Study report	Virucidal activity evaluation EN 14476:2013+A2:2019 - 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)
Reference	AV09213374
Identification of the Sponsor	Sucess Gadget, Nanotecnologia e Novos Materias, Lda Rua Filipa Borges, 1245 4780-823 Barcelos
Sample Identification	Care US Aplicação Batch: AMS210906
Sampling	By the sponsor
Type of sample	Liquid, Biocide
Date of sample reception	15/09/2021

Covilhã, 8th October 2021

Assinado por : **Carlos Alberto da Silva Gaspar**
Num. de Identificação: BI13450736
Data: 2021.10.08 19:44:16 +0100

Study director
(Carlos Gaspar, MSc, Biochemistry)

Document history

Version / Addition	Alterations	Date
v_01	First version of the document	08/10/2021

Proponent and Test Facilities identification

Proponent	Labfit – HPRD: Health Products Research and Development Lda
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Quality Management System

Labfit's quality assurance program is ensured by periodic audits and inspections of the quality management systems implemented: ISO 9001: 2015, ISO 13485: 2016, NP 4457: 2007 and Good Laboratory Practice (GLP) pursuant to Directive 2004/9 / EC. The last GLP inspection took place on 22, 23 and 24/05/2018 and the last external audit took place on 25,26 and 27/08/2021.

The results hereby reported reflect the data registered during the study made for the tested sample.

Test Principle

In December 2019, coronavirus disease 19 (COVID-19), caused by SARS-CoV-2 virus, was first identified in Wuhan, China [1]. By January 2020, it was declared a Public Health Emergency of International Concern by the WHO (World Health Organization) and as a pandemic in March 2020 [2,3]. COVID-19 spreads most often before symptom onset or from asymptomatic people through close proximity, via small droplets or aerosols [4]. Considering this ongoing pandemic, it is clear that there is a need for products with proven virucidal efficacy. This can be achieved by using the EN 14476, which describes the European standard for determining virucidal activity. In this document, modified vaccinia virus Ankara (MVA) is used as a reference virus for all enveloped viruses, meaning that it is a suitable surrogate test virus that can be safely used for testing the virucidal efficacy of products against other enveloped viruses such as SARS-CoV-2 and other coronaviruses [5,6,7]

The test described in EN 14476 aims to determine the virucidal activity of chemical disinfectant and antiseptic products that are used in the medical area in the fields of hygienic handrub, hygienic handwash, instrument disinfection, surface disinfection and textile disinfection [8]. A sample of the product as delivered and/or diluted with hard water (or water for ready to use products) is added to a test suspension of viruses in a solution of an interfering substance. The mixture is maintained at the temperatures and contact times specified previously. At the end of this contact time, an aliquot is taken, the virucidal action of this portion is immediately suppressed by dilutions of the sample in ice-cold cell maintenance medium. The dilutions are transferred into wells in microtiter plates with a monolayer of BHK-21 cells. Infectivity tests are done by quantal tests. After incubation, the titres of infectivity are calculated according to Spearman and Kärber and evaluated. Reduction of virus infectivity is calculated from differences of $\log_{10}$ virus titres before (virus control) and after treatment with the product.

Study conditions

Study beginning date	15/09/2021
Test beginning date	15/09/2021
Test conclusion date	29/09/2021
Study conclusion date	08/10/2021
Sample storage during the test	in the package sent by the sponsor at room temperature in the dark

Materials

Culture Medium and Reagents

Dulbeco's Modified Eagle's Medium	DMEM, Biosera, supplemented with 10 % of FBS
Maintenance medium	DMEM, Biosera, supplemented with 2 % of FBS
Fetal Bovine Serum	FBS, VWR
1.4 % formaldehyde solution	Dilution of folmaldehyde solution 37%, VWR
PBS	PBS tablets 100 mL, VWR
BSA	Albumin from bovine serum, cohn Fration V, Fisher

Virus and cells

The modified vaccinia virus Ankara (MVA) was purchased in ATCC (VR-1508). BHK 21-cells (Baby Hamster Kidney; passage 53) were purchased in ATCC (CCL-10). The cells were inspected regularly for morphological analysis. Before inactivation assays, virus had been passaged four times in BHK 21-cells.

Apparatus, glassware and small items of equipment

Analytical balance	Kern, 770-15
Incubator	Binder, APT.lineTM C150E2
Pipettes	P5000, VWR, Model VE5000 P1000, VWR, Model VE1000 P200, Model VE200 P20, Model VE20
Water bath	VWR, VWB 6
Biosafety cabinet, type 2	Telstar
Equipment	usual laboratory equipment

Reference Substances

Negative control	Wells with only culture medium
Formaldehyde solution	Solution at 1,4 % used as reference test

Methods

The method used to perform this test complies with EN 14476:2013+A2:2019 - 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).

