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

ilac-MRA 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

08/02/2018

Test report L17/0629cM.1

Evaluation of the effectiveness of

Chemisept med

Test virus: murine norovirus (as surrogate of human norovirus)

Method: EN 14476:2013+A1:2015 (clean conditions)

quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in human medicine

Sponsor:
Chemi-Pharm AS
Pollu 132
EST – TALLINN 10917

TEST REPORT

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	Chemi-Pharm AS
Name of product	Chemisept med
Confirmation no.	203850
Product diluent recommended by the manufacturer	-
Batch number	196101017
Application	hand disinfection
Production date	10/10/2017
Expiry date	10/10/2020
Active compound (s) (100 g)	72.5 g ethanol 7.5 g IPA
Appearance, odour	clear, colorless liquid product specific
pH-values	undiluted: 5.38 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	13/10/2017

3. Materials

3.1 Culture medium and reagents

- Dulbecco´s Modified Eagle´s Medium (DMEM, Biozym Scientific GmbH, catalogue no. 880006)
- 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

Murine norovirus (MNV) was obtained from PD. Dr. E. Schreier, Head of FG15 Molecular Epidemiology of Viral Pathogens at the Robert Koch-Institute (RKI) in Berlin. Prior to inactivation, MNV was passaged three times in *RAW 264.7 cells* (a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice, ATCC TIB-71). *RAW 264.7 cells* were cultured with Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and fetal calf serum with low endotoxin. Furthermore, cells (passage 25) 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, 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 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 dilutions	no precipitation
Contact times	15, 20 and 30 seconds and 30 minutes
Interfering substance	0.3 g/l bovine serum albumin (clean conditions, EN 14476)
Procedure to stop action of disinfectant	immediate dilution
Diluent	water
Stability of product in the mix with virus and interfering substance (80.0 % solution)	medium clouding, no precipitation
Virus strain	murine norovirus (Berlin 06 / 06 / DE Isolate S99)
Date of testing	19/12/2017 – 08/02/2018
End of testing	08/02/2018

5. Methods

5.1 Preparation of test virus suspension

To prepare the test virus suspension, *RAW 264.7 cells* which have been cultured with Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and 10 % fetal calf serum with low endotoxin were inoculated with MNV (stock virus solution) in a 175 cm^2 cell culture flask. Once a cytopathic effect had been induced (approx. 1-3 days), freezing and thawing was carried out two times. The cell debris was removed by low speed centrifugation and the supernatant was recovered as test viral suspension, aliquoted and 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 % solutions (demonstrating of non-active range). This solution was prepared with water immediately before the inactivation tests.

5.3 Infectivity assay

Infectivity was determined as endpoint titration according to EN 5.5 transferring 0.1 ml of each dilution into eight wells of a microtitre plate to 0.1 ml of *RAW 264.7 cells* (10-15 x 10^3 cells per well) freshly prepared by scraping, beginning with the highest dilution. Microtitre plates were incubated at 37 °C in a 5 % CO_2-atmosphere. The cytopathic effect was read by using an inverted microscope after five days. Calculation of the infective dose $TCID_{50}$/ml was calculated with the method of Spearman (2) and Kärber (3) with the following formula:

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

meaning

X_0 = $\log_{10}$ 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.

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 four $\log_{10}$ 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 in accordance to EN 5.5. The test product was examined undiluted (80.0 %) and as 50.0 % and 10.0 % (demonstration of non-active range) solutions in water at 20 °C according to EN 14476. 15, 20 and 30 seconds and 30 minutes were chosen as contact times.

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 (EN 5.5.7). 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).

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 5.5.4.1.

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 a volume of double concentrated cell suspension. After 1 h at 37 °C the cells were centrifuged and re-suspended in cell culture medium (EN 5.5.4.2b).

Finally, a comparative titration of the test virus suspension was performed on the pre-treated (disinfectant) and non-pre-treated (PBS) cells as described above.

5.8 Control of efficacy for suppression of disinfectant's activity

Furthermore, a control of efficiency for suppression of disinfectant's activity was included (EN 5.5.5.1).

5.9 Reference virus inactivation test

As reference for test validation a 0.7 % formaldehyde solution according to EN 5.5.6 was included. 5, 15, 30 and 60 minutes were chosen as contact times. In addition, cytotoxicity of formaldehyde test solution was determined following EN 5.5.6.2 with dilutions up to 10^{-5}.

6. Verification of the methodology

The following criteria as mentioned in EN 5.7 were fulfilled:

a) The titre of the test virus suspension allowed the determination of a ≥ 4 log_{10} reduction (maximal virus reduction ≥ 6.88 ± 0.35).

b) The test product (80.0 % solutions) showed no cytotoxicity in the 1:10 dilutions thus allowing the detection of a 4 log_{10} reduction of virus titre.

c) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *RAW 264.7 cells* showed no significant difference (< 1 log_{10}; EN 5.7) of virus titre: 8.00 ± 0.44 (PBS) versus 8.13 ± 0.45 (1:10 dilutions of disinfectant as 80.0 % solution) log_{10} $TCID_{50}$/ml.

d) The control of efficacy for suppression of disinfectant's activity (80.0 % solution) showed no decrease (≤ 0.5 log_{10}; EN 5.5.5.1) in virus titre (8.00 ± 0.38 versus 8.00 ± 0.38 log_{10} $TCID_{50}$/ml).

