



Standard Test Method for Evaluation of Effectiveness of Decontamination Procedures for Air-Permeable Materials when Challenged with Biological Aerosols Containing Human Pathogenic Viruses¹

This standard is issued under the fixed designation E2720; 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

Many communicable diseases are often spread through the aerosol route of exposure. The droplet nuclei formed in these aerosols may infect susceptible individuals directly or contaminate environmental surfaces and render them fomites for further spread of disease. The characteristics of the droplet nuclei (particle size and composition) will influence the viability of microorganisms when exposed to environmental stresses but may also shield them from physical and chemical decontaminants. The wide variations in the types and levels of such protective/shielding ingredients can have impact on the effectiveness of surface decontaminants. This test method is designed to simulate the deposition of droplet nuclei from human respiratory secretions onto and into air-permeable membranes. It is primarily focused on influenza viruses but other respiratory viruses or surrogate viruses could be used. Protocols for assessing the microbicidal activity of disinfectants are also described.

1. Scope

1.1 This test method is designed to evaluate decontamination methods (physical, chemical, self-decontaminating materials) when used on air-permeable materials contaminated with virus-containing droplet nuclei.

1.2 This test method defines the conditions for simulating respiratory droplet nuclei produced by humans.

1.3 The method is specific to influenza viruses but could be adapted for work with other types of respiratory viruses or surrogates ([Appendix X6](#)).

1.4 This test method is suitable only for air-permeable materials.

1.5 This test method does not address the performance of decontaminants against microbes expelled via blood splatter, vomit, or fecal contamination.

1.6 This test method should be performed only by those trained in bioaerosols, microbiology, or virology, or combinations thereof.

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

1.8 This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

2. Referenced Documents

2.1 ASTM Standards:²

[E1052](#) Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension

[E2197](#) Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides

[E2721](#) Test Method for Evaluation of Effectiveness of Decontamination Procedures for Surfaces When Challenged with Droplets Containing Human Pathogenic Viruses

2.2 IEST Standards:

[IEST-RP-CC003.3](#) Garment System Considerations for Clean Rooms and Other Controlled Environments³

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.

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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 Available from Institute of Environmental Sciences and Technology (IEST), Arlington Place One, 2340 S. Arlington Heights Rd., Suite 100, Arlington Heights, IL 60005-4516, <http://www.iest.org>.

2.3 Department of Defense Standards:

CA06PRO411 Method for Evaluating Air Purification Technologies for Collective Protections Using Viable Microbial Aerosols⁴

2.4 EPA Standards:

EPA 600/4-84/013 (N16) USEPA Manual of Methods for Virology⁵

2.5 WHO Standards:

WHO Manual on Animal Influenza Diagnosis and Surveillance⁶

3. Terminology

3.1 Definitions:

3.1.1 *aerosol, n*—a suspension of solid or liquid particles in a gas medium.

3.1.2 *air-permeable material, n*—no standard definition is available; for the purpose of this test method, air-permeable material is described as any membrane that has a pressure drop $\leq$ twice that of high efficiency particulate air (HEPA) media in the same test environment.

3.1.3 *biological aerosol, n*—aerosol comprising particles of biological origin or activity which may affect living things through infectivity, allergenicity, toxicity, or pharmacological and other processes.

3.1.4 *influenza, n*—an infectious disease of birds and mammals caused by RNA viruses of the family *Orthomyxoviridae*.

3.1.5 *protective factor, n*—soluble or insoluble material co-deposited with microorganisms that directly protects the microorganism from environmental stresses or decontaminants.

3.1.6 *respiratory droplet nuclei, n*—evaporatively condensed, pathogen-containing particles of respiratory secretions expelled into the air by coughing, sneezing, or talking, which can remain airborne for long periods of time.

3.1.7 *self-sanitizing material, n*—a substrate containing an antimicrobial agent that collectively acts as a germicide.

4. Summary of Test Method

4.1 The test method describes the steps required to deposit droplet nuclei onto air-permeable membranes and quantitatively assess decontamination efficiency.

4.1.1 Using an aerosol device capable of meeting the data quality objectives set for in this test method, influenza virus or

surrogates are aerosolized to form droplet nuclei that are subsequently applied to air-permeable materials.

4.1.2 The virus-contaminated carriers are subjected to disinfection protocols and incubated for the specified time and conditions. Control samples are incubated under identical conditions but are not exposed to the disinfection protocols.

NOTE 1—Carriers with incorporated microbicides do not receive any additional disinfection treatment. An untreated control is needed to assess antimicrobial efficacy.

4.1.3 Virus particles are eluted from the test and control carriers and viability is assessed by tissue culture 50 % infectious dose assay ($\log_{10}TCID_{50}$).

NOTE 2—Nonviable quantification techniques for viral enumeration such as polymerase chain reaction (PCR) or hemagglutination cannot be used.

4.1.4 The virucidal activity of the decontamination procedure is determined from the log difference in viability between treated and control carriers.

5. Significance and Use

5.1 The efficacy of disinfection technologies can be evaluated on finished products, as well as on developmental items.

5.2 This test method defines procedures for validation of the aerosol generator, preparation of the test specimen, application of the challenge virus, enumeration of viable viruses, assessing data quality, and calculation of decontamination efficacy.

5.3 This test method provides defined procedures for creating droplet nuclei that approximate those produced by human respiratory secretions with particular emphasis on particle size distribution and aerosolization media.

5.4 Safety concerns associated with aerosolizing microbial agents are not addressed as part of this test method. Individual users should consult with their local safety authority, and a detailed biological aerosol safety plan and risk assessment should be conducted prior to using this method. Users are encouraged to consult the manual *Biosafety in Microbiological and Biomedical Laboratories*⁷ published by the U.S. Centers for Disease Control and Prevention (CDC).

