Designation: E 2274 – 03

Standard Test Method for Evaluation of Laundry Sanitizers and Disinfectants[1]

This standard is issued under the fixed designation E 2274; 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 sanitizing/disinfectant laundry detergents/additives for use in top-loading automatic clothes washing operations. This test method is designed predominantly to provide testing with representative vegetative bacteria but can also be designed to accommodate the testing of fungi and viruses.

NOTE 1—This test method does not evaluate sanitizing/disinfectant laundry detergent/additives for use in front-loading, low water volume automatic clothes washing operations.

1.2 Knowledge of microbiological techniques is required for these procedures.

1.3 In this method metric units are used for all applications, except for distance in which case inches are used.

1.4 *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]

D 1193 Specification for Reagent Water

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

2.2 *Other Documents:*

The American Association of Textile Chemists And Colorists (AATCC) Test Method 70-1997 Water Repellency; Tumble Jar Dynamic Absorption Test

Official Methods of Analysis of AOAC International AOAC, Washington, D.C., Chapter 6: Disinfectants, 17th ed., 2000.

DIS/TSS 13 Laundry Additives—Disinfection and Sanitization, U.S. Environmental Protection Agency, Office of Pesticide Programs, April 1980

Federal Standard 40 CFR Part 160, Good Laboratory Practice Standards[3]

Canadian Pest Management Regulatory Standards Trade Memorandum T-1-215

3. Terminology

3.1 *Definitions:*

3.1.1 *active antimicrobial ingredient*—a substance added to a formulation intended specifically for the inhibition or inactivation of microorganisms.

3.1.2 *antimicrobial agent(s)*—an active ingredient designed to suppress the growth or action of microorganisms.

3.1.3 *carrier count control*—procedure used to determine the initial number of microorganisms on a fabric carrier following the inoculation and drying procedure.

3.1.4 *diluent*—sterile deionized water, sterile distilled water or sterile synthetic AOAC hard water that may be used to prepare the active test formulation, vehicle control or product control for use in the test procedure.

3.1.5 *diluted product solution*—test formulation, vehicle control, or product control diluted to use concentration.

3.1.6 *neutralization*—a process that results in quenching the antimicrobial activity of a test formulation. This may be achieved by dilution of the test formulation(s) to reduce the concentration of the antimicrobials, or through the use of chemical agents, called neutralizers, to suppress antibacterial activity.

3.1.7 *numbers control*—in assessing sanitizer level performance, procedure used to determine the number of microorganisms remaining on the fabric carriers and in the wash water following the test procedure in the presence of the diluent. This may also be performed using diluent or phosphate buffer dilution water with surfactant.

3.1.8 *product control*—a formulation with or without an active ingredient(s) used for comparison to the test formulation.

3.1.9 *test formulation*—a formulation containing an antimicrobial agent(s).

3.1.10 *vehicle control*—the test formulation without the active ingredient(s) used for comparison to the test formulation.

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antibacterial 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 U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401.

3.1.11 *wash water*—the liquid contained in the exposure chamber previously exposed to either uninoculated fabric or fabric inoculated with the challenge microorganism.

4. Summary of Test Method

4.1 Under simulated laundry conditions, sets of inoculated fabric swatches are placed into diluted product solution and agitated. After a specified contact time, the wash water and the test fabric are individually cultured either quantitatively (sanitizer efficacy) or qualitatively (disinfectant efficacy).

NOTE 2—See appropriate regulatory guidance document for the minimum number of replicates required to meet a specific claim.

5. Significance and Use

5.1 The procedure in this test method is used to evaluate the activity of a test reagent (antimicrobial agent/active ingredient) or formulation in the reduction or complete kill of the bacterial population in fabric and wash water following a single wash.

6. Apparatus

6.1 *Colony Counter*, any of several types may be used, for example, Quebec.

6.2 *Incubator*, any incubator that can maintain the optimum temperature, ±2°C, for growth of the challenge microorganism(s).

