OECD GUIDELINE FOR THE TESTING OF CHEMICALS

QUANTITATIVE METHOD FOR EVALUATING VIRUCIDAL ACTIVITY OF MICROBICIDES USED ON HARD NON-POROUS SURFACES

INTRODUCTION

Summary

1. This method uses disks (1 cm in diameter) of brushed stainless steel as default carrier to represent hard, non-porous environmental surfaces. Each disk receives 10 µL of the test organism in a soil load. The inoculum is dried and exposed to 50 µL of the use-dilution of the test substance; control carriers receive an equivalent volume of a fluid harmless to the test organism. The contact time and temperature may vary as required. A neutralizer is added at the end of the contact time and the disks then eluted. Most or all of the eluate volume from each disk is assayed for the presence of viable organisms. Log_{10} reductions in the viability of the test organism are calculated in relation to the viability count on the control carriers.

Background and Scope

2. This Test Guideline is designed for testing the bactericidal activity of substances to be used on hard, non-porous surfaces Springthorpe and Sattar (1) (2). Assessments of microbicidal activity using carrier tests give a better indication of the potential of a given microbicide used on surfaces to perform under field conditions. International harmonisation of test methodology has been developed from the OECD workshop (3), reports and ongoing national and international initiatives that mandate such testing be quantitative in nature, and has agreed upon performance criteria. Performance criteria may vary depending on the intended use and label claim of the product. Data from such testing can also provide a basis for classification and labelling of a tested formulation. Statistical techniques are employed to ensure data validity. This test has evolved as a modification of a previous standard of ASTM International (formerly known as American Society for Testing and Materials) (4) following significant international collaboration among OECD member countries. A ring trial to validate five new antimicrobial efficacy methods including this one was carried-out from 2007 to 2009 in which thirty-five laboratories from eight member countries participated and a validation report (5) was produced. For additional background and interpretation of the results, refer to the Guidance Document developed to accompany the Test Guidelines for assessing the activity of microbicides used on hard surfaces (7).

3. Definitions and abbreviations used in this Test Guideline are given in Annex I. Details on relevant materials and reagents and the preparation of the test organisms are found in Annexes III – VI.

Prerequisites for test substance

4. The following information on the test substance should be known before the start of testing:

a) The physical state of test substance, its trade name or identification number (ID), lot number(s), source and receipt date at the testing laboratory.

b) Chemical nature and relative concentrations of active ingredients; such information may come from product label or manufacturer's safety data sheet (SDS).

c) Conditions and duration (shelf-life) for storage of test substance as specified by the manufacturer; depending on label claim and jurisdiction.

d) Directions to dilute the test substance to the level(s) at which it is to be tested; unless otherwise indicated by the manufacturer, hard water, as specified in Annex IV, is to be used as the diluent for test substances requiring dilution in water prior to testing (pH and any other adjustments required to prepare the test substance for testing is to be clearly documented).

Prerequisites for testing

5. The following information should also be known before the start of testing:

a) Specification(s) on test organism(s): source, strain number, growth medium and passage history in test laboratory.

b) The defined performance standard to adapt the number of test organism on dried carriers to be at least 0.5 $\log_{10}$ higher than the defined performance standard, but not higher than 1.5 $\log_{10}$.

c) Directions to prepare suspensions of test organism(s).

d) Specification(s) for default test carriers (and other optional carriers, if to be used).

e) Directions to prepare carriers for inoculation.

f) Directions to inoculate carriers with test organism(s).

g) Specification for numbers of test and control carriers to be used.

h) Directions to apply the test substance to assess microbicidal activity.

i) Directions for neutralisation of test substance and validation of the procedure.

j) Specification for performance criterion (a) when available.

k) Temperature(s) and contact time(s) to be used in testing.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of magnetized stainless steel. Surrogate test organisms are specified herein; however, test organisms more relevant to other settings, e.g., dairy, baking or brewing industries are permitted.

7. The soil load recommended is representative of body secretions and excretions and is also compatible with a wide variety of test organisms that may be used in testing. Other soil loads more relevant for particular applications may be permitted.

8. Certain jurisdictions require additional and/or alternate tests for formulations to be used on medical devices.

9. The method is suitable for testing liquid formulations and the liquid phase of aerosol, pump and trigger spray products. It is also suitable for testing the expressed liquid of towelette products. Refer to the Guidance Document for testing other product forms.

PRINCIPLE OF THE TEST

10. The viability of test organisms is evaluated after disks have been contaminated with test organisms in a soil load and then exposed to the test substance (microbicide) or control fluid (PBS). Disks of brushed stainless steel are used to represent hard, non-porous environmental surfaces. This method consists of the following eight consecutive steps:

a) Preparation of the carriers.

b) Preparation of the test organism and inoculum.

c) Validation of the neutralisation.

d) Inoculation, drying and transfer of the carriers.

e) Exposure of the dried inoculum to the test substance and carrier count control fluid (EBSS or PBS).

f) Neutralisation of the test substance and elution of the test organism.

g) Dilution and recovery of the test organism.

h) Counting the surviving test organisms on test and control carriers and assessing the performance of test substance.

i) Control: Neutralisation control, cytotoxicity control, interference-with-infectivity control and control to assess the influence of the soil on the host cells.

11. This method is fully quantitative and avoids any loss of viable test organisms during the procedure. The level of microbial challenge can also be adjusted in accordance with the desired product performance criterion (a). The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents.

12. The test organism with a soil load is placed at the centre of each carrier. The inoculum is then dried and covered with a defined volume of the test substance equivalent to 641 mL per m^2.

Contaminated control carriers receive an equivalent volume of EBSS or PBS. At the end of the contact time, the test substance is neutralised, the carriers are eluted and the eluates are assayed for viable test organisms. Log_{10} reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean of viable of test organisms on the control carriers.

TEST PROCEDURE

13. Before starting the test procedure a neutraliser is validated for each test organism and each test substance (only the highest concentration under test) using the protocol given in Annex II.

Preparation and sterilisation of carriers

14. The carriers are soaked in a suitable detergent solution (e.g. Blanisol-Pur-Test, 7X or equivalent) free from any antimicrobial activity for two-four hours to degrease and then rinsed thoroughly in distilled water. Four control carriers and three test carriers are used for each test organism and contact time/ temperature.

