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Laboratory identification number	LI-V-023-018
Study Report	Testing the virus-reducing performance of cold plasma against Minute Virus of Mice suspended in water
Sponsor	Kimetec GmbH Gerlinger Str. 36-38 71254 Ditzingen
Test method	Quantitative test on porous and non-porous surfaces
Active substance	Cold plasma
Contact time	20 minutes 60 minutes 90 minutes

Interfering substance	Not applicable	
Storage conditions	20.0 °C ± 2.5 °C, dry	
Project description	<p>Validation of the antiviral activity on textile (porous) and plastic carriers (non-porous) using cold plasma generated by the PlasmaEgg. The test material originated from vein tourniquets.</p> <p>The quantitative determination of the recovered virus inoculum according to the following measures in triplicate:</p> <ul style="list-style-type: none"> - U0: Inoculum control without drying - Ut: Recovery control with drying - At: Test specimen to determine the antiviral activity of cold plasma - Cytotoxicity control 	
Reference documents	<p>modification of the following test methods: DIN EN 17111:2018-12</p> <p>SOP-ST-VIR.M.0070.07</p>	
Reference material	vein tourniquet (porous test specimen) quick lock buckle (non-porous test specimen)	
Written	PD Dr. rer. nat. Maren Eggers	
Test facility	<p>Labor Prof. Dr. G. Enders MVZ GbR Abteilung Virologie Rosenbergstraße 85 70193 Stuttgart</p>	
Dates	Begin of testing:	2023-03-09
	End of testing:	2023-03-20
Technical assistance	Petra Marquart (cell culture) Niels Fellner	

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1. Materials, media and reagents

1.1. Abbreviations

RF	Reduction factor
D-MEM	Dulbecco's Modified Eagle Medium
FCS	Fetal bovine serum
Max	Maximum
Min	Minimum
NEA	Non-essential amino acids
PBS	Phosphates buffered saline (Dulbecco A pH 7.3)
RT	Room temperature
SOP	Standard Operating Procedure

1.2. Apparatus

- Incubator +37 °C ± 2 °C with CO₂ supply
- Fridge 2 - 8 °C
- Laminar Air Flow
- Mixing device
- Vortexer
- Thermometer
- Pipetting aid (Pipet-Boy)
- 5 ml pipettes
- Eppendorf pipette variable 0.5 µl - 10 µl
- Eppendorf pipette variable 10 µl - 100 µl
- Eppendorf pipette variable 100 µl - 1000 µl
- sterile pipette tips (blue, yellow, white)
- sterile disposable pipettes (1 ml, 5 ml, 10 ml)
- 96-well microtiter plates
- Positive Displacement Pipette Tips (sterile) M
- Multidrop (e.g. laboratory systems)
- Neubauer counting chamber
- Water bath
- Centrifuge
- Inverted microscope

1.3. Materials

- Antibiotics
- D-MEM Dulbecco's Modified Eagle Medium
- FCS Fetal calf serum
- NEA Non-essential amino acids
- PBS Phosphate buffered saline solution

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2. Identification of the sample and experimental conditions:

Identification of the sample

Product name	PlasmaEgg	
	Textile carrier (vein tourniquet)	Plastic carrier (quick lock buckle)
Carrier		
	Porous textile carrier: 2 cm x 2 cm	Non-porous plastic carrier: 1 cm x 3 cm
Manufacturer	Kimetec GmbH	
Date of delivery	2022-03-09	
Storage conditions	20.0 °C, dark	

Experimental conditions

Test site	Labor Prof. Dr. G. Enders MVZ GbR Rosenbergstr. 85 70193 Stuttgart Germany
Test period	2023-02-07 – 2023-02-17 2023-03-09 – 2023-03-20
Test method	DIN EN 17111:2018-12
Contact time	20 minutes 60 minutes 90 minutes
Test specimen	Textile carriers Plastic carriers
Temperature of incubation	18.0 °C +/- 1.0 °C to 25.0 °C +/- 1.0 °C

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Identification of the virus

Virus	Minute Virus of mice, strain Crawford
Virus: source	Paul Ehrlich Institute Langen
Virus: batch	0211222 in H ₂ O 10223 8P/5 in H ₂ O
Cell line	A9 cells (mouse fibroblasts)
Cell line: source	ATCC (American Type Culture Collection)
Cell line: number of passage	31 / 21 22 / 12
Temperature of cell incubation	37.0 °C ± 1.0 °C, CO ₂ Incubator (5.0% CO ₂)

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3. Test methods

The tests were performed according to modification of the European standards DIN EN 17111:2018-12, DIN EN 16777:2019-03 and according to the SOP-ST-VIR.M.0070.07.

