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Eurovir® Hygiene-Labor
Antivirale Validierung & Rabies

Virucidal activity of

„PMF-concentrate“

against the Influenza A Virus (H1N1)

Short report of the screening tests S1 and S2

by

PD Dr. Olaf Thraenhart and Dr. Christian Jursch

Study time: August - September 2014

Principal: PMF Natural Products company
Arab Republik of Egypt

Product: ***PMF-concentrate***
[Lot-no.: not specified; product sample as arrived; Arrival: 01.08.2014, Storage at 2-8°C]

Parameter of test:

- 0,75 g of PMF-concentrate solved within 2,95 mL of A. bidest (25,42% [w/v])
- T = 37° C and 60 and 240 min. of exposure

Test system:

- Influenza A Virus (H1N1); Strain: New Caledonia
(Origin: Chiron Behring, Marburg/Lahn, Germany)
- Madin Darby Canine Kidney Cells (MDCK)
(Origin: Robert Koch-Institute, Berlin, Germany)

Test method:

- The testing was performed following the guideline of the DVV and the Robert Koch-Institute (DVV/RKI-guideline [*Bundesgesundhbl. (2008); 51 (8):937-945*]): for the quantitative virucidal suspension test (QST).
- With test S1 the *Spearman & Kärber's* method for virus titration of the main samples was used (cf. DVV/RKI-guideline).
- With test S2 virus titration of the main samples was performed according to *Lycke's* methodolgy *(Arch Ges Virusforsch (1957); 7:483-493).*

Tab. 1: ***Dosage of product*** *(solvent: A. bidest)*

Set	Product(s)	Conc. in Test *(1x)*	Working sol. *(x 1,25)*	Dosage	pH of working sol.
#1	PMF-concentrate	20,34%	25,42%	0,75 g in 2,95 mL	pH 9,54 *(in test: pH 9,52)*

Tab. 2: Results of virus inactivation

Samples	1a + 1b	2a + 2b	1a + 1b	2a + 2b
	Screening test S1 *(virus titration: Spearman & Kärber)*		***Screening test S2*** *(virus titration: acc. Lycke*	
Exposure time	t = 60 min.	t = 240 min.	t = 60 min.	t = 240 min.
Virus input [1] *(per test volume)*	4,47 ± 0,36	4,38 ± 0,23	4,88 ± 0,26	4,88 ± 0,39
Detection limit *(cytotoxicity level)*	2,45		< 1 ID_{50} *(0,0 lg ID_{50})*	
Residual virus [1] (log ID_{50} ± K [95%])	2,19 ± 0,32	≤ 2,45	3,26 ± 0,26	< 1 ID_{50} *(0,0 lg ID_{50})*
Reduction [2] (log ID_{50} ± K [95%])	**2,28 ± 0,48**	**≥ 1,93 ± 0,23**	**1,62 ± 0,37**	**> 4,88 ± 0,55**

[1] = Calculation of 95% confidential interval of virus titer as well as virus reduction following DVV/RKI-Guideline.
[2] = Virus reduction: titer of virus control minus titer of sample (lg ID_{50}).

Results: (cf. Tab. 2)

Control tests

Screening test S1:

- The pH of the 1,25fold concentrated working solution were measured to pH 9,54.
- In the test sample this pH changed only slightly to pH 9,52.
- With S1 the amount of input virus at 37° C was estimated to lg ID_{50} = 4,47 ± 0,36 after 60 min. and to lg ID_{50} = 4,38 ± 0,23 after 240 min. (Δ virus titer = 0,10 ± 0,42).
- Using the *Spearman & Kärber's* titration method a cytotoxicity titer of lg TD_{50} = 2,45 was recorded for the test samples. This is equivalent with the detection limit of screening test S1.

Screening test S2:

- With S2 the amount of input virus at 37° C was estimated to lg ID_{50} = 4,88 ± 0,26 after 60 min. and to lg ID_{50} = 4,88 ± 0,39 after 240 min. (Δ virus titer = 0,0 ± 0,47).
- With the *Lycke's* method a sample dilution was done (VF = 1000). With that dilution no cytotoxicity was visible and the susceptibility of the detection cells was given (Δ titer = 0,18 ± 0,53). In addition, the detection limit of screening test S2 could be improved by 2,45 Log.

