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Standard Test Method for Evaluation of Handwashing Formulations for Virus-Eliminating Activity Using the Entire Hand[1]

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

Physical removal, inactivation *in situ*, elimination and a combination of these actions to reduce the spread of viral infections with effective handwashing has become an achievable goal. Artificial contamination of hands with selected test viruses provides a usable model. This guide is closely related to another ASTM test of handwash agents which restricts contamination and sampling to a smaller area of the hand, Test Method E 1836, Determining the Virus Eliminating Effectiveness of Liquid Hygienic Handwash Agents Using the Fingerpads of Adult Volunteers. This test method tests a larger area of the hands than the fingerpad method; however, reported results are comparable. **(1)**[2]

1. Scope

1.1 This test method is designed to evaluate antimicrobial agents in formulations for utility and effectiveness for virus-eliminating activity using human subjects.[3]

1.2 *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.* **(2, 3)**

2. Referenced Documents

2.1 *ASTM Standards:*

2.1.1 Use the most current editions of the standards referenced herein.

E 1052 Test Method for Efficacy of Virucidal Agents Against Viruses in Suspension[4]

E 1053 Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces[4]

E 1482 Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations[4]

E 1836 Test Method for Determining the Virus - Eliminating Effectiveness of Liquid Hygienic Handwash Agents Using the Fingerpads of Adult Volunteers[4]

2.2 *AOAC Standard:*[5]

AOAC 960.9

3. Summary of Test Method

3.1 This test method is conducted on subjects selected from a group of adult volunteers who have provided a written informed consent and whose hands have been determined to be free from any apparent damage. All subjects should have refrained from the use of any antimicrobials for at least one week prior to initiation of the test and be supplied with selected products free from antimicrobials for use during this week. At least 12 to 15 subjects are selected from this group for the test. The number of required subjects may vary with the virus tested.

3.2 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 washed with the test formulation according to the manufacturer's directions or with a set test regimen.

3.3 The virus titer recovered after washing with the test product is compared to a control titer of virus. For the control, a titer of virus is applied to the hands and recovered from subjects washing with standard hard water (200 ppm as calcium carbonate) or vehicle, or both, instead of the formulation.

3.4 The virus is exposed to the virucide 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. The virus to be recovered after exposure to the test germicide is assayed in a cell culture system appropriate to the test virus. The virus

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides 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 test method.

[3] A knowledge of virological techniques is required for this test method.

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

[5] Available from Association of Organic and Analytical Chemists International, Gaithersburg, MD.

titer of the stock, test samples and controls is determined by the a suitable infectivity assay. Cytotoxicity of the host cell culture system caused by the test germicide at the tested concentration is also determined. The virus-germicide mixture is assayed in numerous units of the host system at a dilution just beyond the cytotoxicity range of the germicide. At least three replicate determinations are performed on controls (untreated) and test samples (treated) to confirm virus elimination by a sample of the test germicide. Results are recorded as the median value of $\log_{10}$ - reduction in virus infectivity.

3.4.1 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 and test virus. For a reference text, see Ref. **(4)**.

4. Significance and Use

4.1 This test method should be used to evaluate the virus-eliminating effectiveness of these formulations after handwashing. Effective formulations can be further evaluated in a clinical trial on human subjects. Published data have shown **(1)** that results of in vitro tests do not accurately reflect what occurs when this class of products is used in the health care facility. This test method involves the incorporation of whole hand exposure and friction from washing, reflecting actual use conditions in human subjects. It is meant to confirm the results of testing with Test Method E 1836. This method gives precise reductions on a limited area of the finger, the fingerpads.

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

5. Equipment and Apparatus

5.1 *Laminar Flow Cabinet*—A Class II biological safety cabinet is required for virus work. The procedures for the proper maintenance and use of such cabinets are given in Ref. **(5)**.

5.2 *Incubator*—An incubator at 36 ± 1°C is needed 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. Work with rhinoviruses will require an incubator at 33 ± 1°C.

5.3 *Positive Displacement Pipette*—A pipette and pipette tips that can accurately dispense 10 to 20 µL volumes is required.

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

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

5.6 *Freezers*—A freezer at –20 ± 2°C is required 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.

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

5.8 *Timer*—Any stop watch that can be read in minutes and seconds is acceptable.

5.9 *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.

5.10 *Handwashing Sink*—A sink of sufficient size to permit subjects to wash hands without touching hands to sink surface is required.

5.10.1 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.

5.10.2 Bland, proven non-antimicrobial soap, preferably liquid, is needed.

5.10.3 Tap water temperature regulator and temperature monitor to monitor and regulate water temperature at 40 ± 2°C is needed.

