
Textiles — Determination of antiviral activity of textile products

Textiles — Détermination de l'activité virucide de produits textiles



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 TCID₅₀ 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 TCID₅₀

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 TCID₅₀ 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 TCID₅₀ 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 $(121 \pm 2) ^\circ\text{C}$.

NOTE The autoclave is described in EN 12353.

7.2 Dry heat sterilizer: Ovens, capable of operating at a temperature of $(180 \pm 2) ^\circ\text{C}$ and $(160 \pm 2) ^\circ\text{C}$.

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 $(37 \pm 1) ^\circ\text{C}$, $(50 \pm 1) ^\circ\text{C}$ and $(56 \pm 1) ^\circ\text{C}$.

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

7.11 Freezer, capable of operating at a temperature of $(-80 \pm 2) ^\circ\text{C}$ or $(-20 \pm 2) ^\circ\text{C}$.

7.12 Liquid nitrogen bath, for the preservation approximately at $-196 ^\circ\text{C}$.

7.13 Membrane filtration device, with a pore size of $0,22 \mu\text{m}$.

7.14 Refrigerator, capable of operating at a temperature between $2 ^\circ\text{C}$ and $8 ^\circ\text{C}$.

7.15 pH meter, having an inaccuracy of calibration $\pm 0,1$ pH units at $(20 \pm 1) ^\circ\text{C}$.

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 $(4 \pm 2) ^\circ\text{C}$, 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 TCID₅₀ 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.

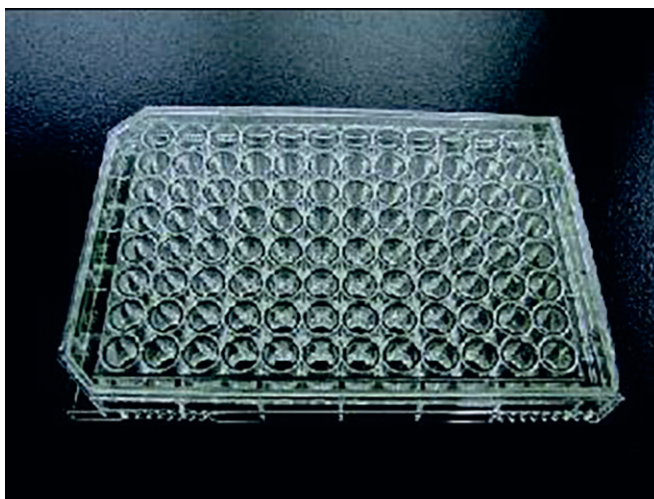


Figure 1 — 96 wells microplate for TCID₅₀ method

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 cm² 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 CO₂ incubator, capable of maintaining an atmosphere with 5 % CO₂, at a temperature of $(34 \pm 1) ^\circ\text{C}$ and $(37 \pm 1) ^\circ\text{C}$.

NOTE Certain CO₂ incubators are described in EN 12353.

7.25 Incubator, capable of maintaining at a temperature of $(25 \pm 1) ^\circ\text{C}$, $(35 \pm 1) ^\circ\text{C}$ and $(37 \pm 1) ^\circ\text{C}$.

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 $121 ^\circ\text{C}$ and a pressure of 103 kPa for 15 min.
- Dry-heat sterilization: by a dry heat sterilizer ([7.2](#)) at a temperature of $180 ^\circ\text{C}$ for 30 min or $160 ^\circ\text{C}$ 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 H₂O, 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 $7,0 \pm 0,2$ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 TCID₅₀ 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 TCID₅₀ method.

