Designation: E 2149 – 01

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 E 2149; 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 resistance of non-leaching antimicrobial treated specimens to the growth of microbes 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. This test also allows for the versatility of testing contamination due to such things as hard water, proteins, blood, serum, various chemicals, and other contaminates or physical/chemical stresses or manipulations of the specimens of interest.

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

1.3 The presence of a leaching antimicrobial is both pre- and post-determined by the presence of a zone of inhibition.

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

1.5 *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:*

E 1054 Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectant, Sanitizer, Antiseptic, or Preserved Products

2.2 *Other Documents:*

AATCC Test Method 147-1998 Antibacterial Activity Assessment of Textile Materials: Parallel Streak Method. American Association of Textile Chemists and Colorists, RTP, NC

AATCC Test Method 100-1999 Antibacterial Finishes on Fabrics, Evaluation of American Association of Textile Chemists and Colorists, RTP, NC

3. Summary of Test Method

3.1 Immobilized antimicrobial agents, such as surface bonded materials, are not free to diffuse into their environment under normal conditions of use. Test methods such as AATCC 147 that are directly dependent on the ready leachability of the antimicrobial agent from the treated fabric are inappropriate for evaluating immobilized antimicrobial agents. The following 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 bacterial suspension during the test period. 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.

4. Significance and Use

4.1 The antimicrobial activity of a substrate-bound antimicrobial is dependent upon direct contact of microbes with the active chemical agent. This test determines the antimicrobial activity of treated specimen by shaking samples of surface bound materials in a concentrated bacterial suspension for a one hour contact time or other contact times as specified by the investigator. The suspension is serially diluted both . before and after contact and cultured. The number of viable organisms in the suspension is determined and the percent reduction is calculated based on initial counts or on retrievals from appropriate untreated controls.

NOTE 2—This method is intended for those surfaces having a percent reduction activity of 50 % to 100 % for the specified contact time.

5. Apparatus

5.1 *Sterilizer*,

5.2 *Incubator*,

5.3 *Spectrophotometer*,

5.4 *Shaker, Wrist Action*—A Wrist Action Shaker is recommended but other means of agitation such as reciprocal action shakers may be satisfactory for routine testing. Shaker must ensure good agitation. Rotary shakers are unacceptable.

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

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5.5 *Water Bath*,

5.6 *Vortex Mixer*,

5.7 *Glassware*,

5.7.1 *Contact Flask*, 250 ml Erlenmeyer flask, capped, autoclavable.

5.7.2 *Dilution Vessels*,

5.7.3 *Pipettes*,

5.8 *Agar*, bore 8-mm diameter.

6. Reagents

6.1 *Buffer Solution*—For test specimen which might alter the pH of the system, Sorensen's Phosphate Buffer (pH 6.8) is recommended.[2] Other appropriate buffers must be shown not to cause a reduction or increase in bacterial numbers by prior testing at the intended use concentration. For all other samples, the following solution is recommended and is prepared from reagent grade chemicals. For buffer stock solution (0.25M KH_2PO_4): Prepare a fresh solution at least once every 6 months as follows: Weigh 34 ± 0.1 g of potassium dihydrogen phosphate into a 100 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 ml ± 0.01 ml of stock buffer solution, with a sterile pipette to flask containing 800 ml of distilled water. Cap and sterilize.

6.2 *Media*:

6.2.1 *Nutrient Broth* (Difco Laboratories, Detroit, MI or equivalent) or media appropriate of organism selected.

6.2.2 *Tryptone Glucose Extract Agar* (Difco Laboratories, Detroit, MI or equivalent) or media appropriate for organism selected.

6.3 *Wetting Agent Surfactant*—Agents must be shown not to cause a reduction or increase in bacterial numbers by prior testing at the intended use concentration.

NOTE 3—Dow Corning, Midland, MI Q2-5211 at 0.01 % final dilution or equivalent can be used.

7. Test Organism

7.1 *Klebsiella pneumoniae*, American Type Culture Collection No. 4352. Other organisms may be used at the discretion of the investigator.

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 and observing for a single species characteristic type of colonies.

NOTE 4—A glossiness in the broth culture of Klebsiella pneumoniae is a sign of reversion and the culture should not be used.

[2] Clinical Chemistry: Principle and Technics, Second Edition 1974, Table A-3e, p 1592.

8. Parameters

8.1 Aerobic organisms and/or contact times used must be specified.

8.2 Surface preparation or conditioning must be specified. Prior manipulation of the specimen in order to demonstrate maximum activity in desired time frame must be recorded and compared to identically handled controls.

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

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

8.5 Wetting agent surfactants must be used with highly hydrophobic specimen.

9. Preparation of Bacterial Inoculum

9.1 Grow a fresh 18 hour shake culture of *Klebsiella pneumoniae*Klebsiella pneumoniae in sterile nutrient broth for each series of samples. If other organisms are specified, they should be prepared in the same manner, unless other media and different calibration techniques are specified.

9.2 Dilute the culture with the sterile buffer solution until the solution has an absorbance of 0.28 ± 0.01 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.

NOTE 5—For other organisms, adjust final concentration to 1.5-3.0 × 10^5 CFU/ml by appropriate methods.

