Henkel AG & Co. KGaA

Microbiology

Test Report

14-02196

Virucidal activity

of

Incidin® pro

tested against

Murine Norovirus (MNV), strain S-99/Berlin/06/DE

according EN 14476
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);
German version EN 14476:2013

The following report consists of 17 pages and may be reproduced only completely.

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Date: 2014-03-24

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Content

1. Identity of the test institute

Henkel AG & Co. KGaA

HSA Corporate Scientific Services

D-40191 Düsseldorf

2. Test substance

2.1.	Product name	Incidin®pro
2.1.1	Batch	IF-13077-S.89-1
2.1.2	Manufacturer / supplier	Ecolab
2.1.3	Date of delivery	2014-01-15
2.1.4	Storage conditions in the laboratory	room temperature
2.1.5	Active ingredient acc. Client	Phenoxyethanol, Bis (3-aminopropyl) dodecylamine, Alkyl dimethyl benzyl ammonium chloride
2.1.6	Appearance	clear, liquid
2.1.7	Production date	2014-01-14 (filled up)
2.1.8	Expiry date	unknown

3. Test method

3.1 DIN EN 14476: 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) German version EN 14476:2013 (Version: October 2013)

3.2 With an additional reworking adapted to
"Studies of the inactivation of Poliomyelitis Virus by Formaldehyde" Effect of initial virus concentration, of mechanical stirring during inactivation and of digestion with trypsine prior to initiation inactivation – E. Lycke (March 30, 1957)

4. Experimental conditions

4.1 Test period
February 2014

4.2 Test concentration
0.5% / 1.0% / 1.25%
The test concentrations were given by the client

4.3 Appearance of the product dilutions

Begin of assay:
Incidin® pro Test with clean load light turbid, liquid
End of assay:
Incidin® pro Test with clean load light turbid, liquid

4.4 Test organisms / Host cells
MNV, strain S-99 /Berlin/06/DE, obtained from Robert Koch Institute (RKI) 2008
Passage 19 from 01 July 2010

TIB -71 (RAW 264.1), obtained from ATCC 2008
Passage 8 from 03 February 2014 and Passage 10 from 18 March 2014 (microspin method)

4.5 Contact time
60 minutes
The contact time were given by the client

4.6 Time table / Test temperature / Test assay structure

Assay 1	MNV	10:00h	20.0°C
Assay 2	MNV	11:00h	20.0°C

Repetition with Microspin columns due to unclear results obtained in the assays from 06.02.14:

Assay 1	MNV (Microspin)	08:30h	20.0°C
Assay 2	MNV (Microspin)	09:00h	20.0°C

Repeated test parameters:
Incidin® pro 1% / 60 Min / clean

All assay ingredients were allowed to adjust to test temperature. The assay was performed in a temperature-controlled water bath.

100µl low organic load (i.e. 0.03% BSA)
+100µl virus inoculum preparation
+800µl test product in 1.25 fold test concentration resp. WSH as blank

For the titer determination of the virus inoculum preparation blanks are run with WSH instead of test product.

After the contact time the reaction is annulled by transfer of 100µl aliquots to 900µl ice-cold supplemented DMEM + 5% FCS and serial 1:10-dilutions up to 10^{-9}. As annulment verification this is already controlled at time 0. The recovery of residual active virus was performed in quantal tests on

the corresponding host cells in microtitre plates using 8 parallels each by transferring 100µl aliquots of each dilution to confluent monolayer cells.

After 6 days the cultures are read out for cytopathic effects (CPEs) with an inverse microscope. The effects are evaluated as follows:

0 =	no cell damage	= no virus activity
1 =	<25% cell damage	= virus activity
2 =	≈50% cell damage	= virus activity
3 =	≈75% cell damage	= virus activity
4 =	≈100% cell damage	= virus activity