10.3.3 Put the 6 wells plate or the microplate in the CO₂ 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 10³ PFU to 10⁴ PFU or TCID₅₀/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 CO₂ 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 CO₂ 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 10⁷ PFU or TCID₅₀/ml by plaque titre assay or TCID₅₀ method. If the concentration is less than 10⁷ PFU or TCID₅₀/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 × 10⁷ PFU or TCID₅₀/ml to 5 × 10⁷ PFU or TCID₅₀/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 10⁵ PFU to 10⁶ PFU or TCID₅₀/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 CO₂ 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 CO₂ 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 10⁷ PFU or TCID₅₀/ml for Feline calicivirus by the plaque assay or TCID₅₀ method.

If the concentration is less than 10⁷ PFU or TCID₅₀/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 × 10⁷ PFU or TCID₅₀/ml to 5 × 10⁷ PFU or TCID₅₀/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 TCID₅₀ 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 10⁷ TCID₅₀/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 TCID₅₀ method

Determine TCID₅₀ 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 $0,40 \text{ g} \pm 0,05 \text{ g}$ 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 $0,40 \text{ g} \pm 0,05 \text{ g}$.

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 $(4 \text{ to } 6) \times 10^4$ PFU/ml or TCID₅₀/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 TCID₅₀ 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:

$\lg(\text{PFU/ml or TCID}_{50}/\text{ml of reference specimen}) - \lg(\text{PFU/ml or TCID}_{50}/\text{ml of antiviral specimen}) \leq 0,5$

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 TCID₅₀/ml), the concentration of the virus becomes 1×10^5 (PFU/ml or TCID₅₀/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 TCID₅₀/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 TCID₅₀/ml.

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

In case of TCID₅₀ 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 TCID₅₀/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 TCID₅₀/ml.

13 Determination of the infectious titre

13.1 Plaque assay

Determine the infectivity titre by plaque assay according to [Annex B](#).

13.2 TCID₅₀ method

Determine TCID₅₀ according to [Annex C](#).

14 Calculation of infectivity titre

14.1 Plaque assay

See [Formula \(1\)](#):

$$P = Z \times R \quad (1)$$

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 \(2\)](#):

$$W = P \times 10 \quad (2)$$

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

Then, the infectivity titre of the virus is calculated using [Formula \(3\)](#):

$$V_p = W \times C \quad (3)$$

where

V_p is the infectivity titre (PFU/vial);

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

14.2 TCID₅₀ method

Other determination methods of TCID₅₀ 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 \(4\)](#).

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

$$a = \sum p - 0,5 \quad (4)$$

where

Y is the infective titre (TCID₅₀/0,1 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 (TCID₅₀/ml) is as shown in [Formula \(5\)](#):

$$A = Y \times 10 \quad (5)$$

And, the infectivity titre (TCID₅₀/vial) is calculated using [Formula \(6\)](#):

$$V = A \times C \quad (6)$$

where

V is the infective titre (TCID₅₀/vial);

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

14.2.2 Example of calculation

See [Figure 4](#).

