INTERNATIONAL STANDARD

ISO 21702

First edition
2019-05

Measurement of antiviral activity on plastics and other non-porous surfaces

Mesure de l'activité antivirale sur les matières plastiques et autres surfaces non poreuses

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Reference number
ISO 21702:2019(E)

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 6, *Ageing, chemical and environmental resistance*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

Antibacterial-treated porous and non-porous products have been widely accepted and used among general consumers as their new choices to purchase for the additional function, which are different from what traditional materials had in terms of material protection.

Recently, antiviral-treated porous and non-porous products have been also in the market.

The measuring test method of antibacterial activity on non-porous products is described in ISO 22196.

The measuring test method of antibacterial activity on porous products (textiles) is described in ISO 20743.

The measuring test method of antiviral activity on porous products (textiles) is described in ISO 18184.

This document is the test method of antiviral activity on non-porous products. It is written based on ISO 22196 and ISO 18184.

In ISO 22196, the scope has been expanded to include surfaces made of plastics and other non-porous materials, thus this document is intended to be applicable to products such as plastics, coating materials, ceramics, natural and artificial leathers, stainless, rubbers, etc.

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Measurement of antiviral activity on plastics and other non-porous surfaces

WARNING — Handling and manipulation of viruses and host cells which are potentially hazardous requires a high degree of technical competence and may be subject to current national legislation and regulations. Only personnel trained in biological techniques should carry out such tests. Appropriate practices for disinfection, sterilization and personal hygiene must be strictly observed.

1 Scope

This document specifies proper methods for measuring antiviral activity on plastics and other non-porous surfaces of antiviral-treated products against specified viruses. Due to the individual sensitivities, the results of one test virus might not be applicable for other viruses.

2 Normative references

There are no normative references in this document.

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 https://www.iso.org/obp

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

3.1
antiviral
state where the number of infectious virus particles on surfaces 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 with 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 in a suspension

3.6
plaque
lysis formed area in a cell monolayer under semisolid medium due to infection by and multiplication of a single infectious virus

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3.7
plaque forming units
PFU
unit expressed as the concentration of the infectious viral particle per unit volume (ml)

3.8
plaque assay
assay to determine the *infectivity titer of virus* (3.5) from PFU by using the series of dilution

4 Materials

4.1 Virus and host cell to be used for the tests

The example species of virus and host cell to be used are shown in Table 1.

Other species of virus and host cells can be used after appropriate validations, as the important virus may differ depending on the target application. If the other species are used, the name of the species and the specific reason for their use shall be included in the test report.

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

Virus name	*Influenza virus*	*Feline calicivirus*
Virus strain	*Influenza A virus* (H3N2): A/Hong Kong/8/68: TC adapted ATCC VR-1679	*Feline calicivirus*; Strain: F-9 ATCC VR-782
Host cell[a]	MDCK cell (dog kidney cell origin) ATCC CCL-34	CRFK cell (cat kidney cell origin) ATCC CCL-94
Growth medium[b]	EMEM (4.3.8)	RPMI 1640 (4.3.8)
[a] The other host cells can be used after appropriate validations regarding the sensitivity against each virus.		
[b] The other media can be used after appropriate validations for the growth of cells.		

4.2 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 ìS/cm.

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

4.3 Culture medium and solutions

4.3.1 General

The culture medium specified below shall be used. The medium may be obtained from commercial suppliers which shall be prepared for use in accordance with the manufacturer's instructions.

4.3.2 Eagle's minimum essential medium (EMEM)

The composition is described in Annex A. If there are any components missing from the composition, they can be added according to Table A.1.

4.3.3 RPMI 1640 medium

The composition is described in Annex A. If there are any components missing from the composition, they can be added according to Table A.2.

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4.3.4 7,5 % sodium bicarbonate solution

Select and prepare the solution using one of the two following options:

— Option 1: Prepare a 7,5 % sodium bicarbonate solution by dissolving 75 g of sodium bicarbonate in 1 000 ml of water. Sterilize the solution by using a 0,22 µm membrane filter.

— Option 2: Prepare a 7,5 % sodium bicarbonate solution by sterilizing 75 g of sodium bicarbonate in a culture container with a cap closed tightly in an autoclave. Sterilize 1 000 ml of water in the autoclave. Dissolve the sterilized sodium bicarbonate in the sterilized water well.

If the solution is not intended to be used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use 7,5 % sodium bicarbonate solution that has been kept for longer than one month after preparation.