15. Up to 20 clean carriers are placed on a sheet of filter paper on the inside bottom surface of a glass Petri dish (150 mm in diameter) or a similar holder. Cover the Petri dish with its lid, wrap the entire dish and sterilise. Extended soaking of the carriers in water or detergent and prolonged rinsing should be avoided to reduce risk of corrosion or rusting. Some extra carriers are always prepared for testing in case a carrier is accidentally dropped or the inoculum on it runs over the edge.

Preparation of test organisms

16. The viruses and their host cells listed in the guidance document could be used for regulated testing. However, other specific viruses and test parameters should be checked before planning the testing to meet relevant regulatory requirements. The strain numbers given are for the American Type Culture Collection (ATCC). Equivalent strains from other established culture collections such as the National Collection of Type Cultures (NCTC) might be acceptable alternatives. The maintenance of virus and host cell stocks is described in Annex III. If other test organism are employed, adapt host cells, incubation requirements and any other test parameters as necessary.

17. Media for growing /maintaining host cells for making virus stocks and for conducting infectivity assays are described generically and may be sold under different trade names depending on the manufacturer (Annex III). Culture conditions (*e.g.* cell culture medium, cell line) for virus recovery should be similar to those for virus propagation.

18. Test viruses are prepared and assayed in permissive cultured host cells. Host cells are maintained in culture by trypsinising confluent monolayers and seeding or reseeding vessels appropriate to culture maintenance or titration of virus infectivity.

19. ***Viruses***: The selection of the test viruses for this method is based on their (a) relative safety for the laboratory staff, (b) ability to grow to titres sufficiently high for testing, (c) property to produce cytopathic effects or plaques, or both, in cell cultures, (d) potential to spread through contaminated environmental surfaces, and (e) relative innate resistance to a variety of chemicals. Other strains or types of viruses may be substituted provided they meet the preceding criteria. Depending on the regulatory agency to be notified and the types of claims to be made, testing against two or more of the following viruses may be required. There is insufficient information on whether the passage history, culture conditions, and strain differences of viruses can influence their susceptibility or resistance to chemicals. Caution must be exercised, however, when substituting viruses as this may lead to variations in results from one laboratory to another.

Proposed list of viruses *

Virus	Host cells
Human Adenovirus Type 5 (VR-1516)	293 cells (CRL-1573) for making virus pools and Vero cells for infectivity assays or A 549 cells for both
Hepatitis A Virus Strain HM-175 (VR-1402)	FRhK-4 (CRL-1688)
Canine Parvovirus-Cornell Strain (VR-2017)	A72 (CRL-1542)
Murine or bovine Parvovirus	PK 13
Murine Norovirus (strain S99 or MNV-1)	RAW 264.7
Human Rhinovirus Type 37 (VR-1147) or Type 14 (VR-284)	MRC-5 (CCL-171) or WI-38 (CCL-75), HeLa T^{4+}
Human Rotavirus Strain Wa (VR-2018)	MA-104 (CRL-2378) or CV-1 (CCL-70)
SV40	

* While only adenovirus 5 was used in the ring trial, the other viruses listed are also all non-enveloped with a potential to spread via contaminated hard, non-porous surfaces. They together represent major classes of enteric and respiratory viruses. Label claims for virucidal activity must be based on testing against at least three of the viruses listed as specified by the target regulatory agency. The host cells given for virus culture and quantitation of infectivity are those in common use. Other cell lines may be used with proper justification.

Method for preparing virus pools (further details are given in Annex III)

20. General: Remove growth medium from the culture flask containing the host cell monolayer. Wash monolayer with a buffered solution suitable for the host cells and inoculate the cells with a suitable volume of thawed virus suspension.

21. Incubate the flask for 60-90 minutes to allow for virus adsorption. Add maintenance medium, *e.g.* Eagle Minimal Essential Medium (EMEM) without serum or with about 2% serum, and incubate the flask at the appropriate temperature until about 75% of the monolayer shows virus-induced cell degeneration (cytopathology) when examined under an inverted microscope.

22. Freeze the flask at -20°C (15-20 minutes) and thaw as described in Annex III at least three times to release virus from infected cells. Centrifuge the contents of the flask at 1000x*g* for 10 minutes to remove gross cell debris and collect the supernatant containing the virus. In case of low yields of infectious virus, material from two or more flasks may be needed to allow for concentration.

Preparation of inocula

23. Add soil load to the virus inoculum before the contamination of carriers. Virus titres are confirmed in each test by determining the numbers of infective units of viruses (PFU/mL or $TCID_{50}$/mL) on each of the control carriers and in the virus suspension.

Inoculation and drying of carriers

24. The test suspension is vortexed for 10-30 seconds or until resuspended, but no more than 60 seconds, to evenly distribute the cells. To obtain 500 µL of the inoculums with a 5% load, 25 µL of BSA, 100 µL of mucin, and 35 µL of yeast extract stocks is added to 340 µL of the virus test suspension (see Table 1). The inoculum is vortexed again for 10 seconds.

Table 1: Volumes of test suspension and soil load to prepare the inoculum

Component	Volume (µL)
Test suspension	340
BSA	25
Mucin	100
Yeast extract	35
Total	**500**

25. 10 µL of the inoculum are withdrawn with a positive-displacement pipette (Figure 1), and deposited at the centre of a carrier (Figure 2), but the inoculum is not spread with the pipette tip. Inoculate all carriers required for the test. For consistency, the same pipette tip is used throughout the inoculation of a batch of carriers (number of carriers/test).

26. The Petri dish is placed in a biological safety cabinet with the laminar flow on at 20-25°C for 60 ± 10 minutes until visible dry. The temperature and humidity are documented.

Exposure of the dried inoculums to the test substance or carrier count control fluid

27. Proper timing is critical to ensure that each carrier receives the exact required exposure time. All carriers are treated the same during the test.