5.5 This test method differs from Test Methods **E1052** and **E2197** in the presentation of the virus to surface. The aforementioned test methods use liquid inoculum to contaminate carrier surfaces, whereas this test method presents the virus in the absence of water as droplet nuclei.

5.6 This test method differs from Test Method **E2721** because (1) smaller particles are being formed, (2) the droplets will be dried, thus forming droplet nuclei, prior to application to air-permeable materials, and (3) unique equipment is required to create the droplet nuclei.

⁴ Foarde, K., Heimbuch, B. K., Maxwell, A., VanOsdell, D., "Method for Evaluating Air Purification Technologies for Collective Protection Using Viable Microbial Aerosols," Test Operating Procedure (TOP) Under the Army Test and Evaluation Command (ATEC), Edgewood Chemical and Biological Center, Edgewood, Md., 2010 in press.

⁵ Available from United States Environmental Protection Agency (EPA), Ariel Rios Bldg., 1200 Pennsylvania Ave., NW, Washington, DC 20460, <http://www.epa.gov>.

⁶ Webster, R., Cox, N., Stohr, K. WHO Manual on Animal Influenza Diagnosis and Surveillance. World Health Organization, Department of Communicable Disease Surveillance and Response. WHO/CDS/CDR/2002.5 Rev. 1.

⁷ CDC-NIH, *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, U.S. Department of Health and Human Services, Washington, D.C., 2009.

6. Apparatus

6.1 *Biological Aerosol Generators*—The apparatus to load microorganisms onto a substrate is composed of several commercially available components and can be readily constructed (see [IEST-RP-CC003.3](#)).^{4,8,9} The overall design of the apparatus can take various forms and can be fashioned in different dimensions while meeting the validation requirements and data quality objectives listed below. [Appendix X1](#) and [Appendix X2](#) contain the description of a prototypical device that can be used to load droplet nuclei onto surfaces. However, it is the responsibility of the user of this standard to validate the performance of the device prior to use.

6.1.1 Validation requirements and baseline testing.

6.1.1.1 *Environmental Conditions*—Generator must be capable of delivering air with a relative humidity of $70 \pm 10\%$.

6.1.1.2 *Leak Test*—The device must maintain a positive pressure of ~ 50 cm of water for at least 10 min.

6.1.1.3 *Flow Rate Consistency*—All ports containing specimen holders must maintain a constant flow with a coefficient of variation (CV) $\leq 10\%$ over the duration of the sampling period.

6.1.1.4 Loading uniformity across the diameter of the test specimen is required to ensure the even distribution of the droplet nuclei over the surface of the carrier. A standard deviation of $\pm 0.5 \log_{10} \text{TCID}_{50}$ is desired.

6.1.1.5 *Sample-to-Sample Variation*—The variability of virus loading for multiple samples loaded for a single test must have a standard deviation of $\pm 0.5 \log_{10} \text{TCID}_{50}$.

6.1.1.6 *Droplet Nuclei Characteristics*—The droplet nuclei generated for this method will have a count median diameter (CMD) of $\sim 0.8 \mu\text{m}$. The virus will be aerosolized in a saliva substitute ([Table 1](#)) that will add the appropriate “protective factors.” This test method would be suitable for simulating other fluids of interest; however, if a different fluid is used, the

formulation and recipe listing the protective factors and particle size must be reported.

6.2 *Other Equipment*—The list is specific for influenza virus. Other equipment may be needed if a different virus is used.

6.2.1 *Autoclave*, capable of maintaining 121 to 123°C and [15 to 17 lbs per in.²—gauge (psig)].

6.2.2 *CO₂ Incubator*, capable of maintaining 35 to 37°C and $5 \pm 0.5\%$ CO₂.

6.2.3 *Vortex Mixer*.

6.2.4 *Analytical Balance*, capable of weighing 0.001 g.

6.2.5 *Refrigerator*, capable of maintaining 2 to 8°C.

6.2.6 *Stopwatch or Electronic Timer*.

6.2.7 *Pipettor*, with a precision of 0.001 mL.

7. Reagents and Materials

7.1 *Reagents*—The list is specific for influenza use. Other reagents may be needed if a different virus is used.

7.1.1 *Influenzavirus (H1N1; A/PR/8/34)—cell culture adapted*, ATCC VR-146.

7.1.1.1 The [WHO Manual](#) on Animal Influenza Diagnosis and Surveillance contains specific procedures for preparing influenza virus and titrating samples. [Appendix X3](#) also has specific information on titrating viable influenza viruses. Other viruses may be used, but conditions for propagation and enumeration are not provided in this method.

7.1.2 *Madin–Darby Canine Kidney (MDCK) Cell Line*, ATCC CRL-34.

7.1.3 *Artificial Saliva*, see [Table 1](#).

7.1.4 *Minimal Essential Medium With Earle’s Balanced Salts (EMEM)*.

7.1.5 *Heat-Inactivated Fetal Bovine Serum (45 min at 56°C)*.

7.1.6 *Penicillin/Streptomycin*, 10 000 units penicillin and 10 mg streptomycin per mL.

7.1.7 *L-Glutamine*, 200 mM in 0.85 % NaCl.

7.1.8 *Crystal Violet*.

7.1.9 *Glutaraldehyde*.

7.1.10 *TPCK–Trypsin*.

7.1.11 *Phosphate Buffered Saline (PBS)*.

7.1.12 *Bovine Serum Albumin*.

7.1.13 *Trypsin–EDTA Solution*, 0.05 % trypsin, 0.53 mM EDTA in Hanks balanced salts solution without sodium bicarbonate, calcium, and magnesium.

7.1.14 *Distilled Water and Purified Water*.

7.1.15 *Ethanol*, laboratory grade.

