Designation: ~~E2721 – 10~~ E2721 – 16

Standard ~~Test Method~~ Practice for Evaluation of Effectiveness of Decontamination Procedures for Surfaces When Challenged with Droplets Containing Human Pathogenic Viruses[1]

This standard is issued under the fixed designation E2721; 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 can often spread through droplets containing infectious agents. Such "contagious droplets" may expose susceptible individuals directly or contaminate environmental surfaces in the immediate vicinity and render them as fomites for further spread of the disease. The characteristics of the droplets (particle size and composition) will influence the viability of the microorganisms when exposed to environmental stresses but also shield them from physical and chemical decontaminants. The wide variations in the types and levels of such protective/shielding ingredients can impact on the effectiveness of surface decontaminants. This ~~test method~~ practice is designed to simulate surface deposition of contagious droplets from human respiratory secretions. It is primarily focused on influenza viruses but other respiratory viruses or surrogates 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 surfaces contaminated with virus-containing droplets.~~

~~1.2 This test method defines the conditions for simulating respiratory droplets produced by humans and depositing the droplets onto surfaces.~~

~~1.3 The method is specific to influenza viruses but could be adapted for work with other types of respiratory viruses or surrogates (Appendix X5).~~

~~1.4 This test method is suitable for working with a wide variety of environmental surfaces.~~

~~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 to Assess the Activity of Microbicides against Viruses in Suspension~~

[1] This ~~test method~~ practice 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, 2010~~April 1, 2016. Published ~~February 2011~~May 2016. Originally approved in 2010. Last previous edition approved in 2010 as E2721–10. DOI: ~~10.1520/E2721–10.~~10.1520/E2721–16.

[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.

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

E2720 Practice for Evaluation of Effectiveness of Decontamination Procedures for Air-Permeable Materials when Challenged with Biological Aerosols Containing Human Pathogenic Viruses

2.2 *EPA Standards:*

EPA 600/4-84/013 (N16) USEPA Manual of Methods for Virology[3]

2.3 *WHO Standards:*

WHO Manual on Animal Influenza Diagnosis and Surveillance[4]

3. Terminology

3.1 *Definitions:*

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

3.1.2 *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.

3.1.3 *contact transmission, n*—infections caused by direct skin-to-skin contact or indirect contact with objects contaminated with pathogens.

3.1.4 *contagious respiratory droplet, n*—respiratory secretions containing infectious microorganisms that form large droplets (≥5 µm) and settle out of the air over short distances.

3.1.5 *droplet transmission, n*—direct transfer of pathogen-containing droplets to conjuncitval or mucous membranes.

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

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

3.1.8 *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 droplets onto surfaces 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 droplets that are subsequently applied to surfaces.

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 50 % tissue culture infectious dose assay ($log_{10}TCID_{50}$).

NOTE 2—Nonviable 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 test 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 droplet generator, preparation of the test specimen, application of the challenge virus, enumeration of viable viruses, assessing data quality, and calculation of decontamination efficiency.

5.3 This test method provides defined procedures for creating droplets that approximate those produced by human respiratory secretions, with particular emphasis on droplet 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*[5] published by the U.S. Centers for Disease Control and Prevention (CDC).

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

[4] 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.

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

5.5 This test method differs from Test Methods E1052 and E2197 in the presentation of virus to the surface. The aforementioned test methods use liquid inoculum to contaminate carrier surfaces, whereas this method presents the virus in droplets that are representative of human respiratory secretions

5.6 This method differs from Test Method E2720, because (*1*) larger droplets are being formed, (*2*) the droplets will not be completely dried prior to application to surfaces, (*3*) the droplets can be applied to any surfaces, not just those that are air permeable, and (*4*) unique equipment is required to create droplets.

6. Apparatus

6.1 *Droplet Apparatus*—The apparatus used to load microorganisms onto a substrate is composed of several commercially available components and can be readily constructed.[6,7,8] 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 contains the description of a prototypical device that can be used to load droplets 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 50 ± 10 %.

6.1.1.2 Loading uniformity across the diameter of the test specimen is required to ensure the even distribution of the droplets over the surface of the carrier. A standard deviation of ±0.5 $log_{10}TCID_{50}$ is desired.

6.1.1.3 *Sample-to-Sample Variation Objective*—The variability of virus loading for multiple samples loaded for a single test must have a standard deviation of ±0.5 log_{10} $TCID_{50}$.

6.1.1.4 *Droplet Characteristics*—The droplets generated for this method will have a number median diameter (CMD) of ~15 ± 5 µm. The virus will be aerosolized in a saliva substitute (Table 1) that will add the appropriate "protective factors." This 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 droplet 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 *CO_2 Incubator,* capable of maintaining 35 to 37°C and 5 ± 0.5 % CO_2.