28. The procedure for exposure of the dried inoculums to the test substance or carrier count control fluid (PBS/EBSS) is as follows:

- using sterile forceps transfer each dried carrier (Figure 3) with the inoculated side up to the flat bottom vial (Figure 4);

- cap the vial;

- repeat until all carriers are transferred; carriers can be stored at 20-25°C for up to 60 minutes;

- use no less than four carriers as controls in each test and at least three test carriers per test organism for each lot of the test substance; additionally three carriers will be loaded with the virus suspension (without the soil load);

- using an air-displacement pipette, deposit 50 µL of the test substance equilibrated to 20-25°C over the dried inoculum on each test carrier, ensuring complete coverage of the inoculum, at predetermined staggered intervals (Figure 5); do not touch pipette tip to carrier; do not cap the vials; for consistency, use the same tip to dispense the test substance on all the disks in a given test.;

- hold test carriers at 20-25°C for selected contact period;

- Treat the control carriers the last by placing on each 50 µL EBSS or PBS (equilibrated to 20-25°C), instead of the test substance; hold the carriers at 20-25°C for selected contact period; and

- remove from testing any carrier where the test substance or control fluid has run over its surface or when it has been touched by the pipette tip.

29. A number of the test organisms on the dried carriers between 0.5 log_{10} and 1.5 log_{10} higher than the defined performance standard is needed. The upper limit of 1.5 log is set to exclude the influence of too high an inoculum on the results to enable a fair comparison of the test substances. The range in the virus titre given above will vary depending upon the number of laboratories used in the testing, as defined in the Guidance Document.

Neutralisation of test substance and elution of test organisms

30. Immediately (within 10 ± 2 seconds) neutralise the test substance at the end of the contact time; the protocol for the validation of the neutraliser is given in Annex II. At the end of the contact period, add 950 µL of validated neutraliser to each vial according to a predetermined schedule. For consistency across laboratories/operators, this should be documented in the test report as the undiluted sample.

31. Cap the vials and vortex each vial for 30 seconds to recover the inoculum. Examine each carrier visually and, in case of incomplete elution, perform further vortexing. Transfer the eluates into separate cryovials for further processing.

32. Complete the dilutions of the eluates and their inoculation into cell cultures within 30 minutes after elution of the carriers. Refer to Annex III for additional to be conducted before hand to rule any interference that the test substance and neutraliser may cause with virus infectivity assays

Dilution and recovery

33. Make 10-fold dilutions of the eluates using plastic cryovials and cell culture maintenance medium (with a suitable fetal bovine serum (FBS) concentration or without FBS) as the diluent. Titrate the samples for virus infectivity using appropriate host cell monolayers. This is done using either a plaque assay system or the most probable number (MPN) method based on tissue culture infective dose 50% ($TCID_{50}$) titration.

34. The elution procedure for control carriers is also the same as that described above for the test carriers. However, eluates from control carriers will always require 10-fold dilutions and processing of the material from dilutions that will provide the numbers of virus (infectivity titres /ml PFU or $TCID_{50/ml}$).

DATA AND REPORTING

Assessing performance of test substance

35. Performance is assessed by measuring the level of virus infectivity on each test carrier and comparing the level obtained to the mean of that on the control carriers. Data is summarized in a tabular form showing raw data for each test and control carrier. Data is to be presented to validate the neutralisation process used in the test and the results of the tests of cytotoxicity and interference.

Requirements the control have to fulfil to pass the test

36. *Neutralisation*: The difference between the virus titre of the samples with addition of the mixture of the test substance and neutraliser in comparison with the virus control should not exceed 0.5 $\log_{10}$.

Interference: The difference between the titre of the disinfectant treated cells in comparison with the virus control should not exceed 0.5 $\log_{10}$.

Cytotoxicity: The soil load must not influence the viability of the host cells. A reduction at least of 4 $\log_{10}$ has to be detectable. If there is a difference between the titres of the control carriers with and without soil load, the quality the soil load have to be checked and if necessary a new batch of soil load have to be used.

Calculating Log_{10} reductions

37. A method for determining $\log_{10}$ reduction in the viability titre of the target organism by the test substance in quantitative carrier tests such as this one has been described (7) (8).

$$\text{Log}_{10}\text{ Reduction} = \text{Average Log}_{10}\text{ Recovery Control} - \text{Average Log}_{10}\text{ Recovery Treatment}$$

Test report

38. The test report includes the following information:

Test and control substances

- A description of the test substance; physical state, colour and pH, trade name or identification number (ID), lot/batch number(s), date of manufacture or expiration date if available.
- Chemical nature and relative concentrations of active ingredients.

Details on the test method

Test virus

- Source
- Scientific name
- Strain number from ATCC or that of another recognised culture collection
- Preparation and passage history

Host cells used for virus growth and/or quantitation

- Source
- ATCC number
- Passage number in the test laboratory
- Culture and maintenance media used

Test conditions

- Temperature
- Contact time

Results

- CFU or $TCID_{50}$ per carrier
- Log_{10} reduction
- Copies of the raw data

Conclusion

REFERENCES

(1) Springthorpe, V.S. and Sattar, S.A. (2005a). *Quantitative Carrier Tests to Assess the Microbicidal Activities of Chemicals: Rationales & Procedures.* ISBN 0-88927-298-0, Centre for Research on Environmental Microbiology (CREM), Univ. of Ottawa, Ottawa, ON, Canada. 100 pages. Available from QCTmanual@webbertraining.com

(2) Springthorpe, V.S. and Sattar, S.A. (2005b). Carrier tests to assess microbicidal activities of chemical disinfectants for use on medical devices and environmental surfaces. J. AOAC International, 88: 182-201.

(3) OECD (2002) Report of the Efficacy Workshop on Certain Antimicrobial Biocides, Arlington, VA, U.S.A., OECD meeting held in April 2002.

(4) ASTM (2006) Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides. Method E-2197-02, Vol. 11.05. ASTM International, West Conshohocken, PA, U.S.A.

(5) OECD (2009) Report on validation of efficacy methods for antimicrobials used on hard surfaces

(6) DeVries, T. A. and Hamilton, M.A (1999). Estimating the antimicrobial log reductions: Part 1: Quantitative assays. Quant. Microbiol. 1: 29-45.

