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EVALUATION OF THE MECHANISM OF ACTION OF ANTIVIRAL COATED PLASTICS AND OTHER COATED NON-POROUS SURFACES

Einführendes Element — Haupt-Element — Ergänzendes Element

Élément introductif — Élément central — Élément complémentaire

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CCMC will prepare and attach the official title page.

Contents

Page

Foreword

This CEN Workshop Agreement (CWA XXXX:YYYY) has been developed in accordance with the CEN-CENELEC Guide 29 "CEN/CENELEC Workshop Agreements – A rapid way to standardization" and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN/WS "Evaluating Antimicrobial Coatings: From Air Filtration Efficiency to Antiviral Mechanism and Ecotoxicology", the secretariat of which is held by UNE (Spanish Association for Standardization) consisting of representatives of interested parties on 2025-07-29, the constitution of which was supported by CEN following the public call for participation made on 2025-06-16. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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The following organizations and individuals developed and approved this CEN Workshop Agreement:

- Politecnico di Torino (POLITO) – Prof. Cristina Balagna (Chair) and Dr. Agelica Luceri
- University of Turin (UNITO) – Prof. Manuela Donalisio, Prof. David Lembo, Mrs. Elisa Feyles and Mrs. Domiziana Porporato
- TEKNIKER – Dr. Rocío Ortiz
- Friedrich Alexander University Erlangen (FAU) – Prof. Aldo R. Boccaccini and Dr. Zoya Hadzhieva and Mrs. Andrada-Ioana Damian
- IRIS Technology Solutions SL. – Dr. Cristina Fernández and Dr. Marina Pellegrino
- AGC Interpane – Dr. Omar Benzine
- SIOEN Industries – Mrs. Sophie Poirier
- GV FILTRI INDUSTRIALI SRL - Mr. Flavio Martina and Mr. Samuele Gentile
- National Technical University of Athens – Prof. Nikolaos Chronis
- Spanish Association for Standardization (UNE) – Mr. Javier Idiago (Secretary)

Introduction

Antibacterial and antiviral-treated non-porous materials have recently become available on the market and are increasingly used in a variety of applications, including healthcare, public facilities, transport, and consumer products. Their development and use reflect the growing demand for surfaces that can reduce the presence of microorganisms and contribute to improved hygiene conditions.

Measuring tests to verify the antibacterial activity and the antiviral activity of non-porous products have been described in ISO 22196 and ISO 21702, respectively.

Understanding the mechanism of action of antiviral coatings is of significant importance for the scientific validation and practical implementation of such technologies. Determining how a treated material interacts with and inactivates viruses enables a more accurate assessment of its performance, stability, and safety under various environmental conditions. Furthermore, knowledge of the underlying mechanisms supports the optimization of material formulations, facilitates reproducibility of results, and contributes to the establishment of robust regulatory and standardization frameworks. Ultimately, elucidating the mechanism of action enhances transparency and confidence in antiviral products, promoting their responsible and effective use in public and private settings.

This CEN Workshop Agreement aims at providing a test method for the investigation of the mechanism of action of coated products that have already demonstrated to be endowed with antiviral activity. This protocol is written based on ISO 21702 and is applicable to non-porous materials such as plastics, ceramics, etc.

This document has been based on the knowledge generated in the EU-funded research project NANOBLOC, which received funding from the European Union's Horizon Europe research and innovation programme under grant agreement No. 101057597.

1 Scope

This document specifies proper methods to investigate the mechanism of action by which a coated specimen reduces viral infectivity titer. This protocol is intended to be used after the verification of the antiviral activity of a coated surface following the ISO 21702:2019 guideline. Two test procedures are described in this document. The first test, the drying test, aims to evaluate whether the antiviral activity of a coated material is due to the direct inactivation of virus particles. The second one, the ion release test aims to assess the potential indirect antiviral activity of ions released by a coated specimen. Due to the individual sensitivities, the results of one test virus might not be applicable for other viruses.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document.

ISO 21702:2019, *Measurement of antiviral activity on plastics and other non-porous surfaces.*

ISO 10993-5:2009, *Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1
Antiviral
State where the number of infectious viral particles on surface of products is reduced.

3.2
Antiviral agent
Agent that reduces the number of infectious virus on surface of products.

3.3
Antiviral activity
Difference in the logarithm of the infectivity titer of virus found on an antiviral-treated product and an untreated product after inoculation and contact to virus.

3.4
Cytopathic effect
Morphological change or destruction of the host cells as a result of the virus multiplication.

3.5
Infectivity titer of virus
Number of infectious viral particles present per unit volume of a suspension.

3.6

Focus

Groups of infected cells in a cell monolayer under liquid medium due to infection by and multiplication of a single infectious virus, identified by the presence of viral antigens.

3.7

Focus forming units (FFU)

Unit expressed as the concentration of the infectious viral particles that form foci per unit volume.

3.8

Focus assay

Assay to determine the infectivity titer of virus from FFU by using a series of dilutions.

3.9

Plaque

Lysis formed in a cell monolayer under semisolid medium due to infection by and multiplication of a single infectious virus.

