MS ISO/IEC 17025
TESTING
SAMM NO. 823

Test Report No.: VX-TR-20-0268
Copy No.: 1

DETERMINATION OF THE VIRUCIDAL ACTIVITY (EN 14476) OF NAOCLEAN

Lab No.:	VX-72-20-0001
Sample Name:	**Naoclean**
Method:	EN 14476:2013+A1:2015 (E) 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)
Client:	PECA Disinfectant Pro (M) Sdn. Bhd. No. 16-3, Jalan Jalil Jaya 6, Jalil Link, Bukit Jalil 57000 Kuala Lumpur Malaysia
Sample Receipt Date:	29 May 2020
Report Date:	9 June 2020

Kuala Lumpur, 9 June 2020

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Materials and Method

Quantitative suspension test for the evaluation of virucidal activity in the medical area according to EN 14476:2013+A1:2015 (E)

1. Testing laboratory identification

Viroxy Sdn. Bhd.
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2. Sample identification

2.1	Sample name:	Naoclean
2.2	Batch no.:	Naoclean20200510
2.3	Product appearance:	Clear colourless solution
2.4	Manufacturer:	PECA Disinfectant Pro (M) Sdn. Bhd. No. 16-3, Jalan Jalil Jaya 6, Jalil Link, Bukit Jalil 57000 Kuala Lumpur Malaysia
2.5	Active substances per 100 g:	Electrolysis Sodium Hypochlorite
2.6	Sample receipt date:	29 May 2020
2.7	Storage conditions:	Room temperature
2.8	Product diluent:	Distilled water

3. Experimental conditions

3.1	Testing period:	29 May – 9 June 2020
3.2	Test organism(s):	*Human coronavirus*, strain 229E, ATCC VR-740
3.3	Concentration/contact time:	100.00 %* / 30 and 60 minutes
3.4	Loading:	0.30 g/L bovine albumin solution
3.5	Test temperature:	20 °C ± 1 °C
3.6	Incubation period:	5 days, 36 °C ± 1 °C

4. Test method and its validation

4.1 Testing method: Quantal test

4.2 Inactivation method: Immediate dilution
Molecular sieving using MicroSpin™ S 400 HR (for formaldehyde only)

The results of validation test A, B, and C proved the viability of the method in all cases.

5. Test results

The results are stated in Tables A and B.

6. Conclusion

Naoclean showed the required virus reduction of ≥4.0 $\log_{10}$ against test strain *Human coronavirus* ATCC VR-740 in accordance with EN 14476:2013+A1:2015 (E) at 100.00 %* concentration after 30 minutes and 60 minutes under the stated condition. According to the simple acceptance decision rule†, there is a < 50 % risk of false acceptance for exposure time of 30 minutes and minimal risk of false acceptance of exposure time of 60 minutes.

Kuala Lumpur, 9 June 2020

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7. Note

Virucidal activity – the capability of a product to produce a reduction in the number of viable viruses belonging to reference strains under defined conditions by at least 4 orders (10^4).

$R = V_C/N_a$ = the reduction in viability, or $\lg R = \lg V_C - \lg N_a$

* The product can only be tested at 97.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