Experimental conditions

Test temperature	20 °C ± 1 °C
Concentration of test product	50.0 %, 5.0 % and 0.5 %
Appearance of product dilutions	No precipitation
Contact time	5 minutes
Interfering substance(s)	0.3 g/L bovine serum albumin
Procedure to stop action of disinfectant	Immediate dilution
Diluent	Distilled water
Stability of product in the mix with virus and interfering substance (highest concentration)	no clouding, no precipitation
Virus strain	Modified vaccinia virus Ankara (MVA) (ATCC-1508)
Cell line	BHK21 [C13] (ATCC® CCL10™)
Cell Line passage	65

Preparation of test virus suspension

For preparation of test virus suspension, BHK-21 were cultivated with DMEM supplemented with 10% of FBS. Cells were infected with a multiplicity of infection of 0.1 in the presence of 2% FBS. After cells showed cytopathic effects, they were subjected to a procedure of freeze/thaw followed by a centrifugation to sediment cell debris. After aliquoting, test virus suspension was stored at -20 °C.

Preparation of disinfectants (dilutions)

The test product was tested as 80.0 %, 8.0 % and 0.8 % solutions for a preliminary cytotoxicity evaluation. Due to the observed cytotoxicity during the assay, the test product was further diluted to the concentrations of 50.0 %, 5.0 % and 0.5 %. These solutions were prepared in distilled water immediately before the inactivation tests.

Inactivation assay (end point titration)

Determination of virucidal activity has been carried out according to EN 14476 (section 5.5). The test product was examined at different solutions concentrations in distilled water at 20 °C according to EN 14476. Immediately at the end of a chosen contact time, activity of the disinfectant was stopped by dilution to 10^{-11}. Titrations of the virus control were performed at the beginning of the test and after the exposure time (EN 14476; section 5.5.7). One part by volume of test virus suspension was mixed with one part interfering substance and eight parts by volume of distilled water. Furthermore, a cell control (only addition of culture medium) was incorporated.

Cytotoxicity control

Reveals the possible alteration in cell structure caused by the test product. Live cells should not manifest a toxic reaction or morphological alterations to a level where achieving 4-log reduction is not possible. To check for cytotoxicity, one part of distilled water and one part of interfering substance were mixed with eight parts of the product test solution. Serial dilutions were prepared in the culture medium and are inoculated into monolayer of cell culture. Results were obtained after 5 days of incubation.

Interference control

Study that the susceptibility of infection in cells is not influenced negatively by the test product. One part of the lowest apparently non-cytotoxic dilution of the product test solution or PBS and 0.1 mL of culture medium were distributed onto wells in a microtitre plate. After 1 h of incubation at 37 °C, the supernatant is discarded. A comparative titration of the virus suspension was performed on the pre-treated (disinfectant) and non-pre-treated (PBS) cells as described above.

Suppression control

This test verifies the efficiency of the neutralizing method in suppressing the virucidal activity of the test product after the required contact time. One part of interfering substance and one part of DMEM + 2 % FBS were added to eight parts of the product test solution. Then, to a container with 4 parts of ice-cold medium, 0.5 parts of the previous mixture and 0.5 parts of the virus test suspension were added. The mixture was then incubated in an ice bath for 30 min ± 10 s. Immediately after incubation, serial ten-fold dilutions were prepared, and virus was titrated.

Reference control

As reference for test validation, a 0.7 % formaldehyde solution was included and 5 and 15 minutes were chosen as contact times. In addition, cytotoxicity of formaldehyde test solution was determined based on EN 14476 (section 5.5.6.2) with dilutions up to 10^{-11}.

Virus control

Determines the infectivity of the test virus suspension. To pass the test, the concentration of virus in the control test must be sufficiently high to enable a 4-log reduction. Infectivity was determined as endpoint titration according to EN 14476 (section 5.5) by transferring 0.1 mL of each dilution into eight wells of a microtitre plate with a monolayer of BHK-21 cells (6-7 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 five days.

Verification of virucidal activity

Calculation of the infective dose $TCID_{50}$/mL was done with the modified method of Spearman and Kärber. The virucidal activity of the test disinfectant was evaluated by calculating the decrease in titre in comparison with the control titration without the product. The difference is given as reduction factor (rf). According to the EN 14476, a disinfectant or a disinfectant solution at a concentration is having virus-inactivating efficacy if the titre is reduced at least by 4 log_{10} within the recommended exposure period.

