DR. BRILL + DR. STEINMANN
INSTITUT FÜR HYGIENE UND MIKROBIOLOGIE

CHLORINEX-60

Evaluation of virucidal efficacy

Test report no.	L23/00600M.1
Method	DIN EN 14476:2019-10
Endpoint	murine norovirus (MNV), strain S99 Berlin (RVB-0651)
Client	Chemi-Pharm AS, Tänassilma tee 11, Tänassilma küla, Saku, EST - HARJU MAAKOND 76406
Laboratory	Dr. Brill + Partner GmbH, Institute for Hygiene and Microbiology Norderoog 2, DE-28259 Bremen

ilac-MRA DAkkS Deutsche Akkreditierungsstelle D-PL-13412-01-01

Dipl.-Biol. Dr. rer. nat. Florian H. H. Brill

c/o Dr. Brill + Partner GmbH
Institut für Hygiene und Mikrobiologie
Norderoog 2, DE 28259 Bremen
Telefon 0049-40/557631-0
Email info@brillhygiene.com
Internet www.brillhygiene.com

Chemi-Pharm AS
Tänassilma tee 11, Tänassilma küla, Saku
EST - HARJU MAAKOND 76406

Bremen, 25 January 2024

Expert opinion

Activity of **CHLORINEX-60** against murine norovirus (MNV), strain S99 Berlin (RVB-0651) in the quantitative suspension test according to DIN EN 14476:2019-10 under dirty conditions

The surface disinfectant **CHLORINEX-60** was tested and evaluated according to DIN EN 14476:2019-10 "Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity in the medical area - Test method and requirements (Phase 2/Step 1); German version EN 14476:2013+A2:2019)".

According to this norm a disinfectant or a disinfectant solution at a particular concentration is considered as having virus-inactivating properties if within the recommended exposure period the titre is reduced by ≥ 4 $\log_{10}$ (inactivation ≥ 99.99 %).

According to the test report L23/00600M.1 dated 25/01/2024, the surface disinfectant **CHLORINEX-60** was examined as solutions with 1 tablet in 1.5 L of WSH, 1 tablet in 3 L of WSH and 1 tablet in 5 L of WSH at 20 °C ± 1 °C and dirty conditions. 5, 10 and 15 minutes were chosen as exposure times. In summary, a virucidal activity against MNV was measured as follows:

1 tab/1.5 L **5 minutes** **dirty conditions (3.0 g/L bovine albumine + 3.0 mL/L sheep erythrocytes)**

Dipl.-Biol. Dr. rer. nat. Florian H. H. Brill

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Test report no L23/00600M.1

Quantitative suspension test for the evaluation of virucidal activity of **CHLORINEX-60** in the medical area (DIN EN 14476:2019-10)

Table of contents

1 General Information and Material

1.1 Client

Client	Chemi-Pharm AS, Tänassilma tee 11, Tänassilma küla, Saku, EST - HARJU MAAKOND 76406
Date of order	29/12/2023
Confirmation no.	233308

1.2 Identification of Test Laboratory

Location	Dr. Brill + Partner GmbH, Institute for Hygiene and Microbiology, Norderoog 2, DE-28259 Bremen, Germany
Study manager	Dr. Britta Becker
Scientific assistant	Dr. Dajana Paulmann
Laboratory technicians	Ilka Wappler, Lena Schüler

1.3 Identification of Sample

Name of product	CHLORINEX-60
Internal product identifier	23/01024-001
Formulation code	not specified
Batch no.	2300953
Production date	15/11/2023
Expiry date	08/2026
Manufacturer	Chemi-Pharm AS, Tänassilma tee 11, Tänassilma küla, Saku, EST - HARJU MAAKOND 76406
Date of delivery	22/11/2023
Storage conditions	room temperature and darkness
Appearance of product	beige tablets
Odour	characteristic
Product type	surface disinfectant
Area of accreditation	DAkkS D-PL-13412-01-01
Recommended diluent	not specified
pH value, undiluted (Manufacturer's data)	not specified

