



**DR. BRILL + DR. STEINMANN**  
INSTITUTE FOR HYGIENE AND MICROBIOLOGY



29/06/2016

## Test report I16L0395A

Efficacy of

# STERISAFE 1.1

TEST REPORT

Test virus: adenovirus type 5

Method: following NF T 72-281:2009 (Phase 2/Step 2)

Quantitative Non-Porous Surface Test for Evaluation of Bactericidal and/or Fungicidal Activity of Chemical Disinfectants and Antiseptics Used in Food, Industrial, Domestic, and Institutional Areas

### Sponsor:

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## 1. Introduction

It was the aim of our study to evaluate the virus-inactivating properties of ozone generated by **STERISAFE 1.1** for room disinfection. The adenovirus type 5 was chosen as test virus. These experiments were performed following the NF T 72-281.

Stainless steel discs are contaminated with a virus inoculum (test virus suspension + soil load) and placed in a suited room at a defined place. Then the inactivation of the test virus as mentioned above by ozone generated by **STERISAFE 1.1** was studied in two runs on two independent days (short cycle and long cycle decontamination). The treated carriers were checked after elution for residual virus at the end of the experiment. The virus-inactivating properties of this procedure under the chosen conditions can be calculated by comparing the virus titres with the controls (carriers in a different room without **STERISAFE 1.1** treatment).

## 2. Test laboratory

Dr. Brill + Partner GmbH Institute for Hygiene and Microbiology, Norderoog 2, DE - 28259 Bremen

## 3. Identification of the device

Manufacturer	Infuser Denmark
Name of device	STERISAFE 1.1
Serial number	0003
System	in-situ generation of ozone
Output	Short cycle: 67.5 ppm ozone Long cycle: 62.5 ppm ozone
Diffusion rate of the test (Bremen)	active circulation of ozone by build-in fan (~550 m <sup>3</sup> /h)
Exit	outlet duct which extends vertically to the top

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## 4. Material

### 4.1 Culture medium and reagents

- Eagle's Minimum Essential Medium with Hank's BSS (MEM, Biozym Scientific GmbH, catalogue no. 880144)
- fetal calf serum (Biochrom AG, article no. S 0115)
- 1.4 % formaldehyde solution (dilution of Roti®-Histofix 4 %, Carl Roth GmbH)
- Aqua bidest. (SG ultrapure water system, type ultra Clear; serial no. 86996-1)
- PBS (Invitrogen, article no. 18912-014)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153)
- skim milk powder (Fluka Analytical; article no. 70166-500).

### 4.2 Virus and cells

The adenovirus type 5 strain adenoid 75 was obtained from PD Dr. A. Heim, Institute of Medical Virology, Hannover Medical School, Hannover, Germany. Before the inactivation assays, the virus had been passaged 3 times in *A549 cells* (human lung epithelial carcinoma cells).

The *A549 cells* (passage 98) originated from Vircell, S.L., Spain, 18320 Santa Fe (now BIOTRIN International GmbH, DE -69126 Heidelberg).

The cells were inspected regularly for morphological alterations and for contamination by mycoplasmas. No morphological alterations of cells and no contamination by mycoplasmas could be detected.

### 4.3 Ozone application unit

The ozone application unit **STERISAFE 1.1** (figure 1) was supplied by Infuser Denmark, Ole Maaløe's vej 5, DK – 2200 Copenhagen.



Figure 1: STERISAFE 1.1

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During the build-up phase the ozone and humidity levels are build-up and actively circulated in the room. The humidity is increased through a fine water mist. In the decontamination phase the ozone concentration in the air is actively regulated to the desired level. During the cleaning phase, the ozone gas is catalytically neutralized and particles are electrostatically precipitated.

