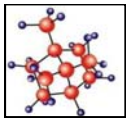
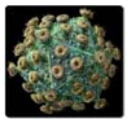

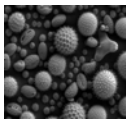

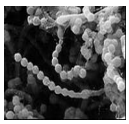
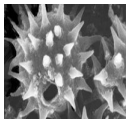


# Ensayos con virus de la Gripe A (H5N8) en superficie. Universidad de Kansas

6

## RCI Inactivation of Avian Influenza

## INTRODUCTION

The influenza virus, a member of the viral family *Orthomyxoviridae*, is characterized as being an enveloped single stranded negative sensed RNA virus (6) that can result in yearly endemic outbreaks and more severe world-wide pandemic outbreaks. Influenza A commonly infects human, swine, equine, and avian isolates. In the case of a pandemic outbreak, highly pathogenic avian influenza (H5N1) is currently the greatest threat due to current epidemic status in Asia, Europe, and Africa and continued threat for pandemic spread. Reassortment of genomic information of the influenza virus can result in a more pathogenic and infectious isolate is heightened during ongoing outbreaks, which could result in a devastating human-to-human transmissibility. Influenza virus is typically spread via aerosols, large droplets, or contact with infectious secretions or fomites (4).

Rapid containment of an outbreak is important for preventing further spread and minimizing the potential for reassortment to occur. Influenza has been shown to survive on nonporous surfaces for up to 48 hours and on material surfaces such as cloth, paper, or tissue for up to 12 hours after being deposited at approximately a $10^5$ $TCID_{50}$/ml level (1). In addition to surface sanitation and disinfection regimens, airborne inactivation of influenza virus is also vital to address predominant modes of transmission such as aerosol and large droplet (4). Environmental contamination with aerosolized droplets containing this pathogen can serve as a reservoir for infection and must be controlled by effective sanitation and disinfection protocols. Minimizing the degree of environmental contamination with highly effective decontamination measures would aid in the overall containment efforts of an outbreak.

The purpose of this study is to validate the complete inactivation of influenza A viruses using a low pathogenic avian influenza (H5N8) as a surrogate virus for the highly pathogenic avian influenza (H5N1) following exposure to the Radiant Catalytic Ionization-Cell™ (RCI-Cell™) system. The RCI-Cell™ system is an advanced oxidation tool which combines UV inactivation in the presence of hydroxical radicals so that synergy between two highly effective inactivation technologies occurs. Efficacy will be determined for dried inoculum on solid surfaces, in cell culture propagated inoculum, and nebulized in a controlled chamber. Efficacy will be determined by reduced or complete loss of infectivity in a cell culture system for treated samples compared to non-treated positive control samples.

## MATERIALS AND METHODS

**Virus and cells.** Low pathogenic avian influenza H5N8 (H5N8, provided generously by the Centers for Disease Control and Prevention, Atlanta, GA) was propagated in 10 day embryonated hen eggs (Kansas State University Department of Poultry Science, Manhattan, KS) to approximately $10^7$ $\log_{10}$ $TCID_{50}$ (as determined in Madin Darby Canine Kidney, MDCK cells). Cells were maintained in Minimal Essential Medium with Earle's salts and L-glutamine (Invitrogen Corporation, Carlsbad, CA) and 2.2 g/L sodium

bicarbonate (Fisher Scientific, Hampton, NH) collectively referred to as MEM containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with antibiotics [2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G (all from Fisher Scientific)]. Infectivity media was made by adding MEM with the addition of 0.1% TPCK treated trypsin (Fisher Scientific) and supplemented with antibiotics (2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G).

**H5N8 inactivation.** Type 302 stainless steel (McMasterCarr, Altanta, GA) coupons (2 x 10 $cm^2$, thickness 0.8 mm) were sterilized by autoclaving for 15 min at 121 C. In a biosafety class II cabinet, 100 µl of egg propagated H5N8 was added to each test coupon and spread to cover the entire surface using the pipette tip and allowed to dry completely for approximately 10-15 min. Then, the inoculated coupons were placed into a sterile transport container and transported to the test chamber. The test coupons were then attached to clips within the test chamber so that all sides of the coupon would be exposed to the RCI-Cell™ treatment. One coupon was removed prior to starting the RCI-Cell™ treatment to be used as the initial control sample. The RCI-Cell™ device was then turned on and samples were taken at various intervals (2, 4, 8, 12, 24 hours) by removing a test coupon and preparing it for virus recovery as described below.