5.11 *Liquid Nitrogen Storage for Cells*—An appropriate liquid nitrogen container and liquid nitrogen for cryopreservation of the stocks of cell lines are needed.

5.12 *Inverted Microscope*—An inverted microscope with 10X eye pieces and 5X, 10X and 40X objectives.

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

5.14 *Cell Culture Flasks*[6] —Plastic cell culture flasks of 25 cm^2 or 75 cm^2 capacity for culturing cells and for preparing virus pools are needed.

NOTE 1—Each flask for growing cell monolayers can be reused by reseeding with new cell cultures 10 or more times before being discarded.

5.15 *Plastic and Glass Vials, Medication (Medicant)*—Sterile screw-capped vials will be required or post test sampling of hands.

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

5.17 *Sterile Glass beads*—Beads that are 3.5 mm in diameter are needed.

6. Materials and Reagents

6.1 *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 2). Antibiotics may be required in the medium to suppress bacterial growth.

6.2 *Organic Load*:

6.2.1 Fetal bovine serum, at a final concentration of 5 % in he virus inoculum (see Note 2), if required for the test.

6.2.2 Peptone, a pancreatic digest of casein as an alternative to serum, is made by dissolving 7.6 g of tryptone powder in 1L physiological saline (0.85 % NaCl). Sterilize by autoclaving or membrane filtration. This peptone solution should contain approximately 2.0 g of total protein/L, which is approximately

[6] Plastic cell culture ware and other related supplies may be purchased from most laboratory supply houses.

equivalent to the protein content of a 5 % solution of fetal bovine serum.

NOTE 2—Fetal bovine serum is considered unsuitable for use as an organic load when working with rotaviruses because of its rotavirus-inhibitory and trypsin-neutralizing activity.

6.3 *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 products. This is the control solution to determine the baseline level of virus elimination, and to rinse the fingerpads after exposure to the test product.

6.4 *Test Products*—Duplicate samples of the product shall be tested.

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

6.6 *Eluent for Virus Recovery from Hands and Fingers*—Use EBSS containing peptone.

7. Test Viruses and Cell Cultures

7.1 The selection of the following test viruses 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*) property 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 handwashing. Other strains or types of viruses may be substituted provided they meet the above criteria.

NOTE 3—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.

NOTE 4—Poliovirus is scheduled for eradication. The goal is to achieve a world free from polio. At that time, laboratory work with the poliovirus will not be possible.

7.2 Human Rotavirus Wa (ATCC VR-201 8) of the cell line CV-1 (ATCC CCL-70) is recommended.

7.2.1 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.

7.3 Human Rhinovirus Type 37 (ATCC VR-I 147) or Rhinovirus 14 (ATCC VR-284) of the cell line MRC-5 (ATCC CCL-171) or WI-38 (ATCC CCL-75) is recommended.

8. Virus Stock Titer Determinations

8.1 Utilize the appropriate host to prepare the virus pool. The host system employed for the virus pool should be the same system to be used for virus recovery following virus challenge of the test germicide. The virus pool should contain not less than 10^8 infective unit/mL.

8.2 *Cell Culture Technique*—1.0 mL of virus is allowed to adsorb on the cell sheet of a 0.75 cm^2 flask for about 1 h at an appropriate incubation temperature after which 25 mL of maintenance media is added. The flask is then re-incubated until about 75 % of the cell monolayer shows virus cytopathology. The flask is frozen and thawed three times in a dry ice-alcohol bath and the disrupted cells centrifuged 20 min at about 1000 xg. The supernatant is collected and divided into appropriate volumes for test and may be used fresh or stored at –70°C.

8.3 Virus titer of the stock is the titer of the test virus after growth and dilution or concentration. A titer of 10^8 infective units/1 mL is required for the test. The control virus titer is determined on subjects using hard water (200 ppm as calcium carbonate) or EBSS (with or without peptone) or the vehicle of the test product for handwashing of virus-contaminated hands. The initial titer of the virus is the virus recovered by sampling the inoculated hands after washing and recovery with EBSS solution.

8.4 The initial or control titers, or both, can be determined using the virus application techniques and recovery samples after washing with buffer, hard water or the vehicle of the test product.

9. Cytotoxicity Testing Antimicrobial

9.1 Prior to studying the effects of the test product on viruses, determine its cytotoxic effect on the host system. Use serial 2-fold dilutions and inoculate a minimum of 4 units/dilution.

9.2 A technique recommended for reduction of cytotoxic effects is based on gel filtration (see Test Method E 1482 and **(5)**). The eluates from virus-contaminated hands are passed through separate columns of Sephadex LH-20 gel[7] to remove residues of the antimicrobial which may be cytotoxic. The filtrates are centrifuged at 10 000 xg to remove bacteria before virus infectivity assay.