(7) OECD Guidance Document on the conduct of quantitative methods for evaluating bactericidal, fungicidal, mycobactericidal and virucidal activities of microbicides used on hard, non-porous surfaces (under publication).

(8) Spearman/Kärber (DVV Guideline 2008. Guideline of “Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V.“ (DVV; German Association for the Control of Virus Diseases) and Robert Koch Institute (RKI; German Federal Health Authority) for testing the virucidal efficacy of chemical disinfectants in the human medical area. Hygiene & Medizin; 34 (7/8), 293-299, 2009) or www.dvg-ev.de

Figure 1 (left): Ten µL of the test organism inoculum being removed with a positive-displacement pipette

Figure 2 (right): The inoculum being placed at the centre of disk carrier

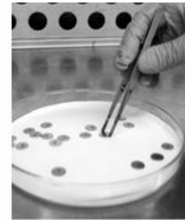

Figure 3 (left): Carrier with dried inoculum being picked up for placement in flat bottom vial

Figure 4 (right): Carrier placed into the flat bottom vial

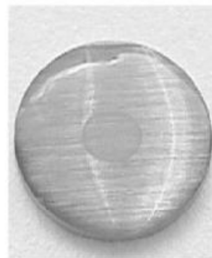

Figure 5: Dried inoculum on carrier covered with 50 µL of test substance or control fluid

ANNEX I

DEFINITIONS AND ABBREVIATIONS

BSA: Bovine Serum Albumin

Carrier an inanimate surface or object to be inoculated with the test organism.

CFU: Colony Forming Unit.

Control fluid (PBS) is the fluid placed on the control carriers in place of the test substance

EBSS: Earle Balanced Salt Solution

Eluate is recovered eluent that contains the test organism.

Eluent is any liquid that is harmless to the test organism(s) and that is added to a carrier to recover these on it.

Inoculum: Test organism in soil load.

Neutralisation is a process to quench microbicidal or microbistatic activity of a test substance remaining at the end of the contact time. This process may be achieved by dilution of the organism-test substance mixture and/or by adding to it one or more chemical neutralisers.

PBS: Phosphate Buffer Saline

PFU: Plaque forming Unit

Soil load is a solution of one or more organic and inorganic substances added to the suspension of the test organism to simulate their presence in body secretions, excretions, or other extraneous substances. It presents the test substance with a challenge to overcome the chemical demand from the soil load and the physical shielding of test organism that it may provide.

Stock culture is the frozen, refrigerated or lyophilized form of the test organism.

$TCID_{50}$: Tissue culture Infective Dose 50%

Test substance is a compound or formulation that is under evaluation for its microbicidal activity.

Test organism is one selected for testing; usually for its susceptibility/resistance characteristics. It also may be referred to as a *surrogate, simulant, target* or *marker microbe.* Ideally, it should be easy and safe to handle, and readily identifiable.

Test suspension: suspension of the test organism used to prepare the stock culture or working culture.

Working culture is the suspension of the test organism prepared for use in the test.

ANNEX II

NEUTRALISATION VALIDATION, REMOVAL OF CYTOTOXICITY, TESTING FOR INTERFERENCE WITH VIRUS INFECTIVITY AND TESTING THE INFLUENCE OF SOIL LOAD TO THE HOST CELLS

Neutralisation validation purpose

The purpose is to determine if the neutralisation of a test substance at the end of the contact time is sufficient to render it inactive against the test virus. Validate the neutralisation process before starting the microbicide tests. Also, determine beforehand the concentration of infective units of the test virus in its stock suspension after adding the soil load so that the required dilutions can be made to achieve the needed level of virus challenge for neutralisation validation.

Procedure

Method

a) Using plaque assay

1. Dilute test virus Dilute test virus in cell culture maintenance medium to give approximately 500-1 000 plaque forming units (PFU)/mL.

2. Prepare the test substance at the level it is to be tested (use-dilution) for virucidal activity.

3. Prepare a further 1:20 and a 1:200 dilution of the use-dilution in the neutraliser to be validated.

4. Add 100 µL of the diluted virus separately to 900 µL of the 1:20 and 1:200 dilutions of the use-dilution as prepared in step #3.

5. As controls, add same volume of the virus separately to 900 µL of the neutraliser alone and EBSS.

6. Allow the vials to stand at room temperature for 5 minutes.

7. Inoculate each host cell monolayer separately with the test and control solutions as prepared in steps 4 and 5.

8. After virus adsorption, place an agar overlay in the inoculated and control cultures.

9. Incubate the cultures as appropriate for the virus and observe them for the development of plaques and/or cytotoxicity

b) Using $TCID_{50}$ assay

1. Dilute test virus in tissue culture maintenance medium to give approximately a $TCID_{50}$ of 10^5/mL.

2. Prepare the test substance at the level it is to be tested (use-dilution) for virucidal activity.

3. Prepare a further 1:20 and a 1:200 dilution of the use-dilution in the neutraliser to be validated.

4. Add 100 µL of the diluted virus separately to 900 µL of the 1:20 and 1:200 dilutions of the use-dilution as prepared in step #3.

5. As controls, add same volume of the virus separately to 900 µL of the neutraliser alone and EBSS.

6. Allow the vials to stand at room temperature for 5 minutes.

7. Prepare serial two-fold dilutions in cell culture maintenance medium

8. Inoculate each host cell monolayer separately with the test and control solutions as prepared in step #s 4 and 5 (100 µL to each well, 8 wells/dilution). It is also possible to use suspension cells in this test especially if such cells are used in the disinfectant test.

9. Incubate the cultures as appropriate for the virus and observe them for any cytopathology (CPE) and/or cytotoxicity.

Possible outcomes

1. Lack of sufficient neutralisation of virucidal activity

When compared to controls, a lower level of virus infectivity in cultures inoculated with the mixture of test substance + neutraliser indicates insufficient neutralization. This requires additional testing to improve the neutralization process. The difference between the virus titre of the samples with addition of the mixture of the test substance and neutraliser in comparison with the virus control should not exceed 0.5 $\log_{10}$.

