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

TEST REPORT

19/12/2018

Test report L18/0650eMV.2

Evaluation of the effectiveness of

Sterisept Wipes

Test virus: modified vaccinia virus Ankara (MVA)

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

Digitally signed by Moraru Grigore
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Sponsor:
Chemi-Pharm AS
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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	Sterisept Wipes
Confirmation no.	207258
Product diluent recommended by the manufacturer	-
Batch number	14300818
Application	surface disinfection
Production date	30/08/2018
Expiry date	30/08/2021
Active compound (s) (100 g)	- 0.45 % didecyl-dimethyl-ammonium chloride (DDAC) (CAS nr: 7173-51-5) - 0.45 % N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine
Appearance, odour	clear, colorless, slightly viscous liquid product specific
pH-values	undiluted: 10.83 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	07/09/2018

3. Materials

3.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Hank`s BSS (MEM, Biozym Scientific GmbH, catalogue no. 880144)
- 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).

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 three times in *BHK 21-cells* (Baby Hamster Kidney).

BHK 21-cells (passage 105) originated from the Friedrich-Löffler-Institut, Bundesforschungsinstitut für Tiergesundheit (formerly Bundesforschungsanstalt für Viruskrankheiten der Tiere, isle of Riems).

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, 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	70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstration of non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	30 seconds and 30 minutes
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 (70.0 % solution)	minor clouding, strong precipitation
Virus strain	modified vaccinia virus Ankara (MVA) (ATCC VR-1508)
Date of testing	05/10/2018 – 19/12/2018
End of testing	19/12/2018

5. Methods

5.1 Preparation of test virus suspension

For preparation of test virus suspension, *BHK 21-cells* were cultivated with MEM and 10 % or 2 % fetal calf serum. *Cells* were infected with a multiplicity of infection of 0.1. 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, test virus suspension was stored at – 80 °C.

5.2 Preparation of disinfectant (dilutions)

The test product was tested as 70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstrating of non-active range) solutions. 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 according to EN 5.5 transferring 0.1 ml of each dilution into eight wells of a microtitre plate to 0.1 ml of freshly trypsinised *BHK 21-cells* (10-15 x 10^3 cells per well), 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 six 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 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 5.5. The test product was examined as 70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstration of non-active range) solutions in Aqua bidest. at 20 °C based on EN 14476. 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 Inactivation assay following the large volume plating method (LVP)

Following the large volume plating method (4) the inactivation assays were further diluted 1:2,500 and 1:2,000, respectively 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 (5, 6). This method is necessary for those products which demonstrate a great cytotoxicity.

Testing the 70.0 % solution, 25 µl of the inactivation assays were added to 62.5 ml medium (total dilution of 1:2,500) and then the total volume was distributed in 12 microtitre plates (54 µl / well, 1152 wells total). Testing the 50.0 % solution, 31.25 µl of the inactivation assays were added to 62.5 ml medium (total dilution of 1:2,000) and then the total volume was distributed in 6 microtitre plates (108 µl / well, 576 wells total). After 6 days of inoculation cultures were observed for cytopathic effects.

The calculation of virus titre without residual virus followed the formula of Poisson:

$$c = \ln p / -V$$

c = number of virus particles

p = the probability to find no virus. The probability to find no virus should not greater than 5 % (p=0.05). By doing so, the number of virus particles can be calculated with a probability of 95 %.

V = test volume (ml)

The titre to be used for calculating the reduction factor (RF) was finally calculated as followed: the determined number of virus particle is first converted with the aid of the dilution factor in the number of particle per ml. Subsequently, the numbers of particles per ml have to be converted in the tissue culture infectious dose per ml ($TCID_{50}$/ml) (1.0 $TCID_{50}$ corresponds to 0.69 infectious virus particles). The common logarithm of this value results in the virus titre (log_{10} $TCID_{50}$/ml) used for calculating the reduction factor (RF).

In assays with residual virus, formula according to Taylor was used for calculating the virus titre:

$$c/ml = \frac{D}{Vw} \times \left(-\ln \frac{n - n_p}{n}\right)$$

c = number of virus particles

D = dilution

V_W = volume per well

n = number of inoculated wells

n_p = number of virus-positive wells

For calculating the reduction factor using the formula according to Taylor the number of virus particles is converted to the logarithmic titre ($log_{10}TCID_{50}$/ml) as described above.

5.7 Determination of cytotoxicity

Determination of cytotoxicity was performed according to EN 5.5.4.1.

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. These mixtures or PBS as control were 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.

