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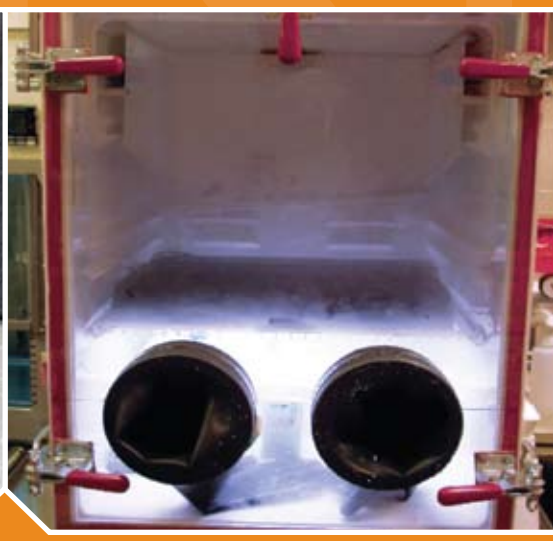
Highly Pathogenic Avian Influenza H5N1 Virus Persistence Testing and Evaluation of Liquid Decontamination Technologies

INVESTIGATION AND TECHNOLOGY EVALUATION REPORT



Office of Research and Development
National Homeland Security Research Center

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INVESTIGATION AND TECHNOLOGY
EVALUATION REPORT

Highly Pathogenic Avian Influenza H5N1 Virus Persistence Testing and Evaluation of Liquid Decontamination Technologies

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Disclaimer

The U.S. Environmental Protection Agency, through its Office of Research and Development's National Homeland Security Research Center, funded, directed, and managed this investigation and technology evaluation through a Blanket Purchase Agreement under General Services Administration contract number GS23F0011L-3 with Battelle. All tests were conducted at Battelle facilities located in West Jefferson, Ohio. It has been reviewed by U.S. Environmental Protection Agency but does not necessarily reflect the Agency's views. No official endorsement should be inferred. U.S. Environmental Protection Agency does not endorse the purchase or sale of any commercial products or services.

Foreword

The U.S. Environmental Protection Agency is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the U.S. Environmental Protection Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the Agency's Office of Research and Development provides data and scientific support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

In September 2002, the Agency announced the formation of the National Homeland Security Research Center. The Center is part of the Office of Research and Development; it manages, coordinates, and supports a variety of research and technical assistance efforts. These efforts are designed to provide appropriate, affordable, effective, and validated technologies and methods for addressing risks posed by chemical, biological, and radiological terrorist attacks. Research focuses on enhancing our ability to detect, contain, and clean up in the event of such attacks.

The Center has developed the Technology Testing and Evaluation Program in an effort to provide reliable information regarding the performance of homeland security related technologies. The Technology Testing and Evaluation Program provides independent, quality assured performance information that is useful to decision makers in purchasing or applying the tested technologies. It provides potential users with unbiased, third-party information that can supplement vendor-provided information. Stakeholder involvement ensures that user needs and perspectives are incorporated into the test design so that useful performance information is produced for each of the tested technologies. The technology categories of interest include detection and monitoring, water treatment, air purification, decontamination, and computer modeling tools for use by those responsible for protecting buildings, drinking water supplies and infrastructure, and for decontaminating structures and the outdoor environment.

The evaluation reported herein was conducted by Battelle as part of Technology Testing and Evaluation Program. Information on National Homeland Security Research Center and Technology Testing and Evaluation Program can be found at http://www.epa.gov/nhsrc.

Acknowledgments

The authors wish to acknowledge the support of all those who helped plan and conduct the evaluation, analyze the data, and prepare this report. We also would like to thank the following U.S. Environmental Protection Agency's personnel for their reviews Dr. Worth Calfee (National Homeland Security Research Center); Dr. Wendy Davis-Hoover (National Risk Management Research Laboratory); and Mr. Jeff Kempter (Office of Pesticide Programs).

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Abbreviations/Acronyms

AOAC	AOAC International (formerly the Association of Analytical Chemists)
°C	degrees Celsius
$CaCO_3$	calcium carbonate
CEK	chicken embryo kidney
CO_2	carbon dioxide
CPE	cytopathic effects
D/E	Dey and Engley
EPA	U.S. Environmental Protection Agency
H5N1	highly pathogenic avian influenza virus
H7N2	low pathogenic avian influenza virus
MDCK	Madin-Darby canine kidney
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Na_2CO_3	sodium carbonate
NHSRC	National Homeland Security Research Center
PBS	phosphate-buffered saline
QA	quality assurance
QC	quality control
QMP	quality management plan
RH	relative humidity
$TCID_{50}$	50% tissue culture infectious dose
TTEP	Technology Testing and Evaluation Program
UV	ultraviolet radiation

Unit Abbreviations

°C	degrees Celsius
cm	centimeter
g	gram
hr	hour
min	minutes
mL	milliliter
mm	millimeter
ppm	parts per million
rpm	revolutions per minute
µL	microliter
µm	micrometer
µW	microwatt

Definitions

$TCID_{50}$	is a quantitative measure of infectivity and growth and is the number of organisms that must be present to infect 50% of the cell culture wells tested.
UV-A/B	ultraviolet radiation within the wavelength range of 280–400 nanometers
UV-A	320–400 nanometers
UV-B	280–320 nanometers
UV-C	ultraviolet radiation within the wavelength range of 200–280 nanometers

Executive Summary

The U.S. Environmental Protection Agency's (EPA) National Homeland Security Research Center (NHSRC) Technology Testing and Evaluation Program (TTEP) helps to protect human health and the environment by carrying out performance tests on homeland security technologies. Under TTEP, the persistence of the highly pathogenic avian influenza H5N1 virus on test coupons prepared from chicken feces, galvanized metal, glass, and soil was investigated. The performance of liquid technologies to decontaminate the H5N1 virus from test coupons of galvanized metal and soil was also investigated at room and low temperatures.

For persistence testing, test coupons were contaminated by spiking each coupon with a quantity of H5N1 virus amounting to a 50% tissue culture infectious dose ($TCID_{50}$) of H5N1 virus (A/Vietnam/1203/04) of at least 1 x 10^6. The persistence of the H5N1 virus was investigated for up to three test durations at five different environmental conditions [each environmental condition is described by a specific temperature, relative humidity (RH) value, and presence/absence of exposure to ultraviolet (UV-A/B) radiation to simulate sunlight]. Please note that all references to and quantifications of the H5N1 virus in this document refer to viable H5N1 virus. The environmental conditions were:

- Room temperature, low RH, no UV
  - Test durations: 1-, 2-, and 4-day
- Room temperature, high RH, no UV
  - Test durations: 1-, 2-, and 4-day
- Low temperature, low RH, no UV
  - Test durations: 4-, 8-, and 13-day
- Low temperature, high RH, no UV
  - Test durations: 4-, 9-, and 13-day
- Low temperature, low RH, UV-A/B
  - Test durations: 24-, 48-, and 96-hours (continuous UV-A/B exposure)

Note that for all experiments, the target room temperature was 22 degrees Celsius (°C), target low temperature was 4 °C, target high RH was 80%, and target low RH was 40%.

The persistence testing yielded the following results. At room temperature (under low RH and high RH, with no UV), the H5N1 virus did not persist on galvanized metal and glass at time periods of one day or greater while generally persisting on chicken feces and soil for less than two days.

At low temperature (under low RH and high RH, with no UV) the H5N1 virus persisted for at least four days on all materials. Following exposure to the low temperature, low RH, no UV environmental condition, the H5N1 virus was detected after 13 days on galvanized metal, glass, and soil. The H5N1 was also viable following exposure to the low temperature, high RH, no UV environmental condition, after nine days on chicken feces, glass, and soil. Although testing was not conducted for durations longer than 13 days for any of the environmental conditions, the H5N1 virus persistence may exceed 13 days, especially on galvanized metal and glass under the low temperature, low RH, no UV environmental condition.

With continuous UV-A/B exposure (and under a low temperature and low RH environmental condition), the H5N1 virus persisted less than 48 hours on galvanized metal and glass but persisted at least 48 hours on chicken feces and soil. A summary of the actual test conditions and the quantities of the H5N1 virus (expressed as individual $TCID_{50}$ values) recovered from chicken feces, galvanized metal, glass, and soil is provided in Table ES-1.

Table ES-1. Summary of Persistence Test Conditions and H5N1 Virus Recoveries[a]

Environmental Condition / Test Duration	Temperature (°C)[b]	RH (%)[b]	Mean Recovered H5N1 Virus ($TCID_{50}$)			
			Chicken feces	Galvanized metal	Glass	Soil
Room Temperature, Low RH, No UV						
1-Day	23	35	6.37×10^{2} [c]	ND	ND	8.82×10^{3} [d]
2-Day	22	36	ND	ND	ND	ND
4-Day	23	49	ND	ND	ND	ND
Room Temperature, High RH, No UV						
1-Day	22	89	7.70×10^{4}	ND	ND	8.26×10^{4}
2-Day	23	90	3.16×10^{3} [c]	ND	ND	ND
4-Day	24	91	ND	ND	ND	ND
Low Temperature, Low RH, No UV						
4-Day	4	28	7.11×10^{4}	3.78×10^{5}	4.98×10^{5}	2.47×10^{4}
8-Day	7	15	3.16×10^{3} [c]	7.33×10^{5}	1.34×10^{6}	1.16×10^{3}
13-Day	7	46	ND	3.44×10^{5}	5.91×10^{5}	1.05×10^{3} [d]
Low Temperature, High RH, No UV						
4-Day	8	89	5.42×10^{4}	4.69×10^{3}	1.37×10^{5}	2.21×10^{5}
9-Day	7	97	5.90×10^{4}	ND	3.16×10^{3}	5.93×10^{5}
13-Day	7	79	7.86×10^{2} [d]	ND	ND	ND
Low Temperature, Low RH, UV-A/B[e]						
24-Hours[e]	4	25	1.06×10^{6}	6.42×10^{4}	2.62×10^{2} [d]	5.93×10^{5}
48-Hours[e]	-1	30	9.56×10^{4}	ND	ND	3.90×10^{4}
96-Hours[e]	0.1	28	3.16×10^{3} [c]	Not Tested	Not Tested	3.11×10^{3} [d]

[a] Spike amount ranged from 5.01×10^{6} to 5.01×10^{7} $TCID_{50}$.
[b] Mean temperature and RH values were based on continuous monitoring at 1-minute intervals, with the exception of the room temperature, low RH, no UV environmental condition, which were derived from the mean of the temperature or RH at the start and end of the tests.
[c] $TCID_{50}$ was detected but at a level ≤ the procedural blank $TCID_{50}$ for chicken feces such that the cytopathic effects observed (and used in the calculation of the $TCID_{50}$) may be attributed to the test material rather than H5N1 virus.
[d] H5N1 virus was not detected on some of the replicate test coupons; a value of 1 $TCID_{50}$ was used for non-detects in the calculation of the mean H5N1 virus recovery.
[e] UV-A/B exposures were continuous (i.e., the UV-A/B lamps did not shut off every 12 hours).
ND = No cytopathic effects detected; the detection limit was 1.31×10^{3} $TCID_{50}$.

The liquid decontamination technologies evaluated included 1% citric acid, pH-amended bleach, a hospital grade 732 parts per million (ppm) quaternary ammonium disinfectant, and 8% sodium carbonate (Na_2CO_3) (all were prepared in water with a hardness of 400 ppm as calcium carbonate [$CaCO_3$]). The H5N1 virus was inoculated onto galvanized metal and soil test coupons and exposed to the decontamination liquid for a 10-minute contact time. The decontamination technology evaluation was implemented at room temperature and at a low temperature; UV-A/B lamps were not used.

The decontamination technology evaluation results indicated that only pH-amended bleach was completely effective at inactivating the H5N1 virus (i.e., H5N1 virus was not detected), and this inactivation only occurred on galvanized metal (at both room and low temperatures). On soil, pH-amended bleach induced mean H5N1 virus log reductions in $TCID_{50}$ of 2.7 at room temperature and 2.9 at low temperature. The mean H5N1 virus log reductions in $TCID_{50}$ associated with 1% citric acid ranged from 1.5 (with soil at room temperature) to 2.1 (with galvanized metal at low temperature). The mean H5N1 virus log reductions in $TCID_{50}$ associated with 732 ppm quaternary ammonium and 8% Na_2CO_3 were less than 1.0.

1.0 Introduction

National Homeland Security Research Center's (NHSRC's) Technology Testing and Evaluation Program (TTEP) works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, scientists, and permitters; and with participation of individual technology developers in carrying out performance tests on homeland security technologies. In response to the needs of stakeholders, TTEP investigates the natural persistence of biological and chemical agents and evaluates the performance of innovative homeland security technologies by developing test plans, conducting evaluations, collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure the generation of high quality data and defensible results. TTEP provides unbiased, third-party information supplementary to vendor-provided information that is useful to decision makers in purchasing or applying the evaluated technologies. Stakeholder involvement ensures that user needs and perspectives are incorporated into the evaluation design to produce useful performance information for each evaluated technology.

Under TTEP, the persistence of viable, highly pathogenic avian influenza H5N1 virus was tested and the performance of liquid decontamination technologies to inactivate the H5N1 virus was evaluated. The primary objectives were to determine how long and under what environmental conditions the H5N1 virus remains viable; and to evaluate the efficacy of four generic liquid decontamination technologies.

Persistence testing investigated the amount of the H5N1 virus remaining on chicken feces, galvanized metal, glass, and soil for one to three different durations under the following five environmental conditions:

- Room temperature, low relative humidity (RH), no ultraviolet radiation (UV)
- Room temperature, high RH, no UV
- Low temperature, low RH, no UV
- Low temperature, high RH, no UV
- Low temperature, low RH, UV-A/B

Note that the target room temperature was 22 degrees Celsius (°C), the target low temperature was 4 °C, the target high RH was 80%, and target low RH was 40%.

Four liquid decontamination technologies (1% citric acid, pH-amended bleach, 732 parts per million (ppm) quaternary ammonium and 8% sodium carbonate [Na_2CO_3]) prepared in hard water were evaluated for their effectiveness in inactivating the H5N1 virus. For this technology evaluation, the H5N1 virus was spiked onto two materials (galvanized metal and soil) and the spiked test coupons were treated with the liquid decontaminant for a 10-minute contact time at room temperature and a low temperature (UV-A/B lamps were not used for the decontamination technology evaluation).

Efforts were conducted according to a peer-reviewed test/QA plan[1] that was developed according to the requirements of the TTEP quality management plan (QMP)[2]. The persistence testing and the technology evaluation both used a $TCID_{50}$ (50% tissue culture infectious dose) assay to quantify the H5N1 virus extracted from test coupons. This report documents the log reductions in $TCID_{50}$ associated with a natural reduction in the H5N1 virus under various environmental conditions and the decrease in the H5N1 virus exposed to various liquid decontamination technologies.

Please note that the test/QA plan[1] also includes testing for the low pathogenic avian influenza virus H7N2. Although persistence testing and evaluation of liquid decontamination technologies were not conducted for the H7N2 virus, preliminary research results associated with the H7N2 virus are included in Appendix A.