7.1.16 *Bleach*.

7.2 *Materials*—The list is specific for influenza use. Other reagents may be needed if a different virus is used.

7.2.1 *Tissue Culture Treated Flasks*—T-25, T-75, T-175, 24-well plate.

7.2.2 *Pipettes*, 1, 5, 10, and 25 mL.

7.2.3 *Test Tube Rack*.

7.2.4 *Micropipettes*, capable of delivering 0.001 mL accurately and consistently.

7.2.5 *1.7-mL Sterile Microcentrifuge Tubes*.

7.2.6 *15-mL Sterile Centrifuge Tubes*.

7.2.7 *50-mL Sterile Centrifuge Tubes*.

7.2.8 *Air-Permeable Test Materials*.

TABLE 1 Artificial Saliva

Reagent	Amount
MgCl ₂ · 7 H ₂ O	0.04 g
CaCl ₂ · H ₂ O	0.13 g
NaHCO ₃	0.42 g
0.2 M KH ₂ PO ₄	7.70 mL
0.2 M K ₂ HPO ₄	12.3 mL
NH ₄ Cl	0.11 g
KSCN	0.19 g
(NH ₂) ₂ CO	0.12 g
NaCl	0.88 g
KCl	1.04 g
Mucin	3.00 g
Distilled water	1000 mL
pH	7

⁸ Heimbuch B. K., Wallace, W. H., Kinney, K., Lumley, A. E., Wu, C-Y, Woo, M-H, Wander, J. D., “A Pandemic Influenza Preparedness Study: Use of Energetic Methods to Decontaminate Filtering Facepiece Respirators Contaminated with H1N1 Aerosols and Droplets,” *American Journal of Infection Control*, 2010, DOI 10.1016/j.ajic.2010.07.004.

⁹ Fisher E, Rengasamy S, Viscusi DJ, Vo E, Shaffer R., Development of a test system to apply virus-containing particles to filtering facepiece respirators for the evaluation of decontamination procedures, *Appl Environ Microbiol*, Vol 75, No. 6, 2009, pp. 1500–1507.

8. Sampling, Test Specimens, and Test Units

8.1 Cut test specimens from finished products or from specimens that can be documented as representative of finished products. The configuration of the particular aerosol device dictates the size and type of each specimen. Seal specimens into the sample holder in the proper orientation. In some cases the complete finished product may be used, which obviates the need for cutting “coupons.” An airtight seal is required to prevent leakage of the aerosol around the sample.

9. Experimental Design

9.1 A minimum of three independent test and control samples must be evaluated so that fundamental statistical analysis of the data can be performed.

10. Test Procedure

10.1 *Apparatus Operation*—Appendix X1 and Appendix X2 describe devices for loading droplet nuclei and detail the standard protocols for operation of the devices. General information that is independent to the aerosol devices is listed below.

10.2 *Perform Neutralizer Effectiveness Test*—The objective of this test is to determine whether toxic effects from the chemical or physical decontamination method have been neutralized by the extraction buffer prior to virus enumeration. Treat a test specimen not exposed to virus with the decontamination procedure following the experimental protocol. Following the completion of the decontamination procedure, place test specimen in 10 mL of the extraction buffer and perform the extraction procedure following the experimental protocol. Remove and discard the test specimen, then split the sample into two equal volumes. Set aside sample A, as it will be used to determine toxicity to the MDCK host cells. Add 10 µL of a virus suspension of known titer (for example, 10^5 TCID₅₀ per mL) to sample B and incubate at room temperature (18 to 24°C) for a minimum of 1 h. Serially dilute sample B (1/10) into serum-free EMEM and determine titer using TCID₅₀ assay. Compare the number of viable viruses recovered from the test specimen extraction buffer to the number recovered from the fresh buffer solution to determine toxicity. Inoculate sample A onto MDCK cells and incubate for 96 ± 4 h at $37^\circ\text{C}/5 \pm 0.5\%$ CO₂. The cells must remain healthy and viable to pass the test.

10.3 *Load Samples With the Droplet Nuclei*—The desired loading should be high enough that no less than $3 \log_{10}$ TCID₅₀/cm² is recovered from the test samples. This value is achieved by altering concentration of the virus in the nebulizer and by adjusting loading times. Appendix X1 and Appendix X2 report the specific test conditions to obtain the required level of loading. If a different test rig is used the values will have to be determined empirically. In general, loading is carried out by diluting the stock of viruses in artificial saliva buffer, which is subsequently added to the nebulizer. After priming, the test articles are exposed to the droplet nuclei for the required amount of time.

10.4 *Decontamination*—Remove samples from the aerosol generator and expose a subset (at least three) to the decontamination method: either a physical or chemical method. Incubate

the samples (treated and control replicates) for the specified amount of time at the required environmental conditions (temperature and humidity). A control set (at least three) is not treated with the decontamination method, but is incubated at the identical conditions (time, humidity, and temperature) as the decontaminated samples.

10.5 Virus Extraction:

10.5.1 *Coupon*—Place the coupons in a 50-mL sterile centrifuge tube containing 10 mL of serum-free EMEM (Sample size may vary depending on the test article being used. An extraction buffer-to-sample ratio of 1.0 mL per cm² should be used). Extract the samples for 20 ± 1 min using a vortex mixer.

10.5.2 *“Large Items”*—Cut representative samples (for example, 38-mm diameter circles) from the device and extract as described in 10.5.1. A minimum of 25 % of the test article should be sampled.