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

6.4 *Timer (Stop-clock)*, any device that can be read for minutes and seconds.

6.5 *Exposure Chamber*, container with closure that can withstand sterilization. Should be large enough to hold a single stainless steel spindle yet allow diluted product solution to completely contact the entire fabric spindle during the tumbling period.

NOTE 3—Standard lids may form a vacuum seal when steam sterilized. To avoid, prior to sterilization place a piece of paper between lid and jar.

6.6 *Stainless Steel spindles*, Spindles are fabricated from a single continuous piece of stainless steel wire, (1/16 in. diameter and bent to contain 3 horizontal extensions, 2 in. long connected by 2 vertical sections approximately 2 in. long.) They are shaped so that vertical sections form 150° angles, free ends of 2 outer horizontal extensions are sharpened to a point. Use as carrier for wrapping fabric ballast. See Fig. 1.

6.7 *Agitator*, tumbling device to rotate Exposure Chamber through 360° vertical orbit of 4 to 8 in. diameter at 45 to 60 rpm or comparable tumbling devices such as, Launderometer or Tumble Jar described in AATCC 70-1997.

6.8 *Micropipettor (and Pipet Tips)*, suitable to deliver 0.01 to 0.03 mL volume.

6.9 *Forceps*, large and small, sterile.

6.10 *Safety Pins*, sterile.

6.11 *Stapler and Staples*.

6.12 *Balance*, with a platform to accommodate 15 ± 0.1 g of test fabric.

6.13 *Sterile Glass Beads*, 3 to 4 mm.

6.14 *Filter Sterilization System for Media and Reagents*, a membrane or cartridge filtration system (0.22 µm pore diameter). Required for sterilizing heat-sensitive solutions.

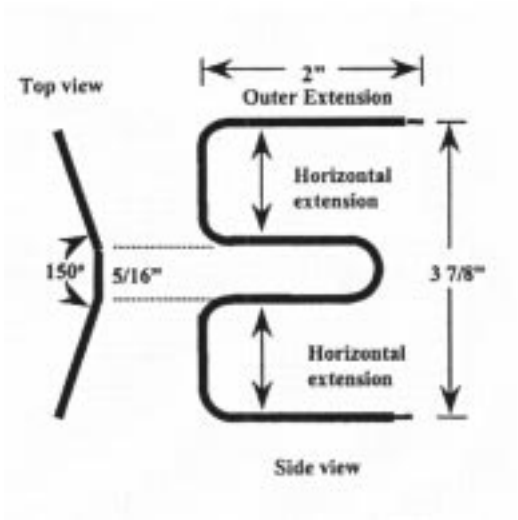

FIG. 1 Stainless Steel Spindel Schematic (top view and side view).

6.15 *Membrane Filtration System for Capture of the Test Organism(s)*, sterile 47 mm diameter membrane filters (0.45 µm pore diameter) and holders for such filters.

7. Reagents and Materials

7.1 *Petri Dishes*, sterile 100 by 15 mm. Required for performing standard plate counts and used in preparation of contaminated fabric carriers.

7.2 *Bacteriological Pipets*, sterile, various sizes.

7.3 *Test Fabric*, approximately 80 by 80 threads/in. bleached, desized, plain-weave cotton print cloth and without bluing or optical brighteners.[4]

NOTE 4—Other test fabrics/blends may be used at the discretion of the investigator.

7.4 *Dilution Fluid*, AOAC Phosphate buffer dilution water[5] or other suitable diluent containing appropriate neutralizers for serial dilution of test samples.

7.5 *Water for Dilution of Formulations under Test*:

7.5.1 Water, sterile, deionized or distilled, equivalent to or better than Type 3, see Specification D 1193.

7.5.2 AOAC Synthetic Hard Water.[5]

7.5.3 All water used for preparation of test solutions shall be sterile.

7.6 *Purity of Reagents*—reagent grade chemicals shall be used in all tests.

