United States
Environmental Protection
Agency

EPA/600/R-10/127 |December 2010 | www.epa.gov/ord

Development of a Methodology to Detect Viable Airborne Virus Using Personal Aerosol Samplers

U.S. EPA, Office of Research and Development
National Homeland Security Research Center

Development of a Methodology to Detect Viable Airborne Virus Using Personal Aerosol Samplers

GANG CAO, FRANCOISE M. BLACHERE,
WILLIAM G. LINDSLEY, JOHN D. NOTI,
AND DONALD H. BEEZHOLD

PREPARED FOR:

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
NATIONAL HOMELAND SECURITY
RESEARCH CENTER
CINCINNATI, OH 45268

AFFILIATION AND ADDRESS:

ALLERGY AND CLINICAL IMMUNOLOGY
BRANCH
HEALTH EFFECTS LABORATORY DIVISION
CENTERS FOR DISEASE CONTROL
NATIONAL INSTITUTE FOR OCCUPATIONAL
SAFETY AND HEALTH
MORGANTOWN, WV 26505

INTERAGENCY AGREEMENT NUMBER
DW7592259701
PROJECT NUMBER TCAD 2.8

"The findings and conclusions in this report have not been formally disseminated by the National Institute for Occupational Safety and Health and should not be construed to represent any agency determination or policy."

U.S. EPA, Office of Research and Development
National Homeland Security Research Center

Disclaimer

U.S. Environmental Protection Agency (EPA), National Homeland Security Research Center and the Centers for Disease Control and Prevention (CDC), National Institute for Occupational Safety and Health (NIOSH), under IA #DW-75-92259701 (CDC IA# Cl10-001), collaborated in the development of the analysis procedure described here.

This report has been peer and administratively reviewed and has been approved for publication as a joint EPA and CDC/NIOSH. Note that approval does not necessarily signify that the contents reflect the views of the Agencies. CDC and EPA do not endorse the purchase or sale of any commercial products or services.

Questions concerning this document or its application should be addressed to:

Don Beezhold

Allergy and Clinical Immunology Branch

Health Effects Laboratory Division

Centers for Disease Control

National Institute for Occupational Safety and Health

Morgantown, WV 26505

304-285-5963

Zec1@cdc.gov

Erin Silvestri

Project Officer

U.S. Environmental Protection Agency

Office of Research and Development

National Homeland Security Research Center

26 W. Martin Luther King Drive, MS NG16

Cincinnati, OH 45268

513-569-7619

Silvestri.Erin@epa.gov

If you have difficulty accessing this PDF document, please contact Kathy Nickel (Nickel.Kathy@epa.gov) or Amelia McCall (McCall.Amelia@epa.gov) for assistance.

Foreword

Following the terrorist events of 2001, the U.S. Environmental Protection Agency's (EPA) mission was expanded to account for critical needs related to homeland security. Presidential directives identified decontamination following a chemical, biological, and/or radiological attack as one of EPA's primary responsibilities. To provide scientific and technical support for EPA's expanded role, EPA's National Homeland Security Research Center (NHSRC) was established. The NHSRC research program is focused on conducting research and delivering products that improve the capability of the Agency to carry out its homeland security responsibilities.

As a part of its responsibility, NHSRC is charged with delivering detection techniques that will enable the rapid characterization of threats, the identification of specific contaminants to protect workers and the public, and the planning for recovery operations. A lot of effort and resources have been allocated to the development of molecular assays and culture techniques applicable to pathogens; however, initial sample collection and preparation technologies lag in development. To bridge this critical gap, EPA collaborated with the Centers for Disease Control and Prevention's (CDC) National Institute for Occupational Safety and Health (NIOSH). EPA and NIOSH worked together to examine the ability of the NIOSH bioaerosol sampler to collect viable airborne viruses and to devise techniques to preserve the viability of airborne viruses during and following after collection. Results of this evaluation will help inform environmental remediation and recovery activities.

This report summarizes the evaluation and the corresponding study results.

Gregory D. Sayles, Ph.D., Acting Director
National Homeland Security Research Center

Project Team

Contributions of the following individuals and organizations to the development of this document are acknowledged:

Centers for Disease Control and Prevention

Gang Cao

Francoise M. Blachere

William G. Lindsley

John D. Noti

Donald H. Beezhold

Stephen Morse

Betsy Weirich

U.S. Environmental Protection Agency (EPA)

Office of Research and Development, National Homeland Security Research Center

Sanjiv Shah

Erin Silvestri

Jacky Rosati (Technical Reviewer)

Sarah Perkins, Student Services Contractor (EP09C000212)

Region 5

Mark Durno (Technical Reviewer)

Region 10 Laboratory

Stephanie Harris (Technical Reviewer)

Contents

List of Figures

List of Tables

List of Acronyms

APS	Aerodynamic particle sizer
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CDC	Centers for Disease Control
cDNA	Complementary DNA
$CEID_{50}$	Chicken embryo infectious dose endpoint 50%
DMEM	Dulbecco's modified Eagle's medium
EMEM	Eagle's modified essential medium
EPA	Environmental Protection Agency
Eq	Equation
HBSS	Hank's balanced salt solution
MDCK	Madin Darby canine kidney
NHSRC	National Homeland Security Research Center
NIOSH	National Institute for Occupational Safety and Health
PBS	Phosphate buffer saline
PFU	Plaque forming unit
PTFE	Polytetrafluoroethylene
qPCR	Quantitative polymerase chain reaction
RCE	Response capability enhancement
RH	Relative humidity
SAM	Standardized Analytical Methods for Environmental Restoration Following Homeland Security Events
$TCID_{50}$	Tissue culture infectious dose endpoint 50%
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
TVP	Total viral particles
VPA	Viral plaque assay
VRA	Viral replication assay

Executive Summary

A unique two-stage cyclone aerosol sampler that can separate bioaerosols into three size fractions was developed by the National Institute for Occupational Safety and Health (NIOSH). The air sampler was tested for the ability to collect viable airborne viruses from a calm air chamber loaded with a surrogate virus. Influenza A was used as the surrogate virus due to the potential for emerging strains of influenza to create a pandemic. The sampler's efficiency at collecting aerosolized particles for 30 min from a calm-air chamber is essentially the same as that from the SKC BioSampler® (SKC Inc., Eighty Four, PA) that collects particles directly into a liquid media (1.2×10^4 total viral particles per liter of air (TVP/L of air) versus 1.3×10^4 TVP/L of air, respectively). The efficiency of the NIOSH air sampler is relatively constant over the collection times of 15, 30, and 60 minutes. The recovery rate for viable viral particles with the NIOSH sampler is approximately 59% of the virus collected and, thus, it surpasses the reported viable recovery rate of most commercial samplers with the exception of the SKC sampler. Under our experimental conditions, viable infectious virus was collected in all three fractions of the NIOSH sampler. The highest number of viable infectious virus was found in the 1-4 μm fraction (48-55%) and the <1 μm fraction (26-41%) while the smallest amount was found in the >4 μm fraction (11-19%). A viral replication assay, which is based on a coupled tissue culture infectious dose endpoint 50%/quantitative polymerase chain reaction ($TCID_{50}$/qPCR) assay, was developed to amplify viral copy number and to increase the sensitivity of the assay to detect viable virus. After normalization for differences in the air-flow volumes collected by the NIOSH and SKC samplers, the NIOSH sampler collected a mean 2.1×10^8 TVP/L of air and the SKC sampler collected a mean 6.4×10^8 TVP/L of air. Results from the viral replication assay verified that the NIOSH sampler retains viable recovery similar to the efficiency obtained by the viral plaque assay. Collection of viral particles onto test tube walls coated with mucin or agar, or into test tubes filled with a various amount of Hank's balanced salt solution, did not further improve viability.

1.0 Introduction

The U.S. Environmental Protection Agency (EPA) has identified the detection of pathogenic airborne microorganisms following a terrorist attack as a critical component of an effective response. Detection of such pathogens would require validated sampling techniques that could be used by multiple laboratories following a homeland security event. To meet this requirement, EPA's National Homeland Security Research Center (NHSRC), along with other EPA divisions and sister agencies, published *Standardized Analytical Methods for Use During Homeland Security Events, Revision 1.0* (September 2004, EPA/600/R-04/126) and Revision *2.0* (September 2005, EPA/600/R-04/126B). It was retitled in 2007 and published as *Standardized Analytical Methods for Environmental Restoration Following Homeland Security Events* (SAM) in r*evision 3.0* (February 2007,EPA/600/R-07/015), *3.1* (November 2007, EPA/600/R-07/136), 4.0 (September 2008, EPA/600/R-07/126D), and 5.0 (September 2009, EPA/600/R-07/126E) which contain suggested assays for use by laboratories tasked with performing confirmatory analysis of environmental samples following a homeland security event.

Concern regarding human exposure to bioaerosols laden with toxic agents has led to the development of a variety of air sampling devices. However, data addressing the efficacy of current samplers to detect viable and infectious airborne viruses is sparse and points to the need of a more efficient sampler (1). A two-stage cyclone bioaerosol sampler has recently been developed by the National Institute for Occupational Safety and Health (NIOSH) (2). The NIOSH sampler is unique in that it size-fractionates bioaerosols and collects them in disposable centrifuge tubes, facilitating direct processing of samples. As air is drawn into an inlet at 3.5 L/min, the first stage of the NIOSH sampler, particles that are >4 µm are collected into a 15 ml centrifuge tube. In the second stage, 1 to 4 µm particles are collected into a 1.5 ml microcentrifuge tube, and particles that are <1 µm are collected onto a 37 mm polytetrafluoroethylene (PTFE) filter.

In previous studies employing a calm air chamber to evaluate the performance of the NIOSH bioaerosol cyclone personal sampler, a nebulized suspension of the FluMist® vaccine (Medimmune, Gaithersburg, MD), which contains live, attenuated influenza virus, (3) was tested. Quantitative polymerase chain reaction (qPCR) analysis results demonstrated that the sampler effectively captured and separated viral-laden particles based on their aerodynamic size. Similarly, while conducting a field study during the February 2008 influenza season, aerosol samples were successfully collected and size-fractionated by both personal and stationary samplers situated in the West Virginia University Hospital Emergency Department (2). With qPCR analysis, 53% of the detectable viral RNA was found in the respirable fraction of the aerosol. Respirable particles are defined as those small enough to be drawn down into the alveolar region of the lungs (4). Collectively, these studies suggest the potential for airborne transmission of influenza. However, the viability and potential infectivity of the captured viral aerosols were not addressed during either study. Development of the methodologies to assess viability and infectivity would directly address any dangers posed by virus-containing particles and improve the utility of the NIOSH bioaerosol sampler for the collection of airborne viruses.

Numerous reports have shown that the viability of airborne viruses is dependent on the virus type, environmental conditions, and on the methods of collection and handling of bioaerosol samples (5). The survival of airborne influenza, for example, has been shown to greatly depend on the relative humidity, as well as on ambient air temperature and ultraviolet radiation levels (6). The number of viable influenza, measles, and mumps virus recovered from a bubbling sampler increases when a virus maintenance fluid is used in the sampler rather than distilled water (7). Airborne bacteriophages have been shown to retain viability longer after collection when they refrigerated rather than stored at room temperature (8).

The objectives of this project were to examine the ability of the NIOSH bioaerosol sampler to collect viable airborne viruses and to devise techniques to preserve the viability of airborne viruses during and following collection. During experimentation, influenza A virus was used as the surrogate virus due to the potential of newly emerging strains to create a pandemic. In this study, we showed that viable infectious virus were present in all three fractions of collected particles. The highest percentages of viable influenza virus were found in the 1-4 µm fraction (48-55%) and the <1 µm fraction (26-41%) while the smallest percentages were found in the >4 um fraction (11-19%). Further, significantly more total and more viable viral particles were collected in the <1 µm range by increasing the air sampling time beyond 15 min. Attempts to increase the viability of aerosolized viruses by collecting them onto test tube walls coated

with mucin or agar, or into test tubes filled with various amounts of Hank's balanced salt solution (HBSS), did not further improve viability.

