Designation: E 2276 – 03

Standard Test Method for Determining the Bacteria-Eliminating Effectiveness of Hygienic Handwash and Handrub Agents Using the Fingerpads of Adult Subjects[1]

This standard is issued under the fixed designation E 2276; 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.

INTRODUCTION

Hands can spread many types of pathogens directly **(1)**[2] or by transfer of such organisms to other surfaces and objects during casual contact **(2,3)**. Therefore, regular and proper decontamination of hands by caregivers and food-handlers in particular is crucial for infection control. Hygienic hand antisepsis is meant to reduce the load of transient microflora on hands, thereby reducing the risk of disease transmission. Such reduction in the bacterial load may be due to a combination of bacterial inactivation and removal of viable bacteria from the skin. In this method the test bacterial suspension is placed on the thumb- and fingerpads of adults to simulate the contamination of hands with transient microflora, the inoculum on the fingerpads is allowed to dry and is then treated with test and control solutions. Since in each test all ten digits on any given subject can be used, the protocol permits the inclusion of the required controls and several replicates of the test formulation in the same sitting.

1. Scope

1.1 This test method is designed to determine the activity of hygienic handwash and handrub **(4)** agents against transient bacterial flora on hands and is not meant for use with surgical hand scrubs or preoperative skin preps.

1.2 Performance of this procedure requires the knowledge of regulations pertaining to the protection of human subjects.[3]

1.3 The test method should be performed by persons with training in microbiology in facilities designed and equipped for work with infectious agents at biosafety level 2 **(5)**.

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:*

D 1129 Terminology Relating to Water[4]

E 1115 Test Method for Evaluation of Surgical Hand Scrub Formulation[5]

E 1173 Test Method for Evaluation of a Pre-Operative Skin Preparation[4]

E 1174 Test Method for Evaluation of Health Care Personnel Handwash Formulation[4]

E 1838 Test Method for Determining the Virus-Eliminating Effectiveness of Liquid Hygienic Handwash and Handrub Agents Using the Fingerpads of Adult Volunteers[4]

3. Terminology

3.1 *Definitiions*—For definitions of general terms used in this test method, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *active ingredient, n*—a substance added to a formulation specifically for the inhibition or inactivation of microorganisms.

3.2.2 *handrub agent, n*—a liquid or gel which is applied by rubbing to decontaminate lightly soiled hands between handwashings; such agents normally contain alcohol alone or with other active ingredients.

3.2.3 *hard water, n*—water with a standard hardness of 200 ppm as calcium carbonate.

3.2.4 *hygienic handwash agent, n*—an agent generally used for handwashing by personnel in hospitals, other health-care

[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] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] *Federal Register*, Vol 46, No. 17, Jan. 27, 1991.

[4] *Annual Book of ASTM Standards*, Vol 11.04.

[5] *Annual Book of ASTM Standards*, Vol 11.05.

facilities, day-care centers, nursing homes, and food-handling establishments should be safe for repeated use, nonirritating, fast-acting, and efficient in eliminating transient microorganisms from intact skin.

3.2.5 *neutralization*, *n*—a process which results in quenching the antimicrobial activity of a test material. This may be achieved through dilution of the test material(s) to reduce the antimicrobial activity, or through the use of chemical agents, called neutralizers, to eliminate antimicrobial activity.

3.2.6 *nonmedicated soap*, *n*—a soap or detergent that is mild to the skin and does not contain any germicidal chemicals.

3.2.7 *soil load*, *n*—a solution of one or more organic and/or inorganic substances added to the suspension of the test organism to simulate the presence of body secretions, excretions or other extraneous substances.

3.2.8 *test formulation*, *n*—a formulation which incorporates antimicrobial ingredients.

3.2.9 *test organism*, *n*—an applied inoculum of an organism that has characteristics which allow it to be readily identified. The test organism is used to simulate a transient topical microbial contaminant. It may also be referred to as a marker organism, bacterial simulant or bacterial contaminant.

3.2.10 *test vehicle*, *n*—the test agent without an active ingredient.

3.2.11 *transient microflora*, *n*—microorganisms from the environment that contaminate but do not normally colonize the skin.

