



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



20/11/2018

Test report L18/0785R.1

Evaluation of the effectiveness of **Bactacid AF**

Test virus: human rotavirus strain Wa

Method: based on 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	Bactid AF
Confirmation no.	207087
Product diluent recommended by the manufacturer	-
Batch number	197251018
Application	surface disinfection
Production date	25/10/2018
Expiry date	25/10/2021
Active compound (s) (100 g)	57 g ethanol 6 g IPA
Appearance, odour	clear, colorless liquid product specific
pH-values	undiluted: 8.23 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	02/11/2018

3. Materials

3.1 Culture medium and reagents

- Eagle's Minimum Essential Medium with Earle's BSS (EMEM, Biozym Scientific GmbH, catalogue no. 880121)
- 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)
- sheep erythrocytes (Fiebig Nährstofftechnik)

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- Trypsin (SERVA Electrophoresis GmbH, article no. 37290).

3.2 Virus and cells

The human rotavirus strain Wa (serotype 1, subgroup II) was obtained by Prof. Dr. Holger Rabenau, Institute of Medical Virology of the Johann Wolfgang Goethe University of Frankfurt, DE - 60596 Frankfurt. Before the described tests, the virus had been passaged in *MA-104 cells* (embryonic rhesus monkey kidney cell line).

The cells (passage 44) 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)
- Polystyrol 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 (80.0 %) and as 50.0 % and 10.0 % (demonstration of non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	15 and 30 seconds
Interfering substance	3.0 g/l bovine serum albumin + 3.0 ml/l erythrocytes (dirty 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 (80.0 % solution)	strong clouding, no precipitation
Virus strain	human rotavirus strain Wa
Date of testing	02/11/2018 – 20/11/2018
End of testing	20/11/2018

5. Methods

5.1 Preparation of test virus suspension

After washing with serum-free Eagle's Minimum Essential Medium twice, cells were incubated with EMEM without fetal calf serum for three hours to eliminate all FCS. This was followed by the addition of virus (stock virus suspension) to MA-104 cells in the presence of trypsin for two hours ± 10 minutes at 37 °C. After this time, medium with trypsin was added. If 90 % of the cells showed a cytopathic effect, cells were subjected to a rapid two-fold freeze-thawing procedure followed by a centrifugation at 1.620 g for 30 minutes at 4 °C in order to sediment cell debris. After aliquotation the supernatant was stored as test virus suspension 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). These solutions were prepared with Aqua bidest. immediately before the inactivation tests.

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5.3 Infectivity assay

Infectivity was determined by means of end point dilution titration in a micro-procedure. For this, samples were serially diluted with ice-cold EMEM with trypsin and 100 µl of each dilution were placed after aspiration of the medium in eight wells of a sterile polystyrene flat bottom 96-well microtitre plate with a preformed *MA-104* monolayer. After one hour at 37 °C, 100 µl EMEM with trypsin were added. Incubation took place at 37 °C in a CO₂-atmosphere (5.0 % CO₂ - content). Finally, cultures were observed for cytopathic effects for six days of inoculation. The infective dose (TCID₅₀) was calculated according to 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 = $\log_{10}$ 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 Aqua bidest. at 20 °C based on EN 14476. 15 and 30 seconds 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.

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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).

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 two parts by volume hard 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 the wells of the microtitre plates with a preformed monolayer of *MA-104-cells*.

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).

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 based on 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:

- The titre of the test virus suspension allowed the determination of a $\geq 4 \log_{10}$ reduction (maximal virus reduction $\geq 5.75 \pm 0.23$).
- The test product showed no cytotoxicity in the 1:10 dilutions (80.0 %) thus allowing the detection of a $4 \log_{10}$ reduction of virus titre.
- The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *MA-104 cells* showed no significant difference ($< 1 \log_{10}$; EN 5.7) of virus titre: 7.50 ± 0.00 (PBS) versus 7.00 ± 0.38 (1:10 dilution of disinfectant as 80.0 % solution) $\log_{10}$ TCID₅₀/ml.
- The control of efficacy for suppression of disinfectant's activity (80.0 % solution) showed no decrease ($\leq 0.5 \log_{10}$; EN 5.5.5.1) in virus titre (7.50 ± 0.35 versus $7.25 \pm 0.33 \log_{10}$ TCID₅₀/ml).
- 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 human rotavirus based on EN 14476 is valid.

7. Results

Results of examination are shown in tables 1 to 7. Tables 1 to 6 demonstrate the raw data, whereas table 7 (a+b) gives a summary of results.

The undiluted test product (80.0 %) was able to inactivate human rotavirus after 15 seconds of exposure time in this quantitative suspension test (table 1). The reduction factor was $\geq 5.38 \pm 0.53$ at this time point. This corresponded to an inactivation of ≥ 99.999 %.

