Aspergillus niger</i>	9642
8.2.2 <i>Aureobasidium pullulans</i> Var.— <i>melanigenum</i>	15233
8.2.3 <i>Chaetomium globosum</i>	6205
8.2.4 <i>Gliocladium virens</i>	9645
8.2.5 <i>Penicillium pinophilum</i>	9644 (See X1.1.6)

NOTE 4—The species listed in 8.2 are used in Practice G 21. The following optional species are also sometimes used: *Aspergillus flavus*, (ATCC No. 9643) and *Aspergillus versicolor* (ATCC No. 11730). See 13.2 and Appendix X1.

8.3 Other pure cultures or mixed cultures of fungal species may be used, if agreed upon between the interested parties and upon the recommendation of the testing laboratories.

9. Sterilization of Equipment and Media

9.1 Follow accepted microbiological practices for sterilizing equipment and media.

NOTE 5—Two references for sterilization methods are TAPPI T 487 (see 2.2) and Ref (1).¹²

10. Preparation of Media

10.1 Potato Dextrose Agar:

10.1.1 Prepare sufficient agar slants and plates for culture propagation and conducting the tests.

10.1.2 Follow the instructions given for preparation of the commercial product. Dissolve using heat and agitation. Transfer an appropriate amount of the agar solution to each flask used for pouring plates, and 10 mL per test tube. Plug flask with appropriate closures. Cap tubes loosely with metal, plastic, or foam caps. Autoclave for 15 min at 103 kPa and a temperature of 121°C (250°F). Allow the agar to cool to 48 to 50°C (118 to 122°F) before pouring the plates, filling to an approximate depth of 3 mm. Allow plates to solidify. Tighten the caps on the tubes and place them in a slanted position to solidify, making a slant of about 51 mm. Store slants and plates in refrigerator until needed.

10.2 *Mineral Salts Agar*—Prepare sufficient medium for tests as described below:

10.2.1 Dissolve in 1 L of water the designated amounts of the following reagents:

	Grams
Potassium phosphate (KH ₂ PO ₄)	0.7
Magnesium sulfate (MgSO ₄ · 7H ₂ O)	0.7
Ammonium nitrate (NH ₄ NO ₃)	1.0
Sodium chloride (NaCl)	0.005
Ferrous sulfate (FeSO ₄ · 7H ₂ O)	0.002
Zinc sulfate (ZnSO ₄ · 7H ₂ O)	0.002
Manganous sulfate (MnSO ₄ · 4H ₂ O)	0.001
Agar	15.0

10.2.2 Adjust the pH of the medium by the addition of 0.01N NaOH solution so that after sterilization the pH is between 6.0 and 6.5, and sterilize by autoclaving at 103 kPa, and 121°C (250°F) for 15 min.

10.2.3 Prepare plates as described in 10.1.2, and store in the refrigerator until needed.

11. Fungal Cultures

11.1 Propagation of Fungal Cultures:

11.1.1 Prepare a fresh culture for each species on PDA and label by species and ATCC Number. Incubate at 25 ± 0.5°C (77 ± 1°F) for a minimum of 10 days or until full sporulation is achieved.

11.1.2 Refrigerate the cultures. Prepare new cultures each month. If contamination occurs, discard the cultures and prepare new ones.

11.2 Preparation of Fungal Inoculum:

11.2.1 Follow the procedure in 11.1.1 to prepare fresh cultures on PDA slants for each species to be used to conduct the tests.

11.2.2 *Harvesting Fungal Cultures and Dislodging Spores*—To one tube of each species of fungi, add 15 mL of sterile distilled or deionized water, containing 0.05 % sorbitan mono-oleate polyoxyethylene. Harvest fungal cultures and dislodge spores by rubbing the growth gently with a sterile inoculating loop or by removing it with a sterile glass rod. Transfer the washings into a sterilized container containing glass beads and shake thoroughly to break up the clumps. Filter through sterile layered cheese cloth or sterile nonabsorbent cotton. Adjust the spore level to 1.0 × 10⁶ per mL, using a hemacytometer and the procedure in Annex. Use this spore suspension of a single species of fungi as the inoculum for the tests described in Sections 14 and 15 when using the option given in 13.1.1.1.

11.2.3 For a mixed culture, obtain a spore count on each fungal species, and adjust each suspension to the level of 1 × 10⁶ per mL. Combine equal portions of the spore suspensions from each of the species in a common sterilized container. Use this mixed spore suspension for the tests described in Sections 14, 15, and 16 when using the option given in 13.1.1.2.

12. Adhesive Sample

12.1 For ready-to-use liquid adhesives, obtain an approximate 250-mL sample. For adhesives to be mixed at the time of use, obtain a sufficient sample of each component, mix in accordance with the manufacturer's instructions, and run the tests on the prepared adhesive mix. For mastics, use the adhesive as packaged for the consumer, directly from the applicator tube.

NOTE 6—The sample size given is for convenience in handling. The test may be run on only a few millilitres of material. When several components

¹² The boldface numbers in parentheses refer to the references at the end of this standard.

are to be mixed to yield the sample to be tested, the amount of each component should be sufficient for remixing should a retest be necessary.

