A protocol for analysis of virucidal properties of antiviral compounds and materials
(Latvian Biomedical Research and Study Centre)

1. Alphavirus-based validation of virucidal properties of surface coatings

WO_3/Cu/WO_3 nanocoatings
Ca-Fe oxide polymer coatings

Virus synthesis and virus quantification

The recombinant Semliki forest virus (SFV) vectors (pSFV1/Ds-Red construct, carrying Discosoma sp. red fluorescent protein gene, and pSFVenh/Luc construct, carrying firefly luciferase gene) were generated as previously described (Kurena et al 2017; Zajakina et al., 2014). Briefly, for the synthesis of infectious but replication deficient vector particles, the BHK-21 cells (Baby hamster kidney cells) were electroporated with respective recombinant viral RNA (pSFV1/Ds-Red and pSFVenh/Luc) together with SFV helper RNA, providing synthesis of SFV structural proteins. After a 48 h incubation virus-containing medium was harvested, rapidly frozen and subsequently used as a virus stock. The virus stocks did not contain the replication competent wild-type virus as confirmed by cell reinfection. The pSFVenh/Luc viral particles were additionally concentrated by ultracentrifugation through two sucrose cushions, as previously described (Hutornojs et al 2008).
The viral titers expressed in infectious units per ml (IFU/mL) were quantified by infecting BHK-21 cells with serial dilutions of viral stock and analyzing either Ds-Red expression via fluorescence microscopy on a Leica DM IL microscope (Leica Microsystems Wetzlar GmbH, Germany), or by immunostaining with rabbit polyclonal antibodies specific to the nsp1 subunit of SFV replicase (generously provided by A. Merits, Institute of Technology, University of Tartu, Estonia), as previously described (Vasilevska et al., 2012).

Determination of virucidal activity on surfaces

10^5-10^6 infectious units (iu) of SFV/enhLuc in DMEM -/-, with total volume of 10-20 µL were incubated on the surface covered with plastic bag for 10-30-60 min at RT (figure 1). Then the virus was resuspended in 600-800 µL PBS +/+ and 200 µL were added to previously washed BHK-21 cells cultivated in 24-well plate till 80% confluency. Cells were incubated with the virus for 1 h at 37 ºC, then 800 µL of 1% BHK medium was added. Glass, plastic and other surfaces, as well as untreated samples served as controls.

Luciferase assay
Antiviral activity of the surfaces was determined using Luciferase assay. The infected cells were incubated overnight at 37 ºC, 5% CO2, and used for the Luciferase assay (Promega), as recommended by manufacturer. Briefly, the cells (24-well) were lysed in 100 µl of the Cell Culture Lysis buffer (Promega), centrifuged at 600 cfr for 5 min, and 1-10 µl of the cell lysate was used immediately for the measurement of the luciferase enzymatic activity luciferase by Luminometer (Luminoskan Ascent, Thermo

Scientific, UK). Relative luciferase units (RLUs) were calculated as an average mean of triplicates. The standard curve, expressing the correlation between the RLUs and the virus titre, was generated using the standard SFV/enhLuc virus dilutions in a range 5×105- 1×103 ifu per infection. The standard virus was not incubated on surfaces. The luciferase assay experiments were performed in duplicates. The respective Log reduction of the virus titer was calculated by the formula:
Log reduction = $Log_{10}(A) - Log_{10}(B)$,
A – virus titer before treatment
B – virus titer after treatment