Test strain virus and cell culture line

Minute virus of mice strain Crawford was used as the test virus. For virus cultivation and the suspension test, A9 cells, a cell line established from mice fibroblasts, were used. The host cells were cultivated at 37.0 °C in a humid atmosphere under 5.0% CO₂. The cells were fed with Dulbeccos Minimum Essential Medium (D-MEM) supplemented with heat-inactivated fetal calf serum (FCS) and non-essential amino acids. For the virus cultivation, confluent monolayers with a maximum age of 2 days were used. The stock virus suspension was produced according to the directive. Cell debris was separated by low speed centrifugation. Aliquots of the virus suspension were stored at -70 °C.

Test procedure

All carriers were inoculated with 5 x 10 µl virus, which was suspended in H₂O after ultrazentrifugation. The titre of the virus suspension in H₂O was $6.17 \pm 0.42 \log_{10} \text{TCID}_{50}/\text{ml}$.

Three carriers were used for each test specimen. Immediately after drying, the carriers were placed in the PlasmaEgg. Then the irradiation by the cold plasma started for the two contact times (20 minutes and 60 minutes).

Immediately after the contact time, the carriers were transferred into 5 ml medium. Each carrier was visually examined for complete elution. For the determination of residual virus titer, a decadal dilution series was prepared. Subsequently, six wells of a microtitre plate containing a confluent monolayer of A9 cells were inoculated with 0.1 ml of each dilution, and the cells were incubated at 37.0°C in a humidified atmosphere under 5.0% CO₂. After 10 days the cell cultures were stained with 50 µl crystal violet per well. The cells were examined microscopically for cytopathic effects (CPE). The cell culture results were recorded as "0" for no CPE and "1" (25.0% CPE) to "4" (100% CPE) depending on the degree of cell damage.

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Determination of the residual virus titre by the large-volume-plating method

After the specified contact time, the virucidal activity was immediately suppressed by a large volume of ice-cold medium (D-MEM + 2.0% FCS) because the textile carriers show a cytotoxic effect. To reach at least a four \log_{10} reduction in the titre of the Minute virus of mice strain Crawford for cytotoxic products, detoxification of the test mixtures by molecular sieving with Microspin S 400 HR columns or using a method described by Large-Volume plating (LVP) assay is necessary. Using the LVP, the lowest apparently non-cytotoxic dilution of the test mixture is added to ice-cold medium after the specified contact time. In addition, the detection of residual virus can be improved by the testing of a large sample volume 1:10. Briefly, 1.0 ml of the test mixture was added to 10.0 ml ice-cold medium after the specified contact time. 100.0 μ l of the diluted sample was added to a defined number of wells (96 wells). The cells were cultivated for a specified incubation period. Then, the cells were inspected microscopically for virus foci (for virus-induced changes in cell morphology).

The viral titre was calculated as follows:

If no virus is observed, the number of infectious virus particles is determined by the Poisson distribution according to CPMP/ICH/295/95 guideline (ICH Q5A, Appendix 3: Statistical considerations for assessing virus assays) using the following formula:

$$c = \ln p/v$$

[("c" concentration of viruses in the test mixture, "p" denoting the 95.0% probability to detect virus, "v" is the plated volume whereas "V" is to be << "V" (total volume)].