Verification of the methodology

The following criteria are mentioned in EN 14476 (section 5.7) and should be fulfilled to validify examination with MVA according the standard.

a) The titre of the test virus suspension must allow the determination of a ≥ 4 log_{10} reduction.
b) The test product (50.0 %) showed cytotoxicity at the highest concentration, failing the requirement of EN 14476. Due to the observed cytotoxicity, test product was submitted to a large volume plating method.
c) The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test was 3.38 ± 0.59 (should be between 0.75 – 3.5) after 5 min and 4.00 ± 0.00 (should be between 2.0 - 4.0) after 15 min for MVA.
d) The comparative titration on pre-treated and non-pre-treated BHK 21-cells showed no significant difference (< 1 log10) of virus titre: 11.50 ± 0.00 in PBS and 11.50 ± 0.00 in disinfectant (0.05 %) log_{10} $TCID_{50}$/mL.
e) One concentration must demonstrate a 4 log_{10} reduction and (at least) one concentration should demonstrate a log_{10} reduction of less than 4.

Test results

The obtained results are described in Table 1 to Table 11.

Table 1: Raw data for Care US Aplicação (50.0 %) tested against MVA at 20 °C (quantal test; 8 wells)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})												
				1	2	3	4	5	6	7	8	9	10	11	12	control
test product	50.0%	clean conditions	5	n.a.	tttt	tttt	1111	1111	0000	0000	0000	0000	0000	0000	0000	0000
					tttt	tttt	1111	1111	0000	0000	0000	0000	0000	0000	0000	0000
test product cytotoxicity	50.0%	clean conditions	n.a.	n.a.	tttt	tttt	0000	0000	0000	0000	0000	0000	0000	0000	0000	0000
					tttt	tttt	0000	0000	0000	0000	0000	0000	0000	0000	0000	0000
virus control	n.a.	clean conditions	0	n.a.	3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	1111	0000
					3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	1111	0000
			5	n.a.	3333	3333	3333	3333	2222	2222	2222	2222	1111	1011	1001	0000
					3333	3333	3333	3333	2222	2222	2222	2222	1111	1101	0001	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)

Table 2: Raw data for Care US Aplicação (5.0 %) tested against MVA at 20 °C (quantal test; 8 wells)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})												
				1	2	3	4	5	6	7	8	9	10	11	12	control
test product	5.0%	clean conditions	5	n.a.	tttt	2222	2222	2222	2222	2222	1111	1111	1111	0000	0000	0000
					tttt	2222	2222	2222	2222	2222	1111	1111	1111	0000	0000	0000
test product cytotoxicity	5.0%	clean conditions	n.a.	n.a.	tttt	0000	0000	0000	0000	0000	0000	0000	0000	0000	n.d.	0000
					tttt	0000	0000	0000	0000	0000	0000	0000	0000	0000	n.d.	0000
virus control	n.a.	clean conditions	0	n.a.	3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	1001	0000
					3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	0001	0000
			5	n.a.	3333	3333	3333	3333	2222	2222	2222	2222	1111	1011	1001	0000
					3333	3333	3333	3333	2222	2222	2222	2222	1111	1101	0001	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)

Table 3. Raw data for Care US Aplicação (0.5 %) tested against MVA at 20 °C (quantal test; 8 wells)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})												
				1	2	3	4	5	6	7	8	9	10	11	12	control
test product	0.5%	clean conditions	5	n.a.	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	0000
					3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	0000
test product cytotoxicity	0.5%	clean conditions	n.a.	n.a.	0000	0000	0000	0000	0000	0000	0000	0000	0000	n.d.	n.d.	0000
					0000	0000	0000	0000	0000	0000	0000	0000	0000	n.d.	n.d.	0000
virus control	n.a.	clean conditions	0	n.a.	3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	1001	0000
					3333	3333	3333	3333	3333	2222	2222	1111	1111	1111	0001	0000
			5	n.a.	3333	3333	3333	3333	2222	2222	2222	2222	1111	1011	1001	0000
					3333	3333	3333	3333	2222	2222	2222	2222	1111	1101	0001	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)

Table 4: Raw data for formaldehyde solution (0.7 %) tested against MVA at 20 °C (quantal test; 8 wells)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})												
				1	2	3	4	5	6	7	8	9	10	11	12	control
formaldehyde	0.7 %	PBS	5	n.a.	tttt	tttt	tttt	1111	1111	1111	0111	1010	0000	0000	0000	0000
					tttt	tttt	tttt	1111	1111	1111	0111	1110	0011	0000	0000	0000
			15	n.a.	tttt	tttt	tttt	1111	1111	1111	1111	0000	0000	0000	0000	0000
					tttt	tttt	tttt	1111	1111	1111	1111	0000	0000	0000	0000	0000
formaldehyde cytotoxicity	0.7 %	PBS	n.a.	n.a.	tttt	tttt	tttt	0000	0000	0000	0000	0000	0000	0000	0000	0000
					tttt	tttt	tttt	0000	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)

Table 5: Raw data for control of efficacy for suppression of disinfectant's activity (50.0 %)

