INTERNATIONAL STANDARD

ISO 18184

Second edition
2019-06

Textiles — Determination of antiviral activity of textile products

Textiles — Détermination de l'activité virucide de produits textiles

Reference number
ISO 18184:2019(E)

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Published in Switzerland

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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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 38, *Textile.*

This second edition cancels and replaces the first edition (ISO 18184:2014), which has been technically revised. The main changes compared to the previous edition are as follows:

— Clause 1 has been updated;

— in Clause 5, viruses and host cells used in this document has been changed to examples of species;

— in 10.6, "Verification of cytotoxic effect" has been removed;

— 11.1, "Preparation of specimen" has been updated;

— 14.3.2, "Calculation of antiviral activity value" has been updated;

— Annex E (Additional virus: Polio virus) has been removed.

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.

Introduction

Recently, along with the global improvement in the level of living, consumers are showing the trend to seek healthcare or health protective products. Also, an increase in the people's interest for protection against epidemic diseases has been noted, as the overcrowded commuting train car where the commuters experience every day, the hospitals, nursing homes, etc.

Being supported by the processing technology of textile products to provide a high performance which has been highly developed recently, the health protective and hygiene relating products have been advancing into the market.

Because those products are relatively new and included the technical aspects out of textile technology, the testing methods have been developed by the individual producers to evaluate the product performance. That has resulted in inexistence of a unified test method, hindering for both consumers and producers a true explanation or understanding of those high functional products.

The antiviral product is one of those products and includes the technical fields of the textile technology and the biotechnology.

The demand to establish an international standard has been growing in the consumers, retailers, producers, etc. as the stakeholders in the market.

Antiviral textile products are textiles capable of reducing the number of infective virus particles that contact the surface of the textile. This document provides a quantitative test method to assess the antiviral performance of such products.

The data obtained in an objective manner by this document give the common knowledge to all the stake holders such as consumers, producers, retailers, etc. to understand the correct performance of the antiviral textile products.

There are two methods to quantify the number of infective virus, as infective virus titre in this document, which are the plaque method and the $TCID_{50}$ method. The method used can be selected by the experience and the convenience of each testing house. Any appropriate cellular system can be used and that the testing conditions when used should be reported.

See Annexes G and H for interlaboratory test results.

Textiles — Determination of antiviral activity of textile products

1 Scope

This document specifies testing methods for the determination of the antiviral activity of the textile products against specified viruses. Due to the individual sensitivities, the results of one test virus cannot be transposed to other viruses.

The textile products include woven and knitted fabrics, fibres, yarns, braids, etc.

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. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 105-F02, *Textiles — Tests for colour fastness — Part F02: Specification for cotton and viscose adjacent fabrics*

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
virus
original biological entity which has a single type of nucleic acid (DNA or RNA), specific structure that opposes the virus to living organisms with a cellular structure (prokaryotes and eukaryotes), and reproduces from its genetic material by replication within the host cell, and leads to absolute intracellular parasitism

Note 1 to entry: The virion is the infectious viral particle.

3.2
virus activity
ability to replicate in the susceptible and permissive host cells

3.3
antiviral activity
property of any substance (chemical or otherwise) producing a modification of one of the elements of the virion structure which induces the latter's inability to replicate

Note 1 to entry: Property that reduces the viral activity, generally through morphological change or structural damage to the surface protein of the virus.

Note 2 to entry: It is not necessarily to imply that the change of antigenic response or the change of constituent element is the reduction of virus infectivity.

3.4
antiviral chemicals
inorganic or organic chemicals able to reduce *virus activity* (3.2)

Note 1 to entry: The organic antiviral chemicals give the change to the surface protein of virus by the chemical adsorption. The inorganic metallic antiviral substances destroy or change the morphology of the virus by the extraction of hydrogen atom in the virus protein by OH radicals which are generated by the radical reaction.

3.5
control fabric
fabric used to verify the stability of the test virus on a textile fabric

Note 1 to entry: The fabrics before the antivirus treatment should be used as a control fabric with the same condition described in 3.5.

Note 2 to entry: In the absence of a control fabric as described in Note 1, the 100 % cotton cloth described in ISO 105-F02 should be used without any chemical treatments such as the fluorescent bleach, etc.

3.6
control test
test to confirm that a specimen does not affect the host cell

Note 1 to entry: This test is performed as same as actual test, but without virus.

Note 2 to entry: Also referred to as control test of specimen,

3.7
cytopathic effect
cytopathic effect (CPE) caused by virus
effect appears as morphological change or destruction of the host cells as a result of the virus multiplication

3.8
infectivity titre of virus
number of infectious viral particles present per unit volume in a cell lysate or in viral suspension

3.9
plaque
area of lysed cells in a monolayer cell culture

3.10
plaque forming units
PFU
unit expressed as the concentration of the infectious virus per unit volume

3.11
plaque assay
assay to determine the infectivity *titre of virus* (3.8) from PFU by using the series of dilution

3.12
$TCID_{50}$
50 % infectious dose of a wash-out virus suspension or the dilution of the virus suspension that induces a CPE in 50 % of cell culture units

Note 1 to entry: See 3.7.

3.13
$TCID_{50}$ method
assay to determine the *infectivity titre of virus* (3.8) from TCID50 by using the series of dilution

3.14
cytotoxicity
morphological alteration of cells and/or their destruction or the reduction of their sensitivity to the multiplication of viruses induced by a product

4 Principle

The viruses are deposited onto a specimen. After specific contact time, the remaining infectious virus is counted, and the reduction rate is calculated by the comparison between the antiviral product test specimen and the control specimen by common logarithm. There are two methods to quantify the infectious virus titre. One method is the plaque assay and the other is the $TCID_{50}$ method. The selection of the method depends on the convenience and experience of the testing organization.

5 Virus and host cell

Examples of species of viruses and host cells are shown in Annex A.

Other species of viruses and host cells can be used after appropriate validations, as the important virus may differ depending on 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.

NOTE Reference viruses are listed in EN 14476 and EN 14675.

6 Warning

This document calls for use of the infectious viruses or substances/procedures that may be injurious to the health/environment if appropriate conditions are not observed. It refers only to technical suitability and does not absolve the user from legal obligations relating to health and safety/environment at any stage.

The warning is extended as the following. The virus in the standard shall be the one of biotechnology safety level class II classified by the directives of WHO as stated. The user of this document shall have enough knowledge and experience of the microbiology. Moreover, users shall comply strictly to the safety standard of the manufacturers and the domestic regulation.

7 Apparatus

7.1 High pressure steam sterilizer: Autoclave, capable of operating at a temperature of (121 ± 2) °C.

NOTE The autoclave is described in EN 12353.

7.2 Dry heat sterilizer: Ovens, capable of operating at a temperature of (180 ± 2) °C and (160 ± 2) °C.

NOTE The hot air ovens are described in EN 12353.

7.3 Measuring flask, with capacity of 1 l.

7.4 Scale, with the available range of 0,01 g to 100 g with accuracy of 1,0 %.

7.5 Pipette, of various capacities with accuracy of 10 % of the nominal volume.

7.6 Washing machine.

7.7 Pipetter, capable of mounting the glass or plastic pipettes.

7.8 Micropipette, having the most suitable volume for each use, with a tip made of glass or plastic, and with a tolerance of 0,5 % or less.

7.9 Water bath, capable of maintaining at a temperature of (37 ± 1) °C, (50 ± 1) °C and (56 ± 1) °C.

7.10 Vortex®[1]-type mixer, used for microbial testing.

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

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

7.13 Membrane filtration device, with a pore size of 0,22 µm.

7.14 Refrigerator, capable of operating at a temperature between 2 °C and 8 °C.

7.15 pH meter, having an inaccuracy of calibration ±0,1 pH units at (20 ± 1) °C.

NOTE The pH meters are described in EN 12353.

