



Designation: D4783 – 01 (Reapproved 2021)

Standard Test Methods for Resistance of Adhesive Preparations in Container to Attack by Bacteria, Yeast, and Fungi¹

This standard is issued under the fixed designation D4783; 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.

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

1. Scope

1.1 These test methods cover the determination of the resistance of liquid adhesive preparations to microbial attack in the container by challenging adhesive specimens with cultures of bacteria, yeast, or fungi, and checking for their ability to return to sterility. These test methods return qualitative results.

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

1.3 *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.* These test methods are designed to be used by persons trained in correct microbiological technique. Specific precautionary statements are given in Section 8.

1.4 *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

D907 Terminology of Adhesives

D4299 Test Method for Effect of Bacterial Contamination on Performance of Adhesive Preparations and Adhesives Films (Withdrawn 1990)³

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.

Current edition approved April 1, 2021. Published April 2021. Originally approved in 1988. Last previous edition approved in 2013 as D4783 – 01 (2013). DOI: 10.1520/D4783-01R21.

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 The last approved version of this historical standard is referenced on www.astm.org.

D4300 Test Methods for Ability of Adhesive Films to Support or Resist the Growth of Fungi
E640 Test Method for Preservatives in Water-Containing Cosmetics

NOTE 1—Test Method E640 is under the jurisdiction of ASTM Committee E35 on Pesticides. The procedure in this method outlines a serial dilution method of determining plate count using a pour plate technique.

2.2 *TAPPI Method:*⁴

T 487 Fungus Resistance of Paper and Paperboard

2.3 *CSMA:*

Cosmetics Preservation, Method 38⁵

3. Terminology

3.1 *Definitions*—Many terms in these test methods are defined in Terminology D907.

3.2 *Definitions of Terms Specific to This Standard:*

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

3.3 *Abbreviations:*

3.3.1 *PBS*—phosphate buffered saline.

3.3.2 *PDA*—potato dextrose agar.

3.3.3 *YMPG*—yeast malt peptone glucose (agar).

4. Summary of Test Method

4.1 The adhesive specimen is challenged by inoculation with a culture of bacteria, yeast, or fungi, which may be a single species or a mixed culture of several species, following the guidelines given in Note 6. The inoculated adhesive specimen is stored at 21 to 27°C (70 to 80°F) for 7 days, during which time cultures (streak plates) are made at preset intervals. See Note 2. At any point in the series of challenges, if the inoculated specimen shows microbial growth on the streak plates made during the week following the challenge (indicating that it has not returned to sterility), the test is discontinued,

⁴ Available from Technological Association of the Pulp and Paper Industry (TAPPI), 15 Technology Parkway South, Suite 115, Peachtree Corners, GA 30092, http://www.tappi.org.

⁵ This method is the same as Test Method E640.

and the sample is reported as *not resistant to attack in the container* by the species or combination of species used as the inoculum. If the cultures show no growth, the test is repeated with up to four challenges. If the specimen tests out as sterile following the fourth challenge, it is reported to be *resistant to attack in the container* by the species or combination of species of bacteria, fungi, or yeast used as the inoculum. At the discretion of the biological laboratory, the test may be discontinued after the second or third challenge. See Section 16 for further interpretation.

4.2 The time necessary to kill is determined by noting the earliest streak plate to read sterile. If the 4-h plate is positive and the 24-h plate is negative, the kill time could be narrowed down further by repeating the challenge and making streak plates at intervals of 4, 8, 12, and 24 h following the challenge.

4.3 The testing laboratory has the option of changing the timing of the challenges, the sterility checks, and the incubation period.

NOTE 2—Two proposed schedules for the challenging and sterility

checks are shown in Table 1 and Table 2, Schedule A for bacteria and yeast, and Schedule B for fungi. The exact format to be followed will vary, according to the convenience of the schedule to the testing laboratory and special circumstances relating to the problem being addressed.

NOTE 3—A serial-dilution plate-count method of checking for sterility may be used when numerical information is needed on the population of viable organisms or the reduction in population with increasing levels of biocide. Lethen broth is recommended for the diluent and Lethen agar for the pour plate. See Note 1.