10. Test Specimen

10.1 *Preparation of Test Specimen*:

10.1.1 *Fabric and Paper*—Samples are selected on weight basis at the discretion of the investigator and weighed to ± 0.1 g.

NOTE 6—Weight, usually between 0.5 and 2.0 g, must ensure strong agitation during contact period. Specimen should be cut into small enough portions to ensure maximum agitation and must be identical in size between treated and untreated controls. Clumping of specimen negatively affects reproducibility.

10.1.2 *Powder and Granular Material*—Weigh to ± 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 9 sq. in. (58 cm^2) of treated surface area. Specimen may also be selected on weight basis at the discretion of the investigator, weigh to ± 0.1 g. Care must be exercised during shaking not to break the flask or bottle. The untreated specimen of the solid must not absorb the solution. The test specimen may be mounted as a seal for the test container so that only the treated surface is in direct contact with the inoculum.

NOTE 7—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 antimicrobial bonded surface specimen to

be tested as in Section 10. One treated piece of each specimen is required. One untreated piece of each specimen of identical composition is required for each series of specimen tested.

11.2 Prepare one sterile 250 ml screw-cap Erlenmeyer flask for each treated and untreated specimen in addition to one "inoculum only" sample. If a series is being run, prepare one flask for each specimen and one for each type of specimen as a control. Add 50 ± 0.1 ml of working dilution of bacterial inoculum prepared in 9.2 to each flask.

11.3 Cap the flasks and place them on a wrist-action shaker. Shake them at maximum speed for 1 min. ± 5 sec. Each flask is considered to be a "0" contact time. Determine bacterial concentration of solution at the "0" time by performing serial dilutions and standard plate count techniques. No series of test flasks should be large enough to require more that 5 minutes between the first and last serial dilution after contact.

NOTE 8—For extra assurance against effects of solution retained antimicrobial, neutralizer containing dilution buffer can be used. See E 1054.

11.4 As soon as the "0" contact time sub-samples have been prepared, place the test and control specimen in their individual flasks. Recap the flasks and place them on the wrist-action shaker. Shake at maximum stroke for 1 hr. ± 5 min. unless a different contact time is specified. Immediately transfer 1 ± 0.01 ml from each flask to a test tube, serial dilute and plate out in duplicate as was done for the "0" contact time subgroup.

NOTE 9—The investigator may run the actual shake flask portion of the procedure at any desired temperature consistent with the test organism, and/or reflective of the temperatures anticipated for the end-use of the specimen, and good microbiological procedures.

NOTE 10—Residual bacterial retention in/on specimen could be tested using appropriate retrieval techniques such as agar imprint tests or buffer extraction and plate count.

11.5 Allow all the petri dishes from both subsets to incubate for 24 to 48 hrs.

11.6 Count the colonies in petri dish. Record the values, average the duplicate petri dish numbers and convert the average to colony forming units per milliliter (CFU/ml) of buffer solution in the flask. If the duplicate counts of any sample do not agree within 15 %, discard that sample and repeat the test.

NOTE 11—The presence of the original test organism may be confirmed by Gram stains and colony morphology.

11.7 Calculate the percent reduction of the organisms resulting from contact with the specimen using the following formula. Results can be presented in either percent reduction when measuring CFU/ml or as a death rate constant when calculating mean $\log_{10}$ density of bacteria.

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

$$\text{Death Rate Constant (mean } \log_{10} \text{ density)} = B - A$$

where:

A = CFU per milliliter (or mean $\log_{10}$ density of bacteria) for the flask containing the treated substrate after the specified contact time, and

B = "0" contact time CFU per milliliter (or mean $\log_{10}$ density of bacteria) for the flask used to determine "A" before the addition of the treated substrate.

11.8 The counts for the flask containing the inoculum only control after specified contact time (C) and counts for the flask containing the untreated control after specified contact time (D) should be within 15 %. If they are not, calculate the percent reduction of organisms from treated sample (A) directly compared to the untreated control (D). Repeat above formula replacing D for B and report accordingly.

where:

C = CFU per milliliter (or mean $\log_{10}$ density of bacteria) for the flask containing the inoculum only control after specified contact time, and

D = CFU per milliliter (or mean $\log_{10}$ density of bacteria) for the flask containing the untreated substrate after the specified contact time.

NOTE 12—If no untreated fabric control 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.

11.9 Record and report the value to the nearest half percent or one-tenth mean $\log_{10}$ density of bacteria if a specific death rate constant is desired.

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 147-1998.

12.2 *Analysis of Supernatant (Post-Test)*:

12.2.1 Inoculate Tryptone Glucose Extract agar plate or agar appropriate for the test organism with confluent lawn of organisms (1×10^5 CFU/ml) 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 required.

12.2.4 Prepare two sterile 250 ml flasks containing 50 ml sterile 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 time specified in 11.4.

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 37 ± 2°C.

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.

NOTE 13—Presence of a zone of inhibition in either of these determinations indicates the unsuitability of the specimen for this method.

13. Precision and Bias

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

NOTE 14—The repeatability (maximum expected difference between two independent results) of the percent reduction should not exceed 5 % at the 95 % confidence level in the range of 75 % to 100 % reduction.