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INSTITUTE FOR HYGIENE AND MICROBIOLOGY

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TEST REPORT

17/06/2021

Test report L21/0485A.2

Evaluation of the effectiveness of

Active Anolyte

Test virus: adenovirus type 5

Method: EN 14476:2013+A2:2019 (clean conditions)

quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in human medicine (phase 2/ step 1)

Sponsor:
Elements of Water GmbH
Unter dem Dostler 4
DE - 54293 Trier

Norderoog 2, DE - 28259 Bremen
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1. Identification of test laboratory

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

2. Identification of sample

Manufacturer	Elements of Water GmbH
Name of product	Active Anolyte
Confirmation no.	222627
Product diluent recommended by the manufacturer	-
Batch number	AA44286-01
Application	surface disinfection
Production date	-
Expiry date	10/2021
Active compound (s) (according to manufacturer´s information)	Active chlorine obtained from hypochloric acid 500 ppm (hypochloric acid 0.045%, hypochlorite ions at 0.005%)
Measured active chlorine concentration[1]	569.24 ppm
Appearance (undiluted product), odour	clear, colorless liquid product specific
pH-values	undiluted: 6.62 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	06/04/2021

3. Materials

3.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Earle`s BSS (EMEM, Biozym Scientific GmbH, catalogue no. 880120)
- fetal calf serum (Thermo Fisher, article no. CH30160.02)
- 1.4 % formaldehyde solution (dilution of Roti®-Histofix 4 %, Carl Roth GmbH)

[1] The active chlorine content of the test solution was determined by titration with sodium thiosulfate (described as in house method of Dr. Brill + Partner GmbH in SOP AA-00277).

- 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).

3.2 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 106) 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.

3.3 Apparatus, glassware and small items of equipment

- CO_2 incubator
- 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 and fixed-volume 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).

4. Experimental conditions

Test temperature	20 °C ± 1.0 °C
Concentration of test product	undiluted (80.0 %) and as 50.0 %, 10.0 % and 0.1 % (demonstration of non-active range) solutions
Appearance of product dilution(s)	no precipitation
Contact time(s)	30 and 60 seconds
Interfering substance	0.3 g/l bovine serum albumin (clean conditions, EN 14476)
Procedure to stop action of disinfectant	immediate dilution
Diluent	Aqua bidest.
Stability of product in the mix with virus and interfering substance (highest effective use solution)	minor clouding, minor precipitation
Virus strain	adenovirus type 5 strain adenoid 75 (ATCC VR-5)
Date of testing	16/04/2021 – 17/06/2021
End of testing	17/06/2021

5. Methods

5.1 Preparation of test virus suspension

For preparation of test virus suspension according to EN 14476 (1) 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 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.

5.2 Preparation of disinfectant (dilutions)

The test product was tested undiluted. Due to the addition of interfering substance and test virus suspension an 80.0 % solution resulted. Furthermore, the product was evaluated as 50.0 %, 10.0 % and 0.1 % (demonstrating of non-active range) solutions (1 part test virus suspension + 1 part interfering substance + 8 parts disinfectant). Due to the addition of interfering substance and test virus suspension the solutions had to be prepared by the factor 1.25.

These solutions were prepared with Aqua bidest. immediately before the inactivation tests.

5.3 Infectivity assay

Infectivity was determined as endpoint titration transferring 0.1 ml of each dilution into eight wells of a microtitre plate containing 0.1 ml of cell suspension. Microtitre plates were incubated at 37 °C in a 5 % CO_2-atmosphere. The cytopathic effect was read by using an inverted microscope. The infective dose $TCID_{50}$/ml was calculated with the method of Spearman (2) and Kärber (3).

5.4 Calculation and verification of virucidal activity

The virucidal activity of the test disinfectant was evaluated by calculating the decrease in titre in comparison with the control titration without disinfectant. The difference is given as reduction factor (RF).

According to the EN 14476, a disinfectant or a disinfectant solution at a particular concentration is having virus-inactivating efficacy if the titre is reduced at least by 4 log_{10} steps within the recommended exposure period. This corresponds to an inactivation of ≥ 99.99 %.

5.5 Inactivation assay

Determination of virucidal activity has been carried out according to EN 14476 point 5.5.

Immediately at the end of a chosen contact time, activity of the disinfectant was stopped by dilution to 10^{-8}.