Active agents (Manufacturer's data)	1 tab = 1.5 g active chlorine if dissolved in water, thus 1 tab/1.5 l = 1000 ppm active chlorine

1.4 Identification of Reference Product Stock Solution

Name of product	Formaldehyde
Batch no.	03893226
Manufacturer	Carl Roth GmbH + Co. KG
Storage conditions	room temperature and darkness

1.5 Experimental Conditions

Test period	10/01/2024 - 15/01/2024
Concentration of test product	1 tab/1.5 L + 1 tab/3 L + 1 tab/5 L
Appearance of product dilutions	no precipitation
Exposure time(s)	5, 10 and 15 minutes
Test temperature	20 °C ± 1 °C
Incubation temperature	36.5 °C – 37.1 °C
Diluent used	water of standardised hardness (WSH, pH 7.0)
Organic load	dirty conditions (3.0 g/L bovine albumine + 3.0 mL/L sheep erythrocytes)
Stability of product in the mix with virus and interfering substance	no clouding, no precipitation
Procedure to stop action of disinfectant	immediate dilution in cell culture medium
Virus Strain	murine norovirus (MNV), strain S99 Berlin (RVB-0651)
Origin of Virus	Friedrich-Löffler-Institut (FLI), Federal Research Institute for Animal Health, Greifswald, Isle of Riems (Dr. S. Reiche)
Passage of Virus	2
Cells name	RAW 264.7 (murine macrophage cell line)
Origin of Cells	American Type Culture Collection (ATCC TIB-71), Manassas, USA
Passage of Cells	30
Cell culture medium	Dulbecco`s Modified Eagle`s Medium (DMEM), biowest, catalogue no L0106-500

2 Methods

2.1 Preparation of test virus suspension

For preparation of test virus suspension according to EN 14476, cells were infected with a multiplicity of infection of 0.1 at 37 °C. 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 of the supernatant, test virus suspension was stored at -80 °C.

2.2 Determination of pH values and/or active concentrations

The following pH values and concentrations of active substances were measured.

Product-conc.	100 %	1 tab/1.5 L	1 tab/3 L	1 tab/5 L
pH in diluent	n.a.	6.84	6.99	7.08
Active conc.	n.d.	n.d.	n.d.	n.d.

2.3 Preparation of disinfectant (dilutions)

The test product was tested as solutions with 1 tablet in 1.5 L of WSH, 1 tablet in 3 L of WSH and 1 tablet in 5 L of WSH (1 part test virus suspension + 1 part interfering substance + 8 parts disinfectant). Due to the addition of interfering substance and test virus suspension the solutions had to be prepared by the factor 1.25. For preparation of the solution with 1 tablet in 1.5 L of WSH, 1 tablet (3.3321 g) was solved in 1L WSH by stirring for approximately 6 minutes at 20 °C. Afterwards, the solution filled up to the final volume of 1.2 L and stirred for another 5 minutes. The resulting slightly cloudy solution was immediately introduced to the inactivation tests. The solutions with 1 tablet in 3 L WSH and 1 tablet in 5 L WSH were prepared by dilution from the 1 tablet in 1.5 L WSH solution.

2.4 Inactivation assay and controls

Determination of virucidal activity has been carried out according to DIN EN 14476:2019-10 section 5.5.

Immediately at the end of a chosen contact time, activity of the disinfectant was stopped by dilution to 1.0E-08.

Titrations of the virus control were performed at the beginning of the test and after the longest exposure time. 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). If a 97.0 % assay was performed, 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.

Determination of cytotoxicity was performed.

For the control of cell sensitivity (interference control), two parts by volume of Aqua bidest. 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 permissive cells for one hour. The disinfectant solution was then removed from the cells, and a comparative titration of the virus suspension was performed on the pre-treated cells.

Furthermore, a control of efficiency for suppression of disinfectant's activity was included.

As reference for test validation a 0.7 % formaldehyde solution was included. 5, 15, 30 and 60 minutes were chosen as contact times.