3.10

Plaque forming units (PFU)

Unit expressed as the concentration of the infectious viral particles that form plaques per unit volume.

3.11

Plaque assay

Assay to determine the infectivity titer of virus from PFU by using a series of dilutions.

4 Materials

4.1 Host cells to be used for the test

The following host cells are recommended for use in the test:

- Human lung fibroblast cells (MRC-5, ATCC® CCL-171™)
- Crandell-Rees Feline Kidney cells (CRFK, ATCC® CCL-94™)

Other host cells can be used after appropriate validations regarding the sensitivity against each virus. If other species are used, the name of the species and the specific reason for their use should be included in the test report.

4.2 Viruses to be used for the test

The species of viruses to be used are as follows:

- Human beta-coronavirus type OC43 (HcoV-OC43), ATCC® VR-1558™
- Feline calicivirus, Strain F-9 ATCC VR-782

Other species of viruses can be used after appropriate validation, since the relevance may differ depending on the application of antiviral coatings. If other species are used, the name of the species and the specific reason for their use should be included in the test report.

The example species of virus, host cells and media to be used are reported in Table 1.

Table 1 — Examples of viruses, virus strains, host cells and media to be used

Virus name	Human coronavirus OC43	Feline calicivirus
Virus strain	Human betacoronavirus type OC43 (HcoV-OC43), ATCC® VR-1558™	Feline calicivirus, Strain F-9 ATCC VR-782
Host cell	Human lung fibroblast cells (MRC-5, ATCC® CCL-171™)	Crandell-Rees Feline Kidney cells (CRFK, ATCC® CCL-94™)
Growth medium	DMEM – high glucose	DMEM – high glucose

4.3 Reagents

Any water used shall be deionized and/or distilled and/or ultra-filtered and/or filtered with RO [reverse osmosis] and have a conductivity of <1 µS/cm.

All reagents shall be of analytical grade and/or of a grade appropriate for microbiological purposes.

4.4 Media and solutions for cell culture

4.4.1 Dulbecco's Modified Eagle Medium (DMEM) – high glucose

The composition is described in Annex A. If there are any components missing from the composition, they can be added according to Annex A.

4.4.2 Inactivated fetal bovine serum (FBS)

Put a cryopreserved fetal bovine serum bottle into a water bath at 37 °C and keep it until it defrosts. Then, raise the temperature of the water bath to 56 °C and keep it for 30 min to inactivate it. Divide it into several tubes. Put them in a freezer at a temperature less than -20 °C. Just before using, put it in the water bath at 37 °C and keep it until it defrosts.

4.4.3 Phosphate buffered saline – Ethylenediaminetetraacetic acid (PBS-EDTA) solution

Prepare phosphate buffered saline (PBS) 10X solution, by dissolving 80 g of sodium chloride, 2 g of potassium chloride, 29 g of phosphoric acid hydrogen 2 sodium 12 hydrate and 2 g of phosphoric acid 2 hydrogen potassium in 1,000 mL of water. Sterilize by autoclaving. Dilute 100 mL of PBS 10X in 900 mL of sterile water to obtain 1,000 mL of PBS 1X solution.

To prepare a PBS-EDTA solution add 1.1 mL of EDTA 0.5M (pH=8) to 1000 mL of PBS 1X solution. Sterilize by autoclaving and keep it at 4°C.

4.4.4 Trypsin – EDTA solution

Initially, to prepare a Trypsin solution add 2.5 g of Trypsin powder to 1,000 mL of PBS 1X solution, agitate until dissolved and sterilize by using a 0.22 µm membrane filter. To obtain a trypsin-EDTA solution add 4 mL of the prepared trypsin solution to 96 mL of PBS-EDTA solution (see 4.4.3), keep it at 4°C.

4.4.5 Penicillin – streptomycin solution[1]

Prepare a penicillin-streptomycin solution by adding 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin to sterilized water.

4.4.6 Methylcellulose-DMEM solution (MTC-DMEM)

Prepare a 1.5% solution of MTC (w/v) adding 0.15 g of MTC powder to 1,000 mL of sterile water. Then prepare a MTC-DMEM solution by mixing 500 mL of DMEM, 10 mL of FBS, 200 mL of sterile water, 10 mL of l-glutamine and 300 mL of heated MTC. Sterilize by autoclaving and store at 4°C.

4.5 Solutions for test procedure

4.5.1 Acetone-methanol solution

Prepare a 50:50 solution of acetone-methanol to fixate cells for the immunocytochemistry staining procedure.

4.5.2 PBS 1X solution

Prepare a PBS 1X solution as described in 4.4.3.

4.5.3 PBS-Triton X-100 solution

Add 1 mL of Triton X-100 to 1,000 mL of PBS 1X solution to permeabilize cells for the immunocytochemistry staining procedure.

4.5.4 PBS – TWEEN 20 solution

Add 0.5 mL of TWEEN 20 to 1,000 mL of PBS 1X solution to perform washes during the immunocytochemistry staining procedure.

4.5.5 PBS – bovine serum albumin (BSA) solution

Dissolve 1 g of BSA in 1,000 mL of PBS 1X solution to incubate antibodies during the immunocytochemistry staining procedure.

