



Standard Test Method for Evaluation of Hygienic Handwash and Handrub Formulations for Virus-Eliminating Activity Using the Entire Hand¹

This standard is issued under the fixed designation E 2011; 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

Mechanical removal and/or in situ inactivation of viruses by hygienic handwash and handrub agents can be assessed using artificially-contaminated hands of adults. This test method uses the entire surface of both hands in contrast to only the fingerpads in the procedure described in Test Method **E 1838**. However, the reported results from these two methods are comparable. **(1, 2)**²

1. Scope

1.1 This test method is designed to evaluate handwash or handrub agents for their ability to reduce viral contamination from hands.

NOTE 1—A knowledge of virological techniques is required for this test method.

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

1.3 *This standard may involve hazardous materials, operations and equipment. This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use. The user should consult a reference for laboratory safety recommendations. (3-5)*

2. Referenced Documents

2.1 *ASTM Standards:*³

E 1482 Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations

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

Current edition approved April 1, 2009. Published April 2009. Originally approved in 1999. Last previous edition approved in 1999 as E 2011 – 99, which was withdrawn in August 2008 and reinstated in April 2009.

² The boldface numbers in parentheses refer to a list of references at the end of this test method.

³ 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.

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

2.2 *AOAC Standard:*

AOAC 960.9 Official Methods of Analysis (1990)⁴

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *hygienic (health-care personnel) handwash agents, n*—agents generally used for handwashing by personnel in hospitals, other health-care facilities, day-care centers, nursing homes, and food-handling establishments; should be safe for repeated use, non-irritating, fast-acting, and efficient in eliminating transient microorganisms from intact skin.

3.1.2 *hygienic handrub agents (that is, hand sanitizers), n*—agents generally used for hand hygiene by personnel in hospitals, other health-care facilities, day-care centers, nursing homes, and food-handling establishments that do not require rinsing; should be safe for repeated use, non-irritating, fast-acting, and efficient in eliminating transient microorganisms from intact skin.

3.1.3 *non-medicated soap, n*—a soap or detergent that is mild to the skin and does not contain any germicidal chemicals.

3.1.4 *soil (organic) 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.

3.1.5 *virus-eliminating (killing/removing) agent, n*—any agent that rids hands of viruses by either killing them on the skin or by dislodging them for subsequent wash-off.

⁴ Available from AOAC International, 481 North Frederick Ave., Suite 500, Gaithersburg, Maryland 20877-2417, <http://www.aoac.org>.

3.1.6 *virus inactivating agent, n*—any agent that renders a virus noninfectious.

4. Summary of Test Method

4.1 This test method uses adult subjects who have provided a written informed consent and whose hands have been determined to be free from any apparent damage at the time of their participation in the study. While no fewer than six subjects are recommended for each virus-test substance combination to be evaluated, the number required may vary depending on the intended use of the data and the target regulatory agency.

4.2 All subjects should refrain from using any antimicrobials starting at least one week prior to the experimental contamination of their hands.

4.3 A prepared suspension of the selected test virus is grown and diluted or concentrated to produce a titer with a minimum of 10^8 infective units/mL. The contaminating virus is applied to the hands and the hands are treated with the test formulation according to the manufacturer's directions or with a set test regimen.

4.4 The virus titer recovered after treatment with the test substance is compared to a control. For the control, the test virus is applied to the hands and recovered after the subject has treated the hands with standard hard water (200 ppm as calcium carbonate) or vehicle, or both, instead of the test substance.

4.5 The virus on experimentally-contaminated hands is exposed to the test substance for the length of time that is representative of actual use conditions of the product, for example, from 10 to 20 s for a handsoap and 20 to 30 s for a handrub. The virus to be recovered after exposure to the test substance is assayed in a cell culture system appropriate to the test virus. The virus titer of the stock, test samples, and controls is determined by a suitable infectivity assay. Cytotoxicity of the host cell culture system caused by the test substance or vehicle at the tested concentration is also determined. The virus-test substance mixture is assayed in numerous units of the host system at a dilution just beyond the cytotoxicity range of the formulation tested. At least three replicate determinations are performed on controls (untreated) and test samples (treated) to confirm the extent of virus elimination by the number of lots required by the target regulatory agency. Results are recorded and $\log_{10}$ and/or percent reduction in virus infectivity are calculated.

4.5.1 This test method is designed to be performed by a trained microbiologist or virologist who is responsible for choosing the appropriate host system for the test virus, and applying the techniques necessary for propagation and maintenance for host system and test virus. For a reference text, see Ref (6).