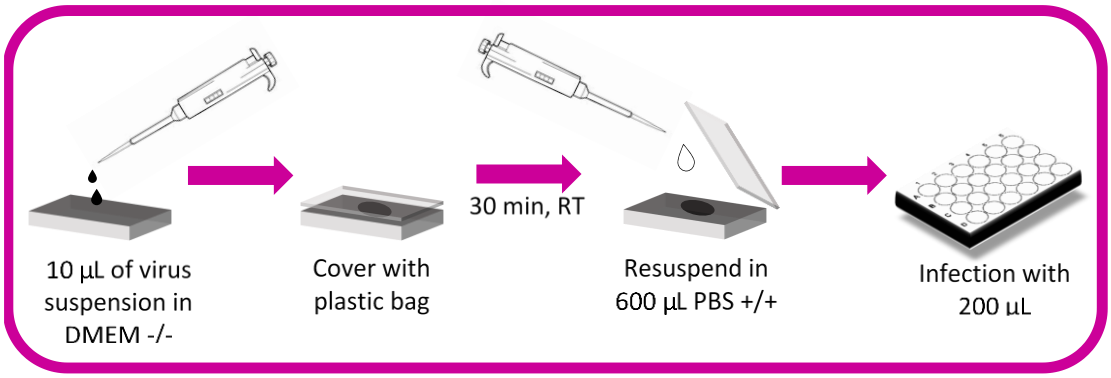

Figure 1. A method for testing of surface antiviral activity.

The example of the test performed on $WO_3/Cu/WO_3$ coated surface is shown on figure 2; Ca-Fe oxide polymer coatings on figure 3.

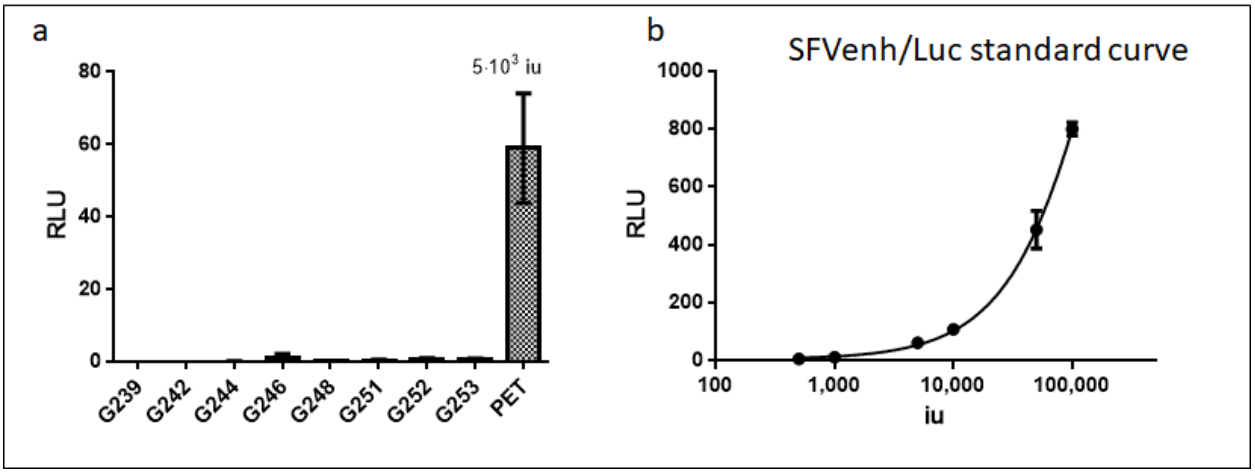

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Figure 2. Relative viral activity after incubation on the surfaces determined using Luciferase assay. The SFVenh/Luc virus ($2×10^5$ iu) was preincubated on tested surfaces and used for BHK cell infection. (a) The infection units (iu) indicated for PET was measured relative to standard SFVenh/Luc virus dilutions. The total virus inhibition was observed for all tested nanocoatings. (b) The SFVenh/Luc standard curve, obtained by serial dilution of the virus stock, RLU = $-2{,}14\cdot10^{-8}ifu^2 + 1{,}009\cdot10^{-2}ifu + 4$; R^2 = 0,9911.