4. Summary of Test Method

4.1 This test method is conducted on a group of adult subjects who have provided informed consent and the skin of whose hands has been determined to be free from any apparent damage. Panelists are to refrain from using any products containing antimicrobial agents for one week prior to the test. A known volume of the test bacterial suspension is placed on a demarcated area on each fingerpad and the inoculum allowed to dry. The contaminated area then is exposed to the control (standard hard water) or test agent for the desired contact time and organisms remaining on the fingerpad are eluted and the eluates are assayed for viable bacteria. Percent reductions in the numbers of viable bacteria after treatment with the control and test agents are then determined. The fingerpad method gives results that are comparable to those obtained using a whole-hand procedure **(6)**. If two different formulations are being compared in the same test, one of them may be designated as a reference and used in place of the hard water control. If desired, one also may use tap water in parallel with the hard water control to determine the influence of water hardness on the test product's bacteria-eliminating activity.

5. Significance and Use

5.1 This in vivo procedure is designed to test the ability of hygienic handwash or handrub agents to eliminate selected types of bacteria from experimentally contaminated skin of the hands of adult subjects. Since the two thumbpads and all eight fingerpads can be used in any given test, it allows for the incorporation of an input control (two), control for viable bacteria remaining after the inoculum has been allowed to dry (two), bacteria eliminated after treatment with a control or reference solution (two), and up to four replicates to assess the bacteria-eliminating efficiency of the product under test. No more than 100 µL of the test bacterial suspension is required to complete one test. The results of testing with this test method may form the basis for confirmatory tests using a suitable whole-hand test protocol, such as Test Method E 1174.

5.2 Whereas, this test method relates to testing with bacteria, it can be readily adapted to work with fungi **(7)**, protozoa and bacteriophages. A similar method for work with viruses of human origin is already an Test Method(E 1838).

5.3 Infectious microorganisms left on hands after washing can be reduced further by drying the washed hands with paper, cloth, or warm air **(8)**. A step for the drying of fingerpads after exposure to the control or test solution, therefore, has not been included to avoid bacterial removal by the drying process itself.

5.4 This test method is not meant for use with surgical hand scrubs or preoperative skin preps.

5.5 The level of contamination with viable bacteria on each fingerpad after the drying of the inoculum should be at least 10^4 colony-forming units (CFU) so that it would permit the detection of up to a 4-$\log_{10}$ reduction in the viability titer of the test organism by a given product under the conditions of this test method.

6. Equipment and Apparatus

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

6.2 *Freezers*—A freezer at -20 ± 2°C is required for the storage of culture media. A second freezer at -70°C or lower is required to store bacterial stocks.

6.3 *Handwashing Sink*—A sink of sufficient size to permit panelists to wash hands without touching hands to sink surface.

6.4 *Incubator*—Any incubator capable of maintaining the following temperatures: *Serratia marcescens* (25 ± 2°C); this temperature is necessary to ensure pigmentation) or *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* (35 ± 2°C).

6.5 *Laminar Flow Cabinet*—A Class II biological safety cabinet is required for this work. The procedures for the proper maintenance and use of such cabinets are given in Ref **(2)**.

6.6 *Magnetic Stirrer and Magnets*—Large enough to hold a 5-L beaker or Erlenmeyer flask for preparing culture media or other solutions.

6.7 *Membrane Filtration System*—A membrane filtration system and membranes with a pore diameter of 0.22-µm are required to sterilize heat-sensitive media/solutions and to capture and culture viable test bacteria in control samples and eluates.

6.8 *Positive Displacement Pipette*—A pipette and pipette tips that accurately can dispense 10-µL volumes.

6.9 *Refrigerator*—A refrigerator at 4 ± 2°C for storage of prepared culture media and reagents.

6.10 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.11 *Timer* (Stop-clock)—One that can be read for minutes and seconds.

6.11.1 *Tap Water Temperature Regulator and Temperature Monitor*—to monitor and regulate water temperature at 40 ± 2°C.

6.11.2 *Water Faucet(s)*—to be located above the sink at a height that permits the hands to be held higher than the elbow during the washing procedure. Faucets with electronic sensors or those that are wrist-, elbow-, knee-, or foot-operated are preferred to avoid recontamination of the washed hands.

7. Materials and Reagents

7.1 *Serological Pipettes*—Sterile reusable or single-use pipettes of 10.0, 5.0, and 1.0-mL capacity.

7.2 *Culture Plates*[6]—Petri plates of 100 mm diameter for culturing bacteria.

7.3 *Culture Media and Supplements*—Culture media and the types and ratios of supplements will vary depending on the type of test bacterium being used.

7.4 *Soil Load*:

7.4.1 *Fetal Bovine Serum*, at a final concentration of 5 % in the bacterial inoculum.

7.4.2 *Tripartite Soil Load*, as an alternative to serum.