If low amount of viruses is detected the most probable average number of TCID₅₀ can be calculated by the use of the following formula which is derived from the Taylor series:

$$c = \frac{D}{V_w} * \left(-\ln \frac{n - n_p}{n} \right)$$

("c" concentration of viruses in the test mixture, "D" dilution factor of prediluted sample, "V_w" is the plated volume per well, "n" number of inoculated wells whereas "n_p" is the number of successfully infected wells).

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Calculation of the virucidal activity of the products

The assessment of the efficacy of the **PlasmaEgg** is carried out by calculating the reduction. Reduction of virus titre was calculated from titre differences between the virus control minus the logarithmic titre of the treated test specimen test virus ($\Delta \log_{10}$ TCID₅₀/ml) at a specifically chosen contact time.

4. Results and Evaluation

The cold plasma technology in association with **Terraplasma** was tested following an exposure time of 20, 60 and 90 minutes.

Validity of the test

The infectivity titer of the virus was determined using the endpoint titration method and the titre was given as \log_{10} TCID₅₀/ml. The titer of the virus suspension was $7.00 \pm 0.45 \log_{10}$ TCID₅₀/ml for the Virus suspended in H₂O.

The textile test specimen caused cytotoxic effects and the plastic carriers caused no cytotoxic effects as shown in Table 1.

Table 1 Verification of cytotoxic effect on host cells

Specimen	Dilution (\log_{10})							
	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Textile carrier	+	-	-	-	-	-	-	-
Plastic carrier	-	-	-	-	-	-	-	-

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Table 2 Inoculation control and recovery control on porous test specimen (vein tourniquet) of Minute Virus of Mice suspended in H₂O

Test date: 2023-02-07 – 2023-02-17

Sample	Contact time	Level of cytotoxicity	Titre of the untreated Interference (\log_{10} TCID _{50/ml}) with 95% confidence interval			
			test 1	test 2	test 3	Mean
inoculation control U_0	0 min	1.50	4.17 ± 0.42	-	-	4.17 ± 0.42
Recovery control after drying U_t 20 min	20 min	1.50	4.67 ± 0.33	4.33 ± 0.33	4.50 ± 0.33	4.50 ± 0.36
Control with drying U_t 60 min	60 min	1.50	4.33 ± 0.33	4.33 ± 0.54	4.83 ± 0.33	4.50 ± 0.40

Test date: 2023-03-09 – 2023-03-20

Sample	Contact time	Level of cytotoxicity	Titre of the untreated Interference (\log_{10} TCID _{50/ml}) with 95% confidence interval			
			test 1	test 2	test 3	Mean
Control without drying U_0	0 min	1.50	5.33 ± 0.33	5.33 ± 0.54	5.50 ± 0.00	5.39 ± 0.29
Control with drying U_t	20 min	1.50	5.00 ± 0.45	5.17 ± 0.42	5.33 ± 0.33	5.17 ± 0.40
Control with drying U_t	60 min	1.50	4.83 ± 0.42	5.33 ± 0.54	5.00 ± 0.45	5.06 ± 0.47

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Table 3 Test results of the activity of the PlasmaEgg against Minute Virus of Mice suspended in H₂O on porous test specimen (vein tourniquet material)

Contact time	Test date	Level of cyto-toxicity	U _t Titre of the virus control (\log_{10} TCID ₅₀ /ml) with 95.0% confidence interval				A _t Titre of the “residual virus” inactivation (\log_{10} TCID ₅₀ /ml) with 95.0% confidence interval				Reduction factor
			Carrier 1	Carrier 2	Carrier 3	Mean value	Carrier 1	Carrier 2	Carrier 3	Mean value	
20 min	2023-02-07	1.50	4.67 +/- 0.33	4.33 +/- 0.33	4.50 +/- 0.33	4.50 +/- 0.36	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≥ 3.00 +/- 0.33
20 min (LVP)	2023-03-09	-	5.00 +/- 0.45	5.17 +/- 0.42	5.33 +/- 0.33	5.17 +/- 0.40	0.66	0.18	0.66	0.50	4.67 ± 0.40
60 min	2023-02-07	1.50	4.33 +/- 0.33	4.33 +/- 0.54	4.83 +/- 0.33	4.50 +/- 0.40	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≤ 1.50 +/- 0.00	≥ 3.00 +/- 0.40
60 min (LVP)	2023-03-09	-	4.83 +/- 0.42	5.33 +/- 0.54	5.00 +/- 0.45	5.05 +/- 0.47	0.66	0.66	0.66	0.66	4.39 ± 0.47