5.9 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).

5.10 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}.

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 ≥ 4.36 ± 0.29, LVP)

b) The test product (70.0 %) showed cytotoxicity in the 1:1,000 dilutions thus allowing the detection of a 4 $\log_{10}$ reduction of virus titre (LVP assay).

c) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *BHK 21-cells* showed no significant difference (< 1 $\log_{10}$; EN 5.7) of virus titre: 6.38 ± 0.25 (PBS, LVP) versus 6.75 ± 0.33 (1:2,500 dilutions of disinfectant as 70.0 % solution, LVP) and 6.50 ± 0.00 (1:2,000 dilutions of disinfectant as 50.0 % solution, LVP) $\log_{10}$ $TCID_{50}$/ml, respectively.

d) The control of efficacy for suppression of disinfectant's activity (70.0 %) showed a decrease of ≥ 2.00 (≤ 4.50 ± 0.00 versus 6.50 ± 0.46 $\log_{10}$ $TCID_{50}$/ml) and failed the requirement of the EN (≤ 0.5 $\log_{10}$; EN 5.5.5.1). In these experiments at the end of the defined exposure time the test mixture was immediately diluted not 1:10 as described in the control of efficacy for suppression of disinfectant's activity but directly 1:2,500 (LVP) and the dilution transferred to the cell culture. For this reason this control is not relevant when using the LVP. Therefore, despite the insufficient control of efficacy for suppression of disinfectant's activity the assay is valid.

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 MVA based on EN 14476 is valid.

7. Results

Results of examination are shown in tables 1 to 10. Tables 1 to 8 demonstrate the raw data, whereas tables 9 (a+b) and 10 give a summary of results.

Since it was not possible to show a reduction in virus titre of 4 $\log_{10}$-steps testing the undiluted test product due to cytotoxicity, a 70.0 % and 50.0 % solution, respectively of the activated product was introduced for the inactivation tests. This solution was tested using the large volume plating method. The further dilutions were examined using the end point dilution method.

The 10.0 % and 1.0 % solutions were not active within 30 minutes of exposure time (tables 1 and 2).

In parallel to the end point dilution method the large volume plating method (LVP) was introduced testing the test product as 70.0 % and 50.0 % solution with 30 seconds of exposure time. The mean virus titre was $\log_{10}$ $TCID_{50}$/ml = 6.50 ± 0.29 (table 6).

The test product as 70.0 % solution was active after 30 seconds of exposure time (table 7). Since no residual virus was found in 1152 cell culture units, the result according to the formula of Poisson was ≤ 2.24 $\log_{10}$ $TCID_{50}$. The reduction factor was therefore ≥ 4.26 ± 0.29 (6.50 ± 0.29 $\log_{10}$ $TCID_{50}$ minus ≤ 2.24 $\log_{10}$ $TCID_{50}$) after 30 seconds of exposure time. This corresponded to an inactivation of ≥ 99.99 %.

The test product as 50.0 % solution was also active after 30 seconds of exposure time (table 8). Since no residual virus was found in 576 cell culture units, the result according to the formula of Poisson was ≤ 2.14 $\log_{10}$ $TCID_{50}$. The reduction factor was therefore ≥ 4.36 ± 0.29 (6.50 ± 0.29 $\log_{10}$ $TCID_{50}$ minus ≤ 2.14 $\log_{10}$ $TCID_{50}$) after 30 seconds of exposure time. This corresponded to an inactivation of ≥ 99.99 %.

8. Conclusion

The surface disinfectant Sterisept Wipes tested as 70.0 % solution demonstrated activity against MVA after an exposure time of 30 seconds under dirty conditions.

Therefore, the surface disinfectant Sterisept Wipes can be declared as active against MVA as follows:

70.0 % 30 seconds dirty conditions

Bremen, 19/12/2018

i.V.

