NARODOWY INSTYTUT ZDROWIA PUBLICZNEGO
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Evaluation of virucidal activity of product

Alco Cid A

Test method according to PN-EN 14476:2013+A1:2015 – „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)"

Warsaw, 2016-03-14

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Warsaw, 14th March 2016.

CID LINES Sp. z o.o.
ul. Świerkowa 20, Niepruszewo,
64-320 Buk

Re.: the virucidal activity of Alco Cid A

Department of Virology NIPH-NIH inform, that according to order of CID LINES Sp. z o.o., the product Alco Cid A for disinfection of surfaces was tested. The aim of this study was to evaluate the virucidal activity of disinfectant Alco Cid A.

The study was performed using quantitative suspension assay according to PN-EN 14476:2013+A1:2015 – „Chemical disinfectants and antiseptics – Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine – Test method and requirements (phase 2, step 1)". Test was done under clean test conditions with poliovirus type 1, adenovirus type 5 and murine norovirus according to guidelines for the products for surface disinfection.

Test was performed using the standard method and the modified method. Final concentration of tested disinfectant Alco Cid A (ready-to-use product) in test procedure was 80% (standard method) or 97% (modified method).

The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control mixture and infectious virus titer in a test mixture containing a specified concentration of the test product and interfering substance, incubated for a specified contact time).

Obtained results of examinations showed that the tested product Alco Cid A, as ready-to-use product, was able to inactivate poliovirus type 1, adenovirus type 5 and murine norovirus under clean test conditions, after 1 minute of contact time.

On the basis of all performed studies and according to guidelines of standard PN-EN 14476:2013+A1:2015 it can be concluded that, the disinfectant Alco Cid A, as a ready-to-use product, has a virucidal activity under clean test conditions after 1 minute of contact time.

KIEROWNIK
Zakładu Wirusologii
Dr hab. Bogumiła Litwińska
Profesor nadzwyczajny w NIZP-PZH

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Evaluation of virucidal activity

of product

Alco Cid A

Poliovirus type 1

(clean test conditions)

Warsaw, 2016-03-14

1. Test Laboratory

Department of Virology
National Institute of Public Health – National Institute of Hygiene
Chocimska 24; 00-791 Warsaw; Poland
tel.: 22 54 21 230
fax: 22 54 21 385
e-mail: wiruspzh@pzh.gov.pl

2. Identification of the customer ordering the test and the identification of sample

Customer (Manufacturer)	CID LINES Sp. z o.o. Świerkowa 20, Niepruszewo, 64-320 Buk
Name of the product	**Alco Cid A**
Batch number	2591511812
Expiry date	September 2017
Date of manufacture	September 2015
Storage conditions	The product should be stored in its original container in a cool, well-ventilated place away from sources of heat and direct sunlight. Packaging should be closed, if the product is not in use.
Active compounds	Ethyl alcohol (80g/100g; 80%).
Appearance of the product	Colorless liquid
Type of the product	The product is designed to disinfect surfaces.

3. Experimental conditions

Date of testing	04.01.2016 – 14.03.2016
Test virus	Poliovirus type 1
Tested concentration	Ready-to-use product
Contact times	30 seconds, 1 minute, 10 minutes
Test temperature	20 ± 1°C
Interfering substance (clean test conditions)	Bovine serum albumin solution (final concentration 0,3g/l)
Procedure to stop action of product	Dilution in ice – cold medium

4. Material and methods

4.1 Virus: poliovirus type 1; attenuated strain LSc2ab (non- envelope RNA virus). The virus suspension, before use, was kept in small volumens in temperature below -70°C.

4.2 Cell line: cell line L20B – recombinant murine fibroblast cell line expressing human poliovirus receptor CD 155. These cells are highly selective for polioviruses, with a characteristic poliovirus cytopathic effect.
Growth culture medium: Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.
Maintenance culture medium: Eagle's minimal essential medium (MEM) supplemented with 2% foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.

4.3 Infectivity assay – quantal test (endpoint titration)

Poliovirus infectious titer was determined as endpoint titration (quantal test) – virus titration on cells in suspension on microtiter plates. 0,1 ml of each virus dilution was transferred into eight wells of a microtiter plate, beginning with the highest dilution. This was followed by the addition of 0,1 ml cell culture suspension in such a density as to enable the formation of a monolayer (>90%) in the cell control, in at least 2 days. Wells with cell culture and without viral suspension served as the cell control.
Microtiter plates with an infected cell culture were incubated at 37°C in a 5% CO_2. - atmosphere for a period of 7 days. The cytopathic effect was read by using an inverted microscope after seven days. Calculation of poliovirus infectious titer was carried out by the Spaerman-Kärber method .

4.4 Determination of cytotoxicity of tested product

To check for possible morphological alteration of cells by the tested product **Alco Cid A**, 1 ml of sterile water was mixed with 1 ml of interfering substance and 8 ml of the product test solution. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. The presence of possible alteration was read by microscope.
Moreover, comparative poliovirus titrations was performed on L20B cells that have or have not been treated with tested product **Alco Cid A** to check the possible reduction of the sensitivity to viruses. In this test the lowest apparently non-cytotoxic dilution of the product was used.

4.5 Control of efficiency for suppresion of disinfectant activity – dilutions in ice-cold medium

Immediately after preparation and mixing of the test mixture (see 4.6) - at 0 time, 0,5 ml of test mixture was added to 4,5 ml of ice-cold MEM + 2 % FCS and was left in the ice bath for 30 min ± 10 s. Then serial dilutions (dilution step 1:10) of this ice-cold mixture were prepared and virus infectious titer was determined according to 4.3. Infectious titer of virus, calculated by the Spaerman-Kärber method, in test mixture (in time 0) was compared to infectious titer of control virus.

4.6 Virucidal testing – determination of ability of tested product Alco Cid A to poliovirus inactivation

Test method – principle: Determining the ability of the product to inactivate the poliovirus based on the reduction of infectious titers. The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4 log was observed (the difference between viral infectious titer in a control mixture and infectious virus titer in a test mixture containing a specified concentration of the test product). The test was performed by the modified method.

Ability of product **Alco Cid A** to poliovirus inactivation was determined by modified method under test conditions described below:

- concentration of tested product – product ready-to-use (during the testing procedure product is diluted and final concentration of the tested product in test mixture was 97%);
- contact times – 30 seconds, 1 minute, 10 minutes;
- test temperature – 20 ± 1°C;
- interfering substance – bovine serum albumin solution (final concentration 0,3g/l – clean conditions).