5. Significance and Use

5.1 These test methods are used to demonstrate whether an adhesive preparation is sufficiently protected with biocide to resist attack by bacteria, yeast, and fungi during its storage life. They are patterned after methods used by biological laboratories serving the adhesive industry.

5.2 These test methods may also be used to determine the efficacy of different biocide systems against specific microorganisms.

5.3 These test methods are especially useful when tested against wild-type microorganisms which have been isolated

TABLE 1 Schedule A—Proposed Bacteria and Yeast Testing, Covering 4-h, 24-h, 48-h, 72-h, and 7-Day Tests

Day of Week	Day no.	First Challenge	Second Challenge	Third Challenge	Fourth Challenge
Monday	(-1)	inoculate fresh bacterial or yeast culture
Tuesday	0	prepare suspension
Tuesday	0	inoculate specimens
Tuesday	(0 + 4 h)	streak 4-h plate
Wednesday	1	streak 24-h plate
Thursday	2	streak 48-h plate
Friday	3	streak 72-h plate
Sat./Sun.	4-5
Monday	6	...	inoculate fresh bacterial or yeast culture
Tuesday	7	...	prepare suspension
Tuesday	7	streak 7-day plate	inoculate specimens
Tuesday	(7 + 4 h)	read 4-h plate	streak 4-h plate
Wednesday	8	read 24-h plate	streak 24-h plate
Thursday	9	read 48-h plate	streak 48-h plate
Friday	10	read 72-h plate	streak 72-h plate
Sat./Sun.	11-12
Monday	13	inoculate fresh bacterial or yeast culture	...
Tuesday	14	prepare suspension	...
Tuesday	14	read 7-day plate	streak 7-day plate	inoculate specimens	...
Tuesday	(14 + 4 h)	...	read 4-h plate	streak 4-h plate	...
Wednesday	15	...	read 24-h plate	streak 24-h plate	...
Thursday	16	...	read 48-h plate	streak 48-h plate	...
Friday	17	...	read 72-h plate	streak 72-h plate	...
Sat./Sun.	18-19
Monday	20	inoculate fresh bacterial or yeast culture
Tuesday	21	prepare suspension
Tuesday	21	...	read 7-day plate	streak 7-day plate	inoculate specimens
Tuesday	(21 + 4 h)	read 4-h plate	streak 4-h plate
Wednesday	22	read 24-h plate	streak 24-h plate
Thursday	23	read 48-h plate	streak 48-h plate
Friday	24	read 72-h plate	streak 72-h plate
Sat./Sun.	25-26
Monday	27
Tuesday	28	read 7-day plate	streak 7-day plate
Tuesday	(28 + 4 h)	read 4-h plate
Wednesday	29	read 24-h plate
Thursday	30	read 48-h plate
Friday	31	read 72-h plate
Sat./Sun.	32-33
Monday	34
Tuesday	35	read 7-day plate

TABLE 2 Schedule B—Proposed Fungi Testing, Covering 4-h, 24-h, 48-h, 72-h, and 7-Day Tests

Day of Week	Day no.	First Challenge	Second Challenge	Third Challenge	Fourth Challenge
Friday	(-10)	inoculate fresh fungal culture
Friday	(-3)	...	inoculate fresh fungal culture
Monday	0	prepare spore suspension
Monday	0	inoculate specimens
Monday	(0 + 4 h)	streak 4-h plate
Tuesday	1	streak 24-h plate
Wednesday	2	streak 48-h plate
Thursday	3	streak 72-h plate
Friday	4	inoculate fresh fungal culture	...
Sat./Sun.	5, 6
Monday	7	...	prepare spore suspension
Monday	7	streak 7-day plate	inoculate specimens
Monday	(7 + 4 h)	read 4-h plate	streak 4-h plate
Tuesday	8	read 24-h plate	streak 24-h plate
Wednesday	9	read 48-h plate	streak 48-h plate
Thursday	10	read 72-h plate	streak 72-h plate	...	inoculate fresh fungal culture
Fri./Sat./Sun.	11, 12, 13
Monday	14	prepare spore suspension	...
Monday	14	read 7-day plate	streak 7-day plate	inoculate specimens	...
Monday	(14 + 4 h)	...	read 4-h plate	streak 4-h plate	...
Tuesday	15	...	read 24-h plate	streak 24-h plate	...
Wednesday	16	...	read 48-h plate	streak 48-h plate	...
Thursday	17	...	read 72-h plate	streak 72-h plate	...
Fri./Sat./Sun.	18, 19, 20
Monday	21	prepare spore suspension
Monday	21	...	read 7-day plate	streak 7-day plate	inoculate specimens
Monday	(21 + 4 h)	read 4-h plate	streak 4-h plate
Tuesday	22	read 24-h plate	streak 24-h plate
Wednesday	23	read 48-h plate	streak 48-h plate
Thursday	24	read 72-h plate	streak 72-h plate
Fri./Sat./Sun.	25, 26, 27
Monday	28	read 7-day plate	streak 7-day plate
Monday	(28 + 4 h)	read 4-h plate
Tuesday	29	read 24-h plate
Wednesday	30	read 48-h plate
Thursday	31	read 72-h plate
Fri./Sat./Sun.	32, 33, 34
Monday	35	read 7-day plate