Virus inactivation

Screening test S1:

- With 20,34% of PMF (final test concentration) and after 60 min. the virus reduction factor was estimated to **RF = 2,28 ± 0,48.** After 240 min. no residual virus could be detected (test virus below detection limit, due to product associated cytotoxicity [lg TD_{50} = 2,45]). The corresponding virus reduction factor was estimated to **RF ≥ 1,93 ± 0,23**.

Screening test S2:

- With 20,34% of PMF (final test concentration) and after 60 min. the virus reduction factor was estimated to **RF = 1,62 ± 0,37.** After 240 min. no residual virus could be detected. The corresponding virus reduction factor was estimated to **RF > 4,88 ± 0,55**.

Conclusions:

- Prolongation of the exposure at 37° C from 60 to 240 min. was not associated with a reduction of input virus. The test virus was stable at the test temperature over the observation period.
- Using the *Lycke's* method of virus titration the results from the first screening test (S1) could be significantly improved.
- After 60 min. at 37° C 20,34% PMF (final concentration) inactivated influenza virus by RF = 1,95 ± 0,30 *(average from S1 and S2)* or by 98,9% under the test conditions. After 240 min. a virus reduction factor of RF > 4,88 ± 0,55 was observed, correspondent to a virus reduction of 99,998%.

Luckenwalde, 12th of November 2014

Dr. Christian Jursch
(Laboratory manager and Managing Director of Eurovir)

Inactivation of influenza A virus by *PMF-concentrate*
- testing with the quantitative virucidal supension test at T = 37 °C -