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

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

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

4. Rabenau HF., Schwebke I., Blümel J., Eggers M., Glebe D., Rapp I., Sauerbrei A., Steinmann E., Steinmann, J., Willkommen H. Wutzler P.: Leitlinie der Deutschen Vereinigung zur Bekämpfung der Viruskrankheiten (DVV) e.V. und des Robert Koch-Instituts (RKI) zur Prüfung von chemischen Desinfektionsmitteln auf Wirksamkeit gegen Viren in der Humanmedizin (Fassung vom 1. Dezember 2014). Bundesgesundheitsbl; 58, 2015, 493–504

5. Bekanntmachung über die Zulassung von Arzneimitteln, Anforderungen an Validierungsstudien zum Nachweis der Virussicherheit von Arzneimitteln aus menschlichem Blut oder Plasma vom 20. Dezember 1993/21. Januar 1994. Bundesanzeiger Nr. 84: 4740-4744 bzw. CPMP/BWP/268/95: Note for Guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses. http://www.ema.europa.eu

6. Taylor JR.: An Introduction to Error Analysis: The study of Uncertainties in Physical Measurements. 2nd ed. University Science Books, 1997, 327 pp

Appendix:

Legend to the Tables

Legend to the Figures

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ZLG-AP-216.11.02

TEST REPORT

19/12/2018

Test report L18/0650eMV.2

Evaluation of the effectiveness of

Sterisept Wipes

Test virus: modified vaccinia virus Ankara (MVA)

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
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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	Sterisept Wipes
Confirmation no.	207258
Product diluent recommended by the manufacturer	-
Batch number	14300818
Application	surface disinfection
Production date	30/08/2018
Expiry date	30/08/2021
Active compound (s) (100 g)	- 0.45 % didecyl-dimethyl-ammonium chloride (DDAC) (CAS nr: 7173-51-5) - 0.45 % N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine
Appearance, odour	clear, colorless, slightly viscous liquid product specific
pH-values	undiluted: 10.83 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	07/09/2018

3. Materials

3.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Hank`s BSS (MEM, Biozym Scientific GmbH, catalogue no. 880144)
- 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).

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 three times in *BHK 21-cells* (Baby Hamster Kidney).

BHK 21-cells (passage 105) originated from the Friedrich-Löffler-Institut, Bundesforschungsinstitut für Tiergesundheit (formerly Bundesforschungsanstalt für Viruskrankheiten der Tiere, isle of Riems).

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, 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	70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstration of non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	30 seconds and 30 minutes
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 (70.0 % solution)	minor clouding, strong precipitation
Virus strain	modified vaccinia virus Ankara (MVA) (ATCC VR-1508)
Date of testing	05/10/2018 – 19/12/2018
End of testing	19/12/2018

5. Methods

5.1 Preparation of test virus suspension

For preparation of test virus suspension, *BHK 21-cells* were cultivated with MEM and 10 % or 2 % fetal calf serum. *Cells* were infected with a multiplicity of infection of 0.1. 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, test virus suspension was stored at – 80 °C.

5.2 Preparation of disinfectant (dilutions)

The test product was tested as 70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstrating of non-active range) solutions. 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 according to EN 5.5 transferring 0.1 ml of each dilution into eight wells of a microtitre plate to 0.1 ml of freshly trypsinised *BHK 21-cells* (10-15 x 10^3 cells per well), 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 six 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 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 5.5. The test product was examined as 70.0 %, 50.0 %, 10.0 % and 1.0 % (demonstration of non-active range) solutions in Aqua bidest. at 20 °C based on EN 14476. 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 Inactivation assay following the large volume plating method (LVP)

Following the large volume plating method (4) the inactivation assays were further diluted 1:2,500 and 1:2,000, respectively 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 (5, 6). This method is necessary for those products which demonstrate a great cytotoxicity.

Testing the 70.0 % solution, 25 µl of the inactivation assays were added to 62.5 ml medium (total dilution of 1:2,500) and then the total volume was distributed in 12 microtitre plates (54 µl / well, 1152 wells total). Testing the 50.0 % solution, 31.25 µl of the inactivation assays were added to 62.5 ml medium (total dilution of 1:2,000) and then the total volume was distributed in 6 microtitre plates (108 µl / well, 576 wells total). After 6 days of inoculation cultures were observed for cytopathic effects.

The calculation of virus titre without residual virus followed the formula of Poisson:

$$c = \ln p / -V$$

c = number of virus particles

p = the probability to find no virus. The probability to find no virus should not greater than 5 % (p=0.05). By doing so, the number of virus particles can be calculated with a probability of 95 %.

V = test volume (ml)

The titre to be used for calculating the reduction factor (RF) was finally calculated as followed: the determined number of virus particle is first converted with the aid of the dilution factor in the number of particle per ml. Subsequently, the numbers of particles per ml have to be converted in the tissue culture infectious dose per ml ($TCID_{50}$/ml) (1.0 $TCID_{50}$ corresponds to 0.69 infectious virus particles). The common logarithm of this value results in the virus titre (log_{10} $TCID_{50}$/ml) used for calculating the reduction factor (RF).