#### 4.4 Apparatus, glassware and small items of equipment

- CO<sub>2</sub> incubator, Nunc GmbH & Co. KG, model QWJ 350
- Agitator (Vortex Genie Mixer, type G 560E)
- pH measurement 315i (WTW, article no. 2A10-100)
- Centrifuge (Sigma-Aldrich-Chemie GmbH, type 113)
- Microscope (Olympus, type CK 30)
- Centrifuge 5804 R (Eppendorf AG)
- Water bath (JULABO, Julabo U 3)
- Adjustable volume automatic pipettes (Eppendorf AG)
- Polysterol 96-well microtitre plate (Nunc GmbH & Co. KG, Wiesbaden)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden)
- Sealed test tubes (Sarstedt AG & Co., Nümbrecht)
- Container, flat bottom, 25 cm, with cap (Sarstedt AG & Co., Nümbrecht)
- Stainless steel discs (2 cm diameter discs) with Grade 2 B finish on both sides (article no. 4174-3000, GK Formblech GmbH, Berlin).

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## 5. Experimental conditions

		short cycle	long cycle
Test temperature	(test room)	25.1 °C	24.2 °C
	(control room)	22.5 °C	25.5 °C
Relative humidity	Start:	50.1 %	54.1 %
	(test room) Average:	64.0 %	75.0 %
	Maximum:	66.5 %	80.1 %
	(control room)	40.5 %	41.1 %
Concentration of test product		67.5 ppm ozone	62.5 ppm ozone
Exposure times	Build-up:	47 min	43 min
	Decontamination:	31 min	70 min
	Cleaning:	48 min	52 min
Position of the carriers		vertical	
Distance: Ozone application unit / carriers		3.60 m (height: 1.0 m from ground) (see figure 2)	
Total quantity used (test product) in 65 m <sup>3</sup>		not applicable	
Total quantity m <sup>3</sup>		not applicable	
Total quantity m <sup>2</sup>		not applicable	
Interfering substance (s)		0.5 % skimmed milk	
Procedure to stop action of product		immediate dilution	
Test virus		adenovirus type 5 strain adenoid 75 (ATCC VR-5)	
Period of analysis		01/06/2016 - 29/06/2016	
End of testing		29/06/2016	

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## 6. Method

### 6.1 Test room

The test room exhibited a ground area of 4.82 x 5.38 m (25.93 m<sup>2</sup>) and a height of 2.53 m with a room volume of 65.61 m<sup>3</sup>. The distance between the front of the machine and the middle of the desk was 3.60 m.

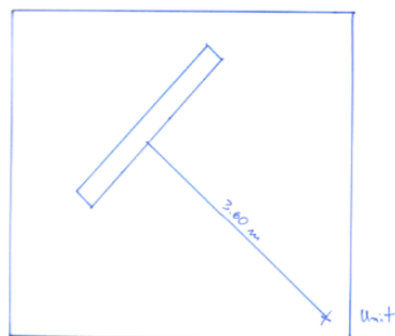


Figure 2: Distance Ozone application unit / carriers

### 6.2 Preparation of test virus suspension

To prepare the test virus suspension, *A549* cells were infected with a multiplicity of infection of 0.1 at 37 °C. After cells showed a cytopathic effect, they were subjected to a threefold freeze/thaw procedure followed by a low speed centrifugation in order to sediment cell debris. After aliquotation of the supernatant, test virus suspension was stored at -80 °C.

### 6.3 Preparation of virus inoculum

For the preparation of virus inoculum 19 parts of the test virus suspension were mixed with 1 part of a 10 % skimmed milk solution (final concentration: 0.5 %).

### 6.4 Preparation of carriers

Prior to use, the carriers (stainless steel discs) were placed in a container with an appropriate quantity of 5 % (v/v) Decon 90® for 60 minutes (at room temperature), in a manner that they do not stick together and the surface gets no damage. Following this, the discs were immediately rinsed off thoroughly with aqua dest. for no less than 10 seconds each. This procedure was repeated once more to remove all surfactants. Afterwards, without drying the carriers, the discs were submerged in 70 % (v/v) isopropyl alcohol for 15 minutes, air-dried by evaporation under the laminar air flow and finally sterilized (steam sterilization). Carriers were only being handled with forceps and were supposed for single use only.