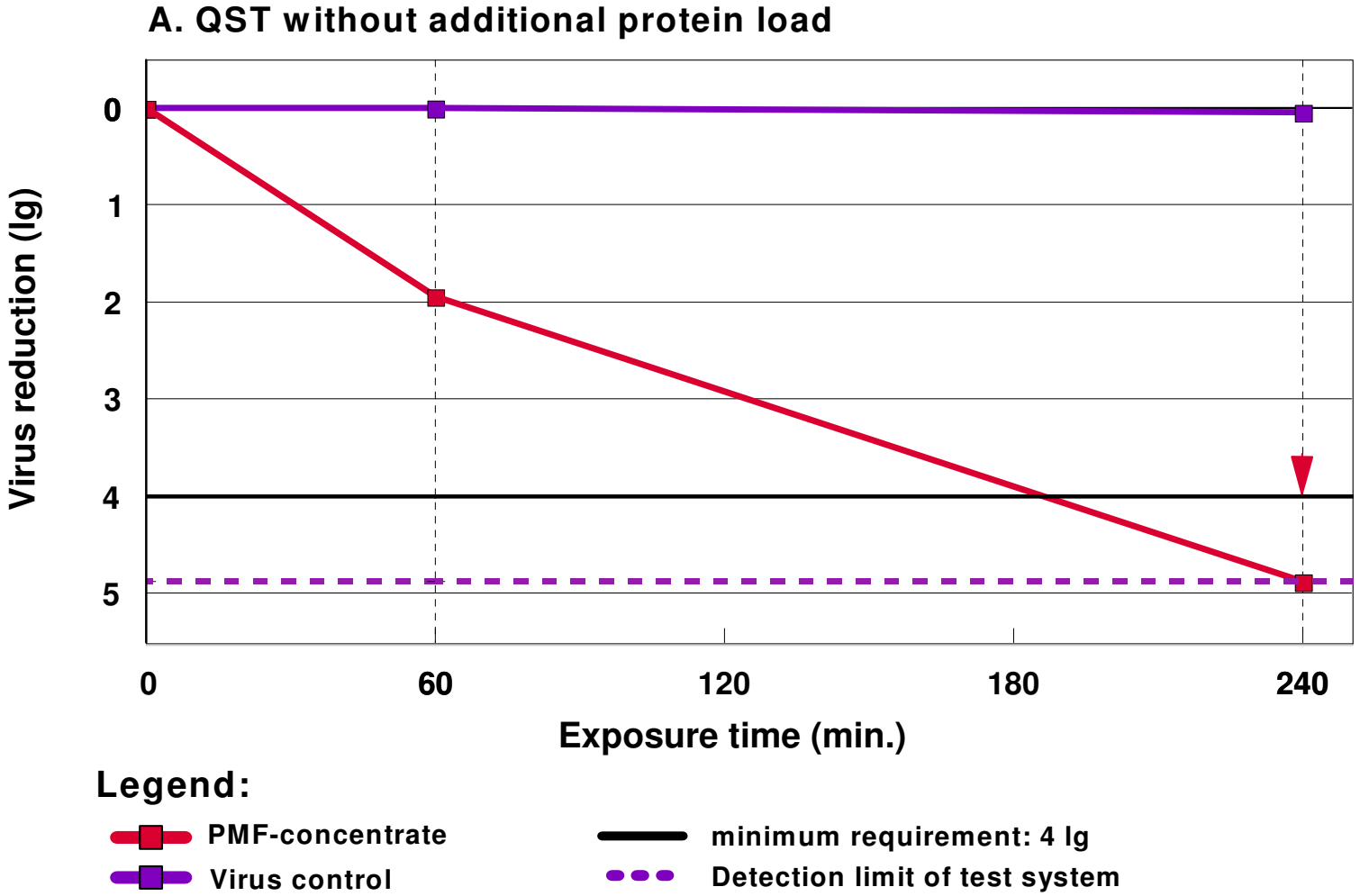

Fig. 1

- Attachment: Experimental protocols -

- Virucidal activity of the product *PMF-concentrate* - Experiment S1 at T = 37° C / testing in the *quantitative suspension test (QST)* against *influenza A virus* using *Spearman & Kärber's* method for virus titration of the main samples.

- Virucidal activity of the product *PMF-concentrate* - Experiment S2 at T = 37° C / testing in the *quantitative suspension test (QST)* against *influenza A virus* using the methodology of *Lycke* for virus titration of the main samples.

Information about the testing

Principal:	PMF Natural Products company	*Test run:*	S1
Product(s):	PMF-concentrate	*Test date:*	26.08.2014
Test system:	Influenza *(H1N1)* + MDCK-cells	*Analysis:*	01.09.2014 *(6 p.i.)*

Test methodology and test parameters

Test method: quantitative virucidal suspensions test according to DVV/RKI-guideline *(Version 08/08)*
Test mixture: 1 VT protein load + 1 VT virus suspension + 8 VT 1,25fold working solution
Protein load: no additional protein load *(PBS)*
Parameter: test temperature: 37° C with the exposure time(s) of: 60 and 240 min.

Tested product sample(s)

1st product: PMF-concentrate *[Product sample: as received (designation: PMF), Arrival: 01.08.2014, Storage at 2-8° C]*

Tab. 1: Weight of content

Set	Product(s)	Conc. in Test *(1x)*	Working sol. *(x 1,25)*	Dosage	pH of working sol.
#1	PMF-concentrate	20,34%	25,42%	0,75 g in 2,95 mL	pH 9,54 *(in test: pH 9,52)*

Tab. 2: Content of samples

Samples	1a	1b	2a	2b
	Virus inactivation			
	Set #1 / 60 min.		Set #1 / 240 min.	
PBS	15µL	15µL	15µL	15µL
Influenza	15µL	15µL	15µL	15µL
PMF / Sol.	120µL	120µL	120µL	120µL
Titration	S&K *(VF = 5)*		S&K *(VF = 5)*	

Samples	3a	3b	4a	4b	5
	Virus control / 60 Min.		Virus control / 240 Min.		Cytotoxicity
	w/o		w/o		Set #1/240 min.
PBS	15µL	15µL	15µL	15µL	15µL
Influenza	15µL	15µL	15µL	15µL	
Medium					15µL
PBS	120µL	120µL	120µL	120µL	
PMF / Sol.					120µL
Titration	S&K *(VF = 5)*		S&K *(VF = 5)*		S&K *(VF = 5)*

Performing of the test

1. Preparation of the product solution: (in the specified sequence)

- ***0,75 g PMF-concentrate*** was solved with agitation and warming to 37°C in 2,95 mL A. bidest.