10.6 Determine the presence of viable virus by performing a TCID₅₀ assay on each sample.

11. Calculation or Interpretation of Results

11.1 *Virus Quantification*—The Spearman–Kärber formula¹⁰ is used to determine the virus titer of each sample (Appendix X4 and Appendix X5 contain sample calculations).

$$L = \log_{10} \text{TCID}_{50} \text{ titer} = X_0 - \left(\frac{d}{2} \right) + d \times \frac{r_i}{n_i} \quad (1)$$

where:

- X_0 = $\log_{10}$ of the reciprocal of the lowest dilution at which all test inocula are positive,
- d = $\log_{10}$ of the dilution factor (that is, the difference between the log dilution intervals),
- n_i = number of test inocula used at each individual dilution,
- r_i = number of positive test inocula (out of n_i), and
- $\sum \left(\frac{r_i}{n_i} \right)$ = sum of the proportion of positive tests beginning at the lowest dilution showing 100 % positive result.

11.2 *Average Loading (TCID₅₀ per cm²)*—Determine the average amount of viable viruses recovered from each test article to ensure the loading specification meet the requirements.

For determining surface loading (L_a) in TCID₅₀/cm²

$$L_a = \bar{L} + \log (V \div A) \quad (2)$$

where:

- V = Volume of extraction media,
- A = Surface area of samples, and
- $\bar{L}$ = mean ($L_{1...N}$) of the untreated sample

11.3 *Data Quality Objectives*—Calculate standard deviation for the control and test populations.

For determining standard deviation:

$$\text{Standard deviation } (\sigma) = \left(\sqrt{\frac{\sum_{i=1}^N (L_i - \bar{L})^2}{N - 1}} \right) \quad (3)$$

where:

¹⁰ Finney, D. J., *Statistical Methods in Biological Assays*. 2nd ed. New York: Hafner Publishing; 1964.

$\bar{L}$ = mean of ($L_{1...N}$), and
 N = number of samples.

11.4 *Decontamination Efficiency*—Efficacy of decontamination is determined by comparing the number of viable viruses recovered from treated test specimens and untreated test specimens. The results are reported as log reduction using the equation below.

For determining log reduction:

$$\Delta \bar{L}_{U-T} = \bar{L}_U - \bar{L}_T \quad (4)$$

where:

$\bar{L}_U$ = Mean of the titers (L , $\log_{10}\text{TCID}_{50}$) recovered from the untreated test specimens, and

$\bar{L}_T$ = Mean of the titers (L , $\log_{10}\text{TCID}_{50}$) recovered from the decontaminated specimens.

11.5 *Statistical Analysis*—An unpaired two-tailed t -test at the 95 % confidence interval is performed to determine if the means of the test and control population are significantly different. p -values ≤ 0.05 indicate that there is a 95 % probability that the differences in the means were not simply due to chance.

12. Report

12.1 The report must, at the minimum, include all of the following specifications:

12.1.1 A statement that the test was conducted as directed in Test Method E2720.

12.1.2 Sample identification—a full description of the material tested.

12.1.3 The microorganism(s) used for conducting the testing.

12.1.4 A description of test device, including the device used to generate the droplet nuclei.

12.1.5 Composition of the buffer used to aerosolize the microorganism.

12.1.6 The surface area (A) for each test specimen.

12.1.7 The actual airflow through the test specimen.

12.1.8 Composition of the neutralization buffer used to extract the virus.

12.1.9 The duration of exposure to aerosol.

12.1.10 The temperature and relative humidity during the exposure.

12.1.11 *The Conditions of Decontamination*—decontaminating agent and concentration, plus any activating factors (for example, intensity, frequency and duration of illumination, voltage applied and time of application, and other applicable parameters).

12.1.12 Results of neutralization tests.

12.1.13 Coefficient of variation for the control and test samples.

12.1.14 Mean viable recoveries in $\log_{10}\text{TCID}_{50}/\text{cm}^2$ for the control and test samples.

12.1.15 Log reduction.

12.1.16 p -value comparing the control and test populations.

NOTE 3—There are no specific pass/fail criteria for this test method. This test method as written is intended to quantify the effectiveness of biological decontamination methods, including antimicrobial technologies that have been incorporated directly into the materials.

13. Precision and Bias

13.1 A precision and bias statement cannot be made for this test method at this time. Round robin testing will be completed within five years following the publication date or the method will be withdrawn.

14. Keywords

14.1 air-permeable materials; bioaerosol; decontamination; droplet nuclei; influenza; TCID_{50} ; virucidal efficacy; virus

APPENDIXES

(Nonmandatory Information)

X1. EXAMPLE DEVICE: OPERATION OF THE LABORATORY-SCALE AEROSOL TUNNEL (LSAT)

X1.1 Diagram of the Laboratory-Scale Aerosol Tunnel (LSAT) (See Fig. X1.1.)

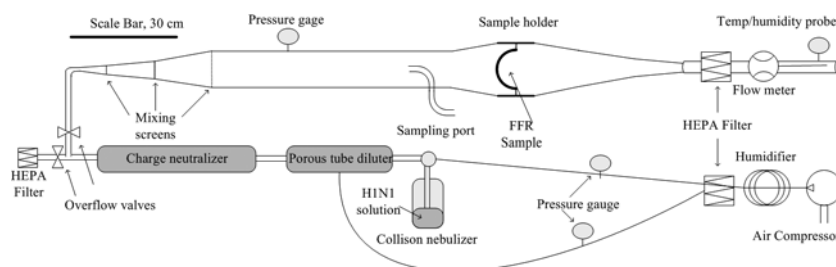


FIG. X1.1 Diagram of the Laboratory-Scale Aerosol Tunnel (LSAT)

X1.2 Parameters of the LSAT

X1.2.1 The test apparatus follows the basic design for the test rig described in **CA06PRO411**, Method for Evaluating Air Purification Technologies for Collective Protections Using Viable Microbial Aerosols. The test duct is composed of 10-cm diameter stainless steel sanitary fittings. A filter holder (15-cm diameter) was loaded into the system to accommodate a filtering facepiece respirator. The biological aerosol is generated using a six-jet Collison nebulizer. Dilution air, which is conditioned by passing the air through a humidifier, is added through the porous tube diluter and charges created on particles are neutralized during passage through a Kr-85 sealed-source charge neutralizer. The biological aerosol travels around the bend and expands in the test duct prior to reaching the test article. The test system contains an upstream sampling port that can be used to characterize the aerosol during or prior to loading experiments. The system contains HEPA filters on the inlet and exit to ensure the air is purified prior to entering or exiting the device. The system was designed to fit into a 6-ft biological safety cabinet to allow for further containment.