4.3.5 Formaldehyde solution

Prepare the formaldehyde solution by adding 100 ml of 37 % formaldehyde solution to 900 ml of water. If it is not intended to be used immediately after preparation, preserve it at 20 °C to 25 °C. Never use the formalin solution that has been kept for longer than one month after preparation.

NOTE The other solution for cell fixation can be used after appropriate validation for cell fixation.

4.3.6 Methylene blue solution

Prepare the methylene blue solution by dissolving 0,375 g of the methylene blue and 62,5 µl of 1 N sodium hydroxide solutions in 1 000 ml of water. If it is not intended to be used immediately after preparation, then preserve it at 20 °C to 25 °C. Never use the methylene blue solution that has been kept for longer than one month after preparation.

4.3.7 Inactivated fetal bovine serum (FBS)

Put a freezed cryopreserved fetal bovine serum in a package 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. 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.

4.3.8 Growth medium

Dissolve 9,53 g of the Eagle's minimum essential medium or 10,4 g of RPMI 1640 medium and 60 mg of kanamycin sulfate in 800 ml of water. Add water to make 1 000 ml of solution in total. Sterilize the solution by using a 0,22 µm membrane filter.

When L-glutamine is not included in the EMEM or RPMI 1640 medium purchased in the market, sterilizing in the autoclave may be applied. Then, before use, add L-glutamine as listed in the example of composition in Annex A.

Add 15 ml of 7,5 % sodium bicarbonate solution and 100 ml of the inactivated fetal bovine serum in the solution and mix well. If it is not intended to be used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a growth medium that has been kept for longer than one month after preparation.

4.3.9 Maintenance medium

Dissolve 9,53 g of the Eagle's minimum essential medium and 60 mg of kanamycin sulfate in 800 ml of water. Add water to make 1 000 ml of solution in total. Sterilize the solution by using a 0,22 µm membrane filter.

When L-glutamine is not included in the EMEM purchased in the market, sterilizing by autoclaving may be applied. Then, before use, add L-glutamine as listed in the example of composition in Annex A.

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Add 15 ml of 7,5 % sodium bicarbonate solution in the solution and mix well. If it is not intended to be used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a maintenance medium that has been kept for longer than one month after preparation.

4.3.10 Double concentration of the maintenance medium

Dissolve 19,06 g of the Eagle's minimum essential medium and 120 mg of kanamycin sulfate in 800 ml of water. Add water till there are 1 000 ml of solution in total. Sterilize the solution by using a 0,22 µm membrane filter. If it is not intended to be used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a growth medium that has been kept for longer than one month after preparation. When L-glutamine is not included in the EMEM purchased on market, sterilizing by autoclaving could be applied. Then, before use, add L-glutamine as listed in the example of composition in Annex A.

4.3.11 Phosphate buffered saline [PBS (-)]

Prepare PBS (-) by dissolving 8,0 g of sodium chloride, 0,2 g of potassium chloride, 2,9 g of phosphoric acid hydrogen 2 sodium 12 hydrate and 0,2 g of phosphoric acid 2 hydrogen potassium in 1 000 ml of water. Sterilize by autoclaving (see 6.2). If it is not intended to be used immediately after preparation, preserve it at 5 °C to 10 °C. Never use a PBS (-) that has been kept for longer than one month after preparation.

4.3.12 Trypsin derived from beef pancreas and PBS (-) solution

4.3.12.1 Dissolve 1,0 g of trypsin derived from pancreas in 100 ml of PBS (-) and mix well for 2 h by using a mixer. Sterilize the solution by using 0,22 µm membrane filter. If it is not intended to be used immediately after preparation, divide the solution in test tubes and preserve them in the freezer at a temperature less than −80 °C. Just before using, put it in the water bath at 37 °C and keep it until it defrosts.

4.3.12.2 Add 1,0 ml of the solution of 4.3.12.1 to 9,0 ml of PBS (-) and mix well. Divide the solution in test tubes and preserve them in the 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.

4.3.13 Trypsin EDTA solution[1)]

Prepare Trypsin EDTA solution by dissolving 2,5 g of trypsin, 0,1 g of kanamycin sulfate, 0,1 g of streptomycin sulfate, 2 mg of amphotericin B and 0,014 mol of EDTA in 1 000 ml of PBS (-). Sterilize the solution by using a 0,22 µm membrane filter. Divide the solution in test tubes and preserve them in the 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.

NOTE Trypsin EDTA solution is available in the market. The products with different components could be used after proper validations.

