PD Dr. Maren Eggers
c/o Labor Prof. Dr. G. Enders MVZ GbR
Rosenbergstraße 85
70193 Stuttgart
Germany
Phone: +49 - (0)711 6357- 130
fax: +49 - (0)711 6357- 202

Laboratory identification number	LI-V-023-029
Study Report	**Testing the virus-reducing performance of photodynamic technology against Adeno virus type 5, Adenoid 75**
Test	Validation of the antiviral activity of parquet flooring coated with **eukula 491 VIROBAC** using photodynamic technology. Quantitative determination of the recovered virus inoculum according to the following measures in duplicate: 1. U_0: Recovery control (– dye / – light / + virus) 2. U_{td}: Dark control (+ dye / – light / + virus) 3. U_{tl}: Light control (– dye / + light / + virus) 4. U_t: Reference control, untreated test specimen (- dye / – light / + virus) 5. A_t: treated test specimen to determine the antiviral activity of **eukula 491 VIROBAC** (+ dye / + light / + virus) 6. Cytotoxicity control
Sponsor	Dr.Schutz GmbH Steinbrinksweg 30 D-31840 Hessisch Oldendorf
Test method	Quantitative test for the evaluation of virucidal activity on coated surfaces
Active substance	Singlet oxygen generated in situ from ambient air
Contact time	4 hours

Interfering substance	Not applicable	
Storage conditions	20.0 °C ± 2.5 °C, dry	
Project description	• Virus contamination of carriers (21.5 cm x 50 cm) coated with **eukula 491 VIROBAC** by the manufacturer • Microbial reduction via light emitting LEDs in combination with **eukula 491 VIROBAC** coated carrier	
Reference documents	modification of the following test methods: • DIN EN 16777:2019-03 • ISO 21702:2019	
	• SOP-ST-VIR.M.0067.09 • SOP-ST-VIR.M.0086.01	
Reference material	FOREX® PVC hard foam	
Written	PD Dr. rer. nat. Maren Eggers	
Test facility	Labor Prof. Dr. G. Enders MVZ GbR Abteilung Virologie Rosenbergstraße 85 70193 Stuttgart	
Dates	Begin of testing:	2023-06-14
	End of testing:	2023-06-21
Technical assistance	Petra Marquart (cell culture) Niels Fellner	

Table of Contents

Page

1. Materials, media and reagents

1.1. Abbreviations

RF	Reduction factor
E-MEM	MEM with Earle´s salts
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
SOPStandard	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
- E-MEM MEM with Earle´s salts
- FCS Fetal calf serum
- NEA Non-essential amino acids
- PBS Phosphate buffered saline solution

2. Identification of the sample and experimental conditions:

Identification of the sample

Product name	**eukula 491 VIROBAC**
Test surface	21.5 cm x 50 cm
Test specimen	Parquet flooring
Reference Material	FOREX classic PVC hard foam
Carrier	
Date of delivery	2023-06-05
Storage conditions	20.0 °C, dark

Experimental conditions

Test site	Labor Prof. Dr. G. Enders MVZ GbR Rosenbergstr. 85 70193 Stuttgart Germany
Test period	2023-06-14 – 2023-06-21
Test method	ISO 21702:2019-05
Contact time	4 hours
Temperature of incubation	21.5 °C ± 3.5 °C

Identification of the virus

Virus	Adeno virus type 5, Adenoid 75
Virus: source	Virus bank of the DVV (Prof. Dr. A. Sauerbrei/University of Jena)
Virus: batch	260322
Virus: number of passage	n+6/3
Cell line	A549 cells (human lung adenocarcinoma epithelial cell line)
Cell line: source	ATCC (American Type Culture Collection)
Cell line: number of passage	128 / 28
Temperature of cell incubation	37.0 °C ± 1.0 °C, CO2 Incubator (5.0% CO2)

Reference material

Material	FOREX classic
Material source	FOREX classic, white matt with foil on one side, material number: SFSFOXC020RWH1F, thyssenkrupp Plastics GmbH

3. Test methods

The tests were performed according to modifications of the European standard DIN EN 16777:2019-03 and ISO 21702:2019 test methods and according to the SOP-ST-VIR.M.0067.09 plus SOP-ST-VIR.M.0086.01.

