

LABOR ENDERS

Prof. Dr. med. Gisela Enders & Kollegen • MVZ

Labor Prof. Gisela Enders MVZ GbR • Rosenbergstraße 85 • 70193 Stuttgart

Expert Opinion

on the efficacy of

Promanum® pure

against the

Murine Norovirus

Labor Prof. Gisela Enders MVZ GbR • Rosenbergstraße 85 • 70193 Stuttgart

B. Braun Medical AG
CoE Infection Control
Seesatz 17

CH-6204 Sempach

Ihre Nachricht

Ihre Zeichen

Unsere Zeichen

Datum

2016-11-16

The efficacy of **Promanum® pure** against the Murine norovirus strain S99 was tested in a suspension test according to the European standard DIN EN 14476:2013/FprA1:2015. The effectiveness of the disinfectant was evaluated under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes) as interfering substance.

Promanum® pure was tested as a 20.0%, 80.0% and 97.0% solution. The exposure times were 15 and 30 seconds.

In conclusion, the product Promanum® pure is effective against Murine norovirus strain S99 at room temperature under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes) as interfering substance with an application time of 15 seconds.


PD Dr. rer. nat. Maren Eggers

Labor Prof. Gisela Enders MVZ GbR • Rosenbergstraße 85 • 70193 Stuttgart

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Efficacy of Promanum® pure against Murine norovirus strain S99 in the virucidal quantitative suspension test for chemical disinfectants and antiseptics.

At the request of B. Braun Medical AG, **Promanum® pure** was tested for its efficacy against Murine norovirus strain S99 in a suspension test according to the European standard DIN EN 14476:2013/FprA1:2015. Under this standard, the product performance is tested against model viruses under defined test conditions, including temperature, contact time, or interfering substances, and the product should demonstrate at least a four log reduction in the titre of the test strain. **Promanum® pure** was examined as a 20.0%, 80.0% and 97.0% solution under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes). The contact times were 15 and 30 seconds.

Laboratory / test site

Labor Prof. Gisela Enders MVZ GbR
Rosenbergstr. 85
70193 Stuttgart
Germany

Identification of the sample

Name of the product	Promanum® pure
Batch number	14074M18
Manufacturer	B. Braun Medical AG
Appearance of the undiluted product	clear, colourless, liquid
Date of manufacture	2014-12-13
Expiry date	2019-01
Date of delivery	2016-10-13
Opened on	2016-11-10
Storage conditions	20°C, dark
Product undiluted pH value	pH 5.20
Active compounds in 100 g	ethanol 73.4% and 2-propanol (Ph. Eur.) 10.0%

Experimental conditions

Test period	2016-11-11 – 2016-11-15
Test temperature	20.0°C +/- 0.5°C
Product test concentrations, pH value	20.0% (pH 5.24), 80.0% (pH 5.22), 97.0% (pH 5.25)
Contact times	15 s and 30 s
Interfering substance	dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes)
Diluent used for product test solution	A. dest.
Appearance of product dilutions	20.0%: strongly turbid
Stability and appearance of the mixture during procedure	20.0%, 80.0% and 97.0%: strongly turbid
Temperature of incubation	37°C ± 1°C, CO ₂ incubator (5% CO ₂)
Virus	Murine norovirus strain S99
Virus: source	Dr. Schreier RKI (Berlin)
Virus: batch	060716
Virus: number of passage	n+5
Cell line	RAW 264.7 (murine macrophage cell line)
Cell line: source	ATCC (American Type Culture Collection)
Cell line: number of passage	35/17

Test strain virus and cell culture line

As test virus Murine norovirus strain S99 was used. RAW 264.7, a cell line established from murine macrophage cells, were used for virus cultivation and the suspension test. The host cells were cultivated at 37.0°C in a humid atmosphere under 5.0% CO₂. The cells were feed with Dulbeccos Minimum Essential Medium (D-MEM) supplemented with heat inactivated fetal calf serum (FCS) and with nonessential amino acids. For virus cultivation confluent monolayers with an age of max. 2 days were used.

The stock virus suspension was produced according to the directive. Cell debris was separated by low speed centrifugation at 2500 rpm for 10 minutes. Aliquots of the virus suspension were stored at -70.0 C.