2.0 Persistence Testing

2.1 Test Materials

Materials considered for H5N1 virus persistence testing are described in Table 2-1. Basswood, concrete, and pine wood were eventually rejected for H5N1 virus persistence testing because of the poor recovery of the H5N1 virus (i.e., <5% of the applied inoculum of approximately 1 x 10^6 $TCID_{50}$ of H5N1 virus per coupon) from these materials (see Section 2.3). Persistence testing was performed using test coupons of uniform size. As indicated in Table 2-1 test coupons for wood, galvanized metal, and glass were each 1.9 centimeter (cm) x 7.5 cm; concrete test coupons were each 1.0 cm x 3.5 cm. Chicken feces and soil test coupons consisted of 3.5 cm diameter Petri dishes, 1 cm high, lined with Parafilm®, and filled with uncompacted material. Straw and feathers were removed from the chicken feces before being placed in the Petri dishes.

Table 2-1. Test Materials

Material	Lot, Batch, or Observation	Manufacturer/ Supplier Name	Approximate Coupon Size, width x length	Approximate Coupon Thickness	Material Preparation
Basswood (a hardwood)	Not applicable	Michaels – Arts and Crafts Store	1.9 cm x 7.5 cm	0.2 cm and 0.7 cm	Autoclaved
Chicken Feces	Not applicable	Veterinarian[a]	--[b]	--[b]	None[c]
Concrete (unpainted)	5 parts sand: 2 parts cement	Wysong Concrete	1.0 cm x 3.5 cm	1 cm	Autoclaved
Galvanized Metal	Heating, ventilation, and air conditioning industry standard 24 gauge galvanized steel	Accurate Fabrication	1.9 cm x 7.5 cm	0.06 cm	Cleaned with acetone; autoclaved
Glass	ASTM C1036	Brooks Brothers	1.9 cm x 7.5 cm	0.3 cm	Autoclaved
Pine Wood (a softwood)	Untreated pine lumber (i.e., no preservative treatment)	Kingswood Lumber	1.9 cm x 7.5 cm	0.2 cm and 1 cm	Gamma irradiation
Soil (topsoil)	Batch No. Py1A0597	GardenScape Brand	--[b]	--[b]	None[c]

[a] Feces was collected from chickens on the day of testing by a veterinarian who raises hens for egg production.

[b] Chicken feces and soil coupons consisted of a 3.5 cm diameter Petri dish with a height of 1 cm lined with Parafilm® and filled with uncompacted material.

[c] Chicken feces and soil were not autoclaved or gamma irradiated given the uncertainties associated with potential side-effects (e.g., potential hydrolysis of humic acids, release of metals, etc.). Instead these materials were filtered (0.2 micrometer [µm]) during the extraction process, which involved agitation and centrifugation of the chicken feces or soil/extraction buffer mixture. The filter was capable of removing bacteria (not viruses) from the supernatant that could interfere with virus quantification. Blanks (non-inoculated coupons) did not indicate any interference with the assay.

2.2 Cytotoxicity of Material Extracts

Prior to persistence testing and the decontamination technology evaluations, the potential for extracts of test coupons (with no virus present) to cause cytotoxicity was determined. Cytotoxic effects caused by test coupon extracts could interfere with the $TCID_{50}$ determination. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic effects of test coupon extracts. For the MTT assay, Madin-Darby canine kidney (MDCK) cells (the cells used to quantitate the $TCID_{50}$) were seeded into each well of a 96-well microtiter plate and exposed to serial dilutions of test material extracts. MDCK cells exposed only to serial dilutions of sterile cell culture medium (the medium in which the virus particles were suspended) were used as controls. The MDCK cells were then incubated in the presence of MTT where mitochondrial succinate dehydrogenase (present in living cells) converts the yellow MTT to a purple formazan salt. The absorbance (optical density) of this purple reaction product is determined using a microplate reader and is proportional to mitochondrial function (i.e., the number of living cells) and is expressed as a percentage of the control cell viability.

The cytotoxicity results showing the lowest dilution able to achieve ≥90% of the control cell viability are summarized in Table 2-2. The cell viability performance criterion (per the test/QA plan[1]) of ≥90% of unexposed control viability with dilution of 1 to 8 (1:8) or less was attained for all materials except soil. However >90% of unexposed soil control viability was obtained with a dilution of 1:16, which was approved as an acceptable level of dilution by the EPA Task Order Project Officer. Although an increased level of dilution was needed for the MTT assay with soil, the $TCID_{50}$ assay was not believed to be affected. This is because cytopathic effects were not generally observed in the MDCK cells exposed to any dilution of non-inoculated soil extract; the detection limit of the $TCID_{50}$ assay was not generally affected by soil. Further, any potential negative bias in the $TCID_{50}$ results due to cytotoxicity of the soil extract would have been indicated through the inadequate positive control recovery, which was not the case.

2.3 H5N1 Virus Recovery From Test Materials

2.3.1 Spiking the Coupons

In order to assess the quantity of virus that could be recovered, following the addition of a known quantity of virus to each of several test coupons, each coupon was spiked with a quantity of virus ranging from 1.98×10^6 to 7.93×10^6 $TCID_{50}$ of H5N1 virus per coupon. One hundred microliters [µL] of stock virus suspension was applied per coupon; the target spike level was 1×10^6 $TCID_{50}$ of H5N1 virus per coupon. The H5N1 virus was a Battelle stock propagated from a parent stock provided by the Centers for Disease Control and Prevention. This H5N1 influenza A virus is the 1,203rd strain isolated from a human in Vietnam in 2004 (A/Vietnam/1203/04). Genetic sequencing was used to verify that propagated stocks matched the parent stock. Spiking of the 1.9 cm x 7.5 cm coupons was conducted using a multichannel micropipette as two rows of five droplets (10 µL per droplet) across the surface of the test coupon. A single channel pipette was used to apply ten 10 µL droplets at separate locations on the surface of each chicken feces and soil test coupon. For concrete, which readily absorbs liquid, the spiking was conducted using a single channel pipette and 100 µL of the inoculum was applied as a single line or streak across the surface of the test coupon.

After spiking, all coupons were allowed to dry undisturbed at 22 ± 2 °C and 40–70% RH for one hour prior to H5N1 virus extraction. Test coupons were placed in an acrylic Compact Glove Box Model 830-ABC (Plas Labs, Inc., Lansing, Mich.), with a volume of 317 liters. Feces collected from chickens on the morning of testing remained moist during the one-hour drying time. The soil, stored in an air-tight container prior to use, also remained moist during the one-hour drying time. Following the one-hour drying time, the H5N1 virus inoculum appeared to absorb into chicken feces and soil and was not visible; however, spots indicative of the dried H5N1 virus inoculum were visible on galvanized metal and glass.

Table 2-2. Material Extract Cytotoxicity Test Results

Material	Mean Control Cell Optical Density[a]	Dilution	Mean Material Extract Optical Density[a]	% Cell Viability
Basswood	0.3612	1:2	0.4604	127[b]
Chicken Feces	0.3108	1:4	0.4114	132[b]
Concrete	0.5557	1:8	0.5075	91.3
Glass[c] Pine Wood[d]	Not Tested	Not Tested	Not Tested	Not Tested
Galvanized Metal	0.2389	1:8	0.2216	92.8
Soil	0.5657	1:16	0.5255	92.9

[a] The optical density (absorbance) is directly proportional to the number of viable cells present in the sample well.
[b] Viability above 100% may reflect non-specific reduction of MTT to formazan from unknown coupon extract components.
[c] The cytotoxicity associated with glass was not evaluated as previous testing indicated there is no interference with MDCK cell viability.
[d] Pine wood was not tested due to insufficient recovery of the H5N1 virus; see section 2.3.3.

2.3.2 H5N1 Virus Extraction and Quantification

For basswood, concrete, galvanized metal, glass, and pine wood, extraction of the H5N1 virus from individual test coupons entailed, following drying, placing a single spiked test coupon into a sterile 50 milliliter (mL) conical vial containing 10 mL of sterile extraction buffer (i.e., phosphate-buffered saline [PBS]). The vials were agitated on an orbital shaker for at room temperature for 15 minutes at approximately 200 revolutions per minute (rpm). The extract was removed from the vial using a pipette and was then serially diluted and plated onto MDCK cells. Quantitation of the H5N1 virus in the extract was based on 10-fold serial dilutions of the material extracts applied to MDCK cells and the subsequent observation of cytopathic effects (CPE) in the MDCK cells to determine the viral concentration. The quantity of virus present was expressed as the $TCID_{50}$ for the H5N1 virus.

The $TCID_{50}$ for H5N1 virus in test coupon extracts was determined by first preparing 10-fold serial dilutions of each extract and then transferring 0.1 mL aliquots of each dilution to five wells (of a 96-well microtiter plate) containing monolayers of MDCK cells. Following transfer of aliquots of the diluted extracts, the 96-well microtiter plate was incubated at 37 ± 2 °C under 5% carbon dioxide (CO_2) in a humidified incubator for 72 to 96 hours or until CPE were visually detected by comparing to the control (cell culture medium only) wells. Using light microscopy each well of the 96-well plate was evaluated and observations documented (+ = positive CPE; 0 = no observed effect) for CPE. The $TCID_{50}$ was calculated using the Spearman-Karber[3] method as follows:

Equation 2-1.

$$\frac{TCID_{50}\ per}{Unit\ Volume} = log\left(\frac{Highest\ Dilution}{with\ 100\%\ CPE}\right) + \left[\frac{1}{2} - \frac{Cumulative\ Number\ of\ Wells\ with\ CPE\ at\ that\ Dilution}{Number\ of\ Wells\ per\ Dilution}\right]$$

For the titer, take the inverse log of $TCID_{50}$ per unit volume. For an inoculating volume of 0.1 mL, the calculated $TCID_{50}$ titer is multiplied by a factor of 10 (10 x 0.1 mL = 1.0 mL) to achieve $TCID_{50}$/mL.

The $TCID_{50}$ assay's limit of quantitation (i.e., detection limit) for all materials using the Spearman-Karber method[3,4] is 131 $TCID_{50}$/mL (i.e., 1.31 x 10^3 $TCID_{50}$ per coupon).

For quantitating the H5N1 virus in chicken feces and soil, each test coupon plus the Parafilm®, which lined each Petri dish (Figure 2-1), were placed in the conical vials containing the PBS extraction buffer. (Figure 2-2). Each vial was shaken on the orbital shaker as described for the other test materials (shaken on an orbital shaker at 200 rpm for 15 minutes), then each vial was centrifuged at 1000 x *g* for 10 minutes. A syringe was used to withdraw a 0.5 to 1 mL aliquot of the extract in each vial, which was then filtered using a 0.2 µm (polyether sulfone, low protein-binding) syringe filter, enabling the H5N1 virus to pass through while excluding larger particles and organisms. The filtered sample was then serially diluted and plated onto the MDCK cells for $TCID_{50}$ determination.

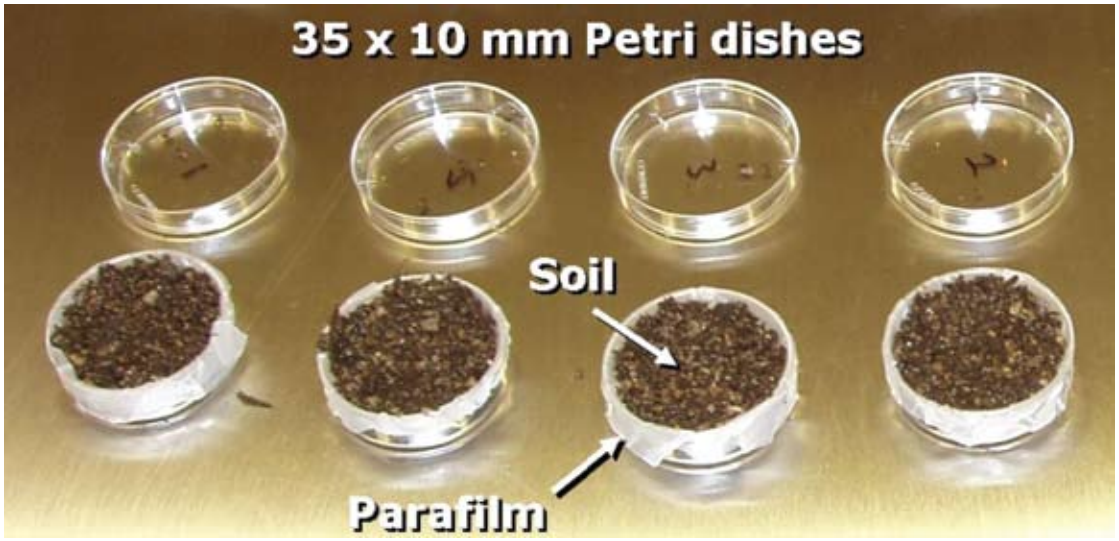

Figure 2-1. Petri Dishes With Soil

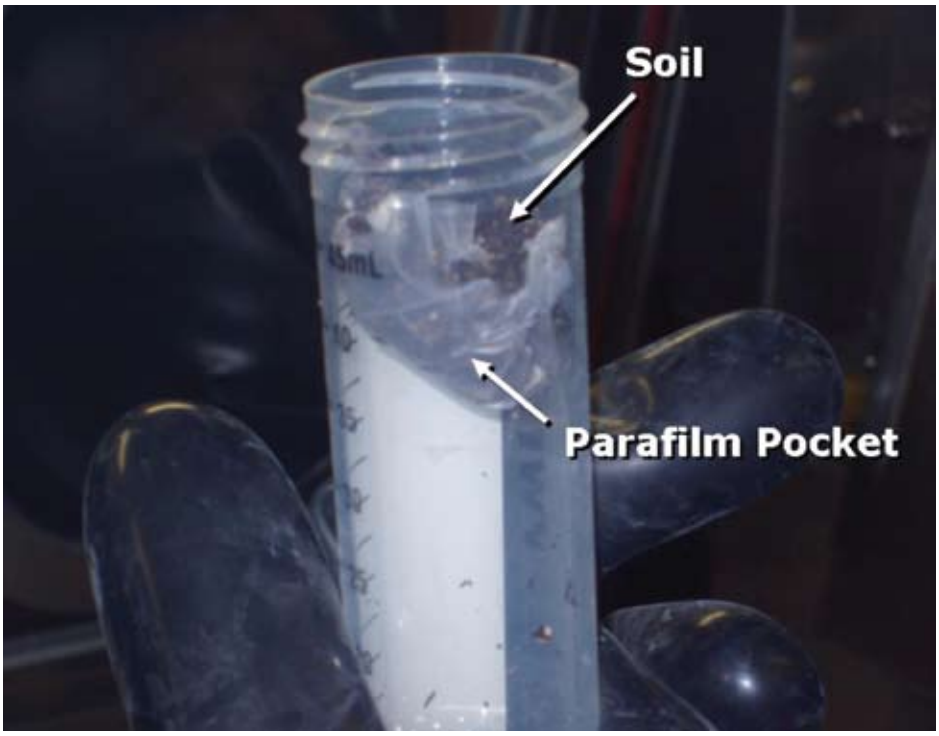

Figure 2-2. Soil and Parafilm® in a Conical Vial

2.3.3 H5N1 Virus Recoveries Based on Results of Virus Spiking Studies

Materials considered for persistence testing were studied to determine if an acceptable level of H5N1 virus (an acceptable level is defined in the test/QA plan[1] as ≥5% of the applied inoculum) could be recovered from the coupons. The coupons were spiked and allowed to dry for one hour; then the H5N1 virus was extracted from the coupons and analyzed for $TCID_{50}$ determinations. Numerous extraction approaches and materials (e.g., the use of surfactants) were considered for use in an attempt to achieve acceptable H5N1 virus recovery. Initial determinations of H5N1 virus recoveries were conducted with three replicate coupons per material; if acceptable recoveries were obtained final demonstrations using five replicate coupons were used to obtain mean recovery and variance data. The H5N1 virus recovery results and associated extraction approaches for each of the materials tested are provided in Tables 2-3 through 2-9. Materials studied include: basswood (a hardwood), chicken feces, concrete, galvanized metal, glass, pine wood (a softwood), and soil. For concrete, special tests were also conducted to investigate the impact of the highly alkaline concrete extraction solution on the viability of the H5N1 virus and the adsorption/deposition of the H5N1 virus into the concrete (Table 2-5). The tabulated data indicate that an acceptable H5N1 virus recovery (≥5% of the applied inoculum) was only obtained from chicken feces, galvanized metal, glass, and soil. Hence, these four materials were used in subsequent persistence testing.

