Designation: E2149 – 13a

Standard Test Method for Determining the Antimicrobial Activity of Antimicrobial Agents Under Dynamic Contact Conditions[1]

This standard is issued under the fixed designation E2149; 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 is designed to evaluate the antimicrobial activity of non-leaching, antimicrobial-treated specimens under dynamic contact conditions. This dynamic shake flask test was developed for routine quality control and screening tests in order to overcome difficulties in using classical antimicrobial test methods to evaluate substrate-bound antimicrobials. These difficulties include ensuring contact of inoculum to treated surface (as in AATCC 100), flexibility of retrieval at different contact times, use of inappropriately applied static conditions (as in AATCC 147), sensitivity, and reproducibility.

1.2 This test method allows for the ability to evaluate many different types of treated substrates and a wide range of microorganisms. Treated substrates used in this test method can be subjected to a wide variety of physical/chemical stresses or manipulations and allows for the versatility of testing the effect of contamination due to such things as hard water, proteins, blood, serum, various chemicals, and other contaminants.

1.3 Surface antimicrobial activity is determined by comparing results from the test sample to controls run simultaneously.

1.4 The presence of an antimicrobial that requires neutralization is determined by the post-test.

1.5 Proper neutralization of all antimicrobials must be confirmed using Test Methods E1054.

1.6 This test method should be performed only by those trained in microbiological techniques.

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

1.8 *This standard may involve hazardous materials, operations, and equipment. 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.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

2.2 *AATCC Documents:*[3]

AATCC 147 Antibacterial Activity Assessment of Textile Materials: Parallel Streak Method

AATCC 100 Antibacterial Finishes on Fabrics

3. Summary of Test Method

3.1 The antimicrobial activity of a substrate-bound, non-leaching antimicrobial agent is dependent upon direct contact of microbes with the active chemical agent. This test determines the antimicrobial activity of a treated specimen by shaking samples of surface-bound materials in a concentrated bacterial suspension for a one hour contact time. The suspension is serially diluted both before and after contact and cultured. The number of viable organisms from the suspension is determined and the percent reduction (or $\log_{10}$ reduction) is calculated by comparing retrievals from appropriate controls.

4. Significance and Use

4.1 Chemically bonded, antimicrobial agents are not free to diffuse into their environment under normal conditions of use. This test method ensures good contact between the bacteria and the treated fiber, fabric, or other substrate, by constant agitation of the test specimen in a challenge suspension during the test period.

[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.

[3] Available from American Association of Textile Chemists and Colorists (AATCC), P.O. Box 12215, Research Triangle Park, NC 27709, http://www.aatcc.org.

4.2 The metabolic state of the challenge species can directly affect measurements of the effectiveness of particular antimicrobial agents or concentrations of agents. The susceptibility of the species to particular biocides could be altered depending on its life stage (cycle). One-hour contact time in a buffer solution allows for metabolic stasis in the population. This test method standardizes both the growth conditions of the challenge species and substrate contact times to reduce the variability associated with growth phase of the microorganism.

4.3 Leaching of an antimicrobial is dependent upon the test conditions being utilized and the ultimate end use of the product. For example, water soluble antimicrobials will be prone to removal from the test surface using the method described in Section 13 but insoluble compounds will not. It is for this reason that the use of the term leaching throughout this document is limited to only the testing conditions described herein. To determine if a compound is immobilized in all conditions or during the end use of the product additional testing may be required.

4.4 This test method cannot determine if a compound is leaching into solution or is immobilized on the substrate. This test method is only intended to determine efficacy as described in 4.5 and subsequent portions of the method.

4.5 This test method is intended to evaluate antimicrobial agents that are not removed from the surface by the aqueous testing conditions, as evaluated by Section 13. If an antimicrobial agent that is shown to be removed from the surface by Section 13 is utilized in this test methodology, controls must be included such that appropriate neutralization steps are including during recovery and enumeration.

4.6 The test is suitable for evaluating stressed or modified specimens, when accompanied by adequate controls.

NOTE 1—Stresses may include laundry, wear and abrasion, radiation and steam sterilization, UV exposure, solvent manipulation, temperature susceptibility, or similar physical or chemical manipulation.

5. Definitions

5.1 *Immobilized:* The antimicrobial remains on the surface of the article throughout the test as determined by the absence of bactericidal activity in Section 13. A neutralizer does not need to be included for this type of antimicrobial

5.2 *Leaching:* Removal of the antimicrobial from the surface by the test conditions being utilized, resulting in a concentration high enough to cause bactericidal activity as defined in Section 13. A valid neutralizer must be utilized for this type of antimicrobial

6. Apparatus

6.1 *Air displacement pipettes,* Eppendorf or equivalent, 100 to 1000 µL with disposable tips.