CI confidence interval

LVP large volume plating

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Table 4 Inoculation control and recovery control on porous test on non-porous test specimen (vein tourniquet plastic buckle) of Minute Virus of Mice suspended in H₂O

Test date: 2023-03-09 – 2023-03-20

Sample	Contact time	Level of cytotoxicity	Titre of the untreated Interference (\log_{10} TCID ₅₀ /ml) with 95% confidence interval			
			test 1	test 2	test 3	Mean
Control without drying U₀	0 min	0.50	5.17 ± 0.42	5.00 ± 0.45	5.00 ± 0.45	5.06 ± 0.44
Control with drying U_t	90 min	0.50	4.83 ± 0.42	4.50 ± 0.47	4.50 ± 0.47	4.61 ± 0.45
Plastic carrier A_t	90 min	0.50	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00

Test date: 2023-02-07 – 2023-02-17

Sample	Contact time	Level of cytotoxicity	Titre of the untreated Interference (\log_{10} TCID ₅₀ /ml) with 95.0% confidence interval			
			test 1	test 2	test 3	Mean
inoculation control U₀	0 min	0.50	4.00 ± 0.45	-	-	4.00 ± 0.45
Recovery control after drying U_{t 20 min}	20 min	0.50	3.83 ± 0.42	3.50 ± 0.00	3.50 ± 0.42	3.61 ± 0.28
Control with drying U_{t 60 min}	60 min	0.50	3.67 ± 0.33	3.50 ± 0.00	3.83 ± 0.33	3.67 ± 0.22

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Table 5 Test results of the activity of the PlasmaEgg against Minute Virus of Mice suspended in H₂O on non-porous test specimen (vein tourniquet quick closure buckle)

Contact time	Test date	Level of cyto-toxicity	U _t Titre of the virus control (\log_{10} TCID ₅₀ /ml) with 95.0% confidence interval				A _t Titre of the “residual virus” inactivation (\log_{10} TCID ₅₀ /ml) with 95.0% confidence interval				Reduction factor
			Carrier 1	Carrier 2	Carrier 3	Mean value	Carrier 1	Carrier 2	Carrier 3	Mean value	
20 min	2023-02-07	0,50	3.83 +/- 0.42	3.50 +/- 0.00	3.50 +/- 0.42	3.61 +/- 0.28	3.00 +/- 0.54	2.83 +/- 0.42	2.33 +/- 0.33	2.72 +/- 0.43	0.89 +/- 0.51
60 min	2023-02-07	0,50	3.67 +/- 0.33	3.50 +/- 0.00	3.83 +/- 0.33	3.67 +/- 0.22	0.67 +/- 0.33	0.83 +/- 0.42	1.17 +/- 0.42	0.89 +/- 0.39	2.78 +/- 0.45
90 min	2023-03-09	0,50	4.83 +/- 0.42	4.50 +/- 0.47	4.50 +/- 0.42	4.61 +/- 0.44	0.50 +/- 0.00	0.50 +/- 0.00	0.50 +/- 0.00	0.50 +/- 0.00	4.11 +/- 0.44

CI confidence interval

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Test results

The data of the virucidal efficacy of cold plasma is presented in Table 3 and 5. The inactivation of the Minute Virus of Mice by cold plasma generated by the PlasmaEgg showed following reductions:

Antiviral activity of cold plasma on porous textile carriers with MVM suspended in H₂O

4.67 log (99.99 % kill rate) compared to the controls within 20 minutes exposure time

4.39 log (99.99 % kill rate) compared to the controls within 60 minutes exposure time

Antiviral activity of cold plasma on non-porous plastic carriers with MVM suspended in H₂O

4.11 log (99.99 % kill rate) compared to the controls within 90 minutes exposure time



31.03.2023

Date

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Head of disinfectant testing and applied / technical hygiene

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- Archiving: The raw data with respect to this test and a copy of the report will be stored in the archive of Labor Enders MVZ.
- Information: The test results exclusively refer to the samples described above. Account of extracts of this test report is only possible by written approval from Labor Enders MVZ.