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## 6.5 Experimental conditions

50 µl of the virus inoculum (suspension of test virus with interfering substance) were applied to the carriers and dried afterwards.

The carriers (in triplicate) were deposited in a magnetic slat with clamps (see figure 3) and transported in the room chosen for surface and air disinfection (vertical position).

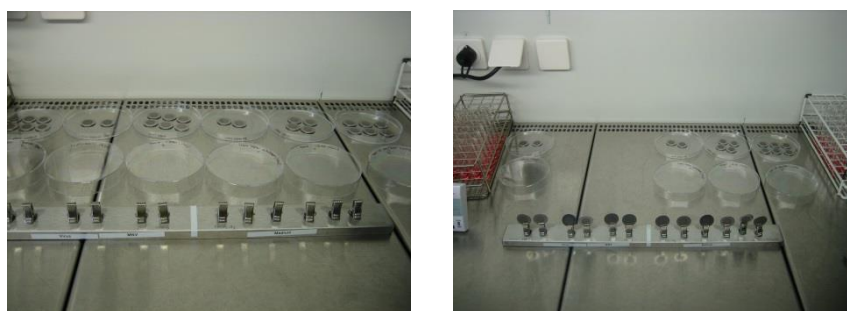


Figure 3: Magnetic slat with clamps (left) and clamps with inoculated carrier (right)

In this room the carriers were placed in a distance of 3.6 m from the ozone application unit (with the contaminated side turned away from device) with a height of 1 to 1.5 m (here 1.0 m). Another two carriers were placed in a petri plate within an open drawer.



Figure 4: position of the magnetic slat with clamps and inoculated carrier

The ozone application unit was prepared according to the instruction of the manufacturer and started (see figure 5).





Figure 5: Position of the STERISAFE 1.1

The build-up phase (increasing of ozone and humidity with active circulation) took place over 47 min and 43 min. The virus-inactivating properties of a treatment with the STERISAFE 1.1 were examined in two runs (short cycle: 31 min at 67.5 ppm ozone and average 64 % relative humidity, long cycle: 70 min at 62.5 ppm and average 75 % relative humidity). After a cleaning phase the carriers were transferred for elution in a 25 ml vial with 10 ml medium without FCS and vortexed for 60 seconds. Directly after elution, series of ten-fold dilutions of the eluate in ice-cold maintenance medium were prepared and inoculated on cell culture.

## 6.6 Controls

All controls were performed as described in 6.5. Determination of VC was done in another room without treatment. Preparations exactly followed the procedure as described in 6.5.

### 6.6.1 Virus controls

For the control of the initial virus titre in the test assay, for determination of the stability after drying and for evaluation of the neutralization of the disinfectant a virus control before drying is needed (VC before). For this control 50 µl virus inoculum was given into 9.950 ml medium without FCS (elution).

In addition, two virus controls directly after drying (VC<sub>t0</sub>) and three carriers for each exposure time tested (VC  $t_{\text{short/long cycle}}$ ) were incorporated. For the VC  $t_0$  the elution took place immediately after drying of virus inoculum in 10 ml medium without FCS. The elution for VC  $t_{\text{short cycle}}$  and VC  $t_{\text{long cycle}}$  was run in parallel to the room disinfection after incubation of the carriers in a separate room without surface and air disinfection. VC  $t_{\text{short/long cycle}}$  are needed as references for the calculation of the reduction factor after treatment with the test product.

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For the formaldehyde control (see 6.6.5) a virus control before drying with phosphate buffer is needed (VC PBS). For this control 100 µl of the test virus suspension were mixed with 900 µl PBS and incubated for 60 min at 20 °C.

### 6.6.2 Control of cytotoxicity

The cytotoxicity control is needed to make a differentiation between cytopathic and cell toxic effects.