2. Preparation of the test samples

- Per test point (concentration/exposure time) 2 redundant test samples were prepared.
- Test mixture: 1 vol. PBS + 1 vol. virus suspension + 8 vol. PMF-working solution (1,25-fold)

3. Dilution of the test sample and estimation of virus titer

- ***Termination of virus inactivation:*** after exposure the test samples were diluted with medium *(cf. virus titration).*
- With the ***virus control*** the virus titer was estimated using the methodology of *Spearman & Kärber* with VF = 5 from 113 µL (out of 150 µL of the test sample).
- With the ***virus inactivation samples*** the virus titer was estimated using the methodology of *Spearman & Kärber* with VF = 5 from 113 µL (out of 150 µL of the test sample).

4. Judgement of the cells / virus detection

- At day 6 p.i. supernatant of the cell cultures were tested with the HA-test (haemagglutination).

Tab. 3.1: Virus control *(virus titration: according to Spearman & Kärber)*

Samples	3a	3b	Ø	4a	4b	Ø	5
	Virus control / 60 min.			Virus control / 240 min.			Cytotoxicity
	w/o			w/o			Set #1
1 / -0,7	4/4 [1]	4/4	**8/8**	4/4 [1]	4/4	**8/8**	4/4
2 / -1,4	4/4	4/4	**8/8**	4/4	4/4	**8/8**	4/4
3 / -2,1	4/4	4/4	**8/8**	4/4	4/4	**8/8**	4/4
4 / -2,8	4/4	4/4	**8/8**	4/4	4/4	**8/8**	0/4
5 / -3,5	4/4	4/4	**8/8**	4/4	4/4	**8/8**	
6 / -4,2	4/4	1/4	**5/8**	3/4	3/4	**6/8**	
7 / -4,9	1/4	0/4	**1/8**	0/4	0/4	**0/8**	
8 / -5,6	1/4		**1/8**				
9 / -6,3	0/4		**0/8**				
10 / -7,0							
11 / -7,7							
ZK	0/4	0/4	**0/8**	0/4	0/4	**0/8**	0/4
Titer/test vol. ($lg\ ID_{50}$)	4,9	4,03	**4,47**	4,38	4,38	**4,38**	2,45
Average ± CI (95%) [2]	4,47 ± 0,36 per 100 µL			4,38 ± 0,23 per 100 µL			-
Reduction [3] $lg\ ID_{50}$ ± CI [95%]	-			**0,10 ± 0,42**			-

[1] = number of virus positive cell culture units to total number of cell culture units
[2] = Calculation of 95% confidental intervall of virus titer as well as virus reduction following DVV/RKI-Guideline.
[3] = Virus reduction: titer of virus control minus titer of sample ($lg\ ID_{50}$).

Tab. 3.2:Virus inactivation *(virus titration: according to Lycke)*

Samples	1a	1b	Ø	2a	2b	Ø
	Virus inactivation / 60 min.			Virus inactivation / 240 min.		
	Set #1			Set #1		
1 / -0,7	tox	tox	**tox**	tox	tox	**tox**
2 / -1,4	tox	tox	**tox**	tox	tox	**tox**
3 / -2,1	0/4 [1]	1/4	**1/8**	tox	tox	**tox**
4 / -2,8	2/4	2/4	**4/8**	0/4	0/4	**0/8**
5 / -3,5	0/4	0/4	**0/8**			
6 / -4,2						
7 / -4,9						
8 / -5,6						
9 / -6,3						
10 / -7,0						
11 / -7,7						
ZK	0/4	0/4	**0/8**	0/4	0/4	**0/8**
Titer/test vol. ($lg\ ID_{50}$)	2,1	2,28	**2,19**	≤ 2,45	≤ 2,45	**≤ 2,45**
Average ± CI (95%) [2]	2,19 ± 0,32 per 100 µL			≤ 2,45 per 100 µL		
Reduction [3] $lg\ ID_{50}$ ± CI [95%]	**2,28 ± 0,48**			**≥ 1,93 ± 0,23**		

[1] = number of virus positive cell culture units to total number of cell culture units
[2] = Calculation of 95% confidental intervall of virus titer as well as virus reduction following DVV/RKI-Guideline.
[3] = Virus reduction: titer of virus control minus titer of sample ($lg\ ID_{50}$).