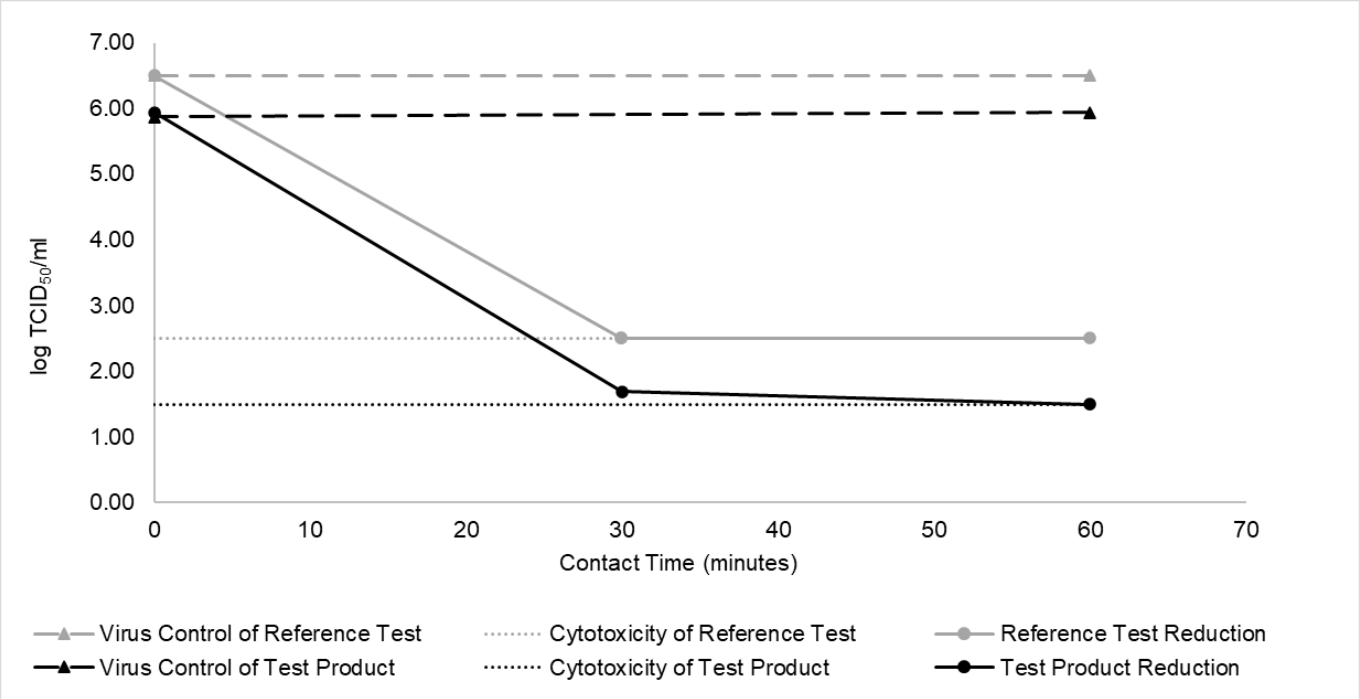
Table A: Evaluation of the virucidal activity of Naoclean on test strains according to EN 14476

Product: Naoclean
Loading: 0.30 g/L bovine albumin solution

Test strain: *Human coronavirus* ATCC VR-740

Virus control, V_C	Cytotoxicity effect, CE
V_{C1}: 6.00± 0.38 V_{C2}: 5.88 ± 0.37	CE_1: 1.50 ± 0.00 CE_2: 1.50 ± 0.00

Test concentration (%) / contact time (min)	First assay, N_{a1}	Second assay, N_{a2}	Average reduction
100.00* / 30	N_{a1}: 1.88 ± 0.37 lg R_1: **4.13 ± 0.53**	N_{a2}: ≤1.50 ± 0.00 lg R_2: **≥4.38 ± 0.37**	lg R: **≥4.26 ± 0.46**
100.00* / 60	N_{a1}: ≤1.50 ± 0.00 lg R_1: **≥4.50 ± 0.38**	N_{a1}: ≤1.50 ± 0.00 lg R_1: **≥4.38 ± 0.37**	lg R: **≥4.44 ± 0.38**



* The product can only be tested at 97.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

Table B: Control tests and method validation for Table A

Test strain	Cell susceptibility control	Suppression efficiency control	Reference test for virus inactivation
Human coronavirus ATCC VR-740	A: 6.13 ± 0.37 A_{PBS}: 6.25 ± 0.33	B: 5.50 ± 0.00 V_C: 5.75 ± 0.33	$C_{30:}$ ≥4.00 ± 0.00 $C_{60:}$ ≥4.00 ± 0.00

Note

$TCID_{50}$: The dilution of the virus suspension that induces a cytopathic effect (CPE) in 50 % of cell culture units

CPE: The morphological alteration of cells and/or their destruction caused by the cytopathic effect of virus multiplication.

V_C: log_{10} $TCID_{50}$ per ml in the viral test suspension at the beginning and at the maximum contact time

N_a: log_{10} $TCID_{50}$ per ml in the test mixture at the end of the contact time

CE: The morphological alteration of cells caused by the cytotoxicity effect of the product test solution.