6.2 *Analytical balance,* to weigh chemicals and substrates and to standardize inoculum delivery volumes by pipettes.

6.3 *Glassware:*

6.3.1 *Contact Flask,* 250 mL Erlenmeyer flask, capped, autoclavable.

6.3.2 *Test tubes,* 18 × 150 mm rimless bacteriological test tubes used for growing test organisms and for serial dilution.

6.4 *Incubator,* capable of maintaining a temperature of 35 ± 2°C.

6.5 *Shaker, wrist action,* capable of aggressive agitation of bacteria and substrate solutions.

6.6 *Spectrophotometer,* capable of measuring an absorbance of 475 nm.

6.7 *Sterile serological pipettes,* capable of 50 and 10 mL capacity.

6.8 *Sterilizer,* any suitable steam sterilizer producing the conditions of sterility.

6.9 *Vortex mixer,* to vortex dilution tubes during serial dilutions.

6.10 *Water bath,* for short term storage of liquefied agar media, capable of maintaining 45 to 50°C.

7. Reagents

7.1 *Buffer Solution*—The following solution is prepared from reagent-grade chemicals. For buffer stock solution (0.25M KH_2PO_4): Prepare a fresh stock solution at least once every 6 months as follows: Weigh 34 ± 0.1 g of potassium dihydrogen phosphate into a 1000 mL beaker. Add 500 mL of distilled water. Adjust pH to 7.2 ± 0.1 with a dilute solution of NaOH. Dilute to 1000 mL; transfer to a flask and store at 4°C. For working buffer solution (0.3mM KH_2PO_4): Prepare a fresh solution at least once every 2 months as follows: Transfer 1 ± 0.01 mL of stock buffer solution with a sterile pipette to flask containing 800 mL of distilled water. Cap, sterilize and store at room temperature.

7.2 *Media:*

7.2.1 *Tryptic Soy Broth,* prepared according to manufacturer's directions.

7.2.2 *Plate Count Agar,* prepared according to manufacturer's directions.

7.3 *Wetting Agent Surfactant*—Agents must be shown by prior testing at the intended use concentration not to cause a reduction or increase in bacterial numbers. DC Q2-5211[4] at 0.01 % final dilution of working buffer solution has been shown to be effective.

8. Test Organism

8.1 *Escherichia coli,* American Type Culture Collection No. 25922.

8.1.1 Cultures of the test organism should be maintained according to good microbiological practice and checked for purity on a routine basis. Consistent and accurate testing requires maintenance of a pure, uncontaminated test culture. Avoid contamination by use of good sterile technique in plating and transferring. Avoid mutation or reversion by strict adherence to monthly stock transfers. Check culture purity by

[4] The sole supplier of DC Q2-5211 known to the committee at this time is Dow Corning, Midland, MI. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

making streak plates periodically, observing for colonies characteristic of *Escherichia coli*, and Gram-staining.

8.1.2 Alternative organisms can be substituted depending on the end use of the product. However, the precision and bias statement has been developed using *Escherichia coli* ATCC 25922. There is are no data to support a precision and bias statement for other organisms at this point. Use of alternate organisms shall be included in the report, in addition to any other modification of media, buffer, bacterial concentration, etc.

9. Parameters

9.1 Surface preparation or conditioning must be specified. Prior manipulation of the specimen may be required in order to demonstrate maximum activity in a desired time frame and must be reported and compared to identically handled controls.

9.2 The weight, size, and material of construction of specimen must be specified.

9.3 Specimens should be prepared such that they can maximize agitation and are reflective of a recordable ratio of surface area to test titer.

10. Preparation of Bacterial Inoculum

10.1 Grow a fresh 18 h shake culture of *Escherichia coli* in sterile Tryptic Soy Broth at 35 ± 2°C prior to performing the test.

10.2 Dilute the culture with the sterile buffer solution until the solution has an absorbance of 0.28 ± 0.02 at 475 nm, as measured spectrophotometrically. This has a concentration of 1.5-3.0 × 10^8 CFU/mL. Dilute appropriately into sterile buffer solution to obtain a final concentration of 1.5-3.0 × 10^5 CFU/mL. This solution will be the working bacterial dilution.

11. Test Specimen

11.1 *Preparation of Test Specimen:*

11.1.1 *Fabric and Paper*—Samples are selected on weight basis and weighed to 1.0 ± 0.1 g.