Study of ability of product **Alco Cid A** to poliovirus inactivation in clean test conditions was conducted in test mixture containing:

- 0,1 ml poliovirus type 1 suspension,
- 0,2 ml 5x concentrated interfering substance (bovine serum albumin solution final concentration 0,3g/l),
- 9,7 ml tested product solution (final concentration of **Alco Cid A** in test mixture was 97%).

The test mixture was incubated in conditions presented above. Immediately at the end of selected contact time, activity of the product was stopped by addition of 0,5 ml of the test mixture into 4,5 ml ice-cold MEM + 2% FCS. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. Poliovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.

4.7 Determination of control virus suspension infectious titer – titration of poliovirus control

Control test: titration of the virus control suspension was performed under test conditions, at contact times 0, 1 and 10 minutes. The product test solution in virus control suspension was substituted by sterile water. Control mixture in modified method containing:

- 0,1 ml poliovirus type 1 suspension,
- 0,2 ml 5x concentrated interfering substance (as described above),
- 9,7 ml sterile water.

At the end of contact time, 0,5 ml of the control mixture was added into 4,5 ml ice-cold MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the poliovirus infectious titer (in control mixture) was determined as endpoint titration (quantal test) according to 4.3.

4.8 Reference virus inactivation test

The aim of this test was the control of the test system's validity. Reference test of poliovirus inactivation was performed with 0,04% glutaraldehyde – determination of virucidal activity of glutaraldehyde against poliovirus type 1. 2 ml of the poliovirus virus suspension was mixed with 8 ml PBS (Phosphate Buffered Saline) and with 10 ml 0,08% glutaraldehyde and incubated at chosen contact times.
Contact times glutaraldehyde with poliovirus suspension were 5, 15, 30 i 60 minutes. At the end of selected contact time, 0,2 ml of the glutaraldehyde test mixture was added into 1,8 ml ice-cold MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the poliovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.
At the same time the control test was performed – determination of control virus suspension infectious titer under test conditions, at contact times 0 and 60 minutes. The glutaraldehyde test solution was substituted by sterile hard water.
Control mixture containing: 1 ml poliovirus suspension, 4 ml PBS and 4 ml sterile hard water. At the end of selected contact time, 0,2 ml of the control test mixture was added into 1,8 ml ice-cold MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the poliovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.
Also, in reference virus inactivation test, the cytotoxicity caused by glutaraldehyde solutions was determined; interactions between glutaraldehyde, poliovirus and cell culture was studied and the control of efficiency for suppresion of glutaraldehyde activity was performed.

5. Verification of the methodology - validation of test results

During the test the following criteria were fulfilled:

a) The titer of the poliovirus type 1 suspension allowed the determination of 4log reduction of virus infectious titer.

b) In clean test conditions the reduction factor of poliovirus titer was:
   - after contact time 30 seconds: 0,87log and 3,87log (I test); 2,50log and 3,75log (II test).
   - after contact time 1 minute: 4,25log and 4,50log (II test); 5,25log (III test).
   - after contact time 10 minutes: 5,00log (I test) and ≥ 5,50log (III test).

c) The reference test solution (0,04 % glutaraldehyde) reduced the poliovirus type 1 titer after 30 and 60 minutes by 1,63log and 2,00log, respectively

d) Observation (in microscope) of morphological changes of cell culture L20B treated with different dilutions of the disinfectant **Alco Cid A** demonstrated that the tested product is not toxic to the cell culture.

e) The comparative poliovirus titration on treated (**Alco Cid A**) and untreated (PBS) L20B cells showed no difference of poliovirus titer. The difference of poliovirus titer was < 1 log (0,12log).

6. Results of product testing – disinfectant Alco Cid A – inactivation of poliovirus type 1 in clean test conditions

The test of disinfectant **Alco Cid A** with poliovirus type 1 was done using a quantitative suspension assay according to PN-EN 14476:2013+A1:2015 – „ 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 was performed in clean test conditions according to guidelines for the products for surface disinfection.

Test was performed using the modified method for ready-to-use products, in which it is possible to test the product in a concentration close to real use concentration. In this method product in test mixture is diluted to a final concentration 97%.

Poliovirus infectious titer was determined as endpoint titration (quantal test). The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control test mixture and infectious virus titer in a test mixture containing a specified concentration of the test product and interfering substance, incubated for a specified contact time).

In clean test conditions reduction of poliovirus infectious titer was:

- after contact time 30 seconds: 0,87log and 3,87log (I test); 2,50log and 3,75log (II test).
- after contact time 1 minute: 4,25log and 4,50log (II test); 5,25log (III test).
- after contact time 10 minutes: 5,00log (I test) and ≥ 5,50log (III test).

The obtained results show that in the clean test conditions disinfectant **Alco Cid A**, as ready-to-use product, inactivated poliovirus type 1, after 1 minute contact time.

Results of testing of disinfectant **Alco Cid A** with poliovirus type 1 in clean test conditions are presented in Table 1, 2 and Figure 1.

7. Conclusions

Based on performed studies it was found that tested disinfectant – **Alco Cid A,** as ready-to-use product, inactivated poliovirus type 1, after 1 minute contact time in clean test conditions. The test of disinfectant **Alco Cid A** was performed by the modified method. In this method product in test mixture is diluted to a final concentration 97%.

Warsaw, 14th March 2016.

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Research report prepared by:

dr Agnieszka Trzcińska
Department of Virology NIPH-NIH

Table 1. Raw data for product **Alco Cid A** tested with poliovirus type 1 in clean test conditions (quantal test; 8 wells).

Product	Concen - tration	Interfering substance	Contact time (minutes)	Dilutions (log)						
				2	3	4	5	6	7	8
Alco Cid A (I test)	97%[1)]	BSA (final concentration 0,3 g/l)	0	44444444	44444444	44444444	44444444	44444444	40040444	04440000
			0	44444444	44444444	44444444	44444444	44444444	44404040	00000000
			0,5	44444444	44444444	44444444	44444444	44444444	00000444	00000000
			0,5	44444444	44444444	04000440	00000000	00000000	00000000	00000000
			10	44444444	00400000	00000000	00000000	00000000	00000000	n.d.
			10	44444444	00400000	00000000	00000000	00000000	00000000	n.d.
Poliovirus - control (I test)	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	00040000
			10	44444444	44444444	44444444	44444444	44444444	44444444	00440044
Alco Cid A (II Test)	97%[1)]		0,5	44444444	44444444	44444444	40444004	00004040	00000000	n.d.
			0,5	44444444	44444444	00004400	00040000	00000000	00000000	n.d.
			1	44444444	44444440	0000000	00000000	00000000	00000000	n.d.
			1	44444444	04440004	04000000	00000000	00000000	00000000	n.d.
Poliovirus - control (II test)	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	00040000
			1	44444444	44444444	44444444	44444444	44444444	44444444	04040040
Alco Cid A (III test)	97%[1)]		0	44444444	44444444	00400404	00000000	00000000	00000000	00000000
			1	44444444	00004000	000000	00000000	00000000	00000000	n.d.
			10	n.d.	00000000	00000000	00000000	00000000	00000000	n.d.
Poliovirus - control (III test)	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	00440404
			10	44444444	44444444	44444444	44444444	44444444	44444444	04440004
Glutaraldehyde (reference test)		PBS	0	44444444	44444444	44444444	44444444	44444444	44444444	04040400
			5	44444444	44444444	44444444	44444444	44444444	04404004	04000400
			15	44444444	44444444	44444444	44444444	44444444	00000400	00000000
			30	44444444	44444444	44444444	44444444	04404004	00000000	00000000
			60	44444444	44444444	44444444	44444444	44000004	00000000	00000000
Poliovirus - control (reference test)	n.a.	PBS	0	44444444	44444444	44444444	44444444	44444444	44444444	40044000
			60	44444444	44444444	44444444	44444444	44444444	44444444	00004404