X1.3 Test Procedure

X1.3.1 Load six test samples into separate sample holders and seal with hot-melt glue to ensure a leak-proof seal.

X1.3.2 Set the overflow valves (7) to direct air through the LSAT.

X1.3.3 Load a six-jet Collison nebulizer with sterile water and attach to the LSAT.

X1.3.4 Apply 30 to 32 psig of pressure to the Collison and allow the test rig to equilibrate for a least 10 min (should reach $70 \pm 10\%$ RH).

X1.3.5 Turn off the pressure to the Collison nebulizer and set the overflow valves to allow air to leave the LSAT through the HEPA filter.

X1.3.6 Attach a new six-jet Collison nebulizer containing ~40 mL of the virus diluted to log 8 TCID₅₀ per mL in mucin buffer to the LSAT.

X1.3.7 Apply 30 to 32 psig of pressure to the Collison and allow it to run for 10 min before exposing the virus to the samples. The flow rate will be ~14 LPM.

X1.3.8 While the Collison nebulizer is equilibrating in step **X1.3.7** load a test sample into the LSAT.

X1.3.9 Open the overflow valves (7) for 10 min to allow exposure of the test article to the virus.

X1.3.10 Close the overflow valves (7), remove the test article, and introduce a new test article.

X1.3.11 Repeat steps **X1.3.8** to **X1.3.10** to load multiple samples.

X1.3.12 Turn off the pressure to the Collison nebulizer and remove the Collison from the LSAT. Seal the opening with an end fitting. Flow air through the porous tube diluter at 30 LPM for at least 30 min to flush the system.

X2. EXAMPLE DEVICE: THE AEROSOL CHAMBER TESTING SYSTEM

X2.1 Diagram of the Aerosol Chamber Testing System

(See **Fig. X2.1**.)

X2.2 Test Parameters of the Aerosol Chamber Testing System

X2.2.1 The total volume of the aerosolization chamber is 43 L. The chamber is equipped with a 25-mm diameter pressure relief port (HEPA exhaust port) and six 25-mm diameter test specimen holder ports. Flowmeters are used to control and measure the nebulizer and dilution inlet air at a total calibrated level of 40 L/min. Another set of six flowmeters is used to measure the flowrate between the vacuum inlet and the test specimen holders at a calibrated level of 4 L/min. A six-jet nebulizer is used to aerosolize viruses into the mixing chamber for subsequent loading onto the test specimens. An airflow regulator upstream to the six-jet nebulizer is used to adjust the nebulizer air flow to ~12 L/min (20 to 22 psig). An airflow regulator is used to adjust the dilution air flow to 36 L/min (13 to 15 psig). A 100-mm (4-in.) diameter fan (107 ± 10 CFM) controlled at 30 V is installed at a 45° angle to mix the

bioaerosol inside the chamber. A vacuum system capable of drawing a minimum of 80 L/min draws the aerosol through the test samples.

X2.3 Test Procedure

X2.3.1 Connect the aerosol chamber testing system, including compressed air supply, one six-jet Collison nebulizer, and dilution air flow to the aerosol chamber, test specimen holders containing test specimens, and a vacuum system (**Appendix X2, Fig. X2.1**).

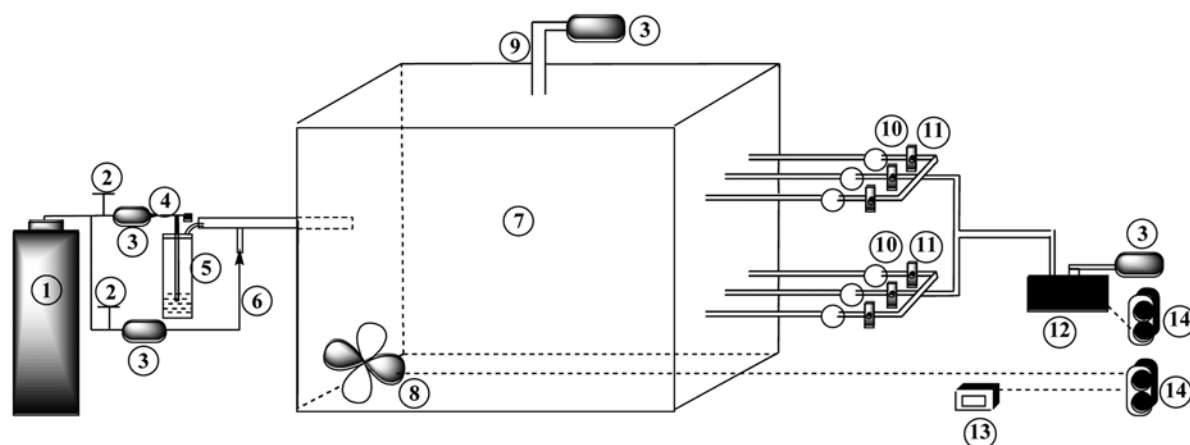
X2.3.2 Use a flow rate of ~40 L/min (~4 L/min for MS2 suspension and ~36 L/min for air dilution) and test duration of 30 min.

