



Standard Test Method for Determining Fungus-Eliminating Effectiveness of Hygienic Handwash and Handrub Agents Using Fingerpads of Adults¹

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INTRODUCTION

Human hands are frequently in contact with other surfaces and can readily acquire transient microbial contamination. Fungi are common among these types of contaminants (**1, 2**),² particularly in healthcare settings and where food is handled. Standardized methods to assess the fungus-eliminating potential of handwash and handrub agents have not been available and this test method addresses the gap.

1. Scope

1.1 This test method is designed to assess the ability of hygienic handwash and handrub agents to reduce levels of fungal contamination on hands (**3**). This test method is not meant for use with surgical hand scrubs (Test Method **E1115**) or preoperative skin preps (Test Method **E1173**).

1.2 Performance of this procedure requires the knowledge of regulations pertaining to human experimentation.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 (**4**).

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

1.5 *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.*

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

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

2. Referenced Documents

2.1 *ASTM Standards*:⁴

D1129 Terminology Relating to Water

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E1115 Test Method for Evaluation of Surgical Hand Scrub Formulations

E1173 Test Method for Evaluation of Preoperative, Precatheterization, or Preinjection Skin Preparations

E1838 Test Method for Determining the Virus-Eliminating Effectiveness of Hygienic Handwash and Handrub Agents Using the Fingerpads of Adults

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporocidal Activities of Chemicals

E2276 Test Method for Determining the Bacteria-Eliminating Effectiveness of Hygienic Handwash and Handrub Agents Using the Fingerpads of Adults

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 *Definitions*—For definitions of general terms used in this test method, refer to Terminologies **D1129** and **E2756**.

3.2 *Definitions of Terms Specific to This Standard*:

⁴ 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.

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

3.2.2 *dry control*—a control to determine the number of colony forming units (CFU) of the test fungus remaining viable after the initial drying of the inoculum on the skin.

3.2.3 *handrub, n*—a liquid, gel, or foam, which is applied by rubbing to decontaminate lightly soiled hands between hand-washings and generally does not require a post-treatment water rinse; such agents usually contain alcohol alone or with other active ingredients.

3.2.4 *hard water, n*—water with a defined level of hardness as calcium carbonate.

3.2.5 *hygienic handwash agent, n*—an agent generally used for handwashing by personnel in hospitals, other health-care facilities, day-care centers, nursing homes, and food-handling establishments to eliminate transient microorganisms from intact skin.

3.2.6 *input control, n*—a control to determine the number of colony forming units of the test fungus placed on each digit.

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

3.2.8 *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.

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

3.2.10 *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 fungal contaminant. It may also be referred to as a marker organism, fungal simulat/surrogate or fungal contaminant.

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

3.2.12 *unmedicated soap, n*—a soap or detergent for handwashing that is mild to the skin and does not contain any antimicrobial chemicals.

4. Summary of Test Method

4.1 This test method uses 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. Subjects are to refrain from using any products containing antimicrobial agents for at least one week prior to the test. A known volume of the test fungal suspension is placed on a demarcated area on each fingerpad and the inoculum dried. The contaminated area is then exposed to the control (water with a standard level of hardness) or test agent for the desired contact time, organisms remaining on the fingerpad are eluted and the eluates assayed for fungal CFU. Reductions in the numbers of CFU after treatment with the control and test agent(s) are then

determined. 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.

5. Significance and Use

5.1 This *in vivo* procedure is designed to test the ability of hygienic handwash or handrub agents to eliminate fungal contamination from experimentally-contaminated hands. 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 cells of the test fungus remaining after the inoculum has dried (two), fungal cells eliminated after treatment with a control or reference solution (two), and up to four replicates to assess the fungus-eliminating efficiency of the formulation under test. No more than 100 μL of the test fungal suspension is required to complete one test.

5.2 Whereas this test method is designed to work with fungi, similar ASTM standards exist for testing against viruses (Test Method E1838) and vegetative bacteria (Test Method E2276).

5.3 Infectious microorganisms left on hands after washing can be reduced further by drying the washed hands with paper, cloth, or warm air (5). A step for the drying of fingerpads after exposure to the control or test solution, therefore, has not been included to avoid fungal removal by the drying process itself.

5.4 This test method is not designed to test surgical hand scrubs or preoperative skin preps.

5.5 The level of contamination with viable fungi on each fingerpad after the drying of the inoculum should be at least 10^4 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 test formulation under the conditions of this test. This in itself does not represent the product performance criterion, which may vary depending on the jurisdiction and the nature of the formulation being evaluated.

6. Equipment and Apparatus

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

6.2 *Freezer*, a freezer at $-70 \pm 2^\circ\text{C}$ or lower is required to store fungal stocks.

6.3 *Handwashing sink*, a sink of sufficient size to permit subjects to wash hands without touching hands to sink surface.

6.4 *Incubator*, any incubator that can maintain a temperature suitable for the growth of the fungal species under test.

6.5 *Laminar flow cabinet*, a Class II biological safety cabinet.