BSA – bovine serum albumin; PBS – buffered phosphate saline; 4 – virus present, 0 – no virus present; n.a. – not applicable; n.d. – not done

1) – In modified method ready-to-use product in test mixture is diluted to a final concentration 97%.

Table 2. Table of results of product Alco Cid A and poliovirus type 1 in clean test conditions

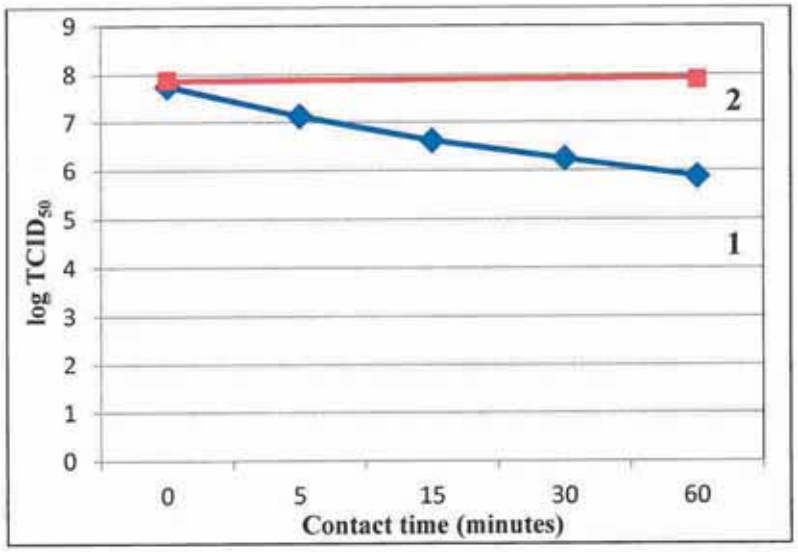
Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log $TCID_{50}$) after different contact times (in min.) with disinfectant				Reduction ≥ 4log after:
				0	0,5	1	10	
Alco Cid A (I test)	**97%**[1)]	BSA (final concentration 0,3 g/l)	0,5	7,50	6,88	n.d.	2,75	30 sec – NO 10 min - YES
				7,13	3,88	n.d.	2,75	
Poliovirus - control (I test)	n.a.		n.a.	7,75	n.d.	n.d.	8,00	
Alco Cid A (II test)	**97%**[1)]		0,5	nb	5,38	3,38	n.d.	30 sec – NO 1 min – YES
				nb	3,88	3,13	n.d.	
Poliovirus - control (II test)	n.a.		n.a.	7,63	n.d.	7,88	n.d.	
Alco Cid A (III test)	**97%**[1)]		0,5	3,88	n.d.	2,75	≤ 2,50	1 min – YES 10 min – YES
Poliovirus - control (III test)	n.a.		n.a.	8,00	n.d.	n.d.	8,00	

Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log $TCID_{50}$) after different contact times (in min) with glutaraldehyde					Reduction of titer after:
				0	5	15	30	60	
Glutaraldehyde (reference test)	0,04 % (v/v)	PBS	3,5	7,75	7,13	6,63	6,25	5,88	30 min - 1,6log 60 min - 2log
Poliovirus - control (reference test)	n.a.		n.a.	7,88	n.d.	n.d.	n.d.	7,88	

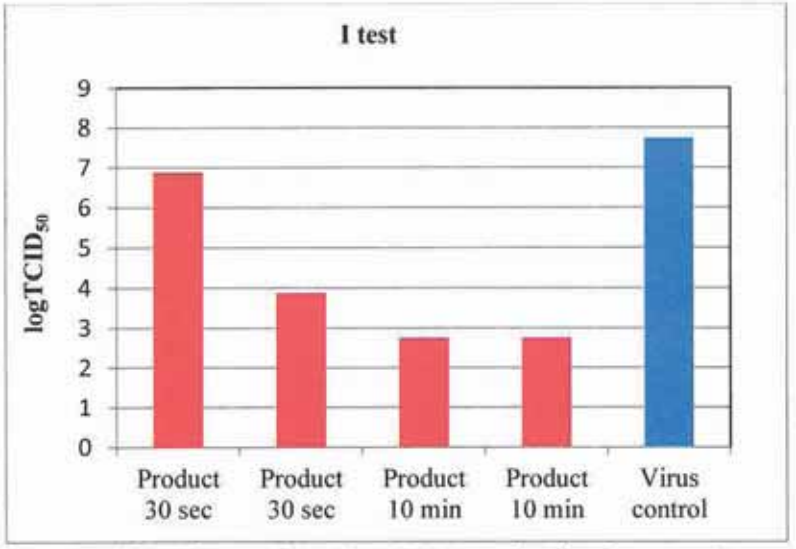
BSA – bovine serum albumin; PBS – buffered phosphate saline; n.a. – not applicable; n.d. – not done

1) – In modified method ready-to-use product in test mixture is diluted to a final concentration 97%.