Table 2-3. H5N1 Virus Recovery From Basswood

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Basswood; 0.7 cm thickness	Placed in PBS, agitated on an orbital shaker for 15 minutes (min) at 200 rpm.	3	2.00×10^6	$5.01 \pm 0.00 \times 10^3$	0.25 ± 0.00
Basswood; 0.2 cm thickness	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	$3.00 \pm 1.74 \times 10^3$	0.15 ± 0.09
Basswood; 0.2 cm thickness	Basswood soaked in 2% skim milk (dry milk reconstituted in deionized water) for 10 min, allowed to dry to the touch, and autoclaved for 15 min at 121 °C. Once cooled, inoculated and allowed to dry for 1 hour (hr), then placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm, coupons were also scraped with a pipette tip, and extraction fluid jetted onto the inoculated surface in an attempt to aid H5N1 virus recovery.	3	2.00×10^6	$4.75 \pm 2.76 \times 10^3$	0.24 ± 0.14
Basswood; 0.2 cm thickness	Placed in PBS, agitated on a plate shaker for 5 min at 1,100 rpm.	3	5.01×10^6	$3.00 \pm 1.73 \times 10^3$	0.06 ± 0.03
Basswood; 0.2 cm thickness	Placed in PBS, agitated on a vortexer for 2 min at 2,500 rpm.	3	5.01×10^6	$4.00 \pm 1.73 \times 10^3$	0.08 ± 0.03

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.

Table 2-4. H5N1 Virus Recovery From Chicken Feces

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Chicken Feces	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm, then centrifuged with the supernatant filtered (0.2 µm) for analysis.	3	2.00×10^6	$1.74 \pm 1.25 \times 10^5$	8.7 ± 6.3
Chicken Feces	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm, then centrifuged with the supernatant filtered (0.2 µm) for analysis.	5	7.92×10^6	$5.23 \pm 1.72 \times 10^5$	6.6 ± 2.2

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.

Table 2-5. H5N1 Virus Recovery From Concrete (Unpainted)

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Concrete	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	1.98×10^6	ND	0.00 ± 0.00
Concrete; extract solution	The PBS extraction of concrete resulted in a highly alkaline solution (i.e., pH > 11). A test was conducted to determine if this solution inactivates the H5N1 virus. Concrete was placed in PBS and shaken to obtain a high alkaline solution, the coupon was removed and the solution was spiked with H5N1 virus then agitated on an orbital shaker for 15 min at 200 rpm.	3	5.01×10^6	$5.34 \pm 4.51 \times 10^5$	11 ± 9.0
Concrete	Placed in PBS and H5N1 virus was spiked into the tube (not onto the coupon), which was then agitated on an orbital shaker for 15 min at 200 rpm. The purpose of this test was to determine if the H5N1 virus was depositing or absorbing into the concrete compounded by the highly alkaline extraction solution.	3	5.01×10^6	$3.00 \pm 1.74 \times 10^5$	6.0 ± 3.5
Concrete	Inoculated with the H5N1 virus and immediately extracted (placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm) to prevent the H5N1 virus from drying out	3	5.01×10^6	$2.44 \pm 2.38 \times 10^4$	0.49 ± 0.47
Concrete	Placed in sodium phosphate dibasic heptahydrate + Tween® 80, agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	ND	0.00 ± 0.00
Concrete	Pulverized immediately after inoculation with the H5N1 virus, placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	ND	0.00 ± 0.00
Concrete	Pulverized immediately after inoculation with the H5N1 virus, placed in sodium phosphate dibasic heptahydrate + Tween® 80 agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	ND	0.00 ± 0.00

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr, except where immediate extraction is noted.
ND = Not detected; the detection limit is 1.31×10^3 $TCID_{50}$.

Table 2-6. H5N1 Virus Recovery From Galvanized Metal

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Galvanized Metal	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	7.92×10^6	$6.96 \pm 1.69 \times 10^5$	8.8 ± 2.1
Galvanized Metal	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	5	5.01×10^6	$12.7 \pm 7.42 \times 10^5$	25 ± 15

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.

Table 2-7. H5N1 Virus Recovery From Glass

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Glass	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	1.98×10^6	$0.75 \pm 1.11 \times 10^6$	38 ± 56
Glass	Placed in PBS + 0.01% Tween® 20 (a non-denaturing surfactant), agitated on an orbital shaker for 15 min at 200 rpm.	3	5.01×10^6	$1.42 \pm 1.52 \times 10^6$	28 ± 30
Glass	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	5	2.00×10^6	$1.86 \pm 1.33 \times 10^5$	9.3 ± 6.7

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.

Table 2-8. H5N1 Virus Recovery From Pine Wood

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)	% Virus Recovery[a]
Pine Wood; 1 cm thickness	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	1.98×10^6	ND	0.00 ± 0.00
Pine Wood; 1 cm thickness	Pine wood soaked in 2% skim milk (dry milk reconstituted in deionized water) for 10 min, allowed to dry to the touch, and autoclaved for 15 min at 121 °C. Once cooled, inoculated and allowed to dry for 1 hr, then placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	5.01×10^6	ND	0.00 ± 0.00
Pine Wood; 0.2 cm thickness	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	ND	0.00 ± 0.00
Pine Wood; 0.2 cm thickness	Pine wood soaked in 2% skim milk (dry milk reconstituted in deionized water) for 10 min, allowed to dry to the touch, and autoclaved for 15 min at 121 °C. Once cooled, inoculated and allowed to dry for 1 hr, then placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm.	3	2.00×10^6	ND	0.00 ± 0.00

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.

Table 2-9. H5N1 Virus Recovery From Soil

Material	Extraction Approach	Replicate Coupons	Inoculum ($TCID_{50}$)	Recovered Virus ($TCID_{50}$)[a]	% Virus Recovery[a]
Soil	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm, then centrifuged with the supernatant filtered (0.2 µm) for analysis.	3	1.98×10^6	$11.0 \pm 8.13 \times 10^5$	56 ± 41
Soil	Placed in PBS, agitated on an orbital shaker for 15 min at 200 rpm, then centrifuged with the supernatant filtered (0.2 µm) for analysis.	5	7.92×10^6	$4.11 \pm 2.52 \times 10^5$	5.2 ± 3.2

[a] Data are expressed as mean ± standard deviation.
The drying time or elapsed time between inoculation and recovery was 1 hr.

2.4 Persistence Testing Approach

The $TCID_{50}$ of the H5N1 virus was measured from the liquid extracts obtained from test coupons (spiked coupons placed in the exposure chamber with temperature, RH, UV-A/B radiation, and contact time treatments) and positive controls (spiked coupons extracted after the one hour drying time [time-zero]). Coupon spiking and H5N1 virus extraction and quantification followed the approach described in Section 2.3.

The log reduction in $TCID_{50}$ was calculated as $\overline{N}/N'$ where $\overline{N}$ is the mean $TCID_{50}$ from five positive controls of a given material and N' is the $TCID_{50}$ from each test coupon replicate of a given material, environmental condition, and contact time. The log reduction in $TCID_{50}$ for each individual test coupon (R) was calculated for each of the five replicate test coupons of each material type, environmental condition, and contact time as:

Equation 2-2. $$R_{ijkl} = \log_{10}\left(\frac{\overline{N}_j}{N'_{ijkl}}\right)$$

Where:

R_{ijkl} = log reduction in $TCID_{50}$ for the ith replicate test coupon, jth test material, kth environmental condition, and lth contact time

$\overline{N}j$ = arithmetic mean $TCID_{50}$ from the five positive controls (which are measured at time-zero) for the jth test material

N'_{ijkl} = $TCID_{50}$ recovered on the ith replicate test coupon, jth test material, kth environmental condition, and lth contact time.

If no $TCID_{50}$ (i.e., no CPE is detected) is measured from a test coupon (N'), the value one was substituted for N'. Since the value one is greater than the observed value of zero, the estimate with this substitution becomes a lower bound for the true log reduction. Next, the mean log reduction in $TCID_{50}$ ($\overline{R}$) for the five replicate test coupons of a given material/ environmental condition/contact time was calculated as:

Equation 2-3. $$\overline{R}_{jkl} = \frac{\sum_{i=1}^{n} R_{ijkl}}{n}$$

Where:

$\overline{R}_{jkl}$ = mean log reduction in $TCID_{50}$ for the jth test material, kth environmental condition, and lth contact time

$\sum_{i=1}^{n} R_{ijkl}$ = sum of the log reductions in $TCID_{50}$ for each individual test coupon for the jth test material, kth environmental condition, and lth contact time n is the number of test coupon replicates (five).

The test matrix and various test conditions that were utilized for H5N1 virus persistence testing are summarized in Table 2-10. The environmental conditions included various combinations of temperature, RH, and UV-A/B radiation. Persistence was measured on four types of test coupons: chicken feces, galvanized metal, glass, and soil, and the test durations ranged from one to 13 days. Initial time points were selected based on comparable data available in the literature, but subsequent time points were adaptively chosen (i.e., shorter or longer durations) based on the initial test results, and tests were not necessarily conducted sequentially from the shortest to longest test duration.

2.4.1 Temperature

Avian influenza viruses, in general, can survive in the environment depending on temperature and humidity conditions, but the various strains of avian influenza may survive longer in cooler and moister conditions[5]. The persistence tests were conducted at room temperature and a lower temperature to better evaluate the influence of temperature on the persistence of the H5N1 virus (e.g., the ability to detect the presence of H5N1 virus). The actual temperatures associated with each test are provided in Table 2-10.

The persistence of the H5N1 virus was tested at the room temperature, low RH, no UV environmental condition by placing test coupons spiked with the virus into a Plas Labs' compact glove box under ambient laboratory conditions; temperature was recorded manually with a digital calibrated thermometer (Fisherbrand™ Traceable Radio-Signal Hygrometer/Thermometer, Fisher Scientific, Pittsburgh, Penn.) at the start and end of the test but not continuously monitored. The temperature for the other environmental conditions was recorded continuously at one-minute intervals with a HOBO® U10 Temperature Data Logger, (Onset Computer Corporation, Bourne, Mass.).

Low temperature persistence tests (with no UV) were conducted by placing the coupons inside a sealed Lock&Lock™ container and the container was then placed inside a refrigerator. For testing at a low temperature with UV-A/B, coupons were placed directly beneath the UV-A/B lamps mounted inside a mini-refrigerator (Marvel® Scientific, Model No. 6CAR, Greenville, MI) modified to include glove ports. Some of the temperatures associated with these low temperature environmental conditions exceeded 20 ºC; this generally occurred during the first two hours of a test as the temperatures inside the Lock&Lock™ container were equilibrating (cooling down) relative to the temperature in the refrigerator. As noted in Section 4.5 and shown in Table 2-10, the range of temperatures often exceeded the allowable test measurement tolerance of ± 2 ºC (as specified in the test/QA plan[1]) for the target temperatures of 22 ºC and 4 ºC. Although some of the mean test temperatures deviated from the target temperatures by a few ºC, the associated $TCID_{50}$ data remain valid and useful.

Table 2-10. Persistence Test Matrix

Target Environmental Condition / Test Duration	Material	Temperature (°C)		RH (%)	
Room Temperature (22 °C), Low RH (40%), No UV[a]	Chicken feces, galvanized metal, glass, and soil	Start	End	Start	End
1-Day		22.7	23.4	32	38
2-Day		22.0	22.9	30	42
4-Day		23.2	22.8	36	61
Room Temperature (22 °C), High RH (80%), No UV	Chicken feces, galvanized metal, glass, and soil	Mean	Range	Mean	Range
1-Day		22.4	19.0 – 23.2	89.1	51.9 – 93.5
2-Day		23.4	22.6 – 24.4	90.4	80.3 – 93.8
4-Day		23.7	22.7 – 24.9	91.2	76.5 – 94.4
Low Temperature (4 °C),Low RH (40%), No UV	Chicken feces, galvanized metal, glass, and soil	Mean	Range	Mean	Range
4-Day		4.0	3.41 – 21.9	27.7	17.5 – 33.3
8-Day		6.7	6.46 – 22.2	15.2	1.00 – 71.3
13-Day		6.7	6.36 – 22.6	46.3	27.4 – 54.6
Low Temperature (4 °C), High RH (80%), No UV	Chicken feces, galvanized metal, glass, and soil	Mean	Range	Mean	Range
4-Day		7.8	7.22 – 23.1	89.5	63.6 – 92.8
9-Day		6.7	6.38 – 22.9	96.9	66.4 – 104
13-Day		6.8	6.48 – 22.6	79.0	24.9 – 100
Low Temperature (4 °C), Low RH (40%), UV-A/B	Chicken feces, galvanized metal, glass, and soil	Mean	Range	Mean	Range
24-Hours		4.1[b]	2.26 – 20.8[c]	24.5[b]	23.1 – 68.3[c]
48-Hours		-0.91	-2.04 – 21.2	29.7	28.0 – 86.2
96-Hours[b]		0.12	-1.76 – 21.7	28.2	25.2 – 65.2

[a] For the room temperature, low RH, no UV test condition, temperature was recorded manually and RH was measured with a digital calibrated hygrometer. Both temperature and RH were recorded at the start and end of the test; neither was continuously monitored.
[b] Galvanized metal and glass were not tested at the 4-day test duration.
[c] Temperature and RH were not recorded during the initial seven hours of the test.

2.4.2 Relative Humidity

Persistence testing was conducted using low and high RH levels to better understand the influence of RH on the persistence of the H5N1 virus. The actual RH levels associated with each test are provided in Table 2-10; the range of RH levels often exceeded the allowable test measurement tolerance of ±10% specified in the test/QA plan[1], as noted in Section 4.5. Deviations from the target RH levels do not invalidate the associated $TCID_{50}$ data.