Inactivation assay

The inactivation tests were run at 20.0°C +/- 1.0°C. The virus suspension was added to the product test solution under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes) as interfering substance. The test assays were mixed in the following way:

Inactivity test

1 part	(0.1 ml)	virus suspension
1 part	(0.1 ml)	3.0 g BSA/97 ml water + 3.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
8 parts	(0.8 ml)	undiluted (80.0% in the test mixture) 25.0% dilution of the test product (20.0% in the test mixture)

virus control

1 part	(0.1 ml)	virus suspension
1 part	(0.1 ml)	3.0 g BSA/97 ml water + 3.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
8 parts	(0.8 ml)	A. dest.

cytotoxicity test

1 part	(0.1 ml)	A. dest.
1 part	(0.1 ml)	3.0 g BSA/97 ml water + 3.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
8 parts	(0.8 ml)	undiluted (80.0% in the test mixture) 25.0% dilution of the test product (20.0% in the test mixture)

Inactivity test (97% in the test mixture)

1 part	(0.1 ml)	virus suspension
2 part	(0.2 ml)	15.0 g BSA/85 ml water + 15.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
97 parts	(9.7 ml)	undiluted test product

virus control

1 part	(0.1 ml)	virus suspension
2 part	(0.2 ml)	15.0 g BSA/85 ml water+ 15.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
97 parts	(9.7 ml)	A. dest

cytotoxicity test

1 part	(0.1 ml)	A. dest.
2 part	(0.2 ml)	15.0 g BSA/85 ml water + 15.0 ml erythrocytes (3.0 g/l BSA + 3.0 ml/l erythrocytes in the test mixture; dirty conditions)
97 parts	(9.7 ml)	undiluted test product

The test product **Promanum® pure** was examined as a 20.0%, 80.0% and 97.0% solution. After the specified contact time, the virucidal activity was immediately suppressed by dilution with nine volumes of ice-cold medium (D-MEM + 2.0% FCS) and without delay the assay was serially diluted 10-fold. Due to the immediate titration, no after-effect of the product could occur. Six wells of a microtitre plate containing a confluent monolayer of RAW cells were inoculated with 0.1 ml of each dilution, and the cells were incubated at 37.0°C in a humidified atmosphere under 5.0% CO₂. After 4 days the cell cultures were stained with 50 µl crystal violet per well. The cells were examined microscopically for cytopathic effects (CPE). The cell culture results were recorded as “0” for no CPE and “1” (25.0% CPE) to “4” (100% CPE) depending on degree of the cell damage. The viral titre was calculated using the Spearman-Kärber-method (Br. J. Psychol. 2 (1908): 227-42, Arch. exp. Path. Pharmac. 162 (1931): 480-87).

Calculation of the virucidal activity of the products

The virucidal activity was determined by the difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus ($\Delta \log_{10} \text{TCID}_{50}/\text{ml}$).

Cytotoxic effect

To check for possible morphological alterations of RAW cells caused by the test product **Promanum® pure**, eight parts of the product test solution were diluted with one part of the interfering substance and one part of A. dest. In the same way as for determining virus infectivity, serial dilutions (1:10) were prepared in culture medium and 100 µl were inoculated onto confluent RAW cell monolayers in a 96 well plates.

Comparative virus titration on cells pre-treated with the test mixture

The comparative virus titration was performed on cells that had been treated with disinfectants to check the reduction in the sensitivity to the virus as follows: Cells were incubated for 1 hour with 100 µl of a 10^{-3} dilution of the 97.0% solution of **Promanum® pure**. Based on the results of the cytotoxicity test, only the lowest apparently non-cytotoxic dilution of the test mixture could be used. After 1 h at 37.0°C the test solution was removed, and the cells were infected with the titrated control virus. The assay is only valid if the virus control of mock-treated cells (no disinfectant) minus the virus control of cells pre-treated with **Promanum® pure** resulted in less than one log difference.

Control of efficiency of suppression of product's activity

100 µl of the undiluted product **Promanum® pure** and 850 µl of ice-cold medium with 2% FCS were mixed. Afterwards 50 µl of the virus inoculum were added and the mixture is incubated in an ice-bath for 30 min \pm 10 s. A serial dilution in \log_{10} steps was prepared and cell cultures were inoculated with the dilutions. The titre was determined. The difference between the titre of the suppression of virucidal activity (SVA) control and the titre of virus control should not exceed 0.50 \log_{10} .

Reference virus inactivation assay

As a validity control of the test system, formaldehyde was selected for inactivation of the reference virus. Contact times were 5 min, 15 min, 30 min and 60 min. The test assays were mixed in the following way:

1 part	(0.2 ml)	virus suspension
4 parts	(0.8 ml)	PBS
5 parts	(1.0 ml)	1.4% formaldehyde solution

To control the cytotoxicity of the formaldehyde test solution 1.0 ml 1.4% formaldehyde is added to 0.8 ml of PBS. The infectivity of the virus control was determined at 0 min and 60 min. The formaldehyde solution was substituted by water. Immediately following the contact time, 0.2 ml of the test mixture was pipetted into a tube containing 1.8 ml ice-cold D-MEM + 2.0% FCS, which was then serially diluted ten-fold.