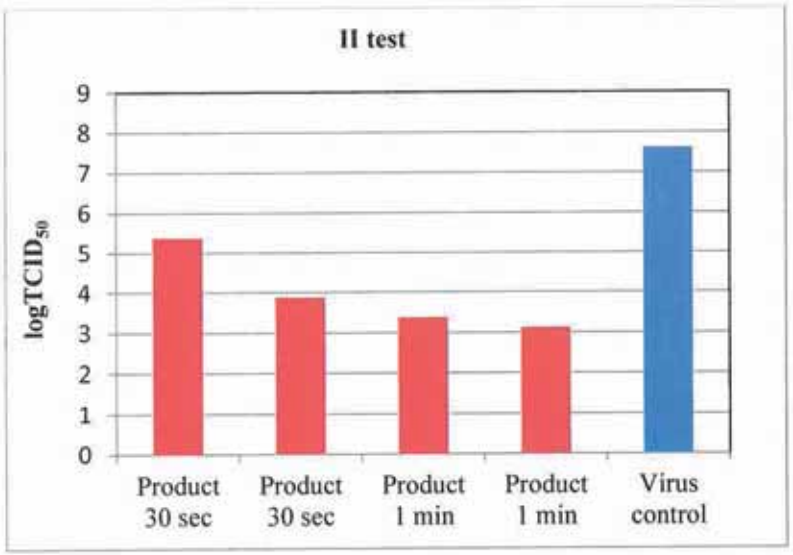
Figure 1. Graphic presentation of results (clean test conditions) for product **Alco Cid A**

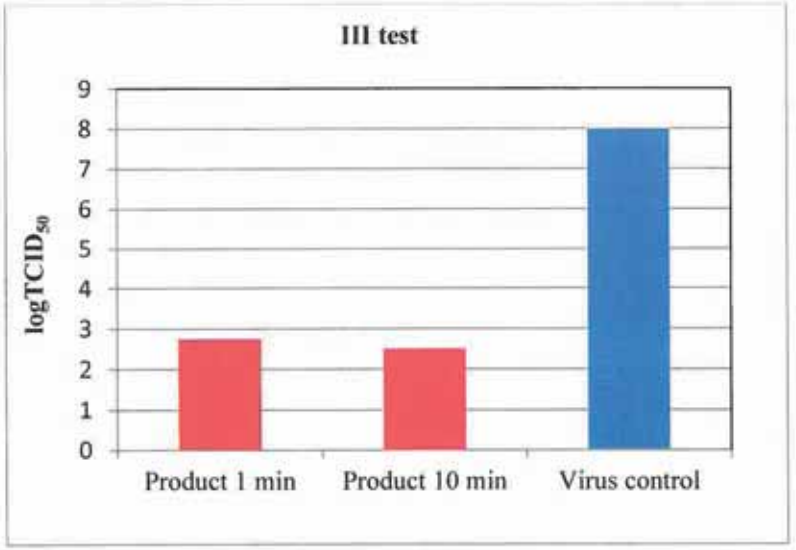


1: Glutaraldehyde (reference test)
2: Poliovirus control (reference test)



Results of test for product **Alco Cid A** and poliovirus type 1





Results of test for product **Alco Cid A** and poliovirus type 1

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Evaluation of virucidal activity

of product

Alco Cid A

Adenovirus type 5

(clean test conditions)

Warsaw, 2016-03-14

1. Test Laboratory

Department of Virology
National Institute of Public Health – National Institute of Hygiene
Chocimska 24; 00-791 Warsaw; Poland
tel.: 22 54 21 230
fax: 22 54 21 385
e-mail: wiruspzh@pzh.gov.pl

2. Identification of the customer ordering the test and the identification of sample

Customer (Manufacturer)	CID LINES Sp. z o.o. Świerkowa 20, Niepruszewo, 64-320 Buk
Name of the product	**Alco Cid A**
Batch number	2591511812
Expiry date	September 2017
Date of manufacture	September 2015
Storage conditions	The product should be stored in its original container in a cool, well-ventilated place away from sources of heat and direct sunlight. Packaging should be closed, if the product is not in use.
Active compounds	Ethyl alcohol (80g/100g; 80%).
Appearance of the product	Colorless liquid
Type of the product	The product is designed to disinfect surfaces.

3. Experimental conditions

Date of testing	04.01.2016 – 14.03.2016
Test virus	Adenovirus type 5
Tested concentration	Ready-to-use product
Contact times	1 minute; 10 minutes
Test temperature	20 ± 1°C
Interfering substance (clean test conditions)	Bovine serum albumin solution (final concentration 0,3g/l)
Procedure to stop action of product	Dilution in ice – cold medium

4. Material and methods

4.1 Virus: adenovirus type 5; strain Adenoid 75, ATCC VR-5 (non-envelope DNA virus). The virus suspension, before use, was kept in small volumens in temperature below -70°C.

4.2 Cell line: A549 cells – human lung carcinoma cells.

Growth culture medium: Dulbecco minimal essential medium (D-MEM) supplemented with 10% inactivated foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.
Maintenance culture medium: Dulbecco minimal essential medium (D-MEM) supplemented with 2% inactivated foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.

4.3 Infectivity assay – quantal test (endpoint titration)

Adenovirus infectious titer was determined as endpoint titration (quantal test) – virus titration on cells in suspension on microtiter plates. 0,1 ml of each virus dilution was transferred into six or eight wells of a microtiter plate, beginning with the highest dilution. This was followed by the addition of 0,1 ml cell culture suspension in such a density as to enable the formation of a monolayer (>90%) in the cell control, in at least 2 days. Wells with cell culture and without viral suspension served as the cell control.
Microtiter plates were incubated ten days at 37°C in a 5% CO_2. - atmosphere. Calculation of adenovirus infectious titer was carried out by the Spaerman-Kärber methods.

4.4 Determination of cytotoxicity of tested product

To check for possible morphological alteration of cells by the tested product **Alco Cid A**, 1 ml of sterile water was mixed with 1 ml of interfering substance and 8 ml of the product test solution. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. The presence of possible alteration was read by microscope. Moreover, comparative adenovirus titrations was performed on A549 cells that have or have not been treated with tested product **Alco Cid A** to check the possible reduction of the cells sensitivity to viruses. In this test the lowest apparently non-cytotoxic dilution of the product was used.

4.5 Control of efficiency for suppresion of disinfectant activity – dilutions in ice-cold medium

Immediately after preparation and mixing of the test mixture (see 4.6) - at 0 time, 0,5 ml of test mixture was added to 4,5 ml of ice-cold D-MEM + 2 % FCS and was left in the ice bath for 30 min ± 10 s. Then serial dilutions (dilution step 1:10) of this ice-cold mixture were prepared and adenovirus infectious titer was determined according to 4.3 (endpoint titration - quantal test). Infectious titer of virus, calculated by the Spaerman-Kärber method, in test mixture (in time 0) was compared to infectious titer of control virus.

4.6 Virucidal testing – determination of ability of tested product Alco Cid A to adenovirus inactivation

Test method – principle: Determining the ability of the product to inactivate the adenovirus based on the reduction of infectious titers. The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control mixture and infectious virus titer in a test mixture containing a specified concentration of the test product).