5. Significance and Use

5.1 This test method is designed to evaluate the virus-eliminating activity of hygienic handwash and handrub agents from experimentally-contaminated hands. Such formulations may be further assessed in a clinical trial for their effectiveness in the field. This test method incorporates whole-hand exposure and reflects actual use conditions such as friction during

hand decontamination, and also enables alternate product forms such as alcohol- or non-alcohol-based liquids, gels, and foams to be tested according to label directions. It is meant to extend, if required, the results of testing with Test Method E 1838, which gives precise reductions in virus infectivity on a limited area of the hands. It may also serve as an alternative test method when product form is not amenable to testing by Test Method E 1838.

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

NOTE 2—Application of viruses on the entire surface of both hands entails a greater risk to the subjects than using fingerpads only. Therefore, greater care is needed to ensure that the hands of the participants are free from any apparent damage. Also, virus preparations must be thoroughly screened for, or documented to be free from, extraneous or adventitious pathogens before use in such tests.

6. Equipment and Apparatus

6.1 *Laminar Flow Cabinet*—a Class II biological safety cabinet. The procedures for the proper maintenance and use of such cabinets are given in Ref (3, 4).

6.2 *Incubator*—an incubator at $36 \pm 1^\circ\text{C}$ for growing host cells and for incubating virus-infected cultures. If an open system is used for cell culture, a CO_2 incubator will be required.

6.3 *Positive Displacement Pipette*—a pipette and pipette tips that can accurately dispense 10 to 20 μL volumes.

6.4 *Sterilizer*—any steam sterilizer suitable for processing cell culture media and reagents. The steam supplied to the sterilizer must be free from additives toxic to cell cultures.

6.5 *Filter Sterilization System*—a membrane or cartridge filtration system (0.22 μm pore diameter) is required for sterilization of heat-sensitive media and solutions.

6.6 *Freezers*—a freezer at $-20 \pm 2^\circ\text{C}$ for the storage of fetal bovine serum and other additives for cell culture media. A second freezer at -70°C or lower is required to store viruses.

6.7 *Refrigerator*—a refrigerator at $4 \pm 2^\circ\text{C}$ is necessary for storage of prepared cell culture media and reagents.

6.8 *Timer*—any stopwatch that can be read in minutes and seconds.

6.9 *Magnetic Stirrer and Magnets*—magnetic stirrer and magnets must be large enough to hold a 5-L beaker or Erlenmeyer flask for preparing cell culture media or other solutions.

6.10 *Handwashing Sink*—a sink of sufficient size to permit subjects to wash hands without touching hands to sink surface.

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

6.10.2 Mild, proven non-antimicrobial soap, preferably liquid.

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

6.11 *Liquid Nitrogen Storage for Cells*—an appropriate liquid nitrogen container and liquid nitrogen for cryopreservation of the stocks of cell lines.

6.12 *Inverted Microscope*—an inverted microscope with 10× eye pieces and 5×, 10×, and 40× objectives.

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

6.14 *Cell Culture Flasks*—plastic cell culture flasks of 25 cm² or 75 cm² capacity for culturing cells and for preparing virus pools.

NOTE 3—Each plastic flask for growing cell monolayers can be reused by reseeding with new cell cultures up to 10 times before being discarded.

NOTE 4—Plastic cell culture ware and other related supplies may be purchased from most laboratory supply houses.

6.15 *Plastic and Glass Vials, Medication (Medicant)*—sterile screw-capped vials will be required for storage of samples. See [Note 4](#).

6.16 *Miscellaneous Labware*—automatic pipettes, pipette tips, plastic vials for storing cell and virus stocks, dilution tubes, cluster plates or flasks for virus titration.

6.17 *Sterile Glass Beads*—3.5 mm in diameter.

6.18 *Glass or Plastic Funnel*—27 cm in diameter.

6.19 *Glass or Plastic Beaker*—200 mL capacity.

7. Materials and Reagents

7.1 *Cell Culture Media and Supplements*—Culture media and the types and ratios of supplements will vary depending on the cell line. Eagle's minimal essential medium (EMEN) with 5 to 10 % fetal bovine serum (virus- and mycoplasma-tested) is used for growing a wide variety of cells (see [Note 5](#)). Antibiotics may be required in the medium to suppress bacterial contamination.