7.6.1 Sodium carbonate.

7.6.2 Alkaline nonionic wetting agent with HLB (hydrophilic-lipophilic balance) value of approximately 13. Prepare solution containing 0.5% nonoxynol-10 class of ethoxylated alkyl phenols, for example Tergitol NP-10 or Triton X-100 an 0.5% Na_2CO_3.

[4] Fabric #400 8-1978) obtained from Test Fabric, Inc., P.O. Box 26, West Pittson, PA 18643 or supplier of comparable specifications.

[5] *Official Methods of Analysis of the AOAC International*, AOAC, Washington, DC, 16th 16th ed, Chapter 6: Disinfectants, 1995.

7.7 *Neutralizing Broths*—growth media appropriate for the challenge microorganism containing chemical agents to suppress antibacterial activity. Alternatively, the neutralizing broths may be of sufficient volume to reduce the concentration of the antimicrobials to below active levels. See 11.8.

7.8 *Challenge Microorganisms*,[6]

7.8.1 *Klebsiella pneumoniae*, ATCC 4352.

7.8.2 *Staphylococcus aureus*, ATCC 6538.

7.8.3 *Pseudomonas aeruginosa*, ATCC 15442.

7.8.4 Other microorganisms, as applicable.

7.9 *Culture Media*:

7.9.1 Nutrient Agar A.[5]

7.9.2 Nutrient Agar B.[5]

7.9.3 Media suitable for identification of microorganism(s) used in the study.

7.9.4 Soybean casein digest medium or other suitable media, with or without specific neutralizers, for recovery of the challenge microorganism(s).

7.10 *Organic Soil Load*—when an organic soil load is to be incorporated in the suspension of the challenge microorganism(s), defibrinated heat-inactivated animal serum may be used or a mixture of the following stock solutions in phosphate buffer dilution water (pH 7.2) may be used (see 7.4):

7.10.1 Add 0.5 g of tryptone to 10 mL phosphate buffer.

7.10.2 Add 0.5 g of bovine serum albumin (BSA) to 10 mL of phosphate buffer.

7.10.3 Add 0.04 g of bovine mucin to 10 mL of phosphate buffer.

7.10.4 Prepare the solutions separately and sterilize by passage through a 0.22 µm pore diameter membrane filter, apportioned and stored at either 4 ± 2°C or -20 ± 2°C for no longer than 3 months.

7.10.5 To obtain a 500 µL inoculum of the challenge microorganism, add to 340 µL of the microbial suspension 25 µL, 100 µL, and 35 µL of BSA, mucin and Tryptone stock solutions, respectively.

NOTE 5—The quality of the above materials may vary among manufacturers or product lots. Therefore, preliminary screening of such items is recommended to ensure compatibility with the test microorganism(s).

NOTE 6—The investigator should confirm the appropriate organic soil usage with the appropriate regulatory agency prior to initiating testing.

8. Fabric and Spindle Preparation

8.1 Scour test fabric by boiling approximately 300 g of material for 1 h in 3 L of distilled or deionized water containing 1.5-g sodium carbonate and 1.5-g nonionic wetting agent. Rinse fabric, first in boiling water and then in cold water, until all visual traces of wetting agent are removed (that is, foaming). Remove as much water as possible from fabric.

8.2 Air dry for at least 24 h at ambient room temperature.

8.3 Cut scoured dry fabric into strips 2 in. (5 cm) wide and weighing 15 ± 0.1 g each. For cotton fabrics, pierce one end of the 15-g test fabric strip and secure onto the outer horizontal extension of a stainless steel spindle. Wind the strip around the three horizontal extensions with sufficient tension to obtain 12 but not 13 laps while using the entire 15 ± 0.1 g of fabric. Staples or a pin may be used to secure the fabric. Fabric wrapped spindles may be sterilized in individual Exposure Chambers. Alternatively, fabric wrapped spindles may be sterilized separately from Exposure Chambers. Ensure dryness of fabric on spindles and Exposure Chambers prior to testing.