For the determination of cytotoxicity 50 µl medium instead of virus inoculum without FCS was deposited onto one carrier. After drying, room disinfection and further dwelling time an elution with 10 ml medium was performed. The cytotoxicity control is needed for definition of the lower detection limit.

### 6.6.3 Cell control

The cells were only treated with cell culture medium.

### 6.6.4 Exclusion of an after-effect (Neutralisation control)

For demonstration that the addition of medium without FCS will contribute to a sufficient neutralization of the activity of the test product 50 µl test virus suspension were added to a second cytotoxicity control and incubated for 30 min on ice. Finally a virus titration was performed.

### 6.6.5 Reference control

As reference for test validation a 0.7 % formaldehyde (v/v) solution was included. Therefore, 100 µl of test virus suspension were mixed with 400 µl phosphate buffer and 500 µl of a 1.4 % formaldehyde solution. 5, 15, 30 and 60 minutes were chosen as contact times. In addition, cytotoxicity of formaldehyde test solution was determined with dilutions up to  $10^{-5}$ .

The difference of the logarithmic titre of the virus control (VC PBS) minus the logarithmic titre of the test virus in the reference inactivation test had to be in the range of the values from different other tests in our lab (mean value), respectively (data not shown).

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## 6.7 Determination of infectivity

Infectivity was determined as endpoint titration transferring 0.1 ml of each dilution into eight wells of a microtitre plate to 0.1 ml of freshly trypsinised *A549 cells* ( $10\text{--}15 \times 10^3$  cells per well), beginning with the highest dilution. Microtitre plates were incubated at 37 °C in a 5 % CO<sub>2</sub>-atmosphere. The cytopathic effect was read by using an inverted microscope after ten days. Calculation of the infective dose TCID<sub>50</sub>/ml was calculated with the method of Spearman (2) and Kärber (3) with the following formula:

$$-\log_{10}\text{TCID}_{50} = X_0 - 0.5 + \sum r/n$$

meaning

$X_0$  = log<sub>10</sub> of the lowest dilution with 100 % positive reaction

$r$  = number of pos. determinations of lowest dilution step with 100 % positive and all higher positive dilution steps

$n$  = number of determinations for each dilution step.

## 7. Calculation of virus-inactivating properties

The virus-inactivating properties of a treatment with the STERISAFE 1.1 were measured by subtracting the mean virus titres (after treatment) from the virus titres resulted in the parallel without surface and air disinfection.

The difference is given as reduction factor (RF) and shown in tables 1 and 2.

## 8. Verification of the methodology

Since all the following criteria were fulfilled, examination with adenovirus type 5 is valid.

- The titre of the test virus suspension allowed the determination of a  $\geq 4 \log_{10}$  reduction (maximal virus reduction  $\geq 5.58$ ).
- The test product showed no cytotoxicity in the 1:10 dilutions thus allowing the detection of a  $4 \log_{10}$  reduction of virus titre.
- The control of efficacy for suppression of disinfectant's activity showed no decrease ( $\leq 0.5 \log_{10}$ ) in virus titre (short cycle: 7.75 versus 7.63 log<sub>10</sub> TCID<sub>50</sub>/ml; long cycle: 7.88 versus 7.63 log<sub>10</sub> TCID<sub>50</sub>/ml).

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- d) The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test (see 6.6.5) were 0.88 after 5 min (between 0.04 - 2.16) and  $\geq 4.25$  after 30 min (between 3.23 – 5.35) for the short cycle and 1.38 after 5 min and  $\geq 4.00$  after 30 min for the long cycle (Table 3). Values were thus in the range of the values from different test in our lab with the adenovirus type 5 (between 0.04 - 2.16 after 5 min and 3.23 – 5.35 after 30 min, data not shown).

## 9. Results

In parallel to the inactivation experiments the temperature and humidity were measured. In the test room the temperature was 25.1 °C (short cycle) and 24.2 °C (long cycle), respectively. The average humidity was 64 % (short cycle) and 75 % (long cycle), respectively.

