KR Biotech Co., Ltd.
Institute of Infectious Disease Control
(BSL3 No. KCDC-09-3-01)

Neungdong-ro 120, Konkuk university
Bld#12, Rm 406, Kwangjin-gu, Seoul

Test Report

Client	Personnel	Seoktae Cheon	Tel. No.	82-10-4236-4560
	Affiliation	PURE O2 Co.	E-mail	suktae72@hanmail.net
	Address	#F334, 45, Jojeong-daero, Hanam-si, Gyeonggi-do, Republic of Korea		
Request	Virucidal Activity Test			
Sample	PURE O2 (Chlorine Dioxide)			
Purpose of Use on the Product	Sterilization, Disinfectant			
Test Virus	COVID-19 (SARS-CoV-2)		Cell Line	Vero E6
Test No.	KR-2007-024-PUR01-C		Test Period	2020.07.17-07.24
Sample State	Liquid: Light green, transparent		Sample Concentration	Stock solution
Reaction Time	30 sec, 1 min, 5 min		Titration	CPE
Test Temperature	Room Temperature (Approx. 20°C)		Tester	Hansam Cho

Test Result

Product Name	Virus Titer $TCID_{50}$	Treatment time	Virus Reduction Rate	
			(log)	(%)
PURE O2 (Chlorine Dioxide)	$3.16x10^6$	30 sec	4.00	99.99%
	$3.16x10^6$	1 min	4.00	99.99%
	$3.16x10^6$	5 min	4.00	99.99%

Result: PURE O2 (Chlorine Dioxide) disinfectant of PURE O2 Co. used in the test showed 99.99% of virucidal effect after 30 seconds of sample treatment on COVID-19 (SARS-CoV-2).

July 31, 2020

Test Manager: Young Bong Kim (Seal)

KR Biotech Co., Ltd.

* This test report is a result limited to the sample and sample name provided by the client and does not guarantee the quality on the overall product.
* This report cannot be used for PR, advertising and litigation purposes, and use of this report other for its original purpose is prohibited.

KR Biotech Co., Ltd.
Institute of Infectious Disease Control
(BSL3 No. KCDC-09-3-01)

Neungdong-ro 120, Konkuk university
Bld#12, Rm 406, Kwangjin-gu, Seoul

TEST REPORT

KR-2007-024-PUR01-C

Virucidal Activity Test

KR Biotech Co., Ltd.

Summary of the Experiment

○ **Test:** Virucidal Activity Test

○ **Test No:** KR-2007-024-PUR01-C

○ **Product Name** PURE O2 (Chlorine Dioxide)

○ **Client**

Affiliation : PURE O2 Co.

Address : #F334, 45, Jojeong-daero, Hanam-si, Gyeonggi-do, Republic of Korea

○ **Institute**

Affiliation : KRBIOTECH Co., Ltd. (ISO13485:2016)

Address : Institute of Infectious Disease Control
Neungdong-ro 120, Konkuk university Bld#12, Rm 406
Kwangjin-gu, Seoul, Korea 05029

Written : Hansam Cho / Ph.D. **Sign**

Approved : Young Bong Kim/Ph.D. Director

Sign **date** July 31, 2020

KR Biotech Co., Ltd

* This test report is a result limited to the sample and sample name provided by the client, and does not guarantee the quality on the overall product.
* This report cannot be used for PR, advertising and litigation purposes, and use of this report other for its original purpose is prohibited.

July 31, 2020

KR BIOTECH

List

Tables

Figure

1. Summary

This test was conducted to measure the efficacy of virus killing of the disinfectant presented by PURE O2 Co. The SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus) virus was used as a test virus, and the sample (liquid) was mixed with the virus culture solution and contacted for a period of time, and then the test was conducted by confirming the activity of the virus. The activity of the virus was confirmed by infecting the host cell with the virus and then measuring by a 50% tissue culture infectious dose assay ($TCID_{50}$). As a result of treating the PURE O2 Co. disinfectant ［PURE O2 (Chlorine Dioxide)］for 30 seconds it was confirmed that 99.99% of COVID-19 was killed.

2. Outline of the test

2.1 Test schedule

Test start date: July 17, 2020

Test end date: July 24, 2020

2.2 Scope of test

This test method aims at demonstrating the virucidal action of a test substance containing a virus existing in a floating (liquid) state. With reference to the Ministry of Environment and the Ministry of Food and Drug Safety, the sterilizing and disinfectant test for COVID-19 virus was conducted in accordance with the test guide for virus external antiseptics modified by KRBIOTECH (KRBOP-0803-03).