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Raw data of Terraplasma PlasmaEgg tested with Minute Virus of Mice Crawford suspended in water on textile carriers

Date: 2023-02-07 – 2023-02-17

Test specimen	replicate	Light Intensity mW/cm ²	Contact time	Dilution (log ₁₀)							
				0	1	2	3	4	5	6	7
Inoculation control without drying U₀		N/A	0 min	xxx xxx	444 444	322 233	011 210	000 000	000 000	000 000	000 000
Recovery control with drying U_{t 20 min}	1	N/A	20 min	xxx xxx	444 444	333 233	221 112	100 000	000 000	000 000	000 000
	2	N/A	20 min	xxx xxx	333 333	333 333	101 222	000 000	000 000	000 000	000 000
	3	N/A	20 min	xxx xxx	333 334	333 323	221 211	000 000	000 000	000 000	000 000
Treated test specimen A_{t 20 min}	1	20	20 min	xxx xxx	000 000						
	2	20	20 min	xxx xxx	000 000						
	3	20	20 min	xxx xxx	000 000						
Recovery control with drying U_{t 60 min}	1	N/A	60 min	xxx xxx	444 433	233 323	112 201	000 000	000 000	000 000	000 000
	2	N/A	60 min	xxx xxx	444 444	232 333	022 022	000 001	000 000	000 000	000 000
	3	N/A	60 min	xxx xxx	444 444	433 333	111 122	010 020	000 000	000 000	000 000
Treated test specimen A_{t 60 min}	1	20	60 min	xxx xxx	000 000						
	2	20	60 min	xxx xxx	000 000						
	3	20	60 min	xxx xxx	000 000						
Cytotoxicity control		N/A	0 min	xxx xxx	000 000						

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

n. a. not applicable

n. d. not done

x cytotoxic

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Raw data of Terraplasma PlasmaEgg tested with Minute Virus of Mice Crawford suspended in water on plastic carriers

Date: 2023-02-07 – 2023-02-17

Test specimen	replicate	Light Intensity mW/cm ²	Contact time	Dilution (log ₁₀)							
				0	1	2	3	4	5	6	7
Inoculation control without drying U₀		N/A	0 min	444 444	444 444	323 334	020 102	000 000	000 000	000 000	000 000
Recovery control with drying U_{t 20 min}	1	N/A	20 min	444 444	444 444	222 222	010 200	000 000	000 000	000 000	000 000
	2	N/A	20 min	444 444	444 444	212 223	000 000	000 000	000 000	000 000	000 000
	3	N/A	20 min	444 444	444 444	121 133	000 000	000 000	000 000	000 000	000 000
Treated test specimen A_{t 20 min}	1	20	20 min	444 444	222 322	000 102	000 001	000 000	000 000	000 000	000 000
	2	20	20 min	444 444	333 233	110 000	000 000	000 000	000 000	000 000	000 000
	3	20	20 min	443 233	221 022	000 000	000 000	000 000	000 000	000 000	000 000
Recovery control with drying U_{t 60 min}	1	N/A	60 min	444 444	444 444	322 222	000 100	000 000	000 000	000 000	000 000
	2	N/A	60 min	444 444	444 444	232 233	000 000	000 000	000 000	000 000	000 000
	3	N/A	60 min	444 444	444 444	213 222	000 120	000 000	000 000	000 000	000 000
Treated test specimen A_{t 60 min}	1	20	60 min	002 000	000 000						
	2	20	60 min	020 010	000 000						
	3	20	60 min	002 112	000 000						
Cytotoxicity control		N/A	0 min	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
Test specimen	replicate	Light Intensity mW/cm ²	Contact time	Dilution (log ₁₀)							
				1	2	3	4	5	6	7	8
Virus suspension		N/A	0 s	444 444	444 444	444 444	333 333	102 012	000 000	000 000	000 000