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

Since all criteria according EN 5.7 were fulfilled, examination with MNV according to EN 14476 is valid.

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 was able to inactivate MNV after 15 seconds under clean conditions in this quantitative suspension test (tables 1 and 2). The reduction factors were ≥ 6.38 ± 0.62 and ≥ 6.38 ± 0.45. The mean value was ≥ 6.38 ± 0.38. This corresponded to an inactivation of ≥ 99.9999 %.

Tested as 50.0 % solution, the test product was not able to inactivate MNV within 30 seconds under clean conditions in this quantitative suspension test (table 3).

Tested as 10.0 % solution, the test product was not active within 30 minutes of exposure time (table 4).

8. Conclusion

The hand disinfectant Chemisept med tested undiluted demonstrated effectiveness against MNV after an exposure time of 15 seconds under clean conditions.

Therefore, the hand disinfectant Chemisept med can be declared as active against MNV as follows:

undiluted **15 seconds** **clean conditions**

Bremen, 08/02/2018

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

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9. 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 (BGBI. I, 1994, page 1703). Appendix revised at 14. 05. 1997 (BGBI. 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 controls incorporated in the inactivation assays.

10. 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.

11. Literature

1. EN 14476:2013+A1:2015: 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 Chemisept med (80.0 %) tested against MNV (1st assay)

Table 2: Raw data for Chemisept med (80.0 %) tested against MNV (2nd assay)

Table 3: Raw data for Chemisept med (50.0 %) tested against MNV

Table 4: Raw data for Chemisept med (10.0 %) tested against MNV

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

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

Table 7: Raw data (MNV) for cell sensitivity (80.0 %)

Table 8 (a+b): Summary of results with Chemisept med and MNV

Legend to the Figures

Figure 1: Virus-inactivating properties of Chemisept med (80.0 %)

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

Table 1: Raw data for Chemisept med (80.0 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5355) (1st assay)

Product	Concentration	Interfering substance	Contact time	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	clean conditions	15 s	4440 4000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			20 s	0000 0040	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			30 s	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60 s	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 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			60 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0444 4400	0000 0304	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 Chemisept med (80.0 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5378) (2nd assay)

Product	Concentration	Interfering substance	Contact time	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	clean conditions	15 s	0400 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			20 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			60 s	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 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0000 0000	0040 0000	0000 0000
			60 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0044 0440	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 Chemisept med (50.0 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5378)

Product	Concentration	Interfering substance	Contact time	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	50.0 %	clean conditions	15 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			20 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30 s	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 4000	n.d.	n.d.
			60 s	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 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0000 0000	0040 0000	0000 0000
			60 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0044 0440	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 Chemisept med (10.0 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5379)

Product	Concentration	Interfering substance	Contact time	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	10.0 %	clean conditions	15 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			20 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30 s	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 0004	0000 0000	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 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0000 0000	0040 0000	0000 0000
			60 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0044 0440	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 MNV at 20 °C (quantal test; 8 wells) (#5378)

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	tttt tttt	tttt tttt	tttt tttt	4444 4444	4444 4444	4444 4444	0400 4000	n.d.	n.d.
			15	tttt tttt	tttt tttt	tttt tttt	4444 4444	4444 4444	0440 4044	0040 0000	n.d.	n.d.
			30	tttt tttt	tttt tttt	tttt tttt	4444 4444	4444 4444	0030 0044	0000 0000	n.d.	n.d.
			60	tttt tttt	tttt tttt	tttt tttt	3443 4444	0000 3044	0000 0000	0000 0000	n.d.	n.d.
formaldehyde cytotoxicity	0.7 % (m/V)	PBS	n.a.	tttt tttt	tttt tttt	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	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4442 4444	4040 0400	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 %) (#5378)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	clean conditions	n.d.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4440 0300	0000 0000	n.d.
corresponding virus control	clean conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0044 0440	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 (MNV) for cell sensitivity (80.0 %) (#5378)

Product	Dilution	Dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
PBS	-	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0400 0044	0004 0000	n.d.
test product	1:10	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4004 4004	0000 0004	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 Chemisept med and MNV

Product*	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after …					> 4 log_{10} reduction after …
				15 s	20 s	30 s	60 s	30 min	
test product (1)	80.0 %	clean conditions	1.50	≤ 2.00±0.38	≤ 1.63±0.25	≤ 1.50±0.00	n.d.	n.d.	15 s (RF ≥ 6.38±0.62)
test product (2)	80.0 %	clean conditions	1.50	≤ 1.63±0.25	n.d.	n.d.	n.d.	n.d.	15 s (RF ≥ 6.38±0.45)
test product (2)	50.0 %	clean conditions	1.50	n.d.	n.d.	7.75±0.33	n.d.	n.d.	> 30 s (RF = 0.25±0.50)
test product (2)	10.0 %	clean conditions	1.50	n.d.	n.d.	n.d.	n.d.	7.75±0.33	> 30 min (RF = 0.25±0.50)