2.0
Materials and Methods

Cell Culture: Madin Darby canine kidney (MDCK) cells (CCL-34) were purchased from the American Type Culture Collection (ATCC™, Manassas, VA). Cells were propagated and maintained in 75-cm^2 flasks (Corning® CellBind® Surface, Corning, NY). Growth medium for MDCK cells consisted of Eagle's minimal essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc, Logan, Utah), 0.4 units/ml penicillin (Invitrogen™, Carlsbad, CA), and 0.4 µg/ml streptomycin (Invitrogen). Cells were incubated at 35°C in a humidified 5% CO_2 incubator until ~90% confluent.

Virus-Influenza A/WS/33 (H1N1) virus (VR-825, Lot#: 58023547 and Lot#: 58772128) was purchased from ATCC. For each experiment, one vial (1 ml) of the virus stock was diluted in 30 ml of Hank's balanced salt solution (HBSS) supplemented with 0.1% bovine serum albumin (BSA), 100 units/ml penicillin, and 100 ug/ml streptomycin (supplemented HBSS). The freshly made virus suspension was placed on ice during the course of the experiments.

Viral Plaque Assay (VPA): MDCK cells were trypsinized, washed and plated at a density of 2.0 x 10^6 per well (CoStar 6-well tissue culture plate, Corning). Cells were incubated at 35°C in a humidified 5% CO_2 incubator overnight. Confluent cellular monolayers were next washed two times with phosphate buffered saline (PBS, Invitrogen) and treated with serial dilutions of the viral aerosol samples. Following 45 min of adsorption, influenza A/WS/33-infected MDCK cells were washed with PBS, overlaid with an agarose medium solution and incubated at 35°C in a humidified 5% CO_2 incubator for 48 h. Plaques were visually enumerated and plaque forming units (PFU)/ml were calculated.

Bioaerosol Samplers*:* A two-stage cyclone bioaerosol sampler developed by NIOSH was used to collect influenza virus-containing aerosols generated in the laboratory. The NIOSH sampler consists of a 15 ml centrifuge tube as the 1st stage, a 1.5 ml centrifuge tube as the 2nd stage, and a backup polytetrafluoroethylene (PTFE filter). As a comparison, the SKC sampler (SKC BioSampler® SKC Inc, Eighty Four, PA) was also used for each experiment. The SKC sampler contained 15 ml of supplemented HBSS in the 20 ml collection vessel.

Calm-Air chamber Aerosolization and Collection of Influenza Virus*:* Diluted influenza virus suspension was aerosolized by a 1-jet Collison nebulizer (BGI, Waltham, MA) for the experiments (EPA01-EPA04) and by an AeroNeb® nebulizer (Aerogen®, Galway, Ireland) for the experiments (EPA05-EPA19). The generated aerosols were mixed in a mixing chamber with 30 L/min of air at 20% relative humidity (RH) through a dispersion nozzle in the top center of a 40 L calm-air chamber. The forces of gravity and inertia caused the aerosols to settle into the bottom of the chamber where the NIOSH sampler (i.e., replicates s and a sample inlet for the SKC samplers (i.e., replicates) were located.

The chamber air was drawn by a Model 3321 Aerodynamic Particle Sizer® (TSI®, Shoreview, MN) at 5 L/min through a vertical probe at the same height as the sampler inlets to monitor the aerosol concentration and size distribution. The aerosols were collected by both NIOSH and SKC samplers. The NIOSH samplers were connected to personal air sampling pumps (Model 224-PCXR4; SKC, Eighty Four, PA, USA) and the SKC samplers were connected to a central vacuum line. The NIOSH samplers were positioned inside and at the bottom of the calm air chamber, whereas, the SKC samplers were placed outside the chamber with the sampler inlet housed inside the chamber. Generally, for each EPA experiment, multiple samplers were placed inside and outside the chamber.

To collect the airborne influenza virus, after operation of the AeroNeb nebulizer for 10 min and once the aerosol concentration in the chamber stabilized, the vacuum pumps for the NIOSH samplers and the vacuum line for the SKC sampler were switched on, simultaneously. The nebulizer provided continuous loading of aerosols in the chamber. The NIOSH samplers collected the aerosols for 15, 30, and 60 mins at 3.5 L/min while the SKC sampler collected the aerosols for 15 mins at 12.5 L/min. After collection, the nebulizer, the pumps and the vacuum line were turned off. The exterior of the samplers were wiped off with a clean, low lint, laboratory tissue to remove the deposited particles. The NIOSH samplers were disassembled for analysis.

The concentrations of aerosols loaded in the calm-air chamber and the viable and total virus particles in the initial viral suspensions varied among experiments, which introduced artificial variations in concentrations of viable and total influenza particles collected by the samplers. To reduce such variations, we normalized the concentrations [conc] of viable and total influenza particles using Equation 1 and Equation (Eq) 2:

$$\text{normalized infectious conc (PFU/L of air) by the sampler i} = \frac{\text{infectious conc (PFU/L of air) by the sampler i}}{\left(\frac{\text{total aerosols collected by the sampler i}}{\text{average total aerosols collected}}\right)\left(\frac{\text{viable particles in the initial viral suspension}}{\text{average viable particles in the initial viral suspension}}\right)}$$

Eq 1

$$\text{normalized total virus conc (TVP/L of air) by the sampler i} = \frac{\text{total virus conc(TVP/L of air) by the sampler i}}{\left(\frac{\text{total aerosols collected by the sampler i}}{\text{average total aerosols collected}}\right)\left(\frac{\text{total viral particles in the initial viral suspension}}{\text{average total viral particles in the initial viral suspension}}\right)}$$

Eq 2

The viability of virus was calculated by dividing the viable influenza viral particles obtained from the VPA by the total viral particles (TVP) per liter of air from quantitative polymerase chain reaction (qPCR).

Viral RNA Isolation and cDNA (complementary DNA) Transcription*:* Viral RNA was isolated from aerosol samples using the MagMax™-96 Viral RNA Isolation Kit (Applied Biosystems®/Ambion®, Austin, TX). Briefly, following collection, aerosol samples were suspended in 500 µl of Lysis/Binding Concentrate (Ambion) and stored at -20°C. Upon thawing, 500 µl of isopropanol was added to each sample to complete the Lysis/Binding Solution preparation and viral RNA was extracted according to the manufacturer's instructions. The final eluted total-RNA volume was 32 µl. RNA was immediately transcribed into cDNA using High Capacity RNA to cDNA Master Mix (Applied Biosystems, Foster City, CA). The final cDNA volume was 40 µl.

Real-Time qPCR Analysis*:* To detect influenza virus, real-time qPCR analysis was performed using the following matrix1 gene primers (corresponding to the 33 amino acids at the n-terminus as described in Spackman et al. (9)): Forward 5'-AGATGAGTCTTCTAACCGAGGTCG-3', Reverse 5'-TGCAAAAACATCTTCAAGTCTCTG-3' and probe: 6FAM-TCAGGCCCCCTCAAA GCC-MGBNFQ. All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 µM and 0.2 µM, respectively. The qPCR (45 cycles) was performed with the Applied Biosystems 7500 Fast Real-Time PCR System as follows: 20 sec at 95°C (initial denaturation), 3 sec at 95°C (amplification), and 30 sec at 60°C (extension). To determine the relative viral genome copy, a standard curve was generated from 10-fold serial dilutions of the influenza M1 matrix gene and analyzed concurrently with all qPCR reactions. A negative control without template was also included in all real-time PCR reactions. All reactions were run in duplicate and averaged.

Viral Replication Assay (VRA)*:* To assess viral replication, a modified tissue culture infectious dose endpoint 50% ($TCID_{50}$) assay (Michael Shaw, PhD[1], personnel communication) was performed. Prior to viral treatment, MDCK cells were trypsinized, washed and re-suspended in (Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 1% bovine serum albumin (BSA, Invitrogen), 25 mM HEPES (Invitrogen), 2 µg/ml L-10 tosylamido-2-phenylethyl chloromethyl ketone (TPCK) Trypsin (Sigma, St. Louis, MO), 0.2 units/ml penicillin (Invitrogen), and 0.2 µg/ml streptomycin (Invitrogen). Cells were plated in quintuplicate at a density of 5.0×10^4 per well (CoStar® flat bottom 96-well plate, Corning) and incubated overnight at 35°C. Plated cells were next treated with five serial dilutions of each viral aerosol sample at 35°C in a humidified 5% CO_2 incubator for 24 h. Following treatment, cell culture supernatants/viral inoculums were removed, cellular monolayers were washed with PBS and cells were lysed in 66 µl Lysis/Binding Solution Concentrate (Ambion) and stored at -20°C until RNA extraction.

VRA RNA Isolation and cDNA Transcription*:* Total RNA was isolated from A/WS/33-infected MDCK cells using the MagMax™-96 Total RNA Isolation Kit (Ambion). Briefly, upon thawing of the lysed cellular solution, 66 µl of isopropanol was added to each sample well to complete the Lysis/Binding Solution preparation and total RNA was extracted according to the manufacturer's instructions. The final eluted total RNA volume was 32 µl. RNA was immediately transcribed into cDNA using High Capacity RNA to cDNA Master Mix (Applied Biosystems). The final cDNA volume was 40 µl.

[1]Michael W. Shaw, PhD, Influenza Division, MS G-16, Centers for Disease Control and Prevention, Atlanta, GA 30333, email: mshaw1@cdc.gov

3.0
Results

Recovery Efficiency of Viral Particles from the NIOSH Sampler: The NIOSH sampler size-fractionates bioaerosols and collects them in disposable collection tubes or on a backup PTFE filter (Figure 1). Experiments were conducted to determine the recovery efficiencies of virus deposited directly onto the wall of a collection tube or the backup filter. One hundred microliters of a stock suspension of influenza A/WS/33 (5.27 X 10^6 virus/ml, ATCC VR-825) was spotted onto replicate backup filters in 10 µl aliquots. The spiked filters were then dried under clean air at a flow rate of 3.5 L/min for 0, 15, 30, or 60 min. The virus was then extracted from each filter and the total number of virus recovered was determined by the qPCR. As control, 100 µl of stock suspension was analyzed by the qPCR. The results (data not shown) revealed that 17.1%, 10.3%, 10.8%, and 11.7% (mean of three replicates under each experimental condition) of the spiked virus was recoverable from filters dried for 0, 15, 30, and 60 min, respectively. The VPA was used to assess the viable virus recovery and the results showed that 60.9%, 57.3%, 52.5%, and 59.5 % (mean of three replicates under each experimental condition) of the virus remained viable after 0, 15, 30, and 60 min of drying before extraction. The recovery of virus from multiple Stage 1 collection tubes was similarly addressed. The recovery of total viral particles was 15.7% and 15.9% from Stage 1 tubes that were extracted immediately or dried under clean air for 30 min, respectively. The viable virus recovery was 65.7% and 50.3% from the Stage 1 tubes that were extracted immediately or dried under the clean air for 30 min, respectively.

Viability Assessment of Collected Viral Particles: A total of 19 calm-air chamber experiments (Figure 2) were performed at room temperature with 20% humidity for this project (Table 1). The objectives of these experiments were to examine the ability of the NIOSH sampler to collect viable airborne viruses and to test the effects of the collection media, collection time and sample storage time on viability of collected airborne influenza virus.