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

7.17 Tweezers, capable of being sterilized.

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

7.19 Biological safety cabinet, class II.

7.20 Vial container, with a capacity of 30 ml and closed with the screw cap. The gasket is made of perfluoroethylene or silicone and the cap is made of polypropylene.

7.21 96 wells microplate with the gamma radiation sterilization, for $TCID_{50}$ method.

96 wells microplates with other sterilization finish may be used after appropriate validation for the growth of cells. See Figure 1.

1) This information is given for the convenience of users of this document and does not constitute an endorsement of ISO by this product.

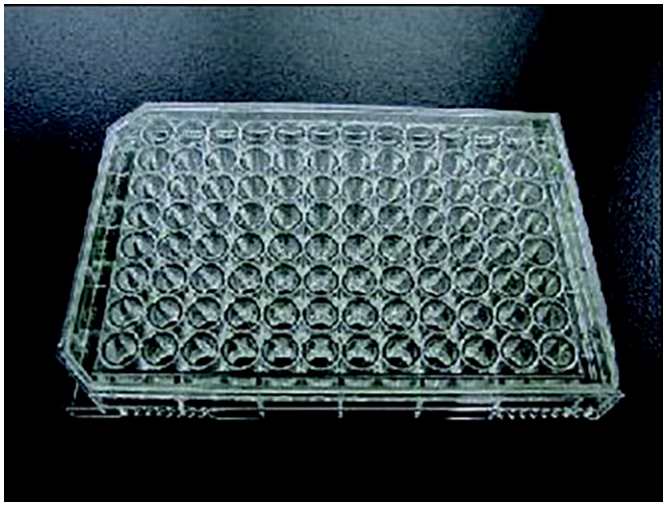


Figure 1 — 96 wells microplate for $TCID_{50}$ method

7.22 6 wells plastic plate with the gamma radiation sterilization, for plaque assay.

6 wells plates with other sterilization finish may be used after appropriate validation for the growth of cells. See Figure 2.

Figure 2 — 6 wells plastic plate for plaque assay

7.23 Flask, for cell culture use with the gamma radiation sterilization finish, with an adherent type, a cell culture area of 75 cm^2 and with the vent cap. The vent cap can exchange abacterial air through 0,2 µm filter. See Figure 3.

Flask with other sterilization finish may be used after appropriate validation for the growth of cells.

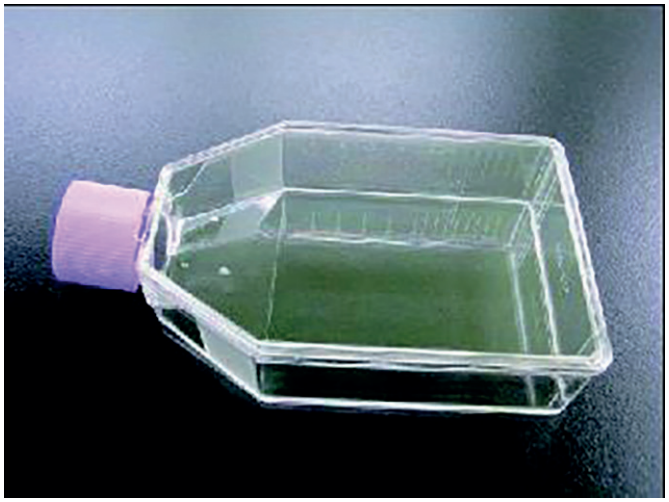


Figure 3 — Flask for cell culture use

7.24 CO_2 incubator, capable of maintaining an atmosphere with 5 % CO_2, at a temperature of (34 ± 1) °C and (37 ± 1) °C.

NOTE Certain CO_2 incubators are described in EN 12353.

7.25 Incubator, capable of maintaining at a temperature of (25 ± 1) °C, (35 ± 1) °C and (37 ± 1) °C.

NOTE Certain incubators are described in EN 12353.

7.26 Centrifuge tube.

7.27 Culture container.

7.28 Test tube.

7.29 Beaker.

8 Sterilization of apparatus

Sterilize all apparatus which come in contact with the cells, the chemicals, or test specimen. The sterilization method shall be used by high pressure steam or dry heat method.

— High pressure steam sterilization: by an autoclave (7.1) at a temperature of 121 °C and a pressure of 103 kPa for 15 min.

— Dry-heat sterilization: by a dry heat sterilizer (7.2) at a temperature of 180 °C for 30 min or 160 °C for 2 h.

In case of plastics products, heat-resistant plastics products or sterilization finish plastics products may be used.

9 Reagent and medium

All reagents shall have the quality suitable for virological needs, i.e. free of toxic substances for testing microorganisms. Some of the media are available in the market.

9.1 Water, which shall be analytical-grade water for microbiological media preparation, which is ion-exchanged and/or freshly distilled and/or ultra-filtered and/or filtered with RO (reverse osmosis).

9.2 Eagle's minimum essential medium (EMEM), Roswell Park Memorial Institute medium (RPMI), available in the market. The composition is described in Annex D. If there are any components missing from the composition, add them according to the composition table.

9.3 7,5 % sodium bicarbonate solution.

9.3.1 Sterilize sodium bicarbonate, 75 g in autoclave in a culture container with a cap closed tightly.

9.3.2 Water is also sterilized by autoclave.

9.3.3 Dissolve sodium bicarbonate in the sterilized water of 1 000 ml well.

Alternative preparation (replacing 9.3.1, 9.3.2, and 9.3.3) as follows. Prepare 7,5 % sodium bicarbonate solution by dissolving 75 g of sodium bicarbonate in 1 000 ml of water. Sterilize the solution by using 0,22 µm membrane filter.

9.4 Formaldehyde solution.

Prepare a formaldehyde solution at the concentration of 3,7 % in water.

The other solution for cell fixation may be used after appropriate validation for the cell fixation.

9.5 Methylene blue solution.

9.5.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the flask:

— Water, 1 000 ml;

— Methylene blue, 0,375 g;

— 1 mol/l sodium hydroxide solutions 62,5 µl.

9.5.2 Dissolve and mix well.

9.6 Inactivated fetal bovine serum (FBS)

9.6.1 Put the freezed cryopreserved fetal bovine serum in a package in the water bath (7.9) at a temperature of 37 °C and keep it until defrosting.

9.6.2 Then, raise the temperature of the water bath to 56 °C and keep it for 30 min to inactivate.

9.6.3 Divide it into several tubes. Put them in the freezer (7.11) at a temperature lower than –20 °C until usage for testing.

9.6.4 Just before use, put it in the water bath at a temperature of 37 °C and keep it until defrosting.

9.7 Growth medium.

9.7.1 Prepare a measuring flask (7.3) of 1 l, and put the following materials into the flask:

— Water, 800 ml;

— Kanamycin sulfate, 60 mg;

— Eagle's minimum essential medium (EMEM) (D.2), 9,53 g, or RPMI 1640 medium (D.3), 10,4 g.

9.7.2 Dissolve and mix well and make up whole solution to 1 000 ml by water.

9.7.3 Sterilize the mixed solution from 9.7.2 by using 0,22 µm filter (7.13).

9.7.4 Add 15 ml of 7,5 % sodium bicarbonate solution (9.3) and 100 ml of the inactivated fetal bovine serum (9.6) in the solution from 9.7.3.

9.8 Maintenance medium.

9.8.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the flask:

— Water, 800 ml;

— Kanamycin sulfate, 60 mg;

— Eagle's minimum essential medium, 9,53 g.

9.8.2 Dissolve them well, then, make up the whole amount to 1 000 ml by adding water.

9.8.3 Sterilize the mixed solution from 9.8.2 by using the filter (7.13) with a pore size of 0,22 µm.

9.8.4 Add 15 ml of 7,5 % sodium bicarbonate solution (9.3) in the solution from 9.8.3.

9.9 Double concentration of the maintenance medium (9.8).

9.9.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the flask:

— Water, 800 ml;

— Kanamycin sulfate, 120 mg;

— Eagle's minimum essential medium, 19,06 g.