For the room temperature, low RH, no UV environmental condition, persistence of the H5N1 virus was tested at ambient laboratory conditions (no active measures were taken to increase or reduce the RH) inside a glove box; RH was measured at the start and end of the test with a digital calibrated hygrometer but not continuously monitored. RH for the persistence testing performed under other environmental conditions was recorded continuously at one-minute intervals with a device that measures and logs temperature and RH data (HOBO® U10, Onset Computer Corporation). For the room temperature, low RH, no UV environmental condition, the RH level appeared to increase with the duration of the test. The moisture content associated with the materials being tested (e.g., soil and chicken feces) likely contributed to the increasing RH inside the sealed glove box as time elapsed.

Persistence testing at the room temperature, high RH, no UV environmental condition was accomplished by placing the coupons directly in a pre-humidified (by using an ultrasonic fogger) glove box. The RH tended to increase with the placement of the coupons into the glove box; consequently during the 1-day test, fresh air was introduced into the glove box to manually lower the RH at the start of the 1-day test. During the 2- and 4-day tests, there were no attempts to adjust the RH, which increased during testing.

Persistence testing at low temperature (with no UV) was conducted by placing the coupons inside a sealed Lock&Lock™ container inside a refrigerator. When testing at a low RH, DRIERITE (W. A. Hammond DRIERITE Co., Xenia, Ohio) was also added to the container. In an attempt to prevent the RH from increasing as the DRIERITE became saturated, fresh DRIERITE was exchanged with the used DRIERITE on the fourth day of the 8-day test and this reduced the RH from 20–30% to <10% (the RH briefly spiked to 71.30% during the exchange). For high RH

persistence testing (target RH 80% per the test/QA plan[1]) inside the Lock&Lock™ container at low temperature, mean RH levels ranging from 79% to 97% were achieved by sealing the inherently moist materials (i.e., soil and chicken feces) in the container.

For testing with UV-A/B (low temperature, low RH), the RH inside the modified mini-refrigerator remained relatively low throughout the testing such that the use of DRIERITE was not required.

2.4.3 Ultraviolet-A/B Radiation

Influenza viruses may be inactivated by UV-B wavelengths in sunlight[6]. UV-A/B radiation was implemented in the tests in an effort to mimic UV radiation levels associated with natural sunlight. The spectrum and intensity of terrestrial UV radiation is highly variable and is affected by time of day, day of year, geographical location, altitude, atmospheric pollution, and clouds. Naturally occurring UV-B levels, observed around noon, range from 19.5 to 150 microwatt ($\mu W/cm^2$, [7–10], and therefore a target exposure chamber condition of 70 $\mu W/cm^2$ UV-B was selected. The amount of UV-A generated during testing was quantitated during the method development phase and actual testing and was kept within the range of UV-A observed in natural sunlight (0 to 4,500 $\mu W/cm^2$)[11]. The target UV-C level to be generated during testing was 0 $\mu W/cm^2$, since UV-C generally does not reach the earth's surface. UV-B, UV-C, and total UV were monitored during the persistence testing (UV-A was calculated as total UV minus the UV-B and UV-C levels).

The level of UV-B radiation generated and the potential of diminishing UV-B output over time was evaluated with data obtained from the lamp manufacturer and data generated during the method development phase of testing.

A ReptiSun™ 10.0 Linear Fluorescent UV-B Lamp was used in the study. The manufacturer, Zoo Med Laboratories, Inc. (San Luis Obispo, Calif.) indicated that after an initial burn in, UV-B levels of the lamp would diminish somewhat over time.

Using a single ReptiSun™ 10.0 Linear Fluorescent UV-B Lamp with a baseline UV-B level of 70 $\mu W/cm^2$, and a Solarmeter® Model 6.2 (Solartech, Inc., Harrison Township, Mich.) the following UV-B levels were measured sequentially over time during preliminary tests:

- 69 $\mu W/cm^2$, 99% of baseline remaining after 1 hour
- 68 $\mu W/cm^2$, 97% of baseline remaining after 2 hours
- 66 $\mu W/cm^2$, 94% of baseline remaining after 17 hours
- 64 $\mu W/cm^2$, 91% of baseline remaining after 64 hours
- 64 $\mu W/cm^2$, 91% of baseline remaining after 89 hours

The UV-A/B lamps were placed inside a scientific-grade mini-refrigerator, which was customized with a faceplate and glove ports (see Figure 2-3). All UV-A/B exposures were continuous; the lamps were not turned off during testing. In addition to measuring UV-B, UV-C was measured with the Solarmeter® Model 8.0 and total UV was measured with Solarmeter® Model 5.0. UV data measured during the persistence testing are summarized in Table 2-11. During the 24-hour test, UV measurements were recorded from the center of the testing area at the start and completion of the test. During the 48- and 96-hour tests, UV measurements were periodically made from five positions beneath the lamps (Figure 2-4). UV measurements were taken at approximately 14 cm below the lamps, which corresponded to the distance of the test coupons from the lamps. All UV-B levels coincided with the target UV level (70 ± 7 $\mu W/cm^2$ UV-B as specified in the test/QA plan).

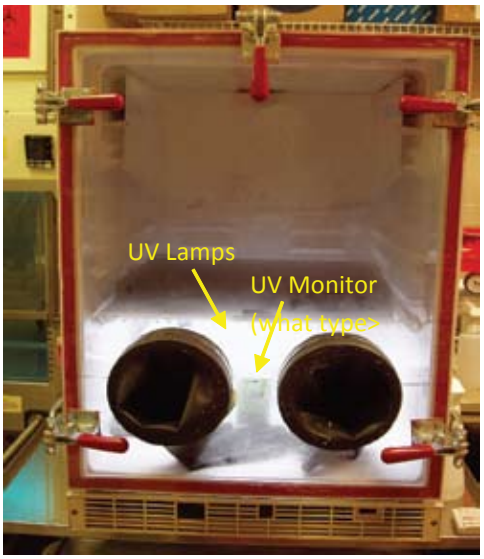

Figure 2-3. Mini-Refrigerator Configured With Ultraviolet A/B Lamps

Table 2-11. Ultraviolet Measurements

Low Temperature, Low RH, UV-A/B Test Durations[a]	UV-A (μW/cm²)		UV-B (μW/cm²)		UV-C (μW/cm²)	
24-Hour[b]	Start 125	End 130	Start 74	End 75	Start 0	End 0
48-Hour[c]	Mean 109	Range 100 – 122	Mean 68	Range 63 – 74	Mean 0	Range 0 – 0
96-Hour[c]	Mean 106	Range 97 – 120	Mean 69	Range 63 – 75	Mean 0	Range 0 – 0

[a] UV-A/B exposures for the 24-, 48-, and 96-hour tests were continuous.
[b] During the 24-hour test, UV measurements were recorded from the center of the testing area at the start and completion of the test.
[c] During the 48- and 96-hour tests, UV measurements were periodically made from five positions beneath the lamps (see Figure 2-4).

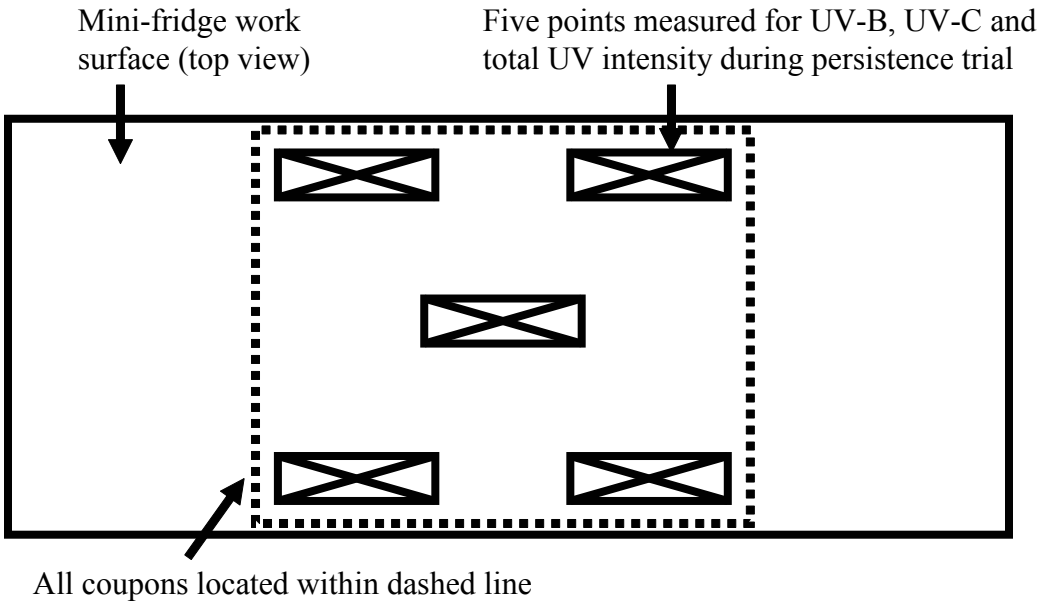

Figure 2-4. Schematic (Top View) of Ultraviolet Sampling Locations

2.5 Test Results

Persistence results for each material/environmental condition combination are summarized in Tables 2-12 through 2-15. A summary of all persistence data obtained is provided in Table 2-16.

2.5.1 Chicken Feces

The results obtained for persistence of the H5N1 virus on chicken feces are summarized in Table 2-12.

The H5N1 virus was least persistent (6.3 mean log reduction in $TCID_{50}$ during the 2-day test) on chicken feces for the room temperature, low RH, no UV environmental condition, and the H5N1 virus was not detected above the $TCID_{50}$ on the laboratory and procedural blanks at any test duration.

Extracts from chicken feces samples not inoculated with the H5N1 virus often induced CPE when testing the most concentrated serial dilution (no CPE observed with subsequent dilutions). This CPE often resulted in a $TCID_{50}$ of 3.16×10^3, and was attributed to the inherent properties of the chicken feces rather than the H5N1 virus. Test coupons with mean recovered $TCID_{50}$ values less than or equal to the associated laboratory and procedural blanks were still used in the calculation of the mean log reductions.

At higher RH (room temperature, high RH, no UV environmental condition), the mean $TCID_{50}$ level recovered from the test coupons after the 1-day test was greater than the $TCID_{50}$ levels associated with the laboratory and procedural blanks. Mold was observed growing on the chicken feces during the 4-day test at the room temperature, high RH, no UV environmental condition.

Table 2-12. H5N1 Virus Persistence on Chicken Feces

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b, c]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature, Low RH, No UV					
1-Day					
Positive Control[d]	7.94×10^6	5/5	$8.05 \pm 4.16 \times 10^5$	10.1 ± 5.2	-
Test Coupon[e]	7.94×10^6	5/5	$6.37 \pm 3.99 \times 10^2$ [h]	0.01 ± 0.01[h]	3.2 ± 0.26[h]
Laboratory Blank[f]	0	1/1	3.16×10^3	-	-
Procedural Blank[g]	0	1/1	3.16×10^3	-	-
2-Day					
Positive Control	7.94×10^6	5/5	$2.11 \pm 1.95 \times 10^6$	26.6 ± 24.5	-
Test Coupon	7.94×10^6	0/5	ND[i]	0.00 ± 0.00[i]	6.3 ± 0.00[i]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
4-Day					
Positive Control	5.01×10^6	5/5	$3.66 \pm 1.87 \times 10^5$	7.30 ± 3.73	-
Test Coupon	5.01×10^6	0/5	ND[i]	0.00 ± 0.00[i]	5.6 ± 0.00[i]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
Room Temperature, High RH, No UV					
1-Day					
Positive Control	5.01×10^7	5/5	$6.96 \pm 3.96 \times 10^6$	13.9 ± 7.90	-
Test Coupon	5.01×10^7	5/5	$7.70 \pm 3.11 \times 10^4$	0.15 ± 0.06	2.0 ± 0.2
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
2-Day					
Positive Control	3.16×10^7	5/5	$2.54 \pm 1.59 \times 10^5$	0.80 ± 0.50	-
Test Coupon	3.16×10^7	5/5	$3.16 \pm 0.00 \times 10^3$ [h]	0.01 ± 0.00[h]	1.9 ± 0.0[h]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
4-Day					
Positive Control	2.00×10^7	5/5	$1.39 \pm 1.17 \times 10^6$	6.96 ± 5.83	-
Test Coupon	2.00×10^7	0/5	ND[i]	0.00 ± 0.00[i]	6.1 ± 0.0[i]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
Low Temperature, Low RH, No UV					
4-Day					
Positive Control	2.00×10^7	5/5	$2.02 \pm 1.83 \times 10^6$	10.1 ± 9.13	-
Test Coupon	2.00×10^7	5/5	$7.11 \pm 3.32 \times 10^4$	0.36 ± 0.17	1.5 ± 0.2
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
8-Day					
Positive Control	5.01×10^7	5/5	$8.05 \pm 4.16 \times 10^5$	1.61 ± 0.83	-
Test Coupon	5.01×10^7	5/5	$3.16 \pm 0.00 \times 10^3$ [h]	0.01 ± 0.00[h]	2.4 ± 0.0[h]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$7.10 \pm 3.94 \times 10^5$	3.55 ± 1.97	-
Test Coupon	2.00×10^7	0/5	ND[i]	0.00 ± 0.00[i]	5.9 ± 0.0[i]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	0/1	ND	-	-

Table 2-12. H5N1 Virus Persistence on Chicken Feces (continued)

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b, c]	% Virus Recovery[b]	Mean Log Reduction[b]
Low Temperature, High RH, No UV					
4-Day					
Positive Control	5.01×10^7	5/5	$8.05 \pm 4.16 \times 10^5$	1.61 ± 0.83	-
Test Coupon	5.01×10^7	5/5	$5.42 \pm 4.09 \times 10^4$	0.11 ± 0.08	1.3 ± 0.3
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
9-Day					
Positive Control	7.94×10^6	5/5	$4.42 \pm 4.86 \times 10^5$	5.57 ± 6.12	-
Test Coupon	7.94×10^6	5/5	$5.90 \pm 4.57 \times 10^4$	0.74 ± 0.58	$1.0 \pm .05$
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$7.10 \pm 3.94 \times 10^5$	3.55 ± 1.97	-
Test Coupon	2.00×10^7	3/5	$7.86 \pm 7.17 \times 10^2$ [i]	0.00 ± 0.00[i]	4.0 ± 2.0[i]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, Low RH, UV-A/B					
24-Hour[j]					
Positive Control	2.00×10^7	5/5	$7.10 \pm 3.94 \times 10^5$	3.55 ± 1.47	
Test Coupon	2.00×10^7	5/5	$1.06 \pm 0.86 \times 10^6$	5.32 ± 4.29	-0.05 ± 0.37
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
48-Hour[j]					
Positive Control	5.01×10^7	5/5	$1.07 \pm 0.26 \times 10^6$	2.14 ± 0.51	-
Test Coupon	5.01×10^7	5/5	$9.58 \pm 4.16 \times 10^4$	0.19 ± 0.08	1.1 ± 0.22
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-
96-Hour[j]					
Positive Control	2.00×10^7	5/5	$2.07 \pm 1.08 \times 10^6$	10.4 ± 5.40	-
Test Coupon	2.00×10^7	5/5	$3.16 \pm 0.00 \times 10^3$ [h]	0.02 ± 0.00[h]	2.8 ± 0.0[h]
Laboratory Blank	0	1/1	3.16×10^3	-	-
Procedural Blank	0	1/1	3.16×10^3	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicate coupons.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] $TCID_{50}$ values for laboratory and procedural blanks are attributed to CPE observed from the test material rather than H5N1 virus.
[d] Positive controls were inoculated, extracted at time-zero (1-hour drying time).
[e] Test coupons were inoculated, exposed to the environmental condition for the test duration.
[f] Laboratory blanks were not inoculated with any virus, and extracted at time-zero.
[g] Procedural blanks were not inoculated, but exposed to the environmental condition for the test duration.
[h] The test coupon $TCID_{50}$ was ≤ the procedural blank $TCID_{50}$ such that the CPE observed may be attributed to the test material rather than H5N1 virus.
[i] A value of 1 $TCID_{50}$ was used for each test coupon replicate with no CPE observed, in the calculation of % virus recovery and mean log reduction.
[j] UV-A/B exposures for 24-, 48-, and 96-hour tests were continuous.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

Lowering the temperature resulted in longer persistence times (1.5 mean log reduction in the $TCID_{50}$ during the 4-day test). In fact, the H5N1 virus was recovered (detected above the laboratory and procedural blank level) from chicken feces after the 4-day test at the low temperature, low RH, no UV environmental condition and after the 9-day test at the low temperature, high RH, no UV environmental condition.

