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Test Report

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To: Nanoclo System Co., Ltd.

Test Report

Test (40L space) of efficacy evaluation of inactivation for viruses by chlorine dioxide “nanoclo2 Case in Type”

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1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa, 252-0329 Japan
Kitasato Research Center for Environmental Science
Chief Director Toshihiro Ito
(Translated by Koichi Kuboyama)

1. Test aims

   To evaluate the inactivation efficacy of chlorine dioxide generating agent "nanoclo2 Case in Type" against Influenza A virus, by setting up the agent in a 40L test chamber and the Influenza A virus as well in the same test space.

2. Client

   N a m e: Nanoclo System Co., Ltd.

   Address: 3-6-2 Nishi-Shinbashi, Minato-ku, Tokyo, 105-0003 Japan

3. Test institution

   N a m e: Kitasato Research Center for Environmental Science

   Address: 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa, 252-0329 Japan

   In charge: Virus Section of Virus Department

4. Test term

   From May 26, 2014 to June 2, 2014

5. Test object

   Chlorine dioxide generating agent "nanoclo2 Case in Type"

   ※Quantity of test object:

   At the diffusion prior to the test start→6g

   After the test start→1.6g

   About 23 hours after the test start, one test object (6g) was added.

6. Test conditions

   Test duration: 0 (beginning), 24 hours

   Temperature: 25 ℃

7. Virus under testing and the compounding method of virus liquid

   Influenza A virus, H1N1, A/PR/8/34, ATCC© VR-95™

   After chorioallantoic inoculation into embryonated egg, the virus was incubated in an incubator. Taking out chorioallantoic liquid, the liquid was purified by density-gradient-centrifugation, which was used as virus liquid for testing.

8. Test method

1) Test of virus inactivation

Virus inactivation test was conducted according to the process below.

The test target was put at the center in a test chamber of 40L.※
After 16 hours diffusion, the chlorine dioxide concentration was adjusted to about 0.04ppm, the target concentration.
The test virus suspension of 4ml on a ϕ60mm laboratory dish with lid open was placed about 10cm from the test target. (Phote-1)
After it had been kept for 24 hours in the chamber which was tightly sealed, quiet and at temperature of about 25℃, the virus suspension was collected from the laboratory dish and used as infectivity titer measuring test raw liquid.
At the same time, the same process was done in a space without placing any test target.

※Placing one piece of the test target, after 16 hours diffusion, the chlorine dioxide gas concentration became four times as high as the target concentration. Then, the quantity of solid agent in the test target was reduced from 6g to 0.16g and the operation began.
Because, after 23 hours, the concentration dropped to 0.01ppm, 6g of solid agent was added and the experiment continued for remaining one hour.

2) Measurement of virus infectivity titer

The virus infectivity titer measuring test raw liquid was serial-diluted 10 times with PBS(phosphate buffered saline), which is call “diluted virus liquid” hereafter.
And 50μL of MDCK(Madin-Darby canine kidney) was suspended into 50μL of the virus infectivity titer measuring raw test liquid or the diluted virus liquid and 5% FBS(fetal bovine serum)　added DMEM(Dulbecco’s modified Eagle’s Medium) .
The 50μL suspension of MDCK was planted into 96 well plate.
Then it was incubated in a carbon dioxide incubator with 37℃ for 4days.
After incubation, we observed the CPE(cytopathic effect) of virus multiplication by inverted microscope, and acquired the virus infectivity titer($TCID_{50}$/mL) using Reed-Muench method.

3) Measurement of chlorine dioxide concentration
The chlorine dioxide concentration inside the test chamber was measured by a portable gas concentration measurement instrument which was provided by the client.

9. Test results
The test results are shown in table-1. The chlorine dioxide concentration inside the test chamber during the test is shown in table-2.

The starting virus infectivity titer was 3.9×10^7 $TCID_{50}$/ml. The virus infectivity titer "without test target (the control)" after 24 hours still standing was 2.9×10^6 $TCDI_{50}$/ml.

On the other hand, the infectivity titer inside the 40L test chamber set with "chlorine dioxide generating agent, nanoclo$_2$ Case in Type" after 24 hours still standing was $6.3 \times 10^2 TCID_{50}$/ml. The comparison of infectivity titer LRV (log reduction value) with the control after 24 hours experiment was 3.7 $\log_{10}$. The reduction ratio calculated from the LRV was 99.98%.

As reference, chlorine dioxide gas concentration during the test is shown. It was kept in the range from 0.01 to 0.13ppm comparing to the target of 0.04ppm.