9.9.2 Dissolve them well, then, make up the whole amount to 1 000 ml by adding water.

9.9.3 Sterilize the mixed solution from 9.9.2 by using the filter (7.13) with a pore size of 0,22 µm.

9.10 0,01 mol/l phosphate buffered saline PBS (-).

9.10.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the flask:

— Sodium chloride, 8 g;

— Potassium chloride, 0,2 g;

— Disodium hydrogen phosphate 12 H_2O, 2,9 g;

— Potassium dihydrogen phosphate, 0,2 g.

9.10.2 Add water by making up whole amount to 1 000 ml and dissolve well.

9.10.3 Then, transfer the solution (9.10.2) to a culture container, then sterilize the solution from 9.10.2 by using an autoclave (7.1) at a temperature of 121 °C for 15 min.

9.11 Trypsin derived from beef pancreas and PBS (-) solution.

9.11.1 Prepare a beaker, then, put the following materials in the beaker:

— 0,01 mol/l phosphate buffered saline PBS (-) (9.10), 100 ml;

— Trypsin derived from beef pancreas, 1,0 g.

9.11.2 Dissolve and mix well by using mixer for 2 h.

9.11.3 Then, sterilize the solution 9.11.2 by using the filter (7.13) with a pore size of 0,22 µm.

The divided solution tubes that are not used immediately are preserved in the freezer at a temperature of lower than −80 °C until usage for testing.

9.11.4 Prepare a test tube and put the following solutions in the test tube:

— 0,01 mol/l phosphate buffered saline PBS (-) (9.10), 9 ml;

— Trypsin derived from beef pancreas and PBS (-) mixed solution (9.11.3), 1 ml.

9.11.5 Dissolve and mix them well.

9.11.6 Divide the solution in test tubes and preserve in the freezer at a temperature of lower than −20 °C until usage for testing.

9.11.7 Just before using, put it in the water bath (7.9) at a temperature of 37 °C and keep it until defrosting.

9.12 Trypsin EDTA solution.

9.12.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the measuring flask:

— 0,01 mol/l phosphate buffered saline PBS (-) (9.10), 1 000 ml;

— Trypsin, 2,5 g;

— Kanamycin sulfate, 0,1 g;

— Streptomycin sulfate, 0,1 g;

— Amphotericin B, 2 mg;

— Ethylenediaminetetraacetic acid (EDTA), 0,014 mol.

9.12.2 Dissolve and mix well.

9.12.3 Then, sterilize the solution from 9.12.2 by using the filter (7.13) with a pore size of 0,22 µm.

9.12.4 Divide the solution in test tubes and preserve in the freezer at a temperature lower than −20 °C until usage for testing.

9.12.5 Before usage, place the test tube in a water bath (7.9) at 37 °C until fully defrosted.

Trypsin EDTA solution is available in the market. The products with the different components from 9.12.1, may be used after proper validation.

9.13 DEAE-dextran solution.

9.13.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the measuring flask:

— Water, 1 000 ml;

— Diethylaminoethyl (DEAE)-dextran, 20 g.

9.13.2 Dissolve and mix well.

9.13.3 Sterilize the solution from 9.13.2 by using the filter (7.13) with a pore size of 0,22 µm.

9.14 Agar medium preparation.

This is prepared with liquid A (9.14.1) and liquid B (9.14.2) and mixed just before using (9.14.3).

9.14.1 Liquid A.

9.14.1.1 Prepare a sterilized culture container (7.27) of 1 l, then, put the following materials in the culture container:

— Double concentration of maintenance medium (9.9), 1 000 ml;

— DEAE-dextran solution (9.13), 10 ml;

— 7,5 % sodium bicarbonate solution (9.3), 40 ml.

Mix well.

9.14.1.2 Only for the influenza virus test and for the plaque assay, add 3,0 ml of the Trypsin from beef pancreas and PBS (-) solution (9.11).

9.14.1.3 Put the solution from 9.14.1.1 or 9.14.1.2 in the water bath (7.9) at a temperature of 37 °C and keep it until using.

9.14.2 Liquid B.

9.14.2.1 Prepare a culture container (7.27) of 2 l, then, put the following materials in the culture container:

— Water, 1 000 ml;

— Cell culture agar, 15 g.

Mix well.

9.14.2.2 Sterilize mixed solution from 9.14.2.1 by using autoclave (7.1) at a temperature of 121 °C and a pressure of 103 kPa for 15 min.

9.14.2.3 Put the solution from 9.14.2.2 in the water bath (7.9) at a temperature of 50 °C and keep it until using.

9.14.3 Mix liquid A and liquid B at 1:1 (vol/vol).

9.15 SCDLP medium, used for washing-out and for suppression of agent activity of the treated textile product.

9.15.1 Prepare a culture container (7.27) of 1 l, then, put the following materials in the culture container:

— Water, 1 000 ml;

— Peptone made of casein, 17,0 g;

— Peptone made of soybean, 3,0 g;

— Sodium chloride, 5,0 g;

— Dipotassium hydrogen phosphate, 2,5 g;

— D-Glucose, 2,5 g;

— Lecithin, 1,0 g.

Mix together and dissolve well, then, add:

— Nonionic surfactant (Tween 80), 7,0 g.

Dissolve and mix well.

9.15.2 Adjust the solution from 9.15.1 to pH 7,0 ± 0,2 by the sodium hydroxide solution or the hydrochloric acid solution in the water bath (7.9) at a temperature of 25 °C.

9.15.3 Sterilize the mixed solution from 9.15.2 by using autoclave (7.1) at a temperature of 121 °C and a pressure of 103 kPa for 15 min.

9.16 Maintenance medium with Trypsin.

9.16.1 Prepare a sterilized culture container (7.27) of 1 l, then, put the following materials in the culture container:

— Maintenance medium (9.8), 1 000 ml;

— Trypsin from beef pancreas and PBS(-) solution (9.11), 3,0 ml.

Dissolve them well.

10 Preparation

10.1 Restoration of host cell from cryopreservation

10.1.1 Put the cryopreserved host cell in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

10.1.2 Prepare a new flask for cell culture (7.23) with a vent cap lid and add 20 ml of growth medium (9.7) in the flask.

10.1.3 Put whole ampule of defrosted host cell (see 10.1.1) in the flask.

10.1.4 Put the flask in the CO_2 incubator (7.24) at a temperature of 37 °C and keep it for (24 ± 2) h to culture the host cell.

10.1.5 Then, observe the flask (see 10.1.4) by microscope if the cells are attached on the bottom of the flask.

If the growth is confirmed, then, go to next step. If not, continue to keep in the incubator.

10.1.6 Drain the remained growth medium in the flask of 10.1.5.

10.1.7 Add 20 ml of the new growth medium (9.7) to the flask of 10.1.6.

10.1.8 Put the flask of 10.1.7 in the CO_2 incubator (7.24) at a temperature of 37 °C for (48 ± 2) h.

10.1.9 Observe the flask of 10.1.8 using a microscope and confirm if the cells are cultured as a confluent growth on the bottom of the flask. If the growth of cells is not enough, continue with the step specified in 10.1.8 until the sufficient growth is confirmed.

10.1.10 Then, proceed to the serial subcultivation by taking the following steps of 10.2.

10.2 Subculture of host cell

10.2.1 Drain an extra growth medium of the flask when 10.1.8 is completed.

10.2.2 Add 5 ml of 0,01 mol/l phosphate buffered saline PBS (-) (9.10) and wash the surface of the grown cells on the bottom of the flask by the solution, then drain the extra PBS (-).

Repeat 3 times of this washing procedure.

10.2.3 Add 0,5 ml of Trypsin EDTA solution (9.12) in the flask of 10.2.2 and spread the solution over the whole surface.

10.2.4 Put the flask of 10.2.3 in the CO_2 incubator (7.24) at a temperature of 37 °C for 10 min ± 1 min to keep warm.

10.2.5 Observe visually the flask of 10.2.4 if the grown cells are starting to come off, if confirmed, tap the side of the flask and disperse the cells.