NOTE 7—Fabric may be purchased in pre-cut strips and then scoured.

8.4 Fabric carriers of approximately 1 by 1.5 in. will be cut from the remaining scoured fabric. Nontoxic permanent marker may be used to place a mark on the edge of each carrier. Alternatively, attach a pin to the short side of each carrier. Place fabric carriers in glass Petri dishes and sterilize. Ensure dryness of fabric prior to testing.

8.5 For each challenge microorganism, prepare at least 3 fabric carriers and 1 fabric wrapped spindle for each active test formulation/product and control/numbers control.

9. Preparation of Challenge Microorganisms

9.1 Subculture microorganism(s) on Nutrient Agar A through at least three daily transfers, incubating at 35 ± 2°C. If only one daily transfer is missed, it is not necessary to repeat the three consecutive transfers prior to use in testing.

9.2 On the day prior to testing, transfer the cells into French square bottles containing 20 mL of solidified Nutrient Agar B. Incubate 18 to 24 h at 35 ± 2°C, agar side down.

9.3 Remove growth from the French square bottles using three-mL dilution fluid and five sterile glass beads to suspend growth. The cultures will be standardized to yield approximately 10^8 colony forming units (CFU) per mL of *S. aureus* and 10^9 CFU/mL of *K. pneumoniae* and *P. aeruginosa*.[5]

NOTE 8—The initial inoculum concentration for different challenge microorganisms may vary and should be determined from carrier and wash water numbers control recovery (see Section 12).

9.4 A soil load may be added to each inoculum (see 7.10).

10. Preparation of Test Sample

10.1 Prepare a sufficient volume of diluted active test formulation and product control (at least 1 L) according to manufacturer instructions, using diluent pre-equilibrated to test temperature.

NOTE 9— Fabric to wash-water ratios based on usage patterns must be considered in this step (see DIS/TSS 13 and Table 1.)

NOTE 10—When appropriate use AOAC hard water in preparation of test product (see 7.5.2).

10.2 Using diluent at test temperature, prepare test product dilution no more than 3 h prior to use and maintain solution at test temperature. Some active ingredients may require preparation and usage in less than 3 h.

11. Procedure

11.1 Inoculate three sterile fabric carriers (in a single sterile Petri dish) with 0.01 to 0.03 mL of prepared inoculum per

TABLE 1 Typical Use Patterns

Usage Pattern	Fabric: Wash-water Ratio
Home or coin-operated laundering	1:10
Industrial laundering	1:5

[6] DIS/TSS 13. Laundry Additives—Disinfection and Sanitization. U.S. Environmental Protection Agency, Office of Pesticide Programs. April 1980.

carrier. Disperse the inoculum over an approximate 1- by 1.5 in. area of each carrier, avoiding the marker, staple, or safety pin. Dry the carriers in a 35 ± 2°C incubator until the carriers are visibly dry, but not longer than 30 min. Use swatches (carriers) within 1 h of drying.

11.2 Using sterile forceps, aseptically place three dried inoculated carriers in an upright position between the sixth and seventh folds of a single wrapped spindle. Do not allow the inoculated carriers to overlap. The marker, staple, or safety pins will allow for easy removal at the end of the procedure.

NOTE 11—Weight of the wetted fabric strip should keep the contaminated swatches intact.

11.3 Aseptically place the spindle into the sterile Exposure Chamber.

11.4 Add prepared test sample (see Section 10).

11.5 Firmly close Exposure Chamber.

NOTE 12—Additional steps may be needed to prevent leaking from Exposure Chamber (that is, seal with Parafilm).

11.6 Place the Exposure Chamber into the Agitator for the specified exposure period (pre- and post-agitation times can be specified in the study protocol separately).

