Designation: E1327 – 07 (Reapproved 2012)ε1

Standard Test Method for Evaluation of Antimicrobial Handwash Formulations by Utilizing Fingernail Regions[1]

This standard is issued under the fixed designation E1327; 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.

ε1 NOTE—Editorial changes were made throughout the document in November 2012.

1. Scope

1.1 This test method can be used to determine the effectiveness of antimicrobial handwashing agents (including handrubs) in the reduction of transient bacterial flora with particular emphasis on the fingernail region.

1.2 A knowledge of microbiological techniques is required for these procedures.

1.3 *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.* For more specific hazard statements, see 7.5.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

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

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

3. Summary of Test Method

3.1 This test method, involving an improved method of recovering bacteria from hands, is used to study the effects of antimicrobial handwashes including health care personnel handwash products. The group of volunteer panelists need not refrain from using topical antimicrobials (such as deodorant soaps) before participating in the study. All subjects wash their hands with a nonantimicrobial hand soap prior to testing to remove any residual hand lotions and to lower the numbers of resident skin flora. Activity of products is measured by comparing the numbers of marker bacteria recovered from artificially contaminated fingernail regions after use of the handwashing formulations to the numbers recovered from the artificially contaminated but unwashed fingernail regions. Broth cultures of *Serratia marcescens* (a red pigmented bacterial species) and *Escherichia coli* (which produces fluorescent colonies on a special agar medium) are used as test bacteria. A spore suspension of *Bacillus subtilis* may be utilized to study (*1*) degree of physical removal by handwashing techniques, and (*2*) the recovery and precision aspects of the test method.

4. Significance and Use

4.1 The procedure should be used to test the degerming effectiveness of antimicrobial hand washing products used by health care personnel that are intended for frequent use, and that are intended to reduce the level of contamination acquired through contact with contaminated objects or people.

4.2 Performance of these procedures requires the knowledge of regulations pertaining to the protection of human subjects (Ref **1**).[3]

5. Apparatus

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

5.2 *Incubators*—One incubator capable of maintaining a temperature of 25 ± 2°C (this temperature is required to ensure pigment production of *Serratia*); a second incubator capable of maintaining 37 ± 2°C used for *E. coli* and *B. subtilis* incubation is acceptable.

5.3 *Water Bath*—Capable of maintaining temperature of 80 ± 2°C for heat shocking of *B. subtilis* spores is needed.

[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.

Current edition approved Oct. 1, 2012. Published November 2012. Originally approved in 1990. Last previous edition approved in 2007 as E1327 – 07. DOI: 10.1520/E1327-07R12E01.

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

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

5.5 *Timer*—Any stop-watch that can be read in minutes and seconds is required.

5.6 *Handwashing Sink*—A sink of sufficient size to permit panelists to wash without touching hands to sink surface or other panelists is needed.

5.6.1 *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.

5.6.2 *Tap Water Temperature Regulator and Temperature Monitor,* to monitor and regulate water temperature of 40 ± 2°C.

5.7 *Quad Petri plates,* 100 by 15 mm, plastic, sterile, disposable.[4]

5.8 *Small Petri Plates,* 60 by 15 mm, glass.

5.9 *Large Petri Plates,* 150 by 15 mm, glass.

5.10 *Tooth Brushes:*
5.10.1 *Young Size.*
5.10.2 *Battery Operated.*

5.11 *Ultraviolet Lamp,* having separate short wave and long wave bulbs.

5.12 *Germicidal Lamp Monitor Strips.*

5.13 *Inoculating Loops or Needles,* sterile.

5.14 *Plate Spreaders or Hockey Sticks,* sterile.

6. Reagents and Materials

6.1 *Bacteriological Pipettes,* 10.0 mL, sterile.

6.2 *Pipettors and Pipette Tips,* Eppendorf, MLA or similar types.

6.3 *Disposable Analyzer Cups,* 2 mL, plastic, not sterile.

6.4 *Sampling Solution*—Dissolve 0.4 g KH_2PO_4, 10.1 g Na_2PO_4 and 1.0 g isooctylphenoxypolyethoxyethanol[5] in 1 L distilled water. Adjust pH to 7.8 with 0.1 *N* HCl or 0.1 *N* NaOH. Dispense in 100 mL-volumes and sterile for 20 min at 121°C.

6.5 *Dilution Fluid*—The sampling fluid may be used for dilutions or use Butterfields sterile phosphate buffered water (**2**) adjusted to pH 7.2 with suitable inactivator for the antimicrobial. Adjust pH with 0.1 *N* HCl or 0.1 *N* NaOH (see Practices E1054).