In assays with residual virus, formula according to Taylor was used for calculating the virus titre:

$$c/ml = \frac{D}{Vw} \times \left(-\ln \frac{n - n_p}{n}\right)$$

c = number of virus particles

D = dilution

V_W = volume per well

n = number of inoculated wells

n_p = number of virus-positive wells

For calculating the reduction factor using the formula according to Taylor the number of virus particles is converted to the logarithmic titre ($log_{10}TCID_{50}$/ml) as described above.

5.7 Determination of cytotoxicity

Determination of cytotoxicity was performed according to EN 5.5.4.1.

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. These mixtures or PBS as control were 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.

5.9 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).

5.10 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}.

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 $\geq 4.36 \pm 0.29$, LVP)

b) The test product (70.0 %) showed cytotoxicity in the 1:1,000 dilutions thus allowing the detection of a 4 $\log_{10}$ reduction of virus titre (LVP assay).

c) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *BHK 21-cells* showed no significant difference (< 1 $\log_{10}$; EN 5.7) of virus titre: 6.38 ± 0.25 (PBS, LVP) versus 6.75 ± 0.33 (1:2,500 dilutions of disinfectant as 70.0 % solution, LVP) and 6.50 ± 0.00 (1:2,000 dilutions of disinfectant as 50.0 % solution, LVP) $\log_{10}$ $TCID_{50}$/ml, respectively.

d) The control of efficacy for suppression of disinfectant's activity (70.0 %) showed a decrease of ≥ 2.00 ($\leq 4.50 \pm 0.00$ versus 6.50 ± 0.46 $\log_{10}$ $TCID_{50}$/ml) and failed the requirement of the EN (≤ 0.5 $\log_{10}$; EN 5.5.5.1). In these experiments at the end of the defined exposure time the test mixture was immediately diluted not 1:10 as described in the control of efficacy for suppression of disinfectant's activity but directly 1:2,500 (LVP) and the dilution transferred to the cell culture. For this reason this control is not relevant when using the LVP. Therefore, despite the insufficient control of efficacy for suppression of disinfectant's activity the assay is valid.

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 MVA based on EN 14476 is valid.

7. Results

Results of examination are shown in tables 1 to 10. Tables 1 to 8 demonstrate the raw data, whereas tables 9 (a+b) and 10 give a summary of results.

Since it was not possible to show a reduction in virus titre of 4 $\log_{10}$-steps testing the undiluted test product due to cytotoxicity, a 70.0 % and 50.0 % solution, respectively of the activated product was introduced for the inactivation tests. This solution was tested using the large volume plating method. The further dilutions were examined using the end point dilution method.

The 10.0 % and 1.0 % solutions were not active within 30 minutes of exposure time (tables 1 and 2).

In parallel to the end point dilution method the large volume plating method (LVP) was introduced testing the test product as 70.0 % and 50.0 % solution with 30 seconds of exposure time. The mean virus titre was $\log_{10}$ $TCID_{50}$/ml = 6.50 ± 0.29 (table 6).

The test product as 70.0 % solution was active after 30 seconds of exposure time (table 7). Since no residual virus was found in 1152 cell culture units, the result according to the formula of Poisson was ≤ 2.24 $\log_{10}$ $TCID_{50}$. The reduction factor was therefore ≥ 4.26 ± 0.29 (6.50 ± 0.29 $\log_{10}$ $TCID_{50}$ minus ≤ 2.24 $\log_{10}$ $TCID_{50}$) after 30 seconds of exposure time. This corresponded to an inactivation of ≥ 99.99 %.

The test product as 50.0 % solution was also active after 30 seconds of exposure time (table 8). Since no residual virus was found in 576 cell culture units, the result according to the formula of Poisson was ≤ 2.14 $\log_{10}$ $TCID_{50}$. The reduction factor was therefore ≥ 4.36 ± 0.29 (6.50 ± 0.29 $\log_{10}$ $TCID_{50}$ minus ≤ 2.14 $\log_{10}$ $TCID_{50}$) after 30 seconds of exposure time. This corresponded to an inactivation of ≥ 99.99 %.

8. Conclusion

The surface disinfectant Sterisept Wipes tested as 70.0 % solution demonstrated activity against MVA after an exposure time of 30 seconds under dirty conditions.