11.7 Using large, sterile forceps, remove spindle from Exposure Chamber and aseptically remove each fabric carrier to a separate wide mouth tube containing 10 mL neutralizing broth.

11.8 Add concentrated neutralizing broth to wash water and mix well.

NOTE 13—The specific neutralizer and concentrations should be determined prior to testing. For addition to wash water, concentration should be increased in order to reduce the amount of neutralizer added to wash water. Otherwise, the volume of neutralizer needed may overflow the Exposure Chamber.

11.9 Addition of fabric carrier to neutralizing broth and concentrated neutralizing broth to wash water completes the exposure time.

11.10 All tubes containing fabric carriers will be mixed on a Vortex-type mixer for approximately ten seconds. Alternatively, other methods such as a foot-arc technique or sonication may be used to extract surviving microorganisms from fabric swatches.

11.10.1 If necessary (see Practices E 1054), after 30 to 60 min from original subculture, transfer carrier to a second tube containing neutralizing broth and mix thoroughly.

NOTE 14—More than one technical person is needed to meet exposure time requirements.

11.11 *Determination of Disinfectant Efficacy*:

11.11.1 Filter entire volume of wash water containing neutralizing broth and plate filter on appropriate agar containing neutralizers as needed.

11.11.2 Incubate plates and tubes containing carriers for 48 to 54 h at 35 ± 2°C.

11.11.3 Results are reported as growth (+) or no growth (−). Positive results should be confirmed by Gram stain and streaking onto an appropriate growth medium for identification.

11.12 *Determination of Sanitizing Efficacy*:

11.12.1 Serially dilute 1.0 mL neutralizing broth containing a single carrier to the 10^{-4} dilution and plate in duplicate in or on an appropriate agar containing neutralizers as needed. Incubate plates at 35 ± 2°C for 48 to 54 h. To determine survivors, count colonies and record as CFU/plate. Average duplicate plates and multiply by the dilution factor to arrive at CFU/carrier. This average count should be converted into $\log_{10}$.

11.12.2 Serially dilute 1.0 mL neutralized wash water to 10^{-2} using dilution fluid containing neutralizers. Plate all dilutions in duplicate in or on agar containing neutralizers as needed. Filter remaining neutralizing broth/wash water combination and plate filter on appropriate agar containing neutralizers as needed. Incubate plates at 35 ± 2°C for 48 to 54 h. To determine survivors, count colonies and record as CFU/plate. Average duplicate plates and multiply by the dilution factor to arrive at CFU/carrier. This average count should be converted into $\log_{10}$.

11.12.3 Results are reported as percent reduction of number of organisms on fabric carriers and in wash water as compared to numbers control.

12. Numbers Control

12.1 In place of the test formulation, use the diluent, or diluent-containing surfactant, and follow steps 11.1-11.9 above. Ensure that carriers used in this control were treated in the same manner as the test carriers.

12.1.1 Serially dilute 1.0 mL neutralizing broth containing a single carrier to 10^{-5} and plate duplicate aliquots of all dilutions in or on an appropriate agar. Incubate plates for 48 to 54 h at 35 ± 2°C. To determine survivors, count colonies and record as CFU/plate. Average duplicate plates and multiply by the dilution factor to arrive at CFU/carrier. This average count should be converted into $\log_{10}$. A minimum average of 1.0×10^4 CFU/carrier must be recovered for a valid test.

12.1.2 Serially dilute 1.0 mL neutralizing broth/wash water combination to 10^{-5} and plate duplicate aliquots of all dilutions in or on an appropriate agar. Incubate plates at 35 ± 2°C for 48 to 54 h. To determine survivors, count colonies and record as CFU/plate. Average duplicate plates and multiply by the dilution factor to arrive at CFU/mL. This average count should be converted into $\log_{10}$. A minimum average of 1.0×10^4 CFU/mL must be recovered fo a valid test.

