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DAkkS Deutsche Akkreditierungsstelle D-PL-13412-01-01 D-PL-13412-01-02
Anerkannt durch/Recognized by Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten
ZLG-AP-216.11.02

TEST REPORT

30/03/2021

Test report L21/0076MV.1

Evaluation of the effectiveness of

Naeve Alcocreme Parfumefri

Test virus: modified vaccinia virus Ankara (MVA)

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:
Mediator A/S
Centervej 2E
DK - 6000 Kolding

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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	Mediator A/S
Name of product	Naeve Alcocreme Parfumefri
Confirmation no.	220788
Product diluent recommended by the manufacturer	-
Batch number	A100-0001
Application	hand rub
Production date	-
Expiry date	-
Active compound (s) (according to manufacturer´s information)	70% Ethanol w/w
Appearance (undiluted product), odour	turbid, white creme product specific
pH-values	undiluted: 10.47 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	18/01/2021

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)
- 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 modified vaccinia virus Ankara (MVA) originated from Dr. Manteufel, Institut für Tierhygiene und Öffentliches Veterinärwesen, DE - 04103 Leipzig. Before inactivation assays, virus had been passaged four times in *BHK 21-cells* (Baby Hamster Kidney).

BHK 21-cells (passage 9) originated from the Leibniz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, DE - 38124 Braunschweig.

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 % and 10.0 % (demonstration of non-active range) solutions
Appearance of product dilution(s)	no precipitation
Contact time(s)	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)	strong clouding, minor precipitation
Virus strain	modified vaccinia virus Ankara (MVA) (ATCC VR-1508)
Date of testing	12/03/2021 – 30/03/2021
End of testing	30/03/2021

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 % and 10.0 % (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 (end point titration)

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 Inactivation assay following the large volume plating method (LVP)

Following the large volume plating method (EN 14476, section 5.5.4.3) the inactivation assays were further diluted 1:5,000 in cell culture medium. The total volume was added (without any further dilution) to the permissive cells. By introducing such a huge dilution, it is possible to eliminate cytotoxicity of the test product in order to demonstrate a 4 $\log_{10}$ reduction of virus titre. Calculation of virus titre follows formula of Taylor or Poisson (EN 14476, section B.3). This method is necessary for those products which demonstrate a great cytotoxicity.

12.5 µl of the inactivation assay were added to 62.5 ml medium and then the total volume was distributed in 6 microtitre plates (108 µl / well, 576 wells total). After 5 days of inoculation cultures were observed for cytopathic effects.

5.7 Determination of cytotoxicity

Determination of cytotoxicity was performed according to EN 14476.

5.8 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.

5.9 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.

5.10 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 using the large volume plating method.

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 $\geq 1.75 \pm 0.48$ (between 0.75 – 3.5) after 5 min and $\geq 2.13 \pm 0.25$ (between 2.0 – 4.0) after 15 min for MVA.

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 (≤ 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 9. Tables 1 to 7 demonstrate the raw data, whereas tables 8 (a+b) and 9 give a summary of results.

Testing the 50.0 % solution with the end point dilution method, no residual virus was found after 60 seconds of exposure time (table 1). The reduction factor was $\geq 2.63 \pm 0.45$.

The 10.0 % solution was not active within 60 seconds of exposure time using the end point dilution method (table 2).

Since it was not possible to show a sufficient log_{10}-reduction testing the undiluted test product using the end point dilution method due to cytotoxicity, in parallel the large volume plating method (LVP) was introduced testing the undiluted test product in an 80.0 % assay with 60 seconds of exposure time. The mean virus titre was log_{10} $TCID_{50}$/ml = 6.81 ± 0.32 (table 6).

The undiluted test product in an 80.0 % assay was active after 60 seconds of exposure time (table 7). Since no residual virus was found in 576 cell culture units at this time point, the result according to the formula of Poisson was ≤ 2.54 log_{10} $TCID_{50}$. The reduction factor was therefore ≥ 4.27 ± 0.32 (6.81 ± 0.32 log_{10} $TCID_{50}$ minus ≤ 2.54 log_{10} $TCID_{50}$). This corresponded to an inactivation of ≥ 99.99 %.