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-1469.

[6] Vo, E., Rengasamy, S., Shaffer, R., "Development of a Test System to Evaluate Decontamination Procedures for Viral Droplets on Respirators." *Applied and Environmental Microbiology*, Vol 75, No. 23, 2009, pp. 7303–7309.

[7] Woo, M. H., Hsu, Y. M., Wu, C. Y., Heimbuch, B. K., Wander, J. D., "A Device for a Consistent and Controlled Delivery of Aerosolized Droplets Containing Viral Agents Onto Surfaces." *Journal of Aerosol Science*, Vol 41, 2010, pp. 941-952.

[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.

TABLE 1 Artificial Saliva[8]

Reagent	Amount
$MgCl_2 \cdot 7 H_2O$	0.04 g
$CaCl_2 \cdot H_2O$	0.13 g
$NaHCO_3$	0.42 g
0.2 M KH_2PO_4	7.70 mL
0.2 M K_2HPO_4	12.3 mL
NH_4Cl	0.11 g
KSCN	0.19 g
$(NH_2)_2CO$	0.12 g
NaCl	0.88 g
KCl	1.04 g
Mucin	3.00 g
Distilled water	1000 mL
*p*H	7

7.1.1.1 The WHO Manual on Animal Influenza Diagnosis and Surveillance contains specific procedures for preparing influenza virus and titering samples. Appendix X2 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 in section 6.1.1.4.

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 m*M* 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 m*M* 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-75, T-175, 12-well, and 96-well plates.

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 *Test Materials.*

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. Place specimens into the droplet loader in the proper orientation. In some cases the complete finished product may be used, which obviates the need for cutting "coupons."

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 describes a droplet loading device and details the standard protocols for operation of the device. General information that is independent of the droplet 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 the 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 $TCID_{50}$ 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 the $TCID_{50}$ 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 35 to 37°C/5 ± 0.5 % CO_2. The cells must remain healthy and viable to pass the test.

10.3 *Load Samples With the Droplets*—The desired loading should be high enough that no less than 3 $log_{10}TCID_{50}/cm^2$ 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 reports these values for the specific test rig. 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 droplets for the required amount of time.

10.4 *Decontamination*—Remove samples from the droplet loader 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 coupon 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 cm^2 should be used). Extract the samples for 20 min using a vortex mixer.

10.5.1.1 *"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 $TCID_{50}$ assay on each sample (Appendix X2).

11. Calculation or Interpretation of Results

11.1 *Virus Quantification*—The Spearman–Karber formula[9] is used to determine the virus titer of each sample (Appendix X3 and Appendix X4 contain sample calculations).

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

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 ($TCID_{50}$ per cm^2)*—Determine the average amount of viable viruses recovered from each test article to ensure the loading specification meets the requirements.

For determining $Log_{10}TCID_{50}/cm^2$:

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

where:

V = Volume of extraction medium,
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:

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

where:

$\bar{L}$ = 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.

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

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, $\log_{10}TCID_{50}$) recovered from the untreated test specimens, and
$\bar{L}_T$ = Mean of the titers (L, $\log_{10}TCID_{50}$) 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 Statement that the test was conducted as directed in Test Method E2721.

12.2 *Sample Identification*—Description of the material tested.

12.3 The microorganism used for conducting the testing.

12.4 Description of test device including the device used to generate the droplets.

12.5 Aerosolization buffer used to aerosolize the microorganism.

12.6 The exposed surface area for each test specimen.

12.7 The liquid flow rate in the droplet loader.

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

12.9 The duration of the exposure to the aerosol.

12.10 The temperature and relative humidity in the chamber during the exposure.

12.11 *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).

12.12 Results of neutralization tests.

12.13 Coefficient of variation for the control and test samples.

12.14 The mean viable recoveries in $\log_{10}TCID_{50}/cm^2$ for the control and test samples.

12.15 Log reduction.

12.16 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 bioaerosol challenge; contagious droplet; decontamination; influenza; virus

APPENDIXES

(Nonmandatory Information)

X1. EXAMPLE DEVICE: OPERATION OF THE DROPLET CHAMBER TESTING SYSTEM

X1.1 Diagram of the Droplet Loader

(See Fig. X1.1.)

X1.2 Parameters of the Droplet Loader

X1.2.1 The system was designed to mimic respiratory droplet transmission of viruses onto any surface. Droplets are created by using an air atomizing nozzle that produces a droplet at the source that has a number median diameter of ~15 ± 5μm. Water evaporates from the droplets as they approach the test samples but they remain as liquid droplets when they impact the test samples. Adequate distribution of the droplets onto the test specimens is achieved by rotating the samples on a turntable at 3 r/min.

