International Standard

ISO 18184

Textiles — Determination of antiviral activity of textile products

Textiles — Détermination de l'activité virucide de produits textiles

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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 document 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, *Textiles.*

This third edition cancels and replaces the second edition (ISO 18184:2019), which has been technically revised.

The main changes are as follows:

— in 14.3.2, the calculation of antiviral activity has been updated;

— a new Annex B has been added for test method for SARS-CoV-2, and subsequent annexes have been moved;

— in Annex E, E.4 has been added for the composition of DMEM medium;

— a new Annex F has been added to give additional examples of the virus strain and host cells.

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 textile 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 I, J and K for interlaboratory test results.

Textiles — Determination of antiviral activity of textile products

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.

1 Scope

This document specifies testing methods for the determination of the antiviral activity of the textile products.

The textile products include woven and knitted fabrics and non-woven fabrics, cotton, fibres, yarns, braids, feathers, 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*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 20743:2021, *Textiles — Determination of antibacterial activity of textile products*

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 https://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: When the fabrics before the antivirus treatment is not applicable, in Note to entry 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 test specimen does not affect the host cell

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

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 $TCID_{50}$ 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

3.15
antiviral textile product
fabric treated with antiviral chemicals

4 Principle

The viruses are deposited onto an antiviral test specimen and control test specimen. After specific contact time, the remaining infectious virus is counted, and the reduction rate is calculated by the comparison between the antiviral test specimen and the control test 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.

Due to the individual sensitivities, the results of one test virus cannot be transposed to other viruses.

5 Example of virus and host cell

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

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

Handling and manipulation of viruses and host cells which are potentially hazardous requires a high degree of technical competence and can be subject to current national legislation and regulations. Appropriate safety precautions should be observed with due consideration given to country-specific regulations. Only personnel trained in biological techniques should carry out such tests. Appropriate practices for disinfection, sterilization and personal hygiene shall be strictly observed.

7 Apparatus

7.1 High pressure steam sterilizer, such as autoclave, capable of operating at a temperature of (121 ± 2) °C, in accordance with ISO 20743:2021, 5.28.

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

7.3 Measuring flask, with capacity of 1 l.

7.4 Balance, with the available range of 0,01 g to 100 g with accuracy of 1,0 % in accordance with ISO 20743:2021, 5.13.

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 Mixer, producing a vortex shaking action in accordance with ISO 20743:2021, 5.4.

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 at approximately –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, 8 °C].

7.15 pH meter, having an inaccuracy of calibration ±0,1 pH units at (20 ± 1) °C in accordance with ISO 20743.

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.

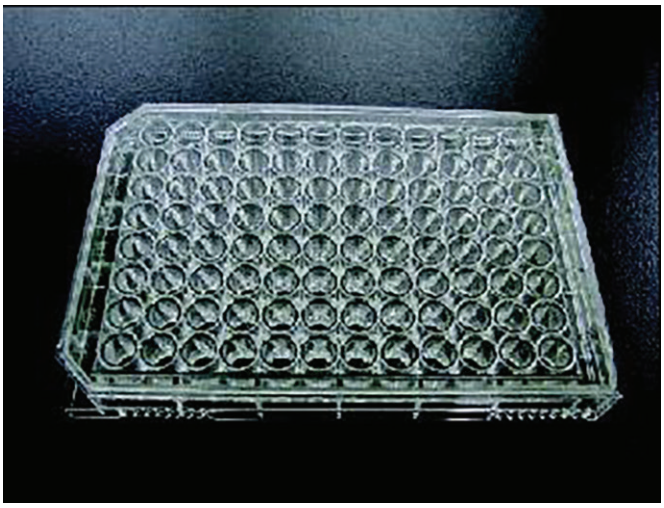


Figure 1 — 96 wells microplate for $TCID_{50}$ method

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

Six 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 bacterial 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.

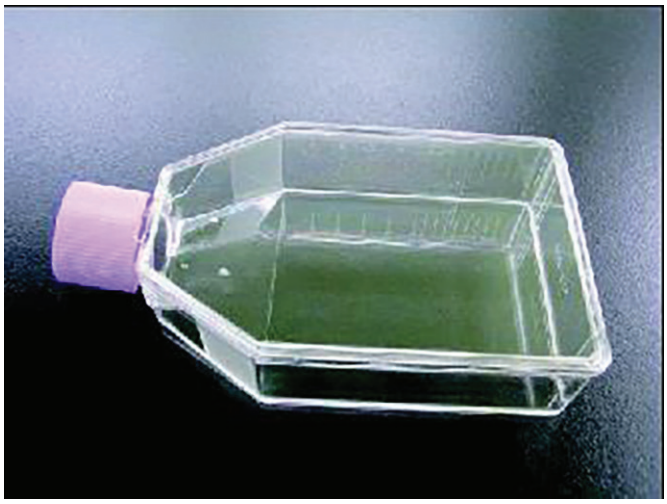


Figure 3 — Flask for cell culture use

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.

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

7.26 Centrifuge tube.

7.27 Culture container.

7.28 Test tube.

7.29 Beaker.

7.30 Glass rod, with approximately 18 mm in diameter.

7.31 Mixer, producing spinning action by a stir bar with a rotating magnetic field.

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 Reagents and materials

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) or grade 3 water in accordance with ISO 3696.

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

9.3 sodium bicarbonate solution

Prepare the solution at the concentration of 7,5 % according to the method 1 or method 2, and mix well just before using.

9.3.1 Method 1

9.3.1.1 Sterilize sodium bicarbonate, 75 g in autoclave in a culture container (7.27) with a cap closed tightly.

9.3.1.2 Water (9.1) is also sterilized by autoclave.

9.3.1.3 Dissolve sodium bicarbonate in the sterilized water (9.3.1.2) of 1 000 ml well.

9.3.2 Method 2

9.3.2.1 Prepare 7,5 % sodium bicarbonate solution by dissolving 75 g of sodium bicarbonate in 1 000 ml of water (9.1).

9.3.2.2 Sterilize the solution by using 0,22 µm membrane filter (7.13).

9.4 Formaldehyde solution

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

9.4.1 Prepare 100 ml of a 37 % formaldehyde solution.

9.4.2 Add 900 ml of water (9.1) into the solution of 9.4.1.

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 (7.3):

— Water (9.1), 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 frozen 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 use 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 Influenza virus and feline calicivirus

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

— water (9.1), 800 ml;

— kanamycin sulfate, 60 mg;

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

9.7.1.2 Dissolve and mix well and make up whole solution to 1 000 ml by water (9.1).

9.7.1.3 Sterilize the mixed solution from 9.7.2 by using filter (7.13).

9.7.1.4 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 9.7.1.3.