from contaminated adhesives as an aid in determining the amount and type of biocide necessary to kill or inhibit the growth of the contaminants. If an isolated microorganism not generally used as a challenge organism, is chosen as the inoculum, it is important to identify the organism and determine on which medium and under what conditions it will grow, in order to demonstrate the efficacy of the biocide.

5.4 The results obtained when using the procedures given in these methods apply only to the species which are used for the testing. The test species listed in Section 9 are frequently used by laboratories to test for antimicrobial properties, but they are not the only ones which could be used. Selection of the species to use for these test methods requires informed judgment by the testing laboratory or by the party requesting the tests. It is also important that species which commonly attack adhesives be used. See 9.4.

5.5 The presence of an active biocide carried over from the adhesive specimen to the agar could have an inhibiting effect on the growth of microorganisms, resulting in no growth

during the span of a normal incubation period, when in fact, viable microorganisms are present, but their growth has been slowed down or held in stasis. The use of Lethen agar and broth is recommended to neutralize the effect of this carry-over.

NOTE 4—Lethen agar may be used for the streak plates, or if another agar is chosen for testing, a Lethen agar plate could be streaked as a control to test against the neutralizing effect. Even more effective would be diluting the challenged adhesive specimen with Lethen broth and running Lethen agar pour plates. See Note 1 and Note 3. Extending the incubation period of negative plates would be another safeguard. To neutralize thiazoline-based preservatives, 10 to 50 ppm of sodium thioglycolate can be added to the medium.

5.6 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 microbiological attack can be drawn by comparing the results to simultaneously run controls of known resistance. See X5.2 for statements regarding test repeatability.

6. Apparatus

6.1 In addition to the standard equipment found in any fully equipped microbiological laboratory, the following items are sometimes needed:

6.1.1 *Autoclave*, capable of producing 103 kPa of steam pressure at 121°C (250°F) and maintaining it for a minimum of 15 min.

6.1.2 *Cell Counting Chamber, Petroff-Hausser*, cell depth 0.02 mm (or equivalent).⁶

6.1.3 *Bottles, Screwcap*, approximately 375 mL, Boston Rounds of flint glass. Mold-A-7232-D, Finish 28-400, and Black Artmold Caps BM-8041, Size 28-400, with rubber ring liners fastened to caps with steamproof adhesive.⁷

6.1.4 *Constant Temperature Chamber*, capable of being maintained at 35 ± 0.5°C (95 ± 1°F) for bacteria, or 30 ± 0.5°C (86 ± 1°F) for fungi, or two chambers if needed simultaneously.

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

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

6.1.7 *Hood*, laminar flow type.⁸

6.1.8 *Jar, Screw Cap*, round, approximately 1 L (1-qt mason type) for samples.

6.1.9 *Pasteur Pipets*.⁶

6.1.10 *Pipet*, 1 mL, disposable, sterile.

6.1.11 *Pipettes*, automatic Oxford or Eppendorf, sterile, with sterile tips.⁶

6.1.12 *Refrigerator*, capable of maintaining temperature of 4 ± 1°C (39 ± 2°F).