In the early stage of this project (EPA01-EPA04, unpublished data), a 1-jet collision nebulizer was used to aerosolize the influenza virus. The results, however, showed a significant loss in viability with this nebulizer. We then tested whether the AeroNeb nebulizer would better preserve viability. The AeroNeb nebulizer was positioned directly on the opening of a stage 1 collection tube and droplets coming out from the bottom of the nebulizer were collected and used for the qPCR and VPA analyses. Samples were collected on ice or at room temperature to determine whether viability could be improved by lowering the collection temperature. As control, a sample of the viral suspension before nebulization was assayed. The nebulizer remained on for 30 min per each collection to mimic the calm-air chamber experimental conditions. The results showed that 42% of the total viral particles aerosolized were recovered in the collection tube placed on ice and essentially no loss in viability was shown. Similarly, 49% of the total viral particles aerosolized were recovered in the tube placed at room temperature and viability did not decrease. These results show that the AeroNeb nebulizer does not reduce viral viability. Experiments using the calm-air chamber for collection of aerosolized virus were previously done at room temperature, and, as such, there is no advantage to performing future experiments at colder temperatures. All of the subsequent experiments described here employed an AeroNeb nebulizer.

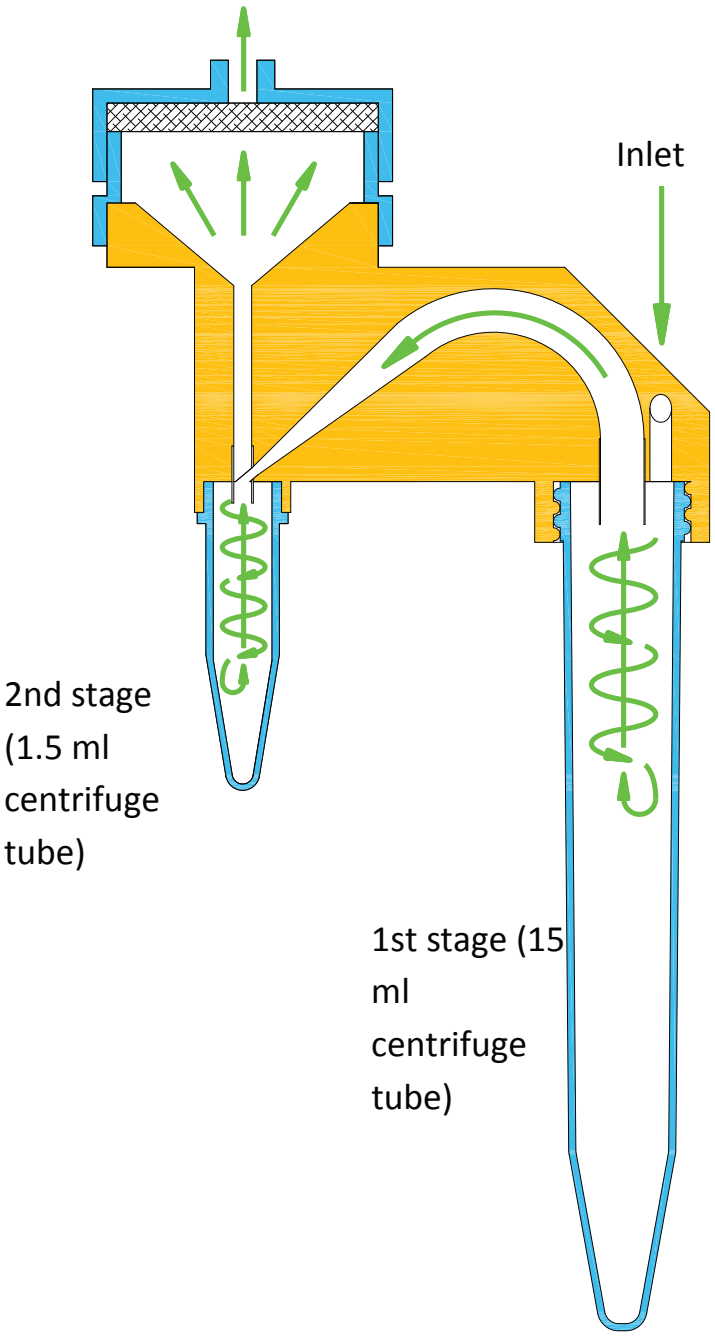


Figure 1. NIOSH two-stage bioaerosol cyclone sampler.

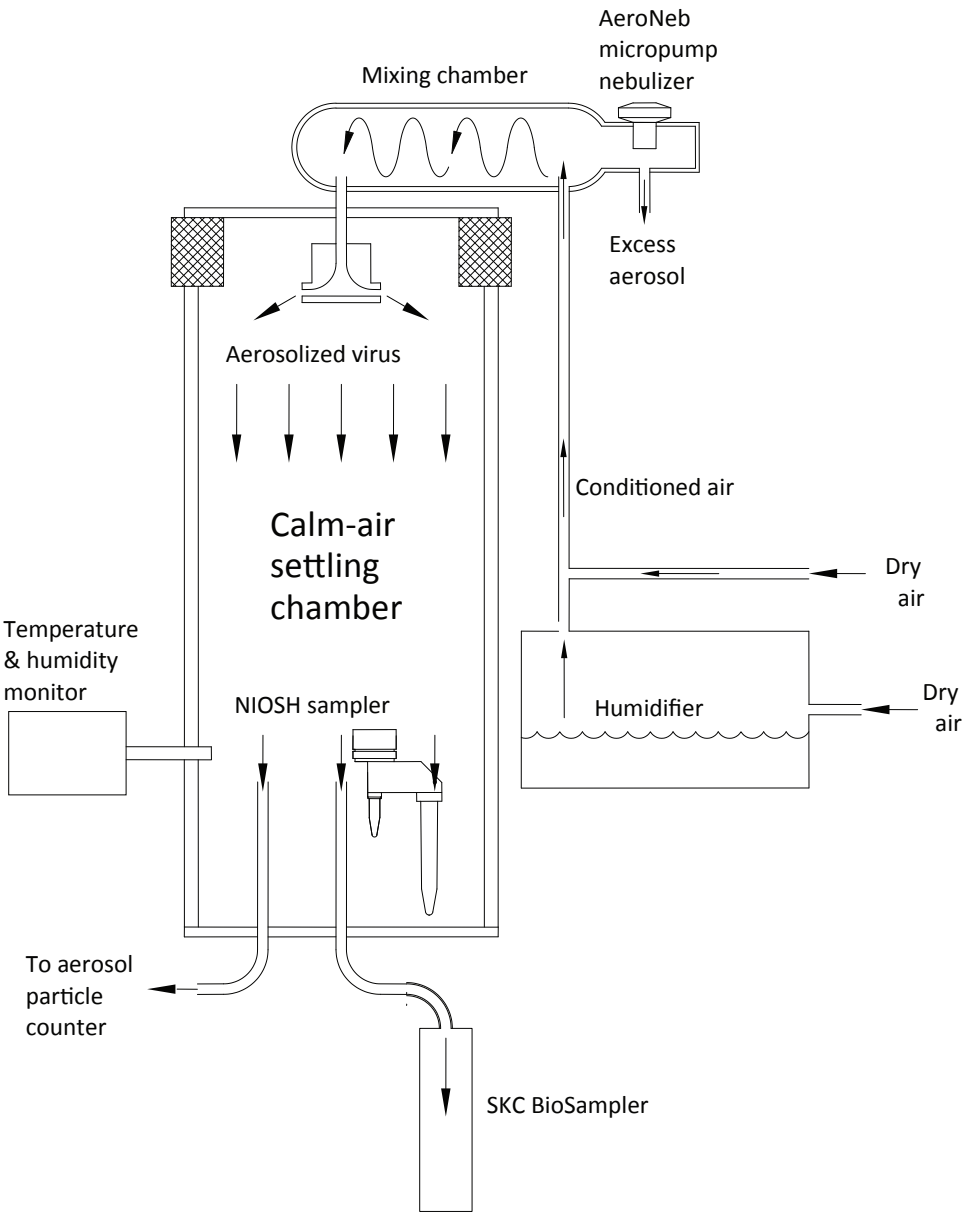


Figure 2. Calm-air settling chamber for collecting aerosolized particles.

Table 1. Summary of Experiments Performed for the EPA Project

Exp#	Nebulizer	Collec. time (min)	# of NIOSH samplers	Viability detection	Note[1]
EPA-01	Collison 1-jet	60	6	VPA	Pilot for detection of viable airborne influenza virus
EPA-02	Collison 1-jet	60	2	VPA	Repeat EPA-01 but with a greater number of aerosolized influenza virus loaded in the calm-air chamber
EPA-03	Collison 1-jet	60	3	VPA	Test the effect of sample storage time on viability of influenza virus collected
EPA-04	Collison 1-jet	60	3	VPA	Test the effect of liquid medium (HBSS) on viability of influenza virus collected
EPA-05	AeroNeb	30	3	VPA	Test a different nebulizer to generate airborne influenza virus
EPA-06	AeroNeb	60	3	VPA	
EPA-07	AeroNeb	30	3	VPA	Stage 1 of NIOSH samplers (T1) was agar-coated
EPA-08	AeroNeb	60; 30	5	VPA	Test the collection time on the performance of NIOSH samplers
EPA-09	AeroNeb	60; 30	4	VPA	
EPA-10	AeroNeb	30; 15	4	VPA	
EPA-11	AeroNeb	20; 30; 40; 50; 60	5	Luciferase	Test the luciferase method for detecting and quantifying airborne influenza virus
EPA-12	AeroNeb	40	3	Luciferase; VPA	
EPA-13	AeroNeb	40	3	Luciferase; VPA	
EPA-14	AeroNeb	30	4	VPA	Test the effect of sample storage time on viability of influenza virus
EPA-15	AeroNeb	30	4	VPA	Stage 1 of NIOSH samplers (T1) was coated with 0.1% mucin solution
EPA-16	AeroNeb	30	4	VPA	Test the new stock VR-825
EPA-17	AeroNeb	30	2	VRA	Test the viral replication assay for detecting and quantifying airborne influenza virus
EPA-18	AeroNeb	30	2	VRA	Stage 1 and Stage 2 of NIOSH samplers (T1, T2) were coated with electret filter
EPA-19	AeroNeb	30	5	VPA	Test the effect of liquid medium (HBSS) on viability of influenza virus collected

Acronyms: EXPP, Experiment; HBSS, Hank's balanced salt solution; VPA, Viral plaque assay

[1]qPCR was performed to determine the total viral particles for all experiments except EPA-01 and EPA-02. Stock VR-825 (Lot#: 58023547) was used for EPA-01-EPA15. Stock VR-825 (Lot#: 58772128) was used for EPA-16 through EPA-19.

We previously demonstrated that the collection of FluMist® vaccine (Medimmune, LLC.; Gaithersburg, MD) with the NIOSH two-stage sampler was linear at least up to 40 min of sampling time (3). In that study, however, the viability of the collected virus particles was not assessed. To determine whether the NIOSH sampler can collect viable virus, aerosolized influenza A/WS/33 was loaded into a calm air chamber equilibrated to 21°C and 20%relative humidity, and air samples were collected for 15, 30, and 60 min in nine separate experiments. For each experiment, 1 to 4 samplers were simultaneously connected to the calm air chamber and the results are shown in Table 2. Over the collection periods of 15, 30, and 60 min, the TVP collected increased linearly (averages of 2.53 X 10^6, 4.4 X 10^6, and 8.74 X 10^6, respectively).