4.7 Neutralisation

instantaneous dilution of the test assays in ice-cold medium

4.8 Reagents

Reagent	**Supplier**	**Batch / LOT**	**Best before**
Blood (Sheep)	Oxoid	69779000	Used for Erythrocytes
Bovine serumalbumine	Serva	130580	04/2016
Clean conditions (BSA)	Serva	0.3%: self preparation	Fresh usage
DMEM (culture medium)	Biochrom	0337B	03/2015
Fetal calf serum (FCS)	Gibco	0429B	04/2019
Formol (as 36.5% formaldehyde)	Sigma&Aldrich	SZBD1000V	07/2015
10xPhosphate-buffered saline (PBS)	Gibco	1135526	03/2014
Trypsine/EDTA	Gibco	1136407	09/2014
Dist. water	HSA Microbiology	2014010205(1)	07/2014
WSH (Hard water) 04.02.2014	HSA Microbiology	2014020401(1)	Fresh usage
WSH (Hard water) 18.03.2014	HSA Microbiology	2014031804(1)	Fresh usage
D-PBS	Gibco	1409684	07/2015
Microspin ™ S-400 HR Columns	GE Healthcare	389605	unknown

4.9 Validation tests

All assays for validation tests are run at test temperature.

4.9.1. Cytotoxicity controls

100µl WSH

+100µl low organic load

+800µl test product in 1.25 fold test concentration

After mixing and direct serial 1:10-dilutions in ice-cold supplemented DMEM + 5% FCS monolayered host cells in microtitre plates are inoculated by 100µl aliquots in 8 parallels.

After 6 days the cultures are read out for cytotoxic effects (CTEs) with an inverse microscope. The effects observed should be distinguished morphologically from CPEs and are evaluated as follows:

0 = no CTE;

X = CTE.

If CTEs cannot be distinguished from CPEs, their degree may not exceed cytopathic cell damage in order to attribute the effect clearly to the virucidal properties of the test product.

4.9.2 Interference controls

100µl of the lowest non-cytotoxic dilution of the test product and 100µl PBS (control) respectively were transferred to 8 parallels monolayered host cells in microtiter plates.

After 1h contact time at 36°C the supernatant is discarded and replaced by 100µl DMEM + 5% FCS -diluted virus inoculum preparation in dilution steps 10^{-2}– 10^{-9}. After another hour an additional volume of 100 µl cellculture media (DMEM + 5% FCS) is pipetted in each well.

After 6 days the cultures are read out for cytopathic effects (CPEs) with an inverse microscope.

Only those dilutions may be used for RF-calculations, where the virus titers in the PBS-treated cells and in the product-treated cells may not differ <1lg.

4.9.3 Disinfection suppression assay

From each of complete test assay a 100µl aliquot is withdrawn already at time 0±5sec. and immediately transferred to ice-cold supplemented DMEM + 5% FCS for 30 minutes. These controls are further processed in analogy to 4.6 and the virus titers may differ from the PBS control titer by ≤0.5lg.

4.9.4 Performance controls with formaldehyde

100µl virus inoculum preparation

+400µl PBS

+500µl 1.4% solution* of formol (= 36.5% formaldehyde)

* the actual concentration of this solution was analysed as 1.5% (w/v) formaldehyde

After contact times of 30 and 60 minutes 100µl aliquots are withdrawn and transferred to ice-cold supplemented DMEM + 5% FCS and serial 1:10 dilutions are prepared. 100µl aliquots of each dilution are plated on monolayered host cells in microtiter plates and incubated 6d / 36°C.

5. Method for decreasing of product initiated host cell-cytotoxicity adapted to the "Lycke method" (1957)

To obviate the product initiated host cell-cytotoxicity of 4.5 - 5.5lg steps (see tab 2), which were observed in the test according to EN14476, test mixtures with the application concentration of 1.25% and controls were additionally reworked after 60 minutes contact time.

Therefore the test mixtures were diluted as follows:

Assay 1) 800µl Incidin® pro (1.25%*1.25) + 100µl clean load + 100µl Virus suspension

Assay 2) 800µl WSH + 100µl clean load + 100µl Virus suspension (=positive control)

Assay 3) 800µl WSH + 100µl clean load + 100µl WSH (=negative control)

→After 60 minutes contact time, a dilution of 1:100 in ice-cold DMEM + 5% FCS was prepared for the test assays 1-3.