4.3.14 DEAE-dextran solution

Prepare DEAE-dextran solution by dissolving 20 g of DEAE-dextran in 1 000 ml of water. Sterilize the solution by using 0,22 µm membrane filter. If it is not intended to be used immediately after preparation, then preserve it at 5 °C to 10 °C. Never use a DEAE-dextran solution that has been kept for longer than one month after preparation.

1) Trypsin EDTA solution is an example of a product available in the market. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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4.3.15 Agar medium for plaque assay

4.3.15.1 A liquid

Add 10 ml of DEAE-dextran solution and 40 ml of 7,5 % sodium bicorbonate solution to 1 000 ml of Double concentration of maintenance medium and mix well. Only for the influenza virus test, add 3,0 ml of the Trypsin from pancreas and PBS (-) solution. Keep the solution in the water bath at 37 °C.

4.3.15.2 B liquid

Add 15 g of cell culture agar to 1 000 ml of water and mix well. Sterilize by autoclaving. Keep the solution in the water bath at 60 °C.

4.3.15.3 Preparation of agar medium

Prepare agar medium for plaque assay with A liquid and B liquid with one to one amount and mix well just before using.

4.3.16 Soybean casein digest broth with lecithin and polyoxyethylene sorbitan monooleate (SCDLP broth)

Prepare the SCDLP broth by dissolving 17,0 g of casein peptone, 3,0 g of soybean peptone, 5,0 g of sodium chloride, 2,5 g of disodium hydrogen phosphate, 2,5 g of glucose and 1,0 g of lecithin in 1 000 ml of water. Mix well and add 7,0 g of nonionic surfactant. Adjust the pH to a value between 6,8 and 7,2 (at 25 °C) with sodium hydroxide or hydrochloric acid. Sterilize by autoclaving. If the broth is not intended to be used immediately after preparation, preserve it at 5 °C to 10 °C. Never use the SCDLP broth if it has been kept for longer than one month after preparation.

NOTE SCDLP is a typical neutralizer. Refer to ASTM E 1054[7] and EN 1040[8] for further information about the other neutralizer.

5 Apparatus

Unless otherwise specified, use the following apparatus and materials.

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.

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

5.3 Hotplate with stirrer, or **hot-water bath**.

5.4 pH-meter, capable of measuring to ±0,2 units.

5.5 Balance, capable of the available range of 100 g ± 0,1 g to 0,01 g ± 0,000 1 g.

5.6 Micro pipetter, sterile, with 1 000 µl and 200 µl tips.

5.7 Pipetter, capable of mounting the plastic pipettes.

5.8 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.

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

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5.10 Liquid nitrogen bath, for the preservation approximately at −196 °C.

5.11 Membrane filter, sterile, with a pore size of 0,22 µm.

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

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

5.14 Six wells plastic plate, sterile, with an adherent type, for plaque assay.

5.15 Flasks for cell culture use, sterile, with an adherent type, a cell culture area of 75 cm^2 and with the 0,2 µm filter vent capwhich can prevent bacterial contamination.

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

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

5.18 Vortex mixer, if required.

5.19 Sonicator, if required.

5.20 Cover film that 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.

NOTE Films cut from stomacher bags are also suitable.

5.21 Test tubes.

5.22 Centrifuge tube.

5.23 Gauze or absorbent cotton.

5.24 1 000 ml volumetric flask.

5.25 Stoppered Erlenmeyer flasks or media bottles, as required for preparation of media.

6 Preparation

6.1 Restoration of host cell from cryopreservation

Put a cryopreserved stock host cell 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 (24 ± 2) h. Then, observe the cell under an inverted microscope if the cells are attached on the bottom of the flask. If the 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 the subculture by taking the following steps of 6.2.

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6.2 Subculture of host cell

Remove the growth medium from the flask and wash the cell monolayer with 5 ml of PBS (-) two times. After removing an extra PBS (-), add 0,5 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 10 min to 20 min. Then, observe the flask if the cells are starting to come off, tap the side of the flask and disperse the cells. Add 5 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 20 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

The cell culture in a 6 wells plate is required for measuring the infectivity titer of virus by the plaque assay. Transfer 1 ml of the subcultured cell suspension prepared in 6.2 into a sterile media bottle with 20 ml of the growth medium.

The ration of the suspension and the growth medium could be changed as needed.