7.2 Soil Load:

7.2.1 Bovine serum, at a final concentration of 5 % in the virus inoculum (see [Note 5](#)), if required for the test.

7.2.2 A tripartite soil load, as an alternative to serum, is prepared from the following stock solutions in phosphate buffer (pH 7.2 to 7.4).

7.2.2.1 Add 0.5 g of tryptone or yeast extract to 10 mL of the buffer.

7.2.2.2 Add 0.5 g of bovine serum albumin (BSA) to 10 mL of the buffer.

7.2.2.3 Add 0.04 g of bovine mucin to 10 mL of the buffer.

7.2.2.4 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.

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

NOTE 5—Fetal bovine serum is considered unsuitable for use as a soil load with rotaviruses because of its rotavirus-inhibitory and trypsin-neutralizing activity.

7.3 *Standard Hard Water*—Water prepared according to [AOAC 960.09 E and F \(4\)](#) to a standard hardness of 200 ppm as calcium carbonate is used for dilution of test substance. This is the control solution to determine the baseline level of virus elimination, and to rinse the hands after exposure to the test substance.

7.4 *Number of Lots of Test Substance to be Used*—The number of separate manufactured lots of each test formulation to be tested will depend on the specific requirements of the target regulatory agency.

7.5 *Diluent for Virus Titration*—Earle's balanced salt solution (EBSS) with a pH of 7.2 to 7.4.

7.6 *Eluent for Virus Recovery from Hands*—EBSS containing 1 % peptone and 0.1 % Tween-80 at final concentrations.

8. Test Viruses and Cell Cultures

8.1 See the Appendix for suggested viruses and host cells.

9. Preparation of Virus Stocks and Determination of Infectivity Titer

9.1 Use appropriate host cells to prepare the virus pool. The virus pool should contain ≥10⁸ infective unit/mL.

9.2 Remove growth or maintenance medium and inoculate 0.1 mL of virus (control flasks receive 0.1 mL of EBSS instead) into each flask (75 cm²) with a confluent cell monolayer and allow 60 min for virus adsorption. Place 15 mL of maintenance medium into each inoculated flask and reincubate till about 75 % of each infected cell monolayer shows virus-induced cytopathology and while control monolayers remain free from any apparent degeneration. Freeze (−20°C) and thaw (room temperature) the infected flasks three times to disrupt host cells for virus release. Centrifuge the cell suspension for 20 min at approximately 1000 xg to sediment the cell debris, collect supernatant and store it at −70°C in suitable aliquots.

9.3 A titer of ≥10⁸ infective units/mL is required for the testing and ultra-centrifugation of virus pools may be needed to achieve such levels of infectivity for the contamination of hands.

9.4 Determine the baseline level of viable virus remaining on the hands of the subject after the rubbing and drying of the experimentally-contaminated skin by first washing or rubbing and then eluting the hands with EBSS-peptone. This represents the 'base' against which the performance of *handwash* agents must be compared. In the case of *handrubs*, no post-treatment rinsing and drying will be used and the hands will be eluted immediately after the handrub has dried.

10. Controls

10.1 *Cell Control*—To ensure that the host cells are not contaminated with bacteria, fungi, or any cytopathogenic viruses other than those used in the test, at least two host cell monolayers are left untreated in each test and examined first at the end of the incubation period. Any obvious contamination or degeneration in such monolayers would invalidate the virus titration.

10.2 *Virus Susceptibility Control*—At least one host cell monolayer receives the test virus at a high enough level to produce cytopathology to ensure that they remain susceptible to it. A lack of obvious and typical virus-induced cytopathic effects in such a monolayer would also invalidate the test.

10.3 *Cytotoxicity Control*—This control applies only in tests with handrub agents. Its objective is to determine if residues of the test substance in the neutralized eluates can produce any apparent degeneration (cytotoxicity) of the cell line for measuring virus infectivity.

Place 0.5 mL of EBSS in the cupped hands of the subject and ask him/her to rub them together in a lathering motion for 20 s. Then place in the cupped hands the same volume of the handrub as required for the test. Elute the hands with 20 mL of an eluent containing an appropriate neutralizer (see 10.5). Inoculate host cell monolayers with the eluate, hold them at room temperature for 60 min, and then observe them in an inverted microscope for any signs of cytotoxicity. Absence of any apparent degeneration of the cells indicates freedom from cytotoxicity that could interfere with the scoring for virus infectivity. In case of cytotoxicity, a different neutralizer or alternative approaches (7) to the removal/reduction of cytotoxicity may be needed.