6.6 *Agar,* Tryptic soy agar or equivalent. Include the appropriate inactivator if needed.

6.7 *Agar with MUG*—Tryptic soy agar with 60 to 80 µg/mL 4-methylumbelliferyl-β-D-glucuronide (MUG) is required.

6.8 *Test Formulations*—Directions for use of test formulation should be included if available. If these are not available, liquid antimicrobial soap formulations are tested by same routine as the nonantimicrobial control (10.5); alcoholic lotion type formulations are rubbed to dryness and then sampled for survivors (10.7).

6.9 *Nonantimicrobial Control Soap,* a liquid castile soap or other liquid soap containing no antimicrobials.

6.10 *Broth*—Tryptic soy broth or equivalent is required.

7. Test Organisms

7.1 *Serratia marcescens* American Type Culture Collection, ATCC No. 14756 is to be used as a marker organism. This is a strain having stable pigmentation. Grow in tryptic soy broth at 25 ± 2°C.

7.2 *Escherichia coli,* ATCC No. 11229 is used as another Gram-negative marker organism. Grow in tryptic soy broth at 35 ± 2°C.

7.3 *Bacillus subtilis,* ATCC No. 19659. Grow in tryptic soy broth at 35 ± 2°C.

7.4 *Preparation of Spore Suspension*—Inoculate each surface of two tryptic soy agar plates (30 mL agar in 150-mm petri plates) with 1 mL of *B. subtilis* tryptic soy broth culture. Spread over the entire surface of the agar. Incubate for 5 to 10 days at 35 ± 2°C. Suspend the growth in 20 mL of 0.1 % tryptone water[6] by rubbing the agar surface with a sterile rubber policeman. Add ethanol to the suspension to a final concentration of 80 % (wt/wt) and store in a refrigerator.

7.5 Other bacteria containing adequate markers to enable distinction from normal flora and of known safety may also be used for testing purposes. (**Warning**—The application of microorganisms to the skin may involve a health risk. Prior to applying *S. marcescens* or other bacteria to the skin, the antibiotic susceptibility profile of the strain should be determined. If the *Serratia* strain is not sensitive to Gentamicin, it should not be used. If an infection occurs, the antibiotic susceptibility profile should be made available to an attending clinician. Following the panelist's contamination and testing for the day, the panelist's hands should be decontaminated with a 70-% ethanol solution. Care should be taken to decontaminate around the fingernail regions.)

7.6 *Preparation of Marker Culture Suspension*—Inoculate a 10-mL tryptic soy broth tube with each of the test bacteria and incubate each tube at the temperature indicated to yield inocula of 10^8–10^9 CFU/mL. When studying mixed inocula, mix equal volumes of the cultures into a sterile test tube; an equivalent volume of *B. subtilis* spore suspension (that is prepared by centrifuging the alcoholic suspension and resuspending cells in water) may be added for bacterial physical removal determinations. Keep mixed suspension on ice during the day's testing.

[4] Presterilized disposable quad plastic petri plates, the two sizes of glass petri plates and other equipment are available from most local laboratory supply houses.

[5] The sole source of supply of the apparatus (Triton X-100) known to the committee at this time is Rohm and Haas Co., Philadelphia, PA. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[6] The sole source of supply of the Bacto Tryptone (Difco) water known to the committee at this time is Difco Laboratories, Detroit, MI. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

8. Panelists

8.1 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 people needed for a trial is dependent on the number of treatments within a study.

8.2 Volunteers are asked to maintain their normal use of soaps, shampoos, and so forth. They are asked to refrain from the use of acids, bases, solvents on the hands during the test period. Gloves should be provided for use where exposure to these agents is unavoidable.

9. Experimental Design

9.1 Each fingernail of a volunteer may be assayed separately; therefore, 10 test determinations (replicates) may be obtained from one volunteer. For the comparison of several products during a single study, a design such as a Latin Square Design may be utilized (**3**). For example, to compare 5 antimicrobial test products, one nonantimicrobial product and unwashed hand control (7 total variables), 7 volunteers, (or multiples of 7) should be recruited. Each person performs one testing of product or other variable on each of 7 test days, according to schedule such as the following; the numbers = day for testing that variable (see Table 1).

9.1.1 *Example:* Volunteer *A* tests Treatment 1 on Day 1, then Treatment 2 on Day 2; Volunteer *B* tests Treatment 2 on Day 1, Treatment 3 on Day 2, and so forth.

