Virucidal Efficacy Assay

Sponsor	AIONX Antimicrobial Technologies
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Viruses Tested:	Influenza A/California/07/09(H1N1) (USU# V3213) Murine Norovirus (USU# V2504)
Cell Line:	MDCK RAW 264.7
Incubation:	4-6 days
Experiment #:	FLU-1316 MNV-004

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Introduction:
Testing was performed to determine if the test system would inactivate an enveloped (influenza A virus) and non-enveloped (murine norovirus) virus when exposed at various time intervals. Virus solutions were placed on the test surface for specified times, then surviving infectious virus was quantified by standard $CCID_{50}$ assays and compared with untreated controls.

Procedures:
The test system was set up as shown in Figure 1, except that it was placed in a biosafety cabinet during testing. A drop of 150-200 μL of either influenza A/California/07/09(H1N1) virus or murine norovirus stock was placed on the active grid area of the black coupon. An untreated virus control was placed on the black plastic coupon outside of the active grid area. For a toxicity control, a drop containing media only (no virus) was placed on the active grid area of a separate coupon. The test was performed in triplicate and the replicates for each virus were tested at the same time with one system set-up for each replicate (3 virus test systems and one toxicity test system were running simultaneously). The two viruses were tested individually at separate times.

The system was turned on and the multimeter set to read at 2000 μA and observed to ensure there was a reading to indicate that the circuit was complete. Readings between 160-400 μA were observed throughout the testing period. The samples were covered with a loose lid to prevent drying out of the droplet due to airflow in the biosafety cabinet. After 10 minutes, 100 μL of the sample was removed and immediately diluted 1/10 in test medium to neutralize, or stop antiviral activity. Samples were stored at -80°C. The test was performed 3 times, collecting samples at 10, 30 and 60 minutes for each virus.

Virus quantitation. Surviving virus from each sample was quantified by standard $CCID_{50}$ end-point dilution assay. Samples were thawed and serially diluted 1/10 in test medium. Then 100 μL of each dilution were plated into quadruplicate wells of 96-well plates containing 80-90% confluent cells. Plates were incubated at 37 ± 2°C with 5% CO_2 for 4 days for norovirus and 6 days for influenza virus. Each well was then scored for presence or absence of virus. The $CCID_{50}$ values were calculated using the Reed-Muench (1948) equation. Three independent replicates of each sample were tested, and the average and standard deviation were calculated. Results were compared with untreated controls by a two-tailed t-test using GraphPad Prism (version 8) software.

Media and cells. The test medium for influenza virus was MEM with 10 IU/mL trypsin, 1 μg/mL EDTA and 50 μg/mL gentamicin. Test medium for mouse norovirus was DMEM with 2% FBS

and 50 μg/mL gentamicin. Cells for titer assays were RAW 264.7 cells for norovirus and MDCK cells for influenza.

Controls: Virus controls were tested on the non-active area of the coupon in parallel and the reduction of virus in test wells compared to virus controls was calculated as the log reduction value (LRV). Toxicity controls were tested with media not containing virus on the active area of the coupon to see if metal ions or other bi-products generated in the test procedure were toxic to cells. Neutralization controls were tested to ensure that virus inactivation did not continue after the exposure time, and that residual sample in the titer assay plates did not inhibit growth and detection of surviving virus. This was done by adding the toxicity samples to titer test plates then spiking each well with a low amount of virus that would produce an observable amount of CPE during the incubation period (about 30 CCID50/well).

Results:
Table 1 shows results for influenza A/CA/07/09 (H1N1) virus at three exposure times. Toxicity in MDCK cells was observed at all three time points in the top (1/10) dilution and therefore the limit of detection of virus for the test wells was 1.7 $\log_{10}$ $CCID_{50}$ per test well containing 0.1 mL of sample. After 10, 30, and 60 minutes, the test system reduced virus titers by 1.7, 4.7, and 4.9 $\log_{10}$ $CCID_{50}$, respectively ($p<0.001$).

Table 2 shows results for murine norovirus at three exposure times. Toxicity was also observed in RAW 264.7 cells at all three time points in the top (1/10) dilution and therefore the limit of detection of virus for the test wells was 1.7 $\log_{10}$ $CCID_{50}$ per test well. After 10, 30, and 60 minutes, the test system reduced virus titers by 1.2 ($p<0.05$), 4.5 ($p<0.0010$), and 4.5 ($p<0.001$) $\log_{10}$ $CCID_{50}$, respectively.

Readings varying from 160-400 μA were observed during the incubation and the readings increased during the course of the incubation, ending with higher reads than at the beginning of the test. Bubbling was observed and a blue tint appeared in the liquid droplet during the longer run times of 30 and 60 minutes.

Virus controls and sterile media controls were as expected in each case. Toxicity controls showed that the system affected the media leading to a toxic effect in both cell types at the 1/10 dilution of the sample. Neutralization controls confirmed that virus was detected in the presence of the test sample on cell plates.

Summary of Results:

The AIONX system effectively reduced virus titers of enveloped (influenza) and non-enveloped (norovirus) viruses. Virucidal activity increased with longer exposure times.

Table 1. Virucidal effect of the AIONX test system against influenza A(H1N1) after 10-, 30-, or 60-minute run times.