X2.3.3 Maintain the temperature in the aerosol chamber between 21 and 25°C and the humidity at $70\% \text{ RH} \pm 10\%$.

X2.3.4 Close all ports except the exhaust port.

X2.3.5 Turn on the circulating fan.

X2.3.6 Connect the nebulizer containing 35 mL of mucin buffer inoculated with influenza virus (log 8 TCID₅₀ per mL) to the aerosol chamber.



- KEY**
- | | |
|-----------------------------------|----------------------------------|
| 1. Compressed air supply | 8. Circulating fan |
| 2. Airflow regulator | 9. Exhaust port with HEPA filter |
| 3. HEPA filter | 10. Test specimen holders |
| 4. Nebulizer air inlet | 11. Flowmeters |
| 5. Six-jet BGI Collison Nebulizer | 12. Vacuum system |
| 6. Dilution air inlet | 13. Timer |
| 7. Aerosol chamber | 14. AC supply |

FIG. X2.1 Diagram of the Aerosol Chamber Testing System

X2.3.7 Open the compressed air to the nebulizer and the dilution air, ensuring the following gauge readings: Compressed air to nebulizer = 20 to 22 psig; dilution air = 13 to 15 psig.

X2.3.8 Start timer and run for ~5 min or until the aerosol concentration has stabilized.

X2.3.9 After the aerosol concentration has stabilized, turn on the vacuum and start timer to begin exposure to the test specimens for 30 min.

X2.3.10 Exposure termination: At the end of 30 min, stop the vacuum system and air supply. Retrieve and save the exposed test specimens for plaque assay and decontamination procedures.

X2.3.11 Disconnect the Collison nebulizer from the apparatus. Add virus suspension to the nebulizer to conduct additional experiments with the same challenge. Otherwise, sterilize the nebulizer by autoclaving.

X2.3.12 Sterilize all apparatus and supplies that come into contact with the challenge viruses to prevent exposure and reduce laboratory contamination.

X3. TCID₅₀ ASSAY FOR INFLUENZA

X3.1 Reagents

X3.1.1 *EMEM–10 % FCS*—460 mL EMEM, 5.0 mL Pen-Strep, 5 mL L-glutamine, 50 mL heat-inactivated fetal calf serum.

X3.1.2 *Serum-Free EMEM*—490 mL EMEM, 5 mL Pen-Strep, 5 mL L-glutamine.

X3.1.3 *EMEM–1 % BSA–Trypsin*—Filter sterilize 100 mL serum-free EMEM, 3.3 mL 30 % BSA, 0.5 mL of 1.0 mg/mL TPCK–trypsin stock solution (this gives 5 µg/mL).

X3.1.4 *Crystal Violet Stain*—2.0 g crystal violet, 300 mL glutaraldehyde, 2700 mL H₂O.

X3.2 Method

X3.2.1 *Prepare Plates with Confluent Monolayer of MDCK Cells*—Plate 1.0 mL per well of 1×10^5 MDCK cells/mL two days before assay in 24-well plates. Observe monolayer daily. Do not use any plate that has been confluent for more than 24 h. Lower cell seeding concentrations can be used, but the cells will take longer to grow to confluence.

X3.2.2 *Wash Three Times with Serum-Free EMEM*—Remove medium by “flicking” the reagent into a reservoir containing a 5 to 10 % bleach solution. Add 1.0 mL of serum-free EMEM/well. Repeat this wash 2 times. Finally, add

1.0 mL of serum-free EMEM and leave the plate in the incubator until the virus dilutions are ready.

X3.2.3 *Titrate Virus Stocks*—Make tenfold dilutions of the virus-containing samples in serum-free EMEM. Always use a new pipette between tubes, as influenza will stick to the plastic. If the tips are not changed, the titer will not be accurate.

X3.2.4 *Inoculate the Cells*—Remove the medium from the plate of MDCK cells and add 1.0 mL of each virus dilution into quadruplicate wells. Incubate for 55 to 65 min at 35 to 37°C, then add 100 µL of EMEM–1 % BSA–trypsin to each well. Incubate for 96 ± 4 h at 35 to 37°C.

NOTE X3.1—The detection limit of the assay can be increased by plating a larger volume of the disinfected sample. Theoretically, a detection limit of one TCID₅₀ infectious dose unit can be achieved by plating the entire volume. This is achievable if a volume of 10 to 15 mL is used for the extraction.

X3.2.5 *Titer Determined by CPE*—Discard the media in each well into a container containing bleach. Add 0.5 mL of crystal violet–glutaldehyde solution and leave to stain for at least 3 h at room temperature. Wash off the dye in running water. Allow to dry before for reading CPE. Infected wells will not stain, whereas wells that did not contain viable virus stain purple.

X4. EXAMPLE CALCULATION FOR TISSUE CULTURE INFECTIOUS DOSE (TCID₅₀) ASSAY

X4.1 *24-Well Plate*, 1.0 mL of sample inoculated into each well. (See Fig. X4.1.)

X4.2 *96-Well Plate*, 100 µL of sample inoculated into each well. (See Fig. X4.2.)