6.1.13 *Spectrophotometer*, capable of measuring cell count at a wavelength of 425 nm.⁶

7. Materials

7.1 *Beef Extract*.

7.2 *Deionized or Distilled Water*, sterile.

7.3 *Disinfectant Solution, Amphyll, Alcide*, or equivalent.⁶

7.4 *Glucose*.

7.5 *Lethen Agar*, (Difco or equivalent).⁶

7.6 *Lethen Broth*, (Difco or equivalent).⁶

7.7 *Mycophil Agar*, pH 4.7 (BBL or equivalent).⁶

7.8 *Nutrient Agar*, (BBL or equivalent).⁶

7.9 *Potato Dextrose Agar (PDA, Dehydrated)*, (Difco or equivalent).⁶

7.10 *Phosphate buffered saline (PBS)*, sterile.

7.11 *Physiological Saline Solution, 0.85 % NaCl*, sterile.

7.12 *Sorbitan mono-oleate polyoxyethylene*.⁹

7.13 *Tryptone*.

7.14 *Tryptone Glucose Extract Agar*, dehydrated (Difco or equivalent).

7.15 *Yeast Malt Peptone Glucose Agar (YMPGA)*, dehydrated (Difco or equivalent).

8. Precautions

8.1 These test methods employ live cultures of bacteria, fungi, and yeast, some of which are capable of causing disease, and others allergic reaction in some humans. In addition to other precautions, assign laboratory personnel trained in correct microbiological techniques to run these tests. Use proper microbiological procedures in order to prevent contamination of the cultures or of the work area. Clean and sterilize in an approved manner all spills and all equipment coming into contact with the cultures and the inoculated adhesive specimens. Also sterilize in an approved manner all cultures and contaminated disposable equipment before discarding. See 1.3.

9. Test Organisms¹⁰

9.1 Cultures of one or more of the following bacterial species are suggested for use:

9.1.1 *Pseudomonas fluorescens* (ATCC 9721).

9.1.2 *Pseudomonas aeruginosa* (ATCC 10145).

9.1.3 *Bacillus subtilis* (ATCC 6984).

9.1.4 *Proteus vulgaris* (ATCC 9920).

9.2 Cultures of one or more of the following yeasts are suggested for use:

9.2.1 *Candida tropicalis* (ATCC 750).

9.2.2 *Kluyveromyces fragilis* (ATCC 8554).

9.2.3 *Candida pseudotropicalis* (ATCC 4135).

9.3 Cultures of one or more of the following species of fungi are suggested for use:

9.3.1 *Aspergillus niger* (ATCC 9642).

9.3.2 *Aspergillus flavus* (ATCC 9643).

9.3.3 *Penicillium pinophilum* (ATCC 9644). See X3.1.6.

9.4 Since a number of other organisms may be of specific interest for certain adhesives or for the environment to which they are to be exposed, such other pure cultures or mixtures of cultures of organisms may be used, if agreed upon between the manufacturer and the purchaser of the adhesive, or upon the recommendation of the testing laboratory.

NOTE 5—See Appendix X1, Appendix X2, Appendix X3, and Appendix X4 for guidelines on selection of species of bacteria, yeast, and fungi, and on problems with use of combined species.

NOTE 6—The use of combined species for the challenge inoculum can result in one species overgrowing the others. When a mixed inoculum is the choice, the recommended groupings are (a) fungi and yeast, and (b) Gram-positive and Gram-negative bacteria. Group (a) organisms prefer a pH of 4.5, while Group (b) organisms prefer a pH of 7. Yeast grow faster than fungi, a fact which should be considered in two steps in the test. The mixed inoculum should be used within 2 h of preparation, and for yeast, the streak plates to determine sterility should be read on Schedule A (same as for bacteria), while for fungi, the plates should be read on Schedule B.

⁶ Available from most laboratory supply houses.

⁷ Screw cap bottles are available from Owens-Illinois Glass Co., OH Building, Toledo, OH, or equivalent bottles and caps have been found suitable for this purpose.

⁸ The Biogard Hood or similar equipment, The Baker Co., Sanford, ME.

⁹ Available commercially as Tween 80.