13. Selection of Conditions of Testing

13.1 Select one option from each of the testing conditions given below:

13.1.1 *Fungal Species for Inoculum*—Select the fungal species to use for the tests based on the informed decision of the testing laboratory or on the requirements of specifications which are to be met, using one of the options below:

13.1.1.1 Testing with spore suspensions of pure cultures of single species, or

13.1.1.2 Testing with a mixed spore suspension of two or more species.

NOTE 7—See Section 8 and Appendix X1 for options and help in selecting the species to use. See Appendix X2 for guidelines on use of mixed cultures.

13.1.2 *Agar Medium*—Select the medium based on the informed decision of the testing laboratory or on specifications to be met:

13.1.2.1 Potato dextrose agar,

13.1.2.2 Mineral salts agar, or

13.1.2.3 Other medium of choice.

13.1.3 *Procedure, Based on Viscosity or Consistency of the Adhesive*:

13.1.3.1 Low-viscosity adhesives, or

13.1.3.2 Mastic-type adhesives.

13.2 *Testing to Meet Government Specifications*—To comply with government specifications, select the following options: the mixed fungal species designated in 13.1.1.2, using the species listed in 8.2, and the MSA designated in 13.1.2.2. Follow the procedure given in Section 16.

14. Film Test for Low Viscosity Adhesives

14.1 *Number of Specimens and Plates per Test*—For each species or mixed species of fungi to be tested against, run two agar plates using three adhesive-coated test specimens described in 14.2, per 150-mm diameter plate and one plate with three uncoated Whatman No. 1 filter disks as a control.

14.2 *Preparation of Adhesive Specimens*—On the day before the tests are to be initiated, prepare the adhesive-coated fiber-glass disks (see 5.1.3). Coat both sides of each disk with the adhesive. Allow the disks to dry until no longer tacky by resting on a sheet of TFE-fluorocarbon-coated paper or a TFE-fluorocarbon-coated grid. Then dry for 24 h at $40 \pm 2^\circ\text{C}$ ($104 \pm 3.6^\circ\text{F}$) to allow the volatiles to dissipate. Store in sterile petri dishes until used.

NOTE 8—The drying time may need to be reduced for some adhesives to avoid curling of the disks. See Appendix X3 for a discussion on handling adhesives.

14.3 *Inoculation and Placement of Specimens*—Seed the duplicate test plates and the control plate, using one of the following procedures:

14.3.1 Inoculate a 150-mm agar plate by placing 0.1 mL of the spore suspension prepared in 11.2.2 on the surface of the plate, dropping from a pipet. Using a sterile L-shaped rod, seed the total surface. Prepare two plates, placing the three adhesive-coated fiber-glass disks equidistant and flat on the

surface. Prepare the control plate by placing three uncoated Whatman No. 1 filter paper disks on the inoculated surface. Using a sterile capillary dropping pipet, place three drops of the spore suspension on the surface of each disk on the test plates and the control plate.

14.3.2 As an alternate method, prepare duplicate plates by placing three adhesive coated fiberglass disks equidistant and flat on the surface of each of two 150-mm agar plates. For the control, place three uncoated Whatman No. 1 filter paper disks on the agar surface of a third plate. Then inoculate the test plates and the control plate by spraying the fungal spore suspension over the entire surface of the agar and the disks. Use the apparatus in 5.1.1 for the spraying, and conduct this operation in the confines of a Class II Type I Biological Safety Cabinet or laminar-flow hood which has been properly monitored by a biological safety officer. Follow the precautions given in 7.2. (**Warning**—In addition to other precautions, use special care with the spray inoculation described in 14.3.2, which has a potential for gross contamination of the work area. Before inoculation place the open plates inside the described biological safety cabinet. Inside the hood, use a secondary containment, such as an empty 10-gal aquarium, partially covered, or a glove bag. Use disposable surgical gloves and a respirator when inoculating by the spray technique and when handling the inoculated plates. Invert the plates (if this will not disturb the disks) and enclose them in a sealed plastic bag to avoid contaminating the incubator. Use of a respirator provides an extra measure of safety when inoculating by the spray technique.)

14.4 *Incubation and Examination*—Seal the plates with parafilm to prevent drying out during incubation. Incubate both the test and control culture plates at $25 \pm 0.5^\circ\text{C}$ ($77 \pm 1^\circ\text{F}$). When PDA is the culture medium, examine at 3, 7, and 14 days for zone of inhibition (ZI) or overgrowth on the adhesive coated disks, and record results using the grading system given in 14.5. For slow growing species, extend the incubation period to 21 days. Examine the control plate and the outer edges of the test plates for presence of a normal confluent growth pattern of the fungal species being tested and an absence of contaminants. When MSA is the culture medium, examine at 7, 14, and 21 days.

NOTE 9—To avoid transfer of developing spores during the incubation period, handle the plates with care for the preliminary readings.

14.5 Grading System:

14.5.1 When PDA is the culture medium use the following grading system:

OC—No growth on disk, clear zone of inhibition

OM—No growth on disk, inhibited growth zone around disk with mycelia, but no spores

NG—No growth on disk, no ZI

SG—Sparse growth on disk

LG—Light growth on disk

MG—Moderate growth on disk

HG—Heavy growth on disk

14.5.2 The passing ratings are OC, OM, and NG. The failing ratings are SG, LG, MG, and HG. Determine whether any peripheral growth around the disk is from the agar or from