Even with exposure to UV-A/B (at a low temperature and low RH), the H5N1 virus was detected above the laboratory and procedural blank level after 48 hours of continuous UV-A/B exposure test on chicken feces.

2.5.2 Galvanized Metal

The test results obtained for persistence of the H5N1 virus on galvanized metal are summarized in Table 2-13. The H5N1 virus was not detected in any of the tests conducted at room temperature. When the tests were conducted at the low temperature, low RH, no UV environmental condition, the H5N1 virus was detected after the 13-day test with only a 0.69 mean log reduction in $TCID_{50}$. At the low temperature, high RH, no UV environmental condition, the H5N1 virus was only detected after a 4-day test; during the 9-day test, the galvanized metal appeared to exhibit oxidation (black spots formed where the H5N1 virus was inoculated), but the H5N1 virus was not detected. When testing under high RH conditions, it was common for the dried inoculum drops to reform (rehydrate) into liquid drops on non-porous surfaces (e.g., galvanized metal). When exposed to UV-A/B (at a low temperature and low RH), the H5N1 virus persisted only for the 24-hour test.

Table 2-13. H5N1 Virus Persistence on Galvanized Metal

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature, Low RH, No UV					
1-Day					
Positive Control[c]	7.94×10^6	5/5	$1.56 \pm 0.41 \times 10^6$	19.6 ± 5.10	-
Test Coupon[d]	7.94×10^6	0/5	ND[g]	0.00 ± 0.00[g]	6.2 ± 0.0[g]
Laboratory Blank[e]	0	0/1	ND	-	-
Procedural Blank[f]	0	0/1	ND	-	-
2-Day					
Positive Control	7.94×10^6	5/5	$2.25 \pm 1.28 \times 10^6$	28.3 ± 16.1	-
Test Coupon	7.94×10^6	0/5	ND[g]	0.00 ± 0.00[g]	6.4 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	
4-Day					
Positive Control	5.01×10^6	5/5	$1.36 \pm 1.03 \times 10^6$	27.2 ± 20.5	-
Test Coupon	5.01×10^6	0/5	ND[g]	0.00 ± 0.00[g]	6.1 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	
Room Temperature, High RH, No UV					
1-Day					
Positive Control	5.01×10^7	5/5	$6.16 \pm 3.69 \times 10^6$	12.3 ± 7.36	-
Test Coupon	5.01×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.8 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	
2-Day					
Positive Control	3.16×10^7	5/5	$1.13 \pm 0.63 \times 10^6$	3.56 ± 1.98	-
Test Coupon	3.16×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.1 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	
4-Day					
Positive Control	2.00×10^7	5/5	$2.76 \pm 2.05 \times 10^6$	13.8 ± 10.3	-
Test Coupon	2.00×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.4 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

Table 2-13. H5N1 Virus Persistence on Galvanized Metal (continued)

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Low Temperature, Low RH, No UV					
4-Day					
Positive Control	2.00×10^7	5/5	$2.92 \pm 1.42 \times 10^6$	14.6 ± 7.11	-
Test Coupon	2.00×10^7	5/5	$3.78 \pm 2.80 \times 10^5$	1.89 ± 1.40	1.0 ± 0.38
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8-Day					
Positive Control	5.01×10^7	5/5	$2.08 \pm 0.68 \times 10^6$	4.16 ± 1.36	-
Test Coupon	5.01×10^7	5/5	$7.33 \pm 3.58 \times 10^5$	1.46 ± 0.71	0.50 ± 0.23
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$1.56 \pm 0.41 \times 10^6$	7.78 ± 2.03	-
Test Coupon	2.00×10^7	5/5	$3.44 \pm 1.51 \times 10^5$	1.72 ± 0.76	0.69 ± 0.20
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, High RH, No UV					
4-Day					
Positive Control	5.01×10^7	5/5	$2.08 \pm 0.68 \times 10^6$	4.16 ± 1.36	-
Test Coupon	5.01×10^7	5/5	$4.69 \pm 3.06 \times 10^3$	0.01 ± 0.01	2.7 ± 0.3
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
9-Day					
Positive Control	7.94×10^6	5/5	$8.81 \pm 6.58 \times 10^5$	11.1 ± 8.28	-
Test Coupon	7.94×10^6	0/5	ND[g]	0.00 ± 0.00[g]	5.9 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$1.56 \pm 0.41 \times 10^6$	7.78 ± 2.03	-
Test Coupon	2.00×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.2 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, Low RH, UV-A/B					
24-Hours[h]					
Positive Control	2.00×10^7	5/5	$1.56 \pm 0.41 \times 10^6$	7.78 ± 2.03	-
Test Coupon	2.00×10^7	5/5	$0.64 \pm 1.41 \times 10^5$	0.32 ± 0.70	2.6 ± 1.1
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
48-Hours[h]					
Positive Control	5.01×10^7	5/5	$1.56 \pm 0.41 \times 10^6$	3.10 ± 0.81	-
Test Coupon	5.01×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.2 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicate coupons.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] Positive controls were inoculated, extracted at time-zero (1-hour drying time).
[d] Test coupons were inoculated, exposed to the environmental condition for the test duration.
[e] Laboratory blanks were not inoculated with any virus, and extracted at time-zero.
[f] Procedural blanks were not inoculated, but exposed to the environmental condition for the test duration.
[g] A value of 1 $TCID_{50}$ was used when no CPE are observed, in the calculation of % virus recovery and mean log reduction.
[h] UV-A/B exposures for the 24- and 48-hour tests were continuous.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

2.5.3 Glass

The H5N1 virus persistence results on glass are summarized in Table 2-14. The H5N1 virus was not detected on any of the coupons tested at room temperature. When the tests were conducted at the low temperature, low RH, no UV environmental condition, the H5N1 virus was detected after a 13-day test with only a 0.59 mean log reduction in $TCID_{50}$. When the tests were conducted at the low temperature, high RH, no UV environmental condition, the H5N1 virus was detected on glass after the 9-day test with a 2.7 mean log reduction in $TCID_{50}$. As noted previously for galvanized metal, when testing under high RH conditions, it was common for the dried inoculums to reform (rehydrate) into liquid drops on non-porous surfaces (e.g., glass). When exposed to UV-A/B (at a low temperature and low RH), the H5N1 virus was detected on only one out of five replicate coupons following the 24-hour test.

Table 2-14. H5N1 Virus Persistence on Glass

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature, Low RH, No UV					
1-Day					
Positive Control[c]	7.94×10^6	5/5	$6.18 \pm 1.60 \times 10^5$	7.79 ± 2.02	-
Test Coupon[d]	7.94×10^6	0/5	ND[g]	0.00 ± 0.00[g]	5.8 ± 0.0[g]
Laboratory Blank[e]	0	0/1	ND	-	-
Procedural Blank[f]	0	0/1	ND	-	-
2-Day					
Positive Control	7.94×10^6	5/5	$3.29 \pm 1.71 \times 10^6$	41.4 ± 21.6	-
Test Coupon	7.94×10^6	0/5	ND[g]	0.00 ± 0.00[g]	6.5 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
4-Day					
Positive Control	5.01×10^6	5/5	$1.01 \pm 0.63 \times 10^6$	20.2 ± 12.7	-
Test Coupon	5.01×10^6	0/5	ND[g]	0.00 ± 0.00[g]	6.0 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Room Temperature, High RH, No UV					
1-Day					
Positive Control	5.01×10^7	5/5	$1.07 \pm 0.26 \times 10^7$	21.4 ± 5.09	-
Test Coupon	5.01×10^7	0/5	ND[g]	0.00 ± 0.00[g]	7.0 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
2-Day					
Positive Control	3.16×10^7	5/5	$2.66 \pm 2.21 \times 10^6$	8.43 ± 7.01	-
Test Coupon	3.16×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.4 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
4-Day					
Positive Control	2.00×10^7	5/5	$2.32 \pm 0.83 \times 10^6$	11.6 ± 4.14	-
Test Coupon	2.00×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.4 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

Table 2-14. H5N1 Virus Persistence on Glass (continued)

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Low Temperature, Low RH, No UV					
4-Day Positive Control	2.00×10^7	5/5	$1.07 \pm 0.59 \times 10^6$	5.35 ± 2.93	-
Test Coupon	2.00×10^7	5/5	$4.98 \pm 2.97 \times 10^5$	2.49 ± 1.49	0.41 ± 0.30
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8-Day Positive Control	5.01×10^7	5/5	$1.10 \pm 0.63 \times 10^6$	2.20 ± 1.25	-
Test Coupon	5.01×10^7	5/5	$1.34 \pm 2.07 \times 10^6$	2.68 ± 4.12	0.26 ± 0.58
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
13-Day Positive Control	2.00×10^7	5/5	$1.79 \pm 0.83 \times 10^6$	8.94 ± 4.16	-
Test Coupon	2.00×10^7	5/5	$5.91 \pm 4.48 \times 10^5$	2.96 ± 2.24	0.59 ± 0.36
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, High RH, No UV					
4-Day Positive Control	5.01×10^7	5/5	$1.34 \pm 1.09 \times 10^6$	2.67 ± 2.17	-
Test Coupon	5.01×10^7	5/5	$1.37 \pm 0.61 \times 10^5$	0.27 ± 0.12	0.63 ± 0.37
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
9-Day Positive Control	7.94×10^6	5/5	$1.58 \pm 1.09 \times 10^6$	19.9 ± 13.8	-
Test Coupon	7.94×10^6	5/5	$3.16 \pm 0.00 \times 10^3$	0.04 ± 0.00	2.7 ± 0.0
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
13-Day Positive Control	2.00×10^7	5/5	$1.79 \pm 0.83 \times 10^6$	8.94 ± 4.16	-
Test Coupon	2.00×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.3 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, Low RH, UV-A/B					
24-Hour[h] Positive Control	2.00×10^7	5/5	$1.79 \pm 0.83 \times 10^6$	1.83 ± 0.66	-
Test Coupon	2.00×10^7	1/5	$2.62 \pm 5.85 \times 10^2$ [g]	0.00 ± 0.00[g]	5.6 ± 1.4[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
48-Hour[h] Positive Control	5.01×10^7	5/5	$1.31 \pm 0.43 \times 10^6$	2.62 ± 0.86	-
Test Coupon	5.01×10^7	0/5	ND[g]	0.00 ± 0.00[g]	6.1 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicate coupons.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] Positive controls were inoculated, extracted at time-zero (1-hour drying time).
[d] Test coupons were inoculated, exposed to the environmental condition for the test duration.
[e] Laboratory blanks were not inoculated with any virus, and extracted at time-zero.
[f] Procedural blanks were not inoculated, but exposed to the environmental condition for the test duration.
[g] A value of 1 $TCID_{50}$ was used when no CPE are observed, in the calculation of % virus recovery and mean log reduction.
[h] UV-A/B exposures for the 24- and 48-hour tests were continuous.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

2.5.4 Soil

The H5N1 virus persistence results in soil are summarized in Table 2-15. When the tests were conducted at room temperature, the H5N1 virus was detected after the 1-day test, but not at the longer durations. When the tests were conducted at the low temperature, low RH, no UV environmental condition, the H5N1 virus was detected from soil after a 13-day test with a 3.1 mean log reduction in $TCID_{50}$. When the tests were conducted at the low temperature, high RH, no UV environmental condition, the H5N1 virus was detected from soil after the 9-day test with no mean log reduction in $TCID_{50}$. When exposed to UV-A/B (at a low temperature and low RH), the H5N1 virus persisted during the 96-hour test with a 3.3 mean log reduction in $TCID_{50}$.

Table 2-15. H5N1 Virus Persistence on Soil

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b, c]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature, Low RH, No UV					
1-Day					
Positive Control[d]	7.94×10^6	5/5	$7.33 \pm 3.58 \times 10^5$	9.23 ± 4.51	-
Test Coupon[e]	7.94×10^6	2/5	$0.88 \pm 1.38 \times 10^4$ [h]	0.11 ± 0.17[h]	4.2 ± 2.4[h]
Laboratory Blank[f]	0	0/1	ND	-	-
Procedural Blank[g]	0	0/1	ND	-	-
2-Day					
Positive Control	7.94×10^6	5/5	$6.40 \pm 2.21 \times 10^5$	8.06 ± 2.78	-
Test Coupon	7.94×10^6	0/5	ND[h]	0.00 ± 0.00[h]	5.8 ± 0.0[h]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
4-Day					
Positive Control	5.01×10^6	5/5	$2.55 \pm 0.88 \times 10^5$	5.09 ± 1.75	-
Test Coupon	5.01×10^6	0/5	ND[h]	0.00 ± 0.00[h]	5.4 ± 0.0[h]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Room Temperature, High RH, No UV					
1-Day					
Positive Control	5.01×10^7	5/5	$1.48 \pm 1.12 \times 10^6$	2.96 ± 2.24	-
Test Coupon	5.01×10^7	5/5	$8.26 \pm 4.31 \times 10^4$	0.16 ± 0.09	1.3 ± 0.26
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
2-Day					
Positive Control	3.16×10^7	5/5	$3.64 \pm 2.80 \times 10^5$	1.15 ± 0.89	-
Test Coupon	3.16×10^7	0/5	ND[f]	0.00 ± 0.00[f]	5.6 ± 0.0[f]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
4-Day					
Positive Control	2.00×10^7	5/5	$1.79 \pm 0.83 \times 10^5$	0.89 ± 0.42	-
Test Coupon	2.00×10^7	0/5	ND[f]	0.00 ± 0.00[f]	5.3 ± 0.0[f]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, Low RH, No UV					
4-Day					
Positive Control	2.00×10^7	5/5	$2.82 \pm 1.57 \times 10^5$	1.41 ± 0.78	-
Test Coupon	2.00×10^7	5/5	$2.47 \pm 3.29 \times 10^4$	0.12 ± 0.16	1.5 ± 0.73
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

Table 2-15. H5N1 Virus Persistence on Soil (continued)

