TEST REPORT
NF EN 14476 - VIRUCIDAL ACTIVITY
OF PRODUCT NOCOLYSE (batch 220709 OS)

N° Report IPL : 1550909MA

on *Influenza virus* type A-H1N1

This report concerns only the product subject to the test

1. Identification of client :

Customer : OXY'PHARM – 917 rue Marcel Paul – ZA des Grands Godets – 94508 Champigny sur Marne

2. Identification of sample

Name of the product : NOCOLYSE

Batch n°: 220709 OS

Manufacturer : OXY'PHARM – 917 rue Marcel Paul – ZA des Grands Godets – 94508 Champigny sur Marne

Storage conditions: Room temperature, darkness

Substances actives : not indicated

Appareance of the product : clear liquid

Product diluents recommended by the manufacter for use: ready to use

3. Périod of testing :

Date of delivery : 2009/09/07

Dates of tests : 2009/09/16 to 2009/09/28

4. Principe of the test

The study consists in determining the virucidal activity of product within test –organism *Influenza virus A-H1N1.* The product is according to the EN 14476 if, in one of the test conditions, it demonstrates at least a 4 log reduction after:

- 60 minutes of contact time for the instrument and surface disinfectants
- 1 minute or 30 s of contact time for the hygienic handrub and handwash

According to European Standard 14476, time additional can be chosen and tested in the conditions of the try.

The realization of this standard comprises several stages, which are :

- Proportion titration of the virus suspension.
- Preliminary test consist in determining the level of disinfecting cytotoxicity according to the cellular line necessary to the detection of the virus. This stage allows defining the cytotoxic concentration of the product, corresponding to the sensitivity of the cells to the tested virus.
- Virus inactivation test by Ethanol 25 % (v/v). The stage is a control of the test system by the test method.
- Virucidal testing.
- Realization of the virus control.

5. Test conditions:

Product diluent :	ppi water
Concentrations of the product tested :	80 %
Test mixtures:	homogeneous
Contact time (obligatory):	60 minutes
Contact time (additionnal) :	/
Test température :	20°C ± 1°C
Incubation température:	37°C ± 1°C +5% CO_2
Neutralization métodology:	Detoxification on Microspin TH S-400.columms
High level soiling condition:	0,3g/l SAB
Monolayer cell :	MDCK

Test-organisms **:**

Influenza virus type A- H1N1- ATCC-UR1469 (10^7 to 10^8 UI/ml).

6. Materiel and method:

6–1 – Determination of infectivity (virus titration)

Infectivity was determinate by means of end point dilution in a micro procedure.

The suspension viral untreated (control and stock suspension) and treated by the product at the test concentration of 80 %, are diluted in series of 10^{-2} to 10^{-10} maximum, in MEM + 2% FCS frozen. 0,1 ml of each diluted is transferred in 8 wells of the micro titration plate containing the confluent cells, from starting with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The reading of the ECP is realized under the inverted microscope daily after 2 days and 5 days of incubation. Calculation of infectivity titer is determined by the Spearman-Karber method.

6-2 – Determination of subcytotoxic dilution of the disinfectant

The aim of this test is to determine the concentration of chemical desinfectant inducing no sign of toxicity with respect to cellular line allowing the description of the virus to be tested.

For elimination of cytotoxicity, two techniques are described later, and are selected according to following conditions :

The dilution method is the first one tested. It is validated if the difference between the log $TCID_{50}$ of virus titre of the stock suspension and the level non cytotoxic level's is $\geq$ at 5 log. If it's not the case, the molecular sieving is carried out. The validation of the conditions method is similar at the first method. If the results are not satisfactory some is the technique employed, the virus test on disinfecting is unrealizable.

1- Dilution method :

The test solution of product, added with 1/5 of water for injectable preparation, ppi water, is diluted in series of 10^{-2} to 10^{-6} in MEM 2 % FCS frozen. Then 0,1 ml of each dilution is transferred in 8 wells of micro titration plate, we start with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The subcytotoxic effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied (5 days).

2- Molecular sieving technique, with a molecular on Microspin^{TM}S-400 HR columns :

Test solution of the product added with water for injectable preparation, ppi water, is filtered on Microspin^{TM}S-400 HR columns . Then, filtrate is diluted in series of 10^{-2} to 10^{-6} in MEM + 2 % FCS frozen. 0,1 ml of each dilution is transferred in 8 wells of micro titration plate. After one hour of incubation at 37°C, with 5 % of CO_2, 0,1 ml of culture medium is added in each well. The subcytotoxicity effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied.

6-3 – Cell sensitivity to virus

The aim of this test is to make sure that MDCK cells with test solution (at the subcytotoxic concentrations) don't alter behavior of virus with the cells. Sensitivity of cells compared to the virus is appreciated by comparison of the virus titre of the stock virus suspension obtained on a cell monolayer treated with the subcytotoxic dilution of disinfectant, with the cell monolayer untreated.