Test strain virus and cell culture line

Adenovirus type 5 strain Adenoid 75 was used as the test virus. A549 cells, a cell line established from a human lung adenocarcinoma epithelial cell, were used for virus cultivation and the suspension test. The host cells of the ATCC (American Type Culture Collection) were cultivated at 37.0 °C in a humid atmosphere under 5.0% CO_2. The cells were fed with MEM with Earle´s salts 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

The treated carriers and control carriers were inoculated with 5 x 10 µl virus, which was diluted in MEM. The titre of the virus suspension was 8.50 ± 0.47 $\log_{10}$ $TCID_{50}$.
Three carriers were tested for each measure. Immediately after drying, the carriers were placed on the sample tray. Then the irradiation by the blue light LED – modules started according to following settings:

- Intensity: 20 mW / cm^2
- voltage: 25 V
- current: 1.089 mA
- contact time: 4 hours

Immediately after the contact time (within 4 hours), the carrier was 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 A549 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% CO_2. After 7 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.

Calculation of the antiviral activity of the products

The viral titre was calculated using the Spearman-Kärber-method (Br. J. Psychol. 2 (1908): 227-42, Arch. exp. Path. Pharmak. 162 (1931): 480-87).

The antiviral activity is calculated by the following formula

$$\mathbf{R= (Ut – U0) – (At – U0) = Ut–At}$$

where

R is the antiviral activity;

U_0 is the average of residual virus recovered from the three untreated test specimens immediately after inoculation; in $TCID_{50}$/ml

U_t is the average of residual virus recovered from the three untreated test specimens after contact time; in $TCID_{50}$/ml

A_t is the average of residual virus recovered from the three treated test specimens after contact time; in $TCID_{50}$/ml

4. Results and Evaluation

The photodynamic technology in association with **eukula 491 VIROBAC** was tested following an exposure time of 4 hours.

Validity of the test

The infectivity titer of the virus was determined using the endpoint titration method and the titre was given as $\log_{10}$ $TCID_{50}$/ml. The titer of the virus suspension was 8.50 ± 0.47 $\log_{10}$ $TCID_{50}$/ml.

The test specimen caused no cytotoxic effects as shown in Table 1. As shown in Table 2 and 3, by comparing the infectivity titer of virus from the negative control $\mathbf{S_n}$ with that from the untreated test specimen $\mathbf{S_u}$ or the treated specimen $\mathbf{S_t}$, the logarithmic value meets the requirements of ≤ 0.5 $\log_{10}$, and therefore, the suppressive efficiency is confirmed. As can be seen from the Table 4, the internal reference control showed an infectivity of 5.89 $\log_{10}$ $TCID_{50}$/ml after drying plus an additional 4 hour incubation time.

The titre recovered immediately after inoculation from the untreated test specimens should be within the range of 5 $\log_{10}$ $TCID_{50}$/ml to 6 $\log_{10}$ $TCID_{50}$/ml. The recovery control U_0 showed that the test is valid since the Adeno virus type 5 titer was ≥ 5 $\log_{10}$ in the inoculum as depicted in table 5. The mean virus titre recovered from each untreated test specimen after contacting for 4 hours was 7.39 $\log_{10}$ $TCID_{50}$/ml.

All conditions for a valid test were fulfilled.