Therefore, the surface disinfectant Sterisept Wipes can be declared as active against MVA as follows:

70.0 % 30 seconds dirty conditions

Bremen, 19/12/2018

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

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

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5. Bekanntmachung über die Zulassung von Arzneimitteln, Anforderungen an Validierungsstudien zum Nachweis der Virussicherheit von Arzneimitteln aus menschlichem Blut oder Plasma vom 20. Dezember 1993/21. Januar 1994. Bundesanzeiger Nr. 84: 4740-4744 bzw. CPMP/BWP/268/95: Note for Guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses. http://www.ema.europa.eu

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Appendix:

Legend to the Tables

Legend to the Figures

Table 1: Raw data for Sterisept Wipes (10.0 %) tested against MVA at 20 °C (quantal test; 8 wells) (#5829)

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	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			10	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	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
test product cytotoxicity	10.0 %	dirty 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.	dirty conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	2322 0433	0000 0002	0000 0000	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	3203 2033	0003 0020	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 Sterisept Wipes (1.0 %) tested against MVA at 20 °C (quantal test; 8 wells) (#5829)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	1.0 %	dirty conditions	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			10	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	tttt tttt	4444 4444	4444 4444	4444 4444	2044 4040	0032 0000	0000 0000	n.d.	n.d.
test product cytotoxicity	1.0 %	dirty 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.	dirty conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	2322 0433	0000 0002	0000 0000	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	3203 2033	0003 0020	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 formaldehyde solution (0.7 %) tested against MVA at 20 °C (quantal test; 8 wells) (#5829)

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	0120 2000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			15	tttt tttt	tttt tttt	tttt tttt	0000 0001	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			30	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			60	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	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	4333 2413	0000 0000	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 4: Raw data for control of efficacy for suppression of disinfectant's activity (70.0 %) (#5829)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	dirty conditions	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
corresponding virus control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	3203 2033	0003 0020	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 5: Raw data (MVA) for cell sensitivity (70.0 % and 50.0 % solution) (#5829) (LVP)

Product	Dilution	Dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
PBS	-	4444 4444	4444 4444	4444 4444	4444 4444	2332 2220	0000 0000	0000 0000	0000 0000	n.d.
test product 70.0 %	1:2,500	4444 4444	4444 4444	4444 4444	4444 4444	3344 3414	0000 2003	0000 0000	0000 0000	n.d.
test product 50.0 %	1:2,000	4444 4444	4444 4444	4444 4444	4444 4444	3433 3344	0000 0000	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 (#5829)

Virus titration	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
1st control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	3203 2033	0003 0020	0000 0000	0000 0000	n.d.
2nd control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	3303 4234	0000 0100	0000 0000	0000 0000	n.d.

n.a. = not applicable t = cytotoxic 0 = no virus detectable
n.d. = not done 1 to 4 = virus detectable (degree of CPE in 8 wells of a microtitre plate)

Table 7: Inactivation of MVA by Sterisept Wipes (70.0 %) at 20 °C (30 seconds) (LVP, 1:2,500) (#5829)

Interfering substance	Row	1	2	3	4	5	6	7	8	9	10	11	12
dirty conditions	plate 1/12	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 2/12	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 3/12	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 4/12	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 5/12	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 6/12	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 7/12	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 8/12	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 9/12	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 10/12	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 11/12	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 12/12	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 8: Inactivation of MVA by Sterisept Wipes (50.0 %) at 20 °C (30 seconds) (LVP, 1:2,000) (#5829)

Interfering substance	Row	1	2	3	4	5	6	7	8	9	10	11	12
dirty 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 9a: Summary of results (end point dilution method) with Sterisept Wipes and MVA

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after …min
				1	5	10	30	60	
test product	10.0 %	dirty conditions	3.50	n.d.	n.d.	n.d.	≤ 3.50±0.00	n.d.	≥ 30 (RF ≥ 3.00±0.33)
test product	1.0 %	dirty conditions	2.50	n.d.	n.d.	n.d.	6.38±0.49	n.d.	> 30 (RF = 0.13±0.67)

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

Table 9b: Summary of results (end point dilution method) with Sterisept Wipes 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±0.37	≤ 4.63±0.25	≤ 4.50±0.00	≤ 4.50±0.00	≥ 30 (RF ≥ 2.00±0.00)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	6.50±0.00	n.a.
virus control	n.a.	dirty conditions	n.a.	6.50±0.35	n.d.	n.d.	n.d.	6.50±0.46	n.a.
suppression control	70.0 %	dirty conditions	4.50	n.d.	n.d.	n.d.	≤ 4.50±0.00	n.d.	n.a.