The total number of viral particles, pooled from the collection tubes and filter, varied with the starting virus concentration in the initial viral suspension (i.e., prior to aerosolization). However, within a given experiment, the number of viral particles collected also increased linearly. For example, in experiment number EPA-10, the average total viral particles collected was 6.41 X 10^5 at 15 min and 1.04 X 10^6 at 30 min. Similarly, in experiment number EPA-8, the average total viral particles collected was 3.97 X 10^5 at 30 min and 8.93 X 10^5 at 60 min. Normalization of the number of collected total virus to the amount loaded into the calm-air chamber, the air flow rate, and the amount of time for collection was performed to determine the efficiency of the NIOSH sampler over the sampling time and also to directly compare its efficiency with the SKC sampler. The results revealed that the NIOSH sampler retained similar collection efficiencies over the 15-60 min sampling period in that 1.32 X 10^4 total viral particles per liter (TVP/L) of air were collected after 15 min of sampling, 1.2 X 10^4 TVP/L were collected after 30 min, and 2.68 X 10^4 TVP/L were collected after 60 min (Table 2). Moreover, the efficiency was essentially the same as that obtained from the SKC sampler (Table 3, average 1.3 X 10^4 TVP/L).

Table 2. Total Influenza Viral Particles Collected by NIOSH Samplers in a Calm-air Chamber

Exp. #	Collect time (min)	Total aerosols collected	Total viral particles (in initial viral suspension)	Total viral particles (TVP) collected	Total virus conc. (TVP/L of air)	Normalized total virus conc. (TVP/L of air) collected
EPA-10	15	2.53E+09	1.03E+09	7.18E+05	1.37E+04	1.48E+04
EPA-10	15	2.53E+09	1.03E+09	5.63E+05	1.07E+04	1.16E+04
					Average (SD)	1.32E+04 (2.26E+03)
EPA-05	30	4.07E+09	2.86E+08	6.20E+05	5.90E+03	2.30E+04
EPA-07	30	4.01E+09	1.94E+08	3.47E+05	3.30E+03	1.93E+04
EPA-08	30	4.85E+09	1.61E+08	3.97E+05	3.78E+03	2.21E+04
EPA-09	30	5.01E+09	3.88E+08	2.89E+05	2.75E+03	6.43E+03
EPA-09	30	5.01E+09	3.88E+08	1.79E+05	1.70E+03	3.99E+03
EPA-10	30	3.82E+09	1.03E+09	9.44E+05	8.99E+03	1.04E+04
EPA-10	30	3.82E+09	1.03E+09	1.13E+06	1.08E+04	1.25E+04
EPA-14	30	4.98E+09	2.10E+09	1.24E+06	1.18E+04	5.11E+03
EPA-14	30	4.98E+09	2.10E+09	2.60E+06	2.48E+04	1.08E+04
EPA-15	30	3.99E+09	3.61E+09	4.87E+06	4.64E+04	1.46E+04
EPA-15	30	3.99E+09	3.61E+09	4.82E+06	4.59E+04	1.45E+04
EPA-16	30	4.72E+09	1.31E+09	1.01E+06	9.66E+03	7.10E+03
EPA-16	30	4.72E+09	1.31E+09	1.27E+06	1.21E+04	8.88E+03
EPA-16	30	4.72E+09	1.31E+09	1.37E+06	1.31E+04	9.60E+03
EPA-16	30	4.72E+09	1.31E+09	9.30E+05	8.86E+03	6.51E+03
EPA-19	30	2.96E+09	8.32E+08	9.86E+05	9.39E+03	1.74E+04
					Average (SD)	1.20E+04 (5.97E+03)
EPA-06	60	7.83E+09	3.36E+08	3.07E+06	1.46E+04	5.40E+04
EPA-08	60	8.96E+09	1.61E+08	8.85E+05	4.21E+03	2.84E+04
EPA-08	60	8.96E+09	1.61E+08	8.76E+05	4.17E+03	2.81E+04
EPA-08	60	8.96E+09	1.61E+08	9.20E+05	4.38E+03	2.95E+04
EPA-09	60	8.85E+09	3.88E+08	8.59E+05	4.09E+03	1.16E+04
EPA-09	60	8.85E+09	3.88E+08	6.88E+05	3.27E+03	9.26E+03
					Average (SD)	2.68E+04 (1.61E+04)

Acronyms: TVP, Total viral particles

To determine whether the viability of aerosolized virus is maintained following collection with the NIOSH sampler, the percentage of viable virus in the initial viral suspension was compared with that in the collected sample (Table 4). In general, as determined by a viral plaque-forming assay (VPA), the average loss of viability was 41% (59% of the recovered virus were viable). In contrast, there was no loss in viability detected with the SKC sampler (Table 5). Prolonged collection times have been shown to result in decreased viral recovery (10). Therefore, to determine whether the NIOSH sampler is able to collect viable virus over an extended sampling period, collected samples were assayed for infective virus using the VPA. As shown in Table 6, the number of viable virus particles collected per liter of air varied with each experiment as a result of variability in the number of viable virus in the initial suspension prior to aerosolization. However, after normalization to correct for these differences, the average PFU/L of air was 14.91 from samples collected for 15 min, 14.26 for samples collected for 30 min, and 11.59 from samples collected for 60 min. The variability among the experiments within each collection period was not statistically significant and more importantly, there was essentially no adverse effect on viability with prolonged collection of up to 60 min.

Table 3. Total Influenza Viral Particles Collected by SKC Samplers in a Calm-air Chamber

Exp.#	Collect time	Total aerosols collected	Total viral particles (in initial viral suspension)	Total viral particles (TVP) collected	Total virus conc. (TVP/L of air)	Normalized total virus conc. (TVP/L of air) collected
EPA-05	15	7.76E+09	2.86E+08	9.25E+05	4.93E+03	1.76E+04
EPA-06	15	6.43E+09	3.36E+08	4.60E+05	2.45E+03	8.98E+03
EPA-07	15	6.54E+09	1.94E+08	7.89E+05	4.21E+03	2.62E+04
EPA-08	15	7.14E+09	1.61E+08	1.18E+06	6.31E+03	4.35E+04
EPA-08	15	7.33E+09	1.61E+08	1.01E+06	5.36E+03	3.60E+04
EPA-09	15	7.99E+09	3.88E+08	7.36E+04	3.93E+02	1.00E+03
EPA-09	15	5.90E+09	3.88E+08	4.48E+05	2.39E+03	8.27E+03
EPA-10	15	9.07E+09	1.03E+09	2.85E+06	1.52E+04	1.29E+04
EPA-10	15	3.95E+09	1.03E+09	1.73E+06	9.20E+03	1.80E+04
EPA-13	15	4.84E+09	6.46E+08	4.11E+05	2.19E+03	5.55E+03
EPA-14	15	8.99E+09	2.10E+09	1.23E+06	6.58E+03	2.75E+03
EPA-14	15	8.58E+09	2.10E+09	3.70E+06	1.97E+04	8.64E+03
EPA-15	15	6.19E+09	3.61E+09	9.59E+06	5.11E+04	1.81E+04
EPA-15	15	8.08E+09	3.61E+09	3.26E+06	1.74E+04	4.72E+03
EPA-16	15	8.14E+09	1.31E+09	1.60E+06	8.52E+03	6.32E+03
EPA-16	15	8.59E+09	1.31E+09	9.21E+05	4.91E+03	3.45E+03
EPA-19	15	5.23E+09	8.32E+08	7.32E+05	3.91E+03	7.11E+03
EPA-19	15	5.35E+09	8.32E+08	4.54E+05	2.42E+03	4.31E+03
					Average (SD)	1.30E+04 (1.18E+04)

Table 4. Viable and Total Influenza Viral Particles in the Initial Viral Suspensions and in the Samples Collected by NIOSH Samplers in a Calm-air Chamber

Exp.#	Collect time (min)	Plaque forming units(PFU) in initial viral suspension)	Total viral particles (TVP) (in initial viral suspension)	Infectious to total (%) (in initial viral suspension)	Infectious conc. (PFU/L of air)	Total virus conc. (TVP/L of air)	Infectious to total (%) (in collected sample)
EPA-10	15	3.90E+06	1.03E+09	0.38	26.90	1.37E+04	0.20
EPA-10	15	3.90E+06	1.03E+09	0.38	28.10	1.07E+04	0.26
EPA-05	30	3.28E+06	2.86E+08	1.15	15.25	5.90E+03	0.26
EPA-07	30	1.61E+06	1.94E+08	0.83	14.96	3.30E+03	0.45
EPA-08	30	1.95E+06	1.61E+08	1.21	15.64	3.78E+03	0.41
EPA-09	30	2.10E+06	3.88E+08	0.54	10.87	2.75E+03	0.40
EPA-09	30	2.10E+06	3.88E+08	0.54	8.33	1.70E+03	0.49
EPA-10	30	3.90E+06	1.03E+09	0.38	25.83	8.99E+03	0.29
EPA-10	30	3.90E+06	1.03E+09	0.38	30.00	1.08E+04	0.28
EPA-14	30	1.91E+06	2.10E+09	0.09	14.64	1.18E+04	0.12
EPA-14	30	1.91E+06	2.10E+09	0.09	19.64	2.48E+04	0.08
EPA-15	30	2.59E+06	3.61E+09	0.07	15.12	4.64E+04	0.03
EPA-15	30	2.59E+06	3.61E+09	0.07	15.12	4.59E+04	0.03
EPA-16	30	7.88E+05	1.31E+09	0.06	5.63	9.66E+03	0.06
EPA-16	30	7.88E+05	1.31E+09	0.06	4.92	1.21E+04	0.04
EPA-16	30	7.88E+05	1.31E+09	0.06	4.88	1.31E+04	0.04
EPA-16	30	7.88E+05	1.31E+09	0.06	6.48	8.86E+03	0.07
EPA-19	30	1.13E+06	8.32E+08	0.14	10.83	9.39E+03	0.12
EPA-06	60	4.80E+06	3.36E+08	1.43	35.35	1.46E+04	0.24
EPA-08	60	1.95E+06	1.61E+08	1.21	12.37	4.21E+03	0.29
EPA-08	60	1.95E+06	1.61E+08	1.21	11.19	4.17E+03	0.27
EPA-08	60	1.95E+06	1.61E+08	1.21	10.65	4.38E+03	0.24
EPA-09	60	2.10E+06	3.88E+08	0.54	8.37	4.09E+03	0.20
EPA-09	60	2.10E+06	3.88E+08	0.54	7.74	3.27E+03	0.24

Table 5. Viable and Total Influenza Viral Particles in the Initial Viral Suspensions and in the Samples Collected by SKC Samplers in a Calm-air Chamber

Exp.#	Collect time (min)	PFU (in initial viral suspension	Total viral particles (in initial viral suspension)	Infectious to total (%) (in initial viral suspension)	Infectious conc. (PFU/L of air)	Total virus conc. (TVP/L of air)	Infectious to total (%) (in collected sample)
EPA-05	15	2.93E+06	2.86E+08	1.02	69.33	4.93E+03	1.41
EPA-06	15	2.40E+06	3.36E+08	0.71	76.27	2.45E+03	3.11
EPA-07	15	1.39E+06	1.94E+08	0.72	38.03	4.21E+03	0.90
EPA-08	15	1.95E+06	1.61E+08	1.21	90.00	6.31E+03	1.43
EPA-08	15	1.95E+06	1.61E+08	1.21	91.00	5.36E+03	1.70
EPA-09	15	2.10E+06	3.88E+08	0.54	64.79	3.93E+02	16.49
EPA-09	15	2.10E+06	3.88E+08	0.54	26.04	2.39E+03	1.09
EPA-10	15	3.90E+06	1.03E+09	0.38	108.00	1.52E+04	0.71
EPA-10	15	3.90E+06	1.03E+09	0.38	55.00	9.20E+03	0.60
EPA-13	15	1.73E+06	6.46E+08	0.27	13.90	2.19E+03	0.63
EPA-14	15	1.91E+06	2.10E+09	0.09	91.00	6.58E+03	1.38
EPA-14	15	1.91E+06	2.10E+09	0.09	56.00	1.97E+04	0.28
EPA-15	15	2.59E+06	3.61E+09	0.07	62.00	5.11E+04	0.12
EPA-15	15	2.59E+06	3.61E+09	0.07	45.00	1.74E+04	0.26
EPA-16	15	7.88E+05	1.31E+09	0.06	18.00	8.52E+03	0.21
EPA-16	15	7.88E+05	1.31E+09	0.06	21.00	4.91E+03	0.43
EPA-19	15	1.13E+06	8.32E+08	0.14	26.00	3.91E+03	0.66
EPA-19	15	1.13E+06	8.32E+08	0.14	28.00	2.42E+03	1.16