Table 1 Verification of cytotoxic effect on host cells

Product	Dilution ($\log_{10}$)							
	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
eukula 491 VIROBAC	–	–	–	–	–	–	–	–

Table 2 Test results for verification of the test according to ISO 21702:2019-05

Sample	Contact time	Level of cytotoxicity	Titre of the residual virus (log_{10} $TCID_{50}$/ml) with 95.0% confidence interval			
			test 1	test 2	test 3	Mean
Sn	30 min	0.50	7.33 ± 0.54	7.50 ± 0.47	7.17 ± 0.42	**7.33 ± 0.48**
Su	30 min	0.50	6.83 ± 0.42	7.17 ± 0.42	7.33 ± 0.33	**7.11 ± 0.39**
St	30 min	0.50	6.83 ± 0.42	6.83 ± 0.42	7.83 ± 0.56	**7.17 ± 0.47**

Table 3 Test validity according to ISO 21702:2019-05

Formulae according to 6.6.3.3 ISO 21702:2019	Negative control S_n	Test specimen	Difference	Pass criteria (≤ 0.5) fulfilled
Sn – Su ≤ 0.5	7.33	7.11	**0.22**	**valid**
Sn – St ≤ 0.5	7.33	7.17	**0.16**	**valid**

S_n is the average of the common logarithm of the infectivity titer of virus, in $TCID_{50}$/ml, from three samples of the negative control;

S_u is the average of the common logarithm of the infectivity titer of virus, in $TCID_{50}$/ml, recovered from three of the untreated test specimens;

S_t is the average of the common logarithm of the infectivity titer of virus, in $TCID_{50}$/ml, recovered from three of the treated test specimens.

Table 4 Internal control on FOREX surface

Sample	Contact time	Level of cytotoxicity	Titre of the residual virus (log_{10} $TCID_{50}$/ml) with 95.0% confidence interval			
			test 1	test 2	test 3	Mean
Dark control U_{td} FOREX classic	4 h	0.50	5.33 ± 0.33	5.83 ± 0.42	6.50 ± 0.47	**5.89 ± 0.41**
Light control U_{tl} FOREX classic	4 h	0.50	5.83 ± 0.47	5.33 ± 0.33	5.17 ± 0.56	**5.44 ± 0.45**

U_0 is the average of residual virus recovered from the three untreated test specimens immediately after inoculation; in $TCID_{50}$/ml

U_t is the average of residual virus recovered from the three untreated test specimens after contact time; in $TCID_{50}$/ml

A_t is the average of residual virus recovered from the three treated test specimens after contact time; in $TCID_{50}$/ml

Table 5 Test results on eukula 491 VIROBAC coated test specimen with Adeno virus type 5, Adenoid 75 according to ISO 22196:2011

Test date: 2023-06-14 – 2023-06-21

Sample	Contact time	Level of cytotoxicity	Titre of the residual virus (log_{10} $TCID_{50}$/ml) with 95.0% confidence interval			
			test 1	test 2	test 3	Mean
Recovery control **U_0**	0 min	0.50	7.67 ± 0.54	7.50 ± 0.47	7.00 ± 0.45	**7.39 ± 0.49**
Reference control **U_t**	4 h	0.50	5.67 ± 0.33	5.50 ± 0.60	5.67 ± 0.56	**5.61 ± 0.50**
Treated test specimen **eukula 491 VIROBAC A_t**	4 h	0.50	4.83 ± 0.42	4.83 ± 0.56	4.33 ± 0.33	**4.67 ± 0.44**

Table 6 Calculation of the antiviral activity

eukula 491 VIROBAC

$R = (U_t - U_{0)} - (A_t - U_0) = (5.61 - 7.39) - (4.67 - 7.39) = -1.78 - (-2.72) =$ **0.94**

R is the antiviral activity;

U_0 is the average of residual virus recovered from the three untreated test specimens immediately after inoculation; in $TCID_{50}$/ml

U_t is the average of residual virus recovered from the three untreated test specimens after contact time; in $TCID_{50}$/ml

A_t is the average of residual virus recovered from the three treated test specimens after contact time; in $TCID_{50}$/ml

Test results

The data of the virucidal efficacy of light emitting LEDs in combination with **eukula 491 VIROBAC** coated parquet flooring is presented in Table 6. The photodynamic inactivation of the Adeno virus type 5, Adenoid 75, by the energy-rich singlet oxygen generated in the test procedure showed a minimal reduction of 0.94 log (88.52 % kill rate) compared to the controls within a 4 hours exposure time. Non-enveloped viruses are relatively resistant.