Then the diluted test assays were proceeded as follows (see tab 6ff):

10 x 100µl aliquots of assay 1

4 x 100µl aliquots of assay 2 (positive control)

1 x 100µl aliquot of assay 5 (negative control)

Every aliquot was added to a tissue culture flask with a surface of 75cm^2, containing 20ml DMEM + 5% FCS and ~5ml fresh suspended host cells

= thus the test mixture were diluted ~1:20000 in each case.

→Afterwards every flask was incubated and observed by microscope until cytopathic effects were detectable.

6. Calculation of the virus titre

From the qualitative results of the residual infectivity the titer m of the infective virus particles in the original 100µl aliquots may be re-calculated as 50% infective dose ($TCID_{50}$) by the method of *Spearman & Kärber*

$$\mathbf{m}\ (TCID_{50}) = \mathbf{X_k} + \mathbf{d/2} - \mathbf{d^*\Sigma p_i}$$

m	=	negative decadal logarithm $TCID_{50}$ in the first 100µl aliquot
$\mathbf{X_k}$	=	decadal logarithm of the first dilution where all parallels (here 8) are positive (1-4)
D	=	decadal logarithm of the dilution factor (here 1)
$\mathbf{P_i}$	=	ratio of positives in one series:

Positives	P_i
0/8	0.000
1/8	0.125
2/8	0.250
3/8	0.375
4/8	0.500
5/8	0.625
6/8	0.750
7/8	0.875
8/8	1.000

The standard deviation $\mathbf{S_m}$ is calculated according to the following formula

$$S_m = \sqrt{d^2 \sum \left[P_i \times (1 - P_i) \div (n - 1)\right]}$$

$\mathbf{S_m}$	=	standard deviation of the logarithmic titer
d	=	decadal logarithm of the dilution factor (here 1)
$\mathbf{P_i}$	=	ratio of positives in the parallels

In many cases it is observed that $P_i = 1$ and $P_{i+2} = 0$. In case of a dilution series with a dilution factor of 10 and 8 parallels the above mentioned formula may then be simplified to

$$S_m = \sqrt{\left[P_{i+1} \times (1 - P_{i+1}) \div 7\right]}$$

Calculation of the virucidal effect (logarithmic reduction factor of the infectivity)

The virucidal effect of the tested product is calculated as logarithmic reduction factor **RF** = difference between the logarithmic virus control titers (corresponding water control at room temperature & test pH) minus the residual logarithmic virus titers found for the product containing assays.

$$\mathbf{RF = lg}\ (\text{Virus control CPE}) - \mathbf{lg}\ (\text{Test product CPE})$$

In cases where the cytotoxic effects (CTE) exceed the cytopathic effects (CPE) the calculated reduction factors are indicated as "≥".The confidence intervals $\mathbf{K_{RF}}$ of the reduction factors are calculated following the formula:

$$K_{RF} = \sqrt{(2S_{m(control)})^2 + (2S_{m(residualtiter)})^2}$$

7. Results

Incidin® pro			
parameter		**logarithmic reduction factors against *MNV***	
test concentration	**contact time temperature**	**clean conditions suspensiontest acc. EN 14476**	**Clean conditions Microspin method acc. EN 14476**
1.25%	60 min / 20°C	>2.00 ± 0.00	>4.00 ± 0.00
1.0%	60 min / 20°C	>3.00 ± 0.00	4.19 ± 0.18
0.5%	60 min / 20°C	2.9 ± 0.13	2.9 ± 0.13
1.25%	60 min / 20°C	RF adapted to "Lycke method" Detection limit **Titre <2.5 / RF >5**	
		Titres of *MNV*	
Control	**Contact time Temperature**	**Clean conditions Suspensiontest acc. EN 14476**	**Clean conditions Microspin method acc. EN 14476**
WSH-control	60 min / 20°C	7.5 ± 0.0	7.5 ± 0.0

Yellow shaded values = results (reduction factors) according to EN14476
Green shaded values = results (reduction factors) according to "Lycke method"

8. Conclusion

Incidin®pro is a Phenoxyethanol, Bis (3-aminopropyl) dodecylamine, Alkyl dimethyl benzyl ammonium chloride - based disinfectant used for surface disinfection in all kind of settings. It was tested for virucidal efficiency acc. to EN 14476 (2013) in a suspension test in standardized hard water against surrogate viruses. For a sufficient efficacy this norm requires a reduction of virus titre of ≥4.0lg. Here we report the results against *Murine Norovirus* (MNV), strain S-99/Berlin/06/DE. Incidin®pro met the requirement with a concentration of 1.0% in 60 minutes under clean conditions (based on the results of the EN14476 suspension test performed with Microspin columns and proved by "Lycke-method").