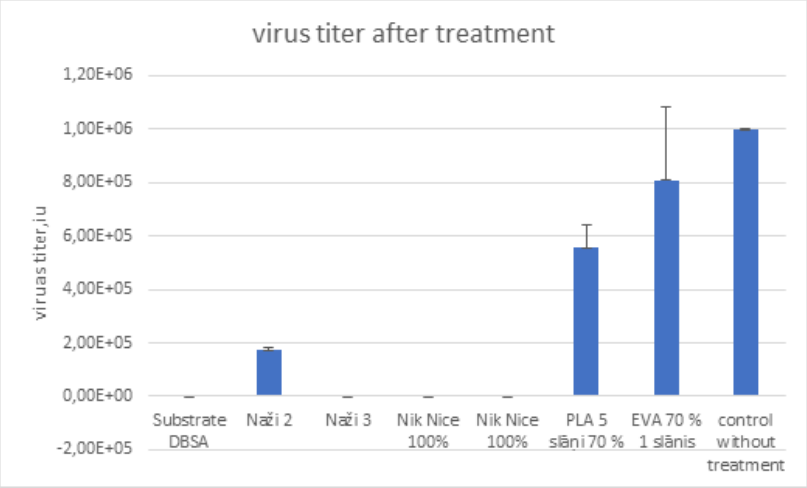
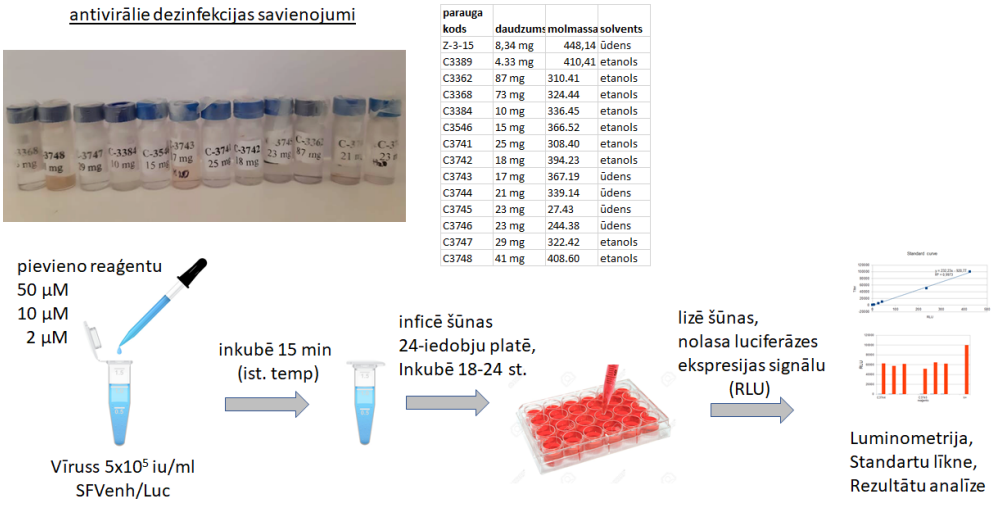


Figure 3. Test 2. relative viral activity after incubation on the surfaces determined using Luciferase assay. Initial number of virus particles incubated on tested surfaces was 10^6 iu (control without treatment).

2. Analysis of potential disinfection compounds in solution

The test compounds were diluted with the solvent (ethanol or water) to a concentration of 25mM. The potential disinfectant reagent was added to a 0.5 ml virus solution (PBS) with a titer of $5x10^5$ iu/ml to achieve concentrations of 50 µM, 10 µM and 2 µM, respectively, and incubated for 15 minutes at room temperature. After incubation, a solution containing viruses and reagents was used for infection of BHK-21 cells (described above). The BHK-21 cell infection was performed on a 24-well plate, in duplicate for each sample (figure 4). In parallel, the BHK-21 cells were infected with standard dilutions of the virus. The virus titers were measured by luciferase assay and the respective virus reduction rates were calculated as described above.