(3) Fetal bovine serum (FBS), Gibco, US

(4) Trypsin-EDTA (0.25% Trypsin), Gibco, US

(5) Penicillin-Streptomycin, Gibco, US

(6) Ethyl Alcohol (EtOH), Deoksan Pharmaceutical, South Korea

(7) Hydrochloric Acid (HCl), Daejung, South Korea

(8) Formaldehyde (HCHO), Deoksan Pharmaceutical, South Korea

(9) Crystal Violet, JUNSEI, Japan

3.3 Equipment and facility

(1) Biological safety cabinet (sterile worktable), Thermo scientific, US

(2) Optical microscope, OPTINITY, China

(3) Centrifuge (LABOGENE1248), Zyrozen, South Korea

(4) Refrigerator (4℃), Samsung Electronics, South Korea

(5) Freezer (-20℃), Samsung Electronics, South Korea

(6) cryogenic freezer (-80℃), Thermo scientific, US

(7) Constant temperature carbon dioxide gas incubator (37℃) BB15,
Thermo scientific, US

(8) Vortex mixer KMC-1300V, Vision Science, South Korea

(9) Dry oven HM-28, Hanil Science, South Korea

(10) LN2 Tank (Locator JR Plus), Thermo scientific, US

(11) Water bath, Korea Science, South Korea

(12) Multi well plate reader, Epoch, US

(13) PE6000, Mettler Instrument, US

(14) BSL-3 (No. KCDC-09-3-01)

4. Methods

4.1 Host cell line and culture

The cell line Vero-E6 is isolated from renal epithelial cells extracted from African green monkeys. Since SARS-CoV-2 can be cultured and causes virus-infected cell lesion (Cytopathic effect), Vero-E6 is used as a host cell in this test for measuring the viral titer.

4.2 Virus

COVID-19 (SARS-CoV-2)

- The Corona Virus COVID-19 (SARS-CoV-2) was first emerged in Wuhan, China in December 2019, and currently in May 21, 2020, there are over 4.8 million people infected worldwide. In addition, over 310,000 people died from COVID-19, and it is still spreading seriously in the US and in South America, etc.

- COVID-19 is included in the beta-corona classification to have positive single-strand RNA as the genome, and it is a spherical form of virus with envelope.

- In March 11, 2020, WHO declared pandemic on this virus, and there is no medicine or vaccine in the present. The resistance to the disinfectant is in mid-grade, but the spreading power is very high to have serious impact globally.

Severe acute respiratory syndrome-related coronavirus (SARS-CoV-2)

- Classification: Coronaviridae family, Betacoronavirus
- Virus genome: ss-RNA
- envelope: Yes
- Resistance: middle
- Titer: 1.43 x 10^6 $TCID_{50}$/mL

4.3 Virucidal test by disinfectant

4.3.1 Cell cytotoxicity

After dissolving in accordance with Chapter 9 or ISO 10993-12 Sample preparation and reference materials of ``Common Standards for Biological Safety of Medical Devices-24-(Korea Food and Drug Administration Notification No. 2006-32)'', the eluate in the colloidal state is not filtered. It is tested according to ISO 10993-5 or Chapter 2 of the "Common Criteria for Biological Safety of Medical Devices (KFDA Notice 2006-32)". Cytotoxicity is usually evaluated according to the test method by direct contact method.

It was carried out according to the cytotoxicity test guideline (document number KRBOP-0803-01, Crystal violet method) of this test institution.

① The cell is cultured in the 96 well plate, and the sample is performed with 10 times serial dilution to be added to the cell culture medium. Here, one column is left for use as the negative control group.

② The cell is cultured in the cell incubator during the designated time (About 3 days) according to the test plan.

③ Optical microscope is used for visual check on the cell state, and 8 channel multi pipette is used to insert 50 μl of crystal violet solution into all wells.

④ The plate is left in room temperature for 30 minutes to wash the plate cleanly in flowing water.

⑤ The plate is dried appropriately to measure the absorbance with the ELISA reader in 575nm wavelength.