¹⁰ Cultures may be purchased from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

10. Sterilization of Equipment and Media

10.1 Follow accepted microbiological practices for sterilizing equipment and media used for the tests. Also, sterilize in an approved manner all cultures and contaminated adhesive specimens before discarding.

NOTE 7—Two references for sterilization methods are TAPPI T487 and Ref (1).¹¹

11. Preparation of Media

11.1 *Potato Dextrose Agar and Tryptone Glucose Agar:*

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

11.1.2 Follow the instructions given for preparation of the commercial product. Dissolve using heat and agitation. Transfer 10 mL of the agar solution to each test tube. 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. Tighten the caps on the tubes and place them in a slanted position to solidify, making a slant of about 51 mm. Store in refrigerator until needed.

11.2 *Other Agar Types—Lethen, Nutrient, and Mycophil—*Prepare as needed to conduct tests, using commercially available product.

11.3 *Tryptone Glucose Extract Broth and Lethen Broth:*

11.3.1 Prepare sufficient media to conduct the tests.

11.3.2 For the tryptone glucose broth add 1.5 g of beef extract, 2.5 g of tryptone, and 0.5 g of glucose to 500 mL of cold distilled water in a 1000-mL beaker. For the Lethen broth, follow directions for the commercial product.

11.3.3 For both types of broth, heat to boiling to dissolve the medium completely, transfer 10 mL of the dissolved medium to each test tube, and autoclave tubes (with caps loose) for 15 min at 103 kPa and a temperature of 121°C (250°F).

11.3.4 Upon removal from the autoclave, allow the tubes to cool to room temperature, tighten the caps, and refrigerate until needed.

12. Bacterial Cultures

12.1 *Propagation of Bacterial Cultures:*

12.1.1 Prepare on a tryptone glucose agar slant a fresh culture for each species to be used for testing, and label it by species and ATCC Number. Incubate at 30 ± 5°C (86 ± 1°F).

12.1.2 When mature and luxuriant growth is achieved, refrigerate the cultures. Prepare new cultures each month. If contamination occurs, discard the cultures and prepare new ones.

12.2 *Preparation of Bacterial Inoculum—*Use the following procedure for each species to be used in the test procedure:

12.2.1 Twenty four hours before starting the test, inoculate a fresh culture on a tryptone glucose agar slant as described in 12.1.1 and incubate at 30 ± 5°C (86 ± 1°F). On the day the test is started, add 10 mL of sterile PBS to the tube, and using a sterile glass rod as an impinger, mix the bacterial growth into

¹¹ The boldface numbers in parentheses refer to the references at the end of this standard.

the PBS. See Note 8, Note 9, and Note 10 for details of preparation. Transfer to a sterile container and adjust the bacterial count to 1 × 10⁷ to 1 × 10⁸, preferably using a spectrophotometer. Set the spectrophotometer at 425 nm. Adjust to an optical density of 0.45 using an optical quality test tube measuring 12.7 mm in diameter. This should give approximately 1.0 × 10⁸ bacteria/mL. For yeast, these settings should give approximately 1.0 × 10⁷ yeasts/mL. Use the product insert provided by the manufacturer for the method of determining cell count.

12.2.2 For a mixed-species inoculum, first adjust each suspension of an individual species to the given level, and then combine equal portions. Use the bacterial suspension as the inoculum for the test in 15.3.1. Prepare a fresh suspension for each subsequent challenge described in 15.3.3.

NOTE 8—Prepare the PBS solution by the following formula:

	grams
NaCl	7.2
Na ₂ HPO ₄ (anhydrous)	1.48
KH ₂ PO ₄ (anhydrous)	0.43

Dilute to 1 L and sterilize. pH should be 7.2.

Instead of PBS, some laboratories use sterile deionized water or sterile physiological saline solution. See 7.2 and 7.11.

NOTE 9—Follow the same general procedure as given in A1.1 to prepare the primary suspension, except for bacteria and yeasts, use PBS as the diluent, do not use a wetting agent, and rinse the pellet only once.

NOTE 10—The alternate method for measuring and adjusting the bacterial and yeast count is use of the Petroff-Hausser counting chamber, which has a depth of 0.02 mm and can handle bacteria and yeasts better than the hemacytometer chamber. Methyl violet may be used to stain the bacteria for better visibility. See the instructions which accompany the counting chamber.