Materials and reagents used:

- ***Testvirus***

Test virus	Influenza A Virus, subtype H1N1
Strain	A/New Caledonia/20/99, IVR-116
Origin	Chiron Behring; Marburg, Germany
Test virus	Seet virus material; Ch.-Bez.: A 04/03; arrived at Eurovir at 18.03.2003 Virus Passage: original virus; Chiron Behring +0 Original seet virus was 10fold diluted with MEM; stored at -80° C

- ***Cells***

Cells	MDCK/63 (Madin Darby canine kidney cells)
Origin	Nationales Referenzzentrum für Influenza; Robert Koch-Institut, Berlin, Germany cells received in living condition with 73th passage at 09.07.2001 (= RKI +0)
Cell passage used	RKI +3 / + 9 / + 28

- ***Additional material and reagents***

Material	*Supplier*	*Order No.*	*Lot*	*Expiry date*
DMEM	Biochrom	F 0435	1272 B	11/2015
Glutamine	Biochrom	K 0283	0978 B	08/2016
Pen./Strept.	Biochrom	A 2213	0018 C	01/2017
FCS	Biochrom	S 0210	0338 T	04/2015
PBS	Biochrom	L 1820	0123 C	01/2017
Trypsine	Invitrogen	25300-096	1437736	09/2015
Hens erythrocytes	Labor Dr. Merk	E-200	E200/1435	03.09.2014

- ***Performing of the experiment and responsibilities***

Part of experiment	Performed by (position)
Supervision	Dr. Ch. Jursch (Laborleiter)
Control of product input	Fr. S. Sachs (Biologielaborantin) und Dr. Ch. Jursch (Laborleiter)
Performing the test	Fr. S. Sachs (Biologielaborantin)
Cell culturing	Fr. S. Sachs (Biologielaborantin)
indirect virus detection / HA-test	Fr. S. Sachs (Biologielaborantin)
Reading of cells & Raw data	Fr. S. Sachs (Biologielaborantin)
Data input & Analysis	Dr. Ch. Jursch (Laborleiter)
Protocol preparation	Dr. Ch. Jursch (Laborleiter)

Information about the testing

Principal:	PMF Natural Products company	*Test run:*	S2
Product(s):	PMF-concentrate	*Test date:*	19.09.2014
Test system:	Influenza *(H1N1)* + MDCK-cells	*Analysis:*	25.09.2014 *(6 p.i.)*

Test methodology and test parameters

Test method: quantitative virucidal suspensions test according to DVV/RKI-guideline *(Version 08/08)*
Test mixture: 1 VT protein load + 1 VT virus suspension + 8 VT 1,25fold working solution
Protein load: no additional protein load *(PBS)*
Parameter: test temperature: 37° C with the exposure time(s) of: 60 and 240 min.

Tested product sample(s)

1st product: PMF-concentrate *[Product sample: as received (designation: PMF), Arrival: 01.08.2014, Storage at 2-8° C]*

Tab. 1: Weight of content

Set	Product(s)	Conc. in Test *(1x)*	Working sol. *(x 1,25)*	Dosage	pH of working sol.
#1	PMF-concentrate	20,34%	25,42%	0,75 g in 2,95 mL	pH 9,54 *(in test: pH 9,52)*

Tab. 2: Content of samples

Samples	1a	1b	2a	2b
	Virus inactivation			
	Set #1 / 60 min.		Set #1 / 240 min.	
PBS	15µL	15µL	15µL	15µL
Influenza	15µL	15µL	15µL	15µL
PMF / Sol.	120µL	120µL	120µL	120µL
Titration	*Lycke (VF = 1000)*		*Lycke (VF = 1000)*	