2. Successful neutralisation of virucidal activity

The presence of comparable levels of infectivity in cultures inoculated with test substance + neutraliser mixture and the controls indicates successful quenching of virucidal activity and thus a validated neutralization process.

3. Cytotoxicity

Apparent degeneration of the cell monolayers receiving the test substance + neutraliser mixture and the neutraliser alone indicates cytotoxicity. The presence of such cytotoxicity precludes the proper detection of any infectious virus. If the cytotoxicity of the test substance is so strong that a decrease of the infectivity titre of 4 $\log_{10}$ cannot be detected, cytotoxicity can be reduced by applying gel filtration, micro-filtration, or appropriate chemical neutralisation agents after the contact time has elapsed. The test laboratory must describe in detail the procedure used to eliminate or reduce to acceptable levels such cytotoxicity.

ANNEX II
(continued)

CHECK FOR INTERFERENCE WITH VIRUS INFECTIVITY (including the influence of soil load to the host cells)

Purpose

The purpose is to determine if sub-cytotoxic levels of the test substance reduces or enhances virus infectivity in host cells. Levels of the test substance which show no obvious cytotoxicity could still reduce or enhance the ability of the challenge virus to infect or replicate in host cells, thus interfering with the estimation of its virucidal activity. An interference control must, therefore, be included to rule out such a possibility. Perform such interference testing before starting the virucide tests and only one lot of the test substance may be used for this purpose.

Procedure

Method

a) Using plaque assay

1. Dilute test virus to give approximately 500-1 000 PFU/mL.

2. Prepare the test substance at the level it is to be tested for virucidal activity.

3. a) Prepare a further 1:20 and a 1:200 dilution of the test substance in the neutraliser to be validated.

   b) Mix 50 µl of the test substance (see point 1.) with 10 µl of the soil load see table 3 and prepare a further 1:20 and a 1:200 dilution of the test substance in the neutraliser to be validated.

Table 3

Component	Volume (µL)
EBSS/PBS	340
BSA	25
Mucin	100
Yeast extract	35
Total	**500**

4. Put 100 µL each of the mixtures a) and b) of the 1:20 and 1:200 dilutions of the test product in the neutraliser into three wells each of a 12-well cell culture plate.

5. In the remaining six wells place 100 µL of the neutraliser each in three and 100 µL of EBSS in three as controls.

6. Incubate plate for 60 minutes.

7. Observe monolayers under an inverted microscope for any obvious signs of toxicity. If damage to the cells is readily visible, it is a sign of cytotoxicity. Use gel filtration or another suitable method to remove cytotoxicity.

8. If monolayers show no observable damage, proceed with the next step to assay for interference with plaque formation.

9. Wash the monolayers once with EBSS/PBS and add virus (diluted to give countable plaques in each well) to 9 wells which had separately received the product (6) and the neutraliser (3). To the two remaining wells add virus with countable number of plaques. Leave the last well as cell culture control.

10. Incubate plates for 60-90 minutes to allow virus to adsorb.

11. Add agar overlay to the monolayers.

12. Incubate plates as appropriate for the virus for development of plaques.

b) Using $TCID_{50}$ assay

1. Prepare the test substance at the level it is to be tested for virucidal activity.

2. Prepare a further 1:20 and a 1:200 dilution of the test substance in the neutraliser to be validated.

3. Mix 50 µl of the test substance (see point 1) with 10 µl of the soil load (see Table 3 below) and prepare a further 1:20 and a 1:200 dilution of the test substance in the neutraliser to be validated.

Table 3

Component	**Volume (µL)**
EBSS/PBS	340
BSA	25
Mucin	100
Yeast extract	35
Total	**500**

4. Put 100 µL each of the mixtures a) and b) of the 1:20 and 1:200 dilutions of the test product in the neutraliser into such a number of wells of a 96-well cell culture plate, which are need for titration see below.

5. As control, put 100 µL EBSS/PBS into such a number of wells of a 96-well cell culture plate, which are needed for titration (see below).

6. Incubate the plates for 60 minutes.

7. Observe monolayers under an inverted microscope for any obvious signs of toxicity. If damage to the cells is readily visible, it is a sign of cytotoxicity. Use gel filtration or another suitable method to remove cytotoxicity.

8. If monolayers show no observable damage, remove the supernatant. Proceed with the next step to assay for interference with $TCID_{50}$.

9. Prepare a serial dilution of the virus suspension in cell culture maintenance medium.

10. Inoculate each host cell monolayer separately with the virus dilution as prepared in step 8 (100 µl to each well, 8 wells per dilution).

11. Incubate the cultures as appropriate for the virus.

Note: To improve the viability of the cells**,** especially in the case of using monolayer, it can be useful to add 100 µl medium after step 8.

Possible outcomes

(1) ***Interference with virus infectivity:*** Any interference by residual amounts of the product will result in significantly lower numbers of plaques/lower $TCID_{50}$ in monolayers pre-treated with its sub-cytotoxic dilution when compared to the number of plaques/$TCID_{50}$ in the control monolayers. Those dilutions that are toxic to the cells or do not exhibit virus replication, or both are not included in the log_{10} reduction calculations of the virucidal activity.

(2) If the titre determined for mixture b) is more than 1.0 log_{10} lower than the titre of mixture a) the soil load influences the test result. A other batch of soil load must be prepared using substances of sufficient quality.

(3) ***Enhancement of virus infectivity:*** In this case, a higher number (as compared to controls) of plaques/higher $TCID_{50}$ in the monolayers treated with a sub-cytotoxic level of the test substance would indicate either deaggregation of virus clumps due to surfactants in the mixture or the alteration of virus receptors on the host cells.

(4) ***Lack of interference:*** An absence of any interference with virus infectivity would be indicated by the appearance of similar numbers of plaques/similar $TCID_{50}$ in treated and control monolayers. The difference between the titre of the disinfectant treated cells in comparison with the virus control shall not exceed 0.5 log_{10}.

ANNEX III

PROCEDURES FOR MAINTENANCE OF STOCKS OF VIRUSES AND HOST CELLS

KEEPING HOST CELL STOCKS BY FREEZING IN LIQUID NITROGEN

Purpose

The procedure describes how to freeze aliquots of cells to be thawed out in case of loss due to contamination or reduction in virus susceptibility with increasing passage number.

