Test Report: BS 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)

Test Laboratory	**BluTest Laboratories Ltd** 5 Robroyston Oval, Nova Business Park, Glasgow, G33 1AP

Identification of sample

Name of the product	NILAQUA
Batch number	9720
Client	Waterless Limited
Client Address	Unit 25, Hope Mills Business Centre, Brimscombe, Stroud, Gloucestershire, GL5 2SQ, United Kingdom.
Project Code	BT-CSS-03
Date of Delivery	09 July 2020
Storage conditions	Ambient
Active substances	Benzalkonium chloride; Didicyldimethyl ammonium chloride.
Appearance	Liquid
Condition upon receipt	Undamaged

Test Method and its validation

Method	1 part interfering substance + 1 part virus suspension + 8 parts biocide were mixed and incubated at the indicated contact temperature for the indicated contact times. Assays were validated by a cytotoxicity control, interference control, neutralisation control and a formaldehyde internal standard.
Neutralisation	Dilution-neutralisation/gel filtration Eagles Minimum Essential Medium + 5.0% v/v foetal bovine serum at 4°C

Experimental Conditions

Period of analysis	10 July – 15 July
Product diluents used	Sterile distilled water
Product test concentrations	10.0% v/v; 50.0% v/v; 80.0% v/v
Appearance product dilutions	No changes noted- stable
Appearance in test mixture	No changes noted- stable
Contact times (minutes)	1 ± 10s
Test temperature	20°C ± 1°C
Interfering substances	0.3g/l bovine albumin
Temperature of incubation	37°C ± 1°C + 5% CO_2
Identification and passage (P) of virus	***Vaccinia virus*** **VR-1549 Elstree strain (P07)**
Identification and passage (P) of cells	Vero Cells (P 32) (*Vaccinia Virus*)

BLUTEST LABORATORIES LIMITED, 5 Robroyston Oval, Nova Business Park, Glasgow, UK, G33 1AP
Telephone: +44 (0)141 558 2782. Email: info@blutest.com. Web site: www.blutest.com.
Company Registration Number: SC364409 VAT Registration Number: GB 979 1131 96 UKAS Number: 4597

PROTOCOL SUMMARY

The basic virucidal efficacy test is set up with three concentrations of test product solution and a1 minute contact time. Virus is exposed to disinfectant in 24-well plates, then neutralised, serially diluted and virus titred in 96-well tissue culture plates to determine the tissue culture infectious dose$_{50}$ ($TCID_{50}$) of surviving virus. *Vaccinia virus* VR-1549 Elstree strain / Vero cells are assayed in each test. $TCID_{50}$ is determined by the method of Karber[1].

Cytotoxicity control
The test product solution is measured for its effects on the host cells used to propagate the virus, to determine the sensitivity of the assay.

Interference control
The effect of the cells after treatment of the test product solution are verified to ensure the cells can show susceptibility for virus infection. This is compared against cells that have not been treated with test product.

Disinfectant suppression control VS1
Virus is added to the highest concentration of test product solution and then the mixture immediately removed and neutralised. The neutralised virus titre is then determined to assess the efficiency of the neutralisation procedure.

Disinfectant suppression control VS2
Internal control which adds virus to neutralised test product solution to assess the efficiency of the neutralisation procedure.

No column Control
Internal control on the highest contact time to assess any impact of the Microspin™ S 400 HR columns.

Virus recovery control
Virus titre is determined for virus in contact with sterile distilled water at t=0, t =1 and at t =15. The virus titre after1 minute is then compared to the recovery of disinfectant-treated virus to measure the log reduction in virus titre. The virus titre at 15 minutes is compared to the reference virus inactivation control.

Reference virus inactivation control
Virus is exposed to 0.7% W/V formaldehyde and the recovery of virus determined by $TCID_{50}$ after 5 and 15 minutes, in order to assess that the test virus has retained reproducible biocide resistance. In addition, the formaldehyde cytotoxicity of neutralised formaldehyde is determined, to measure assay sensitivity.

1Kärber, G.: Beitrag zur Kollektiven Behandlung Pharmakologischer Reihenversuche. Arch. Exp. Path. Pharmak. 162 (1931): 480-487.

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Vaccinia virus (VR-1549) Elstree strain Test Results

EN14476:2013 + A2:2019 Suspension test for the efficacy of NILAQUA, Batch 9720, BT-CSS-03 from Waterless Limited against Vaccinia virus ATCC VR-1549 under CLEAN conditions						
Test Results						
Concentration	**10.0% (v/v)**		**50.0% (v/v)**		**80.0% (v/v)**	
Exposure Time	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml
t = 1 minute	0.00	**3.16E+01**	0.00	**3.16E+01**	0.00	**3.16E+01**
Raw Data		**3.16E+01**		**3.16E+01**		**3.16E+01**
log		**1.50**		**1.50**		**1.50**
log difference		**5.00**		**5.00**		**5.00**

EN14476:2013 + A2:2019 Suspension test for the efficacy of NILAQUA, Batch 9720, BT-CSS-03 from Waterless Limited against Vaccinia virus ATCC VR-1549 under CLEAN conditions									
Summary Table									
Product:	Interfering substance	Concentration	Level of cytotoxicity	lg $TCID_{50}$					>4 lg reduction after 'X' Min
				0 min	1 min	15 min	30 min	60 min	
NILAQUA	0.3 g/l bovine albumin	80.0% (v/v)	1.50	1.50	1.50	1.50	n.a.	n.a.	< 1 minute
		50.0% (v/v)	1.50	n.a.	1.50	1.50	n.a.	n.a.	< 1 minute
		10.0% (v/v)	1.50	n.a.	1.50	1.50	n.a.	n.a.	< 1 minute
Virus Control	CLEAN			6.50	6.50	6.50	6.50	6.50	n.a.
							5 min	15 min	
Formaldehyde	PBS	0.7% (w/v)	3.50				4.67	3.50	>15 mins