10.4 Control for Interference with Virus Infectivity—This control also applies to handrub agents only. Levels of the test substance which show no obvious cytotoxicity could still reduce or enhance the ability of the challenge virus to infect or replicate in host cells, thus interfering with the estimation of its virucidal activity. An interference control must, therefore, be included to rule out such a possibility.

This control can be run using the same eluate as described in 10.3. First expose the cell monolayers to the eluate or EBSS with or without neutralizer (controls) for 60 min and then challenge them with countable numbers of infective units of the test virus. Complete the rest of the procedure for an infectivity assay and incubate the cultures as needed for the challenge virus. Any significant difference in virus infectivity titer is indicative of the test substance's or the neutralizer's ability to affect the virus susceptibility of the host cells. In such a case, a different neutralizer or alternative approaches to the removal of the residues of the test substance in the samples to be titrated for virus infectivity may be needed.

10.5 Validation of Neutralization:

10.5.1 Handwash Agents—While the virucidal activity of the test substance must be neutralized as soon as possible at the end of the exposure period for an accurate measure of the contact time (Test Method E 1482), this is not readily attainable in testing handwash agents as described here. The treatment of the contaminated hands with such test substances is immediately followed by rinsing of the hands with 500 mL of hard water and then drying with paper towels. These additional steps, which are an integral part of a normal handwash procedure, may incrementally reduce the levels of any test substance remaining on the hands with a corresponding reduction in virucidal activity. Therefore, the eluates may not require any active neutralization before infectivity assays.

10.5.2 Handrub Agents—Add a suitable neutralizer to the eluent (EBSS-peptone) when working with handrubs and validate that it can successfully quench the activity of the active(s) against the test virus(es).

10.5.2.1 Add to 10 mL of the eluent with the neutralizer a countable number of infective units of the test virus and hold the suspension for 10 min at room temp. For control, use the eluent without the neutralizer.

10.5.2.2 Assay the suspensions for infectious virus. The neutralization is considered to be validated if the level of infectious virus recovered from the eluents with and without the neutralizer are similar statistically.

11. Cleaning and Decontamination of Hands Before Experimental Contamination

11.1 Immediately prior to the experimental contamination, instruct each subject to wash his/her hands with a mild, proven non-antimicrobial soap for 1 min under running tap water and then dry them thoroughly with paper towels.

11.2 Place approximately 5 mL of 70 % (v/v) ethanol in the cupped hands and instruct subject to rub it over the entire surface of both hands till the hands are thoroughly dry.

12. Procedures for Viral Contamination of Hands and Application of Test Substance/Control Fluids

12.1 Method 1 for Handwash Agents:

12.1.1 Viral Contamination—Immediately after washing hands as directed in Section 11, place 0.5 mL of the test virus suspension onto the palm of the cupped left hand of the subject and distribute the inoculum with washing movements over the entire surface of both hands (not on wrists). Spread suspension for 90 s and allow to dry another 30 s.

12.1.2 Treat hands with the test substance as follows: Moisten the hands with about 10 mL of sterile hard water and then place the amount/volume of the test substance, as specified by the manufacturer or in the set test regimen, into the subject's cupped hands and spread with lathering movements for 20 s in a fashion similar to that described in Ref (8, 9). For a control, use an equivalent volume of EBSS instead.

12.1.3 Gradually pour over both hands of the subject 500 mL of sterile hard water for at least 15 s while the subject rubs hands together to simulate rinsing movements. Such rinsing should be with the hands held over a bowl containing at least 500 mL of a 1:10 dilution of domestic bleach (~5 % sodium hypochlorite) to capture and inactivate the virus. Instruct the subject to dry hands thoroughly with two pieces of paper towel for another 15 s.

12.1.4 Recovery of Virus after Treatment with the Test Agent—Place a sterile funnel (27 cm diameter) in a 200-mL sterile beaker. Instruct the subject to place hands over the funnel and gradually pour 20 mL of EBSS-peptone over them while he/she rubs hands together to elute any remaining virus. No less than 2 mL (~10 %) of the eluate shall be assayed for virus infectivity.

12.1.5 At the end of the elution process, each subject shall decontaminate the hands and dry them thoroughly before leaving the test area.

12.1.6 Place 5 mL of a 1:10 water dilution of domestic bleach (~5 % sodium hypochlorite) into the cupped hands of the subject and require him/her to rub the bleach thoroughly over the surface of both hands for about 2 min. This is to be followed by a thorough washing of hands with plain soap and drying with paper towel.

