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	Crebisol
Batch number	300919
Client	Crebisol Limited
Client Address	1st Floor, 50 Main Street, Newcastle, BT33 0AD
Project Code	BT-CRB-01
Date of Delivery	06 April 2020
Storage conditions	Ambient
Active substances	DDQ50
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 NCTC media + 10.0% v/v horse serum at 4°C

Experimental Conditions

Period of analysis	05 June 2020 to 08 June 2020
Product diluents used	Sterile, synthetic hard water
Product test concentrations	1.0% v/v; 2.0% v/v; 5.0% v/v
Appearance product dilutions	No changes noted- stable
Appearance in test mixture	Sedimentation and Turbidity observed at all concentrations
Contact times (minutes)	2 minutes ± 10s; 5 minutes ± 10s
Test temperature	20°C ± 1°C
Interfering substances	3.0 g/l bovine albumin + 3.0 ml/l erythrocytes
Temperature of incubation	37°C ± 1°C + 5% CO_2
Identification and passage (P) of virus	***Murine coronavirus*** **A59 ATCC VR-764 (P8)**
Identification and passage (P) of cells	NCTC clone 1469 cells (P29)

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 a 2-minute and 5-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. *Murine coronavirus* A59 ATCC VR-764/ NCTC clone 1469 cells are assayed in parallel 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 hard water at t=0, t = 5 and at t =60. The virus titre after 5 minutes is then compared to the recovery of disinfectant-treated virus to measure the log reduction in virus titre. The virus titre at 60 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, 15, 30 and 60 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.

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

Murine coronavirus (A59) Test Results

EN14476:2013 + A2:2019 Suspension test for the efficacy of Crebisol, Batch 300919, BT-CRB-01 from Crebisol Limited against Murine hepatitis virus (A59) under DIRTY conditions						
Test Results						
Concentration	**1.0% (v/v)**		**2.0% (v/v)**		**5.0% (v/v)**	
Exposure Time	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml
t = 2 mins	2.00	**3.16E+03**	1.00	**3.16E+02**	0.00	**3.16E+01**
Raw Data	660000	**3.16E+03**	600000	**3.16E+02**	000000	**3.16E+01**
log		**3.50**		**2.50**		**1.50**
log difference		**2.00**		**3.00**		**4.00**
Exposure Time	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml
t = 5 mins	2.00	**3.16E+03**	1.00	**3.16E+02**	0.00	**3.16E+01**
Raw Data	660000	**3.16E+03**	600000	**3.16E+02**	000000	**3.16E+01**
log		**3.50**		**2.50**		**1.50**
log difference		**2.00**		**3.00**		**4.00**

EN14476:2013 + A2:2019 Suspension test for the efficacy of Crebisol, Batch 300919, BT-CRB-01 from Crebisol Limited against Murine hepatitis virus (A59) under DIRTY conditions									
Summary Table									
Product:	Interfering substance	Concentration	Level of cytotoxicity	lg $TCID_{50}$					>4 lg reduction after 'X' Min
				0 min	2 min	5 min	15 min	60 min	
Crebisol	3.0g/l BSA + 3.0ml/l erythrocytes	5.0% (v/v)	1.50	1.50	1.50	1.50	n.a.	n.a.	>2 mins
		2.0% (v/v)	1.50	n.a.	2.50	2.50	n.a.	n.a.	>5 mins
		1.0% (v/v)	1.50	n.a.	3.50	3.50	n.a.	n.a.	>5 mins
Crebisol	3.0g/l BSA	5.0% (v/v)	1.50	n.a.	1.50	1.50	n.a.	n.a.	<2 mins
		2.0% (v/v)	1.50	n.a.	2.50	2.50	n.a.	n.a.	>5 mins
		1.0% (v/v)	1.50	n.a.	3.50	3.50	n.a.	n.a.	>5 mins
Virus Control	DIRTY			5.50	n.a.	5.50	5.33	n.a.	n.a.
Virus Control	CLEAN			5.50	n.a.	5.50	5.50	n.a.	n.a.
Formaldehyde	PBS	0.7% (w/v)	3.50	n.a.	3.50	3.50	3.50	3.50	>60 mins