4.5.6 PBS – 3,3'-Diaminobenzidine (DAB) solution

Dissolve DAB in PBS 1X solution according to the manufacturer instructions to perform the staining at the end of the immunocytochemistry staining procedure.

4.5.7 Crystal violet (CV) solution

Prepare a 20% ethanol solution adding 200 mL of absolute ethanol in 800 mL of sterile water.

Dissolve 1 g of crystal violet powder in 1,000 mL of 20% ethanol.

[1] Penicillin-streptomycin solutions are available on the market. The products with different components may be used after proper validations.

5 Apparatus

5.1 Dry-heat sterilizer, capable of maintaining the temperature at a value between 160 °C and 180 °C within ±2 °C of the set point at equilibrium conditions, if required.

5.2 Autoclave, capable of maintaining a temperature of (121 ± 2) °C and a pressure of (103 ± 5) kPa.

5.3 Digital PCR reader

5.4 Hotplate with stirrer, or hot-water bath

5.5 Orbital shaker

5.6 pH-meter, capable of measuring to ±0.2 units.

5.7 Balance, capable of the available range of 100 g ± 0.1 g to 0.01g ± 0.0001 g.

5.8 Micro pipettes with sterile 1,000 µL, 200 µL and 10 µL tips.

5.9 Pipettor, capable of mounting the plastic pipettes.

5.10 Plastic pipette, sterile, with capacities of 50 mL ± 0.5 mL, 25 mL ± 0.25 mL, 10 mL ± 0.1 mL and 5 mL ± 0.05 mL, 2 mL ± 0.05 mL, 1 ml ± 0.05 mL.

5.11 Freezer, capable of operating at a temperature of -(80 ± 2) °C or -(20 ± 2) °C.

5.12 Liquid nitrogen bath, used for the preservation approximately at -196 °C.

5.13 Membrane filter, sterile, with a pore size of 0.20/0.45 µm.

5.14 Inverted microscope, capable of being used for the observation of cultured cells.

5.15 Centrifuge, capable of being operated at a temperature of (4 ± 2) °C, and relative centrifugal force of approximately 1,000 g.

5.16 Ninety-six, twenty-four and six wells plastic plate, sterile, with an adherent type, for focus and plaque assays.

5.17 Flasks for cell culture use, sterile, with an adherent type, a cell culture area of 75 cm2 and with the 0.2 µm filter vent cap which can prevent bacterial contamination.

5.18 CO_2 incubator, 5 % CO2, at a temperature of (34 ± 2) °C and (37 ± 2) °C.

5.19 Incubator, capable of maintaining a temperature of (25 ± 1) °C.

5.20 Vortex mixer, if required.

5.21 Sonicator, if required.

5.22 Test tubes

5.23 Centrifuge tube

5.24 Cover film. It does not affect viral stability or absorb water (made of polyethylene, polypropylene or polyester [poly (ethylene terephthalate)]). A film thickness of 0.05 mm to 0.10 mm is recommended. Film cut from stomacher bags are also suitable.

6 Preparation

6.1 Restoration of host cell from cryopreservation

Put a cryopreserved stock host cells in the water bath at 37 °C and defrost rapidly. Transfer the whole ampule of the defrosted host cell into a new flask for cell culture use with 20 mL of growth medium. Incubate the flask at 37 °C in the CO_2 incubator for at least 24 h. Then, observe the cell under an inverted microscope, if the cells are attached on the bottom of the flask and growth is confirmed, go to the next step, if not, continue to keep the flask in the incubator.

Remove the growth medium from the flask of the host cell and add 20 mL of the new growth medium to the flask. Incubate the flask at 37 °C in the CO_2 incubator for 48 (±2) h. Then, observe the monolayer under the inverted microscope and confirm if the cells are cultured as a confluent cell monolayer on the bottom of the flask. If the sufficient growth is not confirmed, continue to incubate until the sufficient growth is confirmed. Then, proceed to step 6.2.

6.2 Subculture of host cell

Remove the growth medium from the flask and wash the cell monolayer with 3 mL of PBS-EDTA solution. After removing the PBS-EDTA add 3 mL of trypsin-EDTA solution to the flask and spread the solution over the whole surface. Incubate the flask at 37 °C in the CO_2 incubator for 5-10 min to 20 min. Then, observe the flask under an inverted microscope to verify if the cells are starting to come off, tap the side of the flask and disperse the cells. Add 7 mL of the growth medium to the flask and pipette the cell suspension gently to avoid the damage to the cells. Transfer 1 mL of the cell suspension into a new flask for cell culture use with 25 mL of the growth medium. The ratio of the suspension and the growth medium may be changed as needed. Incubate the flask at 37 °C in the CO_2 incubator for 3 days to 5 days until confluent cell monolayer is confirmed. The culture period may be changed as needed.

In case of continuously subculturing the cell, repeat the same procedure from the beginning of 6.2

6.3 Cell culture for measuring virus infectivity titer

A seeded 96 well plate is required for measuring the infectivity titer of virus. Transfer 1 mL of the subcultured cell suspension prepared in 6.2 into a sterile media bottle with 12 mL of the growth medium. The ratio of the suspension and the growth medium could be changed as needed to obtain a confluent monolayer of cells in each well 24-48h after seeding.

