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INSTITUTE FOR HYGIENE AND MICROBIOLOGY



Anerkannt durch/Recognized by
Zentralstelle der Länder
für Gesundheitsschutz
bei Arzneimitteln und
Medizinprodukten
ZLG-AP-216.11.02

07/02/2018

Test report L17/0629aM.2

Evaluation of the effectiveness of Bactacid AF

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

Method: EN 14476:2013+A1:2015 (dirty 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

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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	Bactacid AF
Confirmation no.	203850
Product diluent recommended by the manufacturer	-
Batch number	197101017
Application	surface disinfection
Production date	10/10/2017
Expiry date	10/10/2010
Active compound (s) (100 g)	57 g ethanol 6 g IPA
Appearance, odour	clear, colorless liquid product specific
pH-values	undiluted: 7.49 (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)

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- sheep erythrocyte s (Fiebig Nährstofftechnik).

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

- CO₂ 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).

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4. Experimental conditions

Test temperature	20 °C ± 1.0 °C
Concentration of test product	undiluted (97.0 % and 80.0 %) and as 10.0 % (demonstration of non-active range) solution
Appearance of product dilutions	no precipitation
Contact times	15, 20, 30 and 60 seconds and 30 minutes
Interfering substance	3.0 g/l bovine serum albumin + 3.0 g/l erythrocytes (dirty conditions, EN 14476)
Procedure to stop action of disinfectant	immediate dilution
Diluent	water
Stability of product in the mix with virus and interfering substance (97.0 % solution)	medium clouding, minor precipitation
Virus strain	murine norovirus (Berlin 06 / 06 / DE Isolate S99)
Date of testing	19/12/2017 – 07/02/2018
End of testing	07/02/2018

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 cm² 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. Additionally, the test product was examined as 97.0 % solution (0.1 part test virus suspension + 0.2 part interfering substance (5-fold) + 9.7 parts disinfectant).

Furthermore, the product was evaluated as 10.0 % solution (demonstrating of non-active range). This solution was prepared with water immediately before the inactivation tests.

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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* ($10\text{--}15 \times 10^3$ cells per well) freshly prepared by scraping, beginning with the highest dilution. Microtitre plates were incubated at 37 °C in a 5 % CO₂-atmosphere. The cytopathic effect was read by using an inverted microscope after five days. Calculation of the infective dose TCID₅₀/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 = log₁₀ 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 log₁₀ 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 (97.0 % and 80.0 %) and as 10.0 % (demonstration of non-active range) solution in water at 20 °C according to EN 14476. 15, 20, 30 and 60 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.

Titration of the virus control was 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). As virus controls for the 97.0 % assays 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 $20\text{ °C} \pm 1.0\text{ °C}$. 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 0.3 parts by volume of water were mixed with 9.7 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} .

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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 $\geq 4 \log_{10}$ reduction (maximal virus reduction $\geq 5.50 \pm 0.31$, 97.0 % assay).
- b) The test product (97.0 % solutions) showed no cytotoxicity in the 1:10 dilutions thus allowing the detection of a $\log_{10}$ 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: 8.50 ± 0.46 (PBS) versus 7.75 ± 0.44 (1:10 dilutions of disinfectant as 97.0 % solution) $\log_{10}$ TCID₅₀/ml.
- d) The control of efficacy for suppression of disinfectant's activity (97.0 % solution) showed no decrease ($\leq 0.5 \log_{10}$; EN 5.5.5.1) in virus titre (7.13 ± 0.45 versus $6.88 \pm 0.37 \log_{10}$ TCID₅₀/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 in a 97.0 % assay was able to inactivate MNV after 15 seconds under dirty conditions in this quantitative suspension test (tables 1 and 2). The reduction factors were $\geq 5.50 \pm 0.27$ and $\geq 5.38 \pm 0.26$. The mean value was $\geq 5.44 \pm 0.19$. This corresponded to an inactivation of ≥ 99.999 %.

The undiluted test product in an 80.0 % assay was able to inactivate MNV after 60 seconds under dirty conditions in this quantitative suspension test (table 3). The reduction factors was 4.75 ± 0.55 . This corresponded to an inactivation of ≥ 99.99 %.

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

8. Conclusion

The surface disinfectant Bactcid AF tested undiluted demonstrated effectiveness against MNV after an exposure time of 15 seconds under dirty conditions.

Therefore, the surface disinfectant Bactcid AF can be declared as active against MNV as follows:

undiluted 15 seconds dirty conditions

Bremen, 07/02/2018


- 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 (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 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.