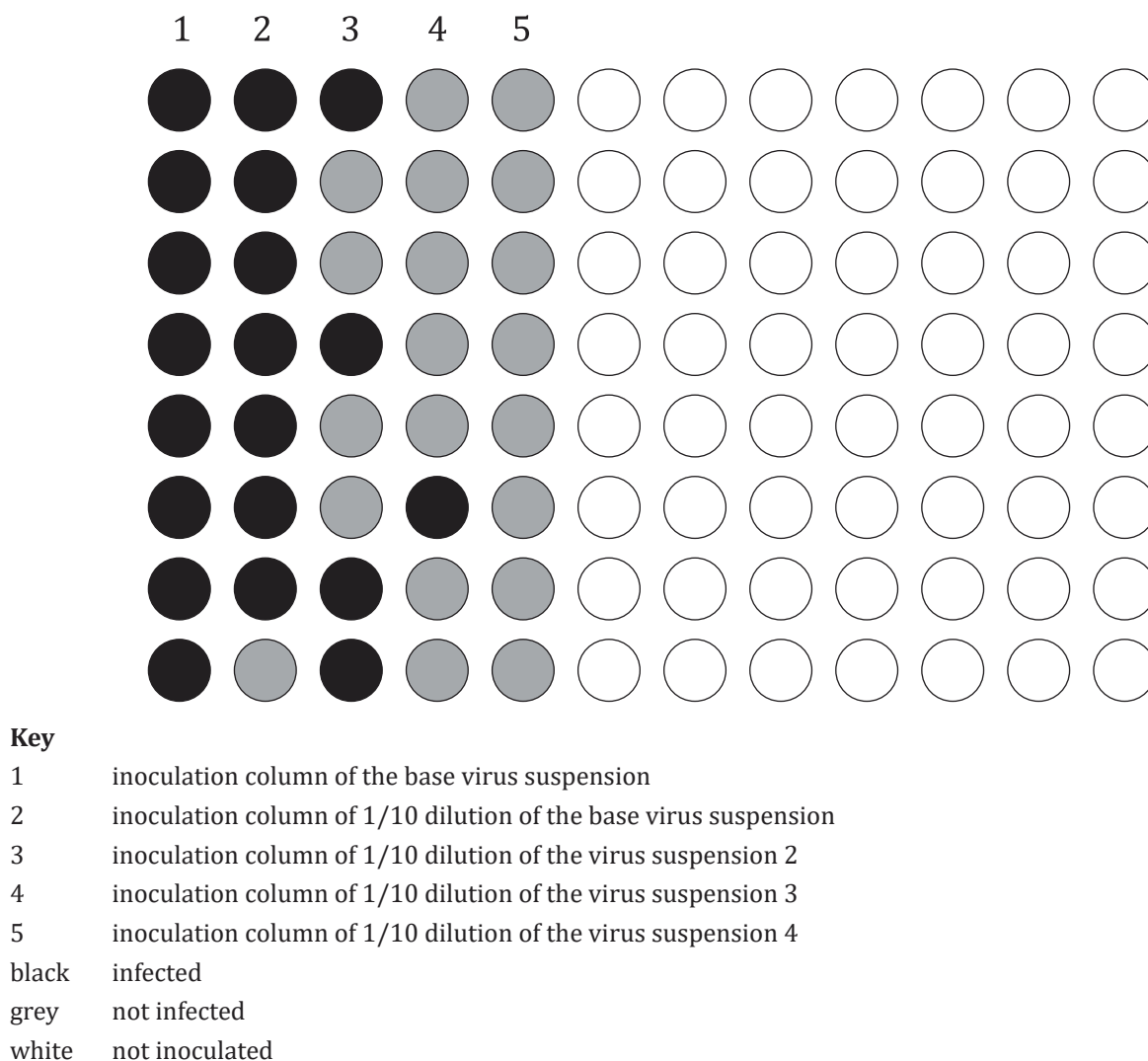


Figure 4 — Example of TCID₅₀ method

$$X = 10^0 = 1,$$

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

$$\text{Then, } a = 2,5 - 0,5 = 2,0$$

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

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

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

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 TCID₅₀/ml
 - Feline calicivirus suspension $> 10^7$ PFU or TCID₅₀/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 $\leq 1,0$.

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 CO₂ 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 CO₂ 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 CO₂ 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](#).