2.5 Determination of infectivity

Infectivity was determined as endpoint titration transferring 0.1 ml of each dilution into eight wells of a microtitre plate containing 0.1 ml of cell suspension. Microtitre plates were incubated at 37 °C in a 5 % CO_2-atmosphere. The cytopathic effect was read by using an inverted microscope. The infective dose $TCID_{50}$/ml was calculated with the method of Spearman (2) and Kärber (3).

2.6 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 DIN 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 %.

3 Results

3.1 Verification of results and pass criteria

Because following criteria were fulfilled, examination according to DIN EN 14476:2019-10 section 5.7 is valid:

a. The titre of the test virus suspension allowed the determination of a ≥ 4 $\log_{10}$ reduction.

b. The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test (see norm section 5.7b) was 1.75 ± 0.48 (between 1.0 – 3.0) after 30 min and 2.50 ± 0.55 (between 2.0 – 4.0) after 60 min for MNV.

c. Cytotoxicity of the test product allowed the detection of a 4 $\log_{10}$ reduction of virus titre.

d. The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) cells showed no significant difference of virus titre (< 1 $\log_{10}$; norm section 5.7).

e. The control of efficacy for suppression of disinfectant's activity showed no decrease in virus titre (≤ 0.5 $\log_{10}$; norm section 5.5.5.1).

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

3.2 Results of Efficacy testing according to DIN EN 14476:2019-10

Results of examination are shown in tables 1 to 4. Table 1 demonstrates a summary of results, whereas tables 2 to 4 represent the raw data.

The test product as solution with 1 tablet in 1.5 L of WSH was able to inactivate MNV after 5 minutes under dirty conditions (tables 1 and 2). The reduction factor was ≥ 4.00 ± 0.55. This corresponded to an inactivation of ≥ 99.99 %.

Tested as solutions with 1 tablet in 3 L of WSH and 1 tablet in 5 L of WSH, the test product was not active within 15 minutes of exposure time, respectively (tables 1 and 2).

3.3 Conclusion

The surface disinfectant CHLORINEX-60 tested as solution with 1 tablet in 1.5 L of WSH demonstrated activity against MNV after an exposure time of 5 minutes under dirty conditions. Therefore, the surface disinfectant CHLORINEX-60 can be declared as active against MNV as follows:

1 tab/1.5 L **5 minutes** **dirty conditions**

4 Signature page

Bremen, 25/01/2024

Dipl.-Biol. Dr. rer. nat. Britta Becker
Director of Laboratory - Virology

Dipl.-Biol. Dr. rer. nat. Dajana Paulmann
Deputy Head of Laboratory - Virology

Table 1: Summary of results

Product name: **CHLORINEX-60** Batch: 2300953
Test organism: MNV Temperature: 20 °C ± 1 °C
Organic load: dirty conditions

Assay	Conc.	Cytotox. /LLOQ [log]	log_{10} $TCID_{50}$/ml after …min					≥4 log_{10} reduction after … min
			5	10	15	30	60	
1	1 tab/ 1.5 L	2.50	≤ 3.75 ± 0.44	≤ 3.13 ± 0.45	≤ 2.75 ± 0.33	n.d.	n.d.	5 RF ≥4.00 ± 0.55
1	1 tab/ 3 L	1.50	6.63 ± 0.41	6.00 ± 0.44	6.00 ± 0.38	n.d.	n.d.	>15 RF =1.75 ± 0.50
1	1 tab/ 5 L	1.50	7.13 ± 0.45	6.75 ± 0.44	6.13 ± 0.37	n.d.	n.d.	>15 RF =1.63 ± 0.50
Controls								
Assay	Conc.	Cytotox. [log]	log_{10} $TCID_{50}$/ml after …min					≥4 log_{10} reduction after … min
			0	5	15	30	60	
Virus control for assay								
1	n.a.	n.a.	8.38 ± 0.41	n.d.	n.d.	n.d.	7.75 ± 0.33	n.a.
Suppression control								
1	1 tab/ 1.5 L	2.50	n.d.	n.d.	n.d.	8.00 ± 0.38	n.d.	n.a.
Virus control for suppression								
1	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.75 ± 0.33	n.a.
Sensitivity control								
1	1 tab/ 1.5 L	n.a.	n.d.	n.d.	n.d.	n.d.	8.00 ± 0.44	n.a.
1	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	7.88 ± 0.37	n.a.
Reference control (Formaldehyde with PBS)								
1	0.7 %	4.50	n.d.	8.13 ± 0.37	7.50 ± 0.46	6.63 ± 0.25	5.88 ± 0.37	> 60 RF =2.50 ± 0.55
Virus control for reference								
1	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	8.38 ± 0.41	n.a.