8. Conclusion

The hand rub Naeve Alcocreme Parfumefri tested undiluted demonstrated activity against MVA after an exposure time of 60 seconds under clean conditions. Therefore, the hand rub Naeve Alcocreme Parfumefri can be declared as active against MVA as follows:

undiluted **60 seconds** **clean conditions**

Bremen, 30/03/2021

- 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

Legend to the Figures

Table 1: Raw data for Naeve Alcocreme Parfumefri (50.0 %) tested against MVA at 20 °C (quantal test; 8 wells) (#7248)

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	1	n.d.	n.a.	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			15	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.	tttt tttt	tttt tttt	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.	4444 4444	4444 4444	4444 4444	4030 2304	0200 0000	0000 0000	n.d.
			60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0340 0340	0020 0000	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 2: Raw data for Naeve Alcocreme Parfumefri (10.0 %) tested against MVA at 20 °C (quantal test; 8 wells) (#7248)

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	1	n.d.	n.a.	n.a.	4444 4444	2244 4344	0000 0000	0000 0000	n.d.	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			15	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.	tttt tttt	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.	4444 4444	4444 4444	4444 4444	4030 2304	0200 0000	0000 0000	n.d.
			60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0340 0340	0020 0000	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 3: Raw data for formaldehyde solution (0.7 %) tested against MVA at 20 °C (quantal test; 8 wells) (#7248)

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.	tttt tttt	0200 0100	0000 0001	0000 0000	0000 0000	n.d.	n.d.
			15	n.d.	n.d.	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			30	n.d.	n.d.	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	n.d.	n.d.	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
formaldehyde cytotoxicity	0.7 % (m/V)	PBS	n.a.	n.d.	n.d.	tttt tttt	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.	4444 4444	4444 4444	4442 4444	0000 0002	0000 0000	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 4: Raw data for control of efficacy for suppression of disinfectant's activity (80.0 %) (#7248)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	clean conditions	n.d.	n.d.	n.a.	4444 4444	4444 4444	0300 0400	0000 0004	0000 0000	n.d.
corresponding virus control	clean conditions	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0340 0340	0020 0000	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 5: Raw data (MVA) for cell sensitivity (80.0 % solution) (LVP) (#7248)

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	0440 3403	0000 0000	0000 0000	n.d.
test product	1:5,000	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0403 0020	0000 0000	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 6: Determination of virus titre (LVP) at 20 °C (#7248)

Virus titration	Interfering substance	Exposure time	dilutions (log_{10})								
			1	2	3	4	5	6	7	8	9
(beginning of test)	clean conditions	0	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4030 2304	0200 0000	0000 0000	n.d.
1st control	clean conditions	60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0340 0340	0020 0000	0000 0000	n.d.
2nd control	clean conditions	60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0000 0000	0000 0000	0000 0000	n.d.

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

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

Table 7: Inactivation of MVA by Naeve Alcocreme Parfumefri (80.0 %) at 20 °C (60 seconds) (LVP, 1:5,000) (#7248)

Interfering substance	Row	1	2	3	4	5	6	7	8	9	10	11	12
clean conditions	plate 1/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
	plate 2/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
	plate 3/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
	plate 4/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
	plate 5/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
	plate 6/6	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000

t = cytotoxic 0 = no virus detectable 1 to 4 = virus detectable (degree of CPE in 8 wells of a microtitre plate)

Table 8a: Summary of results (end point dilution method) with Naeve Alcocreme Parfumefri and MVA

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after …min
				1	2	15	30	60	
test product	50.0 %	clean conditions	3.50	≤ 4.50±0.00	n.d.	n.d.	n.d.	n.d.	≥ 1 (RF ≥ 2.63±0.545)
test product	10.0 %	clean conditions	2.50	6.50±0.00	n.d.	n.d.	n.d.	n.d.	> 1 (RF = 0.63±0.45)

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

Table 8b: Summary of results (end point dilution method) with Naeve Alcocreme Parfumefri and MVA

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	4.50	n.d.	≤ 4.88±0.41	≤ 4.50±0.00	≤ 4.50±0.00	≤ 4.50±0.00	≥ 15 (RF ≥ 2.13±0.25)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	6.63±0.25	n.a.
virus control (+ suppression)	n.a.	clean conditions	n.a.	7.25±0.44	n.d.	n.d.	n.d.	7.13±0.45	n.a.
suppression control	80.0 %	clean conditions	n.d.	n.d.	n.d.	n.d.	6.88±0.41	n.d.	n.a.