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 $0.45 \mu\text{m}$ or $0.22 \mu\text{m}$ are required to sterilize heat-sensitive media/solutions.

6.8 *Positive displacement pipette*, a pipette and pipette tips that accurately can dispense 10- μL volumes.

6.9 *Refrigerator*, a refrigerator at $4 \pm 2^\circ\text{C}$ for storage of prepared culture media and reagents.

6.10 *Sterilizer*, any suitable steam sterilizer that produces the conditions of sterilization is acceptable.

6.11 *Timer (stop clock)*, one that can be read for minutes and seconds.

6.12 *Tap water temperature regulator and temperature monitor*, to monitor and regulate water temperature at $40 \pm 2^\circ\text{C}$.

6.13 *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. Reagents and Materials

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

7.2 *Culture plates*, Petri plates of 100×15 mm diameter for culturing the fungus.

7.3 Soil Load:

7.3.1 A tripartite soil load Test Method E2197, prepared from the following three stock solutions, as an alternative to serum.

7.3.2 Add 0.5 g of tryptone or yeast extract to 10 mL of phosphate buffer.

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

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

7.3.5 Prepare the stock solutions separately and sterilize by passage through a $0.22 \mu\text{m}$ pore diameter membrane filter, aliquot and store at either $4 \pm 2^\circ\text{C}$ or $-20 \pm 2^\circ\text{C}$.

7.3.6 To obtain a 500- μL inoculum of the test inoculum, add to 340 μL of the fungal suspension 35 μL of tryptone or yeast extract (7.3.2), 25 μL BSA (7.3.3), and 100 μL mucin (7.3.4) 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.

7.4 *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 (6) 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 fungal 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 fungus. If water with a higher level of hardness is used for making the use-dilution of the test formulation, this change must be clearly justified and documented in the report.

7.5 *Test agents*, at least two samples of the test formulation shall be evaluated.

NOTE 1—Water with a standard level of hardness is also recommended as a control in this test procedure to determine any mechanical removal of the test organism(s) from the skin.

7.6 *Diluent for fungal titration*, normal saline (0.85 % NaCl) at pH 7.2 to 7.4 or an appropriate buffer.

7.7 *Eluent for fungal recovery from fingerpads*, normal saline.

7.8 *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.

7.9 *Miscellaneous laboratory ware*, automatic pipettes, pipette tips, and plastic vials for storing stock cultures.

7.10 *Broth*, trypticase soy broth (TSB) or equivalent to prepare the inoculum of *Candida albicans*.

7.11 *Agar*, sabouraud dextrose agar or equivalent to prepare the inoculum of *Aspergillus niger* and to recover and count the colonies of the two test organisms in control and test samples. The addition of any neutralizer(s) in such recovery media must first be properly validated (Test Methods E1054).

8. Test Fungi

8.1 Appendix X1 contains a list of suggested test fungi. **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 See Appendix X1.

10. Subjects

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







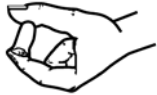



10.2 It is the responsibility of the user of this test method to arrange the necessary clearance for the use of subjects for testing and to obtain informed and written consent from those selected for the study before starting the tests.

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 microbicide treated pools, hot tubs, and spas should also be avoided. Subjects are to be provided with a kit of non-antimicrobial personal care products for exclusive use during the wash-out period and for the duration of the study; rubber gloves to be worn when contact with antimicrobials or harsh chemicals cannot be avoided.

11. Procedure (Flowchart 1)

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

TABLE 1 Flowchart-Procedure for *in vivo* Evaluation of Fungus-eliminating Activity of Handwash and Handrub Agents Using the Fingerpad Method

Step #	
<p>(1) The subject washes hands with non-germicidal soap and tap water and dries them with paper towel. About 3 to 5 mL of 70 % (v/v) ethanol is placed on the hands and they are rubbed together until dry.</p>	
<p>(2) Each digit is pressed against the mouth of an empty plastic vial (8 mm inside diameter) to demarcate the target area.</p>	
<p>(3) 10 µL of the test fungal suspension, with or without soil load, is placed at the center of each demarcated area. Inoculum from the two thumbpads is eluted immediately (Step 8) to act as 'input' control.</p>	
<p>(4) Inoculum on fingerpads allowed to become visibly dry (20 to 30 min). Two randomly selected fingerpads are eluted immediately (Step 8) at the end of drying ('baseline' control).</p>	
<p>(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.</p>	
<p>(6) Skin scraped gently against inside lip of vial to collect as much fluid as possible. For waterless handwash agents or to determine fungal elimination after exposure to the product alone, fingerpads can be eluted (Step 8) without further treatment.</p>	
<p>(7) To simulate post-treatment rinsing of hands for handwash agents, fingerpads are exposed to 1 mL of hard water for 5 to 10 s) The skin is again scraped gently against inside lip of vial to collect as much fluid as possible. The test fungus can be eluted (Step 8).</p>	
<p>(8) To elute the fungi, 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 fungi and log₁₀ reductions calculated.</p>	 
<p>(9) The subject decontaminates the hands by pressing the inoculated areas for 2 to 3 min against a tissue or paper towel soaked in 70 % ethanol. The subject then washes hands thoroughly with soap and water and dries them well before leaving the test area.</p>	

11.1.1 The subject will wash his/her hands with unmedicated 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 subject 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 7.8) to demarcate the area to receive the test inoculum (Step 2).