10.2.6 Add 5 ml of the growth medium (9.7) in the flask of 10.2.5 and pipetting the medium to make it mild. Mix well to avoid the damage to the cells.

10.2.7 Prepare a new flask for cell culture (7.23) and add 20 ml of the growth medium (9.7).

10.2.8 Add 1 ml of the cell suspension of 10.2.6 by the pipette to 10.2.7.

The amount of the cell suspension of 10.2.6 may be changed as needed.

10.2.9 Close the cap of the flask of 10.2.8 and put the flask in the CO_2 incubator (7.24) at a temperature of 37 °C for 5 days to culture.

The culture period may be changed as needed.

10.2.10 To keep the cell repeat the steps from 10.2.1 to 10.2.9.

10.3 Cell culture for the infectious virus titre assay

The cell culture in 6 wells plate or 96 wells microplate is required for the test of the plaque assay or $TCID_{50}$ method.

10.3.1 Put 20 ml of growth medium (9.7) in the culture medium container (7.27) and add 1ml of the subcultured cell suspension of 10.2.6.

10.3.2 Put 3 ml of the cell suspension of 10.3.1 in each hole of the 6 wells plastic plate (7.22) for the plaque assay test. Or put 0,1 ml of the cell suspension of 10.3.1 in each well of the 96 wells microplate (7.21) for $TCID_{50}$ method.

10.3.3 Put the 6 wells plate or the microplate in the CO_2 incubator (7.24) at a temperature of 37 °C and keep it for several days to culture.

The culture period of 3 days to 5 days is usual and may be changed as needed.

10.3.4 Observe the condition of the cells by the inversed microscopy if the multiplied cells are confluent.

10.4 Preparation for test virus

10.4.1 General

The viruses are cryopreserved in the freezer, so the operation to defrost and to grow them for test is required.

10.4.2 Influenza virus

10.4.2.1 Put the cryopreserved base virus in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

10.4.2.2 Drain the growth medium from the flask of 10.2.9 with the cultured cells in the monolayer.

10.4.2.3 Add 5 ml of the maintenance medium (9.8) in the flask of 10.4.2.2. Wash the surface of the cultured cells and drain the maintenance medium. Repeat the washing procedure 2 times.

10.4.2.4 Prepare a new test tube.

10.4.2.5 Put the defrosted base influenza virus of 10.4.2.1 in the test tube of 10.4.2.4, dilute with the maintenance medium (9.8) and adjust the concentration of virus to 10^3 PFU to 10^4 PFU or $TCID_{50}$/ml.

10.4.2.6 Inoculate 1 ml of the adjusted base influenza virus of 10.4.2.5 on the surface of cell in the flask of 10.4.2.3 and spread to the whole surface.

10.4.2.7 Put the flask of 10.4.2.6 in the CO_2 incubator (7.24) at a temperature of 34 °C and keep it for 1 h to adsorb the virus to the cells.

10.4.2.8 Put 20 ml of the maintenance medium (9.8) in the flask (see 10.4.2.7) and add 30 µl of Trypsin derived from beef pancreas and PBS (-) solution (9.11).

10.4.2.9 Put the flask in the CO_2 incubator (7.24) at a temperature of 34 °C for 1 to 3 days to multiply the influenza virus.

10.4.2.10 Observe the cytopathic effect by a microscope and judge the multiplication of influenza virus. If the multiplication of influenza virus is confirmed, proceed to 10.4.2.11.

10.4.2.11 Put the multiplied virus suspension in the centrifugal tube.

10.4.2.12 Centrifuge the multiplied virus suspension of 10.4.2.11 by using a centrifuge at a temperature of 4 °C and 1 000 × g for 15 min.

10.4.2.13 Take the supernatant suspension from the centrifugal tube after the centrifugation. This is to be the influenza virus suspension. Divide the suspension into test tubes appropriately and cryopreserve at −80 °C in the freezer (7.11) or −196 °C in the Liquid nitrogen bath (7.12).

10.4.2.14 Check the concentration of the virus if it is more than 10^7 PFU or $TCID_{50}$/ml by plaque titre assay or $TCID_{50}$ method. If the concentration is less than 10^7 PFU or $TCID_{50}$/ml, prepare it from beginning.

10.4.2.15 Just before use, put the cryopreserved virus suspension of 10.4.2.14 in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

10.4.2.16 This is to be the virus suspension for the test. If not used immediately, preserve in the refrigerator at a temperature of 4 °C.

If needed, just before use, the virus suspension for the test of 10.4.2.16 could be proceeded with 10-fold dilution using water (9.1) as diluent. The concentration of the virus suspension for the test after 10-fold dilution should be adjusted to a titre of 1×10^7 PFU or $TCID_{50}$/ml to 5×10^7 PFU or $TCID_{50}$/ml.

10.4.3 Feline calicivirus

10.4.3.1 Put the cryopreserved base virus in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

10.4.3.2 Drain the growth medium from the flask of 10.2.9 with the cultured cells in the monolayer.

10.4.3.3 Add 5 ml of the maintenance medium (9.8) in the flask of 10.4.3.2. Wash the surface of the cultured cells and drain the maintenance medium. Repeat the washing procedure 2 times.

10.4.3.4 Prepare a new test tube.

10.4.3.5 Put the defrosted base viruses in the test tube of 10.4.3.4, dilute by the maintenance medium (9.8) and adjust the concentration of virus to 10^5 PFU to 10^6 PFU or $TCID_{50}$/ml.

The concentration of virus suspension may be changed as needed.

10.4.3.6 Inoculate 1 ml of the adjusted base viruses of 10.4.3.5 on the surface of the cultured cells in the flask of 10.4.3.3 and spread to the whole surface.

10.4.3.7 Put the flask of 10.4.3.6 in the CO_2 incubator (7.24) at a temperature of 37 °C and keep it for 1 h to absorb the virus into the cells.

10.4.3.8 Add 20 ml of the maintenance medium (9.8) in the flask of 10.4.3.7.

The amount of the maintenance medium may be changed as needed.

10.4.3.9 Put the flask of 10.4.3.8 in the CO_2 incubator (7.24) at a temperature of 37 °C and keep it for 1 to 3 days to multiply the viruses.

10.4.3.10 Observe the cytopathic effect by a microscope and judge the multiplication of the feline calicivirus. If the multiplication of the virus is confirmed.

10.4.3.11 Put the multiplied virus suspension in the centrifugal tube.

10.4.3.12 Centrifuge the multiplied virus suspension of 10.4.3.11 by using the centrifuge (7.18) at a temperature of 4 °C and 1 000 g for 15 min.

10.4.3.13 Take the supernatant fluid from the centrifugal tube after the centrifugation. This is to be the virus suspension. Divide the suspension into test tubes appropriately and cryopreserve at −80 °C in the freezer (7.11) or −196 °C in the liquid nitrogen bath (7.12).

10.4.3.14 Check the concentration of the virus if it is more than 10^7 PFU or $TCID_{50}$/ml for Feline calicivirus by the plaque assay or $TCID_{50}$ method.

If the concentration is less than 10^7 PFU or $TCID_{50}$/ml, prepare it from beginning.

10.4.3.15 Just before use, put the cryopreserved virus suspension of 10.4.3.14 in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting. This is to be a virus suspension for the test. If not used immediately, preserve in the refrigerator at a temperature of 4 °C.

If needed, just before use, the virus suspension for the test of 10.4.3.15 could be proceeded with 10-fold dilution using water (9.1) as diluent. The concentration of the virus suspension for the test after 10-fold dilution should be adjusted to a titre of 1×10^7 PFU or $TCID_{50}$/ml to 5×10^7 PFU or $TCID_{50}$/ml.

10.4.4 Infectivity titre of the test viruses

The infectivity titre of the test virus shall be determined by the following procedure.