9.7.2 Other viruses

In case of SARS-CoV-2, prepare the growth medium according to Annex B. In case of additional example of viruses described in Annex F, select the appropriate growth medium for host cells of each virus.

9.8 Maintenance medium.

9.8.1 Influenza virus and feline calicivirus

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

— water (9.1), 800 ml;

— kanamycin sulfate, 60 mg;

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

9.8.1.2 Dissolve them well, then, make up the whole amount to 1 000 ml by adding water (9.1).

9.8.1.3 Sterilize the mix solution from 9.8.1.2 by using the filter (7.13) with a pore size of 0,22 µm.

9.8.1.4 Add 15 ml of 7,5 % sodium bicarbonate solution (9.3) in the mix solution of 9.8.1.3.

9.8.2 Other viruses

In case of SARS-CoV-2, prepare the maintenance medium according to Annex B. In case of additional example of viruses described in Annex F, select the appropriate maintenance medium for host cells of each virus.

9.9 Double concentration of the maintenance medium.

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

— water (9.1), 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 (9.1).

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 (7.3):

— water (9.1), 800 ml;

— 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 Dissolve the materials of 9.10.1 and mix well and add water (9.1) by making up the whole amount to 1 000 ml and dissolve well.

9.10.3 Then, transfer the mix solution of 9.10.2 into a culture container (7.27), then sterilize the mix solution by using an autoclave (7.1) at a temperature of 121 °C for 15 min.

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

9.11.1 Prepare a beaker (7.29), 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 the materials of 9.11.1 and mix well by using a mixer (7.31) for 2 h.

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

The divided mix solution in tubes that are not used immediately are preserved in the freezer (7.11) at a temperature of lower than −80 °C until usage for testing.

9.11.4 Prepare a test tube (7.28) and put the following solutions in the test tube (7.28):

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

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

9.11.5 Dissolve the solutions of 9.11.4 and mix them well.

9.11.6 Divide the mix solution of 9.11.5 in test tubes (7.28) and preserve in the freezer (7.11) at a temperature of lower than −20 °C until usage for testing.

9.11.7 Just before using, put the mix solution in test tube of 9.11.6 in the water bath (7.9) at a temperature of 37 °C and keep the mix solution in the tube until defrosting.

9.12 Trypsin and ethylenediaminetetraacetic acid (EDTA) solution.

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

— 0,01 mol/l phosphate buffered saline PBS (-) (9.10), 800 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 the materials of 9.12.1 and mix well. Add 0,01 mol/l phosphate buffered saline (PBS (-)) (9.10) into the mix solution of 9.12.1 and make up the total amount to 1 000 ml.

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

9.12.4 Divide the mix solution of 9.12.3 in test tubes (7.28) and preserve in the freezer (7.11) at a temperature lower than −20 °C until use for testing.

9.12.5 Before use, place the test tube of 9.12.4 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 Diethylaminoethyl (DEAE)-dextran solution.

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

— water (9.1), 1 000 ml;

— diethylaminoethyl (DEAE)-dextran, 20 g.

9.13.2 Dissolve the materials of 9.13.1 and mix well. Add water (9.1) and make up the total amount to 1 000 ml.

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

9.14 Agar medium preparation.

9.14.1 Influenza virus and feline calicivirus

9.14.1.1 Liquid A.

9.14.1.1.1 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.

Dissolve the materials of 9.14.1 and mix well.

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

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

9.14.1.2 Liquid B.

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

— water (9.1), 1 000 ml;

— cell culture agar, 15 g.

Dissolve the materials and mix well.

9.14.1.2.2 Sterilize the mix solution of 9.14.1.2.1 by using autoclave (7.1) at a temperature of 121 °C and a pressure of 103 kPa for 15 min.

9.14.1.2.3 Put the mix solution of 9.14.1.2.2 in the water bath (7.9) at a temperature of 50 °C and keep it until using.

9.14.1.3 Mix liquid A and liquid B at 50 : 50 (% volume fraction).

9.14.2 Other viruses

In case of SARS-CoV-2, prepared the agar medium according to Annex B. In case of additional examples of viruses described in Annex F, select the appropriate agar medium for each virus.

9.15 Wash-out solution

9.15.1 General

The wash-out solution is used for the wash-out of the virus from the antiviral test specimens and control test specimens and for suppression of anti-virus activity of the chemicals treated on the anti-virus textile product and prepared as the following.

Other wash-out solution may be used after appropriate validation.

9.15.2 Influenza virus and feline calicivirus

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

— water (9.1), 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 above materials together and dissolve well, then, add:

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

Dissolve the materials all together and mix well.

9.15.2.2 Adjust pH of the mix solution from 9.15.2.1 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.

9.15.2.3 Sterilize the mix solution from 9.15.2.2 by using autoclave (7.1) at a temperature of 121 °C and a pressure of 103 kPa for 15 min.

9.15.3 Other viruses

In case of SARS-CoV-2, prepared the wash-out solution according to Annex B. In case of additional examples of viruses described in Annex F, select the appropriate wash-out solution for each virus and the host cell.

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 (7.27):

— maintenance medium, (9.8), 1 000 ml;

— trypsin from beef pancreas and (PBS (-)) mix solution (9.11), 1,5 ml.

9.16.2 Dissolve the materials of 9.16.1 and mix well.

9.17 Control fabric, or 100 % cotton woven fabric as specified in ISO 105-F02.

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

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

10.1.4 Put the flask of 10.1.3 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 of 10.1.4 by a microscope (7.16) if the cells are attached on the bottom of the flask.

If the growth is confirmed, then, go to next step of 10.1.6. If not, continue to culture the host cell in the incubator.

10.1.6 Drain the remaining growth medium from 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 (7.16) 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 the step of 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 Observe the flask of 10.1.8 by using a microscope (7.16). If the confluent growth of host cell on the bottom of the flask is confirmed, then, drain an extra growth medium of the flask.

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 1 ml of trypsin EDTA solution (9.12) in the flask of 10.2.2 and spread the solution over the whole surface. After this, remove the excess trypsin EDTA solution from the flask.

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 disperse the cells by pipetting by using pipette (7.5) the medium to make it mild. Mix well so as to avoid the damage to the cells.

10.2.7 Prepare a new flask (7.23) for cell culture 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 using the pipette (7.5) to the flask of 10.2.7.

The amount of the cell suspension of 10.2.6 added in 10.2.8 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 When the subculture of host cell is repeated, 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 (7.22) or 96 wells microplate (7.21) is required for the test of the plaque assay and $TCID_{50}$ 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. The amount of subcultured cell suspension of 10.2.6 may be changed as needed.

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. And 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 Place the 6 wells plate (7.22) or the 96 wells microplate (7.21) 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 distributed uniformly and confluent.