Ability of product **Alco Cid A** to adenovirus inactivation was determined by standard method under test conditions described below:

- concentration of tested product – product ready-to-use (during the testing procedure product is diluted and final concentration of the tested product in test mixture was 80%);
- contact times – 1 minute, 10 minutes;
- test temperature – 20 ± 1°C;
- interfering substance – bovine serum albumin solution (final concentration 0,3g/l – clean conditions).

Study of virucidal activity of product **Alco Cid A** against adenovirus in clean conditions was conducted in test mixture containing:

- 1 ml adenovirus type 5 suspension,
- 1 ml interfering substance (bovine serum albumin solution final concentration 0,3g/l),
- 8 ml tested product solution (final concentration of **Alco Cid A** in test mixture was 80%).

The test mixture was incubated in conditions presented above. Immediately at the end of selected contact time, activity of the product was stopped by addition of 0,5 ml of the test mixture into 4,5 ml ice-cold D-MEM + 2% FCS. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. Adenovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.

4.7 Determination of control virus suspension infectious titer – titration of adenovirus control

Control test: titration of the virus control suspension was performed under test conditions, at contact times 1 and 10 minutes. The product test solution in virus control suspension was substituted by sterile water. Control mixture containing:

- 1 ml adenovirus type 5 suspension,
- 1 ml interfering substance (as described above),
- 8 ml sterile water.

At the end of contact time, 0,5 ml of the control mixture was added into 4,5 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the adenovirus infectious titer (in control mixture) was determined as endpoint titration (quantal test) according to 4.3.

4.8 Reference virus inactivation test

The aim of this test was the control of the test system's validity. Reference test of adenovirus inactivation was performed with 0,004% glutaraldehyde – determination of virucidal activity of glutaraldehyde against adenovirus type 5.
2 ml of the adenovirus virus suspension was mixed with 8 ml PBS (Phosphate Buffered Saline) and 10 ml 0,008% glutaraldehyde and incubated at chosen contact times.
Contact times glutaraldehyde with adenovirus suspension were 5, 15, 30 i 60 minutes. At the end of contact time, 0,2 ml of the glutaraldehyde test mixture was added into 1,8 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the adenovirus infectious titer was determined according to 4.3.
At the same time the control test was performed – determination of control virus suspension infectious titer under test conditions, at contact times 0 min and 60 minutes. The glutaraldehyde test solution was substituted by hard water.
Control mixture containing: 1 ml of adenovirus suspension, 4 ml PBS and 4 ml sterile hard water. At the end of contact time, 0,2 ml of the control test mixture was added into 1,8 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the adenovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.
Also, in reference virus inactivation test, the cytotoxicity caused by glutaraldehyde solutions was determined; interactions between glutaraldehyde, adenovirus and cell culture was studied and the control of efficiency for suppresion of glutaraldehyde activity was performed.

5. Verification of the methodology - validation of test results

During the test the following criteria were fulfilled:

a) The titer of the adenovirus type 5 suspension allowed the determination of 4log reduction of virus infectious titer.

b) In clean test conditions the reduction factor of adenovirus titer after contact time 1 minute was: $\geq$ 5,75log (I test) and $\geq$ 5,88log (II test).

c) The reference test solution (0,004% glutaraldehyde) reduced the adenovirus type 5 titer after 30 and 60 minutes by 4,50log and 5,00log, respectively.

d) Observation (in microscope) of morphological changes of cell culture A549 treated with different dilutions of the disinfectant **Alco Cid A** demonstrated that the test product is not toxic to the cell culture.

e) The comparative adenovirus titration on treated (**Alco Cid A**) and untreated (PBS) A549 cells showed no difference of adenovirus titer. The difference of adenovirus titer was < 1log (0,00log).

6. Results of product testing – disinfectant Alco Cid A – inactivation of adenovirus type 5 in clean test conditions

The test of disinfectant **Alco Cid A** with adenovirus type 5 was done using a quantitative suspension assay according to PN-EN 14476:2013+A1:2015 – „ 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 was performed in clean test conditions according to guidelines for the products for surface disinfection. Final concentration tested disinfectant **Alco Cid A** (ready-to-use product) in test procedure was 80% (standard method).

Adenovirus infectious titer was determined as endpoint titration (quantal test). The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control test mixture and infectious virus titer in a test mixture containing a specified concentration of the test product and interfering substance, incubated for a specified contact time).

Reduction of adenovirus infectious titer after contact time 1 minute was: ≥ 5,75log (I test) and ≥ 5,88log (II test). The obtained results show that in clean test conditions disinfectant **Alco Cid A**, as ready-to-use product, inactivated adenovirus type 5, after 1 minute contact time.

Results of testing of disinfectant **Alco Cid A** with adenovirus type 5 in clean test conditions are presented in Table 1, 2 and Figure 1.

7. Conclusions

Based on performed studies it was found that tested disinfectant – **Alco Cid A,** as ready-to-use product, inactivated adenovirus type 5, after 1 minute contact time in clean test conditions. The test of disinfectant **Alco Cid A** was performed by the standard method. In this method product in test mixture is diluted to a final concentration 80%.

Warsaw, 14th March 2016.

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Table 1. Raw data for product **Alco Cid A** tested with adenovirus type 5 in clean test conditions (quantal test; 8 wells).

Product	Concen - tration	Interfering substance	Contact time (minutes)	Dilutions (log)						
				2	3	4	5	6	7	8
Alco Cid A (I test)	**80%**[1)]	BSA (final cocentration 0,3 g/l)	0	n.d.	n.d.	n.d.	00000000	00000000	00000000	00000000
			1	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
			10	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
Alco Cid A (II test)	**80%**[1)]		0	n.d.	n.d.	n.d.	00000000	00000000	00000000	00000000
			1	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
			10	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
Adenovirus - control	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	44044440
			0	44444444	44444444	44444444	44444444	44444444	44444444	40404404
			10	44444444	44444444	44444444	44444444	44444444	44444444	44044444
Glutaraldehyde (reference test)	0,004% (v/v)	PBS	0	44444444	44444444	44444444	44444444	44444444	44404444	04400404
			5	44444444	44444444	44444444	44444444	44444444	04000044	00000040
			15	44444444	44444444	40400004	40000000	00000000	00000000	n.d.
			30	44444444	40000404	00044000	0000000	00000000	n.d.	n.d.
			60	04444444	00000004	00004000	00000000	00000000	n.d.	n.d.
Adenovirus - control (reference test)	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	04000000
			60	44444444	44444444	44444444	44444444	44444444	44444444	00404400

BSA – bovine serum albumin; PBS – buffered phosphate saline; 4 – virus present, 0 – no virus present; n.a. – not applicable; n.d. – not done

1) – In standard method ready-to-use product in test mixture is diluted to a final concentration 80%.