parauga kods	daudzums	molmassa	solvents
Z-3-15	8,34 mg	448,14	ūdens
C3389	4.33 mg	410,41	etanols
C3362	87 mg	310.41	etanols
C3368	73 mg	324.44	etanols
C3384	10 mg	336.45	etanols
C3546	15 mg	366.52	etanols
C3741	25 mg	308.40	etanols
C3742	18 mg	394.23	etanols
C3743	17 mg	367.19	ūdens
C3744	21 mg	339.14	ūdens
C3745	23 mg	27.43	ūdens
C3746	23 mg	244.38	ūdens
C3747	29 mg	322.42	etanols
C3748	41 mg	408.60	etanols

Figure 4. Analysis of antiviral compounds in solution.

The most efficient reagents were tested under highest dilutions (example figure 5.), allowing to calculate IC50 rate.

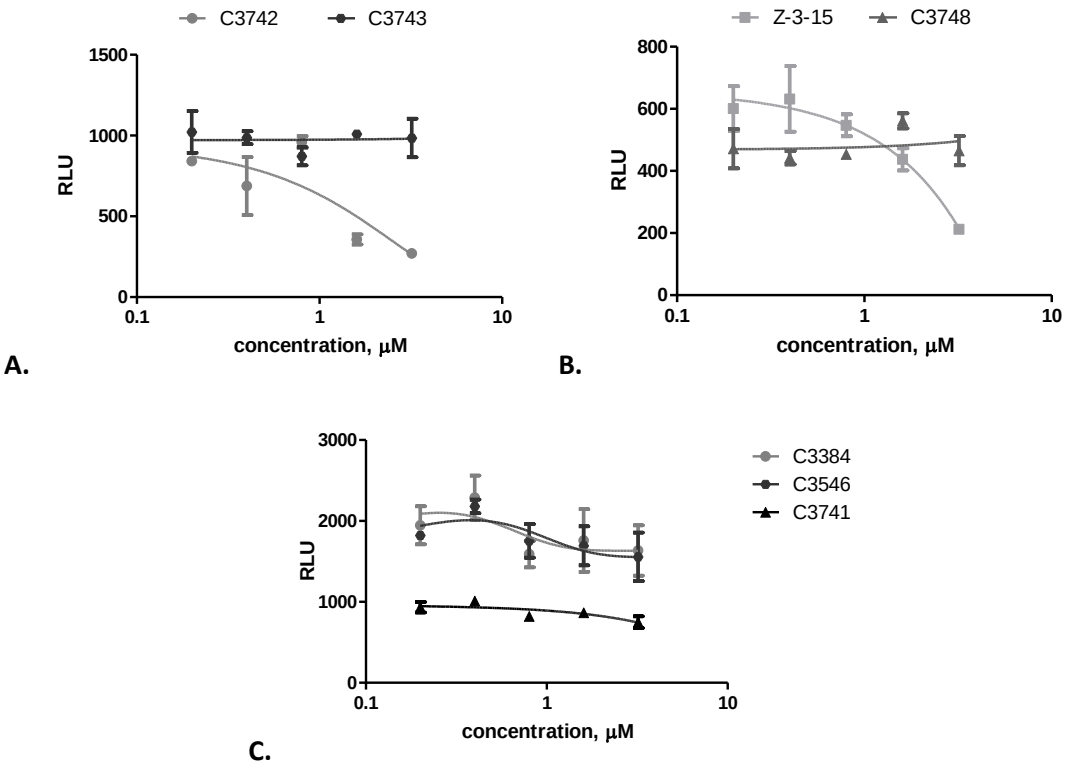


Figure 5. Virus inactivation depending on the reagent concentration. The C3742 and Z-3-15 compounds showed a concentration dependent correlation with inhibition of the virus titers (RLU- relative luciferase units).

3. Virus filtration analysis of biodegradable composite material membranes

The protocol was adjusted to test virus filtration/sorption potential of biodegradable composite membranes and textile materials in a liquid filtration test. The aerosol filtration test was not performed due to the absence of instrumentation for controllable virus containing aerosol generation under BSL2 safety conditions.