10.4 Preparation for test virus

10.4.1 General

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

10.4.2 Influenza virus

10.4.2.1 Place 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 (7.28).

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/ml to 10^4 PFU/ml or $TCID_{50}$/ml.

10.4.2.6 Inoculate 1 ml of the adjusted base influenza virus suspension 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 Place 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 with trypsin (9.16) in the flask of 10.4.2.7.

10.4.2.9 Place the flask of 10.4.2.8 in the CO_2 incubator (7.24) at a temperature of 34 °C for 1 day to 3 days to multiply the influenza virus.

10.4.2.10 Observe the cytopathic effect by an inverted microscope (7.16) 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 (7.26).

10.4.2.12 Centrifuge the multiplied virus suspension of 10.4.2.11 by using a centrifuge (7.18) 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 (7.28) 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 of virus suspension is less than 1×10^7 PFU/ml or $TCID_{50}$/ml, prepare it from 10.4.2.1.

10.4.2.15 Just before use, put the cryopreserved virus suspension of 10.4.2.13 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 used for the test. If not used immediately, preserve in the refrigerator (7.14) at a temperature of 4 °C.

If needed, just before use, the virus suspension for the test of 10.4.2.16 can be preceded 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 Place 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 (7.28).

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/ml to 10^6 PFU/ml 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 Place 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 (9.8) may be changed as needed.

10.4.3.9 Place 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 day to 3 days to multiply the viruses.

10.4.3.10 Observe the cytopathic effect by an inverted microscope (7.16) and judge the multiplication of the feline calicivirus. If the multiplication of the virus is confirmed, proceed to 10.4.3.11.

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 (7.28) 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 of virus suspension is less than 1×10^7 PFU /ml or $TCID_{50}$/ml, prepare it from 10.4.3.1.

10.4.3.15 Just before use, put the cryopreserved virus suspension of 10.4.3.13 in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

10.4.3.16 This is to be the virus suspension used 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 10.4.3.16 can be preceded 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 Other viruses

In case of SARS-CoV-2, prepare the test virus suspension according to Annex B. In case of additional examples of viruses described in Annex F, prepare the test virus suspension in accordance with the appropriate way that produces high titres of infectious viruses.

10.4.5 Infectivity titre of the test viruses

10.4.5.1 Preparation for series of the dilution for the virus suspension

10.4.5.1.1 Put 1,8 ml of the maintenance medium (9.8) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28).

10.4.5.1.2 Add 0,2 ml of the virus suspension for the test of 10.4.2.16 and 10.4.3.16 in the test tubes of 10.4.5.1.1 and agitate the test tubes well by a mixer (7.10).

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

10.4.5.1.3 Put 1,8 ml of the maintenance medium (9.8) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28).

10.4.5.1.4 Add 0,2 ml of the suspension of 10.4.5.1.2 to the test tubes of 10.4.5.1.3 and agitate them well.

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

10.4.5.1.5 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 the observation from the infectious wells for all of 8 wells to 0 wells.

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}.

10.4.5.2 Determination of the infectious titre

10.4.5.2.1 Plaque assay

Determine the infectivity titre according to Annex C.

10.4.5.2.2 $TCID_{50}$ method

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

10.5 Preparation of test specimens

10.5.1 Control fabric

The untreated test sample fabric or the 100% cotton woven fabric specified in ISO 105-F02 shall 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 and 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 them 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. If test specimens tend to curl easily, or it contains wadding or down, place a glass rod (7.30) onto the test specimens in the viral.

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

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

10.5.3 Sterilization of control test specimens and antiviral test specimens

10.5.3.1 Put control test specimens and antiviral test specimens in the vial containers one by one and place 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 Place 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 control test specimens and antiviral test specimens and place 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, close all vial containers by the caps.

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 test specimens and 3 antiviral test specimens sterilized in 10.5.3 in the vial containers, and add 20 ml of the wash-out solution (9.15), in all containers. Then, close the containers by the caps and agitate them by a 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 C or Annex D.

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 control test specimen) – lg (PFU/ml or $TCID_{50}$/ml of antiviral test 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 control test specimens and antiviral test specimens

All control test specimens and antiviral test specimens are prepared in the vial containers with caps in 10.5.

The preparation of control test specimens and antiviral test 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 (7.25) under the testing conditions as described in 11.2 and 11.3. Then, aseptically transfer the control test specimens and antiviral test specimens in sterile vials before proceeding to 11.4 and 11.5.

11.2 Deposit of virus to the control test specimens and antiviral test specimens

Deposit exactly 0,2 ml of the virus suspension prepared in 10.4 onto the control test specimens and antiviral test specimens at several points of the control test specimens and antiviral test specimens in the vial containers by micropipette (7.8) for all. Then put the caps on all vial containers and close them.

11.3 Contacting time

Place 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 can 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 test specimens in 11.2, add 20 ml of wash-out solution (9.15) in the vial containers. Then, close the containers by the caps, and agitate them by mixer (7.10) for 5 s and 5 times for washing out the virus from the control test specimens.

NOTE This virus suspension will be the base wash-out virus suspension of the control test specimen.

11.5 Wash-out of virus after contacting time

After contacting for 2 h in 11.3, add 20 ml of wash-out solution (9.15) in the vial containers, then close the containers by caps, and agitate them by mixer (7.10) for 5 s and 5 times to wash out the virus from the control test specimens and antiviral test specimens.

NOTE These virus suspensions are base wash-out virus suspensions of the antiviral test specimens and the control test 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 wash-out solution (9.15), 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) which is kept in the refrigerator (7.14) at 4 °C in new test tubes (7.28).

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 agitate the test tubes well by a 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) which is kept in the refrigerator (7.14) at 4 °C in new test tubes (7.28). Then put and keep it in the ice bath or refrigerator at temperature of 4 °C.

12.4 Add 0,2 ml of the virus suspension of 12.2 to the test tubes of 12.3 and agitate 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 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 C.

13.2 $TCID_{50}$ method

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

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

The example of testing method using specific pathogen free embryonated hen's eggs is described in Annex G.

14 Calculation of infectivity titre

14.1 Plaque assay

Calculate the infective titre using 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.