A: log_{10} $TCID_{50}$ per ml in the cell susceptibility control as compared to PBS

B: log_{10} $TCID_{50}$ per ml in the suppression efficiency control as compared to the virus control

C: log_{10} $TCID_{50}$ per ml in the reference test for virus inactivation after 30 and 60 minutes (5 and 15 minutes for vaccinia virus)

Table C: Summary of the log reductions of the quantitative suspension test according to EN 14476

Test strain	Test concentration (%) / contact time (min)	Log reduction ($TCID_{50}$/ml)	Associated risk†
Human coronavirus ATCC VR-740	100.00* / 30	**≥4.26 ± 0.46**	< 50 % risk of false acceptance
	100.00* / 60	**≥4.44 ± 0.38**	Minimal risk of false acceptance

* The product can only be tested at 97.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

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Efficacy of Naoclean against *Human coronavirus,* strain 229E, ATCC VR-740*,* in a quantitative suspension test at 20 °C according to EN 14476:2013+A1:2015 (E) under clean condition

EXPERT OPINION*

This expert opinion is based on the test report VX-TR-20-0268 dated 9 June 2020.

The virucidal activity of the disinfectant Naoclean of PECA Disinfectant Pro (M) Sdn. Bhd. against *Human coronavirus* ATCC VR-740 was investigated by a quantitative suspension test according to EN 14476:2013+A1:2015 (E) under clean condition (0.30 g/L bovine albumin solution).

According to this suspension test, a disinfectant or a disinfectant solution at a particular concentration is considered as having virucidal activity if the virus titre is reduced by ≥ 4 $\log_{10}$ (inactivation ≥99.99 %) within the recommended exposure period.

Naoclean was examined at 20 °C at the concentration of 100.00 %** for the exposure times of 30 and 60 minutes. After the exposure times, the viral reduction exceeded 4 $\log_{10}$-steps in all assays. According to the simple acceptance decision rule†, there is a < 50 % risk of false acceptance for exposure time of 30 minutes and minimal risk of false acceptance of exposure time of 60 minutes. Therefore, a virucidal activity against for *Human coronavirus* ATCC VR-740 was measured as follows:

Clean condition	100.00 %**	30 minutes
Clean condition	100.00 %**	60 minutes

Kuala Lumpur, 9 June 2020

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Dr Peter Cheong
Head of Microbiology Laboratories

* Opinions and interpretations expressed here are outside the scope of SAMM (Laboratory Accreditation Scheme of Malaysia) accreditation.

** The product can only be tested at 97.00 % concentration or less, as some dilution always occurs when test organisms and interfering substance are added.

† The decision rule applied is simple acceptance rule with no guard band and up to 50 % risk of false acceptance or rejection. This rule has been determined by the laboratory and agreed with the client prior to testing.

Appendix 1

QAU CERTIFICATE*

The results stated in test report VX-TR-20-0268 dated 9 June 2020 were compared to the raw data of the tests and checked for correct transfer. No deviations were detected.

Kuala Lumpur, 9 June 2020

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Appendix 2 Raw data

Test Method	EN 14476:2013+A1:2015			**Titration Method**	Quantal test		
Product	Naoclean			**Batch No.**	Naoclean20200510		
Product Diluent	Distilled water			**Lab No.**	VX-72-20-0001		
Test Organism	Human coronavirus, strain 229E, ATCC VR-740			**Passage No.**	4		
Cell Line	MRC-5 cells, ATCC CCL-171			**Passage No.**	6		
Interfering Substance	0.30 g/L bovine albumin solution			**Inactivation Method**		Immediate dilution	
Test Temperature (°C)	20	**Incubation Temperature (°C)**	36	**Dilution Method**		Modified	
First Assay Test Date	29/05/2020	**Second Assay Test Date**	04/06/2020	**Analyzed By**	SSU	**Verified By**	PCH

Validation and Control Procedures

	Product Concentration	Dilution	Dilution (log_{10}) 1	2	3	4	5	6	7	8	9	10	log_{10} $TCID_{50}$/ml	$\Delta TCID_{50}$ < 1 lg
Cell Susceptibility Control	PBS	Without	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 0 4 4 3 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	6.25 ± 0.33	Pass? **Yes**
	100.00 %	1:10	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 0 4 4 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	6.13 ± 0.37	