NOTE 11—To meet Schedule A in Table 1, make the inoculation (12.2.1) on Monday, and prepare the suspension (12.2.1 and 12.2.2) on Tuesday, the day the adhesive specimens are inoculated.

13. Yeast Cultures

13.1 Follow the instructions for bacterial cultures in Section 12, except use YMPG agar and determine the cell count using a hemacytometer or a Petroff-Hausser counting chamber. See Note 9.

14. Fungal Cultures

14.1 *Propagation of Fungal Cultures—*Use the following procedure for each species to be used in the tests:

14.1.1 Prepare a fresh culture on potato dextrose agar or other suitable medium for the species, and label by species and ATCC number. Incubate at 25 ± 0.5°C (77 ± 1°F) for 10 days or until full sporulation is achieved.

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

14.2 *Preparation of Fungal Inoculum—*Use the following procedure for each species to be used in the tests:

14.2.1 Ten days before the test is to be started, follow the procedure in 14.1.1 to prepare fresh cultures.

14.2.2 *Harvesting Fungal Cultures and Dislodging Spores—*On the day of the first challenge, to one tube of each species of fungi, add 15 mL of sterile deionized water, containing 0.05 % sorbitan mono-oleate polyoxyethylene. Harvest fungal cultures and dislodge spores by rubbing and

removing the growth gently with a sterile inoculating loop or 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 1.0×10^7 spores/mL, ± 2000 , using a hemacytometer and the procedure in [Annex A1](#). Use this spore suspension of fungi as the inoculum for the test described in [15.4.1](#) and [15.4.3](#) when the option is to test with a single species.

14.2.3 For a mixed culture, obtain a spore count on each fungal species and adjust each suspension to the level of 1×10^6 spores/mL. Combine equal portions of the spore suspensions from each of the species in a common sterilized container. Use this spore suspension for the challenge procedures described in [15.4.1](#) and [15.4.3](#) when the option is to test with a mixed species.

NOTE 12—To meet Schedule B in [Table 2](#) make the inoculation ([14.2.1](#)) on Friday 10 days before first challenge, and prepare the suspension ([14.2.2](#) and [14.2.3](#)) on Monday, the day of the challenge.

15. Test Procedures, Bacterial, Yeast, and Fungal Resistance

15.1 *Adhesive Sample*—Obtain a sufficient sample of adhesive to run the necessary tests, place in a sterilized container, and cap.

15.2 *Adhesive Specimen*—For each test to be run, transfer 300 mL of the adhesive sample to a sterilized 400-mL jar and seal.

NOTE 13—The specimen size may be smaller or larger, but the millilitres of inoculum in [15.3.1](#) should be adjusted to reflect the millilitres of the adhesive specimen.

15.3 Test for Resistance to Bacteria or Yeast:

15.3.1 *Procedure, First Challenge*—Using a sterile pipet, add 5 mL of the aqueous suspension of organisms prepared in [12.2.1](#) or [12.2.2](#) to the 300 mL adhesive specimen prepared in [15.2](#). Mix well using a sterile glass rod or sterile wooden spatula and incubate at $30 \pm 5^\circ\text{C}$ ($86 \pm 1^\circ\text{F}$).

15.3.2 Procedure, Sterility Check:

15.3.2.1 At intervals 4, 24, 48, and 72 h and at 7 days, or at other intervals selected by the testing laboratory, culture the inoculated adhesive specimen onto tryptone glucose agar, making a zig-zag streak plate. For the transfer, use a sterile loop. Incubate each agar plate at $30 \pm 5^\circ\text{C}$ ($86 \pm 1^\circ\text{F}$) for 7 days.

NOTE 14—Some biological laboratories streak with sterile cotton-tipped swabs. Other types of agar are sometimes selected.

15.3.2.2 Examine all streak plates according to Schedule A on [Table 1](#), or the specially designed schedule being used. Record the presence and degree of colonial growth, or the absence of growth on each of the streak plates. To show the comparative degree of growth on the positive plates, use qualitative gradings: light, medium, and heavy.