22.06.2023

Date

PD Dr. rer. nat. Maren Eggers
Head of disinfectant testing and applied / technical hygiene

Raw data of eukula 491 VIROBAC coated test specimen tested with Adeno virus type 5, Adenoid 75

Date: 2023-06-14 – 2023-06-21

Test specimen	replicate	Light Intensity mW/cm2	Contact time	Dilution (log_{10})							
				0	1	2	3	4	5	6	7
S_n	1	N/A	30 min	444 444	444 444	444 444	444 444	444 444	222 222	002 221	002 000
	2	N/A	30 min	444 444	444 444	444 444	444 444	444 444	222 233	120 222	000 200
	3	N/A	30 min	444 444	444 444	444 444	444 444	444 444	443 444	232 222	220 220
S_u	1	N/A	30 min	444 444	444 444	444 444	444 444	333 333	221 222	210 000	000 000
	2	N/A	30 min	444 444	444 444	444 444	444 444	434 444	223 333	210 012	000 000
	3	N/A	30 min	444 444	444 444	444 444	444 444	344 444	223 333	111 220	000 000
S_t	1	N/A	30 min	444 444	444 444	444 444	444 444	444 444	222 223	022 000	000 000
	2	N/A	30 min	444 444	444 444	444 444	444 444	444 444	333 343	200 200	000 000
	3	N/A	30 min	444 444	444 444	444 444	444 444	444 444	232 323	222 022	010 022

Raw data of FOREX carrier tested with Adeno virus type 5, Adenoid 75

Date: 2023-06-14 – 2023-06-21

Test specimen	replicate	Light Intensity mW/cm2	Contact time	Dilution (log_{10})							
				0	1	2	3	4	5	6	7
Dark control U_{td} FOREX classic	1	N/A	4 h	444 444	444 444	444 444	323 222	220 221	000 000	000 000	000 000
	2	N/A	4 h	444 444	444 444	444 444	444 444	422 333	200 200	000 000	000 000
	3	N/A	4 h	444 444	444 444	444 444	444 444	433 333	321 202	000 002	000 000

Test specimen	replicate	Light Intensity mW/cm2	Contact time	Dilution (log_{10})							
				0	1	2	3	4	5	6	7
Light control U_{tl} FOREX classic	1	20	4 h	444 444	444 444	444 444	333 333	121 232	100 000	100 000	000 000
	2	20	4 h	444 444	444 444	444 444	333 333	012 121	000 000	000 000	000 000
	3	20	4 h	444 444	444 444	444 444	333 333	022 003	000 001	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*

Raw data of eukula 491 VIROBAC coated test specimen tested with Adeno virus type 5, Adenoid 75

Date: 2023-06-14 – 2023-06-21

Test specimen	replicate	Light Intensity mW/cm2	Contact time	Dilution (log_{10})							
				0	1	2	3	4	5	6	7
Recovery control U_0	1	N/A	4 h	444 444	444 444	444 444	444 444	444 444	342 333	301 222	300 200
	2	N/A	4 h	444 444	444 444	444 444	444 444	444 444	444 344	212 021	001 000
	3	N/A	4 h	444 444	444 444	444 444	444 444	444 444	222 233	020 032	000 000
Reference control U_t	1	N/A	4 h	444 444	444 444	444 444	333 344	212 133	000 020	000 000	000 000
	2	N/A	4 h	444 444	444 444	444 444	333 333	030 112	020 010	000 000	000 000
	3	N/A	4 h	444 444	444 444	434 444	323 322	121 112	000 002	000 000	000 000
Treated test specimen A_t	1	20	4 h	444 444	444 444	233 334	223 222	000 012	000 000	000 000	000 000
	2	20	4 h	444 444	444 444	433 333	222 033	210 001	000 000	000 000	000 000
	3	20	4 h	444 444	444 444	333 333	031 331	000 000	000 000	000 000	000 000
Cytotoxicity control		N/A	4 h	000 000	000 000	000 000	000 000	000 000	000 000	000 000	000 000
Test specimen	**replicate**	**Light Intensity mW/cm2**	**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	444 444	444 444	444 444	330 122	200 000

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*