Transfer 100 µL of the cell suspension into each well of the 96 well plate, incubate the plate at 37 °C in the CO_2 incubator for 1 to 2 days. The culture period could be changed as needed. Observe the condition of the cells under the inverted microscope and confirm if the cells are cultured as a confluent cell monolayer on the bottom of each well.

6.4 Preparation of test inoculums

6.4.1 Human coronavirus OC43 (HCoV-OC43)

Put the cryopreserved stock of virus suspension in the water bath at 37 °C and defrost rapidly. Remove the growth medium from the flask of the host cell monolayer prepared in 6.2. Inoculate a small volume of the HCoV-

OC43 virus suspension on the surface of the cell monolayer and spread to the whole surface, to reach a Multiplicity of Infection (MOI) of 0.1, and add 10 mL of culture medium to the flask. Incubate the flask at 34 °C in the CO_2 incubator for 1 h to allow the virus to be adsorbed by the cells, then add 10 mL of culture medium to the flask. Incubate the flask at 34 °C in the CO_2 incubator for 1 day to 3 days until about 80 % of the monolayer shows virus-induced cytopathic effect under the inverted microscope. Collect the supernatant from the flask and scrape the bottom of the flask to collect all cells. Transfer the collected cells and supernatants into a centrifuge tube. Centrifuge the virus suspension at 4 °C and 1,200 rpm for 10 min to separate the cell debris. Collect the supernatant from the centrifuge tube and resuspend cellular pellet in 1 mL of culture medium, then rapidly freeze and thaw the resuspended pellet for three times. Centrifuge the resuspended pellet at 4° and 13,000 rpm for 30 sec to separate cellular debris, collect the supernatant and add it to the collected supernatant from the previous centrifugation. Filter the virus suspension with a 0.45 µm filter, this is the stock of HCoV-OC43 suspension. Divide the suspension into new test tubes appropriately and cryopreserve at -80 °C.

To evaluate viral infectivity titer, add 150 µL of undiluted virus suspension to the first well of a round-bottom 96-well plate, and 100 µL of culture medium to the following 11 wells. Perform a serial dilution by moving 50 µL of virus suspension from the first well to the second, mix and repeat the process for at least 11 wells. Take the seeded 96-well plate prepared in 6.3 and inoculate the prepared virus dilutions into the corresponding wells. After 16h at 34°C fix and stain cells with immunocytochemistry staining as in 6.4.2. Count foci at each countable dilution under inverted microscope and express the viral titer as FFU/mL.

Check if the concentration of the infectivity titer of virus is more than 10^8 FFU/mL. If the concentration is less than 10^8 FFU/ml, prepare it from the beginning. Just before using, put the cryopreserved stock suspension in the water bath at 37 °C and defrost it rapidly. Adjust the concentration of the virus suspension to 1-5 x 10^8 FFU/mL with the culture medium. If it is not intended to be used immediately, preserve it in the refrigerator at 4 °C.

6.4.2 Immunocytochemistry staining procedure

All mentioned solutions are described in 4.5.

Remove culture medium from the cell monolayers and add 50 µL of cold acetone-methanol solution, incubate on cells for 1 minute at room temperature, then discard and wash each well with 50 µL of PBS-Triton X-100 solution**.** To allow permeabilization, add 50 µL of PBS-Triton X-100 solution to the cell monolayers and incubate for 5 minutes at 4°C. After that, perform one wash with 50 µL PBS 1X solution and incubate the cells with a proper primary antibody, according to the manufacturer's instructions. Discard the antibody and wash the monolayers once with 50 µL of PBS-Triton X-100 solution and twice with 50 µL of PBS 1X solution. Incubate the cells with a proper secondary antibody (conjugated with Horseradish Peroxidase), according to the manufacturer's instructions. Remove the antibody from the monolayers, perform two washes with 50 µL of PBS – TWEEN 20 solution and one wash with 50 µL of PBS 1X solution. Subsequently, add the PBS – DAB solution according to the manufacturer's instructions. After staining, wash each well with 50 µL PBS 1X solution and add 100 µL PBS 1X solution to preserve the monolayers until counting.

6.4.3 Feline calicivirus (FCV)

Put the cryopreserved stock of virus suspension in the water bath at 37 °C and defrost rapidly. Remove the growth medium from the flask of the host cell monolayer prepared in 6.2. Inoculate a small volume of the FCV virus suspension on the surface of the cell monolayer and spread to the whole surface, to reach a MOI of 0.1, and add 10 mL of culture medium to the flask. Incubate the flask at 34 °C in the CO_2 incubator for 1 h to allow the virus to adsorb to the cells, then add 10 mL of culture medium to the flask. Incubate the flask at 34 °C in the CO_2 incubator for 1 day to 3 days until about 80 % of the monolayer shows virus-induced cytopathic effect under the inverted microscope. Collect the supernatant from the flask and scrape the bottom of the flask to collect all cells. Transfer the collected cells and supernatants into a centrifuge tube. Centrifuge the virus suspension at 4 °C and 1,200 rpm for 10 min to separate the cell debris. Collect the supernatant from the centrifuge tube and resuspend cellular pellet in 1 mL of culture medium, then rapidly freeze and thaw the resuspended pellet three times. Centrifuge the resuspended pellet at 4° and 13,000 rpm for 30 sec to separate

cellular debris, collect the supernatant and add it to the collected supernatant from the previous centrifugation. Divide the viral suspension into new test tubes appropriately and cryopreserve at -80 °C.

