DR. BRILL + DR. STEINMANN
INSTITUTE FOR HYGIENE AND MICROBIOLOGY

TEST REPORT

29/04/2021

Test report 222062-SC1.1

Evaluation of the effectiveness of

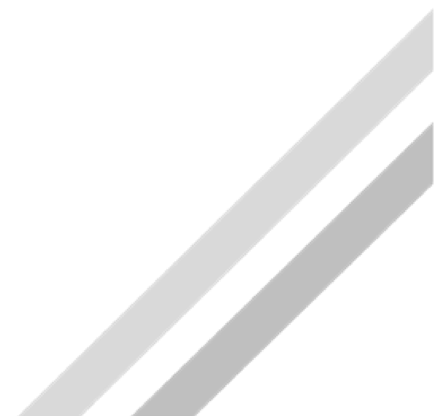
Plasma Liquid Nasensprühgel 20ml

Test virus: SARS-CoV-2 (Severe acute respiratory syndrome coronavirus type 2)

Method: based on EN 14476:2013+A2:2019 (artificial nasal secrete)

quantitative suspension test for the evaluation
of virucidal activity of chemical disinfectants and
antiseptics used in human medicine (phase 2/ step 1)

Sponsor:
Prontomed GmbH
Am Bahndamm 70
DE - 32120 Hiddenhausen



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1. Identification of test laboratories

Molecular & Medical Virology, Ruhr-Universität Bochum, Universitätsstrasse 150, DE - 44801 Bochum

Dr. Brill + Partner GmbH Institute for Hygiene and Microbiology, Norderoog 2, DE - 28259 Bremen

2. Identification of sample

Manufacturer	Prontomed GmbH
Name of product	Plasma Liquid Nasensprühgel 20ml
Confirmation no.	222062
Product diluent recommended by the manufacturer	-
Batch number	-
Application	nasal spray
Production date	-
Expiry date	-
Active compound (s)	<0.06 % sodium hypochlorite / hypochloric acid
Appearance, odour	turbid, colorless gel product specific
pH-values	undiluted: 9.71 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	-

3. Materials

3.1 Culture medium and reagents

- Dulbecco`s Modified Eagle Medium (DMEM, Thermo Fisher, catalogue no. 11965092)
- Fetal Bovine Serum (FBS, Thermo Fisher, article no. 10270106)
- Penicillin-Streptomycin (P/S, Thermo Fisher, catalogue no. 15140122)
- MEM Non-Essential Amino Acids Solution (100X) (NEAAs, Thermo Fisher, catalogue no. 11140035)
- L-Glutamine (200 mM) (L-Glut, Thermo Fisher, catalogue no. 25030024)
- Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo Fisher, catalogue no. 14190250)

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- Trypsin-EDTA (0.5 %), no phenol red (Thermo Fisher, catalogue no. 15400054
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153).
- Crystal Violet (Sigma-Aldrich-Chemie GmbH, article no. C0775)
- Methanol (Carl Roth, article no. KK39)
- bovine mucin (Sigma-Aldrich, article no. M 3895)
- yeast extract (Sigma-Aldrich, article no. Y1625-250 g).

3.2 Virus and cells

The SARS-CoV-2/Germany strain was derived from a patient isolate.

The *Vero E6 cells* were obtained from University Bern, Switzerland.

The cells were inspected regularly for morphological alterations. No morphological alterations could be detected.

4. Experimental conditions

Test temperature	xxxxxxxxxxxx
Concentration of test product	undiluted (80.0 %) and as 50.0 % and 20.0 % (demonstration of non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	60 seconds
Interfering substance	artificial nasal secrete (BSA, mucin and yeast, see 5.3)
Procedure to stop action of disinfectant	immediate dilution
Diluent	Aqua bidest.
Virus strain	SARS-CoV-2/Germany strain
Date of testing	08/03/2021 – 29/04/2021
End of testing	29/04/2021

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5. Methods

5.1 Preparation of test virus suspension

For virus production, 2 x10^6 *Vero E6 cells* were cultivated in a 75 cm^2 flask in DMEM supplemented with 1 % L-Glut, NEAAs, and P/S and 10 % FBS. One day after seeding Medium was changed to 10 mL fresh DMEM inoculated with 100 µl of SARS-CoV-2/Germany virus suspension. The supernatant was harvested after 3 days at 37 °C by centrifugation at 1,500 rpm for 5 min to remove cell debris. Viral titres were determined by plaque assay and endpoint dilution. The supernatant was aliquoted and stored at -80 °C.