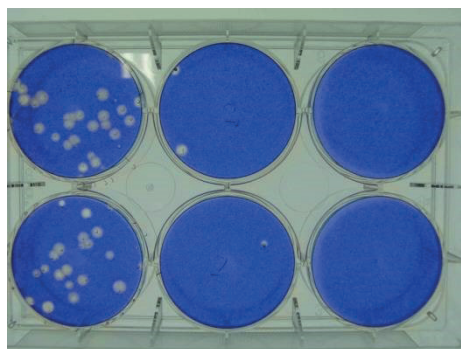


Figure B.1 — Photo of an example of plaque assay

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: TCID₅₀ 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 CO₂ 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 CO₂ 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 TCID₅₀ 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 ₂ O	41,90
	L-Isoleucine	52,50
	L-Leucine	52,50
	L-Lysine HCl	72,50
	L-Methionine	15,00
	L-Phenylalanine	32,50
	L-Threonine	47,60
	L-Tryptophan	10,00
	L-Tyrosine 2Na 2H ₂ O	51,90
	L-Valine	46,80
Vitamins	Choline Chloride	1,00
	Calcium D-Pantothenate	1,00
	Folic Acid	1,00
	Myo Inositol	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 ₂ O	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 ₂ O	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 EID₅₀/0,2 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 centrifugize it by $1\ 000 \times g$ for 10 min.

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

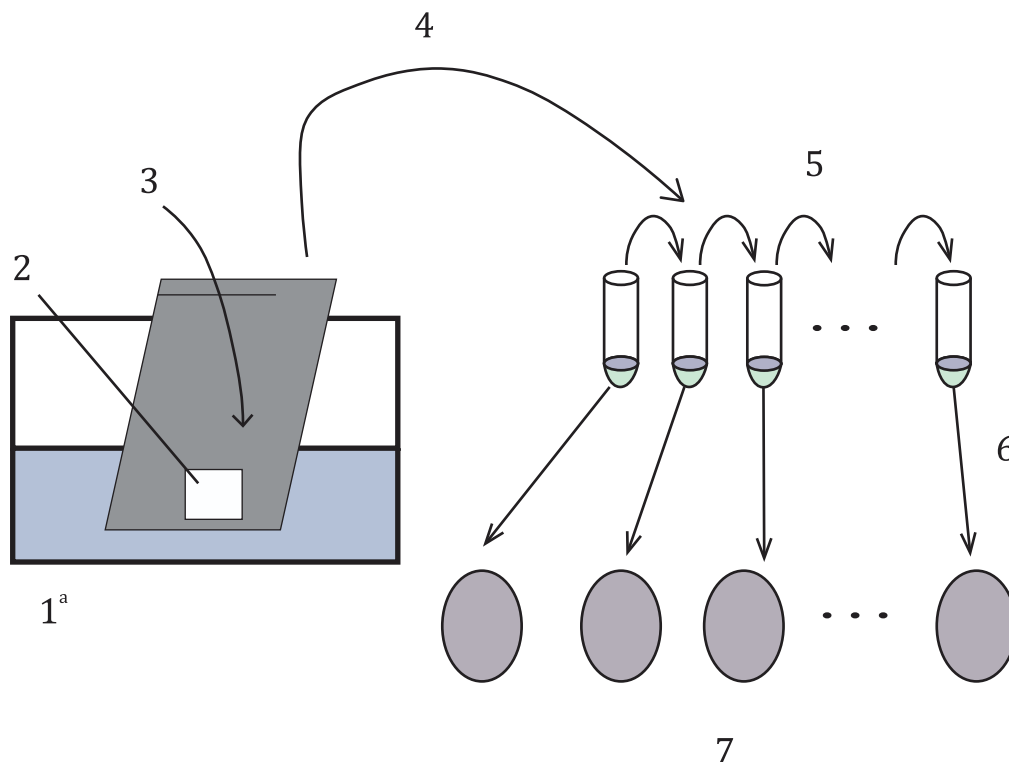
E.6.2.4 Repeat to centrifuge another two times.

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.

E.7.3.4 Incubate them for two days at 35 °C.

E.7.4 Virus assay

E.7.4.1 After two days incubation, take out the allantoic fluid from the eggs and put them into test tubes. Then,

E.7.4.2 Add 0,5 % chicken red blood cell suspension into the test tubes.

E.7.4.3 Observe the test tubes if the haemagglutination is found or not.