Test Duration / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b, c]	% Virus Recovery[b]	Mean Log Reduction[b]
Low Temperature, Low RH, No UV					
8-Day					
Positive Control	5.01×10^7	5/5	$5.23 \pm 1.72 \times 10^5$	1.04 ± 0.34	-
Test Coupon	5.01×10^7	5/5	$1.16 \pm 0.14 \times 10^3$	0.00 ± 0.00	2.7 ± 0.1
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$3.67 \pm 1.31 \times 10^5$	1.83 ± 0.66	-
Test Coupon	2.00×10^7	4/5	$1.05 \pm 0.59 \times 10^3$ [h]	0.01 ± 0.00[h]	3.1 ± 1.4[h]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, High RH, No UV					
4-Day					
Positive Control	5.01×10^7	5/5	$5.23 \pm 1.72 \times 10^5$	1.04 ± 0.34	-
Test Coupon	5.01×10^7	5/5	$2.21 \pm 1.65 \times 10^5$	0.44 ± 0.33	0.46 ± 0.30
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
9-Day					-
Positive Control	7.94×10^6	5/5	$2.82 \pm 3.02 \times 10^5$	3.56 ± 3.81	
Test Coupon	7.94×10^6	5/5	$5.93 \pm 3.95 \times 10^5$	7.46 ± 4.98	-0.25 ± 0.28
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	1/1	2.00×10^4	-	-
13-Day					
Positive Control	2.00×10^7	5/5	$3.67 \pm 1.31 \times 10^5$	1.83 ± 0.66	-
Test Coupon	2.00×10^7	0/5	ND[h]	0.00 ± 0.00[h]	5.6 ± 0.0[h]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature, Low RH, UV-A/B					
24-Hours[i]					
Positive Control	2.00×10^7	5/5	$3.67 \pm 1.31 \times 10^5$	3.55 ± 1.97	-
Test Coupon	2.00×10^7	5/5	$5.93 \pm 3.95 \times 10^5$	2.96 ± 1.98	-0.14 ± 0.28
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
48-Hours[i]					
Positive Control	5.01×10^7	5/5	$5.23 \pm 1.72 \times 10^5$	1.04 ± 0.34	-
Test Coupon	5.01×10^7	5/5	$3.90 \pm 1.01 \times 10^4$	0.08 ± 0.02	1.1 ± 0.1
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
96-Hours[i]					
Positive Control	2.00×10^7	5/5	$1.07 \pm 0.59 \times 10^6$	5.35 ± 2.93	-
Test Coupon	2.00×10^7	4/5	$3.11 \pm 3.28 \times 10^3$ [h]	0.02 ± 0.02[h]	3.3 ± 1.6[h]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicate coupons.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] $TCID_{50}$ values for laboratory and procedural blanks are attributed to CPE observed from the test material rather than H5N1 virus.
[d] Positive controls were inoculated, extracted at time-zero (1-hour drying time).
[e] Test coupons were inoculated, exposed to the environmental condition for the test duration.
[f] Laboratory blanks were not inoculated with any virus, and extracted at time-zero.
[g] Procedural blanks were not inoculated, but exposed to the environmental condition for the test duration.
[h] A value of 1 $TCID_{50}$ was used when no CPE are observed, in the calculation of % virus recovery and mean log reduction.
[i] UV-A/B exposures for the 24-, 48-, and 96-hour test were continuous.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

2.5.5 Comparison of Chicken Feces, Galvanized Metal, Glass, and Soil Results

The H5N1 virus persistence results are summarized in Table 2-16. These data denote the longest duration (days) that the virus was detected and the shortest duration that the virus was not detected to bracket the length of time that the H5N1 virus remained viable for each material and environmental condition. The H5N1 virus persisted for relatively short durations on all materials at room temperature, low RH, no UV and at room temperature, high RH, no UV. The H5N1 virus was not detected after the 1-day test on galvanized metal and glass and was only detected after the 1-day test on soil. For chicken feces, at the room temperature, high RH, no UV environmental condition, the $TCID_{50}$ level measured after the 2-day test was less than the $TCID_{50}$ associated with the procedural blank, possibly indicating an effect of the test material, rather than the H5N1 virus. Similarly, for the room temperature, low RH, no UV environmental condition, the $TCID_{50}$ level measured from chicken feces after the 1-day test was less than the associated procedural blank $TCID_{50}$, indicating a possible test material interference rather than the H5N1 virus.

The H5N1 virus persisted longer on all materials at both low temperature conditions (i.e., low temperature, low RH, no UV and low temperature, high RH, no UV) compared to the room temperature conditions. For the low temperature, low RH, no UV environmental condition, the H5N1 virus persisted on galvanized metal, glass, and soil for 13 days (the longest duration tested). At the low temperature, high RH, no UV environmental condition, the H5N1 virus persisted for shorter durations on galvanized metal (four days), glass (nine days), and soil (nine days). Interestingly, the H5N1 virus persisted on chicken feces longer at the low temperature, high RH, no UV environmental condition (13 days) than at the low temperature, low RH, no UV environmental condition (possibly eight days where the $TCID_{50}$ level detected was less than the $TCID_{50}$ associated with the procedural blank, possibly indicating an effect of the test material).

Table 2-16. Summary of H5N1 Virus Persistence

Material and Environmental Condition	Longest Duration (Days) H5N1 Virus Detected	Shortest Duration (Days) H5N1 Virus Not Detected
Chicken feces		
Room temperature, low RH, no UV	1[a]	2
Room temperature, high RH, no UV	2[a]	4
Low temperature, low RH, no UV	8[a]	13
Low temperature, high RH, no UV	13	NA
Low temperature, low RH, UV-A/B	4[a]	NA
Galvanized metal		
Room temperature, low RH, no UV	NA	1
Room temperature, high RH, no UV	NA	1
Low temperature, low RH, no UV	13	NA
Low temperature, high RH, no UV	4	9
Low temperature, low RH, UV-A/B	1	2
Glass		
Room temperature, low RH, no UV	NA	1
Room temperature, high RH, no UV	NA	1
Low temperature, low RH, no UV	13	NA
Low temperature, high RH, no UV	9	13
Low temperature, low RH, UV-A/B	1	2
Soil		
Room temperature, low RH, no UV	1	2
Room temperature, high RH, no UV	1	2
Low temperature, low RH, no UV	13	NA
Low temperature, high RH, no UV	9	13
Low temperature, low RH, UV-A/B	4	NA

[a] The $TCID_{50}$ measured was at a level ≤ the procedural blank $TCID_{50}$ for chicken feces such that the CPEs observed (and used in the calculation of the $TCID_{50}$) may be attributed to the test material rather than H5N1 virus.
NA = Not available; the H5N1 virus was either detected at all durations or not detected from any duration.

For the low temperature, low RH, UV-A/B environmental condition, the H5N1 virus persisted longer on chicken feces and soil than galvanized metal and glass. The H5N1 virus was detected after only one day (24 hours of continuous exposure to UV-A/B) from galvanized metal and glass. The H5N1 virus was detected after two to four days of continuous exposure to UV-A/B on chicken feces and soil; on chicken feces the H5N1 virus was detected after the 2-day test at a level above the associated procedural blank and potentially detected after the 4-day test but at a level below the associated procedural blank, possibly indicating an effect of the test material rather than the H5N1 virus. In comparing these results to the low temperature, low RH environmental condition (no simulated sunlight), the UV-A/B clearly diminishes the H5N1 persistence.

3.0 Decontamination Technology Evaluation

3.1 Technology Descriptions

The liquid decontamination technologies evaluated consisted of:

- 1% Citric Acid
  - 1% citric acid was prepared by adding 1 gram (g) citric acid, anhydrous, (≥ 99.5% purity) to 99 mL hard water until completely dissolved.
- pH-Amended Bleach
  - pH-amended bleach was prepared by adding 5% acetic acid to household bleach (Clorox®; 5–6% sodium hypochlorite) to obtain a pH-amended bleach solution. The solution was prepared using 9.4 parts hard water, 1 part bleach, and 1 part 5% glacial acetic acid to obtain a solution having a mean pH of 6.81 ± 0.15 and a mean total chlorine content of 6,215 ± 212 ppm.
- 732 ppm Quaternary Ammonium
  - Hospital grade quaternary ammonium disinfectant [n-alkyl dimethyl benzyl ammonium chloride (6.25%), n-alkyl dimethyl ethylbenzyl ammonium chloride (6.25%), inert ingredients (87.5%)] was purchased from a local vendor and prepared per the vendor's guidance (3/4 ounces of disinfectant added to 1 gallon of hard water) to obtain a solution containing 732 ppm of the quaternary ammonium active ingredient. This concentration was not independently verified in this evaluation.
- 8% Na_2CO_3
  - 8% Na_2CO_3 was prepared by adding 8 g Na_2CO_3 (SigmaUltra (Sigma-Aldrich Inc.,St. Louis, Mo.); ≥99.0% purity) to 92 mL hard water until completely dissolved.

All preparations and dilutions were made using AOAC International hard water prepared at 400 ppm hardness as calcium carbonate ($CaCO_3$) (AOAC Official Method 960.09, *Germicidal and Detergent Sanitizing Action of Disinfectants*, Section E. Synthetic Hard-Water, p. 11). All decontamination contact times were 10 minutes.

3.2 Cytotoxicity of Neutralized Decontamination Liquids

During the decontamination technology evaluation, neutralization of each decontamination liquid was required in order to terminate activity at the end of the 10-minute decontamination contact time. For neutralization to be acceptable, the chemicals used could not interfere with the methods used for extraction and quantification of the H5N1 virus. The potential for neutralized decontamination liquids to cause cytotoxicity to the MDCK cells, which could subsequently interfere with the $TCID_{50}$ determination, was assessed with the MTT assay using the approach described in Section 3.2. Neutralization of each decontamination liquid was attempted by dilution with hard water (400 ppm as $CaCO_3$) alone and by the addition of Dey and Engley (D/E) neutralizing broth (solutions ranging from 50-95% D/E broth) followed by dilution with hard water. Fifty percent D/E broth was used for pH-amended bleach (3.0 mL decontamination liquid + 3.0 mL neutralizer), 75% D/E broth was used for 1% citric acid (1.5 mL decontamination liquid + 4.5 mL neutralizer), and 75% D/E broth was used for 732 ppm quaternary ammonium (1.5 mL decontamination liquid + 4.5 mL neutralizer). Sodium carbonate only required a 1:2 dilution of the decontamination liquid itself, with no need for the neutralizer (3.0 mL decontamination liquid + 3.0 mL hard water). The neutralization method for each decontamination liquid was generally selected based on the lowest amount of D/E broth that needed to be added (if any) in order to maintain ≥90% of the mean unexposed control MDCK cell viability after diluting the neutralization solution (with hard water) at a ratio of 1:2 (i.e., equal amounts of neutralization solution and hard water were used). The cytotoxicity test results are provided in Table 3-1 for the decontamination liquids and the selected neutralization approaches.

Table 3-1. Neutralized Decontamination Liquid Cytotoxicity Test Results

Decontamination Liquid	Neutralization Solution	Dilution with Hard Water	Mean Control Cell Optical Density	Mean Neutralized Decontamination Liquid Optical Density	% Cell Viability
1% Citric Acid	75% D/E broth[a]	1:8	0.3336	0.3240	97.1[e]
pH-Amended Bleach	50% D/E broth[b]	1:2	1.0299	1.0795	104.8[e]
732 ppm Quaternary Ammonium	75% D/E broth[c]	1:2	1.0610	1.0216	96.3
8% Na_2CO_3	No neutralizer (D/E broth)[d]	1:2	0.8761	0.8354	95.4

[a] 1.5 mL citric acid + 4.5 mL D/E broth.
[b] 3.0 mL pH-amended bleach + 3.0 mL D/E broth.
[c] 1.5 mL quaternary ammonium + 4.5 mL D/E broth.
[d] 3.0 mL Na_2CO_3 + 3.0 mL hard water.
[e] Viability above 100% may reflect non-specific reduction of MTT to formazan from unknown coupon extract components.

3.3 H5N1 Virus Recovery From Extraction Buffer, Neutralized Decontamination Liquid, and Trough Decontamination Liquid

The decontamination technology evaluation utilized test coupons that were each spiked and dried as described in Section 2.3 for persistence testing. For the evaluation conducted at the lower temperature, the coupons were first dried for one hour at room temperature, then placed in the refrigerator for one hour before adding the decontamination liquids (also placed in the refrigerator one hour prior to testing). In the decontamination technology evaluations, spiked test coupons and procedural blank coupons were inverted (spiked surface down) and placed into separate troughs for galvanized metal and separate vials for soil each holding enough decontamination liquid to cover the spiked surface of the coupon. At the end of the 10-minute decontamination contact time, the coupons were removed, neutralized in separate vials, extracted to recover H5N1 virus, and extracts assayed as described in Section 2.3.

For neutralization to be acceptable, it should not interfere with the methods used for extraction and quantification of the virus, and the neutralization must be effective at inhibiting the virucidal activity of the decontamination liquid. Three method demonstration tests were conducted to determine the applicability of each neutralization approach:

- H5N1 virus recovery from coupons using extraction buffer
- H5N1 virus recovery from neutralized decontamination liquid
- H5N1 virus recovery from trough decontamination liquid.

3.3.1 H5N1 Virus Recovery From Coupons Using Extraction Buffer

The intent of these tests was to demonstrate the ability to recover the H5N1 virus from coupons undergoing the test procedures of decontamination and neutralization by using only extraction buffer (PBS), essentially serving as a recovery control. Spiked coupons (galvanized metal and soil) were added to troughs/dishes containing PBS. After a 10-minute contact time, the test coupons were then removed and extracted and quantified as described in Section 2.3.2. For galvanized metal, the trough solution was also quantified to capture any virus washing into the trough liquid; soil test coupons became a mixture with the PBS in the vial and separate quantification of the trough liquid was not applicable. These tests were conducted three times (Trials A, B and C; each with three replicates) and the mean H5N1 virus recoveries are presented in Table 3-2. Although the recoveries were generally less than the performance criterion of ≥25% of the spiked level, as discussed in the test/QA plan[1], adequate quantities of the H5N1 virus remained and therefore decontamination efficacy (reported as log reduction) could be accurately assessed. Note: EPA's virucidal test guidance does not require a minimum percent recovery, but rather a minimum virus recovery ($TCID_{50}$) of $\geq 10^{4}$ [4, 12].

Table 3-2. H5N1 Virus Recovery From Coupons Using Extraction Buffer

Trial / Material (sample)	Inoculum ($TCID_{50}$)	Mean Recovered Virus ($TCID_{50}$)	Mean % Virus Recovery[a]
Trail A			
Galvanized Metal (total; coupon + trough)	5.01×10^{6}	8.17×10^{5}	13
Galvanized Metal (from coupon)	-	4.39×10^{5}	8.77
Galvanized Metal (from trough)	-	3.78×10^{5}	4.52
Soil	5.01×10^{6}	2.99×10^{5}	3.58
Trial B			
Galvanized Metal (total; coupon + trough)	2.00×10^{6}	5.40×10^{5}	27
Galvanized Metal (from coupon)	-	5.37×10^{5}	26.9
Galvanized Metal (from trough)	-	3.16×10^{3}	0.09
Soil	2.00×10^{6}	3.16×10^{5}	9.48
Trial C			
Galvanized Metal (total; coupon + trough)	5.01×10^{7}	1.52×10^{7}	30
Galvanized Metal (from coupon)	-	9.49×10^{6}	19.0
Galvanized Metal (from trough)	-	5.70×10^{6}	11.4
Soil	5.01×10^{7}	1.75×10^{6}	3.50

[a] The mean % virus recovery is determined by calculating the % virus recovery for each coupon (recovered virus/inoculum) and then computing the mean of these percentages.