11.1.2 *Powder and Granular Material*—Weigh to 1.0 ± 0.1 g. The material must settle after shaking so that no specimen interferes with the retrieval and counting techniques.

11.1.3 *Other Solids (Surface Treatment)*—Reduce the solid in size to fit into the flask or use a sterile wide-mouth bottle. Use a specimen that gives 4 $in.^2$ (25.8 cm^2) of treated surface area. Specimen may also be selected on weight basis, ±0.1 g, at the discretion of the investigator. Care must be exercised during shaking not to break the flask or bottle. The untreated specimen of the solid must not absorb the solution. If appropriate to the nature of the test specimen, it can be mounted as a seal for the test container so that only the treated surface is in direct contact with the inoculum.

NOTE 2—Solids anticipated in this part of the method are plastics, glass beads or chips, ceramics, metal chips, or similar hard surfaces. Sample mass can vary from 0.5g-2.0g depending on sample composition. However, the precision and bias statement has been developed using a one gram sample of a textile material only. There are no data to support a precision and bias statement for other sample masses or sample types at this point. Include in the report the use of alternate sample mass or type, in addition to any other modifications of temperature, method of dynamic contact, etc.

12. Procedure for Determining Antimicrobial Activity

12.1 Prepare the specimen to be tested as described in Section 11. One treated piece of each specimen is required. One untreated piece of each specimen of identical composition is highly recommended for each series of specimen tested.

12.2 Prepare one sterile 250 mL screw-cap Erlenmeyer flask for each treated and untreated specimen, and one "inoculum only" sample for the series being run. Add 50 ± 0.5 mL of working dilution of bacterial inoculum prepared in 10.2 to each flask.

12.3 Determine bacterial concentration of solution at the "0" time by performing serial dilutions and standard plate count techniques from the "inoculum only" sample flask

12.4 Place the test and control specimen in their individual flasks. No series of test flasks should be large enough to require more than 5 min, post-contact, between the first and last serial dilution.

12.5 Place the series of flasks on the wrist-action shaker. Shake at maximum stroke for 1 h ± 5 min. Immediately serial dilute and plate each sample out in triplicate, as was done for the "0" contact time subgroup (12.3).

NOTE 3—Residual bacterial retention in/on specimen could be tested using appropriate retrieval techniques such as agar imprint tests or buffer extraction and plate count.

NOTE 4—Filter solutions in which samples have degraded during shaking. Whatman filter paper Type 1 has been found to be appropriate for this step. Contents of the "inoculum only" flask must be treated in the same manner.

12.5.1 Alternative contact times can be used depending on the end use of the product. However, the precision and bias statement has been developed using a one hour contact time. There are no data to support a precision and bias statement for other contact times at this point. Use of alternate contact times shall be included in the report, in addition to any other modifications of temperature, method of dynamic contact, etc.

12.6 Allow all the Petri dishes from both subsets to incubate for at 35 ± 2°C for 24 h.

12.7 Count the colonies in Petri dish. Record the values, average the triplicate Petri dish numbers and convert the average to colony-forming units per millilitre (CFU/mL).

NOTE 5—The presence of the original test organism may be confirmed by Gram stains and colony morphology.

NOTE 6—When the number of colony-forming units per mL is less than 30 on the lowest dilution plate, report the recovered CFU/mL as "<30", determine the percent reduction and report the reduction as "greater than" the percent found.

12.8 Calculate percent or log bacterial reduction.

12.8.1 If the average CFU/mL values for the untreated control and the "inoculum only" flask agree within 15 % after specified contact time, or if an untreated control is not available, calculate percent reduction of the organisms resulting from treated sample (*A*) directly compared to "inoculum only" flask after specified contact time (*B*) using the following formula. Results can be presented in either percent reduction when measuring CFU/mL or as Log_{10} bacterial reduction when calculating mean log_{10} density of bacteria.

$$\text{Reduction, \% (CFU/mL)} = \frac{B - A}{B} \times 100$$

$$\text{Log}_{10}\text{ bacteria reduction} = \text{Log}_{10}(B) - \text{Log}_{10}(A)$$

where:

A = CFU per millilitre for the flask containing the treated substrate after the specified contact time, and

B = CFU per millilitre for the "inoculum only" flask after the specified contact time.

12.8.2 If the untreated control (if present) and the "inoculum only" flask do not agree within 15 %, calculate the percent reduction of organisms from treated sample (A) directly compared to the untreated control (C).

$$\text{Reduction, \% (CFU/mL)} = \frac{C - A}{C} \times 100$$

$$\text{Log}_{10}\text{ bacteria reduction} = \text{Log}_{10}(C) - \text{Log}_{10}(A)$$

where:

A = CFU per millilitre for the flask containing the treated substrate after the specified contact time, and

C = CFU per millilitre for the flask containing the untreated substrate after the specified contact time.