The test product as 50.0 % solution was not able to inactivate human rotavirus within 30 seconds of exposure time in this quantitative suspension test (table 2).

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The test product as 10.0 % solution was also not able to inactivate human rotavirus within 30 seconds of exposure time in this quantitative suspension test (table 3).

8. Conclusion

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

Therefore, the surface disinfectant Bacticide AF can be declared as active against human rotavirus as follows:

undiluted 15 seconds dirty conditions

Bremen, 20/11/2018


- Dr. Dajana Paulmann -
Scientific Project Manager


- Ilka Wappler -
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 Bacticide AF (80.0 %) tested against human rotavirus
Table 2:	Raw data for Bacticide AF (50.0 %) tested against human rotavirus
Table 3:	Raw data for Bacticide AF (10.0 %) tested against human rotavirus
Table 4:	Raw data for formaldehyde solution (0.7 %) tested against human rotavirus
Table 5:	Raw data for control of efficacy for suppression of disinfectant activity (80.0 %)
Table 6:	Raw data (human rotavirus) for cell sensitivity (80.0 %)
Table 7 (a+b):	Summary of results with Bacticide AF and human rotavirus

Legend to the Figures

Figure 1:	Virus-inactivating properties of Bacticide AF (80.0 %)
Figure 2:	Virus-inactivating properties of formaldehyde (0.7 %)

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Table 1: Raw data for Bactid AF (80.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5776)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log ₁₀)								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	dirty conditions	0.25	0004 0020	0020 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			0.5	0030 3000	0000 0000	0000 0000	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.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	80.0 %	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	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4434 2343	0000 2000	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4430 0344	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 2: Raw data for BactiCid AF (50.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5776)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log ₁₀)									
				1	2	3	4	5	6	7	8	9	
test product	50.0 %	dirty conditions	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			0.5	4444 4444	4444 4444	4444 4444	4323 4404	4000 3320	0030 0000	n.d.	n.d.		
			2	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 %	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	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4434 2343	0000 2000	0000 0000	0000 0000	
			60	4444 4444	4444 4444	4444 4444	4444 4444	4430 0344	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 3: Raw data for Bactid AF (10.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5776)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log ₁₀)									
				1	2	3	4	5	6	7	8	9	
test product	10.0 %	dirty conditions	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			0.5	4444	4444	4444	4444	4444	0330	0000	n.d.	n.d.	
			2	4444	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 %	dirty conditions	n.a.	0000	0000	0000	0000	0000	n.d.	n.d.	n.d.	n.d.	
virus control	n.a.	dirty conditions	0	4444	4444	4444	4444	4444	4434	0000	0000	0000	
			60	4444	4444	4444	4444	4444	4430	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 formaldehyde solution (0.7 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5776)

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	4444 4444	4444 4444	4433 3333	4032 0234	0000 0003	0000 0000	n.d. n.d.
			15	tttt tttt	tttt tttt	4444 4444	4444 4444	4420 0322	3000 0304	0000 0000	0000 0000	n.d. n.d.
			30	tttt tttt	tttt tttt	4444 4444	4303 3323	3302 2020	3002 0000	0000 0000	0000 0000	n.d. n.d.
			60	tttt tttt	tttt tttt	3222 3233	0000 0030	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.	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	4042 0303	0023 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 5: Raw data for control of efficacy for suppression of disinfectant's activity (80.0 %) (#5776)

Product	Interfering substance	dilutions (log ₁₀)								
		1	2	3	4	5	6	7	8	9
test product	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4324 4240	0000 0400	0000 0000	n.d.
corresponding virus control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4430 0344	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 6: Raw data (human rotavirus) for cell sensitivity (80.0 %) (#5776)

Product	Dilution	Dilutions (log ₁₀)								
		1	2	3	4	5	6	7	8	9
PBS	-	4444	4444	4444	4444	4444	4344	0000	0000	n.d.
		4444	4444	4444	4444	4444	4444	0000	0000	
test product	1:10	4444	4444	4444	4444	4444	0004	0000	0000	n.d.
		4444	4444	4444	4444	4444	0444	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 7a: Summary of results with Bacticide AF and human rotavirus

Product	Con- centration	Interfering substance	Level of cytotoxicity	log ₁₀ TCID ₅₀ /ml aftermin					> 4 log ₁₀ reduction after ... min
				0.25	0.5	2	30	60	
test product	80.0 %	dirty conditions	1.50	≤1.88±0.41	≤1.75±0.33	n.d.	n.d.	n.d.	0.25 (RF ≥ 5.38±0.53)
test product	50.0 %	dirty conditions	1.50	n.d.	7.00±0.52	n.d.	n.d.	n.d.	> 0.5 (RF = 0.25±0.61)
test product	10.0 %	dirty conditions	1.50	n.d.	7.25±0.33	n.d.	n.d.	n.d.	> 0.5 (RF = 0.00±0.46)