Table 6. Viable Influenza Viral Particles Collected by NIOSH Samplers in a Calm-air Chamber

Experiment #	Collect time (min)	Total aerosols collected	PFU (in initial viral suspension)	Viable viral particles collected (PFU)	Infectious conc. (PFU/L of air)	Normalized infectious conc. (PFU/L of air) collected
EPA-10	15	2.53E+09	3.90E+06	1.41E+03	26.90	14.59
EPA-10	15	2.53E+09	3.90E+06	1.48E+03	28.10	15.23
					Average (SD)	14.91 (0.46)
EPA-05	30	4.07E+09	3.28E+06	1.60E+03	15.25	9.88
EPA-07	30	4.01E+09	1.61E+06	1.57E+03	14.96	20.05
EPA-08	30	4.85E+09	1.95E+06	1.64E+03	15.64	14.30
EPA-09	30	5.01E+09	2.10E+06	1.14E+03	10.87	8.93
EPA-09	30	5.01E+09	2.10E+06	8.75E+02	8.33	6.84
EPA-10	30	3.82E+09	3.90E+06	2.71E+03	25.83	15.01
EPA-10	30	3.82E+09	3.90E+06	3.15E+03	30.00	17.44
EPA-14	30	4.98E+09	1.91E+06	1.54E+03	14.64	13.29
EPA-14	30	4.98E+09	1.91E+06	2.06E+03	19.64	17.83
EPA-15	30	3.99E+09	2.59E+06	1.59E+03	15.12	12.68
EPA-15	30	3.99E+09	2.59E+06	1.59E+03	15.12	12.68
EPA-16	30	4.72E+09	7.88E+05	5.91E+02	5.63	13.10
EPA-16	30	4.72E+09	7.88E+05	5.17E+02	4.92	11.45
EPA-16	30	4.72E+09	7.88E+05	5.13E+02	4.88	11.36
EPA-16	30	4.72E+09	7.88E+05	6.80E+02	6.48	15.08
EPA-19	30	2.96E+09	1.13E+06	1.14E+03	10.83	28.16
					Average (SD)	14.26 (5.01)
EPA-06	60	7.83E+09	4.80E+06	7.42E+03	35.35	17.38
EPA-08	60	8.96E+09	1.95E+06	2.60E+03	12.37	13.07
EPA-08	60	8.96E+09	1.95E+06	2.35E+03	11.19	11.83
EPA-08	60	8.96E+09	1.95E+06	2.24E+03	10.65	11.26
EPA-09	60	8.85E+09	2.10E+06	1.76E+03	8.37	8.32
EPA-09	60	8.85E+09	2.10E+06	1.63E+03	7.74	7.69
					Average (SD)	11.59 (3.51)

Effect of Collection Medium on the Performance of the NIOSH Sampler: The experimental results showed that the NIOSH sampler is not as efficient at maintaining viral viability as the SKC sampler. Since virus is collected directly in liquid media with the SKC sampler, we suspected that prolonged drying of the collected virus, onto the walls of the collection tubes or onto the backup filter with the NIOSH sampler, could reduce viability. Therefore, the collection environment was modified as follows to address this issue:

Collection into supplemented HBSS: In EPA-04 which used the 1-jet collision nebulizer, one of the three NIOSH samplers contained no collection media and the other two had 1 or 5 ml of supplemented HBSS in the Stage 1 tubes and 0.5 ml of supplemented HBSS in the Stage 2 tubes. No plaques were observed in the Stage 1 tubes of any of the three samplers (data not shown), which suggests that the addition of 1 or 5 ml of supplemented HBSS in the tubes did not improve the collection efficiency presumably because the majority of viral particles were deposited at the top of the collection tubes and cannot reach the collection media in the lower portion of the tubes. Therefore, in EPA-19, we attempted to improve recovery and viability by increasing the amount of supplemented HBSS to either 10 ml or 12 ml in Stage 1 tubes; 0.5 ml supplemented HBSS was also added to the Stage 2 tubes. The results (Figure 3) show that NIOSH samplers, with or without supplemented HBSS, collected similar amounts of total viral particles but viability (assessed using the VPA) was reduced by an average of 54.4 % in tubes containing supplemented HBSS.

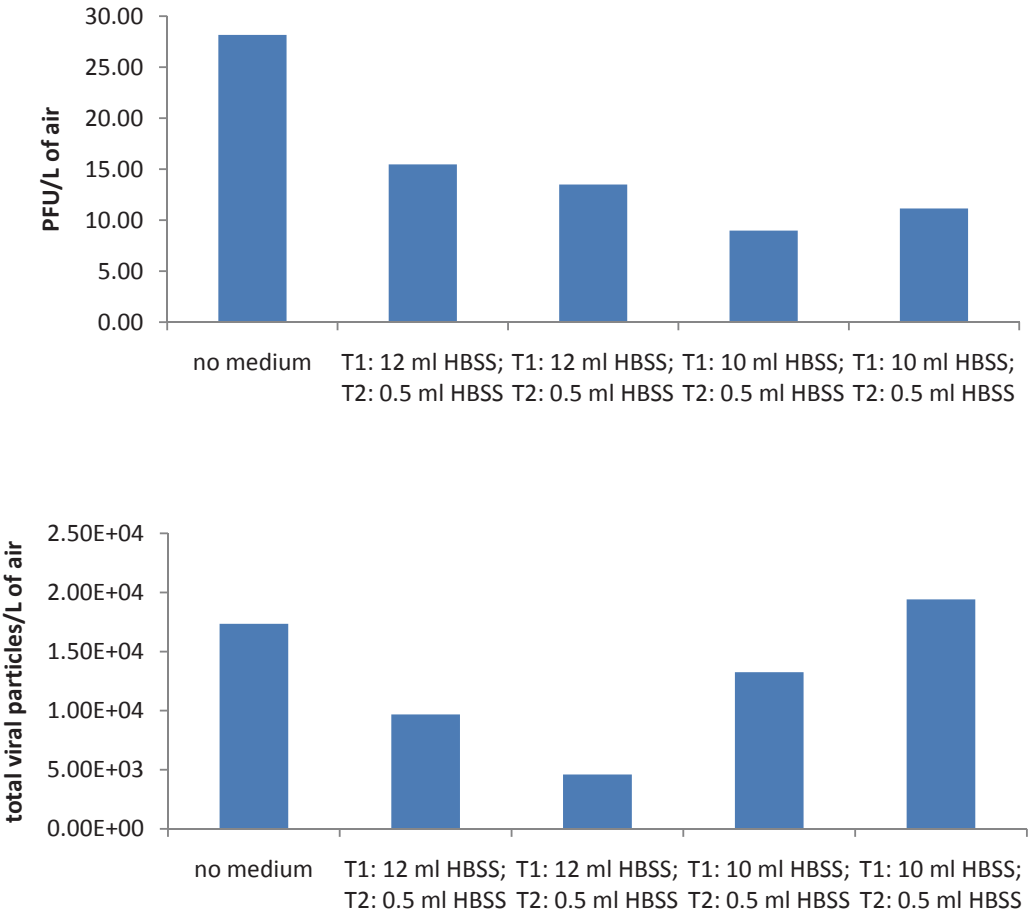


Figure 3. Effect of supplemented HBSS on the collection of viable and total viral particles.

Collection into tubes coated with 2% agar in supplemented HBSS: In EPA-07, the top portion of the stage 1 tube was coated with 2% agar in supplemented HBSS, either by aerosolization or manually. One of the three NIOSH samplers had no coating on the stage 1 tube, one sampler's stage 1 tube was coated with aerosolized agar, and another sampler's stage 1 tube was coated manually with agar. Results demonstrated an average decline in viability of 48% in the tubes coated with agar (Figure 4).

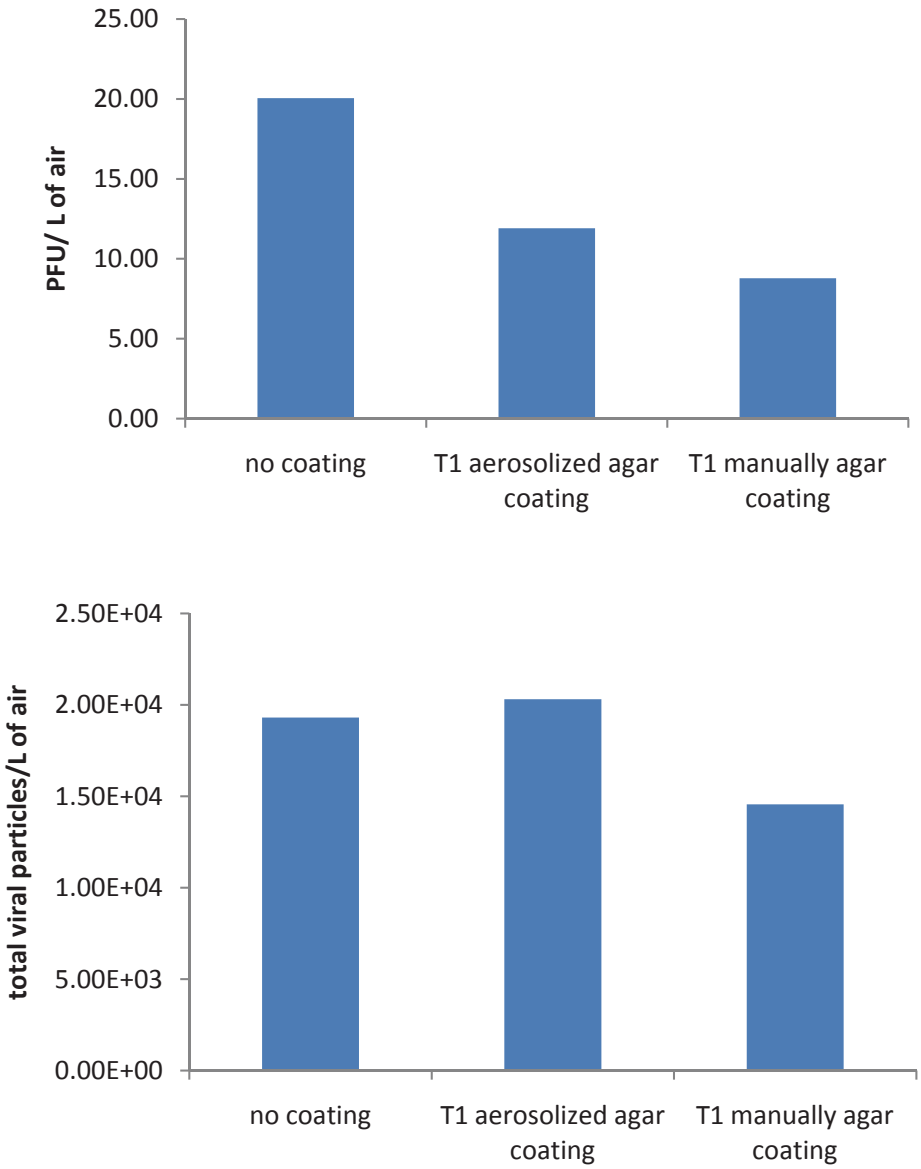


Figure 4. Effect of 2% agar coating on the collection of viable and total viral particles.