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

n. a. not applicable

n. d. not done

x cytotoxic

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Raw data of Terraplasma PlasmaEgg tested with Minute Virus of Mice Crawford suspended in water on textile carriers

2023-03-09 – 2023-03-20

Test specimen	replicate	Light Intensity mW/cm ²	Contact time	Dilution (log ₁₀)							
				0	1	2	3	4	5	6	7
Inoculation control without drying U₀	1	N/A	0 min	xxx xxx	444 444	444 212	223 110	111 000	000 000	000 000	000 000
	2	N/A	0 min	xxx xxx	444 444	444 444	222 232	101 101	100 000	000 000	000 000
	3	N/A	0 min	xxx xxx	444 444	444 444	232 232	111 111	000 000	000 000	000 000
Recovery control with drying U_{t 20 min}	1	N/A	20 min	xxx xxx	444 444	444 444	222 222	010 101	000 000	000 000	000 000
	2	N/A	20 min	xxx xxx	444 444	444 444	222 222	011 110	000 000	000 000	000 000
	3	N/A	20 min	xxx xxx	444 444	444 444	222 222	211 011	000 000	000 000	000 000
Recovery control with drying U_{t 60 min}	1	N/A	60 min	xxx xxx	444 444	444 444	222 212	010 100	000 000	000 000	000 000
	2	N/A	60 min	xxx xxx	444 444	444 444	211 222	010 111	000 001	000 000	000 000
	3	N/A	60 min	xxx xxx	444 444	444 444	322 122	101 010	000 000	000 000	000 000
Cytotoxicity control		N/A	0 min	xxx xxx	000 000						

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

n. a. not applicable

n. d. not done

x cytotoxic

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Raw data of Terraplasma PlasmaEgg tested with Minute Virus of Mice Crawford suspended in water on textile carrier, inactivation assay according to LVP

2023-03-09 – 2023-03-20

Treated test specime n $A_{t\ 20\ min}$	Contact time	Line	1	2	3	4	5	6	7	8	9	10	11	12
	20 min	plate 1 / 3	0000 0000											
	20 min	plate 2 / 3	0000 0000	0000 0002	0000 0000									
	20 min	plate 3 / 3	0000 0000											
	Cytotoxicity control		0000 0000											

Treated test specimen $A_{t\ 60\ min}$	Contact time	Line	1	2	3	4	5	6	7	8	9	10	11	12
	60 min	plate 1 / 3	0000 0000											
	60 min	plate 2 / 3	0000 0000											
	60 min	plate 3 / 3	0000 0000											
	Cytotoxicity control		0000 0000											

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

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Raw data of Terraplasma PlasmaEgg tested with Minute Virus of Mice Crawford suspended in water on plastic carriers

2023-03-09 – 2023-03-20

Test specimen	replicate	Light Intensity mW/cm ²	Contact time	Dilution (log ₁₀)							
				0	1	2	3	4	5	6	7
Inoculation control without drying U₀	1	N/A	0 min	444 444	444 444	444 444	232 222	221 010	000 000	000 000	000 000
	2	N/A	0 min	444 444	444 444	444 444	122 222	011 100	000 000	000 000	000 000
	3	N/A	0 min	444 444	444 444	444 444	122 222	011 001	000 000	000 000	000 000
Recovery control with drying U_{t 90 min}	1	N/A	90 min	444 444	444 444	333 333	222 211	110 000	000 000	000 000	000 000
	2	N/A	90 min	444 444	444 444	223 323	101 111	100 000	000 000	000 000	000 000
	3	N/A	90 min	444 444	444 444	333 333	110 211	000 100	000 000	000 000	000 000
Treated test specimen A_{t 90 min}	1	20	90 min	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
	2	20	90 min	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
	3	20	90 min	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
Cytotoxicity control		N/A	0 min	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
VK Susp				444 444	444 444	444 444	444 444	444 444	222 222	110 100	000 000

1–4 virus present, degree of CPE in cell culture units (6 wells of microtitre plates)

0 no virus present

n. a. not applicable

n. d. not done

x cytotoxic