QACS LAB
QUALITY ASSURANCE & CONTROL SYSTEMS

A: 1 Antigonis str, 14451, Athens, Greece
T: (+30) 210 2934745 | E: info@qacs.gr
F: (+30) 210 2934606 | www.qacslab.com

STUDY REPORT

2021 – 1701 / 21 23 00163

Active chlorine released from hypochlorous acid by electrochemical by activation

ANTIVIRAL ACTIVITY ACCORDING TO THE STANDARD EN 14476

OF THE PRODUCT " ACTIVE CHLORINE RELEASED FROM HYPOCHLOROUS ACID BY ELECTROCHEMICAL BY ACTIVATION " AGAINST

ADENOVIRUS, POLIOVIRUS, MURINE NOROVIRUS AND VACCINIA VIRUS

A: 1 Antigonis str, 14451, Athens, Greece
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PRODUCT IDENTIFICATION

Product code/name	**Active chlorine released from hypochlorous acid by electrochemical by activation**
Date of receipt	17/02/2021
Product Appearance	Clear, colorless liquid
Storage	At room temperature (20°C)
Concentration used in the test	97.0% of the received product
Active compounds	Active chlorine released from hypochlorous acid 0,05%

EXPERIMENTAL CONDITIONS

Test Method	**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 (Phase2/Step 1)"**
Test period	17/02/2021
Strains of viruses	Adenovirus type 5 (ATCC VR-5) Poliovirus 1 Sabin strain, LSc-2ab (WHO) Murine Norovirus (Strain S99 Berlin) Vaccinia virus (Strain MVA)
Cell lines	Human epithelial type 2 (Hep-2) cells Human Rhabdomyosarcoma cells (RD) RAW 264.7 macrophage (ATCC) Baby Hamster Kidney fibroblasts (BHK cells)
Culture medium	DMEM (Dulbecco's Minimal Essential Medium)
Contact times	15 sec
Test temperature	Water bath, 20±1°C
Interfering substance	BSA 0.3 g/L (clean conditions)
Inactivation process	Dilution 1/10 in ice cold maintenance medium
Technical supervisor	Pogka Vasiliki, Ph.D, Kalliaropoulos Antonios
Facilities	BSL-2 facility, Public Health Laboratories, Hellenic Pasteur Institute.

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1. Principle of the test

A 97% dilution of the product " Active chlorine released from hypochlorous acid by electrochemical by activation " was added to a test suspension of titrated viruses in bovine serum albumin solutions of 0.3 g/L (clean conditions). The mixtures were maintained at 20°C for 15 sec. At the end of contact time, an aliquot was taken and the virucidal activity was suppressed by dilutions in ice-cold maintenance medium. The dilutions were then inoculated onto cell monolayers in 96-well culture plates for the titration of the remaining viruses. The titers of the viruses expressed in $TCID_{50}$ values, after 5-days incubation, were determined and expressed in log scale. Reduction of the viruses' infectivity was calculated from the differences of the log virus titers before (control) and after treatment with the product. According to the EN 14476 standard a product has antiviral activity when the reduction of the virus is at least 4 log.

2. Titration of the test viruses

The antiviral activity of the product was tested against four virus strains proposed by the Standard EN 14476, Adenovirus type 5, Poliovirus, Murine norovirus and Vaccinia virus. The viruses were propagated in the appropriate cell culture system to produce a high titer: Hep-2 monolayers for adenovirus titration, BHK for vaccinia virus, RAW cell monolayers for M. norovirus titration and RD cell monolayers for poliovirus titration. Each virus was tested in decimal dilutions 10^{-3} up to 10^{-10}. Each dilution was inoculated 10x in wells of 96-well culture plates with the appropriate cell monolayer. The infected cells were incubated at 37°C in a 5% CO_2 atmosphere for 5 days. The Tissue Culture Infectious Dose ($TCID_{50}$) i.e. the infection dose of a virus suspension inducing a Cytopathic Effect (CPE) in 50% of cell culture units was estimated by the end-point Spearman-Karber method:

$$\textbf{Log } TCID_{50} = L - d(S - 0.5),$$

where *L* is the highest virus concentration used, *d* is the log difference of dilutions, *S* is the sum of % affected (CPE) at each dilution.