Table 2. Table of results of product Alco Cid A and adenovirus type 5 in clean test conditions

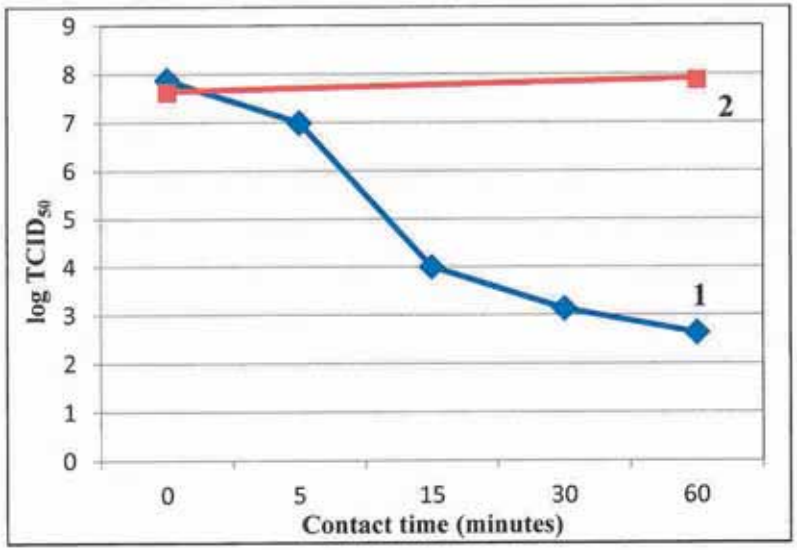
Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log $TCID_{50}$) after different contact times (in min.) with disinfectant			Reduction ≥4log after:
				0	1	10	
Alco Cid A (I test)	**80%**	BSA (final concentration 0,3 g/l)	0,5	≤ 4,50	≤ 2,50	≤ 2,50	1 min – YES 10 min – YES
Alco Cid A (II test)	**80%**		0,5	≤ 4,50	≤ 2,50	≤ 2,50	1 min – YES 10 min – YES
Adenovirus - control	n.a.		n.a.	8,13 8,25	n.d.	8,38	n.a.

Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log $TCID_{50}$) after different contact times (in min) with glutaraldehyde					Reduction of titer after:
				0	5	15	30	60	
Glutaraldehyde (reference test)	0,004 % (v/v)	PBS	3,5	7,88	7,00	4,00	3,13	2,63	30 min - 4,50log 60 min - 5,00log
Adenovirus - control (reference test)	n.a.		n.a.	7,63	n.d.	n.d.	n.d.	7,88	

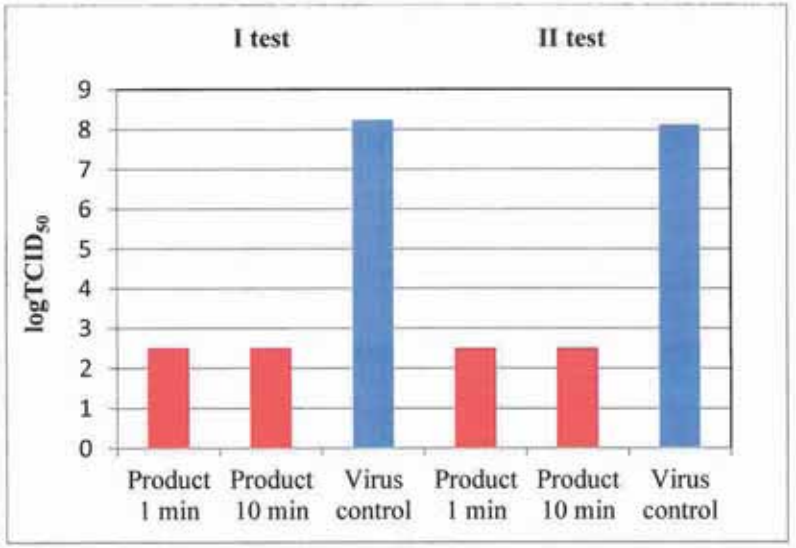
BSA – bovine serum albumin; PBS – buffered phosphate saline; n.a. – not applicable; n.d. – not done

1) – In standard method ready-to-use product in test mixture is diluted to a final concentration 80%.

Figure 1. Graphic presentation of results (clean test conditions) for product **Alco Cid A**



1: Glutaraldehyde (reference test)
2: Adenovirus control (reference test)



Results of test for product **Alco Cid A** and adenovirus type 5

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Evaluation of virucidal activity

of product

Alco Cid A

Murine Norovirus

(clean test conditions)

Warsaw, 2016-03-14

1. Test Laboratory

Department of Virology
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2. Identification of the customer ordering the test and the identification of sample

Customer (Manufacturer)	CID LINES Sp. z o.o. Świerkowa 20, Niepruszewo, 64-320 Buk
Name of the product	**Alco Cid A**
Batch number	2591511812
Expiry date	September 2017
Date of manufacture	September 2015
Storage conditions	The product should be stored in its original container in a cool, well-ventilated place away from sources of heat and direct sunlight. Packaging should be closed, if the product is not in use.
Active compounds	Ethyl alcohol (80g/100g; 80%).
Appearance of the product	Colorless liquid
Type of the product	The product is designed to disinfect surfaces.

3. Experimental conditions

Date of testing	04.01.2016 – 14.03.2016
Test virus	Murine norovirus
Tested concentration	Ready-to-use product
Contact times	1 minute, 10 minuts
Test temperature	20 ± 1°C
Interfering substance (clean test conditions)	Bovine serum albumin solution (final concentration 0,3g/l)
Procedure to stop action of product	Dilution in ice – cold medium

4. Material and methods

4.1 Virus: murine norovirus; strain S99 Berlin; (non-envelope RNA virus). The virus suspension was kept in small volumens in temperature below -70°C before used.

4.2 Cell line: RAW264.7 cell line – monocyte/macrophage; Abelson murine leukemia virus transformed.

Growth culture medium: Dulbecco minimal essential medium (D-MEM) supplemented with 10% inactivated foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.

Maintenance culture medium: Dulbecco minimal essential medium (D-MEM) supplemented with 2% inactivated foetal calf serum (FCS) and 3% L-glutamine with 100 units/ml penicillin and 100 µg/ml streptomycin.

4.3 Infectivity assay – quantal test (endpoint titration)

Murine norovirus infectious titer was determined as endpoint titration (quantal test) – virus titration in cell monolayer on microtiter plates. 0,1 ml of each virus dilution (beginning with the highest dilution) was transferred into eight wells of a microtiter plate coated with a monolayer cell culture, from which the medium was removed previously. Microtiter plate was incubated for 1 h at 37°C, then added 0.1 ml of maintenance medium. Wells with cell culture and without viral suspension served as the cell control.
Microtiter plates were incubated at 37°C in a 5% CO_2 - atmosphere. The cytopathic effect was read by using an inverted microscope after seven days. Calculation of murine norovirus infectious titer was carried out by the Spaerman-Kärber methods.

