ΕΛΛΗΝΙΚΟ ΙΝΣΤΙΤΟΥΤΟ ΠΑΣΤΕΡ
INSTITUT PASTEUR HELLENIQUE
Public Health Laboratories,
Head, Andreas F. Mentis, MD, PhD.

ANTIVIRAL ACTIVITY ACCORDING TO THE STANDARD EN 14476 OF THE PRODUCT "NOVASIN" AGAINST VACCINIA VIRUS

Athens, February 2021

PRODUCT IDENTIFICATION

Product code/name	**NOVASIN**
Date of receipt	December 20th, 2020
Manufacturer	INTRADERMA
Product Appearance	Clear, colorless liquid
Active substances in the composition	HOCl 80% NaClO 20%
Storage	At room temperature (20°C)
Concentration used in the test	97.0% of the received product

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 (Phase 2/Step 1)"**
Test period	January 2021
Strains of viruses	Vaccinia virus (Strain MVA)
Cell lines	Baby Hamster Kidney fibroblasts (BHK cells)
Culture medium	DMEM (Dulbecco's Minimal Essential Medium)
Contact time	1 min
Test temperature	Water bath, 20±1°C
Interfering substance	BSA 3.0 g/L plus 3,0 ml/l erythrocytes (dirty conditions)
Inactivation process	Dilution 1/10 in ice cold maintenance medium
Researchers	Vasiliki Pogka, Ph.D
Facilities	BSL-2 facility, Public Health Laboratories, Hellenic Pasteur Institute.

1. Principle of the test

A 97.0% dilution of the concentrated product was added to a test suspension of titrated virus in bovine serum albumin solutions of 3.0 g/L (dirty conditions). The mixture was maintained at 20°C for 1 min. 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 virus. The titers of the virus 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 vaccinia virus according to the EN 14476 standard. The virus was propagated in the appropriate cell culture system to produce a high titer: BHK cell monolayers were used for vaccinia virus titration. The virus was tested in decimal dilutions 10^{-2} up to 10^{-9}. 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.

CPE results of the vaccinia virus on the BHK cell line are presented in table 1.

Table 1. 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	0	4	4	0	4	4	0	0
10^{-9}	4	0	0	0	0	0	0	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

By using of the Spearman-Karber formula on the aforementioned CPE results, the calculated $TCID_{50}$ of the vaccinia virus was $10^{-7.5}$. Taking into account the standard error of the above calculations, the titer of vaccinia virus used in the tests was:

Initial titer of vaccinia virus	**Log $TCID_{50}$/0.1mL = 7.5±0.188**

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 3.0 g/L BSA were incubated in ice-cold water for 30 min and then 100 µL of each dilution were inoculated onto monolayers of BHK 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 BHK cells in all dilutions of a 97.0% final concentration of the product.

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 BHK cells using serial dilutions of up to 10^{-8} of the aforementioned formaldehyde test solution. Contact times were 5 min and 15 min. The results of the cytotoxicity and the virus

inactivation tests are presented in the tables 2 and 3 respectively (only the results for 5 min contact time are shown).

Table 2: Cytotoxicity test of formaldehyde solution tested on BHK 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 Vaccinia virus

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

A reduction of at least 2 log of the vaccinia virus 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 BHK 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 0.75 log and 3.5 log after 5 min for vaccinia virus to verify the method.

5. Antiviral activity of the product

The antiviral activity of the product against the vaccinia virus was determined for 1 min at 20±1ºC in 3.0 g/L (dirty 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 virus titer was calculated, and the reduction of virus infectivity was determined from the log differences of virus titers before and after treatment with the product. Results are presented in table 4.

Table 4: Vaccinia virus titration after a 1 min contact with 97% final concentration of the product in 3.0 % BSA

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}	0	4	4	0	0	4	0	4	0	4	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: 3.0

Log difference=initial virus titer – virus titer after treatment = 7.5-3.0 = 4.5

CONCLUSION

The antiviral activity of the product "Novasin" against the vaccinia virus was tested according to the 14476:2013+A2:2019 standard: "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)". According to the EN 14476 standard a product has antiviral activity when the reduction of the virus is at least 4 log.

The product "Novasin" in 97.0% final concentration, demonstrated:

- **a 4.5 log reduction of the vaccinia virus after a 1 min contact time in the presence of 3.0 g/L BSA, at 20°**

The product demonstrated antiviral activity against the enveloped DNA vaccinia virus. According to the EN 14476 standard, products that have antiviral activity against the vaccinia virus are considered active against all enveloped viruses.

Athens, February 2021

Andreas F. Mentis, MD, PhD
Clinical Microbiologist

Head, Diagnostic Services Laboratory
Head, National Influenza Reference Laboratory of S. Greece
Head, National Polio Reference Laboratory
Head, National Measles/Rubella Reference Laboratory
Head, Laboratory of Medical Microbiology

Public Health Laboratories
Hellenic Pasteur Institute
127, Vass. Sofias Ave
11521 Athens - GREECE
Tel: +30 210 64 78 816
Fax: +30 210 64 78 832
Email: mentis@pasteur.gr