5.2 Preparation of disinfectant (dilutions)

The test product was tested undiluted. Due to the addition of interfering substance and test virus suspension an 80.0 % solution resulted (1 part test virus suspension + 1 part interfering substance (1-fold) + 8 parts disinfectant).
Furthermore, the product was evaluated as 50.0 % and 20.0 % solutions (demonstrating of non-active range). These solutions were prepared with Aqua bidest. immediately before the inactivation tests.

5.3 Preparation of the soil load

For the preparation of the artificial nasal secrete, 25 µl BSA stock solution (0.5 g of BSA in 10 ml phosphate buffer), 100 µl mucin stock solution (0.04 g bovine mucin in 10 ml of phosphate buffer) and 35 µl yeast extract stock solution (0.5 g yeast extract in 10 ml phosphate buffer) were mixed.
In the inactivation assays, one part of this interfering substance was mixed with 1 part test virus suspension and 8 parts of the test product.

5.4 Infectivity assay

The prepared dilutions of disinfectants were mixed with interfering substance and SARS-CoV2 according to chapter 5.2 and were incubated for the above indicated times (chapter 4). With elapsing exposure time 22 µl of the virus-disinfectant solution was immediately added to the first row of *Vero E6 cells* seeded at 1 x 10^4 cells/well in a 96 well plate one day prior the examination. Following a serial endpoint dilution. After 3 days of incubation at 37 °C in a CO_2-atmosphere (5.0 % CO_2 - content) cultures were observed for cytopathic effects by crystal violet staining. The infectious dose ($TCID_{50}$) was calculated according to the method of Spearman (2) and Kärber (3).

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5.5 Calculation and verification of virucidal activity

The virucidal activity of the test disinfectant was evaluated by calculating the decrease in titre in comparison with the control titration without disinfectant. The difference is given as reduction factor (RF).

According to the EN 14476, a disinfectant or a disinfectant solution at a particular concentration is having virus-inactivating efficacy if the titre is reduced at least by 4 log_{10} steps within the recommended exposure period. This corresponds to an inactivation of ≥ 99.99 %.

5.6 Inactivation assay (end point titration)

Determination of virucidal activity has been carried out according to EN 14476, section 5.5.

Immediately at the end of a chosen contact time, activity of the disinfectant was stopped by dilution to 10^{-8}.

Titrations of the virus control were performed at the beginning of the test and after the longest exposure time (EN 14476, section 5.5.7). One part by volume of test virus suspension was mixed with one part interfering substance and eight parts by volume of WSH or Aqua bidest. (RTU products). If a 97.0 % assay was performed, 0.1 parts by volume of test virus suspension were mixed with 0.2 parts interfering substance and 9.7 parts by volume of Aqua bidest. (RTU products).

Furthermore, a cell control (only addition of medium) was incorporated.

Inactivation tests were carried out in sealed test tubes at 20 °C ± 1.0 °C. Aliquots were retained after appropriate exposure times and residual infectivity was determined.

5.7 Inactivation assay following the large volume plating method (LVP)

Following the large volume plating method (EN 14476, section 5.5.4.3) the inactivation assays were further diluted 1:1,000 in cell culture medium. The total volume was added (without any further dilution) to the permissive cells. By introducing such a huge dilution, it is possible to eliminate cytotoxicity of the test product in order to demonstrate a 4 log_{10} reduction of virus titre. Calculation of virus titre follows formula of Taylor or Poisson (EN 14476, annex B.3). This method is necessary for those products which demonstrate a great cytotoxicity.

62.5 µl of the inactivation assay were added to 62.5 ml medium and then the total volume was distributed in 6 microtitre plates (108 µl / well, 576 wells total). After 3 days of inoculation cultures were observed for cytopathic effects.