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

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

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

7.4.6 Prepare the stock solutions separately and sterilize by passage through a 0.22 µm pore diameter membrane filter, aliquot and store at either 4 ± 2°C or -20±2°C.

7.4.7 To obtain a 500-µL inoculum of the test inoculum, add to 340 µL of the bacterial suspension 25 µL BSA, 100 µL mucin and 35 µL of tryptone stock solutions. This mixture contains approximately 2 g of total protein/L, which is roughly equivalent to the protein content of a 5 % solution of fetal bovine serum.

7.5 *Standard Hard Water*—The quality and disinfectant (for example, chlorine) residual in tap water can vary from site to site and also at different times at the same site. The use of standard hard water, therefore, is recommended here to avoid variations in results due to differences in tap water quality. Water prepared in accordance with AOAC 960.09 E and F **(9)** to a standard hardness of 200 ppm as calcium carbonate is used for dilution of test products, as the control solution to determine the baseline level of bacterial elimination, and to rinse the fingerpads after exposure to the test product. The standard hard water and tap water (if used) must first be tested to ensure that they do not have any activity against the test bacterium.

7.6 *Test Agents*—At least two samples of the product shall be tested.

7.7 *Diluent for Bacterial Titration*—Normal saline (0.85 % NaCl) at pH 7.2-7.4.

7.8 *Eluent for Bacterial Recovery from Fingerpads*—Normal saline.

7.9 *Plastic Vials*—Sterile screw-capped 2.0-mL vials with an inside diameter of about 8 mm will be required for demarcation of the fingerpads and to hold various test solutions.

7.10 *Miscellaneous Laboratory Ware*—Automatic pipettes, pipette tips, plastic vials for storing stock cultures.

7.11 *Broth*—Tryptose phosphate broth (TPB) to prepare the inoculum of the test organisms.

7.12 *Agar*—Trypticase soy agar (TSA) to recover and count the colonies of the test organism in control and test samples. The addition of any neutralizer(s) in such recovery media must first be properly validated. The use of selective-differential media for the detected of the test bacteria in such studies is not recommended because cells stressed or injured after germicide exposure may not form colonies on such agars.

NOTE 1—TSA and TPB, which are based on soybean-casein digests, were used in the development of the method described here. Other media with similar formulations may be used instead.

8. Test Bacteria

8.1 The selection of the following test bacteria is based on their (*a*) relative safety to the volunteers as well as experimenters, (*b*) ability to grow to titers sufficiently high for testing, (*c*) easy to recover and identify in the laboratory, and (*d*) potential to spread through contaminated hands. Other strains or types of bacteria may be substituted provided they meet the preceding criteria.

8.1.1 *Serratia marcescens* (ATCC 14756). This is a strain having stable pigmentation at 25°C.

8.1.2 *Escherichia coli* (ATCC 11229). is an alternative Gram-negative test organism that is considered safe for the experimental contamination of the skin of adult subjects.

8.1.3 *Staphylococcus aureus* (ATCC 6538). This is a normal inhabitant of human body and also an important nosocomial pathogen.

8.1.4 *Staphylococcus epidermidis* (ATCC 14990). is an alternative Gram-positive test organism that is considered safe for the experimental contamination of the skin of adult subjects.

NOTE 2—**Warning:** The application of microorganisms to the skin may involve a health risk. Prior to applying the test organism to the skin, the antibiotic sensitivity profile of the strain should be determined. If the applied organism causes an infection, the antibiotic sensitivity profile should be made available to the attending clinician.

9. Preparation of Test Inoculum

9.1 The stock culture should be at least two, but no more than five, 24-h broth transfers from the original ATCC stock. To prepare the test inoculum, add 0.1 mL of a 24-h broth culture to 100 mL of TSB (7.11) and incubate for 24 ± 4 h at the appropriate temperature.

9.2 Add soil load (7.4), if required.

9.3 Swirl, vortex or shake the test bacterial cell suspension before withdrawal of each aliquot. Assay the in-put suspension for CFU at the beginning and end of the use period. Do not use a test suspension for more than 8 hours on any given day. The titer of the test suspension as CFU may not vary more than ± 0.5 $\log_{10}$ over an 8-h period.

[6] Plastic culture ware may be purchased from most laboratory supply houses.

10. Panelists

10.1 Recruit a sufficient number of healthy adult human volunteers who have no clinical evidence of dermatoses, open wounds, hangnails or other skin disorders. The number of volunteers required for a trial is dependent on the number of treatments within a study.