Collection into tubes coated with 0.1% mucin: Influenza strains bind sialyloligosaccharides in mucin on tracheal epithelium (11). In EPA-15, the top portion of the stage 1 tube was coated manually with a 0.1% mucin solution. The stage 1 tubes in two NIOSH samplers had no coating, and two NIOSH samplers contained coated stage 1 tubes. The results (Figure 5) showed that the NIOSH samplers with and without coating collected essentially the same amount of viable and total viral particles (average 13.22 versus 12.68 PFU/L of air in the coated and non-coated tubes, respectively; average 1.51 X 10^4 versus 1.46 X 10^4 total viral particles/L of air in the coated and non-coated tubes, respectively).

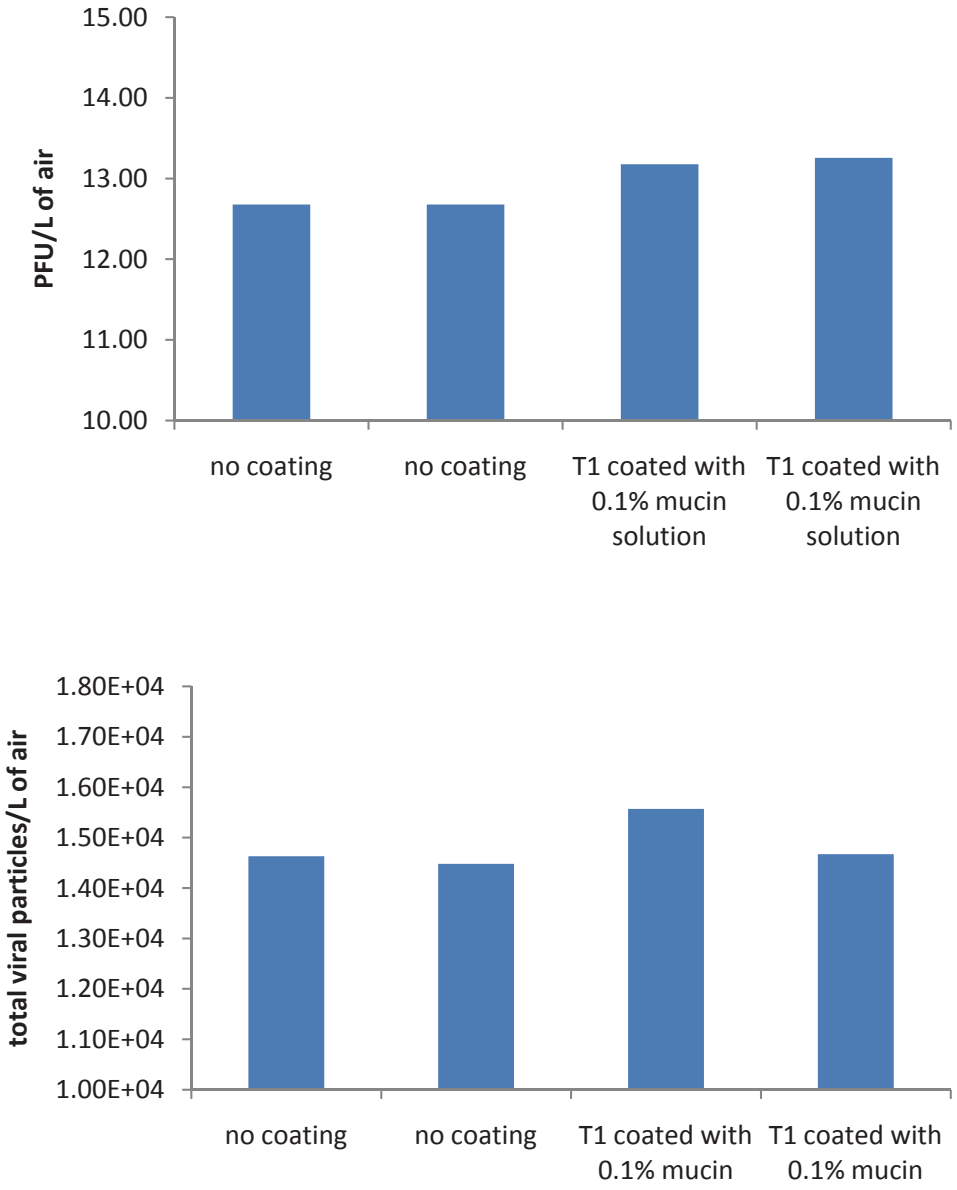
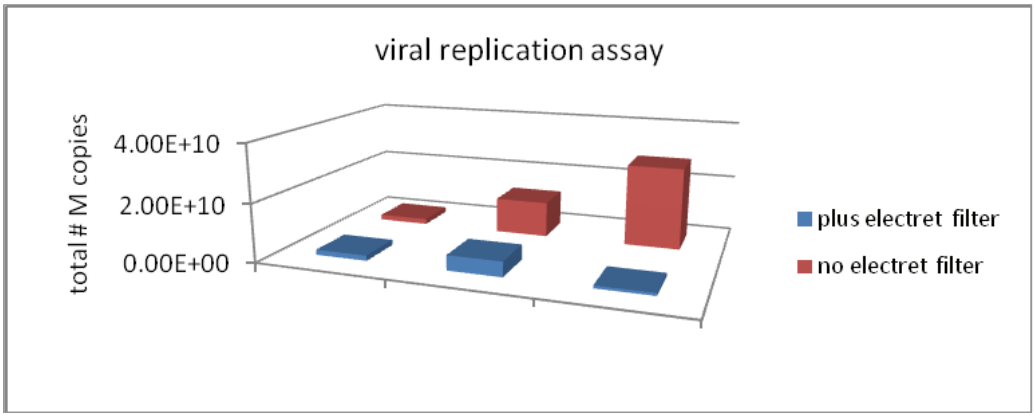
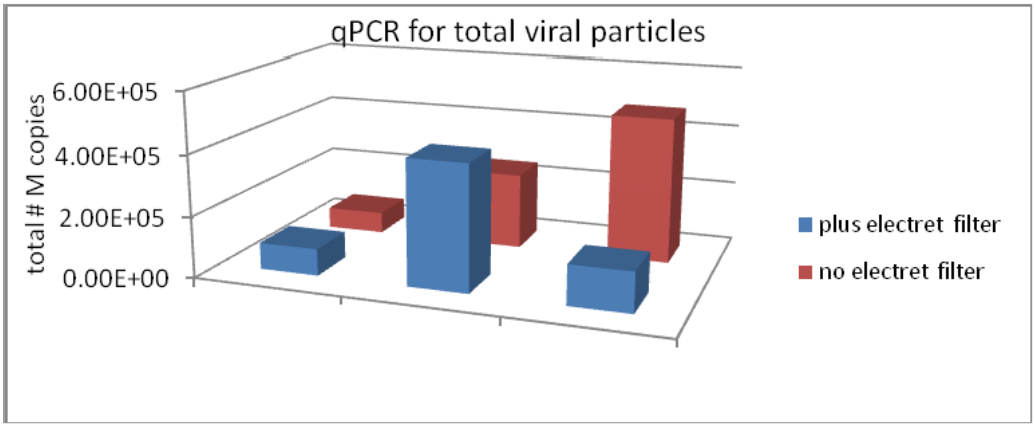


Figure 5. Effect of 0.1% mucin coating on the collection of viable and total viral particles.

Development of an Alternative Viability Assay: To increase sensitivity and improve detection of viable virus, a viral replication assay (VRA) based on a coupled $TCID_{50}$/qPCR assay was developed. In EPA-17 and EPA-18, MDCK cells were infected with the viral samples collected by the NIOSH samplers and the SKC samplers and after 24 h of incubation, the cells were lysed and MDCK cell infection was detected by qPCR analysis. The results from EPA-17 (data not shown) demonstrate high viable viral loads in samples collected from both the NIOSH samplers (total mean 2.1×10^8 virus/L of air) and SKC samplers (total mean 6.4×10^8 virus/L of air). This result supports the above data obtained by the VPA and the concept that viable influenza A can be carried in aerosolized particles.

Coating of electret filters to the Stage 1 and Stage 2 tubes-Since virus is deposited at the tops of the collection tubes, it was possible that the amount of supplemented HBSS added to the collection tubes was still not enough to reach the point of deposition. However, airflow of the sampler would be severely compromised with a further increase in supplemented HBSS to the tubes. As an alternative, in EPA-18, electret filters (12) wetted with supplemented HBSS were inserted inside the top portion of the stage 1 and 2 collection tubes in order to collect virus directly into the liquid environment of the filters. The results (Figure 6) showed that the number of virus collected from the sampler containing the electret filters was about the same as that collected in the absence of the filters (6.3×10^5 versus 8.0×10^5, respectively) but the distribution of virus was significantly affected. The number of virus collected in stage 1 (>4 μm particles) and stage 2 (1-4 μm particles) increased 25% and 64%, respectively, while the amount of virus on the filter (<1 μm particles) decreased 73%. The VRA results, however, revealed a further 67% decrease in viral viability in the presence of the electret filters. The reduced viability of the collected virus may be the result of drying of the electret filters during the sample collection. A method to keep the filters wetted during sampling is under consideration.





viral replication assay			
		total # M copies	
	T1 plus electret filter	1.81E+09	
	T2 plus electret filter	5.20E+09	
	1F	9.46E+08	
	T1 no electret filter	1.87E+09	
	T2 no electret filter	1.24E+10	
	53F	2.83E+10	
qPCR for total viral particles			
		total # M copies	
	T1 plus electret filter	8.77E+04	
	T2 plus electret filter	4.10E+05	
	1F	1.31E+05	6.29E+05
	T1 no electret filter	7.01E+04	
	T2 no electret filter	2.50E+05	
	53F	4.78E+05	7.99E+05

Figure 6. Effect of electret filter coating on the collection of viable and total viral particles.

Effect of storage time on the samples collected by NIOSH samplers and SKC samplers: In field studies, airborne influenza viral particles collected by samplers are generally stored at 4°C for up to 24 h before processing. To investigate whether influenza viral samples remain viable when stored at 4°C prior to processing, we conducted EPA-14 in which four NIOSH samplers and two SKC samplers were used to collect the viral particles. After collection, the samples in two NIOSH samplers were immediately extracted and assayed. The samples in the other two NIOSH samplers were immediately resuspended in supplemented HBSS and then stored at 4°C for 24 h before processing. For the SKC samples, half of the extracted sample from each sampler was processed immediately and the other half was stored at 4°C for 24 h before processing. The results (Figure 7) showed that 24 h storage at 4°C does not significantly affect viral viability.