3.3.2 H5N1 Virus Recovery From Neutralized Decontamination Liquid

The intent of this test was to determine the ability of the neutralization approach (as summarized in table 3-1) to inhibit the virucidal activity of the decontamination liquid. Briefly, spiked coupons (three for each material) were added to the troughs (for galvanized metal) or vials (for soil) of neutralized decontamination liquid (i.e., the neutralization solutions of Table 3-1; subsequent dilution with hard water at 1:2 was only required for the MTT assay) for the 10-minute contact time. The coupons were then removed and extracted and the H5N1 virus quantified as described in Section 2.3.2. For galvanized metal the H5N1 virus in the trough solution was also quantified to account for any virus washing into the trough liquid; soil test coupons became a mixture in the vials with the addition of neutralized decontamination liquid; since no troughs were used, a separate quantitation of the trough liquid was not applicable. The mean $TCID_{50}$ of H5N1 virus recovered from the neutralized decontamination liquids are presented in Table 3-3. Although the recoveries were generally less than the performance criterion of ≥25% of the spiked level (per the test/QA plan), an appreciable amount of the H5N1 virus remained (6.92×10^4 to 5.96×10^6 mean $TCID_{50}$) and therefore decontamination efficacy could be accurately assessed. Note: EPA's virucidal test guidance does not require a minimum percent recovery, but rather a minimum virus recovery ($TCID_{50}$) of $\geq 10^4$ (so that an adequate log reduction could be demonstrated).

3.3.3 H5N1 Virus Recovery From Trough Decontamination Liquid

The intent of these tests was to determine if the H5N1 virus remained in the decontamination liquid after the coupons (galvanized metal only) were removed from the troughs. Spiked galvanized metal coupons were placed in troughs of decontamination liquid for the 10-minute contact time and then removed. Given the possibility of the H5N1 virus being dislodged from the coupon during decontamination, the decontamination liquid in the trough was neutralized, and the H5N1 virus in the trough liquid was quantified as described in Section 2.3.2. The H5N1 virus recovered from the trough decontamination liquid is presented in Table 3-4. In view of the finding that the H5N1 virus was sometimes detected in the trough decontamination solution, the quantification of this residual liquid was incorporated into the actual decontamination technology evaluation approach to ensure the recovery of all of the H5N1 virus.

Table 3-3. H5N1 Virus Recovery From Neutralized Decontamination Liquid

Neutralized Decontamination Liquid / Material (sample)	Inoculum ($TCID_{50}$)	Mean Recovered Virus ($TCID_{50}$)	Mean % Virus Recovery[a]
1% Citric Acid + 75% D/E broth[b]			
Galvanized Metal (total; coupon + trough)	5.01×10^7	5.96×10^6	12
Galvanized Metal (from coupon)	-	5.96×10^6	11.9
Galvanized Metal (from trough)	-	0	0
Soil	5.01×10^7	2.60×10^5	0.52
pH-Amended Bleach + 50% D/E broth[b]			
Galvanized Metal (total; coupon + trough)	2.00×10^6	1.54×10^5	7.6
Galvanized Metal (from coupon)	-	1.51×10^5	7.53
Galvanized Metal (from trough)	-	3.16×10^3	0.09
Soil	2.00×10^6	6.96×10^5	20.9
8% Na_2CO_3 + No D/E broth[b]			
Galvanized Metal (total; coupon + trough)	5.01×10^6	6.96×10^4	1.4
Galvanized Metal (from coupon)	-	6.96×10^4	1.39
Galvanized Metal (from trough)	-	0	0
Soil	5.01×10^6	6.92×10^4	0.83
732 ppm Quaternary Ammonium + 75% D/E broth[b]			
Galvanized Metal (total; coupon + trough)	5.01×10^6	6.66×10^5	13
Galvanized Metal (from coupon)	-	5.96×10^5	11.9
Galvanized Metal (from trough)	-	6.96×10^4	0.83
Soil	5.01×10^6	1.60×10^6	19.1

[a] The mean % virus recovery is determined by calculating the % virus recovery for each coupon (recovered virus/inoculum) and then computing the mean of these percentages.

[b] Unlike the cytoxicity test results of neutralized decontamination liquid shown in Table 3-1, subsequent dilution with hard water was not required for this assay.

Table 3-4. H5N1 Virus Recovery From Galvanized Metal Trough Decontamination Liquid

Decontamination Liquid / Material (sample)	Inoculum ($TCID_{50}$)	Mean Recovered Virus ($TCID_{50}$)	Mean % Virus Recovery[a]
1% Citric Acid	5.01×10^7	0	0
pH-Amended Bleach	2.00×10^6	3.16×10^3	0.09
8% Na2CO_3[b]	Not tested	Not tested	Not tested
732 ppm Quaternary Ammonium	5.01×10^6	0	0

[a] The mean % virus recovery is determined by calculating the % virus recovery for each coupon (recovered virus/inoculum) and then computing the mean of these percentages.
[b] 8% Na_2CO_3 (which did not require D/E broth) for trough neutralization, was not tested.

3.4 Evaluation of Liquid Decontamination Technologies

3.4.1 Test Matrix

Log reductions in the H5N1 virus persistence were measured on two materials: galvanized metal and soil. Based on the relatively longer persistence times for the H5N1 virus under cold temperatures and low RH (especially in the absence of UV-A/B), the efficacy of decontamination liquids was assessed at both room temperature (22 °C) and low temperature (5 °C) for the 10 minute contact times. The experimental treatments performed are shown in Table 3-5.

3.4.2 Test Results

The H5N1 virus recovery and log reduction in $TCID_{50}$ for each decontamination liquid/environmental condition combination are summarized in Tables 3-6 for galvanized metal and Table 3-7 for soil. Table 3-6 and 3-7 also include the number of replicates for positive controls (5), test coupons (5), laboratory blank (1), and procedural blank (1). A summary of the log reductions obtained in all decontamination liquid tests is provided in Table 3-8. For the decontamination evaluations, the mean room temperature was 23.31 °C with a range of 23.11 °C to 23.33 °C and the mean low temperature was 5.34 °C with a range of 4.20 °C to 6.10 °C.

Following a 10-minute contact time, 8% Na_2CO_3 and 732 ppm quaternary ammonium, reduced the H5N1 virus on galvanized metal by less than 1.0 log in $TCID_{50}$ at room temperature and low temperature test conditions (Table 3-6). When 1% citric acid was applied, mean log reductions in H5N1 virus were 2.04 at room temperature and 2.13 at low temperature. Only pH-amended bleach with a 10-minute contact time, completely inactivated the H5N1 virus from galvanized metal (no detectable H5N1 virus), with mean log reductions of 7.1 at room temperature and 6.9 at the low temperature.

Table 3-5. Decontamination Technology Evaluation Matrix

Decontamination Liquid	Material	Environmental Condition
1% Citric Acid	Galvanized metal	Room temperature
	Galvanized metal	Low temperature
	Soil	Room temperature
	Soil	Low temperature
pH-Amended Bleach	Galvanized metal	Room temperature
	Galvanized metal	Low temperature
	Soil	Room temperature
	Soil	Low temperature
8% Na_2CO_3	Galvanized metal	Room temperature
	Galvanized metal	Low temperature
	Soil	Room temperature
	Soil	Low temperature
732 ppm Quaternary Ammonium	Galvanized metal	Room temperature
	Galvanized metal	Low temperature
	Soil	Room temperature
	Soil	Low temperature

Table 3-6. Decontamination Efficacy Against H5N1 Virus on Galvanized Metal

Decontamination Liquid / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature					
1% Citric Acid					
Positive Control[c]	2.00×10^7	5/5	$9.86 \pm 0.98 \times 10^6$	49.3 ± 4.87	-
Test Coupon[d]	2.00×10^7	5/5	$1.00 \pm 0.58 \times 10^5$	0.50 ± 0.29	2.0 ± 0.2
Laboratory Blank[e]	0	0/1	ND	-	-
Procedural Blank[f]	0	0/1	ND	-	-
pH-Amended Bleach					
Positive Control	7.94×10^7	5/5	$2.76 \pm 1.18 \times 10^7$	34.8 ± 14.9	-
Test Coupon	7.94×10^7	0/5	ND	0.00 ± 0.00[g]	7.1 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8% Na_2CO_3					
Positive Control	7.94×10^7	5/5	$2.76 \pm 1.18 \times 10^7$	34.8 ± 14.9	-
Test Coupon	7.94×10^7	5/5	$3.71 \pm 0.96 \times 10^6$	4.67 ± 1.21[h, i]	0.88 ± 0.11[h, i]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
732 ppm Quaternary Ammonium					
Positive Control	7.94×10^7	5/5	$2.76 \pm 1.18 \times 10^7$	34.8 ± 14.9	-
Test Coupon	7.94×10^7	5/5	$5.64 \pm 1.58 \times 10^6$	7.10 ± 1.99	0.70 ± 0.12
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature					
1% Citric Acid					
Positive Control	1.28×10^8	5/5	$5.83 \pm 2.95 \times 10^7$	45.5 ± 23.1	-
Test Coupon	1.28×10^8	5/5	$5.49 \pm 4.27 \times 10^5$	0.43 ± 0.33	2.1 ± 0.4
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
pH-Amended Bleach					
Positive Control	5.01×10^7	5/5	$1.43 \pm 0.53 \times 10^7$	28.5 ± 10.5	-
Test Coupon	5.01×10^7	0/5	ND	0.00 ± 0.00[g]	6.9 ± 0.0[g]
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8% Na_2CO_3					
Positive Control	5.01×10^7	5/5	$1.43 \pm 0.53 \times 10^7$	28.5 ± 10.5	-
Test Coupon	5.01×10^7	5/5	$2.19 \pm 1.86 \times 10^6$	4.37 ± 3.71	0.93 ± 0.35
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
732 ppm Quaternary Ammonium					
Positive Control	5.01×10^7	5/5	$1.43 \pm 0.53 \times 10^7$	28.5 ± 10.5	-
Test Coupon	5.01×10^7	5/5	$6.05 \pm 2.70 \times 10^6$	12.1 ± 5.39	0.43 ± 0.29
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicates.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] Positive controls were inoculated, placed in PBS and exposed to the environmental condition, extracted after the 10-minute contact time. Only one set of positive controls was used for each environmental condition and all four decontamination liquids.
[d] Test coupons were inoculated, exposed to the environmental condition and decontamination liquid for the 10-minute contact time.
[e] Laboratory blanks were not inoculated, extracted at time-zero.
[f] Procedural blanks were not inoculated, placed in PBS, exposed to the environmental condition, extracted after the 10-minute contact time.
[g] A value of 1 $TCID_{50}$ was used for non-detects (from the coupon and the trough liquid, which are summed to determine the total amount of virus) in the calculation of % recovery and mean log reduction.
h Although H5N1 virus was recovered from all five replicate galvanized metal coupons, H5N1 virus was not detected from the associated trough liquid (quantification of virus from the coupon and trough liquid is summed to determine the total amount of virus).
[i] A value of 1 $TCID_{50}$ was used for non-detects in the trough liquid for calculation of % virus recovery and mean log reduction.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

Table 3-7. Decontamination Efficacy Against H5N1 Virus on Soil

Decontamination Liquid / Sample	Inoculum ($TCID_{50}$)	CPE Detections[a]	Mean Recovered Virus ($TCID_{50}$)[b]	% Virus Recovery[b]	Mean Log Reduction[b]
Room Temperature					
1% Citric Acid					
Positive Control[c]	2.00×10^7	5/5	$3.12 \pm 2.80 \times 10^6$	15.62 ± 14.0	-
Test Coupon[d]	2.00×10^7	5/5	$1.25 \pm 0.78 \times 10^5$	0.63 ± 0.39	1.5 ± 0.4
Laboratory Blank[e]	0	0/1	ND	-	-
Procedural Blank[f]	0	0/1	ND	-	-
pH-Amended Bleach					
Positive Control	7.94×10^7	5/5	$4.27 \pm 1.01 \times 10^7$	53.8 ± 12.8	-
Test Coupon	7.94×10^7	5/5	$9.97 \pm 3.05 \times 10^4$	0.13 ± 0.04	2.7 ± 0.2
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8% Na_2CO_3					
Positive Control	7.94×10^7	5/5	$4.27 \pm 1.01 \times 10^7$	53.8 ± 12.8	-
Test Coupon	7.94×10^7	5/5	$6.86 \pm 4.97 \times 10^6$	8.64 ± 6.26	0.95 ± 0.49
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
732 ppm Quaternary Ammonium					
Positive Control	7.94×10^7	5/5	$4.27 \pm 1.01 \times 10^7$	53.8 ± 12.8	-
Test Coupon	7.94×10^7	5/5	$1.63 \pm 0.43 \times 10^7$	20.6 ± 5.36	0.43 ± 0.11
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
Low Temperature					
1% Citric Acid					
Positive Control	1.28×10^8	5/5	$2.83 \pm 1.32 \times 10^7$	22.14 ± 10.28	-
Test Coupon	1.28×10^8	5/5	$7.10 \pm 3.94 \times 10^5$	0.55 ± 0.31	1.7 ± 0.3
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
pH-Amended Bleach					
Positive Control	5.01×10^7	5/5	$2.16 \pm 1.63 \times 10^7$	43.1 ± 32.5	-
Test Coupon	5.01×10^7	5/5	$3.29 \pm 1.68 \times 10^4$	0.07 ± 0.03	2.9 ± 0.3
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
8% Na_2CO_3					
Positive Control	5.01×10^7	5/5	$2.16 \pm 1.63 \times 10^7$	43.1 ± 32.5	-
Test Coupon	5.01×10^7	5/5	$1.15 \pm 1.09 \times 10^7$	22.9 ± 21.8	0.42 ± 0.38
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-
732 ppm Quaternary Ammonium					
Positive Control	5.01×10^7	5/5	$2.16 \pm 1.63 \times 10^7$	43.1 ± 32.5	-
Test Coupon	5.01×10^7	5/5	$8.09 \pm 3.26 \times 10^6$	16.1 ± 6.51	0.45 ± 0.17
Laboratory Blank	0	0/1	ND	-	-
Procedural Blank	0	0/1	ND	-	-

[a] CPE detections: the numerator is the number of coupons with CPE detected and the denominator is the total number of replicate coupons.
[b] Data are expressed as mean ± standard deviation as applicable.
[c] Positive controls were inoculated, placed in PBS and exposed to the environmental condition, extracted after the 10-minute contact time.
[d] Test coupons were inoculated, exposed to the environmental condition and decontamination liquid for the 10-minute contact time.
[e] Laboratory blanks were not inoculated, extracted at time-zero.
[f] Procedural blanks were not inoculated, placed in PBS and exposed to the environmental condition, extracted after 10-minute contact time.
ND = Not detected; the detection limit was 1.31×10^3 $TCID_{50}$.
"-" Not applicable.