Procedure

Test organisms

The source (e.g., ATCC), scientific name, reference number and batch number of the test organism is clearly documented. In addition, records are maintained including dates the test organism was received, subcultured and frozen as initial stock. In addition, the complete passage history is documented and traceable to the initially frozen vials. See Annex V

Culture media and reagents

Commercially prepared culture media and any ingredients purchased to make such media in-house are obtained from reputable sources. Chemicals/reagents are of analytical grade or appropriate for microbiological purposes. See Annex IV.

Method

- freezer and warm up in water bath at 37°C (about 30 minutes if water is already warm).
- Remove flasks with monolayers from incubator and check cell morphology. Label with new passage #, split ratio and date.
- When everything is warmed up, aspirate out spent medium from flask.
- Dispense about 10 mL of PBS into each flask and wash monolayer. Discard the PBS wash.
- Repeat the wash step.
- Dispense 2 mL of trypsin+EDTA into each flask and distribute it evenly over the entire monolayer. Aspirate out the trypsin and incubate flask at 36 ± 1°C for about 10 min.
- Remove flasks from the incubator. Gently tap the flask to break up cell aggregates as much as possible.

- Resuspend cells in each flask in 3 mL of medium; put 2 mL of the cells from each flask into one sterile test tube. Use the remaining 1 mL to reseed flask to grow a monolayer again, if needed.

- Mix cells with foetal bovine serum and DMSO in the ratio of 7:2:1. Tighten caps well.

- Dispense 1 mL of the above mixture into each sterile cryovial already labelled with name of cell line, passage number and date of freezing.

- Put the vials on ice for 30 minutes or so; then move them to -20°C freezer and leave them there for 24 hours.

- Load the vials into labelled metal cranes and immerse them into liquid nitrogen (-196°C).

- Fill out log book with the name of the cell line, passage level, number of vials frozen and the date.

- A week or so later remove a vial from liquid nitrogen. Revive the cells to check their viability and freedom from contamination.

ANNEX III
(continued)

REVIVING CELLS FROM LIQUID NITROGEN STORAGE

Purpose

This procedure is for reviving cells frozen in liquid nitrogen.

Procedure

Method

- Warm up the medium to 37°C in water bath or incubator.
- Dispense 20 mL of medium into each new culture flask.
- Remove vial(s) of cells from liquid nitrogen.
- Immediately hold (but do not immerse) vial(s) in warm water to thaw cells rapidly and avoid damage from ice crystal formation.
- Remove the thawed cells with a pipette and place them in a cell culture flask with pre-warmed growth medium. Shake to evenly distribute the suspension.
- Label the flask with the date and information from the frozen vial.
- Observe cell morphology under an inverted microscope.
- Incubate for 24 hours and then aspirate out the spent media and replace it with fresh medium. This is to get rid of DMSO used as a cryoprotective agent.
- The success of freezing is indicated by the number of cells attaching and dividing in the flask. It is often necessary to change the medium one more before the cells would form a confluent monolayer. They can be split at that stage.

ANNEX III
(continued)

SUB-CULTURING OF HOST CELLS FOR WORK WITH VIRUSES

Purpose

This procedure describes how to split confluent cell monolayers for subculture.

Procedure

Method

1. Remove bottle(s) of medium, and PBS from fridge or cold room, tubes of trypsin from -20°C freezer and warm up in water bath or incubator at 37°C. (about 30 min. if water is already warm).

2. Remove flasks with monolayers from incubator and check cell morphology. Label with new passage #, split ratio and date.

3. When everything is warmed up, aspirate out spent medium from flask.

4. Dispense about 10 mL of PBS into each flask and wash monolayer. Discard the PBS wash.

5. Repeat the wash step.

6. Dispense 2 mL of trypsin+EDTA into each flask and distribute it evenly over the entire monolayer. Aspirate out the trypsin and incubate flask at 36±1°C for about 10 min.

7. Remove flask from the incubator. Gently tap the flask to break up cell aggregates as much as possible.

8. Dispense the amount of medium required for the desired split ratio. For example, for a 1:4 split, collect cells from one flask into 4 mL of medium and leave 1 mL of the cells to reseed the same flask again).

9. Hold the flask at a 45° angle and use a pipette to aspirate the cell suspension in and out repeatedly to break up cell clumps. Swirl the flask. Use the pipette to aspirate the medium in the flask and forcefully expel the liquid to help lift the cell monolayer off of the flask surface. Repeat as needed to harvest the monolayer

10. Put one mL of cell suspension into each new flask and then dispense into it 20 mL fresh growth medium. Label flasks with name of cell line, passage number and date of split.

11. Place the flasks into the incubator and do not disturb then for 2-3 hours to allow for cell adherence to the plastic surface.

12. Monolayers prepared this way can be used for making virus pools, for making plates for virus infectivity assays or for another passage for subsequent experiments.

Example: Test virus adenovirus

TCID50 assay

Virus propagation:

Remove growth medium from confluent monolayer of A549 cells in a 175 cm^2 TC, rinse twice with 40 mL PBS and infect cells with ~200-500 µL virus stock solution (moi of 0.1-1) and 4 mL cell culture medium (e.g., EMEM) (without Fetal Bovine Serum - FBS) for 1-2 h at 37°C (gently shake every 10 to 15 min).

At the end of incubation time remove inoculum and add 25 mL cell culture medium (*e.g.*, EMEM) with 2% FBS. Incubate cells for 2 to 4 days (mostly 3 days) at 37°C and 5% CO_2 (70-95% of all cells will show a distinct CPE at this time).

Virus harvest:

For virus recovery 3 rapid freezing (-20°C) / thawing (37°C) steps are performed to release virus from cells. Transfer the suspension into a 50 mL Falcon tube, centrifuge for 10 min at 1,000 x*g* (4°C) and collect the supernatant as virus stock. Aliquot (1 mL volumes) and store at -80°C with the vials clearly labelled with name of virus, date of preparation of the pool and passage number.