	Product Concentration	Contact Time (minutes)	Dilution (log_{10}) 1	2	3	4	5	6	7	8	9	10	log_{10} $TCID_{50}$/ml	$\lvert TCID_{50} - V_C \rvert$ ≤ 0.5 lg
Suppression Efficiency Control	100.00 %	30	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	5.50 ± 0.00	Pass? **Yes**
	Virus Control (V_C)	30	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 0 0 0 3 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	5.75 ± 0.33	

	Product Concentration	Contact Time (minutes)	Dilution (log_{10}) 1	2	3	4	5	6	7	8	9	10	log_{10} $TCID_{50}$/ml	lg R = V_C - Na
Reference Test	0.70 % Formaldehyde	30	t t t t t t t t	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	2.50 ± 0.00	≥4.00 ± 0.00
		60	t t t t t t t t	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	2.50 ± 0.00	≥4.00 ± 0.00
	Virus Control (V_C)	0	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 3	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	6.50 ± 0.00	
		60	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	6.50 ± 0.00	
	Cytotoxicity Effect (CE)	-	t t t t t t t t	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	2.50 ± 0.00	

Appendix 2 Raw data

Test Procedure

	Product Concentration	Contact Time (minutes)	Dilution (log_{10}) 1	2	3	4	5	6	7	8	9	10	log_{10} $TCID_{50}$/ml	
First Assay (Na_1)	100%	30	4 4 4 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	1.88 ± 0.37	V_{C1} - CE ≥ 4 Pass? **Yes**
	100%	60	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	1.50 ± 0.00	
	Virus Control (V_{C1})	0	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 0 0 0 4 4 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	5.88 ± 0.37	
		60	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 0 0 4 3 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	6.00 ± 0.38	
	Cytotoxicity Effect (CE)	-	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	n.d.	1.50 ± 0.00	

	Product Concentration	Contact Time (minutes)	Dilution (log_{10}) 1	2	3	4	5	6	7	8	9	10	log_{10} $TCID_{50}$/ml	
Second Assay (Na_2)	100%	30	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	1.50 ± 0.00	
	100%	60	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	1.50 ± 0.00	V_{C2} - CE ≥ 4 Pass? **Yes**
	Virus Control (V_{C2})	0	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 3 0 0 4 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	5.88 ± 0.37	
		60	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4	4 4 0 0 4 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	5.88 ± 0.37	
	Cytotoxicity Effect (CE)	-	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0	n.d.	n.d.	n.d.	n.d.	n.d.	1.50 ± 0.00	

Average Reduction (lg R)	Product Concentration	Contact Time (minutes)	First Assay (Na_1) log_{10} $TCID_{50}$/ml	First Assay (Na_1) lg $R_1 = V_{C1}$ - Na_1	Second Assay (Na_2) log_{10} $TCID_{50}$/ml	Second Assay (Na_2) lg $R_2 = V_{C2}$ - Na_2	Average Reduction (lg R)
	100%	30	1.88 ± 0.37	4.13 ± 0.53	≤1.50 ± 0.00	≥4.38 ± 0.37	≥4.26 ± 0.46
	100%	60	≤1.50 ± 0.00	≥4.50 ± 0.38	≤1.50 ± 0.00	≥4.38 ± 0.37	≥4.44 ± 0.38

Note

$TCID_{50}$: The dilution of the virus suspension that induces a CPE in 50 % of cell culture units

CPE: The morphological alteration of cells and/or their destruction caused by the cytopathic effect of virus multiplication. '0' denotes no CPE and '1' (approximately 25 % of cells) to '4' (all cells) denotes the degree of CPE per cell culture units.

V_C: log_{10} $TCID_{50}$ per ml in the viral test suspension at the beginning and at the maximum contact time

N_a: log_{10} $TCID_{50}$ per ml in the test mixture at the end of the contact time

CE: The morphological alteration of cells caused by the cytotoxicity effect of the product test solution. 't' denotes the presence of cytotoxicity per cell culture units.