The standard error was calculated as follows:

$$\sigma_m^2 = d_f^2 \Sigma p_i(1-p_i)/(n_i-1)$$

where *d* is the logarithm of dilution factor, p_i was the observed reaction rate, *n* the number of test objects per dilution and σ_m standard error of the logarithmic titer.

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CPE results of each virus on the appropriate cell line are presented in tables 1a, 1b, 1c and 1d, respectively.

Table 1a. Titration of the adenovirus on Hep-2 cells

Virus dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-4}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-5}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-6}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-7}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-8}	4	4	0	4	4	0	4	4	4	4	0	0
10^{-9}	4	0	4	0	0	0	4	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE

Table 1b. Titration of the Vaccinia virus on BHK cells

Virus dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-4}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-5}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-6}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-7}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-8}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-9}	0	4	4	0	0	0	4	0	4	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE

Table 1c. Titration of the Murine norovirus on RAW cells

Virus dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-4}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-5}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-6}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-7}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-8}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-9}	0	4	0	4	0	0	0	4	0	0	0	0

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10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE

Table 1d. Titration of the Poliovirus on RD cells

Virus dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-4}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-5}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-6}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-7}	4	4	4	4	4	4	4	4	4	4	0	0
10^{-8}	4	4	4	4	0	4	0	4	4	4	0	0
10^{-9}	0	0	0	0	4	0	4	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE

By using of the Spearman-Karber formula on the aforementioned CPE results, the calculated $TCID_{50}$ of the Adenovirus, Vaccinia virus, Norovirus and Poliovirus strains were $10^{-7.6}$, $10^{-7.9}$, $10^{7.8}$ and $10^{-7.5}$ respectively. Taking into account the standard error of the above calculation, the titers of the viral strains used in the tests were:

Initial titer of Adenovirus type 5	**Log $TCID_{50}$/0.1mL = 7.6±0.224**
Initial titer of Vaccinia virus	**Log $TCID_{50}$/0.1mL = 7.9±0.163**
Initial titer of Murine norovirus	**Log $TCID_{50}$/0.1mL = 7.8±0.153**
Initial titer of Poliovirus	**Log $TCID_{50}$/0.1mL = 7.5±0.189**

3. Cytotoxic effect of the product

We determined the highest concentration of the product (97.0% final concentration) not having toxic effect on the cells used for the virus culture. Dilutions 10^{-1} to 10^{-8} of the product in culture medium with 0.3 g/L BSA were incubated in ice-cold water for 30 min and then 100 µL of each dilution were inoculated onto monolayers of Hep-2, BHK, RAW and RD cells in the wells of culture plates. Any microscopic changes in the cells after 5-days incubation were recorded.

No cytotoxic effect was observed on Hep-2, BHK, RD and RAW cells in all dilutions of a 97.0% final concentration of the product.

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4. Reference test for virus inactivation

Formaldehyde 0.7% (w/v) was included as reference for test validation according to the Standard EN 14476. Cytotoxicity test as well as antiviral activity determination was performed on RD cells using serial dilutions of up to 10^{-8} of the aforementioned formaldehyde test solution. Contact times were 30 min and 60 min. The results of the cytotoxicity and the virus inactivation tests are presented in the tables 2 and 3 respectively (only the results for 30 min contact time are shown).

Table 2: Cytotoxicity test of formaldehyde solution tested on RD cells

Product Dilutions	Presence or absence of cell cytotoxicity of the product (*)										Cell control	
10^{-1}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-2}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-3}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-4}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-5}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0

(*) tox= cytotoxicity, 0 = absence of cytotoxicity

Table 3: Data of formaldehyde solution inactivation tested against Poliovirus

Virus Dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-4}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-5}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-6}	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox	0	0
10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE. Tox=cytotoxicity

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A reduction of at least 2.0 log of the poliovirus titer was recorded in the presence of 0.7% (w/v) formaldehyde. Higher log reduction could not be observed due to toxicity of formaldehyde on RD cells. According to the EN 14476 standard, the difference between the logarithmic titer of the virus control and the logarithmic titer of the test organism in the reference inactivation test should be between 0.5 and 2.5 log after 30 min for poliovirus to verify the method.

5. Antiviral activity of the product

The antiviral activity of the product against the adenovirus, vaccinia virus, murine norovirus and poliovirus strains was determined for 15 sec at 20±1°C in 0.3 g/L (clean conditions). Immediately at the end of contact time, a 1/10 dilution was made in ice-cold cell maintenance medium and 30 min later, subsequent serial dilutions (step 1:10) were inoculated onto cell culture monolayers. After incubation, the titer of each virus was calculated, and the reduction of the virus infectivity was determined from the log differences of virus titers before and after treatment with the product. Results are presented in tables 4, 5, 6 and 7 for adenovirus, vaccinia virus, murine norovirus and poliovirus, respectively.