Titrations of the virus control were performed at the beginning of the test and after the longest exposure time. One part by volume of test virus suspension was mixed with one part interfering substance and eight parts by volume of WSH or Aqua bidest. (RTU products). If a 97.0 % assay was performed, 0.1 parts by volume of test virus suspension were mixed with 0.2 parts interfering substance and 9.7 parts by volume of Aqua bidest. (RTU products).

Furthermore, a cell control (only addition of medium) was incorporated.

Inactivation tests were carried out in sealed test tubes in a water bath at 20 °C ± 1.0 °C. Aliquots were retained after appropriate exposure times and residual infectivity was determined.

5.6 Determination of cytotoxicity

Determination of cytotoxicity was performed according to EN 14476.

5.7 Cell sensitivity to virus

For the control of cell sensitivity to virus two parts by volume of water were mixed with eight parts by volume of the lowest apparently non-cytotoxic dilution of the product. This mixture or PBS as control was added to permissive cells for one hour. The disinfectant solution was then removed from the cells, and a comparative titration of the virus suspension was performed on the pre-treated cells.

5.8 Control of efficacy for suppression of disinfectant's activity

Furthermore, a control of efficiency for suppression of disinfectant's activity was included as described in EN 14476.

5.9 Reference virus inactivation test

As reference for test validation a 0.7 % formaldehyde solution was included. 5, 15, 30 and 60 minutes were chosen as contact times.

6. Verification of the methodology

Because following criteria were fulfilled, examination according to EN 14476 is valid:

a) The titre of the test virus suspension allowed the determination of a ≥ 4 $\log_{10}$ reduction.

b) The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test (see EN 14476, section 5.7b) was $\geq 3.75 \pm 0.33$ (between 3.0 – 5.0) after 30 min and $\geq 4.00 \pm 0.00$ (between 3.5 – 5.5) after 60 min for adenovirus type 5.

c) The cytotoxicity of the product solution does not affect cell morphology and cell growth or the susceptibility of the test organism in the dilutions of the test product used for demonstration of a 4 log reduction.

d) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) cells showed no significant difference of virus titre (< 1 $\log_{10}$; EN 14476, section 5.7).

e) The control of efficacy for suppression of disinfectant's activity showed no decrease in virus titre (≤ 0.5 log_{10}; EN 14476, section 5.5.5.1).

f) One concentration demonstrated a 4 log_{10} reduction and (at least) one concentration demonstrated a log_{10} reduction of less than 4.

7. Results

Results of examination are shown in tables 1 to 8. Tables 1 to 7 demonstrate the raw data, whereas table 8 (a+b) gives a summary of results.

The undiluted test product in an 80.0 % assay was active after 30 seconds of exposure time (table 1). The reduction factor was ≥ 6.25 ± 0.33. This corresponded to an inactivation of ≥ 99.9999 %.

The test product as 50.0 % solution was also able to inactivate MNV after 30 seconds of exposure time under clean conditions (table 2). The reduction factor was ≥ 5.25 ± 0.33. This corresponded to an inactivation of ≥ 99.999 %.

The test product as 10.0 % solution was also able to inactivate MNV after 30 seconds of exposure time under clean conditions (table 3). The reduction factor was ≥ 4.25 ± 0.33. This corresponded to an inactivation of ≥ 99.99 %.

The test product as 0.1 % solution was not active within 30 seconds of exposure time (table 4).

8. Conclusion

The surface disinfectant Active Anolyte tested undiluted demonstrated activity against adenovirus type 5 after an exposure time of 30 seconds under clean conditions. Therefore, the surface disinfectant Active Anolyte can be declared as active against adenovirus type 5 as follows:

undiluted 30 seconds clean conditions

Bremen, 17/06/2021

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

9. Literature

1. EN 14476:2013+A2:2019: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity of chemicals disinfectants and antiseptics in human medicine test - Test method and requirements (phase 2, step 1)

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 Pharmak; 162, 1931, 480-487

Appendix:

Legend to the Tables

Table 1: Raw data for Active Anolyte (80.0 %) tested against adenovirus type 5

Table 2: Raw data for Active Anolyte (50.0 %) tested against adenovirus type 5

Table 3: Raw data for Active Anolyte (10.0 %) tested against adenovirus type 5

Table 4: Raw data for Active Anolyte (0.1 %) tested against adenovirus type 5

Table 5: Raw data for formaldehyde solution (0.7 %) tested against adenovirus type 5