9.1.2 Each product or variable is tested once on each day, unless multiple numbers of volunteers are in the study.

9.1.3 The number of fingers, which are inoculated and then assayed after using the product, should be kept standard throughout. Although the number can be as high as 10, three fingers on one hand is a more convenient and cost savings approach. The ring, middle, and index fingers of the left hand have been selected for several studies; however, an operator may select the number and particular fingers to assay as long as they are held constant throughout.

10. Procedure

10.1 Before tests for the day, sterilize the analyzer cups by placing in suitable rack (24-well culture plates with lids are convenient) and placing the open cups under short-wave ultraviolet lamp for 15 to 30 min. To each sterile disposable analyzer cup, add 0.9 mL of sterile diluent: set up sufficient cups only for each day's testing.

10.2 Place 7 mL of sampling solution into each of 21 small petri plates.

TABLE 1 Latin Square Design for Testing Seven Variables

	Volunteer						
Day	A	B	C	D	E	F	G
1	T_1	T_2	T_3	T_4	T_5	T_6	T_7
2	T_2	T_3	T_4	T_5	T_6	T_7	T_1
3	T_3	T_4	T_5	T_6	T_7	T_1	T_2
4	T_4	T_5	T_6	T_7	T_1	T_2	T_3
5	T_5	T_6	T_7	T_1	T_2	T_3	T_4
6	T_6	T_7	T_1	T_2	T_3	T_4	T_5
7	T_7	T_1	T_2	T_3	T_4	T_5	T_6

10.3 Place 0.02 mL of marker culture suspension on the region surrounding the cuticle and under the fingernails of three fingers of the left hand of a volunteer. The volunteer then holds the hand in front of an electric fan for 5 min for complete drying of the suspension.

10.4 For unwashed hand determinations, proceed directly to 10.8.

10.5 When testing nonantimicrobial soap (controls), wet both hands under flowing warm tap water (40 ± 2°C). Add 2.5 to 3.0 mL of the liquid soap to hands, rub hands together in normal washing manner for 15 s (no additional water), then rinse under the flowing water for 15 s to remove suds. Do not dry hands, proceed directly to 10.8.

10.6 For testing liquid antimicrobial soap formulations, follow the use directions on the label or follow the routine of 10.5. After washing, proceed to 10.8 without drying hands.

10.7 Alcoholic formulations are tested by placing the recommended volume on the hands and then rubbing the hands together until the alcohol has evaporated. Proceed to 10.8.

10.8 After performing the procedure for the day designated in the Latin Square Design, the technician scrubs with a toothbrush for 1 min each fingernail into a separate petri plate containing 7 mL of sampling solution.

NOTE 1—Although manual toothbrushes may be used for this purpose, greater uniformity between scrubbings may be obtained with less operator fatigue if an electric toothbrush such as the GE model TB-9 or another type is used. A brush which operates parallel with the handle is preferred because of less splashing.

10.9 After each scrubbing, the brushes are dropped into a beaker containing 70 % ethyl or isopropyl alcohol and allowed to stand for at least 10 min. The brushes are then rinsed in sterile distilled water and allowed to dry. The brushes are not sterilized.

10.10 Perform serial 10-fold dilutions. Place 0.1 mL amounts of the appropriate dilutions onto the surface of agar sections of quad plates. These drops of liquid are spread with sterile inoculation loops, needle spreaders, or hockey sticks to completely cover the quads. Allow drops to completely absorb.

10.11 Incubate inverted plates at 35 ± 2°C for 12 to 18 h. Count the *E. coli* colony-forming units (CFU) that fluoresce under long-wave ultraviolet light. Transfer the plates to a 25°C incubator and incubate for another day.

10.12 Count the red-pigmented *S. marcescens* CFU. Record the CFU per countable sections of the plates and convert values to the CFU obtained per finger by multiplying by the appropriate dilution factors.

10.13 Convert each CFU-per-finger determination to the $\log_{10}$ value.

10.14 Determine the mean $\log_{10}$ CFU per finger value. This is the mean $\log_{10}$ value for that variable and subject for that day. These $\log_{10}$ values are used for statistical comparisons.

10.15 If an estimation of the degree of physical removal caused by a product is desired, *B. subtilis* spores may be

included in the test strain(s) inoculum. Portions of the collection fluids are heated at 80°C for 10 min to kill the vegetative test strains; the fluids are then diluted and plated on tryptic soy agar.

11. Statistical Evaluations

11.1 The completed table of data obtained from the Latin Square Design described should be analyzed by Analysis of Variance Tests (3).

11.2 In tests where a small number of variables and large numbers of people are involved, for example, 20 people testing 2 antimicrobial products, a nonantimicrobial product and unwashed hand controls, use a Newman-Keuls multiple range analysis (4), or the parametric Student's *t* test.