10.2 It is the responsibility of the user of this test method to arrange the necessary clearance for the use of adult panelists/volunteers for testing and to obtain informed and written consent from those selected for the study before starting the tests.

10.3 Instruct subjects to avoid contact with antimicrobial products, other than the test material(s), for the duration of their involvement in the study and for at least one week prior to the first test. This restriction includes antimicrobial-containing antiperspirants, deodorants, shampoos, lotions, and soaps, also such materials as acids, bases and solvents. Bathing in biocide treated pools, hot tubs, spas should also be avoided. Subjects are to be provided with a kit of non-antimicrobial personal care products for exclusive use for the duration of the study and rubber gloves to be worm when contact with antimicrobials or hard chemicals cannot be avoided.

11. Procedure

11.1 Fig. 1 shows the main steps in the performance of this test method.

11.1.1 The volunteer will wash his/her hands with a non-medicated soap for at least 10 s, rinse, and then dry them thoroughly with a clean paper or cloth towel. This procedure reduces variability in the test results by removing accumulated oil and dirt from the hands. Place about 3 to 5 mL of 70 % (v/v) ethanol in the palm of one of the washed hands and instruct the volunteer to rub it well over the entire surface of both hands until the alcohol and water have evaporated completely (*Step 1*).

11.1.2 Press a thumbpad or fingerpad over the mouth of an empty plastic vial (see 7.9) to demarcate the area to receive the test inoculum (*Step 2*).

11.1.3 Using a positive displacement pipette, deposit 10 µL of the bacterial suspension, with or without a soil load, at the center of each demarcated area (*Step 3*).

11.1.4 It is recommended that thumbpads be used to determine the amount of viable bacteria placed in each demarcated area (Input Control). Once a thumbpad has been contaminated, do not allow the inoculum to dry, but immediately elute it in accordance with 11.1.9.

11.1.5 Allow the inoculum on all fingerpads to become visibly dry (~20 to 30 min) under ambient conditions (*Step 4*).

11.1.6 To determine the number of viable bacteria remaining viable after this drying period elute the bacteria from two randomly selected fingerpads in accordance with 11.1.9 (*Step 8*).

11.1.7 Expose the dried inoculum on the required number of randomly selected fingerpads, and control or reference solution by placing 1.0 mL of the in-use dilution of the test product, control, or reference solution in a plastic vial (7.9). Place a contaminated fingerpad over the mouth of the vial and invert it. Allow the contents of the vial to remain in contact with the contaminated area for 10 to 15 s (*Step 5*) while subjecting the vial to 10 full inversions. For viscous formulations, invert the vial and keep its contents in contact with the contaminated area for 20 sec without any inversions. Scrape the fingerpad on the inside rim of the vial to recover as much of the fluid as possible (*Step 6*). Such scraping also simulates the friction often applied in hand antisepsis.

11.1.8 To simulate post-treatment rinsing of hands, expose two fingerpads to 1 mL of hard water for 5 to 10 s (*Step 7*). Eluate the bacteria from the fingerpads (*Step 8*).

11.1.9 For bacterial elution, place the contaminated area of the thumb/finger over the mouth of a plastic vial (see 7.9) containing 1 mL of the eluent. Invert the vial with the pad still over it, and allow the eluent to remain in contact with the inoculated contaminated area for 5 to 10 s. Invert the vial 20 times with the pad still in place. Repeat the soak and inversion step once more. Finally, turn the vial upright and scrape the pad against the inside rim of the vial to recover as much of the fluid as possible (*Step 8*).

11.1.10 Decontaminate the thumbpad by pressing it for 2 to 3 min over tissue paper or paper towel soaked in 70 % (v/v) ethanol (*Step 9*).

11.1.11 Instruct the panelists to decontaminate their hands (11.1.10), wash them thoroughly with soap and water and dry them well before leaving the test area.

11.1.12 Titrate the eluates and controls for viable bacteria using a minimum of three culture plates for each dilution tested. If titrations cannot be carried out within 3 to 4 h of collection, store samples overnight at 4 to 10°C.

11.2 Neutralization of any antibacterial activity in the eluates must be properly validated. Add the test bacteria to the neutralized eluate and titrate it along with appropriate controls to demonstrate that there is no detectable loss in bacterial viability after exposure to the neutralized eluates.

12. Enumeration of Bacteria in Control and Test Samples

12.1 Process the control and test samples using either spread plating or membrane filtration to detect CFU of the test bacterium. The membrane filtration method (*a*) has the advantage of allowing larger sample volumes to be processed especially when the viable counts in the eluates may be low, and (*b*) permits the rinsing of the filters to reduce germicide residues, which may interfere with bacterial recovery. The pour-plate technique is not recommended for *S. marcescens* because subsurface colonies may not exhibit the characteristic red pigment.