11.1.3 Using a positive displacement pipette, deposit 10 μL of the fungal suspension at the center of each demarcated area (Step 3).

11.1.4 It is recommended that thumbpads be used to determine the number of viable organism 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 (approximately 20 to 30 min) under ambient conditions (Step 4).

11.1.6 To determine the number of organism remaining viable after this drying period, elute the inoculum from two randomly selected (one on each hand) fingerpads in accordance with 11.1.9 (Step 8).

11.1.7 Expose the dried inoculum on the required number of randomly selected fingerpads to the test formulation, control or reference solution by placing 1.0 mL of the in-use dilution of it in a plastic vial (7.8). 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 no less than 10 and no longer than 30 s (Step 5) while subjecting the vial to ten (10) full inversions. For viscous formulations, invert the vial and keep its contents in contact with the contaminated area for the desired contact time 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 antiseptics.

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). Elute the organisms from the fingerpads (Step 8).

11.1.9 For fungus elution, place the contaminated area of the thumb/finger over the mouth of a plastic vial (see 7.8) 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 fingerpads by pressing them for 2 to 3 min over tissue paper or paper towel soaked in 70 % (v/v) ethanol (Step 9).

11.1.11 Instruct the subjects 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 fungal cells. 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 fungicidal activity in the eluates must be properly validated. Add the test fungus to the neutralized eluate and titrate it along with appropriate controls to demonstrate that there is no detectable loss in fungal viability after exposure to the neutralized eluates.

12. Enumeration of Fungi in Control and Test Samples

12.1 The control and test samples may be processed using spread-plating, membrane filtration or spiral plating to detect CFU of the test fungus. 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 fungal recovery.

12.2 Incubate the inoculated plates and sterility controls of the recovery medium 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 subjects.

13.2 This test method is designed to include the two thumbpads to determine the viable units of the fungus placed on the digits (input control), two fingerpads to assess the number of organisms remaining viable after the drying of the inoculum (dry control), two fingerpads to determine the extent of fungal elimination after treatment with standard hard water alone, and four fingerpads to assess the level of fungal 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 required.

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

14. Precision and Bias

14.1 *Precision*—This test method has been successfully applied to determine the activity of hand hygiene agents against *C. albicans* (7) and the efficiency of fungal elution from untreated fingerpads was found to be higher than 80 %. The method has also been applied for work with *A. niger* with an efficiency of recovery of the conidia from untreated fingerpads being about 92 %.

14.2 Since there is no accepted reference material, method, or laboratory suitable for determining the bias for the procedure in this test method more measuring \log_{10} reduction or \log_{10} transfer, no statement on bias can be made at this stage.

15. Keywords

15.1 antiseptics; *Aspergillus niger*; *Candida albicans*; conidia; eluent; fingerpads; fungus-eliminating activity; hygienic handrubs; hygienic handwashing; infection control; *in vivo* testing; non-germicidal soap; soil load; standard hard water; subjects

APPENDIX

XI. FUNGI RECOMMENDED FOR USE IN THIS TEST

X1.1 The selection of the following test fungi is based on their (a) relative safety to the subjects as well as experimenters, (b) ability to grow to titers sufficiently high for testing, (c) ease of recovery and identification in the laboratory, and (d) potential to spread through contaminated hands. Other strains or types of fungi may be substituted provided they meet the preceding criteria and are approved by the relevant institutional review board.

X1.2 *Candida albicans* (ATCC 10231). This represents non-filamentous fungi (yeast-like) and is among the most common species of *Candida* recovered as clinical isolates (7).

X1.3 *Aspergillus niger* (ATCC 64958). This is an example of a filamentous fungus that is considered safe for the experimental contamination of the skin of adult subjects.

X1.4 Other fungal species may be used provided they are safe for contamination of the hands of adult subjects and only

after permission has been received for their use from the relevant institutional review board.

X1.5 To prepare *C. albicans* test inoculum, add 0.1 mL of a frozen stock culture to 10 mL of TSB (7.10) and incubate for 48 ± 4 h at the appropriate temperature.

X1.6 To prepare a suspension of the conidia of *A. niger*, inoculate the center of a Sabouraud dextrose agar plate with 0.1 mL of a frozen stock suspension and incubate for 10 days at 28°C. Harvest mycelial mats from the agar surface, homogenize with sterile glass beads in normal saline, and filter through sterile gauze to remove the hyphae.

X1.7 Add soil load (7.3) to the test fungal cell suspension, if required.

X1.8 Swirl, vortex, or shake the test fungal cell suspension before withdrawal of each aliquot.

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