Results

The product **Promanum® pure** was tested as a 20.0%, 80.0% and 97.0% solution under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes) as interfering substance and following exposure times of 15 and 30 seconds, the reduction of viral titre was measured.

Validity of the test

The test product dilutions of 1:10 and 1:100 caused cytotoxic effects (Table 1). As shown in Table 2, the comparative virus titration on cells treated with test mixture dilution or without resulted in a difference of less than one log. The after-effect control, which measures the efficiency of suppression of product's activity, shall be ≤ 0.50 lg. As shown in Table 3, the control was 0.33 lg.

The results of the reference virus inactivation using formaldehyde are given in Table 4. The 0.7% formaldehyde solution was toxic for RAW cells at the 1:1000 dilution. The difference of the logarithmic titre of the virus control minus the test virus was 3.33 ± 0.56 logs after 60 min.

Test results

The data of the virucidal efficacy of the three dilutions of **Promanum® pure** is presented in Table 5. **Promanum® pure** was tested as a 20.0%, 80.0% and 97.0% solution under dirty conditions (3.0 g/l BSA + 3.0 ml/l erythrocytes), and following an exposure time of 15 and 30 seconds. The 20.0% concentration of **Promanum® pure** showed no virucidal activity under dirty conditions. The 80.0% and 97.0% concentration of **Promanum® pure** was virucidal active after a 15 seconds contact time under dirty conditions.

Table 1: Cytotoxic factor of Promanum® pure

Concentration	Interfering substance	Dilution (log ₁₀)						
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
20.0%	dirty conditions	–	–	–	–	–	–	–
80.0%	dirty conditions	+	–	–	–	–	–	–
97.0%	dirty conditions	+	+	–	–	–	–	–

Table 2: Comparative virus titration on cells treated with the test mixture dilution

Concentration	Interfering substance	Titre of the virus control (log ₁₀ TCID ₅₀ /ml) PBS	Titre of the comparative virus titration (log ₁₀ TCID ₅₀ /ml)	Difference in virus titre (log ₁₀ TCID ₅₀ /ml)
97.0%	dirty conditions	8.50 +/- 0.47	8.50 +/- 0.60	0.00

Table 3: Control of efficiency of suppression of product's activity

Concentration	Titre of the virus control (log ₁₀ TCID ₅₀ /ml)	Titre of the after effect control titration (log ₁₀ TCID ₅₀ /ml)	Difference in virus titre (log ₁₀ TCID ₅₀ /ml)
97.0%	8.33 +/- 0.33	8.00 +/- 0.45	0.33

Table 4: Reference virus inactivation of Murine norovirus strain S99 with 0.7% formaldehyde

Cyto-toxicity	Titre of the virus control (0 min) (log ₁₀ TCID ₅₀ /ml) with 95% confidence interval	Titre of the virus control (60 min) (log ₁₀ TCID ₅₀ /ml) with 95% confidence interval	Titre of the “residual virus” inactivation (log ₁₀ TCID ₅₀ /ml) with 95% confidence interval				Reduction factor with 95% confidence interval			
			5 min	15 min	30 min	60 min	5 min	15 min	30 min	60 min
4.50 +/- 0.00	8.17 +/- 0.42	8.00 +/- 0.45	7.67 +/- 0.33	7.17 +/- 0.42	6.00 +/- 0.45	4.67 +/- 0.33	0.33 +/- 0.56	0.83 +/- 0.61	2.00 +/- 0.63	3.33 +/- 0.56

Table 5: Virucidal activity of Promanum® pure against Murine norovirus strain S99 (dirty conditions)

Concentration	Interfering substance	Titre of the virus control (log ₁₀ TCID ₅₀ /ml) with 95% confidence interval	Level of cyto-toxicity	Titre of the “residual virus” inactivation (log ₁₀ TCID ₅₀ /ml) with 95% confidence interval		Reduction factor with 95% confidence interval	
				15 s	30 s	15 s	30 s
20.0%	dirty conditions	8.00 +/- 0.45	1.50	8.50 +/- 0.47	8.17 +/- 0.42	-0.50 +/- 0.65	-0.17 +/- 0.61
80.0%	dirty conditions	8.00 +/- 0.45	2.50	3.83 +/- 0.56	≤ 2.50 +/- 0.00	4.17 +/- 0.71	≥ 5.50 +/- 0.45
97.0%	dirty conditions	8.00 +/- 0.45	3.50	≤ 3.50 +/- 0.00	≤ 3.50 +/- 0.00	≥ 4.50 +/- 0.45	≥ 4.50 +/- 0.45

Conclusion

The product **Promanum® pure** efficiently inactivates Murine norovirus strain S99 at room temperature under dirty conditions within 15 seconds exposure time.