BLUTEST LABORATORIES LIMITED, 5 Robroyston Oval, Nova Business Park, Glasgow, UK, G33 1AP
Tel: +44 (0)141 558 2782. Email: info@blutest.com. Web site: www.blutest.com. Company No.: SC364409. VAT No.: GB 979 1131 96. UKAS No.: 4597

Murine coronavirus (A59) Control Data

EN14476:2013 + A2:2019 Suspension test for the efficacy of Crebisol, Batch 300919, BT-CRB-01 from Crebisol Limited against Murine hepatitis virus (A59) under DIRTY conditions

Controls

Virus Recovery 0 min		Virus Recovery 5 min		Virus Recovery 60 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
4.00	**3.16E+05**	4.00	**3.16E+05**	3.83	**2.15E+05**	0.00	**3.16E+01**	0.00	**3.16E+01**	3.50	**1.00E+05**
666600	**3.16E+05**	666600	**3.16E+05**	666500	**2.15E+05**	000000	**3.16E+01**	000000	**3.16E+01**	666210	**1.00E+05**
	5.50		**5.50**		**5.33**		**1.50**		**1.50**		**5.00**
									4.00		**0.50**

Formaldehyde reference inactivation controls

Cytotoxicity			0.7% Formaldehyde								
		Exposure time	5 mins		15 mins		30 mins		60 mins		
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	
2.00	**3.16E+03**		2.00	**3.16E+03**	2.00	**3.16E+03**	2.00	**3.16E+03**	2.00	**3.16E+03**	
660000	**3.16E+03**		660000	**3.16E+03**	660000	**3.16E+03**	660000	**3.16E+03**	660000	**3.16E+03**	
	3.50	log		**3.50**		**3.50**		**3.50**		**3.50**	
		log difference		**1.83**		**1.83**		**1.83**		**1.83**	

Interference control	Virus dilution						No column Control	
	-3	-4	-5	-6	-7	-8	5 mins	
PBS Control	1	1	1	0.33	0	0	raw data	$TCID_{50}$/ml
	3.16E+02	3.16E+02	3.16E+02	6.76E+01	3.16E+01	3.16E+01	4.17	**4.64E+05**
	2.50	**2.50**	**2.50**	**1.83**	**1.50**	**1.50**	666610	**4.64E+05**
Raw Data	**6**	**6**	**6**	**2**	**0**	**0**		**5.67**
Product	1	1	1	0.5	0	0		
	3.16E+02	3.16E+02	3.16E+02	1.00E+02	3.16E+01	3.16E+01		
	2.50	**2.50**	**2.50**	**2.00**	**1.50**	**1.50**		
Raw Data	**6**	**6**	**6**	**3**	**0**	**0**	**Stock Virus ($TCID_{50}$)**	
Log Difference	**0.00**	**0.00**	**0.00**	**-0.17**	**0.00**	**0.00**	5.50	
Product Cyt Dilution	-1	-1	-1	-1	-1	-1	**1.00E+07**	
PBS Dilution	Neat	Neat	Neat	Neat	Neat	Neat	6666630000	

BLUTEST LABORATORIES LIMITED, 5 Robroyston Oval, Nova Business Park, Glasgow, UK, G33 1AP
Tel: +44 (0)141 558 2782. Email: info@blutest.com. Web site: www.blutest.com. Company No.: SC364409. VAT No.: GB 979 1131 96. UKAS No.: 4597

Murine coronavirus (A59) Control Data

Parallel Control Test												
Controls						Test Results						
Virus Recovery 0 min		Virus Recovery 5 min		Virus Recovery 60 min		Concentration	1.0% (v/v)		2.0% (v/v)		5.0% (v/v)	
raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	raw data	$TCID_{50}$/ml	**Exposure Time**	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml
4.00	**3.16E+05**	4.00	**3.16E+05**	4.00	**3.16E+05**	**t = 2 mins**	2.00	**3.16E+03**	1.00	**3.16E+02**	0.00	**3.16E+01**
666600	**3.16E+05**	666600	**3.16E+05**	666600	**3.16E+05**	Raw data	660000	**3.16E+03**	600000	**3.16E+02**	000000	**3.16E+01**
	5.50		**5.50**		**5.50**	log		**3.50**		**2.50**		**1.50**
						log difference		**2.00**		**3.00**		**4.00**
						Exposure Time	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml	data	$TCID_{50}$/ml
						t = 5 mins	2.00	**3.16E+03**	1.00	**3.16E+02**	0.00	**3.16E+01**
						Raw data	660000	**3.16E+03**	600000	**3.16E+02**	000000	**3.16E+01**
						log		**3.50**		**2.50**		**1.50**
						log difference		**2.00**		**3.00**		**4.00**