A: log_{10} $TCID_{50}$ per ml in the cell susceptibility control as compared to PBS

B: log_{10} $TCID_{50}$ per ml in the suppression efficiency control as compared to the virus control

C: log_{10} $TCID_{50}$ per ml in the reference test for virus inactivation after 30 and 60 minutes (5 and 15 minutes for vaccinia virus)

Appendix 3 Summary of test description

1. Virus and cells

1.1. *Human coronavirus,* strain 229E, ATCC VR-740

1.1.1. Passage no.: 4

1.1.2. Cell line: MRC-5 cells, ATCC CCL-171

1.1.3. Cell line passage no.: 6

1.1.4. Culture medium: EMEM

2. Materials and reagents

2.1. Eagle's Minimal Essential Medium (EMEM, Sigma, catalogue no. M3024)

2.2. Fetal Bovine Serum (FBS, Sigma, catalogue no. F7524)

2.3. Formaldehyde (Merck, catalogue no. 1.0.4003.2500)

2.4. Dulbecco's Phosphate Buffered Saline (PBS, Sigma, catalogue no. P3813)

2.5. Bovine albumin fraction V (Merck, catalogue no. K49238418733)

3. Apparatus and glassware

3.1. CO_2 incubator (Memmert, model ICO 105)

3.2. Cooling water bath (Memmert, model WNB7 with CDP115)

3.3. Inverted microscope (Optika, IM-2)

3.4. Vortex® mixer (Biosan model Biosan V-1 Plus)

3.5. Microtitre plate (NEST)

3.6. Tissue culture flask (JET Biofil)

4. Test procedure

4.1. Preparation of test virus suspension

4.1.1. Cell monolayers shall be >90 % confluent before inoculation. Cell lines are selected in accordance with their sensitivity to the test organisms.

4.1.2. The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353:2013 (E).

4.1.3. The stock virus suspension is multiplied in an appropriate cell line that produces high titres of infectious viruses for 1 hour at 36 °C with intermittent tilting every 15 minutes.

4.1.4. The cells are subjected to 3 freeze/thaw cycles once cytopathic effect (CPE) is observed in 80 % of the cell population.

4.1.5. Separate the cells debris is by centrifugation at 400 g_N for 15 minutes.

4.1.6. Aliquot the supernatant containing the test virus suspension and store at -80 °C.

4.2. Test Na – Determination of virucidal concentrations

4.2.1. Pipette 0.1 ml of interfering substance into a container of suitable capacity for appropriate mixing.

4.2.2. Add 0.1 ml of the virus test suspension to the container, carefully avoiding the upper part of the sides. Mix well.

4.2.3. Add 0.97 ml of the product test solution to the container.

4.2.4. Mix, start a stopwatch at once, and place the container in a water bath controlled at the chosen test temperature.

4.2.5. Immediately at the end of the chosen contact time, mix, pipette 0.5 ml of the test mixture (virus suspension, interfering substance, and product test solution) into 4.5 ml ice-cold maintenance medium and put into an ice bath.

4.2.6. Within 30 minutes, prepare a series of ten-fold dilutions of this mixture (text mixture and maintenance medium).

4.2.7. Transfer 0.1 ml of each dilution into six or eight wells of a microtitre plate containing a confluent (>90 %) cell monolayer without any medium.

4.2.8. The last row of six or eight wells will receive 0.1 ml of culture medium and will serve as the cell control.

4.2.9. After 1 hour of incubation at 37 °C, 0.1 ml of cell culture medium is added to each well.

4.2.10. After incubation, the virus titre is calculated. The reduction of virus infectivity is determined from differences of $\log_{10}$ virus titres before and after treatment with the product.

4.3. Cytotoxicity effect – determination of the morphological alteration of cells caused by the product test solution

4.3.1. Mix 0.1 part of hard water and 0.1 part of interfering substances with9.78 parts of the product test solution.

4.3.2. Serial dilutions are prepared in the culture medium and are inoculated into cell monolayers.

4.3.3. This test is done in parallel with Section 4.2.

4.3.4. Any microscopic changes in the cells are recorded when reading the tests for CPE.

4.3.5. If the cytotoxicity is so great that the residual infectivity titre is smaller than the required $\log_{10}$ $TCID_{50}$, special techniques have to be used, such as molecular sieving or ultrafiltration. Follow the instructions of the manufacturer.

4.4. **Cell susceptibility control A – Verification of the susceptibility of the cells for virus infection is not influenced negatively by the treatment with the product test solution**

4.4.1. Comparative virus titrations are performed on cells that have or have not been treated with product test solution to check the reduction of the sensitivity to viruses.

