

Enozo Human Coronavirus Testing

Enozo Technologies, Inc. Aqueous Ozone Spray Bottle demonstrates greater than 99.9% inactivation within 30 seconds of Human Coronavirus 229E/ATCC VR-740, a commercially available surrogate virus for the Human Coronavirus SARS-Cov-2 which causes the COVID-19 Disease.

Overview: Testing of the Enozo Aqueous Ozone (AO) Spray Bottle against virus surrogate 229E shows >99.9% inactivation when treated with aqueous ozone generated by the Enozo Aqueous Ozone Spray Bottle (SB100). The surrogate virus 229E/ATCC VR-740* is an appropriate test virus commercially available to mimic the SARS-CoV-2 virus responsible for development of the disease COVID-19. The test protocol is based on the ASTM E1052 Standard†, with considerations for use of ozone outlined below.

This study‡ evaluated the effectiveness of the Enozo AO spray bottle to remove virus 229E/ATCC VR-740 from hard surfaces such as countertops and doorknobs. The bottle is designed as a handheld device to generate “on-demand” AO spray which kills bacteria, viruses, and fungal spores on hard surfaces. For this study, the Enozo bottle was challenged with 229E which is a preferred surrogate for SARS-CoV-2 for use in Biosafety Level 2 laboratories. The surrogate virus is deemed to be ‘harder to kill’ than SARS-CoV-2 by ASTM. Previous testing has shown the AO spray bottle to be effective against the virus MS-2 bacteriophage (99.9% reduction in 30 seconds E1052), as well as Feline Calicivirus§ (99% in 5 minutes).

The study is based on ASTM test method E1052. In this method the virus is prepared in sufficient quantity and then mixed into a solution. The ozonated water is sprayed into the viral solution at a 9:1 ratio (9-parts AO to 1-part viral solution). After 30 seconds a neutralizer is added to the mix which halts the germ-killing action of the ozone. The mix is then incubated using standard cell culture methods and allowed to reproduce for an appropriate time after which visual assessment of virus population remaining is made. This population is compared to that of control samples prepared using un-ozonated water and reported using appropriate statistical methods.

The results indicated greater than 99.9% (> 3 net log reduction) in the Human Coronavirus 229E/ATCC VR-740 virus.

Disclaimer: The Enozo Aqueous Ozone Spray Bottle is categorized as a pesticidal device within the EPA classification structure. The EPA does not routinely include pesticidal devices in its review and therefore EPA has not confirmed whether, or under what circumstances, such products might be effective against the spread of COVID-19.

* https://www.astm.org/COMMIT/GuidanceCOVID19SurrogateSel_April242020press.pdf

† ATL Modified ASTM E1052 Study Report NG4136 01MAY2013

‡ This study was conducted in compliance with Good Laboratory Practices (GLP) as defined in 21 CFR, Part 58.

§ ATL Modified AOAC GST FCV Study Report NG3660 22OCT2012 (2)

Procedures

The ASTM E1052 Standard Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension protocol was followed as closely as possible, with special considerations for ozone as the test chemical outlined below. In brief, 1 ml virus was combined with 9 ml aqueous ozone from the Enozo SB100 Ozone Generating Spray Bottle, incubated at room temperature for 30 seconds, and diluted in the viral growth medium Eagle's Minimal Essential Medium with 2% Fetal Bovine Serum (EMEM + 2% FBS) to neutralize. Serial dilutions of inactivated ozone + virus were added to 24-hour old cultured human lung fibroblast cells (ATCC CCL-171) and scored for cytotoxic effects at 6 days post-infection. Virus control, cytotoxicity control, and neutralization control were performed in parallel. After 30 seconds a neutralizer is added to the mix which halts the germ-killing action of the ozone. The mix is then incubated using standard cell culture methods and allowed to reproduce for an appropriate time after which visual assessment of virus population remaining is made. This population is compared to that of control samples prepared using un-ozonated water and reported using appropriate statistical methods.

The results indicated greater than 99.9% (>3 net log reduction) in virus.

This study was conducted in compliance with Good Laboratory Practices (GLP) as defined in 21 CFR, Part 58.

Procedural Considerations

- The ASTM E1052 was chosen as the test method because it is the antiviral test method that best preserves the initial viability of low titer viruses, of which the human coronavirus surrogate 229E is representative.
- The Human Coronavirus 229E is a BL-2 virus that has the same structure, and therefore chemical reactivity, of the SARS-CoV-2 that causes COVID-19. It is the closest surrogate to SARS-CoV-2 that is available for testing, and the US EPA recognizes

tests with this surrogate as likely representative of results with SARS-CoV-2.