The results show a loss of virus titre of 0.67 log<sub>10</sub>-steps for the short cycle and 0.88 log<sub>10</sub>-steps for the long cycle in comparison to the virus titre on the carrier without drying (VC before).

The cytotoxicity was 1.50 CD<sub>50</sub>/ml on A549 cells calculated in parallel to the infective dose TCID<sub>50</sub>/ml showing the lower detection limit.

Our experiments show that after the short cycle decontamination residual adenovirus type 5 could be detected. The calculated reduction factors (RF) after a decontamination time of 31 minutes at 67.5 ppm and average 64 % of humidity in a short cycle were 2.33 (carriers placed on the table) and 1.71 (carriers placed in a drawer) (Table 1).

After a decontamination time of 70 minutes at 62.5 ppm and average 75 % humidity (long cycle) still a very small amount of virus particles could be detected. The reduction factors were  $\geq 4.25$  (carriers placed on the table) and  $\geq 4.31$  (carriers placed in a drawer) (Table 2).

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## 10. Conclusions

Under the defined conditions a sufficient activity (4 log<sub>10</sub> reduction) of ozone generated by **STERISAFE 1.1** against adenovirus type 5 was found. Therefore, the room disinfectant devices **STERISAFE 1.1** can be declared as active against adenovirus type 5 as follows:

70 minutes of decontamination with 62.5 ppm and average 75 % humidity

**Bremen, 29/06/2016**

**- Dr. Britta Becker -**

Head of Laboratory

**- Birte Bischoff -**

Deputy Head of Laboratory

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## 11. Quality control

The Quality Assurance of the results was maintained by performing the determination of the virus-inactivating properties of the disinfectant in accordance with Good Laboratory Practice regulations:

- 1) Chemicals Act of Germany, Appendix 1, dating of 01.08 1994 (BGBl. I, 1994, page 1703). Appendix revised at 14. 05. 1997 (BGBl. I, 1997, page 1060)
- 2) OECD Principles of Good Laboratory Practice (revised 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Organization for Economic Co-operation and Development, Paris 1998.

The plausibility of the results was additionally confirmed by different controls incorporated in the inactivation assays.

## 12. Records to be maintained

All testing data, protocol, protocol modifications, the final report, and correspondence between Dr. Brill + Partner GmbH and the sponsor will be stored in the archives at Dr. Brill + Partner GmbH.

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The test results in this test report relate only to the items examined.

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### 13. Literature

- 1) NF T 72-281:2011: Methods of airborne disinfection of surfaces – Determination of bactericidal, fungicidal, yeasticidal and sporicidal activity (English version of French standard NF T 72-281 :2009 : Procédés de désinfection des surfaces par voie aérienne – Détermination de l'activité bactériode, fongicide, levuricide et sporicide)
- 2) Spearman, C.: The method of `right or wrong cases` (constant stimuli) without Gauss's formulae. Brit J Psychol; 2 1908, 227-242
- 3) Kärber, G.: Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Path Pharmac; 162, 1931, 480-487

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## Appendix:

### Legend to the Tables

- Table 1: Results with adenovirus type 5, short cycle decontamination (31 minutes at 67.5 ppm ozone and 64 % humidity) (2<sup>nd</sup> day)
- Table 2: Results with adenovirus type 5, long cycle decontamination (70 minutes at 62.5 ppm ozone and 75 % humidity) (1<sup>st</sup> day)
- Table 3: Results with formaldehyde solution (0.7 %) (quantal test; 8 wells)