Calculate the infectivity titre (PFU/ml), W, using Formula (2):

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

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 $TCID_{50}$ method

14.2.1 General

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

14.2.2 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):

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

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

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

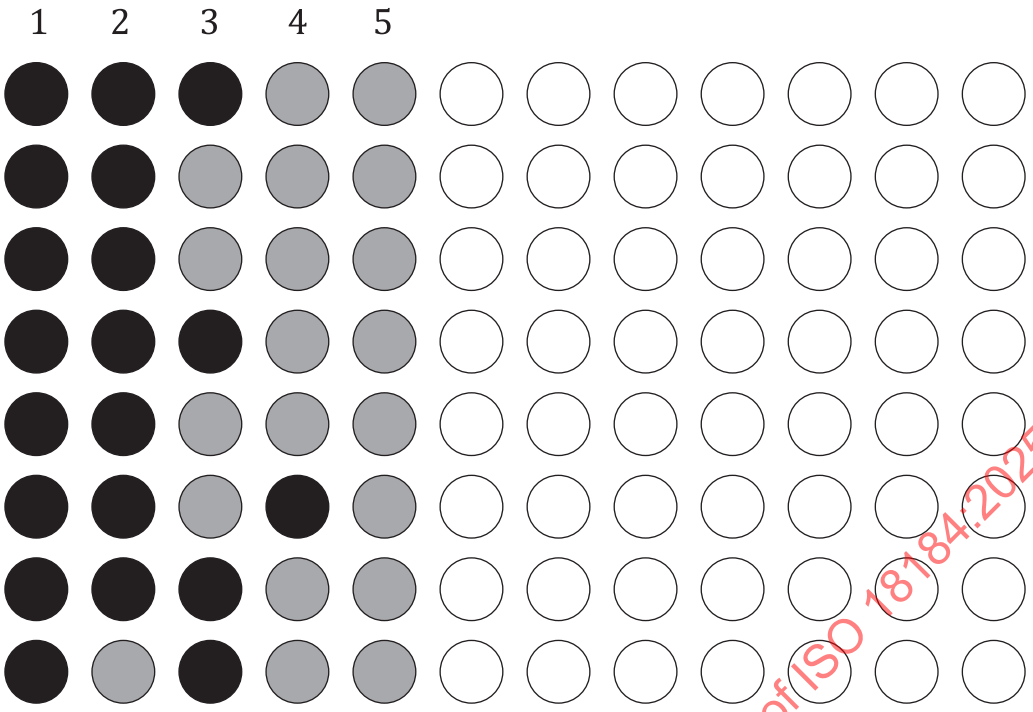
where

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

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

14.2.3 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

Verify the test according to following validation criteria of this test.

a) The virus infective titre of inoculated concentration shall have the following conditions for the test:

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

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

b) Confirmed the efficiency for suppression of agent activity of test specimen in 10.6.

c) Calculate the logarithm reduction value of infective titre of control test specimen using Formula (7) which is rounded to one decimal place. It shall be 2 or less

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

where

M is the reduction value;

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

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

14.3.2 Calculation of antiviral activity value

In case the logarithm reduction value of infective titre of control test specimen, M of the Formula (7) is less than 1,0 (M <1,0), calculate the antiviral activity value by Formula (8), and round to one decimal place.

$$M_v = \lg(V_a/V_c) = \lg(V_a) - \lg(V_c) \quad (8)$$

In case the logarithm reduction value of infective titre of control test specimen, M of Formula (7) is between 1,0 and 2,0 including 1, 0 and 2,0 ($1{,}0 \leq M \leq 2{,}0$), calculate the antiviral activity value by Formula (9), and round to one decimal place.

$$M_v = \lg(V_b/V_c) = \lg(V_b) - \lg(V_c) \quad (9)$$

where

M_v is the antiviral activity value;

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

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

$\lg(V_c)$ Is the common logarithm average of 3 infectivity titre value after 2 h contacting with the antiviral test specimen.

15 Example of test results

The interlaboratory test has been executed and the test results are shown in Annex I, Annex J and Annex K.

16 Judgement of antiviral efficacy

The obtained value may be judged as the level of antiviral efficacy as shown in Annex H.

17 Test report

The test report shall contain the following information:

a) a reference to this document, i.e. ISO 18184:2025;

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;

g) date of the test.

Annex A
(informative)

Virus strains and host cells

Examples of virus strains and the host cells used in this document is shown in Table A.1.

Table A.1 — Examples of 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 can be used after appropriate validation regarding its sensitivity against each viruses. [b] Other media can be used after appropriate validation for the growth of cells.		

Annex B
(normative)

Test method for SARS-CoV-2

B.1 General

This method specifies the testing method for antiviral activity assay of the textile products by using SARS-CoV-2. The host cell and media for SARS-CoV-2 used in this document is shown in Table B.1. The SARS-CoV-2 strain to be used in this document can be identified by Real-time RT-PCR as SARS-CoV-2 if needed. As an example of primers and probes for Real time RT-PCR detection, see "2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Panel Primers and Probes" (Effective: 24 Jan 2020) as shown in Table B.2.

Table B.1 — The host cell and media for SARS-CoV-2 used for this document

Virus kind	Severe respiratory syndrome coronavirus 2 (SARS-CoV-2) [a]
Virus strain	Virus strain identified by Real-time RT-PCR as SARS-CoV-2
Host cell	VeroE6/TMPRSS2 (African green monkey kidney cell origin)
Growth medium	DMEM (B.2.5)
[a] SARS-CoV-2 strains are routinely used for this method in Japan.	

Table B.2 — Example of primers and probes for Real-time RT-PCR detection of SARS-CoV-2

	Name	Base sequence (5' to 3')
Primer	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT
	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG
Probe	2019-nCoV_N1-P	ACCCCGCATTACGTTTGGTGGACC
Primer	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA
	2019-nCoV_N2-R	GCGCGACATTCCGAAGAA
Probe	2019-nCoV_N2-P	ACAATTTGCCCCCAGCGCTTCAG

B.2 Preparation of reagent and medium

B.2.1 Growth medium

B.2.1.1 Prepare a measuring flask (7.3) of 1 l, and put the following materials into the flask (7.3):

— Dulbecco's Modified Eagle Medium (DMEM) (low glucose) (B.2.5) 1 000 ml;

— G418 Sulfate Solution (B.2.6) 10,5 ml.

B.2.1.2 Dissolve the materials of B.2.1.1 and mix well.

B.2.1.3 Add 52,5 ml of Inactivated fetal bovine serum (9.6) into the mix solution of B.2.1.2.

B.2.2 Maintenance medium

B.2.2.1 Prepare a measuring flask (7.3) of 1 l, then, put the following materials in the flask:

— water (9.1), 800 ml;

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

B.2.2.2 Dissolve the materials of B.2.2.1 well, then, make up the whole amount to 1 000 ml by adding water (9.1).

B.2.2.3 Sterilize the mix solution from B.2.2.2 by using the filter (7.13) with a pore size of 0,22 µm.

B.2.2.4 Add 15 ml of 7,5 % sodium bicarbonate solution (9.3) in the mix solution of B.2.2.3.