Transfer 3 ml of the cell suspension into each hole of the 6 wells plastic plate for the plaque assay. Incubate the plate at 37 °C in the CO_2 incubator for 3 days to 5 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 hole.

6.4 Preparation of test inoculums

6.4.1 Influenza virus

Put the cryopreserved stock virus suspension in the water bath at 37 °C and defrost rapidly. Dilute the defrosted stock influenza virus suspension with the maintenance medium to obtain a virus suspension of 10^3 PFU/ml to 10^4 PFU/ml.

Remove the growth medium from the flask of the host cell monolayer prepared in 6.2, wash the monolayer with 5 ml of the maintenance medium two times after removing an extra maintenance medium. Inoculate with 1 ml of the adjusted influenza virus suspension on the surface of the cell monolayer and spread to the whole surface. Incubate the flask at 34 °C in the CO_2 incubator for 1 h to allow the virus to adsorb to the cells. Add 20 ml of the maintenance medium and 30 µl of Trypsin derived from pancreas and PBS (-) solution 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. Transfer the contents of the flask into a centrifugal tube. Centrifuge the virus suspension at 4 °C and 1 000 g for 15 min to separate the cell debris. Take the supernatant from the centrifugal tube after the centrifugation. This is to be the stock influenza virus suspension. Divide the suspension into new test tubes appropriately and cryopreseave at −80 °C in the freezer. Check the concentration of the infectivity titer of virus if it 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 influenza virus suspension in the water bath at 37 °C and defrost it rapidly. Adjust the concentration of the virus suspension to 1×10^8 PFU/ml to 5×10^8 PFU/ml with the maintenance medium. Then, dilute the adjusted virus suspension with water to obtain 10-fold dilution suspension. This is to be the test inoculum. If it is not intended to be used immediately, preserve it in the refrigerator at 4 °C.

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6.4.2 Feline calicivirus

Put the cryopreserved stock virus suspension in the water bath at 37 °C and defrost rapidly. Dilute the defrosted stock feline calicivirus suspension with the maintenance medium to obtain a virus suspension of about 10^7 PFU/ml.

The concentration of the virus suspension could be changed as needed.

Remove the growth medium from the flask of the host cell monolayer prepared in 6.2, wash the monolayer with 5 ml of the maintenance medium two times after removing an extra maintenance medium. Inoculate with 1 ml of the adjusted feline calicivirus suspension on the surface of the cell monolayer and spread to the whole surface. Incubate the flask at 37 °C in the CO_2 incubator for 1 h to allow the virus to adsorb to the cells. Add 5 ml to 20 ml of the maintenance medium to the flask. Incubate the flask at 37 °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. Transfer the contents of the flask into a centrifugal tube. Centrifuge the virus suspension at 4 °C and 1 000 g for 15 min to separate the cell debris. Take the supernatant from the centrifugal tube after the centrifugation. This is to be the stock feline calicivirus suspension. Divide the suspension into new test tubes appropriately and cryopreseave at −80 °C in the freezer. Check the concentration of the infectivity titer of virus if it 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 feline calicivirus suspension in the water bath at 37 °C and defrost it rapidly. Adjust the concentration of the virus suspension to 1×10^8 PFU/ml to 5×10^8 PFU/ml with the maintenance medium. Then, dilute the adjusted virus suspension with water to obtain 10-fold dilution suspension. This is to be the test inoculum. If it is not intended to be used immediately, preserve it in the refrigerator at 4 °C.

6.5 Preparation of test specimens

Main testing shall be performed using at least three specimens from each treated test material. At least twelve specimens of the untreated material and nine specimens of treated material are required. Three untreated test specimens are used to measure the infectivity titer of virus immediately after inoculation. Three untreated test specimens and three treated test specimens are used to measure the infectivity titer of virus after contacting for 24 h. Six untreated test specimens and six treated test specimens are used for the control test in 6.6.

NOTE Use of more than three replicate specimens of the treated test 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 untreated specimens if all tests are conducted at the same time using the same test inoculum.

Prepare flat (50 ± 2) mm × (50 ± 2) mm specimens of the treated and the untreated test materials. Specimens should be no thicker than 10 mm. If it is difficult or impossible to cut the product into a square of this size, then test specimens in other sizes and shapes may be used, as long as they can be covered with a film which surface area is between 400 mm^2 and 1 600 mm^2. 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. If the test specimen is not a 50 mm × 50 mm square dimension, the used dimensions shall be included in the test report.