The following concentration and exposure times are recommended for Murine norovirus strain S99:

dirty conditions

undiluted

15 seconds


PD Dr. rer. nat. Maren Eggers

Archiving: *The raw data with respect to this test and a copy of the report will be stored in the archive of Labor Enders MVZ.*

Information: *The test results exclusively refer to the samples described above. Account of extracts of this test report is only possible by written approval from Labor Enders MVZ.*

Raw data for the test product Promanum® pure tested against Murine norovirus strain S99 under dirty conditions (quantal test; 6 wells)

2016-11-11 – 2016-11-15

Product	Con- centra- tion	Interfering substance	Contact time	Dilution (log ₁₀)							
				1	2	3	4	5	6	7	8
Promanum® pure	20.0%	dirty conditions	15 s	444	444	444	444	444	444	440	400
				444	444	444	444	444	444	444	000
			30 s	444	444	444	444	444	444	444	000
				444	444	444	444	444	444	004	000
Virus control			30 s	444	444	444	444	444	444	444	000
				444	444	444	444	444	444	000	000
Cytotoxicity				000	000	000	000	000	000	000	000
				000	000	000	000	000	000	000	000
Promanum® pure	80.0%	dirty conditions	15 s	xxx	044	004	000	000	000	000	000
				xxx	444	404	000	000	000	000	000
			30 s	xxx	000	000	000	000	000	000	000
				xxx	000	000	000	000	000	000	000
Virus control			30 s	444	444	444	444	444	444	444	000
				444	444	444	444	444	444	000	000
Cytotoxicity				xxx	000	000	000	000	000	000	000
				xxx	000	000	000	000	000	000	000

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

x cytotoxic

Raw data for the test product Promanum® pure tested against Murine norovirus strain S99 under dirty conditions (quantal test; 6 wells)

2016-11-11 – 2016-11-15

Product	Con- centra- tion	Interfering substance	Contact time	Dilution (log ₁₀)							
				2	3	4	5	6	7	8	9
Promanum® pure	97.0%	dirty conditions	15 s	xxx	000	000	000	000	000	000	000
				xxx	000	000	000	000	000	000	000
			30 s	xxx	000	000	000	000	000	000	000
				xxx	000	000	000	000	000	000	000
Virus control			30 s	444	444	444	444	444	404	000	000
				444	444	444	444	444	400	000	000
Cytotoxicity				xxx	000	000	000	000	000	000	000
				xxx	000	000	000	000	000	000	000
Cell sensitivity to virus after preincubation with the test product				444	444	444	444	444	444	404	000
				444	444	444	444	444	004	000	000
Cell sensitivity to virus after preincubation with PBS				444	444	444	444	444	440	400	000
				444	444	444	444	444	444	000	000
After-effect control without product			30 min	444	444	444	444	444	444	000	000
				444	444	444	444	444	440	000	000
After-effect control with product			30 min	xxx	xxx	444	444	444	440	000	000
				xxx	xxx	444	444	444	400	000	000

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

x cytotoxic

**Raw data for 0.7% formaldehyde tested against Murine norovirus strain S99
(quantal test; 6 wells)**

2016-11-11 – 2016-11-15

Product	Con- centra- tion	Interfering substance	Con- tact time	Dilution (log ₁₀)								
				1	2	3	4	5	6	7	8	9
Formaldehyde	0.7%	PBS	5 min	xxx xxx	xxx xxx	xxx xxx	444 444	444 444	444 444	000 004	000 000	000 000
			15 min	xxx xxx	xxx xxx	xxx xxx	444 444	444 444	044 440	000 000	000 000	000 000
			30 min	xxx xxx	xxx xxx	xxx xxx	444 444	004 044	000 000	000 000	000 000	000 000
			60 min	xxx xxx	xxx xxx	xxx xxx	000 400	000 000	000 000	000 000	000 000	000 000
Virus control			0 min	444 444	444 444	444 444	444 444	444 444	444 444	044 044	000 000	000 000
Virus control			60 min	444 444	444 444	444 444	444 444	444 444	444 444	400 440	000 000	000 000
Cytotoxicity				xxx xxx	xxx xxx	xxx xxx	000 000	000 000	000 000	000 000	000 000	000 000

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)
0 no virus present
x cytotoxic