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

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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 Pharmac; 162, 1931, 480-487

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Appendix:

Legend to the Tables

Table 1:	Raw data for Bactacid AF (97.0 %) tested against MNV (1 st assay)
Table 2:	Raw data for Bactacid AF (97.0 %) tested against MNV (2 nd assay)
Table 3:	Raw data for Bactacid AF (80.0 %) tested against MNV
Table 4:	Raw data for Bactacid AF (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 (97.0 %)
Table 7:	Raw data (MNV) for cell sensitivity (97.0 %)
Table 8 (a+b):	Summary of results with Bactacid AF and MNV

Legend to the Figures

Figure 1:	Virus-inactivating properties of Bactacid AF (97.0 %)
Figure 2:	Virus-inactivating properties of formaldehyde (0.7 %)



Table 2: Raw data for Bactid AF (97.0 %) 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	97.0 %	dirty conditions	15 s	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			20 s	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	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	97.0 %	dirty conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	
virus control	n.a.	dirty conditions	0 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0040 0400	0000 0000	0000 0000	
			60 min	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0444 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 3: Raw data for Bactid AF (80.0 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5355)

Product	Concentration	Interfering substance	Contact time	Dilutions (log ₁₀)								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	dirty conditions	15 s	4444 4444	4444 4444	4444 4444	4404 0440	4000 0000	0000 0000	n.d.	n.d.	
			20 s	4444 4444	4444 4444	4444 4444	4040 0004	0000 0000	0000 0000	n.d.	n.d.	
			30 s	4444 4444	4444 4444	0044 4404	0000 0400	0000 0000	0000 0000	n.d.	n.d.	
			60 s	4444 4444	4440 0444	0040 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	
test product cytotoxicity	80.0 %	dirty conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.	
virus control	n.a.	dirty conditions	0 min 60 min	n.d. 4444 4444	n.d. 4444 4444	n.d. 4444 4444	n.d. 4444 4444	n.d. 4444 4444	n.d. 4404 4040	n.d. 0000 0000	n.d. 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 BactiCid AF (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 %	dirty 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	0444 0044	0000 0004	n.d.
test product cytotoxicity	10.0 %	dirty 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.	dirty 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	4400 4443	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 4044	0040 0000	n.d.	n.d.	
			30	tttt tttt	tttt tttt	tttt tttt	4444 4444	4444 0030	0000 0000	n.d.	n.d.	
			60	tttt tttt	tttt tttt	tttt tttt	3443 4444	0000 3044	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 0 = no virus present; t = cytotoxic

n.d. = not done 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 (97.0 %) (#5378)

Product	Interfering substance	dilutions (log ₁₀)								
		1	2	3	4	5	6	7	8	9
test product	dirty conditions	n.d.	4444 4444	4444 4444	4444 4444	4444 4444	0444 4000	0040 0000	0000 0000	n.d.
corresponding virus control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0444 0000	0000 0000	0000 0000	0000 0000

n.a. = not applicable

0 = no virus present; t = cytotoxic

n.d. = not done

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 (97.0 %) (#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	4444 4400	0000 4400	n.d.
test product	1:10	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4440 4444	0004 0440	0000 0000	n.d.

n.a. = not applicable

0 = no virus present; t = cytotoxic

n.d. = not done

1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)



Table 8a: Summary of results with Bactioid AF 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)	97.0 %	dirty conditions	1.50	≤ 1.50±0.00	≤ 1.50±0.00	≤ 1.50±0.00	≤ 1.50±0.00	n.d.	15 s (RF ≥ 5.50±0.27)
test product (2)	97.0 %	dirty conditions	1.50	≤ 1.50±0.00	≤ 1.50±0.00	n.d.	n.d.	n.d.	15 s (RF ≥ 5.38±0.26)
test product (3)	80.0 %	dirty conditions	1.50	5.25±0.44	4.88±0.37	4.25±0.44	3.38±0.41	n.d.	60 s (RF = 4.75±0.55)
test product (4)	10.0 %	dirty conditions	1.50	n.d.	n.d.	n.d.	n.d.	8.25±0.44	> 30 min (RF = 0.00±0.55)

*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 BactiCID AF and MNV

Product	Con- centration	Interfering substance	Level of cytotoxicity	log ₁₀ TCID ₅₀ /ml aftermin					> 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. (97.0 % assay)	dirty conditions	n.a.	n.d.	n.d.	n.d.	n.d.	7.00±0.38	n.a.
virus control (2) (+ suppression)	n.a. (97.0 % assay)	dirty conditions	n.a.	6.75±0.33	n.d.	n.d.	n.d.	6.88±0.37	n.a.
virus control (3)	n.a. (80.0 % assay)	dirty conditions	n.a.	n.d.	n.d.	n.d.	n.d.	8.13±0.37	n.a.
virus control (4)	n.a. (80.0 % assay)	dirty conditions	n.a.	n.d.	n.d.	n.d.	n.d.	8.25±0.33	n.a.
suppression control	97.0 %	dirty conditions	2.50	n.d.	n.d.	n.d.	7.13±0.45	n.d.	n.a.
sens. control PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	8.50±0.46	n.a.
sens. control test product	97.0 % → 1:10	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.75±0.44	n.a.