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**Table 1: Results with adenovirus type 5, short cycle decontamination (31 minutes at 67.5 ppm ozone and 64 % humidity) (#4426)**

virus control	carrier	log <sub>10</sub> TCID <sub>50</sub> /ml	average log (geometric)	RF
VC before drying	carrier - 1	7.75	7.75	n.a.
	carrier - 2	n.d.		
	carrier - 3	n.d.		
VC t <sub>0</sub>	carrier - 1	7.25	7.44	0.31
	carrier - 2	7.63		
	carrier - 3	n.d.		
VC t <sub>short cycle</sub>	carrier - 1	6.88	7.08	0.67
	carrier - 2	7.13		
	carrier - 3	7.25		

neutralization control	log <sub>10</sub> TCID <sub>50</sub> /ml	RF
VC before drying	7.75	n.a.
disinfectant	7.63	0.13

decontamination time	disinfectant	concentration	carrier	log <sub>10</sub> TCID <sub>50</sub> /ml	average log (geometric)	RF
short cycle (31 min), table	ozone	67.5 ppm	carrier - 1	4.75	4.75	2.33
			carrier - 2	4.75		
			carrier - 3	4.75		
short cycle (31 min), drawer	ozone	67.5 ppm	carrier - 1	5.25	5.38	1.71
			carrier - 2	5.50		
			carrier - 3	n.d.		

n.a. = not applicable    n.d. = not done

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**Table 2: Results with adenovirus type 5, long cycle decontamination (70 minutes at 62.5 ppm ozone and 75 % humidity) (#4422)**

virus control	carrier	log <sub>10</sub> TCID <sub>50</sub> /ml	average log (geometric)	RF
VC before drying	carrier - 1	7.88	7.88	n.a.
	carrier - 2	n.d.		
	carrier - 3	n.d.		
VC t <sub>0</sub>	carrier - 1	7.38	7.44	0.44
	carrier - 2	7.50		
	carrier - 3	n.d.		
VC t <sub>long cycle</sub>	carrier - 1	7.13	7.00	0.88
	carrier - 2	7.00		
	carrier - 3	6.88		

neutralization control	log <sub>10</sub> TCID <sub>50</sub> /ml	RF
VC before drying	7.88	n.a.
disinfectant	7.63	0.25

decontamination time	disinfectant	concentration	disinfectant	log <sub>10</sub> TCID <sub>50</sub> /ml	average log (geometric)	RF
long cycle (70 min), table	ozone	62.5 ppm	carrier - 1	≤ 2.88	≤ 2.75	≥ 4.25
			carrier - 2	≤ 2.63		
			carrier - 3	≤ 2.75		
long cycle (70 min), drawer	ozone	62.5 ppm	carrier - 1	≤ 2.75	≤ 2.69	≥ 4.31
			carrier - 2	≤ 2.63		
			carrier - 3	n.d.		

n.a. = not applicable      n.d. = not done

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**Table 3: Results for formaldehyde solution (0.7 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells)**

Product	Con- centration	test number	Level of cytotoxicity	log <sub>10</sub> TCID <sub>50</sub> /ml after ....min				
				0	5	15	30	60
formaldehyde	0.7 % (w/v)	#4426 (short cycle)	4.50	n.d.	7.00	6.13	≤ 3.63	≤ 3.50
formaldehyde	0.7 % (w/v)	#4422 (long cycle)	4.50	n.d.	6.88	5.75	≤ 4.25	≤ 3.50
virus control (VC PBS)	n.a.	#4426 (short cycle)	n.a.	n.d.	n.d.	n.d.	n.d.	7.88
virus control (VC PBS)	n.a.	#4422 (long cycle)	n.a.	n.d.	n.d.	n.d.	n.d.	8,25

n.a. = not applicable      n.d. = not done

\* Test procedure accredited according to DIN EN ISO/IEC 17025. Test report issued by Dr. Brill + Partner GmbH, Norderoog 2, DE – 28259 Bremen, Germany, Telephone +49. 40. 55763164, Telefax +49. 40. 55763161, www.brillhygiene.com. No copying or transmission, in whole or in part, of this test report without the explicit prior written permission. The test results exclusively apply to the tested samples. Information on measurement uncertainty on request. © Dr. Brill + Partner GmbH 2016