**Virus Recovery.** H5N8 virus was recovered from the stainless steel surfaces by adding the test coupon to a sterile 50 ml conical vial (Fisher Scientific) containing 5 ml infectivity media. Tubes were then vortexed for 1 min. Endpoint dilution titration was conducted in MDCK cells by adding 220 µl from the 5 ml infectivity media containing any suspended virus to the first dilution well in a minimum of 6 wells of a 96 well microtiter plate containing confluent MDCK cells. Then, serial 1:10 dilutions were prepared by adding 20 µl from the first well into the next 6 wells each containing 180 µl infectivity media. The final well contained only 200 µl infectivity media to serve as a negative cellular control. Plates were incubated at 37 C, 5% CO2 for 48 hours. Cytopathic effect (CPE) was determined for each well and viral counts were reported as TCID50/ml as calculated by Reed and Muench (3).

**Real-Time Reverse Transcription Polymerase Chain Reaction *(r*RT-PCR*).*** Viral RNA was recovered using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Quantitative detection of the extracted influenza RNA was conducted using *r*RT-PCR using a fluorescently labeled TaqMan probe. The *r*RT-PCR primer and probe sequences were provided generously by the Molecular Genetics Influenza Branch, Centers for Disease Control and Prevention in Atlanta, GA. The detection threshold for successfully detecting influenza RNA was a FAM fluorescence signal ≥ 3 using the SmartCycler.

## RESULTS

The average amount of H5N8 recovered from the stainless steel coupons in all experiments was 5.35 $\log_{10}$ $TCID_{50}$/ml. Following treatment with the RCI-Cell™, the average log reductions of the H5N8 virus were 1.85, 2.79, 4.16, 5.35, and 5.35 $\log_{10}$ $TCID_{50}$/ml following 2, 4, 8, 12, and 24 hour treatments (Figure 1) based on the recovery of infectious virus.

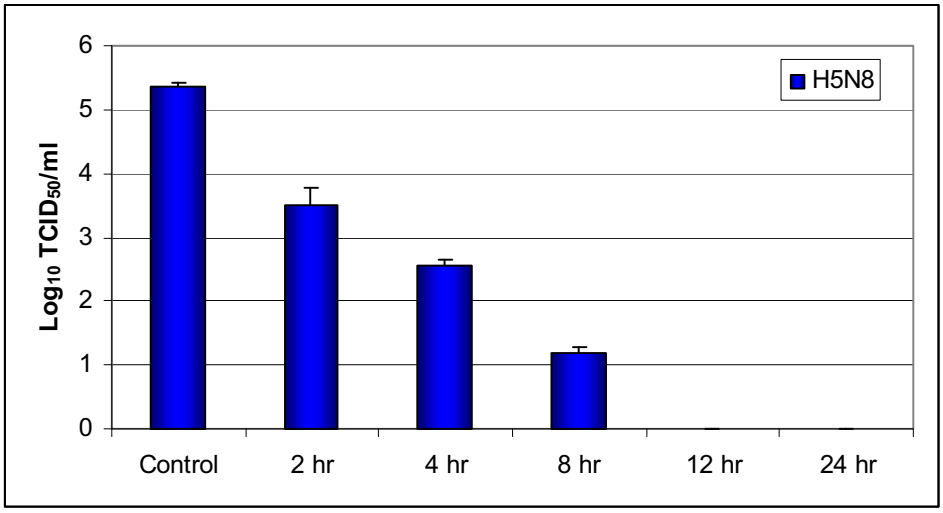


**Figure 1: Recovery of H5N8 post-treatment with RCI-Cell™ based on $TCID_{50}$/ml in MDCK cells.**

The average amount of viral H5N8 RNA recovered from the stainless steel coupons in all experiments was 4.00 $log_{10}$ based on a quantitative RT-PCR available for influenza A viruses. Following treatment with the RCI-Cell™, the average log reductions of the H5N8 virus based on the amount of RNA recovered varied between 0.23 to 0.54 $log_{10}$ following all exposure times (2, 4, 8, 12, and 24 hour) indicating that the mechanism of action for loss of infectivity was more likely due to disruption of the lipid envelope or structural proteins than with degradation of the viral nucleic acid (Figure 2).