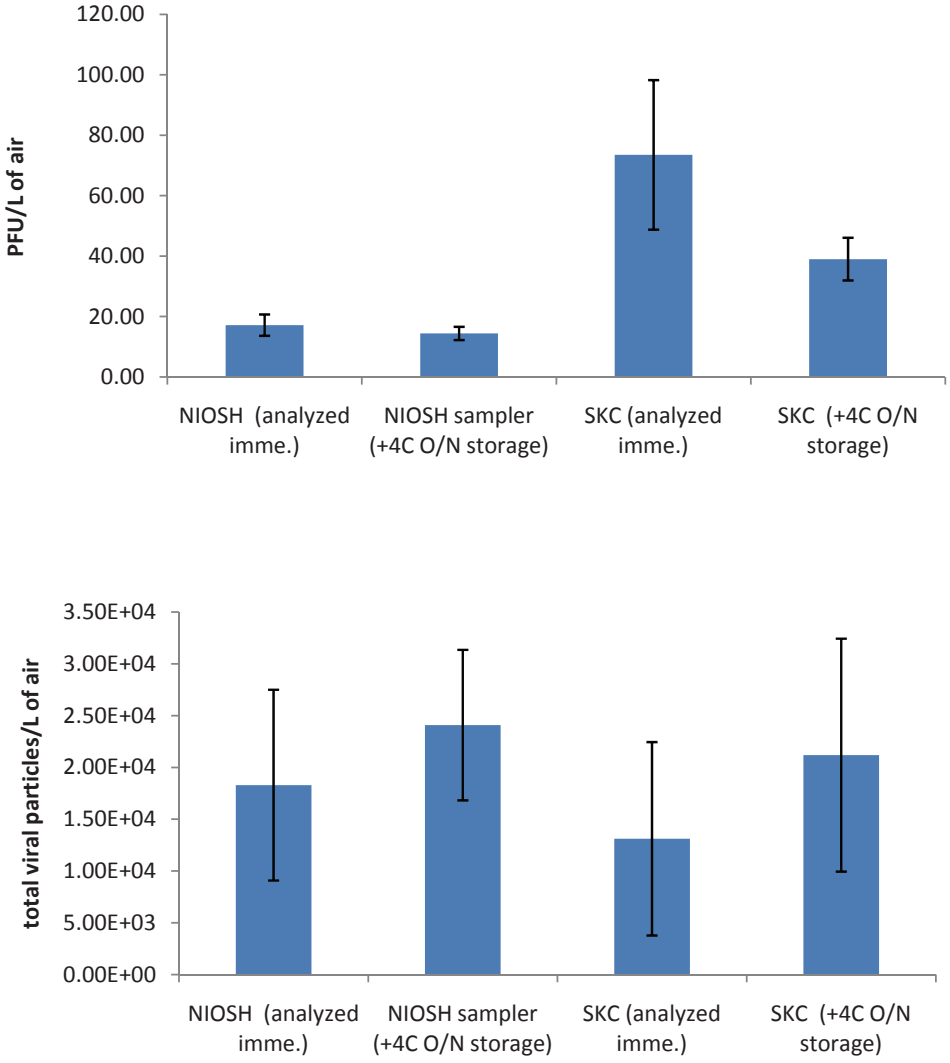


Figure 7. Effect of storage time on the samples collected by NIOSH and SKC samplers

Distribution of collected airborne influenza virus in different sampling stages: The particle sizes and concentrations of aerosols loaded in the calm-air chamber were monitored by an aerodynamic particle sizer (APS) for each experiment. A typical particle size distribution of aerosols (mass based) loaded in the calm-air chamber is shown in Figure 8. The mass median aerodynamic diameter was 1.0 µm. Our previous studies (3) showed that when chamber air loaded with viral-laden aerosols is drawn into an inlet at 3.5 L/min, particles with diameter larger than 4 µm are collected in the Stage 1 tube of the NIOSH sampler, 1-4 µm particles are collected in the Stage 2 tube, and particles with diameter smaller than 1 µm are collected on a backup filter. The average distribution of collected viable and total viral particles in the collection tubes and backup filter from all calm air chamber experiments is shown (Table 7). Eighty-six percent of viable particles (mean of the samples collected at three collection times) were contained in aerosols with diameters ≤ 4 µm. When calm air experimental results are averaged, the distribution of the viable viral particles in the two stages and on the backup filter was similar regardless of collection times though the distribution of the collected total viral particles appeared to vary with collection times. When compared to the 15 min collection time, the fraction of the collected total viral particles after a 60 min collection decreased from 21% to 3% in the Stage 1 tubes and increased from 37% to 63% on the backup filter (Table 7).

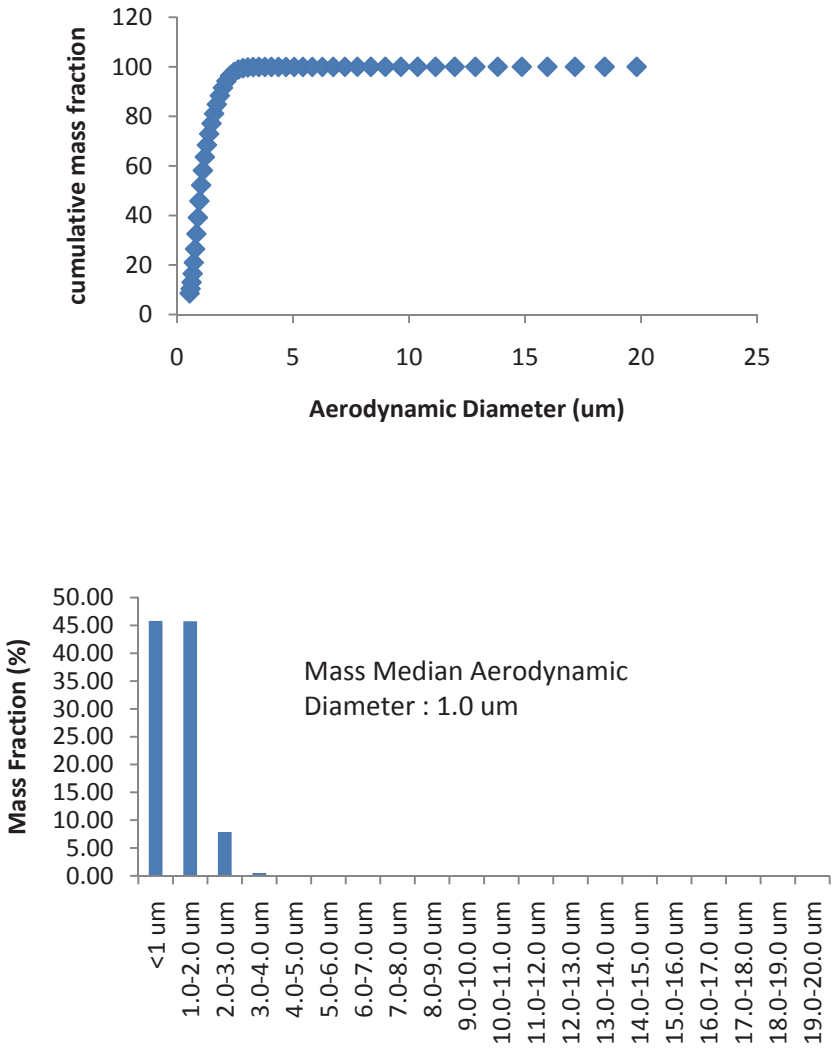


Figure 8. A typical size distribution of aerosols in the calm-air chamber.

However, examination of individual experiments emphasizes the variability observed among experiments. This can be seen, for example, by comparing EPA-08 and EPA-09 where collection occurred for 30 and 60 min in each experiment, with EPA-10 where collection occurred for 15 and 30 min (Table 8). Within the same experiment, the samplers with different collection times showed less variability when comparing the distribution of viable with total viral particles, indicating that the NIOSH samplers performed consistently over time within a given experiment. In contrast, in EPA-10 a higher fraction of the total viral particles was found in the Stage 1 tubes than that found in EPA-08 and EPA-09. This variation may be the result from using different concentrations of the initial viral stocks for generation of the airborne influenza viral particles. EPA-10 had 6.4 times more viral particles in the nebulizer solution than EPA-08, which may have led to a concentration-dependent distribution of viral particles.

Table 7. Distribution of Collected Viable and Total Airborne Influenza Virus in the Stage 1, Stage 2 and Backup Filter of the NIOSH Samplers (Average of all the NIOSH Samplers Used in the Project)[a]

Collection time (min)	Distribution of collected viable influenza virus			Distribution of collected total viral particles		
	T1	T2	F	T1	T2	F
15	19(1)%	55(2)%	26(1)%	21(3)%	42(2)%	37(1)%
30	12(5)%	43(11)%	45(12)%	11(7)%	31(12)%	58(13)%
60	11(6)%	48(9)%	41(11)%	3(2)%	34(5)%	63(7)%

[a] The number in parentheses is the standard deviation.

Table 8. Distribution of Collected Viable and Total Airborne Influenza Virus in the Stage 1, Stage 2 and Backup Filter of the NIOSH Samplers (The Individual Experiments for the Effect of Collection Times)

Experiment #	Sampler stages	30 min		60 min	
		Viable	Total	Viable	Total
EPA-08	T1	7.91%	4.18%	13.60%	3.3%
	T2	57.08%	32.13%	52.11%	36.24%
	F	35.01%	63.70%	34.29%	60.46%
EPA-09	T1	11.42%	0.33%	9.29%	0.81%
	T2	35.72%	20.63%	39.84%	28.61%
	F	52.86%	79.03%	50.87%	70.58%
Experiment #	**Sampler stages**	**15 min**		**30 min**	
		Viable	**Total**	**Viable**	**Total**
EPA-10	T1	18.60%	21.34%	22.11%	25.12%
	T2	55.01%	41.82%	51.19%	39.83%
	F	26.39%	36.84%	26.70%	35.05%

4.0 Discussion

Concerns regarding the vulnerability of large populations to potentially deadly pathogens such as viruses, and the emergence of pandemic strains of influenza A, have prompted a number of studies into the mechanisms of their transmission (13-20). Of the various modes of transmission possible, aerosol transmission poses a profound threat since it has the greatest potential for widespread dissemination. Aerosolized virus in particles less than 4 μm in diameter can be easily inhaled into the alveolar region of the lungs and can also remain airborne for an extended period of time (4). Recent studies revealed that 42-53% of influenza virus in healthcare facilities was associated with airborne particles less than 4 μm in size, but whether the viability of the virus was not determined (2,21). There are a number of aerosol samplers in use, yet these vary greatly in their efficiency of collection and in their ability to maintain the viability of collected viruses. In addition, the determination of the magnitude of a potential epidemic would require a determination of the presence of infectious aerosolized virus. The presence of infectious aerosolized virus, in turn, would requires size-fractionation. However, none of the samplers can size-fractionate particles. Therefore, the technical limitations of the samplers interfere with the ability to determine the magnitude of a potential epidemic.

In a recent study (1), the efficiencies of four commercial air samplers were compared. In that study, the SKC sampler was superior to 37 mm cassette samplers containing either a Teflon filter (74% recovery) or a gelatin filter (63% recovery), and the CCI sampler that contains a polyurethane foam filter (32% recovery). The NIOSH sampler's efficiency at collecting aerosolized particles for 30 min from a calm-air chamber is essentially the same as that from the SKC sampler that collects particles directly into a liquid media (1.2×10^4 TVP/L of air versus 1.3×10^4 TVP/L of air, respectively). Further, the efficiency is relatively constant over the collection times of 15, 30, and 60 min.

In the initial assessment of the NIOSH sampler, the efficiency of extracting virus that had been spiked directly into the Stage 1 collection tube and backup filter was addressed. On average, 11% of the virus could be recovered from the filter and 15.8% could be recovered from the Stage 1 tube. The viability of virus extracted from the filter and the Stage 1 tube was 56.7% and 58%, respectively. These results are in stark contrast to the >95% recovery of virus that was first aerosolized and collected by the NIOSH sampler. Fabian et al. (1) reported a similar finding in that particle extraction efficiency of spiked or aerosolized virus from a particular sampler was not consistent. They emphasized the importance of conducting sampler experiments using test aerosols rather than spiking experiments.