4.3.2 Virucidal Test

This test was conducted on the basis of ASTM E1052-11, the virus killing test in the sample stock solution. In addition, to neutralize the cytotoxicity of the disinfectant itself, the neutralizing agent (10% FBS) suggested in the "Sterilization Disinfectant Efficacy Test Method Data Collection (NIER-GP2018-170)" was used.

① One day before the test, prepare Vero-E6 cells in a 96 well plate.

② Sample stock solution and SARS-CoV-2 virus were mixed and reacted at room temperature for 30 seconds, 1 minute, and 5 minutes.

③ Neutralizing agent (10% FBS) was added and allowed to stand at room temperature for 10~20 minutes, and then diluted 10 times. To reduce cytotoxicity, a gel filtration method (E-1482) is proposed, but it was omitted in consideration of the virus risk, and the cytotoxicity was evaluated based on the step dilution without cytotoxicity. For example, in 10^{-1}, cytotoxicity was observed, but in 10^{-2}, it did not show cytotoxicity, and when the virus was killed and all cells were healthy, it was evaluated that the virus was killed in 10^{0},10^{-1}.

④ Each diluent was infected with Vero-E6 cells, and cultured at 5% CO_2 at 37°C. At this time, normal physiological saline was used as a control.

⑤ After 3 days of culture, cytopathic effect (CPE) was observed under a microscope.

⑥ Crystal violet staining reagent was treated with cells and stained at room temperature for 30 minutes.

⑦ The titer of the virus was calculated by counting the number of stained wells.

4.4 Data reading and calculation

4.4.1 Cytotoxicity Test

The results of the cytotoxicity test are judged to have no cytotoxicity when the

absorbance (value) is 50% or more based on the absorbance (100%) of the cell negative control.

4.4.2 Virucidal Test by disinfectant

To evaluate the virus killing efficacy, each diluent was inoculated into a host cell, and virus titers of the control group and the test group were measured after 3 days.

The number of wells stained with Crystal violet dyeing reagent was counted to calculate the titer by Reed & Muench method. Virus titers were calculated according to 4.4.3 and reduction rates were determined according to 4.4.4.

4.4.3 Calculate viral titer

The virus titers can be confirmed by observing the morphological changes (CPE) of cultured cells caused by virus growth for a period of time. The virus infectious value is obtained by inoculating, cultivating, and observing the cultured cells seeded in a plurality of incubators by preparing a 10^n dilution series of the virus solution. After the CPE observation for a certain period of time (four days post infection), the virus infection value ($TCID_{50}$) is calculated according to ICH Q5A (R1), which is indicated by taking the commercial log value.

The number of wells determined to be positive is cumulatively calculated from the high diluent side to obtain the cumulative positive rate (%) of each diluent.

$$TCID_{50}: N=10^{[(A-50)/(A-B)]-(a)}$$

How to calculate viral titer

1) Calculate the cumulative for number of well which had decided to be positive from high

diluted solution and obtain the cumulated positivity rate (%) of each diluted solution.
2) Obtain 50% of cumulative positivity rate and cumulative positivity rate of high diluted solution is called as A; cumulative positivity rate of low diluted solution is called as B; and the natural logarithm value of diluted solution with A obtained is called as a.
3) Obtain the viral titer according to the following formula.

However, if overall well became negative even for the diluted solution having the lowest magnification, assume that overall well become positive in the diluted solution that is one step lower than that diluted solution and then calculate; add a sign of inequality to obtained value and then write down. And make the valid number to have 2 digits by rounding the 3rd number of calculated value for valid digit number of viral titer.

4.4.4 How to calculate the viral reduction factor (Ri)

Regarding the combustion process, the viral reduction factor (Ri) can be calculated with natural logarithm for ratio of viral titer in the test solution, whether the sample underwent combustion process or not for test solution. However, in case of reduction of viral titer in the test solution is less than 10^1 ($\log_{10}$= natural logarithm value 1), it is not determined as the reduction of viral titer and not used for calculation of viral clearance factor.