10. Comment
In this test, we placed, inside 40L test chamber, "chlorine dioxide generating agent, nanoclo$_2$ Case in Type" that the client provided, and evaluated the disinfection efficacy against A type influenza virus.

As the test results, after 24 hours still standing, 3.7$\log_{10}$ (reduction ratio 99.98%) decrease was observed.

It is reported that the virus inactivation effect by chlorine dioxide gas was affected by humidity or organic matters existence.[1)] Suitable using methods considering the effects from humidity or organic matters are required when it is actually utilized.

Reference books

1) Morino H et al., Inactivation of feline calicivirus by chlorine dioxide gas-generating gel. Yakugaku Zasshi. 133(9):1017-22,2013

Table-1 Disinfection efficacy against A type influenza virus by "chlorine dioxide generating agent, nanoclo$_2$ Case in Type"

Test object	Experiment time		Infectivity titer LRV(log reduction value)※1 (reduction ratio※2)
	0(start)	24 hours	After 24 hours
Without test object (control)	3.9×10^7	2.9×10^6	
chlorine dioxide generating agent, "nanoclo2 portable type"		6.3×10^2	3.7 (99.98)

Virus used: Influenza A virus (H1N1, A/PR/8/34, ATCC® VR-95™)

Virus infectivity titer of raw virus liquid for testing: 3.9×10^7 $TCID_{50}$/ml

Infectivity titer unit: $TCID_{50}$/ml

Detection limit value: 6.3 $TCID_{50}$/ml

※1: Difference from the control after 24 hour experiment: $\log_{10}$ (infectivity titer of the control / infectivity titer of chlorine dioxide generating agent taken after experiment time)

※2: Figure acquired from conversion of LRV into reduction ratio: $(1-1/10^{LRV}) \times 100$ (%)

Table-2 Chlorine dioxide gas concentration inside 40L test chamber

Measured item	Experiment time (hour)	
	0(start)	24
Chlorine dioxide gas concentration	0.040	0.024

※ Measurement instrument: Portable gas concentration measurement instrument (J・M・S、provided by the client)

※ Unit: ppm

※ Regarding measured results of chlorine dioxide gas concentration during the tests, please refer to the reference data.
Measurement instrument: Chlorine dioxide detecting tube (No.23L, Gastec)