When preparing specimens, be sure to avoid contamination with microorganisms or extraneous organic debris. At the same time, be sure to avoid the specimens to contact each other. If any metal apparatus is used to avoid cross-contamination, it is necessary to ensure that the metal does not have any antiviral effect. If necessary, test specimens can be cleaned/disinfected/sterilized prior to the testing (e.g. by wiping with 70 % ethanol resolved in water). Cleaning of the test specimen shall not be allowed to avoid softening, dissolution of the surface coating or elution of components. If cleaning is required due to gross contamination, the cleaning method shall be included in the test report.

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6.6 Control test

6.6.1 General

The purpose of the control test is to confirm the suppressive efficiency of agent's activity on the test specimen. The suppressive efficiency means no cytotoxic effect on the host cell, no reduction of the cell sensitivity to virus, and inactivation of the antiviral activity in the SCDLP broth (4.3.16). There is a case that the agent which does not obviously show the cytotoxicity still reduces the infectious or replicating ability of the virus, thus the agent interferes the evaluation of the antiviral activity. Therefore, the control test shall be performed to exclude such a possibility of failing to evaluate properly.

6.6.2 Verification of cytotoxic effect on host cell

Add 10 ml of either the SCDLP broth or a suitable, validated neutralizer to each Petri dish with the test specimens prepared in 6.5. It is important to ensure that the neutralizer completely washes the specimens with pipetting the SCDLP broth at least four times. Three untreated test specimens and three treated test specimens are used for this test.

Remove the growth medium from the 6 wells plastic plates prepared in 6.3, wash the monolayer once with 3 ml of maintenance medium. After removing the extra maintenance medium, inoculate 0,1 ml of the SCDLP broth recovered from the test specimen in the 2 wells. Put the plate at 34 °C for influenza virus or at 37 °C for feline calicivirus in the CO_2 incubator for 1 h, in the meantime, tilte the plates every 15 min so the cell monolayer will be kept moist. Then, wash the monolayer once with 3 ml of the maintenance medium. After removing the extra maintenance medium, pour 3 ml of the agar medium for plaque assay (4.3.15) into the wells. Close a lid and keep it at room temperature for about 10 min. After confirming the agar coagulates, incubate the plate at 34 °C for influenza virus or at 37 °C for feline calicivirus in the CO_2 incubator for 2 days to 3 days.

After the incubation, add 3 ml of the formalin solution (4.3.5) to the wells, then keep it at room temperature for more than 1 h to fix the cells. Remove the formalin solution and the agar medium from the wells, add 3 ml of the methylene blue solution (4.3.6), then keep it at room temperature for 15 min to dye the cells. Wash the extra methylene blue solution with tapped water.

If no cytotoxicity is observed, proceed to the next step (see 6.6.3).

When cytotoxicity is observed, neutralizer should be carefully modified, changed or the amount of neutralizer be carefully increased. When it is difficult to proceed with 10 ml of the neutralizer due to the size and characteristics of the test specimen, then the volume of the solution may be increased.

If the neutralizer is modified, changed or the amount of neutralizer is increased, the same condition on neutralizer shall be applied at the next step (6.6.3).

6.6.3 Verification of cell sensitivity to virus and the inactivation of antiviral activity

6.6.3.1 Procedure for verification

Add 10 ml of either the SCDLP broth (4.3.16) or a suitable, validated neutralizer to each Petri dish with the test specimens prepared in 6.5. It is important to ensure that the neutralizer completely washes the specimens by pipetting the SCDLP broth at least four times. Three untreated test specimens and three treated test specimens are used for this test.

Take 5 ml of the SCDLP broth from each Petri dish recovered from the test specimen into new test tubes, and as for negative control, take 5 ml of the new SCDLP broth into new three test tubes. Then, add 50 µl of a virus suspension prepared to be a concentration of (4 to 6) × 10^4 PFU/ml into the 9 tubes and keep them at 25 °C for 30 min.

Remove the growth medium from the 6 wells plastic plates prepared in 6.3, wash the monolayer once with 3 ml of maintenance medium. After removing the extra maintenance medium, inoculate 0,1 ml of each test suspension in the 2 wells. Put the plate at 34 °C for influenza virus or at 37 °C for feline

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calicivirus in the CO_2 incubator for 1 h to allow the virus to adsorb to the cells, in the meantime, tilt the plates every 15 min so the monolayer keeps moisture. Then, wash the monolayer once with 3 ml of maintenance medium. After removing the extra maintenance medium, pour 3 ml of agar medium for plaque assay (4.3.15) into the wells. Close a lid and keep at room temperature for about 10 min. After confirming the agar coagulates, incubate the plate at 34 °C for influenza virus or at 37 °C for feline calicivirus in the CO^2 incubator for 2 days to 3 days.