To evaluate viral infectivity titer, add 150 µL of undiluted virus suspension to the first well of a round-bottom 96-well plate, and 100 µL of culture medium to the following 11 wells. Perform a serial dilution by moving 50 µL of virus suspension from the first well to the second, mix and repeat the process for at least 11 wells. Take the seeded 96-well plate prepared in 6.3 and inoculate the prepared virus dilutions into the corresponding wells for 1h at 34°C. Next, remove viral inoculum, add MTC-DMEM solution to cells and incubate the plate. After 24h at 34°C fix and stain cells with crystal violet staining as in 6.4.4. Count plaques at each countable dilution under inverted microscope and express the viral titer as PFU/mL.

Check if the concentration of the infectivity titer of virus is more than 10^8 PFU/mL. If the concentration is less than 10^8 PFU/mL, prepare it from the beginning. Just before using, put the cryopreserved stock suspension in the water bath at 37 °C and defrost it rapidly. Adjust the concentration of the virus suspension to 1-5 x 10^8 PFU/mL with the culture medium. If it is not intended to be used immediately, preserve it in the refrigerator at 4 °C.

6.4.4 Crystal violet staining procedure

Remove the MTC-DMEM solution and add to each well 100 µL of CV solutions as prepared in 4.5.7. Incubate the monolayers for 20 minutes at room temperature in agitation on an orbital shaker. Next, remove the CV solution and wash the cells three times with deionized and distilled water, let the monolayer dry before counting.

6.5 Preparation of specimens

Main testing shall be performed using at least three uncoated specimens and three coated specimens (for each tested coating). Three uncoated specimens and three coated specimens are used to measure the infectivity titer of virus after contacting the material, use of more than three replicate specimens of the coated material can help reduce variability, especially for materials that show lower antiviral effects.

When testing a series of antiviral treatments for a single polymer, each antiviral treatment may be compared to a single set of uncoated specimens if all tests are conducted at the same time using the same test inoculum.

Prepare flat specimens of the coated and the uncoated materials, with a surface area not smaller than 2.5 x 2.5 cm, unless otherwise specified. Specimens should be no thicker than 10 mm. It is preferable to prepare test specimens from the final product itself. If not, then the test specimens may be prepared in a format suitable for the testing using the same raw materials and processing methods as are normally used for the product.

When preparing specimens, be sure to avoid contamination with microorganisms or extraneous organic debris. Test specimens can be disinfected/sterilized prior to the testing (e.g. by exposing the coated and uncoated materials to UV rays for at least 15 minutes per side). Cleaning of the test specimen should not be allowed to avoid softening, dissolution of the surface coating or elution of components.

6.6 Control tests

6.6.1 General

The purpose of the control test is to confirm the antiviral activity of the coated materials, indeed, the drying test and ion release test that are reported in the following sections are designed to be performed on coatings that already demonstrated to be endowed with antiviral activity against the tested viruses, according to ISO 21702:2019. Moreover, if the coated material is intended to be used in strict contact with people, it is necessary to verify cytotoxicity following ISO 10993-5:2009. Additionally, the control test involves also the evaluation of a possible cytotoxic effect of tested materials on cells.

6.6.2 Verification of antiviral activity following the ISO 21702:2019

The antiviral activity of the coated specimen should be measured following the **ISO 21702:2019** [1].

The present protocols to study the mechanism of action should be followed only if the coated specimen achieved an R value ≥ 2 (99.99% inhibition of viral infectivity) following the indications for antiviral activity measurement listed in ISO 21702:2019.

6.6.3 Verification of cytotoxic effect on host cell

If the coated material is intended to be used in strict contact with people, the *in vitro* cytotoxicity of the test specimen should be measured by following the **ISO 10993-5:2009** [2].

The present protocols to study the mechanism of action should be followed only if the coated specimen demonstrated to reduce cell viability by less than 30 % following the indications listed in ISO 10993-5:2009.

7 Test procedure

7.1 Preparation of specimens

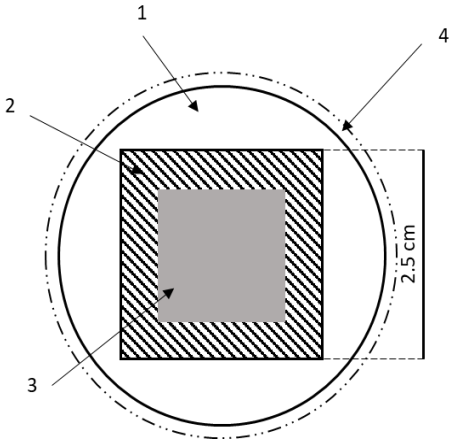
All specimens are prepared in sterile Petri dishes as described in 6.5. Use sterile tweezers to handle specimens. Put the coated side of the product on top when placed in the sterile Petri dish. Do not test cross-sections of the product.