Samples	3a	3b	4a	4b	5
	Virus control / 60 Min.		Virus control / 240 Min.		Cytotoxicity
	w/o		w/o		Set #1/240 min.
PBS	15µL	15µL	15µL	15µL	15µL
Influenza	15µL	15µL	15µL	15µL	
Medium					15µL
PBS	120µL	120µL	120µL	120µL	
PMF / Sol.					120µL
Titration	S&K *(VF = 5)*		S&K *(VF = 5)*		*Lycke (VF = 1000)*

Performing of the test

1. Preparation of the product solution: (in the specified sequence)

- ***0,75 g PMF-concentrate*** was solved with agitation and warming to 37°C in 2,95 mL A. bidest.

2. Preparation of the test samples

- Per test point (concentration/exposure time) 2 redundant test samples were prepared.
- Test mixture: 1 vol. PBS + 1 vol. virus suspension + 8 vol. PMF-working solution (1,25-fold)

3. Dilution of the test sample and estimation of virus titer

- ***Termination of virus inactivation:*** after exposure the test samples were diluted with medium *(cf. virus titration).*
- With the ***virus control*** the virus titer was estimated using the methodology of *Spearman & Kärber* with VF = 5 from 113 µL (out of 150 µL of the test sample).
- With the ***virus inactivation samples*** the virus titer was estimated using the methodology of *Lycke.* For each of the test samples (a and b) 48 µL was added to 96 mL Medium, corresponding to a dilution of VF = 1000. All of the 96 mL were then transferred to cell cultures with 200 µL per well (480 wells).

4. Susceptibility control

- Sample 5 (cytotoxicity sample) was diluted 1000fold and was then distributed to cell cultures (cf. virus inactivation). Afterwards a virus dilution serie (VK/E) was transferred to these cells.

5. Judgement of the cells / virus detection

- At day 6 p.i. supernatant of the cell cultures were tested with the HA-test (haemagglutination).

Tab. 3.1: Virus control + Susceptibility control *(virus titration: according to Spearman & Kärber)*

Samples	3a	3b	Ø	4a	4b	Ø	5	VK/E
	Virus control / 60 min.			Virus control / 240 min.			Susceptibility Control	
1 / -0,7	4/4 [1]	4/4	**8/8**	4/4 [1]	4/4	**8/8**	8/8	8/8
2 / -1,4	4/4	4/4	**8/8**	4/4	4/4	**8/8**	8/8	8/8
3 / -2,1	4/4	4/4	**8/8**	4/4	4/4	**8/8**	8/8	8/8
4 / -2,8	4/4	4/4	**8/8**	4/4	4/4	**8/8**	8/8	8/8
5 / -3,5	4/4	4/4	**8/8**	4/4	4/4	**8/8**	8/8	8/8
6 / -4,2	4/4	4/4	**8/8**	3/4	4/4	**7/8**	7/8	8/8
7 / -4,9	2/4	2/4	**4/8**	1/4	2/4	**3/8**	3/8	3/8
8 / -5,6	0/4	0/4	**0/8**	1/4	1/4	**2/8**	2/8	3/8
9 / -6,3				0/4	0/4	**0/8**	0/8	0/8
10 / -7,0								
11 / -7,7								
ZK	0/4	0/4	**0/8**	0/4	0/4	**0/8**	0/8	0/8
Titer/test vol. ($lg\ ID_{50}$)	4,9	4,9	**4,9**	4,73	5,08	**4,9**	4,91 ± 0,39	5,08 ± 0,23
Average ± CI (95%) [2]	4,90 ± 0,26 per 100 µL *(≃4,88 lg ID_{50} pro 96 µL)*			4,90 ± 0,39 per 100 µL *(≃4,88 lg ID_{50} pro 96 µL)*			Δ titer = 0,18 ± 0,53	
Reduction [3] lg ID_{50} ± CI [95%]	-			**0,0 ± 0,47**			**cells susceptible: yes [2]**	

[1] = number of virus positive cell culture units to total number of cell culture units
[2] = Calculation of 95% confidental intervall of virus titer as well as virus reduction following DVV/RKI-Guideline.
[3] = Virus reduction: titer of virus control minus titer of sample ($lg\ ID_{50}$).
[4] = Susceptibility of the detection cells is to be assumed when Δ virus titer is ≤ lg 0,5 [DVV/RKI-Guideline].