Time (min)	Test volume (µL)	CNTL volume (µL)	Test sample ($CCID_{50}$/0.1 mL)[b]	Virus Control $CCID_{50}$/0.1 mL	LRV[c]
10	150	150	4.7	6.5	1.8
10	150	150	4.7	6.3	1.6
10	150	150	4.7	6.5	1.8
		Average[a]	4.7 ± 0.0***	6.4 ± 0.12	1.7
30	150	150	2	6.7	4.7
30	150	150	2	6.5	4.5
30	150	150	1.7	6.5	4.8
		Average[a]	1.9 ± 0.19***	6.6 ± 0.10	4.7
60	200	200	<1.7	6.5	>4.8
60	200	200	1.7	6.7	5.0
60	200	200	<1.7	6.5	>4.8
		Average[a]	1.7 ± 0.0***	6.6 ± 0.10	4.9

[a] Average of 3 replicates $\pm$ standard deviation

[b] Toxicity to cells was observed in the 1/10 dilution of all time points and therefore the limit of detection in test wells was 1.7 $\log_{10}$ $CCID_{50}$ of virus per 0.1 mL.

[c] LRV (log reduction value) is the $\log_{10}$ reduction of virus compared to the virus control

***P < 0.001 by two-tailed t-test compared with untreated virus control (water). For statistical analysis "<" signs were ignored.

Table 2. Virucidal effect of the AIONX test system against murine norovirus after 10-, 30-, or 60-minute run times.

Time (min)	Test volume (µL)	CNTL volume (µL)	Test sample ($CCID_{50}$/0.1 mL)[b]	Virus Control $CCID_{50}$/0.1 mL	LRV[c]
10	150	150	5.3	7.3	2.0
10	150	150	5.7	6.3	0.6
10	150	150	5.7	6.5	0.8
		Average[a]	5.5 ± 0.21*	6.7 ± 0.53	1.2
30	150	150	<1.7	6	>4.3
30	150	150	2	6.7	4.7
30	150	150	<1.7	6.3	>4.6
		Average[a]	1.8 ± 0.19***	6.3 ± 0.34	4.5
60	200	200	<1.7	6.3	>4.6
60	200	200	<1.7	6	>4.3
60	200	200	<1.7	6.3	>4.6
		Average[a]	1.7 ± 0.00***	6.2 ± 0.17	4.5

[a] Average of 3 replicates $\pm$ standard deviation

[b] Toxicity to cells was observed in the 1/10 dilution of all time points and therefore the limit of detection in test wells was 1.7 $\log_{10}$ $CCID_{50}$ of virus per 0.1 mL.

[c] LRV (log reduction value) is the $\log_{10}$ reduction of virus compared to the virus control

*P < 0.05, ***P < 0.001 by two-tailed t-test compared with untreated virus control (water). For statistical analysis "<" signs were ignored.

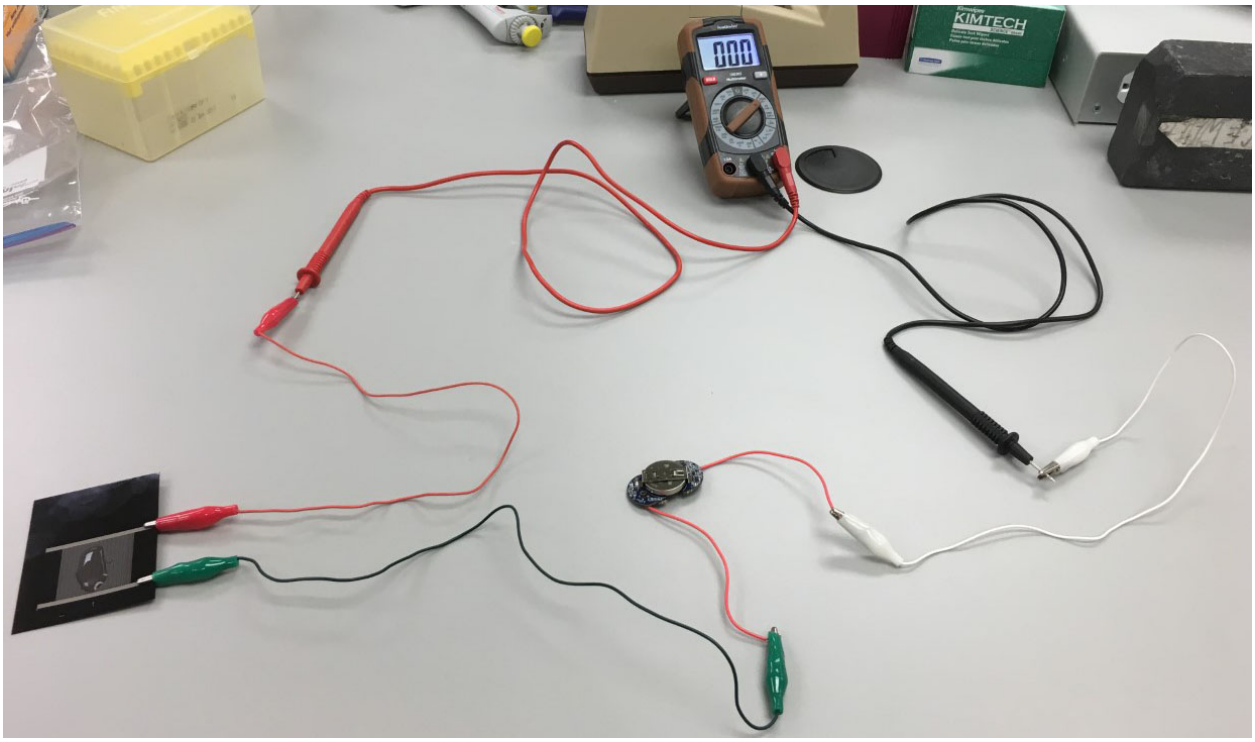

Figure 1. Example of AIONX system set-up. A 3V battery was used to power the printed circuit board (PCB). The multimeter was set to measure μA and was part of the complete circuit; measurements ranged from 160-400 during the test. A droplet of media or water was required on the active grid area of the test coupon (black) to complete the circuit.