n.a. = not applicable n.d. = not done n.c. = not calculable conc = concentration cytotox = cytotoxicity LLOQ = lower limit of quantification

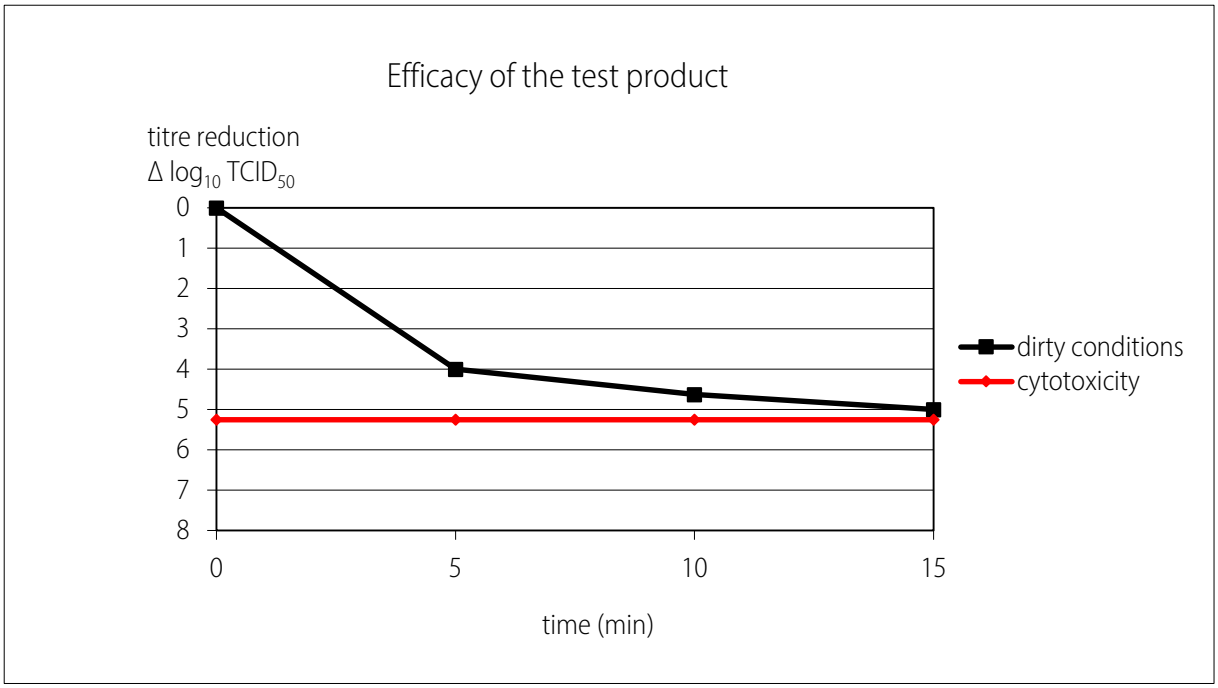

Figure 1: Virus-inactivating properties of CHLORINEX-60 (1 tab/1.5 L) against MNV