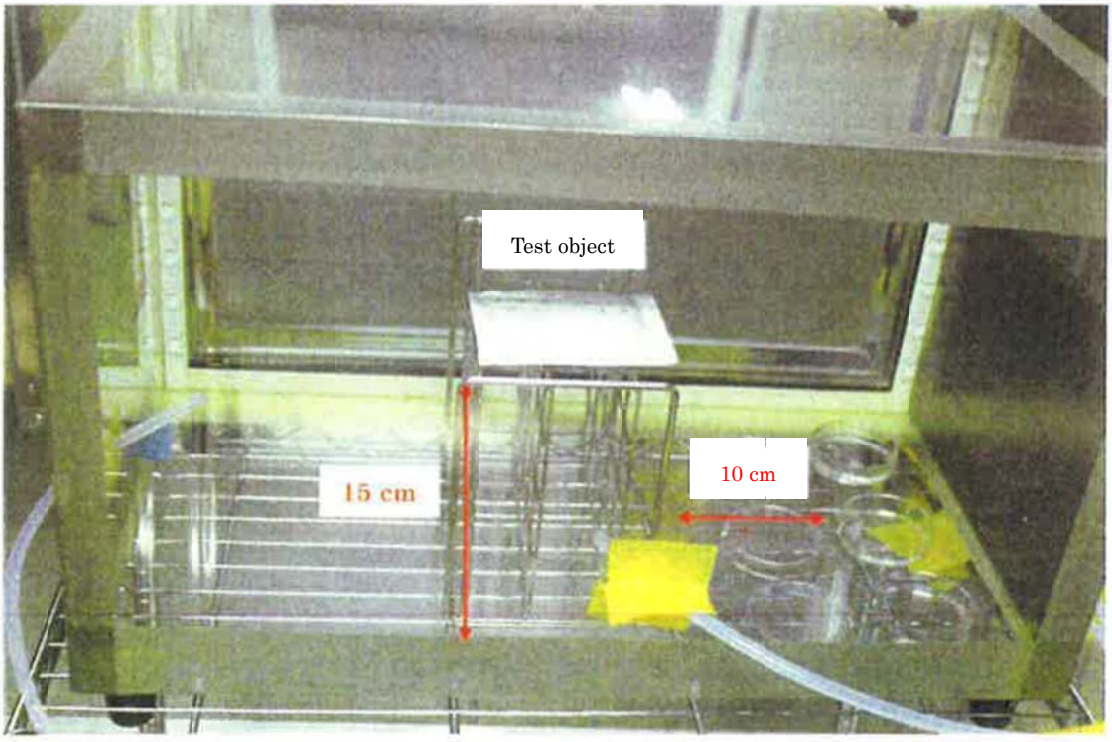


Photo-1 Test conditions inside 40L test chamber

Reference data

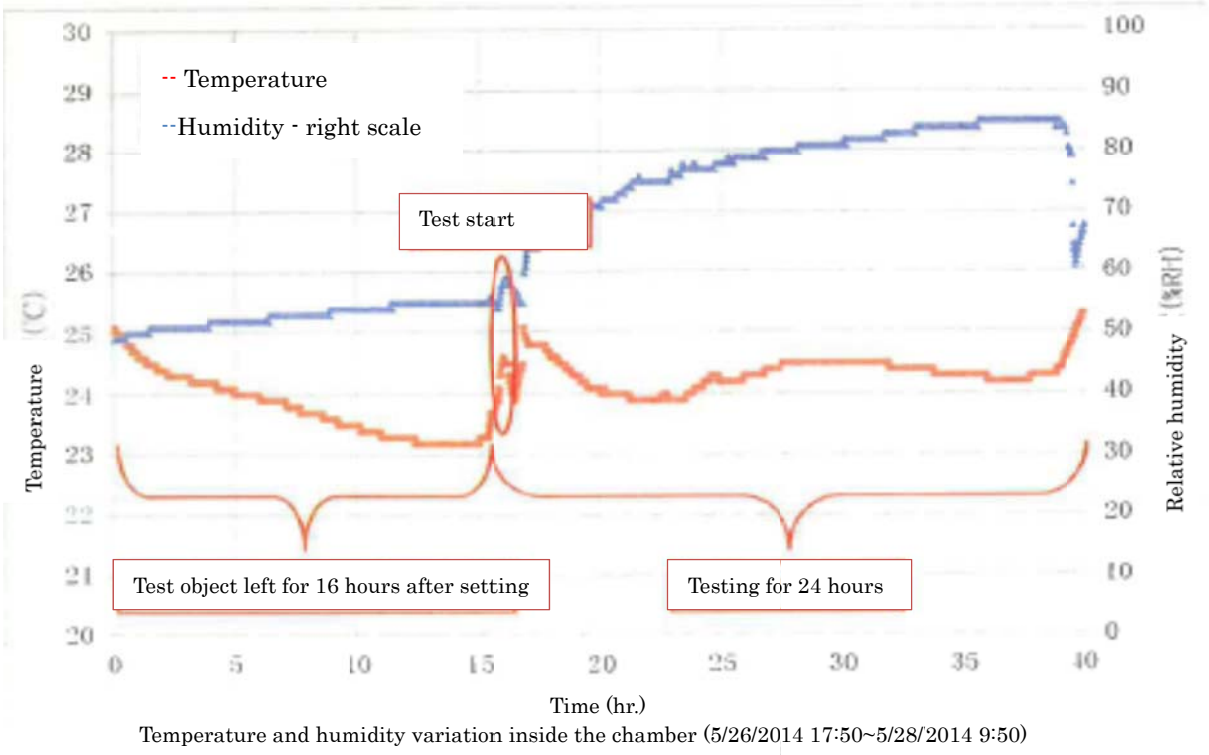
Chlorine dioxide gas concentration and temperature/humidity inside the test chamber during the testing are shown below:

Table-3 Measurement results of chlorine dioxide gas concentration

Date	Time	Chlorine dioxide concentration(ppm)	Remarks
May 27th	9:00	0.160	
	Test start→9:50	0.040	Solid agent decreased from 6g to 1.6g.
	14:30	0.057→0.041	Ventilated from 0.057 to 0.041.
	17:00	0.037	
	18:00	0.035	
May 28th	9:00	0.01	Added one solid agent (6g)
	9:20	0.13→0.04	Ventilated to 0.04ppm
	Test finish→9:50	0.024	

※ Measurement instrument: Portable gas concentration measurement instrument (J・M・S、provided by the client)

※ Test start: May 27th 9:50 (Experiment time 0 hour)
Test finish: May 28th 9:50 (Experiment time 24 hours)



Temperature and humidity variation inside the chamber (5/26/2014 17:50~5/28/2014 9:50)

Chart-1 Temperature and humidity variation inside the test chamber during the testing