Procedure

From each sample material, a piece of 1.5 cm to 1.5 cm in size was cut out and in the form of a cone was placed in a 1,5 ml tube. 50 µl recombinant SFV-1 Luc virus with a titer of 10^7 iu/ml were added into each cone of the test material (figure 6). The tubes were centrifuged for 20 seconds, 500 x g (3500 rpm) (FVL-2400N Combi-Spin, Mini-Centrifuge/Vortex, Biosan) to allow the liquid pass through the material. Standards for virus dilution were also prepared for the assessment of virus titre. The 10 µl of the passed solution (filtrate) was used for cell infection and luciferase-based quantification of the virus titers (as described above - luciferase assay). The example of the results is presented on figure 7.

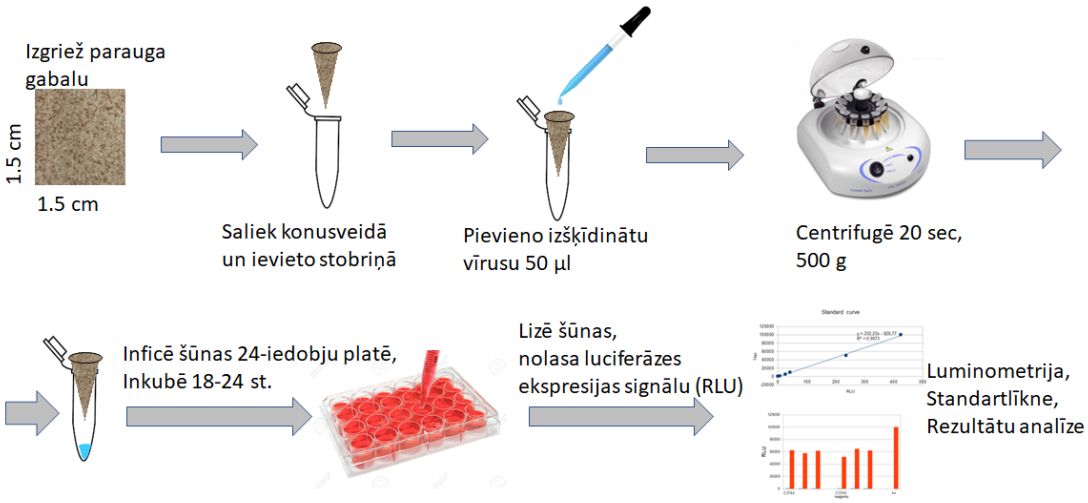


Figure 6. Schematic representation of the virus pressure filtration test.

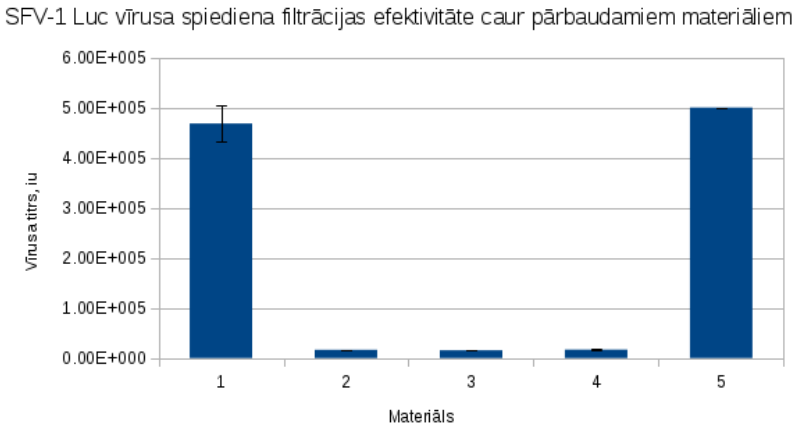


Figure 7. SFV1 / Luc virus pressure filtration efficiency through test materials. 1 - surgical mask; 2-cellulose fiber material; 3. cellulose and mushroom fiber composite material; 4. basidium mushroom fiber material; 5. control SFV-1 Luc virus 5x105 iu, without filtration.