4.4 Determination of cytotoxicity of tested product

To check for possible morphological alteration of cells by the tested product **Alco Cid A**, 1 ml of sterile water was mixed with 1 ml of interfering substance and 8 ml of the product test solution. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. The presence of possible alteration was read by microscope.

Moreover, comparative murine norovirus titrations was performed on RAW264.7 cells that have or have not been treated with tested product **Alco Cid A** to check the reduction of the sensitivity to murine norovirus. In this test the lowest apparently non-cytotoxic dilution of the product was used.

4.5 Control of efficiency for suppresion of disinfectant activity – dilutions in ice-cold medium

Immediately after preparation and mixing of the test mixture (see 4.6) - at 0 time, 0,5 ml of test mixture was added to 4,5 ml of ice-cold D-MEM + 2 % FCS and was left in the ice bath for 30 min ± 10 s. Then serial dilutions (dilution step 1:10) of this ice-cold mixture were prepared and murine norovirus infectious titer was determined according to 4.3. Infectious titer of virus, calculated by the Spaerman-Kärber method, in test mixture (in time 0) was compared to infectious titer of control virus.

4.6 Virucidal testing – determination of ability of tested product Alco Cid A to murine norovirus inactivation

Test method – principle: Determining the ability of the product to inactivate the murine norovirus based on the reduction of infectious titers. The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control mixture and infectious virus titer in a test mixture containing a specified concentration of the test product).

Ability of product **Alco Cid A** to murine norovirus inactivation was determined by standard method under test conditions described below:

- concentration of tested product – product ready-to-use (during the testing procedure product is diluted and final concentration of the tested product in test mixture was 80%);
- contact times – 1 minute, 10 minutes;
- test temperature – 20 ± 1°C;
- interfering substance – bovine serum albumin solution (final concentration 0,3g/l – clean conditions).

Study of virucidal activity of product **Alco Cid A** against murine norovirus was conducted in test mixture containing:

- 1 ml of murine norovirus suspension,
- 1 ml of interfering substance (bovine serum albumin solution final concentration 0,3g/l),
- 8 ml of tested product solution (final concentration of product in test mixture was 80%).

The test mixture was incubated in conditions presented above. Immediately at the end of selected contact time, activity of the product was stopped by addition of 0,5 ml of the test mixture into 4,5 ml ice-cold D-MEM + 2% FCS. This was followed by preparing serial dilutions (dilution step 1:10) and inoculation into cell cultures. Murine norovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.

4.7 Determination of control virus suspension infectious titer – titration of murine norovirus control

Control test: titration of the murine norovirus control was performed under test conditions, at contact times 0 and 10 minutes. The product test solution was substituted by sterile hard water. Control mixture containing:

- 1 ml of murine norovirus suspension,
- 1 ml of interfering substance (as described above),
- 8 ml sterile hard water.

At the end of contact time, 0,5 ml of the control mixture was added into 4,5 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the murine norovirus infectious titer (in control mixture) was determined as endpoint titration (quantal test) according to 4.3.

4.8 Reference virus inactivation test

The aim of this test was the control of the test system's validity. Reference test of murine norovirus inactivation was performed with 0,04% glutaraldehyde – determination of virucidal activity of glutaraldehyde against murine norovirus. 2 ml of the murine norovirus suspension was mixed with 8 ml PBS (Phosphate Buffered Saline) and 10 ml 0,08% glutaraldehyde and incubated at chosen contact times. Contact times glutaraldehyde with murine norovirus suspension were 5, 15, 30 i 60 minutes. At the end of contact time, 0,2 ml of the glutaraldehyde test mixture was added into 1,8 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the murine norovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.
At the same time the control test was performed – determination of control virus suspension infectious titer under test conditions, at contact times 0 min and 60 minutes. The glutaraldehyde test solution was substituted by hard water. Control mixture containing: 1 ml of murine norovirus suspension, 4 ml PBS and 4 ml sterile hard water. At the end of contact time, 0,2 ml of the control test mixture was added into 1,8 ml ice-cold D-MEM + 2% FCS. Then the serial dilutions (dilution step 1:10) were prepared and the murine norovirus infectious titer was determined as endpoint titration (quantal test) according to 4.3.

Also, in reference virus inactivation test, the cytotoxicity caused by glutaraldehyde solutions was determined; interactions between glutaraldehyde, murine norovirus and cell culture was studied and the control of efficiency for suppresion of glutaraldehyde activity was performed.

5. Verification of the methodology - validation of test results

During the test the following criteria were fulfilled:

a) The titer of the murine norovirus suspension allowed the determination of 4log reduction of virus infectious titer.

b) In clean test conditions the reduction factor of murine norovirus titer after 1 minute and 10 minutes contact times was $\geq$ 5,00log (I i II test).

c) The reference test solution (0,04 % glutaraldehyde) reduced the murine norovirus titer after 30 and 60 minutes by 4,37log and 5,12log, respectively.

d) Observation (in microscope) of morphological changes of cell culture RAW264.7 treated with different dilutions of the disinfectant **Alco Cid A** demonstrated that the tested product is not toxic to the cell culture.

e) The comparative murine norovirus titration on treated (**Alco Cid A**) and untreated (PBS) RAW264.7 cells showed no difference of murine norovirus titer. The difference of murine norovirus titer was $<$ 1 log (0,13log).

6. Results of product testing – disinfectant Alco Cid A – inactivation of murine norovirus in clean test conditions

The test of disinfectant **Alco Cid A** with murine norovirus was done using a quantitative suspension assay according to PN-EN 14476:2013+A1:2015 – „ 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 was performed in clean test conditions according to guidelines for the products for surface disinfection. Final comcentration tested disinfectant **Alco Cid A** (ready-to-use products) in test procedure was 80% - standard method.

Murine norovirus infectious titer was determined as endpoint titration (quantal test). The product was considered as having virucidal activity when reduction of infectious virus titer ≥ 4log was observed (the difference between viral infectious titer in a control test mixture and infectious virus titer in a test mixture containing a specified concentration of the test product and interfering substance, incubated for a specified contact time).

In clean test conditions disinfectant **Alco Cid A,** as ready-to-use product**,** was able to inactivate murine norovirus, after 1 minute contact time. Reduction of murine norovirus infectious titer after contact time 1 minute was: ≥ 5,00log (I and II test).

Results of testing of disinfectant **Alco Cid A** with murine norovirus type 1 in clean test conditions are presented in Table 1, 2 and Figure 1.