Virus titration:

Virus titration is performed on A549-cells. Cells in a 175 cm^2 TC are detached with trypsin/EDTA, suspended in 180 mL cell culture medium (e.g., EMEM) (10 % FBS) and 100 µL of this cell suspension are transferred into each well of a 96-well flat bottom microtitre plate.

Infectivity titre is determined by means of end-point dilution titration in a microtitre-procedure. For this, samples are diluted 1:10 with ice-cold EMEM with 2% FBS and 100 µL of each dilution are placed in eight wells of a sterile polystyrene flat bottomed microtitre plate (Nunc GmbH & Co. KG). 100 µL (adjusted to provide approx. 10-15 x 10^3 cells) of freshly trypsinized A549 cells had been added the day before. It is also possible to use 100µl of suspended cells. Cultures are incubated at 37°C and 5.0 % CO_2 in order to follow the development of cytopathic effects for a period of 10 (to 14) days after inoculation.

Notes:

a) In some cases the A549 cells grow better with a lower $NaHCO_3$-content (e.g. 0.11%), then replace EBSS by PBS, because the cells are very sensitive against higher $NaHCO_3$-contents.

b) For neutralisation and interference tests, the concentration of FBS should be between 2 and 5%.

ANNEX IV

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

Dimethyl sulfoxide (DMSO)

Fetal Bovine serum (FBS same as FCS – Fetal Calf Serum):

Trypsin (0.05%)-EDTA, 1X

Earl's Balanced Salts (EBSS) powder: Make 1 L solution by dissolving in deionized water, filter to sterilize.

Neutraliser in eluent

Chemical neutralisers vary with test substance and should be manufacturer-specified whenever possible. Tween-80 is used in the eluent to help dissociate any microbial clumps that may have formed during testing. The neutraliser is sterilised with or aseptically added to Tween-80 in the PBS eluent prior to use. The final concentration of Tween-80 in the eluent is typically 0.1% v/v. Other concentrations of polysorbate 80 or other neutralisers may be used providing they are validated. When the neutraliser is heat-sensitive and is aseptically added, the neutraliser and Tween-80 should be prepared sterile at double strength in PBS (pH 7.2-7.4) and then mixed in equal volumes.

Phosphate buffer saline (PBS)

Add 1.25 mL of PB stock solution and 8.75 g of NaCl to a volumetric flask, fill with deionised water to the 1000 mL mark, and mix; adjust pH to 7.2 ± 0.2, if necessary. Sterilise by filtration or autoclaving at 121°C for 15-20 min. Alternative PBS formulations with the same pH may be used.

Phosphate buffer (PB) stock solution

Dissolve 34.0 g of potassium dihydrogen phosphate (KH2PO4) in 500 mL of water. Adjust pH to 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with deionised water. Alternative phosphate buffers with the same pH may be used.

Soil load

The recommended standard soil load to be incorporated in the test microbial suspension is a mixture of the following stock solutions in PBS (pH 7.2-7.4):

a. Add 0.5 g yeast extract to 10 mL of PBS (*low* molecular weight component), mix, and pass through a 0.45 µm pore diameter membrane filter, aliquot and store at either 4 ± 2°C or -20 ± 2°C.

b. Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS (*high* molecular weight component), mix and pass through a 0.45 µm pore diameter membrane filter, aliquot and

store at either 4 ± 2°C or -20 ± 2°C.

c. Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS (mucoid substance), mix, and autoclave (15-20 minutes at 121°C), aliquot. Store at either 4 ± 2°C or -20 ± 2°C.

The stock solutions of the soil load have a self-life of at least one year when stored between 4 ± 2°C and -20 ± 2°C.

Note: The method permits additional or alternate soil loads, depending on label claims and use sites, providing it can be demonstrated that they have at least as great a disinfectant demand as the default soil load. Many materials derived from animal sources may contain substances inhibitory to test microbes. Therefore, each purchased lot of such materials should be screened to ensure no such interference exists.

Test substance

Dilute it first if required for testing and bring it to the test temperature prior to use.

Test substance diluent

The test substance diluent is hard water. The following is based on CEN method prEN-13727: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area – Test method and requirements. The procedure is as follows for preparing one litre of hard water:

a. Preparation of Solution A: dissolve 19.84 g magnesium chloride ($MgCl_2$) and 46.24 g calcium chloride ($CaCl_2$) in water and dilute to 1000 mL. Sterilise by membrane filtration or in the autoclave. Store the solution in the refrigerator for no longer than one month;

b. Preparation of Solution B: dissolve 35.02 g sodium bicarbonate ($NaHCO_3$) in water and dilute to 1000 mL. Sterilise by membrane filtration. Store the solution in the refrigerator for no longer than one week;

c. Place 600-700 mL of water in a 1000 mL volumetric flask and add 6.0 mL of Solution A and then 8.0 mL of Solution B. Mix and add more water to the flask to reach 1000 mL. The pH of the hard water should be 7.0 ± 0.2 when measured at 20 ± 1°C. If necessary, adjust the pH by using a solution of 40 g/L (about 1 mol/L) of sodium hydroxide (NaOH) or 36.5 g/L (about 1 mol/L) of hydrochloric acid (HCl).

d. The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

Note: The final hardness expressed as calcium carbonate ($CaCO_3$) is 375 mg/L. Other levels of water hardness may be used as appropriate.

Water

Use either deionised distilled water or water with equivalent quality for making reagent solutions and culture media. One reference document for preparing, storing and testing reagent-grade water is *Standard Methods for the Examination of Water and Wastewater* (http://www.standardmethods.org/).

ANNEX V

MATERIAL AND EQUIPMENT

Sterilise all labware and equipment as appropriate. Sterilisation can be achieved by moist heat in an autoclave, by dry heat in a hot-air oven or other appropriate, validated sterilisation process.

Air Displacement Pipettes: Eppendorf or equivalent, 50–1000 µL with disposable tips – to measure test substance, eluents and diluents as appropriate.

Analytical balance: to weigh chemicals and to calibrate inoculum delivery volumes by pipettes. Analytical balances should be calibrated at least annually.

Biological Safety Cabinet: suitable for the containment of the test organisms used. Such cabinets require periodic recertification.

Bucket (of ice and of water at 37°C).