4. Analysis of virucidal capacity of different spectrum UV radiation

Experimental UV light sources (lamps) with different UV spectrum characteristics provided by plumbum (Pb), thallium (Tl), arsenicum (As) and selenium (Se) were used in the tests (figure 8). As a control, a mercury (Hg) based lamp was used. Lamps of this type are widely used for sterilization of various surfaces.

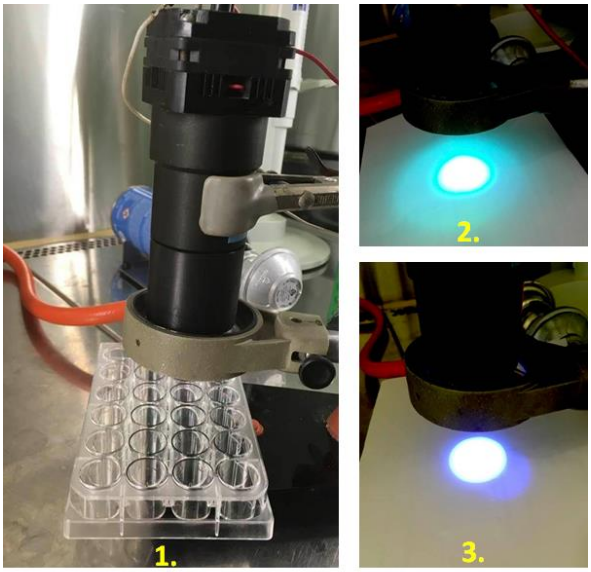


Figure 8. (1) Experimental installation, (2) Tl irradiation, (3) Pb irradiation.

The effect of these lamps on virus samples in liquid and dry form (dried virus-containing solution) was tested. In a PBS buffer 200 µl of a virus sample at a concentration of $1x10^7$/ml was placed in a 24-well plate (surface area 1,9 cm2). Alternatively, the dry virus sample was prepared by drying for 1 hour at room temperature in a DMEM medium of 20 µl of virus solution at a concentration of 1x108/ml. The prepared samples of the virus were placed under the respective lamp at a distance of 11 cm and irradiated for 5 or 1 minute (figure 9, an example). Next, BHK-21 cell infection was performed in a 24-well plate in duplicates. In parallel, the BHK-21 cells were infected with two replicates of the standard dilutions of the virus, which were prepared from the same solution of the virus source. After 18-24 hours, the activity of the luciferase reporter gene in cellular lysates was determined as described above (luciferase assay) and the respective virus reduction rates were calculated.

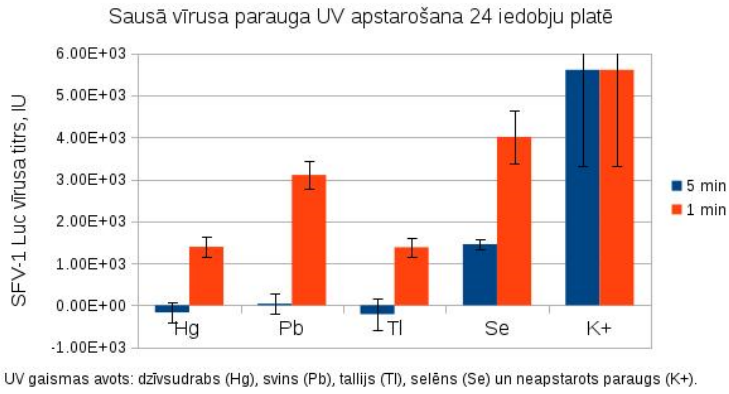


Figure 9. Virucidal effect of the UV irradiation (1 min, 5 min treatment)