10.4.4.1 Preparation for series of the dilution for the virus suspension

10.4.4.1.1 Put 1,8 ml of the maintenance medium (9.8) in a new test tubes, keep it in the water bath with ice.

10.4.4.1.2 Add 0,2 ml of the virus suspension for the test of 10.4.2.16 and 10.4.3.15 in the test tubes of 10.4.4.1.1, and shake the test tubes well by Vortex mixture (7.8).

NOTE The dilution of 1/10 (10^{-1}) is prepared.

10.4.4.1.3 Put 1,8 ml of the maintenance medium (9.8) in new test tubes, keep in the water bath with ice.

10.4.4.1.4 Add 0,2 ml of the suspension of 10.4.4.1.2 to the test tubes of 10.4.4.1.3 and shake them well.

NOTE The dilution of 1/100 (10^{-2}) is prepared.

10.4.4.1.5 Repeat this procedure to prepare the series of the dilution for the virus suspension.

NOTE 1 In case of $TCID_{50}$ method, the series of the dilution for the virus suspension is required to prepare for the observation from the infectious wells for all of 8 wells to 0 wells.

NOTE 2 In case of the infective virus titre of 10^7 $TCID_{50}$/ml for the test, the series of the dilution for the virus suspension can be required to prepare by 10^{-7}.

10.4.4.2 Determination of the infectious titre

10.4.4.2.1 Plaque assay

Determine the infectivity titre according to Annex B.

10.4.4.2.2 $TCID_{50}$ method

Determine $TCID_{50}$ according to Annex C.

10.5 Preparation for test specimen

10.5.1 Control fabric

The untreated test sample fabric or the cotton 100 % woven fabric specified in ISO 105-F02 should be used. Before testing, wash in the washing machine the 100 % cotton woven fabric using tap water for 10 cycles (the washing cycle consist of washing for 10 min at 60 °C without detergent, fluorescent bleaching agent or any other chemicals and 2 time-rinsing for 5 min).

10.5.2 Preparation of test specimens

10.5.2.1 Obtain test specimens with mass of 0,40 g ± 0,05 g and cut pieces with approximately 20 mm by 20 mm a piece and make up the mass with the several pieces. In case of yarns, prepare the yarns in bundle and then cut approximately 20 mm with the same mass of 0,40 g ± 0,05 g.

10.5.2.2 Obtain 9 specimens for the control fabric and 6 specimens for the antiviral test sample.

NOTE Three (3) control fabric specimens and three (3) antiviral test specimens are used for the control test of the effect of test specimen without virus. Three (3) control fabric specimens are used for the infectivity titre measurement immediately after inoculation of virus. The remaining 3 control specimens and 3 antiviral specimens are used for the main test of this document.

10.5.3 Sterilization of specimens

10.5.3.1 Put specimens in the vial containers one by one and put all vial containers in a metal wire basket and cover them by aluminium foil. Put caps of vial containers in the basket with wrapping of aluminium foil separately from containers.

10.5.3.2 Put the basket of 10.5.3.1 in the autoclave (7.1) at 121 °C and 103 kPa to sterilize for 15 min.

10.5.3.3 After sterilization, remove the foil and take out all vial containers with the specimens and put them in a safety cabinet (7.16) and keep for 60 min for cooling down, then after checking of no dew condensation in the vial containers, put the caps on all vial containers and close them.

If the high-pressure steam sterilization is not recommended because of the property of the antiviral agents or the characteristic of textile products, the other appropriate sterilization method may be chosen.

10.6 Control test

10.6.1 General

The purpose of the control test is to confirm the efficiency for suppression of agent activity of test specimen. The efficiency for suppression of agent activity of test specimen means no cytotoxicity and no reduction of cell sensitivity to virus and inactivation of antiviral activity.

10.6.2 Verification of cytotoxicity by cell sensitivity to virus and the inactivation of antiviral activity

10.6.2.1 Put 3 control specimens and 3 antiviral test specimens sterilized in 10.5.3 in the vial containers, and add 20 ml of the washing out solution, SCDLP medium (9.15) in all containers. Then, put the caps on the containers and agitate them by Vortex mixer (7.10) for 5 s and 5 times.

10.6.2.2 Take 5 ml of washing out solution from a vial container to a new tube for all vial containers.

10.6.2.3 Add 50 µl of virus suspension prepared to be a concentration of (4 to 6) × 10^4 PFU/ml or $TCID_{50}$/ml into the tubes.

10.6.2.4 Keep them at 25 °C in the incubator (7.25) for 30 min.

10.6.2.5 Determine infective titre according to Annex B or Annex C.

In case of $TCID_{50}$ method, the washout solution of 10.6.2.1 shall be used for preparation of series of the dilution.

10.6.2.6 A condition for verification for this test:

lg (PFU/ml or $TCID_{50}$/ml of reference specimen) – lg (PFU/ml or $TCID_{50}$/ml of antiviral specimen) ≤ 0,5

If the above value is over 0,5, wash-out solution should be carefully modified or changed or the amount of wash-out solution should be carefully increased.

If wash-out solution is modified, changed or the amount of wash-out solution is increased, the same condition on wash-out solution shall be applied at 11.4 and 11.5.

11 Test procedure

11.1 Preparation of specimen

All specimens are prepared in the vial containers with caps in 10.5.

The preparation of specimens in sterile Petri dishes is permitted provided that the moisture is ensured (by placing a cover on each Petri dish) when the Petri dishes are placed in the incubator under the testing conditions as described in 11.2 and 11.3. Then, aseptically transfer the specimens in sterile vials before proceeding to 11.4 and 11.5.

11.2 Deposit of virus to the specimens

Deposit exactly 0,2 ml of the virus suspension prepared in 10.4 onto the specimen at several points of the specimen in the vial containers by micropipette for all. Then put the caps on all vial containers and close them.

11.3 Contacting time

Put the vials of 11.2 in the incubator (7.25) and keep for 2 h as a standard time at a temperature of 25 °C.

The contacting time could be varied and may be determined by the concerned party, but not longer than 24 h.

11.4 Wash-out of virus immediately after deposit

Immediately after deposit of virus on 3 control specimens in 11.2, add 20 ml of SCDLP (9.15) medium in the vial containers. Then, put the caps on the containers, close them and agitate them by Vortex mixer (7.8) for 5 s and 5 times for washing out the virus from the specimens.

NOTE This virus suspension is to be base wash-out virus suspension of the reference specimen.

11.5 Wash-out of virus after contacting time

After contacting for 2 h in 11.3, add 20 ml of SCDLP medium in the vial containers, then put the caps on the containers, close them and agitate them by Vortex mixer (7.8) for 5 s and 5 times to wash out the virus from the specimens.

NOTE These virus suspensions are base wash-out virus suspensions of the antiviral specimens and the reference specimens after contacting. If the virus concentration of inoculation is 10^7 (PFU/ml or $TCID_{50}$/ml), the concentration of the virus becomes 1×10^5 (PFU/ml or $TCID_{50}$/ml) (because wash-out virus suspension contains 0,2 ml virus suspension and 20 ml of the SCDLP medium, then $0{,}2/20{,}2 \approx 0{,}01 = 1 \times 10^{-2}$ and $10^7 \times 1 \times 10^{-2} = 1 \times 10^5$).

12 Preparation of the series of the dilution for the virus suspension

12.1 Put 1,8 ml of the maintenance medium (9.8) in new test tubes, then put and keep it in the ice bath or refrigerator at temperature of 4 °C.

12.2 Add 0,2 ml of the wash-out virus suspension of 11.4 and 11.5 in the test tubes of 12.1, and shake the test tubes well by Vortex-type mixer (7.10).

NOTE The dilution of 1/10 (10^{-1}) is prepared. The concentration of the virus suspension for this dilution is $1 \times 10^5 \times 10^{-1} = 1 \times 10^4$ PFU/ml or $TCID_{50}$/ml.