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

Table 9: Summary of results (LVP, 1:5,000) with Naeve Alcocreme Parfumefri and MVA

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after …min
				0	0.5	1	2	60	
test product	80.0 %	clean conditions	n.a.	n.d.	n.d.	≤ 2.54	n.d.	n.d.	1 (RF ≥ 4.27±0.32)
virus control	n.a.	clean conditions	n.a.	7.25±0.44	n.d.	n.d.	n.d.	7.13±0.45 6.50±0.00 (Ø6.81±0.32)	n.a.
sens. PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.13±0.37	n.a.
sens. product	80.0 % → 1:5,000	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.88±0.37	n.a.

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

Figure 1: Virus-inactivating properties of Naeve Alcocreme Parfumefri (80.0 %) (LVP)

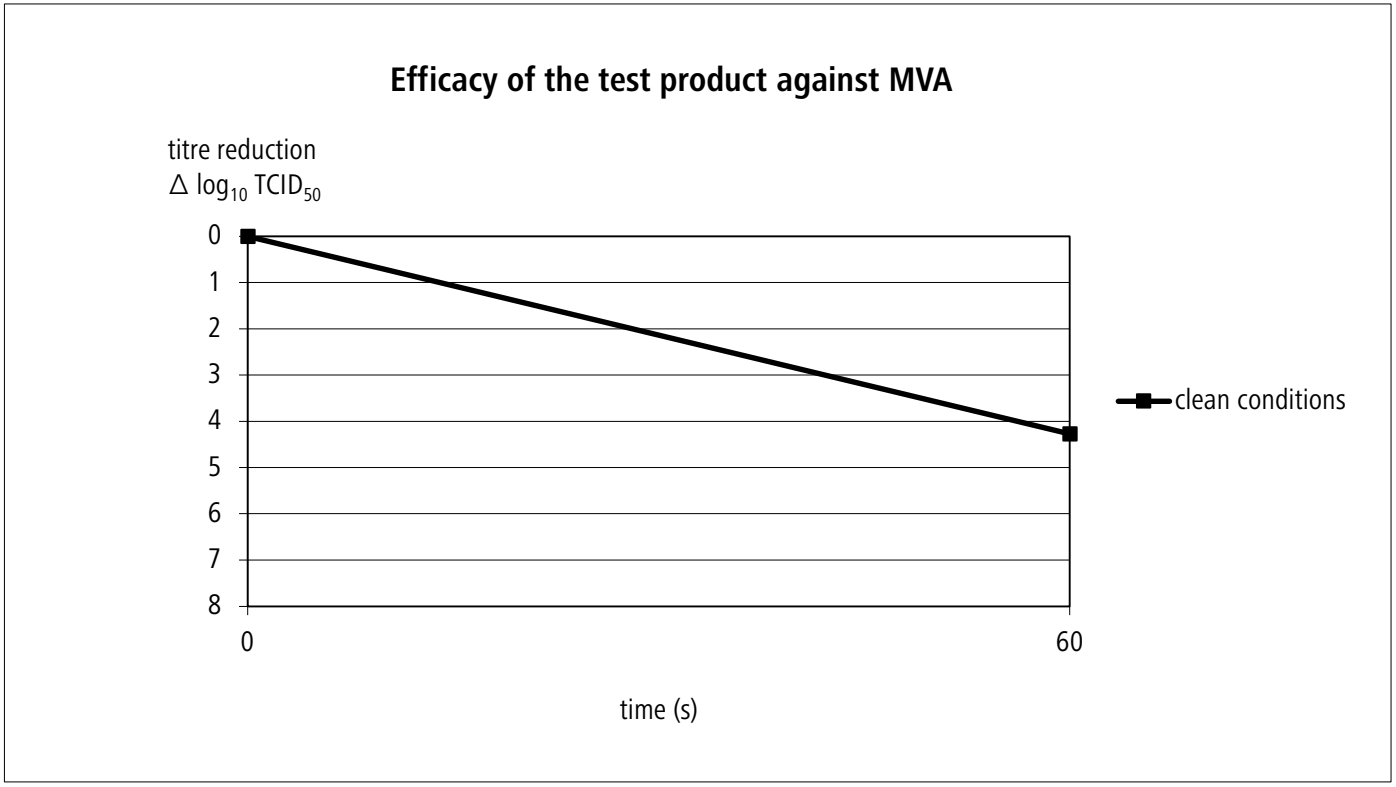

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)