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



Table 7b: Summary of results with Bactioid AF and human rotavirus

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	3.50	n.d.	7.38±0.41	6.63±0.49	6.25±0.55	4.63±0.25	> 60 (RF = 2.75±0.55)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	7.38±0.49	n.a.
virus control	n.a.	dirty conditions	n.a.	7.63±0.25	n.d.	n.d.	n.d.	7.25±0.33	n.a.
suppression control	80.0 %	dirty conditions	1.50	n.d.	n.d.	n.d.	7.50±0.35	n.d.	n.a.
sens.control PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.50±0.00	n.a.
sens. control test product	80.0 % → 1:10	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.00±0.38	n.a.

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



Figure 1: Virus-inactivating properties of Bactacid AF (80.0 %)

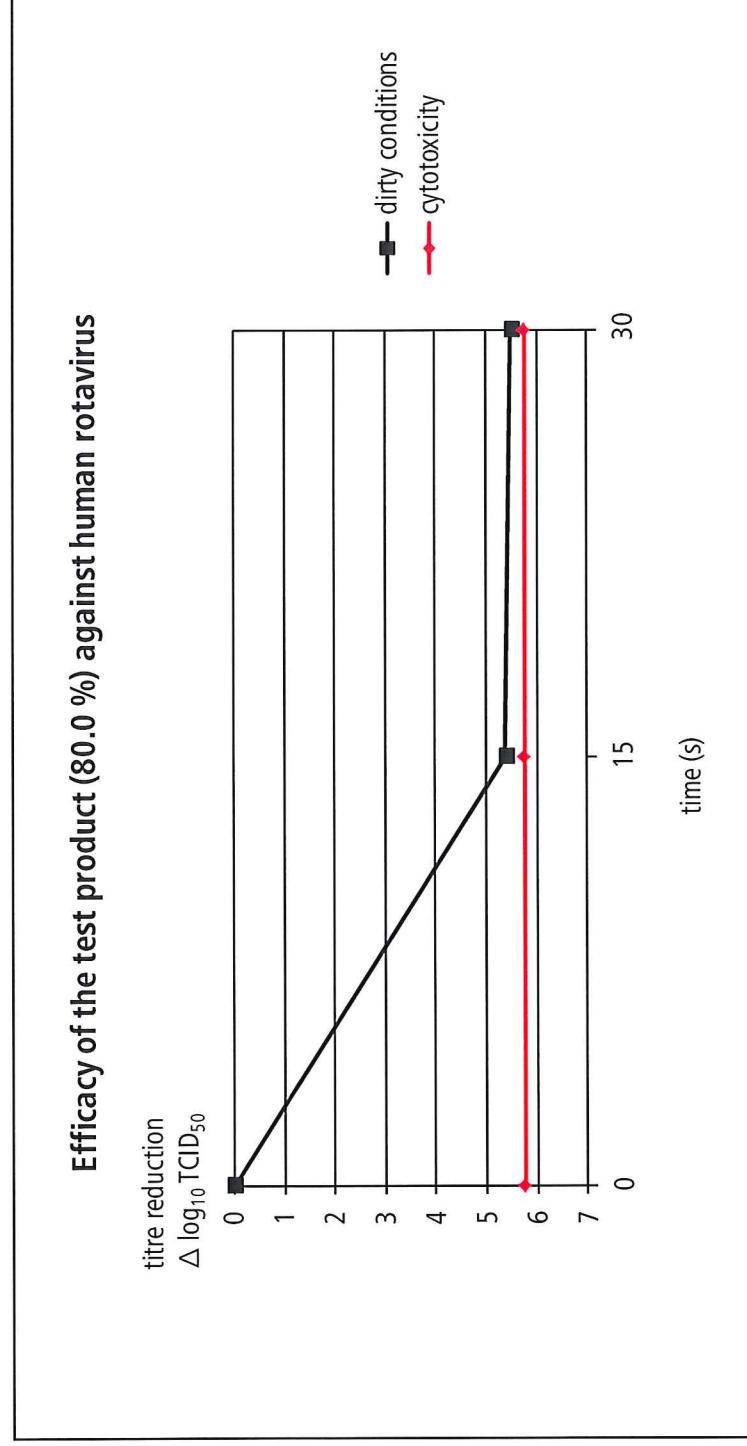




Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)