Table 6: Raw data for control of efficacy for suppression of disinfectant's activity (80.0 %)

Table 7: Raw data (adenovirus type 5) for cell sensitivity (80.0 %)

Table 8 (a+b): Summary of results with Active Anolyte and adenovirus type 5

Legend to the Figures

Figure 1: Virus-inactivating properties of Active Anolyte (80.0 %)

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)

Table 1: Raw data for Active Anolyte (80.0 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells) (#7361)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	clean conditions	0.5	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			1	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	80.0 %	clean conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0403 3040	0000 0000	0000 0000
			60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4433 4444	0040 0300	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 2: Raw data for Active Anolyte (50.0 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells) (#7361)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	50.0 %	clean conditions	0.5	n.d.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	50.0 %	clean conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0403 3040	0000 0000	0000 0000
			60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4433 4444	0040 0300	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 3: Raw data for Active Anolyte (10.0 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells) (#7361)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	10.0 %	clean conditions	0.5	n.d.	n.d.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	10.0 %	clean conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0403 3040	0000 0000	0000 0000
			60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4433 4444	0040 0300	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 4: Raw data for Active Anolyte (0.1 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells) (#7376)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	0.1 %	clean conditions	0.5	n.d.	n.d.	4444 4444	4444 4444	4444 4444	2322 1232	2000 0201	0000 0000	n.d.
			1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	0.1 %	clean conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	n.d.	n.d.	n.d.	4444 4444	4444 4444	3330 0223	0001 0202	0000 0000	0000 0000
			60	n.d.	n.d.	n.d.	4444 4444	4444 4444	3303 2323	0200 0002	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 5: Raw data for formaldehyde solution (0.7 %) tested against adenovirus type 5 at 20 °C (quantal test; 8 wells) (#7361)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
formaldehyde	0.7 % (m/V)	PBS	5	n.d.	n.d.	4444 4444	4444 4444	4433 4344	0040 3000	0000 0000	n.d.	n.d.
			15	n.d.	n.d.	4444 4444	4020 4033	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			30	n.d.	n.d.	0100 0020	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	n.d.	n.d.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
formaldehyde cytotoxicity	0.7 % (m/V)	PBS	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	PBS	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4443 4233	0000 0000	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 6: Raw data for control of efficacy for suppression of disinfectant's activity (80.0 %) (#7361)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	clean conditions	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4443 3044	0030 0000	0000 0000	n.d.
corresponding virus control	clean conditions	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4433 4444	0040 0300	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 7: Raw data (adenovirus type 5) for cell sensitivity (80.0 % solution) (#7361)

Product	Dilution	Dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
PBS	-	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4404 4444	0003 3000	0000 0000	n.d.
test product	1:10	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4434 4444	0030 2030	0000 0000	n.d.

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 8a: Summary of results with Active Anolyte and adenovirus type 5

Product*	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after …min
				0.5	1	5	30	60	
test product (1)	80.0 %	clean conditions	1.50	≤ 1.50±0.00	≤ 1.50±0.00	n.d.	n.d.	n.d.	0.5 (RF ≥ 6.25±0.33)
test product (1)	50.0 %	clean conditions	1.50	≤ 2.50±0.00	n.d.	n.d.	n.d.	n.d.	0.5 (RF ≥ 5.25±0.33)
test product (1)	10.0 %	clean conditions	1.50	≤ 3.50±0.00	n.d.	n.d.	n.d.	n.d.	0.5 (RF ≥ 4.25±0.33)
test product (2)	0.1 %	clean conditions	1.50	7.88±0.37	n.d.	n.d.	n.d.	n.d.	> 0.5 (RF = 0.00±0.25)

*The number in brackets gives the number of the corresponding virus control, see table 8b

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

Table 8b: Summary of results with Active Anolyte and adenovirus type 5

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after … min
				0	5	15	30	60	
formaldehyde	0.7 % (w/v)	PBS	3.50	n.d.	6.75±0.33	5.13±0.37	≤3.75±0.33	≤3.50±0.00	60 (RF ≥ 4.00±0.00)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	7.50±0.00	n.a.
virus control (1) (+ suppression)	n.a.	clean conditions	n.a.	8.00±0.38	n.d.	n.d.	n.d.	7.75±0.33	n.a.
virus control (2)	n.a.	clean conditions	n.a.	7.63±0.49	n.d.	n.d.	n.d.	7.63±0.41	n.a.
suppression control	80.0 %	clean conditions	n.d.	n.d.	n.d.	n.d.	7.50±0.35	n.d.	n.a.
sens. PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.63±0.41	n.a.
sens. product	80.0 % → 1:10	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.88±0.37	n.a.