Düsseldorf, 24 March 2014

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Head of laboratory

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Laboratory

A copy of the original signed version of this report and the raw data are filed at HSA Corporate Scientific Services / Microbiology for a storage period of 10 years

Table appendix

Tab. 1 Interference controls for Incidin® pro on *TIB* cells (assay 1 + 2)

m = Titer as log_{10}-$TCID_{50}$/100µl acc. *Spearman* & *Kärber*; S_m = Standard deviation; P_i = Ratio of positives in a series of parallels

Test	product	load	Product dilution %	log dilution								m	S_m
				-2	-3	-4	-5	-6	-7	-8	-9		
Interference-control (1)	Incidin®pro	With-out	1:10000 = 0.000125 %	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44000000 Pi = 0.250	00000000 Pi = 0	7.7	0.164
Interference-control (1)	PBS	With-out	-	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Test	**product**	**load**	**Product dilution %**	**log dilution**								**m**	**S_m**
				-2	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**	**-9**		
Interference-control (2)	Incidin®pro	With-out	1:10000 = 0.000125 %	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	40000000 Pi = 0.125	00000000 Pi = 0	7.6	0.125
Interference-control (2)	PBS	With-out	-	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	44444444 Pi = 1.0	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000

Tab. 2 Cytotoxicity controls (x) on *TIB* cells (assay 1)

Assay	ac (%)	load	log dilution								Cell control
			-2	-3	-4	-5	-6	-7	-8	-9	
Incidin®pro	1.25	Clean	xxxxxxxx	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000
Incidin®pro	1.0	Clean	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000
Incidin®pro	0.5	Clean	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000
PBS	-	Clean	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000
Formaldehyde	0.75%	without	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000

Tab. 2.1 Cytotoxicity controls (x) on *TIB* cells (assay 2)

Assay	ac (%)	load	log dilution								Cell control
			-2	-3	-4	-5	-6	-7	-8	-9	
Incidin®pro	1.25	Clean	xxxxxxxx	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000
Incidin®pro	1.0	Clean	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000
Incidin®pro	0.5	Clean	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000
PBS	-	Clean	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000	00000000
Formaldehyde	0.75%	without	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000

Tab. 3 Formaldehyde control against *MNV*, strain S-99, (assay 1&2)

m = Titer as log_{10}-$TCID_{50}$/100µl acc. *Spearman & Kärber*

Test	t (min.)	log dilution								Cell-control	m	log RF
		-2	-3	-4	-5	-6	-7	-8	-9			
1	30	xxxxxxxx	xxxxxxxx	xxxxxxxx	44444444	40000000	00000000	00000000	00000000	00000000	5.5	2.0
	60	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000	<4.5	>3.0
Test	t (min.)	log dilution								Cell-control	m	log RF
		-2	-3	-4	-5	-6	-7	-8	-9			
2	30	xxxxxxxx	xxxxxxxx	xxxxxxxx	44444444	40000000	00000000	00000000	00000000	00000000	5.6	1.9
	60	xxxxxxxx	xxxxxxxx	xxxxxxxx	00000000	00000000	00000000	00000000	00000000	00000000	<4.5	>3.0

The formaldehyde concentration was analytically determined as 1.5% resulting to an assay concentration of 0.75%.