Tab. 3.2:Virus inactivation *(virus titration: according to Lycke)*

Samples	1a + 1b	2a + 2b
	Virus inactivation *(VF = 1000)*	
	Set #1 / 60 min.	Set #1 / 240 min.
analysed sample vol.	2 x 48 = 96 µL	2 x 48 = 96 µL
Cell culture units	480	480
Virus positive	448	0
Ratio p [2]	0,9333	0,0
Residual virus *(lg ID_{50} per 96 µL)*	3,26 ± 0,26	< 1 ID_{50} *(0,0 lg ID_{50})*
Virus input *(lg ID_{50} per 96 µL)*	4,88 ± 0,26	4,88 ± 0,39
Reduction [3] ***(lg ID_{50} ± CI [95%])***	**1,62 ± 0,37**	**> 4,88 ± 0,55**

[1] = sample volume transferred onto cell cultures: 48 µL from test mix a. plus 48 µL from test mix b. resulting in 2 x 48 = 96 µL
[2] = ratio of virus positive cell culture units to total number of cell cultures.
[3] = Virus reduction: titer of virus control (cf. Tab. 3.1) minus titer of sample (lg ID_{50})

Estimation of virus titer by LYCKE´s method (Arch Ges Virusforsch (1957); 7:483-493)

Calculation of virus titer by using the following formula:

$- ID_{50} = [1,4 \times \ln (1-p)]$ p = ratio of positive cell cultures to total number of cell cultures

- ***Example:*** 51 out of 100 cell culture units was virus positive → p = 51/100 = 0,51

p put into formula: $- ID_{50} = [1,4 \times \ln (1- 0,51)]$

with ln (0,49): $- ID_{50} = 1,4 \times -0,71$

resulting in: $- ID_{50} = -0,998$ or $ID_{50} = 0,998$

That means that per single cell culture unit 0,998 or 1 ID_{50} of residual virus was present.

When this content of virus was multiplied with the number of cell culture units (= 100) the complete amount of residual virus was obtained: 1,0 ID_{50} x 100 = 100 ID_{50} or lg ID_{50} = 2,0

Result of the example: the total quantity of residual virus which was present in the examined sample of liquid was estimated to **lg ID_{50} = 2,0**

Materials and reagents used:

- ***Testvirus***

Test virus	Influenza A Virus, subtype H1N1
Strain	A/New Caledonia/20/99, IVR-116
Origin	Chiron Behring; Marburg, Germany
Test virus	Seet virus material; Ch.-Bez.: A 04/03; arrived at Eurovir at 18.03.2003 Virus Passage: original virus; Chiron Behring +0 Original seet virus was 10fold diluted with MEM; stored at -80° C

- ***Cells***

Cells	MDCK/63 (Madin Darby canine kidney cells)
Origin	Nationales Referenzzentrum für Influenza; Robert Koch-Institut, Berlin, Germany cells received in living condition with 73th passage at 09.07.2001 (= RKI +0)
Cell passage used	RKI +3 / + 9 / + 35

- ***Additional material and reagents***

Material	*Supplier*	*Order No.*	*Lot*	*Expiry date*
DMEM	Biochrom	F 0435	1272 B	11/2015
Glutamine	Biochrom	K 0283	0978 B	08/2016
Pen./Strept.	Biochrom	A 2213	0018 C	01/2017
FCS	Biochrom	S 0210	0338 T	04/2015
PBS	Biochrom	L 1820	0123 C	01/2017
Trypsine	Invitrogen	25300-096	1437736	09/2015
Hens erythrocytes	Labor Dr. Merk	E-200	E200/1439	01.10.2014

- ***Performing of the experiment and responsibilities***

Part of experiment	Performed by (position)
Supervision	Dr. Ch. Jursch (Laborleiter)
Control of product input	Fr. S. Sachs (Biologielaborantin) und Dr. Ch. Jursch (Laborleiter)
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