12. Precision and Bias

12.1 *Precision*—The precision of this test method within one laboratory has been determined (5); precision of recovery is comparable with other accepted methods such as the glove juice technique.

12.2 Using *B. subtilis* spore tracers, this test method recovers almost all inoculated spores that were placed on the fingernails as inert tracers and then scrubbed into collection fluid versus placing the equivalent amount of spores in the equivalent volume of collection fluid and assaying directly (5). The precision aspects of this test method can be expressed as the coefficient of variation by using replicate spore recovery data, described in the reference.

13. Keywords

13.1 antimicrobial; *Escherichia coli*; fingernail; handwash or handrub; health care personnel handwash; *Serratia marcescens*; testing individual fingers; toothbrush

APPENDIX

(Nonmandatory Information)

X1. GUIDE FOR METHODS OF TESTING ANTIMICROBIAL FORMULATIONS BY SAMPLING FINGERS AND THE FINGERNAIL REGION

X1.1 Introduction

X1.1.1 Assessing the effectiveness of antimicrobials for use on the hands has been problematic and controversial since Semmelweiss and Price. We know that the hands are instruments of contamination and dissemination of infectious microorganisms and we also know now that the microflora and contamination of hands are very different and not representative of other areas of skin on the body. There are few sebaceous glands, intersticies in the cuticle area and skin folds that can secrete bacteria, and fingernails of varying length that can collect dirt, skin fragments, and microorganisms.

X1.1.2 The recognition of these differences has prompted the development of sampling and enumeration methods that capsulize the challenging areas of the fingers and hands. Several methods to sample skin and cuticle areas of the fingers have been published as standards and articles in the literature. This guide describes some of these methods. They are practical because there are 10 individual fingers and cuticle areas to sample. Also, these methods are valuable in screening actives, and treatments for predicted effectiveness in reaching these difficult to sample and treat areas.

X1.1.3 Recently published ASTM standards and publications by Sattar et al. (Test Method E2276 and Test Method E1838) have utilized the fingerpad area to apply contaminants, viruses, and bacteria that samples limited areas. Rotter has described methods (also described in EN1500) in which the whole hand is contaminated, usually with *Escherichia coli*, but only the forefingers are sampled, usually to estimate reduction after use of alcohol-based handrubs.

X1.1.4 These publications offer additional means of testing the microbial flora of the hands with concentration on the individual fingers as a test area. Many literature sources have emphasized the fingers as the major instrument of transmission of transient flora so critical in the spread of infections.

X1.2 Nail Space

X1.2.1 The fingernails have been challenging because they often vary in length and in the variety of accumulation found under the nail. Most surgical scrub regimens have promoted the use of nail cleaning during the scrub, but again, it can be variable. Leyden et al. have contributed to our understanding of the effect of the material and microbial population under the nails by describing a procedure for sealing the nail space. This technique could be included in a test protocol in the following test methods. This procedure can isolate the flora (either natural or artificial contamination) under the nails and permit determination with and without sealing method.

X1.2.2 McGinley et al. (6) have studied the composition of the subungual space and concluded that the microbial level is approximately 10^5 CFUs under the nail. In this same vein, Hann redid Price's study using a basin and showed that if the distal fingers (phalanges) were eliminated from the collection device used, much lower numbers of bacteria were recovered.

X1.2.3 Leyden examined the effect of sealing the nail space on the log reduction in conventional recovery modes in a glove

or in a plastic bag. The technique he used was to apply a coat of acrylic nail polish, allow it to dry, and re-apply to finish with three coats. His study showed the collection of bacteria in the nail space is not easily removed with the use of detergent-based antimicrobials. These formulations showed a powerful influence on the hand surface, but not as significant on the bacteria in the subungual space.

X1.2.4 This test method (Test Method E1327) has been published as a standard test method since 1990 (reapproved in 2000 and 2007). The procedures are spelled out in detail. However, the basic idea of testing an individual finger or group of them is valuable. Some aspects of the sampling method could be simplified. The use of an electric toothbrush to ensure sampling of the cuticle and nail region is useful.

X1.3 Casewell Method

X1.3.1 British Public Health Laboratories (Ayliffe and staff (**7**)) have used a procedure for sampling the microflora of the hands for many years that involves the use of a basin containing glass beads with a surfactant solution to ensure removal of bacteria from the hands. Ayliffe et al. have described this and used the method in numerous publications. Casewell (**8**) adapted the methodology to better simulate conditions in the wards by using *Klebsiella aerogenes* K21, neutralizing the effect of the contaminating organism on the palmar surface and recording results as median number of organisms recoverable. Normal precautions for volunteers were followed. An 18-hour peptone water culture was used to inoculate (0. 02 ml) the palmar surfaces of the distal phalanges. The opposed fingertips were rubbed together for 30 s to spread the inoculum. The 10 inoculated fingers could then be used to sample the fingers at increasing intervals.