12.2 Incubate the inoculated plates and sterility controls of the recovery medium for 48 ± 4 h at the appropriate temperature.

12.3 Count colonies and convert data to $\log_{10}$ units.

13. Repetitions and Statistical Evaluations

13.1 For each formulation to be tested, repeat this method at least two times with at least three panelists in each.

13.2 This test method is designed to include the two thumbpads to determine the viable bacteria placed on the fingerpads (inoculum control), two fingerpads to assess the number of bacteria remaining viable after the drying of the

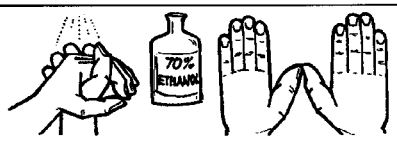
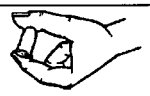
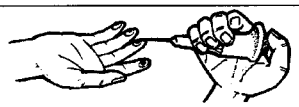
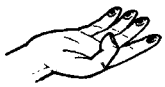
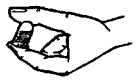
Step #	
(1) The panelist washes hands with non-germicidal soap and tap water and dries them with paper towel. About 3-5 mL of 70% (v/v) ethanol is placed on the hands and they are rubbed together till dry.	
(2) Each digit is pressed against the mouth of an empty plastic vial (8 mm inside diam.) to demarcate the target area.	
(3) 10 µL of the test bacterial suspension, with or without soil load, is placed at center of each demarcated area. Inoculum from the two thumbpads is eluted immediately (Step 8 below) to act as 'input' control.	
(4) Inoculum on fingerpads allowed to become visibly dry (20-25 minutes). Two randomly selected fingerpads are eluted immediately (Step 8) at the end of drying ('baseline' control).	
(5) Dried inoculum on at least two randomly selected fingerpads is exposed to 1 mL of test product or control fluid in a plastic vial for desired contact time, with specified number of full inversions.	
(6) Skin scraped against inside lip of vial to collect as much fluid as possible. For waterless handwash agents or to determine bacterial elimination after exposure to the product alone, fingerpads can be eluted (Step 8) without further treatment	
(7) To simulate post-treatment rinsing of hands, fingerpads are exposed to 1 mL of hard water for 5-10 seconds. The test bacteria can be eluted (Step 8) at this stage or after drying of hands. To determine bacterial removal after the drying of washed hands, they can be dried in air or with paper or cloth towel for specified time and the bacteria recovered from them.	
(8) To elute the bacteria, the digit is placed on the mouth of a plastic vial with 1 mL of eluent and subjected to 20 full inversions; skin is scraped against inside lip of vial to collect as much fluid as possible. The eluates and controls are titrated for viable bacteria and $\log_{10}$ reductions calculated.	 
(9) The panelist decontaminates the hands by pressing the inoculated areas for 2-3 minutes against a tissue or paper towel soaked in 70% ethanol. The panelist then washes hands thoroughly with soap and water and dries them well before leaving the test area.	

FIG. 1 Procedure For In Vivo Evaluation Of The Bacteria-Eliminating Activity Of Handwash And Handrub Agent Using The Fingerpad Test

inoculum (dry control), two fingerpads to determine the extent of bacterial elimination after treatment with standard hard water alone, and four fingerpads to assess the level of bacterial elimination after the combined effect of exposure to the test product and the post-treatment rinse with the standard hard water. For waterless agents, the hard water rinse is not called for.

13.3 The difference in the number of viable bacteria in the inoculum control and the dry control represents the loss in bacterial viability due to the drying of the inoculum. The number of viable bacteria remaining after the drying of the inoculum must be used as the baseline to determine the extent of bacterial elimination after treatment with the control solution or the test product.

14. Precision and Bias

14.1 *Precision*—The precision of this test method within two laboratories has been determined and compared with that

of a whole-hand protocol **(6)**. The efficiency of the test method of bacterial elution from the fingerpads has been tested with two different types of bacteria and has been found to be about 90 %.

15. Keywords

15.1 bacteria-eliminating activity; eluent; *Escherichia coli*; fingerpads; germicidal soap; hygienic handwashing; infection control; in vivo testing; *Serratia marcescens*; skin flora; soil load; standard hard water; *Staphylococcus aureus*; *Staphylococcus epidermidis*

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