15.3.2.3 If colonial growth of the species used for the inoculum appears on the surface of the 7-day streak plate at the end of the 7-day incubation period, the test is complete, and report the adhesive sample to be *not resistant* to the species or group of species of bacteria used for the challenge.

15.3.2.4 If colonial growth of bacteria is not observed at the end of the incubation period for the 7-day streak plate, continue the test as described in [15.3.3](#).

15.3.3 *Procedure, Subsequent Challenges*—For any specimen testing sterile to the first challenge, repeat the procedure described in [15.3.1](#) – [15.3.2.4](#), reinoculating the same adhesive specimen, following the selected schedule. Discontinue testing at any point in the schedule on a specimen which shows colonial growth on the 7-day streak plate. See Sections [16](#) and [17](#) for further guidance on additional challenges, interpretation of results, and reporting.

15.3.4 *Control Streak Plate*—Test the adhesive specimen for presence of microorganisms before challenge by streaking on potato dextrose and tryptone glucose agar plates.

15.3.5 As a control to any test run, challenge an unprotected adhesive specimen.

15.3.6 *Standard Inhibitory Control*—Include in any ladder series or in any test of a single adhesive specimen, a control specimen protected with a level of biocide known to be adequate.

NOTE 15—An inhibitory control is useful as a check on the procedural process, since its performance is predictable. Preferably this should be the same formulation as the test adhesive, but containing a sufficient biocide level to protect. If this is not available, an adequately protected unrelated adhesive formulation which has been pretested may be used.

15.4 Test for Resistance to Fungi:

15.4.1 *Procedure, First Challenge*—Follow the procedure given in [15.3.1](#), except use the aqueous fungal spore suspension prepared in [14.2.2](#) or [14.2.3](#) for the inoculum and incubate the challenged specimen at $25 \pm 0.5^\circ\text{C}$ ($77 \pm 1^\circ\text{F}$).

15.4.2 *Procedure, Sterility Check*—Follow the procedure given [15.3.2.1](#) and [15.3.2.2](#), except use potato dextrose agar plates or other suitable medium chosen for the species. Incubate the streak plates at $25 \pm 0.5^\circ\text{C}$ ($77 \pm 1^\circ\text{F}$), and follow Schedule B on [Table 2](#).

15.4.3 *Procedure, Subsequent Challenges*—Follow the procedure given in [15.3.3](#), except use a freshly prepared aqueous suspension of fungal spores.

15.4.4 *Controls*—See [15.3.4](#), [15.3.5](#), and [15.3.6](#).

16. Interpretation of Test Results

16.1 The highest level of biocidal protection is shown by a specimen returning to sterility by the fourth hour following the challenge, holding this sterility for 7 days, and performing in the same manner after four challenges. Consider a specimen that passes this test, however, as protected only against the species used for the challenge. Return to sterility 24 h after the challenge and holding this sterility for seven days, and showing this performance after two to four challenges is considered adequate microbial protection for some adhesives by many biological laboratories.

17. Report

17.1 Report the following information:

17.1.1 The species of bacteria, yeast, or fungi used for each test,

17.1.2 The schedule followed, A or B, or a summary of the testing procedure if it varies from the ones given,

17.1.3 A separate report for each test which used an individual species and each test which used a mixed culture,

17.1.4 The final readings on all streak plates, recording the presence of colonial growth, graded as light, medium or heavy, as positive, and absence of growth as negative,

17.1.5 The time required for the specimen to return to sterility,

17.1.6 The presence or absence of colonial growth on the control and inhibitory streak plates, and

17.1.7 Based on the presence or absence of growth, in accordance with the guidelines given, report the adhesive specimen to be *resistant* or *not resistant* to the organisms used

for the challenge, when tested by the procedure as summarized according to 15.3 and 15.4.

18. Precision and Bias

18.1 No information is presented about either the precision or bias of Test Methods in D4783 for measuring the resistance of adhesive preparations to attack by bacteria, yeast, and fungi since the test results are non quantitative.