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

Table 10: Summary of results (LVP) with Sterisept Wipes 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	5	10	30	60	
test product	70.0 % (1:2,500)	dirty conditions	n.a.	≤ 2.24	n.d.	n.d.	n.d.	n.d.	0.5 (RF ≥ 4.26±0.29)
test product	50.0 % (1:2,000)	dirty conditions	n.a.	≤ 2.14	n.d.	n.d.	n.d.	n.d.	0.5 (RF ≥ 4.36±0.29)
virus control	n.a.	dirty conditions	n.a.	n.d.	n.d.	n.d.	n.d.	6.50±0.46 6.50±0.35 (Ø6.50±0.29)	n.a.
sens. PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.38±0.25	n.a.
sens. product	70.0 % → 1:2,500	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.75±0.33	n.a.
sens. product	50.0 % → 1:2,000	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.50±0.00	n.a.

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

Figure 1: Virus-inactivating properties of Sterisept Wipes (70.0 %)

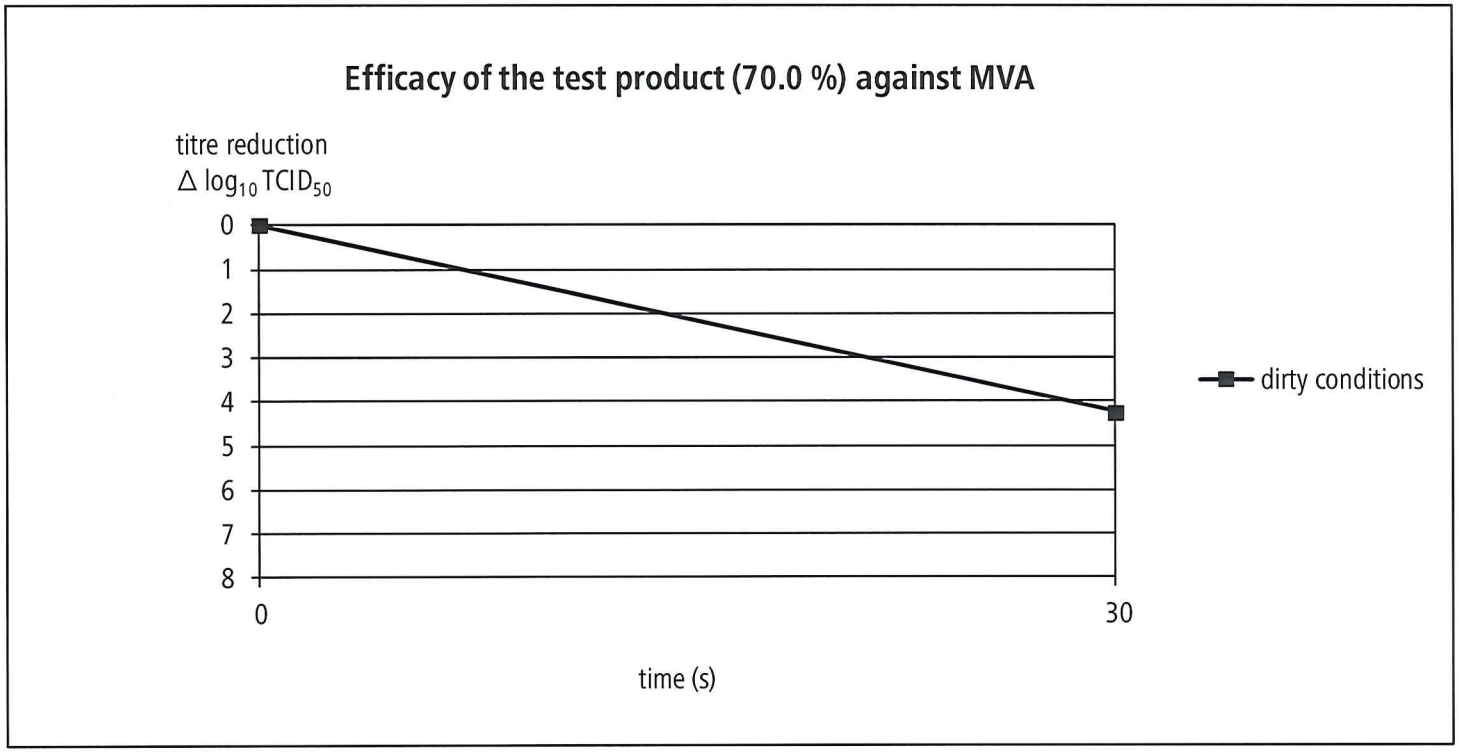

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)