After incubation, add 3 ml of the formalin solution (4.3.5) to the wells, then keep it at room temperature for more than 1 h to fix the cells. Remove the formalin solution and the agar medium from the wells, add 3 ml of the methylene blue solution (4.3.6), then keep it at room temperature for 15 min to dye the cells. Wash the extra methylene blue solution with tapped water.

Select the wells containing 6 to 60 plaques. If the number of plaques in the wells containing the 0,1 ml aliquots of the test suspension is less than 6, then count and record the number of plaques in these wells. If there is no plaque recovered in any of the wells in the dilution series, record the number of plaques as "<1".

6.6.3.2 Determination of the infectivity titer of virus

For each test suspension, determine the infectivity titer of virus in accordance with Formula (1):

$$S = (10 \times P) \tag{1}$$

where

S is the infectivity titer of virus per ml per test suspension;

P is the average plaque count for the duplicate wells.

6.6.3.3 Condition for verification for this test

By comparing the infectivity titer of virus from the SCDLP broth for negative control with that from the untreated test specimen or the treated specimen, the logarithmic value shall satisfy the requirements of Formulae (2) and(3):

$$|S_n - S_u| \leq 0,5 \tag{2}$$

$$|S_n - S_t| \leq 0,5 \tag{3}$$

where

S_n is the average of the common logarithm of the infectivity titer of virus, in PFU/ml, from three of the SCDLP broth for negative control;

S_u is the average of the common logarithm of the infectivity titer of virus, in PFU/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 PFU/ml, recovered from three of the treated test specimens.

If the above value is >0,5, the neutralizer should be carefully modified, changed or the amount of neutralizer should be carefully increased.

If the neutralizer is modified, changed or the amount of neutralizer is increased, the same condition about neutralizer shall be applied at 7.4.

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7 Test procedure

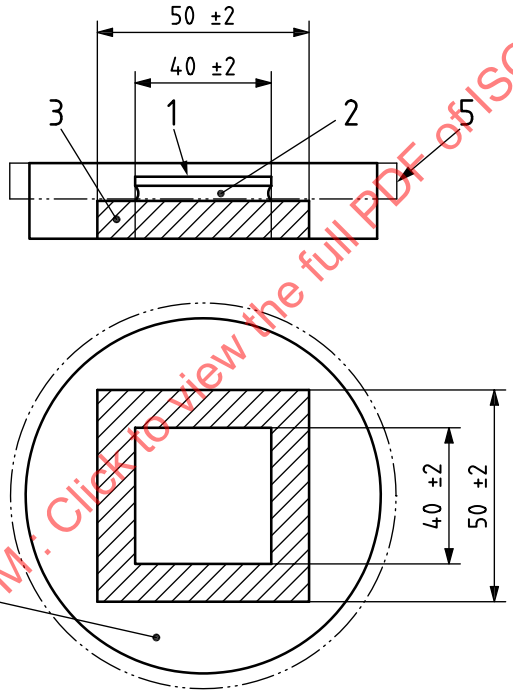
7.1 Preparation of test specimen

All specimens are prepared in the sterile Petri dish in 6.5. Put the treated side of the product on top when placed in the sterile Petri dish. Do not test crosssections of the product.

7.2 Inoculation of test specimens

Pipette 0,4 ml of the test inoculum prepared in 6.4 onto the test surface. Cover the test inoculum with a piece of film (5.20) that measures 40 mm × 40 mm and gently press down on the film so that the test inoculum spreads to the edges. Make sure that the test inoculum does not leak beyond the edges of the film. After the specimen has been inoculated and the cover film applied, close with the lid of the Petri dish (see Figure 1).

Dimensions in millimetres



Key

1 cover film
2 test inoculum (0,4 ml)
3 test specimen
4 Petri dish
5 lid of Petri dish

Figure 1 — Inoculation of test specimen and placement of cover film

Unless otherwise specified, the standard size of the cover film shall be a square of (40 ± 2) mm × (40 ± 2) mm for the 50 mm × 50 mm test specimen. If the test specimen is not a standard size, then the size of the film shall be modified in direct proportion. Do not, however, modify the size of the film to less than 400 mm^2, and the gap of each edge between the cover film and the test specimen shall always be 2,5 mm to 5,0 mm. If the size of the cover film differs from 40 mm × 40 mm, the actual used size shall be included in the test report. The volume and concentration of the inoculum used shall also be adjusted to the cover film in direct proportion. Include the modification in the test report.