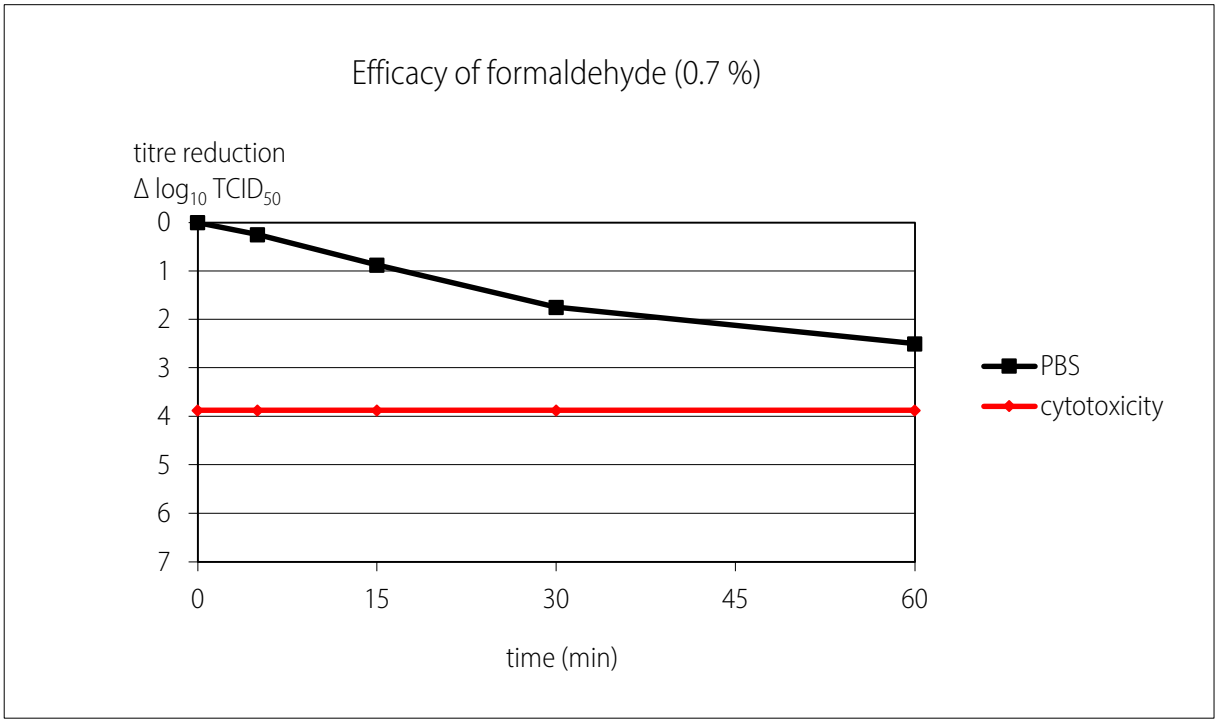

Figure 2: Virus-inactivating properties of the formaldehyde (0.7 %) against MNV

Table 2: Raw data for efficacy test

Product name: **CHLORINEX-60** Batch: 2300953
Test organism: MNV Temperature: 20 °C ± 1 °C
Organic load: dirty conditions Test Run: 1st assay
No. of assay: #8763

Conc.	Contact time [min]	Dilutions [log_{10}]								
		1	2	3	4	5	6	7	8	9
1 tab/ 1.5 L	5	n.d.	4444 4044	4000 4040	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
	10	n.d.	4040 0044	0000 0004	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
	15	n.d.	4004 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
1 tab/ 3 L	5	n.d.	4444 4444	4444 4444	4444 4444	0444 4444	0400 0040	0000 0000	n.d.	n.d.
	10	n.d.	4444 4444	4444 4444	4444 4444	4400 0040	0000 0400	0000 0000	n.d.	n.d.
	15	n.d.	4444 4444	4444 4444	4444 4444	0440 0044	0000 0000	0000 0000	n.d.	n.d.
1 tab/ 5 L	5	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4044 4000	0000 0400	0000 0000	n.d.
	10	n.d.	n.d.	4444 4444	4444 4444	4440 4444	4040 4000	0000 0000	0000 0000	n.d.
	15	n.d.	n.d.	4444 4444	4444 4444	4400 4404	0000 0000	0000 0000	0000 0000	n.d.
Virus control										
n.a.	0	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4404 4440	0000 0400	0000 0000
	60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0040 4000	0000 0000	0000 0000
Cytotoxicity control										
1 tab/ 1.5 L	n.a.	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.	n.d.
1 tab/ 3 L	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.	n.d.
1 tab/ 5 L	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.	n.d.