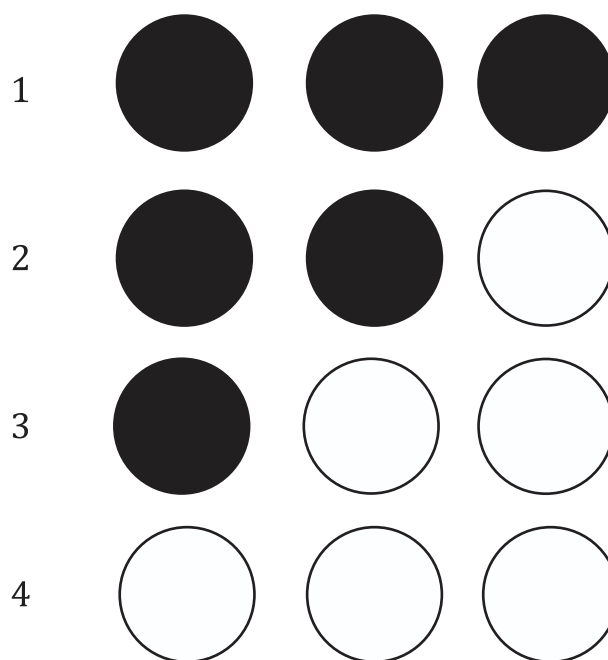
- Virus infection (–): Red cell precipitated is observed but no condensation.
- Virus infection (+): Red cell condensation observed.

E.7.5 Calculation of the virus titre

Calculate the virus titre EID_{50} by the method of Reed and Muench.

50 % EID is calculated from the cumulative infected number of each dilution at 50 %.

The example of calculation is shown in the [Figure E.2](#).



Key

- | | |
|-------|--|
| 1 | inoculation row of the virus suspension dilution 10^{-4} |
| 2 | inoculation row of the virus suspension dilution 10^{-5} |
| 3 | inoculation row of the virus suspension dilution 10^{-6} |
| 4 | inoculation row of the virus suspension dilution 10^{-7} |
| black | infected |
| white | not infected |

Figure E.2 — Example of the embryonated eggs method

In the example, [Table E.1](#) was determined.

Table E.1 — Example of the EID test

Dilution	Infected eggs	Cumulative count		Cumulative (%)	EID ₅₀
		Infected	Not infected		
10 ⁻⁴	3	6	0	100	Proportional, (75-50)/(75-25)
10 ⁻⁵	2	3	1	75	
10 ⁻⁶	1	1	3	25	
10 ⁻⁷	0	0	6	0	
EID ₅₀ = -5 - (75 - 50)/(75 - 25) = -5 - 0,5 = -5,5					

E.7.6. Antiviral efficacy value

The efficacy value is calculated from [Formula \(E.1\)](#):

$$M_v = -\lg(Vb/Va) = -[\lg(Vb) - \lg(Va)] \quad (\text{E.1})$$

where

M_v is the antiviral efficacy value;

$\lg(Vb)$ is the EID₅₀ of the viral fluid without contact to a specimen after contacting time;

$\lg(Va)$ is the EID₅₀ of the viral fluid with contact to the specimen after contacting time.

In the example, if the concentration without specimen is 10⁻⁷, then

$$\text{EID}_{50} = \text{Lg } 10^{-7} = -7,$$

$$M_v = - [(-7) - (-5,5)] = 1,5$$

See also [Annex F](#).

Annex F (informative)

Antiviral efficacy — Antiviral performance of the products

The antiviral textile products may be evaluated by the categories according to [Table F.1](#) from the result of this test.

Table F.1 — Antiviral performance standard

Item	Antiviral efficacy value, M_v	Standard
Tested textile product	$3,0 > M_v \geq 2,0$	Good effect
	$M_v \geq 3,0$	Excellent effect

Annex G (informative)

Interlaboratory test result (1)

G.1 Participants

One (1) testing house and one (1) laboratory in Japan.

G.2 Sample

Seven (7) samples are tested. The details are shown in [Table G.1](#).