X1.2.2 The chamber is composed of a stainless steel shell that is has a dimension of 60 by 60 by 70 cm (L by W by H). The chamber has six ports on the bottom and top of the chamber to allow for introduction and exit of the droplets and dilution air. The ports are 0.93-cm NPT threaded openings spaced 15 cm from the center of the chamber. The ports are spaced 15 cm apart in a circular pattern. The rear panel of the chamber also contains two 0.93-cm NPT threaded ports, which are used to install humidity and temperature probes. The chamber contains an access door (55 by 32.5 cm) located 12.5 cm from the bottom of the chamber. A fractional-horsepower DC gear motor is mounted on the bottom of the chamber that is attached to a 55-cm diameter circular turntable and perforated with 0.31-cm holes. The turntable is positioned 15 cm above the bottom of the chamber. The motor is wired to a DC speed controller that is used to set the speed of the turntable. An air atomizing nozzle (Passche, Chicago, IL; part number SA 2000), is mounted into a 0.93-cm diameter fitting using epoxy. The nozzle is fitted into the forwardmost port in the top of the chamber. The other ports on the top of the chamber are fitted with set screws. All six ports on the bottom of the chamber

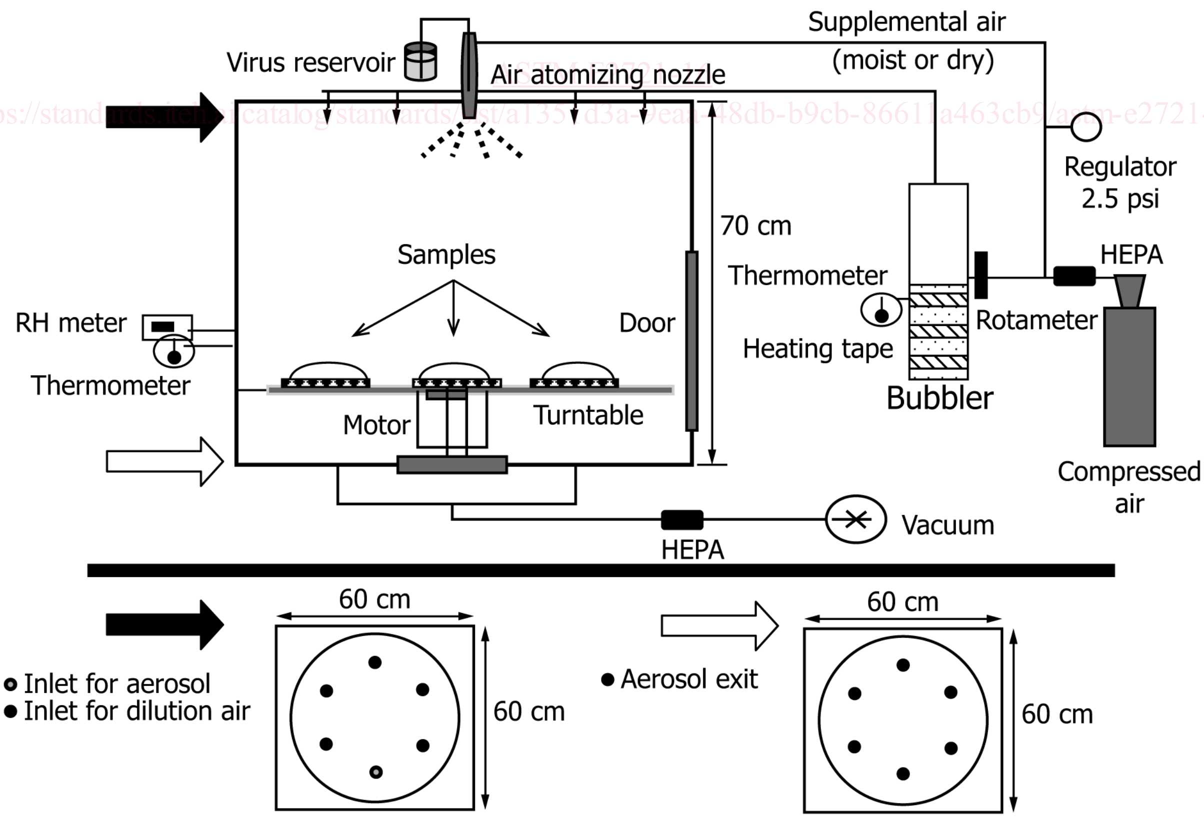

FIG. X1.1 Diagram of the Droplet Loader

are fitted with high-efficiency particulate air (HEPA) filters. An external compressor and vacuum pump capable of moving 2 CFM of air are needed to operate the droplet loader. A bubbler or other humidifying device is required for operating at high humidity.

X1.3 Test Procedure

X1.3.1 Plumb HEPA-filtered air line to the top of the droplet loader and set flow to 2 CFM. Flush the chamber for at least 1 h prior to beginning test.