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

Figure 1: Virus-inactivating properties of Active Anolyte (80.0 %)

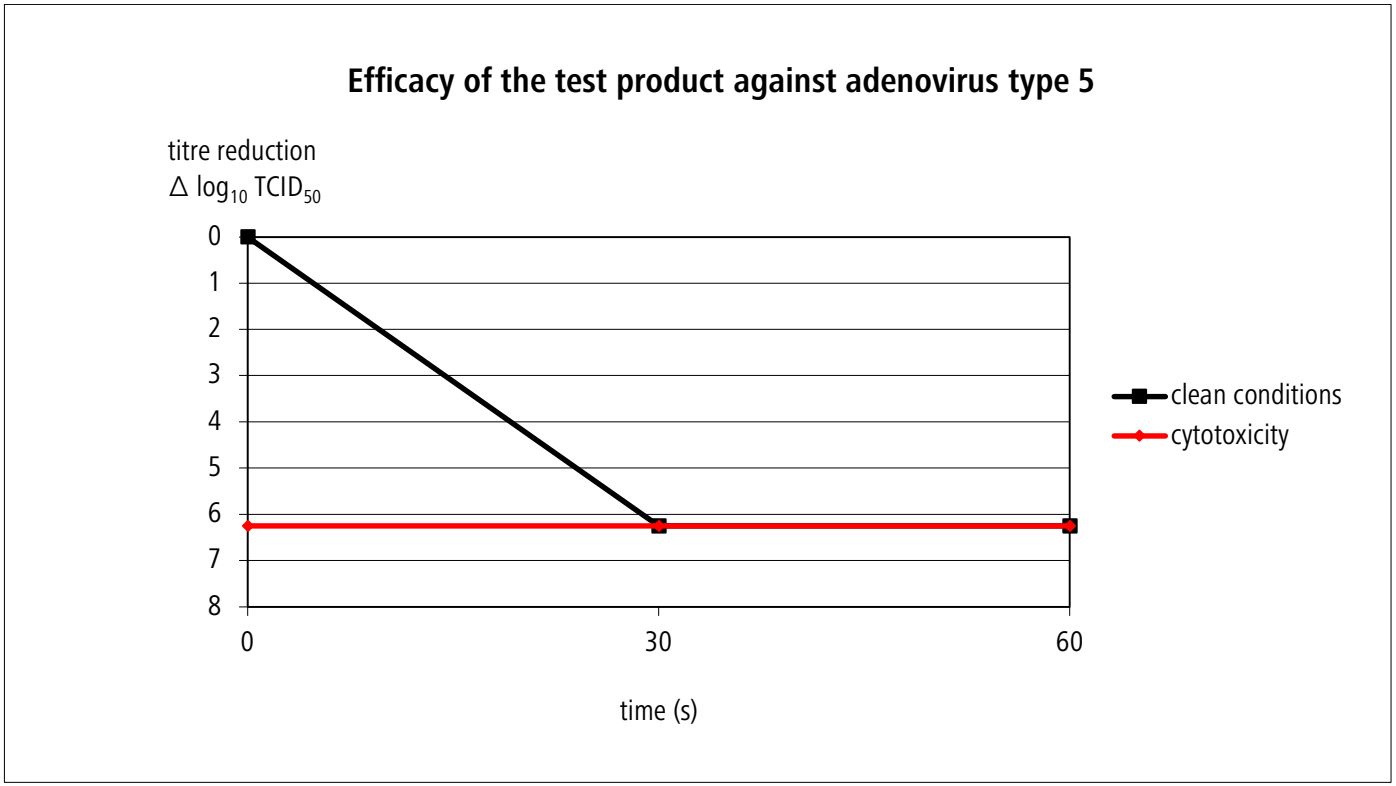

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)