Table G.1 — Sample details for interlaboratory test

No.	Material and composition	Antivirus treatment	Remarks
A	Cotton 100 % woven fabric: Plain	Control (non)	A ₀ : time 0, A ₂ : time 2 h
B	Cotton 100 % woven fabric: Twill	Non	
C		Normal	
D		Strong	
E	Polyester 65 %/ cotton 35 % woven fabric: Broadcloth	Non	
F		Normal	
G		Strong	

G.3 Test condition

- Test virus: Influenza A virus (H1N1) or Influenza A virus (H3N2)
- Host cell: Dog kidney cell origin (MDCK cell) ATCC CCL-34
- Infective titre measurement: Plaque assay method

G.4 Test result

G.4.1 Test result of a testing house

See [Table G.2](#).

Table G.2 — Test result of a testing house

Sample	A ₀	A ₂	B	C	D	E	F	G	Remarks
Sampling time	0	2	2	2	2	2	2	2	
Lg PFU/vial	7,77	7,31	7,08	< 2,0	< 2,0	7,35	6,43	6,45	
	7,7	7,28	7,31	< 2,0	< 2,0	7,31	6,31	6,46	
	7,72	7,36	7,23	< 2,0	< 2,0	7,2	6,43	6,28	
Mean	7,73	7,32	7,21	< 2,0	< 2,0	7,29	6,39	6,40	
Designation	Lg Va	Lg Vb	Lg Vc						
Verification (A ₀ – A ₂)		0,41							≤ 2,0: OK
Antiviral activity value			0,1	> 5,3	> 5,3	0,0	0,9	0,9	

— Test virus: Influenza A virus (H1N1)

As the verification of the test virus infective titre of inoculated concentration shall be:

— Influenza virus suspension > 10⁷ PFU/ml: test virus was 2,8 × 10⁸ PFU/ml

G.4.2 Test result of a laboratory

See [Table G.3](#).

Table G.3 — Test result of a laboratory

	A ₀	A ₂	B	C	D	E	F	G	Remarks
Sampling time	0	2	2	2	2	2	2	2	
Lg PFU/vial	7,36	7,18	7,2	< 2,0	< 2,0	7,08	6,6	6,51	
	7,45	7,23	6,97	< 2,0	< 2,0	7,23	> 6	> 6	
	7,61	7,04	7,08	< 2,0	< 2,0	7,15	6,63	6,59	
Mean	7,47	7,15	7,08	< 2,0	< 2,0	7,15	6,62	6,55	
Designation	Lg Va	Lg Vb	Lg Vc						
Verification (A ₀ – A ₂)		0,32							≤ 2,0: OK
Antiviral activity value			0,1	> 5,1	> 5,1	0,0	0,5	0,6	

— Test virus: Influenza A virus (H3N2)

As the verification of the test virus infective titre of inoculated concentration shall be:

— Influenza virus suspension > 10⁷ PFU/ml: test virus was 1,2 × 10⁸ PFU/ml

Annex H (informative)

Interlaboratory test result (2)

H.1 Participants

Four (4) testing houses and one (1) laboratory in Japan.

H.2 Sample

Seven (7) samples are tested. The details are as shown in [Table H.1](#).

Table H.1 — Sample details for interlaboratory test

No.	Material and composition	Antivirus treatment	Remarks
	Cotton 100 % woven fabric	Control (non)	A ₀ : time 0, A ₂ : time 2 h
A	Cotton 100 % woven fabric	Non	
		Antivirus treatment	
B	Cotton 100 % woven fabric	Non	
		Antivirus treatment	
C	Polypropylene/Polyethylene/Rayon Nonwoven	Non	
		Antivirus treatment	

H.3 Test condition

H.3.1 Virus, host cell, etc.