How to calculate the viral reduction factor (Ri)

- Viral titer appeared in the experimental group before the combustion: 10^A
  Total amount of test solution before the combustion: V^A
  - → Viral titer of test solution before the combustion $V^A \times 10^A = N_A$
- Viral titer appeared in the experimental group after the combustion: 10^B
  Total amount of test solution after the combustion: V^B
  - → Viral titer of test solution after the combustion $V^B \times 10^B = N_B$

Viral titer (Ri) of test solution is

$$10^{Ri} = V^A \times 10^A / V^B \times 10^B = N_A / B_A$$

$$Ri = \log_{10}(N_A / B_A) = \log_{10} N_A - \log_{10} N_B$$

5. Results

5.1 Cytotoxicity by disinfectant

As a result of confirming cytotoxicity by treating the sample stock solution with Vero-E6 cells, no toxicity was observed in Vero-E6 cells.

Table 1. Cytotoxicity test results

Cell	The highest dilution factor that does not show host cell toxicity
Vero-E6	10^{-1}

5.2 Disinfectant test

The initial virus titer of SARS-CoV-2 for the disinfectant test is 6.16 $\log_{10}$ $TCID_{50}$/ml.

PBS was used as a control to evaluate the efficacy of the disinfectant in this test. After mixing the virus and the control (PBS) and neutralizing it after 30 seconds, the virus titer was calculated through cell infection, and the titer of the control group was 6.50 $\log_{10}TCID_{50}$/ml. After mixing the requested disinfectant (PURE O2 (Chlorine Dioxide)) and the virus and neutralizing it after 30 seconds, the virus titer was calculated and the titer of the test group was 2.50 $\log_{10}$ $TCID_{50}$/ml. Therefore, the reduction rate of SARS-CoV-2 by disinfectant (PURE O2 (Chlorine Dioxide)) was confirmed to be 4.00 after 30 seconds.

As a result of processing SARS-CoV-2 in the sample disinfectant solution for 30 seconds the virus killing efficacy of 99.99% or more was confirmed.

Table 2. Virus titer calculation

(unit: $log_{10}TCID_{50}$/ml)

Virus	Treatment	Virus titer	Control (PBS)	Test
SARS-CoV-2	30 sec	6.16	6.50	2.50
	1 min	6.16	6.50	2.50
	5 min	6.16	6.50	2.50

Table 3. Virus reduction rate

Virus	Treatment	Log reduction (LR)
SARS-CoV-2	30 sec	4.00
	1 min	4.00
	5 min	4.00

$LR = L_U - L_T$

L_U : Virus titer of the control (untreated)

L_T : Virus titer of the test (treated)

Table 4. Disinfectant test results

Disinfectant	Virus	Treatment	Virus reduction (log)	Virus reduction (%)
PURE O2 (Chlorine Dioxide)	SARS-CoV-2	30 sec	≥4.00	99.99%
		1 min	≥4.00	99.99%
		5 min	≥4.00	99.99%

* Interpretation of results

Log reduction	Percent (%) reduction
≥1	≥90 %
≥2	≥99 %
≥3	≥99.9 %
≥4	≥99.99 %
≥5	≥99.999 %

6. Conclusion

The SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus) virus reduction rate (virucidal rate) for PURE O2 Co. disinfectant (PURE O2 (Chlorine Dioxide)) samples under guideline test conditions was 4.00 after 30 seconds of sample treatment, confirming the virus killing efficacy of 99.99% or more.

7. References

(1) ASTM E1052-11, Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension

(2) Schmidt, N. J. et. Al., Diagnostic Procedures for Viral, Rickettsial and Chlamydial infection, 7th edition, Am. Pub. Hlth. Assoc., Washington, DC, 1995.

(3) BS EN 14476:2013 A1:2015, Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity in the medical area

(4) Test method for the evaluation of virucidal efficacy of three common liquid surface disinfectants on a simulation environmental surface. Appl Microbiol, 28(1974), pp.748-752

(5) In vitro evaluation of antiviral and virucidal activity of a high molecular weight hyaluronic acid. Virology Journal 8, Article number:141(2011)

(6) Virucidal and Neutralizing Activity Tests for Antiviral Substances and Antibodies 10.21769/BioProtoc.2855 Vol 8, Iss 10, May 20, 2018

(7) Guidelines for disinfectants for external use (non-pharmaceutical products) Effectiveness Evaluation Act 2014.8. Food and Drug Safety Evaluation Institute

(8) Sterilization. Disinfectant Efficacy Test Method Data Collection 2018. 12. National Institute of Environmental Science

* This report is limited to the sample and sample name presented by the sponsor, and does not guarantee the quality of the entire product.
* This report cannot be used for publicity, advertising, and litigation, and is not for use.