Tab. 4 Efficacy data Incidin® pro against *MNV*, strain S99 (assay 1)

m = Titer as log_{10}-$TCID_{50}$/100µl acc. *Spearman & Kärber*; S_m = Standard deviation; P_i = Ratio of positives in a series of parallels

Assay 1	Ac (%)	load	log dilution								m	S_m
			-2	-3	-4	-5	-6	-7	-8	-9		
Incidin®pro 0 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 0 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444440 Pi = 0.875	00000000 Pi = 0	00000000 Pi = 0	7.4	0.125
Incidin®pro 0 min / 20°C	0.5	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
WSH 0 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
PBS 0 min / 20°C	-	without	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 60 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<5.5	0.000
Incidin®pro 60 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<4.5	0.000
Incidin®pro 60 min / 20°C	0.5	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	40000000 Pi = 0.125	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	4.6	0.125
WSH 60 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
PBS 60 min / 20°C	-	without	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000

Assay 1 Microspin	Ac (%)	load	log dilution								m	S_m
			-2	-3	-4	-5	-6	-7	-8	-9		
Incidin®pro 0 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444400 Pi = 0.750	00000000 Pi = 0	00000000 Pi = 0	7.2	0.164
Incidin®pro 0 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444440 Pi = 0.875	00000000 Pi = 0	00000000 Pi = 0	7.4	0.125
Incidin®pro 0 min / 20°C	0.5	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
WSH 0 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 60 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<3.5	0.000
Incidin®pro 60 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	3.5	0.125
Incidin®pro 60 min / 20°C	0.5	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	4.5	0.000
WSH 60 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000

Tab. 4.1 Efficacy data Incidin® against *MNV*, strain S99 (assay 2)

m = Titer as log_{10}-$TCID_{50}$/100µl acc. *Spearman & Kärber*; S_m = Standard deviation; P_i = Ratio of positives in a series of parallels

Assay 2	Ac (%)	load	log dilution								m	S_m
			-2	-3	-4	-5	-6	-7	-8	-9		
Incidin®pro 0 min / 20°C	3.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444000 Pi = 0.625	00000000 Pi = 0	00000000 Pi = 0	7.1	0.183
Incidin®pro 0 min / 20°C	2.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444000 Pi = 0.625	00000000 Pi = 0	00000000 Pi = 0	7.1	0.183
Incidin®pro 0 min / 20°C	0.5	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444000 Pi = 0.625	00000000 Pi = 0	00000000 Pi = 0	7.1	0.183
WSH 0 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
PBS 0 min / 20°C	-	without	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 60 min / 20°C	3.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<5.5	0.000
Incidin®pro 60 min / 20°C	2.0	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<4.5	0.000
Incidin®pro 60 min / 20°C	0.5	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	4.5	0.000
WSH 60 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
PBS 60 min / 20°C	-	without	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000

Assay 2 Microspin	Ac (%)	load	log dilution								m	S_m
			-2	-3	-4	-5	-6	-7	-8	-9		
Incidin®pro 0 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 0 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444400 Pi = 0.750	00000000 Pi = 0	00000000 Pi = 0	7.2	0.164
Incidin®pro 0 min / 20°C	0.5	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
WSH 0 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000
Incidin®pro 60 min / 20°C	1.25	clean	xxxxxxxx Pi = 1	xxxxxxxx Pi = 1	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	<3.5	0.000
Incidin®pro 60 min / 20°C	1.0	clean	xxxxxxxx Pi = 1	Xxxxx000 Pi = 0.625	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	3.1	0.183
Incidin®pro 60 min / 20°C	0.5	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	40000000 Pi = 0.125	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	00000000 Pi = 0	4.6	0.125
WSH 60 min / 20°C	-	clean	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	44444444 Pi = 1	00000000 Pi = 0	00000000 Pi = 0	7.5	0.000

Tab. 5 Calculated virus titres and logarithmic reduction factors for Incidin®pro against *MNV*, strain S99 (assay 1+2)