- Human Coronavirus was prepared by removing growth medium from 24-hour sub cultured human lung fibroblast cells (ATCC CCL-171) in a 75-cm² flask, washing 3 times with sterile phosphate buffered saline (PBS), and covering the cell sheet with 3-ml PBS. Cells were frozen at -80 °C for 20 minutes and thawed at 37 °C for 5 minutes for a total of 3 freeze-thaw cycles. Cells were then scraped into the PBS and centrifuged at 2000 rpm for 20 minutes to remove cells. This preparation results in a high titer of active virus and removes virus-inactivating serum from the cell growth medium.
- Virus and test substance were combined by spraying 9 ml undiluted, ozonated water onto 1 ml virus sample rather than by pre-spraying test substance and then adding virus. This consideration reduces the impact of ozone's short half-life by minimizing the time between ozone generation and testing. It also mimics the field use case of spraying directly onto a contaminated area without altering the dynamics of the test that would be performed on a diluted chemical substance.
- Neutralization was performed by 10-fold dilution in growth medium, followed by additional serial 10-fold dilutions prior to plating on healthy host cells, as described in the ASTM E1052 protocol.
- Neutralization and cytotoxicity controls were performed according to the ASTM E1052 protocol. In addition, a complete 24-well plate of healthy, untreated normal cells was grown to control for any

Figure 1. Enozo Technologies, Inc. SB100 Aqueous Ozone Spray Bottle



effects of closer exposure of edge and corner wells to atmospheric conditions.

Results

Activity of virus after spray with either ozonated or un-ozonated water from the Enozo SB100 spray bottle was tested by adding 2 ml of serial 10-fold dilutions, in quadruplicate, to a 24-well culture plate that had 24 hours growth of cultured human fibroblast cells (ATCC CCL-171). Cells were incubated at 35 °C with 5-10% carbon dioxide in air for 6 days and scored for cytotoxic effects. An overview of results is presented below. Wells marked with an “X” showed cytotoxicity. There was no cytotoxicity observed in healthy, untreated cells, in the cytotoxicity controls, or in the neutralization controls. The lack of toxicity in any of the control conditions is consistent with the nonspecific reactivity and short half-life of aqueous ozone. These properties make the active ingredient susceptible to a wide range of neutralizers and minimize its toxic effects on host cells during the time frame of the test.

Table 1. Virus treated with un-ozonated water (ozone off), sprayed through Enozo SB100 Bottle

10 ²	X	X	X	X
10 ³	X	X	X	X
10 ⁴	X	X	X	X
10 ⁵	X	X	X	X
10 ⁶	X	X		
10 ⁷				

Median Tissue Culture Infectious Dose (TCID50): 106/2ml = 5 x 10⁵/ml in 6 days.

Table 2. Virus treated with ozonated water (1 ppm ozone), sprayed through Enozo SB100

10 ²			X	X
10 ³				
10 ⁴				
10 ⁵				
10 ⁶				
10 ⁷				

Median Tissue Culture Infectious Dose (TCID50): 102/2ml = 5 x 10¹/ml in 6 days. Viral inactivation with ozone: 50 TCID50 / 500000 TCID50 = 1 x 10⁻⁴.
This corresponds to 1 – 1 x 10⁻⁴ = 99.99% inactivation of virus at 1 ppm ozone.

Reproducibility

The test was repeated to ensure reproducibility, with the following results.

Table 3. Virus treated with un-ozonated water (ozone off), sprayed through Enozo SB100

10 ²	X	X	X	X
10 ³	X	X	X	X
10 ⁴	X	X	X	X
10 ⁵	X	X	X	X
10 ⁶	X		X	
10 ⁷	X			

Median Tissue Culture Infectious Dose (TCID₅₀): 106 / 2ml = 5 x 10⁵/ml in 6 days.

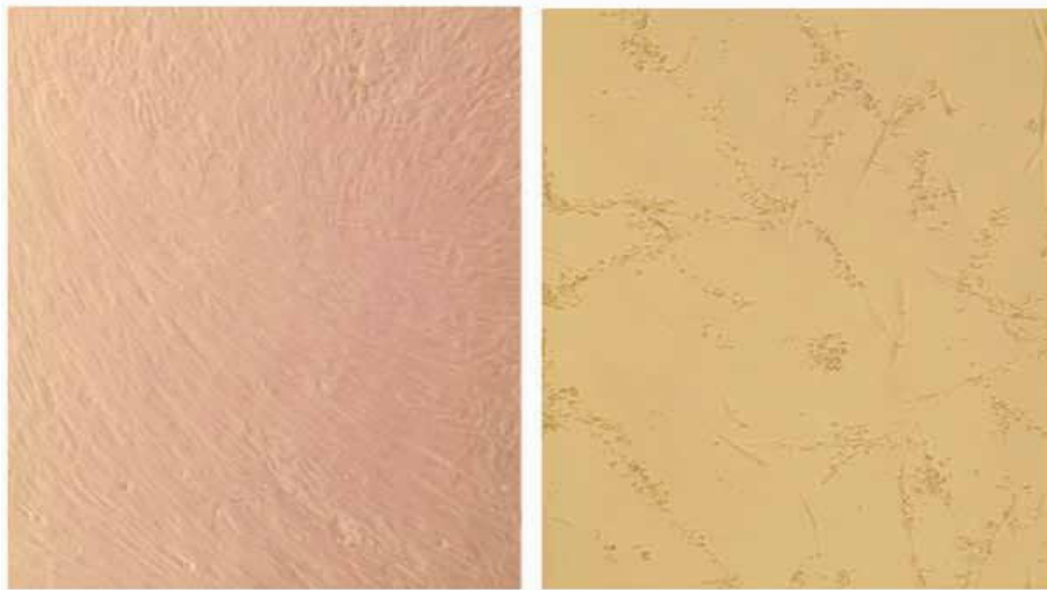
Table 4. Virus Treated with Ozonated Water (1 Ppm Ozone), Sprayed Through Enozo SB100