BLUTEST LABORATORIES LIMITED, 5 Robroyston Oval, Nova Business Park, Glasgow, UK, G33 1AP
Tel: +44 (0)141 558 2782. Email: info@blutest.com. Web site: www.blutest.com. Company No.: SC364409. VAT No.: GB 979 1131 96. UKAS No.: 4597

CONCLUSION

Verification of the methodology

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

a) The titre of the test suspension of at least 10^8 TCID50 /ml 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 0.5 and 2.5 after 30 min and between 2.0 and 4.5 after 60 min for poliovirus
    - Between 3.0 and 5.0 after 30 min and between 3.5 and 5.5 after 60 min for adenovirus
    - Between 1.0 and 3.0 after 30 min and between 2.0 and 4.0 after 60 min for murine norovirus
    - Between 0.0 and 2.0 after 30 min and between 0.5 and 2.5 after 60 min for parvovirus
    - 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 5.0% v/v for VS1. This neutralisation validation has been verified by VS2, which shows the product has been successfully neutralised.

According to EN 14476:2013 + A2:2019, **Crebisol POSSESSES VIRUCIDAL** activity at a concentration of **5.0% v/v** as tested after **2 MINUTES** at **20°C** under (**DIRTY** conditions (3.0 g/l bovine albumin + 3.0 ml/l erythrocytes) against *Murine coronavirus* (A59) ATCC VR-764/ NCTC clone 1469 cells, a surrogate for SARS-CoV-1,2 and MERS CoV.

Murine coronavirus (also known as murine hepatitis virus) as a surrogate for SARS-CoV-2/Covid-19 is the type species of the Betacoronavirus genus that includes SARS-CoV-1&2; MERS-CoV.
Genus Betacoronavirus; Type species: Murine coronavirus
Species: Betacoronavirus 1, Human coronavirus HKU1, Murine coronavirus, Pipistrellus bat coronavirus HKU5, Rousettus bat coronavirus HKU9, Severe acute respiratory syndrome-related coronavirus 1, Severe acute respiratory syndrome-related coronavirus-2, Tylonycteris bat coronavirus HKU4, Middle East respiratory syndrome-related coronavirus, Human coronavirus OC43, Hedgehog coronavirus 1 (EriCoV)

This genus includes (source) bat coronaviruses, pre-existing identified human coronaviruses not associated with severe acute respiratory distress, SARS-CoV 1,2 and MERS-CoV.

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

Authorised signatory

Dr Chris Woodall, Director
BluTest Laboratories Ltd
Glasgow, UK
Date: 09 JUNE 2020

DISCLAIMER

The results in this test report only pertain to the sample supplied.
BluTest (BT) has performed the testing detailed in this report using reasonable skill and care and has used reasonable endeavours to carry out the testing in accordance with an EN 14476 protocol. All forecasts, recommendations and results contained in this report are submitted in good faith. However, other than as expressly set out in this report, no warranty is given (i) in relation to the testing or the use(s) to which any results or deliverables produced in the course of the testing are or may be put by the Client or their fitness or suitability for any particular purpose or under any special conditions notwithstanding that any such purpose or conditions may have been made known to BT or (ii) that the intended results or deliverables from the testing can be achieved or (iii) that the Client can freely make use of the results or the deliverables without infringing any third party intellectual property rights and the Client will be deemed to have satisfied itself in this regard. BT shall have no liability (which is hereby excluded to the fullest extent permissible by law) in respect of any loss, liability or damage, including without limitation any indirect and/or consequential loss such as loss of profit or loss of business, market or goodwill, that the Client may suffer directly or indirectly as a result of or in connection with: (i) the performance of the testing; (ii) the use of any materials, samples or other information provided by the Client for use in the testing; and (iii) the Client's reliance upon or use of any results or deliverables provided as part of the testing.

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