12.2 Method 2 for Handwash Agents:

12.2.1 In this alternative procedure, only the eight fingerpads are contaminated and eluted after treatment allowing the use of a smaller volume of eluent.

12.2.2 Viral Contamination of the Hands—Instruct the subject to wash and decontaminate hands as described in Section 11. Face the palms upward and with fingers and thumbs

outstretched, apply 20 μ L of virus suspension on each fingerpad. The subject will then rub each fingerpad against the thumbpad of the same hand over a total period of 40 ± 5 s. Allow the inoculum to dry for 90 ± 5 s before exposure to the test substance or control fluid.

12.2.3 Perform application of test substance or control fluid in the same manner as described in 12.1.2.

12.2.4 After the hands are dry, place one finger at a time along with the thumb into a sterile vial containing 10 glass beads in 10 mL of EBSS-peptone. While holding several of the beads between the digits, rub the entire surface of the fingerpad against the thumbpad vigorously for 20 s. Drain as much of the eluent as possible back into the vial. Repeat the procedure with the remaining fingerpads. Assay at least 2 mL (~20 %) of the eluate for infectious virus.

12.2.5 Instruct the subject to decontaminate hands (12.1.5) before leaving the test area.

12.3 Method for Handrubs:

12.3.1 Contaminate the subject's hands using one of the two methods described in 12.1.1 or 12.2.2.

12.3.2 Do not pre-moisten the hands.

12.3.3 Place the volume of the test substance, as specified by the manufacturer or in the set test regimen, into the subject's cupped hands and instruct the subject to rub the test substance over the entire surface of both hands for 20 s. For a control, use an equivalent volume of EBSS instead.

12.3.4 Do not wash hands in water or dry them with paper towel.

12.3.5 Elute any remaining virus from the hands in accordance with the whole-hand (12.1.4) or fingerpad (12.2.4) contamination procedure described.

12.3.6 Decontaminate the subject's hands as described in 12.1.6.

12.4 *Assaying for Infectious Virus* —Assay the eluates and controls of infectious virus in monolayers of susceptible host cells using a plaque assay or a method based on 50 % cell culture infective dose (TCID₅₀).

13. Calculation of Reduction in Virus Infectivity

13.1 For handwash agents, the log₁₀ reduction on the hands after the application of the test substance and post-treatment water-rinse is compared to that recovered after the application of hard water only. In the case of handrub agents, the comparison should be between the hands treated with test substance and the EBSS control.

13.2 At this time there are no criteria for the log₁₀ reduction required for a claim for effectiveness.

14. Keywords

14.1 antiseptics; adenovirus; calicivirus; handrubbing; handwashing; skin sampling; rhinovirus; rotavirus; viral infection; virucidal activity; virus-eliminating activity

APPENDIX

(Nonmandatory Information)

X1. VIRUSES AND THEIR HOST CELLS RECOMMENDED FOR USE IN THIS TEST PROTOCOL

X1.1 The selection of the following test viruses 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) ability to produce cytopathic effects or plaques, or both, in cell cultures, (d) potential to spread through contaminated hands, and (e) relative resistance to agents used in hygienic handwash and handrub agents. Other strains or types of viruses may be substituted provided they meet the above criteria.

NOTE X1.1—There is insufficient information on whether the passage history, culture conditions, and strain differences of viruses can influence the efficiency of their elimination by hygienic handwash agents. Therefore, caution must be exercised when substituting viruses as this may lead to variations in results from one laboratory to another.

X1.2 Human Rotavirus Wa (ATCC VR-201 8) with CV-1 (ATCC CCL-70) or MA-104 (ATCC CRL-2378) cells as hosts.

X1.3 Prior to rotavirus inoculation, cell monolayers must

be washed at least three times with EBSS to remove the serum from the growth medium. All diluents, maintenance media and agar overlays must also be free from serum. Most rotaviruses also require the presence of trypsin in the medium for growth and plaques formation or development of cytopathic effects.

X1.4 Human Rhinovirus Type 37 (ATCC VR-I 147) or 14 (ATCC VR-284) and MRC-5 (ATCC CCL-171) or WI-38 (ATCC CCL-75) cells for culture and infectivity assays.

X1.5 Feline calicivirus (ATCC VR-782); the cell line recommended is CrFK (ATCC CCL-94). This virus is currently widely accepted as a surrogate for human norovirus.

X1.6 Human Adenovirus Type 5 (ATCC VR-1516) and cell line 293 (ATCC CRL-1573) for making virus pools and Vero cells (ATCC CCL-81) for infectivity titrations.



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