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It is essential that the test inoculum does not leak beyond the edges of the cover 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 shall be included in the test report.

— Option 1: Reduce the volume of the test inoculum applied to the test surface. However, do not use less than 0,1 ml of the test inoculum. When the volume of the test inoculum is reduced, the concentration of the infectivity titer of virus in the inoculum shall be increased so as to provide the same number of the infectivity titer of virus 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.

7.3 Contact of the inoculated test specimens

Unless otherwise specified, keep each of the Petri dish with the inoculated test specimens (including the untreated test specimens) at (25 ± 1) °C and a relative humidity of not less than 90 % for 24 h.

Other contact time up to 24 h may be applied if agreed upon by the relevant parties. In this case, the modified contact time shall be included in the test report.

7.4 Recovery of virus from test specimens

7.4.1 Test specimens immediately after inoculation

Immediately after inoculation, process the three untreated test specimens by adding 10 ml of either the SCDLP broth (4.3.16) or a suitable, validated neutralizer to the Petri dish. The number of the infectivity titer of virus recovered from the untreated test specimens will be used to determine the recovery rate of the virus. It is important to ensure that the neutralizer completely washes the specimens with pipetting the SCDLP broth at least four times.

If there are any changes on volume or component to be applied to the neutralizer, it shall be included in the test report. The volume change shall be taken account of the calculation described in 8.1.

Special considerations may be required to achieve a sufficient recovery, in case of option 2 in 7.2 and the viscosity of the inoculum is increased. In this case, mechanical agitations may be required, such as stomaching, vortexing or sonicating. If one of these shows a recovery rate equivalent to or superior to that of obtained when tested without adding viscosity, such methods may be used. If one of the alternative recovery methods is used, it shall be included in the test report. Use of alternative recovery methods may affect the antiviral activity and shall therefore be fully validated.

7.4.2 Test specimens after contact

After the contact as described in 7.3, process three untreated test specimens and three treated test specimens with the same procedure described in 7.4.1. Proceed immediately to measure the infectivity titer of virus recovered from the test specimen (see 7.5).

7.5 Determining the infectivity titer of virus by plaque assay

Enumerate plaques by performing 10-fold serial dilutions of the suspension recovered in 7.4.1 and 7.4.2 in the maintenance medium (4.3.9). Remove the growth medium from 6 wells plastic plates with the host cell monolayer prepared in 6.3, wash the monolayer once with 3 ml of maintenance medium. After removing extra maintenance medium, inoculate 0,1 ml of the suspension recovered in 7.4.1. and 7.4.2 and each dilution into the 2 wells of the plastic plates. Put the plates at 34 °C for influenza virus or at 37 °C for feline calicivirus in the CO_2 incubator for 1 h to allow the virus to adsorb to the cells, in the meantime, tilt the plates every 15min so the cell monolayer keep moisture. Then, wash the monolayer once with 3 ml of the maintenance medium. After removing the extra maintenance medium, pour 3 ml of the agar medium into the wells for plaque assay (4.3.15). Close a lid and keep at room temperature for about 10 min. After confirming the agar coagulates, incubate the plate at 34 °C for influenza virus or at 37 °C for feline calicivirus in the CO2 incubator for 2 days to 3 days.

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After incubation, add 3 ml of the formalin solution (4.3.5) to the wells and keep it at room temperature for more than 1 h to fix the cells. Remove the formalin solution and the agar medium from the wells, add 3 ml of the methylene blue solution (4.3.6), then keep it at room temperature for 15 min to dye the cells. Wash the extra methylene blue solution with tapped water.

Select the two wells containing 6 to 60 plaques in the same dilution series. For each dilution series, record the number of plaques, together with the dilution factor. If the number of plaques in the wells containing the 0,1 ml of the recovered suspension is less than 6, then count and record the number of the plaques in these wells. If there is no plaque recovered in the wells containing the recovered suspension, record the number of plaques as "<1".

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

8 Expression of results

8.1 Determination of the infectivity titer of virus

For each test specimen, determine the infectivity titer of virus recovered in accordance with Formula (4):

$$N = (10 \times C \times D \times V) / A \quad (4)$$

where

N is the infectivity titer of virus recovered per cm^2 of test specimen;

C is the average number of plaque counted for the duplicate wells;

D is the dilution factor for the wells counted;

V is the volume of the SCDLP added to the specimen, in ml;

A is the surface area of the cover film, in cm^2.