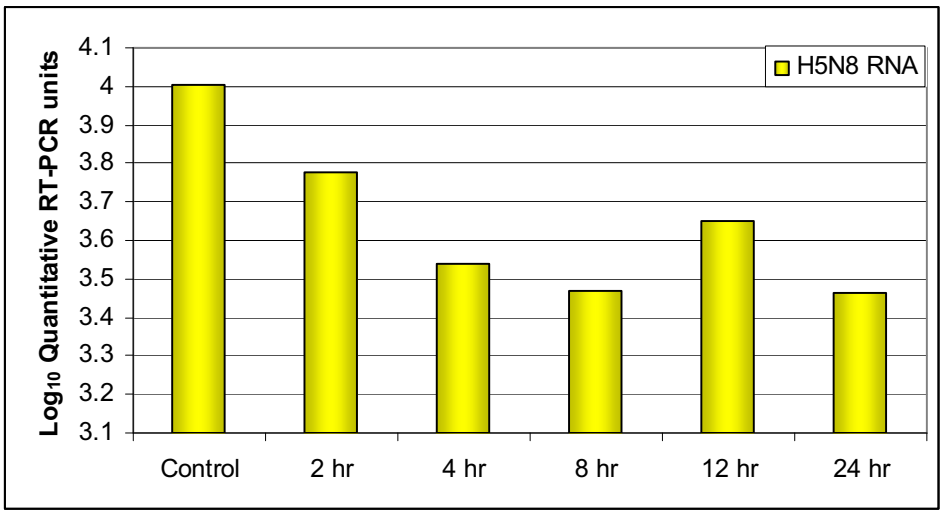


**Figure 2: Recovery of H5N8 RNA post-treatment with RCI-Cell™ based on quantitative RT-PCR.**

## DISCUSSION

In an effort to better understand the inactivation of the influenza virus using the RCI-Cell™, the efficacy was evaluated using a low pathogenic avian influenza isolate, H5N8 inoculated onto stainless steel surfaces. Inactivation efficacy was determined following

the current EPA guidelines for determining virus disinfection (2) which allows the recovery of treated virus as endpoint dilution including a $TCID_{50}$ recovery assay of infectious virus. In addition to the recovery of infectious virus, we wanted to determine if any disruption of viral RNA was occurring by using a quantitative RT-PCR assay specific for influenza A viruses in our experiments.

Based on the current EPA guidelines to achieve a > 4.0 log10 reduction in starting virus titer (2), RCI-Cell™ treatment for 8 hours or more resulted in the successful inactivation of the H5N8 isolate (Figure 1) for a starting contamination level of 5.35 $\log_{10}$ $TCID_{50}$/ml. Additional testing would be required to determine if lower exposure times would result in complete inactivation for contamination levels lower than 5.35 $\log_{10}$ $TCID_{50}$/ml, which might be more representative in a real outbreak (1, 5).

The quantitative RT-PCR results indicate that degradation of viral RNA (Figure 2) was not the major mechanism for viral inactivation, as the levels of RNA recovered after each treatment time were not significantly different from each other, $P > 0.05$. Other possible viral targets include the lipid envelope and structural proteins which were likely affected by the RCI-Cell™ treatment. The oxidative mechanism of this treatment likely disrupted the relatively susceptible envelope and could have resulted in denaturing the surface structural proteins of the influenza virus necessary for successful attachment and entry mechanism vital for infectivity.