B.2.3 Agar medium preparation

B.2.3.1 Liquid A

B.2.3.1.1 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.

Dissolve the materials and mix well.

B.2.3.1.2 Only for the plaque assay of the SARS-CoV-2 test, add 40 ml of the inactivated fetal bovine serum (FBS) (9.6).

B.2.3.1.3 Put the mix solution of B.2.3.1.1 and B.2.3.1.2 in the water bath (7.9) at a temperature of 37 °C and keep it until using.

B.2.3.2 Liquid B

See 9.14.2.

B.2.4 Wash-out solution

B.2.4.1 Prepare a sterilized culture container (7.27) of 1 l, and put the following materials into the culture container (7.27) and dissolve the materials and mix well:

— Dulbecco's Modified Eagle Medium (DMEM) (low glucose) (B.2.5) 1 000 ml;

— Inactivated fetal bovine serum (FBS) (9.6) 20,4 ml.

B.2.4.2 Then, add 50 ml of SCDLP medium 450 ml of the mix solution of B.2.4.1.

B.2.5 Dulbecco's modified Eagle medium (DMEM) (low glucose)

The composition of DMEM is specified in Annex E and this is available in the market. When use of commercial DMEM, check the composition and if there are lack of components comparing to Annex E, add the components to use.

B.2.6 G418 Sulfate solution

Dissolve 5 g of G418 sulfate into 50 ml of water (9.1). Then, sterilize the mix solution by using the filter (7.13) with a pore size of 0,22 µm.

B.2.7 Maintenance medium with FBS

Prepare a sterilized culture container (7.27) of 1 l, and put the following materials into the culture container (7.27) and dissolve the materials and mix well:

— Dulbecco's modified Eagle medium (DMEM) (low glucose) (B.2.5) 1 000 ml;

— inactivated fetal bovine serum (FBS) (9.6) 20,4 ml.

B.3 Preparation

B.3.1 Restoration of host cell from cryopreservation

B.3.1.1 Put the cryopreserved host cell in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

B.3.1.2 Prepare a new flask (7.23) for cell culture with a vent cap lid and put 20 ml of growth medium (B.2.1) in the flask.

B.3.1.3 Put whole ampule of the defrosted host cell of B.3.1.1 in the flask.

B.3.1.4 Put the flask of B.3.1.3 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.

B.3.1.5 Then, observe the flask of B.3.1.4 by microscope (7.16) if the cells are attached on the bottom of the flask.

If the growth is confirmed, then proceed to B.3.1.6. If not, continue to culture the host cell in the incubator.

B.3.1.6 Drain the remained growth medium in the flask of B.3.1.5.

B.3.1.7 Add 20 ml of the new growth medium (B.2.1) to the flask of B.3.1.6.

B.3.1.8 Put the flask of B.3.1.7 in the CO_2 incubator (7.24) at a temperature of 37 °C for (48 ± 2) h.

B.3.1.9 Observe the flask of B.3.1.8 using a microscope (7.16) 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 the step of B.3.1.8 until the sufficient growth is confirmed.

B.3.1.10 Then, proceed to the serial subcultivation by taking the following steps of B.3.2.

B.3.2 Subculture of host cell

B.3.2.1 Observe the flask of B.3.1.8 by using a microscope (7.16). If the confluent growth of host cell on the bottom of the flask is confirmed, then, drain an extra growth medium of the flask.

B.3.2.2 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.

B.3.2.3 Add 1 ml of Trypsin EDTA solution (9.12) in the flask of B.3.2.2 and spread the solution over the whole surface. After this, remove the excess Trypsin EDTA solution from the flask.

B.3.2.4 Put the flask of B.3.2.3 in the CO_2 incubator (7.24) at a temperature of 37 °C for 10 min ± 1 min to keep warm.

B.3.2.5 Observe visually the flask of B.3.2.4 if the grown cells are starting to come off, if confirmed, tap the side of the flask and disperse the cells.

B.3.2.6 Add 5 ml of the growth medium (B.2.1) in the flask of B.3.2.5 and disperse the cells by pipetting by using pipette (7.5) the medium to make it mild. Mix well so as to avoid the damage to the cells.

B.3.2.7 Prepare a new flask (7.23) for cell culture and add 20 ml of the growth medium (B.2.1).

B.3.2.8 Add 1 ml of the cell suspension of B.3.2.6 by using the pipette (7.5) to the flask of B.3.2.7.

The amount of the cell suspension of B.3.2.6 added in B.3.2.8 may be changed as needed.

B.3.2.9 Close the cap of the flask of B.3.2.8 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.

B.3.2.10 When the subculture of host cell is repeated, repeat the steps from B.3.2.1 to B.3.2.9.

B.3.3 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 and $TCID_{50}$ method.

B.3.3.1 Put 20 ml of growth medium (B.2.1) in the culture medium container (7.27) and add 1ml of the subcultured cell suspension of B.3.2.6. The amount of subcultured cell suspension of B.3.2.6 may be changed as needed.

B.3.3.2 Put 3 ml of the cell suspension of B.3.3.1 in each hole of the 6 wells plastic plate (7.22) for the plaque assay test. And put 0,1 ml of the cell suspension of B.3.3.1 in each well of the 96 wells microplate (7.21) for $TCID_{50}$ method.

B.3.3.3 Place 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.

B.3.3.4 Observe the condition of the cells by the inversed microscopy if the multiplied cells are distributed uniformly and confluent.

B.3.4 Preparation of test virus

B.3.4.1 Place the cryopreserved base virus in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

B.3.4.2 Drain the growth medium from the flask of B.3.2.9 with the cultured cells in the monolayer.

B.3.4.3 Add 5 ml of the maintenance medium (B.2.2) in the flask of B.3.4.2. Wash the surface of the cultured cells and drain the maintenance medium. Repeat the washing procedure 2 times.

B.3.4.4 Prepare a new test tube (7.28).

B.3.4.5 Put the defrosted base SARS-CoV-2 of B.2.4.1 in the test tube of B.3.4.4 dilute with the maintenance medium (B.2.2) and adjust the concentration of virus to 10^3 PFU/ml to 10^4 PFU/ml or $TCID_{50}$/ml.

The concentration of virus suspension may be changed as needed.

B.3.4.6 Inoculate 1 ml of the adjusted base SARS-CoV-2 suspension of B.3.4.5 on the surface of cell in the flask of B.3.4.3 and spread to the whole surface.

B.3.4.7 Place the flask of B.3.4.6 in the CO_2 incubator (7.24) at a temperature of 37 °C and keep it for 1,5 h to adsorb the virus to the cells.

B.3.4.8 Put 20 ml of the maintenance medium (B.2.2) in the flask of B.3.4.7.