Calculate the arithmetic mean of the number of plaque recovered in each set of the test specimens and express this value in two significant figures. If no plaque is recovered in any of the wells, then record as "<10 V" (where V is the volume, in ml, of the SCDLP added to the specimen). For calculating the average when there is no plaque recovered, consider the number of plaque to be "10 V".

EXAMPLE In the case of V = 10 ml, the number used for calculating the average will be 100.

8.2 Conditions for a valid test

8.2.1 When the four conditions given in 8.2.2, 8.2.3, 8.2.4 and 8.2.5, respectively, 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.

8.2.2 The logarithmic value of the number of plaques recovered immediately after inoculation from the untreated test specimens shall satisfy the requirement of Formula (5):

$$(L_{max} - L_{min}) / (L_{mean}) \leq 0,2 \quad (5)$$

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where

L_{max} is the common logarithm (i.e. base 10 logarithm) of the maximum number of plaques recovered from a specimen;

L_{min} is the common logarithm of the minimum number of plaques recovered from a specimen;

L_{mean} is the common logarithm of the mean number of plaques recovered from the three specimens.

8.2.3 The average number of plaques recovered immediately after inoculation from the untreated test specimens shall be within the range of $2{,}5 \times 10^5$ PFU/cm^2 to $1{,}2 \times 10^6$ PFU/cm^2.

8.2.4 The number of plaques recovered from each untreated test specimen after contacting for 24 h shall not be less than $6{,}2 \times 10^2$ PFU/cm^2.

8.2.5 The suppressive efficiency of the agent's activity described in 6.6 is to be confirmed.

8.3 Calculation of the antiviral activity

When the test is deemed as valid, calculate the antiviral activity using Formula (6) recording the result to one decimal place.

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \quad (6)$$

where

R is the antiviral activity;

U_0 is the average of the common logarithm of the number of plaques recovered from the three untreated test specimens immediately after inoculation, in PFU/cm^2;

U_t is the average of the common logarithm of the number of plaques recovered from the three untreated test specimens after 24 h, in PFU/cm^2;

A_t is the average of the common logarithm of the number of plaques recovered from the three treated test specimens after 24 h, in PFU/cm^2.

8.4 Effectiveness of the antiviral agent

The value of the antiviral activity can be used to characterize the effectiveness of an antiviral agent. The antiviral-activity values used to define the effectiveness shall be agreed upon by all interested parties.

9 Repeatability and reproducibility

Repeatability and reproducibility are discussed quantitatively in Annex B.

10 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 21702:2019;

b) the type of plastics used for the treated and untreated test specimens and the size, shape and thickness of the specimens;

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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 cell used and their strain numbers, indicating the reason if other species of virus or host cell were used;

e) the volume of test inoculum used;

f) the number of plaques in the test inoculum;

g) the values of U_0, U_t and A_t used in 8.3;

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.

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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 EMEM

The example of composition of the EMEM is described in Table A.1. The EMEM is available on the market.

Table A.1 — Composition of EMEM

Composition in 1 000 ml water		mg
Amino acids	L-Arginine HCl	126,40
	L-Cystine 2HCl	31,20
	L-Glutamine	292,00
	L-Histidine HCl · H_2O	41,90
	L-Isoleucine	52,50
	L-Leucine	52,50
	L-Lysine HCl	72,50
	L-Methionine	15,00
	L-Phenylalnine	32,50
	L-Threonine	47,60
	L-Tryptophan	10,00
	L-Tyrosine 2Na · $2H_2O$	51,90
	L-Valine	46,80
Vitamins	Choline Chloride	1,00
	D Calcium Pantothenate	1,00
	Folic Acid	1,00
	Myo Insitol	2,00
	Nicotinamide	1,00
	Pyridoxine HCl	1,00
	Riboflavin	0,10
	Thiamine HCl	1,00
Inorganic salts	Calcium Chloride [$CaCl_2$]	200,00
	Magnesium Sulfate [$MgSO_4$]	97,70
	Potassium Chloride [KCl]	400,00
	Sodium Chloride [NaCl]	6 800,00
	Sodium Phosphate Monobasic [NaH_4PO_4, H_2O] Monohydrate	140,00
Others	Dextrose	1 000,00
	Phenol Red Sodium Salt	10,00

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