The SKC sampler is reported to be the most efficient aerosol sampler at maintaining essentially 100% viability of collected virus (1). In contrast, recoveries of viable virus from samplers with a gelatin filter, Teflon® filter, or polyurethane filter, were only 10%, 7%, and 22%, respectively (1). The NIOSH sampler maintains viable recovery at an average 26% and, thus, surpasses all but the SKC sampler. In this study, the viability of virus in the initial suspension used to generate the aerosolized particles was only 0.06-1.43% (average 0.53%) of the total viral particles. Viral stocks with similarly low (0.3-0.5%) viability were used in the study by Fabian et al. (1). Conceivably the viability is actually much higher as viability assessed by the chicken embryo infectious dose endpoint 50% ($CEID_{50}$) from the manufacturer (ATCC) is 10-50X higher. This discrepancy may be due to a lack of sensitivity of the VPA for determining viability or due to the decline in stock viability with storage time. Whatever the cause, there is a need to increase the sensitivity of the viability assessments for future studies. To address this issue, a VRA based on a coupled $TCID_{50}$/qPCR assay to amplify viral particle number and increase sensitivity was developed in the later stage of this project. The VRA was used to analyze the initial viral solutions and the aerosol samples in EPA17 and EPA18. The results showed highly viable viral loads in the viral suspensions and the samples, yet how the viable viral loads detected by the VRA relate to the actual number of viable virus prior to viral replication is unknown. This is because the VRA detects virus that has infected and replicated from an unknown copy number within MDCK cells during the 24 h incubation period. The amount of virus per cell is expected to exponentially increase and then plateau at some point during the 24 h incubation. Therefore, the number of viral replications/cell is not easily determined. Additionally, virus secreted from an infected MDCK cell can subsequently infect MDCK cells that were not initially infected, further complicating the quantification of starting virus in each collection sample. Further studies are needed to correlate the number of total viral particles collected and detected by the qPCR with the viability results of the VRA. In the future, an assay with sufficient sensitivity, and a viral suspension with a large percentage of viable

viral particles for nebulization, will certainly reduce the experimental uncertainties and improve viability studies on airborne virus.

A previous study on collection of viable airborne virus (1) has suggested that samplers containing collection medium can preserve the viability of collected virus better than those without collection medium. The NIOSH sampler retains viability at 26% of the total viral population; however, we attempted to improve this by collecting viral particles into a moist environment. To this end, we added supplemented HBSS media to the collection tubes. We found that the addition of supplemented HBSS did not improve viability, possibly because the virus is deposited at the top of the tube and above the media rather than into the media. Adding more media, however, would negatively alter the aerodynamics of collection. Alternatively, the collected viral particles may have become too dilute and the sensitivity of the plaque assay may have been compromised. Diverting the deposition of viral particles to a lower part of the collection tube would reduce the amount of supplemented HBSS needed for collection, without adversely altering the aerodynamics of the sampler. To circumvent the above problems, the top portion of the Stage 1 collection tubes were coated with 2% agar or 0.1% mucin, or supplemented HBSS-soaked electrets filters were inserted near the top of the tubes. However, neither viability nor the total number of recovered viral particles increased over the tubes lacking these materials. It is possible that the extraction of virus from these coatings was insufficient and, as a result, the coatings dried-out during the collection period. Alternatively, the extraction of virus could have been too harsh and, as a result, viability was negatively affected.

The results from these studies support the use of the NIOSH sampler for analysis of air quality in an outside environment or within occupational environments such as hospital emergency rooms. Analyses of outside air for aerosolized virus may be complicated by the presence of other microorganisms, dust, or pollen that may alter the aerodynamics of fractionation or provide a hitch-hiking mode of transport for the virus. We have investigated whether the NIOSH sampler can fractionate co-aerosolized particles. We showed that, while efficient separation of influenza A and *Aspergillus versicolor* fungal spores was possible, there was a shift in the overall deposition of virus to the Stage 1 collection tube, and fewer viral particles were found on the backup filter (3). Further, other environmental parameters such as humidity likely influence whether fractionation is shifted or whether viable virus is recovered. An early study showed that the stability of influenza A is minimal at 50% relative humidity (RH), high at 60-80% RH, and maximal at 20-40% RH (22). A more recent study essentially confirmed those results and showed that transmission of influenza does not occur at 80% RH, is low at 50% RH, high at 65% RH, and maximal at 20% and 35% RH (23). The stability of other viruses including Semliki forest virus (24), HIV (25), and respiratory syncytial virus (26) were shown to be affected by the RH. For this study, we maintained the RH at 20% for all experiments; future work would include an investigation into the role of humidity on viability.

An important distinction between the NIOSH sampler and the SKC sampler is the ability to fractionate aerosols and identify which fractions contain viable virus. Coughing, sneezing, and talking generate airborne particles ranging in size from a few millimeters to less than one micrometer. Particles less than 10 µm are, arguably, the most problematic as they can remain airborne for hours and are readily inhaled deeply into the respiratory tract. Knowing whether viable influenza A is present on these small particles provides better assessments for risk of infection and precautionary guidelines for prevention. Such information would dictate the type of particle mask or respirators to use, enable appropriate adjustments to air handling systems, and determine which aerosol-generating medical procedures to avoid during influenza outbreaks. Unlike the SKC BioSampler, the NIOSH sampler is easier for people to wear as a personal sampler because it does not require aqueous buffers for capturing airborne virus and, therefore, potential spillage onto clothing is eliminated. Additionally, field use is simplified as the collection tubes of the NIOSH sampler can be capped and stored after air sampling, and virus deposited on the dry walls of the tubes can be later recovered into liquid media. In contrast, virus collected directly into liquid media is potentially more labile and the samples would need to be maintained at cooler temperatures. Lastly, air sampling with the NIOSH sampler occurs at 3.5 L/min versus 12.5 L/min with the SKC sampler, and, thus, a smaller and lighter sampling pump could be worn.

5.0
Conclusions

The use of the NIOSH aerosol sampler for investigation of real-world environmental samples holds much promise. The sampler is a lightweight device that could be used either as an area sampler (i.e. hospital room), or as a personal breathing zone air-sampler that could be worn on the clothing of healthcare workers or others. The NIOSH sampler eliminates sample loss, and minimizes the sample contamination and degradation that is associated with most other aerosol samplers. The demonstrated ability of the sampler to collect a viable surrogate virus, influenza A, leads us to be cautiously optimistic about its potential use in the detection of other viruses and microorganisms. Increased acceptance of the NIOSH sampler as a better alternative to other air samplers requires improvements in viability retention, particularly from the backup filter that collects the smallest particles, i.e., those most likely suited for long-range dissemination and deep lung inhalation.

6.0 References

1. Fabian P, McDevitt JJ, Houseman EA, and Milton DK. Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: considerations for a new infectious virus aerosol sampler. *Indoor Air* 19(5):433-41, 2009.
2. Blachere FM, Lindsley WG, Pearce TA, Anderson SE, Fisher M, Khakoo R, Meade BJ, Lander O, Davis S, Thewlis RE, Celik I, Chen BT, and Beezhold DH. Measurement of Airborne Influenza in a Hospital Emergency Department. *Clin Infect Dis* 48:438-40, 2009.
3. Blachere FM, Lindsley WG, Slaven JE, Green BJ, Anderson SE, Chen BT, and Beezhold DH. Bioaerosol sampling for the detection of aerosolized influenza virus. *Influenza and Other Respiratory Viruses* 1(3): 113-120, 2007.
4. Hinds WC. In *Aerosol Technology*: *Properties, Behavior and Measurement of Airborne Particles* 2nd Ed John Wiley & Sons, NY pp 233-257, 1999.
5. Sattar SA and Ljaz MK. Airborne viruses. In: *Manual of Environmental Microbiology* (Hurst CJ, Crawford RL, McInerney MJ, Knudsen GR, Stetzenbach LD, eds) ASM Press, Washington DC, pp 871-883, 2002.
6. Weber TP and Stilianakis NI. Inactivation of influenza A viruses in the environment and modes of transmission: a critical review. *J Infect* 57(5):361-73, 2008.
7. Agranovski IE, Safatov AS, Borodulin AI, Pyankov OV, Petrishchenko VA, Sergeev AN, Agafonov AP, Ignatiev GM, Sergeev AA, and Agranovski V. Inactivation of viruses in bubbling processes utilized for personal bioaerosol monitoring. *Appl Environ Microbiol* 70(12):6963-7, 2004.
8. Tseng C and Li C. Collection efficiencies of aerosol samplers for virus-containing aerosols. *J Aerosol Sci* 36:593-607, 2005.
9. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, and Suarez DL. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 40(9):3256-60, 2002.
10. Medaglia SP. SpinCo® based air sampler foot and mouth disease virus (FMDV) test report. Kansas City, Missouri: Spector Industries, 2001.
11. Couceiro JN, Paulson JC, and Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res* 2:155-65, 1993.
12. Lee, M-H, Otani Y, Namiki N, and Emi H. Prediction of Collection Efficiency of High-Performance Electret Filters. *J Chem Eng Jpn* 35(1):57-62, 2002.
13. Johnson N, Phillpotts R, and Fooks AR. Airborne transmission of lyssaviruses. *J Med Microbiol* 55(6):785-90, 2006.
14. Booth TF, Kournikakis B, Bastien N, Ho J, Kobasa D, Stadnyk L, Li Y, Spence M, Paton S, Henry B, Mederski B, White D, Low DE, McGeer A, Simor A, Vearncombe M, Downney J, Jamieson FB, Tang P, and Plummer F. Detection of airborne severe acute respiratory syndrome (SARS) coronavirus and environmental contamination in SARS outbreak units. *J Infect Dis* 191(9):1472-7, 2005.
15. Myatt TA, Johnston SL, Zuo Z, Wand M, Kebadze T, Rudnick S, and Milton DK. Detection of airborne rhinovirus and its relation to outdoor air supply in office environments. *Am J Respir Crit Care Med* 169(11):1187-90, 2004.
16. Yu IT, Li Y, Wong TW, Tam W, Chan AT, Lee JH, Leung DY, and Ho T. Evidence of airborne transmission of the severe acute respiratory syndrome virus. *N Engl J Med* 350(17):173-9, 2004.
17. Bray M. Defense against filoviruses used as biological agents. *Antiviral Res* 57(1-2):53-60, 2003.
18. Salomon R and Webster RG. The influenza virus enigma. *Cell* 136:402-10, 2009.

19. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F et al. Pandemic potential of a strain of influenza A (H1N1): early findings. *Science* 324:1557-61, 2009.

20. Tellier R. Aerosol transmission of influenza A virus: a review of new studies. *J R Soc Interface* 6:S783-S790, 2009.

21. Lindsley WG, Blachere FM, Davis KA, Pearce TA, Fisher MA, Khakoo R, Davis SM, Rogers ME, Thewlis RE, Posada JA, Redrow JB, Celik IB, Chen BT, and Beezhold DH. Distribution of airborne influenza virus and respiratory syncytial virus in an urgent care medical clinic. *Clin Infect Dis* 50(5):693-8, 2010.

22. Schaffer FL, Soergel ME, and Straube DC. Survival of airborne influenza virus: effects of propagating host, relative humidity, and composition of spray fluids. *Arch Virol* 51:263-273, 1976.

23. Lowen AC, Mubareka S, Steel J, and Palese P. Influenza virus transmission is dependent on relative humidity and temperature. *PLoS Pathog* 3(10):1470-6, 2007.

24. De Jong JC, Harmsen M, Plantinga AD, and Trouwbrost T. Inactivation of Semliki forest virus in aerosols. *Appl Environ Microb* 32(3):315-9, 1976.

25. Hearps AC, Ryan CE, Morris LM, Plate MM, Greengrass V, and Crowe SM. Stability of dried blood spots for HIV-1 drug resistance analysis. *Curr HIV Res* 8(2):134-40, 2010.

26. Welliver RC Sr. Temperature, humidity, and ultraviolet B radiation predict community respiratory syncytial virus activity. *Pediatr Infect Dis J* 26(11 Suppl):S29-35, 2007.

EPA
United States
Environmental Protection
Agency

PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development (8101R)
Washington, DC 20460

Official Business
Penalty for Private Use
$300