Product	Interfering substance	Dilutions (log_{10})												
		1	2	3	4	5	6	7	8	9	10	11	12	control
test product	clean conditions	3333	3333	3333	2222	1111	1111	1111	1111	1111	0111	1111	n.a.	0000
		3333	3333	3333	2222	1111	1111	1111	1111	1111	1111	0111	n.a.	0000
virus control	clean conditions	n.a.	3333	3333	3333	3333	2222	2222	2222	2222	1111	1011	1001	0000
			3333	3333	3333	3333	2222	2222	2222	2222	1111	1101	0001	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)

Table 6. Raw data for control of cell sensitivity (0.05 %)

Product	Dilution	Dilutions (log_{10})											
		1	2	3	4	5	6	7	8	9	10	11	control
test product	n.a.	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	0000
		3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	0000
PBS	n.a.	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	0000
		3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	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)

Table 7: Determination of virus titre at 20 °C

Virus titration	Dilutions (log_{10})																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	control
MVA	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	2222	2222	2222	2222	2222	2222	2222	2222	1111	1111	0000
	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	3333	2222	2222	2222	2222	2222	2222	2222	2222	1111	1111	0000
Virus titration	**Dilutions (log_{10})**																						
	23		**24**		**25**		**26**		**27**		**28**		**29**		**30**		**31**		**32**		**33**		**control**
MVA	1111		1111		1111		1111		1111		1111		1111		1111		1111		1111		0000		0000
	1111		1111		1111		1111		1111		1111		1111		1111		1111		1111		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)

Table 8. Raw data for the inactivation of MVA by Care US Aplicação (50.0 %) at 20 °C (5 minutes) (LVP, 1:10 000)

Interfering substance	Row	1	2	3	4	5	6	7	8	9	10	11	Control
clean 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

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)

Table 9. Summary of results (end point dilution method) by Care US Aplicação and MVA

Product	Concentration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after…min					> 4 log_{10} reduction after….min
				0	0.5	2	5	15	
test product	50.00%	clean conditions	2	n.a.	n.d.	n.d.	5.50 ± 0.00	n.d.	-
	5.00%		1	n.a.	n.d.	n.d.	10.50 ± 0.00	n.d.	-
	0.50%		0	n.a.	n.d.	n.d.	12.50 ± 0.00	n.d.	-
virus control	n.a.	clean conditions	n.a.	12.50 ± 0.00	n.d.	n.d.	11.63 ± 0.25	n.d.	n.a.

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

Table 10: Summary of results (end point dilution method) by Care US Aplicação and MVA

Product	Concentration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after…min					> 4 log_{10} reduction after….min
				0	0.5	2	5	15	
formaldehyde	0.7 %	PBS	3	n.a.	n.a.	n.a.	9.13 ± 0.30	8.50 ± 0.00	15 (rf ≥ 4.00 ± 0.00)
supression control	50.00%	clean conditions	2	n.a.	n.d.	n.d.	11.25 ± 0.48	n.d.	n.a.
sensitivity product	0.05%	n.a.	n.a.	n.a.	n.d.	n.d.	11.50 ± 0.00	n.d.	n.a.
sensitivity PBS	n.a.	n.a.	n.a.	n.a.	n.d.	n.d.	11.50 ± 0.00	n.d.	n.a.

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

Table 11: Summary of results (LVP) by Care US Aplicação and MVA

Product	Concentration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after…min					> 4 log_{10} reduction after….min
				0	0.5	2	5	15	
test product	50.0 % → 1:10 000	clean conditions	0	n.a.	n.d.	n.d.	≤ 2.75	n.d.	≥ 8.87 ± 0.25

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

If one applies the following formula[9] commonly used to quantify the antiviral effect in percentage, a result of **> 99.99 %**viral reduction is obtained.

$$P = (1 - 10^{-L}) \times 100$$

Where:

P is the percent reduction

L is the log reduction

Results discussion and conclusion

The product was tested at 50.0 % of its concentration for 5 minutes but was shown to be cytotoxic against the cell line used in the test (Table 1). Due to this, we proceeded to apply the large volume plating method (LVP) approach in parallel to the end point dilution method, by testing the 50.0 % solution at a dilution of 1:10 000 with 5 minutes of exposure time. Since no residual virus was found in 528 out of 528 cell culture units at this timepoint, the results according to Poisson formula was ≤ 2.75 log_{10} $TCID_{50}$. The reduction factor was ≥ 8.87 ± 0.25, accomplishing to the standard criteria considered for active products (log reduction ≥ 4; Table 11).

According to the EN 14476, a disinfectant is effective in inactivating the virus if the titre is reduced at least by 4 log_{10} steps within the recommended exposure time.

The product **Care US Aplicação** demonstrated virucidal activity after an exposure time of **5 minutes** under **clean conditions**.

Study records storage and general data storage

All records related to the study (study plan, raw data, spreadsheets and report) will be kept in the file, at Labfit facilities.

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