Product	Application concentration (%)	Organic load	Cyto-toxicity (log steps)	lg-TCID 50 (100 µl) after ... min					>4 lg decrease or complete inactivation of virus particles after... min
				0	5	15	30	60	
Assay 1 Incidin®pro	1.25	Clean conditions	5.5	7.5				<5.5	60
				RF 0				RF >2.0	
Assay 1 Incidin®pro	1.0	Clean conditions	4.5	7.4				>4.5	60
				RF 0.1				RF >3.0	
Assay 1 Incidin®pro	0.5	Clean conditions	2.5	7.5				4.6	Not sufficient within 60 minutes
				RF 0				RF 2.9	
Assay 1 Virus control	-	PBS	-	7.5				7.5	
		Clean conditions	-	7.5				7.5	
Assay 1 Formaldehyde	0.7%	without	4.5				5.5	<4.5	60
							RF2.0	RF >3.0	
Assay 2 Incidin®pro	1.25	Clean conditions	5.5	7.1				<5.5	60
				RF 0.4				RF >2.0	
Assay 2 Incidin®pro	1.0	Clean conditions	4.5	7.1				>4.5	60
				RF 0.4				RF >3.0	
Incidin 2 Incidin®pro	0.5	Clean conditions	2.5	7.1				4.5	Not sufficient within 60 minutes
				RF 0.4				RF 3.0	
Assay 2 Virus control	-	PBS	-	7.5				7.5	
		Clean conditions	-	7.5				7.5	
Assay 2 Formaldehyde	0.7%	without	4.5				5.6	<4.5	60
							RF 1.9	RF >3.0	

Tab. 5.1 Calculated virus titres and logarithmic reduction factors for Incidin®pro against *MNV*, strain S99 (assay 1+2) Microspin method

Product / method: Microspin acc. EN 14476	Application concentration (%)	Organic load	Cyto-toxicity (log steps)	lg-TCID 50 (100 µl) after ... min					>4 lg decrease or complete inactivation of virus particles after... min
				0	5	15	30	60	
Assay 1 Incidin®pro	1.25	Clean conditions	3.5	7.2				<3.5	60
				RF 0.3				RF >4.0	
Assay 1 Incidin®pro	1.0	Clean conditions	2.5	7.4				3.5	60
				RF 0.1				RF 4.0	
Assay 1 Incidin®pro	0.5	Clean conditions	1.5	7.5				4.5	Not sufficient within 60 minutes
				RF 0				RF 3.0	
Assay 1 WSH control	-	Clean conditions	-	7.5				7.5	

Product / method: Microspin acc. EN 14476	Application concentration (%)	Organic load	Cyto-toxicity (log steps)	lg-TCID 50 (100 µl) after ... min					>4 lg decrease or complete inactivation of virus particles after... min
				0	5	15	30	60	
Assay 2 Incidin®pro	1.25	Clean conditions	3.5	7.5				<3.5	60
				RF 0				RF >4.0	
Assay 2 Incidin®pro	1.0	Clean conditions	2.5	7.2				3.1	60
				RF 0.3				RF 4.4	
Assay 2 Incidin®pro	0.5	Clean conditions	1.5	7.5				4.6	Not sufficient within 60 minutes
				RF 0				RF 2.9	
Assay 2 WSH control	-	Clean conditions	-	7.5				7.5	

Tab 6. Disinfection suppression assay (1&2)

Assay	Product	Application conc.	Load	Contact time	Titre/lg
1	PBS	-	Clean conditions	30 Min	7.6
	Incidin®pro	1.25%	Clean conditions		7.5
		1.0%	Clean conditions		7.5
		0.5%	Clean conditions		7.5
Assay	**Product**	**Application conc.**	**Load**	**Contact time**	**titre**
2	PBS	-	Clean conditions	30 Min	7.5
	Incidin®pro	1.25%	Clean conditions		7.5
		1.0%	Clean conditions		7.5
		0.5%	Clean conditions		7.5

Tab 7. Results "Lycke"-method / Efficacy of 1.25% Incidin®pro against *MNV*, strain S99 (60 Min / clean conditions)

Test mixture: 800 µl 1.25%*1.25 Incidin®pro +100µl clean load +100µl Virus

Contact time: 60 Min → 10x100µl Aliquot of test mixture inoculate in 75cm² tissue flask with 20ml DMEM+5%FCS and ~5ml fresh suspended host cells

1. Flask	2. Flask	3. Flask	4. Flask	5. Flask
Negative =no Virus	Negative =no Virus	Negative =no Virus	Negative =no Virus	Negative =no Virus
theoretical titre <1.5	*theoretical titre <1.5*	*theoretical titre <1.5*	*theoretical titre <1.5*	*theoretical titre <1.5*
6. Flask	**7. Flask**	**8. Flask**	**9. Flask**	**10. Flask**
Negative =no Virus	Negative =no Virus	Negative =no Virus	Negative =no Virus	Negative =no Virus
theoretical titre <1.5	*theoretical titre <1.5*	*theoretical titre <1.5*	*theoretical titre <1.5*	*theoretical titre <1.5*