The results obtained in this research experiment show that exposure to the RCI-Cell™ system for 8 hours results in the required level of inactivation of an avian influenza isolate, H5N8 which was used as a safe surrogate for the highly pathogenic H5N1 isolate. The mechanism of action of this technology is likely due to the oxidative chemistry resulting in both disruption of the lipid envelope and the denaturing effect on the structural viral proteins necessary for virus replication.

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1. **Bean, B., B. M. Moore, B. Sterner, L. R. Peterson, D. N. Gerding, and H. H. J. Balfour.** 1982. Survival of Influenza Viruses on Environmental Surfaces. The Journal of Infectious Diseases **146:**47-51.
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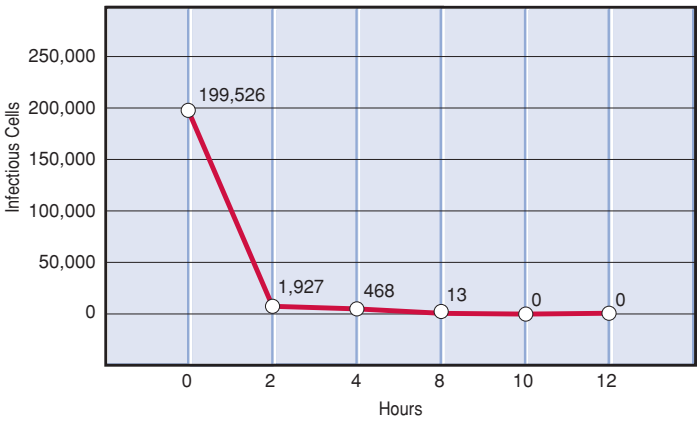
6. **Wright, P. F., and R. G. Webster.** 2001. Orthomyxoviruses, Fourth ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.

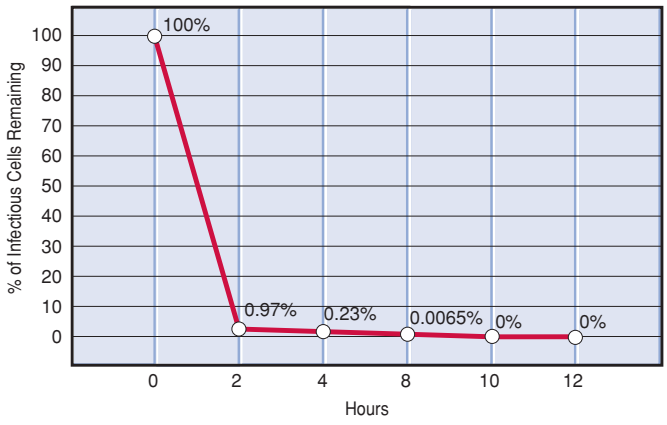
# Effects of RCI Technology

on reducing Avian Influenza A (HSN1) on **surfaces*** in 12-hour testing.

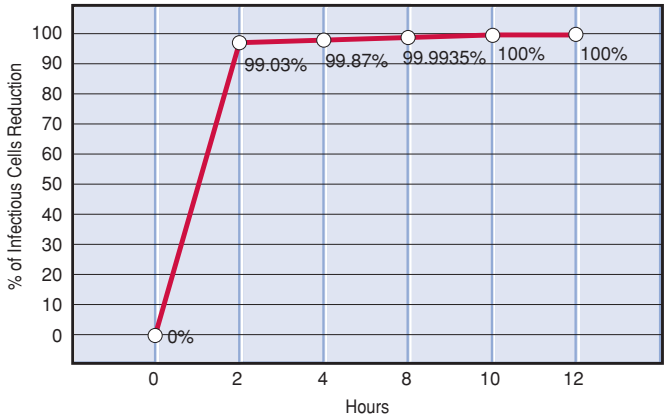
**Avian Influenza A (H5N8) Inactivation with RCI**
Infectious Cells vs Time




**Avian Influenza A (H5N8) Inactivation with RCI**
Percent of Infectious Cells Remaining vs Time




**Avian Influenza A (H5N8) Inactivation with RCI**
Percent of Infectious Cells Reduced vs Time




Testing by Kansas State University. Field results may vary based on environmental conditions.