Treatment of cells (with the subcytotoxic concentration of disinfectant)

0,1 ml of the lowest apparently non cytotoxic dilution of the test solution are distributed on to each 8 wells established cell cultures in microtitre plates. Plates are incubated at 37°C for 1 h with 5 % CO_2. In the same time, the stock virus suspension is diluted to 10^{-2} to 10^{-10}. Then, 0,1 ml of each dilution is added in each wells. Plates are again incubated at 37°C for 1 h with 5 % CO_2. 0,1 ml of cell media is added in each wells. The reading of ECP is realized thanks to calculation of infectivity titer are determined by the Spearman-Karber method.

Cells untreated with the subcytotoxic concentration of disinfectant

It's the same procedure as cells treated. Only difference is that the dilution subcytotoxic is removed by MEM 2 % fœtal calf serum. Only these dilutions of the product can be used for the determination of the residual infectivity which produces a titer reduction of the virus of < 1 log.

Validation of test :

Difference between log $_{DICT50}$ and titrate of cells treated and untreated must be < 1 log for the test of cells sensibility of virus will be valided.

6-4 – Control of efficiency for suppression of disinfectant activity

Dilution in ice cold medium

Immediately after preparation of the test mixture (virus + interfering substance + test solution) ($d = 10^{-1}$), reaction is stopped : 0,5 ml of the test mixture is placed into 4,5 ml of ice cold MEM + 2 % fœtal calf serum ($d = 10^{-2}$).
Dilutions are realized in the same media from 10^{-3} to 10^{-8}.
The *Influenza virus* A H1N1 is titrated as described in 6-1.

Filtration technique

Just after the preparation of the test mixture, reaction is stopped putting 1 ml on Microspin^{TM}S-400 HR columns.
After a centrifugation, filtrate is diluted in ice cold MEM + 2 % fœtal calf serum from 10^{-2} to 10^{-8}.
The *Influenza virus* A H1N1 is titrated as described in 6-1.

6-5 – Virucidal testing

The aim of a virucidy test for a disinfectant is to put in contact the viral suspension with an interfering substance and a test solution.
Reaction is stopped after the time of contact notified :

- By dilution on ice cold medium (MEM)
- By filtration technique on Microspsin^{TM}S-400HR columns. Filtrate is diluted form 10 to 10 in a ice cold medium.

Titer of each test is determined as described in 6-1.

6-6 – Inactivation test of the virus at Ethanol of 25 % (v/v)

Virucidy tests are realized on the stock suspension of *Influenza virus* A H1N1 with Ethanol in order to control the behavior of our strain with chemical agents.
This control requires realization of the following procedures :

- Control of the Ethanol cytotoxicity opposite our cell line
- Control of efficiency of the stopped activity
- Virucidal testing of ethanol (5, 15, 30 and 60 minutes)

6-7 – Titration of the virus standard

The infectivity of the test virus suspension shall be determined under test conditions at contact times 0 min and 60 min.
The product test solution is substituted by water.
The *Influenza virus* A H1N1 is titred as described in 6-1.

7. RESULTS

7.1 Virus titration:

The infectivity titer of the suspension viral is: 7,625 in logUI/ml

7.2 Cytotoxicity of product

Examinations showed that, without treatment, the disinfectant had a toxicity of 3,5 logCD50/ml For elimination of cytotoxicity, the technique by Detoxification on Microspin TH S-400.columms is choice (see table 1)

Table 1	**Level of cytotoxicity (log)**
dilution technical C1	3,5
ultrafiltration on microspinTM S-400 columns C2	2,5

7.3 Sensibility of cells

Difference between the virus titrations on treated cell (A) and on untreated cell (B) is lower than 1 (see table2), consequently the test is valided.

Table 2	**Treated cell : A**	**Untreated cell : B**
Log $_{DICT50}$	7,75	8,00

7.4 Virucidal activity of product

The table 3 will gather the results obtained by the following points:

- contol of efficiency for suppression o disinfectant activity
- Virucidal testing
- Inactivation test of the virus
- Titration of the virus

Product	Concentrations	interfering substance	Level of cytotoxicity Log $_{DICT50}$	neutralization control Log $_{DICT50}$	Log $_{DICT50}$ after ……. min					> 4 log réduction after ……. min
					0	5	15	30	60	
NOCOLYSE	80 %	0,3g/l SAB	2,5	7,825	< 2,5	n.d.	n.d.	n.d.	< 2,5	Reduction superior of 5,375 log in 60 min
Ethanol	25%	PBS	1,5	n.a.	8,125	7,75	7,5	6,625	5,75	Reduction inferior of 4 log in 60 min
Virus control	n.a.	PBS	n.a.	n.a.	8,00	n.d.	n.d.	n.d.	7,75	n.a.
Virus control test	n.a.	0,3g/l SAB	n.a.	n.a.	8,125	n.d.	n.d.	n.d.	7,875	n.a.

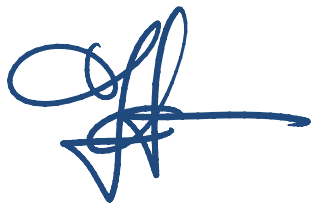
Calcul of virucidal activity : Réduction = LogDICT 50 test - LogDICT 50 viral control

8. CONCLUSION

The product NOCOLYSE, batch n° 2200709, is active at 80 % after a contact time of 60 minutes in accordance with NF EN 14476 under clean conditions on *Influenza virus type A-H1N1*.

Lille, October 12th 2009

Modified, it October 21th 2009



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