*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 Chemisept med and MNV

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.	7.75±0.33	7.25±0.44	6.88±0.37	5.88±0.37	> 60 (RF = 2.00±0.52)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	7.88±0.57	n.a.
virus control (1)	n.a.	clean conditions	n.a.	n.d.	n.d.	n.d.	n.d.	8.38±0.49	n.a.
virus control (2) (+ suppression)	n.a.	clean conditions	n.a.	7.63±0.25	n.d.	n.d.	n.d.	8.00±0.38	n.a.
suppression control	80.0 %	clean conditions	1.50	n.d.	n.d.	n.d.	8.00±0.38	n.d.	n.a.
sens.control PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	8.00±0.44	n.a.
sens. control test product	80.0 %→ 1:10	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	8.13±0.45	n.a.

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

Figure 1: Virus-inactivating properties of Chemisept med (80.0 %)

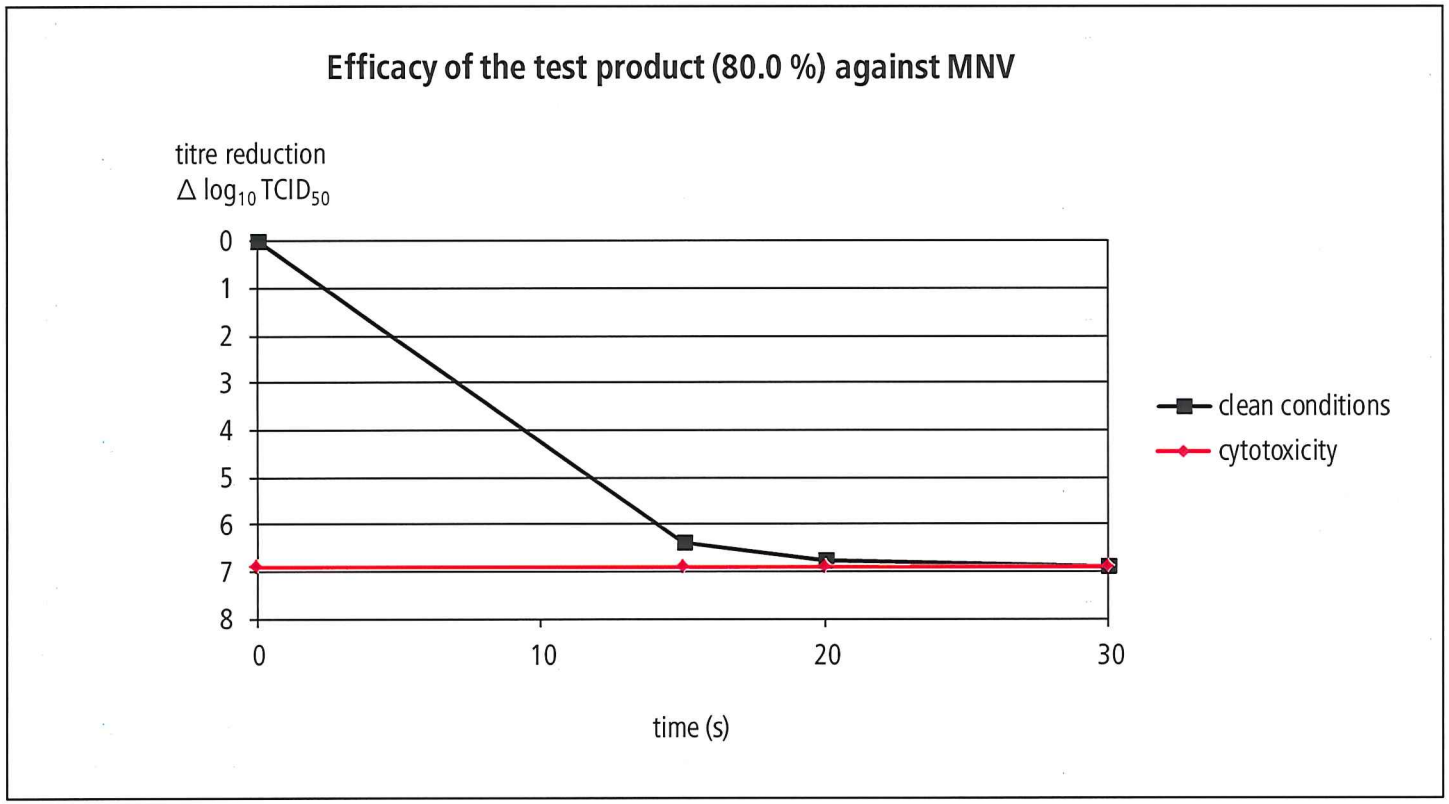

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)