Contact time: 60 Min **Negative and positive Controls**

1. Flask	2. Flask	3. Flask	4. Flask	5. Flask
100µl clean load + 100µl Virus + 800µl WSH	100µl clean load + 100µl Virus + 800µl WSH	100µl clean load + 100µl Virus + 800µl WSH	100µl clean load + 100µl Virus + 800µl WSH	100µl clean load + 100µl WSH + 800µl WSH
Virus positive	*Virus positive*	*Virus positive*	*Virus positive*	*Virus negative*
6. Flask	**7. Flask**	**8. Flask**	**9. Flask**	**10. Flask**
100µl dirty load + 100µl Virus + 800µl WSH	100µl dirty load + 100µl Virus + 800µl WSH	100µl dirty load + 100µl Virus + 800µl WSH	100µl dirty load + 100µl Virus + 800µl WSH	100µl dirty load + 100µl WSH + 800µl WSH
Virus positive	*Virus positive*	*Virus positive*	*Virus positive*	*Virus negative*

tab. 8 Efficacy of Incidin® pro against *MNV,* strain S-99 (assay 1 & 2)

Product	Ac (%)	load	Log $_{10}$ TCID/ml incl. 95% Confidence interval after				RF incl. 95% Confidence interval after				≥4 log$_{10}$ Reduction /complete inactivation after...min
			0 min	5 min	30 min	60 min	0 min	5 min	30 min	60 min	
Incidin®pro ① + ②	1.25	Clean load	7.313 ± 0.183			<5.500 ± 0.000	0.188 ± 0.183			>2.000 ± 0.000	**60**
	1.0	Clean load	7.250 ± 0.222			<4.500 ± 0.000	0.250 ± 0.222			>3.000 ± 0.000	**60**
	0.5	Clean load	7.313 ± 0.183			4.563 ± 0.125	0.188 ± 0.183			2.938 ± 0.125	Not sufficient within 60 min
Virus control ① + ②	-	Clean load	7.500 ± 0.000			7.500 ± 0.000					
Micospin method Incidin®pro ① + ②	1.25	Clean load	7.375 ± 0.164			<3.500 ± 0.000	0.125 ± 0.164			>4.000 ± 0.000	**60**
	1.0	Clean load	7.313 ± 0.206			3.313 ± 0.183	0.188 ± 0.206			4.188 ± 0.183	**60**
	0.5	Clean load	7.500 ± 0.000			4.563 ± 0.125	0.000 ± 0.000			2.938 ± 0.125	Not sufficient within 60 min
Micospin method Virus control ① + ②	-	Clean load	7.500 ± 0.000			7.500 ± 0.000					
PBScontrol ① + ②	-	without	7.500 ± 0.000			7.500 ± 0.000					
Interference control ① + ② Incidin®pro	0.000125 %	without				7.688 ± 0.206					
Interference control ① + ② PBS	-	without				7.500 ± 0.000					
Formaldehyde Control ① + ②	0.75%	without			5.563 ± 0.125	<4.500 ± 0.000			1.938 ± 0.125	>3.000 ± 0.000	**60**

Disinfection suppression assay (1&2)					
Load	Contact time	Incidin®pro 1.25%	Incidin®pro 1.0%	Incidin®pro 0.5%	PBS
Clean conditions	30 min	7.500 ± 0.000	7.500 ± 0.000	7.500 ± 0.000	-
Cytotoxicity controls (assay 1 & 2)					
Load	formaldehyde	Incidin® 1.25%	Incidin® 1.0%	Incidin® 0.5%	PBS
Clean conditions		5.50 ± 0.00	4.50 ± 0.00	2.50 ± 0.00	<1.50 ± 0.00
Load	formaldehyde	Incidin® 1.25% Microspin	Incidin® 1.0% Microspin	Incidin® 0.5% Microspin	PBS
Clean conditions	-	3.50 ± 0.00	2.50 ± 0.00	1.50 ± 0.00	Not done
Without load	4.50 ± 0.00	-	-	-	-