19. Keywords

19.1 adhesive; bacteria; biological testing; culture; fungi; growth; yeast

ANNEX

(Mandatory Information)

A1. PROCEDURE FOR DETERMINING FUNGAL SPORE CONCENTRATION IN THE INOCULUM

A1.1 *Preparation of Primary Suspension*—Follow the procedure in 14.2 to harvest the spores. Transfer the washings from an agar slant of each species into a different sterile flask. Using a sterile 50-mL centrifuge tube, wash the spores of each species separately three times in sterile distilled water with the first wash containing a wetting agent, such as Tween 80 or the equivalent, 0.05 % sorbitan mono-oleate polyoxyethylene in deionized water. Centrifuge, decant and re-suspend. Do not use a wetting agent in the final rinse or for the final dilution. After the final centrifugation, leave the pellet in 5 to 6 mL of liquid. Re-suspend with a mechanical shaker, and use this primary suspension to make the spore count, using the hemacytometer.

A1.2 *Loading the Hemacytometer Chamber*—Place the special cover slip for the hemacytometer over the counting areas by centering the slip so that it touches the “V’s.” Using a sterile Pasteur pipet (6.1.11), or a disposable 1-mL pipet, charge by capillary action both sides of the chamber introducing the spore suspension at the “V’s.” If the liquid flows into the trough, wash the counting chamber and recharge. Wait 5 min to allow the spores to settle before counting. Make sure the spores are evenly distributed to assure an accurate count.

A1.3 *Dimensions of Hemacytometer Chamber:*

A1.3.1 For the purposes of these test methods, the dimensions given are for the subdivisions of the center primary square. This square is ruled into 25 secondary squares, which are further divided into 16 tertiary squares.

A1.3.2 The Levy hemacytometer chamber is $\frac{1}{10}$ mm deep and is divided into squares of the following dimensions:

$$\begin{aligned} \text{Primary square} &= 1 \text{ by } 1 \text{ by } 1/10 \text{ mm} = 1/10 \text{ mm}^3 \\ \text{Secondary square} &= 1/5 \text{ by } 1/5 \text{ by } 1/10 \text{ mm} = 1/250 \text{ mm}^3 \\ \text{Tertiary square} &= 1/20 \text{ by } 1/20 \text{ by } 1/10 \text{ mm} = 1/4000 \text{ mm}^3 \end{aligned}$$

A1.4 *Counting the Spores*—Use a 10× ocular and a 25× objective on the microscope. Focus on the center subdivided primary square, and count the spores in 5 secondary squares, 4

corners and the middle. This will total 80 tertiary squares. It may be necessary to dilute a portion of the primary suspension to make the count. Count all of the spores in the designated areas, but count only the spores that are on the right and lower boundary lines of each square, the center line of triple lines demarking the squares. Typically the primary suspension should have 6 to 10 times the desired 1×10^6 spores per millilitre, making it possible to dilute to the desired concentration.

NOTE A1.1—For a graphic depiction of the grids of the chamber, see the instructions which come with the hemacytometer. These directions give procedures for counting white blood cells and red blood cells, the original use of the chamber. The procedure given here is the one used for the red blood cell count, except the dilution may vary, and a factor of 1000 is used to convert cubic millimetres to millilitres. For further discussion on the spore count, see Refs (2) and (3).

A1.5 *Calculation:*

A1.5.1 The volume of 80 tertiary squares equals $80 \times \frac{1}{4000} \text{ mm}^3$.

A1.5.2 When no dilution is used, the dilution factor is one.

A1.5.3 When the spores in 80 tertiary squares are counted, use the two-part formula below:

$$\begin{aligned} \text{spores per mm}^3 &= \frac{(\text{total spores counted}) \times (\text{dilution}) \times 4000}{80} \\ (\text{spores per mm}^3) \times 1000 &= (\text{spores per mL}) \end{aligned}$$

Example: When 200 spores are counted, and the dilution factor is one:

$$1.0 \times 10^4 \text{ spores per mm}^3 = \frac{(200) \times (1) \times 4000}{80}$$

$$1.0 \times 10^4 \text{ spores per mm}^3 \times 1000 = 1.0 \times 10^7 \text{ spores per mL}$$

A1.6 After determining the number of spores per millilitre in the primary spore suspension, transfer 1 mL of the suspension to a sterile tube and make the proper dilution to achieve