12.3 Put 1,8 ml of the maintenance medium (9.8) in new test tubes, then put and keep it in the water bath with ice.

12.4 Add 0,2 ml of the virus suspension of 12.2 to the test tubes of 12.3 and shake them well.

NOTE The dilution of 1/100 (10^{-2}) is prepared. The concentration of the virus suspension for this dilution is $1 \times 10^4 \times 10^{-1} = 1 \times 10^3$ PFU/ml or $TCID_{50}$/ml.

12.5 Repeat this procedure to prepare the series of the dilution for the virus suspension.

In case of $TCID_{50}$ method, the series of the dilution for the virus suspension is required to prepare for observation from the dilution point in which all of 8 wells are infected to the dilution point in which all of 8 wells are not infected. In case that all of 8 wells are not infected at base wash-out virus suspension, the infectivity titre should be calculated assuming that all of 8 wells are infected in the previous dilution series, and give the result an inequality sign.

NOTE 1 In case of the infective virus titre of 10^7 $TCID_{50}$/ml, the virus suspension of 0,2 ml is diluted by the wash-out virus suspension of 20 ml. Then the base wash-out virus suspension becomes 1×10^5 $TCID_{50}$/ml.

13 Determination of the infectious titre

13.1 Plaque assay

Determine the infectivity titre by plaque assay according to Annex B.

13.2 $TCID_{50}$ method

Determine $TCID_{50}$ according to Annex C.

14 Calculation of infectivity titre

14.1 Plaque assay

See Formula (1):

$$P = Z \times R \tag{1}$$

where

P is the infective titre (PFU/0,1 ml);

Z is the arithmetic average of plaques of 2 wells (number of plaques per 0,1 ml);

R is the dilution factor.

See Formula (2):

$$W = P \times 10 \tag{2}$$

where W is the infectivity titre (PFU/ml).

Then, the infectivity titre of the virus is calculated using Formula (3):

$$V_p = W \times C \tag{3}$$

where

V_p is the infectivity titre (PFU/vial);

C is the wash-out virus suspension amount (ml).

14.2 $TCID_{50}$ method

Other determination methods of $TCID_{50}$ are permitted. The use of another determination method shall be reported.

NOTE Methods, such as Spearman-Kärber method, are described in EN 14476 or in EN 14675.

14.2.1 Behrens and Karber method

In case of a logarithm dilution series as shown in Formula (4).

$$Y = X \times 10^{a}$$

$$a = \sum p - 0{,}5 \tag{4}$$

where

Y is the infective titre ($TCID_{50}$/0,1 ml);

X is the dilution rate of the base virus suspension;

P is the ratio of the cytopathic effect at the respective dilution of the virus suspension.

$\sum p$ is the sum of values of p.

Then, infectivity titre: A ($TCID_{50}$/ml) is as shown in Formula (5):

$$A = Y \times 10 \tag{5}$$

And, the infectivity titre ($TCID_{50}$/vial) is calculated using Formula (6):

$$V = A \times C \tag{6}$$

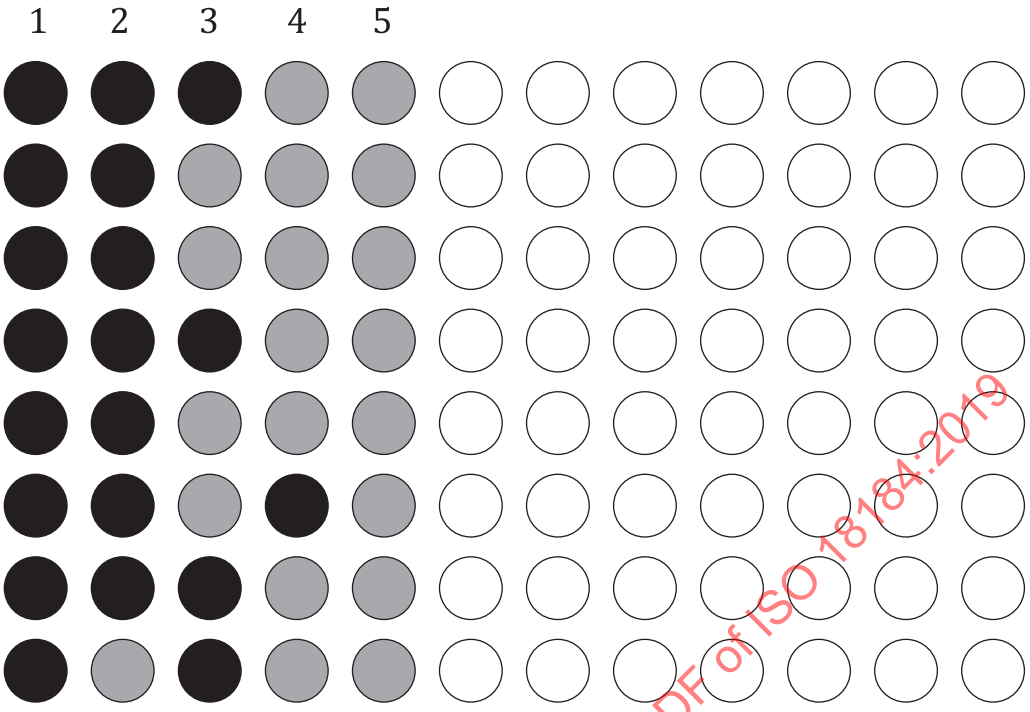
where

V is the infective titre ($TCID_{50}$/vial);

C is the amount of wash-out virus suspension (ml);

14.2.2 Example of calculation

See Figure 4.



Key

1	inoculation column of the base virus suspension
2	inoculation column of 1/10 dilution of the base virus suspension
3	inoculation column of 1/10 dilution of the virus suspension 2
4	inoculation column of 1/10 dilution of the virus suspension 3
5	inoculation column of 1/10 dilution of the virus suspension 4
black	infected
grey	not infected
white	not inoculated

Figure 4 — Example of $TCID_{50}$ method

$X = 10^0 = 1,$

$\sum p = 8/8 + 7/8 + 4/8 + 1/8 + 0/8 = 2,5$

Then, $a = 2,5 - 0,5 = 2,0$

$Y = 10^{2,0} = 1,0 \times 10^2$

$A = Y \times 10 = 1,0 \times 10^3$

$V = A \times C = A \times 20 = 1,0 \times 10^3 \times 20 = 2,0 \times 10^4$

14.3 Test result

14.3.1 Verification of this test

a) The virus infective titre of inoculated concentration for the test,

— Influenza virus suspension > 10^7 PFU or $TCID_{50}$/ml

— Feline calicivirus suspension > 10^7 PFU or $TCID_{50}$/ml

b) To be confirmed the efficiency for suppression of agent activity of test specimen in 10.6.

c) Logarithm reduction value of infective titre of control specimen ≤ 1,0.

In case 24 h is applied for the contact time in 11.3, the logarithm reduction value of infective titre of control specimen shall be less than 2,0. See Formula (7):

$$M = \lg(Va/Vb) = \lg(Va) - \lg(Vb) \quad (7)$$

where

M is the reduction value;

$\lg(Va)$ is the common logarithm average of 3 infectivity titre value immediate after inoculation of the control specimen;

$\lg(Vb)$ is the common logarithm average of 3 infectivity titre value after 2 h contacting with the control specimen.

14.3.2 Calculation of antiviral activity value

See Formula (8) for the calculation of antiviral activity value.

$$Mv = \lg(Va/Vc) = \lg(Va) - \lg(Vc) \quad (8)$$

where

Mv is the antiviral activity value;

$\lg(Va)$ is the common logarithm average of 3 infectivity titre value immediate after inoculation of the control specimen;

$\lg(Vc)$ is the common logarithm average of 3 infectivity titre value after 2 h contacting with the antiviral fabric specimen.

15 Test report

The test report shall contain the following information:

a) a reference to this document, i.e. ISO 18184:2019;

b) the identification of sample;

c) the details of virus strain and host cell;

d) the method to determine the infectious titre;

e) test result;

f) any deviation from the specified procedures.

Annex A
(informative)

Virus strains and host cells

Examples of viruses and host cells used in this document is shown in Table A.1.