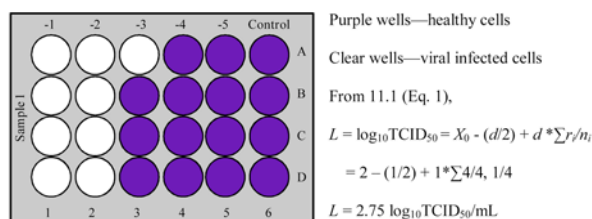


FIG. X4.1 24-Well Plate

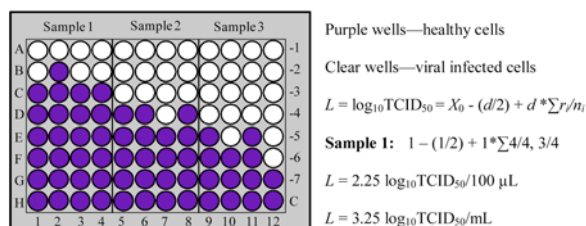


FIG. X4.2 96-Well Plate

Sample 2:

$$3 - \left(\frac{1}{2}\right) + 1 \times \sum \frac{4}{4}, \frac{1}{4}$$

$$L = 3.75 \log_{10} \text{TCID}_{50}/100 \mu\text{L} = 4.75 \log_{10} \text{TCID}_{50}/\text{mL}$$

Sample 3:

$$4 - \left(\frac{1}{2}\right) + 1 \times \sum \frac{4}{4}, \frac{2}{4}, \frac{1}{4}$$

$$L = 5.25 \log_{10} \text{TCID}_{50}/100 \mu\text{L} = 6.25 \log_{10} \text{TCID}_{50}/\text{mL}$$

X5. EXAMPLE CALCULATIONS FOR DATA QUALITY OBJECTIVES AND DECONTAMINATION EFFICIENCY

X5.1 Example Data Set

$\log_{10}\text{TCID}_{50}/\text{mL}$

Untreated samples: 3.75, 4.0, 4.0; mean = $\bar{L}_U = 3.92$
 Decontaminated samples: 1, 0.75, 1; mean = $\bar{L}_T = 0.92$
 Sample size: A = 11.0 cm^2
 Extraction volume: V = 10.0 mL

X5.2 Average PFU per cm^2

For determining surface loading (L_a , $\log_{10}\text{TCID}_{50}/\text{cm}^2$):

From 11.2 (Eq 2):

$$L_a = \bar{L} + \log(V \div A) \quad (\text{X5.1})$$

$$3.92 + \log(10 \div 11) = 3.87 \log_{10}\text{TCID}_{50}/\text{cm}^2$$

X5.3 Data Quality Objectives

X5.3.1 Determine standard deviation for the control and test populations.

For determining standard deviation: From 11.3 (Eq 3):

$$\sigma = \sqrt{\frac{\sum_{i=1}^N (L_i - \bar{L})^2}{N - 1}} \quad (\text{X5.2})$$

$$\sigma_u = \sqrt{\frac{(3.75 - 3.92)^2 + (4.00 - 3.92)^2 + (4.00 - 3.92)^2}{3 - 1}} \\ = 0.144 \log_{10}\text{TCID}_{50}$$

$$\sigma_d = \sqrt{\frac{(1.00 - 0.92)^2 + (0.75 - 0.92)^2 + (1.00 - 0.92)^2}{3 - 1}} \\ = 0.144 \log_{10}\text{TCID}_{50}$$

X5.4 Decontamination Efficiency

For determining log reduction: From 11.4 (Eq 4):

$$\Delta \bar{L}_{U-T} = \bar{L}_U - \bar{L}_T = 3.92 - 0.92 = 3.00 \quad (\text{X5.3})$$

X6. METHOD ADAPTATION FOR USING BACTERIOPHAGE MS2

As mentioned in subsection 1.3, this test method could be used with a surrogate virus. It is easier and cheaper to work with surrogate viruses. Also, surrogates are often BSL-I microorganisms, which typically require lower levels of containment. Described in this appendix are the method modifications required if bacteriophage MS2 is used as the surrogate virus. Other surrogate viruses not covered in this appendix may also be considered.

X6.1 Scope (No changes.)

X6.2 Referenced Documents (No changes.)

X6.3 Terminology

X6.3.1 *coli phage*, n —any bacteriophage that infects Gram-negative coliform bacteria.

X6.3.2 *plaque*, n —a clearing formed on a lawn of cells due to viral replication within the host cells.

X6.3.3 *plaque forming unit (PFU)*, n —any entity that gives rise to a plaque.

X6.4 Summary of Test Method

X6.4.1 Viability will be quantified using a viable plaque assay.

X6.5 Significance and Use (No changes.)

X6.6 Additional Equipment

X6.6.1 *Water Baths*, capable of maintaining 45 ± 2 to $80 \pm 2^\circ\text{C}$.

X6.6.2 *Orbital Shaker/Incubator*, capable of achieving 100 to 250 r/min and $37 \pm 2^\circ\text{C}$.

X6.6.3 *Centrifuge*, capable of 10 000 r/min.

X6.7 Additional Reagents and Materials

X6.7.1 *MS2 Coli Phage*—(ATCC 15597-B1).

X6.7.1.1 Methods for preparing and quantifying viable MS2 coli phage can be found in the EPA 600/4-84/013 (N16). Appendix X7 also provides information on how to perform a plaque assay.

X6.7.2 *Escherichia coli* (ATCC 15597).

X6.7.3 *Yeast Extract*.

X6.7.4 *Agar*.

X6.7.5 *Tryptone*.

X6.7.6 *Sodium Chloride* (NaCl).

X6.7.7 *Glucose*.

X6.7.8 *Calcium Chloride* (CaCl_2), 1.0 M solution.

X6.7.9 *HCl Solution* (2.0 M).

X6.7.10 *NaOH Solution* (2.0 M).

X6.7.11 *Thiamine*, 10 % w/w solution.

X6.7.12 *Lysozyme*.

X6.7.13 *Distilled Water and Purified Water*.

X6.7.14 *Trypticase Soy Broth* (TSB), Difco 211825.

X6.7.15 *Trypticase Soy Agar* (TSA), Difco 236590.

X6.7.16 *Petri Dishes*, sterile 15×100 mm.

X6.7.17 *Test Tubes*, 16×100 mm.

X6.7.18 *Test Tube Rack*.

