Designation: E2149 – 10

# Standard Test Method for Determining the Antimicrobial Activity of Immobilized 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 100-2004), flexibility of retrieval at different contact times, use of inappropriately applied static conditions (as in AATCC 147-2004), 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 a leaching antimicrobial is determined both pre- and post-test.

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

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

1.7 *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 *AATCC Documents:*[2]

AATCC 147-2004 Antibacterial Activity Assessment of Textile Materials: Parallel Streak Method

AATCC 100-2004 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 Immobilized, as chemically bonded, antimicrobial agents are not free to diffuse into their environment under normal conditions of use. Textile test methods, such as AATCC 147-2004, that are directly dependent on the ready leachability of the antimicrobial agent from the treated fabric are inappropriate for evaluating immobilized antimicrobial agents. 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.

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

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

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 Liquid analysis of antimicrobial activity of non-leaching agents provides the ability to completely wet-out a test substrate. With the use of wetting-agent surfactants, false negatives observed when comparing hydrophobic and hydrophilic substrates can be reduced.

4.4 This test method is not intended for directly comparing the activities of leaching and non-leaching antimicrobial agents. In liquid environments, leaching biocides may release the active ingredient at differential rates. Furthermore, residual antimicrobial activity of leaching biocides may be present in serial dilution and may exert additional activity after desired contact time, unless adequately sequestered at end of test. Controls for both of these factors are not included in this test method; therefore, screening protocols are introduced to identify the presence of leaching biocides.

4.5 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. Apparatus

5.1 *Agar bore,* 8-mm diameter.

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

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

5.4 *Glassware:*

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

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

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

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

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

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

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

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

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

## 6. Reagents

6.1 *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.

6.2 *Media:*

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

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

6.3 *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[3] at 0.01 % final dilution of working buffer solution has been shown to be effective.

## 7. Test Organism

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

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

NOTE 2—Original method, ASTM E2149-01, specified *Klebsiella pneumoniae* as test organism. *Escherichia coli* is used in this test method as it is easier to handle and is a more universally accepted test type organism.

## 8. Parameters

8.1 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.

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

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

## 9. Preparation of Bacterial Inoculum

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

[3] The sole source of supply of the apparatus 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.

9.2 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.

## 10. Test Specimen

10.1 *Preparation of Test Specimen:*

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

10.1.2 *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.

10.1.3 *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 3—Solids anticipated in this part of the method are plastics, glass beads or chips, ceramics, metal chips, or similar hard surfaces.

## 11. Procedure for Determining Antimicrobial Activity

11.1 Prepare the specimen to be tested as described in Section 10. 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.

11.2 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 9.2 to each flask.

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

11.4 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.

11.5 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 (11.3).

NOTE 4—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 5—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.

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

11.7 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 6—The presence of the original test organism may be confirmed by Gram stains and colony morphology.

NOTE 7—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.

11.8 Calculate percent or log bacterial reduction.

11.8.1 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.

11.8.2 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.

11.9 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 8—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, the test must be repeated.

## 12. Procedure for Determining Presence of Leaching Antimicrobial

12.1 *Analysis of specimen (Pre-Test):*

12.1.1 Measure presence or absence of zone of inhibition using AATCC 147-2004.

12.2 *Analysis of Supernatant (Post-Test Zone of Inhibition (ZOI)):*

12.2.1 Inoculate plate count agar plate with confluent lawn of organisms and allow to dry.

12.2.2 Bore 8 mm diameter hole in center of inoculated agar plate and remove plug.

12.2.3 Prepare the specimen to be tested as in Section 10. One treated and one untreated piece of each specimen is highly recommended.

12.2.4 Prepare two sterile 250 mL flasks containing 50 mL sterile working buffer solution.

12.2.5 Place test and control specimen in their individual flasks. Cap the flasks and place them on the wrist-action shaker. Shake for 1 h ± 5 min.

12.2.6 Add 100 µL of solution directly from each flask tested to the agar hole and allow to dry. Set each plate for incubation at 35 ± 2°C for 24 h.

12.2.7 Record and report presence or absence of zone of bacterial inhibition surrounding 8 mm diameter hole. Presence of a zone of inhibition indicates leaching.

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

12.3.1 Prepare test solution as directed in 12.2.3 to 12.2.5.

12.3.2 After agitation, remove all specimens from solution and filter if large particulates are present.

12.3.3 Add organism prepared in Section 9 to filtered buffer to obtain a final concentration of 1.5-3.0 × $10^5$ CFU/mL.

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

12.3.5 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.

12.3.6 Calculate percent reduction from initial "0" time as directed in 11.7.

12.3.7 Record and report presence of solution activity. Presence of residual antimicrobial activity indicates the presence of a leaching type of antimicrobial agent.

NOTE 9—Presence of leaching antimicrobial activity in either of these determinations indicates the unsuitability of the specimen for this test method.

## 13. Precision and Bias

13.1 A precision and bias statement cannot be made for this test method at this time.

## 14. Keywords

14.1 antimicrobial; antibacterial; non-leaching; shake flask test; textile