10 ²	X	X		X
10 ³	X			
10 ⁴				
10 ⁵				
10 ⁶				
10 ⁷				

Median Tissue Culture Infectious Dose (TCID₅₀): 102.5 / 2ml = 1.58 x 10²/ml in 6 days.

Viral inactivation with ozone: 158 TCID₅₀ / 500000 TCID₅₀ = 3.16 x 10⁴ (This corresponds to 1 – 3.16 x 10⁴ = 99.97% inactivation of virus at 1 ppm ozone.)

Figure 2. Representative images (100x magnified) of healthy MRC-5 human lung fibroblast cells (left) and of the same cell type showing cytotoxic effects (right) due to viral infection. Microscopic images such as these were used to score infectivity.



Healthy, elongated human lung fibroblasts after addition of ozone-treated virus (10^3 dilution of virus).

Human lung fibroblasts showing cytotoxic effects after infection with unozonated virus (10^6 dilution of virus).

Table 5. Study ID No. NG4136: Modified ASTM E1052 Test for Activity of Chemicals in Suspension

Microorganism	Suspension Type	Contact Time	PFU/ml*	Geometric Mean PFU/ ml	% Reduction vs Time Zero	Log10 Reduction vs. Time Zero
MS-2 Bacteriophage	MS-2 Bacteriophage	Time Zero	3.75E+07	3.72E+07	N/A	N/A
			3.75E+07			
	Test Suspension	30 Seconds	≤ 5.00E+01	≤ 5.00E+01	≥ 99.9998%	≥ 5.87
			≤ 5.00E+01			
		2 Minutes	≤ 5.00E+01	≤ 1.00E+02	≥ 99.9997%	≥ 5.57
			1.50E+02			
		5 Minutes	≤ 5.00E+01	≤ 5.00E+01	≥ 99.9998%	≥ 5.87
			≤ 5.00E+01			

Note: No virus detected therefore virus levels were at or below the limit of detection (≤5.00E+01).

Figure 3. Modified ASTM E1052 Test: MS-2 Bacteriophage Data

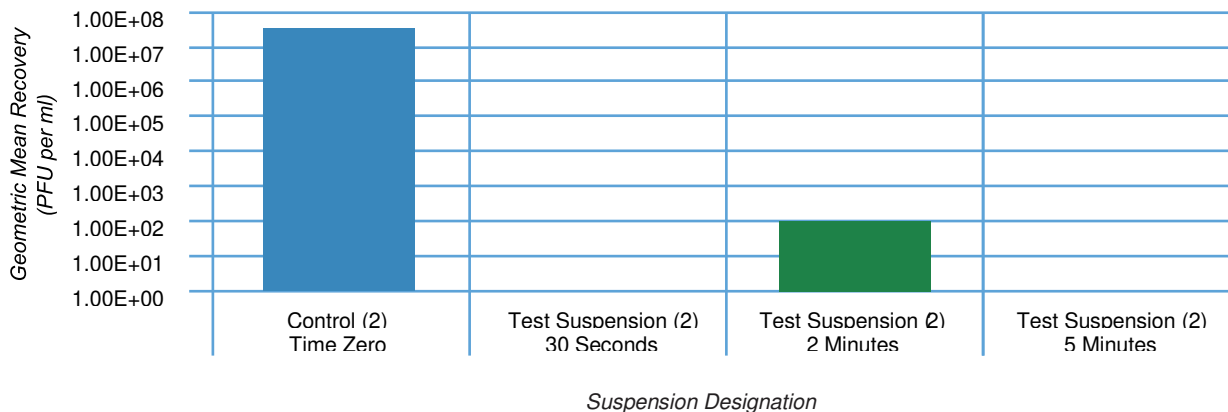


Table 6. Neutralization Control Data

Suspension Composition	Count 1 (PFU/ml)	Count 2 (PFU/ml)	Count Average (PFU/ml)	Neutralization Validated? (≤ 0.50 log10)
RO Water (Control)	824	832	828	Yes
Ozonated Water (Test)	776	960	868	