13. Neutralizer Efficacy Control

13.1 For each challenge microorganism, prepare three 10 mL neutralizing broth tubes. Inoculate each tube with less than 100 CFU/mL of the challenge microorganism and confirm the number of CFU added to each tube. For each challenge microorganism, follow steps 11.2-11.9 using three sterile carriers. When spindle is removed from wash water and prior to addition of concentrated neutralizing broth, add less than 100 CFU/mL of the challenge microorganism to the wash water.

13.1.1 Filter the entire volume of the neutralizing broth/wash water combination and plate the filter on the appropriate agar containing neutralizers as needed.

13.1.2 Incubate plates and tubes containing carriers for 48 to 54 h at 35 ± 2°C. Check for growth and perform Gram stain to confirm presence of challenge microorganism. See Practices E 1054.

NOTE 15—This control should be conducted prior to testing. At the discretion of the investigator, this control may be performed again on the test date.

13.2 CFU recovered should be within a Log_{10} of 0.20 of the confirmed CFU for the neutralizer to be considered effective. (see Practice 1054

14. Carrier Count Control

14.1 Repeat step 11.1 for each challenge microorganism.

NOTE 16—Carrier Count Control should be performed on swatches dried and held for the same amount of time as swatches used for the test procedure.

14.2 Place each carrier into a tube containing 10 mL diluent utilized for step 12.1.

14.3 Mix each tube on a Vortex-type mixer for ten seconds. Other methods such as a foot-arc technique or sonication may be used to extract microorganisms from fabric swatches.

14.4 Perform serial ten-fold dilutions to the 10^{-5} dilution and plate duplicate aliquots of all dilutions in or on the appropriate agar. Incubate plates at 35 ± 2°C for 48 to 54 h.

NOTE 17—The control is optional and should be performed at the discretion of the investigator. Various microorganisms respond differently to the inoculum preparation, carrier inoculation and drying procedures. This procedure may serve as a troubleshooting tool if a microorganism does not perform as expected in the numbers control and test procedure.

15. Calculation

15.1 Percent reduction is calculated as follows:

$$\%\text{reduction} = (a - b)/a \times 100 \quad (1)$$

where:

a = average number of organisms surviving in the fabric carriers of the numbers control, or the wash water of the numbers control.

b = average of the number of organisms surviving in the fabric carriers or wash water.

16. Report

16.1 For sanitizer efficacy, report the percent reduction for all microorganisms and product lots tested.

16.2 For disinfectant efficacy, report the number of carriers showing growth of the test microorganism out of the total number of carriers. Also report the number of wash water samples showing growth of the test microorganism out of the total number of samples.

16.3 Also report the following information:

16.3.1 Test agent identity under test.

16.3.2 Chemical class of product(s) under test.

16.3.3 Concentration(s) and class of active ingredient(s) tested.

16.3.4 Water employed to dilute product (if AOAC hard water employed, report hardness levels).

16.3.5 Whether or not soil was employed.

16.3.6 Test microorganisms.

16.3.7 Media and reagents employed.

16.3.8 Neutralizing broth and neutralizer concentration employed.

16.3.9 Growth/no growth determination from neutralizer efficacy control.

16.3.10 Number of microorganisms surviving on each of the three test carriers.

16.3.11 Number of microorganisms surviving on each of the three numbers control carriers.

16.3.12 Number of microorganisms surviving in the test wash water.

16.3.13 Number of microorganisms surviving in the numbers control wash water.

16.3.14 If applicable, number of microorganisms surviving on each of the carrier count controls.

16.3.15 Statement that the test was done in accordance with this method.

17. Precision and Bias

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

18. Keywords

18.1 launderometer; laundry additives; laundry disinfectant; laundry sanitizer; Petrocci and Clarke; tumble jar

Bibliography

(**1**) Petrocci, A.N. and Clarke, P. Proposed Test Methods for Antimicrobial Laundry Additives. *Journal of the AOAC* Vol. 52. No.4, pp. 836–842, 1969.