Carriers: Disks (1 cm in diameter) made from 0.7 mm thick sheets of brushed and magnetised stainless steel (AISI #430). Both sides of the carriers are identical in their topography and finish.

Cell culture flasks and plasticware

Cell culture Media and supplements: Media Media for growing and maintaining cell cultures can be purchased from any major biological supply house as ready-to-use products or as powders that can be readily dissolved in water and sterilised for use. Foetal bovine serum, agar, antibiotics, amino acids, trypsin-EDTA, EBSS and most other needed supplements are also readily available commercially.

Cells: frozen vial and as complete monolayers ready for subculture.

Centrifuge: to sediment virus-infected host cells or cell debris, when needed.

Centrifuge Tubes (Polypropylene) with Caps: 50 mL capacity.

Cryovials for holding eluates and for making dilutions: suitable to hold 2.0 mL easily.

Dry heat sterilizer for sterilisation of metal carrier disks instead of autoclaving.

Flasks or beakers: 250-mL capacity for preparing culture media.

Forceps, straight or curved a) with smooth flat tips to handle membrane filters; and b) appropriate to pick up the carriers for placement in vials. Using multiple sterile forceps is recommended. If multiple forceps are not available, a single pair of forceps can be decontaminated between uses by dipping the tips in ethanol and flaming it with a burner. Exercise caution to avoid contamination and any fire hazards from igniting the alcohol.

Freezers: a freezer at -20 ± 2°C for the storage of media and additives. A second freezer at -70°C or lower to store the stocks of test organisms.

Freezing jar

Gas Cylinders for the CO_2 incubator: A regular supply of gas cylinders to maintain CO_2 in the incubator for cell culture and virus infectivity assays.

Glassware: One-L flask with a side-arm and appropriate tubing to capture the filtrates from 47 mm diameter membrane filters; alternatively, a suitable commercial manifold can be used. Erlenmeyer flasks to hold 250 mL of culture media or reagents.

Glass or ceramic beads: 3 mm to 4 mm in diameter.

Gloves: sterile, disposable, for handling test items.

Hot Air Oven: an oven at 60°C to dry clean and wrapped sterile glassware.

Incubator: to maintain a temperature of 36 ± 1°C; if an open system is to be used for cell culture and virus assays, a CO_2 incubator will be required to maintain an atmosphere with 5% CO_2.

Inverted microscope: to observe cell culture monolayers for cytopathology or cytotoxicity.

Liquid nitrogen equipment

Magnet: strong enough to hold the carrier in place in the vial while the liquid is being poured out of it for membrane filtration.

Magnetic Stir Plate and Stir Bars: large enough for a 5-L beaker or Erlenmeyer flask for preparing culture media or other solutions.

Markers: permanent labware marking pens.

Membrane Filtration System for Media and Reagents: a membrane or cartridge filtration system (0.22 µm pore diameter) for sterilising heat-sensitive solutions. Reusable or disposable filtration systems may be used.

Membrane Filtration System for Recovery of the Test organisms: sterile 47 mm diameter membrane filters and sterile glass, plastic or metal holders for such filters. Membranes with either 0.22 µm or 0.45 µm pore diameter may be used as appropriate for the test organism. Reusable or disposable filtration systems may be used.

Miscellaneous Laboratory Ware: pipette tips, plastic vials for storing stocks of microbes, dilution tubes.

Multi-well disposable plastic cell culture plates (12-well, 24-well or 96-well): to grow host cell monolayers for plaque assays or other means of titrating virus infectivity.

Petri plates (Pyrex glass) 150 mm in diameter: for holding and autoclave sterilisation of metal carriers.

Petri plates (plastic): 100 mm X 15 mm for microbial growth and recovery media.

pH meter: having an accuracy of calibration of no more than ± 0.1 pH units to measure pH of buffers, eluents and test substance. Note: A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media.

Pipettes (Graduated): of nominal capacities of 10 mL and 1 mL and 0.1 mL

Pipette and pipette tips (Air Displacement): Eppendorf or equivalent, 50-1000 µL with disposable tips – to measure test substance, eluents and diluents as appropriate.

Pipette and tips (electronic or non-Electronic Positive Displacement): 10-100 µL pipette and appropriate pipette tips fitted with "plungers" that can dispense accurately 10 µL volumes for inoculation of carriers without the aerosol generation.

Plastic flasks (25 cm^2, 75 cm^2 and 175 cm^2 surface area) for growing and maintaining cell cultures.

Plastic Vials to Hold Test Carriers: flat bottom and wide-mouth to accommodate addition and removal of the carriers, for holding inoculated carriers to be exposed to the test substance and for accommodating neutraliser/eluent. Suitable vials should be at least 25 mm in neck diameter and hold at least 30 mL of liquid.

Refrigerator: 4 ± 2°C; for storage of media, culture plates and reagents.

Serological Pipettes: sterile reusable or single-use pipettes of 1.0, 5.0 and 10.0 mL capacity.

Silicone grease for desiccators.

Spectrophotometer: for measuring turbidity of microbial suspensions.

Steriliser: any steam steriliser suitable for processing culture media, reagents and labware; the steam supplied to the steriliser should be free from additives toxic to the test organisms.

Test Organisms: Obtain ATCC organisms directly from ATCC or other credible sources.

Timer: any laboratory timer that can be read in minutes and seconds.

Vacuum Source: a vacuum pump or access to an in-house vacuum line to pull the samples through membrane filters and to evacuate desiccators to dry inoculated carriers.

Vials or Tubes for Dilution: wide-mouthed and suitable to hold 30 mL easily.

Vials (plastic) to Hold Test Carriers: flat bottom and wide-mouth to accommodate addition and removal of the carriers, for holding inoculated carriers to be exposed to the test substance and for accommodating neutraliser/ eluent. Suitable vials should be at least 25 mm in neck diameter and hold at least 30 mL of liquid. Transparent vial are more desirable to allow the viewing of the carriers for removal of inoculum.

Vortex Mixer: to vortex the eluate and rinsing fluid in the carrier vial to ensure efficient recovery of the test organism(s).

Water bath: to attain temperatures up to 60°C to warm up media and reagents or to thaw frozen cells.