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6. Results

Results of examination are shown in tables 1 and 2. Table 1 shows the results using the end point dilution method, whereas table 2 shows the results using large volume plating method.

Testing the undiluted test product in an 80 % assay, no residual virus could be detected after 60 seconds of exposure time using the end point dilution method. The reduction factor was ≥ 3.33. Due to cytotoxicity, a reduction of 4 log_{10}-steps could not be shown and the large volume plating method was introduced.

The test product as 50.0 % and 20.0 % solutions was not active within 60 seconds of exposure time using the end point dilution method.

Since it was not possible to show a sufficient log_{10} reduction testing the undiluted test product due to cytotoxicity with the end point dilution method, in parallel the large volume plating method (LVP) was introduced with 60 seconds of exposure time. The virus titre was log_{10} $TCID_{50}$/ml = 6.53.

The undiluted test product in an 80 % assay was not active within 60 seconds of exposure time. Since residual was found in 35 of 576 cell culture units at this time point, the result according to the formula of Taylor was 2.92 log_{10} $TCID_{50}$. The reduction factor was therefore 3.61 (6.53 log_{10} $TCID_{50}$ minus 2.92 log_{10} $TCID_{50}$).

7. Conclusion

The nasal spray Plasma Liquid Nasensprühgel 20ml tested undiluted is not to be classified as an antiseptic, as it did not achieve the required reduction of 4 log_{10} steps against SARS-CoV-2 after an exposure time of 60 seconds with artificial nasal secrete as interfering substance.

But the achieved present results confirm a reduction of the SARS-CoV-2 virus by 3.33 log_{10} steps, which corresponds a reduction to over 99.9 %.

Bremen, 29/04/2021

- Dr. Britta Becker -
Head of Laboratory

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8. Literature

1. EN 14476:2013+A2:2019: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity of chemicals disinfectants and antiseptics in human medicine test - Test method and requirements (phase 2, step 1)
2. Spearman, C.: The method of `right or wrong cases` (constant stimuli) without Gauss's formulae.
   Brit J Psychol; 2 1908, 227-242
3. Kärber, G.: Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche.
   Arch Exp Path Pharmak; 162, 1931, 480-487

Appendix:

Legend to the Tables

Table 1: Results (end point dilution method) with Plasma Liquid Nasensprühgel 20ml and SARS-CoV-2

Table 2: Results (LVP) with Plasma Liquid Nasensprühgel 20ml and SARS-CoV-2

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Table 1: Results (end point dilution method) with Plasma Liquid Nasensprühgel 20ml and SARS-CoV-2

Product	Conc.	Soil load	Cytotoxicity $log_{10} CD_{50}/ml$	Titre virus control ($log_{10} TCID_{50}/ml$)	Virus titre ($log_{10} TCID_{50}/ml$) after				Reduction factor after			
					30 s	60 s	1 min	5 min	30 s	60 s	1 min	5 min
Plasma Liquid Nasensprühgel 20ml	80.0%	artificial nasal secrete	3.20	6.53	n.d.	≤ 3.20	n.d.	n.d.	n.d.	≥ 3.33	n.d.	n.d.
Plasma Liquid Nasensprühgel 20ml	50.0%	artificial nasal secrete	3.20	6.53	n.d.	≤ 4.03	n.d.	n.d.	n.d.	≥ 2.50	n.d.	n.d.
Plasma Liquid Nasensprühgel 20ml	20.0%	artificial nasal secrete	3.20	6.53	n.d.	6.20	n.d.	n.d.	n.d.	0.33	n.d.	n.d.

n.a. = not applicable n.d. = not done

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Table 2: Results (LVP, 1:1,000) with Plasma Liquid Nasensprühgel 20ml and SARS-CoV-2

Product	Conc.	Soil load	Exposure time	Ø Titre virus control (log_{10} $TCID_{50}$/ml)	Total number of positive wells after titration with the LVP	Virus titre (LVP) (log_{10} $TCID_{50}$/ml)	Reduction factor
Plasma Liquid Nasensprühgel 20ml	80.0%	artificial nasal secrete	60 s	6.53	35	2.92	3.61

n.a. = not applicable n.d. = not done

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