The amount of the maintenance medium (B.2.2) may be changed as needed.

B.3.4.9 Place the flask of B.3.4.8 in the CO_2 incubator (7.24) at a temperature of 37 °C for 1 day to 3 days to multiply the SARS-CoV-2.

B.3.4.10 Observe the cytopathic effect by an inverted microscope (7.16) and judge the multiplication of new corona virus. If the multiplication of SARS-CoV-2 is confirmed, proceed to B.3.4.11.

B.3.4.11 Put the multiplied virus suspension in the centrifugal tube (7.26).

B.3.4.12 Centrifuge the multiplied virus suspension of B.3.4.11 by using a centrifuge (7.18) at a temperature of 4 °C and 1 000 ×g for 15 min.

B.3.4.13 Take the supernatant suspension from the centrifugal tube after the centrifugation. This is to be the SARS-CoV-2 suspension. Divide the suspension into test tubes (7.28) appropriately and cryopreserve at −80 °C in the freezer (7.11) or −196 °C in the liquid nitrogen bath (7.12).

B.3.4.14 Check the concentration of the virus if it is more than 10^7 PFU or $TCID_{50}$/ml for SARS-CoV-2 by the plaque assay or $TCID_{50}$ method. If the concentration of virus suspension is less than 1×10^7 PFU/ml or $TCID_{50}$/ml, prepare it from B.3.4.1.

B.3.4.15 Just before use, place the cryopreserved virus suspension of B.3.4.14 in the water bath (7.9) at a temperature of 37 °C and keep it for rapid defrosting.

B.3.4.16 This is the virus suspension used 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 can be preceded 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.

B.3.5 Infectivity titre of the test viruses

B.3.5.1 Preparation for series of the dilution for the virus suspension

B.3.5.1.1 Put 1,8 ml of the maintenance medium with FBS (B.2.7) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28).

B.3.5.1.2 Add 0,2 ml of the virus suspension for the test of B.3.4.16 in the test tubes of B.3.5.1.1 and agitate the test tubes well by a mixer (7.10).

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

B.3.5.1.3 Put 1,8 ml of the maintenance medium with FBS (B.2.7) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28).

B.3.5.1.4 Add 0,2 ml of the suspension of B.3.5.1.2 to the test tubes of B.3.5.1.3 and agitate them well.

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

B.3.5.1.5 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 the observation from the infectious wells for all of 8 wells to 0 wells.

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}.

B.3.5.2 Determination of the infectious titre

B.3.5.2.1 Plaque assay— Test procedure

B.3.5.2.1.1 Using a microscope (7.16), observe the confluency of the cell cultures prepared in 6 well plates prepared in B.2.3. If the cells are confluent, drain the growth medium from the plate.

B.3.5.2.1.2 Add 3 ml of the maintenance medium (B.2.2) and gently swirl to wash the cell surface. Remove the medium and repeat the washing procedure 2 times.

B.3.5.2.1.3 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 maintenance medium with FBS (B.2.7) for validation of the medium.

B.3.5.2.1.4 Place the plate of B.2.5.2.1.3 in the CO_2 incubator (7.24) at the temperature listed in Table B.2 and keep it for 1,5 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.3.5.2.1.5 Put 3 ml of the maintenance medium (B.2.2) in the plate and wash the surface, then drain the extra maintenance medium.

B.3.5.2.1.6 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.3.5.2.1.7 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.3 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.

B.3.5.2.1.8 Drain the agar medium from B.3.5.2.1.7, add 3 ml of the methylene blue solution (9.5), then keep it at room temperature for 15 min to dye the cells.

B.3.5.2.1.9 Wash the extra methylene blue solution with tap water. Confirm the dyeing of the cells.

B.3.5.2.1.10 Count the number of plaques (white blotches).

B.3.5.2.1.11 Take average of two counts.

See Figure B.1.

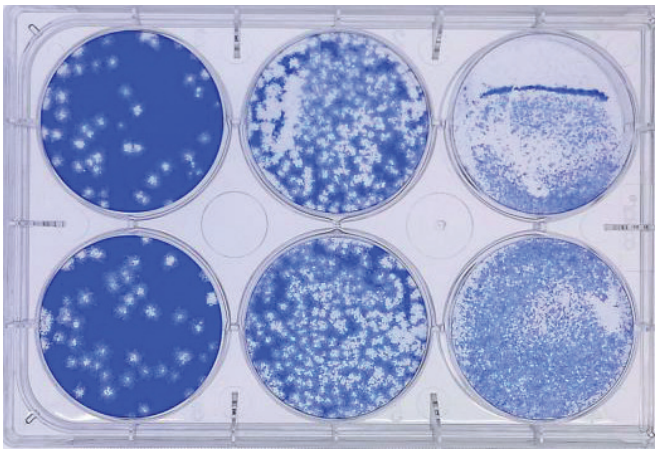


Figure B.1 — Photo of an example of plaque assay

Table B.3 — Carbon dioxide incubator condition

Virus for examination	SARS-CoV-2
Clause applied	B.3.5.2.1 and B.6.1
Adsorption temperature setting °C	37
Culture temperature setting °C	

B.3.5.2.2 Determination of PFU

B.3.5.2.2.1 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 plaque 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.3.5.2.2.2 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.4.

Table B.4 — Interpretation of the data

Dilution	Wash-out virus suspension	1st dilution	2nd dilution	3rd dilution	Nth 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.

B.3.5.2.3 $TCID_{50}$ method — Test procedure

B.3.5.2.3.1 Observe at the microscope (7.16) the confluency of the cell cultures prepared in 96 wells microplates prepared in B.3.3. If the cells are confluent, drain the growth medium from the plate.

B.3.5.2.3.2 Add 0,1 ml of the maintenance medium (B.2.2) and gently swirl to wash the cell surface. Remove the medium and repeat the washing procedure 2 times.

B.3.5.2.3.3 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 maintenance medium (B.2.2) for validation of the cells.

B.3.5.2.3.4 Put the microplate of B.3.5.2.3.3 in the CO_2 incubator (7.24) at a temperature of the listed in Table B.4 and keep it for 1,5 h to let the cells absorb the virus.

B.3.5.2.3.5 Drain the supernatant from the plate.

B.3.5.2.3.6 Add 0,1 ml of the maintenance medium (B.2.2), wash the surface by the medium and drain the extra maintenance medium.

B.3.5.2.3.7 Add 0,2 ml of the maintenance medium with FBS (B.2.7), then place the microplate in the CO_2 incubator (7.24) at a temperature of the listed in Table B.5 and keep it for 7 days to culture.

B.3.5.2.3.8 Observe each cell in the wells by inverted microscopy if the cytopathic effect of the cell is occurred.