Table A.1 — Viruses, host cells and media used for this document

Virus kind	Influenza virus	Feline calicivirus
Virus strain	Influenza A virus(H3N2): A/Hong Kong/8/68: TC adapted ATCC VR-1679 Influenza A virus (H1N1): A/PR/8/34: TC adapted ATCC VR-1469,	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 (9.7)	RPMI 1640 (9.7)
[a] Other host cells could be used after appropriate validation regarding its sensitivity against each viruses.		
[b] Other media could be used after appropriate validation for the growth of cells.		

Annex B
(normative)

Infectivity titre test: Plaque assay

B.1 Test procedure

B.1.1 Using a microscope, observe the confluency of the cell cultures prepared in 6 well plates prepared in 10.3. If the cells are confluent, drain the growth medium from the plate.

B.1.2 Add 3 ml of the maintenance medium (9.8) and gently swirl to wash the cell surface. Remove the medium and repeat the washing procedure 2 times.

B.1.3 Inoculate 0,1 ml of the washing out virus suspension and the diluted virus suspension for the test in the 2 wells for one virus suspension, such as the base virus suspension into the first 2 wells and the 1/10 diluted virus suspension into the second 2 wells, as such. In the last 2 wells, inoculate the pure maintenance medium (9.8) for validation of the medium.

B.1.4 Put the plate of B.1.3 in the CO_2 incubator (7.24) at the temperature listed in Table B.1 and keep it for 1 h, to let the cells absorb the virus. Tilt the plate every 15 min to let the whole area of the cells absorb the virus.

B.1.5 Put 3 ml of the maintenance medium (9.8) in the plate and wash the surface, then drain the extra maintenance medium.

B.1.6 Add 3 ml of the agar medium (9.14) for the plaque assay. Close a lid and keep at room temperature for 10 min.

B.1.7 Confirm the agar solidifies, then invert the plate upside down and put it in the CO_2 incubator (7.24) at a temperature of the listed in Table B.1 and keep it for 2 days to 3 days to culture.

After taking it out from the CO_2 incubator (7.24), put it upright and add 3 ml of the formaldehyde solution for cell fixation (9.4), then keep it at room temperature for more than 1 h to fix the cells.

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

B.1.8 Drain the agar medium from B.1.7, add 3 ml of the methylene blue solution (9.5), then keep it at room temperature for 15 min to dye the cells.

B.1.9 Wash the extra methylene blue solution with tap water. Confirm the dyeing of the cells.

B.1.10 Count the number of plaques (white blotches).

B.1.11 Take average of two counts.

See Figure B.1.

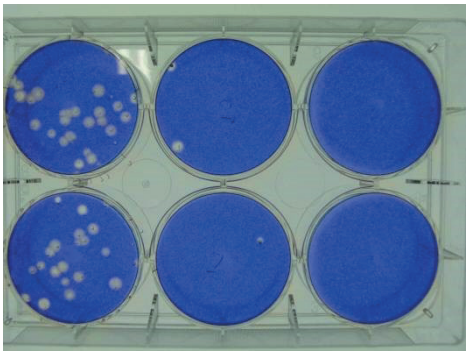


Figure B.1 — Photo of an example of plaque assay

Table B.1 — Carbon dioxide incubator condition

Virus for examination	Influenza virus	Feline calicivirus
Clause applied	10.4.4.2.1 and 13.1	
Adsorption temperature setting °C	34	37
Culture temperature setting °C		

B.2 Determination of PFU

B.2.1 General

The plaques (white brotches) are countable by around 60 points. Over 60 points, the separation of plaques (white brotches) becomes unclear. There is another case which no plaques is observed, then as a possibility the average of two becomes less than 1. So, the determination of PFU is defined as the following in this document.

B.2.2 Determination of PFU

As described in this document, the plaques are counted on the dyed cells for the dilution series wells. The number of the plaques is obtained an average of two data on each dilution as Table B.2.

Table B.2 — Interpretation of the data

Dilution	Wash-out virus suspension	1st dilution	2nd dilution	3rd dilution	N^{th} dilution
Dilution rate	1	1/10	1/100	1/1 000	$1/10^N$
Average number of plaques	C1	C2	C3	C4	CN

The number of the plaques is determined in this document as follows.

— If one of C1 to CN shows the number of plaques with 6 approximately 60, use data of 6 to 60 as PFU of the test.

— If C1 is 1 to less than 6, use C1 as PFU of the test.

— If C1 is less than 1, including zero, use 1 for calculation for the test.

Annex C
(normative)

Infectivity titre test: $TCID_{50}$ method

C.1 Test procedure

C.1.1 Observe at the microscope the confluency of the cell cultures prepared in 96 wells microplates prepared in 10.3. If the cells are confluent, drain the growth medium from the plate.

C.1.2 Add 0,1 ml of the maintenance medium (9.8) and gently swirl to wash the cell surface. Remove the medium and repeat the washing procedure 2 times.

C.1.3 Inoculate 0,1 ml of the wash-out virus suspension and the diluted virus suspension of the test in the 8 wells for one virus suspension, such as the base virus suspension to the first 8 wells and the 1/10 dilution for the virus suspension to the second 8 wells, as such. In the final 8 wells, inoculate the pure maintenance medium (9.8) for validation of the medium.

C.1.4 Put the microplate of C.1.3 in the CO_2 incubator (7.24) at a temperature of the listed in Table C.1 and keep it for 1 h, to let the cells absorb the virus.

C.1.5 Drain the supernatant from the plate.

C.1.6 Add 0,1 ml of the maintenance medium (9.8), wash the surface by the medium and drain the extra maintenance medium.

C.1.7 Add 0,2 ml of the maintenance medium (9.8) for Feline calicivirus or 0,2 ml of the maintenance medium with Trypsin (9.16) for Influenza virus and put the microplate of C.1.4 in the CO_2 incubator (7.24) at a temperature of the listed in Table C.1 and keep it for 7 days to culture.

C.1.8 Observe each cell in the wells by inverted microscopy if the cytopathic effect of the cell is occurred.

C.1.9 Confirm the cytopathic effect and calculate $TCID_{50}$ by Behrens and Karber method.

Table C.1 — Carbon dioxide incubator condition

Virus for test	Influenza virus	Feline calicivirus
Clause applied	10.4.4.2.2 and 13.2	
Adsorption temperature setting °C	34	37
Culture temperature setting °C		

Annex D
(informative)

Composition of media

D.1 General

Media culture are commercially available. As long as the cell cultures present an expected behaviour and growth, other media can be used

D.2 Composition of EMEM

An example of composition of EMEM is described in Table D.1. The EMEM is available in the market, however, if there are lack of components of the composition listed in Table D.1, add them accordingly to the Table D.1.

Table D.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
	Calcium D-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

Table D.1 *(continued)*

Composition in 1 000 ml water		mg
Inorganic Salts	Calcium Chloride	200,00
	Magnesium Sulfate	97,70
	Potassium Chloride	400,00
	Sodium Chloride	6 800,00
	Sodium Phosphate Monobasic Monohydrate	140,00
Others	Dextrose	1 000,00
	Phenol Red Sodium Salt	10,00

D.3 Composition of RPMI 1640 medium

RPMI 1640 is also available in market. The example of composition is described in the Table D.2.