- Test virus: Influenza virus (H3N2) (ATCC VR-1679)
- Host cell: Dog kidney cell origin (MDCK cell) ATCC CCL-34
- Infective titre measurement: Plaque assay method

H.3.2 Plaque assay condition

- 0,3 % bovine serum albumin added to test virus suspension
- Specimen mass: 0,4 g
- Virus suspension inoculation amount: 0,2 ml
- Dying condition: 25 °C, 2 h
- Washout solution: SCDLP medium

H.4 Test result

H.4.1 Sample A

See Table H.2.

Table H.2 — Interlaboratory test result for sample A

		Sample				Antiviral activity	Initial validation
		Control		A			
		Not treated		Not treated	Treated		
						16 $M_V = \text{Lg } V_b - \text{Lg } V_c$	$\times 10^8$
Sampling time (h)		0	2	2	2		
Lg PFU/vial	TH 1	7,41	6,71	6,74	3,56	3,2	1,5
	TH 2	7,01	6,84	6,76	< 2,0	> 4,84	0,47
	TH 3	7,13	6,48	6,09	< 2,0	> 4,48	0,65
	TH 4	7,72	7,38	7,33	< 2,0	> 5,4	3,5
	lab 1	7,76	7,34	7,31	< 2,0	> 5,34	3,6
Mean		7,47	7,01	6,91	—	> 4,65	
Designation		Lg V_a	Lg V_b		Lg V_c		
Verification ($A_0 - A_2$)			0,46				
Standard value			$\leq 2,0$				$\geq 10^7$

H.4.2 Sample B

See [Table H.3](#).

Table H.3 — Interlaboratory test result for sample B

		Sample				Antiviral activity	Initial validation
		Control		B			
		Not treated		Not treated	Treated	17 $M_V = \text{Lg } V_b - \text{Lg } V_c$	$\times 10^8$
Sampling time (h)		0	2	2	2		
Lg PFU/vial	TH 1	7,41	6,71	6,89	6,42	0,3	1,5
	TH 2	7,01	6,84	6,93	6,25	0,59	0,47
	TH 3	7,13	6,48	6,35	5,91	0,57	0,65
	TH 4	7,72	7,38	7,30	6,54	0,8	3,5
	lab 1	7,76	7,34	7,41	6,57	0,77	3,6
Mean		7,47	7,01	7,04	6,40	0,6	
Designation		Lg V_a	Lg V_b		Lg V_c		
Verification ($A_0 - A_2$)			0,46				

H.4.3 Sample C

See [Table H.4](#).

Table H.4 — Interlaboratory test result for sample C

		Sample				Antiviral activity	Initial validation
		Control		C			
		Not treated		Not treated	Treated	18 $M_V = L_g V_b - L_g V_c$	$\times 10^8$
Sampling time (h)		0	2	2	2		
Lg PFU/vial	TH 1	7,41	6,71	—	< 2	> 4,7	1,5
	TH 2	7,01	6,84	—	< 2	> 4,84	0,47
	TH 3	7,13	6,48	—	< 2	> 4,48	0,65
	TH 4	7,72	7,38	7,40	< 2	> 5,4	3,5
	lab 1	7,76	7,34	7,70	< 2,56	> 4,78	3,6
Mean		7,47	7,01	7,55	—	> 4,84	
Designation		Lg V_a	Lg V_b		Lg V_c		
Verification ($A_0 - A_2$)			0,46				

H.4.4 Summary for interlaboratory test results

See [Table H.5](#).

Table H.5 — Summary of antiviral activity

Testing house or laboratory	Antiviral activity value		
	Sample		
	A	B	C
TH 1	3,2	0,3	> 4,7
TH 2	> 4,84	0,59	> 4,84
TH 3	> 4,48	0,57	> 4,48
TH 4	> 5,4	0,8	> 5,4
Lab 1	> 5,34	0,77	> 4,78
Mean	> 4,65	0,6	> 4,84

Bibliography

- [1] EN 14476, *Chemical disinfectants and antiseptics — Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine-Test method and requirements (phase 2, step 1)*
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