X1.3.2 Sampling of individual fingers was performed by washing an extended finger for 30 s in a sterile (disposable) galley pot (7 cm diameter) containing 30 mL of sterile glass beads (3 to 5 mm diam.) and 20 mL of 0.1 % Tween 80; 0.3 % lecithin and 0.1 % histidine (in this case for chlorhexidine) or another appropriate neutralizer could be used. Samples could be taken at different times. Twenty mL of double strength isolation medium (broth) was added immediately to each 20 mL of finger washings. Agar spread plates were prepared to determine low numbers of surviving organisms. (Salzman, Clark and Klemm, 1968 (**9**) and Casewell 1977 (**10**)). Results were recorded as CFU/mL and converted to $\log_{10}$ CFU for data analysis.

X1.3.3 A different method for blocking the exposure of a finger(s) to wash formulations was used in Casewell's procedure. He used finger cots or cut-off surgical gloves to cover some fingers during a test. With 10 sites available, it is easy to examine variables. It may be useful to increase the level of contamination on the fingers. Some other test organisms will survive drying on the skin and show a lower level of die-off.

X1.3.4 Casewell recommended use of this model in "in use" settings as well as urging the use of relevant clinical organisms. These advantages are clear, but his methodology can be useful with more often tested marker strains in laboratory subjects. When strains (often Gram negative) more sensitive to drying on the skin are used, an adequate count to estimate reductions reliably may be difficult.

REFERENCES

(1) *Federal Register*, Vol 46, No. 17, Jan. 27, 1981.

(2) Butterfield's Phosphate Buffer. *Journal of the Association of Official Analytical Chemists*, Vol 22, No. 625, 1939.

(3) Bennett, C. A., and Franklin, N. L., *Statistical Analysis in Chemistry and the Chemical Industry*, John Wiley and Sons, New York, 1954.

(4) Miller, R. G., *Simultaneous Statistical Inference*, McGraw Hill, New York, 1966.

(5) Mahl, M. C., *Journal of Clinical Microbiology*, Vol 27, 1989, pp. 2295–2299.

(6) McGinley, K. J., Larson E. L., and Leyden, J. J., "Composition and density of microflora in the subungual space of the hand," *J. Clin. Microbial*, Vol 26, 1988, pp. 950–953.

(7) Ayliffe, G. A. J., Babb, J. R., and Quaraishi, A. H., "A test for 'hygienic' hand disinfection," *J. Clin. Path*, 31, 1978, pp. 923–928.

(8) Casewell, N. W., Law, M. M. and Desai, N., "A laboratory model for testing agents for hygienic hand disinfection: hand washing and chlorhexidine for the removal of Klebsiella," *J. Hosp. Infec*, Vol 12, 1988, pp. 163–175.

(9) Salzman, T. C., Clark, J.J. and Klemm, L., "Hand contamination of personnel as a mechanism of cross-infection in nasocomial infections with antibiotic-resistant Escherichia coli and a. Klebsiell aerobacter," *Antimic Agents and Chemother*, 1968, pp. 97–100.

(10) Casewell, M. W. and Phillips, I., "Hands as a route of transmission for Klebsiella species," *Brit. Med. J*, Vol 2, 1977, pp. 1315–1317.

BIBLIOGRAPHY

(1) Ansari, S. A., Sattar, S. A., Springthrope, V. S., Wells, G. A. and Tostowaryk, W., "In vivo protocol for testing efficacy of hand-washing agents against viruses and bacteria: Experiments with Rotavirus and Escherichia coli," *Appl. And Environ. Microbiol*, Vol 55, 1989, pp. 3113–3118.

(2) Rotter, M. L., "Hygienic hand disinfection," *Infec. Control*, Vol 5, 1984, pp. 18–22.

(3) Rotter, M., Koller, W., Wewalka, G., Werner, H. P., Ayliffe, G.A.J. and Babb, J. R., "Evaluation of procedures for hygienic hand disinfection controlled parallel experiments on the Vienna test model," *J. Hyg. Comb*, Vol 96, 1986, pp. 27–37.

(4) Rotter, M., Koller, W. and Wewalka, G., "Povidone-iodine and chlorhexidine gluconate-containing detergents for disinfection of hands," *J. Hosp. Infec*, Vol 1, 1980, pp. 149–158.