10. Conditioning

10.1 Prior to testing the virus-eliminating activity of the test product or agent, wash the subject's hands with a mild, proven non-antimicrobial soap for 1 min under running water to remove transient microflora. Rinse thoroughly and dry with two paper towels.

11. Procedures for Application of the Test Virus to the Hands and Washing Techniques

11.1 *Method 1*:

11.1.1 *Virus Innoculation of the Hands*—Immediately after washing as directed in 10.1, apply 0.02 mL of virus stock to each fingerpad and spread with the pipette tip. 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.

11.1.2 Wash the virus-contaminated hands with the test agent as follows: Moisten the hands with about 10 mL of sterile distilled water and then carefully pour 3 to 5 mL or the amount indicated in the test product labeling into the subjects' cupped

[7] The sole source of supply of the apparatus known to the committee at this time is Pharmacia, Uppsala, Sweden. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

hands and spread with washing movements. Rub with handwashing movements for time period in directions or for 30 s in the controlled fashion described in Ref. **(6)** as follows:

> "The preparation was applied in a procedure consisting of five strokes backwards and forwards: palm to palm, right palm over left dorsum, left palm over right dorsum, palm to palm with fingers interlaced, backs of fingers to opposing palm with fingers interlocked, rotational rubbing of right thumb clasped in left palm and left thumb clasped in right palm, rotational rubbing backwards and forwards with clasped fingers of right hand in palm of left hand and clasped fingers of left hand in palm of right hand: hands and wrists are rubbed until the end of the 30 sec. period, then rinsed under a running tap for 15 sec."

Rinsing should be thorough with either procedure (15 s) followed by drying and by patting with two paper towels for 15 s.

11.1.3 *Recovery of Virus after Treatment with the Test Agent*—Use 20 mL of standard hard water in a small bowl containing sterile glass beads (3.5 mm). To recover virus from the fingertips and palms, place the fingers and thumbs in the bowl and press, rub, and move the hands around in the solution for 1 min. The eluate from the hands is treated as described previously and the appropriate assay is performed for the test virus. Only a small fraction of the eluate can be titrated.

11.2 *Method 2*:

11.2.1 This alternative procedure which uses innoculated fingertips, can be used to conserve high titer inocula. Use of a vial with glass beads to sample following exposure to the test product reduces the sampling volume and permits recovery of lower titer levels of virus.

11.2.2 *Virus Inoculation of the Hands*—Wash the hands briefly before application of the virus as described in 10.1. Face the palms upward and with fingers outstretched, apply 20 µL of virus suspension to each fingertip and thumb. Spread the virus over each fingertip by rubbing together opposing fingertips and thumbs for 40 ± 5 s. Dry the inoculum for 90 ± 5 s before testing.

11.2.3 Perform application of test product in the same manner described in 11.1.2 followed by the washing with the test product, rinsing, and drying after application.

11.2.4 *Recovery of Virus after Treatment with the Test Agent*:

11.2.4.1 After the hands are dry, place one finger at a time and the thumb into a glass medicine vial (medicant tube) containing glass beads (3.5 mm) and 2 to 3 mL of standard hard water and rub vigorously up and down for 1 min. Agitate the eluate in turn **(7)**. Repeat the procedure on the other hand using a fresh vial. Assay the eluate from the hands for infectious virus. After the titration, incubate the inoculated cultures at the appropriate temperature for the required time period and record the results. About 25 % of the eluate should be titrated.

11.2.4.2 At the end of the elution process, each subject should disinfect the hands with 75 % ethanol by rubbing thoroughly using two applications of 5 mL each for 30 s.

11.3 It should be stressed that after completion of the test, all test specimens and test equipment shall be autoclaved according to the autoclave manufacturer's instructions to ensure destruction of the viruses and to prevent accidental contamination of the laboratory and environment.

12. Calculation of Effectiveness

12.1 The log reduction on each hand can be compared to either the virus recovered when washed with product and water, buffer (EBSS) or vehicle, or with the virus recovered in the eluate after application of the virus stock. Since, at this time, there are no criteria concerning the log reduction required for a claim of effectiveness, a judgement of effectiveness needs to be developed. However, when dealing with the normal flora of the skin, historically a one- to two-log reduction has been considered effective. It appears from the literature that the non-enveloped viruses are more difficult to inactivate or remove, or both, than the enveloped ones.

13. Precision and Bias

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

14. Keywords

14.1 antisepsis; handwashing; poliovirus; skin sampling; rhinovirus; rotavirus; viral infection; virucidal activity; virus-eliminating activity

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