7.2 Drying test

7.2.1 General

The aim of the drying test is to assess whether the antiviral coating has the ability to directly inactivate viral particles, either by trapping them within the coating or by damaging or destroying the structure of the virion.

7.2.2 Virus inoculation on specimens



1. Petri dish
2. Specimen
3. Viral inoculum
4. Petri dish lid

Figure 1 – Schematic representation of the inoculation of the specimen

Unless otherwise specified, the standard size of the specimen should be a square of 2.5 cm × 2.5 cm. If the size of the specimen differs from the standard, the actual size should be included in the test report. The volume and the concentration of the inoculum used should also be adjusted in direct proportion. Include the modification in the test report.

Pipette 100µL of the virus inoculum prepared in 6.4 onto the test surface. Make sure that the test inoculum does not leak beyond the edges of the specimen. If there are difficulties in preventing such leakage on hydrophilic surfaces, process using option 1 below. If it does not help, apply option 2. If one of these options is used to prevent leakage, it should be included in the test report.

Option 1: Reduce the volume of the test inoculum applied to the test surface. When the volume of the test inoculum is reduced, the concentration of the viral particles in the inoculum should be increased so as to provide the same number of virions as when the normal volume of the test inoculum is applied.

Option 2: Increase the viscosity of the test inoculum by adding an inert thickener such as agar.

Keep each of the Petri dishes with the inoculated test specimens (including the uncoated specimens) under a biological hood for 3h at room temperature, allowing the drying of the viral inoculum. Other contact times may be applied if agreed upon by the relevant parties. In this case, the modified contact time should be included in the test report.

7.2.3 Recovery of virus from specimens

After 3 hours, recover the dried viral inoculum from both the coated and uncoated specimens by adding 500 µL of culture medium to each Petri dish. Rinse the specimens four times with the medium to collect the residual virus. The final volume of the collected virus suspension should be at least 300 µL. If the test specimen has high absorbency and the recovered volume is less than the required minimum, increase the volume of the recovery medium accordingly. Any changes to the recovery volume should be documented in the test report. Such volume adjustments should also be considered in the calculations described in section 8.3. Proceed immediately with the measurement of the infectivity titer of the virus recovered from the test specimens.

7.2.4 Determination of virus infectivity titer

Seed the appropriate cell lines in 96-well plastic plates at a sub-optimal confluency. The following day, prepare serial dilutions of the virus suspensions using a separate 96-well plate, suitable for resuspension. Add 150 µL of undiluted virus suspension to the first well, and 100 µL of culture medium to the subsequent 11 wells. Perform a 3-fold serial dilution by transferring 50 µL from the first well to the second, mixing thoroughly, and repeating the process across the remaining wells, resulting in 12 dilutions per condition. Each well should contain a final volume of 100 µL. The titration should be performed in duplicate. Remove the growth medium from the cell plates and wash the monolayer once with 100 µL of culture medium. After discarding the wash, inoculate the prepared virus dilutions into the corresponding wells. Incubate the plates at 34 °C in a CO_2 incubator for 1h to allow viral adsorption. Following incubation, wash the monolayer once with 100 µL of culture medium.

For human coronavirus, the viral infectivity titer shall be determined by focus assay. After removing the wash medium, add 100 µL of fresh culture medium to each well and incubate the plates for 16 hours at 34 °C. Detect foci by immunocytochemistry staining. Further details regarding the immunocytochemistry staining procedure can be found in 6.4.2. Count foci microscopically, and express viral infectivity titer as focus-forming unit (FFU) per mL.

For feline calicivirus, the viral infectivity titer shall be determined by plaque assay.

After removing the wash medium, overlay the infected cells with MTC-DMEM solution and incubate the plates for 24 hours at 34 °C. After incubation, fix and stain the cells with crystal violet staining as described in 6.4.4. Count the resulting plaques microscopically and express viral infectivity titer as plaque-forming units (PFU) per mL.

NOTE: To determine the infectivity titer of virus, other methods like $TCID_{50}$ can be used after appropriate validations.

7.2.5 Determination of the number of viral genome copies harvested from specimens

To discriminate whether viral particles are retained within the tested coating or released into the collecting medium with a structural damage that affects infectivity, the number of viral genome copies can be evaluated by quantitative real-time PCR (qRT-PCR). The starting material consists of the virus suspensions harvested as described in section 7.2.2. Extract viral genome using an appropriate cell-free virus DNA/RNA extraction kit, and perform qRT-PCR according to the manufacturer's instructions. If the number of viral genome copies recovered from the coated specimens is significantly lower than that from the uncoated specimens, it indicates that intact/damaged viral particles are being retained within the coating or destroyed. Conversely, if the number of genome copies is not significantly different between coated and uncoated specimens, this suggests that the damaged virions are being released.