n.a. = not applicable 0 = no virus present t = cytotoxic conc = concentration
n.d. = not done 1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 3: Raw data for controls

Product name: **CHLORINEX-60** Batch: 2300953
Test organism: MNV Temperature: 20 °C ± 1 °C
Organic load: dirty conditions Test Run: 1st assay
No. of assay: #8763

Conc.	Contact time [min]	Dilutions [log_{10}]								
		1	2	3	4	5	6	7	8	9
Suppression control										
1 tab/ 1.5 L	30	n.d.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 4404	0000 0000	n.d.
Virus control for suppression										
n.a.	60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	0040 4000	0000 0000	0000 0000
Sensitivity control										
1 tab/ 1.5 L	60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4444 4444	4400 0004	0000 4000	n.d.
PBS	60	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4444 4444	0440 4000	0000 0000	n.d.

n.a. = not applicable 0 = no virus present t = cytotoxic conc = concentration
n.d. = not done 1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 4: Raw data for reference test

Reference:	Formaldehyde	Batch:	03893226
Test organism:	MNV	Temperature:	20 °C ± 1 °C
Organic load:	PBS	Test Run:	1st assay
No. of assay:	#8763		

Conc. [%]	Contact time [min]	Dilutions [log_{10}]								
		1	2	3	4	5	6	7	8	9
0.7	5	n.d.	tttt tttt	tttt tttt	4444 4444	4444 4444	4444 4444	4404 0404	n.d.	n.d.
	15	n.d.	tttt tttt	tttt tttt	4444 4444	4444 4444	4044 0444	0040 0400	n.d.	n.d.
	30	n.d.	tttt tttt	tttt tttt	4444 4444	4444 4444	0000 0400	0000 0000	n.d.	n.d.
	60	n.d.	tttt tttt	tttt tttt	4444 4444	4000 0404	0000 0000	0000 0000	n.d.	n.d.
Virus control										
n.a.	60	n.d.	n.d.	n.d.	4444 4444	4444 4444	4444 4444	4044 4044	0000 4000	0000 0000
Cytotoxicity control										
0.7	n.a.	tttt tttt	tttt tttt	tttt tttt	0000 0000	n.d.	n.d.	n.d.	n.d.	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)

5 Appendix

5.1 Version history

Previous versions are replaced by most recent version.

Version	Date	Reason of change and changelog	Author
01	25/01/2024	-	BBi

5.2 Accreditation and certificates

DIN EN 14476:2019-10 is included in our accreditation according to DIN EN ISO/IEC 17025.

5.3 Terms of use

No copying or transmission, in whole or in part, of this test report without the explicit prior written permission. The test results exclusively apply to the tested samples. Information on measurement uncertainty and Version history on request.

5.4 Literature

1 DIN EN 14476:2019-10 "Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity in the medical area - Test method and requirements (phase 2, step 1); German version EN 14476:2013+A2:2019)"
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

5.5 List of Abbreviations

DIN	=	Norm by German institute for normation (German: Deutsches Institut für Normung)
EN	=	European norm (issued by European Committee for Standardization – CEN)
IEC	=	International Electrotechnical Commission
ISO	=	International Organization for Standardization
$TCID_{50}$	=	Tissue Culture Infectious Dose 50
PBS	=	Phosphate buffered saline (see norm section 5.2.2.3 for recipe)
n.d.	=	Not detected
n.a.	=	Not applicable