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



Figure 1: Virus-inactivating properties of Bactacid AF (97.0 %)

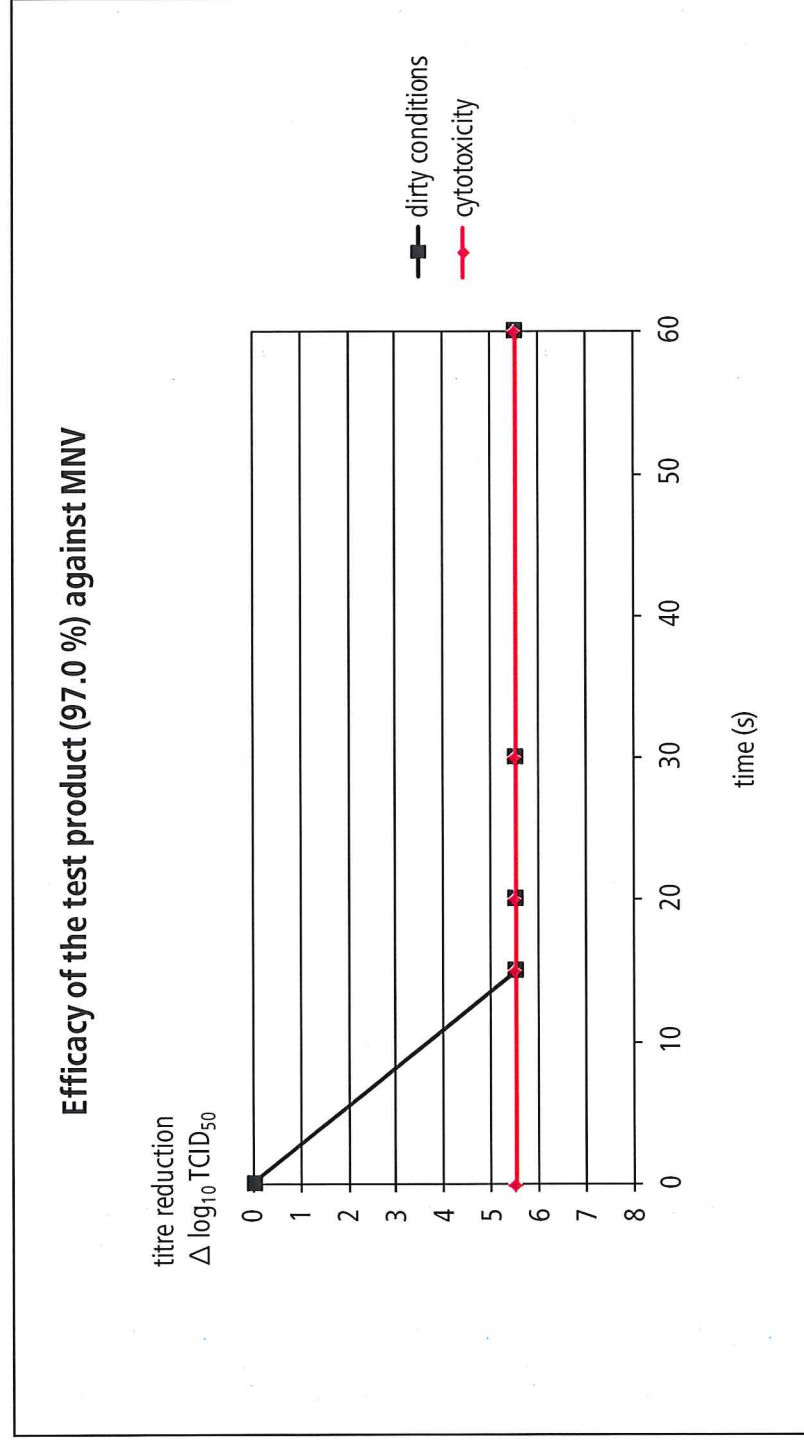




Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)