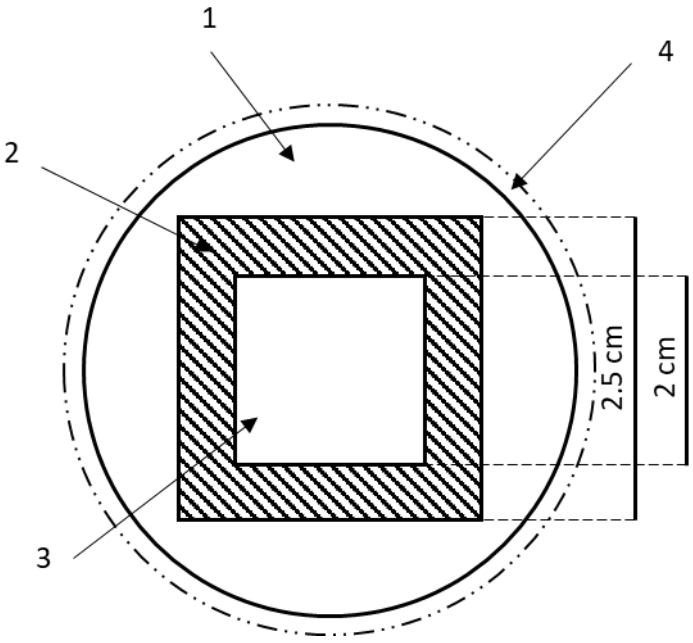
Additional tests analyzing the collecting medium from the drying test by electron microscopy, could verify the presence/absence of viral particles and the entity of the structural damage.

7.3 Ion release test

7.3.1 General

The aim of the ion release test is to assess whether the ions released from the antiviral coating inhibit virus infectivity.

7.3.2 Culture medium deposition on specimens and placement of cover film



1. Petri dish
2. Specimen
3. Cover film over medium
4. Petri dish lid

Figure 2 – Schematic representation of culture medium addition and placement of the cover film onto the specimen

Unless otherwise specified, the standard size of the test specimen should be a square of 2.5 cm × 2.5 cm. If the size of the test specimen differs from the standard, the actual size should be included in the test report. The volume and the concentration of the culture medium used should also be adjusted in direct proportion. Include the modification in the test report.

Pipette 100μL of culture medium onto the test surface. Cover the medium with a piece of film that measures 2 cm × 2 cm and gently press down on the film so that the medium spreads to the edges. The cover film allows to avoid medium evaporation during the incubation time. Make sure that the medium does not leak beyond the edges of the film. If there are difficulties in preventing such leakage on hydrophilic surfaces, process using option 1 below. If it does not help, apply option 2. If one of these options is used to prevent leakage, it should be included in the test report.

Option 1: Reduce the volume of the test inoculum applied to the test surface. When the volume of the test inoculum is reduced, the concentration of the viral particles in the inoculum should be increased so as to provide the same number of virions as when the normal volume of the test inoculum is applied.

Option 2: Increase the viscosity of the test inoculum by adding an inert thickener such as agar.

After the medium has been added and the cover film applied, close the lid of the Petri dish. Keep each of the Petri dish with the medium (both coated and uncoated specimens) in a CO_2 incubator at (25 ± 1) °C and a relative humidity of not less than 90% for 3 hours or 24 hours, allowing the release of ions from the test specimens. Other contact times may be applied if agreed upon by the relevant parties. In this case, the modified contact time should be included in the test report.

7.3.3 Recovery of culture medium from specimens

After 3 hours or 24 hours of incubation, recover the ions released into the medium from both coated and uncoated specimens by adding 800 μL of culture medium to each Petri dish. Rinse each specimen four times using the culture medium to ensure thorough collection of the released ions. The final volume of the ion suspension should be at least 300 μL. If the test specimen has high absorbency and the recovered volume is less than the required minimum, increase the volume of the recovery medium accordingly. Any changes to the recovery volume should be documented in the test report. Such volume adjustments should also be considered in the calculations described in section 8.3.

7.3.4 Virus inoculation on wash-out solutions

Transfer 300 μL of each ion suspension into separate 1.5 mL microcentrifuge tubes. Add 100 μL of virus inoculum (prepared in 6.4) to each tube, mixing thoroughly to ensure uniform distribution. Incubate the tubes in a CO_2 incubator at (25 ± 1) °C with ≥90% relative humidity for either 3 hours or 24 hours, depending on the prior incubation time of the culture medium on the coated and uncoated specimens. Immediately after incubation, proceed with the measurement of the infectivity titer of the virus.

7.3.5 Determination of virus infectivity titer

Viral infectivity titer shall be determined as described in 7.2.3.

8 Expression of results

8.1 Calculation of virus infectivity titer

8.1.1 Calculation of virus infectivity titer for drying test

For each test specimen, determine the infectivity titer of virus recovered in accordance with the following formula:

Virus infectivity titer (FFU/mL or PFU/mL) = (CxDx10x5)

where

C is the average number of foci/plaques counted for the duplicate wells;

D is the dilution factor for the wells counted;

If no foci/plaques are recovered in any of the wells, then record as 1 focus/plaque.