X1.3.2 Place test articles into settling chamber so they are equally spaced relative to one another and so they are 1 in. from the outer edge of the turntable.

X1.3.3 Set the turntable to rotate at 3 r/min.

X1.3.4 Add 25 mL of the virus diluted to log 8 $TCID_{50}$ per mL in mucin buffer to the reservoir.

X1.3.5 Connect the virus reservoir to the air atomizing nozzle and apply 2.5 to 3.0 psig of pressure.

X1.3.6 Adjust the liquid flow rate into the air atomizing nozzle to ~2 mL per min. Expose the samples until the entire volume in the reservoir is consumed.

X1.3.7 Turn off the pressure to the air atomizing nozzle.

X1.3.8 Evacuate vagrant aerosols remaining in the chamber by drawing vacuum at the bottom chamber at a rate of ~1.5 ft^3 per min for a minimum of 15 min.

X1.3.9 Remove the samples from the droplet loader and perform decontamination tests. Control and test sample should be spaced alternately.

X1.3.10 Flow dilution air into the chamber at >2 CFM for at least 4 h to dry the chamber.

X2. $TCID_{50}$ ASSAY FOR INFLUENZA

X2.1 Reagents

X2.1.1 *EMEM–10 % FCS*—440 mL EMEM, 5.0 mL Pen-Strep, 5 mL L -glutamine, 50 mL heat-inactivated fetal calf serum.

X2.1.2 *Serum-Free EMEM*—490 mL EMEM, 5 mL Pen-Strep, 5 mL L-glutamine.

X2.1.3 *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).

X2.1.4 *Crystal Violet Stain*—2.0 g crystal violet, 300 mL glutaraldehyde, 2700 mL H_2O.

X2.2 Method

X2.2.1 *Prepare Plates With Confluent Monolayer of MDCK Cells*—Plate 1.0 mL per well of $1{\times}10^5$ 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.

X2.2.2 *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.

X2.2.3 *Titrate Virus Stocks*—Make tenfold serial 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.

X2.2.4 *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 X2.1—The detection limit of the assay can be increased by plating a larger volume of the disinfected sample. Theoretically, a detection limit of one $TCID_{50}$ 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.

X2.2.5 *Titer Determined by CPE*—Discard the medium 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 virus stain purple.

X3. EXAMPLE CALCULATION FOR TISSUE CULTURE INFECTIOUS DOSE ($TCID_{50}$) ASSAY

X3.1 **24-Well Plate**,1.0 mL of sample inoculated into each well. (See Fig. X3.1.)

X3.2 **96-Well Plate**,100 µL of sample inoculated into each well. (See Fig. X3.2.)

X4. EXAMPLE CALCULATION FOR DATA QUALITY OBJECTIVES AND DECONTAMINATION EFFICIENCY

X4.1 Example Data Set

$\log_{10}TCID_{50}$/mL

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

X4.2 Average $\log_{10}TCID_{50}/cm^2$

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

From 11.2 (Eq 2):

$$L_a = \bar{L} + \log(V \div A) \quad (X4.1)$$

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

X4.3 Data Quality Objectives

X4.3.1 Determine the standard deviation for the control and test populations.

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

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(L_i - L)^2}{N-1}} \quad (X4.2)$$

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

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

X4.4 Decontamination Efficiency X4.4

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

$$\Delta \bar{L}_{U-T} = \bar{L}_U - \bar{L}_T = 3.92 - 0.92 = 3.00 \qquad (X4.3)$$

X5. METHOD ADAPTATION FOR USING BACTERIOPHAGE MS2

As mentioned in 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 annex are the method modifications required if bacteriophage MS2 is used as the surrogate virus. Other surrogate viruses not covered in the appendix may also be considered.

X5.1 Scope (No changes.)

X5.2 Referenced Documents (No changes.)

X5.3 Terminology

X5.3.1 *coli phage, n*—any bacteriophage that infects Gram-negative coliform bacteria.

X5.3.2 *plaque, n*—a clearing formed on a lawn of cells due to viral replication within the host cells.

X5.3.3 *plaque forming unit (PFU), n*—any entity that gives rise to a plaque.

X5.4 Summary of Test Tethod

X5.4.1 Viability will be quantified using a viable plaque assay.

X5.5 Significance and Use (No changes.)

X5.6 Additional Equipment

X5.6.1 *Water Baths,* capable of maintaining 45 ± 2 to 60 ± 2°C.

X5.6.2 *Orbital Shaker/Incubator,* capable of achieving 100 to 250 r/min and 37 ± 2°C.

X5.6.3 *Centrifuge,* capable of 10 000 r/min.

X5.7 Additional Reagents and Materials

X5.7.1 *MS2 Coli Phage*—(ATCC 15597-B1).