X6.7.19 *Erlenmeyer Flasks*, 250 mL.

X6.7.20 *Micropipettes*, capable of delivering 0.001 mL accurately and consistently.

X6.7.21 *Sterile Cotton Swabs*.

X6.7.22 *Sterile Centrifuge Tubes*, 15 mL.

X6.7.23 *Autoclave Bottles*, 1 L.

X6.8 Sampling, Test Specimens, and Test Units (No changes.)

X6.9 Experimental Design (No changes.)

X6.10 Test Procedure

X6.10.1 Virus viability will be quantified using a plaque assay.

X6.11 Calculation and Interpretation of Results

X6.11.1 *Data Quality Objectives*—Perform CV calculation on the control and test populations. A CV $\geq 40\%$ for the negative control population indicates that the data are questionable.

For determining coefficient of variation:

$$\text{Standard Deviation } [(ARC_1, ARC_2, ARC_3) \div \text{Mean } (ARC_1, ARC_2, ARC_3)] \quad (X6.1)$$

where:

ARC = Average raw counts of triplicate plates for each independent sample.

X6.11.2 *Average PFU per cm²*—Determine the average amount of viable viruses recovered from each test article to ensure the loading specification meet the requirements.

For determining PFU/cm²:

$$(\text{Mean } ARC_n \times PF \times D^{-1} \times V) \div SA \quad (X6.2)$$

where:

PF = Plating factor (if plating 1 mL of solution, *PF* = 1; if plating 0.1 mL, *PF* = 10),

V = Volume of extraction media,

D = Dilution, and

SA = Surface area of samples.

X6.11.3 *Decontamination Efficiency*—Antimicrobial efficacy is determined by comparing the number of viable viruses recovered from treated test specimens to untreated test specimens. The results are reported as log reduction using the equation below.

For determining log reduction:

$$\text{log reduction} = \log_{10} (C \div T) \quad (X6.3)$$

where:

C = Mean number of viable viruses recovered from the untreated test specimens, and

T = Mean number of viable viruses recovered from specimens after decontamination.

X6.11.4 *Statistical Analysis*—An unpaired two-tailed *t*-test at the 95 % confidence interval is performed to determine if the means of the test and control population are significantly different. *P*-values ≤ 0.05 indicate that there is a 95 % probability that the differences in the means were not simply due to chance.

X6.12 Additional Reporting

X6.12.1 State the test was conducted as directed using **Appendix X6** of Test Method E2720.

X6.12.2 Mean viable recoveries in PFU/cm² for the control and test samples.

X6.13 Precision and Bias (No changes.)

X6.14 Additional Keywords coli phage; MS2

X7. MS2 PLAQUE ASSAY

X7.1 Preparation of *E. coli* (Day 1)

X7.1.1 Streak *E. coli* from the frozen stock or from a “transfer” plate onto a TSA plate and incubate overnight at 37 $\pm$ 2°C.

X7.2 Inoculation of TSB With *E. coli* (Day 2)

X7.2.1 Use a swab to inoculate 50 mL of sterile TSB (in a 250-mL Erlenmeyer flask) with the overnight *E. coli* growth.

X7.2.2 Incubate the flask at 37 $\pm$ 2°C with shaking at 200 to 250 r/min overnight.

X7.3 Media Preparation and Plating (Day 3)

X7.3.1 Dilute the overnight *E. coli* culture 1:10 in 1X PBS (OD₅₅₀ should be $\approx$ 1) and mix.

X7.3.2 Add 3 mL of the above dilution to $\sim$ 50 mL (in a 250-mL Erlenmeyer flask) of sterile MS2 medium (1 % tryptone, 0.5 % yeast extract, 1 % sodium chloride, pH 7).

X7.3.3 Incubate the flask at 37 $\pm$ 2°C with shaking at 200 to 250 r/min for 2.5 to 3 h.

NOTE X7.1—If more than one flask is needed due to sample volume, stagger the inoculations for optimum use at 2.5 to 3 h old.

X7.4 Prepare MS2 Soft-Agar Media

X7.4.1 Prepare the appropriate volume of 1 % tryptone, 0.1 % yeast extract, 0.8 % sodium chloride, 0.1 % dextrose, 1 % agar then adjust pH to 7 $\pm$ 0.2. Pour 500 mL of media into 1-L bottles.

X7.4.2 Autoclave media for 15 $\pm$ 1 min, then place the bottles in a 60 $\pm$ 2°C water bath and allow the media to cool for at least 1 h.

X7.4.3 Add 0.5 mL of 1 % thiamine and 2 mL of 1M CaCl₂ per 500 mL of medium. Mix and pipette 9 mL of soft-agar medium into sterile 15-mL screw cap tubes. Store the media tubes in the 60 $\pm$ 2°C water bath. When ready to plate the samples, transfer the media tubes to a 50 $\pm$ 2°C water bath.

X7.5 Plating of Samples

X7.5.1 Remove three MS2 soft-agar media tubes from the 50 $\pm$ 2°C water bath and place in a small rack in the hood.

X7.5.2 Uncap the tubes and add $\sim$ 0.25 mL of the *E. coli* suspension to each MS2 soft-agar media tube.

X7.5.3 Add 1 mL of the test solution containing MS2 coli phage to each MS2 soft-agar medium tube.

X7.5.4 Replace the caps and invert the tubes three times to thoroughly mix sample before pouring into sterile Petri dishes. Gently swirl the Petri dishes for uniform coverage.

NOTE X7.2—It is important to work quickly, but carefully, once the tubes are placed in the hood. If they are left in the hood too long, they will

solidify and cannot be used.

X7.5.5 Once the plates have hardened, invert, then incubate at $37 \pm 2^{\circ}\text{C}$ for 18 to 24 h.

X7.5.6 Count plaques.

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