7. Conclusions

Based on performed studies it was found that tested disinfectant – **Alco Cid A,** as ready-to-use product, inactivated murine norovirus, after 1 minute contact time in clean test conditions. The test of disinfectant **Alco Cid A** was performed by the standard method. In this method product in test mixture is diluted to a final concentration 80%.

Warsaw, 14th March 2016.

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Department of Virology NIPH-NIH

Table 1. Raw data for product **Alco Cid A** tested with murine norovirus in clean test conditions (quantal test; 8 wells).

Product	Concen - tration	Interfering substance	Contact time (minutes)	Dilutions (log)						
				2	3	4	5	6	7	8
Alco Cid A (I test)	**80%**[1)]	BSA (final concentration 0,3 g/l)	0	nb	nb	nb	00000000	00000000	00000000	00000000
			1	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
			10	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
Alco Cid A (II test)	**80%**[1)]		0	n.d.	n.d.	n.d.	00000000	00000000	00000000	00000000
			1	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
			10	00000000	00000000	00000000	00000000	00000000	n.d.	n.d.
Murine norovirus - control	n.a.		0	44444444	44444444	44444444	44444444	44444444	40444444	00400000
			10	44444444	44444444	44444444	44444444	44444444	40044444	00004040
Glutaraldehyde (reference test)	0,04% (v/v)	PBS	0	44444444	44444444	44444444	44444444	44444444	40000444	00000044
			5	44444444	44444444	44444444	04444444	04000400	00000000	00000000
			15	44444444	44444444	44404004	00004400	00000000	00000000	n.d.
			30	44444444	44440440	40000000	0000000	00000000	n.d.	n.d.
			60	44444444	04000000	00000000	00000000	00000000	n.d.	n.d.
Murine norovirus - control (reference test)	n.a.		0	44444444	44444444	44444444	44444444	44444444	44444444	00000404
			60	44444444	44444444	44444444	44444444	44444444	44444444	44040040

BSA – bovine serum albumin; PBS – buffered phosphate saline; 4 – virus present, 0 – no virus present; n.a. – not applicable; n.d. – not done

1) – In standard method ready-to-use product in test mixture is diluted to a final concentration 80%.

Table 2. Table of results of product Alco Cid A and murine norovirus in clean test conditions

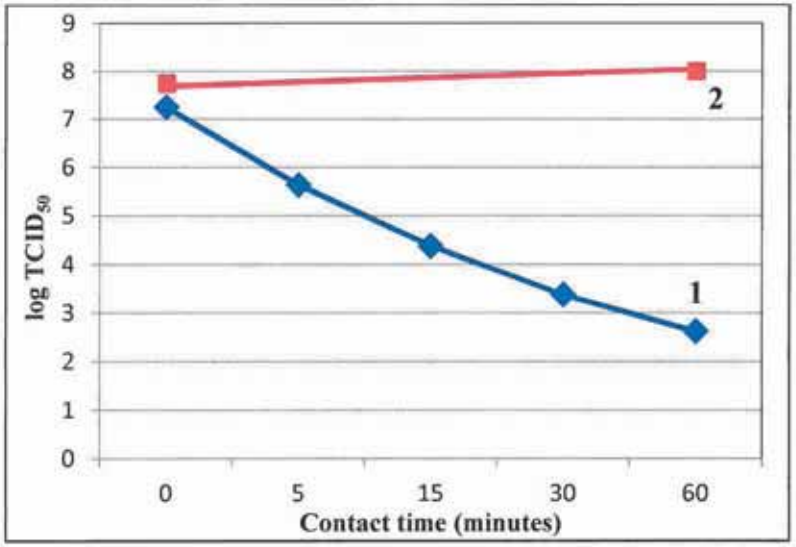
Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log TCID50) after different contact times (in min.) with disinfectant			Reduction ≥4log after:
				0	1	10	
Alco Cid A (I test)	**80%**[1)]	BSA (final concentration 0,3 g/l)	0,5	≤ 4,50	≤ 2,50	≤ 2,50	1 min – YES 10 min – YES
Alco Cid A (II test)	**80%**[1)]		0,5	≤ 4,50	≤ 2,50	≤ 2,50	1 min – YES 10 min – YES
Murine norovirus - control	n.a.		n.a.	7,50	n.d.	7,50	n.a.

Tested product	Concentration	Interfering substance	Level of cytotoxicity	Virus titer (log $TCID_{50}$) after different contact times (in min) with glutaraldehyde					Redukction of titer after:
				0	5	15	30	60	
Glutaraldehyde (reference test)	0,04 % (v/v)	PBS	3,5	7,25	5,63	4,38	3,38	2,63	30 min - 4,37log 60 min - 5,12log
Murine norovirus - control (reference test)	n.a.		n.a.	7,75	n.d.	n.d.	n.d.	8,00	

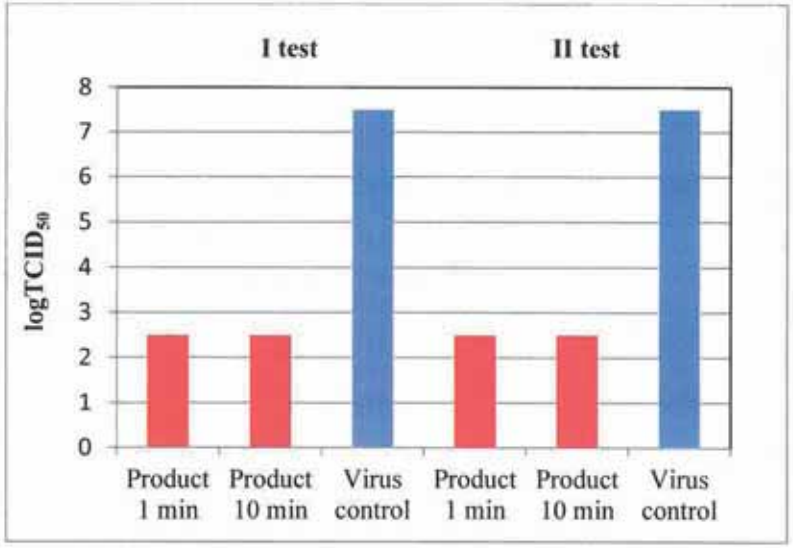
BSA – bovine serum albumin; PBS – buffered phosphate saline; n.a. – not applicable; n.d. – not done

1) – In modified method ready-to-use product in test mixture is diluted to a final concentration 97%.

Figure 1. Graphic presentation of results (clean test conditions) for product **Alco Cid A**



1: Glutaraldehyde (reference test)
2: Murine norovirus – control (reference test)



Results of test for product **Alco Cid A** and murine norovirus