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Vaccinia virus (VR-1549) Elstree strain Control Data

EN14476:2013 + A2:2019 Suspension test for the efficacy of NILAQUA, Batch 9720, BT-CSS-03 from Waterless Limited **against Vaccinia virus ATCC VR-1549 under CLEAN conditions**

Controls

Virus Recovery 0 min		Virus Recovery 1 min		Virus Recovery 15 min		Cytotoxicity		Disinfectant Suppression VS		Disinfectant Suppression VS2	
raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml
5.00	**3.16E+06**	5.00	**3.16E+06**	5.00	**3.16E+06**	0.00	**3.16E+01**	0.00	**3.16E+01**	4.67	**1.48E+06**
	3.16E+06		**3.16E+06**		**3.16E+06**		**3.16E+01**		**3.16E+01**		**1.48E+06**
	6.50		**6.50**		**6.50**		**1.50**		**1.50**		**6.17**
									5.00		**0.33**

Formaldehyde reference inactivation controls

Cytotoxicity		Exposure time	0.7% Formaldehyde			
			5 mins		15 mins	
raw data	$TCID_{50}$/ml		raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml
2.00	**3.16E+03**		3.17	**4.68E+04**	2.00	**3.16E+03**
	3.16E+03			**4.68E+04**		**3.16E+03**
	3.50	log		**4.67**		**3.50**
		log difference		**1.83**		**3.00**

No column Control

1 min

raw data	$TCID_{50}$/ml
5.33	**6.76E+06**
	6.76E+06
	6.83

Interference control	Virus dilution					
	-3	**-4**	**-5**	**-6**	**-7**	**-8**
PBS Control	1	1	1	1	0	0
	3.16E+02	3.16E+02	3.16E+02	3.16E+02	3.16E+01	3.16E+01
	2.50	**2.50**	**2.50**	**2.50**	**1.50**	**1.50**
Raw Data	**6**	**6**	**6**	**6**	**0**	**0**
Product	1	1	1	1	0.17	0
	3.16E+02	3.16E+02	3.16E+02	3.16E+02	4.68E+01	3.16E+01
	2.50	**2.50**	**2.50**	**2.50**	**1.67**	**1.50**
Raw Data	**6**	**6**	**6**	**6**	**1**	**0**
Log Difference	**0.00**	**0.00**	**0.00**	**0.00**	**-0.17**	**0.00**
Product Cyt Dilution	-1	-1	-1	-1	-1	-1
PBS Dilution	Neat	Neat	Neat	Neat	Neat	Neat

Stock Virus ($TCID_{50}$)
6.17
4.68E+07

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CONCLUSION

Verification of the methodology

A test is only valid if the following criteria are fulfilled:

a) The titre of the test suspension is sufficiently high to at least enable a titre reduction of 4 lg to verify the method.

b) Detectable titre reduction is at least 4 $\log_{10}$.

c) Difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test is between:

- Between 0.75 and 3.5 after 5 min and between 2.0 and 4.0 after 15 min for Vaccinia virus

d) Cytotoxicity of the product solution does not affect cell morphology and growth or susceptibility for the test virus in the dilutions of the test mixtures which are necessary to demonstrate a 4 $\log_{10}$ reduction of the virus.

e) The interference control result does not show a difference of > 1.0 $\log_{10}$ of virus titre for test product treated cells in comparison to the non-treated cells.

f) Neutralisation validation. This is called the disinfectant suppression test in this protocol. The disinfectant was neutralised by column chromatography through an Illustra Microspin S-400 HR column to achieve the best possible neutralisation available for this test. The difference for virus is greater than 0.5 $\log_{10}$ indicating rapid irreversible virucidal activity of the disinfectant by dilution at a concentration of 80.0% v/v for VS1. This neutralisation validation has been verified by VS2, which shows the product has been successfully neutralised. The difference for virus is not greater than 0.5 $\log_{10}$ indicating effective neutralisation of the virucidal activity of the disinfectant by dilution at a concentration of 80.% v/v.

According to EN 14476:2013 + A2:2019, **NILAQUA POSSESSES VIRUCIDAL** activity at a concentration of **10.0, 50.0 and 80.0 % v/v** of the working concentration as tested after **1 MINUTE** at **20°C** under **CLEAN** conditions (0.3 g/l bovine albumin) against *Vaccinia virus* VR-1549 Elstree strain / Vero cells.

This product therefore is effective against all enveloped viruses as defined in EN 14476:2013 + A2:2019 Annex A*. This therefore includes all coronaviruses and SARS-CoV-2.

Authorised signatory

Dr Chris Woodall, Director
BluTest Laboratories Ltd
Glasgow, UK
Date: 24 JULY 2020

BLUTEST LABORATORIES LIMITED, 5 Robroyston Oval, Nova Business Park, Glasgow, UK, G33 1AP
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*EN 14476 2013 + A2 2019 Annex A (informative – Enveloped viruses)

Poxviridae
Herpesviridae
Filoviridae (e.g. Ebola, Marburg)
Flavivirus
Hepatitis C Virus (HCV)
Hepatitis Delta Virus (HDV)
Influenza Virus
Paramyxoviridae
Rubella Virus
Measles Virus
Rabies Virus
Coronavirus (e.g. SARS, MERS)
Human Immunodeficiency Virus (HIV)
Human T Cell Leukemia Virus (HTLV)
Hepatitis B virus (HBV)

Reference: Van Regenmortel MHV et al.,Eds.: Virus Taxonomy, Classification and Nomenclature of Viruses, seventh report of the international committee on taxonomy of viruses. Academic Press, San Diego, 2000

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