None of the evaluated decontamination technologies completely inactivated the H5N1 virus on soil (Table 3-7). For 8% Na_2CO_3 and 732 ppm quaternary ammonium (at room and the cold temperature), mean H5N1 log reductions on soil were less than 1.0. When 1% citric acid was applied, mean log reductions in H5N1 virus were 1.5 at room temperature and 1.7 at low temperature. When pH-amended bleach was used the mean H5N1 virus log reductions on soil were 2.7 at room temperature and 2.9 at the low temperature.

Table 3-8. Summary of Decontamination Efficacy Against H5N1 Virus

Material and Environmental Condition	H5N1 Virus Mean Log Reduction in $TCID_{50}$[a]			
	1% Citric Acid	pH-Amended Bleach	8% Na_2CO_3	732 ppm Quaternary Ammonium
Galvanized Metal				
Room Temperature	2.0 ± 0.2	7.1 ± 0.0[b]	0.88 ± 0.11[c]	0.70 ± 0.12
Low Temperature	2.1 ± 0.4	6.9 ± 0.0[b]	0.93 ± 0.35	0.43 ± 0.29
Soil				
Room Temperature	1.5 ± 0.4	2.7 ± 0.2	0.95 ± 0.49	0.43 ± 0.11
Low Temperature	1.7 ± 0.3	2.9 ± 0.3	0.42 ± 0.38	0.45 ± 0.17

[a] Data are expressed as mean ± standard deviation.
[b] H5N1 virus was not detected from any of the replicate test coupons or associated trough liquid; a value of 1 $TCID_{50}$ was used for these non-detects in the calculation of the H5N1 virus mean log reduction in $TCID_{50}$.
[c] H5N1 virus was not detected from the associated trough liquid; a value of 1 $TCID_{50}$ is used for non-detects in the calculation of H5N1 virus mean log reduction in $TCID_{50}$.

4.0 Quality Assurance/Quality Control

Quality assurance/quality control (QC) procedures were performed in accordance with the test/QA plan[1] and the TTEP QMP[2] for the persistence testing and decontamination technology evaluation. QA/QC procedures are summarized below. Some experimental procedures could not be conducted in accordance with the test/QA plan; those deviations are summarized in Section 4.5.

4.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, biological safety cabinets) used at the time of use was verified as being certified, calibrated, or validated.

4.2 Audits

4.2.1 Performance Evaluation Audit

No performance evaluation audit was performed for biological agents and organisms because quantitative standards for these biological materials do not exist. Performance evaluation audits for analytical measurements (e.g., spectrophotometric absorbance, temperature, RH, and contact time), will be conducted and reported in conjunction with the virus persistence/decontamination testing associated with freeze-dried vaccinia virus.

4.2.2 Technical Systems Audit

A technical systems audit was conducted on April 16, 2009. The assessment addressed all steps from removal from the cold storage (low temperature) followed by application of the decontamination technologies, neutralization, extraction, preparation of serial dilutions, and seeding onto cells. Only steps missed were staining of plates and reading of final plaques. All equipment used was within calibration and no other items were noted. In addition, all known deviations from the test procedures specified in the test/QA plan have been documented in this listing of deviations in Section 4.5.

4.2.3 Data Quality Audit

At least 10% of the data acquired during the persistence testing and decontamination technology evaluation were audited. A Battelle QA auditor traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.3 Quality Assurance/Quality Control Reporting

The audit described in Section 4.2.3 was documented in accordance with the QMP[2]. Quality control samples including laboratory blanks, procedural blanks, and positive controls are reported along with the test coupon results in Sections 2 and 3 for each persistence and decontamination test conducted.

4.4 Data Review

Records and data generated from the persistence testing and decontamination technology evaluation received a QC/technical review. All data were recorded by Battelle staff. The person performing the QC/technical review was not involved in the experiments and added his/her initials and the date to a hard copy of the record being reviewed. This hard copy was returned to the Battelle staff member who stored the record.

4.5 Deviations

Deviations from the test/QA plan[1] occasionally arose once work in the laboratory was initiated; these deviations were not expected to adversely affect data quality and included:

1. Soil and chicken feces were not autoclaved (avoiding potential effects on the soil matrix, such as hydrolysis of humic acids), but instead filtered through a 0.2 µm filter during the extraction process to remove bacteria (not viruses) from the supernatant that could potentially interfere with virus quantification. Blanks (non-inoculated coupons) confirmed that any naturally occurring viruses did not interfere with the $TCID_{50}$ assay (i.e., the $TCID_{50}$ was generally not detected). For soil, there was only one occasion (the 9-day test at low temperature, high RH, no UV) where $TCID_{50}$ was detected in the procedural blank.
2. Unpainted concrete and soil coupons were too small to spike with a multichannel pipette, so a single channel pipette was used for these coupons. There were no adverse impacts on the testing.
3. Regarding the assessment of potential cytotoxic effects of materials extracts, that could potentially interfere with the $TCID_{50}$ assay, the cell viability performance criterion of ≥90% of unexposed control cell viability with dilution of 1:8 or less was not attained for soil; however >90% of unexposed control cell viability was obtained with a dilution of 1:16, which was approved as an acceptable level of dilution by the EPA Task Order Project Officer. As noted in Section 2.2, although an increased level of dilution was needed for the MTT assay with soil, the $TCID_{50}$ assay was not affected as cytopathic effects were generally not observed in the MDCK cells exposed to any dilution of soil extract. There were no adverse impacts on the testing.
4. Alternative approaches were used for generating high RH and low temperature environmental conditions than proposed in the test/QA plan[1]. Specifically, instead of using a nebulizer to generate a high RH at the room temperature condition, the coupons were placed in a glove box pre-humidified by using an ultrasonic-fogger. The moist test material (i.e., soil

and chicken feces) maintained a high RH level. For conducting low temperature test, rather than running tubing from a re-circulating chiller through the glove box, testing was conducted inside a refrigerator. When testing at a low temperature with no UV the coupons were placed inside a Lock&Lock™ plastic storage container which was placed in the refrigerator. When testing at a low temperature with UV-A/B the coupons were placed directly into a refrigerator modified to include glove ports. There were no adverse impacts on the testing.

5. Continuous monitoring of temperature and RH was inadvertently not conducted during the H5N1 virus persistence testing at the room temperature, low RH, no UV environmental condition and during the initial seven hours of the 1-day test at the low temperature, low RH, UV-A/B environmental condition. No unusual events occurred that would lead one to expect that the temperature and RH of these tests deviated from those of comparable tests.
6. Temperature and RH inside the test chamber were found to be difficult to control (especially when trying to test non-ambient conditions over relatively long periods of time, i.e. days) and were often outside of the target environmental conditions (±2 °C for temperature and ±10% for RH), as documented in Section 2.4. However, these deviations from target environmental conditions do not invalidate the $TCID_{50}$ data. The actual (measured) temperature and RH data are all reported.
7. The performance criteria of no observed CPE in MDCK cells for laboratory and procedural blanks often failed for chicken feces; however laboratory and procedural blanks with CPE were not rejected because they were attributed to the test material itself. Relatively low detections of $TCID_{50}$ (i.e., $\leq 3.16 \times 10^3$ $TCID_{50}$) in chicken feces test coupons were often flagged as not being detected above the associated blank of 3.16×10^3 $TCID_{50}$ (i.e., it could not be determined if the CPE were attributed to the H5N1 virus or simply the chicken feces).
8. Contrary to the method development tests, most of the positive control virus recoveries were below the performance criterion (>20% and <120% virus recovery). However, even the lowest percent virus recovery (0.8%) had a mean of 2.54×10^5 $TCID_{50}$ recovered, which is an appreciable amount of virus to assess persistence. EPA's virucidal test guidance does not require a minimum percent recovery, but rather a minimum virus recovery ($TCID_{50}$) of $\geq 10^4$. It is possible that having a lower virus titer initially may make the virus seem less persistent than it would have been with a higher initial titer.
9. Regarding the decontamination technology evaluation, the positive controls (at the 10-minute contact time) were exposed to PBS rather than being exposed only to air, which better mimicked the actual application of the decontamination liquid.
10. Although the H5N1 virus recoveries from neutralized decontamination liquid and extraction buffer were generally <25%, the H5N1 virus recoveries were $>6 \times 10^4$ $TCID_{50}$, which is an appreciable amount of virus to assess decontamination efficacy. Note: EPA's virucidal test guidance does not require a minimum percent recovery, but rather a minimum virus recovery ($TCID_{50}$) of $\geq 10^4$. The H5N1 virus was detected in the trough decontamination liquid, such that the H5N1 virus recovery in the actual decontamination technology evaluation was quantified from the coupon and the liquid in the trough (for galvanized metal only) to ensure that all of the viable H5N1 virus was quantified.

5.0 Summary

5.1 H5N1 Virus Persistence

The persistence of the H5N1 virus (A/Vietnam/1203/04) was investigated on four materials (chicken feces, galvanized metal, glass, and soil) under five environmental conditions (each condition consisted of various combinations of temperature, RH, and exposure to UV-A/B radiation [to simulate sunlight]). Under room temperatures at both low and high RH (no UV), the H5N1 virus persisted for less than two days on chicken feces, galvanized metal, glass, and soil. At low temperatures under both low and high RH (no UV) the H5N1 virus persisted for at least four days on all materials. The H5N1 virus was detected following exposure to the low temperature, low RH, no UV environmental condition after 13 days on galvanized metal, glass, and soil. The H5N1 virus was detected on chicken feces, glass, and soil following exposure to the low temperature, high RH, no UV environmental condition for nine days. Testing was not conducted for durations longer than 13 days for any of the environmental conditions; the duration of H5N1 virus persistence may exceed 13 days, especially on galvanized metal and glass under the low temperature, low RH, no UV environmental condition.

With continuous UV-A/B exposure to simulate sunlight and under a low temperature and low RH environmental condition, the H5N1 virus persisted less than 48 hours on galvanized metal and glass. H5N1 persisted 96 hours on soil and chicken feces, although for chicken feces, the H5N1 level recovered from the test coupons could not be distinguished from the interference of the test material itself.

5.2 H5N1 Virus Liquid Decontamination

Four decontamination liquids (1% citric acid, pH-amended bleach, 732 ppm quaternary ammonium, and 8% Na_2CO_3) prepared with hard water were applied to galvanized metal and soil coupons inoculated with the H5N1 virus, and tested at room and low temperature conditions for 10-minute contact times. The decontamination technology evaluation indicated that only pH-amended bleach was effective at completely inactivating the H5N1 virus (i.e., the H5N1 virus was not detected), and this result was only obtained for galvanized metal (at both room and low temperatures). For soil, pH-amended bleach induced a mean H5N1 virus log reductions in $TCID_{50}$ of 2.7 at room temperature and 2.9 at low temperature. The mean H5N1 virus log reductions in $TCID_{50}$ associated with 1% citric acid ranged from 1.5 (with soil at room temperature) to 2.1 (with galvanized metal at low temperature). The mean log reductions in the H5N1 virus, determined for 732 ppm quaternary ammonium and 8% Na_2CO_3, were all less than 1.0 for both temperature conditions tested.

6.0 References

1. *Technology Testing and Evaluation Program Test/QA Plan for Persistence Testing of Avian Influenza Viruses on Outdoor Materials,* Version 1, Prepared for EPA by Battelle, Columbus, Ohio, March 2007.
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Appendix A:
H7N2 Virus Testing

In an effort to generate paired persistence and decontamination efficacy data for highly pathogenic avian influenza virus (i.e., H5N1 virus, strain A/Vietnam/1203/04) and low pathogenic avian influenza virus (i.e., H7N2 virus strain A/chicken/Maryland/Minh Ma/04), preliminary research was also conducted with the H7N2 virus.

To improve comparability of the test results for both viruses, it was desired to quantify both viruses using the same approach (i.e., the $TCID_{50}$ assay). However, initial efforts at propagating the H7N2 virus resulted in virus concentrations that were less than the target titer of 1×10^7 $TCID_{50}$ (using MDCK cells for virus quantitation, as with the H5N1 virus). Re-propagation of the H7N2 virus continued to yield apparently low titers; however, this was eventually attributed to the use of a mammalian cell line (MDCK) to quantify the H7N2 virus. Attempts were thus made to quantify the H7N2 virus using chicken embryo kidney (CEK) cells to conduct the $TCID_{50}$ assay. Initial results indicated that CEK cells may be used to quantify the H7N2 virus and yielded results indicating that the propagated H7N2 virus stock had a titer of 1.26×10^7 $TCID_{50}$/mL. However unacceptably high variability of this assay was obtained with repeated assays. Research was conducted to evaluate the effect of different aged CEK cells with the $TCID_{50}$ assay to reduce the assay variability, but the titer results remained too variable to draw conclusions (see Table A-1). It was determined that additional research was needed to develop a reliable CEK assay for H7N2 virus, which was beyond the scope of the current project.

Table A-1. H7N2 Virus Titers Obtained Using Chicken Embryo Kidney Cells[a]

H7N2 Virus Stock	7/16/07 Titer: Cells Plated 1 Day After Receiving ($TCID_{50}$/mL)	7/31/07 Titer: Cells Plated 1 Day After Receiving ($TCID_{50}$/mL)	8/1/07 Titer: Cells Plated 2 Days After Receiving ($TCID_{50}$/mL)
4/13/07 H7N2 Stock (1:500)[b]	1.26×10^6	5.01×10^5	1.26×10^6
4/13/07 H7N2 Stock (1:1000)[c]	2.00×10^4	2.00×10^5	3.16×10^5
4/13/07 H7N2 Stock (1:5000)[d]	5.01×10^3	3.16×10^5	5.01×10^5
5/4/07 H7N2 Stock	2.00×10^3	1.26×10^5	2.00×10^6
5/25/07 H7N2 Stock[e]	2.00×10^3	2.00×10^5	2.00×10^6

[a] The results of using the fresh (plated within 48 hours of receipt from vendor) CEK cells (7/31/07 titer) and the aged (plated after 48 hours after receipt from vendor) CEK cells (8/1/07 titer) were compared with initial titer results; all results were based on the fifth day of post-infection.

[b] 1:500 = initial 1:10 dilution of H7N2 virus stock (0.01 mL virus stock + 0.09 mL diluent), final 1:50 dilution of initial dilution (0.03 mL 1:10 virus dilution + 1.47 mL diluent) for a 1:500 quantity of virus needed to infect eggs.

[c] 1:1000 = initial 1:100 dilution of H7N2 virus stock (0.01 mL virus stock + 0.99 mL diluent), final 1:10 dilution of initial dilution (0.15 mL 1:10 virus dilution + 1.35 mL diluent) for a 1:1000 quantity of virus needed to infect eggs.

[d] 1:5000 = initial 1:100 dilution of H7N2 virus stock (0.01 mL virus stock + 0.99 mL diluent), final 1:50 dilution of initial dilution (0.03 mL 1:10 virus dilution + 1.47 mL diluent) for a 1:5000 quantity of virus needed to infect eggs.

[e] Titered at 1.26×10^7 $TCID_{50}$/mL on 6/28/07; cells plated two days after receiving.

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