Table 4: Adenovirus titration after a 15 sec contact with 97.0% final concentration of the product in 0.3 % BSA

Virus Dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-4}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-5}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE. Tox=cytotoxicity

The titer of the Adenovirus remaining after the treatment with the product is:

Log $TCID_{50}$after treatment: ≤1.5 Log difference=initial virus titer – virus titer after treatment = 7.6-(≤1.5) = ≥6.1

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Table 5: Vaccinia virus titration after a 15 sec contact with 97.0% final concentration of the product in 0.3 % BSA

Virus Dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-4}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-5}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE. Tox=cytotoxicity

The titer of the Vaccinia virus remaining after the treatment with the product is:

Log $TCID_{50}$after treatment: ≤1.5

Log difference=initial virus titer – virus titer after treatment = 7.9-(≤1.5) = ≥6.4

Table 6: M. norovirus titration after a 15 sec contact with 97% final concentration of the product in 0.3 % BSA

Virus Dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
10^{-3}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-4}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-5}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE. Tox=cytotoxicity

The titer of the murine norovirus remaining after the treatment with the product is:

Log $TCID_{50}$ after treatment: ≤1.5

Log difference=initial virus titer – virus titer after treatment = 7.8-(≤1.5) = ≥6.3

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Table 7. Poliovirus titration after a 15 sec contact with 97% final concentration of the product in 0.3 % BSA

	Virus Dilutions	CPE in cell culture wells of culture plate (*)										Cell control	
	10^{-3}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-4}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-5}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-6}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-7}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-9}	0	0	0	0	0	0	0	0	0	0	0	0
	10^{-10}	0	0	0	0	0	0	0	0	0	0	0	0

(*) CPE grading in each well: 0= no CPE, 1= 25% CPE, 2=50% CPE, 3= 75% CPE 4= 100% CPE. Tox=cytotoxicity

The titer of the poliovirus remaining after the treatment with the product is:

Log $TCID_{50}$ after treatment: ≤1.5

Log difference=initial virus titer – virus titer after treatment = 7.5-(≤1.5) = ≥6.0

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6. Conclusion

The antiviral activity of the product " Active chlorine released from hypochlorous acid by electrochemical by activation " against the Adenovirus type 5, Vaccinia virus, Murine norovirus and Poliovirus was tested according to the EN 14476 standard: “Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity in the medical area- test method and requirements (Phase2/Step1).” According to the EN 14476 standard a product has antiviral activity when a reduction of at least 4 log of the virus is observed.

The product " Active chlorine released from hypochlorous acid by electrochemical by activation " in 97.0% final concentration, demonstrated:

- a ≥6.1 log reduction of the Adenovirus type 5 (ATCC VR-5) after 15 sec contact time in the presence of 0.3 g/L BSA, at 20°C
- a ≥6.4 log reduction of the Vaccinia virus (Strain MVA) after 15 sec contact time in the presence of 0.3 g/L BSA, at 20°C
- a ≥6.3 log reduction of the Murine Norovirus (Strain S99 Berlin) after 15 sec contact time in the presence of 0.3 g/L BSA, at 20°C
- a ≥6.0 log reduction of the Poliovirus type 1 after 15 sec contact time in the presence of 0.3 g/L BSA, at 20°C

The product demonstrated antiviral activity against the non-enveloped DNA adenovirus, the nonenveloped RNA murine norovirus, the non-enveloped RNA poliovirus and the enveloped DNA vaccinia virus. According to the EN 14476 standard, products that have antiviral activity against the adenovirus, the poliovirus and the murine norovirus are considered active against all viruses (enveloped and non-enveloped).

Study Manager:

QACS Laboratories
1 Antigonis str 14451 Melamorfossi Greece
VAT no EL 998709411, email: info@qacs.gr
Tel +30-2102934745 fax +30-210 2934606
www. qacs.gr

Lagiopoulos Giorgos
Technical Manager of Microbiological Dpt
Agronomist – Food Technologist, MSc
Pharmaceutical Microbiology PgCert
Date: 19/04/2021

End of Study Report