B.3.5.2.3.9 Confirm the cytopathic effect and calculate $TCID_{50}$ by Behrens and Karber method.

Table B.5 — Carbon dioxide incubator condition

Virus for test	SARS-CoV-2
Clause applied	**B.3.5.2.3 and B.7.2**
Adsorption temperature setting °C	**37**

B.3.6 Preparation of test specimens

B.3.6.1 Control fabric

See 10.5.1.

B.3.6.2 Preparation of test specimens

See 10.5.2.

B.3.6.3 Sterilization of control test specimens and antiviral test specimens

See 10.5.3.

B.3.7 Control test

See 10.6.

B.4 Test procedure

B.4.1 Preparation of control test specimens and antiviral test specimens

See 11.1.

B.4.2 Deposit of virus to the control test specimens and antiviral test specimens

See 11.2.

B.4.3 Contacting time

See 11.3.

B.4.4 Wash-out of virus immediately after deposit

See 11.4.

B.4.5 Wash-out of virus after contacting time

See 11.5.

B.5 Preparation of the series of the dilution for the virus suspension

B.5.1 Put 1,8 ml of the maintenance medium with FBS (B.2.7) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28).

B.5.2 Add 0,2 ml of the wash-out virus suspension of B.4.4 and B.4.5 in the test tubes of B.5.1, and agitate the test tubes well by a 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.

B.5.3 Put 1,8 ml of the maintenance medium with FBS (B.2.7) which is kept in the refrigerator (7.14) at 4 °C in a new test tube (7.28). then put and keep it in the ice bath or refrigerator at temperature of 4 °C.

B.5.4 Add 0,2 ml of the virus suspension of B.5.2 to the test tubes of B.5.3 and agitate 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.

B.5.5 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.

B.6 Determination of the infectious titre

B.6.1 Plaque assay

Determine the infectivity titre by plaque assay according to B.3.5.2.1.

B.6.2 $TCID_{50}$ method

Determine $TCID_{50}$ according to B.3.5.2.3.

B.7 Calculation of infectivity titre

B.7.1 Plaque assay

See 14.1.

B.7.2 $TCID_{50}$ method

See 14.2.

B.7.3 Test result

B.7.3.1 Verification of this test

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

   — SARS-CoV-2 suspension > 10^7 PFU/ml or $TCID_{50}$/ml

b) See 14.3.1 b).

c) See 14.3.1 c).

B.7.3.2 Calculation of antiviral activity value

See 14.3.2.

Annex C
(normative)

Infectivity titre test — Plaque assay

C.1 Test procedure

C.1.1 Using a microscope (7.16), 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.

C.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.

C.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.

C.1.4 Place the plate of C.1.3 in the CO_2 incubator (7.24) at the temperature listed in Table C.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.

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

C.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.

C.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 C.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.

C.1.8 Drain the agar medium from C.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.

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

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

C.1.11 Take average of two counts.

See Figure C.1.

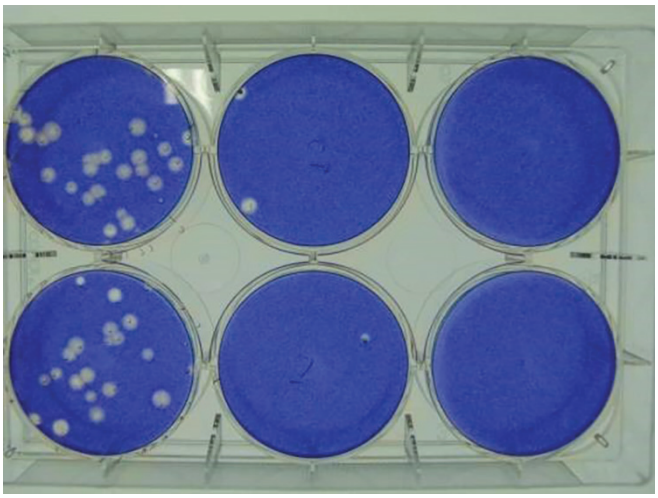


Figure C.1 — Photo of an example of plaque assay

Table C.1 — Carbon dioxide incubator condition

Virus for examination	Influenza virus	Feline calicivirus
Clause applied	10.4.5.2.1 and 13.1	
Adsorption temperature setting °C	34	37
Culture temperature setting °C		

C.2 Determination of PFU

C.2.1 General

The plaques (white brotches) are countable by around 60 points. Over 60 points, the separation of plaques (white brotches) becomes unclear. In another case where no plaques are observed, then as a possibility the average of two becomes less than 1. The determination of PFU is defined in C.2.2.

C.2.2 Determination of PFU

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 C.2.

Table C.2 — Interpretation of the data

Dilution	Wash-out virus suspension	1st dilution	2nd dilution	3rd dilution	Nth 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 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 D
(normative)

Infectivity titre test: $TCID_{50}$ method — Test procedure

D.1 Observe at the microscope (7.16) 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.

D.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.

D.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 cells.

D.4 Put the microplate of D.3 in the CO_2 incubator (7.24) at a temperature of the listed in Table D.1 and keep it for 1 h to let the cells absorb the virus.

D.5 Drain the supernatant from the plate.

D.6 Add 0,1 ml of the maintenance medium (9.8), wash the surface by the medium and drain the extra maintenance medium.

D.7 In case of the influenza virus, add 0,2 ml of the maintenance medium with trypsin (9.16) and in case of the feline calicivirus, add 0,2 ml of the maintenance medium (9.8). Then place the microplate in the CO_2 incubator (7.24) at a temperature of the listed in Table D.1 and keep it for 7 days to culture.

D.8 Observe each cell in the wells by inverted microscopy if the cytopathic effect of the cell is occurred.

D.9 Confirm the cytopathic effect and calculate $TCID_{50}$ by Behrens and Karfber method.

Table D.1 — Carbon dioxide incubator condition

Virus for test	Influenza virus	Feline calicivirus
Clause applied	10.4.5.2.2 and 13.2	
Adsorption temperature setting °C	34	37
Culture temperature setting °C		

Annex E
(informative)

Composition of media

E.1 General

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

E.2 Composition of EMEM

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

Table E.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
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

E.3 Composition of RPMI 1640 medium

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

Table E.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
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

E.4 Composition of DMEM medium

The composition of DMEM (low glucose) is shown in Table E.3. The DMEM medium is available in the market, however, if there are lack of components of the composition listed in Table E.3, add them accordingly to the Table E.3.