8.1.2 Calculation of virus infectivity titer for ion release test

For each test specimen, determine the infectivity titer of virus recovered in accordance with the following formula:

Virus infectivity titer (FFU/mL or PFU/mL) = (CxDx10x4)

where

C is the average number of foci/plaques counted for the duplicate wells;

D is the dilution factor for the wells counted;

If no foci/plaques are recovered in any of the wells, then record as 1 focus/plaque.

8.2 Conditions for a valid test

When the two following conditions are satisfied, the test is deemed valid. If all conditions are not met, the test is considered as invalid and the specimens shall be retested.

1. The logarithmic value of the number of foci/plaques recovered from the uncoated specimens shall satisfy the requirement of the following formula:

$(L_{max} - L_{min}) / (L_{mean}) \leq 0.2$

where

L_{max} is the common logarithm (i.e. base 10 logarithm) of the maximum number of foci/plaques among the two replicates counted per dilution;

L_{min} is the common logarithm of the minimum number of foci/plaques among the two replicates counted per dilution;

L_{mean} is the common logarithm of the mean number of foci/plaques among the two replicates counted per dilution.

2. The number of foci/plaques recovered from each uncoated specimen shall not be less than 1×10^2 FFU/mL or PFU/mL.

8.3 Calculation of antiviral activity

When the test is deemed as valid, calculate the antiviral activity using the following formula:

$R = UC - C$

where

R is the antiviral activity;

UC is the average of the common logarithm of the viral infectivity titers calculated from the three uncoated test specimens, in FFU/mL or PFU/mL;

C is the average of the common logarithm of the viral infectivity titers calculated from the three coated test specimens, in FFU/mL or PFU/mL.

8.4 Effectiveness of antiviral agent

Text of subclause. The value of the antiviral activity can be used to characterize the effectiveness of an antiviral agent. The R value has to be higher ≥1 to define a coating as effective.

9 Repeatability and reproducibility

Repeatability is the closeness of agreement between successive measurements of the same measurand carried out under the same conditions of measurement. These conditions include the same measurement procedure, same operator, same measuring instrument, same location, and repetition over a short period of time.

Reproducibility is the closeness of agreement between measurements of the same measurand carried out under changed conditions. These changes may include different operators, different measuring instruments, different laboratories, and different time intervals [3].

The herein described protocols shall ensure high repeatability and reproducibility of results, in accordance with the definitions provided above.

10 Test report

The test report should include the following information:

a) a reference to this document;

b) the type of plastics/non-porous surfaces used for the coated and uncoated specimens and the size, shape and thickness of the specimens;

c) the type of polymer used for the cover film and the size, shape and thickness of the film;

d) the species of test virus and host cells used, indicating the reason if other species of virus or host cell were used;

e) the volume of test inoculum used;

f) the number of foci/plaques in the test inoculum;

g) the viral titers from the uncoated and test specimens calculated in 8.1;

h) the antiviral activity calculated;

i) details of any deviation from this document as well as details of any alternative procedures, if used, including cleaning method of the test specimens, the use of inert thickeners, the type and volume of neutralizer used, the use of an alternative recovery method and the use of an alternative incubation temperature;

j) identification of the test laboratory, and the name and signature of the head of the laboratory;

k) the date of commencement of the experiments;

l) the date of the test report.

Annex A
(informative)

Composition of media

A.1 General

The media are available on the market and used for cell culture.

A.2 Composition of DMEM

The example of composition of the DMEM-high glucose is described in Table A.1. The DMEM is available on the market.

Table A.1 — Composition of DMEM-high glucose

Composition [1×] g/L		g
Inorganic salts	Calcium chloride	0.2
	Ferric nitrate • $9H_2O$	0.0001
	Magnesium sulfate (anhydrous)	0.09767
	Potassium chloride	0.4
	Sodium bicarbonate	3.7
	Sodium chloride	6.4
	Sodium phosphate monobasic (anhydrous)	0.109
Amino acids	L-Arginine • HCl	0.084
	L-Cystine • 2HCl	0.0626
	L-Glutamine	0.584
	Glycine	0.03
	L-Histidine • HCl • H_2O	0.042
	L-Isoleucine	0.105
	L-Leucine	0.105
	L-Lysine • HCl	0.146
	L-Methionine	0.03
	L-Phenylalanine	0.066

	L-Serine	0.042
	L-Threonine	0.095
	L-Tryptophan	0.016
	L-Tyrosine • 2Na • $2H_2O$	0.10379
	L-Valine	0.094
Vitamins	Choline chloride	0.004
	Folic acid	0.004
	myo-Inositol	0.0072
	Niacinamide	0.004
	D-Pantothenic acid (hemicalcium)	0.004
	Pyridoxine • HCl	0.004
	Riboflavin	0.0004
	Thiamine • HCl	0.004
Other	D-Glucose	4.5
	Phenol red • Na	0.0159

Bibliography

[1] ISO 21702:2019(E), *Measurement of antiviral activity on plastics and other non-porous surfaces.*

[2] ISO 10993-5:2009, *Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity.*

[3] ISO 5725-1:2023, *Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.*