*Scientific tests have demonstrated the use of EcoQuest air purifiers substantially reduce microbial populations on **surfaces**. Presently EcoQuest does not make a similar claim with respect to airborne microbial. These statements have not been evaluated by the FDA. These products are not intended to diagnose, treat, cure, or prevent any disease.



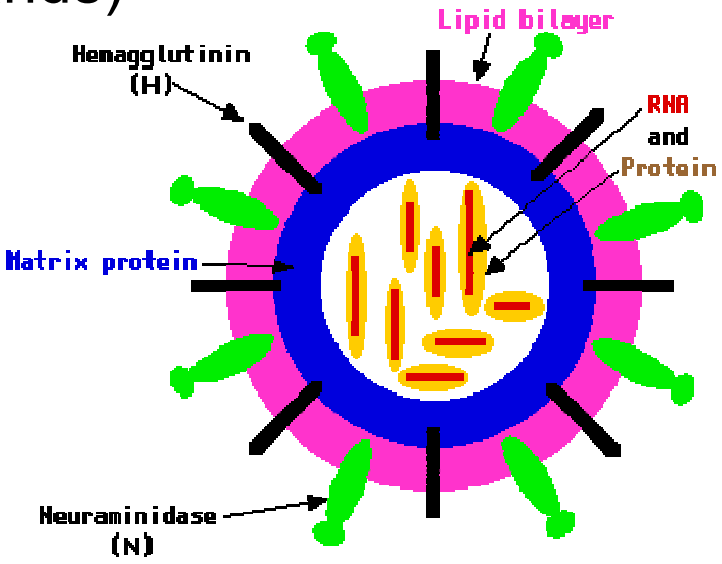
IM_AF_Avain Flu Charts_0906

# RCI Inactivation of Avian Influenza on **surfaces**

Jill Bieker, PhD
James L, Marsden, PhD
Kansas State University

# Avian Influenza

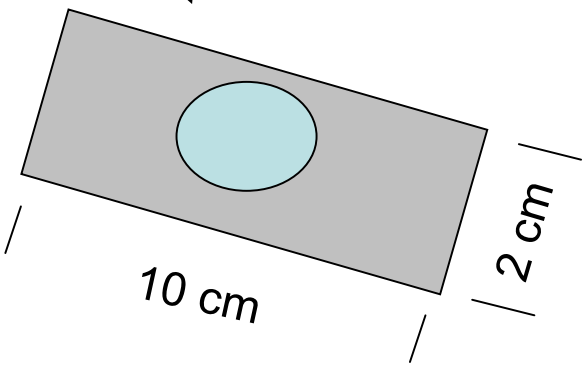
- Influenza virus is made up of:
  - Hemagglutinin (16 subtypes ie. H5)
  - Neuraminidase (9 subtypes ie. N1)
  - Matrix protein
  - RNA (8 negative sense strands)
  - Lipid envelope