Table E.3 — Composition of DMEM medium (low glucose)

Composition in 1 000 ml water		mg
Amino acids	L-Arginine HCl	84,00
	L-Cystine 2HCl	62,60
	L-Glutamine	584,00
	Glycine	30,00
	L-Histidine HCl H_2O [Free Base]	42,00
	L-Isoleucine	105,00
	L-Leucine	105,00
	L-Lysine HCl	146,00
	L-Methionine	30,00
	L-Phenylalanine	66,00
	L-Serine	42,00
	L-Threonine	95,00
	L-Tryptophan	16,00
	L-Tyrosine 2Na $2H_2O$	103,79
	L-Valine	94,00
Vitamins	Choline Chloride	4,00
	Folic acid	4,00
	myo-Inositol	7,20
	Niacinamide	4,00
	D-Pantothenic acid Hemicalcium	4,00
	Pyridoxine HCl	4,04
	Riboflavin	0,40
	Thiamine HCl	4,00
Inorganic salts	Calcium Chloride	200,00
	Iron (3) Nitrate Nonahydrate	0,10
	Magnesium Sulfate [Anhydrous]	97,67
	Potassium Chloride	400,00
	Sodium Dihydrogen Phosphate	3 700,00
	Sodium Chloride	6 400,00
	Disodium hydrogen phosphate anhydrous	109,00
Others	D-Glucose	1 000,00
	Phenol Red Na	15,90
	Pyruvic acid Na	110,00

Annex F
(informative)

Additional examples of viruses and host cells

F.1 General

The main flame of this document chooses two viruses as influenza in category of with envelope and feline calicivirus in category of without envelope. Because of outbreak of the new corona virus, SARS-CoV-2 has been added to this test procedure. However, the safety level of SARS-CoV-2 is higher and the handling of this virus is limited in higher safety level cabinet. Under this situation, the following viruses are added as examples of viruses for using in this test procedure. The purpose of the test using these viruses shall have the consensus among concerned parties.

F.2 Virus strain and host cell

Table F.1 — Additional examples of the virus strain and host cell

Virus kind	**Human enterovirus 71** [a]	**Human coronavirus (229E)** [b]	**Human coronavirus (OC43)** [b]	**Feline coronavirus** [c]
Virus strain	*Human Enterovirus* 71 (EV71) ATCC VR-1432	*Human coronavirus* 229E Strain 229E ATCC VR-740	*Human coronavirus* OC43 Strain OC43 ATCC VR-1558	*Feline coronavirus* Strain Munich
Host cell	Vero cell (African green monkey kidney cell origin)	MRC-5 cell (Human lung fibroblast) ATCC CCL-171	HCT-8 [HRT-18] cell (Human large intestine epithelial) ATCC CCL-244	CRFK cell (Cat kidney cell origin) ATCC CCL-94
Culture medium	Eagle's Minimal Essential Medium (EMEM)	Dulbecco's Modified Eagle Medium (DMEM)	Dulbecco's Modified Eagle Medium (DMEM)	Eagle's Minimal Essential Medium (EMEM)

[a] Human Enterovirus 71 are routinely used in China.

[b] Human coronavirus 229E and OC43 are routinely used in US.

[c] Feline coronavirus is used in UK and Europe.

F.3 Preparation of the test

The preparation of the viruses shall meet the test requirements.

Annex G
(informative)

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

G.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.

G.2 Overview

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

G.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.

G.4 Preparation of test specimen

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

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

G.4.3 Put the test specimen into the polyethylene bag with vinyl zipper.

NOTE There is no need for sterilization of test specimen.

G.5 Preparation of the embryonated hen's eggs

Choose 10-day old embryonated hen's eggs.

G.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.

G.6.1 Virus cultivation

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

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

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

G.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.

G.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.

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

G.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 (7.28).

G.6.2.2 Mount the test tube on the centrifuge and centrifugalize (7.18) it by 1 000 × g for 10 min.

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

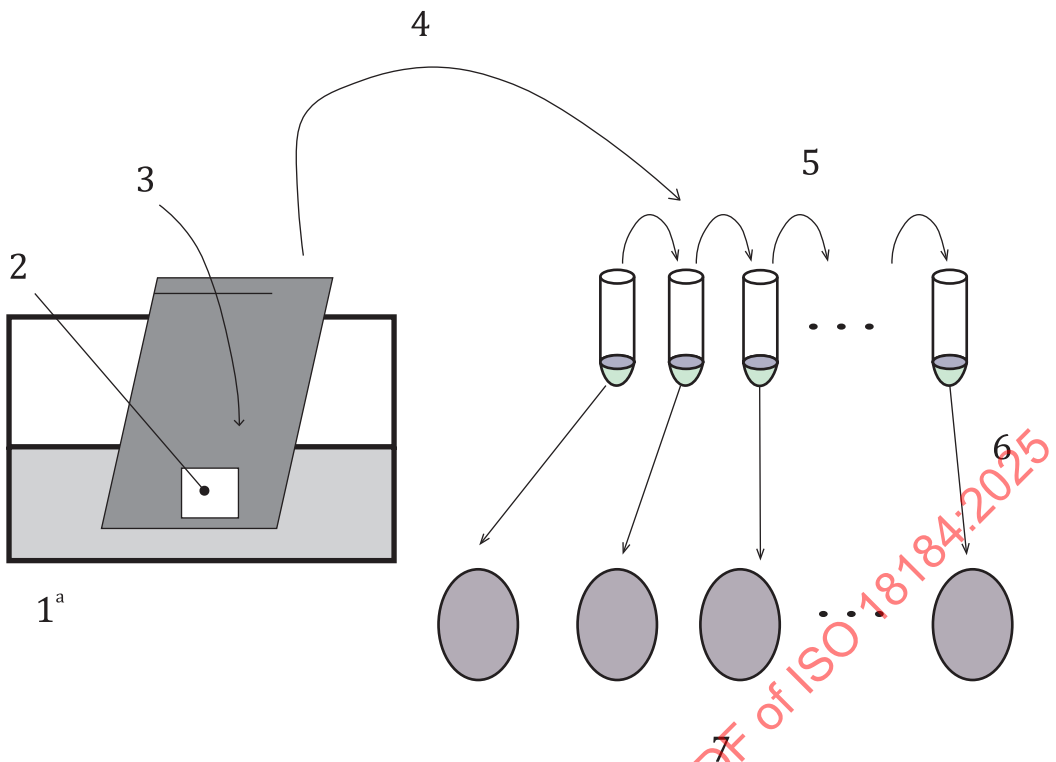
G.6.2.4 Repeat to centrifuge another two times.

G.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.

G.7 Test procedure

G.7.1 General

General process image is shown in Figure G.1.



Key

1 water bath 4 °C
2 test 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 G.1 — General process image of embryonated eggs method

G.7.2 Contact the virus suspension to the test specimen

G.7.2.1 Put the test specimen in a polyethylene bag with zipper.

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

G.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.

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

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

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

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