Table D.2 — Composition of RPMI 1640

Composition in 1 000 ml water		mg
Amino Acids	L-Arginine[Free Base]	200,00
	L-Asparagine[Anhydrous]	50,00
	L-Aspartic Acid	20,00
	L-Cystine 2HCl	65,20
	L-Glutamic Acid	20,00
	L-Glutamine	300,00
	Glycine	10,00
	L-Histidine[Free Base]	15,00
	Hydroxy-L-Proline	20,00
	L-Isoleucine	50,00
	L-Leucine	50,00
	L-Lysine HCl	40,00
	L-Methionine	15,00
	L-Phenylalanine	15,00
	L-Proline	20,00
	L-Serine	30,00
	L-Threonine	20,00
	L-Tryptophan	5,00
	L-Tyrosine 2Na $2H_2O$	28,83
	L-Valine	20,00

Table D.2 *(continued)*

Composition in 1 000 ml water		mg
Vitamins	Biotin	0,20
	Choline Chloride	3,00
	Folic Acid	1,00
	myo-Inositol	35,00
	Niacinamide	1,00
	D-Pantothenic Acid Hemicalcium	0,25
	p-Aminobenzoic Acid	1,00
	Pyridoxine HCl	1,00
	Riboflavin	0,20
	Thiamine HCl	1,00
	Cyanocobalamin	0,005
Inorganic Salts	Calcium Nitrate $4H_2O$	100,00
	Magnesium Sulfate[Anhydrous]	48,84
	Potassium Chloride	400,00
	Sodium Chloride	6 000,00
	Disodium hydrogen phosphate anhydrous	800,00
Others	D-Glucose	2 000,00
	Glutathione, Reduced	1,00
	Phenol Red Na	5,30

Annex E
(informative)

Testing method using specific pathogen free (SPF) embryonated hen's eggs

E.1 General

The embryonated hen's egg has been used in the traditional biotechnology testing method, especially for the production of the vaccine for epidemic disease caused by infectious viruses. As stated in this document, the technology to use a cultured cell is becoming a more common method to biotechnology testing to avoid the use of living body such as an embryonated hen. However, this method is still existing and used in some testing houses, so this technology is described in this annex.

E.2 Overview

This method specifies to testing method for antiviral activity assay of the textile products by using SPF embryonated hen's eggs.

E.3 Specific pathogen free (SPF) embryonated hen's eggs

SPF embryonated hen's eggs are eggs without experience to be infected by the pathogenic microorganism used for production of vaccine. It is used through this document.

E.4 Preparation of specimen

E.4.1 Take a specimen with the weight of 0,2 g each and take 3 specimens from one sample.

E.4.2 For the sheet like sample, such as woven, knitted fabric or nonwoven fabric, cut 0,2 g specimen into 1,5 cm square and pile up them.

E.4.3 Put the specimen into the polyethylene bag with vinyl zipper.

NOTE There is no need for sterilization of specimen.

E.5 Preparation of the embryonated hen's eggs

Choose 10-day old embryonated hen's eggs.

E.6 Preparation of virus

Influenza virus: chick-embryo-adapted strain of A/PR/8/34 (H1N1): ATCC VR-1469 or A/Hong Kong/8/68 (H3N2):ATCC VR-1679 is used in this test.

E.6.1 Virus cultivation

E.6.1.1 Inoculate a 0,2 ml to 0,6 ml aliquot of the virus suspension into allantoic cavity of the eggs.

E.6.1.2 Incubate the eggs in the incubator at a temperature of 35 °C, for 3 days.

E.6.1.3 After incubation, put the eggs into refrigerator to cool down overnight, and then take the allantoic fluid from the eggs.

E.6.1.4 Examine the all fluids taken from the eggs to confirm multiplication of influenza virus. If infected, the haemagglutination is observed. If confirmed, the fluids are considered as virus fluids, or virus media.

E.6.1.5 Just before testing, dilute the virus fluids with the phosphate buffered saline solution (PBS) (pH 7,2) to make the concentration of virus as 10^7 EID_{50}/0,2 ml.

E.6.2 Preparation of 0,5 % chicken red blood cell suspension

E.6.2.1 Draw a 4,0 ml aliquot of chicken venous blood by using a syringe which is containing 1,0 ml of the sterilized 2 % sodium citrate, and put it into a test tube.

E.6.2.2 Mount the test tube on the centrifuge and centrifugalize it by 1 000 × g for 10 min.

E.6.2.3 Drain the supernatant and then add a 5 ml aliquot of PBS to the test tube.

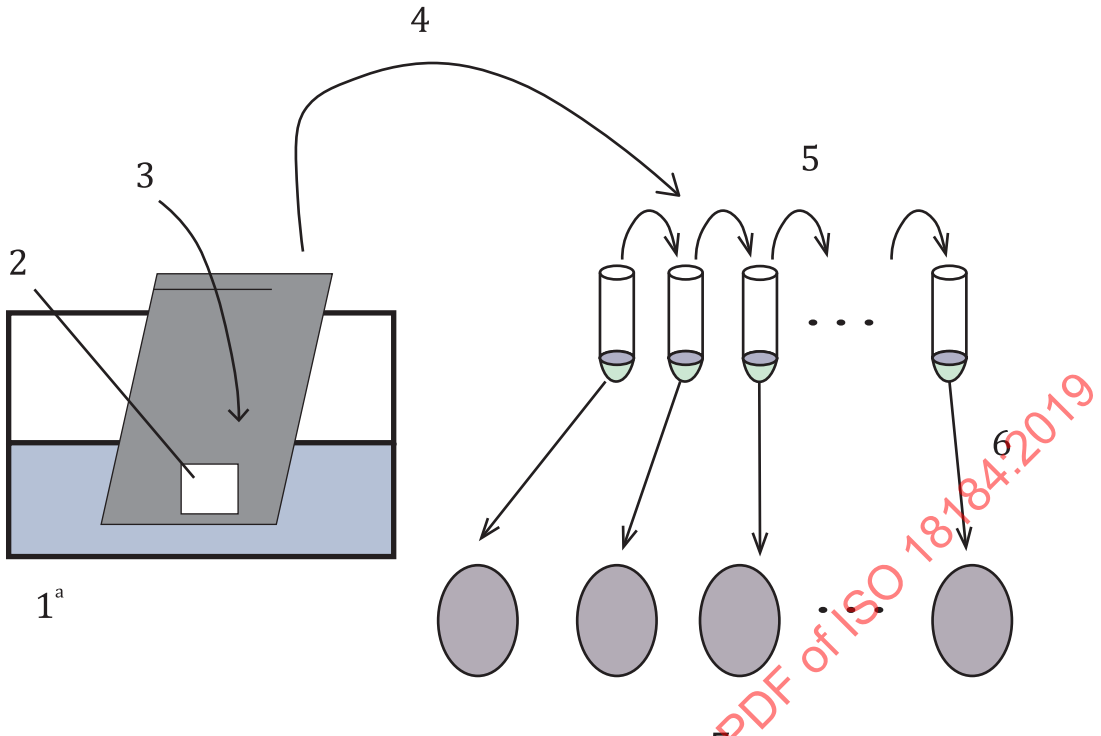
E.6.2.4 Repeat to centrifuge another two times.

E.6.2.5 Mix 0,5 ml aliquot of the settled-out chicken red blood and 99,5 ml aliquot of PBS and shake the test tube well.

E.7 Test procedure

E.7.1 General

General process image is shown in Figure E.1.



Key

1 water bath 4 °C
2 specimen in a plastic bag, 3
3 plastic bag with virus suspension
4 recover virus suspension after contacting
5 dilution series of virus suspension by PBS
6 inoculation of virus suspension in the eggs
7 eggs for incubation
a Alternatively, an open air room with a temperature of 25 °C can be used.

Figure E.1 — General process image of embryonated eggs method

E.7.2 Contact the virus suspension to the specimen

E.7.2.1 Put the specimen in a polyethylene bag with zipper.

E.7.2.2 Put the virus solution diluted by PBS into the polyethylene bag with the specimen.

E.7.2.3 Put the bags into the water bath with 4 °C or place the bags in open air in room with 25 °C and keep for 10 min or 2 h as contacting time.

E.7.3 Inoculation of the reacted virus suspension to 10 day old embryonated hen's eggs

E.7.3.1 Recover the reacted virus suspension from the plastic bag of E.7.2.3.

E.7.3.2 Dilute the reacted virus suspension by series of dilution, such as 10^{-1}, 10^{-2}, 10^{-3}, etc.

E.7.3.3 Inoculate the diluted virus suspension of 0,2 ml aliquot into allantoic cavity of the eggs, three eggs for each dilution.