# Methodology

- Inoculum: low pathogenic avian influenza H5N8 *isolated from turkey

100 µl cell propagated H5N8 added to coupon



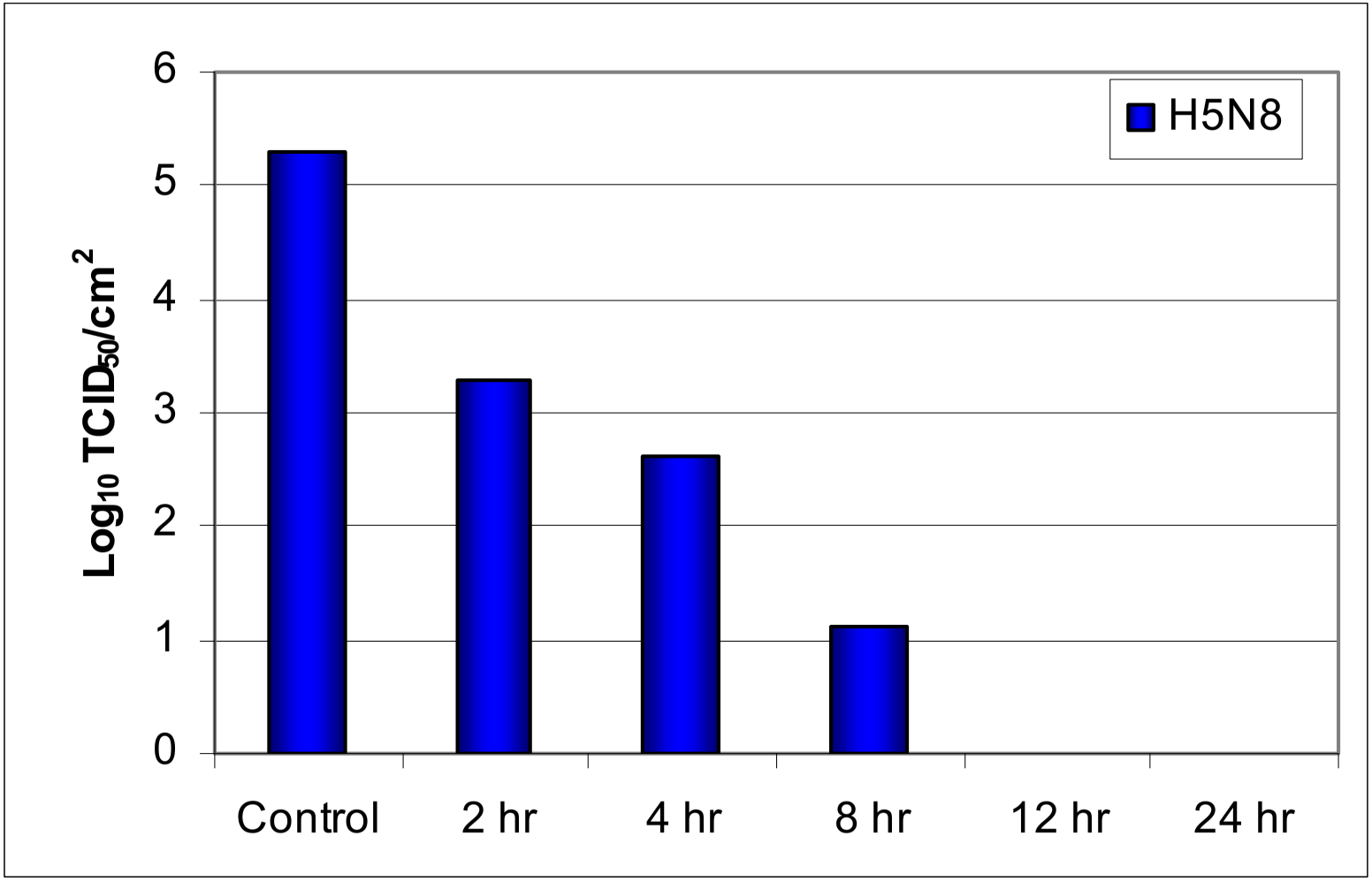

Inoculum spread with pipette tip to cover entire surface & dried in biosafety hood for 10 minutes

Stainless steel coupons exposed to RCI cell for 2, 4, 8, 12, & 24 hours

# Methodology

- Following contact times, stainless steel coupons were added to 50 ml conical tubes with 10 ml MEM infectivity media and vortexed
- Serial 1:10 dilutions were prepared in MEM infectivity media and monolayered Madin Darby Canine Kidney (MDCK) cells were infected for 48 hrs at 37 C with 5% $CO_2$
- Titer reported as tissue culture infective dose 50 ($TCID_{50}/cm^2$)

# Results



# Conclusions

- Based on these tests, treatment with the RCI-Cell™ system against the avian H5N8 virus showed continual inactivation of the infectious virus on **surfaces***
- After 12 hrs, no infectious virus was detected at all
- RCI is a highly effective system for the inactivation of this virus

*These scientific tests have demonstrated the use of EcoQuest air purifiers substantially reduce microbial populations on **surfaces** - including but not limited to avian H5N8 virus. At this point, product testing does not make a similar demonstration with respect to airborne microbials. These statements have not been evaluated by the FDA. These products are not intended to diagnose, treat, cure, or prevent any disease.