12.9 Record and report the value to the nearest one-hundredth percent or $\log_{10}$ reduction of bacteria. Whether reduction calculations are based on values from an untreated control or inoculum control shall be indicated on report.

NOTE 7—Utilizting a one hour time point, if no untreated control substrate is available, and the counts for the flask containing the "inoculum only" control after specified contact time (C) are not within 15 % of original count, repeat the test. If longer time frames are utilized, such as 24h, it is possible to have growth within the "inoculum only" flask that would exceed the allowable 15%, in this case the test would not be repeated.

13. Procedure for Determining Presence of Leaching Antimicrobial

13.1 *Analysis of Supernatant (Post Test-Solution Test):*

13.1.1 Prepare test specimen as directed in Section 11.

13.1.2 After agitation, remove all specimens from solution and filter according to Note 4.

13.1.3 Add organism prepared in Section 10 to filtered buffer to obtain a final concentration of $1.5\text{-}3.0 \times 10^5$ CFU/mL.

13.1.4 Immediately determine bacterial concentration of solution at the "0" time by performing serial dilutions and standard plate count techniques.

13.1.5 Place the series of flasks on the wrist-action shaker. Shake at maximum stroke for 1 h ± 5 min. Immediately serial dilute and plate out in triplicate as was done for the "0" contact time subgroup.

13.1.6 Calculate percent reduction from initial "0" time as directed in 12.7.

13.1.7 Record and report presence of solution activity. Presence of residual antimicrobial activity indicates the presence of an antimicrobial agent in a solution that must be neutralized during testing.

NOTE 8—If it is determined that a neutralizer is required, a suitable neutralizer will be selected based on the Test Methods E1054 and will be incorporated during 12.5. Immediately after shaking samples will be diluted in the appropriate neutralizing solution.

14. Precision and Bias[5]

14.1 *Precision:*

14.1.1 An interlaboratory study (ASTM ILS #715) of this test method was conducted at ten laboratories testing four sample types (an untreated control, a low efficacy material, a high efficacy hydrophobic material, and a high efficacy hydrophilic material). An ANOVA model was fit with random effects to determine the repeatability and reproducibility of the log density of organisms in the inocula, the repeatability and reproducibility of the organisms associated with the untreated controls, and the repeatability and reproducibility of the log reductions of organisms associated with the treated sample results.

14.1.2 For the inoculum for this protocol, the repeatability standard deviation was 0.28; and the reproducibility standard deviation was also 0.28, with 0.00% of the variability due to among lab sources.

14.1.3 For the untreated control material for this protocol, the repeatability standard deviation was 0.21; and the reproducibility standard deviation was 0.29, with 50% of the variability due to among lab sources.

14.1.4 The repeatability and reproducibility of the log reductions (calculated with respect to the inoculum) for each sample type are summarized in the Table 1.

14.1.5 The repeatability and reproducibility of the log reductions (calculated with respect to the untreated controls) for each sample type are summarized in Table 2.

14.1.6 For the high efficacy hydrophobic and hydrophilic samples considered, the method was statistically significantly responsive to the increase in efficacy compared to the low efficacy sample.

14.2 *Bias*—Since an accepted reference value is not available, randomization is used whenever possible to reduce the potential for systematic bias.

[5] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:E35-1007. Contact ASTM Customer Service at service@astm.org.

TABLE 1 Repeatability and Reproducibility Standard Deviations as a Function of the LR with Respect to the Inocula at the T1 Time Point for the Three Sample Types Tested

Sample	Description	Mean LR	Repeatability SD	Among lab %	Reproducibility SD
2	low efficacy	1.70	0.3581	13%	0.8775
3	high efficacy, hydrophobic	5.40	0.7042	63%	1.15
4	high efficacy, hydrophilic	5.98	0.3186	24%	0.3643

TABLE 2 Repeatability and Reproducibility Standard Deviations as a Function of the LR with Respect to the Untreated Controls for the Three Sample Types Tested.

Sample	Description	Mean LR	Repeatability SD	Among lab %	Reproducibility SD
2	low efficacy	1.40	0.2111	93%	0.8255
3	high efficacy, hydrophobic	5.10	0.6940	62%	1.13
4	high efficacy, hydrophilic	5.68	0.2369	57%	0.3596

15. Keywords

15.1 antimicrobial; antibacterial; shake flask test; textile