4.4.2. 0.1 ml of the lowest apparently non-cytotoxic dilution (no microscopic alteration) of the product test solution or PBS and 0.1 ml of culture medium are distributed onto each of 6 established cell cultures in 96-well microtitre plates.

4.4.3. After 1 hour of incubation at 37 °C, the supernatant is discarded.

4.4.4. The virus is diluted from 10^{-1} to 10^{-10} and titrated on the treated or untreated cells.

4.4.5. Verify according to Section 4.8.

4.5. **Suppression efficiency control B – Immediate dilution method validation**

4.5.1. Immediately after preparation of the test mixture in Section 4.2, pipette 0.5 ml of the test mixture (virus suspension, interfering substance, and product test solution) into 4.5 ml of ice-cold maintenance medium.

4.5.2. Mix again and start the clock. Incubate the mixture in the ice bath for 30 minutes ± 10 seconds.

4.5.3. Immediately prepare dilutions up to 10^{-8} and titrate the virus.

4.5.4. This control is performed in parallel to the test.

4.5.5. Verify according to Section 4.8.

4.6. **Reference test for virus inactivation C – Validation of the test system**

4.6.1. 2 ml of the test suspension shall be mixed with 8 ml of PBS and 10 ml of 1.4 % (w/v) formaldehyde.

4.6.2. Contact times are 30 and 60 minutes.

4.6.3. Immediately at the end of the contact time, mix and pipette 0.2 ml of the test mixture into a tube containing 1.8 ml ice-cold maintenance medium followed by a further 10-fold dilution.

4.6.4. Leave the mixture in the ice bath.

4.6.5. Dilutions up to 10^{-6} are prepared by pipetting the diluted test mixture into another tube containing ice-cold maintenance medium in the ice bath.

4.6.6. In exceptional cases, smaller volumes of the reagents and of the test suspension could be used, ensuring that the relative proportions are maintained.

4.6.7. The cytotoxic control of the formaldehyde shall be performed according to Section 4.3 whereby 8 ml of 1.4 % (w/v) formaldehyde is used instead of the product.

4.6.8. The mixture is further diluted to 10^{-5} in an ice bath.

4.6.9. Verify according to Section 4.8.

4.7. **Titration of the virus control**

4.7.1. The infectivity of the test suspension shall be determined under test conditions at the beginning of the contact time and at the maximum contact time used in the test.

4.7.2. Section 4.2 is repeated by substituting the product test solution with hard water or water for ready-to-use products.

4.7.3. Verify according to Section 4.8.

4.8. **Verification of methodology**

4.8.1. The titre of the test suspension (virus control) of at least 10^8 $TCID_{50}$/mL is sufficiently high to at least enable a titre reduction of 4 log to verify the method. The detectable titre reduction shall be at least 4 log.

4.8.2. Cytotoxicity of the product test solution does not affect cell morphology and growth or susceptibility for the test organism in the dilutions of the test mixtures which are necessary to demonstrate a 4-log reduction of the virus.

4.8.3. Comparative virus titration on cells cultures treated with test mixture dilutions and in parallel with PBS (cell susceptibility control) result in a difference of <1 log of virus titre.

4.8.4. The difference to the test suspension in the control of efficiency for suppression of products' activity shall be ≤0.5 log.

4.8.5. The difference between the logarithmic titre of the virus control and the logarithmic titre of the test organism in the reference inactivation test is:

4.8.5.1. Between -0.5 and -2.5 after 30 minutes and between -2 and -4.5 after 60 minutes for poliovirus

4.8.5.2. Between -3 and -5 after 30 minutes and between -3.5 and -5.5 after 60 minutes for adenovirus

4.8.5.3. Between 0.0 and -2.0 after 30 minutes and between -0.5 and -2.5 after 60 minutes for parvovirus

4.8.5.4. Between -0.75 and -3.5 after 20 and 30 minutes and between -2.0 and ≥-4.0 after 120 and 30 minutes for vacciniavirus.

5. Literature

5.1. EN 14476:2013+A1:2015 (E): 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)

5.2. EN 14885:2015 (E): Chemical disinfectants and antiseptics – Application of European Standards for chemical disinfectants and antiseptics

5.3. EN 12353:2013 (E): Chemical disinfectants and antiseptics – Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity

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